

POPULATION DIVERGENCE AND EVOLUTION  
OF THE ENDANGERED *Sesbania tomentosa* (FABACEAE)

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## ABSTRACT

*Sesbania tomentosa* (Fabaceae) is an endemic flowering plant primarily adapted to coastal strand and dry lowland habitat in the Hawaiian Islands, now extant in relicts of its former range. Efforts have been made to delineate distinct taxa from among the remaining populations. In the most recent treatment of Hawaiian Fabaceae, however, *S. tomentosa* was recognized as a single variable species. In an attempt to address issues of taxonomy, the present study compared phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses (DNA sequence and microsatellite marker variation) and assessed their relative level of congruence. A complete lack of variation between eight putative taxa from six islands at two nuclear DNA regions (1035 bp) contrasts with the highly differentiated population structure of the nine microsatellite loci sampled, while confidence in the relationships proposed in morphological phylogenies based on putative taxonomy was low. Instead, Bayesian genetic clustering assignments and associated private alleles occurred in a distinct phylogeographic pattern. As a result, populations from Nihoa, Kaua‘i and O‘ahu are distinguished as a separate subspecies of *S. tomentosa*, populations from Maui Nui and Hawai‘i Island (respectively) form two additional subspecies, and a fourth subspecies endemic to SE Moloka‘i distinguishes itself from the rest of Maui Nui.

Naturally-occurring populations of *Sesbania tomentosa* plus a substantial number of outplanted individuals were analyzed for levels of allelic diversity, heterozygosity and inbreeding. Evidence of genetic bottlenecks in populations was also investigated, as well as an analysis of population sub-structuring. Natural ecological dynamics affecting population differentiation often leave lasting genetic signatures, and are addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants. The

molecular data can be interpreted to support the hypothesis that distinctive-appearing remnant populations of this highly variable species have diverged at an accelerated rate due to human induced habitat fragmentation within the larger context of the speciation process itself. This study also provides examples of increasing genetic diversity in outplantings when intentional mixing of populations to augment diversity was practiced, as well as in situations where the genepools of natural populations are dynamic over time.

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## CHAPTER 1

### **Phylogenetic relationships and population structuring within the *Sesbania tomentosa* species complex; relevance for restoration management of relict plant populations**

#### **Introduction**

*Sesbania tomentosa* Hook. and Arn. is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. *Sesbania tomentosa* is currently recognized as a single species (Geesink et al., 1999) although it is highly variable for many important characters across its range. This led Rock (1920), Degener and Degener (1978) and Char (1983) to delimit up to nine distinct putative taxa. Two major groups emerged in a genetic analysis of Hawaiian *Sesbania* measuring variation at ten isozyme loci across the geographical range of the species Gemmill et al. (1995). An analysis of *S. tomentosa* with both sequencing and population genetic markers would lend justification at the molecular level for one or more separate taxonomic entities.

Twenty-nine of the fifty-two populations of *Sesbania tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826, largely the result of intense ungulate grazing pressure across its range. As a result, this species was federally listed as Endangered by the U.S. Fish and Wildlife Service in 1992. The relictual nature of the present range of the species is thought to have accentuated morphological differentiation of populations (Geesink et al., 1999). On the other hand, natural ecological dynamics affecting population differentiation (e.g., pollination syndromes, plant maturation rate, seedbank dynamics, population flush-crash cycles) often leave lasting genetic signatures, and can be addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants.

Understanding both the nature of population differentiation and its extent (in terms of putative speciation) has important implications for restoration management of this Endangered plant. For example, three of Char's (1983) putative taxa occur within 5–10 km of one another in Hawai'i Volcanoes National Park. Two of these putative taxa are endemic to the park, each represented by less than 50 naturally-occurring individuals. Two other species delimited by Char

were once found to occur within 4 km of one other on the west coast of Kaua‘i. Today one of these putative species can be found in a small population of 20–30 individuals *in situ*, the other *ex situ* at the National Tropical Botanical Garden. Given the close proximity of populations in both these instances, separate putative Hawaiian *Sesbania* taxa had likely exchanged genes in the past when the plant’s range was more substantial. Restoration managers need to address the genetic structuring of this apparent species complex before considering the translocation of propagules to enhance genetic variation within reproductive populations. This is important, as mixing populations representing separate putative taxa may also put their genetic and taxonomic integrity at risk.

#### Primary Objectives of Investigation

- 1) Investigate morphological relationships between putative taxa of Hawaiian *Sesbania* using phylogenetic analysis of a morphological character dataset
- 2) Investigate the distinctiveness of putative taxa of Hawaiian *Sesbania* on a molecular level using DNA sequencing of nuclear regions
- 3) Levels of genetic variation within and between populations (and putative taxa) will be examined using microsatellite marker analysis
- 4) Compare genetic diversity of naturally-occurring individuals and populations with their counterpart outplanted individuals and populations

#### Primary Hypotheses

- 1) Hawaiian *Sesbania* form a monophyletic group and represent a recent radiation among the Hawaiian Islands
- 2) The formal recognition of additional taxa of Hawaiian *Sesbania* is warranted based on genetic and morphological evidence
- 3) Populations will exhibit high levels of genetic structure with evidence of inbreeding within and divergence among populations

- 4) Natural selection in different environments over time combined with contemporary fragmentation (isolation) of populations caused Hawaiian *Sesbania* to separate into the distinctive appearing populations found today
- 5) Levels of inbreeding will be higher, and genetic diversity lower, in outplanted populations than in their naturally-occurring counterparts

## Materials and Methods

In an attempt to address issues of taxonomy, the present study compared phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses (DNA sequence and microsatellite marker variation) and assessed their relative level of congruence. Morphometric measures from the dataset developed by Char (1983) were used to construct morphological phylogenies. Phylogenetic inference at the molecular level used sequences from two nuclear DNA regions (1035 bp sampled): the non-coding internal transcribed spacer (ITS) of the ribosomal DNA cistron (Baldwin, 1993) and the highly variable gene-coding region triosephosphate translocator (TRPT) (Choi et al., 2004, 2006). Nine microsatellite marker loci were also used to assess within and among population variation found in individuals and to assess the degree of population differentiation. Together, sequence and microsatellite variation provide an estimate of phylogenetic relationships among the species and populations previously identified by Char (1983) and others.

Leaf samples of 539 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 38 sub-populations (separate clusters of plants 1 to 3 km apart within a population) comprising 18 populations from seven islands were sampled. An additional 141 individuals (collected from 8 populations on four islands) were sampled from *S. tomentosa* outplantings and restoration nursery stock. Twelve individuals were sampled from herbarium specimens to provide historical DNA from various populations for comparison. In order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, one population was sampled in three separate years (seasons), and the genetic diversity of the standing populations of each year are herein compared. The long term viability of populations actively managed for restoration was addressed using microsatellite markers, by comparing the genetic diversity of naturally-occurring populations of *Sesbania tomentosa* with those of their outplanted

counterparts to assess rates of inbreeding and impacts of genetic drift. As various numbers of founding individuals (from 1 to more than 10) have been used to assemble the outplanted populations measured, the genetic effects of seed sourcing practices were also examined.

## CHAPTER 2

### Phylogenetic relationships within the *Sesbania tomentosa* species complex

#### Introduction

The boundaries of species that have recently and rapidly diverged are difficult to determine when species-specific traits (morphological and/or genetic) have not had sufficient time to coalesce (Glor, 2010). Even if the morphology of the species in question seems to suggest such boundaries, DNA sequence divergence often will not have occurred due to insufficient time for accumulation of mutations within the different types (Mort et al., 2007). Hawaiian plant radiations are well recognized for morphological variation disconnected from genetically detectable differences (e.g., Gemmill et al., 2002, Lindqvist et al., 2003, Knope et al., 2012, Cantley et al., 2014). On the other hand, population genetic markers, those tied to allele frequencies diverging at a much more rapid pace, are able to distinguish genetically-isolated populations and groups of populations (Zhang and Hewitt, 2003). According to the unified species concept of de Queiroz (2007), a species is defined as a separately evolving metapopulation lineage (ancestral sequence of populations). Given this, the ability of population genetic markers to identify the boundaries of isolated gene pools makes them a suitable choice for analyzing recent and rapid plant radiations.

An investigation into the evolution of the Hawaiian endemic *Sesbania tomentosa* Hook. & Arn. (Fabaceae) is warranted, as past taxonomic history suggests there are relationships to resolve within this highly variable species. In the most recent treatment of Hawaiian Fabaceae, however, *S. tomentosa* was recognized as a single species with one form (f. *arborea* Rock) (Geesink et al., 1999). A previous genetic study by Gemmill et al. (1995) demonstrated that two major groups of Hawaiian *Sesbania* emerged when measuring variation at ten isozyme loci across the geographical range of the species. An analysis of *S. tomentosa* with both sequencing and population genetic markers may lend justification at the molecular level for one or more separate taxonomic entities.

*Sesbania tomentosa* is adapted to coastal strand and dry to mesic upland habitat. Geesink et al. (1999) described the species as a sprawling shrub with branches up to 14 meters long or

alternatively found as a small tree up to 6 meters in height. Leaves are even-pinnate and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. Leaflets are usually sparsely to densely covered with silky hairs, as referred to by the specific epithet. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds. The chromosome number reported is  $2n = 24$  (Geesink et al., 1999) suggesting the species is diploid (base chromosome number  $x = 12$ ).

G.T. Lay and A. Collie were the first to collect *Sesbania tomentosa* during the voyage of the HMS Blossom (under Captain Frederick William Beechey) through the Hawaiian Islands from 1826–1827, and their specimen was later described by Hooker and Arnott (1838). However, the type locality was erroneously listed as Acapulco, Mexico, this later corrected by Gray (1854). Since the botanists on the expedition were only believed to have collected on O‘ahu, the type locality is presumed to be from somewhere on that island (Gray, 1854; Feipel, 1914). Gray (1854) described *S. tomentosa* as a woody plant with decumbent (semi-prostrate) stems, having branches and foliage silky-tomentose when young, but turning glabrate with age. Gray noted that these plants occurred on the Wai‘anae coast of O‘ahu and on the coast of Hawai‘i east of Kīlauea Crater. Hillebrand (1888) described *S. tomentosa* in much the same way as Gray, only he found it occurring as a multi-branched shrub, 6 to 12 feet (2 to 4 m) in height. His specimens were also collected from the Wai‘anae coast of O‘ahu and on the southern shores of Moloka‘i, Lāna‘i and Hawai‘i.

Rock (1920) proposed an alternate form of *Sesbania tomentosa*, forma *arborea*, an arborescent type he had collected at Mahana (west Moloka‘i) growing 12 to 15 feet in height. He described the leaves as being longer, and the leaflets smaller and more numerous than the creeping variety he found growing nearby in the dunes at Mo‘omomi. Rock lists his arborescent form as also being present on the islands of Kaua‘i, O‘ahu and Hawai‘i.

Degener (1938) was the first to consider that *S. tomentosa* represents a poorly understood species complex and is probably composed of a number of forms on most of the islands (delineated primarily in terms of plant habit and leaf pubescence). Degener and Sherff (1949) considered the prostrate form at Mo‘omomi, Moloka‘i to be sufficiently distinct to warrant its own variety (*S. tomentosa* var. *molokaiensis*), due in part to the dense sericeous tomentum found on both surfaces of the leaflets. St. John (1973) concurred with Rock (1920) and with Degener



and Sherff (1949), and listed one endemic species of *Sesbania* with one variety (var. *molokaiensis* Degener & Sherff) and one form (f. *arborea* Rock). Degener and Degener (1978) recognized four new species of Hawaiian *Sesbania* elevating *S. tomentosa* var. *molokaiensis* and f. *arborea* to *S. molokaiensis* (Degener & Sherff) Degener & I. Degener and *S. arborea* (Rock) Degener & I. Degener, respectively. They also described *S. hawaiiensis* Degener & I. Degener from the South point region of Hawai‘i (mainly on the basis of slight variations in flower, stem and seed color) and *S. hobyi* Degener & I. Degener (a small erect tree with long extending branches and only a minor pubescence on lower surface of leaflets) from the island of Lāna‘i.

Char’s (1983) taxonomic thesis is the most recent and extensive survey of the morphological variation among Hawaiian *Sesbania* populations, making the important observation that the presence of hairs on leaflets is a useful taxonomic character. *Sesbania tomentosa* was split by Char into two varieties, the geographically widespread “var. *tomentosa*” (a highly polymorphic taxon in terms of leaf tomentum and flower color) and a minor variant from a single population, “var. *hobyi*” from Lāna‘i. Char also recognized *S. molokaiensis* from Mo‘omomi Moloka‘i (noting dense tomentum on both surfaces of leaflets) and *S. arborea* (noting sparse hairs confined to midrib of lower surface of leaflet) from the islands of Moloka‘i, Maui and Hawai‘i. Char named five additional putative taxa as well (none of which were ever validly published): “*polihalensis*” from the islands of Kaua‘i and Nihoa (erect shrubs with hairs on upper surface of leaflets confined to the midrib and veins), “*manaensis*” from the Mānā plain of Kaua‘i, “*oricola*” from the islands of O‘ahu, Ni‘ihau and Necker (erect shrubs with both surfaces of leaflets covered with dense tomentum) and “*kauensis* var. *kauensis*” and “*kauensis* var. *intermedia*” (erect shrubs with extremely long trailing lower branches and large leaflets with conspicuous reddish-brown pigmentation on stipules and leaflet margins) from the Ka‘ū district of Hawai‘i Island (Char, 1983). Char compiled morphometric datasets based on her observations of both plants in the field as well as herbarium specimens to elucidate relationships among populations of *Sesbania*. Her research reported that while a certain degree of phenotypic plasticity is apparent in varieties of Hawaiian *Sesbania*, cultivated individuals of the different varieties in a common garden retained the same morphological characters as their counterparts in the field (Char, 1983).

The purpose of the present study was to compare phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses

(DNA sequence and microsatellite marker variation) to assess their relative level of congruence. Morphometric measures from the dataset developed by Char (1983) were used here to construct morphological phylogenies. For the sake of simplicity in identifying the various morphotypes, Char's (1983) unpublished nomenclature is used throughout since it had covered the broadest spectrum of variation across Hawaiian *Sesbania*. Phylogenetic inference at the molecular level used sequences from two nuclear DNA regions: the non-coding internal transcribed spacer (ITS) of the ribosomal DNA cistron (Baldwin, 1993) and the highly variable gene-coding region triosephosphate translocator (TRPT) (Choi et al., 2006). Microsatellite markers were used to assess within and among population variation found in individuals and to assess the degree of population differentiation. Together, sequence and microsatellite variation will provide an estimate of phylogenetic relationships among the species and populations previously identified by Char (1983) and others from which character evolution can be estimated.

The ITS region has been the most extensively used nuclear region for phylogenetic analyses in plants since first used by Baldwin et al. (1995). Many legume groups have been sampled for ITS (Allan and Porter 2000; Lavin et al., 2003, Schrire et al., 2003; McMahon and Hufford 2004); ITS even varies below the species level within some taxa (Lavin et al., 2003). ITS sequence variation has been shown to provide better resolution of closely related legumes compared to the plastid region *trnL-F* (Wojciechowski et al., 1999; Lavin et al., 2001). In addition, *trnK-matK* showed little nucleotide variation across *Sesbania* taxa worldwide (Farruggia, 2009) and no variation among the four Hawaiian accessions (from three separate submissions) on GenBank (accession #s JX295926, JQ669637, JQ669638, HQ730420). It is for these reasons, and because of the eventual outcome of nDNA sequencing, that the plastid genome was not sampled for the present study.

Variation at the exon-derived TRPT gene was also examined as Choi et al. (2004) provided evidence that this region is suitable for phylogenetic analysis in legumes at the specific and subspecific levels. The divergence of this region between six legume genera (*Medicago*, *Pisum*, *Lotus*, *Glycine*, *Vigna* and *Phaseolus*) was shown to range as high as 42.7% (Choi et al., 2006) and Farruggia et al. (2009) found that the TRPT region concurred with species level resolution of ITS and *trnK-matK* topologies of *Sesbania* worldwide.

Microsatellite markers have a more rapid mutation rate than DNA sequence data (Jarne and Lagoda, 1996), and were another tool used to study relationships between Hawaiian

*Sesbania* populations and the various morphological types. Analytical methods such as STRUCTURE use multilocus microsatellite genotypes to assign individuals to genetic clusters without their *a priori* designation into populations. These methods were complemented by pairwise comparisons of allele frequencies in geographical populations as well as among the different morphological types to clarify their relationships.

## Materials and Methods

### Collection of morphological character data

Eighteen morphological characters discussed by Char (1983) in terms of their taxonomic significance for Hawaiian *Sesbania* were coded as discrete data for input into a matrix (Tables 2.1 and 2.2). Seven of these 18 characters were highly variable within putative taxa, therefore average values of characters over a range of sample sizes (20 to over 300) were used. The other 11 characters were less variable within putative taxa and were classified on the basis of personal observations made in the field and from reading Char's concise descriptions of each putative taxon.

Sachet (1987) examined the morphology of the South Pacific species of *Sesbania* and considered that the French Polynesian species *S. coccinea* (L.f.) Poir. was undoubtedly a close relative of *S. tomentosa* and, thus, was used as the outgroup in the phylogenetic analysis of morphological data. Character states were measured from 20 herbarium specimens at the B. P. Bishop Museum Herbarium (BISH; Honolulu, HI) and were used along with the taxonomic description of Sachet (1987) to develop the data matrix entry (Table 2.1).

### Phylogenetic analysis of morphological character dataset

The exhaustive search algorithm was used in PAUP v. 4.0 (Swofford, 2002) to infer maximum parsimony phylogenetic hypotheses. All character state changes were treated as unordered and unweighted. Bayesian analysis was also carried out on the data matrix using the standard discrete morphology model (Lewis, 2001) in MrBayes v. 3.1 (Ronquist et al., 2005) using 100,000 MCMC replications following a burn-in of 40,000 replicates. Posterior

Table 2.1. Character state matrix of putative species of Char (1983) plus outgroup (*S. coccinea*).

Putative species	Morphological characters																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>S. coccinea</i> (outgroup)	2	0	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0
“ <i>arborea</i> ”	0	0	1	1	1	0	0	0	0	1	0	0	0	0	1	1	1	0
“ <i>molokaiensis</i> ”	3	0	2	1	1	2	2	0	1	1	0	0	1	0	2	1	1	1
“ <i>manaensis</i> ”	1	0	2	1	1	1	1	1	1	0	0	1	0	0	1	1	1	0
“ <i>polihalensis</i> ”	1	0	1	0	0	1	1	0	1	0	0	1	0	0	1	0	0	0
“ <i>oricola</i> ”	1	0	2	0	0	2	2	0	1	1	0	0	1	0	1	1	1	0
“ <i>tomentosa</i> var. <i>tomentosa</i> ”	3	0	1	0	1	0	1	0	1	1	0	1	1	0	1	1	1	0
“ <i>kauensis</i> var. <i>kauensis</i> ”	2	1	1	0	1	0	1	1	0	1	0	0	1	0	3	1	1	0
“ <i>kauensis</i> var. <i>intermedia</i> ”	2	1	0	1	1	0	1	1	0	0	1	2	1	1	3	1	1	0

Table 2.2. Characters and coding key used for phylogenetic analysis of Hawaiian *Sesbania*.

Character	Character #	Code
HABIT:	1	0 = small tree (1–3 m); 1 = erect shrub; 2 = erect shrub with trailing lower branches; 3 = procumbent shrub
LEAVES:		
Mean leaf length:	2	0 = 10.0–13.0 cm; 1 = 13.0–17.0 cm
Mean number leaflet pairs/leaf:	3	0 = 17–15; 1 = 14–12; 2 = 11–9
Mean leaflet length:	4	0 = 23–30mm; 1 = 16–23mm
Mean leaflet width:	5	0 = 9–11mm; 1 = 6–9mm
Indument on upper leaf surface:	6	0 = entirely glabrate; 1 = partially tomentose; 2 = densely tomentose
Indument on lower leaf surface:	7	0 = sparsely tomentose; 1 = moderately tomentose; 2 = densely tomentose
Pigmentation:	8	0 = obscured / not readily recognizable; 1 = dark, prominent
INFLORESCENCE:		
Color	9	0 = gradations of yellow-orange-red; 1 = strictly red
Mean Flower length:	10	0 = 3–4cm; 1 = 2–3 cm
Number of flowers/raceme:	11	0 = 1–6 flowers/raceme; 1 = 7–9 flowers/raceme
Mean peduncle length:	12	0 = 1–3cm; 1 = 3–5cm; 2 = 5–8cm
Mean pedicel length	13	0 = 0–1.5cm; 1 = 1.5–3.0cm
Calyx lobe length:	14	0 = less than 1/2 as long as corolla; 1 = 1/2–2/3 as long as corolla
Appendages on standard petal:	15	0 = 0.5–1.5mm; 1 = 1.5–2.5mm; 2 = 2.5–3.0mm 3 = absent
PODS:		
Length of beak:	16	0 = long beak (2–3 cm) 1 = short beak (0.5–2 cm)
Surface:	17	0 = tomentose; 1 = glabrous
Seed length:	18	0 = $\geq$ 5mm; 1 = < 5mm

probabilities were calculated by Mr. Bayes as a means to test branch support. Both phylogenetic trees were visualized using Fig Tree v. 1.3.1.

## DNA sample collection

Leaf samples of 459 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 16 populations from seven islands were sampled (Table 2.3). An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. I recorded GPS coordinates for the locations of all samples each individual plant sample collected. Samples at 'Āpua point, Kawela–Kamiloloa, Pu'u Koa'e and Nihoa comprise a subset of their respective populations (individuals collected arbitrarily from throughout each population. At Pu'u Koa'e and Nihoa samples were obtained by surrogate collectors [Ken Wood, National Tropical Botanical Garden (NTBG) and Beth Flint (USFWS)] and no GPS coordinates were logged. An attempt to distinguish groups of naturally occurring vs. out-planted individuals at Ka'ena point was made with the assistance of Betsy Gagné [Hawai'i Division of Forestry and Wildlife (DOFAW)]. Except where noted above, only naturally occurring plants and all known individuals known extant at the time of collection were sampled for analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4° to 8° C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNeasy tissue kits (QIAGEN; Valencia, CA) according to the manufacturer's specifications and the purified sample, along with negative and positive controls, were visually checked using electrophoresis.

Additional sampling of historically-collected tissue from the Mo'omomi dunes population on Moloka'i was conducted with loaned specimens from the herbarium of the New York Botanical Garden (NY), the B. P. Bishop Museum Herbarium (BISH) and the U. S. National Herbarium (US) (Table 2.4). DNA was extracted from 10 specimens using QIAGEN's QiaAmp Stool minikits, modified CTAB protocols (Drábková et al., 2002) and a PTB (N-phenacylthiazolium bromide) protocol (Asif and Cannon, 2005). For each of the 10 specimens at least one of the extraction protocols listed proved successful. These historically-collected

Table 2.3. Origin of DNA samples analyzed of *Sesbania tomentosa*, using the putative species designations for populations of Char (1983). Duplicate genotypes in cases where plants had occurred less than 10 m apart were removed prior to running the various analyses (and are not listed here). Unique genotypes obtained from cultivated individuals were added into the Kīpuka Nēnē–Hilina pali, Mānā, Papanalaho–Nākālele, Polihale, Pu‘u Pīmoe, Waiaka‘īlio population datasets. Unique genotypes obtained from herbarium specimens augment the Kāohikaipu & Mōkapu and Mo‘omomi population datasets.

Putative species designation	Island	Population	# individuals analyzed
“ <i>tomentosa</i> var. <i>tomentosa</i> ”	Hawai‘i	‘Āpua point	50
	Hawai‘i	Kamilo point–Ka Lae	67
	Kaho‘olawe	Pu‘u Koa‘e	25
	Maui	Papanalaho–Nākālele	46
			Total = 188
“ <i>kauensis</i> var. <i>kauensis</i> ”	Hawai‘i	Pepeiau–Kukalau‘ula pali	19
	Hawai‘i	Kamo‘oali‘i–Kū‘ē‘ē	18
			Total = 37
“ <i>kauensis</i> var. <i>intermedia</i> ”	Hawai‘i	Kīpuka Nēnē–Hilina pali	33
“ <i>arborea</i> ”	Moloka‘i	Kawela–Kamiloloa	35
	Maui	Pu‘u Pīmoe	12
	Hawai‘i	Waiaka‘īlio	14
			Total = 61
“ <i>molokaiensis</i> ”	Moloka‘i	Mo‘omomi	36
“ <i>oricola</i> ”	O‘ahu	Kāohikaipu & Mōkapu	5
	O‘ahu	Ka‘ena point	17
			Total = 20
“ <i>polihalensis</i> ”	Kaua‘i	Polihale	38
	Nihoa	Nihoa	49
			Total = 87
“ <i>manaensis</i> ”	Kaua‘i	Mānā	5

Total Overall = 469

Table 2.4. DNA collected from herbarium sheets (one sample per sheet) loaned from B. P. Bishop Museum Herbarium (BISH), New York Botanical Garden (NY) and the U. S. National Herbarium (US).

Barcode/ID #	Collector	Date	Location notes from herbarium sheet
990804 (NY)	J.F.C. Rock	3-1909	Molokai. Moomomi.
990808 (NY)	J.F.C. Rock	3-1910	Molokai. Moomomi.
990809 (NY)	C.N. Forbes	3-24-1915	Molokai. Moomomi.
55944 (BISH)	G.C. Munro	7-22-1926	Moomomi sandhills.
990820 (NY)	O. Degener	4-19-1928	Kalani, Moomomi. creeping branches take root, single large plant in sand dunes several hundred feet above sea.
990817 (NY)	O. Degener	4-25-1928	Moomomi, Molokai arid sand dunes.
55933 (BISH)	M.C. Neal	4-1-1934	Mokapu Crater, Oahu, edge of cliff.
990810 (NY)	F.R. Fosberg	12-26-1936	Molokai. Moomomi prostrate shrub, base of sand dunes.
14052 (US)	F.R. Fosberg	6-13-1937	Oahu. Kaohikaipu.
990811 (NY)	C.S. Judd	9-16-1937	Molokai. Moomomi procumbent shrub, sand hills alt. 10m.
177376 (BISH)	H. St.John	1-3-1939	Moomomi, Kaluahoi on sand dunes.
488514 (BISH)	H. St.John	12-24-1948	Moomomi, Kaluahoi, trailing on sand dunes near shore.



samples were included in the analysis of microsatellite fragment sizes to supplement the allelic diversity of my 2006 collection of extant plants at Mo‘omomi.

The scant demographics of certain populations necessitated augmentation of the dataset in order to provide marginally larger sample sizes for comparison. DNA from a herbarium specimen collected in 1934 from “Ulupa‘u Crater” on the Mōkapu peninsula (O‘ahu) was extracted, which supplemented a DNA sample collected in 2008 from the Mōkapu peninsula at Nu‘upia Ponds. Another herbarium specimen collected in 1937 from the islet of Kāohikaipu (O‘ahu) was extracted to supplement total extant diversity represented by two Kāohikaipu-derived individuals in cultivation at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu). These five samples were combined into a single Windward O‘ahu population for this analysis. The unique genotypes of cultivated individuals (derived from their respective natural populations) were also used to augment the Kīpuka Nēnē–Hilina pali (Hawai‘i Island), Pu‘u Pīmoe and Papanalahoa (Maui) and Polihale and Mānā (Kaua‘i) populations. All five individuals comprising the Mānā population are cultivated specimens of the National Tropical Botanical Garden (F<sub>1</sub> and F<sub>2</sub> generation derived from a single wild plant, now extirpated). The Polihale population is composed of groups of unique genotypes collected over 3 sampling years (2006–2010), in addition to several unique cultivated genotypes. For the Waiaka‘īlio population, extant in only a single surviving individual at the time sampling was undertaken, DNA was successfully extracted with the PTB protocol of Asif and Cannon (2005) using the woody core of eight plants that had been standing dead for approximately one year. In addition, the seedbank surrounding the standing dead plants was examined, producing an additional ten *Sesbania tomentosa* plants for genotyping.

Within each population sampled, duplicate genotypes derived from plants occurring less than 10 m from one another were identified and were omitted from all subsequent analyses. I hypothesize that these are either branches of the same plant that over time separated from one another or else artifacts of extreme genetic sub-structuring within certain populations, and the full dataset was analyzed in detail in the population genetic analysis of Chapter 2. The exceptions were the Windward O‘ahu and Mānā (Kaua‘i) populations, where duplicate genotypes (progeny of the same parent plants) were maintained in the dataset to support slightly larger sample sizes in these remnant groups of plants.

In addition, a sample of *Sesbania marchionica* F. Br. from Marquesas (in cultivation at the McBryde Garden of the National Tropical Botanical Garden) was collected to provide DNA for inclusion in the molecular phylogeny. This species (listed as a variety of *S. coccinea* before Lorence resurrected the taxon *S. marchionica*) is purported to have a close relationship with Hawaiian *Sesbania* (Fosberg, 1948; Sachet, 1987). Four additional taxa were used for outgroup comparison at the two nuclear regions, selected from Genbank submissions based on the phylogenetic analysis of Farruggia (2009) [the American taxa *S. herbacea* (Mill.) McVaugh and *S. vesicaria* (Jacq.) Elliott] and the presumed origin of Hawaiian *Sesbania* determined by Fosberg (1948) [the Austral taxon *S. formosa* (F.Muell) N.T. Burb. and the Indo Pacific taxon *S. grandiflora* (L.) Pers.]. Genbank accession numbers are as follows (ITS and TRPT accessions, respectively): JX453682 and KC254800 (*S. herbacea*), AF398761 and EU258899 (*S. vesicaria*), JX453678 and HQ730391 (*S. formosa*), AF536354 and HQ730392 (*S. grandiflora*).

### Phylogenetic Analysis

Two individuals from one or two populations of eight putative taxa of Hawaiian *Sesbania* plus one individual of *S. marchionica* (23 samples total; Table 2.5) were chosen to be amplified and sequenced at the two nuclear regions using primers described in the literature (ITS: White et al., 1990, TRPT: Choi et al., 2006). ITS amplifications were carried out in 25.0  $\mu$ L reaction volumes with final concentrations of: 0.5  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM  $MgCl_2$ , 0.8 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 unit *Taq* polymerase (Promega); 20–30 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, ending with a final extension of 72°C for 7 min. PCR products were electrophoresed on 1% agarose to verify amplified product, cleaned with ExoSAP (USB Corp., Cleveland, Ohio, USA) following manufacturer specifications and then bi-directionally sequenced on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA) at the Center for Genomic, Proteomic and Bioinformatic Research (CGPBR) facility at UH Mānoa.

Table 2.5. Twenty-two DNA samples sequenced from *Sesbania tomentosa* populations in the Hawaiian Islands, using the putative species designations for populations of Char (1983), plus *S. marchionica*. Voucher representations of populations sampled stored at B. P. Bishop Museum Herbarium (BISH), Joseph F. Rock Herbarium (HAW), Hawai‘i Volcanoes National Park Herbarium (HVNP) and National Tropical Botanical Garden Herbarium (PTBG).

Putative species designation	Island	Population	Voucher representations of populations sampled
<i>“tomentosa var. tomentosa”</i>	Hawai‘i Hawai‘i Maui Maui	‘Āpua point Ka Lae Nākālele point Papanalaho	Herat & Higashino 884 (BISH) Herbst 938 (BISH) Hobdy 809 (BISH) Oppenheimer 109902 (BISH)
<i>“kauensis var. kauensis”</i>	Hawai‘i Hawai‘i	Pepeiau Kū‘ē‘ē	Banko 1 (HVNP) Char 74 (BISH)
<i>“kauensis var. intermedia”</i>	Hawai‘i Hawai‘i	Kīpuka Nēnē Hilina pali	Char 71 (BISH) Reeser June 1975 (HAW)
<i>“arborea”</i>	Maui Maui Moloka‘i Moloka‘i	Pu‘u Pīmoe Pu‘u Pīmoe Kawela Kamiloloa	Davis 52 (BISH)  Pekelo 27 (BISH) Degener, Degener & Pekelo 32430 (NY)
<i>“molokaiensis”</i>	Moloka‘i Moloka‘i	Mo‘omomi Mo‘omomi	Degener 17954 (NY)
<i>“oricola”</i>	O‘ahu O‘ahu	Ka‘ena point Ka‘ena point	Char 83015 (BISH)
<i>“polihalensis”</i>	Kaua‘i Kaua‘i Nihoa Nihoa	Polihale Polihale Nihoa Nihoa	Char 76023 (BISH)  Yen 1016 (BISH)
<i>“manaensis”</i>	Kaua‘i Kaua‘i	Mānā Mānā	Char 76001 (BISH)
<i>S. marchionica</i> F. Br.	Ua Huka	Te kohai	Wood 10556 (PTBG)

The resultant 23 sequences for each of the two regions were edited using CHROMAS LITE v. 2.11 (Technelysium Pty Ltd., 2012) and aligned (with the addition of the four outgroup taxa) using MEGA v. 6.0 (Tamura et al., 2007). Maximum likelihood (ML) heuristic search algorithm was used in PAUP v. 4.0 (Swofford, 2002) to infer phylogenetic hypotheses. In this analysis *S. marchionica* was included in the Hawaiian *Sesbania* ingroup, while the four GenBank accessions were placed in the outgroup. Branch support was estimated using 1,000 bootstrap replicates.

Bayesian analysis was carried out using the GTR model in MrBayes v. 3.1 using 10 million MCMC replications following a burn-in of 2 million replicates. Posterior probabilities were calculated by Mr.Bayes and were used to construct the phylogenetic tree. The American species *S. herbacea* was used as the sole outgroup species in this analysis, as Faruggia (2009) placed it with Hawaiian *Sesbania* in a well-supported clade. Both phylogenetic trees were visualized using Fig Tree v. 1.3.1.

#### Microsatellite analysis of population structure

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* under contract with the United States Geological Survey (USGS). Ninety-six dinucleotide ( $CA_n$ ) and tetranucleotide ( $CATC_n$ ,  $TACA_n$ , and  $TAGA_n$ ) microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 2.6) based on their range of polymorphism and ease of scoring in a screening of eight DNA samples, one from each of the putative taxa of Char (1983). Each sample was amplified in a 25.0  $\mu$ L volume with final concentrations of 0.6  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM  $MgCl_2$ , 0.8 mM dNTPs (Promega), 1 unit *Taq* polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler with the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 2.6) for 40 s, and 72°C for 30 s, ending with a final extension of 72°C for 4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were

Table 2.6. Nine microsatellite primer pairs developed for *Sesbania tomentosa*.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 469 individuals sampled for this study. Range, allele size range in base pairs (bp). Prefixes in italics before forward primer sequence indicate dye used for poolplexing.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG R: TAG-GTG-TGG-CGT-GCA-TAA-C	57.3 58.1	10	205–223
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A R: CCC-TTC-CCC-TCC-TCT-TAG	56.0 56.2	9	264–280
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	55.8 56.3	14	198–236
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC R: TAG-CCA-TGC-TTC-ATC-AAT-CG	58.9 59.8	21	288–328
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.8 56.9	13	163–187
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG R: GGC-AAC-ATT-TGA-GTG-GAG-G	58.6 59.1	16	196–276
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1 55.1	14	180–236
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.7 55.2	11	212–252
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.1 54.8	14	265–321

included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on the ABI Prism 377XL sequencer at the CGPBR facility at UH Mānoa. The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (ABI). Stutter peaks were identified, and then the program MICROCHECKER (Van Oosterhout et al., 2004) was used to identify possible genotyping errors due to non-amplified (null) alleles and short allele dominance (large allele dropout). A maximum likelihood estimate of the frequency of null alleles (Expectation Maximization algorithm of Dempster et al., 1977) was then calculated for each locus and geographic population using the program FREENA (Chapuis and Estoup 2007). The microsatellite dataset was analyzed to assess linkage (genotypic) disequilibrium in GENEPOP v. 4.0 (updated from Raymond and Rousset, 1995) using log-likelihood ratio statistics (*G*-tests).

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was calculated using GENALEX v. 6.4 (Peakall and Smouse, 2006) at three hierarchical levels: within populations, among populations, and among the putative specific designations of Char (1983). This test partitions total genetic variance and calculates  $\Phi_{PT}$ , an analogue of  $F_{ST}$ . Significance was tested against a null distribution of 10,000 random permutations. Private alleles (alleles exclusive to a given population) were also calculated in GENALEX v. 6.4.

Population structure was examined using a full Bayesian-clustering approach, implemented in the program STRUCTURE v. 2.3.3 (Pritchard et al., 2000), which assigned individual genotypes to populations, irrespective of geographical location of origin. Default settings of the program were used (admixture model, independence among loci) using the putative specific designations of Char (1983) as prior information for the model to consider (Hubisz et al., 2009). To determine the most likely number of populations or groups (*K*) in the data, a series of analyses were performed from *K* = 1 (all populations represent a single panmictic unit) to 15 (the maximum number of populations allowable) using 40,000 burn-in and 100,000 repetitions, with ten iterations per *K*. These results were examined using the  $\Delta K$  method (Evanno et al., 2005) to identify the most likely number of groups in the data. Ten additional

iterations at the identified  $K$  were computed using 100,000 burn-in and 300,000 repetitions. The program CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize these last ten iterations. Cluster membership coefficients for each individual and pre-defined population were obtained (permuted across replicates using *FullSearch* algorithm) and used as input files for the cluster visualization program DISTRUCT v. 1.1 (Rosenberg, 2004).

Each individual was assigned to a particular genetic cluster when its coefficient of membership was greater than 50%. Geographic populations were assigned to a particular genetic cluster when 72–100% of their individuals were assigned to that genetic cluster. The initial analysis was repeated on each  $K$  separately to detect sub-structuring in the two genetic groups; no information about specific designation was used as *a priori* in this subsequent analysis. The number of genetic sub-clusters was estimated for each group using the  $\Delta K$  method, ten additional iterations were performed at the appropriate  $K$  (100,000 burn-in and 300,000 repetitions) and both the *FullSearch* and *Greedy* (10,000 random input orders of runs) algorithms were used in CLUMPP. Individuals were then assigned to genetic sub-clusters when their coefficient of membership was greater than 0.5; geographic populations assigned to sub-clusters based on 58–100% individual assignment.

The extent and significance of the genetic differentiation among geographic populations was investigated with MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötter, 2003) by calculating global and pairwise  $F_{ST}$  values (averaged over multiple loci) among the geographic populations. Global and pairwise  $F_{ST}$  values were also obtained for the eight synonyms of *S. tomentosa* by combining distinct geographic populations into the taxa they were purported to represent. The significance of  $F_{ST}$  values was tested with 10,000 permutations using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ). FREENA (Chapuis and Estoup 2007) was also used to estimate pairwise  $F_{ST}$  values ( $F_{ST(ENA)}$ ) from genotype frequencies corrected for the presence of null alleles [using the excluding null alleles (ENA) method of Chapuis and Estoup 2007] that tend to positively bias  $F_{ST}$  estimates. Most of the non-visible genotypes in the dataset were assumed to be due to technical problems (e.g., degraded or low quantity of DNA or PCR amplification inconsistencies) and were specified in the FREENA dataset. These were distinguished from the null homozygous genotypes at locus A122 in 16 out of 17 individuals of the Ka'ena point population, probably due to a mutated flanking sequence that prevented that particular locus from amplifying.

A principal coordinates analysis (PCA) was used to examine the extent of genetic clustering of populations (and putative taxa) throughout Hawai‘i using co-dominant genotypic distances ( $\Phi_{PT}$ ) between individuals (Smouse and Peakall, 1999) and the chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967) between populations in GENALEX v. 6.4 (Peakall and Smouse, 2006). Lastly, a neighbor-joining (NJ) tree was constructed using the chord distance ( $D_C$ ) with 1,000 bootstrap replications in POPULATIONS v. 1.2.31 (Langella, 2000) and graphically displayed with TREEVIEW (Page, 1996). The chord distance of Cavalli-Sforza and Edwards (1967) was chosen in both cases because the null allele bias for this genetic distance is low (Chapuis and Estoup, 2007), and because it is the most efficient distance for obtaining a correct tree topology using microsatellite data (Takezaki and Nei, 1996).

## Results

### Phylogenetic analysis of morphological character dataset

Maximum parsimony analysis based on the morphological character dataset evaluated 135,135 trees retaining one. Two of Char’s (1983) taxa from Kaua‘i, “*polihalensis*” and “*manaensis*”, were identified as the basal-branching sisters to a clade containing the remainder of Hawaiian *Sesbania* (Figure 2.1). As a means for comparison, Bayesian analysis revealed a topology similar to that of the maximum parsimony analysis except for the inclusion of “*manaensis*” in the clade with the remaining putative taxa of Hawaiian *Sesbania*. Other than this discrepancy, posterior probabilities suggested varying levels of confidence (mostly below 50%; exceptions labeled on tree) in the same relationships proposed in parsimony analysis (Figure 2.2). Two sub-clades emerged, one joining the putative taxa “*kauensis* var. *kauensis*” with “*kauensis* var. *intermedia*”, both from the Ka‘ū district of Hawai‘i Island, and the other joining “*oricola*” from O‘ahu with “*molokaiensis*” from northwest Moloka‘i (Figures 2.1 and 2.2).

### Phylogenetic analysis of molecular datasets

Approximately 1035 base pairs (bp) were sequenced (720 bp of ITS and 315 bp of TRPT) of 22 samples of *Sesbania tomentosa* from 16 populations on 7 Hawaiian Islands



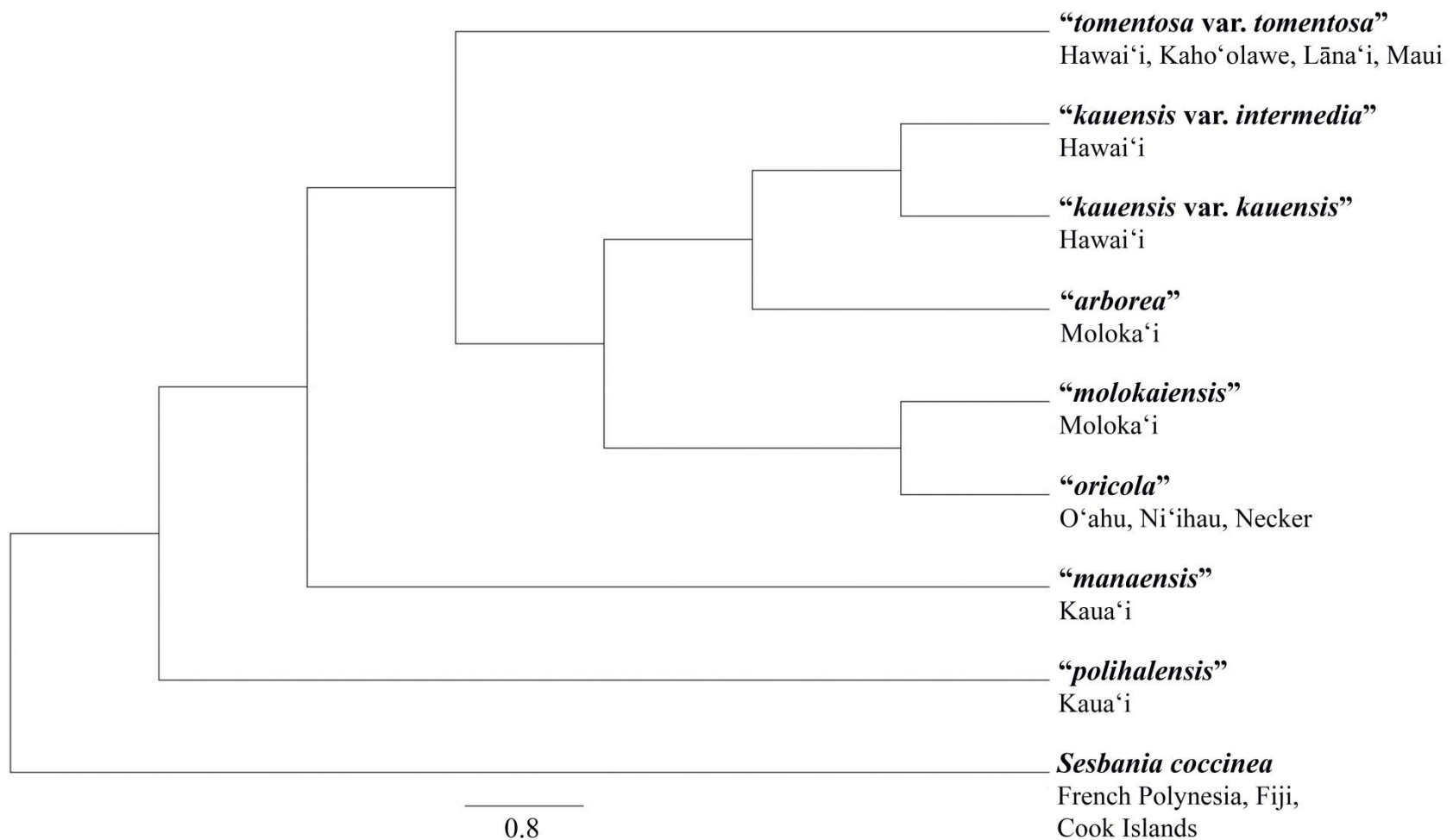


Figure 2.1. Exhaustive maximum parsimony phylogeny of Char's (1983) morphological character dataset of *Sesbania tomentosa* populations using *S. coccinea* as an outgroup. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

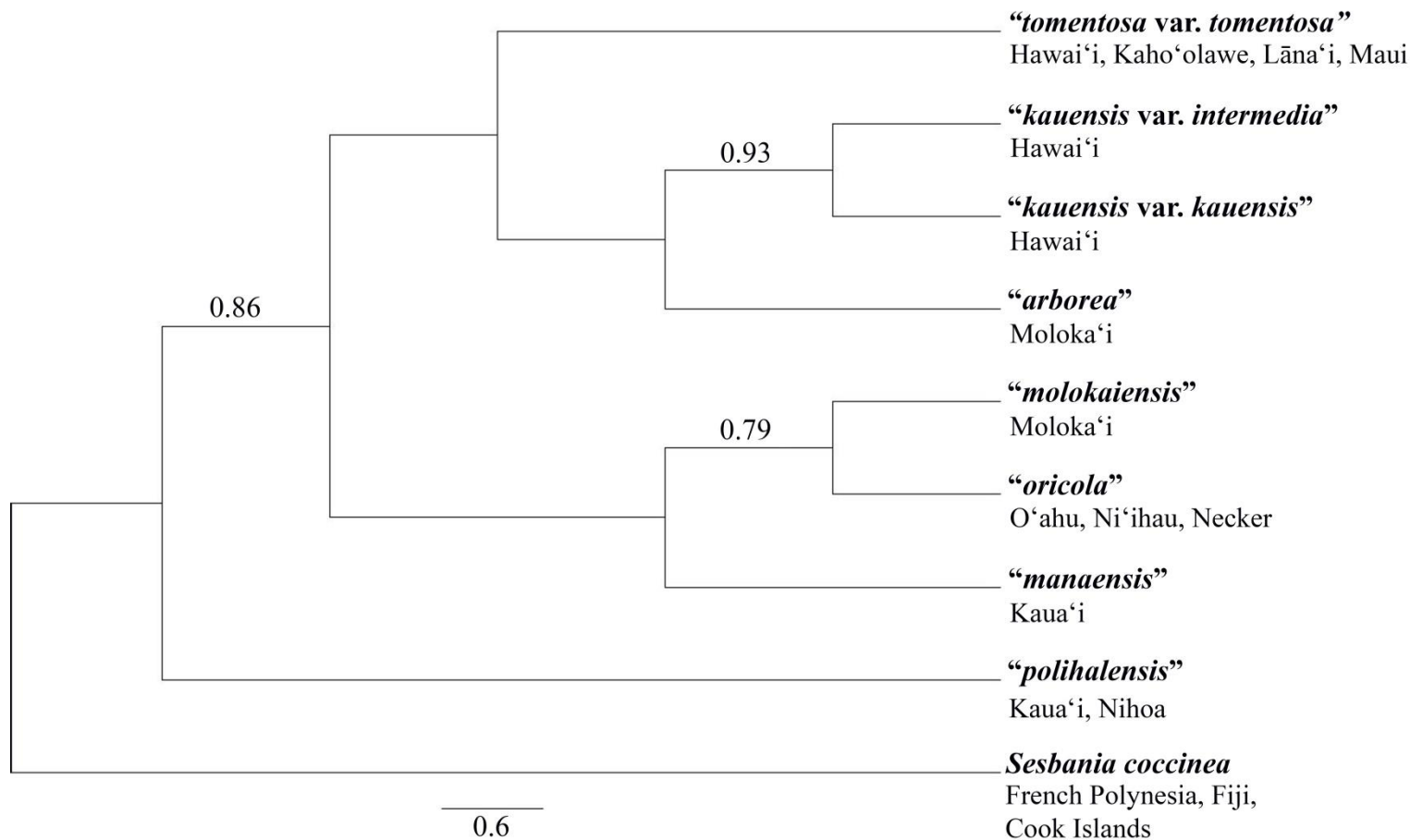


Figure 2.2. Bayesian analysis (standard discrete morphology model; Lewis, 2001) of Char's (1983) morphological character dataset of *Sesbania tomentosa* populations using *S. coccinea* as an outgroup. 100,000 MCMC replications were analyzed following a burn-in of 40,000 replicates. Posterior probabilities listed above branches where they offer greater than 50% support for nodes. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

plus *S. marchionica* (Marquesas). There was no sequence divergence whatsoever across the 22 Hawaiian samples sequenced for ITS. However, *S. marchionica* was divergent from the Hawaiian samples in 5 out of 720 bp at the ITS region. For TRPT, 6 out of 315 bp were divergent among the Hawaiian samples. Six samples sequenced from three populations on O‘ahu, Kaua‘i and Nihoa (designated “*oricola*”, “*polihalensis*” and “*manaensis*” by Char, 1983) were the only ones to diverge at these positions. Two Nihoa samples (designated “*polihalensis*” by Char, 1983) shared four of the same six base pair substitutions. Divergence was represented by within-individual polymorphic states (sequences showing equal peaks for two nucleotides) becoming non-polymorphic (a single peak). Polymorphic states were coded as ambiguities (with standard IUPAC coding) and were not considered to be phylogenetically informative. *Sesbania marchionica* was divergent at two of the same 6 positions as the Hawaiian samples at the TRPT region.

Both the maximum likelihood and Bayesian phylogenies for each gene region analyzed separately were identical to their respective combined analyses (ITS plus TRPT) therefore only combined gene region phylogenies are presented. In both the combined likelihood and Bayesian analyses *S. marchionica* was sister to the Hawaiian *Sesbania* clade (Figures 2.3 and 2.4). In the maximum likelihood phylogeny, where four taxa were used as outgroup species, the American species *S. herbacea* appeared to be the closest relative (according to the scale) to the Hawaiian-Marquesan species (Figure 2.3). A similar result was observed in the Bayesian phylogeny, where *S. herbacea* was used as the sole outgroup species (Figure 2.4).

#### Microsatellite analysis of population structure

At the nine microsatellite loci examined, the number of alleles per locus averaged 13.5 (ranging 9–21), for a total of 122 alleles among the 459 samples. Each locus had two to four alleles with a frequency greater than 0.1, and these most-common alleles had average frequencies per locus that ranged from 0.17 to 0.28 (with a maximum across loci of 0.50). None of the 35 tests for multiple comparisons between loci (genotypic disequilibrium) in GENEPOP were significant at the 5% nominal level after Bonferroni

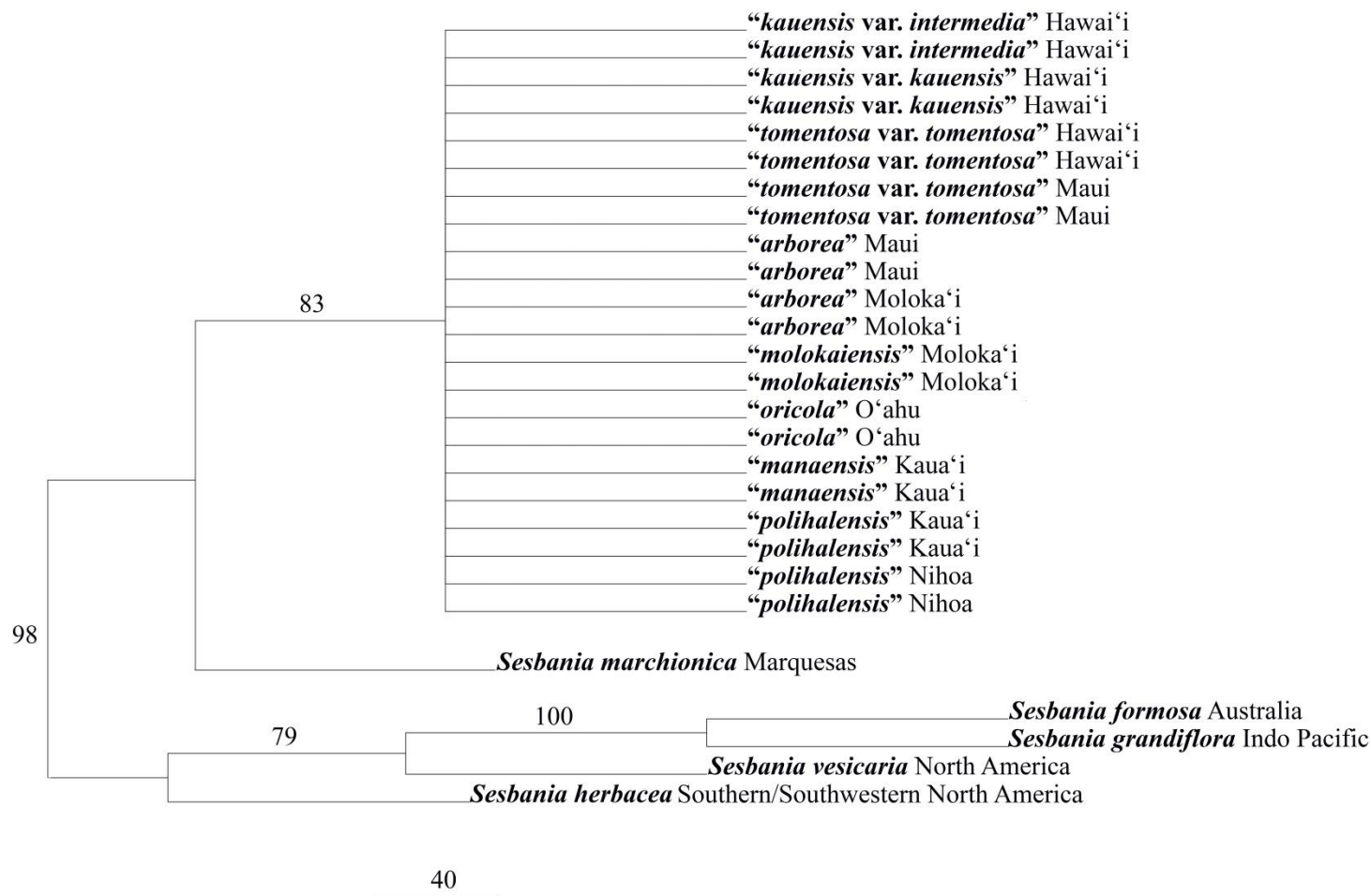


Figure 2.3. Maximum likelihood analysis of the combined ITS and TRPT datasets of *Sesbania tomentosa* and *S. marchionica* samples using *S. herbaceae*, *S. vesicaria*, *S. formosa* and *S. grandiflora* as the outgroup. Branch support was estimated using 1,000 bootstrap replicates. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

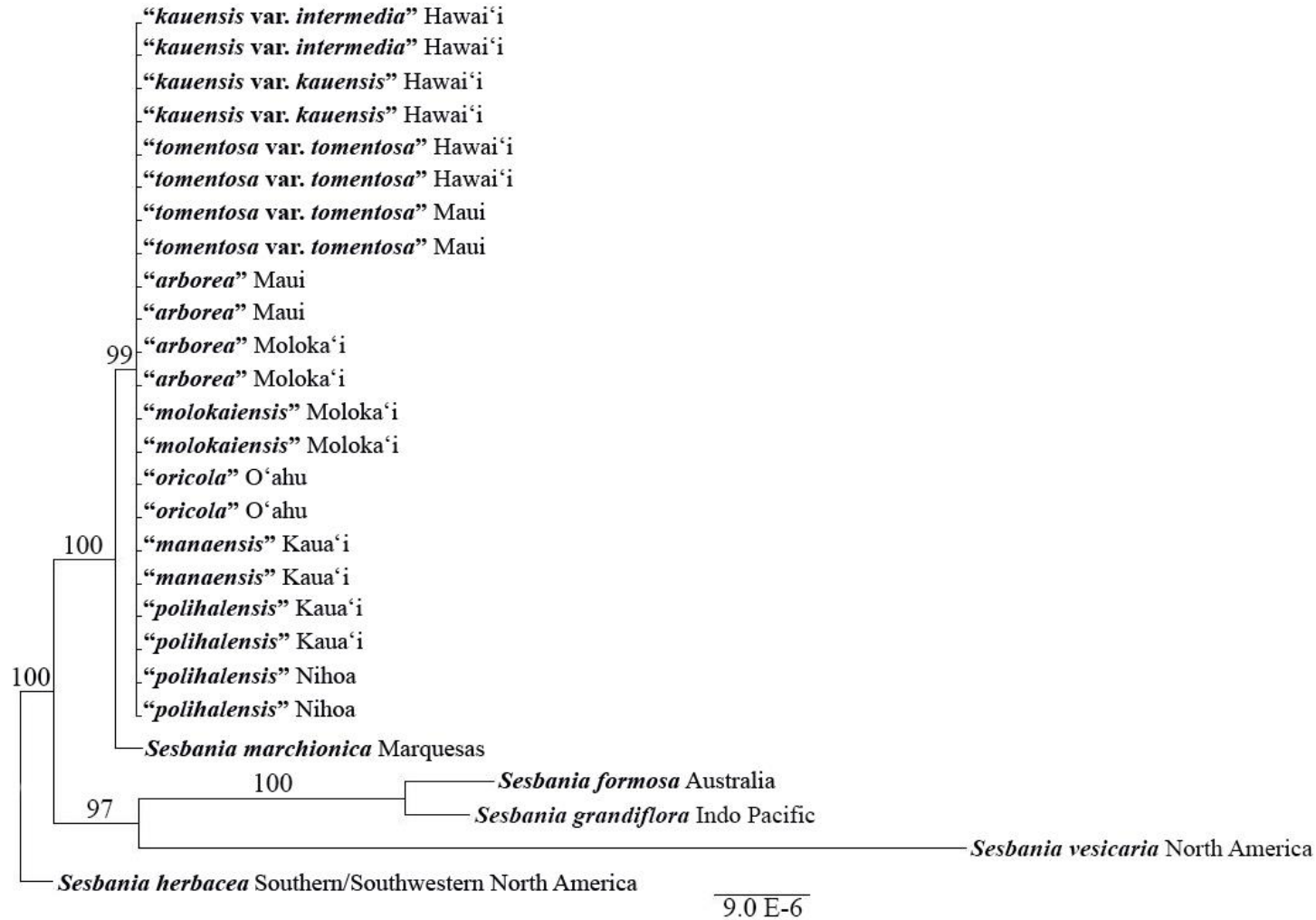


Figure 2.4. Bayesian analysis (GTR Model) of the combined ITS and TRPT datasets of *Sesbania tomentosa*, *S. marchionica*, *S. vesicaria*, *S. formosa* and *S. grandiflora* samples using *S. herbacea* as the outgroup. Posterior probabilities listed above branches. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

corrections when averaged over all populations. Thus, the different microsatellite loci can be considered to provide independent information on population structure.

MICROCHECKER indicated that there was a general excess of homozygotes evenly distributed across allele size classes at all 9 loci in an average of 11 out of 16 populations per locus, an indication of possible null alleles or false homozygotes in the data set (data not shown). Estimated frequencies of null alleles per locus in each population (using the ENA method implemented in FREENA) ranged from 0.000 to 0.404 (the exception being the Ka‘ena point populations that ranged from 0.980 to 1.000 at locus A122). When averaged over loci, the frequency of null alleles in the 16 populations varied from 0.040 to 0.340. The mean null allele frequency over all populations and loci was 0.149.

An analysis of molecular variance (AMOVA) revealed that the majority of genetic variation was found within Hawaiian *Sesbania* populations (56%) with 40% distributed among populations. Only 4% was found among the eight putative species (Char, 1983) tested (significant at the 1% nominal level, Table 2.7).

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Table 2.7. Results of AMOVA (Excoffier et al., 1992) at three hierarchical levels: among putative species (Char, 1983), among populations, and within populations of Hawaiian *Sesbania*. Significance was tested against a null distribution of 10,000 random permutations

Source of variation	d.f.	Sum of squares	Fixation index	% variation	P-value
Among putative species	7	1230.067	$\Phi_{RT} = 0.042$	4	0.000
Among populations	8	1309.236	$\Phi_{PR} = 0.418$	40	0.000
Within populations	453	3277.650	$\Phi_{PT} = 0.443$	56	0.000

Using the program STRUCTURE and following the method of Evanno et al. (2005), two distinct genetic clusters were found among *Sesbania tomentosa* individuals sampled across all islands (Figures 2.5 and 2.6). The largest increase in the posterior probability occurred at  $K = 2$ , suggesting that this was the best model for the data. One genetic cluster corresponded to the Hawai‘i Island samples (red cluster) and the other comprised individuals sampled from the remaining islands (orange cluster; Figure 2.7). Most of the geographic populations sampled showed a high proportion of individuals assigned to one cluster only, generally from 90% to 100%. Populations of “*arborea*” and “*molokaiensis*” sampled from Moloka‘i had proportions much lower (0.86 and 0.72 assigned to the orange cluster, respectively) levels of admixture much higher than the 5% threshold which might be attributed to stochastic noise. In addition, cluster membership coefficients of Maui Nui (referring to the prehistorically contiguous island composed of Kaho‘olawe, Maui, Moloka‘i, and Lāna‘i; Price and Elliott-Fisk, 2004) individuals assigned to the orange cluster also averaged low (0.68 for “*tomentosa* var. *tomentosa*” on Kaho‘olawe; 0.74 for “*arborea*” on Maui, 0.69 for “*arborea*” on Moloka‘i and 0.72 for “*molokaiensis*” on Moloka‘i).

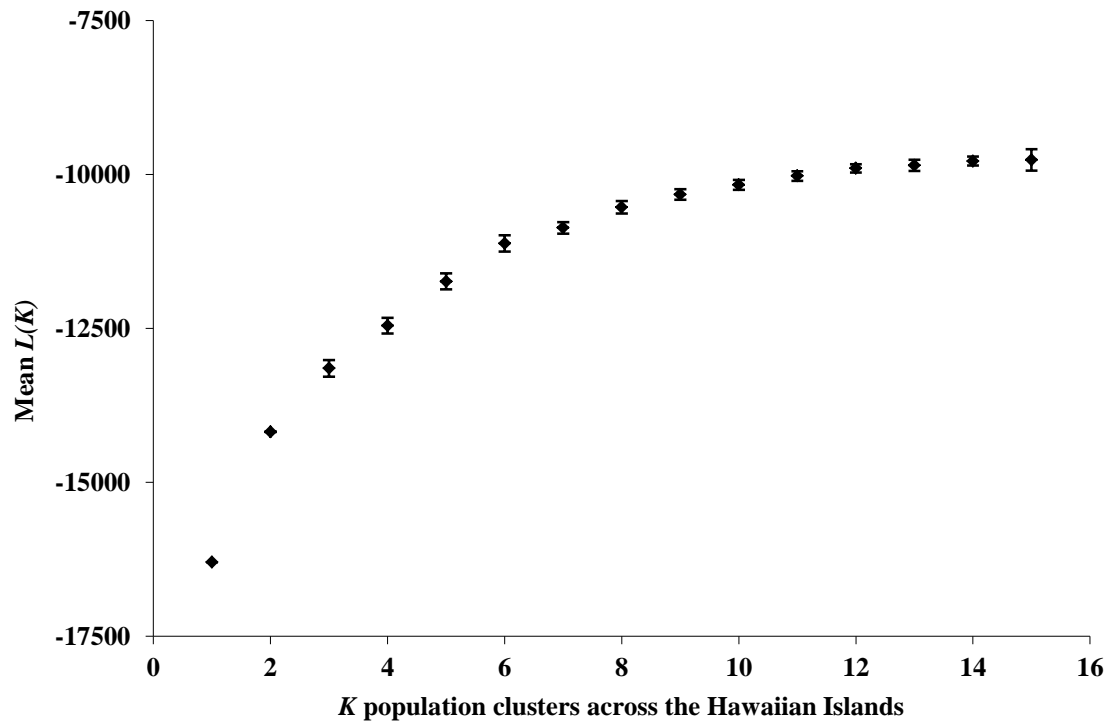


Figure 2.5. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

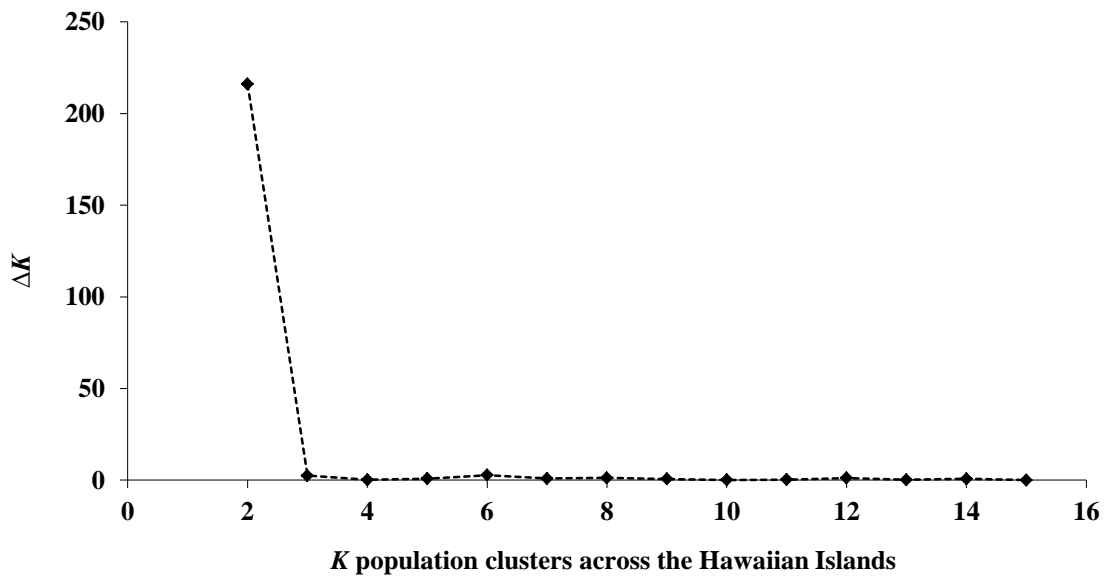


Figure 2.6. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).



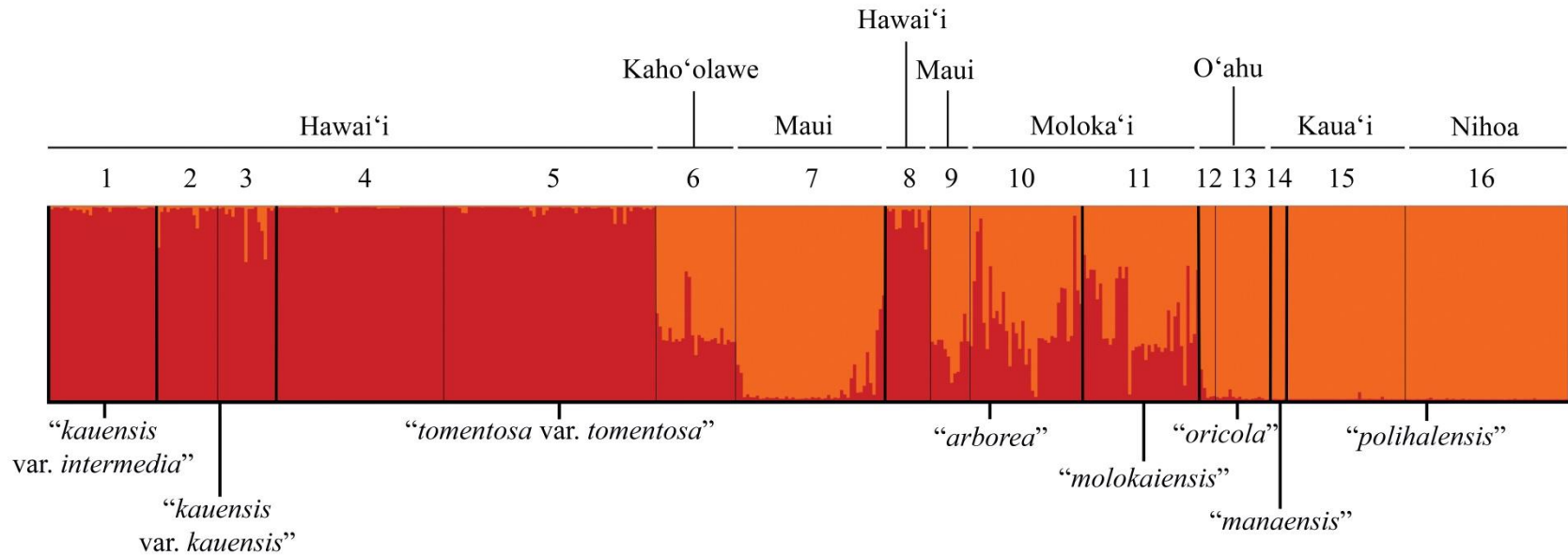


Figure 2.7. STRUCTURE graph for the most likely number of clusters of Hawaiian *Sesbania* according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic clusters (red and orange). Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 1. Kīpuka Nēnē–Hilina pali, 2. Pepeiau–Kukalau‘ula pali, 3. Kamo‘oali‘i–Kū‘ē‘ē, 4. ‘Āpua point, 5. Kamilo point–Ka Lae, 6. Pu‘u Koa‘e, 7. Papanalahoa–Nākālele, 8. Waiaka‘īlio, 9. Pu‘u Pīmoē, 10. Kawela–Kamiloloa, 11. Mo‘omomi, 12. Kāohikaipu & Mōkapu, 13. Ka‘ena point, 14. Mānā, 15. Polihale, 16. Nihoa. Island of origin for each population listed at top of figure.

When considering the population (or in this case, species) cluster membership coefficients, indications of admixture were even more prevalent (e.g., proportion of membership of “*tomentosa* var. *tomentosa*” in the red cluster was 0.70; proportion of membership of “*arborea*” in the orange cluster was 0.59; proportion of membership of “*molokaiensis*” in the orange cluster was 0.51; data not shown).

Additional analysis of the two genetic demes described above found  $K = 2$  within the red cluster of Figure 2.7 (Figures 2.8 and 2.9) and  $K = 4$  within the orange cluster of Figure 2.7 (Figures 2.10 and 2.11). Within the red cluster the first sub-cluster comprised the Hawai‘i Volcanoes National Park populations (orange) plus the small remnant population in North Kohala (Waiaka‘īlio) and the second sub-cluster (yellow) comprised the combined populations from the South point Region (Kamilo point–Ka Lae; Figure 2.12).

Two relatively distinct groups, comprising two genetic demes each, characterize the STRUCTURE plot in Figure 2.13 split between Maui Nui and the remaining Islands to the northwest. Populations from O‘ahu and Kaua‘i separate out into a distinct sub-cluster (pink) from the relatively large population on Nihoa, 250 km to the northwest of Kaua‘i (mauve). Secondly, levels of admixture were highest in the populations from Moloka‘i. For example, the combined (modern plus historical) Mo‘omomi population of “*molokaiensis*” was not definitively assigned to any one particular genetic group, the highest proportion of individuals (44%) being assigned to the red cluster, shared with “*tomentosa* var. *tomentosa*” from Kaho‘olawe and “*arborea*” from Maui. While the “*arborea*” population at Kawela–Kamiloloa was definitively assigned to the red cluster, the proportion of individuals assigned to that cluster was relatively low (0.80), and three individuals failed to be assigned to any cluster at the 0.50 cut-off. Cluster membership coefficients for the Moloka‘i individuals (with respect to their assigned cluster) averaged moderately low as well (0.70 for combined Mo‘omomi and 0.87 for Kawela–Kamiloloa), similar to “*arborea*” individuals on Maui (0.85) where another individual failed to be assigned to any cluster at the 0.50 cut-off. When considering the ten historically collected samples from Mo‘omomi separately, cluster membership coefficients averaged low at 0.72, and individual cluster assignments varied widely (indicating admixture).

