ANALYSIS OF RELATIONSHIPS AMONG ENDEMIC HAWAIIAN *HIBISCUS*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPY

IN

HORTICULTURE

May 2013

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Keywords: *Hibiscus,* RAPD markers, nectar, pollination

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DEDICATION

This dissertation is dedicated to the memory of my parents who were avid gardeners and naturalists.

ACKNOWLEDGEMENTS

I would like to thank the Garden Club of Honolulu, the Morden Lab in Botany at the University of Hawaii at Manoa and the Harold L. Lyon Arboretum for assisting with the funding for this study that made it possible. I would also like to thank my committee members for their direction and support during this project and Robert Paull for his assistance with nectar analysis and use of his lab. I am very grateful for invaluable assistance in the lab from Mitsuko Yorkston and the student assistants in the Morden Lab: Chelsea Osaki, Dylan Morden, Bao Chen, Allegra Pan, and Gabby Cravalho, and to Richard Pender for assistance with nectar analysis. I thank Susan Takehashi Shirley Ishihara and Eileen Herring for their technical support and Cliff Smith for his encouragement and support of the value of this study. David Orr, Steve Perlman, Hank Oppenheimer, Mashuri Waite, Matt Keir, Lauren Weisenberger, Alvin Yoshinaga, Leon Marcus, Joel Lau, John Obata, Ron Fenstemacher, Carol Nakamura, Karen Shigematsu, Patty Moriasu, Nellie Sugii and Heidi Bornhorst were invaluable in assisting with collection of plant material and technical support. I would like to thank Waimea Arboretum, the Army Natural Resources, Environmental Division, Conservation and Restoration Branch, and the National Tropical Botanical Garden for access to their collections.

Finally, I thank my family and friends for their constant encouragement and support during this project especially Steve Kramer.

ABSTRACT

There are 9 taxa of endemic *Hibiscus* section *Lilibiscus* in the Hawaiian Islands putatively derived from a single colonizing ancestor. Three of these taxa are federally listed as endangered. In the two complete taxonomic treatments of this group based on morphology, there is disagreement over the names and numbers of species and subspecies. This study was undertaken to examine the genetic relationships of these plants in an effort to clarify species boundaries. Randomly amplified polymorphic DNA (RAPD) markers were used to assess variation within and among populations and species. RAPD analysis demonstrated that the four previously recognized red-flowered taxa were genetically distinct as well as morphologically distinct from each other, and the extent of differentiation in *H. kokio* subsp. *kokio* (*H. kahilii*) and *H. kokio* subsp. *saintjohnianus* supports their recognition as separate taxa from *H. kokio.* The RAPD data indicates that the four red-flowered taxa should be recognized by their previously used names, *H. clayi, H. kahilii, H. kokio* and *H. saintjohnianus.*

RAPD analysis of the white-flowered taxa demonstrated that five genetically distinct taxa were evident and that *H. arnottianus* subsp. *immaculatus* on Molokai was more similar to *H. waimeae* subsp. *waimeae* on Kauai than to *H. arnottianus* subsp. *arnottianus* on Oahu. Also, *H. arnottianus* subsp. *immaculatus* was more closely related to *H. waimeae* subsp. *waimeae* than *H. waimeae* subsp. *hannerae* was to *H. waimeae* subsp. *waimeae* even though the latter two are both from Kauai. *H. arnottianus* subsp. *punaluuensis* was unexpectedly very distinct genetically from all the other white-flowered taxa. The Manoa Cliffs population of *H. arnottianus* subsp. *punaluuensis,*

whose range overlaps with *H. arnottianus* subsp. *arnottianus* at this location, was more similar to the other white-flowered taxa than to the Pali, Oahu population of *H. arnottianus subsp. punaluuensis*. However, the Manoa Cliffs *H. arnottianus* subsp. *punaluuensis* was genetically distinct from *H. arnottianus* and *H. waimeae*, and may represent an isolated hybrid population of *H. arnottianus* x *H. punaluuensis*. Because of these genetic distinctions, in addition to morphological distinctions described in earlier treatments, each of these four taxa is being recognized as a distinct species: *H. arnottianus* A. Gray, *H. immaculatus* M. Roe, *H. hannerae* (O. Degener & I. Degener) Huppman (new combination), and *H. waimeae* A. Heller.