Global  $F_{ST}(\theta)$  over all populations (averaged over loci) was 0.396 ( $P < 0.001$ ); correction for null alleles using the ENA method (Chapuis and Estoup, 2007) reduced this value slightly to 0.370 ( $P < 0.001$ , Table 2.8). On the other hand, global  $F_{ST}(\theta)$  over all putative species tested

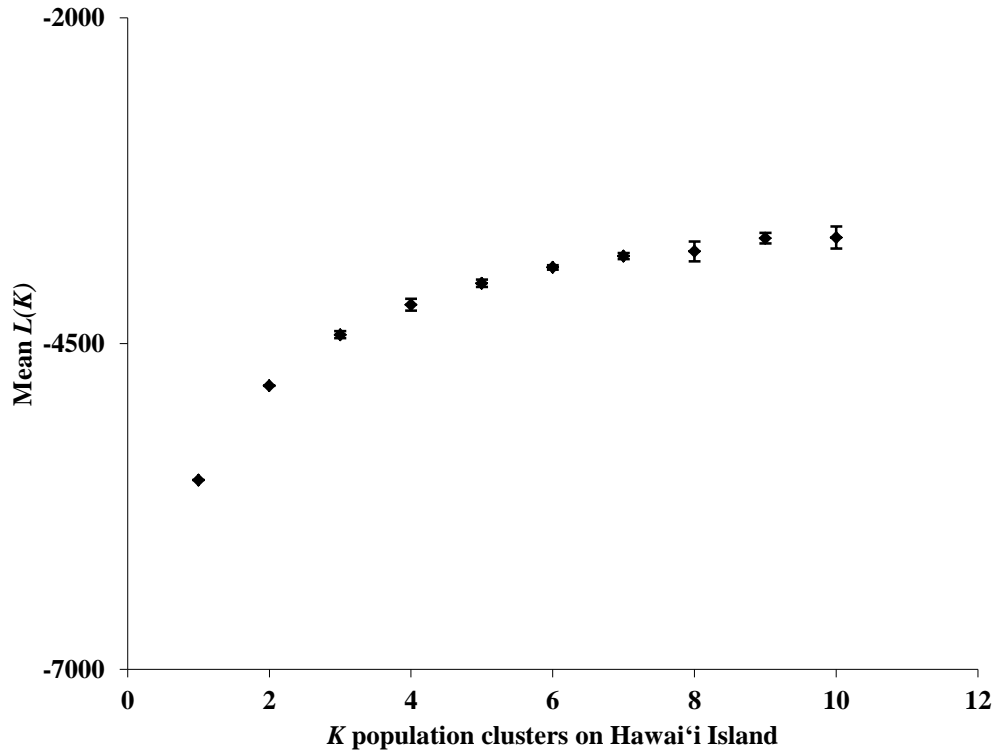


Figure 2.8. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

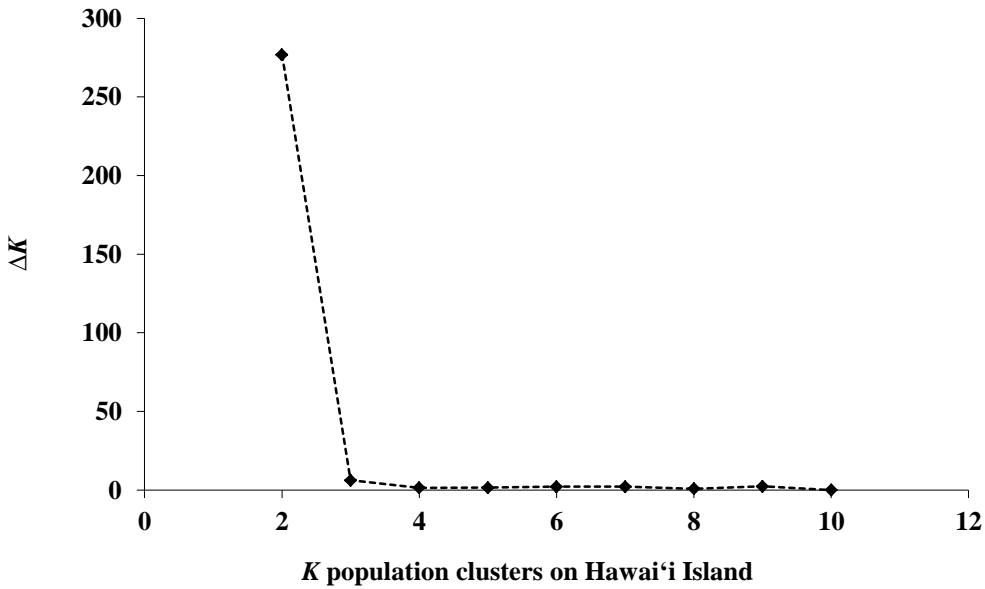


Figure 2.9. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

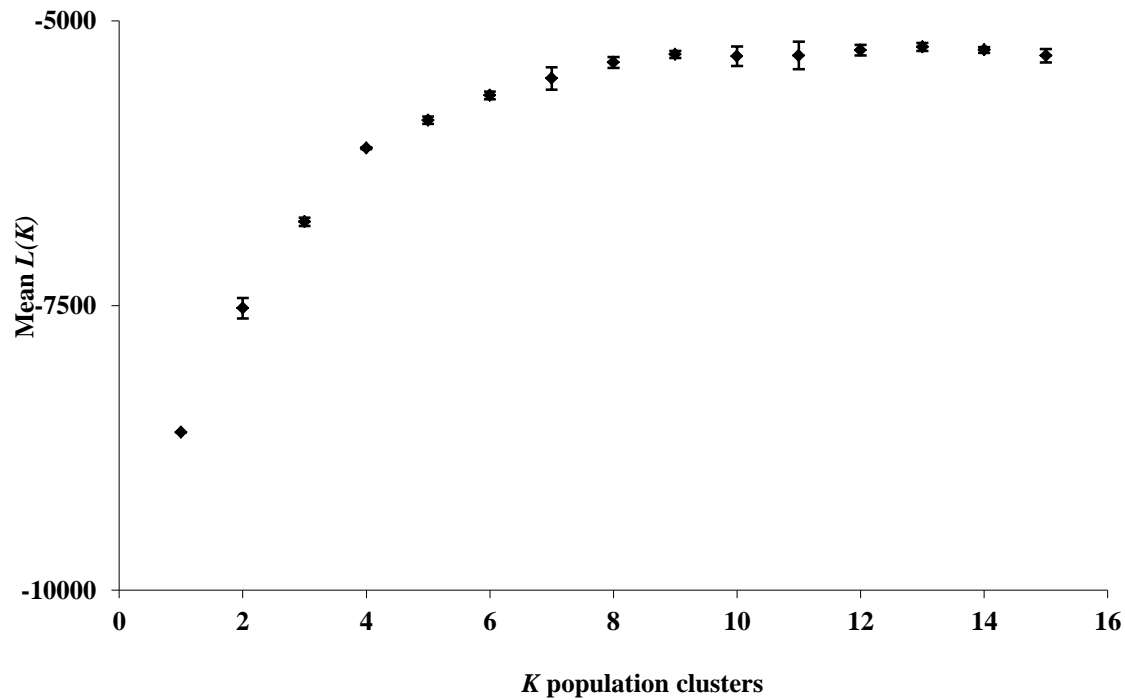


Figure 2.10. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

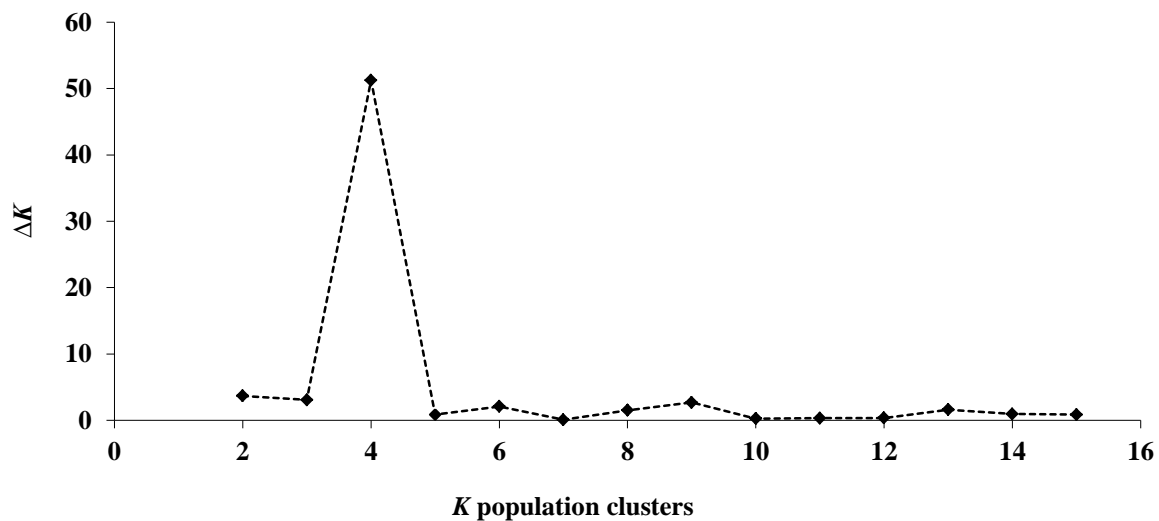


Figure 2.11. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

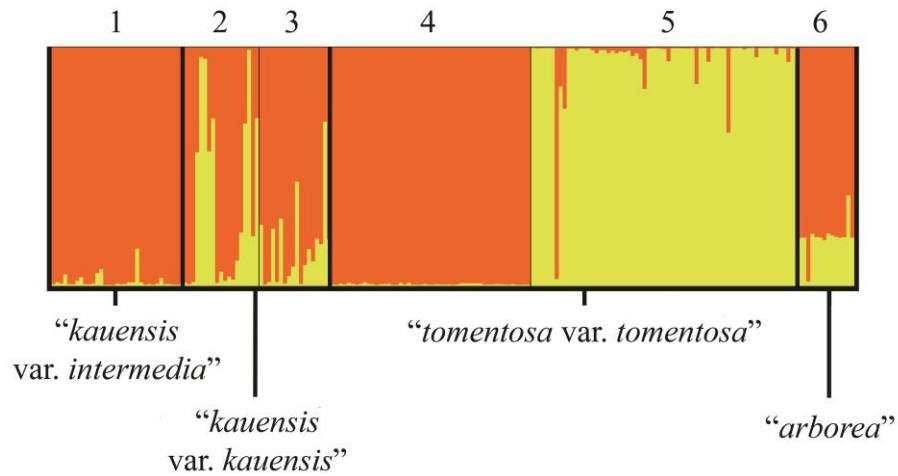


Figure 2.12. STRUCTURE graph for the most likely number of sub-clusters on Hawai‘i Island (red cluster of Figure 2.7) according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 1. Kīpuka Nēnē–Hilina pali, 2. Pepeiau–Kukalau‘ula pali, 3. Kamo‘oali‘i–Kū‘ē‘ē, 4. ‘Āpua point, 5. Kamilo point–Ka Lae, 6. Waiaka‘īlio.

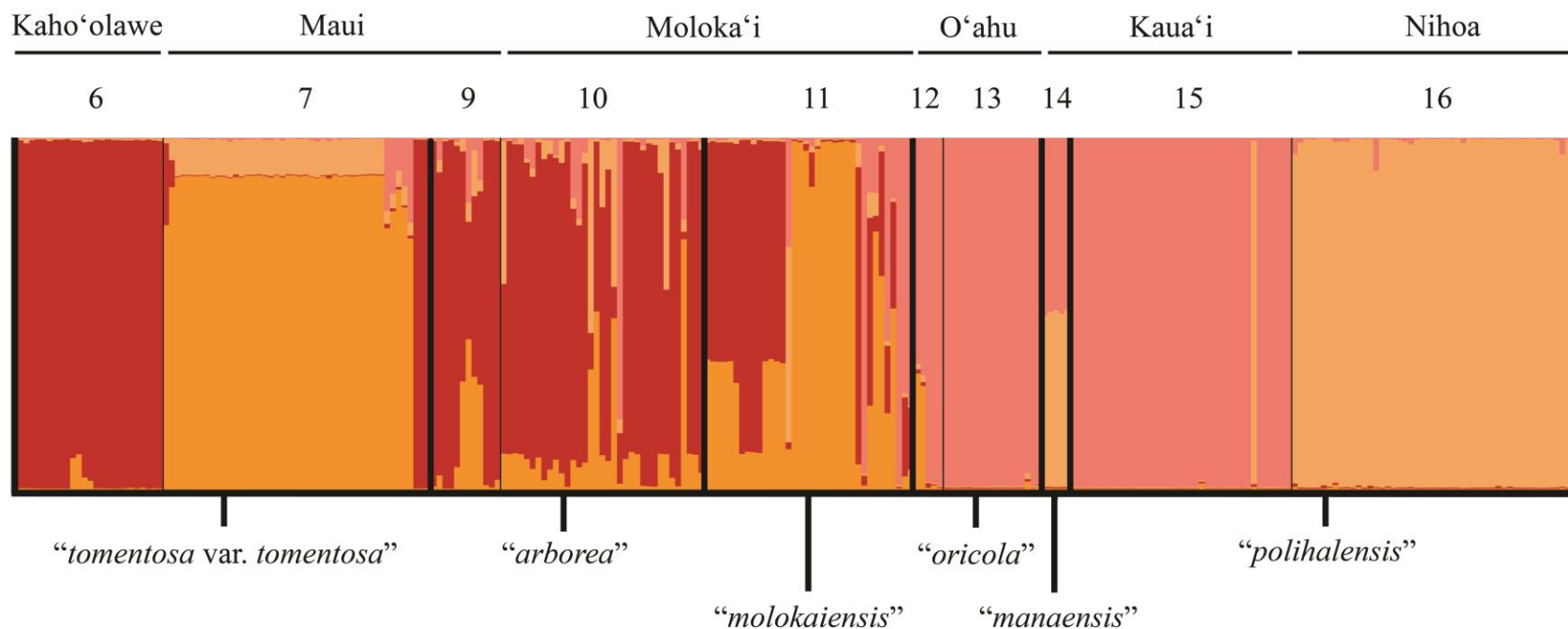


Figure 2.13. STRUCTURE graph for the most likely number of sub-clusters in the orange cluster of Figure 2.7 according to the  $\Delta K$  method ( $K = 4$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 6. Pu'u Koa'e, 7. Papanalaho-Nākālele, 9. Pu'u Pīmoe, 10. Kawela-Kamiloloa, 11. Mo'omomi, 12. Kāohikaipu & Mōkapu, 13. Ka'ena point, 14. Mānā, 15. Polihale, 16. Nihoa. Island of origin for each population listed at top of figure.

Table 2.8.  $F_{ST}$  ( $\theta$ ; Weir and Cockerham, 1984) per locus and global over all populations ( $F_{ST\ POP}$ ) and over all 8 putative species (Char, 1983) of Hawaiian *Sesbania* tested ( $F_{ST\ SPECIES}$ ).  $F_{ST}$  values corrected for the possible presence of null alleles using the ENA method (Chapuis and Estoup, 2007) included for comparison [ $F_{ST\ POP\ (ENA)}$  and  $F_{ST\ SPECIES\ (ENA)}$ , respectively]. Significant  $P$ -values ( $\alpha = 0.01$ ) listed in bottom row of table apply to all four analyses listed above.

	<b>Locus:</b>									
	<b>C5</b>	<b>A105</b>	<b>A123</b>	<b>C3</b>	<b>A122</b>	<b>A119</b>	<b>A128</b>	<b>C103</b>	<b>C106</b>	<b>Global</b>
$F_{ST\ POP}$	0.406	0.402	0.346	0.425	0.254	0.387	0.400	0.481	0.447	0.396
$F_{ST\ POP\ (ENA)}$	0.397	0.384	0.343	0.420	0.220	0.335	0.335	0.440	0.439	0.370
$F_{ST\ SPECIES}$	0.234	0.246	0.111	0.289	0.172	0.188	0.215	0.197	0.243	0.211
$F_{ST\ SPECIES\ (ENA)}$	0.224	0.230	0.110	0.486	0.140	0.134	0.150	0.156	0.233	0.207
<b><math>P</math>-value</b>	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

was 0.211 ( $P < 0.001$ ); correction for null alleles using the ENA method (Chapuis and Estoup, 2007) again reduced this value only slightly to 0.207 ( $P < 0.001$ , Table 2.8). This analysis indicates that of the total genetic variation found across the range of the species, 37–40% is ascribable to genetic difference (differences in allele frequencies) among geographic populations, while 21% of the total variation is ascribable to genetic differences among the putative species (when geographic populations are pooled together as species).

In addition, correction for null alleles only marginally decreased pairwise  $\theta$ -values, indicating that null alleles were not strongly biasing the analysis of genetic differentiation among populations (Tables 2.9, 2.10, 2.11 and 2.12). One hundred and eleven of the 120 pairwise comparisons were significant at the 1% nominal level and an additional 5 comparisons were significant at the 5% level after Bonferroni corrections (Tables 2.9 and 2.10). When distinct geographic populations were combined into the putative taxa of *Sesbania tomentosa*, all pairwise comparisons were significant at the 1% nominal level after Bonferroni corrections (Tables 2.11 and 12). Besides the two closely related “*kauensis*” varieties, “*tomentosa* var. *tomentosa*” and “*arborea*” appeared the least differentiated from all the other putative taxa, and from each other. On the other hand, the group of putative taxa from O‘ahu, Kaua‘i and Nihoa (“*oricola*”, “*manaensis*” and “*polihalensis*”, respectively) appeared the most differentiated from putative taxa on the remaining Hawaiian Islands (Tables 2.11 and 2.12).

Co-dominant genotypic distances ( $\Phi_{PT}$ ) were also used in a principal coordinates analysis (PCA) to examine the extent of genetic clustering of Hawaiian *Sesbania* populations (Figure 2.14) and individuals (Figure 2.15) throughout the state. The first two principal coordinates (PC) axes of Figure 2.14 explained 39.2 and 18.2% of the genetic variation among populations, respectively, for a total of 57.4%. A scattergram of these two axes showed strong geographical correlation, with populations from O‘ahu, Kaua‘i and Nihoa separated from all other populations (displaced along PC axis 1; Figure 2.14). While an apparent cohesion existed among “*arborea*” populations from three separate Islands (Moloka‘i, Maui and Hawai‘i Island), “*tomentosa* var. *tomentosa*” populations were displaced along PC axis 2 in a geographical pattern; populations from Maui and Kaho‘olawe were separated from populations on Hawai‘i Island (Figure 2.14). The first two principal coordinates (PC) axes of Figure 2.15 explained 29.6 and 21.3% of the genetic variation among populations, respectively, for a total of 50.9%. The scattergram of these two axes again showed strong geographical correlation among individuals, respective of their



Table 2.9. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between populations of Hawaiian *Sesbania* on top half of matrix, Bonferroni-corrected  $P$ -values ( $\alpha_{0.01} = 0.012$ ) listed in bottom half. n.s. indicates pairwise comparisons insignificant at the 0.05 level.

Kīpuka Nēnē–Hilina pali	0.000	0.063	0.046	0.281	0.214	0.404	0.364	0.303	0.387	0.187	0.317	0.335	0.514	0.530	0.489	0.459
Pepeiau–Kukalau‘ula pali	0.036	0.000	0.035	0.272	0.145	0.401	0.358	0.266	0.334	0.148	0.273	0.347	0.516	0.542	0.507	0.427
Kamo‘oali‘i–Kū‘ē‘ē	n.s.	n.s.	0.000	0.247	0.162	0.391	0.339	0.255	0.347	0.143	0.309	0.329	0.517	0.550	0.501	0.444
‘Āpua point	0.012	0.012	0.012	0.000	0.343	0.493	0.476	0.473	0.489	0.322	0.449	0.473	0.626	0.597	0.651	0.576
Kamilo Point–Ka Lae	0.012	0.012	0.012	0.012	0.000	0.379	0.352	0.296	0.368	0.178	0.310	0.352	0.454	0.490	0.462	0.429
Waiaka‘īlio	0.012	0.012	0.012	0.012	0.012	0.000	0.521	0.478	0.456	0.272	0.404	0.599	0.753	0.753	0.763	0.565
Pu‘u Koa‘e	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.318	0.424	0.207	0.298	0.458	0.598	0.606	0.516	0.454
Pu‘u Pīmoe	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.326	0.126	0.315	0.349	0.539	0.605	0.541	0.387
Papanalahoā–Nākālele	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.258	0.323	0.380	0.519	0.577	0.529	0.421
Kawela–Kamiloloa	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.190	0.233	0.340	0.410	0.343	0.266
Mo‘omomi	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.416	0.568	0.583	0.522	0.405
Kāohikaipu & Mōkapu	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.334	0.482	0.550	0.466
Ka‘ena point	0.012	0.012	0.024	0.012	0.012	0.012	0.012	0.036	0.012	0.012	0.012	n.s.	0.000	0.585	0.663	0.526
Polihale	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.621	0.559
Mānā	0.012	0.012	0.024	0.012	0.012	0.012	0.012	0.048	0.012	0.012	0.012	0.012	n.s.	0.012	0.000	0.495
Nihoa	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000

Table 2.10. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between populations of Hawaiian *Sesbania*, corrected for the presence of null alleles [ $F_{ST(ENA)}$ ].

Kīpuka Nēnē–Hilina pali	0.000	0.058	0.035	0.258	0.177	0.407	0.340	0.287	0.338	0.178	0.288	0.323	0.502	0.533	0.517	0.426
Pepeiau–Kukalau‘ula pali		0.000	0.030	0.238	0.125	0.398	0.323	0.240	0.287	0.138	0.239	0.324	0.492	0.532	0.514	0.387
Kamo‘oali‘i–Kū‘ē‘ē			0.000	0.219	0.138	0.394	0.316	0.236	0.296	0.134	0.269	0.312	0.504	0.542	0.522	0.407
‘Āpua point				0.000	0.313	0.485	0.432	0.435	0.427	0.294	0.384	0.445	0.598	0.567	0.637	0.531
Kamilo Point–Ka Lae					0.000	0.407	0.317	0.274	0.310	0.132	0.260	0.343	0.451	0.497	0.501	0.399
Waiaka‘īlio						0.000	0.505	0.457	0.433	0.296	0.410	0.585	0.734	0.744	0.756	0.533
Pu‘u Koa‘e							0.000	0.296	0.361	0.199	0.229	0.434	0.574	0.593	0.513	0.410
Pu‘u Pīmoe								0.000	0.281	0.125	0.285	0.334	0.515	0.592	0.539	0.350
Papanalahoa–Nākālele									0.000	0.219	0.257	0.328	0.468	0.538	0.503	0.363
Kawela–Kamiloloa										0.000	0.162	0.243	0.350	0.426	0.390	0.262
Mo‘omomi											0.000	0.398	0.543	0.561	0.519	0.357
Kāohikaipu & Mōkapu												0.000	0.323	0.458	0.548	0.415
Ka‘ena point													0.000	0.553	0.649	0.484
Polihale														0.000	0.619	0.533
Mānā															0.000	0.479
Nihoa																0.000

Table 2.11. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between 8 putative species (Char, 1983) of Hawaiian *Sesbania* on top half of matrix, Bonferroni corrected  $P$ -values ( $\alpha_{0.01} = 0.0028$ ) listed in bottom half.

“ <i>kauensis</i> var. <i>intermedia</i> ”	0.0000	0.0572	0.1357	0.2001	0.2613	0.4335	0.3792	0.5026
“ <i>kauensis</i> var. <i>kauensis</i> ”	0.0028	0.0000	0.0725	0.1407	0.2092	0.4029	0.3345	0.4746
“ <i>tomentosa</i> var. <i>tomentosa</i> ”	0.0028	0.0028	0.0000	0.0854	0.1239	0.2691	0.2432	0.3521
“ <i>arborea</i> ”	0.0028	0.0028	0.0028	0.0000	0.1349	0.2998	0.2367	0.3643
“ <i>molokaiensis</i> ”	0.0028	0.0028	0.0028	0.0028	0.0000	0.3962	0.2740	0.4296
“ <i>oricola</i> ”	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000	0.3155	0.5011
“ <i>polihalensis</i> ”	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000	0.3155
“ <i>manaensis</i> ”	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000

Table 2.12. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between 8 putative species (Char, 1983) of Hawaiian *Sesbania*, corrected for the presence of null alleles [ $F_{ST(ENA)}$ ].

“ <i>kauensis</i> var. <i>intermedia</i> ”	0.0000	0.0455	0.1374	0.1906	0.2323	0.4070	0.3382	0.5250
“ <i>kauensis</i> var. <i>kauensis</i> ”		0.0000	0.0798	0.1281	0.1768	0.3690	0.2933	0.4916
“ <i>tomentosa</i> var. <i>tomentosa</i> ”			0.0000	0.0686	0.1056	0.2755	0.2215	0.4216
“ <i>arborea</i> ”				0.0000	0.1160	0.2904	0.2098	0.4126
“ <i>molokaiensis</i> ”					0.0000	0.3572	0.2310	0.4419
“ <i>oricola</i> ”						0.0000	0.2258	0.4856
“ <i>polihalensis</i> ”							0.0000	0.3395
“ <i>manaensis</i> ”								0.0000

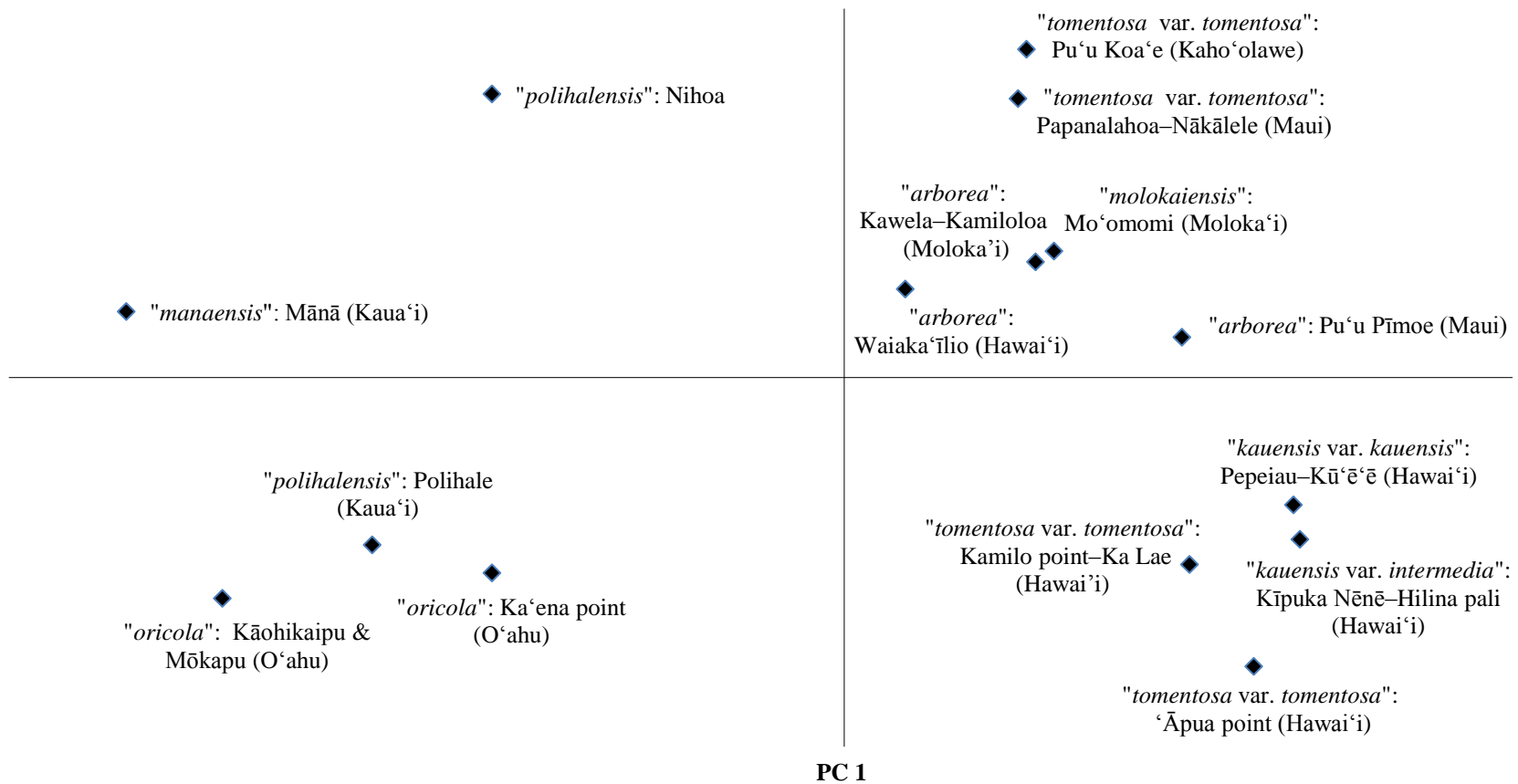


Figure 2.14. Principal Coordinate Analysis (PCA) of the chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967) between populations of Hawaiian *Sesbania*. Each population is identified by the putative species designations of Char (1983).

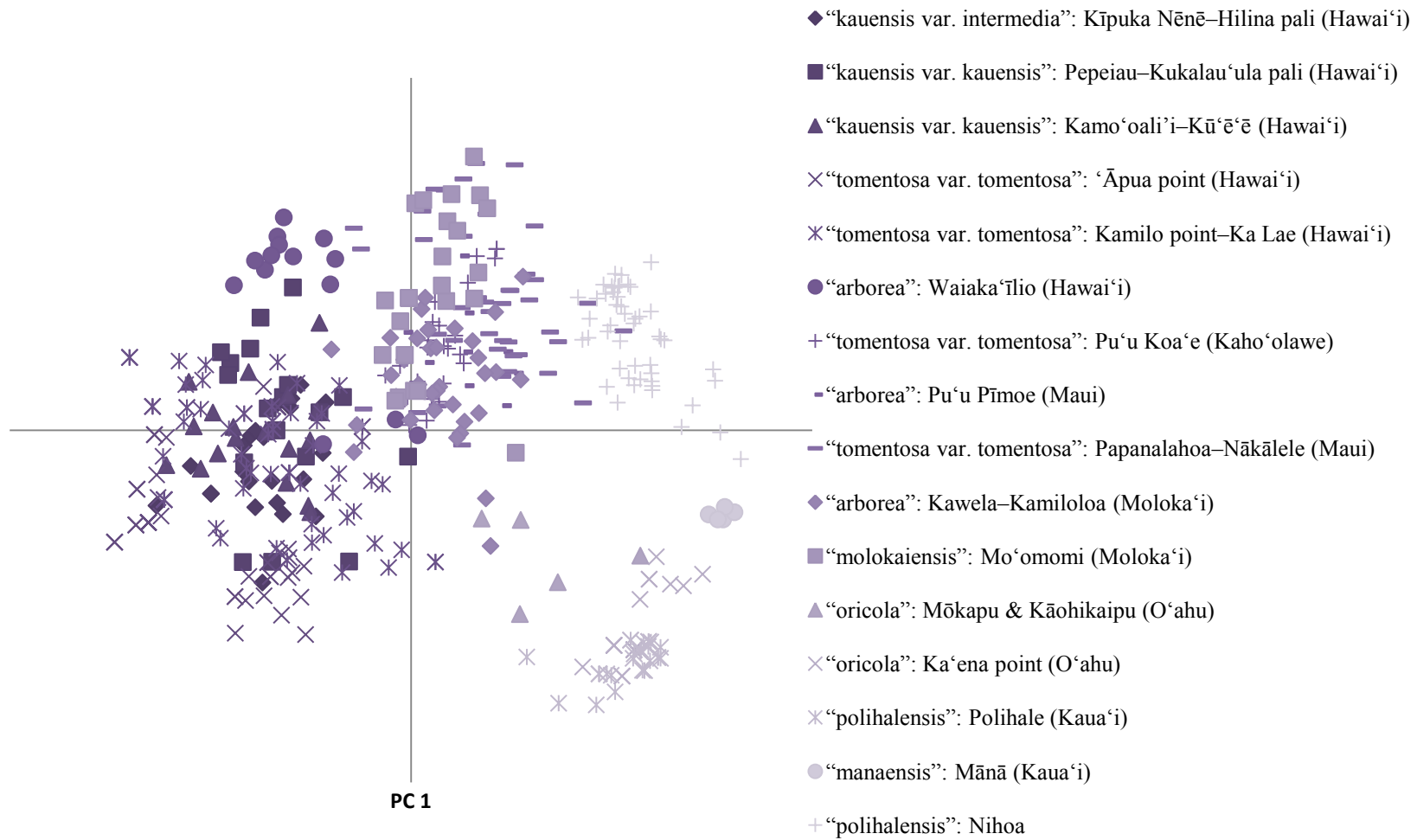


Figure 2.15. Principal Coordinate Analysis (PCA) of the codominant genotypic distances (Smouse and Peakall, 1999) between individuals of Hawaiian *Sesbania*. Population of origin for each individual distinguished by shaded symbols. Each population is identified by the putative species designations of Char (1983).

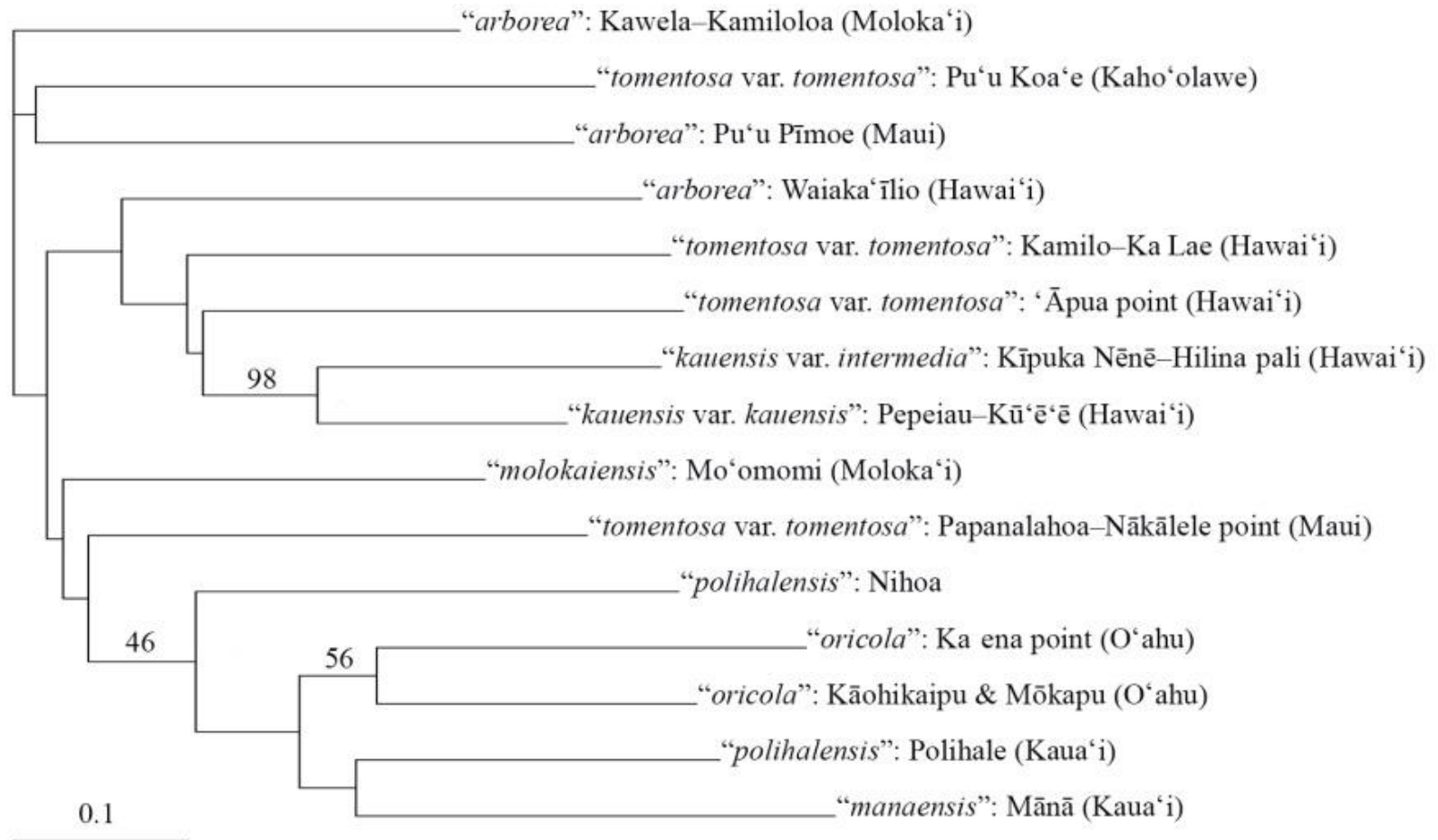


Figure 2.16. Neighbor-joining tree of Hawaiian *Sesbania* populations based on chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967). Each population is identified by the putative species designations of Char (1983). Bootstrap support values (1,000 replicates) are shown only where support exceeded 40%.

population and island of origin; the Maui Nui individuals were particularly cohesive, as well as the individuals from O‘ahu and Kaua‘i (Figure 2.15).

Support was low for most of the branches of the microsatellite NJ phenogram due to high variance in bootstrapped distance estimates (Figure 2.16). Relatively few loci were examined (nine) so there may not have been sufficient resolution to recover the correct topology (Takezaki and Nei 1996). In contrast to PCA, NJ analysis showed the “*tomentosa* var. *tomentosa*” population from Papanalaho–Nākālele (Maui) and the “*molokaiensis*” population from Mo‘omomi (Moloka‘i) more closely related to the populations on the Islands of O‘ahu, Kaua‘i and Nihoa than they were to the rest of the Maui Nui populations. Support was also relatively strong on both morphological phylogenies at the nodes which paired “*molokaiensis*” with “*oricola*” (O‘ahu) (Figures 2.1 and 2.2). Other than this discrepancy, as with the PCA and STRUCTURE analysis, there appeared to be a consistent geographical pattern to the topology.

## Discussion

### Inconclusive morphological and molecular phylogenies

Results from the morphological analysis suggest that many of the characters used to develop the data set do not support the relationships among taxa in any meaningful way. In the morphological phylogenies, the high homoplasy index, and the extremely low rescaled consistency index (values) indicate that autapomorphies are inflating the consistency index and that many of the characters constructing the phylogenies are homoplastic. The morphological analysis is consistent with the conclusion drawn by Geesink et al. (1999) that Char’s characters vary independently and that differences among populations are based on differing means and not discrete quantitative or qualitative differences. In addition, the standard deviations around some of the means are larger than the discrete categories used to code that particular character (Char, 1983). As such, attempting to ascertain phylogenetic relationships among the various populations using morphological characters was confounded and any purported clarification such an analysis offers of the manner in which these populations evolved is misleading.

Similarly, the molecular DNA phylogeny was unable to suggest any meaningful relationships among populations of Hawaiian *Sesbania*, besides sharing a sister relationship with



*S. marchionica* from the Marquesas. Fosberg (1948) suggested that the presumed origin of Hawaiian *Sesbania* is from somewhere in the South Pacific given the morphological similarity to other Pacific (*S. coccinea*, *S. marchionica* and *S. grandiflora*) and Austral (*S. formosa*) species. However, the data here show evidence for an American origin, consistent with the cosmopolitan *Sesbania* phylogenies of Farruggia (2009).

In contrast to the isozyme phenogram of Gemmill et al. (1995) (discussed below), sequence diversity was virtually non-existent at the two nuclear regions sampled for this study. The ITS sequences obtained herein were identical to sequences submitted to GenBank [from Kaua‘i (“*polihalensis*”): AF536355 and AF536356; from O‘ahu (“*oricola*”): AF536357, AF536358 and AF536359; from Moloka‘i (“*arborea*”): JX453663]. Therefore, DNA sequence data, at least with the genes used here, will not be able to resolve phylogenetic relationships among the morphologically variable Hawaiian populations, and provides no evidence (by itself) for splitting *S. tomentosa* into additional taxa. In spite of this, it appears that all Hawaiian *Sesbania* populations form a monophyletic group and represent a recent, incipient evolutionary radiation among the Hawaiian Islands. In this case, close analysis of the population genetic dataset is necessary to infer connections between the observed morphologies of distinct populations.

#### Resolution of taxonomic groups with population genetic markers

Overall, STRUCTURE provided less resolution in identifying distinct clusters (or lineages) than  $F_{ST}(\theta)$ . This might be explained by a poor fit between assumptions of the STRUCTURE model, which assumes Hardy-Weinberg equilibrium within populations, and the empirical data (see Chapter 2). As a point of comparison, Wright’s (1978) guidelines state that values of  $F_{ST}$  above 0.25 indicate “very great” genetic differentiation. Many of the putative species and populations analyzed here far exceed this level of differentiation, suggesting that the sequence markers used above were unable to detect the more recent, dramatic divergence evident in microsatellite loci.

Since STRUCTURE is useful in determining the lower bounds of potential species (Shaffer and Thomson, 2007), the results presented herein provide a basis for beginning to understand the apparent diversification of Hawaiian *Sesbania* populations. The first division of

Hawai‘i Island in a separate cluster from the rest of the populations to the northwest is an important lower bound. The fact that geographic populations of “*arborea*” and “*tomentosa* var. *tomentosa*” from different islands failed to cluster together genetically is evidence of morphological homoplasy among populations. The strong phylogeographic pattern present in the STRUCTURE analysis at both hierarchical levels (whereby geographically proximate populations cluster together regardless of their putative species designations) is also seen in the PCA and NJ results. This pattern also indicates that Maui Nui (situated in the middle of the high islands of the Hawaiian archipelago) might be the center of origin and diversity for Hawaiian *Sesbania*. Strong indications of admixture in Maui Nui populations, and in particular in the populations of “*arborea*” and “*molokaiensis*”, lend support to this assertion. The closest relatives to Hawaiian *Sesbania* are all arborescent, thus the arborescent “*arborea*” could be seen as a primitive type and peripatric divergence of the more prostrate and tomentose “*molokaiensis*” and “*tomentosa* var. *tomentosa*” morphotypes formed the basis for the wide range of variation we observe across the Hawaiian Islands. Two of these three types were observed by Rock in 1919 and all three were observed by Degener in 1918 within 10 km of one another on the island of Moloka‘i (Hawai‘i Biodiversity and Mapping Program). Relatively low pairwise  $F_{ST}(\theta)$ -values (average 0.10; ranging from 0.08–0.13) between these three putative taxa as compared with pairwise  $F_{ST}(\theta)$ -values between these three and the remaining five taxa (average 0.3; ranging from 0.07–0.43) corroborate this scenario. Arguably the two most morphologically distinct populations analyzed here occur within 25 km of one another on the Island of Moloka‘i (“*molokaiensis*” and “*arborea*”), yet STRUCTURE analysis and PCA grouped these two populations together.

Global  $F_{ST}$  among the eight putative species of Hawaiian *Sesbania* tested (0.211) was roughly half that among geographic populations (0.396). In addition, the AMOVA analysis suggested there was much more variation being distributed among geographic populations (40%) than there was among the eight putative species (4%), and that over half of the total variation (56%) was found within each population. As a means of comparison, in the widespread wind-dispersed *Metrosideros* (Myrtaceae) of the Hawaiian Islands, up to 91% of the variation was found within populations and 4% of the total variation was partitioned among taxa on a single island (Wright and Ranker, 2010; Stacy et al., 2014).

The “unified species concept” defines a species as a “separately evolving metapopulation lineage” (de Queiroz 2007), the term “lineage” referring to an ancestor-descendent sequence of populations. When two or more loci indicate that a lineage is distinct (i.e., harboring a set of unique or “private” alleles), that lineage or group of populations should become a candidate for species recognition (Shaffer and Thomson, 2007). There were two private alleles at two loci (at frequencies of 0.01 and 0.11) in one of the “*kauensis*” populations of Hawai‘i Volcanoes National Park (Kamo‘oali‘i–Kū‘ē‘ē). However, because one of the two alleles occurs at low frequency, and this population did not cluster independently of other populations on Hawai‘i Island in the STRUCTURE analysis, this example illustrates only a minor distinction to this population of “*kauensis*”. On the other hand, the small population of “*tomentosa* var. *tomentosa*” on Hawai‘i Island at Ka Lae (29 individuals when sampled in 2006) exhibited four private alleles at three loci, again at relatively low frequencies (average 0.03; ranging from 0.01–0.45). The second hierarchical layer of STRUCTURE analysis had separated this population (and 3 other nearby populations) out from the others on Hawai‘i Island. This population was recognized by Degener (1978) as “*hawaiiensis*”, yet was subsumed by Char (1983), who included it instead with other “*tomentosa* var. *tomentosa*” samples collected (from five islands) in her morphometric analysis. Thus, its relative distinction was not analyzed in the morphological and genetic comparisons made for this study. However, if you consider all of the populations of Hawai‘i Island together (as did the first layer of STRUCTURE analysis) there were eleven private alleles at seven loci (average frequency 0.020; ranging from 0.005–0.065).

The largest number of private alleles (16 occurring at 8 loci) were found in the “*arborea*” population of SE Moloka‘i (Kawela–Kamiloloa), albeit at low frequencies (average 0.05; ranging from 0.01–0.16) and occurring in only 60% of the individuals sampled. When all of the remaining populations of Maui Nui were considered together (excluding the “*arborea*” population of SE Moloka‘i) there were six private alleles at four loci (average frequency 0.090; ranging from 0.004–0.292). Considering all 3 populations of “*arborea*” (from 3 islands) together added only 1 more private allele, therefore the uniqueness of the SE Moloka‘i population is stressed.

The large census size of the SE Moloka‘i population (1,000 plants in 2006; USFWS, 2010) might be preserving rare alleles more efficiently, yet the same should also be true in the even larger population on Nihoa (5,000 plants; USFWS, 2010) which was found to harbor only

one private allele (at a frequency of 0.01). The large number of private alleles may indeed be strong indications of a separately evolving lineage of Hawaiian *Sesbania* in SE Moloka‘i, and to a lesser extent at Ka Lae on Hawai‘i Island. In Chapter 2 the latter example is discussed in terms of the fact that large census size may not be the only factor in harboring unique alleles in populations of Hawaiian *Sesbania*; the Ka Lae population appears to have been fenced in (to the exclusion of ungulates) since 1908 (Love, 1991). In this regard, it is also interesting to note the observation that the tall arborescent form seems more resistant to browsing by deer in SE Moloka‘i (Degener, 1978), which would also allow that particular population to maintain alleles (as well as a large population size) more effectively.

In pairwise  $F_{ST}$  analysis the putative taxa from O‘ahu, Kaua‘i and Nihoa appeared the most differentiated from putative taxa on the remaining Hawaiian Islands. STRUCTURE also hints at separately evolving lineages comprised of the populations from O‘ahu, Kaua‘i and Nihoa. There were two alleles (at 2 loci) private to these three islands combined as well (average frequency 0.090; ranging from 0.004–0.173). While O‘ahu and Kaua‘i populations separated into a distinct sub-cluster from the population on Nihoa, a distinction reflected in the PCA and NJ tree, this phylogeographic trend is expected due to Nihoa’s more remote location 250 km to the NW of Kaua‘i. In addition, samples from Nihoa, Kaua‘i and O‘ahu all diverged slightly from the rest of the Hawaiian samples sequenced at the TRPT region. Lastly, a possible mutated flanking sequence at microsatellite locus A122 in the Ka‘ena point O‘ahu plants and three monomorphic loci in plants originating from O‘ahu and Kaua‘i (one fixed locus in plants from Nihoa) are additional indications of a separate lineage/species of *Sesbania* in the main Hawaiian Islands to the northwest of Maui Nui. The isozyme analysis of Gemmill et al. (1995) suggested this pattern of relationships as well, with a single (fixed) allele separating populations from these three islands from Maui Nui and Hawai‘i Island by a mean genetic identity (genetic similarity rather than distance; Nei, 1972) of 0.58.

#### Taxonomic recommendations for the *Sesbania tomentosa* species complex

While the revisions of Char (1983) were here considered to represent the narrowest rendering of distinct Hawaiian *Sesbania* taxa, analyses here suggest that it needs to be broadened. According to Stuessy (1990), subspecies should be regarded as subdivisions of a

species complex, and represent variation that is genetically controlled. They usually have several conspicuous morphological differences between them and their ‘parent’ species, a cohesive geographical distribution of populations and multiple loci that are genetically divergent. While the morphological distinctions are not clear-cut in my opinion, the latter two conditions appear to be met in several cases pertaining to Hawaiian *Sesbania*. By this definition, populations of *Sesbania* on O‘ahu, Kaua‘i and Nihoa strongly support a distinct northwestern lineage in the process of divergence, and therefore a separate subspecies of *S. tomentosa*. Populations on Maui Nui appear to form another separately evolving metapopulation lineage, a second subspecies. There is also strong support for recognizing “*arborea*” from SE Moloka‘i as a third subspecies, apart from the larger Maui Nui lineage, while evidence is lacking to broaden this circumscription to include the populations of semi-arborescent individuals on Maui and Hawai‘i Island. Populations occurring on Hawai‘i Island form a fourth subspecies of *S. tomentosa*, while any distinction of the Degener’s taxon from Ka Lae within a larger Hawai‘i Island lineage appears to be an artifact of its historical isolation and remoteness.

## Conclusion

Since populations of *Sesbania tomentosa* are in most cases readily distinguishable by the morphology of their representative individuals, this indicates that certain traits (e.g., leaf pubescence and plant habit) have a more rapid rate of evolution than the DNA sequences that were sampled. Natural selection in different environments, along with random drift and mutation in fragmented (isolated) populations may have caused Hawaiian *Sesbania* to separate out into the distinctive appearing populations we see today. Over the past century, an overlap of morphological characters observed in what was once a much more contiguous range of the species has largely been erased. With inbreeding comes a loss of genetic diversity, hence higher  $F_{ST}$  values and overall genetic structuring. The results presented here could indicate a recent phenomenon due to rarity or an ancient one due to divergence (or a combination). An investigation of population fragmentation and sub-structuring will be explored further in Chapter 3. In this case, an assessment of the occurrence of inbreeding and drift among populations will be essential. Microsatellite loci respond to random genetic drift and mutation much more rapidly than the regions sequenced herein; certainly within the time period when populations of

Hawaiian *Sesbania* became increasingly isolated from one another. On a final note, testing whether or not  $F_1$ ,  $F_2$  and  $F_3$  (and backcrosses) have markedly reduced fertility would be the next step in addressing the issues of taxonomy presented, (a fourth condition for sub-specific recognition according to Stuessy, 1990), and should be a focus for future research attempting to discriminate Hawaiian *Sesbania*.

## CHAPTER 3

### **The influence of inbreeding and genetic drift on the differentiation of *Sesbania tomentosa* populations, a rare plant species of the Hawaiian Islands**

#### **Introduction**

Contemporary impacts on the genetic makeup of plant populations and the influence of prehistoric evolutionary phenomena can be difficult to distinguish (e.g., Muir and Schlötterer, 2005; Edwards et al., 2008). The genetic effects of contemporary fragmentation of habitat and decline in numbers of individuals are important to separate from the long term effects of genetic drift, which ultimately can lead to divergence within a species (Ashley et al., 2003). Population subdivision, genetic founder effects, bottlenecks and inbreeding are also expected to have played important roles over the long run in natural processes of differentiation and speciation (Wright, 1931, 1942, 1977; Mayr, 1954; Carson, 1975; Templeton, 1980). Plant reproductive syndromes will be influential over the long run as well, with populations of predominately self-pollinating species having less genetic variation and greater divergence among populations than that associated with more outcrossing species (Hamrick and Godt, 1996). Genetic drift is thought to take place at an accelerated rate in smaller populations (Kimura, 1983), therefore the size of natural populations over time is an additional consideration. Natural ecological dynamics affecting population differentiation often leave lasting genetic signatures, and should be addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants.

*Sesbania tomentosa* Hook. and Arn. is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. This species was federally listed as Endangered by the U.S. Fish and Wildlife Service in 1992. Twenty-nine of the fifty-two populations of *S. tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826 (Table 3.1). Seven populations have been extirpated over the 10 years since this study began, and others have experienced severe demographic decline due to drought, pest outbreaks, etc. (personal communications and observations). A hermaphroditic breeding system, conspicuous flowers and autochorous dispersal of dry fruit have made *S. tomentosa* acutely

Table 3.1. Evidence for the catastrophic decline of *Sesbania tomentosa* populations in the main Hawaiian Islands. Biological surveys since the plant's original description in 1826 are tallied along with cultural indicators of the plant's physical presence at selected locations. Place/land division names are included here only when the species occurrence at a given location was not recorded by biological surveys, and when corresponding locations are > 2 km apart. Both extant and extinct occurrences refer only to naturally-occurring groups of plants (separated by > 1 km). Extant vs. extinct status verified via personal communication with private land managers and conservation workers, Federal employees and Hawai'i State personnel. 'Ohai is the Hawaiian name for *Sesbania tomentosa* (Andrews, 1922).

Island	Extant population (as of 2015)	Extinct population (as of 2015)	Place names / type of location	Division names / type of division
Hawai'i	<p>Āpua point Pepeiau Kukalau'ula Kīpuka Nēnē Hilina pali 1 Hilina pali 2 Hilina pali 3 Fuel Break Rd. Kamo'oali'i Kū'ē'ē</p>	<p>Kamilo point Mahana bay Kīpuka Hanalua Ka Lae Waiaka'īlio Ka'ūpūlehu</p>	<p><sup>e</sup>Ohai'ula / beach <sup>e</sup>Kalae'ohai / point <sup>e</sup>Moku'ohai / bay <sup>b</sup>Pu'u 'ohai / hill</p>	<p><sup>f</sup>Kalae'ohai / boundary <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / kīhāpai <sup>f</sup>Opū'ohai / 'ili 'āina <sup>f</sup>Pū'ohai / ahupua'a <sup>e</sup>Ohaieka / 'ili 'āina</p>
Maui	<p>Papanalahoa Kahakuloa Mōkōlea</p>	<p>Pu'u Pīmoe Nākālele Līhau</p>	<p><sup>a</sup>Maka'ohai / fishing site <sup>b</sup>Kalae'ohai / point</p>	<p><sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Pū'ohai / 'ili 'āina</p>
Kaho'olawe	<p>Pu'u Koa'e</p>	<p>Kaho'olawe</p>		
Lāna'i		<p>Maunalei Kahinahina Mānele Kaumālapa'u Kamoku Paoma'i Kūāhua</p>		<p><sup>f</sup>Ka'ohai / ahupua'a</p>



Table 3.1. (Continued) Evidence for the catastrophic decline of *Sesbania tomentosa* populations in the main Hawaiian Islands.

Island	Extant population (as of 2015)	Extinct population (as of 2015)	Place names / type of location	Division names / type of division
Molokaʻi	Moʻomomi Kawela Kamiloloa Makakupaʻia	Kalaeokaʻilio Maunaloa Kalaeokalāʻau Waiahewahewa Pālāʻau Mahana	<sup>f</sup> Loko ʻOhaipilo / pond	<sup>f</sup> ʻOhaipilo / ʻili ʻāina <sup>f</sup> Kaʻiliʻohai / ʻili ʻāina
Oʻahu	Kaʻena point Mōkapu Kāohikaipu	Waiʻanae Mokulua Manini pali	<sup>f</sup> Loko Kaʻohai / pond <sup>f</sup> Kaʻohai / tree grove	<sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ku pono <sup>f</sup> Kaʻohai / ʻokipuʻu <sup>f</sup> Kaʻohai / moʻo ʻāina
Kauaʻi	Polihale Hanapēpē	<sup>c</sup> Mānā Plain	<sup>b/d</sup> ʻOhaiʻula / ridge <sup>d</sup> ʻOhaiʻula / valley <sup>d</sup> ʻOhaiʻula / point <sup>a</sup> Waiʻohai / beach	<sup>f</sup> Kaʻohai / moʻo ʻāina <sup>f</sup> Waiʻohai / ʻili ʻāina <sup>f</sup> Haleʻohai / ʻili ʻāina
Niʻihau		<sup>c</sup> Leeward Niʻihau <sup>c</sup> Kawaihoa		
Total	23	29	13	23

<sup>a</sup> Clark, J.R.K. 2002. Hawaiʻi Place Names. University of Hawaiʻi Press, Honolulu, HI. 412 p.

<sup>b</sup> Coulter, J.W. 1935. A Gazetteer of the Territory of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 241 p.

<sup>c</sup> Hawaiʻi Biodiversity and Mapping Program: Hawaiʻi Natural Heritage Program

<sup>d</sup> Juvik, S.P. and Juvik, J.O. 1998. Atlas of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 333 p.

<sup>e</sup> Pukui, M.K., Elbert, S.H. and Mookini, E.T. 1974. Place Names of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 289 p.

<sup>f</sup> Soehren, L.J. 2002–2010. A Catalog of Hawaiian Place Names accessed at <http://ulukau.org/cgi-bin/hpn?l=haw>

vulnerable to extinction compared with other dry forest taxa, according to the analysis of Pau et al. (2009). On the other hand, entirely new occurrences of this species have been discovered since this study began near Nu‘upia Pond (Mōkapu, O‘ahu) and at Pa‘akahi Point (Hanapēpē, Kaua‘i) after heavy winter rains, indicating an important role of the seedbank within the metapopulation as a whole as well as the ephemeral nature of the plant as a component of the vegetation.

The habit of *Sesbania tomentosa* is highly variable, often with island specific forms. Plants may grow as sprawling shrubs with prostrate to decumbent branches (reportedly up to 14 meters long, and possibly longer) or as a small bush or tree up to six meters in height. Leaves are even-pinnately compound and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. The species is named for the leaves, that are usually sparsely to densely covered with silky hairs. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet to deep red. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds. The chromosome number for *S. tomentosa* is  $2n = 24$  (Geesink et al., 1999), suggesting the species is diploid (base chromosome number  $x = 12$ ). *Sesbania tomentosa* is currently recognized as a single species (Geesink et al., 1999) although it is highly variable for many important characters across its range. This led Rock (1920), Degener and Degener (1978) and Char (1983) to delimit up to nine distinct putative taxa. According to Andrews (1922), the Hawaiian name for *S. tomentosa* is ‘ohai.

Cultural knowledge can be used to hypothesize the prior distribution of *Sesbania tomentosa* in the Polynesian era. The Hawaiians named the various features and places in their environment, and often incorporated plant descriptions in names (Pukui et al., 1974). Geographic place names (beaches, points, hills, ridges, etc.) often mention a specific plant, likely reflecting an observable element of the geography at the time that place was named (Sam Gon, The Nature Conservancy, personal communication; Coulter, 1935). The names and boundaries of parcels of land, often named for observable elements of the environment and landscape as well, are known through oral tradition originating as far back as the 15<sup>th</sup> century (Kamakau, 1961; 1976). The use of land division names to infer past geographical extent of a plant species in Hawai‘i was used by McEldowney (1983) to map the extent of ‘ōhi‘a lehua (*Metrosideros polymorpha*) forest across the prehistoric Waimea (Kohala, Hawai‘i) plain. If place and land division names referring

to 'ohai are considered indications of past occurrences of *S. tomentosa*, then the total number of populations ever recorded would increase by 41% (adding 36 additional occurrences; Table 3.1).

The methods of Price et al. (2007) were used to predict the natural range for *Sesbania tomentosa*. This was accomplished by demarcating a general bioclimatic envelope, built upon a database that includes information on the known distribution of the species by geographic region, major habitat type, and elevation range. In this model, most of the main Hawaiian Islands (excepting the islands of Maui and Hawai'i) are almost completely encircled by the range of *S. tomentosa*, which extends along the coasts and well inland in dry-mesic areas (Figure 3.1). Anecdotaly, MacCaughey (1916) remarked, "the bush is often to be found in the vicinity of the little beach settlements, particularly along the arid leeward shores." Degener (1978) commented on the decline of populations of *S. tomentosa* on O'ahu and Hawai'i Island as compared to his observations 50 years prior.

On the other hand, some evidence suggests that the decline of *Sesbania tomentosa* has been progressing for centuries. Based on extensive palynological core data on O'ahu (Athens 1997, 2002), by A.D. 1600 the entire landscape below 460 m had been extensively altered, indicated in part by a catastrophic decline in the pollen of native species. For example, *S. tomentosa* disappeared from the 'Ewa plain pollen record around 1300 AD, where it has not been observed in historic times. Athens et al. (2002) correlate the destruction of lowland vegetation with the arrival of the Polynesian rat, *Rattus exulans*. At Hawai'i Volcanoes National Park, extensive rat damage of seedpods of *S. tomentosa* has been documented, and the presence of fruits on plants rapidly rebounds when rats were controlled in the species habitat (Pratt et al., 2011). Rat, ungulate, and arthropod predation, along with human disturbance, is listed as the main contemporary factors in the fragmentation and decline of reproductive populations of *S. tomentosa* [US Fish and Wildlife Service (USFWS), 2010].

Lack of adequate pollination services has also been deemed another threat in populations of *S. tomentosa* (USFWS, 2010). The results of two pollination studies of *S. tomentosa* show a mixed-mating system (Goodwillie et al., 2005) where some plant seeds are derived from outcrossing and some are derived from either pollinator-mediated or autonomous self-fertilization. Working at Ka'ena point on O'ahu, Hopper (2002) found that *S. tomentosa* is fully self-compatible and self-pollen, as well as non-self-pollen, was equally likely to result in fertilization and fruit set. The species is pollinator-limited (the flower's protective wing and keel

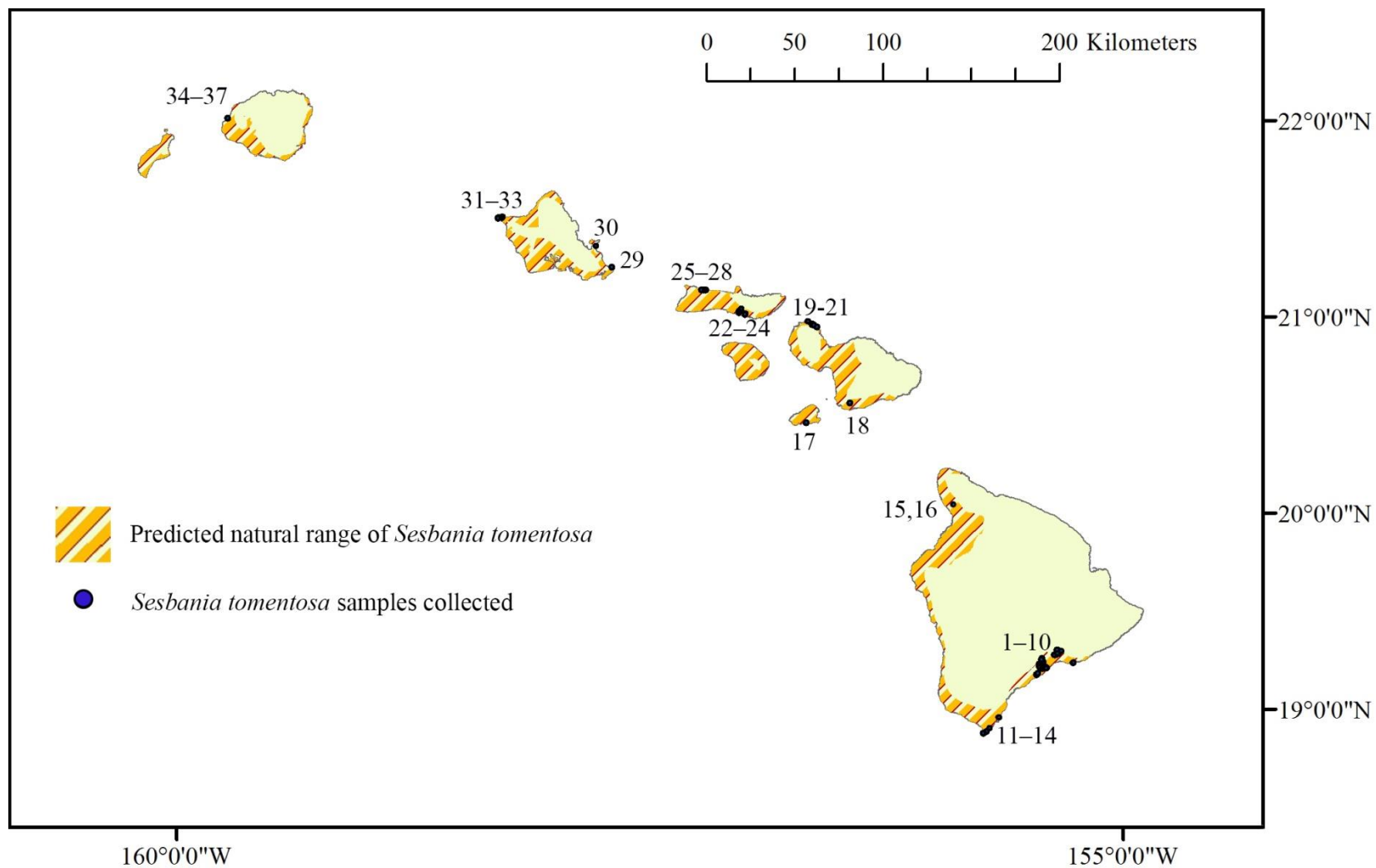


Figure 3.1. Location of DNA samples collected in 2006–2010; numbers on map correspond to sub-populations/populations listed in Table 3.2. Predicted natural range of *Sesbania tomentosa* provided by Jonathan Price, University of Hawai‘i at Hilo.

petals necessitate mechanical pollination), yet in the absence of a pollinator the proximity of the stigma and the anthers ensure that selfing is still possible. While rates of autogamy were shown to be low (0.8%), this rate might be high enough to maintain low levels of reproduction in a species where individuals have the potential to produce 1,000 flowers over the course of a season (Hopper, 2002). The endemic *Hylaeus* pollinators (accounting for 86.4% of all floral visitations and 99.6% of observed pollen transport) were noted to spend most of their time around single plants, and Hopper believed that a large proportion of the pollination and fruit set he observed at Ka‘ena point, as well as in his observations of the species at Hawai‘i Volcanoes National Park, could be the result of geitonogamy (Hopper, 2002). *Hylaeus* are thought to be important pollinators for native Hawaiian plants in general because of the frequency of their visitation (Magnacca, 2007; Koch and Sahli, 2013; Krushelnycky, 2014). In a more recent study, Pratt et al. (2011) observed *Hylaeus flavipes* and *H. laetus* to be the most abundant visitors of *S. tomentosa* at the upland population at Kīpuka Nēnē (Hawai‘i Volcanoes National Park), and found the species’ pollen on the bodies of *Hylaeus* (accounting for 60.2% of total visits, 25.0% of which involving observed pollen transport). Again, geitonogamy was purported to be the main mechanism of pollination for this plant at Kīpuka Nēnē (Pratt et al., 2011). Therefore, it is unclear whether a lack of pollination services would be a threat to *S. tomentosa* populations or would alter their genetic makeup at all, as inbreeding and a high degree of relatedness between adjacent individuals would seem to be a natural consequence of the plant’s ecology.

This chapter will address population-level processes that might be affecting the rapid differentiation of populations discussed in Chapter 2. Levels of genetic variation within and among populations of *S. tomentosa* were measured using microsatellite marker analysis to investigate inbreeding and population sub-structuring and to examine evidence for genetic bottlenecks. The genetic diversity of a naturally-occurring extant population (Mo‘omomi, Moloka‘i) was also compared with a molecular sampling of herbarium specimens collected there 60–100 years prior to the sampling of 2006, to illustrate the consequences of one such bottleneck directly. Another population for which census size had been known to fluctuate from year to year (Polihale, Kaua‘i) was repeatedly sampled over a four-year period to observe how population genetic diversity might be dynamic over time, and also add an additional dimension to a discussion of natural vs. human induced genetic bottlenecks.

## Materials and Methods

### DNA sample collection

Leaf samples of 539 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 38 sub-populations (separate clusters of plants 1 to 3 km apart within a population) comprising 18 populations from seven islands were sampled (Table 3.2, Figure 3.1). An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. I recorded GPS coordinates for each individual plant sample collected. Samples at ‘Āpua point, Kawela–Kamiloloa, Pu‘u Koa‘e and Nihoa comprise a subset of their respective populations (individuals collected arbitrarily from throughout each population). At Pu‘u Koa‘e and Nihoa, samples were obtained by surrogate collectors [Ken Wood, National Tropical Botanical Garden (NTBG) and Beth Flint (USFWS)] and no GPS coordinates were logged. An attempt to distinguish groups of naturally occurring *vs.* out-planted individuals at Ka‘ena point was made with the assistance of Betsy Gagné (Hawai‘i Division of Forestry and Wildlife). Except where noted above, only naturally occurring plants and all known individuals extant at the time of collection were sampled for analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4 to 8°C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNeasy tissue kits (QIAGEN; Valencia, CA) according to the manufacturer’s protocol and the purified sample, along with negative and positive controls, were visually checked using electrophoresis.

Additional sampling of historically-collected tissue from the Mo‘omomi dunes population on Moloka‘i was conducted with loaned specimens from the herbarium of the New York Botanical Garden (NY), the B. P. Bishop Museum Herbarium (BISH) and the U. S. National Herbarium (US) (Table 3.3). DNA was extracted from 10 specimens using the QIAGEN QiaAmp Stool minikit, modified CTAB protocols (Drábková et al., 2002) and a PTB (N-phenacylthiazolium bromide) protocol (Asif and Cannon, 2005). For each of the 10 specimens at least one of the extraction protocols listed proved successful (samples checked via electrophoresis). These historically collected samples were included in analyses of microsatellite

Table 3.2. Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands. Sub-populations are listed as combined into population aggregate groups for subsequent analysis; distances between clusters of plants designated as sub-populations within a given population are listed in parentheses. ID numbers code for sub-populations listed on Figures 3.1, 3.3, 3.6 and 3.7. *n* and *N*, sample size of sub-populations and populations, respectively.

Island	ID #	Sub-population/population	<i>n/N</i>
Hawai‘i	1	Kīpuka Nēnē makai	12
	2	Kīpuka Nēnē mauka	6
	3	Hilina pali cluster 1	8
	4	Hilina pali cluster 2	6
	5	Hilina pali fuel break rd.	3
		(2 km apart)	Kīpuka Nēnē–Hilina pali population total: 27
	6	Pepeiau	10
	7	Kukalau‘ula pali	9
		(2 km apart)	Pepeiau–Kukalau‘ula pali population total: 19
	8	Kamo‘oali‘i	13
	9	Kū‘ē‘ē	5
	10	‘Āpua point	58
	11	Kamilo point	9
	12	Mahana bay	29
	13	Kīpuka Hanalua	12
	14	Ka Lae	29
		(2 km apart)	Mahana bay–Ka lae population total: 70
	15	Waiaka‘īlio	8
	16	Waiaka‘īlio seedbank	10
			Waiaka‘īlio population total: 18

Table 3.2. (Continued) Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands.

Island	ID #	Sub-population/population	n/N
Kaho‘olawe	17	Pu‘u Koa‘e	25
Maui	18	Pu‘u Pīmoe	9
	19	Papanalahoa point	37
	20	Mōkōlea point	5
	21	Nākālele point	2
		(1–2 km apart) Papanalahoa–Nākālele point population total:	46
Moloka‘i	22	Kawela	17
	23	Kamiloloa	14
	24	Makakupa‘ia	4
		(2–3 km apart) Kawela–Kamiloloa population total:	35
	25	Moloka‘i ranch rd.	14
	26	Nature Conservancy preserve	3
	27	Mo‘omomi pavillion	9
		(1–2 km apart) Mo‘omomi population total:	26
	28	Mo‘omomi herbarium	10
O‘ahu	29	Kāohikaipu	2
	30	Mōkapu (Nu‘upia pond)	4
		(15 km apart) Kāohikaipu & Mōkapu population total:	6
	31	Ka‘ena point State Park	15
	32	Ka‘ena point outplantings	32
	33	Ka‘ena point NAR	18
		(1–2 km apart) Ka‘ena point population total:	65



Table 3.2. (Continued) Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands.

Island	ID #	Sub-population/population	n/N
Kaua'i	34	Polihale State Park (2006)	16
	35	Polihale State Park (2009)	11
	36	Polihale State Park (2010)	12
		Polihale State Park population total:	39
	37	Mānā plain	4
Nihoa	38	Nihoa	49
			Total = 539

Table 3.3. DNA collected off herbarium sheets of *Sesbania tomentosa* loaned from B. P. Bishop Museum Herbarium (BISH), New York Botanical Garden (NY) and the U. S. National Herbarium (US).

Barcode/ID #	Collector	Date	Location notes from herbarium sheet
990804 (NY)	J.F.C. Rock	3-1909	Molokai. Moomomi.
990808 (NY)	J.F.C. Rock	3-1910	Molokai. Moomomi.
990809 (NY)	C.N. Forbes	3-24-1915	Molokai. Moomomi.
55944 (BISH)	G.C. Munro	7-22-1926	Moomomi sandhills.
990820 (NY)	O. Degener	4-19-1928	Kalani, Moomomi. creeping branches take root, single large plant in sand dunes several hundred feet above sea.
990817 (NY)	O. Degener	4-25-1928	Moomomi, Molokai arid sand dunes.
55933 (BISH)	M.C. Neal	4-1-1934	Mokapu Crater, Oahu, edge of cliff.
990810 (NY)	F.R. Fosberg	12-26-1936	Molokai. Moomomi prostrate shrub, base of sand dunes.
14052 (US)	F.R. Fosberg	6-13-1937	Oahu. Kaohikaipu.
990811 (NY)	C.S. Judd	9-16-1937	Molokai. Moomomi procumbent shrub, sand hills alt. 10m.
177376 (BISH)	H. St.John	1-3-1939	Moomomi, Kaluahoi on sand dunes.
488514 (BISH)	H. St.John	12-24-1948	Moomomi, Kaluahoi, trailing on sand dunes near shore.

fragment sizes to compare genetic diversity of modern vs. historical plants collected from the Mo‘omomi population.

The demographics of certain populations necessitated augmentation of the dataset in order to provide marginally larger sample sizes for comparison. One cultivated individual derived from Kāohikaipu (1 plant extant in 2009) and one cultivated individual derived from Nu‘upia Ponds (3 plants extant in 2009) at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu) augmented the extant individuals in these two sub-populations, combined together in a single Windward O‘ahu population for statistical purposes. In addition, all four individuals comprising the Mānā, Kaua‘i population were cultivated specimens at the National Tropical Botanical Garden (F<sub>1</sub> and F<sub>2</sub> generation derived from a single wild plant, now extirpated). For the Waiaka‘īlio, Hawai‘i population, consisting of only a single surviving individual at the time sampling was undertaken, DNA was extracted from the woody core of eight plants that had been standing dead for approximately one year using the PTB protocol of Asif and Cannon (2005). In addition, the seedbank surrounding the dead plants was examined, producing an additional 10 *S. tomentosa* plants for genotyping. Lastly, in order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, the Polihale (Kaua‘i) population was sampled in 2006 (16 plants), 2009 (11 plants) and 2010 (12 plants), and the genetic diversity of the standing populations of each year are herein compared. GPS coordinates accompanied each DNA collection, yet in many cases it was impossible to determine whether or not the same individual was collected multiple times (in successive years) due to the close clustering of individuals.

### Microsatellite analysis

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* under contract with the United States Geological Survey (USGS). Ninety-six dinucleotide (CA<sub>n</sub>) and tetranucleotide (CATC<sub>n</sub>, TACA<sub>n</sub>, and TAGA<sub>n</sub>) microsatellite repeats. Ninety-six microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 3.4) based on their range of polymorphism and ease of scoring in a screening of

eight DNA samples (collected from eight populations on six islands). Each sample was amplified in a 25.0  $\mu$ L volume with final concentrations of: 0.6  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 unit *Taq* DNA polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 3.4) for 40 s, 72°C for 30 s; ending with a final extension of 72°C for 4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA) at the Center for Genomic, Proteomic and Bioinformatic Research (CGPBR) facility at UH Mānoa. The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (Applied Biosystems). Stutter peaks were identified, and the program MICROCHECKER (Van Oosterhout et al., 2004) was then used to identify possible genotyping errors due to non-amplified alleles (null alleles) and short allele dominance (large allele dropout). A maximum likelihood estimate of the frequency of null alleles (Expectation Maximization algorithm of Dempster et al., 1977) was then calculated for each locus and geographic population using the program FREENA (Chapuis and Estoup 2007).

The microsatellite dataset was analyzed to assess linkage (genotypic) disequilibrium (both globally as well as at the level of geographic population) in GENEPOP v. 4.0 (Rousset, 2008) using log-likelihood ratio statistics (*G*-tests). Significance was assessed using 200 batches of 10,000 iterations and Bonferroni-corrected *P*-values at significance level ( $\alpha = 0.05$ ).

Population structure was first examined using a full Bayesian-clustering approach, implemented in the program STRUCTURE v. 2.3.3 (Pritchard et al., 2000), which assigned individual genotypes to populations, irrespective of geographical location of origin. Default settings of the program were used (admixture model, independence among loci, no prior information included). To determine the most likely number of populations or groups (*K*) in the

Table 3.4. Nine microsatellite primer pairs developed for *Sesbania tomentosa*.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 539 individuals sampled for this study. Range, allele size range in base pairs (bp). Prefixes in italics before forward primer sequence indicate dye used for poolplexing.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG R: TAG-GTG-TGG-CGT-GCA-TAA-C	57.3 58.1	10	205–223
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A R: CCC-TTC-CCC-TCC-TCT-TAG	56.0 56.2	9	264–280
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	55.8 56.3	14	198–236
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC R: TAG-CCA-TGC-TTC-ATC-AAT-CG	58.9 59.8	21	288–328
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.8 56.9	13	163–187
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG R: GGC-AAC-ATT-TGA-GTG-GAG-G	58.6 59.1	16	196–276
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1 55.1	14	180–236
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.7 55.2	11	212–252
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.1 54.8	14	265–321

data, a series of analyses were performed from  $K = 1$  (all populations represent a single panmictic unit) to 15 (the maximum number of populations allowable) using 40,000 burn-in and 100,000 repetitions, with ten iterations per  $K$ . These results were examined using the  $\Delta K$  method (Evanno et al., 2005) to identify the most likely number of groups in the data. Ten additional iterations at the identified  $K$  were computed using 100,000 burn-in and 300,000 repetitions. The program CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize these last ten iterations. Cluster membership coefficients for each individual and pre-defined population were obtained (permuted across replicates using FullSearch algorithm) and used as input files for the cluster visualization program DISTRUCT v. 1.1 (Rosenberg, 2004) and for additional chart analysis.

Each individual was assigned to a particular genetic cluster when its coefficient of membership was greater than 50%. Geographic populations and sub-populations were assigned to a particular genetic cluster when 67–100% of their individuals were assigned to that genetic cluster. The initial analysis was repeated on each  $K$  separately to detect sub-structuring within the genetic groups previously inferred. The number of genetic sub-clusters was estimated for each group using the  $\Delta K$  method, ten additional iterations were performed at the appropriate  $K$  (100,000 burn-in and 300,000 repetitions) and both the *Greedy* and *FullSearch* algorithms (10,000 random input orders of runs) were used in CLUMPP. Individuals were then assigned to genetic sub-clusters when their coefficient of membership was greater than 0.5; geographic populations assigned to sub-clusters based on 70–100% individual assignment.

Diversity indices were estimated for the geographic populations using MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötterer, 2003). Diversity indices include expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ), mean number of alleles per locus ( $A$ , a measure of diversity not corrected for sample size), allelic richness ( $A_R$ , allelic diversity corrected for sample size) and monomorphic loci (loci harboring only one allele in a given population) within each population. Private alleles (alleles exclusive to a given population) were calculated in GENALEX v. 6.4 (Peakall and Smouse, 2006). GENEPOP was also used to test for a hypothesis of heterozygote deficiency within each geographic population at each locus and combined across loci using  $U$ -tests. Significance was assessed using 200 batches of 10,000 iterations and Bonferroni-corrected  $P$ -values at significance level ( $\alpha = 0.05$ ). Estimates were

obtained for  $f$ , the within population inbreeding coefficient or the correlation of allele frequencies among individuals within populations, in FSTAT v. 2.9.3.2 (Goudet, 2002).

The extent and significance of the genetic differentiation among geographic populations was investigated with MSA by calculating global and pairwise  $F_{ST}$  values (averaged over multiple loci), with 100,000 permutations to assess significance using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ). FREENA was also used to estimate pairwise  $F_{ST}$  values ( $F_{ST(ENA)}$ ) from genotype frequencies corrected for the presence of null alleles [using the excluding null alleles (ENA) method of Chapuis and Estoup 2007], which tend to positively bias  $F_{ST}$  estimates. Most of the non-visible genotypes in the dataset were assumed to be due to technical problems (e.g., degraded or low quantity of DNA or PCR amplification inconsistencies) and were specified in the FREENA dataset. These were distinguished from the null homozygous genotypes at locus A122 in 64 out of 65 individuals of the combined Ka'ena point population, probably due to a mutated flanking sequence which prevented that particular locus from amplifying.

The presence of a pattern of isolation by distance (IBD; Wright, 1943) between the populations across the Hawaiian Islands was investigated by testing the correlation of the matrix of pairwise log-transformed  $F_{ST}$  (Weir and Cockerham 1984) and  $F_{ST(ENA)}$  (Chapuis and Estoup, 2007) values against the matrix of log-transformed geographic distances using a Mantel test with 10,000 permutations in IBDWS v. 3.16 (Jensen et al., 2005).

Strong spatial genetic structure (i.e., nonrandom spatial distribution of genotypes) would be expected in a plant species with restrictions on the movement of pollen throughout the population (and beyond). In this scenario, genetic similarity is higher among neighboring individuals than more distant individuals (IBD). Kinship coefficients are based on the probability of identity of alleles for two homologous genes sampled in some particular way. In the case of a kinship coefficient between two individuals, the two genes are randomly sampled within each of the two individuals. SPAGEDI v. 1.3 (Hardy and Vekemans, 2002) was used to compute the kinship coefficients of Loiselle et al. (1995) for all pairs of individuals in a given population (some populations are grouped into larger aggregate populations based on their proximity) in order to analyze the individuals and populations at various levels of genetic structure. Only those samples accompanied by GPS location coordinates were used in this analysis (this excluding samples from Waiaka'ilio, Pu'u Koa'e, Kāohikaipu, Mōkapu, Mānā and Nihoa). In order to test for a significant pattern of isolation by distance, the multi-locus kinship coefficient for each pair

of individuals was plotted against the matrix of log-transformed Euclidean distance separating them using a Mantel test with 10,000 permutations. Average kinship coefficients were calculated for 18 distance classes as in a spatial autocorrelation analysis. For each comparison, short intervals (5–25 m) were used for the first distance classes to obtain a detailed picture at a small spatial scale, and then wider intervals (100–10,000 m) were used at larger spatial scales because kinship is expected to vary less. Null hypothesis of no spatial genetic structure was tested using a one-sided Mantel test.

After a severe reduction in effective population size ( $N_E$ ), there should be a transient excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (Piry et al., 1999). Bottlenecks generate transient heterozygosity excess because rare alleles are generally lost faster than heterozygosity during a bottleneck (Luikart and Cornuet, 1998). Wilcoxon sign-rank tests of heterozygosity excess (10,000 iterations) were implemented in BOTTLENECK v. 1.2.02 (Luikart and Cornuet, 1998; Piry et al., 1999). This program used allele frequency data to detect recent reductions in effective population size (i.e., within the past  $0.2N_E$ – $4N_E$  generations) under a 100% stepwise mutation model (SMM), an infinite alleles model (IAM) and a two-phase mutation model (TPM with 70% SMM, 30% IAM). A second approach (also implemented in BOTTLENECK) tested a mode shift away from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium, whereby alleles at low frequency become less abundant than alleles at intermediate frequency (Luikart et al., 1998). FREENA produced alternate allele frequency datasets for each population corrected for the presence of null alleles (using the Expectation Maximization algorithm of Dempster et al., 1977) that were subsequently run in BOTTLENECK for an alternative analysis. A third approach, utilized by the program AGAR<sub>ST</sub> v. 3.3 (Harley, 2003), measured the mean ratio ( $M$ -ratio) of number of alleles in a population ( $k$ ) divided by the range in allele size ( $r$ ) according to the method described by Garza and Williamson (2001). This ratio was calculated as  $M = k/r + 1$  to avoid dividing by zero in monomorphic populations (Excoffier et al., 2005). During a population decline, the number of alleles decreases more rapidly than does the range in allele size, leading to a decrease of  $M$ . Since the recovery time of  $M$  is longer than that of the measures tested in BOTTLENECK (not all mutations will increase  $M$ ), this method tests for population reductions over a longer period of time. A comparison of a population's  $M$ -ratio with its allelic diversity will also distinguish between populations recently reduced from populations that have been small for a long time ( $M$

will recover after a population decline without the maintenance of rare alleles, allelic diversity will not; Garza and Williamson, 2001).

Coalescent models link demographic history with population genealogy and provide a measure of how much the data supports one scenario over other possible scenarios that might have produced that data. The program 2<sub>MOD</sub> (Ciofi et al., 1999) was used to compare the relative likelihoods of two coalescent models: gene flow (equilibrium between gene flow and drift) vs. genetic drift (ancestral population fragmented into isolated sub-populations that then diverge purely by drift) in populations of *Sesbania tomentosa* across the Ka‘ū district of Hawai‘i Island. The Markov Chain Monte Carlo (MCMC) simulation employed by 2<sub>MOD</sub> ran 3 times with 100,000 iterations each. Results across runs were combined, and the probability of each model calculated.

## Results

### Microsatellite allele frequencies

There was an average of 13.8 alleles per locus at the nine microsatellite loci examined, ranging from 9 to 21, for a total of 124 alleles among the 539 samples of *Sesbania tomentosa*. Each locus had only three to four alleles with a frequency greater than 0.1, and these most common alleles had average frequencies per locus that ranged from 0.17–0.28 (with a maximum across loci of 0.46). None of the 36 tests for multiple comparisons between loci (genotypic disequilibrium) in GENEPOP were significant at the 5% nominal level after Bonferroni corrections when averaged over all populations. Thus, the different microsatellite loci can be considered to provide independent information on population structure. Significant genotypic disequilibrium was detected for 27 out of 36 pairs of loci when each population was analyzed separately. This was most predominately found in the populations at ‘Āpua point (12 pairs of loci) and Mahana bay (8 pairs), and to a lesser extent in populations at Ka Lae (3 pairs), Pu‘u Koa‘e (2 pairs) and Ka‘ena point (2 pairs; data not shown).

MICROCHECKER indicated that there was a general excess of homozygotes evenly distributed across allele size classes in 280 out of 342 (38 × 9) population-locus combinations, an indication of possible null alleles or false homozygotes in the data set (data not shown).



Estimated frequencies of null alleles per locus per population (using the ENA method implemented in FREENA) ranged from 0.00 to 0.42 (the exception being the Ka‘ena point populations that ranged from 0.97 to 1.00 at locus A122). When averaged over loci, the frequency of null alleles in the 38 populations varied from 0.0006 to 0.2950. The mean null allele frequency over all populations and loci was 0.12.

#### Non-random mating and genetic diversity within populations

After Bonferroni corrections, all nine loci had significant heterozygote deficiencies at the 5% nominal level as compared to Hardy-Weinberg equilibrium (HWE) within 9 to 25 out of 38 populations. In total, there were 39 instances where a locus showed significant departure from HWE within a population and 103 instances where a locus, variable in other populations, became fixed for an allele (data not shown). When averaged over all nine loci, 22 out of 38 populations had significant heterozygote deficiencies at the Bonferroni corrected nominal level ( $\alpha_{0.05} = 0.00015$ ; Table 3.5). Inbreeding coefficients averaged over nine loci ranged from a relatively low level ( $f = 0.188$ ) in the large population on Nihoa (estimated 3,000–5,000 individuals; USFWS, 2010), to extremely high rates of inbreeding ( $f = 0.791$ – $0.943$ ) in the small remnant sub-populations (9–29 individuals extant in each at the time of sampling) scattered along the southern coast of Hawai‘i Island from Kamilo point to Ka Lae (Table 3.5). Another population that exhibited high inbreeding was that at ‘Āpua point ( $f = 0.7$ ), a much larger population along the southern coast of Hawai‘i Island (58 individuals sampled out of a total of 125 extant plants).

Expected/observed heterozygosities ranged from 0.148/0.000 (Nākālele point, Maui) to 0.778/0.583 (Makakupa‘ia, Moloka‘i). Mean number of alleles per locus/mean allelic richness (averaged over loci) ranged from 1.1/1.2 (Ka‘ena point NAR, O‘ahu) to 7.56/2.8 (Kawela, Moloka‘i); Table 3.6). These four populations of *Sesbania tomentosa* are therefore at either extremes of the range of genetic diversity observed. The 21 populations exhibiting the lowest levels of diversity ( $H_E \leq 0.2$ ) harbored 79 out of 89 of the monomorphic loci observed in this study (Table 3.5). On the other end of the spectrum, private alleles occurred in 10 out of 38 populations, most notably in the Ka Lae, Kawela and Kamiloloa populations (Table 3.6).

Table 3.5. Heterozygote deficiency and inbreeding statistics of *Sesbania tomentosa* populations.  $n/N$ , sample size.  $f$ , Weir and Cockerham's (1984) inbreeding coefficient. Significant  $P$ -values for a test of the hypothesis of heterozygote deficiency in GENEPOP combined across loci are indicated in bold using Bonferroni corrected  $P$ -values ( $\alpha_{0.05} = 0.00015$ ). Number of loci significant in GENEPOP test at  $\alpha_{0.05}$ . ML, monomorphic loci; loci harboring only one allele in a given population, and is out of a total of nine loci.

Population	Island	$n/N$	$f$	$P$ -value (GENEPOP)	# of loci significant	ML
Kīpuka Nēnē makai	Hawai'i	12	-1.000	1.0000		5
Kīpuka Nēnē mauka	Hawai'i	6	0.074	0.1705		1
Hilina pali cluster 1	Hawai'i	8	0.509	<b>0.0000</b>	4	1
Hilina pali cluster 2	Hawai'i	6	0.634	<b>0.0000</b>	3	2
Hilina pali fuel break rd.	Hawai'i	3	0.286	0.3351		5
Pepeiau	Hawai'i	10	0.297	<b>0.0000</b>	2	1
Kukalau'ula pali	Hawai'i	9	0.430	<b>0.0000</b>	6	
Kamo'oali'i	Hawai'i	13	0.524	<b>0.0000</b>	7	
Kū'ē'ē	Hawai'i	5	0.500	<b>0.0000</b>	4	
Āpua point	Hawai'i	58	0.700	<b>0.0000</b>	7	1
Kamilo point	Hawai'i	9	0.847	<b>0.0000</b>	1	1
Mahana bay	Hawai'i	29	0.922	<b>0.0000</b>	9	
Kīpuka Hanalua	Hawai'i	12	0.943	<b>0.0000</b>	9	
Ka Lae	Hawai'i	29	0.791	<b>0.0000</b>	9	
Waiaka'īlio	Hawai'i	8	0.153	0.0929		
Waiaka'īlio seedbank	Hawai'i	10	0.605	<b>0.0000</b>	2	4
Pu'u Koa'e	Kaho'olawe	25	0.467	<b>0.0000</b>	8	
Pu'u Pīmoe	Maui	9	0.306	0.0004	2	
Papanalaoa	Maui	37	0.258	<b>0.0000</b>	4	1
Mōkōlea point	Maui	5	0.091	0.2062		6
Nākālele point	Maui	2	1.000	0.1116		7
Kawela	Moloka'i	17	0.387	<b>0.0000</b>	7	
Kamiloloa	Moloka'i	14	0.517	<b>0.0000</b>	8	
Makakupa'ia	Moloka'i	4	0.280	0.0011	1	
Moloka'i ranch rd.	Moloka'i	14	0.666	<b>0.0000</b>	5	
Nature Conservancy preserve	Moloka'i	3	0.507	0.0032		
Mo'omomi pavillion	Moloka'i	9	0.479	0.0002	1	5
Mo'omomi herbarium	Moloka'i	10	0.326	<b>0.0000</b>	4	
Kāohikaipu	O'ahu	2	-0.500	1.0000		7
Mōkapu	O'ahu	4	0.468	0.0037		3
Ka'ena point State Park	O'ahu	15	0.599	<b>0.0000</b>	3	4
Ka'ena point outplantings	O'ahu	32	0.415	<b>0.0000</b>	3	5
Ka'ena point NAR	O'ahu	18	-0.299	1.0000		8
Polihale State Park (2006)	Kaua'i	16	0.331	0.0168		6
Polihale State Park (2009)	Kaua'i	11	0.698	<b>0.0000</b>	6	3
Polihale State Park (2010)	Kaua'i	12	0.734	<b>0.0000</b>	4	5

Table 3.5. (Continued) Heterozygote deficiency and inbreeding statistics of *Sesbania tomentosa* populations.

Population	Island	$n/N$	$f$	$P$ -value (GENEPOP)	# of loci significant	ML
Mānā	Kauaʻi	4	0.600	0.1244		7
Nihoa	Nihoa	49	0.188	0.0005	3	1

Table 3.6. Genetic diversity statistics of *Sesbania tomentosa* populations.  $n$ , sample size;  $A$  and  $A_R$ , mean number of alleles per locus and mean allelic richness (averaged over loci) respectively;  $H_E$  and  $H_O$ , expected and observed heterozygosity respectively.

Population	Island	$n$	$A$	$A_R$	Private alleles	$H_O$	$H_E$
Kīpuka Nēnē makai	Hawai‘i	12	1.44	1.36		0.444	0.232
Kīpuka Nēnē mauka	Hawai‘i	6	2.22	1.77		0.370	0.397
Hilina pali cluster 1	Hawai‘i	8	2.89	2.11		0.278	0.546
Hilina pali cluster 2	Hawai‘i	6	2.67	1.91		0.185	0.476
Hilina pali fuel break rd.	Hawai‘i	3	1.44	1.38		0.185	0.244
Pepeiau	Hawai‘i	10	3.44	2.01		0.356	0.498
Kukalau‘ula pali	Hawai‘i	9	5.00	2.48		0.395	0.675
Kamo‘oali‘i	Hawai‘i	13	5.11	2.26	1	0.291	0.598
Kū‘ē‘ē	Hawai‘i	5	3.33	2.29	1	0.333	0.630
‘Āpua point	Hawai‘i	58	2.56	1.70		0.117	0.387
Kamilo point	Hawai‘i	9	2.00	1.39		0.037	0.230
Mahana bay	Hawai‘i	29	2.67	1.65		0.031	0.388
Kīpuka Hanalua	Hawai‘i	12	3.11	1.88		0.028	0.463
Ka Lae	Hawai‘i	29	4.33	1.98	4	0.103	0.488
Waiaka‘īlio	Hawai‘i	8	2.78	1.55	1	0.276	0.322
Waiaka‘īlio seedbank	Hawai‘i	10	1.89	1.21		0.060	0.145
Pu‘u Koa‘e	Kaho‘olawe	25	3.78	1.95	1	0.271	0.504
Pu‘u Pīmoe	Maui	9	3.78	2.21	1	0.420	0.594
Papanalahoa	Maui	37	2.56	1.70		0.294	0.395
Mōkōlea point	Maui	5	1.44	1.25		0.111	0.121
Nākālele point	Maui	2	1.22	1.22		0.000	0.148
Kawela	Moloka‘i	17	7.56	2.80	7	0.480	0.773
Kamiloloa	Moloka‘i	14	6.56	2.74	5	0.360	0.732
Makakupa‘ia	Moloka‘i	4	4.11	2.73		0.583	0.778
Moloka‘i ranch rd.	Moloka‘i	14	2.56	1.74		0.143	0.417
Nature Conservancy preserve	Moloka‘i	3	2.44	2.16		0.333	0.607
Mo‘omomi pavillion	Moloka‘i	9	1.89	1.43	1	0.123	0.230
Mo‘omomi herbarium	Moloka‘i	10	4.56	2.55		0.485	0.705
Kāohikaipu	O‘ahu	2	1.22	1.22		0.167	0.130
Mōkapu	O‘ahu	4	2.11	1.66		0.194	0.341
Ka‘ena point State Park	O‘ahu	15	1.67	1.42		0.089	0.217
Ka‘ena point outplantings	O‘ahu	32	1.56	1.32		0.097	0.166
Ka‘ena point NAR	O‘ahu	18	1.11	1.12		0.076	0.059
Polihale State Park (2006)	Kaua‘i	16	1.33	1.22		0.083	0.123
Polihale State Park (2009)	Kaua‘i	11	2.33	1.55		0.092	0.294
Polihale State Park (2010)	Kaua‘i	12	1.67	1.41		0.065	0.236
Mānā	Kaua‘i	4	1.22	1.17		0.056	0.127
Nihoa	Nihoa	49	4.00	1.82	1	0.320	0.393

## Genetic structure of populations

Global  $F_{ST}(\theta)$  over all populations and loci was 0.509 ( $P \leq 0.0001$ ); correction for null alleles reduced this value slightly to 0.488 (Table 3.7). This analysis indicates that of the total genetic variation found across the range of the species, roughly half is ascribable to genetic difference (differences in allele frequencies) among populations, and the other half is found within any given population.

Using the program STRUCTURE and following the method of Evanno et al. (2005), two distinct genetic clusters were found among *Sesbania tomentosa* individuals sampled across all islands (Figures 3.2 and 3.3). The largest increase in the posterior probability occurred at  $K = 2$ , suggesting that this was the best model for the data. One genetic cluster corresponded to populations from Hawai‘i Island, Kaho‘olawe, Maui (excepting populations at Papanalaoa and Mōkōlea) and Moloka‘i (red cluster) and the other comprised individuals sampled from the Islands of O‘ahu, Kaua‘i and Nihoa, plus the populations at Papanalaoa and Mōkōlea, Maui (orange cluster; Figure 3.4). Most of the geographic populations sampled showed a high proportion of individuals assigned to a given cluster, generally from 95% to 100%. Populations sampled from Maui Nui (referring to the prehistorically contiguous island composed of Kaho‘olawe, Maui, Moloka‘i, and Lāna‘i; Price and Elliott-Fisk, 2004) assigned to the red cluster had proportions much lower (0.89 for Pu‘u Pīmoe; 0.86 for Kamiloloa; 0.84 for Makakupa‘ia; 0.60 for Mo‘omomi herbarium samples). These are levels of admixture higher than the 5% threshold that may be attributed to stochastic noise. In addition, cluster membership coefficients of Maui Nui individuals assigned to the red cluster also averaged low (0.83 for Pu‘u Pīmoe; 0.70 for Nākālele point; 0.83 for Kawela; 0.87 for Kamiloloa; 0.84 for Makakupa‘ia; 0.78 for Mo‘omomi herbarium samples). As a point of reference, 100% of Hawai‘i Island individuals were assigned to the red cluster with an average cluster membership coefficient of 0.97. When considering the populations comprising the orange cluster from O‘ahu, Kaua‘i and Nihoa, 100% of these individuals were assigned to the orange cluster with an average cluster membership coefficient of 0.97. The Maui populations assigned to the orange cluster were comprised of individuals whose average cluster membership coefficient was 0.86, and this coefficient was 0.90 when considering the individuals comprising the Nihoa population, so indications of admixture are also to be found in the orange cluster (Figure 3.4).

Table 3.7. Global  $F_{ST}(\theta)$  and  $F_{ST(ENA)}$  over all populations and loci.

<b>Locus</b>	<b>C5</b>	<b>A105</b>	<b>A123</b>	<b>C3</b>	<b>A122</b>	<b>A119</b>	<b>A128</b>	<b>C103</b>	<b>C106</b>	<b>Global</b>
$F_{ST}(\theta)$	0.516	0.521	0.472	0.588	0.321	0.451	0.509	0.613	0.572	0.509
$F_{ST(ENA)}$	0.521	0.497	0.452	0.575	0.314	0.413	0.457	0.59	0.541	0.488
$P$ -value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

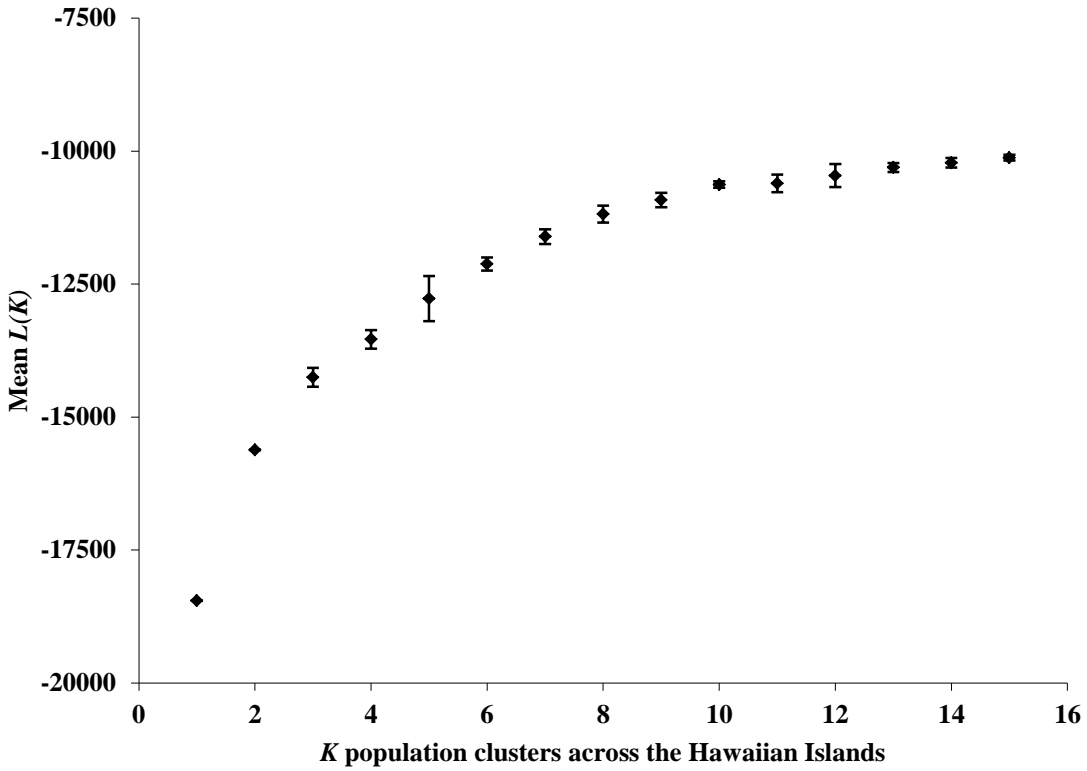


Figure 3.2. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

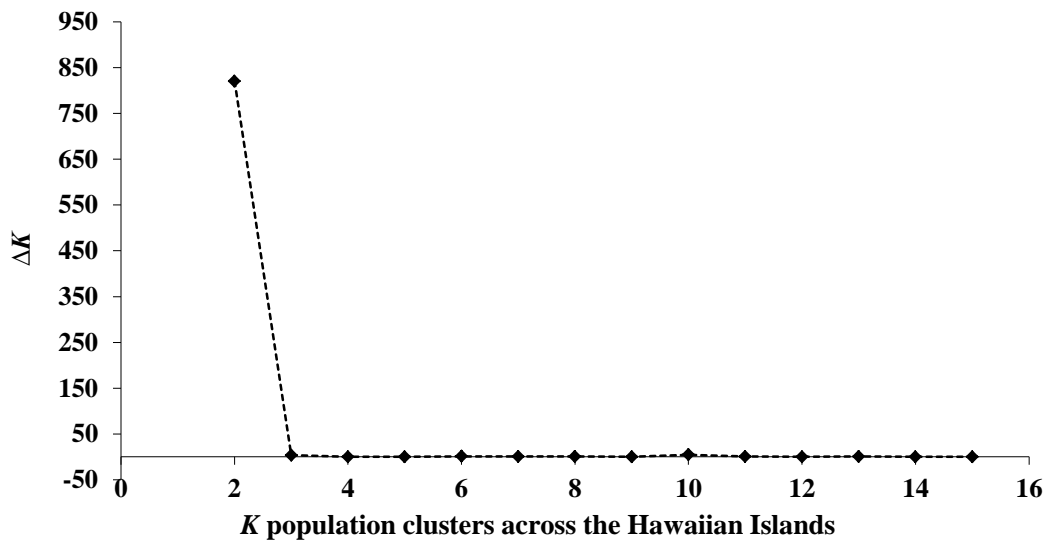


Figure 3.3. Magnitude of  $\Delta K$  (second-order rate of change of STRUcTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

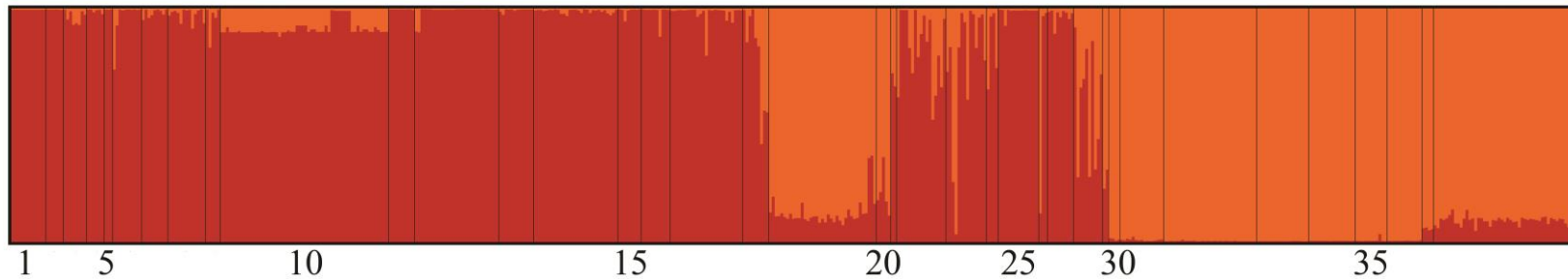


Figure 3.4. STRUCTURE graph for the most likely numbers of clusters of Hawaiian *Sesbania* according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic clusters (red and orange). Thin black lines distinguish the 38 sub-populations and populations: 1. Kīpuka Nēnē makai, 2. Kīpuka Nēnē mauka, 3. Hilina pali cluster 1, 4. Hilina pali cluster 2, 5. Hilina pali fuel break rd., 6. Pepeiau, 7. Kukalau‘ula pali, 8. Kamo‘oali‘i, 9. Kū‘ē‘ē, 10. ‘Āpua point, 11. Kamilo point, 12. Mahana bay, 13. Kīpuka Hanalua, 14. Ka Lae, 15. Waiaka‘īlio, 16. Waiaka‘īlio seedbank, 17. Pu‘u Koa‘e, 18. Pu‘u Pīmoe, 19. Papanalahoa, 20. Mōkōlea point, 21. Nākālele point, 22. Kawela, 23. Kamiloloa, 24. Makakupa‘ia, 25. Moloka‘i ranch rd., 26. Nature Conservancy preserve, 27. Mo‘omomi pavillion, 28. Mo‘omomi herbarium, 29. Kāohikaipu, 30. Mōkapu, 31. Ka‘ena point State Park, 32. Ka‘ena point NAR outplantings, 33. Ka‘ena point NAR, 34. Polihale State Park (2006), 35. Polihale State Park (2009), 36. Polihale State Park (2010), 37. Mānā, 38. Nihoa.



Further analysis of the two genetic clusters described above found additional levels of structure. Within the red cluster (of Figure 3.4), the largest increase in posterior probability occurred at  $K = 3$  (Figures 3.5 and 3.6) while the largest increase in the orange cluster (of Figure 3.4) occurred at  $K = 2$  (Figures 3.7 and 3.8). Within the red cluster, the first sub-cluster comprised populations from Hawai‘i Volcanoes National Park (excepting the population at Pepeiau), and hereafter referred to as the Hawai‘i Volcanoes sub-cluster. The second sub-cluster comprised populations on Hawai‘i Island in the South point Region (Kamilo point to Ka Lae) plus Pepeiau, hereafter the South point sub-cluster. The third sub-cluster comprised the small remnant North Kohala population on Hawai‘i Island (Waiaka‘īlio) plus the populations from Kaho‘olawe, Maui (excepting Papanalaho and Mōkōlea) and Moloka‘i, hereafter the Maui Nui sub-cluster (Figure 3.9).

Levels of admixture were relatively high in the populations at Pepeiau (proportion of individuals assigned to South point sub-cluster was 0.70) and Kukalau‘ula pali (proportion of individuals assigned to Hawai‘i Volcanoes sub-cluster was 0.78) with average individual cluster membership coefficients of 0.76 and 0.84, respectively. Indications of admixture were also high in the Kamo‘oali‘i and Kū‘ē‘ē populations (proportion of individuals assigned to Hawai‘i Volcanoes sub-cluster were 0.85 and 0.80 with average individual cluster membership coefficients of 0.81 and 0.64, respectively). At Mahana bay, 96% of individuals were assigned to the South point subcluster, although the average individual cluster membership coefficient was only 0.77 (Figure 3.9). Indications of admixture were also apparent in populations on Moloka‘i (average individual cluster membership coefficients for the Kawela, Makakupa‘ia, Moloka‘i ranch road and Mo‘omomi Nature Conservancy preserve populations in the Maui Nui sub-cluster were 0.89, 0.78, 0.75 and 0.90, respectively). At Moloka‘i Ranch Rd., the proportion of individuals assigned to Maui Nui sub-cluster was 0.78, plus two individuals failed to be assigned to any cluster at the 0.5 cut-off. When considering the ten historically collected samples from Mo‘omomi individual cluster assignments varied widely (indicating admixture). Taken as a whole, these ten samples were not definitively assigned to any one particular genetic sub-cluster (again, two individuals failed to be assigned to any sub-cluster at the 0.5 cut-off; Figure 3.9).

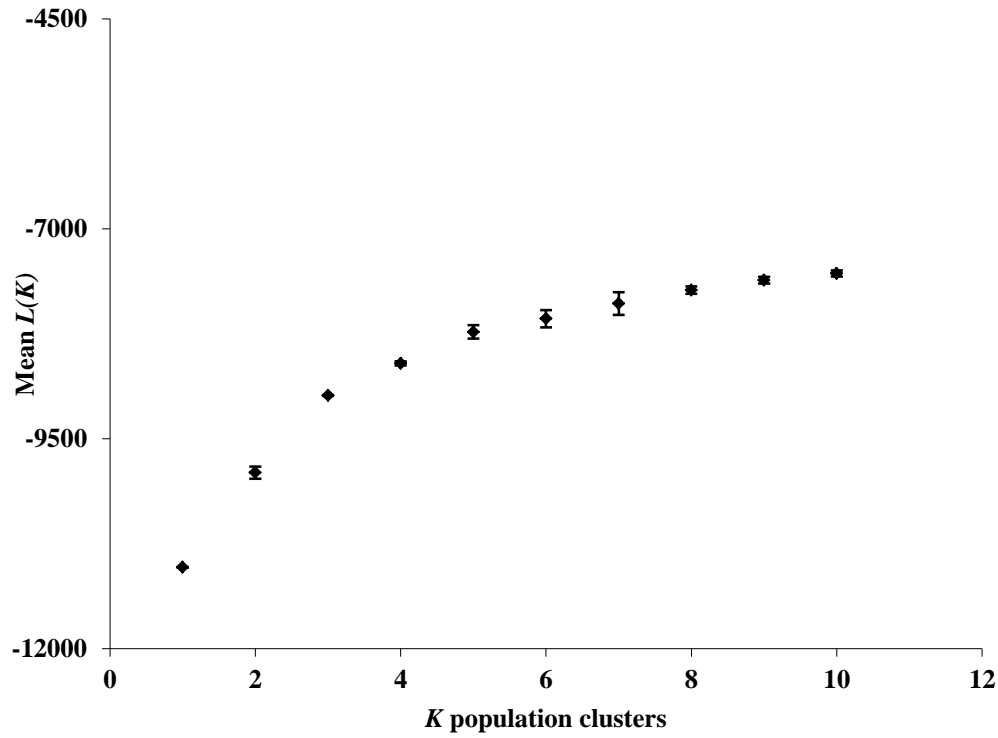


Figure 3.5. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

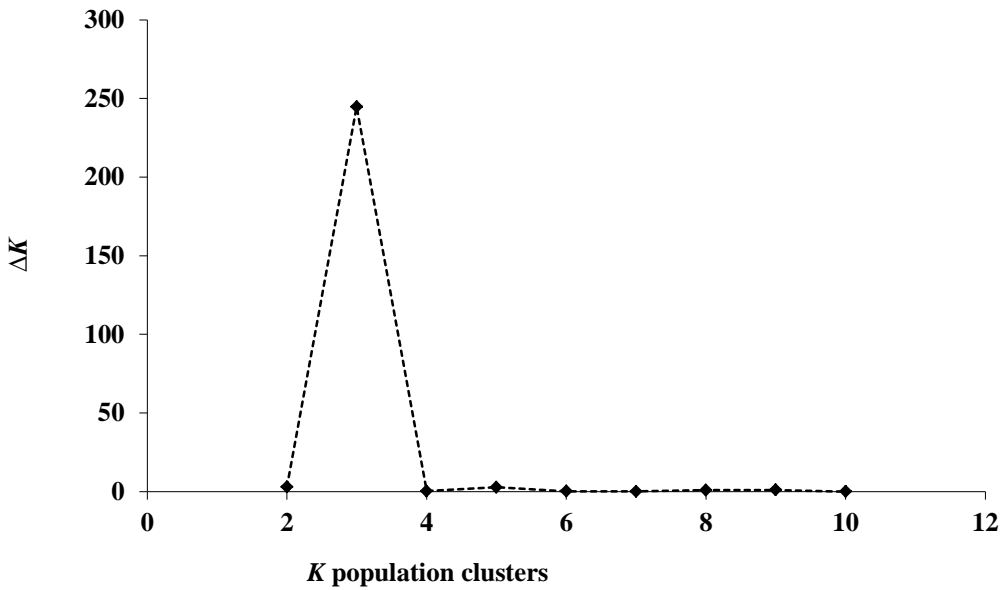


Figure 3.6. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

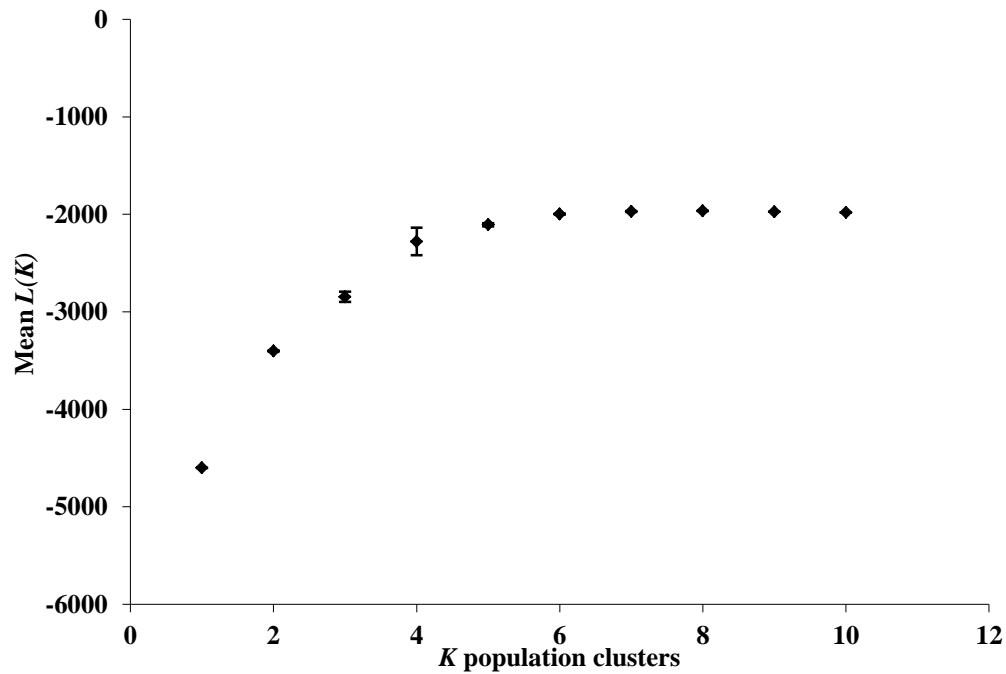


Figure 3.7. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

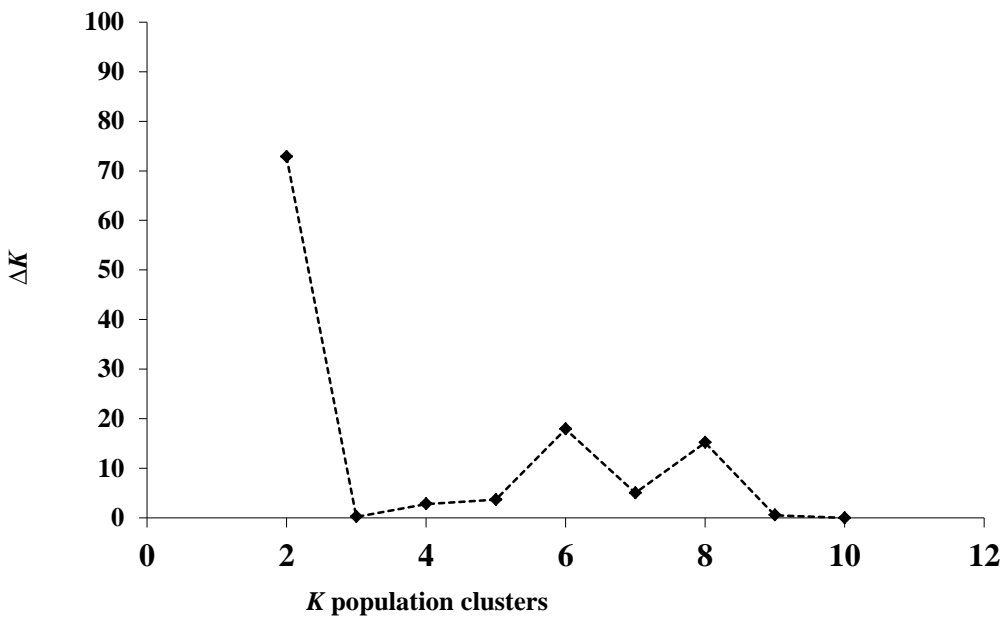


Figure 3.8. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

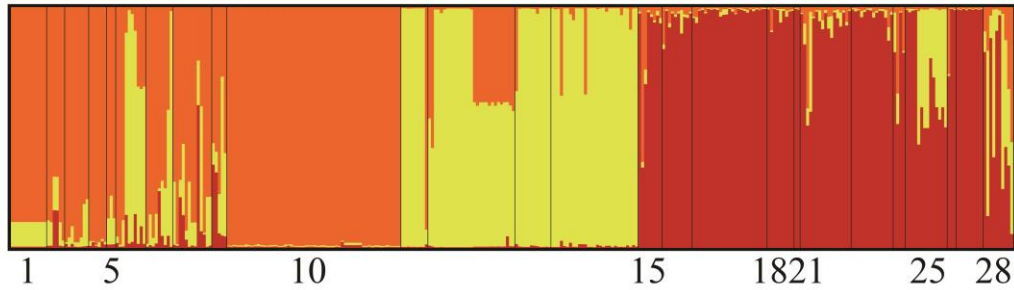


Figure 3.9. STRUCTURE graph for the most likely number of sub-clusters in the red cluster of Figure 3.4 according to the  $\Delta K$  method ( $K = 3$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 3 genetic sub-clusters. Thin black lines distinguish the 26 sub-populations and populations: 1. Kīpuka Nēnē makai, 2. Kīpuka Nēnē mauka, 3. Hilina pali cluster 1, 4. Hilina pali cluster 2, 5. Hilina pali fuel break rd., 6. Pepeiau, 7. Kukalau‘ula pali, 8. Kamo‘oali‘i, 9. Kū‘ē‘ē, 10. ‘Āpua point, 11. Kamilo point, 12. Mahana bay, 13. Kīpuka Hanalua, 14. Ka Lae, 15. Waiaka‘īlio, 16. Waiaka‘īlio seedbank, 17. Pu‘u Koa‘e, 18. Pu‘u Pīmoe, 21. Nākālele point, 22. Kawela, 23. Kamiloloa, 24. Makakupa‘ia, 25. Moloka‘i ranch rd., 26. Nature Conservancy preserve, 27. Mo‘omomi pavillion, 28. Mo‘omomi herbarium.

It is important to note that the height of the modal value  $\Delta K$  in Figure 3.8 ( $\Delta K = 72.8$  at  $K = 2$ ) is an indicator of the strength of the signal detected by STRUCTURE (Evanno et al., 2005), in this case significantly weaker than the previous two analyses ( $\Delta K = 244.7$  at  $K = 3$  in Figure 3.6 and  $820.4$  at  $K = 2$  in Figure 3.3). Two relatively distinct groups characterize the STRUCTURE plot: the O‘ahu populations cluster with the Polihale (Kaua‘i) population, and the NW Maui populations (Papanalaoa and Mōkōlea) cluster with the Mānā (Kaua‘i) and Nihoa population (Figure 3.10).

#### Isolation by distance between and within populations

There was a significant correlation between genetic and geographic distances ( $r^2 = 0.363$ ,  $P < 0.0001$ ), indicating a pattern of isolation by distance (IBD) among populations of *Sesbania tomentosa* across the Hawaiian Islands (Figure 3.11). Using spatial analysis of kinship coefficients between individuals, there was agreement with the model of isolation by distance in that a significant linear decrease of estimated pairwise kinship coefficients with the logarithm of increasing geographical distance was detected in all nine aggregate (combined) populations tested ( $P < 0.01$ ; Table 3.8). When looking at the individual populations on a smaller scale (i.e., within individual population clusters separated by  $> 2$  km), 10 of the 27 populations tested significantly for the relationship at the 0.01 level, and an additional four were significant at the 0.05 level. With the exception of lower (and in a few cases, higher) average kinship coefficients between adjacent individuals, none of these test results differed when duplicate individuals were omitted from the analysis (data not shown). The 13 remaining (non-significant) populations had low census sizes ( $\leq 18$  individuals were compared in each), which are expected to have substantially biased the estimator (Ritland, 1996).

#### Indirect estimates of genetic bottlenecks

The Wilcoxon tests carried out in BOTTLENECK revealed evidence for a rapid loss of genetic diversity in four populations (Kīpuka Nēnē makai, Hilina pali cluster 1, Hilina pali fuel break road and Polihale (2010) populations based on the three mutation models examined). These same populations revealed a mode shift away from an L-shaped distribution of alleles, a

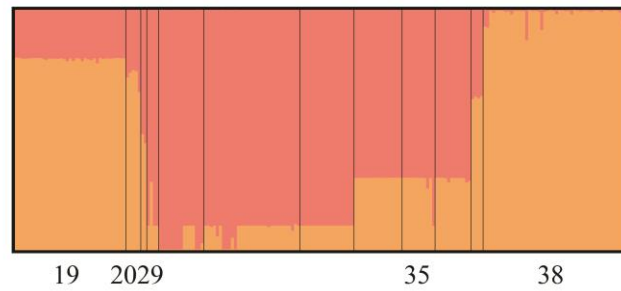


Figure 3.10. STRUCTURE graph for the most likely number-of sub-clusters in the orange cluster of Figure 3.4 according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Thin black lines distinguish the 12 sub-populations/populations: 19. Papanalahoa, 20. Mōkōlea point, 29. Kāohikaipu, 30. Mōkapu, 31. Ka‘ena point State Park, 32. Ka‘ena point NAR outplantings, 33. Ka‘ena point NAR, 34. Polihale State Park (2006), 35. Polihale State Park (2009), 36. Polihale State Park (2010), 37. Mānā, 38. Nihoa.

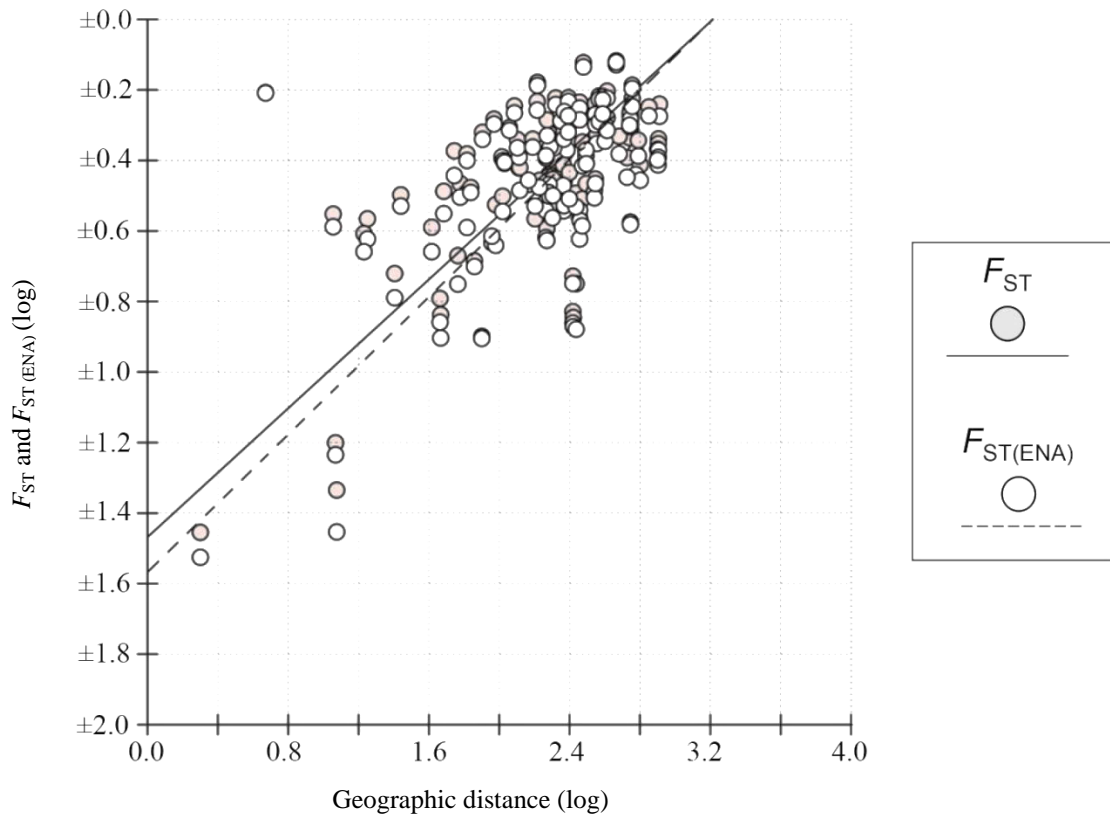


Figure 3.11. Significant correlation of log-transformed  $F_{ST}$  (Weir and Cockerham, 1984) and  $F_{ST(ENA)}$  (Chapuis and Estoup, 2007) over all loci with log-transformed geographic distance (km). Mantel test,  $r^2 = 0.363$ ,  $P < 0.0001$  (both analyses).

Table 3.8. Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.  $F_1$ , average kinship coefficient between adjacent individuals (i.e. first distance interval);  $b_{r0}$ , slope of the regression of pairwise kinship coefficients on the logarithm of geographical distance;  $P$ -value of the one-sided Mantel test with  $H_0$ : observed  $b_{r0} = 0$ , significant values (at 0.01 level) listed in bold. NA indicates analysis not applicable due to uniform genotypes across a given population.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{r0}$	$P$ -value
Hawai‘i	Kīpuka Nēnē–Hilina pali		595	0–5	0.3252	-0.0912	<b>0.0001</b>
		Kīpuka Nēnē makai	66	0–5	1.0000	NA	NA
		Kīpuka Nēnē mauka	15	20–25	0.3748	-0.3523	0.0562
		Hilina pali cluster 1	28	15–20	0.4090	-0.2907	<b>0.001</b>
		Hilina pali cluster 2	15	20–25	-0.2094	0.0533	0.6379
		Hilina pali fuel break rd.	3	5–10	0.3818	-0.4013	0.3336
	Pepeiau–Kukalau‘ula pali		171	10–15	0.1469	-0.0459	<b>0.0001</b>
		Pepeiau	45	20–25	0.1748	-0.0992	<b>0.001</b>
		Kukalau‘ula pali	36	200–500	0.1088	-0.0528	<b>0.01</b>



Table 3.8. (Continued) Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{ro}$	$P$ -value
Hawai‘i	Kamo‘oali‘i–Kū‘ē‘ē	Kamo‘oali‘i Kū‘ē‘ē	153	50–75	0.3707	-0.0300	<b>0.007</b>
			78	100–200	0.0829	0.0118	0.66
			10	50–75	0.1111	-0.1386	0.02
		‘Āpua point	1653	0–5	0.7661	-0.2724	<b>0.0001</b>
	Hawai‘i Volcanoes National Park TOTAL		8385	0–5	0.6355	-0.0714	<b>0.0001</b>
		Kamilo point	36	0–5	0.2371	-0.1102	0.0395
	Mahana bay–Ka Lae	Mahana bay Kīpuka Hanalua Ka Lae	2415	0–5	0.5815	-0.0925	<b>0.0001</b>
406			0–5	0.9311	-0.3553	<b>0.0001</b>	
66			0–5	0.1715	-0.0914	0.05	
406			0–5	0.0061	-0.0240	0.024	
Maui		Pu‘u Pīmoē	36	0–5	-0.0135	-0.0106	0.3506
	Papanalaho–Nākālele point	Papanalaho point Mōkōlea point Nākālele point	903	0–5	0.3139	-0.0561	<b>0.0001</b>
			630	0–5	0.1762	-0.0711	<b>0.0001</b>
			10	0–5	-0.0215	0.0094	0.6989
			1	0–5	-0.6667	NA	NA

Table 3.8. (Continued) Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{ro}$	$P$ -value
Moloka'i	Kawela–Kamiloloa		595	0–5	0.0779	-0.0172	<b>0.0001</b>
		Kawela	136	0–5	-0.0874	-0.0138	<b>0.01</b>
		Kamiloloa	91	0–5	-0.2540	-0.0096	0.208
		Makakupaia	6	15–20	0.0614	-0.0786	0.1697
	Mo'omomi		231	0–5	0.8278	-0.1039	<b>0.0001</b>
		Moloka'i ranch rd.	91	0–5	0.7488	-0.3432	<b>0.0002</b>
		Nature Conservancy preserve	3	10–15	0.4256	-0.1910	0.3362
		Mo'omomi pavillion	10	15–20	0.6111	-0.0437	0.1583
O'ahu	Ka'ena point		2016	0–5	0.4470	-0.1166	<b>0.0001</b>
		Ka'ena point State Park	105	0–5	0.7154	-0.1717	<b>0.001</b>
		Ka'ena point NAR outplantings	465	0–5	0.1726	-0.0957	<b>0.0003</b>
		Ka'ena point NAR	153	0–5	-0.0022	-0.0048	0.3229
Kaua'i		Polihale State Park (2006)	120	0–5	0.1225	-0.0270	0.1813
		Polihale State Park (2010)	66	0–5	0.0370	-0.0425	0.076

trend observed in an additional 15 populations as well (Table 3.9). When the dataset was corrected for the presence of null alleles, none of the Wilcoxon tests was significant and only three populations remained divergent from the L-shaped distribution. On the other hand, 31 out of 38 populations had an  $M$ -ratio suggestive of a history of bottlenecks.  $M$ -ratios below 0.68 were found in every population where the number of sampled individuals was sufficiently large ( $M$ -ratios above 0.68 were only found in populations  $\leq 14$  individuals), with the exception of the Ka‘ena point NAR outplantings ( $n = 32$ ) and ‘Āpua point ( $n = 58$ ).

#### Modeling genetic drift in Ka‘ū

The largest natural landscape left in Hawai‘i where some degree of connectivity between populations of *Sesbania tomentosa* could potentially still occur is in the Ka‘ū district of Hawai‘i Island, including the populations within the boundaries of Hawai‘i Volcanoes National Park down into the South point region. Using the coalescent modeling program 2<sub>MOD</sub>, a genetic drift model for populations of *S. tomentosa* across the Ka‘ū district was seven times more likely than the gene flow model [ $P$  (genetic drift) =  $0.88 \pm 0.0004$ , Bayes factor = 6].

#### Direct observations of genetic drift at Mo‘omomi, Moloka‘i

Mean expected and observed heterozygosity in the modern collections of *Sesbania tomentosa* at the three Mo‘omomi populations ( $n = 26$ ) declined when referenced against the historically collected samples ( $n = 10$ ; Table 3.6). Mean number of alleles per locus and allelic richness also both declined. The historic samples revealed seven more alleles total (across the nine loci) than did the three contemporary population samples combined. In addition, there are 20 “ghost alleles” across the nine loci, alleles that occurred in the samples collected 60–100 years ago that were not present at Mo‘omomi during an entire census collection in 2006 (Figure 3.12).

Table 3.9. Three tests for genetic bottlenecks in *Sesbania tomentosa* populations. *M*-ratio (Garza and Williamson 2001) is the number of alleles divided by range in allele size, averaged over 9 loci. Mode shift indicates deviation from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium. Wilcoxon tests for heterozygote excess (Piry et al., 1999) under three mutation models (step-wise mutation, SMM; two phase model, TPM; infinite alleles model, IAM). The latter two tests were duplicated using alternate allele frequency datasets corrected for the presence of null alleles (using the Expectation Maximization algorithm of Dempster et al., 1977). Values highlighted in bold are those indicative of bottlenecks ( $P \leq 0.05$  for the Wilcoxon tests and an *M*-ratio  $< 0.68$ )

Island	Population	<i>M</i> -ratio	Mode shift	Wilcoxon tests:			CORRECTED FOR NULL ALLELES			
				SMM	TPM	IAM	Mode shift	SMM	TPM	IAM
Hawai'i Island	Kīpuka Nēnē makai	<b>0.683</b>	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	<b>shifted</b>	0.935	0.935	0.935
	Kīpuka Nēnē mauka	<b>0.695</b>	<b>shifted</b>	0.527	0.422	0.320	normal	0.613	0.511	0.432
	Hilina pali cluster 1	<b>0.559</b>	<b>shifted</b>	<b>0.021</b>	<b>0.011</b>	<b>0.006</b>	<b>shifted</b>	0.918	0.787	0.787
	Hilina pali cluster 2	<b>0.595</b>	normal	0.148	0.148	0.148	normal	0.986	0.981	0.936
	Hilina pali fuel break rd.	0.767	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	<b>shifted</b>	0.935	0.935	0.935
	Pepeiau	<b>0.649</b>	normal	0.809	0.473	0.229	normal	0.999	0.999	0.997
	Kukalau'ula pali	0.718	normal	0.918	0.545	0.149	normal	0.988	0.711	0.223
	Kamo'oali'i	<b>0.579</b>	normal	0.999	0.981	0.633	normal	1.000	1.000	0.999
	Kū'ē'ē	<b>0.602</b>	<b>shifted</b>	0.248	0.082	0.064	normal	0.353	0.211	0.167
	'Āpua point	0.716	normal	0.319	0.156	0.014	normal	0.589	0.410	0.101
	Kamilo point	<b>0.635</b>	<b>shifted</b>	1.000	0.996	0.994	normal	1.000	1.000	1.000
	Mahana bay	<b>0.656</b>	normal	0.500	0.213	0.082	normal	0.752	0.285	0.082
	Kīpuka Hanalua	<b>0.566</b>	normal	0.918	0.715	0.455	normal	0.986	0.898	0.715
	Ka Lae	<b>0.674</b>	normal	0.981	0.849	0.326	normal	0.990	0.918	0.455
	Waiaka'īlio	<b>0.485</b>	normal	0.998	0.997	0.981	normal	1.000	1.000	1.000
	Waiaka'īlio seedbank	<b>0.489</b>	normal	0.984	0.984	0.969	normal	0.999	0.999	0.999

Table 3.9. (Continued) Three tests for genetic bottlenecks in *Sesbania tomentosa* populations.

Island	Population	<i>M</i> -ratio	Mode shift	Wilcoxon tests:			CORRECTED FOR NULL ALLELES			
				SMM	TPM	IAM	Mode shift	SMM	TPM	IAM
Maui Nui	Pu‘u Koa‘e	<b>0.552</b>	normal	0.849	0.455	0.125	normal	0.918	0.545	0.367
	Pu‘u Pīmoē	<b>0.567</b>	<b>shifted</b>	0.918	0.411	<b>0.024</b>	normal	0.999	0.998	0.995
	Papanalahoa	<b>0.460</b>	normal	0.231	0.019	<b>0.006</b>	normal	0.935	0.715	0.326
	Mōkōlea point	<b>0.556</b>	<b>shifted</b>	0.937	0.937	0.813	normal	1.000	1.000	1.000
	Nākālele point	0.750	<b>shifted</b>	0.125	0.125	0.125	normal	0.912	0.912	0.912
	Kawela	<b>0.651</b>	normal	0.367	0.248	0.082	normal	0.997	0.976	0.684
	Kamiloloa	0.714	normal	0.545	0.326	0.179	normal	0.986	0.898	0.82
	Makakupa‘ia	<b>0.595</b>	<b>shifted</b>	0.367	0.326	0.213	normal	0.532	0.511	0.489
	Moloka‘i ranch rd.	<b>0.659</b>	<b>shifted</b>	0.411	0.179	<b>0.018</b>	normal	0.633	0.500	0.326
	Nature Conservancy preserve	<b>0.616</b>	<b>shifted</b>	0.064	0.064	0.064	normal	0.912	0.912	0.912
	Mo‘omomi pavillion	<b>0.429</b>	<b>shifted</b>	0.437	0.437	0.094	normal	0.999	0.999	0.997
	Mo‘omomi herbarium	<b>0.620</b>	<b>shifted</b>	0.082	0.064	0.064	normal	0.934	0.911	0.911
O‘ahu	Kāohikaipu	<b>0.533</b>	<b>shifted</b>	0.25	0.25	0.25	normal	0.900	0.900	0.900
	Mōkapu	<b>0.411</b>	<b>shifted</b>	0.578	0.578	0.578	normal	0.950	0.950	0.950
	Ka‘ena point State Park	<b>0.630</b>	<b>shifted</b>	0.313	0.109	0.109	normal	0.935	0.935	0.935
	Ka‘ena point outplantings	0.719	<b>shifted</b>	0.906	0.438	0.063	normal	1.000	0.612	0.450
	Ka‘ena point NAR	<b>0.510</b>	normal	1.0	0.25	0.25	normal	1.000	0.900	0.900
Kaua‘i	Polihale State Park (2006)	<b>0.611</b>	<b>shifted</b>	0.125	0.063	0.063	normal	0.999	0.998	0.997
	Polihale State Park (2009)	0.783	normal	0.781	0.656	0.344	normal	0.997	0.995	0.986
	Polihale State Park (2010)	<b>0.588</b>	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	normal	0.936	0.936	0.936
	Mānā	<b>0.643</b>	<b>shifted</b>	0.125	0.125	0.125	normal	0.922	0.922	0.922
Nihoa	Nihoa	<b>0.695</b>	normal	0.991	0.578	0.371	normal	0.999	0.993	0.787

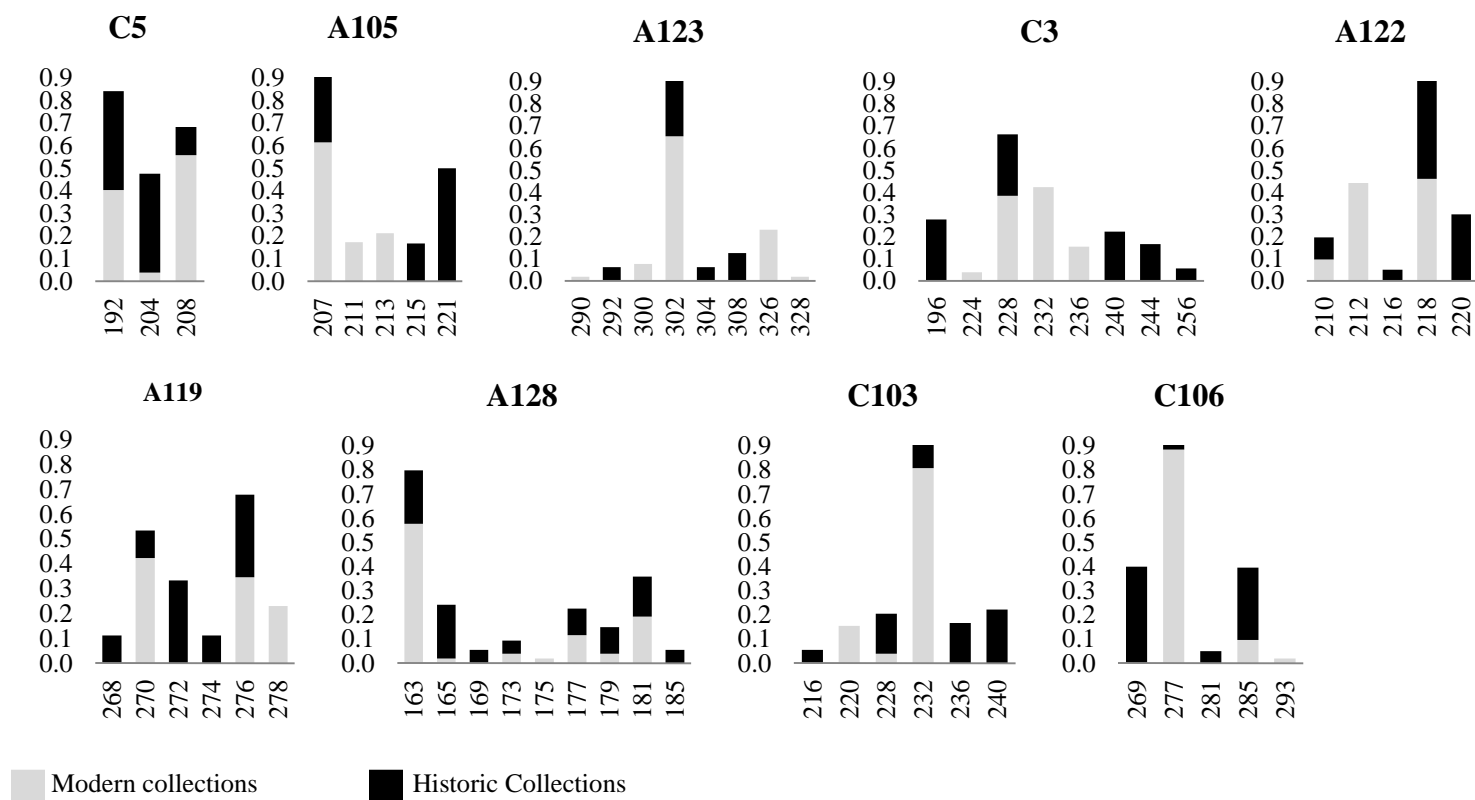


Figure 3.12. A comparison of allele frequencies for *Sesbania tomentosa* at nine microsatellite loci (C5, A105, A123, C3, A122, A119, A128, C103 and C106) sampled from 26 individuals at Mo'omomi Moloka'i (2006) vs. 10 historical samples collected 60–100 years prior. Frequencies listed on y-axes; alleles listed on x-axes.

## The dynamic nature of the gene pool at Polihale, Kauaʻi

Since all extant individuals of *Sesbania tomentosa* were sampled from the Polihale population on two out of three occasions (spanning 4 years), it is possible to observe changes in the genetic makeup of populations of this rapidly reproducing plant species over time. All measures of genetic diversity rose from levels seen in 2006 when sampling of the Polihale Kauaʻi population was repeated in 2009, yet then dropped again slightly in 2010 (Table 3.6). While the number of monomorphic loci (i.e., zero diversity at a locus) dropped from six in 2006 to three in 2009, this number rose again to five loci fixed for a single allele in 2010 (Table 3.5). More importantly, extant individuals sampled from 2009 contained nine additional alleles (at 6 loci) not found in individuals comprising the population in 2006 (Figure 3.13). By 2010, seven of these nine alleles were again lost, yet a completely new allele not seen in the previous two sampling years emerged to join the standing gene pool. All three mutation models employed in the BOTTLENECK program, plus an allele frequency mode shift and microsatellite repeat size range *M*-ratio were sensitive to and reflect this rapid real-time record of population decline at Polihale from 2009 to 2010 (Table 3.9).