There are marked morphological differences between the red and white-flowered endemic *Hibiscus* section *Lilibiscus* with the reds having floral characteristics associated with bird pollination and the whites having characteristics associated with hawk moth pollination. The flower nectar sugars of these two groups were compared to ascertain whether there were differences in nectar concentration and the relative percentages of fructose, glucose and sucrose in the nectar that could be correlated with generally accepted pollination syndromes reported for these two pollinator types. High-performance liquid chromatography (HPLC) and refractometry were used to analyze floral nectar. The nectar concentration averages (± one SD) for the red-flowered and white-flowered taxa were very similar, 15 ± 5.7% soluble sugars in the reds and 17 ± 6.9% in the whites. The HPLC data was also very similar across species in the two flower types, averaging 45% fructose, 55% glucose and negligible amounts, if any, of sucrose. The nectar in all endemic Hawaiian *Hibiscus* section *Lilibiscus* is characteristic of what has been generally observed in passerine bird pollinated flowers, dilute and

predominately glucose and fructose (hexose-dominant). These results do not entirely follow the general concept that hawkmoth pollinated (white, fragrant) flowers have nectar that is more dilute and sucrose-dominant rather than hexose-dominant. Though the nectar was dilute in the white-flowered species (as expected for pollination by hawkmoths), it was hexose- rather than sucrose-rich. Selection pressure appears to have been considerably stronger on flower morphology than on nectar sugar content. Another possibility is that the genetic factors controlling adaptation of nectar sugars to pollinators in this group are phylogenetically constrained whereas those controlling characters in flower morphology, such as size, color and fragrance are more variable.

Hibiscus brackenridgei is a federally endangered Hawaiian endemic species and Hawaii's state flower. It occurs on Oahu, Lanai, Maui and Hawaii Island, mostly in very small, isolated populations in dry shrublands where it is vulnerable to fire and invasive species. Conservation managers require correct taxonomic classification to plan management strategies for this rare species. Randomly amplified polymorphic DNA (RAPD) markers were used to assess variation within and among populations in the two extant currently recognized subspecies, *H. brackenridgei* subsp. *brackenridgei* and *H. brackenridgei* subsp. *mokuleianus*, and to examine the boundaries between the two subspecies. Two more recently discovered populations on Oahu (Makua and Keaau) were also investigated to examine their relationships to the two previously described subspecies. RAPD analysis demonstrated that three of the four Oahu populations clustered closely together, including the one at Keaau, and were clearly distinct from all the populations on the other islands as well as the Makua Oahu population. The Makua plants clustered most closely with the populations from the other islands, particularly the

Keomuku, Lanai population. The analysis of the relationship of the two Lanai populations indicates that, though they fall within *H. brackenridgei* subsp. *brackenridgei,* they are more closely related to populations on other islands than to each other: Kanepuu to Hawaii Island individuals, and Keomuku to Maui individuals. The RAPD data are mostly in agreement with the current circumscription of extant *H. brackenridgei* subspecies: *H. brackenridgei* subsp. *brackenridgei* on Lanai, Maui and Hawaii Island, and *H. brackenridgei* subsp. *mokuleianus* on Oahu. The one exception is the Makua, Oahu population that clearly does not align closely with the three other Oahu populations sampled (Keaau, Kealia-Kawaiu, and Waialua). A more in depth study of the Makua plants based on morphology and molecular analyses is needed to determine if this population should be included in *H. brackenridgei* subsp. *brackenridgei* with the plants from Lanai, Maui and Hawaii Island or be placed in possibly another subspecies. Management for conservation of this species is discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