## Discussion

### Maintenance of genetic diversity in spite of high levels of inbreeding

While private alleles occurred in 10 out of 38 populations, the three populations where the highest amount of exclusive genetic diversity was found exhibit interesting associations with accompanying levels of inbreeding. Limited sampling ( $n = 35$ ) of the demographically large Kawela and Kamiloloa populations [the combined sub-populations of SE Molokaʻi were believed to comprise 1,500–2,000 individuals in 2006 (USFWS, 2010)] exhibited the greatest number of private alleles, more than all the other populations combined (Table 3.6). Mean number of alleles per locus and allelic richness were also highest in these two population samples. High allelic diversity observed in the limited sampling of the SE Molokaʻi populations was accompanied by lower (yet still relatively high) rates of inbreeding and might be explained by a combination of two factors. The high population density of *Sesbania tomentosa* over a large

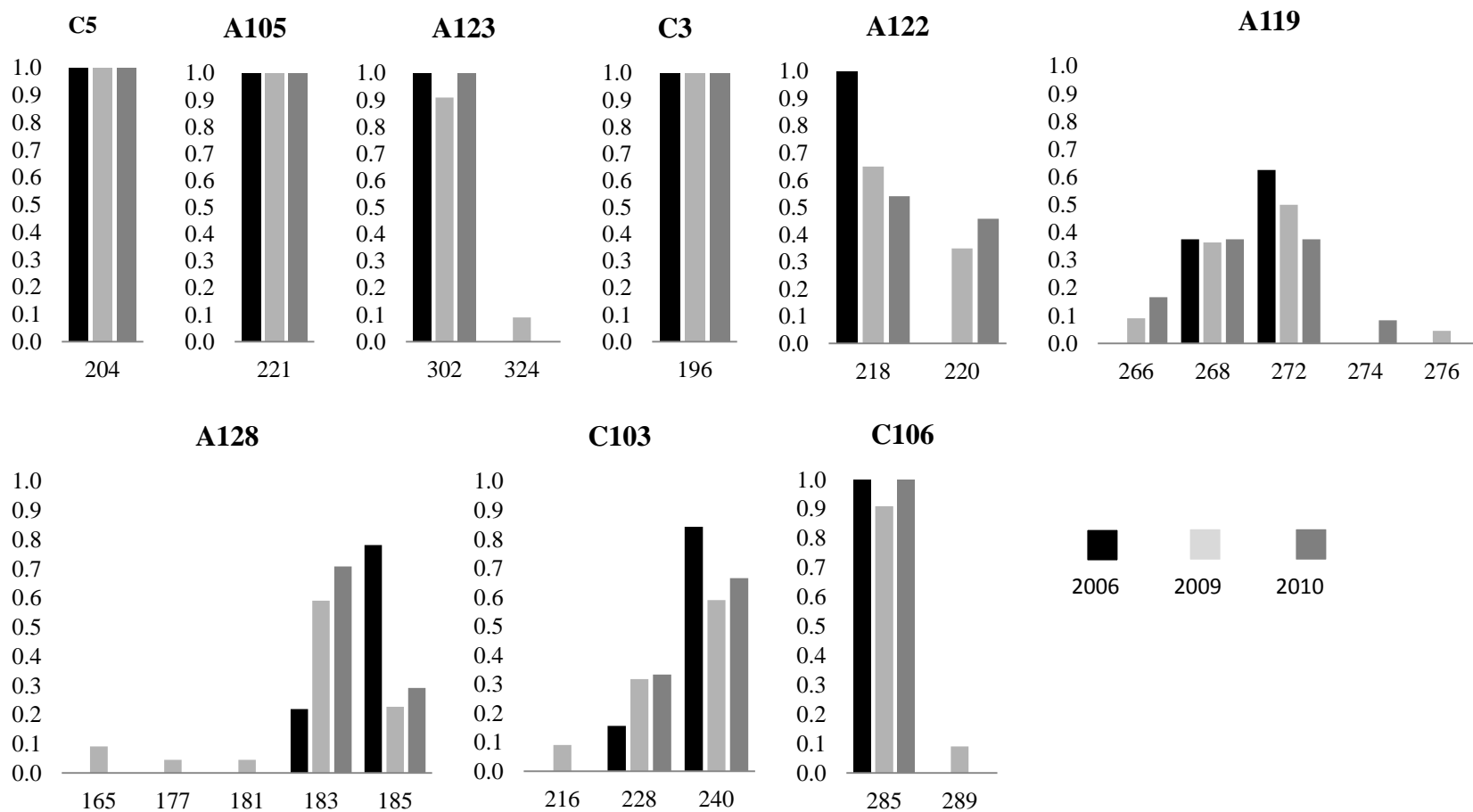


Figure 3.13. A comparison of allele frequencies for *Sesbania tomentosa* at nine microsatellite loci (C5, A105, A123, C3, A122, A119, A128, C103 and C106) sampled from all extant individuals of the Polihale Kaua'i population during visits in 2006, 2009 and 2010. Frequencies listed on y-axes; alleles listed on x-axes.



area ( $7 \times 3$  km; USFWS, 2010) would maintain higher allelic diversity than would a comparatively smaller population (Hamrick and Godt, 1989), yet high rates of genetic sub-structuring (as discussed below) would still result in a preponderance of non-random mating in the form of inbreeding.

The Ka Lae (Hawai'i Island) population is also interesting in that extremely high levels of inbreeding were accompanied by unexpectedly high levels of allelic diversity and the third highest occurrence of private alleles (on par with the previous two examples discussed). In order to explain this, reviewing the history of land use at Ka Lae is in order. On several occasions, Herbst (1972) found *Sesbania tomentosa* occurring exclusively within the stone fence that surrounded the SW corner of the point, a barrier that he felt protects the plants from cattle (*Bos taurus*) that have historically grazed nearby. This fence was erected circa 1908 when 10 acres of land were set aside for the lighthouse service (Love, 1991). In 1991, a similar observation was made noting 85 plants found exclusively within the stone enclosure (Hawai'i Biodiversity and Mapping Program). In 2006, samples were collected from plants both within the stone enclosure (17 plants extant at that time) as well as up to 100 m outside the stone enclosure (12 plants extant at that time), as cattle grazing in proximity to the Ka Lae enclosure ceased 20 years prior. Protection from grazing over the past hundred years within the enclosure might have preserved genotypes that would have otherwise been lost, maintaining allelic diversity over time beyond that of unprotected populations of similar size. The highest rates of inbreeding observed for *S. tomentosa* across the Hawaiian Islands were found at Ka Lae, as well as in small clusters of plants scattered along 10 km of coastline to the east.

#### Potential causes and impacts of high levels of inbreeding observed

Deficiency of heterozygotes is measured against the proportion of heterozygotes expected if the population's allele frequencies were in Hardy-Weinberg equilibrium, an ideal state providing a baseline against which to measure genetic change (Hartl and Clark, 2007). While 22 of the 38 populations had significant heterozygote deficiencies at the Bonferroni corrected nominal level, the 16 populations lacking detectable heterozygote deficiencies had an average sample size of 9.6 compared to 17.5 for the remaining 22 populations exhibiting significant heterozygote deficiencies at the nominal level. Small census (sample) size of certain populations

might be influencing these higher (insignificant)  $P$ -values. The exception is the large reproductive population at Nihoa, the only population with a large ( $> 20$ ) sample size ( $n = 49$  out of 3,000–5,000 individuals) that did not have a significant deficiency of heterozygotes (Table 3.5). There is reason to believe that non-random mating is the norm within *Sesbania tomentosa* populations across the main Hawaiian Islands, yet it is important to determine whether this is predominantly a natural or unnaturally-exacerbated phenomenon.

While there was a general excess of homozygotes evenly distributed across allele size classes in 280 out of 342 population-locus combinations, the presence of null alleles would only be suspected when some loci show significant excess of homozygotes while others do not deviate from Hardy-Weinberg proportions. In the present study, the consistency of the homozygote excess across the nine loci indicates that nonrandom mating (e.g., mating of close neighbors or self-pollination, both of which increase inbreeding) might be playing a role in amplifying estimates of null alleles.

The mean null allele frequency over all populations and loci was 0.12, interpreted as a “moderate” null allele frequency by Chapuis and Estoup (2007). Since the algorithms developed to estimate null alleles assume random mating (Dempster et al., 1977; Brookfield, 1996), these frequencies are probably overestimated as the evidence for non-random mating in populations of *Sesbania tomentosa* is overwhelming.

A correlation between the occurrence of linkage disequilibrium and levels of inbreeding was observed at ‘Āpua point (12 pairs of loci in linkage disequilibrium) and Mahana bay (8 pairs of loci in linkage disequilibrium). These populations also exhibit the sixth ( $f = 0.7$ ) and second ( $f = 0.922$ ) highest rates of inbreeding in this study, respectively. Diversity was excessively low (many monomorphic loci) to adequately address genotypic disequilibrium in many populations sampled, predominately those on O‘ahu and Kaua‘i (Table 3.5). Since the test of linkage disequilibrium assumes Hardy-Weinberg equilibrium, it is likely that these results are due to deviations from this assumption within each population to varying degrees (dependent upon levels of inbreeding taking place within each).

The high rates of inbreeding observed (as high as 0.94) would seem extremely detrimental to the survival of this species into the future, yet evidence for inbreeding depression has so far been inconclusive. For example, manual supplemental hand cross pollination failed to significantly increase reproduction in plants at the small Kīpuka Nēnē makai population ( $n = 12$ ),

yet testing pollen viability and stigma receptivity confirmed male and female vigor (Pratt et al., 2011). It was discovered in the course of the present research that all 12 plants had identical microsatellite genotypes where four out of nine loci were heterozygous (and thus not likely to be the product of selfing). Therefore, it became apparent that what was originally believed to be 12 separate individuals comprises only a single large sprawling individual (genet), which may or may not have become fragmented into separate clonal individuals (ramets). As a result, Pratt et al. (2011) speculated that self-incompatibility mechanisms in *S. tomentosa* might account for the low seed set observed at this population. Over the three-year study period, none of the 380 buds and flowers that were tagged at this population matured into fruit. In contrast, fruit production appeared much higher in a larger (150+ individuals) coastal population 12 km to the southeast at 'Āpua point (Pratt et al., 2011), however, the remoteness of this location precluded the monthly monitoring of buds and flowers. Hopper (2002) observed lower seed set in more isolated/smaller groups of plants at Ka'ena point, yet he also observed periodic seedling recruitment around isolated individuals, and seeds derived from his self-fertilization treatments were viable. Therefore, it is conceivable that inbreeding depression has not been an issue in all cases. Hopper (2002) also measured the genetic fitness (seed viability and pollen fertility) of two Ka'ena point populations of *Sesbania tomentosa* (one a small isolated group of plants and the other a large contiguous population of plants). He found that there was no difference between seed germination success of seeds from the two populations, and that pollen fertility was actually (inexplicably) higher in the isolated plants.

The rapid growth and reproduction of *Sesbania tomentosa*, along with a “persistent” seedbank [seeds proven viable after 10 years in storage (Lilleeng-Rosenberger, 2005) and after 3 years in the soil (Pratt et al., 2011)] and short life span are characteristics of pioneer species associated with harsh environments (Odum, 1971). Repeated colonization of open habitat would have been accompanied by a high rate of self-fertilization that would have purged many deleterious alleles (Charlesworth and Charlesworth, 1987; Barrett and Charlesworth, 1991; Barrett 1998). Plant populations with a history of inbreeding and that readily self-fertilize typically do not exhibit inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). Factors that promote the evolution of selfing include a lack of effective pollination and repeated colonization of new areas by single individuals (Schemske and Lande,

1985). Other *Sesbania* taxa are also known to be extremely successful in establishing themselves by producing fertile seed from selfing (Jamnandass et al., 2005)

### Genetic structure of populations across Hawai‘i

Global  $F_{ST}(\theta)$  over all populations and loci was estimated to be approximately 0.5; Wright’s (1978) guidelines state that values above 0.25 indicate “very great” genetic differentiation. These  $F_{ST}$  results are strong indication of reduced and/or ineffective gene flow between populations of *Sesbania tomentosa*. This was corroborated by the coalescent modeling which suggested that in Ka‘ū (the largest natural landscape left in Hawai‘i where some degree of connectivity between populations of *S. tomentosa* could potentially still occur) the contemporary population structure of *S. tomentosa* has been predominantly influenced by genetic drift in isolation rather than gene flow.

Overall, STRUCTURE provided less resolution in identifying distinct clusters than  $F_{ST}(\theta)$ . This might be explained by a poor fit between assumptions of the STRUCTURE model (Hardy-Weinberg equilibrium within populations) with the reality of *Sesbania tomentosa* reproduction in nature. The results presented here offer an alternative view of the relationships between populations purported in previous STRUCTURE and  $F_{ST}(\theta)$  analyses (see Chapter 2), this time using a more natural sampling strategy where duplicate (identical) genotypes derived from plants occurring < 10 m from one another were included (whereas in Chapter 2 they were excluded). These identical genotypes are believed to be either samples inadvertently taken from branches of the same plant (branches which over time physically separated from one another), or are an artifact of extreme genetic sub-structuring within certain populations. For example, as a result of including duplicate genotypes occurring less than 10 m apart, global  $F_{ST}(\theta)$  over all populations increased from 0.39 to 0.50.

Populations of *Sesbania tomentosa* also clustered together in different ways as a result of this alternate analysis. For example, the Maui Nui populations shifted from being in a cluster associated with O‘ahu, Kaua‘i and Nihoa in the analysis (see Chapter 2) to being associated with populations on Hawai‘i Island (Figure 3.4). The apparent drifting apart of populations at either end of the Island chain (with Maui Nui situated in the middle) is an expected phylogeographic pattern that correlates well with the IBD results presented above. Despite the highly restricted

gene flow between populations estimated above (global and pairwise  $F_{ST}$ ), a significant pattern of isolation by distance suggests that historical gene flow among contiguous populations existed or that dispersal and establishment of populations occurred in a linear, rather than random, order to give rise to the much larger and continuous distribution. The PCA and NJ results from Chapter 2 (Figures 2.14 and 2.16; Chapter 2) corroborate this as well. In spite of the somewhat unexpected assignment of the two largest NW Maui populations in the same cluster as populations from O‘ahu, Kaua‘i (350 km apart) and Nihoa (600 km apart), these cluster assignments follow the observed phylogeographic trends (Figure 3.4).

### Genetic sub-structure of populations across Hawai‘i

The extremely high inbreeding coefficients observed in this study may be due in part to a Wahlund effect in which heterozygosity in populations is reduced due to sub-population structure (Wahlund, 1928). The larger the sub-population and the more recently it has been isolated, the smaller the inbreeding effect of population subdivision (Hartl and Clark, 2007). Strong evidence of population sub-structuring is apparent throughout most populations of *Sesbania tomentosa*, indicating that adjacent plants are more closely related than non-adjacent plants.

Accompanying this trend are cases in which the clonal nature of populations comes in to question. For example, at Kīpuka Nēnē in Hawaii Volcanoes National Park, Pratt et al. (2011) reported that branches of monitored plants grew 1 to 4 m during their three-year study period. They also noted the tendency for the plant to sprawl at ground level and to root from branch nodes adding to the dynamic nature of *S. tomentosa* populations. What was believed by the authors to be groups of separate *S. tomentosa* plants became a tangled mass in subsequent years of monitoring (with all DNA samples turning up the same genotype). On the other hand, a large plant might break up over time into several apparently distinct patches. Each time plant fragmentation occurs, this could potentially increase the maximum distance between clonal pairs, perhaps explaining why pairs of identical genotypes were collected from branches attached to seemingly separate individuals 30 m apart at Kīpuka Nēnē (data not shown).

In considering the role of self-fertilization in the reproductive dynamics of populations, this sub-structuring might also be explained by examining the behavior of the *Hyla*

pollinators. Hopper (2002) observed *Hylaeus* to spend most of their time around a single plant (resulting in most of the pollination and fruit set he observed to be the result of geitonogamy) and he believed they will not forage far unless there is native dominated vegetation containing both nectar and pollen and sites for resting and nesting. Grasses surrounding plants are believed to serve as isolating barriers as they are not used by any species of bee (Hopper, 2002). *Hylaeus* bees on Haleakalā were inferred to have visited multiple (separate individual) plants when foraging only when plants were located very close to one another (Krushelnycky, 2014). Indeed, a single *Sesbania tomentosa* plant in full bloom would supply much more pollen than an individual bee could carry back to its burrow, reducing the need for visits to multiple plants (which would have otherwise facilitated cross-pollination). Therefore, the vectors most responsible for effecting pollination in *S. tomentosa* (playing a much larger role than all other floral visitors combined; Hopper, 2002, Pratt et al., 2011) are not adequate for facilitating outcrossing, and the plant would therefore be more dependent upon the less common occurrence of seed dispersal for gene flow. Increased spatial gene flow would otherwise have a homogenizing effect, reducing the genetic differentiation between populations (Wright, 1969; Slatkin, 1987). On the other hand, germination from the seed bank would help to preserve strong spatial genetic structure in a predominately selfing species via temporal gene flow (Honnay et al., 2008).

#### A prolonged history of genetic bottlenecks

There are apparent differences in heterozygosity and allelic diversity between populations, many of which have several loci that are either monomorphic or are approaching fixation within a population. The loci and alleles involved vary between neighboring populations, strongly suggesting the influence of bottlenecks. When correcting for the presence of null alleles in the dataset, there does not appear to be a lack of low frequency alleles in most populations and evidence for recent bottlenecks in the form of a transitory heterozygote excess was also largely unsubstantiated in the BOTTLENECK analysis. When population size becomes very small (~10 individuals) and when generation times are short, as with most populations of *Sesbania tomentosa* [e.g., Hopper (2002) reported a longevity of 3–10 years at Ka‘ena point], a new mutation-drift equilibrium should be arrived at quite rapidly (Watterson, 1984).

Analysis of the  $M$ -ratio was much more successful in detecting bottlenecks, suggesting they have been occurring for a longer period of time than BOTTLENECK could detect (i.e., not within the past  $0.2N_E$ – $4N_E$  generations). Garza and Williamson (2001) suggested that  $M$ -ratios lower than 0.68 would indicate evidence of a bottleneck, whereas values greater than 0.8 would denote no bottleneck history whatsoever. As the  $M$ -ratio is predicted to recover following a reduction in population size, the rebound in size of the Polihale (Kaua‘i) population from 2006 ( $n = 10$ ) to 2009 ( $n = 50$ ) is seen here to have been accompanied by an increase in the  $M$ -ratio. When the Polihale population was sampled again in 2010 (after it declined back to 12 individuals) genetic signatures of recent bottlenecks were also evident in the BOTTLENECK Wilcoxon tests. The only other populations providing evidence for recent bottlenecks were along the Hilina pali in Hawai‘i Volcanoes National Park, an area known to have been heavily grazed by feral goats (*Capra aegagrus hircus*) over the last century (see below). *Sesbania tomentosa* in that area is quite distinct in that it forms relatively large and apparently long-lived patches (Linda Pratt, US Geological Survey, personal communication); with longer generations, the signatures of recent bottlenecks would be expected to persist for longer periods of time.

Two subsets of samples were compared from the Mo‘omomi population to test for changes in allele frequencies over time. DNA samples from 10 historically-collected herbarium samples and 26 samples representing the entire extant population in 2006 were both genotyped. This strategy has been used in other studies to observe the genetic effects of demographic bottlenecks in a direct manner, in contrast to the indirect methods employed above (Bouzat et al., 1998; Larson et al., 2002; Nyström et al., 2006; Larsson et al., 2008). Twenty alleles (out of a total of 55) in the 10 historic samples were not found in any of the plants extant at the Mo‘omomi population in 2006, a possible indication of a genetic bottleneck having taken place. The loss of these alleles also suggests that genetic drift, and loss of genetic diversity overall, may be occurring at Mo‘omomi. While the alleles that were lost may have been rare to begin with (and thus were the first to be lost during population contraction), it is still important to recognize their loss from the population completely. On the other hand, it remains possible that some of those lost alleles were maintained in the soil seed bank *in situ* during sampling there in 2006, with the potential to subsequently germinate and again contribute their alleles to the population.

Studying the history of land use of the sites surveyed for this study is another means to examine population bottlenecks, particularly where intensive animal grazing is known to have

taken place. For example, sheep (*Ovis aries*) were penned near the beach at Mo‘omomi, Moloka‘i (Cooke, 1949). Degener and Degener (1978) reported that *Sesbania tomentosa* was on the verge of extinction at Mo‘omomi in 1928, and cattle (*Bos taurus*) and axis deer (*Axis axis*) were taking a toll on the plants at Mo‘omomi as late as 1990 (Hawai‘i Biodiversity and Mapping Program). Similarly, cattle grazing at Ka‘ena point on O‘ahu in the early 1900’s severely impacted *S. tomentosa* there (Degener and Degener, 1978). At ‘Āpua point in Hawai‘i Volcanoes National Park, feral goats (*Capra aegagrus hircus*) were driven down off the mountain and penned at the lush ‘ohai habitat during the 1920’s and 30’s (Clark, 1985). Cattle breached the stone wall enclosing the lighthouse at Ka Lae in the mid-1970’s, completely denuding the ground of *S. tomentosa* (yet the soil remained stocked with its seeds for many years after; Degener and Degener, 1978). Approximately 70,000 animals were extricated from the park, yet 15,000 persisted in the area around Hilina pali as recently as the 1970’s (Baker and Reeser, 1972; Katahira and Stone, 1982). While levels of inbreeding were high and genetic diversity low in the above-mentioned populations, evidence for genetic bottlenecks in the dataset was lacking in most cases (the exception being Hilina pali). Extensive wildfires burned through Kīpuka Nēnē twice in the last 40 years, and can also be expected to have caused dramatic declines in the *S. tomentosa* population there as well.

Arthropod grazing pressure has also resulted in catastrophic drop in numbers of *Sesbania tomentosa* plants in recent times. In the 1960’s, a stink bug (*Comptosoma xanthagramma*) outbreak devastated the Ka‘ena point (O‘ahu) plants where a natural seedbank provided the recovery (Howarth, 1985). From 2002 to 2004, the grey bird grasshopper (*Schistocerca nitens*) outbreak completely defoliated the *S. tomentosa* on Nihoa (Latchinsky, 2008) and, as a result, many plants had perished when the population was again observed in 2006 (Beth Flint, USFWS, personal communication). Magnolia scale (*Neolecanium cornuparyum*) were first observed on the *S. tomentosa* at Polihale (Kaua‘i) in August of 2004. Almost all of the larger plants (ca. 2 m tall) died, yet there were many new seedlings after a wet year in 2009 that seemed less susceptible to the scale (USFWS, 2010). While the Ka‘ena point and Polihale populations both had high levels of inbreeding and relatively low diversity, only the Polihale population tested positive for evidence of recent genetic bottlenecks. The Nihoa population, on the other hand, had levels of diversity lower than would be expected given its large size (as compared with much



smaller populations that exhibited the same or higher levels of diversity) that is possibly related to the grasshopper outbreak and subsequent decline.

On the islet of Pu‘u Koa‘e (near Kaho‘olawe), cycles of drought appear to have caused dramatic fluctuations in *Sesbania tomentosa* numbers providing another means to verify population bottlenecks having taken place (USFWS, 2010). Following a drought in 2000, the population shifted from having 70 mature individuals, 15 juveniles and 15 seedlings to consisting of one surviving mature individual accompanied by 300 seedlings. A single mature individual was later observed accompanied by up to 70 dead individuals. In 2003, 100 mature individuals plus 200 seedlings were reported. This rose to 300–400 individuals in 2008, and back down again to 50 in 2010. The last observations made were in 2011 when 10 large plants along with 400 young healthy plants approximately 10–45 cm tall were found (Ken Wood, NTBG, personal communication). No evidence for genetic bottlenecks was detected at Pu‘u Koa‘e and levels of diversity remain moderate.

Similar fluctuations in population size were seen at Ka Lae where 85 *Sesbania tomentosa* plants in 1991 were reduced to only 2 plants in 1992 (Hawai‘i Biodiversity and Mapping Program). This population had rebounded to 29 when surveyed in 2006, although other fluctuations might have taken place in the 14-year gap between recorded observations. Fluctuations in numbers of plants have also been recorded for the Polihale (Kaua‘i) population. Only five plants persisted in the 1980’s, but numbers increased to 30 plants reported in 1992 although dwindling again to seven in 2001. In 2005 that number exceeded 30 again and by late 2006 the population was down to less than 20, and by 2008 the number was hovering around 10. There were 50 plants reported in 2009, but this number subsequently dropped back to 12 by 2010 (a genetic bottleneck was detected for the Polihale population in 2010). It is of interest to note that the genetic diversity of this population was highest in 2009 when the population size had reached a 30-year high. Out of a total of 22 alleles (at 9 loci) occurring at Polihale over the 3 sample years, seven were private to the Polihale population in 2009 (Figure 3.13) in spite of sampling during that year (as opposed to other years) not being exhaustive (only 11 out of 30 plants extant at time of collection were genotyped due to degraded plant tissue collections).

Experiments done at the Lyon Arboretum Seed Conservation Lab found that *Sesbania tomentosa* seeds have no light requirement for germination once they become imbibed with water (Alvin Yoshinaga, Lyon Arboretum, personal communication). Therefore, these seeds are

capable of surviving through drought yet germinate immediately once rains have returned. This limits the temporal range of gene flow that the soil seed bank provides, thus genetic drift is still able to progress through the bottleneck albeit at a slower pace than it would be able to otherwise (Templeton and Levin, 1979; Honnay et al., 2008).

The above records are actual, observed population flush-crash cycles (Carson, 1975) that were not all detected by the indirect methods employed herein. Perhaps these populations are able to recover in that the maintenance of a seed bank in the soil would allow low frequency alleles to remain in the genepool (through the bottleneck). Another scenario would be that some of these have historically been small, fluctuating populations, and have experienced no rapid decline in numbers. Populations suffering a reduction in census size may not suffer a severe reduction of  $N_E$  (a genetic bottleneck) if historical  $N_E$  has always been low due to fluctuations in population size, inbreeding, or metapopulation structure involving cycles of extinction and recolonization (Cornuet and Luikart, 1996; Watterson, 1984). At the Polihale (Kaua‘i) population, the effective population size calculated using the fluctuating census numbers listed above was 12 (roughly half the average annual population size over the past 30 years). In either case, a very rapid intrinsic rate of increase following a population bottleneck would minimize genetic loss (Nei et al., 1975). Accordingly, the most rapidly-rebounding, abundant population year sampled at Polihale (2009) exhibited the highest levels of diversity.

*Sesbania tomentosa* has thus been shown to maintain an ample seedbank for future colonization of the plant metapopulation, and the rapid maturation of *S. tomentosa* plants (from seed to seed in less than 1 year) is also coming into play. For example, genetic drift is thought to be accelerated in species with shorter generation times (Kimura, 1983). Seeds sprouting from a seed bank represent migration from the past, and have the potential to buffer against a loss of diversity while at the same time slowing genetic drift (Templeton and Levin, 1979; Honnay et al., 2008).

## Conclusion

Populations of *Sesbania tomentosa* exhibit high levels of genetic structure with extensive inbreeding within and divergence among individual populations. Corresponding with previous observations suggesting geitonogamy commonly taking place in this species, the high  $F_{ST}$  values

observed among *S. tomentosa* populations are comparable to rates of differentiation seen in other predominately selfing, short-lived perennial species (Hamrick and Godt, 1996). The significant pattern of isolation by distance across the Hawaiian Islands indicates that the underlying structure derives from ancient timescale processes (migration and gene flow) as well as from drift in the contemporary populations. The consistently high levels of inbreeding observed, accompanied by strong spatial genetic sub-structure, are also indications of a species predominately reliant on selfing to maintain reproduction. There is little sign of futile selfing occurring in this species (inbreeding depression leading to the loss of selfed progeny; Robertson et al., 2011) as many populations of mature individuals are composed of highly homozygous genotypes. Therefore, low levels of gene flow between populations (and a high occurrence of selfing) can be presumed to have been a trend in the past that has been accentuated by more recent fragmentation and decline. Indeed, the meta-analysis of Aguilar et al. (2008) suggested that fragmentation of plant populations has the effect of shifting mating patterns towards increased selfing.

The original immigration of *Sesbania* to Hawai‘i need not have taken place very far back in the past to account for the morphological differentiation observed today, as is probably the case given the low levels of nDNA sequence divergence (see Chapter 2). The microsatellite loci examined in this study appear to have responded to genetic drift much more rapidly than the regions that were sequenced. Natural selection in different environments, along with random drift and mutation, would cause morphological variation to accumulate in the species as a whole. Rates of adaptation and morphological change in isolated breeding populations would be impacted by the rapid maturation of *S. tomentosa* and the maintenance of an ample, viable seedbank. Ecologically, this species also appears prone to inbreeding and repeated bottlenecks, adding additional efficiency to a trend of divergence. It is entirely plausible that both the microsatellite as well as the morphological differentiation observed have been accentuated within the time period when populations of *S. tomentosa* became increasingly fragmented and isolated from one another. In other words, more modern impacts on the range of the species have probably only accelerated what was already naturally-occurring. For example, three distinct morphotypes were observed before the era of ranching within 10 km of one another on the island of Moloka‘i (Hawai‘i Biodiversity and Mapping Project). On the other hand, lava flows of the

past 400–700 years (Sherrod et al., 2007) have separated three additional morphotypes within Hawai‘i Volcanoes National Park by 5–10 km distance as well.

On a final note, there may have been specialist honeycreeper finches (Fringillidae) foraging in the range of *Sesbania* prior to the introduction of avian diseases (circa 1800’s) that would have provided the large proportion of pollination services for this plant (nectar-rich, scentless, showy flowers are suggestive of this; S. Conant personal communication). As such, the birds would have provided for greater outcrossing within and among populations than is seen at present. The shift to insect pollination (with *Hylaeus*) would have severely limited gene flow within and between populations, further separating them out into the distinctive appearing populations found today.

## CHAPTER 4

### Genetic diversity and the role of seed sourcing practices in restoration outplantings of the rare Hawaiian plant *Sesbania tomentosa*

#### Introduction

Under ideal circumstances, efforts to restore populations of rare plants aim to maintain levels of genetic diversity found in natural populations among individuals that will be used for replanting. The restored population is likely to be more self-sustaining if the plant material used is diverse, by ensuring successive generations of progeny will be free from the deleterious effects of inbreeding (Charlesworth and Charlesworth, 1987; Huenneke, 1991; Fenster and Dudash, 1994; Knapp and Dyer 1998). Over the long term, increased adaptive potential imparted by genetic diversity improves successful responses to future environmental change and reduces the risk of extinction (Ellstrand and Elam, 1993; Frankham, 2005). On the other hand, restoration projects often use only locally collected planting material following a precautionary notion that such material might comprise locally adapted genotypes (Millar and Libby, 1989; Hufford and Mazer, 2003; McKay et al., 2005). If there are limits to the harvesting of local planting material (such as in cases of low reproduction of rare plant populations in Hawai‘i), collections made might only provide a restricted sample of the source population and the genetic base of the outplanted population would be narrow (e.g., Burgarella et al., 2007; Kettle et al., 2008). It has been suggested that for rare species with few remaining individuals the central focus of restoration efforts should be to maximize genetic diversity in restored populations regardless of the origin of planting material (Frankham et al., 2011; Maschinski et al., 2013). Although outbreeding depression may be a consequence of this, Frankham et al. (2011) suggest that the probability of this is low in most plant and animal populations and mitigating the effects of inbreeding depression are much more relevant in preventing extinction.

Knowledge of population genetic structure and diversity at the outset of any restoration effort would help determine whether it would be safe to mix different source populations in an outplanted population (Hamrick et al., 1991; Keller and Waller, 2002). Mixing material collected from multiple populations should increase genetic variation in the outplanted population. Several

studies have found that using seed from more than one source population resulted in outplanted populations with more genetic variation (e.g., Smulders et al., 2000; Gustafson et al., 2004; Dolan et al., 2008) and others have found them to be more resilient and reproductively fit when compared with single-source outplanted populations (Vergeer et al., 2005; Maschinski et al., 2013; Weisenberger et al., 2014). Weisenberger et al. (2014) determined that mixing was an important strategy in the recovery of Hawaiian *Schiedea* with 1 to 2 plants per population, with outplantings derived from between population crosses exhibiting a strong heterotic effect.

*Sesbania tomentosa* Hook. and Arn. (Fabaceae) is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. The habit of *S. tomentosa* is highly variable, often with island specific forms. Plants may grow as sprawling shrubs with prostrate to decumbent branches (reportedly up to 14 meters long, though possibly reaching much longer in extreme examples) or as a small bush or tree up to six meters in height. Leaves are even-pinnately compound and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. The species is named for the leaves, that are sparsely to densely covered with silky hairs. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet to deep red. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds.

*Sesbania tomentosa* was listed as Endangered by the U.S. Fish and Wildlife Service in 1992, and has been a focus species for outplanting by various state and federal agencies tasked with its recovery. Fifty-six percent of all populations of *S. tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826 (Hawai'i Biodiversity and Mapping Program). In fact, at least seven populations have been extirpated since DNA collections for this study began (in 2006) and others have experienced severe demographic decline due to drought, pest outbreaks, or other natural or anthropogenic causes (personal observations). A hermaphroditic breeding system, conspicuous flowers and autochorous dispersal of dry fruit have made *S. tomentosa* acutely vulnerable to extinction compared with other dry forest taxa according to the analysis of Pau et al. (2009). On the other hand, entirely new occurrences of this species have been discovered since this study began near Nu'upia pond (Mōkapu, O'ahu) and at Pa'akahi point (Hanapēpē, Kaua'i) after heavy winter rains, indicating an important role of the seedbank within the metapopulation as a whole as well as the ephemeral nature of the species as a component of the vegetation.

Chapter 3 explored the structure of microsatellite diversity in populations of *Sesbania tomentosa* throughout its known range. Genetic analysis with microsatellite markers is here used to compare the genetic diversity of naturally-occurring populations of *S. tomentosa* with those of their outplanted counterpart populations in Hawai‘i, to assess rates of inbreeding and impacts of genetic drift. Examples where molecular markers were used to gain valuable insight in guiding restoration management of rare plant populations are plentiful (e.g., Knapp and Connors, 1999; Mattner et al., 2002; Rottenberg and Parker, 2003), including a number of examples from Hawai‘i (Morden and Loeffler, 1999; Friar et al., 2000, 2001; Kwon and Morden, 2002). As various numbers of founding individuals (from 1 to more than 10) have been used to assemble the outplanted populations measured, seed sourcing practices will also be examined. To the degree that sampling bottlenecks occur, restored populations should be observed to be genetically depauperate compared with their natural population counterpart, and might be subject to additional negative effects of inbreeding and genetic drift in the future. The hypothesis that genetic diversity should impart resilience in *S. tomentosa* populations was tested using data on the survivorship of outplantings.

## **Materials and Methods**

### **DNA sample collection**

Leaf samples of 166 individuals of *Sesbania tomentosa* were collected between 2006 and from eight naturally occurring populations throughout the Hawaiian Islands. These eight populations were used as sources of seed to propagate an additional 141 individuals whose leaves were also sampled for this study, examining 307 samples total. An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4 to 8°C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNEASY tissue kits (QIAGEN; Valencia, CA) according to the manufacturer’s protocol and then visually checked using electrophoresis. Propagative material had been collected from source populations previous to this study (several years previous in some cases) and it cannot be ruled out that additional individuals may have been

present at that time. Varying degrees of mixture and number of original founders were used to comprise the outplanted populations, and are listed in Table 4.2.

In most cases, outplanted individuals of *Sesbania tomentosa* were sampled more than one year post-planting. Kāohikaipu and Mōkapu-derived individuals were in cultivation at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu) and were sampled prior to their outplanting at Ka Iwi State Scenic Shoreline and Ka‘ena point State Park (O‘ahu). In addition, the two individuals comprising the Mānā (Kaua‘i) population (now extirpated) were cultivated specimens of the National Tropical Botanical Garden (F<sub>1</sub> generation derived from a single wild plant). Lastly, in order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, the Polihale (Kaua‘i) population was sampled in 2006 (16 plants), 2009 (11 plants) and 2010 (12 plants), and the genetic diversity of the standing populations of each year are herein compared. GPS coordinates accompanied each DNA collection, yet in many cases it was impossible to determine whether or not the same individual was collected multiple times (in successive years) due to the close clustering of individuals.

## Microsatellite Analysis

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* using magnetic bead capture molecules for dinucleotide (CA<sub>n</sub>) and tetranucleotide (CATC<sub>n</sub>, TACA<sub>n</sub>, and TAGA<sub>n</sub>) microsatellite repeats. Ninety-six microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 4.1) based on their range of polymorphism and ease of scoring in a screening of eight DNA samples (collected from eight populations on six islands). Each sample was amplified in a 25.0 µL volume with final concentrations of: 0.6 µM each of forward and reverse primers, 1X PCR Buffer, 2.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs (Promega), 1 unit *Taq* polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 4.1) for 40 s, 72°C for 30 s; ending with a final extension of 72°C for



Table 4.1. Nine microsatellite primer pairs developed for *Sesbania tomentosa*. Prefixes in italics before forward primer sequence indicate dye used for poolplexing.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 307 individuals sampled for this study. Range, allele size range in base pairs.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG	57.3	6	207–221
		R: TAG-GTG-TGG-CGT-GCA-TAA-C	58.1		
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A	56.0	8	264–278
		R: CCC-TTC-CCC-TCC-TCT-TAG	56.2		
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC	55.8	8	206–230
		R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	56.3		
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC	58.9	11	290–326
		R: TAG-CCA-TGC-TTC-ATC-AAT-CG	59.8		
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C	56.8	10	163–185
		R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.9		
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG	58.6	7	196–248
		R: GGC-AAC-ATT-TGA-GTG-GAG-G	59.1		
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA	55.1	9	192–236
		R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1		
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C	55.7	11	212–252
		R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.2		
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G	54.1	7	265–321
		R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.8		

4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA). The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (Applied Biosystems).

Diversity indices were estimated for the geographic populations (both natural and outplanted) using MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötterer, 2003). Diversity indices include expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ), mean number of alleles per locus ( $A$ , a measure of diversity not corrected for sample size) and allelic richness ( $A_R$ , allelic diversity corrected for sample size). Private alleles (alleles exclusive to a given population) were calculated in GENALEX v. 6.4 (Peakall and Smouse, 2006). Inbreeding ( $F_{IS}$ ) was calculated with INEST (using the “individual inbreeding model”), which estimates inbreeding while simultaneously accounting for the presence of null alleles (Chybicki and Burczyk, 2009). The extent and significance of the genetic differentiation between natural and the outplanted counterpart populations was investigated with MSA by calculating pairwise  $F_{ST}$  ( $\theta$  Weir and Cockerham, 1984) averaged over multiple loci, with 100,000 permutations to assess significance using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ).

A loss of rare alleles is an expected genetic signature resulting from a population bottleneck (Cornuet and Luikart, 1996; Luikart et al., 1998). To test for loss of rare alleles in the outplanted populations, the proportions of rare alleles (frequency  $< 0.1$ ) in each of the populations (natural and representative) were calculated.

After a severe reduction in effective population size ( $N_E$ ), there should be a transient excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (Piry et al., 1999). Bottlenecks generate transient heterozygosity excess because rare alleles are generally lost faster than heterozygosity during a bottleneck (Luikart and Cornuet, 1998). Wilcoxon sign-rank tests of heterozygosity excess (10,000 iterations) were implemented in BOTTLENECK v. 1.2.02 (Luikart and Cornuet, 1998; Piry et al., 1999). This program used

allele frequency data to detect recent reductions in effective population size (i.e., within the past  $0.2N_E$ – $4N_E$  generations) under a 100% stepwise mutation model (SMM), an infinite alleles model (IAM) and a two-phase mutation model (TPM with 70% SMM, 30% IAM). A second approach (also implemented in BOTTLENECK) tested a mode shift away from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium, whereby alleles at low frequency become less abundant than alleles at intermediate frequency (Luikart et al., 1998).

## Results

### Microsatellite allele frequencies

There was an average of 8.5 alleles per locus at the nine microsatellite loci examined, ranging from 6 to 11, for a total of 77 alleles among the 307 samples of *Sesbania tomentosa*. Each locus had only two to four alleles with a frequency greater than 0.10, and these most common alleles had average frequencies per locus that ranged from 0.20 to 0.41 (with a maximum across loci of 0.59).

### Genetic diversity and nonrandom mating of natural vs. outplanted populations

Of the eight natural (source) populations of *Sesbania tomentosa* sampled, Kīpuka Nēnē–Hilina pali ( $n = 35$ ) and Pu‘u Pīmoe ( $n = 9$ ) exhibited the highest values of allelic diversity (Table 4.2). Accordingly, the highest values of allelic diversity of all nine outplanted populations were observed in Kanaio U.S. Army Training Area (allelic richness = 2.67; expected/observed heterozygosity = 0.542/0.241) and Kulanaokuaiki (allelic richness = 2.43; expected/observed heterozygosity = 0.387/0.122), their outplanted counterparts (Table 4.2). The six Kanaio outplantings were sourced from only three founding individuals, with twelve alleles failing to be captured from the natural population at Pu‘u Pīmoe (data not shown). The Kanaio outplantings are notable for having two private alleles (average frequency = 0.21) not found in its source population and for lacking significant genetic differentiation from its source population (Tables 4.2 and 4.3). On the other hand, the outplanted population at Kulanaokuaiki ( $n = 35$ ) was composed of material sourced from a comparatively large number of separate founders ( $> 10$ )

Table 4.2. Genetic diversity statistics of natural vs. outplanted representative populations of *Sesbania tomentosa*.  $n$ , sample size;  $n_F$ , number of founders from natural population used to source the seeds comprising the outplanted representative population.  $A$  and  $A_R$ , mean number of alleles per locus and mean allelic richness (averaged over loci) respectively. Private alleles are alleles found in a given population not found in its counterpart population; average allele frequencies subsequently listed. Percentage of rare alleles is the proportion of rare alleles (frequency < 0.1) to total number of alleles in a given population.  $H_O$  and  $H_E$ , observed and expected heterozygosity.  $F_{IS}$ , inbreeding coefficient. ML is number of monomorphic loci and is out of a total of nine loci.

Population	Island	$n$	$n_F$	$A$	$A_R$	Private Alleles	% rare alleles	$H_O$	$H_E$	$F_{IS}$	ML
Natural population:											
Kīpuka Nēnē–Hilina pali	Hawai‘i	35		4.7	3.6	11/0.05	48.7	0.328	0.571	0.264	1
Outplanted representative populations:											
Kīpuka Nēnē	Hawai‘i	7	2–3 <sup>a</sup>	2.0	2.0		17.6	0.095	0.246	0.161	1
Kulanaokuaiki	Hawai‘i	21	> 10 <sup>a</sup>	2.9	2.4		36.0	0.122	0.387	0.621	1
Natural population:											
Pu‘u Pīmoe	Maui	9		3.8	3.4	12/0.12	23.5	0.420	0.594	0.157	
Outplanted representative population:											
Kanaio U.S. Army Training Area	Maui	6	3 <sup>b</sup>	2.7	2.7	2/0.21	12.5	0.241	0.542	0.212	1
Natural population:											
Papanalahoā–Nākālele	Maui	44		3.2	3.1	14/0.32	34.5	0.260	0.473	0.309	
Outplanted representative population:											
Kanahā County Beach Park	Maui	28	4 <sup>c</sup>	1.7	1.7		20.0	0.107	0.091	0.020	4

Table 4.2. (Continued) Genetic diversity statistics of natural vs. outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	<i>n<sub>F</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>	Private Alleles	% rare alleles	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>F<sub>IS</sub></i>	ML
Natural population:											
Nu‘upia Ponds (Mōkapu)	O‘ahu	3		2.0	1.5	8/0.34	0.0	0.222	0.500	0.652	3
Mokulē‘ia State Tree Nursery (2009):											
origin: Nu‘upia ponds	O‘ahu	8	2 <sup>d</sup>	1.2	1.1	1/0.12	0.0	0.125	0.084	0.046	7
Natural population:											
Kāohikaipu	O‘ahu	1		1.1	1.1		0.0	0.111	0.111	NA	8
Mokulē‘ia State Tree Nursery (2009):											
origin: Kāohikaipu	O‘ahu	11	1 <sup>d</sup>	1.2	1.1	1/0.14	0.0	0.141	0.085	0.031	7
Natural population:											
Ka‘ena point State Park–Natural Area Reserve (NAR)	O‘ahu	33		1.7	1.7	1/0.01	7.1	0.077	0.219	0.649	3
Outplanted representative population:											
Ka‘ena point NAR	O‘ahu	32	> 10 <sup>d</sup>	1.7	1.7	1/0.01	7.1	0.098	0.166	0.400	3

Table 4.2. (Continued) Genetic diversity statistics of natural *vs.* outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	<i>n<sub>F</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>	Private Alleles	% rare alleles	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>F<sub>IS</sub></i>	ML
Natural population:											
Polihale State Park (2006)	Kaua‘i	16		1.3	1.3		0.0	0.083	0.123	0.006	6
Polihale State Park (2009)	Kaua‘i	11		2.3	2.3	5/0.12	38.1	0.092	0.294	0.328	3
Polihale State Park (2010)	Kaua‘i	12		1.7	1.7		6.6	0.065	0.236	0.214	5
Outplanted representative population:											
Lāwa‘i Kai (NTBG)	Kaua‘i	14	14 <sup>e</sup>	2.4	2.3	5/0.06	40.9	0.182	0.267	0.070	1
Origin of <i>ex-situ</i> source population:											
Mānā	Kaua‘i	2		1.1	1.1		0.0	0.056	0.056	0.000	8
Outplanted representative population:											
Makauwahi (NTBG)	Kaua‘i	14	3 <sup>f</sup>	1.2	1.0	1/0.91	0.0	0.008	0.026	0.049	7

<sup>a</sup> Thomas Belfield, Hawai‘i Volcanoes National Park, personal communication (2007)

<sup>b</sup> Chuck Chimera, U.S. Army Natural Resources, personal communication (2006)

<sup>c</sup> Forest Starr, US Geological Survey, personal communication (2006)

<sup>d</sup> Greg Manscur, Hawai‘i Division of Forestry and Wildlife, personal communication (2007)

<sup>e</sup> Mike Demotta, National Tropical Botanical Garden, personal communication (2007)

<sup>f</sup> David Burney, National Tropical Botanical Garden, personal communication (2007)

Table 4.3. Genetic differentiation between natural populations and their outplanted counterpart populations. Pairwise  $F_{ST}$  values ( $\theta$ , Weir and Cockerham 1984) averaged over nine microsatellite loci on top half of matrices; Significant Bonferroni-corrected  $P$ -values listed in bottom half of matrices. n.s. indicates pairwise comparisons non-significant at the 0.05 level.

Natural population: Kīpuka Nēnē–Hilina pali	0.00000	0.23846	0.12839
Outplanted population: Kīpuka Nēnē	0.00003	0.00000	0.14749
Outplanted population Kulanaokuaiki	0.00003	0.04143	0.00000
Natural population: Pu‘u Pīmoe	0.00000	-0.02124	
Outplanted population: Kanaio Army Training Area	n.s.	0.00000	
Natural population: Papanalahoa–Nākālele	0.00000	0.63501	
Outplanted population: Kanahā County Beach Park	0.00001	0.00000	
Natural population: Nu‘upia ponds	0.00000	0.39183	
Mokulē‘ia State Tree Nursery (2009)	0.01043	0.00000	
Natural population: Kāohikaipu	0.00000	-0.05902	
Mokulē‘ia State Tree Nursery (2009)	n.s.	0.00000	
Natural population: Ka‘ena point	0.00000	0.03266	
Outplanted population: Ka‘ena point NAR	n.s.	0.00000	
Natural population: Polihale State Park (2006)	0.00000	0.15612	0.21813
Natural population: Polihale State Park (2009)	0.00006	0.00000	-0.05130
Natural population: Polihale State Park (2010)	0.00306	n.s.	0.00000
Outplanted population: Lāwa‘i Kai	0.00930	n.s.	n.s.
Natural population: Mānā	0.00000	0.82904	
Outplanted population: Makauwahi	0.00472	0.00000	

originating from a larger geographical area than any other natural population sampled for this study (source plants occurred up to 4 km apart in separate sub populations; Belfield et al., 2011). The lowest levels of allelic diversity observed in source (natural, remnant) populations, Mānā ( $n = 2$ ) and Kāohikaipu ( $n = 1$ ) had outplanted counterpart populations that were also the least diverse (Makauwahi allelic richness = 1.05; expected/observed heterozygosity = 0.026/0.008; Kāohikaipu outplantings allelic richness = 1.09; expected/observed heterozygosity = 0.085/0.141)

Expected/observed heterozygosities averaged over loci were compared between natural and outplanted populations of *Sesbania tomentosa* and in most cases the values calculated for the natural populations were reduced in their outplanted counterparts. The exceptions were at Lāwa‘i Kai outplantings and, to a lesser extent, in Ka‘ena point State Park–NAR outplantings, both being derived from greater than 10 source plant individuals. Expected/observed heterozygosity averaged over three sampling years at Polihale (2006, 2009 and 2010) rose from 0.217/0.080 to 0.267/0.182 in Lāwa‘i Kai outplantings (seeds sourced from Polihale 2004). Expected/observed heterozygosity at Ka‘ena point rose from 0.219/0.077 to 0.166/0.098 in the outplantings (Table 4.2). Mean number of alleles per locus and mean allelic richness (averaged over loci) were mostly all reduced in a similar manner, again the exception being at Lāwa‘i Kai outplantings (rising from 1.77 to 2.30 alleles per locus), and to a lesser extent, in Ka‘ena point NAR outplantings (rising from 1.66 to 1.75 alleles per locus). Private alleles and the percentage of rare alleles (frequency < 0.1) also declined in outplanted populations, except for at Lāwa‘i Kai where private alleles remained the same and the percentage of rare alleles rose slightly from 38.1 to 40.9%. The only instance where the number of monomorphic loci declined in the outplanted population was Lāwa‘i Kai (monomorphic loci averaged over three sampling years at Polihale declined from 4.67 to 1.00 in Lāwa‘i Kai). Neither the Lāwa‘i Kai nor Ka‘ena point NAR outplantings were significantly differentiated from their source populations (Table 4.3).

Notable instances of genetic decline in outplanted populations of *Sesbania tomentosa* include Kīpuka Nēnē, where only two to three founders were used to source a population. These outplantings lost twice as many rare alleles and had lower allelic richness and observed heterozygosity values than their sister outplanted population Kulanaokuaiki, where more than 10 founders from the Kīpuka Nēnē-Hilina pali population were used as a source. Both of these outplanted populations were missing eleven low-frequency private alleles found in their source



population (Table 4.2), and both exhibited significant genetic differentiation from their source population at the 1% nominal level after Bonferroni corrections (Table 4.3).

The Kanahā County Beach Park outplanted population (founded from four individuals) is also notable in its dramatic decline, where 14 alleles were lost from the source population at Papanalahoā–Nākālele (alleles with an average frequency of 0.32), four previously polymorphic loci became fixed for a single allele, and measures of heterozygosity and allelic diversity were all cut in half (Table 4.2). One of the highest rates of genetic differentiation detected (0.635) was observed between Kanahā and its source population at Papanalahoā–Nākālele (Table 4.3). Four genotypes, each having eight out of nine loci fixed for a single allele, made up 89% of the Kanahā outplantings; the remaining 3 individuals had completely unique genotypes (data not shown).

Inbreeding coefficients ( $F_{IS}$ ) within populations were measured where  $F_{IS} = -1.0$  indicates 100% heterozygosity of individuals,  $F_{IS} = 0.0$  indicates the observed number of heterozygotes equals the number expected based on allele frequencies, and  $F_{IS} = 1.0$  indicates the complete absence of heterozygotes in a population with multiple alleles per locus. Coefficients averaged over nine loci were compared between natural and outplanted populations and in three cases (Kulanaokuaiki, Kanaio U.S. Army Training Area, and Makauwahi) the inbreeding coefficients of outplanted populations were greater than those of their corresponding source (natural) populations (Table 4.2). At Kīpuka Nēnē, Ka‘ena point, Kanahā and Lāwa‘i Kai, inbreeding coefficients in outplanted populations were reduced compared to those of their corresponding source (natural) populations (Table 4.2).

#### Indirect estimates of genetic bottlenecks

The Wilcoxon tests carried out in BOTTLENECK revealed evidence for recent, rapid losses of genetic diversity in only three populations of *Sesbania tomentosa*, two of which were natural (source) populations. A significant population decline is estimated to have taken place in the Polihale (2010) populations based on all three mutation models examined, and in the Ka‘ena point State Park–NAR based on two out of three models examined (Table 4.4). Kanaio U.S. Army Training Area was the only outplanted population to show evidence for a recent, significant population decline, based on all three mutation models ( $P \leq 0.05$ ). The distribution of

Table 4.4. Tests for genetic bottlenecks in natural *vs.* outplanted representative populations of *Sesbania tomentosa*. Mode shift indicates deviation from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium. Wilcoxon tests for heterozygote excess (Piry et al., 1999) under three mutation models (step-wise mutation, SMM; two phase model, TPM; infinite alleles model, IAM). Values highlighted in bold are those indicative of a bottleneck ( $P \leq 0.050$ ).

Population	Island	<i>n</i>	Mode Shift	Wilcoxon tests:		
				SMM	TPM	IAM
Natural population:						
Kīpuka Nēnē–Hilina pali	Hawai‘i	35	normal	0.900	0.320	<b>0.009</b>
Outplanted representative populations:						
Kīpuka Nēnē	Hawai‘i	7	shifted	0.973	0.945	0.945
Kulanaokuaiki	Hawai‘i	21	normal	0.629	0.473	0.273
Natural population:						
Pu‘u Pīmoē	Maui	9	shifted	0.918	0.411	<b>0.024</b>
Outplanted representative population:						
Kanaio U.S. Army Training Area	Maui	6	shifted	<b>0.037</b>	<b>0.014</b>	<b>0.009</b>
Natural population:						
Papanalahoā–Nākālele	Maui	44	normal	0.326	0.082	<b>0.007</b>
Outplanted representative population:						
Kanahā County Beach Park	Maui	28	normal	0.984	0.969	0.953
Natural population:						
Nu‘upia Ponds (Mōkapu)	O‘ahu	2	shifted	0.578	0.578	0.578
Mokulē‘ia State Tree Nursery (2009): origin: Nu‘upia Ponds	O‘ahu	8	shifted	0.932	0.921	0.910

Table 4.4. (Continued) Tests for genetic bottlenecks in natural vs. outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	Mode Shift	Wilcoxon Tests:		
				SMM	TPM	IAM
Natural population:						
Kāohikaipu	O‘ahu	1	shifted	1.000	1.000	1.000
Mokulē‘ia State Tree Nursery (2009):						
origin: Kāohikaipu	O‘ahu	11	shifted	0.986	0.955	0.432
Natural populations:						
Ka‘ena point State Park–Natural Area Reserve (NAR)	O‘ahu	33	shifted	0.313	<b>0.047</b>	<b>0.031</b>
Outplanted representative population:						
Ka‘ena point NAR	O‘ahu	32	shifted	0.906	0.438	0.063
Natural populations						
Polihale State Park (2006)	Kaua‘i	16	shifted	0.125	0.063	0.063
Polihale State Park (2009)	Kaua‘i	11	normal	0.781	0.656	0.344
Polihale State Park (2010)	Kaua‘i	12	shifted	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>
Outplanted representative populations:						
Lāwa‘i Kai	Kaua‘i	14	normal	1.000	1.000	0.875
Origin of <i>ex-situ</i> source population:						
Mānā	Kaua‘i	2	shifted	1.000	1.000	1.000
Outplanted representative populations:						
Makauwahi	Kaua‘i	14	normal	1.000	1.000	1.000

alleles in these same populations was also indicative of a bottleneck, as alleles at low frequency were found to be less abundant than alleles at intermediate frequency (a “mode shift”), a trend observed in an additional five source (natural) and four outplanted populations as well (Table 4.4).

## Discussion

An analysis of molecular variance (AMOVA) in Chapter 2 revealed that 56% of the genetic variation was found within populations of *Sesbania tomentosa* (Table 2.7), and efforts to create new populations should take care to maintain this variation. Extreme cases of genetic erosion in many of the restored populations sampled, as measured by increased inbreeding and loss of alleles, imply that material used for outplanting was the offspring of very few outcrossed parents. On the other hand, loss of heterozygosity might be explained by seed collectors having inadvertently obtained groups of progeny that were the result of selfing (geitonogamous or otherwise). Low inbreeding coefficients were observed at Kanahā Beach Park, the Mokulē‘ia state tree nursery seedlings originating from Kāohikaipu and Nu‘upia and in the *ex situ* source population for the plants originating from Mānā, yet these were probably due to extremely low sample size and/or allelic diversity constraints on calculations.

Evidence for recent bottlenecks in the form of transitory heterozygote excess was largely unsubstantiated in the BOTTLENECK analysis. When population size becomes very small (~10 or fewer individuals) and when generation times are short as with most populations of *Sesbania tomentosa* [e.g., Hopper (2002) reported a longevity of 3 to 10 years at Ka‘ena point], a new mutation-drift equilibrium should be arrived at quite rapidly (Watterson, 1984).

Of the eight source (natural) populations of *Sesbania tomentosa*, Kīpuka Nēnē–Hilina pali and Pu‘u Pīmoe exhibited the highest values of allelic diversity. Accordingly, the highest values of allelic diversity of all nine outplanted populations were observed in the outplanted counterparts of these two populations, suggesting that the standing variation of a founding population is an important baseline determination of possible levels of diversity that can potentially be captured in an outplanted representative population.

The strategy used at Hawai‘i Volcanoes National Park to source the Kulanaokuaiki outplantings involved mixing five separate subpopulations located 2 km apart (combined

together here as the Kīpuka Nēnē–Hilina pali population). This is the sole case of material collected from separate subpopulations being combined into a single outplanted population that was genetically analyzed in this study. The average  $F_{ST}(\theta)$  among these five subpopulations was 0.39 (see Table 2.8). This relatively high amount of differentiation between subpopulations has significant management implications. If it had arisen from genetic drift acting over a long period of time, there would be more reason for maintaining subpopulations separately instead of mixing them *in situ*. On the other hand, if the differentiation was due to more recent and rapid genetic drift due to population decline and fragmentation of habitat, random allele loss is playing the predominate role in high  $F_{ST}(\theta)$  values. The subpopulations would then be more appropriately managed by mixing to regenerate much of the original genetic diversity. Similar to other legumes, *Sesbania tomentosa* is a preferred food for feral goats (*Capra aegagrus hircus*) that have long been a problem in this area of the park; 15,000 animals persisted in the area around Hilina pali as recently as the 1970's (Baker and Reeser, 1972; Katahira and Stone, 1982). In addition, extensive wildfires burned through Kīpuka Nēnē twice in the last 40 years, and can also be expected to have caused dramatic declines in the *S. tomentosa* population there as well. It appears that the decision to mix subpopulations on the part of park resource management personnel was therefore guided and sound.

At the Kanaio U.S. Army training area, it appears that a small number of *Sesbania tomentosa* individuals captured levels of diversity above normal when compared with the other examples listed in Table 4.2. For example, two private alleles (average frequency = 0.21) were found in these outplantings that were not found in their source population at Pu'u Pīmoe at the time of sampling there in 2006. This was entirely by chance, as only three founding individuals were used to source the seed for Kanaio U.S. Army training area (seeds collected at Pu'u Pīmoe 2002), yet emphasizes the need to maximize founders in case such genetically unique individuals happen to be present.

The individuals comprising these small remnant source populations of *Sesbania tomentosa* may vary over time due to seed bank recruitment and/or extirpation of individual plants. For example, the Nu'upia ponds outplantings were derived from only two of the three plants extant in the natural population at the time of sampling (due to the lack of fruit set on one individual at the time of seed collection). All measures of genetic diversity declined, including the loss of 8 alleles from the omission of a single (possible) founding individual (Table 4.2). The

opposite occurred in the Makauwahi outplantings, derived from three *ex situ* founding individuals (natural population previously extirpated), only two of which survived to collect DNA from for this study in 2006. As a result, the outplantings preserve an additional allele and slightly higher measures of genetic diversity overall than the existing *ex situ* source. Similarly, the Kāohikaipu individual extant at the time of collection in 2009 (here representing the entire natural source population) happened to be slightly less genetically diverse than the two individuals on that islet at the time seeds were collected for outplantings (two years prior), in that a single allele was missing in the extant natural source population (Table 4.2).

Lāwa‘i Kai is another example of where the seed sourcing practices used maintained levels of genetic diversity above that contained in the natural standing population of *Sesbania tomentosa* at Polihale State Park during the years 2006, 2009 and 2010 (Table 4.2). Over 400 pods were collected from 14 founding individuals during the summer of 2004 to create the outplantings for Lāwa‘i Kai (Mike Demotta, NTBG, personal communication). This is one of the best cases of maximizing founders analyzed for this study, which fortunately happened one reproductive cycle (approximately 1 year) after the population had “flushed”. Similarly, 2009 was another year in which the Polihale population had rebounded in numbers and the levels of diversity rose (and indications of bottlenecks fell) when compared to diversity levels among 2006 collections. From this, it was deduced that the population “flush” of 2003 produced a similar result in standing genetic diversity of the founding population in 2004, which led to the results obtained here.

A relatively high percentage of the alleles in both the Kulanaokuaiki (36.0%) and Lāwa‘i Kai (40.9%) populations occurred at a frequency less than 0.1, yet the importance of rare alleles in the restoration of plant populations has been debated. Some argue it is not necessary to capture all the genetic variation in a species as rare alleles may in fact be recent mutations or deleterious and are likely to be lost in a few generations of random mating (Brown and Briggs, 1991; Holsinger and Gottlieb, 1991). In the case of *Sesbania tomentosa*, it is possible that inbreeding has purged deleterious alleles long ago (see Chapter 2) and that we need not leave it to random mating *in situ* to preserve such alleles. For instance, rare alleles might become more common in outplanted populations if an effort to cross fertilize individual plants to maximize genetic diversity in offspring took place; this study has helped to identify individuals appropriate for such measures.

Regardless of the advantages that maximizing genetic diversity should theoretically impart over the long run, mortality of *Sesbania tomentosa* outplantings has been high in some of the sites that were shown to have captured the highest amounts of genetic diversity in this study. For example, of the 177 outplantings at Kulanaokuaiki and Kīpuka Nēnē, 12.9% survived 50 months post-planting (Belfield et al., 2011) yet only 3–4 outplantings (approximately 1.6%) survived as of 2015 (Joshua VanDeMark, Plant Extinction Prevention Program, personal communication). The preponderance of the exotic natal redtop grass (*Melinis repens* (Willd.) Zizka) may be affecting water balance and increasing competition with *S. tomentosa* in these two sites (Belfield et al., 2011). In addition, as of 2014 all individuals at both the Pu‘u Pīmoe population and Kanaio U.S. Army Training Area had been extirpated (Keahi Bustamente, Plant Extinction Prevention Program, personal communication), probably due to an extended period of drought in SE Maui since 2006. The outplanted individuals at Lāwa‘i Kai also suffered from high mortality (possibly due to root knot nematodes (David Burney, NTBG, personal communication) and had to be periodically replaced by new plantings on site. On the other hand, the outplantings at Ka‘ena point (O‘ahu) are some of the most successful in Hawai‘i- often up to 94% of outplantings survive 6 months post planting, and a substantial amount have seemed to survive indefinitely (15 years later; David Smith, Hawai‘i Department of Land and Natural Resources, personal communication). This success is notable given the relative lack of genetic diversity observed in this study in both the outplantings and the standing natural population at Ka‘ena point.

## Conclusion

In all of the above examples of outplanted populations of *Sesbania tomentosa*, it will be important to monitor the occurrence of the plants that establish themselves from seeds produced by the reintroduced plants, and their progeny for impact of genetic drift in the future generations. The outplanting efforts and strategies of the National Tropical Botanical Garden and Hawai‘i Volcanoes National Park were the most successful at capturing genetic diversity in this species when compared with other examples. This comparison is most notable at Kanahā County Beach Park where a large population was ineffectively used to source a relatively large number of outplantings (only 4 founders out of 44 potential source plants) resulting in extremely low

genetic diversity and one of the highest rates of genetic differentiation from source to founder population. In spite of the low diversity at Kanahā (and therefore low theoretical expectations for long-term success), almost all of the outplantings survived and flourished in the coastal dune habitat for over five years until all of them were killed in a six foot tidal surge event caused by the 2011 Tōhoku Earthquake in Japan. Twelve new plants were recorded at Kanahā in 2014, apparent seedlings sprouted from the seedbank after all the plants had perished in 2011 (Forest Starr, USGS, personal communication). This was a true test of the population's health and resilience. The only other instance of seedling recruitment in an outplanted population was at the Makauwahi site (David Burney, NTBG, personal communication), also a population with low genetic diversity. The Kanahā site receives ca. 400 mm of rain per year (vs. the Lāwa'i Kai/Makauwahi sites which receive ca. 980 mm and Kulanaokuaiki/Kīpuka Nēnē which receive ca. 1860 mm of rain per year; Giambelluca et al., 2014). Drought conditions at each site immediately post planting are probably more relevant for mortality, as is the consistency of rainfall in the years that followed. In each case, it would appear that lack of adequate rainfall at the less successful sites can be ruled out as a factor in their decline.

The long-term survival of outplantings of *Sesbania tomentosa* might not depend upon genetic diversity as much as other unforeseen factors. For example, a comparison of horticultural methods used to raise and outplant this species is likely to offer more insight and assistance than information on genetic diversity for resource managers across the state. Regardless, it has been shown that the allelic diversity changes from year to year in the Polihale population of *S. tomentosa*, and this pattern is likely to be true in other populations that fluctuate and resprout intermittently from a seedbank. Collecting from a large number of founders, as widely spaced apart as is possible (on a local geographic scale), is shown to play an important role in preserving genetic diversity in outplanted populations. Collecting from founders spaced apart temporally (e.g., the same population in subsequent years) might produce additional, unique founders and may be an important strategy to consider, as well as focusing collection activities during years in which natural populations are rebounding in numbers ("flushing"). As expected, the standing variation of founders being collected from in any given year is of primary importance in raising genetically diverse seedlings.



## CHAPTER 5

### Synthesis of hypotheses and findings

The hypothesis that Hawaiian *Sesbania* form a monophyletic group and represent a recent radiation among the Hawaiian Islands was accepted as sequence diversity was shown to be virtually non-existent at the two nuclear regions sampled for this study. In addition, the monophyletic group containing Hawaiian *Sesbania* was also found to include *S. marchionica* from the Marquesas. The hypothesis that the formal recognition of additional taxa of Hawaiian *Sesbania* may be warranted was also accepted, although DNA sequence data provided no evidence (by itself) for splitting *S. tomentosa* into additional species. The evidence was found instead in the microsatellite analysis where Bayesian genetic clustering assignments and associated private alleles occurred in a distinct phylogeographic pattern. As a result, populations from Nihoa, Kaua‘i and O‘ahu are distinguished as a separate subspecies of *S. tomentosa*, populations from Maui Nui and Hawai‘i Island (respectively) form two additional subspecies, and a fourth subspecies endemic to SE Moloka‘i distinguishes itself from the rest of Maui Nui.

The hypothesis that populations will exhibit high levels of genetic structure with evidence of inbreeding within and divergence among populations was also accepted. Global  $F_{ST}$  over all populations and loci was estimated at 0.39 (or as high as 0.50 if including apparent clonal genotypes in the dataset) and inbreeding coefficients ( $f$ ) were estimated at 0.56 (ranging as high as 0.94). Strong spatial genetic sub-structure was also observed within populations and sub-populations. These results, considered in light of previously published observations of pollination in *Sesbania tomentosa*, infer that this species is predominately inbreeding due to sib and/or self-mating. The hypothesis that levels of inbreeding will be higher, and genetic diversity lower, in outplanted populations than in their naturally-occurring counterparts is not accepted, as inbreeding coefficients were shown to be extremely high in natural as well as outplanted populations. In certain cases, genetic diversity rose and inbreeding coefficients fell when outplanted populations were compared with their naturally-occurring counterparts. Genetic diversity was shown to be dynamic over time in natural populations whose members fluctuate and resprout intermittently from a seedbank. The standing variation of founders being collected from in any given year is therefore of primary importance in raising genetically diverse seedlings.

The hypothesis that natural selection in different environments over time combined with contemporary fragmentation (isolation) of populations caused Hawaiian *Sesbania* to separate into the distinctive appearing populations found today is also tentatively accepted. Ecologically, this rapidly maturing species appears prone to inbreeding and repeated bottlenecks, adding efficiency to a natural trend of divergence. Yet it is entirely plausible that both the microsatellite as well as the morphological differentiation observed have been accentuated within the time period when populations of *S. tomentosa* became increasingly isolated from one another. In other words, more modern impacts on the range of the species have probably only accelerated what was already naturally-occurring. The relative contributions of contemporary vs. long term impacts on population differentiation are impossible to completely disentangle, yet evidence is herein presented which points to differentiation that was largely underway prior to historic fragmentation of populations.

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POPULATION DIVERGENCE AND EVOLUTION  
OF THE ENDANGERED *Sesbania tomentosa* (FABACEAE)

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## ABSTRACT

*Sesbania tomentosa* (Fabaceae) is an endemic flowering plant primarily adapted to coastal strand and dry lowland habitat in the Hawaiian Islands, now extant in relicts of its former range. Efforts have been made to delineate distinct taxa from among the remaining populations. In the most recent treatment of Hawaiian Fabaceae, however, *S. tomentosa* was recognized as a single variable species. In an attempt to address issues of taxonomy, the present study compared phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses (DNA sequence and microsatellite marker variation) and assessed their relative level of congruence. A complete lack of variation between eight putative taxa from six islands at two nuclear DNA regions (1035 bp) contrasts with the highly differentiated population structure of the nine microsatellite loci sampled, while confidence in the relationships proposed in morphological phylogenies based on putative taxonomy was low. Instead, Bayesian genetic clustering assignments and associated private alleles occurred in a distinct phylogeographic pattern. As a result, populations from Nihoa, Kaua‘i and O‘ahu are distinguished as a separate subspecies of *S. tomentosa*, populations from Maui Nui and Hawai‘i Island (respectively) form two additional subspecies, and a fourth subspecies endemic to SE Moloka‘i distinguishes itself from the rest of Maui Nui.

Naturally-occurring populations of *Sesbania tomentosa* plus a substantial number of outplanted individuals were analyzed for levels of allelic diversity, heterozygosity and inbreeding. Evidence of genetic bottlenecks in populations was also investigated, as well as an analysis of population sub-structuring. Natural ecological dynamics affecting population differentiation often leave lasting genetic signatures, and are addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants. The

molecular data can be interpreted to support the hypothesis that distinctive-appearing remnant populations of this highly variable species have diverged at an accelerated rate due to human induced habitat fragmentation within the larger context of the speciation process itself. This study also provides examples of increasing genetic diversity in outplantings when intentional mixing of populations to augment diversity was practiced, as well as in situations where the genepools of natural populations are dynamic over time.

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## CHAPTER 1

### **Phylogenetic relationships and population structuring within the *Sesbania tomentosa* species complex; relevance for restoration management of relict plant populations**

#### **Introduction**

*Sesbania tomentosa* Hook. and Arn. is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. *Sesbania tomentosa* is currently recognized as a single species (Geesink et al., 1999) although it is highly variable for many important characters across its range. This led Rock (1920), Degener and Degener (1978) and Char (1983) to delimit up to nine distinct putative taxa. Two major groups emerged in a genetic analysis of Hawaiian *Sesbania* measuring variation at ten isozyme loci across the geographical range of the species Gemmill et al. (1995). An analysis of *S. tomentosa* with both sequencing and population genetic markers would lend justification at the molecular level for one or more separate taxonomic entities.

Twenty-nine of the fifty-two populations of *Sesbania tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826, largely the result of intense ungulate grazing pressure across its range. As a result, this species was federally listed as Endangered by the U.S. Fish and Wildlife Service in 1992. The relictual nature of the present range of the species is thought to have accentuated morphological differentiation of populations (Geesink et al., 1999). On the other hand, natural ecological dynamics affecting population differentiation (e.g., pollination syndromes, plant maturation rate, seedbank dynamics, population flush-crash cycles) often leave lasting genetic signatures, and can be addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants.

Understanding both the nature of population differentiation and its extent (in terms of putative speciation) has important implications for restoration management of this Endangered plant. For example, three of Char's (1983) putative taxa occur within 5–10 km of one another in Hawai'i Volcanoes National Park. Two of these putative taxa are endemic to the park, each represented by less than 50 naturally-occurring individuals. Two other species delimited by Char

were once found to occur within 4 km of one other on the west coast of Kaua‘i. Today one of these putative species can be found in a small population of 20–30 individuals *in situ*, the other *ex situ* at the National Tropical Botanical Garden. Given the close proximity of populations in both these instances, separate putative Hawaiian *Sesbania* taxa had likely exchanged genes in the past when the plant’s range was more substantial. Restoration managers need to address the genetic structuring of this apparent species complex before considering the translocation of propagules to enhance genetic variation within reproductive populations. This is important, as mixing populations representing separate putative taxa may also put their genetic and taxonomic integrity at risk.

#### Primary Objectives of Investigation

- 5) Investigate morphological relationships between putative taxa of Hawaiian *Sesbania* using phylogenetic analysis of a morphological character dataset
- 6) Investigate the distinctiveness of putative taxa of Hawaiian *Sesbania* on a molecular level using DNA sequencing of nuclear regions
- 7) Levels of genetic variation within and between populations (and putative taxa) will be examined using microsatellite marker analysis
- 8) Compare genetic diversity of naturally-occurring individuals and populations with their counterpart outplanted individuals and populations

#### Primary Hypotheses

- 6) Hawaiian *Sesbania* form a monophyletic group and represent a recent radiation among the Hawaiian Islands
- 7) The formal recognition of additional taxa of Hawaiian *Sesbania* is warranted based on genetic and morphological evidence
- 8) Populations will exhibit high levels of genetic structure with evidence of inbreeding within and divergence among populations

- 9) Natural selection in different environments over time combined with contemporary fragmentation (isolation) of populations caused Hawaiian *Sesbania* to separate into the distinctive appearing populations found today
- 10) Levels of inbreeding will be higher, and genetic diversity lower, in outplanted populations than in their naturally-occurring counterparts

## Materials and Methods

In an attempt to address issues of taxonomy, the present study compared phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses (DNA sequence and microsatellite marker variation) and assessed their relative level of congruence. Morphometric measures from the dataset developed by Char (1983) were used to construct morphological phylogenies. Phylogenetic inference at the molecular level used sequences from two nuclear DNA regions (1035 bp sampled): the non-coding internal transcribed spacer (ITS) of the ribosomal DNA cistron (Baldwin, 1993) and the highly variable gene-coding region triosephosphate translocator (TRPT) (Choi et al., 2004, 2006). Nine microsatellite marker loci were also used to assess within and among population variation found in individuals and to assess the degree of population differentiation. Together, sequence and microsatellite variation provide an estimate of phylogenetic relationships among the species and populations previously identified by Char (1983) and others.

Leaf samples of 539 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 38 sub-populations (separate clusters of plants 1 to 3 km apart within a population) comprising 18 populations from seven islands were sampled. An additional 141 individuals (collected from 8 populations on four islands) were sampled from *S. tomentosa* outplantings and restoration nursery stock. Twelve individuals were sampled from herbarium specimens to provide historical DNA from various populations for comparison. In order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, one population was sampled in three separate years (seasons), and the genetic diversity of the standing populations of each year are herein compared. The long term viability of populations actively managed for restoration was addressed using microsatellite markers, by comparing the genetic diversity of naturally-occurring populations of *Sesbania tomentosa* with those of their outplanted

counterparts to assess rates of inbreeding and impacts of genetic drift. As various numbers of founding individuals (from 1 to more than 10) have been used to assemble the outplanted populations measured, the genetic effects of seed sourcing practices were also examined.

## CHAPTER 2

### Phylogenetic relationships within the *Sesbania tomentosa* species complex

#### Introduction

The boundaries of species that have recently and rapidly diverged are difficult to determine when species-specific traits (morphological and/or genetic) have not had sufficient time to coalesce (Glor, 2010). Even if the morphology of the species in question seems to suggest such boundaries, DNA sequence divergence often will not have occurred due to insufficient time for accumulation of mutations within the different types (Mort et al., 2007). Hawaiian plant radiations are well recognized for morphological variation disconnected from genetically detectable differences (e.g., Gemmill et al., 2002, Lindqvist et al., 2003, Knope et al., 2012, Cantley et al., 2014). On the other hand, population genetic markers, those tied to allele frequencies diverging at a much more rapid pace, are able to distinguish genetically-isolated populations and groups of populations (Zhang and Hewitt, 2003). According to the unified species concept of de Queiroz (2007), a species is defined as a separately evolving metapopulation lineage (ancestral sequence of populations). Given this, the ability of population genetic markers to identify the boundaries of isolated gene pools makes them a suitable choice for analyzing recent and rapid plant radiations.

An investigation into the evolution of the Hawaiian endemic *Sesbania tomentosa* Hook. & Arn. (Fabaceae) is warranted, as past taxonomic history suggests there are relationships to resolve within this highly variable species. In the most recent treatment of Hawaiian Fabaceae, however, *S. tomentosa* was recognized as a single species with one form (f. *arborea* Rock) (Geesink et al., 1999). A previous genetic study by Gemmill et al. (1995) demonstrated that two major groups of Hawaiian *Sesbania* emerged when measuring variation at ten isozyme loci across the geographical range of the species. An analysis of *S. tomentosa* with both sequencing and population genetic markers may lend justification at the molecular level for one or more separate taxonomic entities.

*Sesbania tomentosa* is adapted to coastal strand and dry to mesic upland habitat. Geesink et al. (1999) described the species as a sprawling shrub with branches up to 14 meters long or

alternatively found as a small tree up to 6 meters in height. Leaves are even-pinnate and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. Leaflets are usually sparsely to densely covered with silky hairs, as referred to by the specific epithet. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds. The chromosome number reported is  $2n = 24$  (Geesink et al., 1999) suggesting the species is diploid (base chromosome number  $x = 12$ ).

G.T. Lay and A. Collie were the first to collect *Sesbania tomentosa* during the voyage of the HMS Blossom (under Captain Frederick William Beechey) through the Hawaiian Islands from 1826–1827, and their specimen was later described by Hooker and Arnott (1838). However, the type locality was erroneously listed as Acapulco, Mexico, this later corrected by Gray (1854). Since the botanists on the expedition were only believed to have collected on O‘ahu, the type locality is presumed to be from somewhere on that island (Gray, 1854; Feipel, 1914). Gray (1854) described *S. tomentosa* as a woody plant with decumbent (semi-prostrate) stems, having branches and foliage silky-tomentose when young, but turning glabrate with age. Gray noted that these plants occurred on the Wai‘anae coast of O‘ahu and on the coast of Hawai‘i east of Kīlauea Crater. Hillebrand (1888) described *S. tomentosa* in much the same way as Gray, only he found it occurring as a multi-branched shrub, 6 to 12 feet (2 to 4 m) in height. His specimens were also collected from the Wai‘anae coast of O‘ahu and on the southern shores of Moloka‘i, Lāna‘i and Hawai‘i.

Rock (1920) proposed an alternate form of *Sesbania tomentosa*, forma *arborea*, an arborescent type he had collected at Mahana (west Moloka‘i) growing 12 to 15 feet in height. He described the leaves as being longer, and the leaflets smaller and more numerous than the creeping variety he found growing nearby in the dunes at Mo‘omomi. Rock lists his arborescent form as also being present on the islands of Kaua‘i, O‘ahu and Hawai‘i.

Degener (1938) was the first to consider that *S. tomentosa* represents a poorly understood species complex and is probably composed of a number of forms on most of the islands (delineated primarily in terms of plant habit and leaf pubescence). Degener and Sherff (1949) considered the prostrate form at Mo‘omomi, Moloka‘i to be sufficiently distinct to warrant its own variety (*S. tomentosa* var. *molokaiensis*), due in part to the dense sericeous tomentum found on both surfaces of the leaflets. St. John (1973) concurred with Rock (1920) and with Degener



and Sherff (1949), and listed one endemic species of *Sesbania* with one variety (var. *molokaiensis* Degener & Sherff) and one form (f. *arborea* Rock). Degener and Degener (1978) recognized four new species of Hawaiian *Sesbania* elevating *S. tomentosa* var. *molokaiensis* and f. *arborea* to *S. molokaiensis* (Degener & Sherff) Degener & I. Degener and *S. arborea* (Rock) Degener & I. Degener, respectively. They also described *S. hawaiiensis* Degener & I. Degener from the South point region of Hawai‘i (mainly on the basis of slight variations in flower, stem and seed color) and *S. hobyi* Degener & I. Degener (a small erect tree with long extending branches and only a minor pubescence on lower surface of leaflets) from the island of Lāna‘i.

Char’s (1983) taxonomic thesis is the most recent and extensive survey of the morphological variation among Hawaiian *Sesbania* populations, making the important observation that the presence of hairs on leaflets is a useful taxonomic character. *Sesbania tomentosa* was split by Char into two varieties, the geographically widespread “var. *tomentosa*” (a highly polymorphic taxon in terms of leaf tomentum and flower color) and a minor variant from a single population, “var. *hobyi*” from Lāna‘i. Char also recognized *S. molokaiensis* from Mo‘omomi Moloka‘i (noting dense tomentum on both surfaces of leaflets) and *S. arborea* (noting sparse hairs confined to midrib of lower surface of leaflet) from the islands of Moloka‘i, Maui and Hawai‘i. Char named five additional putative taxa as well (none of which were ever validly published): “*polihalensis*” from the islands of Kaua‘i and Nihoa (erect shrubs with hairs on upper surface of leaflets confined to the midrib and veins), “*manaensis*” from the Mānā plain of Kaua‘i, “*oricola*” from the islands of O‘ahu, Ni‘ihau and Necker (erect shrubs with both surfaces of leaflets covered with dense tomentum) and “*kauensis* var. *kauensis*” and “*kauensis* var. *intermedia*” (erect shrubs with extremely long trailing lower branches and large leaflets with conspicuous reddish-brown pigmentation on stipules and leaflet margins) from the Ka‘ū district of Hawai‘i Island (Char, 1983). Char compiled morphometric datasets based on her observations of both plants in the field as well as herbarium specimens to elucidate relationships among populations of *Sesbania*. Her research reported that while a certain degree of phenotypic plasticity is apparent in varieties of Hawaiian *Sesbania*, cultivated individuals of the different varieties in a common garden retained the same morphological characters as their counterparts in the field (Char, 1983).

The purpose of the present study was to compare phylogenetic hypotheses of Hawaiian *Sesbania* determined by morphological markers with those determined by molecular analyses

(DNA sequence and microsatellite marker variation) to assess their relative level of congruence. Morphometric measures from the dataset developed by Char (1983) were used here to construct morphological phylogenies. For the sake of simplicity in identifying the various morphotypes, Char's (1983) unpublished nomenclature is used throughout since it had covered the broadest spectrum of variation across Hawaiian *Sesbania*. Phylogenetic inference at the molecular level used sequences from two nuclear DNA regions: the non-coding internal transcribed spacer (ITS) of the ribosomal DNA cistron (Baldwin, 1993) and the highly variable gene-coding region triosephosphate translocator (TRPT) (Choi et al., 2006). Microsatellite markers were used to assess within and among population variation found in individuals and to assess the degree of population differentiation. Together, sequence and microsatellite variation will provide an estimate of phylogenetic relationships among the species and populations previously identified by Char (1983) and others from which character evolution can be estimated.

The ITS region has been the most extensively used nuclear region for phylogenetic analyses in plants since first used by Baldwin et al. (1995). Many legume groups have been sampled for ITS (Allan and Porter 2000; Lavin et al., 2003, Schrire et al., 2003; McMahon and Hufford 2004); ITS even varies below the species level within some taxa (Lavin et al., 2003). ITS sequence variation has been shown to provide better resolution of closely related legumes compared to the plastid region *trnL-F* (Wojciechowski et al., 1999; Lavin et al., 2001). In addition, *trnK-matK* showed little nucleotide variation across *Sesbania* taxa worldwide (Farruggia, 2009) and no variation among the four Hawaiian accessions (from three separate submissions) on GenBank (accession #s JX295926, JQ669637, JQ669638, HQ730420). It is for these reasons, and because of the eventual outcome of nDNA sequencing, that the plastid genome was not sampled for the present study.

Variation at the exon-derived TRPT gene was also examined as Choi et al. (2004) provided evidence that this region is suitable for phylogenetic analysis in legumes at the specific and subspecific levels. The divergence of this region between six legume genera (*Medicago*, *Pisum*, *Lotus*, *Glycine*, *Vigna* and *Phaseolus*) was shown to range as high as 42.7% (Choi et al., 2006) and Farruggia et al. (2009) found that the TRPT region concurred with species level resolution of ITS and *trnK-matK* topologies of *Sesbania* worldwide.

Microsatellite markers have a more rapid mutation rate than DNA sequence data (Jarne and Lagoda, 1996), and were another tool used to study relationships between Hawaiian

*Sesbania* populations and the various morphological types. Analytical methods such as STRUCTURE use multilocus microsatellite genotypes to assign individuals to genetic clusters without their *a priori* designation into populations. These methods were complemented by pairwise comparisons of allele frequencies in geographical populations as well as among the different morphological types to clarify their relationships.

## Materials and Methods

### Collection of morphological character data

Eighteen morphological characters discussed by Char (1983) in terms of their taxonomic significance for Hawaiian *Sesbania* were coded as discrete data for input into a matrix (Tables 2.1 and 2.2). Seven of these 18 characters were highly variable within putative taxa, therefore average values of characters over a range of sample sizes (20 to over 300) were used. The other 11 characters were less variable within putative taxa and were classified on the basis of personal observations made in the field and from reading Char's concise descriptions of each putative taxon.

Sachet (1987) examined the morphology of the South Pacific species of *Sesbania* and considered that the French Polynesian species *S. coccinea* (L.f.) Poir. was undoubtedly a close relative of *S. tomentosa* and, thus, was used as the outgroup in the phylogenetic analysis of morphological data. Character states were measured from 20 herbarium specimens at the B. P. Bishop Museum Herbarium (BISH; Honolulu, HI) and were used along with the taxonomic description of Sachet (1987) to develop the data matrix entry (Table 2.1).

### Phylogenetic analysis of morphological character dataset

The exhaustive search algorithm was used in PAUP v. 4.0 (Swofford, 2002) to infer maximum parsimony phylogenetic hypotheses. All character state changes were treated as unordered and unweighted. Bayesian analysis was also carried out on the data matrix using the standard discrete morphology model (Lewis, 2001) in MrBayes v. 3.1 (Ronquist et al., 2005) using 100,000 MCMC replications following a burn-in of 40,000 replicates. Posterior

Table 2.1. Character state matrix of putative species of Char (1983) plus outgroup (*S. coccinea*).

Putative species	Morphological characters																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>S. coccinea</i> (outgroup)	2	0	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0
“ <i>arborea</i> ”	0	0	1	1	1	0	0	0	0	1	0	0	0	0	1	1	1	0
“ <i>molokaiensis</i> ”	3	0	2	1	1	2	2	0	1	1	0	0	1	0	2	1	1	1
“ <i>manaensis</i> ”	1	0	2	1	1	1	1	1	1	0	0	1	0	0	1	1	1	0
“ <i>polihalensis</i> ”	1	0	1	0	0	1	1	0	1	0	0	1	0	0	1	0	0	0
“ <i>oricola</i> ”	1	0	2	0	0	2	2	0	1	1	0	0	1	0	1	1	1	0
“ <i>tomentosa</i> var. <i>tomentosa</i> ”	3	0	1	0	1	0	1	0	1	1	0	1	1	0	1	1	1	0
“ <i>kauensis</i> var. <i>kauensis</i> ”	2	1	1	0	1	0	1	1	0	1	0	0	1	0	3	1	1	0
“ <i>kauensis</i> var. <i>intermedia</i> ”	2	1	0	1	1	0	1	1	0	0	1	2	1	1	3	1	1	0

Table 2.2. Characters and coding key used for phylogenetic analysis of Hawaiian *Sesbania*.

Character	Character #	Code
HABIT:	1	0 = small tree (1–3 m); 1 = erect shrub; 2 = erect shrub with trailing lower branches; 3 = procumbent shrub
LEAVES:		
Mean leaf length:	2	0 = 10.0–13.0 cm; 1 = 13.0–17.0 cm
Mean number leaflet pairs/leaf:	3	0 = 17–15; 1 = 14–12; 2 = 11–9
Mean leaflet length:	4	0 = 23–30mm; 1 = 16–23mm
Mean leaflet width:	5	0 = 9–11mm; 1 = 6–9mm
Indument on upper leaf surface:	6	0 = entirely glabrate; 1 = partially tomentose; 2 = densely tomentose
Indument on lower leaf surface:	7	0 = sparsely tomentose; 1 = moderately tomentose; 2 = densely tomentose
Pigmentation:	8	0 = obscured / not readily recognizable; 1 = dark, prominent
INFLORESCENCE:		
Color	9	0 = gradations of yellow-orange-red; 1 = strictly red
Mean Flower length:	10	0 = 3–4cm; 1 = 2–3 cm
Number of flowers/raceme:	11	0 = 1–6 flowers/raceme; 1 = 7–9 flowers/raceme
Mean peduncle length:	12	0 = 1–3cm; 1 = 3–5cm; 2 = 5–8cm
Mean pedicel length	13	0 = 0–1.5cm; 1 = 1.5–3.0cm
Calyx lobe length:	14	0 = less than 1/2 as long as corolla; 1 = 1/2–2/3 as long as corolla
Appendages on standard petal:	15	0 = 0.5–1.5mm; 1 = 1.5–2.5mm; 2 = 2.5–3.0mm 3 = absent
PODS:		
Length of beak:	16	0 = long beak (2–3 cm) 1 = short beak (0.5–2 cm)
Surface:	17	0 = tomentose; 1 = glabrous
Seed length:	18	0 = $\geq$ 5mm; 1 = < 5mm

probabilities were calculated by Mr. Bayes as a means to test branch support. Both phylogenetic trees were visualized using Fig Tree v. 1.3.1.

## DNA sample collection

Leaf samples of 459 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 16 populations from seven islands were sampled (Table 2.3). An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. I recorded GPS coordinates for the locations of all samples each individual plant sample collected. Samples at 'Āpua point, Kawela–Kamiloloa, Pu'u Koa'e and Nihoa comprise a subset of their respective populations (individuals collected arbitrarily from throughout each population. At Pu'u Koa'e and Nihoa samples were obtained by surrogate collectors [Ken Wood, National Tropical Botanical Garden (NTBG) and Beth Flint (USFWS)] and no GPS coordinates were logged. An attempt to distinguish groups of naturally occurring vs. out-planted individuals at Ka'ena point was made with the assistance of Betsy Gagné [Hawai'i Division of Forestry and Wildlife (DOFAW)]. Except where noted above, only naturally occurring plants and all known individuals known extant at the time of collection were sampled for analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4° to 8° C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNeasy tissue kits (QIAGEN; Valencia, CA) according to the manufacturer's specifications and the purified sample, along with negative and positive controls, were visually checked using electrophoresis.

Additional sampling of historically-collected tissue from the Mo'omomi dunes population on Moloka'i was conducted with loaned specimens from the herbarium of the New York Botanical Garden (NY), the B. P. Bishop Museum Herbarium (BISH) and the U. S. National Herbarium (US) (Table 2.4). DNA was extracted from 10 specimens using QIAGEN's QiaAmp Stool minikits, modified CTAB protocols (Drábková et al., 2002) and a PTB (N-phenacylthiazolium bromide) protocol (Asif and Cannon, 2005). For each of the 10 specimens at least one of the extraction protocols listed proved successful. These historically-collected

Table 2.3. Origin of DNA samples analyzed of *Sesbania tomentosa*, using the putative species designations for populations of Char (1983). Duplicate genotypes in cases where plants had occurred less than 10 m apart were removed prior to running the various analyses (and are not listed here). Unique genotypes obtained from cultivated individuals were added into the Kīpuka Nēnē–Hilina pali, Mānā, Papanalaho–Nākālele, Polihale, Pu‘u Pīmoe, Waiaka‘īlio population datasets. Unique genotypes obtained from herbarium specimens augment the Kāohikaipu & Mōkapu and Mo‘omomi population datasets.

Putative species designation	Island	Population	# individuals analyzed
“ <i>tomentosa</i> var. <i>tomentosa</i> ”	Hawai‘i	‘Āpua point	50
	Hawai‘i	Kamilo point–Ka Lae	67
	Kaho‘olawe	Pu‘u Koa‘e	25
	Maui	Papanalaho–Nākālele	46
			Total = 188
“ <i>kauensis</i> var. <i>kauensis</i> ”	Hawai‘i	Pepeiau–Kukalau‘ula pali	19
	Hawai‘i	Kamo‘oali‘i–Kū‘ē‘ē	18
			Total = 37
“ <i>kauensis</i> var. <i>intermedia</i> ”	Hawai‘i	Kīpuka Nēnē–Hilina pali	33
“ <i>arborea</i> ”	Moloka‘i	Kawela–Kamiloloa	35
	Maui	Pu‘u Pīmoe	12
	Hawai‘i	Waiaka‘īlio	14
			Total = 61
“ <i>molokaiensis</i> ”	Moloka‘i	Mo‘omomi	36
“ <i>oricola</i> ”	O‘ahu	Kāohikaipu & Mōkapu	5
	O‘ahu	Ka‘ena point	17
			Total = 20
“ <i>polihalensis</i> ”	Kaua‘i	Polihale	38
	Nihoa	Nihoa	49
			Total = 87
“ <i>manaensis</i> ”	Kaua‘i	Mānā	5

Total Overall = 469

Table 2.4. DNA collected from herbarium sheets (one sample per sheet) loaned from B. P. Bishop Museum Herbarium (BISH), New York Botanical Garden (NY) and the U. S. National Herbarium (US).

Barcode/ID #	Collector	Date	Location notes from herbarium sheet
990804 (NY)	J.F.C. Rock	3-1909	Molokai. Moomomi.
990808 (NY)	J.F.C. Rock	3-1910	Molokai. Moomomi.
990809 (NY)	C.N. Forbes	3-24-1915	Molokai. Moomomi.
55944 (BISH)	G.C. Munro	7-22-1926	Moomomi sandhills.
990820 (NY)	O. Degener	4-19-1928	Kalani, Moomomi. creeping branches take root, single large plant in sand dunes several hundred feet above sea.
990817 (NY)	O. Degener	4-25-1928	Moomomi, Molokai arid sand dunes.
55933 (BISH)	M.C. Neal	4-1-1934	Mokapu Crater, Oahu, edge of cliff.
990810 (NY)	F.R. Fosberg	12-26-1936	Molokai. Moomomi prostrate shrub, base of sand dunes.
14052 (US)	F.R. Fosberg	6-13-1937	Oahu. Kaohikaipu.
990811 (NY)	C.S. Judd	9-16-1937	Molokai. Moomomi procumbent shrub, sand hills alt. 10m.
177376 (BISH)	H. St.John	1-3-1939	Moomomi, Kaluahoi on sand dunes.
488514 (BISH)	H. St.John	12-24-1948	Moomomi, Kaluahoi, trailing on sand dunes near shore.



samples were included in the analysis of microsatellite fragment sizes to supplement the allelic diversity of my 2006 collection of extant plants at Mo‘omomi.

The scant demographics of certain populations necessitated augmentation of the dataset in order to provide marginally larger sample sizes for comparison. DNA from a herbarium specimen collected in 1934 from “Ulupa‘u Crater” on the Mōkapu peninsula (O‘ahu) was extracted, which supplemented a DNA sample collected in 2008 from the Mōkapu peninsula at Nu‘upia Ponds. Another herbarium specimen collected in 1937 from the islet of Kāohikaipu (O‘ahu) was extracted to supplement total extant diversity represented by two Kāohikaipu-derived individuals in cultivation at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu). These five samples were combined into a single Windward O‘ahu population for this analysis. The unique genotypes of cultivated individuals (derived from their respective natural populations) were also used to augment the Kīpuka Nēnē–Hilina pali (Hawai‘i Island), Pu‘u Pīmoe and Papanalahoā (Maui) and Polihale and Mānā (Kaua‘i) populations. All five individuals comprising the Mānā population are cultivated specimens of the National Tropical Botanical Garden (F<sub>1</sub> and F<sub>2</sub> generation derived from a single wild plant, now extirpated). The Polihale population is composed of groups of unique genotypes collected over 3 sampling years (2006–2010), in addition to several unique cultivated genotypes. For the Waiaka‘īlio population, extant in only a single surviving individual at the time sampling was undertaken, DNA was successfully extracted with the PTB protocol of Asif and Cannon (2005) using the woody core of eight plants that had been standing dead for approximately one year. In addition, the seedbank surrounding the standing dead plants was examined, producing an additional ten *Sesbania tomentosa* plants for genotyping.

Within each population sampled, duplicate genotypes derived from plants occurring less than 10 m from one another were identified and were omitted from all subsequent analyses. I hypothesize that these are either branches of the same plant that over time separated from one another or else artifacts of extreme genetic sub-structuring within certain populations, and the full dataset was analyzed in detail in the population genetic analysis of Chapter 2. The exceptions were the Windward O‘ahu and Mānā (Kaua‘i) populations, where duplicate genotypes (progeny of the same parent plants) were maintained in the dataset to support slightly larger sample sizes in these remnant groups of plants.

In addition, a sample of *Sesbania marchionica* F. Br. from Marquesas (in cultivation at the McBryde Garden of the National Tropical Botanical Garden) was collected to provide DNA for inclusion in the molecular phylogeny. This species (listed as a variety of *S. coccinea* before Lorence resurrected the taxon *S. marchionica*) is purported to have a close relationship with Hawaiian *Sesbania* (Fosberg, 1948; Sachet, 1987). Four additional taxa were used for outgroup comparison at the two nuclear regions, selected from Genbank submissions based on the phylogenetic analysis of Farruggia (2009) [the American taxa *S. herbacea* (Mill.) McVaugh and *S. vesicaria* (Jacq.) Elliott] and the presumed origin of Hawaiian *Sesbania* determined by Fosberg (1948) [the Austral taxon *S. formosa* (F.Muell) N.T. Burb. and the Indo Pacific taxon *S. grandiflora* (L.) Pers.]. Genbank accession numbers are as follows (ITS and TRPT accessions, respectively): JX453682 and KC254800 (*S. herbacea*), AF398761 and EU258899 (*S. vesicaria*), JX453678 and HQ730391 (*S. formosa*), AF536354 and HQ730392 (*S. grandiflora*).

### Phylogenetic Analysis

Two individuals from one or two populations of eight putative taxa of Hawaiian *Sesbania* plus one individual of *S. marchionica* (23 samples total; Table 2.5) were chosen to be amplified and sequenced at the two nuclear regions using primers described in the literature (ITS: White et al., 1990, TRPT: Choi et al., 2006). ITS amplifications were carried out in 25.0  $\mu$ L reaction volumes with final concentrations of: 0.5  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 unit *Taq* polymerase (Promega); 20–30 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, ending with a final extension of 72°C for 7 min. PCR products were electrophoresed on 1% agarose to verify amplified product, cleaned with ExoSAP (USB Corp., Cleveland, Ohio, USA) following manufacturer specifications and then bi-directionally sequenced on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA) at the Center for Genomic, Proteomic and Bioinformatic Research (CGPBR) facility at UH Mānoa.

Table 2.5. Twenty-two DNA samples sequenced from *Sesbania tomentosa* populations in the Hawaiian Islands, using the putative species designations for populations of Char (1983), plus *S. marchionica*. Voucher representations of populations sampled stored at B. P. Bishop Museum Herbarium (BISH), Joseph F. Rock Herbarium (HAW), Hawai‘i Volcanoes National Park Herbarium (HVNP) and National Tropical Botanical Garden Herbarium (PTBG).

Putative species designation	Island	Population	Voucher representations of populations sampled
<i>“tomentosa var. tomentosa”</i>	Hawai‘i Hawai‘i Maui Maui	‘Āpua point Ka Lae Nākālele point Papanalaho	Herat & Higashino 884 (BISH) Herbst 938 (BISH) Hobdy 809 (BISH) Oppenheimer 109902 (BISH)
<i>“kauensis var. kauensis”</i>	Hawai‘i Hawai‘i	Pepeiau Kū‘ē‘ē	Banko 1 (HVNP) Char 74 (BISH)
<i>“kauensis var. intermedia”</i>	Hawai‘i Hawai‘i	Kīpuka Nēnē Hilina pali	Char 71 (BISH) Reeser June 1975 (HAW)
<i>“arborea”</i>	Maui Maui Moloka‘i Moloka‘i	Pu‘u Pīmoe Pu‘u Pīmoe Kawela Kamiloloa	Davis 52 (BISH)  Pekelo 27 (BISH) Degener, Degener & Pekelo 32430 (NY)
<i>“molokaiensis”</i>	Moloka‘i Moloka‘i	Mo‘omomi Mo‘omomi	Degener 17954 (NY)
<i>“oricola”</i>	O‘ahu O‘ahu	Ka‘ena point Ka‘ena point	Char 83015 (BISH)
<i>“polihalensis”</i>	Kaua‘i Kaua‘i Nihoa Nihoa	Polihale Polihale Nihoa Nihoa	Char 76023 (BISH)  Yen 1016 (BISH)
<i>“manaensis”</i>	Kaua‘i Kaua‘i	Mānā Mānā	Char 76001 (BISH)
<i>S. marchionica</i> F. Br.	Ua Huka	Te kohai	Wood 10556 (PTBG)

The resultant 23 sequences for each of the two regions were edited using CHROMAS LITE v. 2.11 (Technelysium Pty Ltd., 2012) and aligned (with the addition of the four outgroup taxa) using MEGA v. 6.0 (Tamura et al., 2007). Maximum likelihood (ML) heuristic search algorithm was used in PAUP v. 4.0 (Swofford, 2002) to infer phylogenetic hypotheses. In this analysis *S. marchionica* was included in the Hawaiian *Sesbania* ingroup, while the four GenBank accessions were placed in the outgroup. Branch support was estimated using 1,000 bootstrap replicates.

Bayesian analysis was carried out using the GTR model in MrBayes v. 3.1 using 10 million MCMC replications following a burn-in of 2 million replicates. Posterior probabilities were calculated by Mr.Bayes and were used to construct the phylogenetic tree. The American species *S. herbacea* was used as the sole outgroup species in this analysis, as Faruggia (2009) placed it with Hawaiian *Sesbania* in a well-supported clade. Both phylogenetic trees were visualized using Fig Tree v. 1.3.1.

#### Microsatellite analysis of population structure

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* under contract with the United States Geological Survey (USGS). Ninety-six dinucleotide ( $CA_n$ ) and tetranucleotide ( $CATC_n$ ,  $TACA_n$ , and  $TAGA_n$ ) microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 2.6) based on their range of polymorphism and ease of scoring in a screening of eight DNA samples, one from each of the putative taxa of Char (1983). Each sample was amplified in a 25.0  $\mu$ L volume with final concentrations of 0.6  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM  $MgCl_2$ , 0.8 mM dNTPs (Promega), 1 unit *Taq* polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler with the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 2.6) for 40 s, and 72°C for 30 s, ending with a final extension of 72°C for 4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were

Table 2.6. Nine microsatellite primer pairs developed for *Sesbania tomentosa*.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 469 individuals sampled for this study. Range, allele size range in base pairs (bp). Prefixes in italics before forward primer sequence indicate dye used for poolplexing.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG R: TAG-GTG-TGG-CGT-GCA-TAA-C	57.3 58.1	10	205–223
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A R: CCC-TTC-CCC-TCC-TCT-TAG	56.0 56.2	9	264–280
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	55.8 56.3	14	198–236
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC R: TAG-CCA-TGC-TTC-ATC-AAT-CG	58.9 59.8	21	288–328
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.8 56.9	13	163–187
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG R: GGC-AAC-ATT-TGA-GTG-GAG-G	58.6 59.1	16	196–276
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1 55.1	14	180–236
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.7 55.2	11	212–252
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.1 54.8	14	265–321

included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on the ABI Prism 377XL sequencer at the CGPBR facility at UH Mānoa. The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (ABI). Stutter peaks were identified, and then the program MICROCHECKER (Van Oosterhout et al., 2004) was used to identify possible genotyping errors due to non-amplified (null) alleles and short allele dominance (large allele dropout). A maximum likelihood estimate of the frequency of null alleles (Expectation Maximization algorithm of Dempster et al., 1977) was then calculated for each locus and geographic population using the program FREENA (Chapuis and Estoup 2007). The microsatellite dataset was analyzed to assess linkage (genotypic) disequilibrium in GENEPOP v. 4.0 (updated from Raymond and Rousset, 1995) using log-likelihood ratio statistics (*G*-tests).

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was calculated using GENALEX v. 6.4 (Peakall and Smouse, 2006) at three hierarchical levels: within populations, among populations, and among the putative specific designations of Char (1983). This test partitions total genetic variance and calculates  $\Phi_{PT}$ , an analogue of  $F_{ST}$ . Significance was tested against a null distribution of 10,000 random permutations. Private alleles (alleles exclusive to a given population) were also calculated in GENALEX v. 6.4.

Population structure was examined using a full Bayesian-clustering approach, implemented in the program STRUCTURE v. 2.3.3 (Pritchard et al., 2000), which assigned individual genotypes to populations, irrespective of geographical location of origin. Default settings of the program were used (admixture model, independence among loci) using the putative specific designations of Char (1983) as prior information for the model to consider (Hubisz et al., 2009). To determine the most likely number of populations or groups (*K*) in the data, a series of analyses were performed from *K* = 1 (all populations represent a single panmictic unit) to 15 (the maximum number of populations allowable) using 40,000 burn-in and 100,000 repetitions, with ten iterations per *K*. These results were examined using the  $\Delta K$  method (Evanno et al., 2005) to identify the most likely number of groups in the data. Ten additional

iterations at the identified  $K$  were computed using 100,000 burn-in and 300,000 repetitions. The program CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize these last ten iterations. Cluster membership coefficients for each individual and pre-defined population were obtained (permuted across replicates using *FullSearch* algorithm) and used as input files for the cluster visualization program DISTRUCT v. 1.1 (Rosenberg, 2004).

Each individual was assigned to a particular genetic cluster when its coefficient of membership was greater than 50%. Geographic populations were assigned to a particular genetic cluster when 72–100% of their individuals were assigned to that genetic cluster. The initial analysis was repeated on each  $K$  separately to detect sub-structuring in the two genetic groups; no information about specific designation was used as *a priori* in this subsequent analysis. The number of genetic sub-clusters was estimated for each group using the  $\Delta K$  method, ten additional iterations were performed at the appropriate  $K$  (100,000 burn-in and 300,000 repetitions) and both the *FullSearch* and *Greedy* (10,000 random input orders of runs) algorithms were used in CLUMPP. Individuals were then assigned to genetic sub-clusters when their coefficient of membership was greater than 0.5; geographic populations assigned to sub-clusters based on 58–100% individual assignment.

The extent and significance of the genetic differentiation among geographic populations was investigated with MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötter, 2003) by calculating global and pairwise  $F_{ST}$  values (averaged over multiple loci) among the geographic populations. Global and pairwise  $F_{ST}$  values were also obtained for the eight synonyms of *S. tomentosa* by combining distinct geographic populations into the taxa they were purported to represent. The significance of  $F_{ST}$  values was tested with 10,000 permutations using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ). FREENA (Chapuis and Estoup 2007) was also used to estimate pairwise  $F_{ST}$  values ( $F_{ST(ENA)}$ ) from genotype frequencies corrected for the presence of null alleles [using the excluding null alleles (ENA) method of Chapuis and Estoup 2007] that tend to positively bias  $F_{ST}$  estimates. Most of the non-visible genotypes in the dataset were assumed to be due to technical problems (e.g., degraded or low quantity of DNA or PCR amplification inconsistencies) and were specified in the FREENA dataset. These were distinguished from the null homozygous genotypes at locus A122 in 16 out of 17 individuals of the Ka'ena point population, probably due to a mutated flanking sequence that prevented that particular locus from amplifying.

A principal coordinates analysis (PCA) was used to examine the extent of genetic clustering of populations (and putative taxa) throughout Hawai‘i using co-dominant genotypic distances ( $\Phi_{PT}$ ) between individuals (Smouse and Peakall, 1999) and the chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967) between populations in GENALEX v. 6.4 (Peakall and Smouse, 2006). Lastly, a neighbor-joining (NJ) tree was constructed using the chord distance ( $D_C$ ) with 1,000 bootstrap replications in POPULATIONS v. 1.2.31 (Langella, 2000) and graphically displayed with TREEVIEW (Page, 1996). The chord distance of Cavalli-Sforza and Edwards (1967) was chosen in both cases because the null allele bias for this genetic distance is low (Chapuis and Estoup, 2007), and because it is the most efficient distance for obtaining a correct tree topology using microsatellite data (Takezaki and Nei, 1996).

## Results

### Phylogenetic analysis of morphological character dataset

Maximum parsimony analysis based on the morphological character dataset evaluated 135,135 trees retaining one. Two of Char’s (1983) taxa from Kaua‘i, “*polihalensis*” and “*manaensis*”, were identified as the basal-branching sisters to a clade containing the remainder of Hawaiian *Sesbania* (Figure 2.1). As a means for comparison, Bayesian analysis revealed a topology similar to that of the maximum parsimony analysis except for the inclusion of “*manaensis*” in the clade with the remaining putative taxa of Hawaiian *Sesbania*. Other than this discrepancy, posterior probabilities suggested varying levels of confidence (mostly below 50%; exceptions labeled on tree) in the same relationships proposed in parsimony analysis (Figure 2.2). Two sub-clades emerged, one joining the putative taxa “*kauensis* var. *kauensis*” with “*kauensis* var. *intermedia*”, both from the Ka‘ū district of Hawai‘i Island, and the other joining “*oricola*” from O‘ahu with “*molokaiensis*” from northwest Moloka‘i (Figures 2.1 and 2.2).

### Phylogenetic analysis of molecular datasets

Approximately 1035 base pairs (bp) were sequenced (720 bp of ITS and 315 bp of TRPT) of 22 samples of *Sesbania tomentosa* from 16 populations on 7 Hawaiian Islands



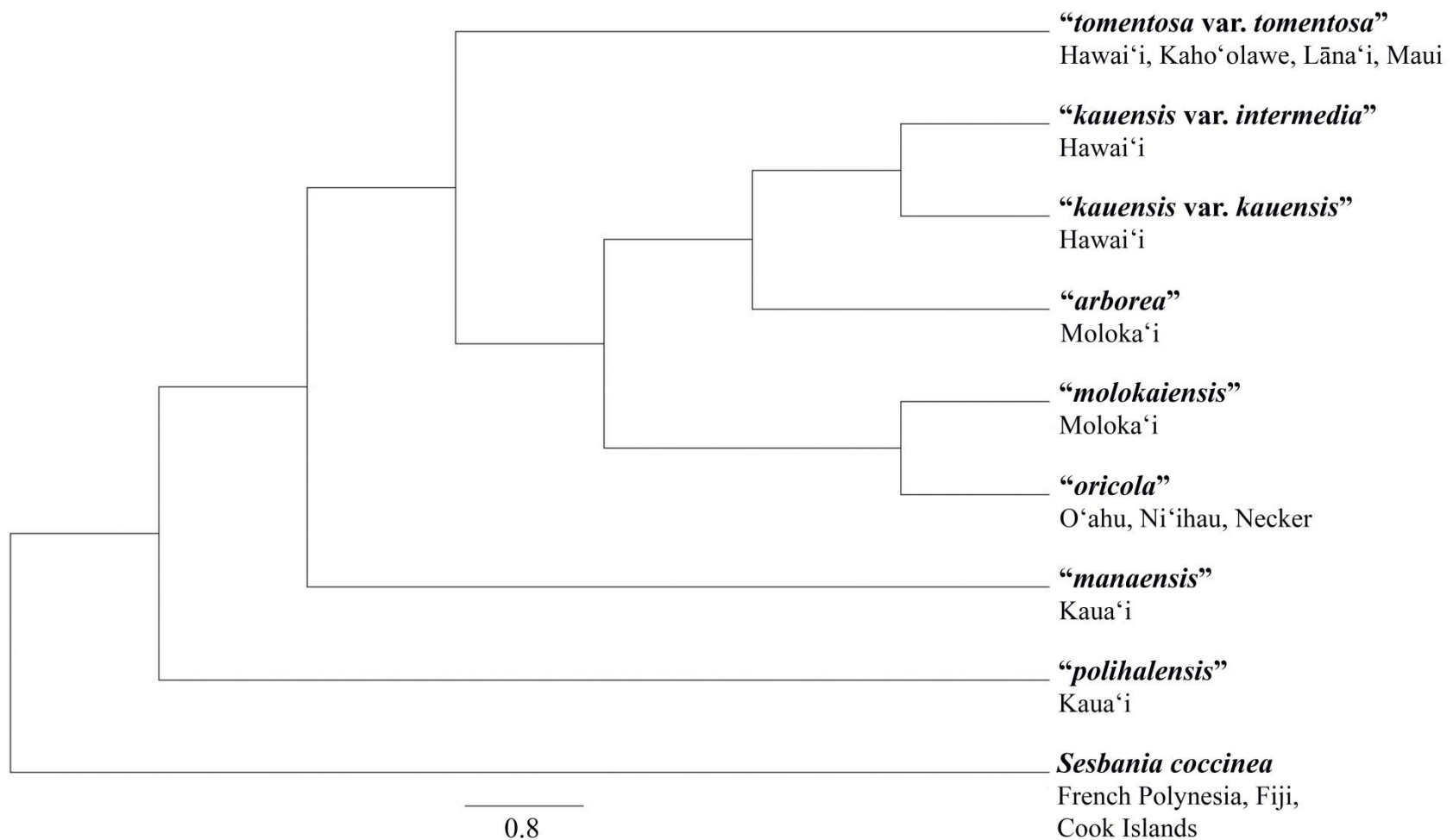


Figure 2.1. Exhaustive maximum parsimony phylogeny of Char's (1983) morphological character dataset of *Sesbania tomentosa* populations using *S. coccinea* as an outgroup. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

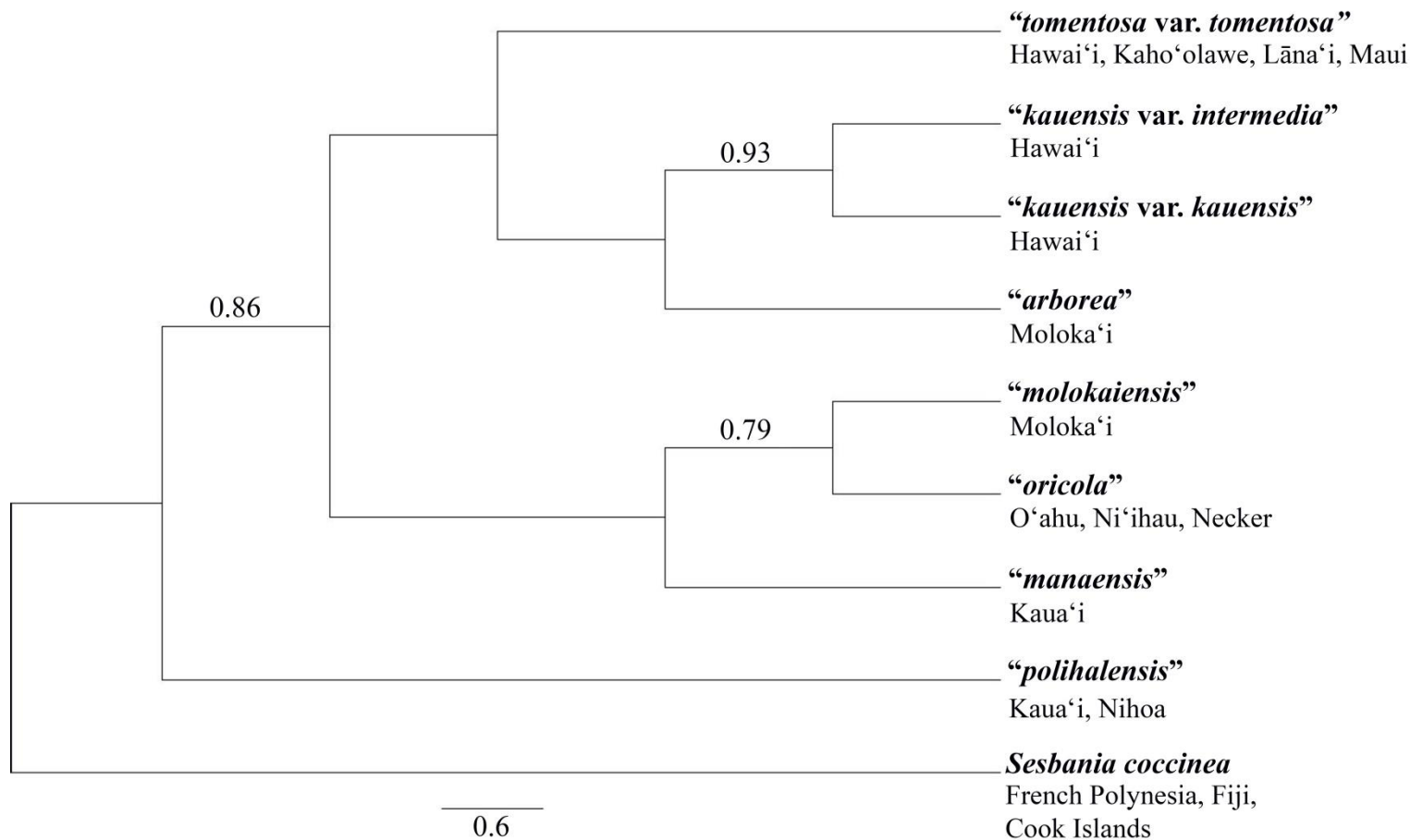


Figure 2.2. Bayesian analysis (standard discrete morphology model; Lewis, 2001) of Char’s (1983) morphological character dataset of *Sesbania tomentosa* populations using *S. coccinea* as an outgroup. 100,000 MCMC replications were analyzed following a burn-in of 40,000 replicates. Posterior probabilities listed above branches where they offer greater than 50% support for nodes. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

plus *S. marchionica* (Marquesas). There was no sequence divergence whatsoever across the 22 Hawaiian samples sequenced for ITS. However, *S. marchionica* was divergent from the Hawaiian samples in 5 out of 720 bp at the ITS region. For TRPT, 6 out of 315 bp were divergent among the Hawaiian samples. Six samples sequenced from three populations on O‘ahu, Kaua‘i and Nihoa (designated “*oricola*”, “*polihalensis*” and “*manaensis*” by Char, 1983) were the only ones to diverge at these positions. Two Nihoa samples (designated “*polihalensis*” by Char, 1983) shared four of the same six base pair substitutions. Divergence was represented by within-individual polymorphic states (sequences showing equal peaks for two nucleotides) becoming non-polymorphic (a single peak). Polymorphic states were coded as ambiguities (with standard IUPAC coding) and were not considered to be phylogenetically informative. *Sesbania marchionica* was divergent at two of the same 6 positions as the Hawaiian samples at the TRPT region.

Both the maximum likelihood and Bayesian phylogenies for each gene region analyzed separately were identical to their respective combined analyses (ITS plus TRPT) therefore only combined gene region phylogenies are presented. In both the combined likelihood and Bayesian analyses *S. marchionica* was sister to the Hawaiian *Sesbania* clade (Figures 2.3 and 2.4). In the maximum likelihood phylogeny, where four taxa were used as outgroup species, the American species *S. herbacea* appeared to be the closest relative (according to the scale) to the Hawaiian-Marquesan species (Figure 2.3). A similar result was observed in the Bayesian phylogeny, where *S. herbacea* was used as the sole outgroup species (Figure 2.4).

#### Microsatellite analysis of population structure

At the nine microsatellite loci examined, the number of alleles per locus averaged 13.5 (ranging 9–21), for a total of 122 alleles among the 459 samples. Each locus had two to four alleles with a frequency greater than 0.1, and these most-common alleles had average frequencies per locus that ranged from 0.17 to 0.28 (with a maximum across loci of 0.50). None of the 35 tests for multiple comparisons between loci (genotypic disequilibrium) in GENEPOP were significant at the 5% nominal level after Bonferroni

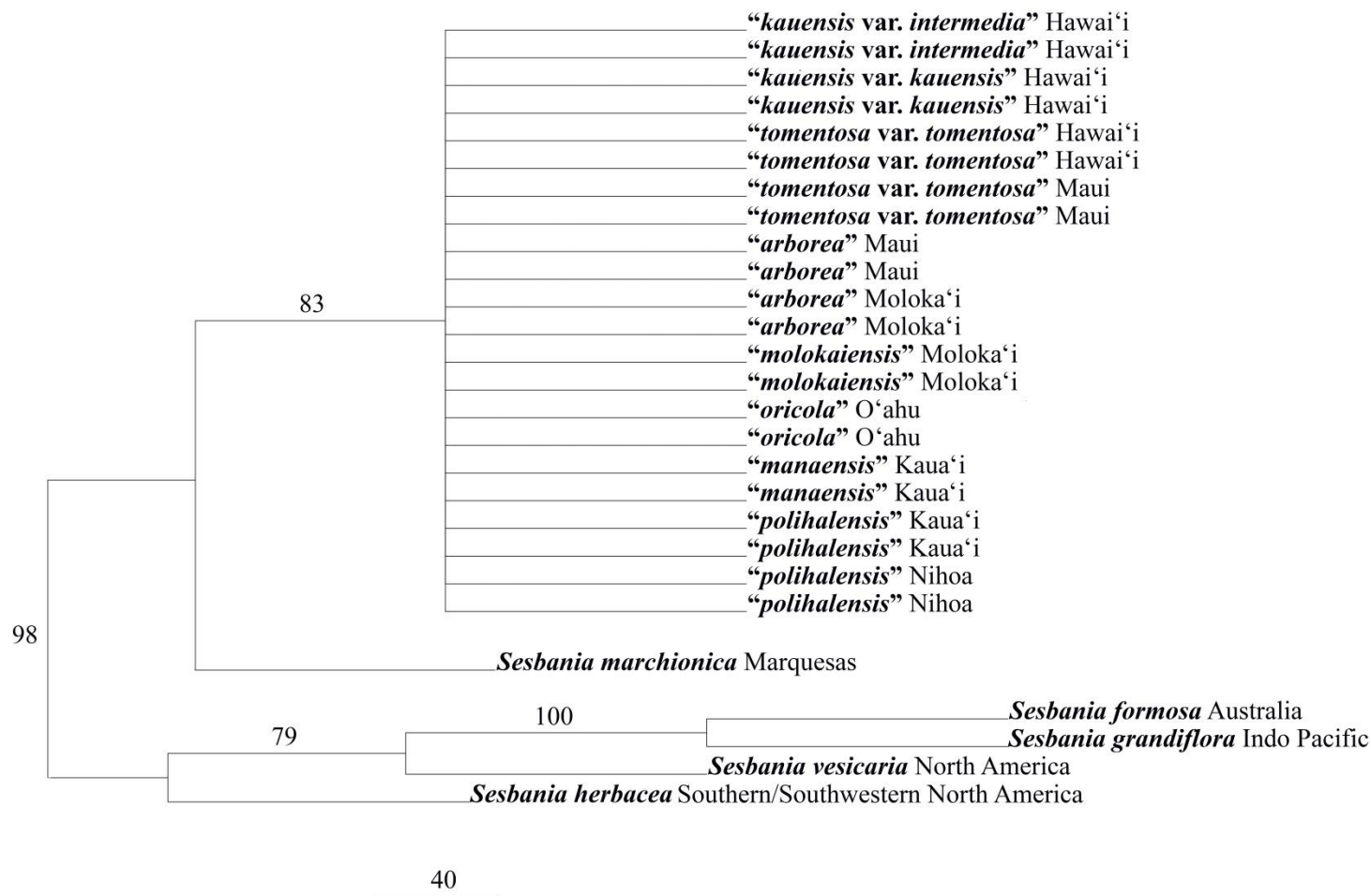


Figure 2.3. Maximum likelihood analysis of the combined ITS and TRPT datasets of *Sesbania tomentosa* and *S. marchionica* samples using *S. herbaceae*, *S. vesicaria*, *S. formosa* and *S. grandiflora* as the outgroup. Branch support was estimated using 1,000 bootstrap replicates. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

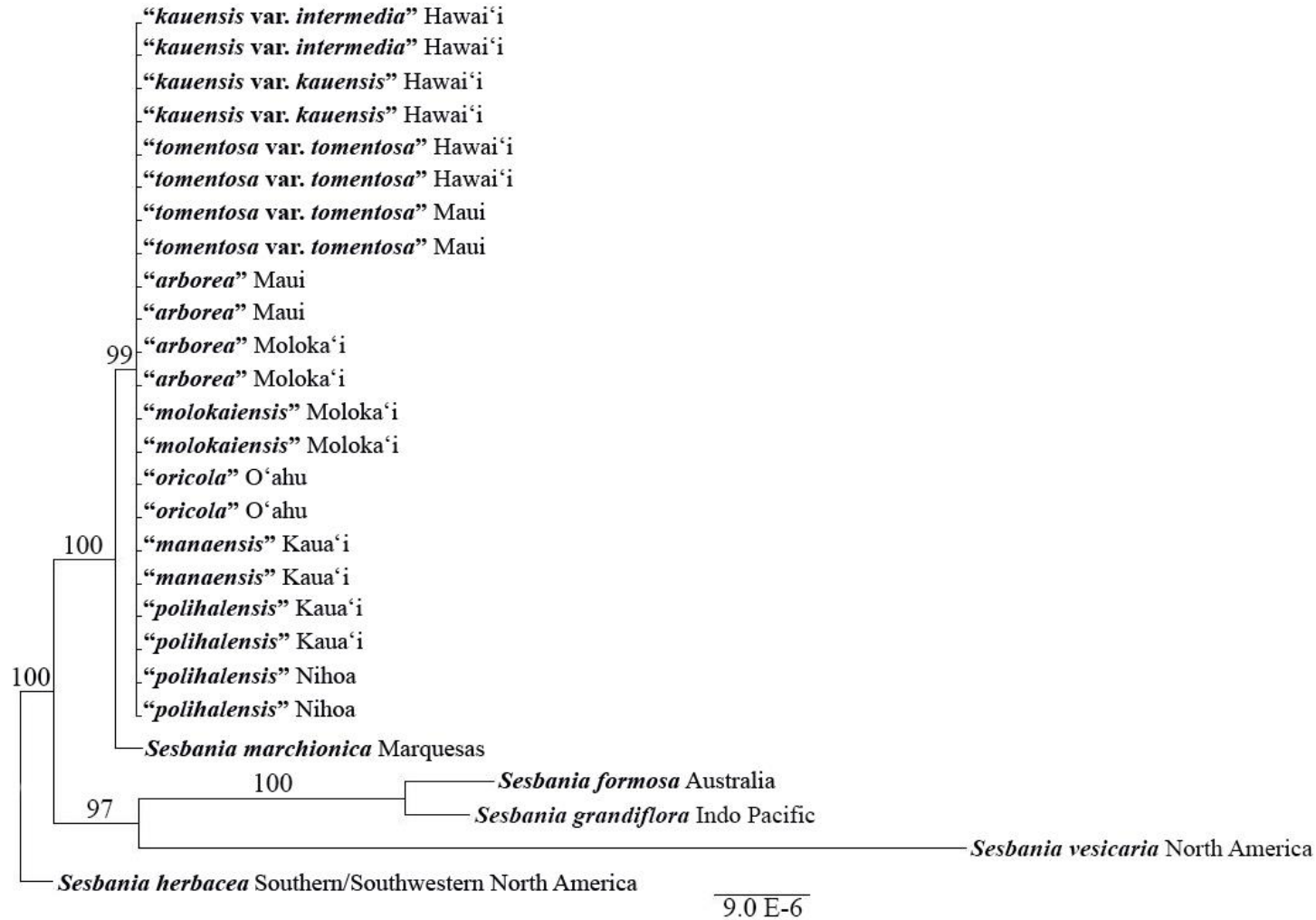


Figure 2.4. Bayesian analysis (GTR Model) of the combined ITS and TRPT datasets of *Sesbania tomentosa*, *S. marchionica*, *S. vesicaria*, *S. formosa* and *S. grandiflora* samples using *S. herbacea* as the outgroup. Posterior probabilities listed above branches. Hawaiian samples identified by the putative taxa designations for populations of Char (1983).

corrections when averaged over all populations. Thus, the different microsatellite loci can be considered to provide independent information on population structure.

MICROCHECKER indicated that there was a general excess of homozygotes evenly distributed across allele size classes at all 9 loci in an average of 11 out of 16 populations per locus, an indication of possible null alleles or false homozygotes in the data set (data not shown). Estimated frequencies of null alleles per locus in each population (using the ENA method implemented in FREENA) ranged from 0.000 to 0.404 (the exception being the Ka‘ena point populations that ranged from 0.980 to 1.000 at locus A122). When averaged over loci, the frequency of null alleles in the 16 populations varied from 0.040 to 0.340. The mean null allele frequency over all populations and loci was 0.149.

An analysis of molecular variance (AMOVA) revealed that the majority of genetic variation was found within Hawaiian *Sesbania* populations (56%) with 40% distributed among populations. Only 4% was found among the eight putative species (Char, 1983) tested (significant at the 1% nominal level, Table 2.7).

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Table 2.7. Results of AMOVA (Excoffier et al., 1992) at three hierarchical levels: among putative species (Char, 1983), among populations, and within populations of Hawaiian *Sesbania*. Significance was tested against a null distribution of 10,000 random permutations

Source of variation	d.f.	Sum of squares	Fixation index	% variation	P-value
Among putative species	7	1230.067	$\Phi_{RT} = 0.042$	4	0.000
Among populations	8	1309.236	$\Phi_{PR} = 0.418$	40	0.000
Within populations	453	3277.650	$\Phi_{PT} = 0.443$	56	0.000

Using the program STRUCTURE and following the method of Evanno et al. (2005), two distinct genetic clusters were found among *Sesbania tomentosa* individuals sampled across all islands (Figures 2.5 and 2.6). The largest increase in the posterior probability occurred at  $K = 2$ , suggesting that this was the best model for the data. One genetic cluster corresponded to the Hawai‘i Island samples (red cluster) and the other comprised individuals sampled from the remaining islands (orange cluster; Figure 2.7). Most of the geographic populations sampled showed a high proportion of individuals assigned to one cluster only, generally from 90% to 100%. Populations of “*arborea*” and “*molokaiensis*” sampled from Moloka‘i had proportions much lower (0.86 and 0.72 assigned to the orange cluster, respectively) levels of admixture much higher than the 5% threshold which might be attributed to stochastic noise. In addition, cluster membership coefficients of Maui Nui (referring to the prehistorically contiguous island composed of Kaho‘olawe, Maui, Moloka‘i, and Lāna‘i; Price and Elliott-Fisk, 2004) individuals assigned to the orange cluster also averaged low (0.68 for “*tomentosa* var. *tomentosa*” on Kaho‘olawe; 0.74 for “*arborea*” on Maui, 0.69 for “*arborea*” on Moloka‘i and 0.72 for “*molokaiensis*” on Moloka‘i).

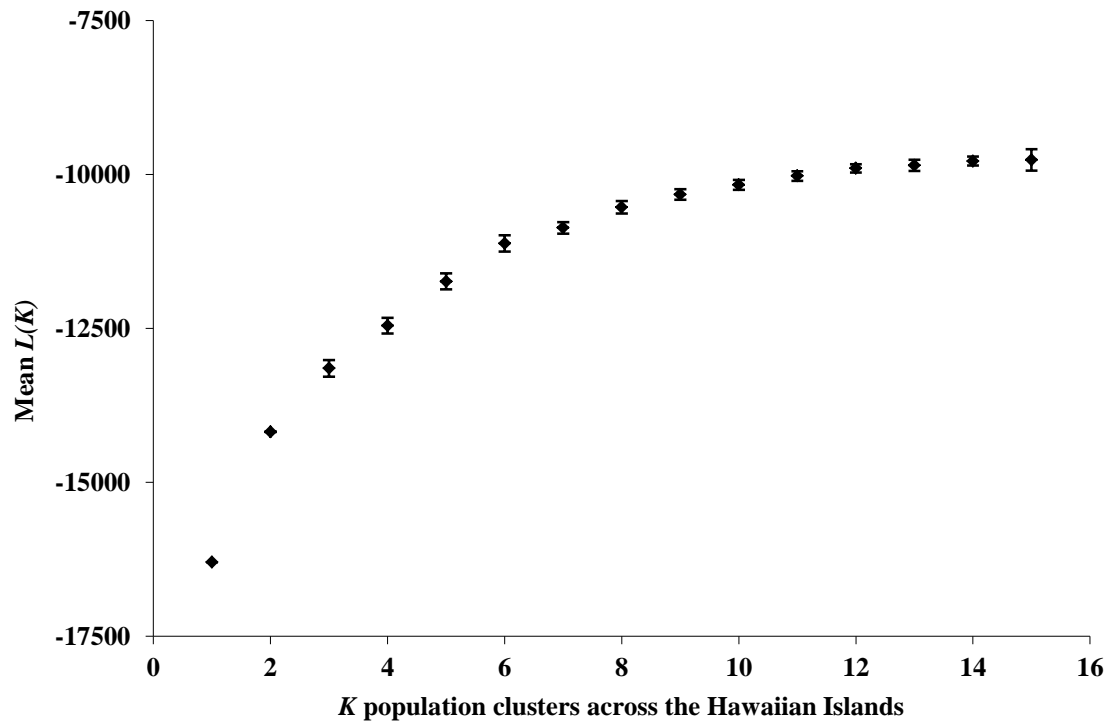


Figure 2.5. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

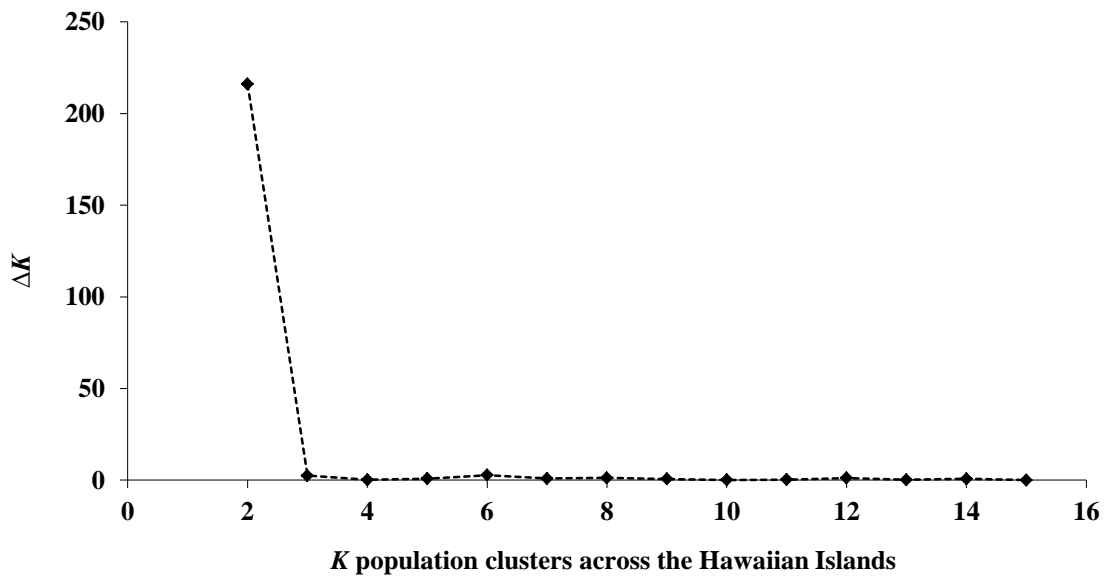


Figure 2.6. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).



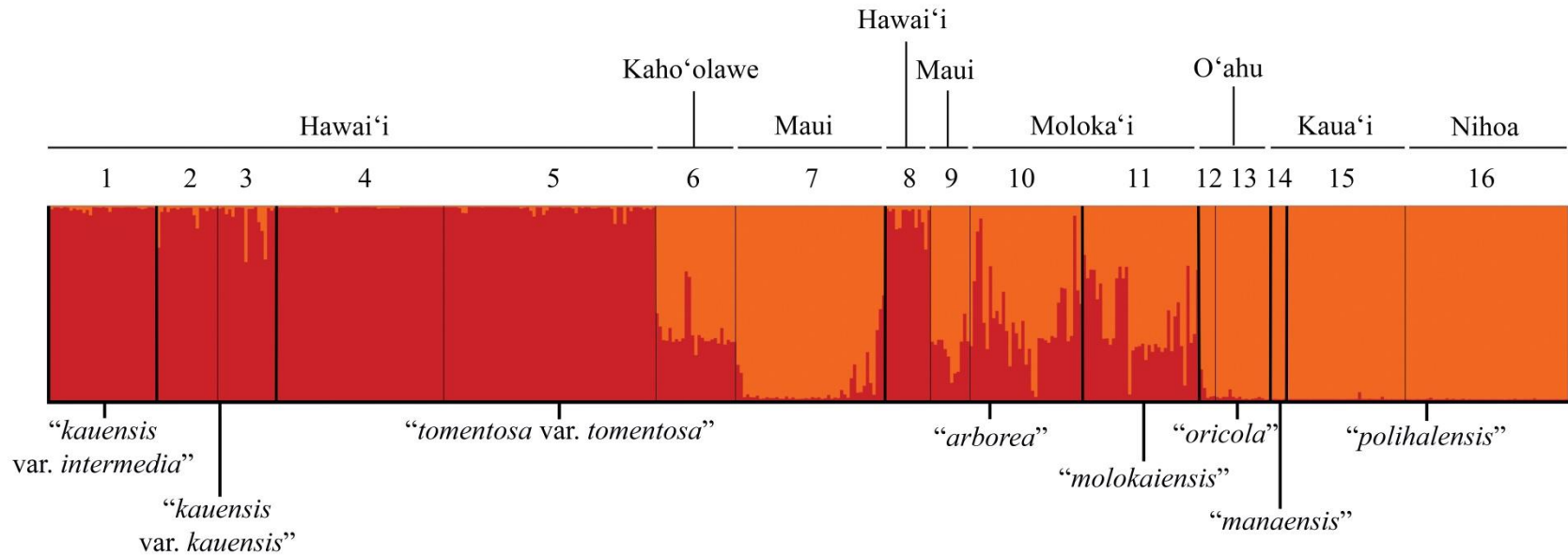


Figure 2.7. STRUCTURE graph for the most likely number of clusters of Hawaiian *Sesbania* according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic clusters (red and orange). Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 1. Kīpuka Nēnē–Hilina pali, 2. Pepeiau–Kukalau‘ula pali, 3. Kamo‘oali‘i–Kū‘ē‘ē, 4. ‘Āpua point, 5. Kamilo point–Ka Lae, 6. Pu‘u Koa‘e, 7. Papanalahoa–Nākālele, 8. Waiaka‘īlio, 9. Pu‘u Pīmoē, 10. Kawela–Kamiloloa, 11. Mo‘omomi, 12. Kāohikaipu & Mōkapu, 13. Ka‘ena point, 14. Mānā, 15. Polihale, 16. Nihoa. Island of origin for each population listed at top of figure.

When considering the population (or in this case, species) cluster membership coefficients, indications of admixture were even more prevalent (e.g., proportion of membership of “*tomentosa* var. *tomentosa*” in the red cluster was 0.70; proportion of membership of “*arborea*” in the orange cluster was 0.59; proportion of membership of “*molokaiensis*” in the orange cluster was 0.51; data not shown).

Additional analysis of the two genetic demes described above found  $K = 2$  within the red cluster of Figure 2.7 (Figures 2.8 and 2.9) and  $K = 4$  within the orange cluster of Figure 2.7 (Figures 2.10 and 2.11). Within the red cluster the first sub-cluster comprised the Hawai‘i Volcanoes National Park populations (orange) plus the small remnant population in North Kohala (Waiaka‘īlio) and the second sub-cluster (yellow) comprised the combined populations from the South point Region (Kamilo point–Ka Lae; Figure 2.12).

Two relatively distinct groups, comprising two genetic demes each, characterize the STRUCTURE plot in Figure 2.13 split between Maui Nui and the remaining Islands to the northwest. Populations from O‘ahu and Kaua‘i separate out into a distinct sub-cluster (pink) from the relatively large population on Nihoa, 250 km to the northwest of Kaua‘i (mauve). Secondly, levels of admixture were highest in the populations from Moloka‘i. For example, the combined (modern plus historical) Mo‘omomi population of “*molokaiensis*” was not definitively assigned to any one particular genetic group, the highest proportion of individuals (44%) being assigned to the red cluster, shared with “*tomentosa* var. *tomentosa*” from Kaho‘olawe and “*arborea*” from Maui. While the “*arborea*” population at Kawela–Kamiloloa was definitively assigned to the red cluster, the proportion of individuals assigned to that cluster was relatively low (0.80), and three individuals failed to be assigned to any cluster at the 0.50 cut-off. Cluster membership coefficients for the Moloka‘i individuals (with respect to their assigned cluster) averaged moderately low as well (0.70 for combined Mo‘omomi and 0.87 for Kawela–Kamiloloa), similar to “*arborea*” individuals on Maui (0.85) where another individual failed to be assigned to any cluster at the 0.50 cut-off. When considering the ten historically collected samples from Mo‘omomi separately, cluster membership coefficients averaged low at 0.72, and individual cluster assignments varied widely (indicating admixture).

Global  $F_{ST}(\theta)$  over all populations (averaged over loci) was 0.396 ( $P < 0.001$ ); correction for null alleles using the ENA method (Chapuis and Estoup, 2007) reduced this value slightly to 0.370 ( $P < 0.001$ , Table 2.8). On the other hand, global  $F_{ST}(\theta)$  over all putative species tested

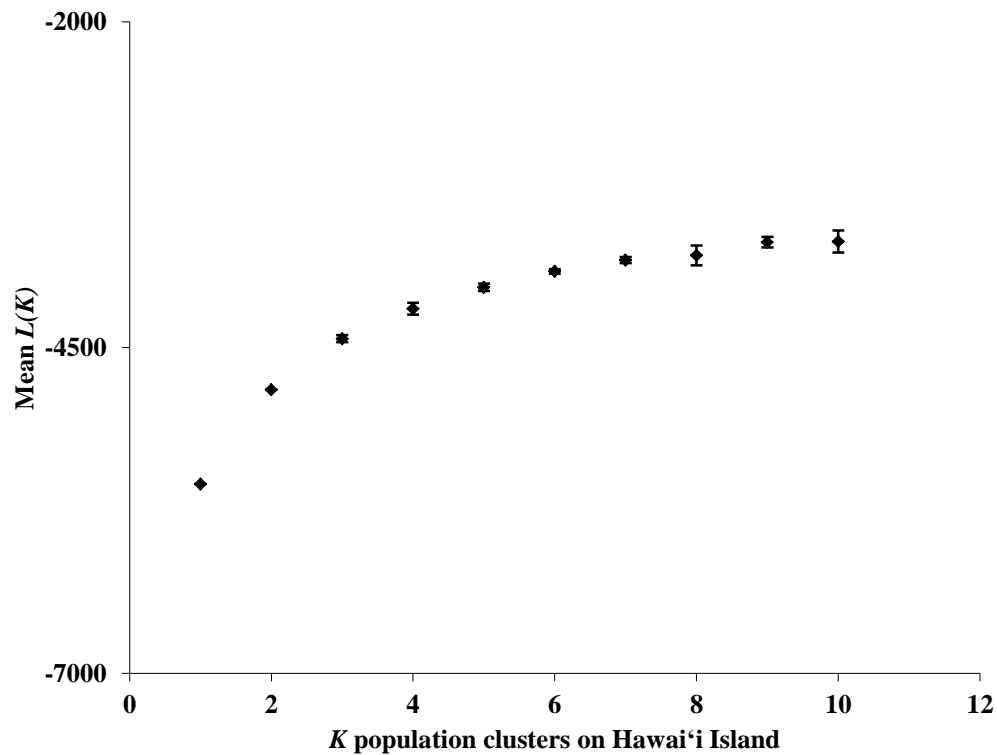


Figure 2.8. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

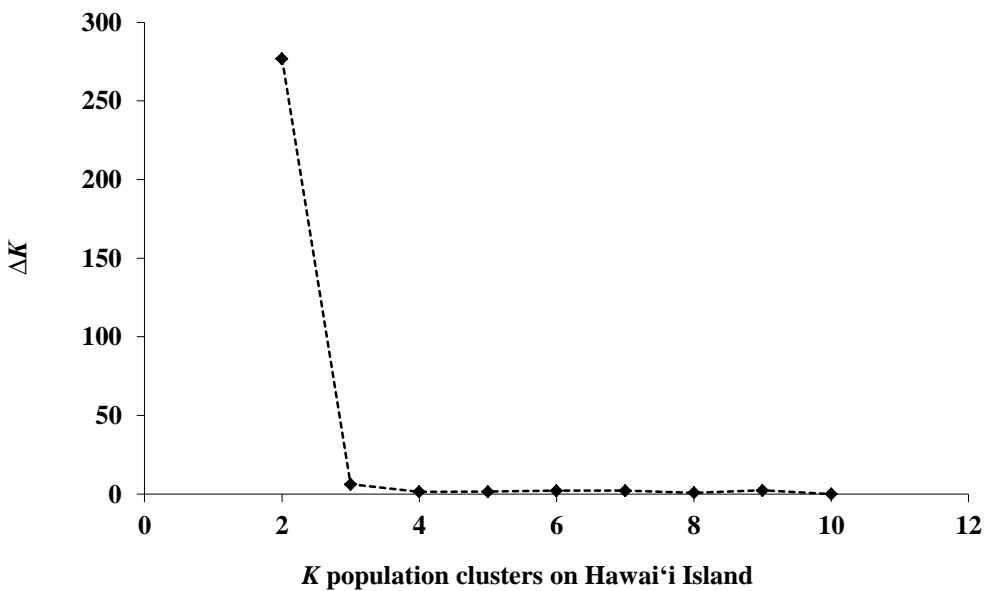


Figure 2.9. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

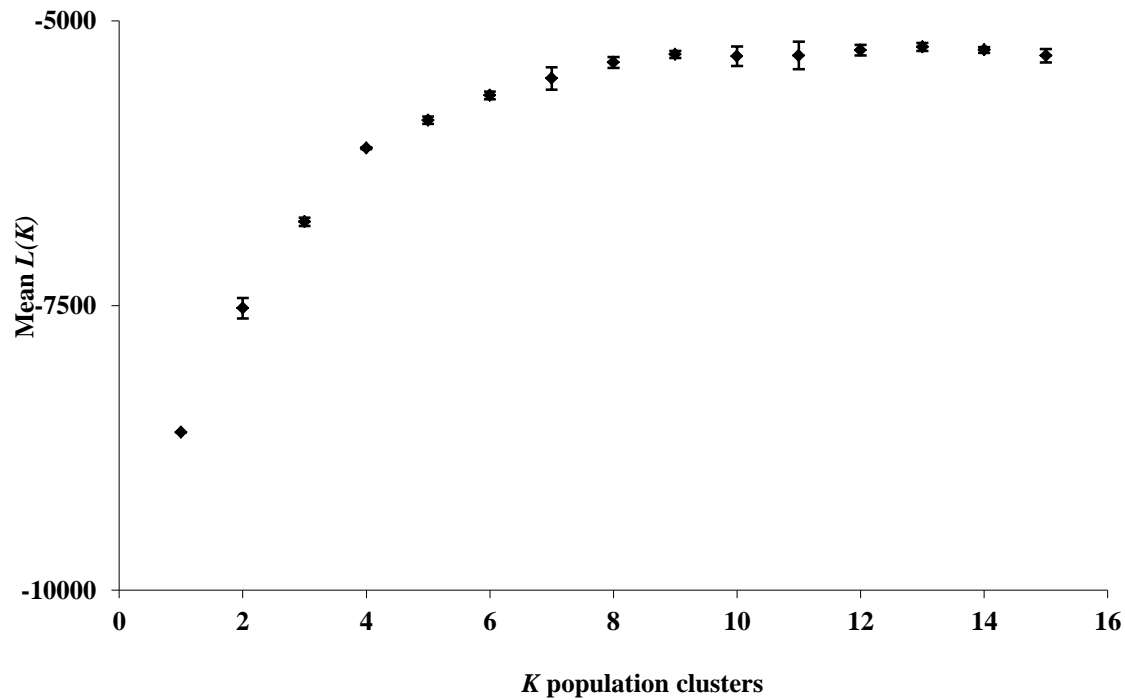


Figure 2.10. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

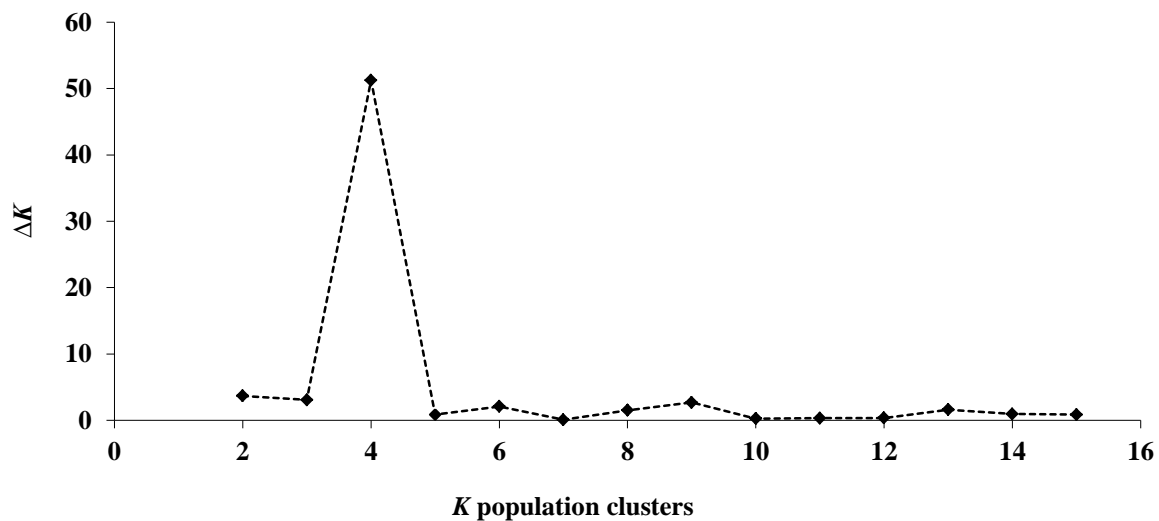


Figure 2.11. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

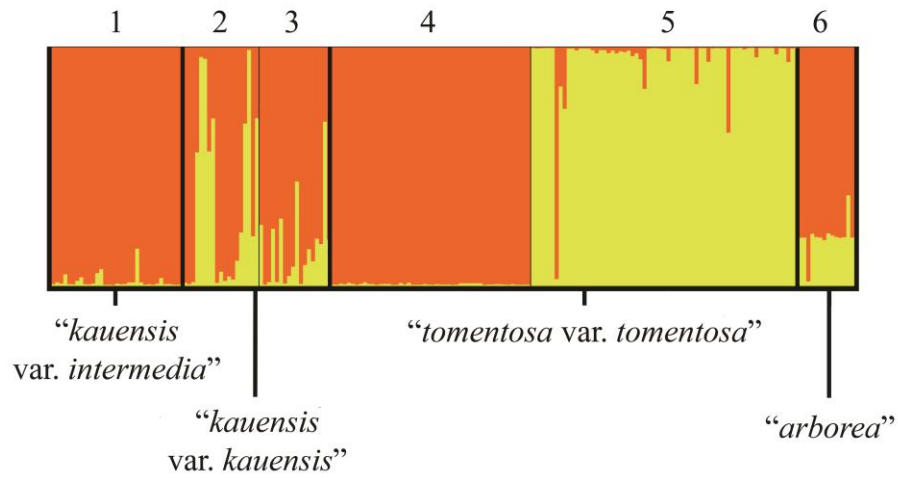


Figure 2.12. STRUCTURE graph for the most likely number of sub-clusters on Hawai‘i Island (red cluster of Figure 2.7) according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 1. Kīpuka Nēnē–Hilina pali, 2. Pepeiau–Kukalau‘ula pali, 3. Kamo‘oali‘i–Kū‘ē‘ē, 4. ‘Āpua point, 5. Kamilo point–Ka Lae, 6. Waiaka‘īlio.

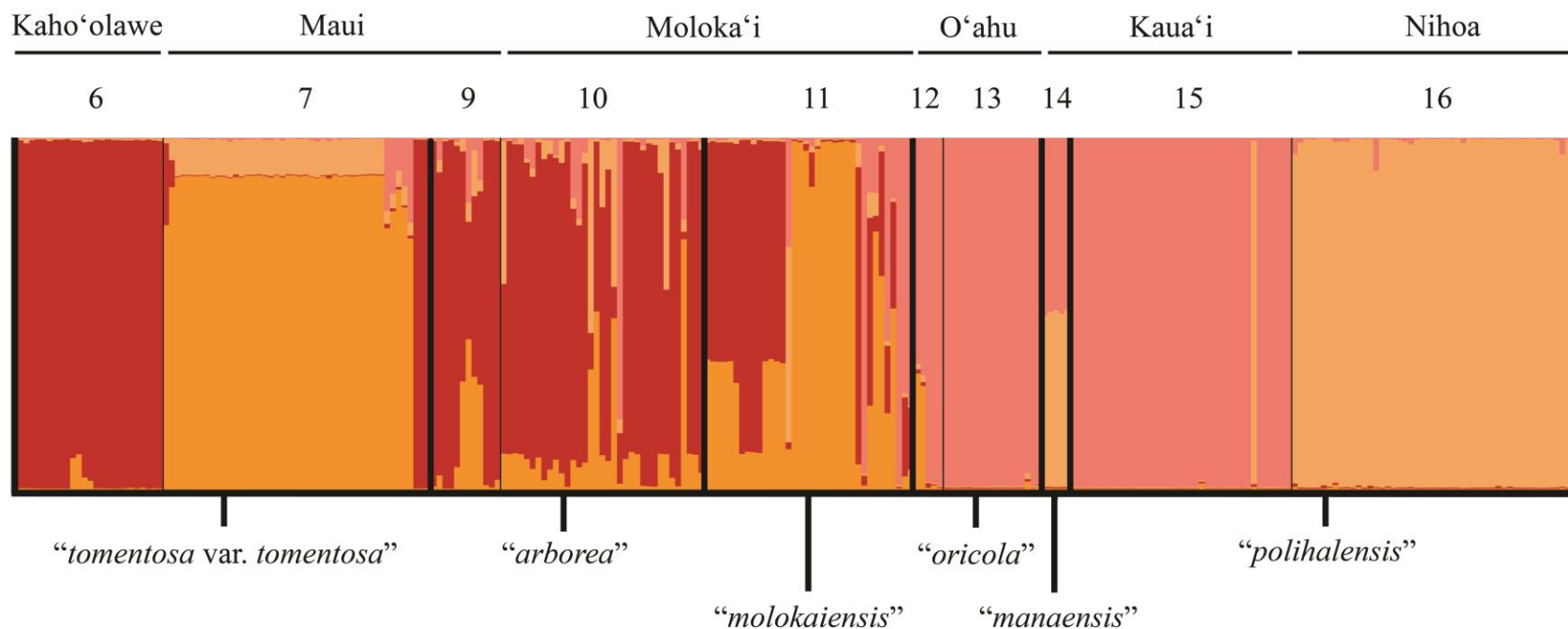


Figure 2.13. STRUCTURE graph for the most likely number of sub-clusters in the orange cluster of Figure 2.7 according to the  $\Delta K$  method ( $K = 4$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Black brackets identify the putative species designations for populations of Char (1983). Thin black lines additionally distinguish populations within a given putative taxon: 6. Pu'u Koa'e, 7. Papanalaho-Nākālele, 9. Pu'u Pīmoe, 10. Kawela-Kamiloloa, 11. Mo'omomi, 12. Kāohikaipu & Mōkapu, 13. Ka'ena point, 14. Mānā, 15. Polihale, 16. Nihoa. Island of origin for each population listed at top of figure

Table 2.8.  $F_{ST}$  ( $\theta$ ; Weir and Cockerham, 1984) per locus and global over all populations ( $F_{ST\ POP}$ ) and over all 8 putative species (Char, 1983) of Hawaiian *Sesbania* tested ( $F_{ST\ SPECIES}$ ).  $F_{ST}$  values corrected for the possible presence of null alleles using the ENA method (Chapuis and Estoup, 2007) included for comparison [ $F_{ST\ POP\ (ENA)}$  and  $F_{ST\ SPECIES\ (ENA)}$ , respectively]. Significant  $P$ -values ( $\alpha = 0.01$ ) listed in bottom row of table apply to all four analyses listed above.

	<b>Locus:</b>									
	<b>C5</b>	<b>A105</b>	<b>A123</b>	<b>C3</b>	<b>A122</b>	<b>A119</b>	<b>A128</b>	<b>C103</b>	<b>C106</b>	<b>Global</b>
$F_{ST\ POP}$	0.406	0.402	0.346	0.425	0.254	0.387	0.400	0.481	0.447	0.396
$F_{ST\ POP\ (ENA)}$	0.397	0.384	0.343	0.420	0.220	0.335	0.335	0.440	0.439	0.370
$F_{ST\ SPECIES}$	0.234	0.246	0.111	0.289	0.172	0.188	0.215	0.197	0.243	0.211
$F_{ST\ SPECIES\ (ENA)}$	0.224	0.230	0.110	0.486	0.140	0.134	0.150	0.156	0.233	0.207
<b><math>P</math>-value</b>	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

was 0.211 ( $P < 0.001$ ); correction for null alleles using the ENA method (Chapuis and Estoup, 2007) again reduced this value only slightly to 0.207 ( $P < 0.001$ , Table 2.8). This analysis indicates that of the total genetic variation found across the range of the species, 37–40% is ascribable to genetic difference (differences in allele frequencies) among geographic populations, while 21% of the total variation is ascribable to genetic differences among the putative species (when geographic populations are pooled together as species).

In addition, correction for null alleles only marginally decreased pairwise  $\theta$ -values, indicating that null alleles were not strongly biasing the analysis of genetic differentiation among populations (Tables 2.9, 2.10, 2.11 and 2.12). One hundred and eleven of the 120 pairwise comparisons were significant at the 1% nominal level and an additional 5 comparisons were significant at the 5% level after Bonferroni corrections (Tables 2.9 and 2.10). When distinct geographic populations were combined into the putative taxa of *Sesbania tomentosa*, all pairwise comparisons were significant at the 1% nominal level after Bonferroni corrections (Tables 2.11 and 12). Besides the two closely related “*kauensis*” varieties, “*tomentosa* var. *tomentosa*” and “*arborea*” appeared the least differentiated from all the other putative taxa, and from each other. On the other hand, the group of putative taxa from O‘ahu, Kaua‘i and Nihoa (“*oricola*”, “*manaensis*” and “*polihalensis*”, respectively) appeared the most differentiated from putative taxa on the remaining Hawaiian Islands (Tables 2.11 and 2.12).

Co-dominant genotypic distances ( $\Phi_{PT}$ ) were also used in a principal coordinates analysis (PCA) to examine the extent of genetic clustering of Hawaiian *Sesbania* populations (Figure 2.14) and individuals (Figure 2.15) throughout the state. The first two principal coordinates (PC) axes of Figure 2.14 explained 39.2 and 18.2% of the genetic variation among populations, respectively, for a total of 57.4%. A scattergram of these two axes showed strong geographical correlation, with populations from O‘ahu, Kaua‘i and Nihoa separated from all other populations (displaced along PC axis 1; Figure 2.14). While an apparent cohesion existed among “*arborea*” populations from three separate Islands (Moloka‘i, Maui and Hawai‘i Island), “*tomentosa* var. *tomentosa*” populations were displaced along PC axis 2 in a geographical pattern; populations from Maui and Kaho‘olawe were separated from populations on Hawai‘i Island (Figure 2.14). The first two principal coordinates (PC) axes of Figure 2.15 explained 29.6 and 21.3% of the genetic variation among populations, respectively, for a total of 50.9%. The scattergram of these two axes again showed strong geographical correlation among individuals, respective of their



Table 2.9. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between populations of Hawaiian *Sesbania* on top half of matrix, Bonferroni-corrected  $P$ -values ( $\alpha_{0.01} = 0.012$ ) listed in bottom half. n.s. indicates pairwise comparisons insignificant at the 0.05 level.

Kīpuka Nēnē–Hilina pali	0.000	0.063	0.046	0.281	0.214	0.404	0.364	0.303	0.387	0.187	0.317	0.335	0.514	0.530	0.489	0.459
Pepeiau–Kukalau‘ula pali	0.036	0.000	0.035	0.272	0.145	0.401	0.358	0.266	0.334	0.148	0.273	0.347	0.516	0.542	0.507	0.427
Kamo‘oali‘i–Kū‘ē‘ē	n.s.	n.s.	0.000	0.247	0.162	0.391	0.339	0.255	0.347	0.143	0.309	0.329	0.517	0.550	0.501	0.444
‘Āpua point	0.012	0.012	0.012	0.000	0.343	0.493	0.476	0.473	0.489	0.322	0.449	0.473	0.626	0.597	0.651	0.576
Kamilo Point–Ka Lae	0.012	0.012	0.012	0.012	0.000	0.379	0.352	0.296	0.368	0.178	0.310	0.352	0.454	0.490	0.462	0.429
Waiaka‘īlio	0.012	0.012	0.012	0.012	0.012	0.000	0.521	0.478	0.456	0.272	0.404	0.599	0.753	0.753	0.763	0.565
Pu‘u Koa‘e	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.318	0.424	0.207	0.298	0.458	0.598	0.606	0.516	0.454
Pu‘u Pīmoe	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.326	0.126	0.315	0.349	0.539	0.605	0.541	0.387
Papanalahoā–Nākālele	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.258	0.323	0.380	0.519	0.577	0.529	0.421
Kawela–Kamiloloa	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.190	0.233	0.340	0.410	0.343	0.266
Mo‘omomi	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.416	0.568	0.583	0.522	0.405
Kāohikaipu & Mōkapu	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.334	0.482	0.550	0.466
Ka‘ena point	0.012	0.012	0.024	0.012	0.012	0.012	0.012	0.036	0.012	0.012	0.012	n.s.	0.000	0.585	0.663	0.526
Polihale	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000	0.621	0.559
Mānā	0.012	0.012	0.024	0.012	0.012	0.012	0.012	0.048	0.012	0.012	0.012	0.012	n.s.	0.012	0.000	0.495
Nihoa	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.000

Table 2.10. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between populations of Hawaiian *Sesbania*, corrected for the presence of null alleles [ $F_{ST(ENA)}$ ].

Kīpuka Nēnē–Hilina pali	0.000	0.058	0.035	0.258	0.177	0.407	0.340	0.287	0.338	0.178	0.288	0.323	0.502	0.533	0.517	0.426
Pepeiau–Kukalau‘ula pali		0.000	0.030	0.238	0.125	0.398	0.323	0.240	0.287	0.138	0.239	0.324	0.492	0.532	0.514	0.387
Kamo‘oali‘i–Kū‘ē‘ē			0.000	0.219	0.138	0.394	0.316	0.236	0.296	0.134	0.269	0.312	0.504	0.542	0.522	0.407
‘Āpua point				0.000	0.313	0.485	0.432	0.435	0.427	0.294	0.384	0.445	0.598	0.567	0.637	0.531
Kamilo Point–Ka Lae					0.000	0.407	0.317	0.274	0.310	0.132	0.260	0.343	0.451	0.497	0.501	0.399
Waiaka‘īlio						0.000	0.505	0.457	0.433	0.296	0.410	0.585	0.734	0.744	0.756	0.533
Pu‘u Koa‘e							0.000	0.296	0.361	0.199	0.229	0.434	0.574	0.593	0.513	0.410
Pu‘u Pīmoe								0.000	0.281	0.125	0.285	0.334	0.515	0.592	0.539	0.350
Papanalahoa–Nākālele									0.000	0.219	0.257	0.328	0.468	0.538	0.503	0.363
Kawela–Kamiloloa										0.000	0.162	0.243	0.350	0.426	0.390	0.262
Mo‘omomi											0.000	0.398	0.543	0.561	0.519	0.357
Kāohikaipu & Mōkapu												0.000	0.323	0.458	0.548	0.415
Ka‘ena point													0.000	0.553	0.649	0.484
Polihale														0.000	0.619	0.533
Mānā															0.000	0.479
Nihoa																0.000

Table 2.11. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between 8 putative species (Char, 1983) of Hawaiian *Sesbania* on top half of matrix, Bonferroni corrected  $P$ -values ( $\alpha_{0.01} = 0.0028$ ) listed in bottom half.

<i>“kauensis var. intermedia”</i>	0.0000	0.0572	0.1357	0.2001	0.2613	0.4335	0.3792	0.5026
<i>“kauensis var. kauensis”</i>	0.0028	0.0000	0.0725	0.1407	0.2092	0.4029	0.3345	0.4746
<i>“tomentosa var. tomentosa”</i>	0.0028	0.0028	0.0000	0.0854	0.1239	0.2691	0.2432	0.3521
<i>“arborea”</i>	0.0028	0.0028	0.0028	0.0000	0.1349	0.2998	0.2367	0.3643
<i>“molokaiensis”</i>	0.0028	0.0028	0.0028	0.0028	0.0000	0.3962	0.2740	0.4296
<i>“oricola”</i>	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000	0.3155	0.5011
<i>“polihalensis”</i>	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000	0.3155
<i>“manaensis”</i>	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0000

Table 2.12. Pairwise  $F_{ST}$ -values ( $\theta$ ; Weir and Cockerham, 1984) between 8 putative species (Char, 1983) of Hawaiian *Sesbania*, corrected for the presence of null alleles [ $F_{ST(ENA)}$ ].

“ <i>kauensis</i> var. <i>intermedia</i> ”	0.0000	0.0455	0.1374	0.1906	0.2323	0.4070	0.3382	0.5250
“ <i>kauensis</i> var. <i>kauensis</i> ”		0.0000	0.0798	0.1281	0.1768	0.3690	0.2933	0.4916
“ <i>tomentosa</i> var. <i>tomentosa</i> ”			0.0000	0.0686	0.1056	0.2755	0.2215	0.4216
“ <i>arborea</i> ”				0.0000	0.1160	0.2904	0.2098	0.4126
“ <i>molokaiensis</i> ”					0.0000	0.3572	0.2310	0.4419
“ <i>oricola</i> ”						0.0000	0.2258	0.4856
“ <i>polihalensis</i> ”							0.0000	0.3395
“ <i>manaensis</i> ”								0.0000

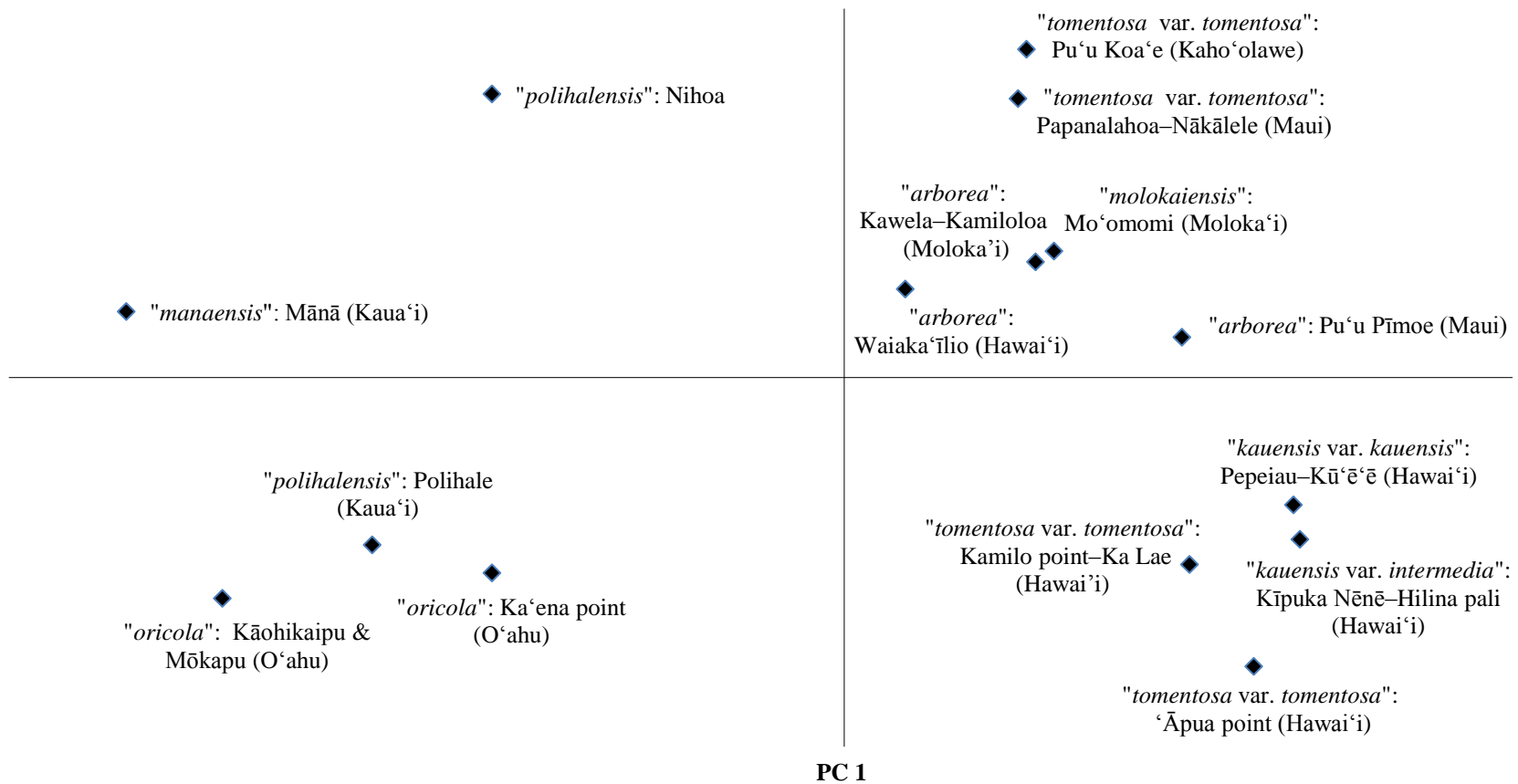


Figure 2.14. Principal Coordinate Analysis (PCA) of the chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967) between populations of Hawaiian *Sesbania*. Each population is identified by the putative species designations of Char (1983).

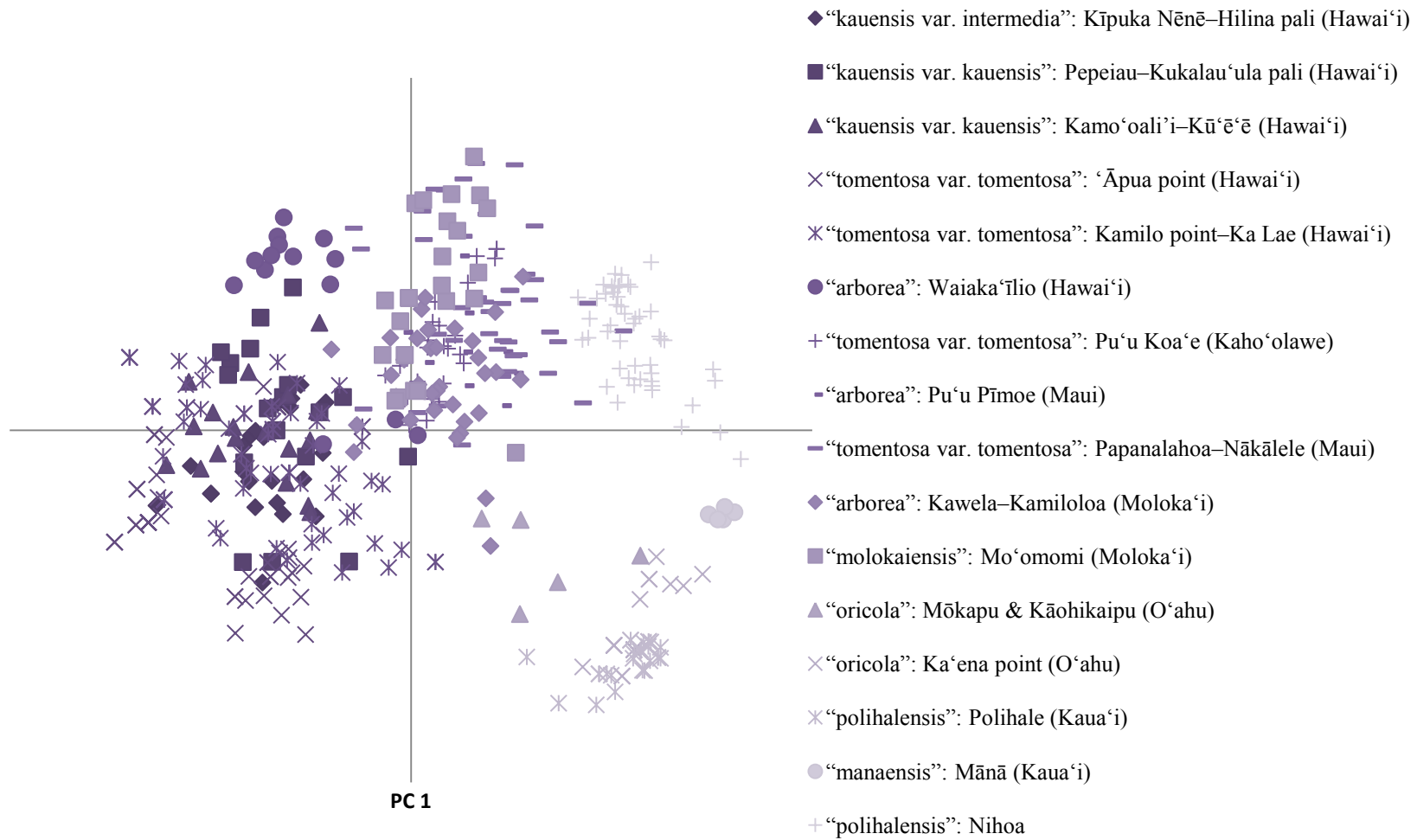


Figure 2.15. Principal Coordinate Analysis (PCA) of the codominant genotypic distances (Smouse and Peakall, 1999) between individuals of Hawaiian *Sesbania*. Population of origin for each individual distinguished by shaded symbols. Each population is identified by the putative species designations of Char (1983).

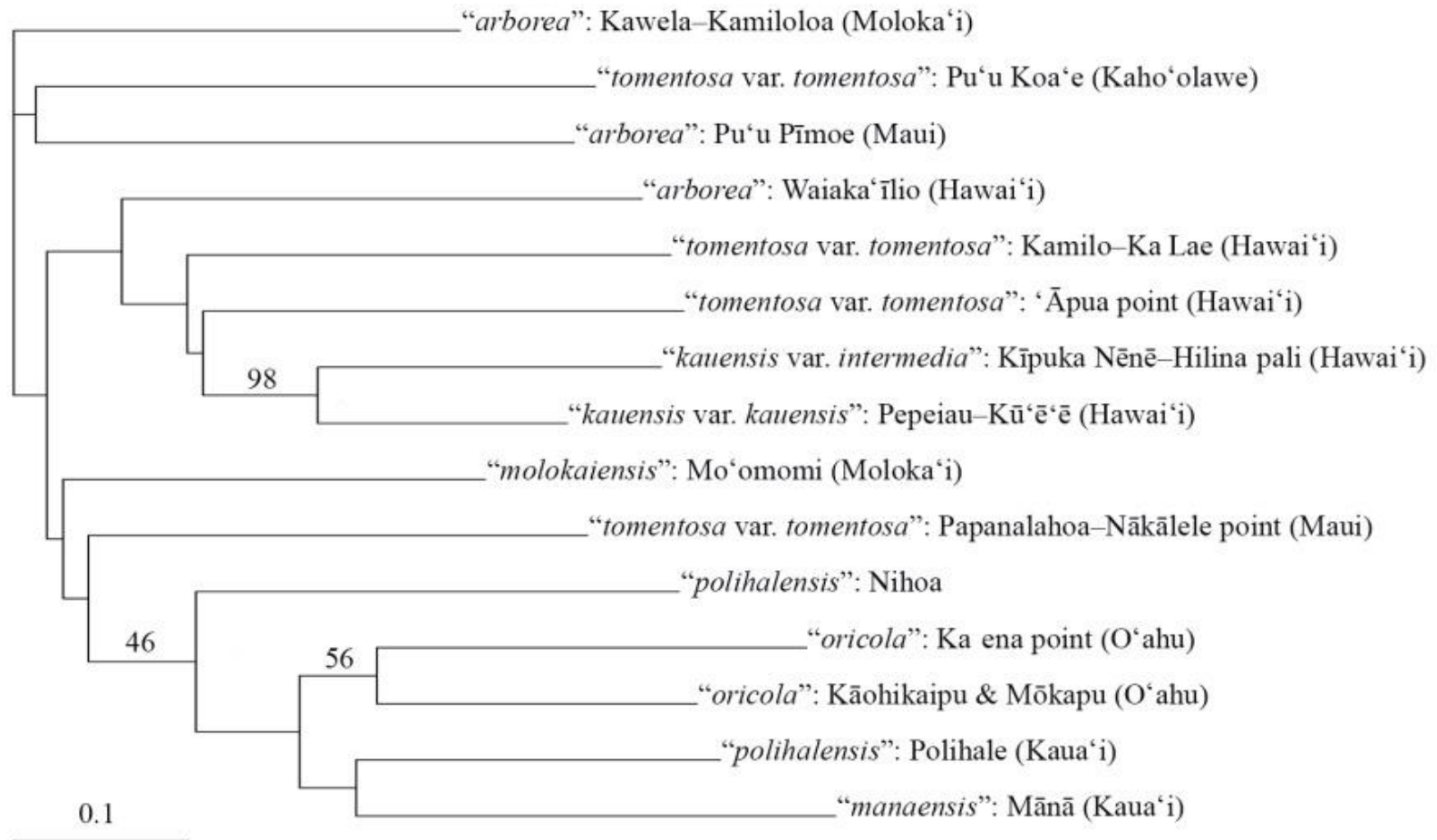


Figure 2.16. Neighbor-joining tree of Hawaiian *Sesbania* populations based on chord distance ( $D_C$ ; Cavalli-Sforza and Edwards, 1967). Each population is identified by the putative species designations of Char (1983). Bootstrap support values (1,000 replicates) are shown only where support exceeded 40%.

population and island of origin; the Maui Nui individuals were particularly cohesive, as well as the individuals from O‘ahu and Kaua‘i (Figure 2.15).

Support was low for most of the branches of the microsatellite NJ phenogram due to high variance in bootstrapped distance estimates (Figure 2.16). Relatively few loci were examined (nine) so there may not have been sufficient resolution to recover the correct topology (Takezaki and Nei 1996). In contrast to PCA, NJ analysis showed the “*tomentosa* var. *tomentosa*” population from Papanalaho–Nākālele (Maui) and the “*molokaiensis*” population from Mo‘omomi (Moloka‘i) more closely related to the populations on the Islands of O‘ahu, Kaua‘i and Nihoa than they were to the rest of the Maui Nui populations. Support was also relatively strong on both morphological phylogenies at the nodes which paired “*molokaiensis*” with “*oricola*” (O‘ahu) (Figures 2.1 and 2.2). Other than this discrepancy, as with the PCA and STRUCTURE analysis, there appeared to be a consistent geographical pattern to the topology.

## Discussion

### Inconclusive morphological and molecular phylogenies

Results from the morphological analysis suggest that many of the characters used to develop the data set do not support the relationships among taxa in any meaningful way. In the morphological phylogenies, the high homoplasy index, and the extremely low rescaled consistency index (values) indicate that autapomorphies are inflating the consistency index and that many of the characters constructing the phylogenies are homoplastic. The morphological analysis is consistent with the conclusion drawn by Geesink et al. (1999) that Char’s characters vary independently and that differences among populations are based on differing means and not discrete quantitative or qualitative differences. In addition, the standard deviations around some of the means are larger than the discrete categories used to code that particular character (Char, 1983). As such, attempting to ascertain phylogenetic relationships among the various populations using morphological characters was confounded and any purported clarification such an analysis offers of the manner in which these populations evolved is misleading.

Similarly, the molecular DNA phylogeny was unable to suggest any meaningful relationships among populations of Hawaiian *Sesbania*, besides sharing a sister relationship with



*S. marchionica* from the Marquesas. Fosberg (1948) suggested that the presumed origin of Hawaiian *Sesbania* is from somewhere in the South Pacific given the morphological similarity to other Pacific (*S. coccinea*, *S. marchionica* and *S. grandiflora*) and Austral (*S. formosa*) species. However, the data here show evidence for an American origin, consistent with the cosmopolitan *Sesbania* phylogenies of Farruggia (2009).

In contrast to the isozyme phenogram of Gemmill et al. (1995) (discussed below), sequence diversity was virtually non-existent at the two nuclear regions sampled for this study. The ITS sequences obtained herein were identical to sequences submitted to GenBank [from Kaua‘i (“*polihalensis*”): AF536355 and AF536356; from O‘ahu (“*oricola*”): AF536357, AF536358 and AF536359; from Moloka‘i (“*arborea*”): JX453663]. Therefore, DNA sequence data, at least with the genes used here, will not be able to resolve phylogenetic relationships among the morphologically variable Hawaiian populations, and provides no evidence (by itself) for splitting *S. tomentosa* into additional taxa. In spite of this, it appears that all Hawaiian *Sesbania* populations form a monophyletic group and represent a recent, incipient evolutionary radiation among the Hawaiian Islands. In this case, close analysis of the population genetic dataset is necessary to infer connections between the observed morphologies of distinct populations.

#### Resolution of taxonomic groups with population genetic markers

Overall, STRUCTURE provided less resolution in identifying distinct clusters (or lineages) than  $F_{ST}(\theta)$ . This might be explained by a poor fit between assumptions of the STRUCTURE model, which assumes Hardy-Weinberg equilibrium within populations, and the empirical data (see Chapter 2). As a point of comparison, Wright’s (1978) guidelines state that values of  $F_{ST}$  above 0.25 indicate “very great” genetic differentiation. Many of the putative species and populations analyzed here far exceed this level of differentiation, suggesting that the sequence markers used above were unable to detect the more recent, dramatic divergence evident in microsatellite loci.

Since STRUCTURE is useful in determining the lower bounds of potential species (Shaffer and Thomson, 2007), the results presented herein provide a basis for beginning to understand the apparent diversification of Hawaiian *Sesbania* populations. The first division of

Hawai‘i Island in a separate cluster from the rest of the populations to the northwest is an important lower bound. The fact that geographic populations of “*arborea*” and “*tomentosa* var. *tomentosa*” from different islands failed to cluster together genetically is evidence of morphological homoplasy among populations. The strong phylogeographic pattern present in the STRUCTURE analysis at both hierarchical levels (whereby geographically proximate populations cluster together regardless of their putative species designations) is also seen in the PCA and NJ results. This pattern also indicates that Maui Nui (situated in the middle of the high islands of the Hawaiian archipelago) might be the center of origin and diversity for Hawaiian *Sesbania*. Strong indications of admixture in Maui Nui populations, and in particular in the populations of “*arborea*” and “*molokaiensis*”, lend support to this assertion. The closest relatives to Hawaiian *Sesbania* are all arborescent, thus the arborescent “*arborea*” could be seen as a primitive type and peripatric divergence of the more prostrate and tomentose “*molokaiensis*” and “*tomentosa* var. *tomentosa*” morphotypes formed the basis for the wide range of variation we observe across the Hawaiian Islands. Two of these three types were observed by Rock in 1919 and all three were observed by Degener in 1918 within 10 km of one another on the island of Moloka‘i (Hawai‘i Biodiversity and Mapping Program). Relatively low pairwise  $F_{ST}(\theta)$ -values (average 0.10; ranging from 0.08–0.13) between these three putative taxa as compared with pairwise  $F_{ST}(\theta)$ -values between these three and the remaining five taxa (average 0.3; ranging from 0.07–0.43) corroborate this scenario. Arguably the two most morphologically distinct populations analyzed here occur within 25 km of one another on the Island of Moloka‘i (“*molokaiensis*” and “*arborea*”), yet STRUCTURE analysis and PCA grouped these two populations together.

Global  $F_{ST}$  among the eight putative species of Hawaiian *Sesbania* tested (0.211) was roughly half that among geographic populations (0.396). In addition, the AMOVA analysis suggested there was much more variation being distributed among geographic populations (40%) than there was among the eight putative species (4%), and that over half of the total variation (56%) was found within each population. As a means of comparison, in the widespread wind-dispersed *Metrosideros* (Myrtaceae) of the Hawaiian Islands, up to 91% of the variation was found within populations and 4% of the total variation was partitioned among taxa on a single island (Wright and Ranker, 2010; Stacy et al., 2014).

The “unified species concept” defines a species as a “separately evolving metapopulation lineage” (de Queiroz 2007), the term “lineage” referring to an ancestor-descendent sequence of populations. When two or more loci indicate that a lineage is distinct (i.e., harboring a set of unique or “private” alleles), that lineage or group of populations should become a candidate for species recognition (Shaffer and Thomson, 2007). There were two private alleles at two loci (at frequencies of 0.01 and 0.11) in one of the “*kauensis*” populations of Hawai‘i Volcanoes National Park (Kamo‘oali‘i–Kū‘ē‘ē). However, because one of the two alleles occurs at low frequency, and this population did not cluster independently of other populations on Hawai‘i Island in the STRUCTURE analysis, this example illustrates only a minor distinction to this population of “*kauensis*”. On the other hand, the small population of “*tomentosa* var. *tomentosa*” on Hawai‘i Island at Ka Lae (29 individuals when sampled in 2006) exhibited four private alleles at three loci, again at relatively low frequencies (average 0.03; ranging from 0.01–0.45). The second hierarchical layer of STRUCTURE analysis had separated this population (and 3 other nearby populations) out from the others on Hawai‘i Island. This population was recognized by Degener (1978) as “*hawaiiensis*”, yet was subsumed by Char (1983), who included it instead with other “*tomentosa* var. *tomentosa*” samples collected (from five islands) in her morphometric analysis. Thus, its relative distinction was not analyzed in the morphological and genetic comparisons made for this study. However, if you consider all of the populations of Hawai‘i Island together (as did the first layer of STRUCTURE analysis) there were eleven private alleles at seven loci (average frequency 0.020; ranging from 0.005–0.065).

The largest number of private alleles (16 occurring at 8 loci) were found in the “*arborea*” population of SE Moloka‘i (Kawela–Kamiloloa), albeit at low frequencies (average 0.05; ranging from 0.01–0.16) and occurring in only 60% of the individuals sampled. When all of the remaining populations of Maui Nui were considered together (excluding the “*arborea*” population of SE Moloka‘i) there were six private alleles at four loci (average frequency 0.090; ranging from 0.004–0.292). Considering all 3 populations of “*arborea*” (from 3 islands) together added only 1 more private allele, therefore the uniqueness of the SE Moloka‘i population is stressed.

The large census size of the SE Moloka‘i population (1,000 plants in 2006; USFWS, 2010) might be preserving rare alleles more efficiently, yet the same should also be true in the even larger population on Nihoa (5,000 plants; USFWS, 2010) which was found to harbor only

one private allele (at a frequency of 0.01). The large number of private alleles may indeed be strong indications of a separately evolving lineage of Hawaiian *Sesbania* in SE Molokaʻi, and to a lesser extent at Ka Lae on Hawaiʻi Island. In Chapter 2 the latter example is discussed in terms of the fact that large census size may not be the only factor in harboring unique alleles in populations of Hawaiian *Sesbania*; the Ka Lae population appears to have been fenced in (to the exclusion of ungulates) since 1908 (Love, 1991). In this regard, it is also interesting to note the observation that the tall arborescent form seems more resistant to browsing by deer in SE Molokaʻi (Degener, 1978), which would also allow that particular population to maintain alleles (as well as a large population size) more effectively.

In pairwise  $F_{ST}$  analysis the putative taxa from Oʻahu, Kauaʻi and Nihoa appeared the most differentiated from putative taxa on the remaining Hawaiian Islands. STRUCTURE also hints at separately evolving lineages comprised of the populations from Oʻahu, Kauaʻi and Nihoa. There were two alleles (at 2 loci) private to these three islands combined as well (average frequency 0.090; ranging from 0.004–0.173). While Oʻahu and Kauaʻi populations separated into a distinct sub-cluster from the population on Nihoa, a distinction reflected in the PCA and NJ tree, this phylogeographic trend is expected due to Nihoa's more remote location 250 km to the NW of Kauaʻi. In addition, samples from Nihoa, Kauaʻi and Oʻahu all diverged slightly from the rest of the Hawaiian samples sequenced at the TRPT region. Lastly, a possible mutated flanking sequence at microsatellite locus A122 in the Kaʻena point Oʻahu plants and three monomorphic loci in plants originating from Oʻahu and Kauaʻi (one fixed locus in plants from Nihoa) are additional indications of a separate lineage/species of *Sesbania* in the main Hawaiian Islands to the northwest of Maui Nui. The isozyme analysis of Gemmill et al. (1995) suggested this pattern of relationships as well, with a single (fixed) allele separating populations from these three islands from Maui Nui and Hawaiʻi Island by a mean genetic identity (genetic similarity rather than distance; Nei, 1972) of 0.58.

#### Taxonomic recommendations for the *Sesbania tomentosa* species complex

While the revisions of Char (1983) were here considered to represent the narrowest rendering of distinct Hawaiian *Sesbania* taxa, analyses here suggest that it needs to be broadened. According to Stuessy (1990), subspecies should be regarded as subdivisions of a

species complex, and represent variation that is genetically controlled. They usually have several conspicuous morphological differences between them and their ‘parent’ species, a cohesive geographical distribution of populations and multiple loci that are genetically divergent. While the morphological distinctions are not clear-cut in my opinion, the latter two conditions appear to be met in several cases pertaining to Hawaiian *Sesbania*. By this definition, populations of *Sesbania* on O‘ahu, Kaua‘i and Nihoa strongly support a distinct northwestern lineage in the process of divergence, and therefore a separate subspecies of *S. tomentosa*. Populations on Maui Nui appear to form another separately evolving metapopulation lineage, a second subspecies. There is also strong support for recognizing “*arborea*” from SE Moloka‘i as a third subspecies, apart from the larger Maui Nui lineage, while evidence is lacking to broaden this circumscription to include the populations of semi-arborescent individuals on Maui and Hawai‘i Island. Populations occurring on Hawai‘i Island form a fourth subspecies of *S. tomentosa*, while any distinction of the Degener’s taxon from Ka Lae within a larger Hawai‘i Island lineage appears to be an artifact of its historical isolation and remoteness.

## Conclusion

Since populations of *Sesbania tomentosa* are in most cases readily distinguishable by the morphology of their representative individuals, this indicates that certain traits (e.g., leaf pubescence and plant habit) have a more rapid rate of evolution than the DNA sequences that were sampled. Natural selection in different environments, along with random drift and mutation in fragmented (isolated) populations may have caused Hawaiian *Sesbania* to separate out into the distinctive appearing populations we see today. Over the past century, an overlap of morphological characters observed in what was once a much more contiguous range of the species has largely been erased. With inbreeding comes a loss of genetic diversity, hence higher  $F_{ST}$  values and overall genetic structuring. The results presented here could indicate a recent phenomenon due to rarity or an ancient one due to divergence (or a combination). An investigation of population fragmentation and sub-structuring will be explored further in Chapter 3. In this case, an assessment of the occurrence of inbreeding and drift among populations will be essential. Microsatellite loci respond to random genetic drift and mutation much more rapidly than the regions sequenced herein; certainly within the time period when populations of

Hawaiian *Sesbania* became increasingly isolated from one another. On a final note, testing whether or not  $F_1$ ,  $F_2$  and  $F_3$  (and backcrosses) have markedly reduced fertility would be the next step in addressing the issues of taxonomy presented, (a fourth condition for sub-specific recognition according to Stuessy, 1990), and should be a focus for future research attempting to discriminate Hawaiian *Sesbania*.

## CHAPTER 3

### **The influence of inbreeding and genetic drift on the differentiation of *Sesbania tomentosa* populations, a rare plant species of the Hawaiian Islands**

#### **Introduction**

Contemporary impacts on the genetic makeup of plant populations and the influence of prehistoric evolutionary phenomena can be difficult to distinguish (e.g., Muir and Schlötterer, 2005; Edwards et al., 2008). The genetic effects of contemporary fragmentation of habitat and decline in numbers of individuals are important to separate from the long term effects of genetic drift, which ultimately can lead to divergence within a species (Ashley et al., 2003). Population subdivision, genetic founder effects, bottlenecks and inbreeding are also expected to have played important roles over the long run in natural processes of differentiation and speciation (Wright, 1931, 1942, 1977; Mayr, 1954; Carson, 1975; Templeton, 1980). Plant reproductive syndromes will be influential over the long run as well, with populations of predominately self-pollinating species having less genetic variation and greater divergence among populations than that associated with more outcrossing species (Hamrick and Godt, 1996). Genetic drift is thought to take place at an accelerated rate in smaller populations (Kimura, 1983), therefore the size of natural populations over time is an additional consideration. Natural ecological dynamics affecting population differentiation often leave lasting genetic signatures, and should be addressed alongside contemporary impacts on plant habitat when discussing the divergence of plant population remnants.

*Sesbania tomentosa* Hook. and Arn. is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. This species was federally listed as Endangered by the U.S. Fish and Wildlife Service in 1992. Twenty-nine of the fifty-two populations of *S. tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826 (Table 3.1). Seven populations have been extirpated over the 10 years since this study began, and others have experienced severe demographic decline due to drought, pest outbreaks, etc. (personal communications and observations). A hermaphroditic breeding system, conspicuous flowers and autochorous dispersal of dry fruit have made *S. tomentosa* acutely

Table 3.1. Evidence for the catastrophic decline of *Sesbania tomentosa* populations in the main Hawaiian Islands. Biological surveys since the plant's original description in 1826 are tallied along with cultural indicators of the plant's physical presence at selected locations. Place/land division names are included here only when the species occurrence at a given location was not recorded by biological surveys, and when corresponding locations are > 2 km apart. Both extant and extinct occurrences refer only to naturally-occurring groups of plants (separated by > 1 km). Extant vs. extinct status verified via personal communication with private land managers and conservation workers, Federal employees and Hawai'i State personnel. 'Ohai is the Hawaiian name for *Sesbania tomentosa* (Andrews, 1922).

Island	Extant population (as of 2015)	Extinct population (as of 2015)	Place names / type of location	Division names / type of division
Hawai'i	<p>Āpua point Pepeiau Kukalau'ula Kīpuka Nēnē Hilina pali 1 Hilina pali 2 Hilina pali 3 Fuel Break Rd. Kamo'oali'i Kū'ē'ē</p>	<p>Kamilo point Mahana bay Kīpuka Hanalua Ka Lae Waiaka'īlio Ka'ūpūlehu</p>	<p><sup>e</sup>Ohai'ula / beach <sup>e</sup>Kalae'ohai / point <sup>e</sup>Moku'ohai / bay <sup>b</sup>Pu'u 'ohai / hill</p>	<p><sup>f</sup>Kalae'ohai / boundary <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / kīhāpai <sup>f</sup>Opū'ohai / 'ili 'āina <sup>f</sup>Pū'ohai / ahupua'a <sup>e</sup>Ohaieka / 'ili 'āina</p>
Maui	<p>Papanalahoa Kahakuloa Mōkōlea</p>	<p>Pu'u Pīmo'e Nākālele Līhau</p>	<p><sup>a</sup>Maka'ohai / fishing site <sup>b</sup>Kalae'ohai / point</p>	<p><sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Ka'ohai / 'ili 'āina <sup>f</sup>Pū'ohai / 'ili 'āina</p>
Kaho'olawe	<p>Pu'u Koa'e</p>	<p>Kaho'olawe</p>		
Lāna'i		<p>Maunalei Kahinahina Mānele Kaumālapa'u Kamoku Paoma'i Kūāhua</p>		<p><sup>f</sup>Ka'ohai / ahupua'a</p>



Table 3.1. (Continued) Evidence for the catastrophic decline of *Sesbania tomentosa* populations in the main Hawaiian Islands.

Island	Extant population (as of 2015)	Extinct population (as of 2015)	Place names / type of location	Division names / type of division
Molokaʻi	Moʻomomi Kawela Kamiloloa Makakupaʻia	Kalaeokaʻilio Maunaloa Kalaeokalāʻau Waiahewahewa Pālāʻau Mahana	<sup>f</sup> Loko ʻOhaipilo / pond	<sup>f</sup> ʻOhaipilo / ʻili ʻāina <sup>f</sup> Kaʻiliʻohai / ʻili ʻāina
Oʻahu	Kaʻena point Mōkapu Kāohikaipu	Waiʻanae Mokulua Manini pali	<sup>f</sup> Loko Kaʻohai / pond <sup>f</sup> Kaʻohai / tree grove	<sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ʻāina <sup>f</sup> Kaʻohai / ʻili ku pono <sup>f</sup> Kaʻohai / ʻokipuʻu <sup>f</sup> Kaʻohai / moʻo ʻāina
Kauaʻi	Polihale Hanapēpē	<sup>c</sup> Mānā Plain	<sup>b/d</sup> ʻOhaiʻula / ridge <sup>d</sup> ʻOhaiʻula / valley <sup>d</sup> ʻOhaiʻula / point <sup>a</sup> Waiʻohai / beach	<sup>f</sup> Kaʻohai / moʻo ʻāina <sup>f</sup> Waiʻohai / ʻili ʻāina <sup>f</sup> Haleʻohai / ʻili ʻāina
Niʻihau		<sup>c</sup> Leeward Niʻihau <sup>c</sup> Kawaihoa		
Total	23	29	13	23

<sup>a</sup> Clark, J.R.K. 2002. Hawaiʻi Place Names. University of Hawaiʻi Press, Honolulu, HI. 412 p.

<sup>b</sup> Coulter, J.W. 1935. A Gazetteer of the Territory of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 241 p.

<sup>c</sup> Hawaiʻi Biodiversity and Mapping Program: Hawaiʻi Natural Heritage Program

<sup>d</sup> Juvik, S.P. and Juvik, J.O. 1998. Atlas of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 333 p.

<sup>e</sup> Pukui, M.K., Elbert, S.H. and Mookini, E.T. 1974. Place Names of Hawaiʻi. University of Hawaiʻi Press, Honolulu, HI. 289 p.

<sup>f</sup> Soehren, L.J. 2002–2010. A Catalog of Hawaiian Place Names accessed at <http://ulukau.org/cgi-bin/hpn?l=haw>

vulnerable to extinction compared with other dry forest taxa, according to the analysis of Pau et al. (2009). On the other hand, entirely new occurrences of this species have been discovered since this study began near Nu‘upia Pond (Mōkapu, O‘ahu) and at Pa‘akahi Point (Hanapēpē, Kaua‘i) after heavy winter rains, indicating an important role of the seedbank within the metapopulation as a whole as well as the ephemeral nature of the plant as a component of the vegetation.

The habit of *Sesbania tomentosa* is highly variable, often with island specific forms. Plants may grow as sprawling shrubs with prostrate to decumbent branches (reportedly up to 14 meters long, and possibly longer) or as a small bush or tree up to six meters in height. Leaves are even-pinnately compound and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. The species is named for the leaves, that are usually sparsely to densely covered with silky hairs. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet to deep red. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds. The chromosome number for *S. tomentosa* is  $2n = 24$  (Geesink et al., 1999), suggesting the species is diploid (base chromosome number  $x = 12$ ). *Sesbania tomentosa* is currently recognized as a single species (Geesink et al., 1999) although it is highly variable for many important characters across its range. This led Rock (1920), Degener and Degener (1978) and Char (1983) to delimit up to nine distinct putative taxa. According to Andrews (1922), the Hawaiian name for *S. tomentosa* is ‘ohai.

Cultural knowledge can be used to hypothesize the prior distribution of *Sesbania tomentosa* in the Polynesian era. The Hawaiians named the various features and places in their environment, and often incorporated plant descriptions in names (Pukui et al., 1974). Geographic place names (beaches, points, hills, ridges, etc.) often mention a specific plant, likely reflecting an observable element of the geography at the time that place was named (Sam Gon, The Nature Conservancy, personal communication; Coulter, 1935). The names and boundaries of parcels of land, often named for observable elements of the environment and landscape as well, are known through oral tradition originating as far back as the 15<sup>th</sup> century (Kamakau, 1961; 1976). The use of land division names to infer past geographical extent of a plant species in Hawai‘i was used by McEldowney (1983) to map the extent of ‘ōhi‘a lehua (*Metrosideros polymorpha*) forest across the prehistoric Waimea (Kohala, Hawai‘i) plain. If place and land division names referring

to 'ohai are considered indications of past occurrences of *S. tomentosa*, then the total number of populations ever recorded would increase by 41% (adding 36 additional occurrences; Table 3.1).

The methods of Price et al. (2007) were used to predict the natural range for *Sesbania tomentosa*. This was accomplished by demarcating a general bioclimatic envelope, built upon a database that includes information on the known distribution of the species by geographic region, major habitat type, and elevation range. In this model, most of the main Hawaiian Islands (excepting the islands of Maui and Hawai'i) are almost completely encircled by the range of *S. tomentosa*, which extends along the coasts and well inland in dry-mesic areas (Figure 3.1). Anecdotaly, MacCaughey (1916) remarked, "the bush is often to be found in the vicinity of the little beach settlements, particularly along the arid leeward shores." Degener (1978) commented on the decline of populations of *S. tomentosa* on O'ahu and Hawai'i Island as compared to his observations 50 years prior.

On the other hand, some evidence suggests that the decline of *Sesbania tomentosa* has been progressing for centuries. Based on extensive palynological core data on O'ahu (Athens 1997, 2002), by A.D. 1600 the entire landscape below 460 m had been extensively altered, indicated in part by a catastrophic decline in the pollen of native species. For example, *S. tomentosa* disappeared from the 'Ewa plain pollen record around 1300 AD, where it has not been observed in historic times. Athens et al. (2002) correlate the destruction of lowland vegetation with the arrival of the Polynesian rat, *Rattus exulans*. At Hawai'i Volcanoes National Park, extensive rat damage of seedpods of *S. tomentosa* has been documented, and the presence of fruits on plants rapidly rebounds when rats were controlled in the species habitat (Pratt et al., 2011). Rat, ungulate, and arthropod predation, along with human disturbance, is listed as the main contemporary factors in the fragmentation and decline of reproductive populations of *S. tomentosa* [US Fish and Wildlife Service (USFWS), 2010].

Lack of adequate pollination services has also been deemed another threat in populations of *S. tomentosa* (USFWS, 2010). The results of two pollination studies of *S. tomentosa* show a mixed-mating system (Goodwillie et al., 2005) where some plant seeds are derived from outcrossing and some are derived from either pollinator-mediated or autonomous self-fertilization. Working at Ka'ena point on O'ahu, Hopper (2002) found that *S. tomentosa* is fully self-compatible and self-pollen, as well as non-self-pollen, was equally likely to result in fertilization and fruit set. The species is pollinator-limited (the flower's protective wing and keel

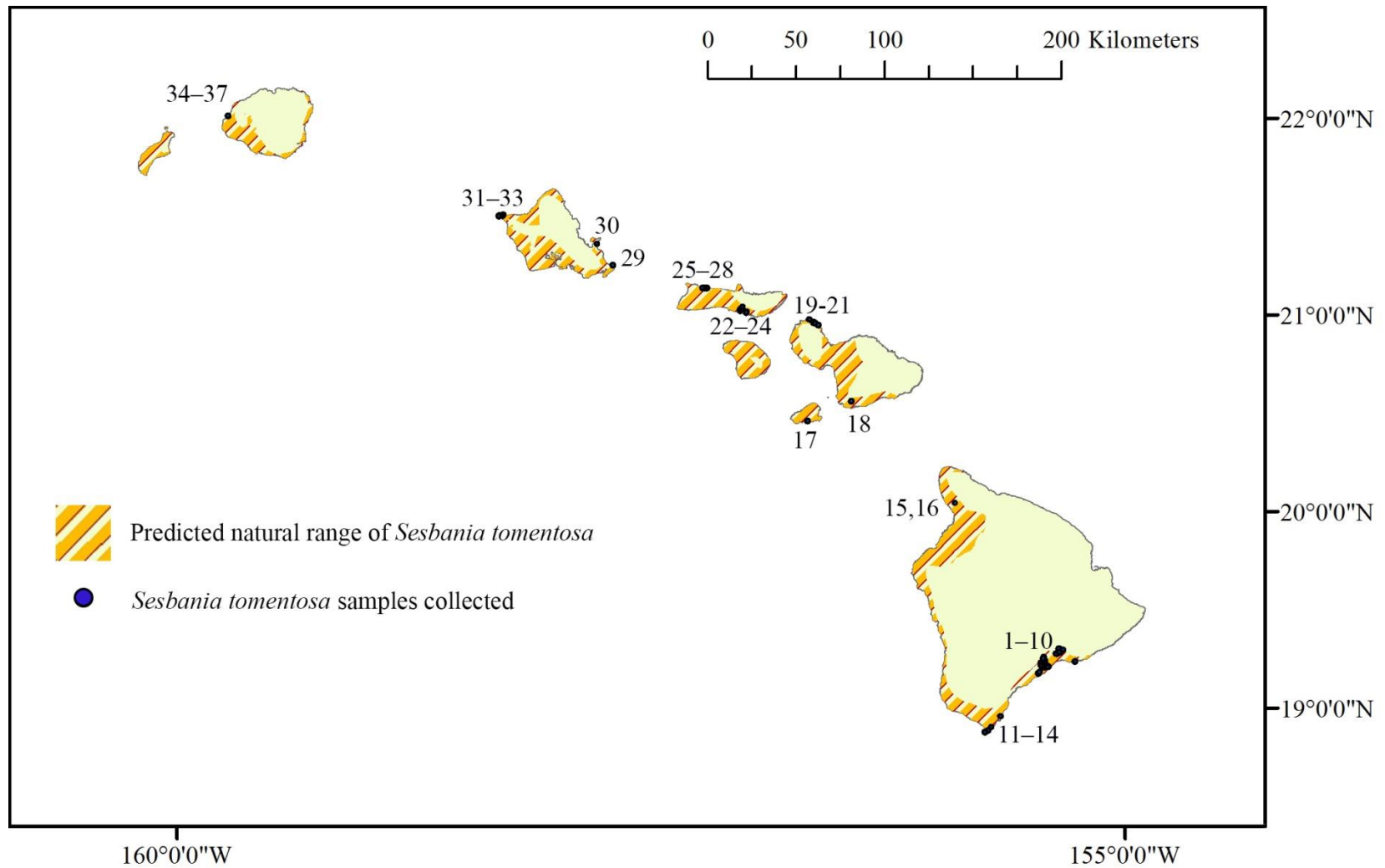


Figure 3.1. Location of DNA samples collected in 2006–2010; numbers on map correspond to sub-populations/populations listed in Table 3.2. Predicted natural range of *Sesbania tomentosa* provided by Jonathan Price, University of Hawai‘i at Hilo.

petals necessitate mechanical pollination), yet in the absence of a pollinator the proximity of the stigma and the anthers ensure that selfing is still possible. While rates of autogamy were shown to be low (0.8%), this rate might be high enough to maintain low levels of reproduction in a species where individuals have the potential to produce 1,000 flowers over the course of a season (Hopper, 2002). The endemic *Hylaeus* pollinators (accounting for 86.4% of all floral visitations and 99.6% of observed pollen transport) were noted to spend most of their time around single plants, and Hopper believed that a large proportion of the pollination and fruit set he observed at Ka‘ena point, as well as in his observations of the species at Hawai‘i Volcanoes National Park, could be the result of geitonogamy (Hopper, 2002). *Hylaeus* are thought to be important pollinators for native Hawaiian plants in general because of the frequency of their visitation (Magnacca, 2007; Koch and Sahli, 2013; Krushelnycky, 2014). In a more recent study, Pratt et al. (2011) observed *Hylaeus flavipes* and *H. laetus* to be the most abundant visitors of *S. tomentosa* at the upland population at Kīpuka Nēnē (Hawai‘i Volcanoes National Park), and found the species’ pollen on the bodies of *Hylaeus* (accounting for 60.2% of total visits, 25.0% of which involving observed pollen transport). Again, geitonogamy was purported to be the main mechanism of pollination for this plant at Kīpuka Nēnē (Pratt et al., 2011). Therefore, it is unclear whether a lack of pollination services would be a threat to *S. tomentosa* populations or would alter their genetic makeup at all, as inbreeding and a high degree of relatedness between adjacent individuals would seem to be a natural consequence of the plant’s ecology.

This chapter will address population-level processes that might be affecting the rapid differentiation of populations discussed in Chapter 2. Levels of genetic variation within and among populations of *S. tomentosa* were measured using microsatellite marker analysis to investigate inbreeding and population sub-structuring and to examine evidence for genetic bottlenecks. The genetic diversity of a naturally-occurring extant population (Mo‘omomi, Moloka‘i) was also compared with a molecular sampling of herbarium specimens collected there 60–100 years prior to the sampling of 2006, to illustrate the consequences of one such bottleneck directly. Another population for which census size had been known to fluctuate from year to year (Polihale, Kaua‘i) was repeatedly sampled over a four-year period to observe how population genetic diversity might be dynamic over time, and also add an additional dimension to a discussion of natural vs. human induced genetic bottlenecks.

## Materials and Methods

### DNA sample collection

Leaf samples of 539 individuals of *Sesbania tomentosa* were collected between 2006 and 2009 from naturally occurring populations throughout the Hawaiian Islands. In total, 38 sub-populations (separate clusters of plants 1 to 3 km apart within a population) comprising 18 populations from seven islands were sampled (Table 3.2, Figure 3.1). An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. I recorded GPS coordinates for each individual plant sample collected. Samples at 'Āpua point, Kawela–Kamiloloa, Pu'u Koa'e and Nihoa comprise a subset of their respective populations (individuals collected arbitrarily from throughout each population). At Pu'u Koa'e and Nihoa, samples were obtained by surrogate collectors [Ken Wood, National Tropical Botanical Garden (NTBG) and Beth Flint (USFWS)] and no GPS coordinates were logged. An attempt to distinguish groups of naturally occurring *vs.* out-planted individuals at Ka'ena point was made with the assistance of Betsy Gagné (Hawai'i Division of Forestry and Wildlife). Except where noted above, only naturally occurring plants and all known individuals extant at the time of collection were sampled for analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4 to 8°C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNeasy tissue kits (QIAGEN; Valencia, CA) according to the manufacturer's protocol and the purified sample, along with negative and positive controls, were visually checked using electrophoresis.

Additional sampling of historically-collected tissue from the Mo'omomi dunes population on Moloka'i was conducted with loaned specimens from the herbarium of the New York Botanical Garden (NY), the B. P. Bishop Museum Herbarium (BISH) and the U. S. National Herbarium (US) (Table 3.3). DNA was extracted from 10 specimens using the QIAGEN QiaAmp Stool minikit, modified CTAB protocols (Drábková et al., 2002) and a PTB (N-phenacylthiazolium bromide) protocol (Asif and Cannon, 2005). For each of the 10 specimens at least one of the extraction protocols listed proved successful (samples checked via electrophoresis). These historically collected samples were included in analyses of microsatellite

Table 3.2. Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands. Sub-populations are listed as combined into population aggregate groups for subsequent analysis; distances between clusters of plants designated as sub-populations within a given population are listed in parentheses. ID numbers code for sub-populations listed on Figures 3.1, 3.3, 3.6 and 3.7. *n* and *N*, sample size of sub-populations and populations, respectively.

Island	ID #	Sub-population/population	<i>n/N</i>
Hawai‘i	1	Kīpuka Nēnē makai	12
	2	Kīpuka Nēnē mauka	6
	3	Hilina pali cluster 1	8
	4	Hilina pali cluster 2	6
	5	Hilina pali fuel break rd.	3
		(2 km apart)	Kīpuka Nēnē–Hilina pali population total: 27
	6	Pepeiau	10
	7	Kukalau‘ula pali	9
		(2 km apart)	Pepeiau–Kukalau‘ula pali population total: 19
	8	Kamo‘oali‘i	13
	9	Kū‘ē‘ē	5
	10	‘Āpua point	58
	11	Kamilo point	9
	12	Mahana bay	29
	13	Kīpuka Hanalua	12
	14	Ka Lae	29
		(2 km apart)	Mahana bay–Ka lae population total: 70
	15	Waiaka‘īlio	8
	16	Waiaka‘īlio seedbank	10
			Waiaka‘īlio population total: 18

Table 3.2. (Continued) Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands.

Island	ID #	Sub-population/population	n/N
Kaho‘olawe	17	Pu‘u Koa‘e	25
Maui	18	Pu‘u Pīmoe	9
	19	Papanalaho point	37
	20	Mōkōlea point	5
	21	Nākālele point	2
		(1–2 km apart) Papanalaho–Nākālele point population total:	46
Moloka‘i	22	Kawela	17
	23	Kamiloloa	14
	24	Makakupa‘ia	4
		(2–3 km apart) Kawela–Kamiloloa population total:	35
	25	Moloka‘i ranch rd.	14
	26	Nature Conservancy preserve	3
	27	Mo‘omomi pavillion	9
		(1–2 km apart) Mo‘omomi population total:	26
	28	Mo‘omomi herbarium	10
O‘ahu	29	Kāohikaipu	2
	30	Mōkapu (Nu‘upia pond)	4
		(15 km apart) Kāohikaipu & Mōkapu population total:	6
	31	Ka‘ena point State Park	15
	32	Ka‘ena point outplantings	32
	33	Ka‘ena point NAR	18
		(1–2 km apart) Ka‘ena point population total:	65



Table 3.2. (Continued) Population of origin for DNA collections made of *Sesbania tomentosa* in Hawaiian Islands.

Island	ID #	Sub-population/population	n/N
Kauaʻi	34	Polihale State Park (2006)	16
	35	Polihale State Park (2009)	11
	36	Polihale State Park (2010)	12
		Polihale State Park population total:	39
	37	Mānā plain	4
Nihoa	38	Nihoa	49
			Total = 539

Table 3.3. DNA collected off herbarium sheets of *Sesbania tomentosa* loaned from B. P. Bishop Museum Herbarium (BISH), New York Botanical Garden (NY) and the U. S. National Herbarium (US).

Barcode/ID #	Collector	Date	Location notes from herbarium sheet
990804 (NY)	J.F.C. Rock	3-1909	Molokai. Moomomi.
990808 (NY)	J.F.C. Rock	3-1910	Molokai. Moomomi.
990809 (NY)	C.N. Forbes	3-24-1915	Molokai. Moomomi.
55944 (BISH)	G.C. Munro	7-22-1926	Moomomi sandhills.
990820 (NY)	O. Degener	4-19-1928	Kalani, Moomomi. creeping branches take root, single large plant in sand dunes several hundred feet above sea.
990817 (NY)	O. Degener	4-25-1928	Moomomi, Molokai arid sand dunes.
55933 (BISH)	M.C. Neal	4-1-1934	Mokapu Crater, Oahu, edge of cliff.
990810 (NY)	F.R. Fosberg	12-26-1936	Molokai. Moomomi prostrate shrub, base of sand dunes.
14052 (US)	F.R. Fosberg	6-13-1937	Oahu. Kaohikaipu.
990811 (NY)	C.S. Judd	9-16-1937	Molokai. Moomomi procumbent shrub, sand hills alt. 10m.
177376 (BISH)	H. St.John	1-3-1939	Moomomi, Kaluahoi on sand dunes.
488514 (BISH)	H. St.John	12-24-1948	Moomomi, Kaluahoi, trailing on sand dunes near shore.

fragment sizes to compare genetic diversity of modern vs. historical plants collected from the Mo‘omomi population.

The demographics of certain populations necessitated augmentation of the dataset in order to provide marginally larger sample sizes for comparison. One cultivated individual derived from Kāohikaipu (1 plant extant in 2009) and one cultivated individual derived from Nu‘upia Ponds (3 plants extant in 2009) at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu) augmented the extant individuals in these two sub-populations, combined together in a single Windward O‘ahu population for statistical purposes. In addition, all four individuals comprising the Mānā, Kaua‘i population were cultivated specimens at the National Tropical Botanical Garden (F<sub>1</sub> and F<sub>2</sub> generation derived from a single wild plant, now extirpated). For the Waiaka‘īlio, Hawai‘i population, consisting of only a single surviving individual at the time sampling was undertaken, DNA was extracted from the woody core of eight plants that had been standing dead for approximately one year using the PTB protocol of Asif and Cannon (2005). In addition, the seedbank surrounding the dead plants was examined, producing an additional 10 *S. tomentosa* plants for genotyping. Lastly, in order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, the Polihale (Kaua‘i) population was sampled in 2006 (16 plants), 2009 (11 plants) and 2010 (12 plants), and the genetic diversity of the standing populations of each year are herein compared. GPS coordinates accompanied each DNA collection, yet in many cases it was impossible to determine whether or not the same individual was collected multiple times (in successive years) due to the close clustering of individuals.

### Microsatellite analysis

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* under contract with the United States Geological Survey (USGS). Ninety-six dinucleotide (CA<sub>n</sub>) and tetranucleotide (CATC<sub>n</sub>, TACA<sub>n</sub>, and TAGA<sub>n</sub>) microsatellite repeats. Ninety-six microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 3.4) based on their range of polymorphism and ease of scoring in a screening of

eight DNA samples (collected from eight populations on six islands). Each sample was amplified in a 25.0  $\mu$ L volume with final concentrations of: 0.6  $\mu$ M each of forward and reverse primers, 1X PCR Buffer, 2.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs (Promega, Madison, Wisconsin, USA), 1 unit *Taq* DNA polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 3.4) for 40 s, 72°C for 30 s; ending with a final extension of 72°C for 4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA) at the Center for Genomic, Proteomic and Bioinformatic Research (CGPBR) facility at UH Mānoa. The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (Applied Biosystems). Stutter peaks were identified, and the program MICROCHECKER (Van Oosterhout et al., 2004) was then used to identify possible genotyping errors due to non-amplified alleles (null alleles) and short allele dominance (large allele dropout). A maximum likelihood estimate of the frequency of null alleles (Expectation Maximization algorithm of Dempster et al., 1977) was then calculated for each locus and geographic population using the program FREENA (Chapuis and Estoup 2007).

The microsatellite dataset was analyzed to assess linkage (genotypic) disequilibrium (both globally as well as at the level of geographic population) in GENEPOP v. 4.0 (Rousset, 2008) using log-likelihood ratio statistics (*G*-tests). Significance was assessed using 200 batches of 10,000 iterations and Bonferroni-corrected *P*-values at significance level ( $\alpha = 0.05$ ).

Population structure was first examined using a full Bayesian-clustering approach, implemented in the program STRUCTURE v. 2.3.3 (Pritchard et al., 2000), which assigned individual genotypes to populations, irrespective of geographical location of origin. Default settings of the program were used (admixture model, independence among loci, no prior information included). To determine the most likely number of populations or groups (*K*) in the

Table 3.4. Nine microsatellite primer pairs developed for *Sesbania tomentosa*.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 539 individuals sampled for this study. Range, allele size range in base pairs (bp). Prefixes in italics before forward primer sequence indicate dye used for poolplexing.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG R: TAG-GTG-TGG-CGT-GCA-TAA-C	57.3 58.1	10	205–223
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A R: CCC-TTC-CCC-TCC-TCT-TAG	56.0 56.2	9	264–280
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	55.8 56.3	14	198–236
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC R: TAG-CCA-TGC-TTC-ATC-AAT-CG	58.9 59.8	21	288–328
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.8 56.9	13	163–187
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG R: GGC-AAC-ATT-TGA-GTG-GAG-G	58.6 59.1	16	196–276
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1 55.1	14	180–236
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.7 55.2	11	212–252
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.1 54.8	14	265–321

data, a series of analyses were performed from  $K = 1$  (all populations represent a single panmictic unit) to 15 (the maximum number of populations allowable) using 40,000 burn-in and 100,000 repetitions, with ten iterations per  $K$ . These results were examined using the  $\Delta K$  method (Evanno et al., 2005) to identify the most likely number of groups in the data. Ten additional iterations at the identified  $K$  were computed using 100,000 burn-in and 300,000 repetitions. The program CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize these last ten iterations. Cluster membership coefficients for each individual and pre-defined population were obtained (permuted across replicates using FullSearch algorithm) and used as input files for the cluster visualization program DISTRUCT v. 1.1 (Rosenberg, 2004) and for additional chart analysis.

Each individual was assigned to a particular genetic cluster when its coefficient of membership was greater than 50%. Geographic populations and sub-populations were assigned to a particular genetic cluster when 67–100% of their individuals were assigned to that genetic cluster. The initial analysis was repeated on each  $K$  separately to detect sub-structuring within the genetic groups previously inferred. The number of genetic sub-clusters was estimated for each group using the  $\Delta K$  method, ten additional iterations were performed at the appropriate  $K$  (100,000 burn-in and 300,000 repetitions) and both the *Greedy* and *FullSearch* algorithms (10,000 random input orders of runs) were used in CLUMPP. Individuals were then assigned to genetic sub-clusters when their coefficient of membership was greater than 0.5; geographic populations assigned to sub-clusters based on 70–100% individual assignment.

Diversity indices were estimated for the geographic populations using MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötterer, 2003). Diversity indices include expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ), mean number of alleles per locus ( $A$ , a measure of diversity not corrected for sample size), allelic richness ( $A_R$ , allelic diversity corrected for sample size) and monomorphic loci (loci harboring only one allele in a given population) within each population. Private alleles (alleles exclusive to a given population) were calculated in GENALEX v. 6.4 (Peakall and Smouse, 2006). GENEPOP was also used to test for a hypothesis of heterozygote deficiency within each geographic population at each locus and combined across loci using  $U$ -tests. Significance was assessed using 200 batches of 10,000 iterations and Bonferroni-corrected  $P$ -values at significance level ( $\alpha = 0.05$ ). Estimates were

obtained for  $f$ , the within population inbreeding coefficient or the correlation of allele frequencies among individuals within populations, in FSTAT v. 2.9.3.2 (Goudet, 2002).

The extent and significance of the genetic differentiation among geographic populations was investigated with MSA by calculating global and pairwise  $F_{ST}$  values (averaged over multiple loci), with 100,000 permutations to assess significance using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ). FREENA was also used to estimate pairwise  $F_{ST}$  values ( $F_{ST(ENA)}$ ) from genotype frequencies corrected for the presence of null alleles [using the excluding null alleles (ENA) method of Chapuis and Estoup 2007], which tend to positively bias  $F_{ST}$  estimates. Most of the non-visible genotypes in the dataset were assumed to be due to technical problems (e.g., degraded or low quantity of DNA or PCR amplification inconsistencies) and were specified in the FREENA dataset. These were distinguished from the null homozygous genotypes at locus A122 in 64 out of 65 individuals of the combined Ka'ena point population, probably due to a mutated flanking sequence which prevented that particular locus from amplifying.

The presence of a pattern of isolation by distance (IBD; Wright, 1943) between the populations across the Hawaiian Islands was investigated by testing the correlation of the matrix of pairwise log-transformed  $F_{ST}$  (Weir and Cockerham 1984) and  $F_{ST(ENA)}$  (Chapuis and Estoup, 2007) values against the matrix of log-transformed geographic distances using a Mantel test with 10,000 permutations in IBDWS v. 3.16 (Jensen et al., 2005).

Strong spatial genetic structure (i.e., nonrandom spatial distribution of genotypes) would be expected in a plant species with restrictions on the movement of pollen throughout the population (and beyond). In this scenario, genetic similarity is higher among neighboring individuals than more distant individuals (IBD). Kinship coefficients are based on the probability of identity of alleles for two homologous genes sampled in some particular way. In the case of a kinship coefficient between two individuals, the two genes are randomly sampled within each of the two individuals. SPAGEDI v. 1.3 (Hardy and Vekemans, 2002) was used to compute the kinship coefficients of Loiselle et al. (1995) for all pairs of individuals in a given population (some populations are grouped into larger aggregate populations based on their proximity) in order to analyze the individuals and populations at various levels of genetic structure. Only those samples accompanied by GPS location coordinates were used in this analysis (this excluding samples from Waiaka'ilio, Pu'u Koa'e, Kāohikaipu, Mōkapu, Mānā and Nihoa). In order to test for a significant pattern of isolation by distance, the multi-locus kinship coefficient for each pair

of individuals was plotted against the matrix of log-transformed Euclidean distance separating them using a Mantel test with 10,000 permutations. Average kinship coefficients were calculated for 18 distance classes as in a spatial autocorrelation analysis. For each comparison, short intervals (5–25 m) were used for the first distance classes to obtain a detailed picture at a small spatial scale, and then wider intervals (100–10,000 m) were used at larger spatial scales because kinship is expected to vary less. Null hypothesis of no spatial genetic structure was tested using a one-sided Mantel test.

After a severe reduction in effective population size ( $N_E$ ), there should be a transient excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (Piry et al., 1999). Bottlenecks generate transient heterozygosity excess because rare alleles are generally lost faster than heterozygosity during a bottleneck (Luikart and Cornuet, 1998). Wilcoxon sign-rank tests of heterozygosity excess (10,000 iterations) were implemented in BOTTLENECK v. 1.2.02 (Luikart and Cornuet, 1998; Piry et al., 1999). This program used allele frequency data to detect recent reductions in effective population size (i.e., within the past  $0.2N_E$ – $4N_E$  generations) under a 100% stepwise mutation model (SMM), an infinite alleles model (IAM) and a two-phase mutation model (TPM with 70% SMM, 30% IAM). A second approach (also implemented in BOTTLENECK) tested a mode shift away from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium, whereby alleles at low frequency become less abundant than alleles at intermediate frequency (Luikart et al., 1998). FREENA produced alternate allele frequency datasets for each population corrected for the presence of null alleles (using the Expectation Maximization algorithm of Dempster et al., 1977) that were subsequently run in BOTTLENECK for an alternative analysis. A third approach, utilized by the program AGAR<sub>ST</sub> v. 3.3 (Harley, 2003), measured the mean ratio ( $M$ -ratio) of number of alleles in a population ( $k$ ) divided by the range in allele size ( $r$ ) according to the method described by Garza and Williamson (2001). This ratio was calculated as  $M = k/r + 1$  to avoid dividing by zero in monomorphic populations (Excoffier et al., 2005). During a population decline, the number of alleles decreases more rapidly than does the range in allele size, leading to a decrease of  $M$ . Since the recovery time of  $M$  is longer than that of the measures tested in BOTTLENECK (not all mutations will increase  $M$ ), this method tests for population reductions over a longer period of time. A comparison of a population's  $M$ -ratio with its allelic diversity will also distinguish between populations recently reduced from populations that have been small for a long time ( $M$

will recover after a population decline without the maintenance of rare alleles, allelic diversity will not; Garza and Williamson, 2001).

Coalescent models link demographic history with population genealogy and provide a measure of how much the data supports one scenario over other possible scenarios that might have produced that data. The program 2<sub>MOD</sub> (Ciofi et al., 1999) was used to compare the relative likelihoods of two coalescent models: gene flow (equilibrium between gene flow and drift) vs. genetic drift (ancestral population fragmented into isolated sub-populations that then diverge purely by drift) in populations of *Sesbania tomentosa* across the Ka‘ū district of Hawai‘i Island. The Markov Chain Monte Carlo (MCMC) simulation employed by 2<sub>MOD</sub> ran 3 times with 100,000 iterations each. Results across runs were combined, and the probability of each model calculated.

## Results

### Microsatellite allele frequencies

There was an average of 13.8 alleles per locus at the nine microsatellite loci examined, ranging from 9 to 21, for a total of 124 alleles among the 539 samples of *Sesbania tomentosa*. Each locus had only three to four alleles with a frequency greater than 0.1, and these most common alleles had average frequencies per locus that ranged from 0.17–0.28 (with a maximum across loci of 0.46). None of the 36 tests for multiple comparisons between loci (genotypic disequilibrium) in GENEPOP were significant at the 5% nominal level after Bonferroni corrections when averaged over all populations. Thus, the different microsatellite loci can be considered to provide independent information on population structure. Significant genotypic disequilibrium was detected for 27 out of 36 pairs of loci when each population was analyzed separately. This was most predominately found in the populations at ‘Āpua point (12 pairs of loci) and Mahana bay (8 pairs), and to a lesser extent in populations at Ka Lae (3 pairs), Pu‘u Koa‘e (2 pairs) and Ka‘ena point (2 pairs; data not shown).

MICROCHECKER indicated that there was a general excess of homozygotes evenly distributed across allele size classes in 280 out of 342 (38 × 9) population-locus combinations, an indication of possible null alleles or false homozygotes in the data set (data not shown).



Estimated frequencies of null alleles per locus per population (using the ENA method implemented in FREENA) ranged from 0.00 to 0.42 (the exception being the Ka‘ena point populations that ranged from 0.97 to 1.00 at locus A122). When averaged over loci, the frequency of null alleles in the 38 populations varied from 0.0006 to 0.2950. The mean null allele frequency over all populations and loci was 0.12.

#### Non-random mating and genetic diversity within populations

After Bonferroni corrections, all nine loci had significant heterozygote deficiencies at the 5% nominal level as compared to Hardy-Weinberg equilibrium (HWE) within 9 to 25 out of 38 populations. In total, there were 39 instances where a locus showed significant departure from HWE within a population and 103 instances where a locus, variable in other populations, became fixed for an allele (data not shown). When averaged over all nine loci, 22 out of 38 populations had significant heterozygote deficiencies at the Bonferroni corrected nominal level ( $\alpha_{0.05} = 0.00015$ ; Table 3.5). Inbreeding coefficients averaged over nine loci ranged from a relatively low level ( $f = 0.188$ ) in the large population on Nihoa (estimated 3,000–5,000 individuals; USFWS, 2010), to extremely high rates of inbreeding ( $f = 0.791$ – $0.943$ ) in the small remnant sub-populations (9–29 individuals extant in each at the time of sampling) scattered along the southern coast of Hawai‘i Island from Kamilo point to Ka Lae (Table 3.5). Another population that exhibited high inbreeding was that at ‘Āpua point ( $f = 0.7$ ), a much larger population along the southern coast of Hawai‘i Island (58 individuals sampled out of a total of 125 extant plants).

Expected/observed heterozygosities ranged from 0.148/0.000 (Nākālele point, Maui) to 0.778/0.583 (Makakupa‘ia, Moloka‘i). Mean number of alleles per locus/mean allelic richness (averaged over loci) ranged from 1.1/1.2 (Ka‘ena point NAR, O‘ahu) to 7.56/2.8 (Kawela, Moloka‘i); Table 3.6). These four populations of *Sesbania tomentosa* are therefore at either extremes of the range of genetic diversity observed. The 21 populations exhibiting the lowest levels of diversity ( $H_E \leq 0.2$ ) harbored 79 out of 89 of the monomorphic loci observed in this study (Table 3.5). On the other end of the spectrum, private alleles occurred in 10 out of 38 populations, most notably in the Ka Lae, Kawela and Kamiloloa populations (Table 3.6).

Table 3.5. Heterozygote deficiency and inbreeding statistics of *Sesbania tomentosa* populations.  $n/N$ , sample size.  $f$ , Weir and Cockerham's (1984) inbreeding coefficient. Significant  $P$ -values for a test of the hypothesis of heterozygote deficiency in GENEPOP combined across loci are indicated in bold using Bonferroni corrected  $P$ -values ( $\alpha_{0.05} = 0.00015$ ). Number of loci significant in GENEPOP test at  $\alpha_{0.05}$ . ML, monomorphic loci; loci harboring only one allele in a given population, and is out of a total of nine loci.

Population	Island	$n/N$	$f$	$P$ -value (GENEPOP)	# of loci significant	ML
Kīpuka Nēnē makai	Hawai'i	12	-1.000	1.0000		5
Kīpuka Nēnē mauka	Hawai'i	6	0.074	0.1705		1
Hilina pali cluster 1	Hawai'i	8	0.509	<b>0.0000</b>	4	1
Hilina pali cluster 2	Hawai'i	6	0.634	<b>0.0000</b>	3	2
Hilina pali fuel break rd.	Hawai'i	3	0.286	0.3351		5
Pepeiau	Hawai'i	10	0.297	<b>0.0000</b>	2	1
Kukalau'ula pali	Hawai'i	9	0.430	<b>0.0000</b>	6	
Kamo'oali'i	Hawai'i	13	0.524	<b>0.0000</b>	7	
Kū'ē'ē	Hawai'i	5	0.500	<b>0.0000</b>	4	
Āpua point	Hawai'i	58	0.700	<b>0.0000</b>	7	1
Kamilo point	Hawai'i	9	0.847	<b>0.0000</b>	1	1
Mahana bay	Hawai'i	29	0.922	<b>0.0000</b>	9	
Kīpuka Hanalua	Hawai'i	12	0.943	<b>0.0000</b>	9	
Ka Lae	Hawai'i	29	0.791	<b>0.0000</b>	9	
Waiaka'ilio	Hawai'i	8	0.153	0.0929		
Waiaka'ilio seedbank	Hawai'i	10	0.605	<b>0.0000</b>	2	4
Pu'u Koa'e	Kaho'olawe	25	0.467	<b>0.0000</b>	8	
Pu'u Pīmoe	Maui	9	0.306	0.0004	2	
Papanalaoa	Maui	37	0.258	<b>0.0000</b>	4	1
Mōkōlea point	Maui	5	0.091	0.2062		6
Nākālele point	Maui	2	1.000	0.1116		7
Kawela	Moloka'i	17	0.387	<b>0.0000</b>	7	
Kamiloloa	Moloka'i	14	0.517	<b>0.0000</b>	8	
Makakupa'ia	Moloka'i	4	0.280	0.0011	1	
Moloka'i ranch rd.	Moloka'i	14	0.666	<b>0.0000</b>	5	
Nature Conservancy preserve	Moloka'i	3	0.507	0.0032		
Mo'omomi pavillion	Moloka'i	9	0.479	0.0002	1	5
Mo'omomi herbarium	Moloka'i	10	0.326	<b>0.0000</b>	4	
Kāohikaipu	O'ahu	2	-0.500	1.0000		7
Mōkapu	O'ahu	4	0.468	0.0037		3
Ka'ena point State Park	O'ahu	15	0.599	<b>0.0000</b>	3	4
Ka'ena point outplantings	O'ahu	32	0.415	<b>0.0000</b>	3	5
Ka'ena point NAR	O'ahu	18	-0.299	1.0000		8
Polihale State Park (2006)	Kaua'i	16	0.331	0.0168		6
Polihale State Park (2009)	Kaua'i	11	0.698	<b>0.0000</b>	6	3
Polihale State Park (2010)	Kaua'i	12	0.734	<b>0.0000</b>	4	5

Table 3.5. (Continued) Heterozygote deficiency and inbreeding statistics of *Sesbania tomentosa* populations.

<b>Population</b>	<b>Island</b>	<b><i>n/N</i></b>	<b><i>f</i></b>	<b><i>P</i>-value (GENEPOP)</b>	<b># of loci significant</b>	<b>ML</b>
Mānā	Kauaʻi	4	0.600	0.1244		7
Nihoa	Nihoa	49	0.188	0.0005	3	1

Table 3.6. Genetic diversity statistics of *Sesbania tomentosa* populations.  $n$ , sample size;  $A$  and  $A_R$ , mean number of alleles per locus and mean allelic richness (averaged over loci) respectively;  $H_E$  and  $H_O$ , expected and observed heterozygosity respectively.

Population	Island	$n$	$A$	$A_R$	Private alleles	$H_O$	$H_E$
Kīpuka Nēnē makai	Hawai‘i	12	1.44	1.36		0.444	0.232
Kīpuka Nēnē mauka	Hawai‘i	6	2.22	1.77		0.370	0.397
Hilina pali cluster 1	Hawai‘i	8	2.89	2.11		0.278	0.546
Hilina pali cluster 2	Hawai‘i	6	2.67	1.91		0.185	0.476
Hilina pali fuel break rd.	Hawai‘i	3	1.44	1.38		0.185	0.244
Pepeiau	Hawai‘i	10	3.44	2.01		0.356	0.498
Kukalau‘ula pali	Hawai‘i	9	5.00	2.48		0.395	0.675
Kamo‘oali‘i	Hawai‘i	13	5.11	2.26	1	0.291	0.598
Kū‘ē‘ē	Hawai‘i	5	3.33	2.29	1	0.333	0.630
‘Āpua point	Hawai‘i	58	2.56	1.70		0.117	0.387
Kamilo point	Hawai‘i	9	2.00	1.39		0.037	0.230
Mahana bay	Hawai‘i	29	2.67	1.65		0.031	0.388
Kīpuka Hanalua	Hawai‘i	12	3.11	1.88		0.028	0.463
Ka Lae	Hawai‘i	29	4.33	1.98	4	0.103	0.488
Waiaka‘īlio	Hawai‘i	8	2.78	1.55	1	0.276	0.322
Waiaka‘īlio seedbank	Hawai‘i	10	1.89	1.21		0.060	0.145
Pu‘u Koa‘e	Kaho‘olawe	25	3.78	1.95	1	0.271	0.504
Pu‘u Pīmoe	Maui	9	3.78	2.21	1	0.420	0.594
Papanalahoa	Maui	37	2.56	1.70		0.294	0.395
Mōkōlea point	Maui	5	1.44	1.25		0.111	0.121
Nākālele point	Maui	2	1.22	1.22		0.000	0.148
Kawela	Moloka‘i	17	7.56	2.80	7	0.480	0.773
Kamiloloa	Moloka‘i	14	6.56	2.74	5	0.360	0.732
Makakupa‘ia	Moloka‘i	4	4.11	2.73		0.583	0.778
Moloka‘i ranch rd.	Moloka‘i	14	2.56	1.74		0.143	0.417
Nature Conservancy preserve	Moloka‘i	3	2.44	2.16		0.333	0.607
Mo‘omomi pavillion	Moloka‘i	9	1.89	1.43	1	0.123	0.230
Mo‘omomi herbarium	Moloka‘i	10	4.56	2.55		0.485	0.705
Kāohikaipu	O‘ahu	2	1.22	1.22		0.167	0.130
Mōkapu	O‘ahu	4	2.11	1.66		0.194	0.341
Ka‘ena point State Park	O‘ahu	15	1.67	1.42		0.089	0.217
Ka‘ena point outplantings	O‘ahu	32	1.56	1.32		0.097	0.166
Ka‘ena point NAR	O‘ahu	18	1.11	1.12		0.076	0.059
Polihale State Park (2006)	Kaua‘i	16	1.33	1.22		0.083	0.123
Polihale State Park (2009)	Kaua‘i	11	2.33	1.55		0.092	0.294
Polihale State Park (2010)	Kaua‘i	12	1.67	1.41		0.065	0.236
Mānā	Kaua‘i	4	1.22	1.17		0.056	0.127
Nihoa	Nihoa	49	4.00	1.82	1	0.320	0.393

## Genetic structure of populations

Global  $F_{ST}(\theta)$  over all populations and loci was 0.509 ( $P \leq 0.0001$ ); correction for null alleles reduced this value slightly to 0.488 (Table 3.7). This analysis indicates that of the total genetic variation found across the range of the species, roughly half is ascribable to genetic difference (differences in allele frequencies) among populations, and the other half is found within any given population.

Using the program STRUCTURE and following the method of Evanno et al. (2005), two distinct genetic clusters were found among *Sesbania tomentosa* individuals sampled across all islands (Figures 3.2 and 3.3). The largest increase in the posterior probability occurred at  $K = 2$ , suggesting that this was the best model for the data. One genetic cluster corresponded to populations from Hawai‘i Island, Kaho‘olawe, Maui (excepting populations at Papanalaoa and Mōkōlea) and Moloka‘i (red cluster) and the other comprised individuals sampled from the Islands of O‘ahu, Kaua‘i and Nihoa, plus the populations at Papanalaoa and Mōkōlea, Maui (orange cluster; Figure 3.4). Most of the geographic populations sampled showed a high proportion of individuals assigned to a given cluster, generally from 95% to 100%. Populations sampled from Maui Nui (referring to the prehistorically contiguous island composed of Kaho‘olawe, Maui, Moloka‘i, and Lāna‘i; Price and Elliott-Fisk, 2004) assigned to the red cluster had proportions much lower (0.89 for Pu‘u Pīmoe; 0.86 for Kamiloloa; 0.84 for Makakupa‘ia; 0.60 for Mo‘omomi herbarium samples). These are levels of admixture higher than the 5% threshold that may be attributed to stochastic noise. In addition, cluster membership coefficients of Maui Nui individuals assigned to the red cluster also averaged low (0.83 for Pu‘u Pīmoe; 0.70 for Nākālele point; 0.83 for Kawela; 0.87 for Kamiloloa; 0.84 for Makakupa‘ia; 0.78 for Mo‘omomi herbarium samples). As a point of reference, 100% of Hawai‘i Island individuals were assigned to the red cluster with an average cluster membership coefficient of 0.97. When considering the populations comprising the orange cluster from O‘ahu, Kaua‘i and Nihoa, 100% of these individuals were assigned to the orange cluster with an average cluster membership coefficient of 0.97. The Maui populations assigned to the orange cluster were comprised of individuals whose average cluster membership coefficient was 0.86, and this coefficient was 0.90 when considering the individuals comprising the Nihoa population, so indications of admixture are also to be found in the orange cluster (Figure 3.4).

Table 3.7. Global  $F_{ST}(\theta)$  and  $F_{ST(ENA)}$  over all populations and loci.

<b>Locus</b>	<b>C5</b>	<b>A105</b>	<b>A123</b>	<b>C3</b>	<b>A122</b>	<b>A119</b>	<b>A128</b>	<b>C103</b>	<b>C106</b>	<b>Global</b>
$F_{ST}(\theta)$	0.516	0.521	0.472	0.588	0.321	0.451	0.509	0.613	0.572	0.509
$F_{ST(ENA)}$	0.521	0.497	0.452	0.575	0.314	0.413	0.457	0.59	0.541	0.488
$P$ -value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

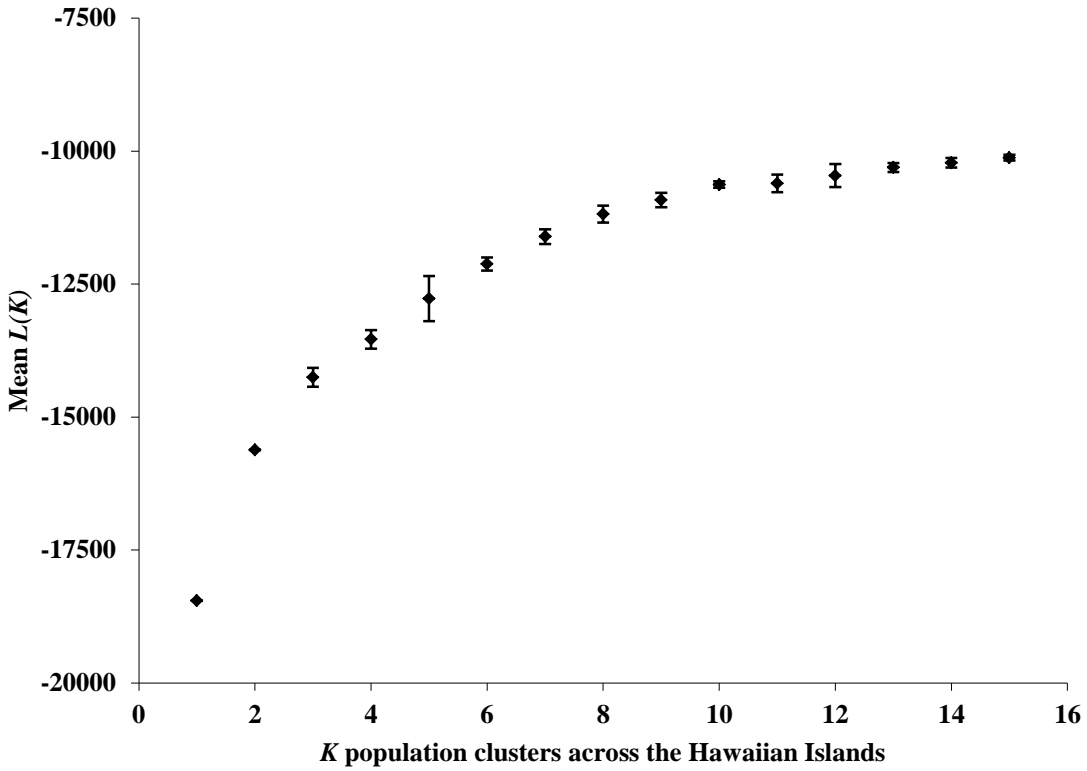


Figure 3.2. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

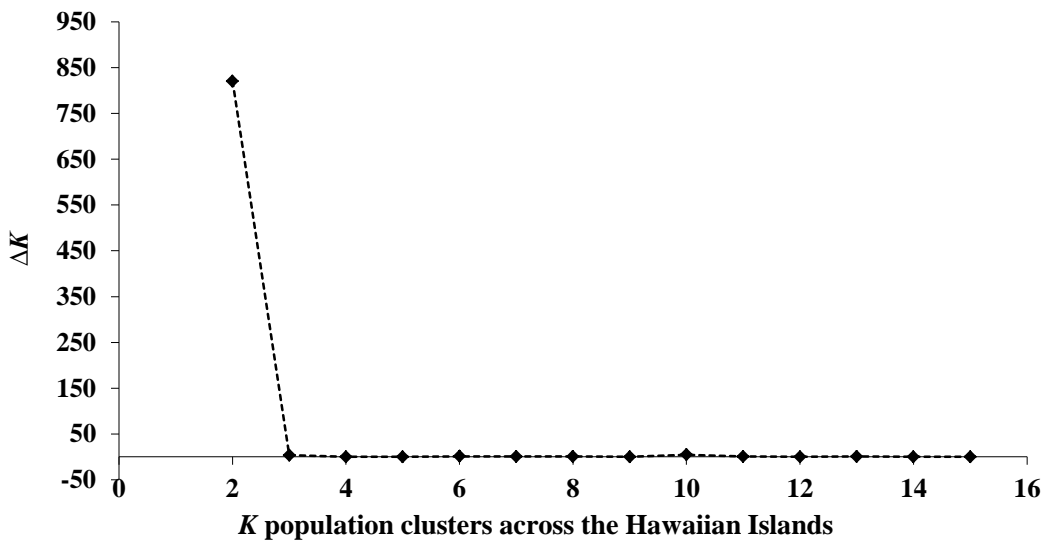


Figure 3.3. Magnitude of  $\Delta K$  (second-order rate of change of STRUcTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

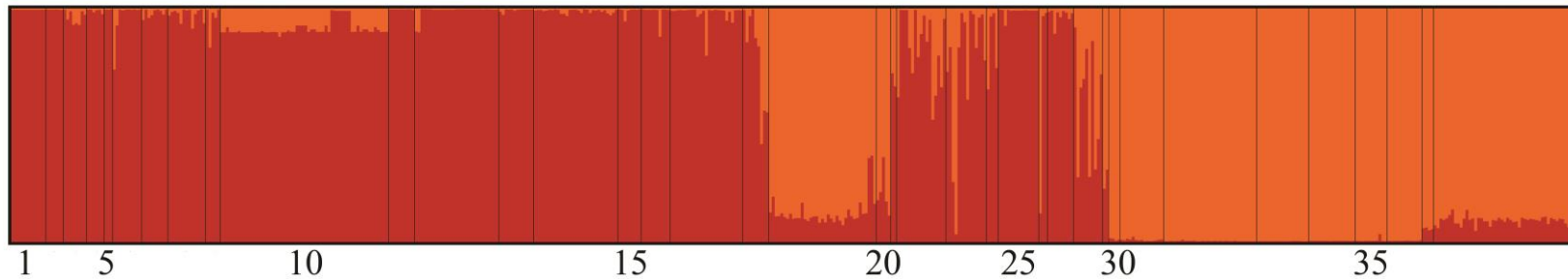


Figure 3.4. STRUCTURE graph for the most likely numbers of clusters of Hawaiian *Sesbania* according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic clusters (red and orange). Thin black lines distinguish the 38 sub-populations and populations: 1. Kīpuka Nēnē makai, 2. Kīpuka Nēnē mauka, 3. Hilina pali cluster 1, 4. Hilina pali cluster 2, 5. Hilina pali fuel break rd., 6. Pepeiau, 7. Kukalau‘ula pali, 8. Kamo‘oali‘i, 9. Kū‘ē‘ē, 10. ‘Āpua point, 11. Kamilo point, 12. Mahana bay, 13. Kīpuka Hanalua, 14. Ka Lae, 15. Waiaka‘īlio, 16. Waiaka‘īlio seedbank, 17. Pu‘u Koa‘e, 18. Pu‘u Pīmoe, 19. Papanalahoa, 20. Mōkōlea point, 21. Nākālele point, 22. Kawela, 23. Kamiloloa, 24. Makakupa‘ia, 25. Moloka‘i ranch rd., 26. Nature Conservancy preserve, 27. Mo‘omomi pavillion, 28. Mo‘omomi herbarium, 29. Kāohikaipu, 30. Mōkapu, 31. Ka‘ena point State Park, 32. Ka‘ena point NAR outplantings, 33. Ka‘ena point NAR, 34. Polihale State Park (2006), 35. Polihale State Park (2009), 36. Polihale State Park (2010), 37. Mānā, 38. Nihoa.



Further analysis of the two genetic clusters described above found additional levels of structure. Within the red cluster (of Figure 3.4), the largest increase in posterior probability occurred at  $K = 3$  (Figures 3.5 and 3.6) while the largest increase in the orange cluster (of Figure 3.4) occurred at  $K = 2$  (Figures 3.7 and 3.8). Within the red cluster, the first sub-cluster comprised populations from Hawai‘i Volcanoes National Park (excepting the population at Pepeiau), and hereafter referred to as the Hawai‘i Volcanoes sub-cluster. The second sub-cluster comprised populations on Hawai‘i Island in the South point Region (Kamilo point to Ka Lae) plus Pepeiau, hereafter the South point sub-cluster. The third sub-cluster comprised the small remnant North Kohala population on Hawai‘i Island (Waiaka‘īlio) plus the populations from Kaho‘olawe, Maui (excepting Papanalaho and Mōkōlea) and Moloka‘i, hereafter the Maui Nui sub-cluster (Figure 3.9).

Levels of admixture were relatively high in the populations at Pepeiau (proportion of individuals assigned to South point sub-cluster was 0.70) and Kukalau‘ula pali (proportion of individuals assigned to Hawai‘i Volcanoes sub-cluster was 0.78) with average individual cluster membership coefficients of 0.76 and 0.84, respectively. Indications of admixture were also high in the Kamo‘oali‘i and Kū‘ē‘ē populations (proportion of individuals assigned to Hawai‘i Volcanoes sub-cluster were 0.85 and 0.80 with average individual cluster membership coefficients of 0.81 and 0.64, respectively). At Mahana bay, 96% of individuals were assigned to the South point subcluster, although the average individual cluster membership coefficient was only 0.77 (Figure 3.9). Indications of admixture were also apparent in populations on Moloka‘i (average individual cluster membership coefficients for the Kawela, Makakupa‘ia, Moloka‘i ranch road and Mo‘omomi Nature Conservancy preserve populations in the Maui Nui sub-cluster were 0.89, 0.78, 0.75 and 0.90, respectively). At Moloka‘i Ranch Rd., the proportion of individuals assigned to Maui Nui sub-cluster was 0.78, plus two individuals failed to be assigned to any cluster at the 0.5 cut-off. When considering the ten historically collected samples from Mo‘omomi individual cluster assignments varied widely (indicating admixture). Taken as a whole, these ten samples were not definitively assigned to any one particular genetic sub-cluster (again, two individuals failed to be assigned to any sub-cluster at the 0.5 cut-off; Figure 3.9).

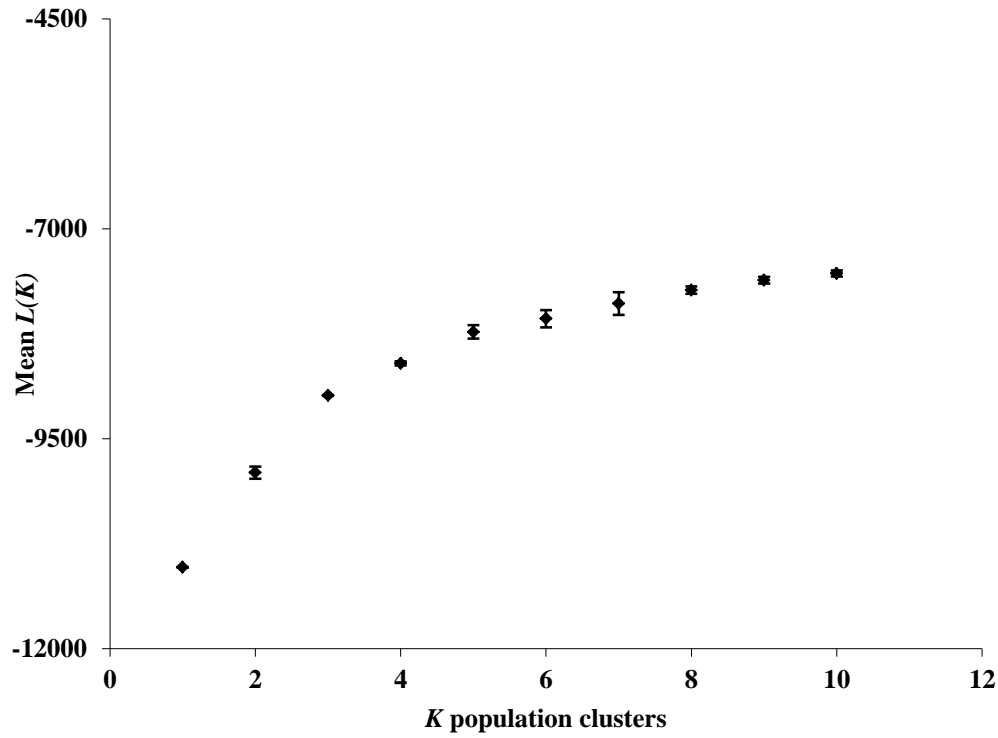


Figure 3.5. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

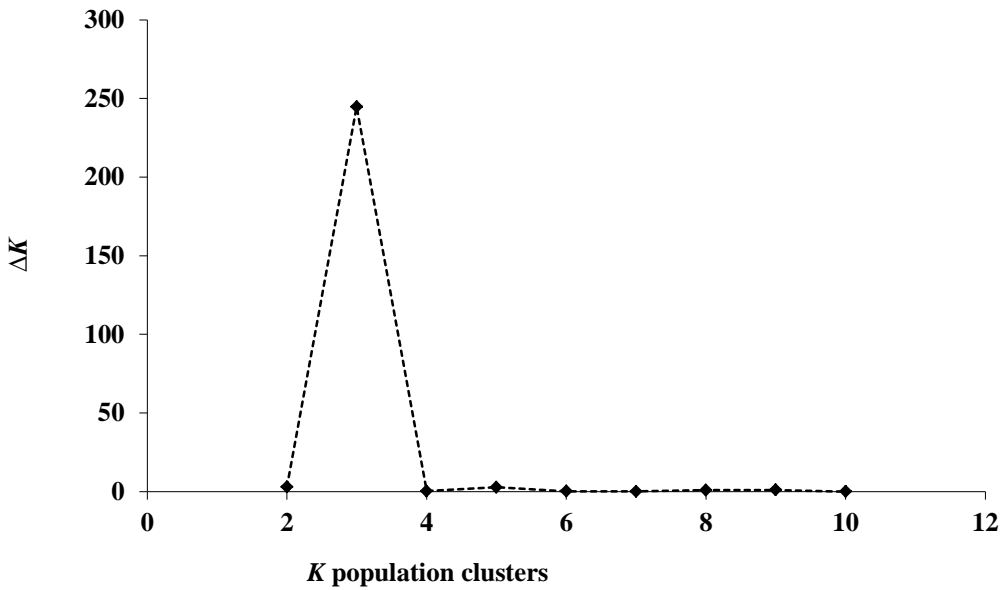


Figure 3.6. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

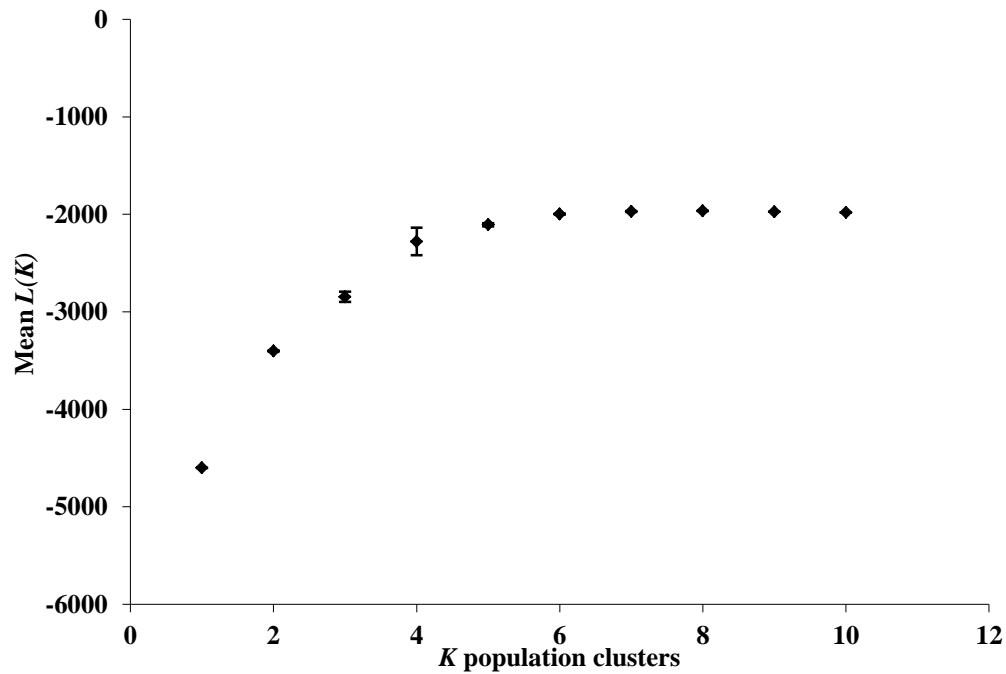


Figure 3.7. Log probability of data  $L(K)$  as a function of  $K$  averaged over the 10 replicates at each  $K$ . Bars indicate standard deviation around mean  $L(K)$ .

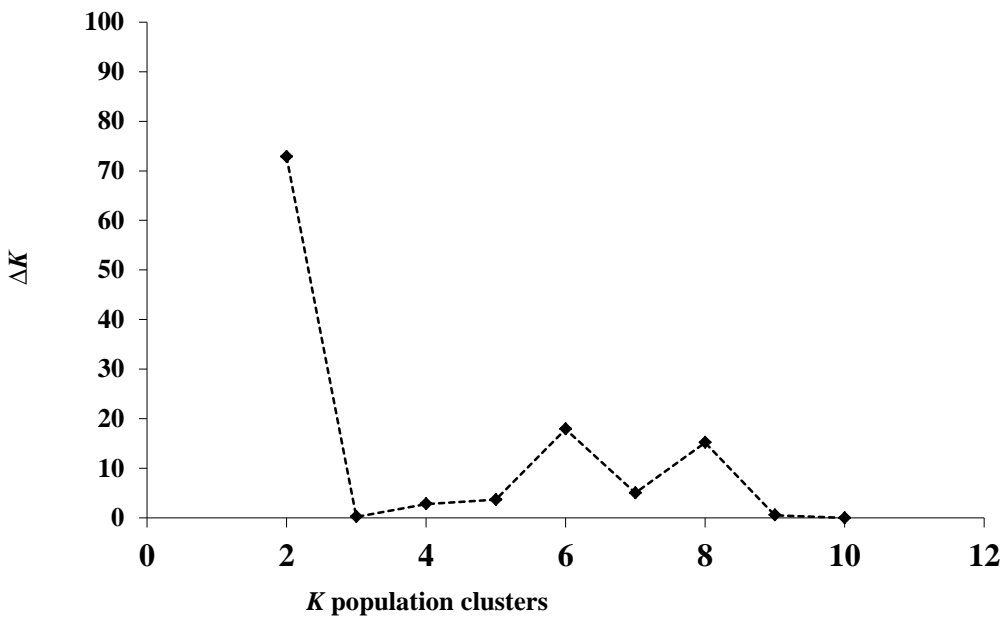


Figure 3.8. Magnitude of  $\Delta K$  (second-order rate of change of STRUCTURE likelihood values) as a function of  $K$ , following the method of Evanno et al. (2005).

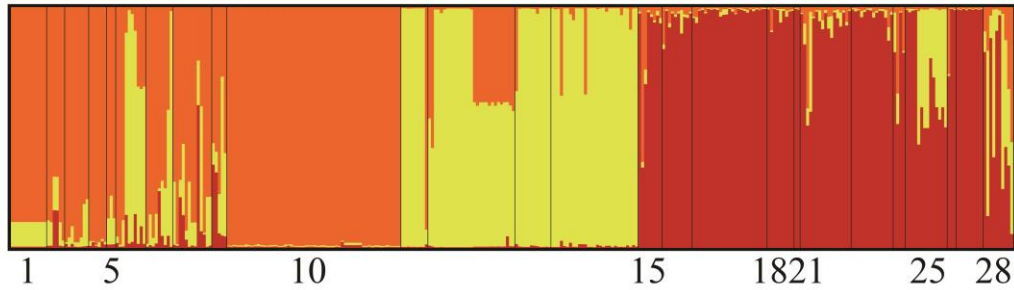


Figure 3.9. STRUCTURE graph for the most likely number of sub-clusters in the red cluster of Figure 3.4 according to the  $\Delta K$  method ( $K = 3$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 3 genetic sub-clusters. Thin black lines distinguish the 26 sub-populations and populations: 1. Kīpuka Nēnē makai, 2. Kīpuka Nēnē mauka, 3. Hilina pali cluster 1, 4. Hilina pali cluster 2, 5. Hilina pali fuel break rd., 6. Pepeiau, 7. Kukalau‘ula pali, 8. Kamo‘oali‘i, 9. Kū‘ē‘ē, 10. ‘Āpua point, 11. Kamilo point, 12. Mahana bay, 13. Kīpuka Hanalua, 14. Ka Lae, 15. Waiaka‘īlio, 16. Waiaka‘īlio seedbank, 17. Pu‘u Koa‘e, 18. Pu‘u Pīmoe, 21. Nākālele point, 22. Kawela, 23. Kamiloloa, 24. Makakupa‘ia, 25. Moloka‘i ranch rd., 26. Nature Conservancy preserve, 27. Mo‘omomi pavillion, 28. Mo‘omomi herbarium.

It is important to note that the height of the modal value  $\Delta K$  in Figure 3.8 ( $\Delta K = 72.8$  at  $K = 2$ ) is an indicator of the strength of the signal detected by STRUCTURE (Evanno et al., 2005), in this case significantly weaker than the previous two analyses ( $\Delta K = 244.7$  at  $K = 3$  in Figure 3.6 and  $820.4$  at  $K = 2$  in Figure 3.3). Two relatively distinct groups characterize the STRUCTURE plot: the O‘ahu populations cluster with the Polihale (Kaua‘i) population, and the NW Maui populations (Papanalaoa and Mōkōlea) cluster with the Mānā (Kaua‘i) and Nihoa population (Figure 3.10).

#### Isolation by distance between and within populations

There was a significant correlation between genetic and geographic distances ( $r^2 = 0.363$ ,  $P < 0.0001$ ), indicating a pattern of isolation by distance (IBD) among populations of *Sesbania tomentosa* across the Hawaiian Islands (Figure 3.11). Using spatial analysis of kinship coefficients between individuals, there was agreement with the model of isolation by distance in that a significant linear decrease of estimated pairwise kinship coefficients with the logarithm of increasing geographical distance was detected in all nine aggregate (combined) populations tested ( $P < 0.01$ ; Table 3.8). When looking at the individual populations on a smaller scale (i.e., within individual population clusters separated by  $> 2$  km), 10 of the 27 populations tested significantly for the relationship at the 0.01 level, and an additional four were significant at the 0.05 level. With the exception of lower (and in a few cases, higher) average kinship coefficients between adjacent individuals, none of these test results differed when duplicate individuals were omitted from the analysis (data not shown). The 13 remaining (non-significant) populations had low census sizes ( $\leq 18$  individuals were compared in each), which are expected to have substantially biased the estimator (Ritland, 1996).

#### Indirect estimates of genetic bottlenecks

The Wilcoxon tests carried out in BOTTLENECK revealed evidence for a rapid loss of genetic diversity in four populations (Kīpuka Nēnē makai, Hilina pali cluster 1, Hilina pali fuel break road and Polihale (2010) populations based on the three mutation models examined). These same populations revealed a mode shift away from an L-shaped distribution of alleles, a

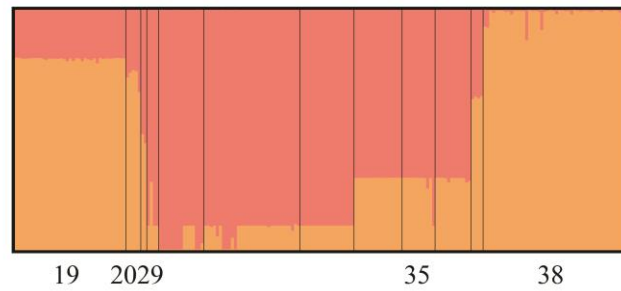


Figure 3.10. STRUCTURE graph for the most likely number-of sub-clusters in the orange cluster of Figure 3.4 according to the  $\Delta K$  method ( $K = 2$ ). Individuals are presented as thin vertical lines, and colors indicate the degree of membership of each individual in each of 2 genetic sub-clusters. Thin black lines distinguish the 12 sub-populations/populations: 19. Papanalahoa, 20. Mōkōlea point, 29. Kāohikaipu, 30. Mōkapu, 31. Ka'ena point State Park, 32. Ka'ena point NAR outplantings, 33. Ka'ena point NAR, 34. Polihale State Park (2006), 35. Polihale State Park (2009), 36. Polihale State Park (2010), 37. Mānā, 38. Nihoa.

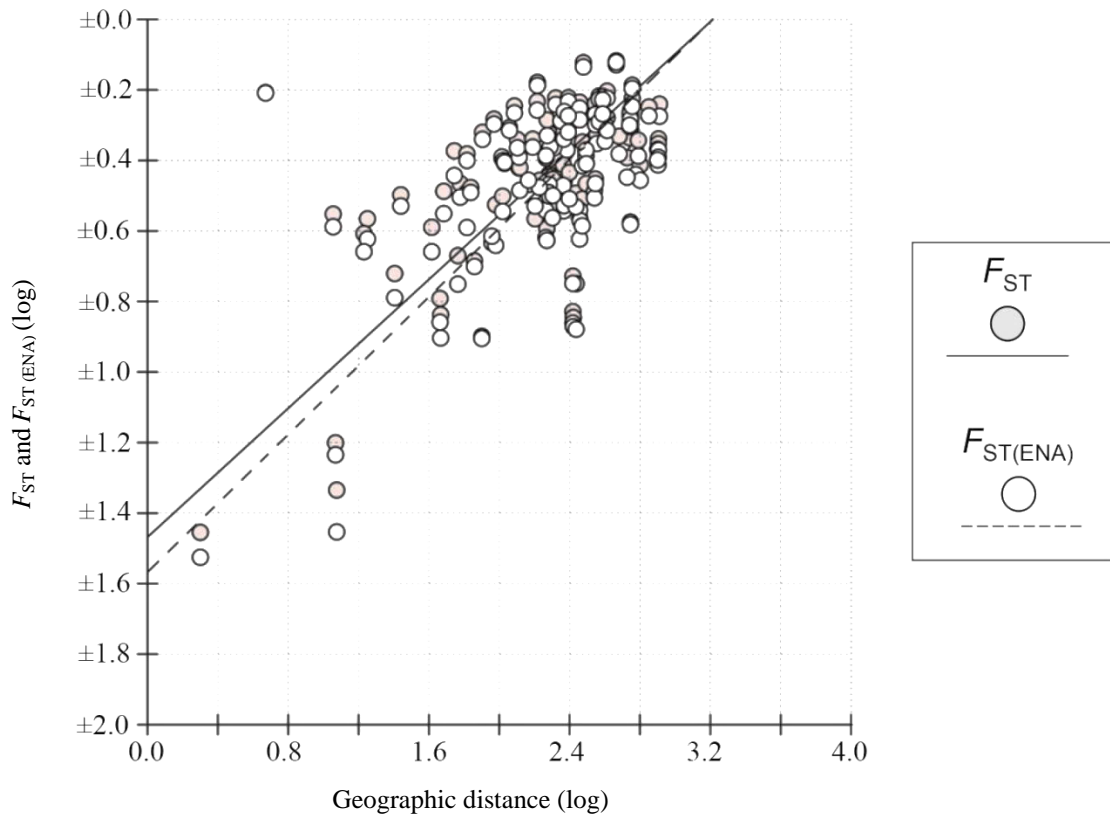


Figure 3.11. Significant correlation of log-transformed  $F_{ST}$  (Weir and Cockerham, 1984) and  $F_{ST(ENA)}$  (Chapuis and Estoup, 2007) over all loci with log-transformed geographic distance (km). Mantel test,  $r^2 = 0.363$ ,  $P < 0.0001$  (both analyses).

Table 3.8. Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.  $F_1$ , average kinship coefficient between adjacent individuals (i.e. first distance interval);  $b_{r0}$ , slope of the regression of pairwise kinship coefficients on the logarithm of geographical distance;  $P$ -value of the one-sided Mantel test with  $H_0$ : observed  $b_{r0} = 0$ , significant values (at 0.01 level) listed in bold. NA indicates analysis not applicable due to uniform genotypes across a given population.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{r0}$	$P$ -value
Hawai‘i	Kīpuka Nēnē–Hilina pali		595	0–5	0.3252	-0.0912	<b>0.0001</b>
		Kīpuka Nēnē makai	66	0–5	1.0000	NA	NA
		Kīpuka Nēnē mauka	15	20–25	0.3748	-0.3523	0.0562
		Hilina pali cluster 1	28	15–20	0.4090	-0.2907	<b>0.001</b>
		Hilina pali cluster 2	15	20–25	-0.2094	0.0533	0.6379
		Hilina pali fuel break rd.	3	5–10	0.3818	-0.4013	0.3336
	Pepeiau–Kukalau‘ula pali		171	10–15	0.1469	-0.0459	<b>0.0001</b>
		Pepeiau	45	20–25	0.1748	-0.0992	<b>0.001</b>
		Kukalau‘ula pali	36	200–500	0.1088	-0.0528	<b>0.01</b>



Table 3.8. (Continued) Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{ro}$	$P$ -value
Hawai‘i	Kamo‘oali‘i–Kū‘ē‘ē		153	50–75	0.3707	-0.0300	<b>0.007</b>
		Kamo‘oali‘i	78	100–200	0.0829	0.0118	0.66
		Kū‘ē‘ē	10	50–75	0.1111	-0.1386	0.02
		‘Āpua point	1653	0–5	0.7661	-0.2724	<b>0.0001</b>
	Hawai‘i Volcanoes National Park TOTAL		8385	0–5	0.6355	-0.0714	<b>0.0001</b>
		Kamilo point	36	0–5	0.2371	-0.1102	0.0395
	Mahana bay–Ka Lae		2415	0–5	0.5815	-0.0925	<b>0.0001</b>
Mahana bay Kīpuka Hanalua Ka Lae		406	0–5	0.9311	-0.3553	<b>0.0001</b>	
		66	0–5	0.1715	-0.0914	0.05	
		406	0–5	0.0061	-0.0240	0.024	
Maui		Pu‘u Pīmoē	36	0–5	-0.0135	-0.0106	0.3506
	Papanalaho–Nākālele point		903	0–5	0.3139	-0.0561	<b>0.0001</b>
		Papanalaho point	630	0–5	0.1762	-0.0711	<b>0.0001</b>
		Mōkōlea point	10	0–5	-0.0215	0.0094	0.6989
		Nākālele point	1	0–5	-0.6667	NA	NA

Table 3.8. (Continued) Spatial genetic structure in populations of *Sesbania tomentosa* at various scales of analysis.

Island	Population aggregate	Population	Pairs of individuals	Distance interval of $F_1$ (m)	$F_1$	$b_{ro}$	$P$ -value
Moloka'i	Kawela–Kamiloloa		595	0–5	0.0779	-0.0172	<b>0.0001</b>
		Kawela	136	0–5	-0.0874	-0.0138	<b>0.01</b>
		Kamiloloa	91	0–5	-0.2540	-0.0096	0.208
		Makakupaia	6	15–20	0.0614	-0.0786	0.1697
	Mo'omomi		231	0–5	0.8278	-0.1039	<b>0.0001</b>
		Moloka'i ranch rd.	91	0–5	0.7488	-0.3432	<b>0.0002</b>
		Nature Conservancy preserve	3	10–15	0.4256	-0.1910	0.3362
		Mo'omomi pavillion	10	15–20	0.6111	-0.0437	0.1583
O'ahu	Ka'ena point		2016	0–5	0.4470	-0.1166	<b>0.0001</b>
		Ka'ena point State Park	105	0–5	0.7154	-0.1717	<b>0.001</b>
		Ka'ena point NAR outplantings	465	0–5	0.1726	-0.0957	<b>0.0003</b>
		Ka'ena point NAR	153	0–5	-0.0022	-0.0048	0.3229
Kaua'i		Polihale State Park (2006)	120	0–5	0.1225	-0.0270	0.1813
		Polihale State Park (2010)	66	0–5	0.0370	-0.0425	0.076

trend observed in an additional 15 populations as well (Table 3.9). When the dataset was corrected for the presence of null alleles, none of the Wilcoxon tests was significant and only three populations remained divergent from the L-shaped distribution. On the other hand, 31 out of 38 populations had an  $M$ -ratio suggestive of a history of bottlenecks.  $M$ -ratios below 0.68 were found in every population where the number of sampled individuals was sufficiently large ( $M$ -ratios above 0.68 were only found in populations  $\leq 14$  individuals), with the exception of the Ka‘ena point NAR outplantings ( $n = 32$ ) and ‘Āpua point ( $n = 58$ ).

#### Modeling genetic drift in Ka‘ū

The largest natural landscape left in Hawai‘i where some degree of connectivity between populations of *Sesbania tomentosa* could potentially still occur is in the Ka‘ū district of Hawai‘i Island, including the populations within the boundaries of Hawai‘i Volcanoes National Park down into the South point region. Using the coalescent modeling program 2<sub>MOD</sub>, a genetic drift model for populations of *S. tomentosa* across the Ka‘ū district was seven times more likely than the gene flow model [ $P$  (genetic drift) =  $0.88 \pm 0.0004$ , Bayes factor = 6].

#### Direct observations of genetic drift at Mo‘omomi, Moloka‘i

Mean expected and observed heterozygosity in the modern collections of *Sesbania tomentosa* at the three Mo‘omomi populations ( $n = 26$ ) declined when referenced against the historically collected samples ( $n = 10$ ; Table 3.6). Mean number of alleles per locus and allelic richness also both declined. The historic samples revealed seven more alleles total (across the nine loci) than did the three contemporary population samples combined. In addition, there are 20 “ghost alleles” across the nine loci, alleles that occurred in the samples collected 60–100 years ago that were not present at Mo‘omomi during an entire census collection in 2006 (Figure 3.12).

Table 3.9. Three tests for genetic bottlenecks in *Sesbania tomentosa* populations. *M*-ratio (Garza and Williamson 2001) is the number of alleles divided by range in allele size, averaged over 9 loci. Mode shift indicates deviation from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium. Wilcoxon tests for heterozygote excess (Piry et al., 1999) under three mutation models (step-wise mutation, SMM; two phase model, TPM; infinite alleles model, IAM). The latter two tests were duplicated using alternate allele frequency datasets corrected for the presence of null alleles (using the Expectation Maximization algorithm of Dempster et al., 1977). Values highlighted in bold are those indicative of bottlenecks ( $P \leq 0.05$  for the Wilcoxon tests and an *M*-ratio  $< 0.68$ )

Island	Population	<i>M</i> -ratio	Mode shift	Wilcoxon tests:			CORRECTED FOR NULL ALLELES			
				SMM	TPM	IAM	Mode shift	SMM	TPM	IAM
Hawai'i Island	Kīpuka Nēnē makai	<b>0.683</b>	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	<b>shifted</b>	0.935	0.935	0.935
	Kīpuka Nēnē mauka	<b>0.695</b>	<b>shifted</b>	0.527	0.422	0.320	normal	0.613	0.511	0.432
	Hilina pali cluster 1	<b>0.559</b>	<b>shifted</b>	<b>0.021</b>	<b>0.011</b>	<b>0.006</b>	<b>shifted</b>	0.918	0.787	0.787
	Hilina pali cluster 2	<b>0.595</b>	normal	0.148	0.148	0.148	normal	0.986	0.981	0.936
	Hilina pali fuel break rd.	0.767	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	<b>shifted</b>	0.935	0.935	0.935
	Pepeiau	<b>0.649</b>	normal	0.809	0.473	0.229	normal	0.999	0.999	0.997
	Kukalau'ula pali	0.718	normal	0.918	0.545	0.149	normal	0.988	0.711	0.223
	Kamo'oali'i	<b>0.579</b>	normal	0.999	0.981	0.633	normal	1.000	1.000	0.999
	Kū'ē'ē	<b>0.602</b>	<b>shifted</b>	0.248	0.082	0.064	normal	0.353	0.211	0.167
	'Āpua point	0.716	normal	0.319	0.156	0.014	normal	0.589	0.410	0.101
	Kamilo point	<b>0.635</b>	<b>shifted</b>	1.000	0.996	0.994	normal	1.000	1.000	1.000
	Mahana bay	<b>0.656</b>	normal	0.500	0.213	0.082	normal	0.752	0.285	0.082
	Kīpuka Hanalua	<b>0.566</b>	normal	0.918	0.715	0.455	normal	0.986	0.898	0.715
	Ka Lae	<b>0.674</b>	normal	0.981	0.849	0.326	normal	0.990	0.918	0.455
	Waiaka'īlio	<b>0.485</b>	normal	0.998	0.997	0.981	normal	1.000	1.000	1.000
	Waiaka'īlio seedbank	<b>0.489</b>	normal	0.984	0.984	0.969	normal	0.999	0.999	0.999

Table 3.9. (Continued) Three tests for genetic bottlenecks in *Sesbania tomentosa* populations.

Island	Population	<i>M</i> -ratio	Mode shift	Wilcoxon tests:			CORRECTED FOR NULL ALLELES			
				SMM	TPM	IAM	Mode shift	SMM	TPM	IAM
Maui Nui	Pu‘u Koa‘e	<b>0.552</b>	normal	0.849	0.455	0.125	normal	0.918	0.545	0.367
	Pu‘u Pīmoē	<b>0.567</b>	<b>shifted</b>	0.918	0.411	<b>0.024</b>	normal	0.999	0.998	0.995
	Papanalahoa	<b>0.460</b>	normal	0.231	0.019	<b>0.006</b>	normal	0.935	0.715	0.326
	Mōkōlea point	<b>0.556</b>	<b>shifted</b>	0.937	0.937	0.813	normal	1.000	1.000	1.000
	Nākālele point	0.750	<b>shifted</b>	0.125	0.125	0.125	normal	0.912	0.912	0.912
	Kawela	<b>0.651</b>	normal	0.367	0.248	0.082	normal	0.997	0.976	0.684
	Kamiloloa	0.714	normal	0.545	0.326	0.179	normal	0.986	0.898	0.82
	Makakupa‘ia	<b>0.595</b>	<b>shifted</b>	0.367	0.326	0.213	normal	0.532	0.511	0.489
	Moloka‘i ranch rd.	<b>0.659</b>	<b>shifted</b>	0.411	0.179	<b>0.018</b>	normal	0.633	0.500	0.326
	Nature Conservancy preserve	<b>0.616</b>	<b>shifted</b>	0.064	0.064	0.064	normal	0.912	0.912	0.912
	Mo‘omomi pavillion	<b>0.429</b>	<b>shifted</b>	0.437	0.437	0.094	normal	0.999	0.999	0.997
	Mo‘omomi herbarium	<b>0.620</b>	<b>shifted</b>	0.082	0.064	0.064	normal	0.934	0.911	0.911
O‘ahu	Kāohikaipu	<b>0.533</b>	<b>shifted</b>	0.25	0.25	0.25	normal	0.900	0.900	0.900
	Mōkapu	<b>0.411</b>	<b>shifted</b>	0.578	0.578	0.578	normal	0.950	0.950	0.950
	Ka‘ena point State Park	<b>0.630</b>	<b>shifted</b>	0.313	0.109	0.109	normal	0.935	0.935	0.935
	Ka‘ena point outplantings	0.719	<b>shifted</b>	0.906	0.438	0.063	normal	1.000	0.612	0.450
	Ka‘ena point NAR	<b>0.510</b>	normal	1.0	0.25	0.25	normal	1.000	0.900	0.900
Kaua‘i	Polihale State Park (2006)	<b>0.611</b>	<b>shifted</b>	0.125	0.063	0.063	normal	0.999	0.998	0.997
	Polihale State Park (2009)	0.783	normal	0.781	0.656	0.344	normal	0.997	0.995	0.986
	Polihale State Park (2010)	<b>0.588</b>	<b>shifted</b>	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>	normal	0.936	0.936	0.936
	Mānā	<b>0.643</b>	<b>shifted</b>	0.125	0.125	0.125	normal	0.922	0.922	0.922
Nihoa	Nihoa	<b>0.695</b>	normal	0.991	0.578	0.371	normal	0.999	0.993	0.787

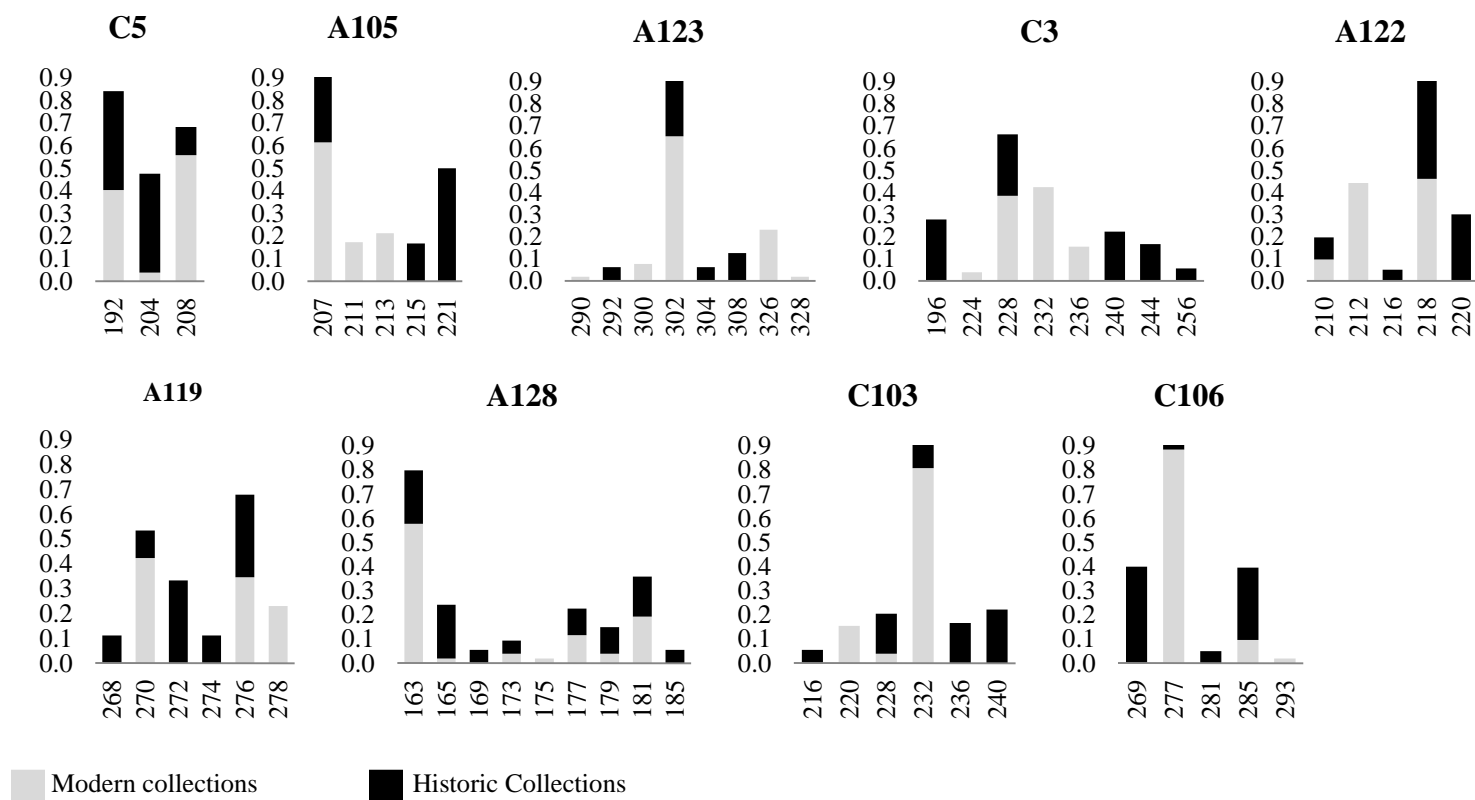


Figure 3.12. A comparison of allele frequencies for *Sesbania tomentosa* at nine microsatellite loci (C5, A105, A123, C3, A122, A119, A128, C103 and C106) sampled from 26 individuals at Mo'omomi Moloka'i (2006) vs. 10 historical samples collected 60–100 years prior. Frequencies listed on y-axes; alleles listed on x-axes.

## The dynamic nature of the gene pool at Polihale, Kauaʻi

Since all extant individuals of *Sesbania tomentosa* were sampled from the Polihale population on two out of three occasions (spanning 4 years), it is possible to observe changes in the genetic makeup of populations of this rapidly reproducing plant species over time. All measures of genetic diversity rose from levels seen in 2006 when sampling of the Polihale Kauaʻi population was repeated in 2009, yet then dropped again slightly in 2010 (Table 3.6). While the number of monomorphic loci (i.e., zero diversity at a locus) dropped from six in 2006 to three in 2009, this number rose again to five loci fixed for a single allele in 2010 (Table 3.5). More importantly, extant individuals sampled from 2009 contained nine additional alleles (at 6 loci) not found in individuals comprising the population in 2006 (Figure 3.13). By 2010, seven of these nine alleles were again lost, yet a completely new allele not seen in the previous two sampling years emerged to join the standing gene pool. All three mutation models employed in the BOTTLENECK program, plus an allele frequency mode shift and microsatellite repeat size range *M*-ratio were sensitive to and reflect this rapid real-time record of population decline at Polihale from 2009 to 2010 (Table 3.9).

## Discussion

### Maintenance of genetic diversity in spite of high levels of inbreeding

While private alleles occurred in 10 out of 38 populations, the three populations where the highest amount of exclusive genetic diversity was found exhibit interesting associations with accompanying levels of inbreeding. Limited sampling ( $n = 35$ ) of the demographically large Kawela and Kamiloloa populations [the combined sub-populations of SE Molokaʻi were believed to comprise 1,500–2,000 individuals in 2006 (USFWS, 2010)] exhibited the greatest number of private alleles, more than all the other populations combined (Table 3.6). Mean number of alleles per locus and allelic richness were also highest in these two population samples. High allelic diversity observed in the limited sampling of the SE Molokaʻi populations was accompanied by lower (yet still relatively high) rates of inbreeding and might be explained by a combination of two factors. The high population density of *Sesbania tomentosa* over a large

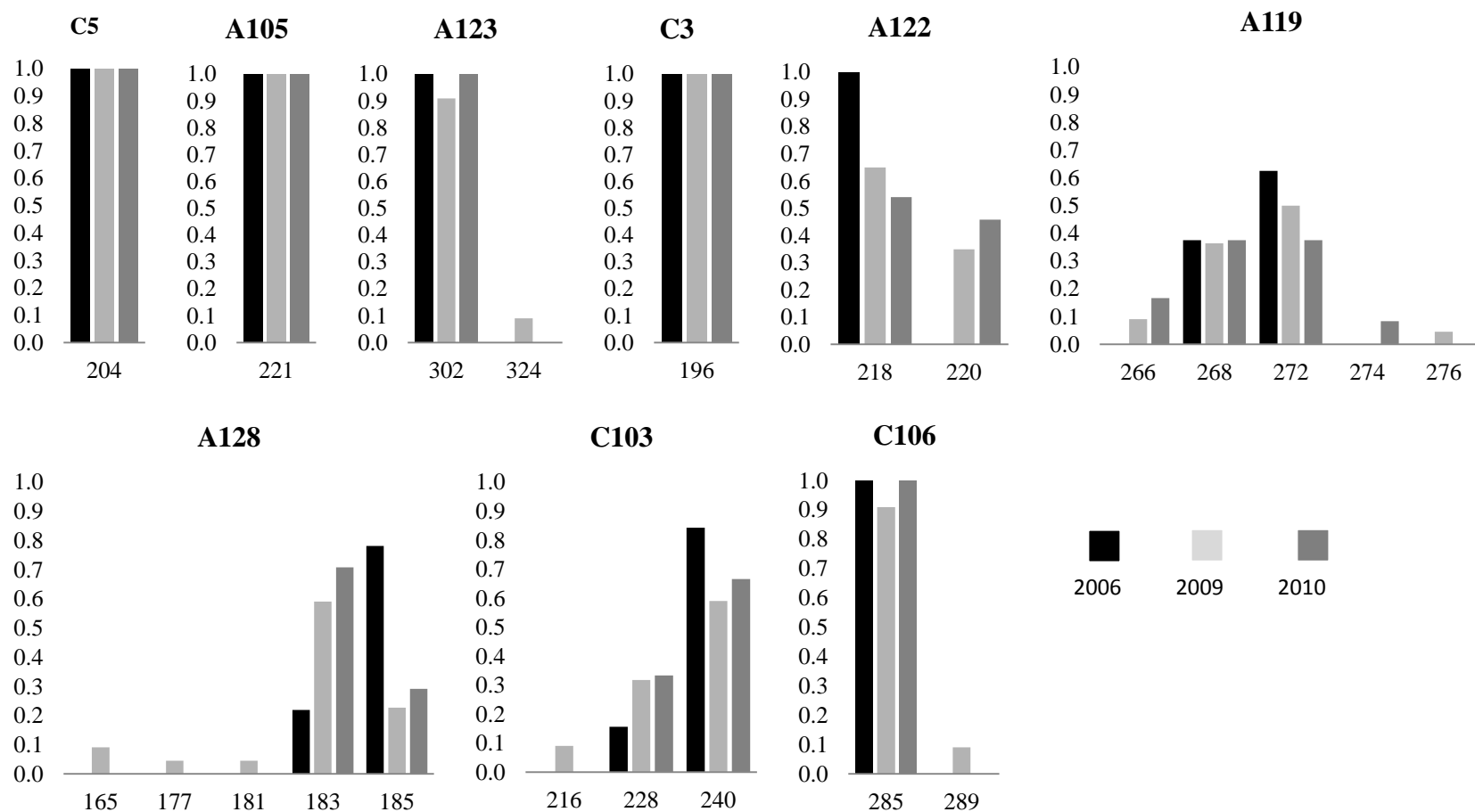


Figure 3.13. A comparison of allele frequencies for *Sesbania tomentosa* at nine microsatellite loci (C5, A105, A123, C3, A122, A119, A128, C103 and C106) sampled from all extant individuals of the Polihale Kaua'i population during visits in 2006, 2009 and 2010. Frequencies listed on y-axes; alleles listed on x-axes.



area ( $7 \times 3$  km; USFWS, 2010) would maintain higher allelic diversity than would a comparatively smaller population (Hamrick and Godt, 1989), yet high rates of genetic sub-structuring (as discussed below) would still result in a preponderance of non-random mating in the form of inbreeding.

The Ka Lae (Hawai'i Island) population is also interesting in that extremely high levels of inbreeding were accompanied by unexpectedly high levels of allelic diversity and the third highest occurrence of private alleles (on par with the previous two examples discussed). In order to explain this, reviewing the history of land use at Ka Lae is in order. On several occasions, Herbst (1972) found *Sesbania tomentosa* occurring exclusively within the stone fence that surrounded the SW corner of the point, a barrier that he felt protects the plants from cattle (*Bos taurus*) that have historically grazed nearby. This fence was erected circa 1908 when 10 acres of land were set aside for the lighthouse service (Love, 1991). In 1991, a similar observation was made noting 85 plants found exclusively within the stone enclosure (Hawai'i Biodiversity and Mapping Program). In 2006, samples were collected from plants both within the stone enclosure (17 plants extant at that time) as well as up to 100 m outside the stone enclosure (12 plants extant at that time), as cattle grazing in proximity to the Ka Lae enclosure ceased 20 years prior. Protection from grazing over the past hundred years within the enclosure might have preserved genotypes that would have otherwise been lost, maintaining allelic diversity over time beyond that of unprotected populations of similar size. The highest rates of inbreeding observed for *S. tomentosa* across the Hawaiian Islands were found at Ka Lae, as well as in small clusters of plants scattered along 10 km of coastline to the east.

#### Potential causes and impacts of high levels of inbreeding observed

Deficiency of heterozygotes is measured against the proportion of heterozygotes expected if the population's allele frequencies were in Hardy-Weinberg equilibrium, an ideal state providing a baseline against which to measure genetic change (Hartl and Clark, 2007). While 22 of the 38 populations had significant heterozygote deficiencies at the Bonferroni corrected nominal level, the 16 populations lacking detectable heterozygote deficiencies had an average sample size of 9.6 compared to 17.5 for the remaining 22 populations exhibiting significant heterozygote deficiencies at the nominal level. Small census (sample) size of certain populations

might be influencing these higher (insignificant)  $P$ -values. The exception is the large reproductive population at Nihoa, the only population with a large ( $> 20$ ) sample size ( $n = 49$  out of 3,000–5,000 individuals) that did not have a significant deficiency of heterozygotes (Table 3.5). There is reason to believe that non-random mating is the norm within *Sesbania tomentosa* populations across the main Hawaiian Islands, yet it is important to determine whether this is predominantly a natural or unnaturally-exacerbated phenomenon.

While there was a general excess of homozygotes evenly distributed across allele size classes in 280 out of 342 population-locus combinations, the presence of null alleles would only be suspected when some loci show significant excess of homozygotes while others do not deviate from Hardy-Weinberg proportions. In the present study, the consistency of the homozygote excess across the nine loci indicates that nonrandom mating (e.g., mating of close neighbors or self-pollination, both of which increase inbreeding) might be playing a role in amplifying estimates of null alleles.

The mean null allele frequency over all populations and loci was 0.12, interpreted as a “moderate” null allele frequency by Chapuis and Estoup (2007). Since the algorithms developed to estimate null alleles assume random mating (Dempster et al., 1977; Brookfield, 1996), these frequencies are probably overestimated as the evidence for non-random mating in populations of *Sesbania tomentosa* is overwhelming.

A correlation between the occurrence of linkage disequilibrium and levels of inbreeding was observed at ‘Āpua point (12 pairs of loci in linkage disequilibrium) and Mahana bay (8 pairs of loci in linkage disequilibrium). These populations also exhibit the sixth ( $f = 0.7$ ) and second ( $f = 0.922$ ) highest rates of inbreeding in this study, respectively. Diversity was excessively low (many monomorphic loci) to adequately address genotypic disequilibrium in many populations sampled, predominately those on O‘ahu and Kaua‘i (Table 3.5). Since the test of linkage disequilibrium assumes Hardy-Weinberg equilibrium, it is likely that these results are due to deviations from this assumption within each population to varying degrees (dependent upon levels of inbreeding taking place within each).

The high rates of inbreeding observed (as high as 0.94) would seem extremely detrimental to the survival of this species into the future, yet evidence for inbreeding depression has so far been inconclusive. For example, manual supplemental hand cross pollination failed to significantly increase reproduction in plants at the small Kīpuka Nēnē makai population ( $n = 12$ ),

yet testing pollen viability and stigma receptivity confirmed male and female vigor (Pratt et al., 2011). It was discovered in the course of the present research that all 12 plants had identical microsatellite genotypes where four out of nine loci were heterozygous (and thus not likely to be the product of selfing). Therefore, it became apparent that what was originally believed to be 12 separate individuals comprises only a single large sprawling individual (genet), which may or may not have become fragmented into separate clonal individuals (ramets). As a result, Pratt et al. (2011) speculated that self-incompatibility mechanisms in *S. tomentosa* might account for the low seed set observed at this population. Over the three-year study period, none of the 380 buds and flowers that were tagged at this population matured into fruit. In contrast, fruit production appeared much higher in a larger (150+ individuals) coastal population 12 km to the southeast at 'Āpua point (Pratt et al., 2011), however, the remoteness of this location precluded the monthly monitoring of buds and flowers. Hopper (2002) observed lower seed set in more isolated/smaller groups of plants at Ka'ena point, yet he also observed periodic seedling recruitment around isolated individuals, and seeds derived from his self-fertilization treatments were viable. Therefore, it is conceivable that inbreeding depression has not been an issue in all cases. Hopper (2002) also measured the genetic fitness (seed viability and pollen fertility) of two Ka'ena point populations of *Sesbania tomentosa* (one a small isolated group of plants and the other a large contiguous population of plants). He found that there was no difference between seed germination success of seeds from the two populations, and that pollen fertility was actually (inexplicably) higher in the isolated plants.

The rapid growth and reproduction of *Sesbania tomentosa*, along with a “persistent” seedbank [seeds proven viable after 10 years in storage (Lilleeng-Rosenberger, 2005) and after 3 years in the soil (Pratt et al., 2011)] and short life span are characteristics of pioneer species associated with harsh environments (Odum, 1971). Repeated colonization of open habitat would have been accompanied by a high rate of self-fertilization that would have purged many deleterious alleles (Charlesworth and Charlesworth, 1987; Barrett and Charlesworth, 1991; Barrett 1998). Plant populations with a history of inbreeding and that readily self-fertilize typically do not exhibit inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). Factors that promote the evolution of selfing include a lack of effective pollination and repeated colonization of new areas by single individuals (Schemske and Lande,

1985). Other *Sesbania* taxa are also known to be extremely successful in establishing themselves by producing fertile seed from selfing (Jamnandass et al., 2005)

### Genetic structure of populations across Hawai‘i

Global  $F_{ST}(\theta)$  over all populations and loci was estimated to be approximately 0.5; Wright’s (1978) guidelines state that values above 0.25 indicate “very great” genetic differentiation. These  $F_{ST}$  results are strong indication of reduced and/or ineffective gene flow between populations of *Sesbania tomentosa*. This was corroborated by the coalescent modeling which suggested that in Ka‘ū (the largest natural landscape left in Hawai‘i where some degree of connectivity between populations of *S. tomentosa* could potentially still occur) the contemporary population structure of *S. tomentosa* has been predominantly influenced by genetic drift in isolation rather than gene flow.

Overall, STRUCTURE provided less resolution in identifying distinct clusters than  $F_{ST}(\theta)$ . This might be explained by a poor fit between assumptions of the STRUCTURE model (Hardy-Weinberg equilibrium within populations) with the reality of *Sesbania tomentosa* reproduction in nature. The results presented here offer an alternative view of the relationships between populations purported in previous STRUCTURE and  $F_{ST}(\theta)$  analyses (see Chapter 2), this time using a more natural sampling strategy where duplicate (identical) genotypes derived from plants occurring < 10 m from one another were included (whereas in Chapter 2 they were excluded). These identical genotypes are believed to be either samples inadvertently taken from branches of the same plant (branches which over time physically separated from one another), or are an artifact of extreme genetic sub-structuring within certain populations. For example, as a result of including duplicate genotypes occurring less than 10 m apart, global  $F_{ST}(\theta)$  over all populations increased from 0.39 to 0.50.

Populations of *Sesbania tomentosa* also clustered together in different ways as a result of this alternate analysis. For example, the Maui Nui populations shifted from being in a cluster associated with O‘ahu, Kaua‘i and Nihoa in the analysis (see Chapter 2) to being associated with populations on Hawai‘i Island (Figure 3.4). The apparent drifting apart of populations at either end of the Island chain (with Maui Nui situated in the middle) is an expected phylogeographic pattern that correlates well with the IBD results presented above. Despite the highly restricted

gene flow between populations estimated above (global and pairwise  $F_{ST}$ ), a significant pattern of isolation by distance suggests that historical gene flow among contiguous populations existed or that dispersal and establishment of populations occurred in a linear, rather than random, order to give rise to the much larger and continuous distribution. The PCA and NJ results from Chapter 2 (Figures 2.14 and 2.16; Chapter 2) corroborate this as well. In spite of the somewhat unexpected assignment of the two largest NW Maui populations in the same cluster as populations from O‘ahu, Kaua‘i (350 km apart) and Nihoa (600 km apart), these cluster assignments follow the observed phylogeographic trends (Figure 3.4).

### Genetic sub-structure of populations across Hawai‘i

The extremely high inbreeding coefficients observed in this study may be due in part to a Wahlund effect in which heterozygosity in populations is reduced due to sub-population structure (Wahlund, 1928). The larger the sub-population and the more recently it has been isolated, the smaller the inbreeding effect of population subdivision (Hartl and Clark, 2007). Strong evidence of population sub-structuring is apparent throughout most populations of *Sesbania tomentosa*, indicating that adjacent plants are more closely related than non-adjacent plants.

Accompanying this trend are cases in which the clonal nature of populations comes in to question. For example, at Kīpuka Nēnē in Hawaii Volcanoes National Park, Pratt et al. (2011) reported that branches of monitored plants grew 1 to 4 m during their three-year study period. They also noted the tendency for the plant to sprawl at ground level and to root from branch nodes adding to the dynamic nature of *S. tomentosa* populations. What was believed by the authors to be groups of separate *S. tomentosa* plants became a tangled mass in subsequent years of monitoring (with all DNA samples turning up the same genotype). On the other hand, a large plant might break up over time into several apparently distinct patches. Each time plant fragmentation occurs, this could potentially increase the maximum distance between clonal pairs, perhaps explaining why pairs of identical genotypes were collected from branches attached to seemingly separate individuals 30 m apart at Kīpuka Nēnē (data not shown).

In considering the role of self-fertilization in the reproductive dynamics of populations, this sub-structuring might also be explained by examining the behavior of the *Hyla*

pollinators. Hopper (2002) observed *Hylaeus* to spend most of their time around a single plant (resulting in most of the pollination and fruit set he observed to be the result of geitonogamy) and he believed they will not forage far unless there is native dominated vegetation containing both nectar and pollen and sites for resting and nesting. Grasses surrounding plants are believed to serve as isolating barriers as they are not used by any species of bee (Hopper, 2002). *Hylaeus* bees on Haleakalā were inferred to have visited multiple (separate individual) plants when foraging only when plants were located very close to one another (Krushelnycky, 2014). Indeed, a single *Sesbania tomentosa* plant in full bloom would supply much more pollen than an individual bee could carry back to its burrow, reducing the need for visits to multiple plants (which would have otherwise facilitated cross-pollination). Therefore, the vectors most responsible for effecting pollination in *S. tomentosa* (playing a much larger role than all other floral visitors combined; Hopper, 2002, Pratt et al., 2011) are not adequate for facilitating outcrossing, and the plant would therefore be more dependent upon the less common occurrence of seed dispersal for gene flow. Increased spatial gene flow would otherwise have a homogenizing effect, reducing the genetic differentiation between populations (Wright, 1969; Slatkin, 1987). On the other hand, germination from the seed bank would help to preserve strong spatial genetic structure in a predominately selfing species via temporal gene flow (Honnay et al., 2008).

#### A prolonged history of genetic bottlenecks

There are apparent differences in heterozygosity and allelic diversity between populations, many of which have several loci that are either monomorphic or are approaching fixation within a population. The loci and alleles involved vary between neighboring populations, strongly suggesting the influence of bottlenecks. When correcting for the presence of null alleles in the dataset, there does not appear to be a lack of low frequency alleles in most populations and evidence for recent bottlenecks in the form of a transitory heterozygote excess was also largely unsubstantiated in the BOTTLENECK analysis. When population size becomes very small (~10 individuals) and when generation times are short, as with most populations of *Sesbania tomentosa* [e.g., Hopper (2002) reported a longevity of 3–10 years at Ka‘ena point], a new mutation-drift equilibrium should be arrived at quite rapidly (Watterson, 1984).

Analysis of the  $M$ -ratio was much more successful in detecting bottlenecks, suggesting they have been occurring for a longer period of time than BOTTLENECK could detect (i.e., not within the past  $0.2N_E$ – $4N_E$  generations). Garza and Williamson (2001) suggested that  $M$ -ratios lower than 0.68 would indicate evidence of a bottleneck, whereas values greater than 0.8 would denote no bottleneck history whatsoever. As the  $M$ -ratio is predicted to recover following a reduction in population size, the rebound in size of the Polihale (Kaua‘i) population from 2006 ( $n = 10$ ) to 2009 ( $n = 50$ ) is seen here to have been accompanied by an increase in the  $M$ -ratio. When the Polihale population was sampled again in 2010 (after it declined back to 12 individuals) genetic signatures of recent bottlenecks were also evident in the BOTTLENECK Wilcoxon tests. The only other populations providing evidence for recent bottlenecks were along the Hilina pali in Hawai‘i Volcanoes National Park, an area known to have been heavily grazed by feral goats (*Capra aegagrus hircus*) over the last century (see below). *Sesbania tomentosa* in that area is quite distinct in that it forms relatively large and apparently long-lived patches (Linda Pratt, US Geological Survey, personal communication); with longer generations, the signatures of recent bottlenecks would be expected to persist for longer periods of time.

Two subsets of samples were compared from the Mo‘omomi population to test for changes in allele frequencies over time. DNA samples from 10 historically-collected herbarium samples and 26 samples representing the entire extant population in 2006 were both genotyped. This strategy has been used in other studies to observe the genetic effects of demographic bottlenecks in a direct manner, in contrast to the indirect methods employed above (Bouzat et al., 1998; Larson et al., 2002; Nyström et al., 2006; Larsson et al., 2008). Twenty alleles (out of a total of 55) in the 10 historic samples were not found in any of the plants extant at the Mo‘omomi population in 2006, a possible indication of a genetic bottleneck having taken place. The loss of these alleles also suggests that genetic drift, and loss of genetic diversity overall, may be occurring at Mo‘omomi. While the alleles that were lost may have been rare to begin with (and thus were the first to be lost during population contraction), it is still important to recognize their loss from the population completely. On the other hand, it remains possible that some of those lost alleles were maintained in the soil seed bank *in situ* during sampling there in 2006, with the potential to subsequently germinate and again contribute their alleles to the population.

Studying the history of land use of the sites surveyed for this study is another means to examine population bottlenecks, particularly where intensive animal grazing is known to have

taken place. For example, sheep (*Ovis aries*) were penned near the beach at Mo‘omomi, Moloka‘i (Cooke, 1949). Degener and Degener (1978) reported that *Sesbania tomentosa* was on the verge of extinction at Mo‘omomi in 1928, and cattle (*Bos taurus*) and axis deer (*Axis axis*) were taking a toll on the plants at Mo‘omomi as late as 1990 (Hawai‘i Biodiversity and Mapping Program). Similarly, cattle grazing at Ka‘ena point on O‘ahu in the early 1900’s severely impacted *S. tomentosa* there (Degener and Degener, 1978). At ‘Āpua point in Hawai‘i Volcanoes National Park, feral goats (*Capra aegagrus hircus*) were driven down off the mountain and penned at the lush ‘ohai habitat during the 1920’s and 30’s (Clark, 1985). Cattle breached the stone wall enclosing the lighthouse at Ka Lae in the mid-1970’s, completely denuding the ground of *S. tomentosa* (yet the soil remained stocked with its seeds for many years after; Degener and Degener, 1978). Approximately 70,000 animals were extricated from the park, yet 15,000 persisted in the area around Hilina pali as recently as the 1970’s (Baker and Reeser, 1972; Katahira and Stone, 1982). While levels of inbreeding were high and genetic diversity low in the above-mentioned populations, evidence for genetic bottlenecks in the dataset was lacking in most cases (the exception being Hilina pali). Extensive wildfires burned through Kīpuka Nēnē twice in the last 40 years, and can also be expected to have caused dramatic declines in the *S. tomentosa* population there as well.

Arthropod grazing pressure has also resulted in catastrophic drop in numbers of *Sesbania tomentosa* plants in recent times. In the 1960’s, a stink bug (*Comptosoma xanthagramma*) outbreak devastated the Ka‘ena point (O‘ahu) plants where a natural seedbank provided the recovery (Howarth, 1985). From 2002 to 2004, the grey bird grasshopper (*Schistocerca nitens*) outbreak completely defoliated the *S. tomentosa* on Nihoa (Latchinsky, 2008) and, as a result, many plants had perished when the population was again observed in 2006 (Beth Flint, USFWS, personal communication). Magnolia scale (*Neolecanium cornuparyum*) were first observed on the *S. tomentosa* at Polihale (Kaua‘i) in August of 2004. Almost all of the larger plants (ca. 2 m tall) died, yet there were many new seedlings after a wet year in 2009 that seemed less susceptible to the scale (USFWS, 2010). While the Ka‘ena point and Polihale populations both had high levels of inbreeding and relatively low diversity, only the Polihale population tested positive for evidence of recent genetic bottlenecks. The Nihoa population, on the other hand, had levels of diversity lower than would be expected given its large size (as compared with much



smaller populations that exhibited the same or higher levels of diversity) that is possibly related to the grasshopper outbreak and subsequent decline.

On the islet of Pu‘u Koa‘e (near Kaho‘olawe), cycles of drought appear to have caused dramatic fluctuations in *Sesbania tomentosa* numbers providing another means to verify population bottlenecks having taken place (USFWS, 2010). Following a drought in 2000, the population shifted from having 70 mature individuals, 15 juveniles and 15 seedlings to consisting of one surviving mature individual accompanied by 300 seedlings. A single mature individual was later observed accompanied by up to 70 dead individuals. In 2003, 100 mature individuals plus 200 seedlings were reported. This rose to 300–400 individuals in 2008, and back down again to 50 in 2010. The last observations made were in 2011 when 10 large plants along with 400 young healthy plants approximately 10–45 cm tall were found (Ken Wood, NTBG, personal communication). No evidence for genetic bottlenecks was detected at Pu‘u Koa‘e and levels of diversity remain moderate.

Similar fluctuations in population size were seen at Ka Lae where 85 *Sesbania tomentosa* plants in 1991 were reduced to only 2 plants in 1992 (Hawai‘i Biodiversity and Mapping Program). This population had rebounded to 29 when surveyed in 2006, although other fluctuations might have taken place in the 14-year gap between recorded observations. Fluctuations in numbers of plants have also been recorded for the Polihale (Kaua‘i) population. Only five plants persisted in the 1980’s, but numbers increased to 30 plants reported in 1992 although dwindling again to seven in 2001. In 2005 that number exceeded 30 again and by late 2006 the population was down to less than 20, and by 2008 the number was hovering around 10. There were 50 plants reported in 2009, but this number subsequently dropped back to 12 by 2010 (a genetic bottleneck was detected for the Polihale population in 2010). It is of interest to note that the genetic diversity of this population was highest in 2009 when the population size had reached a 30-year high. Out of a total of 22 alleles (at 9 loci) occurring at Polihale over the 3 sample years, seven were private to the Polihale population in 2009 (Figure 3.13) in spite of sampling during that year (as opposed to other years) not being exhaustive (only 11 out of 30 plants extant at time of collection were genotyped due to degraded plant tissue collections).

Experiments done at the Lyon Arboretum Seed Conservation Lab found that *Sesbania tomentosa* seeds have no light requirement for germination once they become imbibed with water (Alvin Yoshinaga, Lyon Arboretum, personal communication). Therefore, these seeds are

capable of surviving through drought yet germinate immediately once rains have returned. This limits the temporal range of gene flow that the soil seed bank provides, thus genetic drift is still able to progress through the bottleneck albeit at a slower pace than it would be able to otherwise (Templeton and Levin, 1979; Honnay et al., 2008).

The above records are actual, observed population flush-crash cycles (Carson, 1975) that were not all detected by the indirect methods employed herein. Perhaps these populations are able to recover in that the maintenance of a seed bank in the soil would allow low frequency alleles to remain in the genepool (through the bottleneck). Another scenario would be that some of these have historically been small, fluctuating populations, and have experienced no rapid decline in numbers. Populations suffering a reduction in census size may not suffer a severe reduction of  $N_E$  (a genetic bottleneck) if historical  $N_E$  has always been low due to fluctuations in population size, inbreeding, or metapopulation structure involving cycles of extinction and recolonization (Cornuet and Luikart, 1996; Watterson, 1984). At the Polihale (Kaua‘i) population, the effective population size calculated using the fluctuating census numbers listed above was 12 (roughly half the average annual population size over the past 30 years). In either case, a very rapid intrinsic rate of increase following a population bottleneck would minimize genetic loss (Nei et al., 1975). Accordingly, the most rapidly-rebounding, abundant population year sampled at Polihale (2009) exhibited the highest levels of diversity.

*Sesbania tomentosa* has thus been shown to maintain an ample seedbank for future colonization of the plant metapopulation, and the rapid maturation of *S. tomentosa* plants (from seed to seed in less than 1 year) is also coming into play. For example, genetic drift is thought to be accelerated in species with shorter generation times (Kimura, 1983). Seeds sprouting from a seed bank represent migration from the past, and have the potential to buffer against a loss of diversity while at the same time slowing genetic drift (Templeton and Levin, 1979; Honnay et al., 2008).

## Conclusion

Populations of *Sesbania tomentosa* exhibit high levels of genetic structure with extensive inbreeding within and divergence among individual populations. Corresponding with previous observations suggesting geitonogamy commonly taking place in this species, the high  $F_{ST}$  values

observed among *S. tomentosa* populations are comparable to rates of differentiation seen in other predominately selfing, short-lived perennial species (Hamrick and Godt, 1996). The significant pattern of isolation by distance across the Hawaiian Islands indicates that the underlying structure derives from ancient timescale processes (migration and gene flow) as well as from drift in the contemporary populations. The consistently high levels of inbreeding observed, accompanied by strong spatial genetic sub-structure, are also indications of a species predominately reliant on selfing to maintain reproduction. There is little sign of futile selfing occurring in this species (inbreeding depression leading to the loss of selfed progeny; Robertson et al., 2011) as many populations of mature individuals are composed of highly homozygous genotypes. Therefore, low levels of gene flow between populations (and a high occurrence of selfing) can be presumed to have been a trend in the past that has been accentuated by more recent fragmentation and decline. Indeed, the meta-analysis of Aguilar et al. (2008) suggested that fragmentation of plant populations has the effect of shifting mating patterns towards increased selfing.

The original immigration of *Sesbania* to Hawai‘i need not have taken place very far back in the past to account for the morphological differentiation observed today, as is probably the case given the low levels of nDNA sequence divergence (see Chapter 2). The microsatellite loci examined in this study appear to have responded to genetic drift much more rapidly than the regions that were sequenced. Natural selection in different environments, along with random drift and mutation, would cause morphological variation to accumulate in the species as a whole. Rates of adaptation and morphological change in isolated breeding populations would be impacted by the rapid maturation of *S. tomentosa* and the maintenance of an ample, viable seedbank. Ecologically, this species also appears prone to inbreeding and repeated bottlenecks, adding additional efficiency to a trend of divergence. It is entirely plausible that both the microsatellite as well as the morphological differentiation observed have been accentuated within the time period when populations of *S. tomentosa* became increasingly fragmented and isolated from one another. In other words, more modern impacts on the range of the species have probably only accelerated what was already naturally-occurring. For example, three distinct morphotypes were observed before the era of ranching within 10 km of one another on the island of Moloka‘i (Hawai‘i Biodiversity and Mapping Project). On the other hand, lava flows of the

past 400–700 years (Sherrod et al., 2007) have separated three additional morphotypes within Hawai‘i Volcanoes National Park by 5–10 km distance as well.

On a final note, there may have been specialist honeycreeper finches (Fringillidae) foraging in the range of *Sesbania* prior to the introduction of avian diseases (circa 1800’s) that would have provided the large proportion of pollination services for this plant (nectar-rich, scentless, showy flowers are suggestive of this; S. Conant personal communication). As such, the birds would have provided for greater outcrossing within and among populations than is seen at present. The shift to insect pollination (with *Hylaeus*) would have severely limited gene flow within and between populations, further separating them out into the distinctive appearing populations found today.

## CHAPTER 4

### Genetic diversity and the role of seed sourcing practices in restoration outplantings of the rare Hawaiian plant *Sesbania tomentosa*

#### Introduction

Under ideal circumstances, efforts to restore populations of rare plants aim to maintain levels of genetic diversity found in natural populations among individuals that will be used for replanting. The restored population is likely to be more self-sustaining if the plant material used is diverse, by ensuring successive generations of progeny will be free from the deleterious effects of inbreeding (Charlesworth and Charlesworth, 1987; Huenneke, 1991; Fenster and Dudash, 1994; Knapp and Dyer 1998). Over the long term, increased adaptive potential imparted by genetic diversity improves successful responses to future environmental change and reduces the risk of extinction (Ellstrand and Elam, 1993; Frankham, 2005). On the other hand, restoration projects often use only locally collected planting material following a precautionary notion that such material might comprise locally adapted genotypes (Millar and Libby, 1989; Hufford and Mazer, 2003; McKay et al., 2005). If there are limits to the harvesting of local planting material (such as in cases of low reproduction of rare plant populations in Hawai'i), collections made might only provide a restricted sample of the source population and the genetic base of the outplanted population would be narrow (e.g., Burgarella et al., 2007; Kettle et al., 2008). It has been suggested that for rare species with few remaining individuals the central focus of restoration efforts should be to maximize genetic diversity in restored populations regardless of the origin of planting material (Frankham et al., 2011; Maschinski et al., 2013). Although outbreeding depression may be a consequence of this, Frankham et al. (2011) suggest that the probability of this is low in most plant and animal populations and mitigating the effects of inbreeding depression are much more relevant in preventing extinction.

Knowledge of population genetic structure and diversity at the outset of any restoration effort would help determine whether it would be safe to mix different source populations in an outplanted population (Hamrick et al., 1991; Keller and Waller, 2002). Mixing material collected from multiple populations should increase genetic variation in the outplanted population. Several

studies have found that using seed from more than one source population resulted in outplanted populations with more genetic variation (e.g., Smulders et al., 2000; Gustafson et al., 2004; Dolan et al., 2008) and others have found them to be more resilient and reproductively fit when compared with single-source outplanted populations (Vergeer et al., 2005; Maschinski et al., 2013; Weisenberger et al., 2014). Weisenberger et al. (2014) determined that mixing was an important strategy in the recovery of Hawaiian *Schiedea* with 1 to 2 plants per population, with outplantings derived from between population crosses exhibiting a strong heterotic effect.

*Sesbania tomentosa* Hook. and Arn. (Fabaceae) is an endemic Hawaiian flowering plant adapted to coastal strand and dry to mesic upland habitat. The habit of *S. tomentosa* is highly variable, often with island specific forms. Plants may grow as sprawling shrubs with prostrate to decumbent branches (reportedly up to 14 meters long, though possibly reaching much longer in extreme examples) or as a small bush or tree up to six meters in height. Leaves are even-pinnately compound and consist of 18 to 38 oblong to elliptic leaflets, each 15 to 38 millimeters long and 5 to 18 millimeters wide. The species is named for the leaves, that are sparsely to densely covered with silky hairs. The flowers, in clusters of 2 to 9, are salmon tinged with yellow, orange-red or scarlet to deep red. Fruits are slightly flattened pods 7 to 23 centimeters long and about 5 millimeters wide, and contain 6 to 27 olive to pale or dark brown seeds.

*Sesbania tomentosa* was listed as Endangered by the U.S. Fish and Wildlife Service in 1992, and has been a focus species for outplanting by various state and federal agencies tasked with its recovery. Fifty-six percent of all populations of *S. tomentosa* recorded by naturalists have gone extinct since Lay and Collie first collected the plant in 1826 (Hawai'i Biodiversity and Mapping Program). In fact, at least seven populations have been extirpated since DNA collections for this study began (in 2006) and others have experienced severe demographic decline due to drought, pest outbreaks, or other natural or anthropogenic causes (personal observations). A hermaphroditic breeding system, conspicuous flowers and autochorous dispersal of dry fruit have made *S. tomentosa* acutely vulnerable to extinction compared with other dry forest taxa according to the analysis of Pau et al. (2009). On the other hand, entirely new occurrences of this species have been discovered since this study began near Nu'upia pond (Mōkapu, O'ahu) and at Pa'akahi point (Hanapēpē, Kaua'i) after heavy winter rains, indicating an important role of the seedbank within the metapopulation as a whole as well as the ephemeral nature of the species as a component of the vegetation.

Chapter 3 explored the structure of microsatellite diversity in populations of *Sesbania tomentosa* throughout its known range. Genetic analysis with microsatellite markers is here used to compare the genetic diversity of naturally-occurring populations of *S. tomentosa* with those of their outplanted counterpart populations in Hawai‘i, to assess rates of inbreeding and impacts of genetic drift. Examples where molecular markers were used to gain valuable insight in guiding restoration management of rare plant populations are plentiful (e.g., Knapp and Connors, 1999; Mattner et al., 2002; Rottenberg and Parker, 2003), including a number of examples from Hawai‘i (Morden and Loeffler, 1999; Friar et al., 2000, 2001; Kwon and Morden, 2002). As various numbers of founding individuals (from 1 to more than 10) have been used to assemble the outplanted populations measured, seed sourcing practices will also be examined. To the degree that sampling bottlenecks occur, restored populations should be observed to be genetically depauperate compared with their natural population counterpart, and might be subject to additional negative effects of inbreeding and genetic drift in the future. The hypothesis that genetic diversity should impart resilience in *S. tomentosa* populations was tested using data on the survivorship of outplantings.

## **Materials and Methods**

### **DNA sample collection**

Leaf samples of 166 individuals of *Sesbania tomentosa* were collected between 2006 and from eight naturally occurring populations throughout the Hawaiian Islands. These eight populations were used as sources of seed to propagate an additional 141 individuals whose leaves were also sampled for this study, examining 307 samples total. An approximately 4 cm<sup>2</sup> square leaflet tip from each plant was collected for DNA analysis. Leaf tissue was placed in paper envelopes and zip-lock bags with silica gel desiccant in an airtight container, and then transferred into cold storage (4 to 8°C) prior to DNA extraction. All extractions were carried out using 0.5 to 1.0 g of leaf material with DNEASY tissue kits (QIAGEN; Valencia, CA) according to the manufacturer’s protocol and then visually checked using electrophoresis. Propagative material had been collected from source populations previous to this study (several years previous in some cases) and it cannot be ruled out that additional individuals may have been

present at that time. Varying degrees of mixture and number of original founders were used to comprise the outplanted populations, and are listed in Table 4.2.

In most cases, outplanted individuals of *Sesbania tomentosa* were sampled more than one year post-planting. Kāohikaipu and Mōkapu-derived individuals were in cultivation at the Hawai‘i State nursery (Mokulē‘ia, O‘ahu) and were sampled prior to their outplanting at Ka Iwi State Scenic Shoreline and Ka‘ena point State Park (O‘ahu). In addition, the two individuals comprising the Mānā (Kaua‘i) population (now extirpated) were cultivated specimens of the National Tropical Botanical Garden (F<sub>1</sub> generation derived from a single wild plant). Lastly, in order to track changes in the genetic makeup of the species seedbank (and the associated extant population) over time, the Polihale (Kaua‘i) population was sampled in 2006 (16 plants), 2009 (11 plants) and 2010 (12 plants), and the genetic diversity of the standing populations of each year are herein compared. GPS coordinates accompanied each DNA collection, yet in many cases it was impossible to determine whether or not the same individual was collected multiple times (in successive years) due to the close clustering of individuals.

## Microsatellite Analysis

Genetic Identification Services (Chatsworth, CA, USA) constructed libraries and isolated potential microsatellite primer loci for *Sesbania tomentosa* using magnetic bead capture molecules for dinucleotide (CA<sub>n</sub>) and tetranucleotide (CATC<sub>n</sub>, TACA<sub>n</sub>, and TAGA<sub>n</sub>) microsatellite repeats. Ninety-six microsatellite-containing clones were identified after sequencing, for which 54 sets of primers were developed using DESIGNERPCR v. 1.03 (Research Genetics, Huntsville, Alabama, USA). Nine microsatellite loci were subsequently chosen (Table 4.1) based on their range of polymorphism and ease of scoring in a screening of eight DNA samples (collected from eight populations on six islands). Each sample was amplified in a 25.0 µL volume with final concentrations of: 0.6 µM each of forward and reverse primers, 1X PCR Buffer, 2.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs (Promega), 1 unit *Taq* polymerase (Promega); 2–4 ng of DNA sample was then added. Amplification took place using an MJ Research Thermocycler (Waltham, Massachusetts, USA) with the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 40 s, the primer specific annealing temperature (listed in Table 4.1) for 40 s, 72°C for 30 s; ending with a final extension of 72°C for



Table 4.1. Nine microsatellite primer pairs developed for *Sesbania tomentosa*. Prefixes in italics before forward primer sequence indicate dye used for poolplexing.  $T_A$ , annealing temperature in °C.  $N_A$ , number of alleles found in all 307 individuals sampled for this study. Range, allele size range in base pairs.

Locus	Repeat motif	Primer sequence (5'–3')	$T_A$	$N_A$	Range
A105	TG <sub>11</sub>	F: <i>VIC</i> -CGG-TAA-TGA-CTT-TGA-GGA-GG	57.3	6	207–221
		R: TAG-GTG-TGG-CGT-GCA-TAA-C	58.1		
A119	TG <sub>13</sub>	F: <i>6FAM</i> -GAA-CTT-GAA-CCC-CAA-CTG-A	56.0	8	264–278
		R: CCC-TTC-CCC-TCC-TCT-TAG	56.2		
A122	CA <sub>11</sub>	F: <i>VIC</i> -AAC-AGG-ATT-AAC-GTG-GTT-CTC	55.8	8	206–230
		R: GCT-TTC-CAA-TAT-AGA-CAT-GGT-G	56.3		
A123	TG <sub>12</sub>	F: <i>6FAM</i> -TGC-CAC-AGT-TTA-TCA-CTA-CGC	58.9	11	290–326
		R: TAG-CCA-TGC-TTC-ATC-AAT-CG	59.8		
A128	CA <sub>13</sub>	F: <i>6FAM</i> -GGA-CCA-ATT-TTG-GAG-TTT-ACT-C	56.8	10	163–185
		R: CCT-GGT-GTT-GAA-TGT-GTC-ATA	56.9		
C3	TGTA <sub>20</sub>	F: <i>PET</i> -CGC-TGT-TCT-CTG-CGC-TAG	58.6	7	196–248
		R: GGC-AAC-ATT-TGA-GTG-GAG-G	59.1		
C5	TGTA <sub>14</sub>	F: <i>PET</i> -CTG-AAG-CCT-TGC-TGA-AGA	55.1	9	192–236
		R: GGA-GGA-GGA-TTT-GTA-GAA-AGA	55.1		
C103	TACA <sub>3</sub> TATA TACA <sub>11</sub>	F: <i>PET</i> -CTA-GCC-ACA-TCA-GGA-GTT-ATT-C	55.7	11	212–252
		R: GTT-GGA-TAG-TTC-CCA-AAA-ATC	55.2		
C106	TACA <sub>8</sub>	F: <i>VIC</i> -TGC-ATT-TTG-CTT-ATG-TGT-G	54.1	7	265–321
		R: CCC-TCT-TCA-AAC-TAC-ATG-ATG	54.8		

4 min. PCR products were electrophoresed on 1% agarose to verify amplification. One negative and four positive controls (samples with known genotypes) were included in each run of 96 PCRs to check for potential contamination and standardize genotyping.

For each of three fluorescently-labeled primer pair multiplex combinations, 1.0  $\mu$ L of pooled PCR product was visualized on an Applied Biosystems (ABI) Prism 377XL sequencer (Waltham, Massachusetts, USA). The complete dataset of allele sizes was constructed using ABI PEAK SCANNER and GENEMARKER v. 1.4 (Softgenetics; State College, PA, USA) software, and through visual inspection of the PCR peak sizes generated in comparison with LIZ500 molecular size marker (Applied Biosystems).

Diversity indices were estimated for the geographic populations (both natural and outplanted) using MICROSATELLITE ANALYZER (MSA) v. 4.05 (Dieringer and Schlötterer, 2003). Diversity indices include expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ), mean number of alleles per locus ( $A$ , a measure of diversity not corrected for sample size) and allelic richness ( $A_R$ , allelic diversity corrected for sample size). Private alleles (alleles exclusive to a given population) were calculated in GENALEX v. 6.4 (Peakall and Smouse, 2006). Inbreeding ( $F_{IS}$ ) was calculated with INEST (using the “individual inbreeding model”), which estimates inbreeding while simultaneously accounting for the presence of null alleles (Chybicki and Burczyk, 2009). The extent and significance of the genetic differentiation between natural and the outplanted counterpart populations was investigated with MSA by calculating pairwise  $F_{ST}$  ( $\theta$  Weir and Cockerham, 1984) averaged over multiple loci, with 100,000 permutations to assess significance using Bonferroni corrected  $P$ -values at ( $\alpha = 0.01$ ).

A loss of rare alleles is an expected genetic signature resulting from a population bottleneck (Cornuet and Luikart, 1996; Luikart et al., 1998). To test for loss of rare alleles in the outplanted populations, the proportions of rare alleles (frequency  $< 0.1$ ) in each of the populations (natural and representative) were calculated.

After a severe reduction in effective population size ( $N_E$ ), there should be a transient excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (Piry et al., 1999). Bottlenecks generate transient heterozygosity excess because rare alleles are generally lost faster than heterozygosity during a bottleneck (Luikart and Cornuet, 1998). Wilcoxon sign-rank tests of heterozygosity excess (10,000 iterations) were implemented in BOTTLENECK v. 1.2.02 (Luikart and Cornuet, 1998; Piry et al., 1999). This program used

allele frequency data to detect recent reductions in effective population size (i.e., within the past  $0.2N_E$ – $4N_E$  generations) under a 100% stepwise mutation model (SMM), an infinite alleles model (IAM) and a two-phase mutation model (TPM with 70% SMM, 30% IAM). A second approach (also implemented in BOTTLENECK) tested a mode shift away from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium, whereby alleles at low frequency become less abundant than alleles at intermediate frequency (Luikart et al., 1998).

## Results

### Microsatellite allele frequencies

There was an average of 8.5 alleles per locus at the nine microsatellite loci examined, ranging from 6 to 11, for a total of 77 alleles among the 307 samples of *Sesbania tomentosa*. Each locus had only two to four alleles with a frequency greater than 0.10, and these most common alleles had average frequencies per locus that ranged from 0.20 to 0.41 (with a maximum across loci of 0.59).

### Genetic diversity and nonrandom mating of natural vs. outplanted populations

Of the eight natural (source) populations of *Sesbania tomentosa* sampled, Kīpuka Nēnē–Hilina pali ( $n = 35$ ) and Pu‘u Pīmoe ( $n = 9$ ) exhibited the highest values of allelic diversity (Table 4.2). Accordingly, the highest values of allelic diversity of all nine outplanted populations were observed in Kanaio U.S. Army Training Area (allelic richness = 2.67; expected/observed heterozygosity = 0.542/0.241) and Kulanaokuaiki (allelic richness = 2.43; expected/observed heterozygosity = 0.387/0.122), their outplanted counterparts (Table 4.2). The six Kanaio outplantings were sourced from only three founding individuals, with twelve alleles failing to be captured from the natural population at Pu‘u Pīmoe (data not shown). The Kanaio outplantings are notable for having two private alleles (average frequency = 0.21) not found in its source population and for lacking significant genetic differentiation from its source population (Tables 4.2 and 4.3). On the other hand, the outplanted population at Kulanaokuaiki ( $n = 35$ ) was composed of material sourced from a comparatively large number of separate founders ( $> 10$ )

Table 4.2. Genetic diversity statistics of natural vs. outplanted representative populations of *Sesbania tomentosa*.  $n$ , sample size;  $n_F$ , number of founders from natural population used to source the seeds comprising the outplanted representative population.  $A$  and  $A_R$ , mean number of alleles per locus and mean allelic richness (averaged over loci) respectively. Private alleles are alleles found in a given population not found in its counterpart population; average allele frequencies subsequently listed. Percentage of rare alleles is the proportion of rare alleles (frequency < 0.1) to total number of alleles in a given population.  $H_O$  and  $H_E$ , observed and expected heterozygosity.  $F_{IS}$ , inbreeding coefficient. ML is number of monomorphic loci and is out of a total of nine loci.

Population	Island	$n$	$n_F$	$A$	$A_R$	Private Alleles	% rare alleles	$H_O$	$H_E$	$F_{IS}$	ML
Natural population:											
Kīpuka Nēnē–Hilina pali	Hawai‘i	35		4.7	3.6	11/0.05	48.7	0.328	0.571	0.264	1
Outplanted representative populations:											
Kīpuka Nēnē	Hawai‘i	7	2–3 <sup>a</sup>	2.0	2.0		17.6	0.095	0.246	0.161	1
Kulanaokuaiki	Hawai‘i	21	> 10 <sup>a</sup>	2.9	2.4		36.0	0.122	0.387	0.621	1
Natural population:											
Pu‘u Pīmoē	Maui	9		3.8	3.4	12/0.12	23.5	0.420	0.594	0.157	
Outplanted representative population:											
Kanaio U.S. Army Training Area	Maui	6	3 <sup>b</sup>	2.7	2.7	2/0.21	12.5	0.241	0.542	0.212	1
Natural population:											
Papanalahoā–Nākālele	Maui	44		3.2	3.1	14/0.32	34.5	0.260	0.473	0.309	
Outplanted representative population:											
Kanahā County Beach Park	Maui	28	4 <sup>c</sup>	1.7	1.7		20.0	0.107	0.091	0.020	4

Table 4.2. (Continued) Genetic diversity statistics of natural vs. outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	<i>n<sub>F</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>	Private Alleles	% rare alleles	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>F<sub>IS</sub></i>	ML
Natural population:											
Nu‘upia Ponds (Mōkapu)	O‘ahu	3		2.0	1.5	8/0.34	0.0	0.222	0.500	0.652	3
Mokulē‘ia State Tree Nursery (2009):											
origin: Nu‘upia ponds	O‘ahu	8	2 <sup>d</sup>	1.2	1.1	1/0.12	0.0	0.125	0.084	0.046	7
Natural population:											
Kāohikaipu	O‘ahu	1		1.1	1.1		0.0	0.111	0.111	NA	8
Mokulē‘ia State Tree Nursery (2009):											
origin: Kāohikaipu	O‘ahu	11	1 <sup>d</sup>	1.2	1.1	1/0.14	0.0	0.141	0.085	0.031	7
Natural population:											
Ka‘ena point State Park–Natural Area Reserve (NAR)	O‘ahu	33		1.7	1.7	1/0.01	7.1	0.077	0.219	0.649	3
Outplanted representative population:											
Ka‘ena point NAR	O‘ahu	32	> 10 <sup>d</sup>	1.7	1.7	1/0.01	7.1	0.098	0.166	0.400	3

Table 4.2. (Continued) Genetic diversity statistics of natural *vs.* outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	<i>n<sub>F</sub></i>	<i>A</i>	<i>A<sub>R</sub></i>	Private Alleles	% rare alleles	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>F<sub>IS</sub></i>	ML
Natural population:											
Polihale State Park (2006)	Kaua‘i	16		1.3	1.3		0.0	0.083	0.123	0.006	6
Polihale State Park (2009)	Kaua‘i	11		2.3	2.3	5/0.12	38.1	0.092	0.294	0.328	3
Polihale State Park (2010)	Kaua‘i	12		1.7	1.7		6.6	0.065	0.236	0.214	5
Outplanted representative population:											
Lāwa‘i Kai (NTBG)	Kaua‘i	14	14 <sup>e</sup>	2.4	2.3	5/0.06	40.9	0.182	0.267	0.070	1
Origin of <i>ex-situ</i> source population:											
Mānā	Kaua‘i	2		1.1	1.1		0.0	0.056	0.056	0.000	8
Outplanted representative population:											
Makauwahi (NTBG)	Kaua‘i	14	3 <sup>f</sup>	1.2	1.0	1/0.91	0.0	0.008	0.026	0.049	7

<sup>a</sup> Thomas Belfield, Hawai‘i Volcanoes National Park, personal communication (2007)

<sup>b</sup> Chuck Chimera, U.S. Army Natural Resources, personal communication (2006)

<sup>c</sup> Forest Starr, US Geological Survey, personal communication (2006)

<sup>d</sup> Greg Manscur, Hawai‘i Division of Forestry and Wildlife, personal communication (2007)

<sup>e</sup> Mike Demotta, National Tropical Botanical Garden, personal communication (2007)

<sup>f</sup> David Burney, National Tropical Botanical Garden, personal communication (2007)

Table 4.3. Genetic differentiation between natural populations and their outplanted counterpart populations. Pairwise  $F_{ST}$  values ( $\theta$ , Weir and Cockerham 1984) averaged over nine microsatellite loci on top half of matrices; Significant Bonferroni-corrected  $P$ -values listed in bottom half of matrices. n.s. indicates pairwise comparisons non-significant at the 0.05 level.

Natural population: Kīpuka Nēnē–Hilina pali	0.00000	0.23846	0.12839	
Outplanted population: Kīpuka Nēnē	0.00003	0.00000	0.14749	
Outplanted population Kulanaokuaiki	0.00003	0.04143	0.00000	
Natural population: Pu‘u Pīmoe	0.00000	-0.02124		
Outplanted population: Kanaio Army Training Area	n.s.	0.00000		
Natural population: Papanalahoa–Nākālele	0.00000	0.63501		
Outplanted population: Kanahā County Beach Park	0.00001	0.00000		
Natural population: Nu‘upia ponds	0.00000	0.39183		
Mokulē‘ia State Tree Nursery (2009)	0.01043	0.00000		
Natural population: Kāohikaipu	0.00000	-0.05902		
Mokulē‘ia State Tree Nursery (2009)	n.s.	0.00000		
Natural population: Ka‘ena point	0.00000	0.03266		
Outplanted population: Ka‘ena point NAR	n.s.	0.00000		
Natural population: Polihale State Park (2006)	0.00000	0.15612	0.21813	0.12597
Natural population: Polihale State Park (2009)	0.00006	0.00000	-0.05130	-0.00327
Natural population: Polihale State Park (2010)	0.00306	n.s.	0.00000	0.05404
Outplanted population: Lāwa‘i Kai	0.00930	n.s.	n.s.	0.00000
Natural population: Mānā	0.00000	0.82904		
Outplanted population: Makauwahi	0.00472	0.00000		

originating from a larger geographical area than any other natural population sampled for this study (source plants occurred up to 4 km apart in separate sub populations; Belfield et al., 2011). The lowest levels of allelic diversity observed in source (natural, remnant) populations, Mānā ( $n = 2$ ) and Kāohikaipu ( $n = 1$ ) had outplanted counterpart populations that were also the least diverse (Makauwahi allelic richness = 1.05; expected/observed heterozygosity = 0.026/0.008; Kāohikaipu outplantings allelic richness = 1.09; expected/observed heterozygosity = 0.085/0.141)

Expected/observed heterozygosities averaged over loci were compared between natural and outplanted populations of *Sesbania tomentosa* and in most cases the values calculated for the natural populations were reduced in their outplanted counterparts. The exceptions were at Lāwa‘i Kai outplantings and, to a lesser extent, in Ka‘ena point State Park–NAR outplantings, both being derived from greater than 10 source plant individuals. Expected/observed heterozygosity averaged over three sampling years at Polihale (2006, 2009 and 2010) rose from 0.217/0.080 to 0.267/0.182 in Lāwa‘i Kai outplantings (seeds sourced from Polihale 2004). Expected/observed heterozygosity at Ka‘ena point rose from 0.219/0.077 to 0.166/0.098 in the outplantings (Table 4.2). Mean number of alleles per locus and mean allelic richness (averaged over loci) were mostly all reduced in a similar manner, again the exception being at Lāwa‘i Kai outplantings (rising from 1.77 to 2.30 alleles per locus), and to a lesser extent, in Ka‘ena point NAR outplantings (rising from 1.66 to 1.75 alleles per locus). Private alleles and the percentage of rare alleles (frequency < 0.1) also declined in outplanted populations, except for at Lāwa‘i Kai where private alleles remained the same and the percentage of rare alleles rose slightly from 38.1 to 40.9%. The only instance where the number of monomorphic loci declined in the outplanted population was Lāwa‘i Kai (monomorphic loci averaged over three sampling years at Polihale declined from 4.67 to 1.00 in Lāwa‘i Kai). Neither the Lāwa‘i Kai nor Ka‘ena point NAR outplantings were significantly differentiated from their source populations (Table 4.3).

Notable instances of genetic decline in outplanted populations of *Sesbania tomentosa* include Kīpuka Nēnē, where only two to three founders were used to source a population. These outplantings lost twice as many rare alleles and had lower allelic richness and observed heterozygosity values than their sister outplanted population Kulanaokuaiki, where more than 10 founders from the Kīpuka Nēnē–Hilina pali population were used as a source. Both of these outplanted populations were missing eleven low-frequency private alleles found in their source



population (Table 4.2), and both exhibited significant genetic differentiation from their source population at the 1% nominal level after Bonferroni corrections (Table 4.3).

The Kanahā County Beach Park outplanted population (founded from four individuals) is also notable in its dramatic decline, where 14 alleles were lost from the source population at Papanalahoā–Nākālele (alleles with an average frequency of 0.32), four previously polymorphic loci became fixed for a single allele, and measures of heterozygosity and allelic diversity were all cut in half (Table 4.2). One of the highest rates of genetic differentiation detected (0.635) was observed between Kanahā and its source population at Papanalahoā–Nākālele (Table 4.3). Four genotypes, each having eight out of nine loci fixed for a single allele, made up 89% of the Kanahā outplantings; the remaining 3 individuals had completely unique genotypes (data not shown).

Inbreeding coefficients ( $F_{IS}$ ) within populations were measured where  $F_{IS} = -1.0$  indicates 100% heterozygosity of individuals,  $F_{IS} = 0.0$  indicates the observed number of heterozygotes equals the number expected based on allele frequencies, and  $F_{IS} = 1.0$  indicates the complete absence of heterozygotes in a population with multiple alleles per locus. Coefficients averaged over nine loci were compared between natural and outplanted populations and in three cases (Kulanaokuaiki, Kanaio U.S. Army Training Area, and Makauwahi) the inbreeding coefficients of outplanted populations were greater than those of their corresponding source (natural) populations (Table 4.2). At Kīpuka Nēnē, Ka‘ena point, Kanahā and Lāwa‘i Kai, inbreeding coefficients in outplanted populations were reduced compared to those of their corresponding source (natural) populations (Table 4.2).

#### Indirect estimates of genetic bottlenecks

The Wilcoxon tests carried out in BOTTLENECK revealed evidence for recent, rapid losses of genetic diversity in only three populations of *Sesbania tomentosa*, two of which were natural (source) populations. A significant population decline is estimated to have taken place in the Polihale (2010) populations based on all three mutation models examined, and in the Ka‘ena point State Park–NAR based on two out of three models examined (Table 4.4). Kanaio U.S. Army Training Area was the only outplanted population to show evidence for a recent, significant population decline, based on all three mutation models ( $P \leq 0.05$ ). The distribution of

Table 4.4. Tests for genetic bottlenecks in natural *vs.* outplanted representative populations of *Sesbania tomentosa*. Mode shift indicates deviation from the L-shaped distribution of allele frequencies expected under mutation-drift equilibrium. Wilcoxon tests for heterozygote excess (Piry et al., 1999) under three mutation models (step-wise mutation, SMM; two phase model, TPM; infinite alleles model, IAM). Values highlighted in bold are those indicative of a bottleneck ( $P \leq 0.050$ ).

Population	Island	<i>n</i>	Mode Shift	Wilcoxon tests:		
				SMM	TPM	IAM
Natural population:						
Kīpuka Nēnē–Hilina pali	Hawai‘i	35	normal	0.900	0.320	<b>0.009</b>
Outplanted representative populations:						
Kīpuka Nēnē	Hawai‘i	7	shifted	0.973	0.945	0.945
Kulanaokuaiki	Hawai‘i	21	normal	0.629	0.473	0.273
Natural population:						
Pu‘u Pīmoē	Maui	9	shifted	0.918	0.411	<b>0.024</b>
Outplanted representative population:						
Kanaio U.S. Army Training Area	Maui	6	shifted	<b>0.037</b>	<b>0.014</b>	<b>0.009</b>
Natural population:						
Papanalahoā–Nākālele	Maui	44	normal	0.326	0.082	<b>0.007</b>
Outplanted representative population:						
Kanahā County Beach Park	Maui	28	normal	0.984	0.969	0.953
Natural population:						
Nu‘upia Ponds (Mōkapu)	O‘ahu	2	shifted	0.578	0.578	0.578
Mokulē‘ia State Tree Nursery (2009): origin: Nu‘upia Ponds	O‘ahu	8	shifted	0.932	0.921	0.910

Table 4.4. (Continued) Tests for genetic bottlenecks in natural vs. outplanted representative populations of *Sesbania tomentosa*.

Population	Island	<i>n</i>	Mode Shift	Wilcoxon Tests:		
				SMM	TPM	IAM
Natural population:						
Kāohikaipu	O‘ahu	1	shifted	1.000	1.000	1.000
Mokulē‘ia State Tree Nursery (2009):						
origin: Kāohikaipu	O‘ahu	11	shifted	0.986	0.955	0.432
Natural populations:						
Ka‘ena point State Park–Natural Area Reserve (NAR)	O‘ahu	33	shifted	0.313	<b>0.047</b>	<b>0.031</b>
Outplanted representative population:						
Ka‘ena point NAR	O‘ahu	32	shifted	0.906	0.438	0.063
Natural populations						
Polihale State Park (2006)	Kaua‘i	16	shifted	0.125	0.063	0.063
Polihale State Park (2009)	Kaua‘i	11	normal	0.781	0.656	0.344
Polihale State Park (2010)	Kaua‘i	12	shifted	<b>0.031</b>	<b>0.031</b>	<b>0.031</b>
Outplanted representative populations:						
Lāwa‘i Kai	Kaua‘i	14	normal	1.000	1.000	0.875
Origin of <i>ex-situ</i> source population:						
Mānā	Kaua‘i	2	shifted	1.000	1.000	1.000
Outplanted representative populations:						
Makauwahi	Kaua‘i	14	normal	1.000	1.000	1.000

alleles in these same populations was also indicative of a bottleneck, as alleles at low frequency were found to be less abundant than alleles at intermediate frequency (a “mode shift”), a trend observed in an additional five source (natural) and four outplanted populations as well (Table 4.4).

## Discussion

An analysis of molecular variance (AMOVA) in Chapter 2 revealed that 56% of the genetic variation was found within populations of *Sesbania tomentosa* (Table 2.7), and efforts to create new populations should take care to maintain this variation. Extreme cases of genetic erosion in many of the restored populations sampled, as measured by increased inbreeding and loss of alleles, imply that material used for outplanting was the offspring of very few outcrossed parents. On the other hand, loss of heterozygosity might be explained by seed collectors having inadvertently obtained groups of progeny that were the result of selfing (geitonogamous or otherwise). Low inbreeding coefficients were observed at Kanahā Beach Park, the Mokulē‘ia state tree nursery seedlings originating from Kāohikaipu and Nu‘upia and in the *ex situ* source population for the plants originating from Mānā, yet these were probably due to extremely low sample size and/or allelic diversity constraints on calculations.

Evidence for recent bottlenecks in the form of transitory heterozygote excess was largely unsubstantiated in the BOTTLENECK analysis. When population size becomes very small (~10 or fewer individuals) and when generation times are short as with most populations of *Sesbania tomentosa* [e.g., Hopper (2002) reported a longevity of 3 to 10 years at Ka‘ena point], a new mutation-drift equilibrium should be arrived at quite rapidly (Watterson, 1984).

Of the eight source (natural) populations of *Sesbania tomentosa*, Kīpuka Nēnē–Hilina pali and Pu‘u Pīmoe exhibited the highest values of allelic diversity. Accordingly, the highest values of allelic diversity of all nine outplanted populations were observed in the outplanted counterparts of these two populations, suggesting that the standing variation of a founding population is an important baseline determination of possible levels of diversity that can potentially be captured in an outplanted representative population.

The strategy used at Hawai‘i Volcanoes National Park to source the Kulanaokuaiki outplantings involved mixing five separate subpopulations located 2 km apart (combined

together here as the Kīpuka Nēnē–Hilina pali population). This is the sole case of material collected from separate subpopulations being combined into a single outplanted population that was genetically analyzed in this study. The average  $F_{ST}(\theta)$  among these five subpopulations was 0.39 (see Table 2.8). This relatively high amount of differentiation between subpopulations has significant management implications. If it had arisen from genetic drift acting over a long period of time, there would be more reason for maintaining subpopulations separately instead of mixing them *in situ*. On the other hand, if the differentiation was due to more recent and rapid genetic drift due to population decline and fragmentation of habitat, random allele loss is playing the predominate role in high  $F_{ST}(\theta)$  values. The subpopulations would then be more appropriately managed by mixing to regenerate much of the original genetic diversity. Similar to other legumes, *Sesbania tomentosa* is a preferred food for feral goats (*Capra aegagrus hircus*) that have long been a problem in this area of the park; 15,000 animals persisted in the area around Hilina pali as recently as the 1970's (Baker and Reeser, 1972; Katahira and Stone, 1982). In addition, extensive wildfires burned through Kīpuka Nēnē twice in the last 40 years, and can also be expected to have caused dramatic declines in the *S. tomentosa* population there as well. It appears that the decision to mix subpopulations on the part of park resource management personnel was therefore guided and sound.

At the Kanaio U.S. Army training area, it appears that a small number of *Sesbania tomentosa* individuals captured levels of diversity above normal when compared with the other examples listed in Table 4.2. For example, two private alleles (average frequency = 0.21) were found in these outplantings that were not found in their source population at Pu'u Pīmoe at the time of sampling there in 2006. This was entirely by chance, as only three founding individuals were used to source the seed for Kanaio U.S. Army training area (seeds collected at Pu'u Pīmoe 2002), yet emphasizes the need to maximize founders in case such genetically unique individuals happen to be present.

The individuals comprising these small remnant source populations of *Sesbania tomentosa* may vary over time due to seed bank recruitment and/or extirpation of individual plants. For example, the Nu'upia ponds outplantings were derived from only two of the three plants extant in the natural population at the time of sampling (due to the lack of fruit set on one individual at the time of seed collection). All measures of genetic diversity declined, including the loss of 8 alleles from the omission of a single (possible) founding individual (Table 4.2). The

opposite occurred in the Makauwahi outplantings, derived from three *ex situ* founding individuals (natural population previously extirpated), only two of which survived to collect DNA from for this study in 2006. As a result, the outplantings preserve an additional allele and slightly higher measures of genetic diversity overall than the existing *ex situ* source. Similarly, the Kāohikaipu individual extant at the time of collection in 2009 (here representing the entire natural source population) happened to be slightly less genetically diverse than the two individuals on that islet at the time seeds were collected for outplantings (two years prior), in that a single allele was missing in the extant natural source population (Table 4.2).

Lāwa‘i Kai is another example of where the seed sourcing practices used maintained levels of genetic diversity above that contained in the natural standing population of *Sesbania tomentosa* at Polihale State Park during the years 2006, 2009 and 2010 (Table 4.2). Over 400 pods were collected from 14 founding individuals during the summer of 2004 to create the outplantings for Lāwa‘i Kai (Mike Demotta, NTBG, personal communication). This is one of the best cases of maximizing founders analyzed for this study, which fortunately happened one reproductive cycle (approximately 1 year) after the population had “flushed”. Similarly, 2009 was another year in which the Polihale population had rebounded in numbers and the levels of diversity rose (and indications of bottlenecks fell) when compared to diversity levels among 2006 collections. From this, it was deduced that the population “flush” of 2003 produced a similar result in standing genetic diversity of the founding population in 2004, which led to the results obtained here.

A relatively high percentage of the alleles in both the Kulanaokuaiki (36.0%) and Lāwa‘i Kai (40.9%) populations occurred at a frequency less than 0.1, yet the importance of rare alleles in the restoration of plant populations has been debated. Some argue it is not necessary to capture all the genetic variation in a species as rare alleles may in fact be recent mutations or deleterious and are likely to be lost in a few generations of random mating (Brown and Briggs, 1991; Holsinger and Gottlieb, 1991). In the case of *Sesbania tomentosa*, it is possible that inbreeding has purged deleterious alleles long ago (see Chapter 2) and that we need not leave it to random mating *in situ* to preserve such alleles. For instance, rare alleles might become more common in outplanted populations if an effort to cross fertilize individual plants to maximize genetic diversity in offspring took place; this study has helped to identify individuals appropriate for such measures.

Regardless of the advantages that maximizing genetic diversity should theoretically impart over the long run, mortality of *Sesbania tomentosa* outplantings has been high in some of the sites that were shown to have captured the highest amounts of genetic diversity in this study. For example, of the 177 outplantings at Kulanaokuaiki and Kīpuka Nēnē, 12.9% survived 50 months post-planting (Belfield et al., 2011) yet only 3–4 outplantings (approximately 1.6%) survived as of 2015 (Joshua VanDeMark, Plant Extinction Prevention Program, personal communication). The preponderance of the exotic natal redtop grass (*Melinis repens* (Willd.) Zizka) may be affecting water balance and increasing competition with *S. tomentosa* in these two sites (Belfield et al., 2011). In addition, as of 2014 all individuals at both the Pu‘u Pīmoe population and Kanaio U.S. Army Training Area had been extirpated (Keahi Bustamente, Plant Extinction Prevention Program, personal communication), probably due to an extended period of drought in SE Maui since 2006. The outplanted individuals at Lāwa‘i Kai also suffered from high mortality (possibly due to root knot nematodes (David Burney, NTBG, personal communication) and had to be periodically replaced by new plantings on site. On the other hand, the outplantings at Ka‘ena point (O‘ahu) are some of the most successful in Hawai‘i- often up to 94% of outplantings survive 6 months post planting, and a substantial amount have seemed to survive indefinitely (15 years later; David Smith, Hawai‘i Department of Land and Natural Resources, personal communication). This success is notable given the relative lack of genetic diversity observed in this study in both the outplantings and the standing natural population at Ka‘ena point.

## Conclusion

In all of the above examples of outplanted populations of *Sesbania tomentosa*, it will be important to monitor the occurrence of the plants that establish themselves from seeds produced by the reintroduced plants, and their progeny for impact of genetic drift in the future generations. The outplanting efforts and strategies of the National Tropical Botanical Garden and Hawai‘i Volcanoes National Park were the most successful at capturing genetic diversity in this species when compared with other examples. This comparison is most notable at Kanahā County Beach Park where a large population was ineffectively used to source a relatively large number of outplantings (only 4 founders out of 44 potential source plants) resulting in extremely low

genetic diversity and one of the highest rates of genetic differentiation from source to founder population. In spite of the low diversity at Kanahā (and therefore low theoretical expectations for long-term success), almost all of the outplantings survived and flourished in the coastal dune habitat for over five years until all of them were killed in a six foot tidal surge event caused by the 2011 Tōhoku Earthquake in Japan. Twelve new plants were recorded at Kanahā in 2014, apparent seedlings sprouted from the seedbank after all the plants had perished in 2011 (Forest Starr, USGS, personal communication). This was a true test of the population's health and resilience. The only other instance of seedling recruitment in an outplanted population was at the Makauwahi site (David Burney, NTBG, personal communication), also a population with low genetic diversity. The Kanahā site receives ca. 400 mm of rain per year (vs. the Lāwa'i Kai/Makauwahi sites which receive ca. 980 mm and Kulanaokuaiki/Kīpuka Nēnē which receive ca. 1860 mm of rain per year; Giambelluca et al., 2014). Drought conditions at each site immediately post planting are probably more relevant for mortality, as is the consistency of rainfall in the years that followed. In each case, it would appear that lack of adequate rainfall at the less successful sites can be ruled out as a factor in their decline.

The long-term survival of outplantings of *Sesbania tomentosa* might not depend upon genetic diversity as much as other unforeseen factors. For example, a comparison of horticultural methods used to raise and outplant this species is likely to offer more insight and assistance than information on genetic diversity for resource managers across the state. Regardless, it has been shown that the allelic diversity changes from year to year in the Polihale population of *S. tomentosa*, and this pattern is likely to be true in other populations that fluctuate and resprout intermittently from a seedbank. Collecting from a large number of founders, as widely spaced apart as is possible (on a local geographic scale), is shown to play an important role in preserving genetic diversity in outplanted populations. Collecting from founders spaced apart temporally (e.g., the same population in subsequent years) might produce additional, unique founders and may be an important strategy to consider, as well as focusing collection activities during years in which natural populations are rebounding in numbers ("flushing"). As expected, the standing variation of founders being collected from in any given year is of primary importance in raising genetically diverse seedlings.



## CHAPTER 5

### Synthesis of hypotheses and findings

The hypothesis that Hawaiian *Sesbania* form a monophyletic group and represent a recent radiation among the Hawaiian Islands was accepted as sequence diversity was shown to be virtually non-existent at the two nuclear regions sampled for this study. In addition, the monophyletic group containing Hawaiian *Sesbania* was also found to include *S. marchionica* from the Marquesas. The hypothesis that the formal recognition of additional taxa of Hawaiian *Sesbania* may be warranted was also accepted, although DNA sequence data provided no evidence (by itself) for splitting *S. tomentosa* into additional species. The evidence was found instead in the microsatellite analysis where Bayesian genetic clustering assignments and associated private alleles occurred in a distinct phylogeographic pattern. As a result, populations from Nihoa, Kaua‘i and O‘ahu are distinguished as a separate subspecies of *S. tomentosa*, populations from Maui Nui and Hawai‘i Island (respectively) form two additional subspecies, and a fourth subspecies endemic to SE Moloka‘i distinguishes itself from the rest of Maui Nui.

The hypothesis that populations will exhibit high levels of genetic structure with evidence of inbreeding within and divergence among populations was also accepted. Global  $F_{ST}$  over all populations and loci was estimated at 0.39 (or as high as 0.50 if including apparent clonal genotypes in the dataset) and inbreeding coefficients ( $f$ ) were estimated at 0.56 (ranging as high as 0.94). Strong spatial genetic sub-structure was also observed within populations and sub-populations. These results, considered in light of previously published observations of pollination in *Sesbania tomentosa*, infer that this species is predominately inbreeding due to sib and/or self-mating. The hypothesis that levels of inbreeding will be higher, and genetic diversity lower, in outplanted populations than in their naturally-occurring counterparts is not accepted, as inbreeding coefficients were shown to be extremely high in natural as well as outplanted populations. In certain cases, genetic diversity rose and inbreeding coefficients fell when outplanted populations were compared with their naturally-occurring counterparts. Genetic diversity was shown to be dynamic over time in natural populations whose members fluctuate and resprout intermittently from a seedbank. The standing variation of founders being collected from in any given year is therefore of primary importance in raising genetically diverse seedlings.

The hypothesis that natural selection in different environments over time combined with contemporary fragmentation (isolation) of populations caused Hawaiian *Sesbania* to separate into the distinctive appearing populations found today is also tentatively accepted. Ecologically, this rapidly maturing species appears prone to inbreeding and repeated bottlenecking, adding efficiency to a natural trend of divergence. Yet it is entirely plausible that both the microsatellite as well as the morphological differentiation observed have been accentuated within the time period when populations of *S. tomentosa* became increasingly isolated from one another. In other words, more modern impacts on the range of the species have probably only accelerated what was already naturally-occurring. The relative contributions of contemporary vs. long term impacts on population differentiation are impossible to completely disentangle, yet evidence is herein presented which points to differentiation that was largely underway prior to historic fragmentation of populations.

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