Genetic Variation in Endemic Species of Hawaiian *Hibiscus* section *Lilibiscus* (Malvaceae) Based on RAPD Analysis

Introduction

The Genus Hibiscus

The genus *Hibiscus* (Malvaceae) is a large and diverse group of herbs, shrubs and trees found in a wide variety of habitats around the world. Linnaeus named the genus after the Greek word for mallow, *hibiskos*, and described the genus in 1737 in *Genera Plantarum* (Bates 1965, Staples and Herbst 2005). The genus has a complicated taxonomic history due to disagreement over the value of various morphological characters used to describe the group which has resulted in the number of species in the genus varying greatly from 200 to 675 depending on the author (Bates 1965, Fryxell 1997, Mabberley 2008). A phylogeny of *Hibiscus* and the tribe Hibisceae (Pfeil 2002), using the chloroplast DNA sequences *ndhF* and the *rpl16* intron, found that some genera in Hibisceae were nested within *Hibiscus* and also that *Hibiscus* was a paraphyletic group, requiring that the genus *Hibiscus* be either expanded to include all these genera or be split up into ten or more new genera. The larger number of species above (675) is the result of recent treatments that now include *Abelmoschus, Decaschistia, Goethea, Kosteletzkya, Malvaviscus, Pavonia, Senra* and *Taliparitì* in *Hibiscus* (Mabberley 2008). The smaller number is based on species that share at least the following characters: 5-celled loculicidally dehiscent capsules with many reniform seeds, a 5-branched style and a regularly lobed, non-spathelike calyx (Bates 1965, Fryxell 1997). However, other morphological characters are also often used to characterize the genus including alternate,

usually palmately veined leaves, flowers with five petals, and a tubular staminal column formed by fusion of the filaments that encloses a style that terminates in 5 branches (Roe 1961, Bates 1965, Beers and Howie 1987, Bates 1990, Staples and Herbst 2005).

Most *Hibiscus* species are found in the tropics and subtropics, but some are from temperate climates and many are important economic crops providing fiber or timber (*H. cannabinus, H. elatus, H. hamabo, H. macrophyllus, H. tiliaceus*), food or drinks (*H. acetocella, H. esculentus, H. heterophyllus, H. manihot,* and *H. sabdariffa*), shoeshine or dye (*H. rosa-sinensis*), and paper (*H. mutabilis*) (Bates 1965; Pfeil 2002; Schlueter 2003; Mabberley 2008). The Hawaiians had multiple traditional uses for the indigenous *H. tiliaceus* (*Talipariti tiliaceum*), *hau* in Hawaiian (Bates 1965, Neal 1965, Abbot 1992, Krauss 1993, Krauss 2001). The lightweight wood could be used for starting fires, booms for canoes and floats for fishing nets, and the bark could be made into cordage or rope for nets, sandals or *lei.* In traditional Hawaiian medicine the slimy sap found in the inner bark or flowers of *H. tiliaceus* was used as a laxative, to aid in childbirth or to reduce chest congestion. The flower petals of *H. tiliaceus* and other Hawaiian native *Hibiscus* (*kokio*) were used to make pink dyes.

Hibiscus species are economically important in the ornamental plant industry in tropical and temperate regions where they are grown as garden, container or greenhouse plants. Two temperate species that have been used in breeding programs for ornamental use are *H. syriacus* native to eastern Asia and often referred to as the rose-of-Sharon or shrub althea, and *H. moscheutos*, the rose mallow from North America (Bates 1965, Staples and Herbst 2005, Mabberley 2008). The large, colorful flowers of hibiscus have become a classic symbol of the tropics, called by some “the Queen of the Tropics,” and

numerous cultivars of tropical ancestry are readily available in garden centers in the tropics as well as in temperate regions where they are considered annuals such as Yoder Brothers, Inc.'s series "TradeWinds Everblooming Hibiscus" (cultivars of *H. rosa-sinensis*) bred to be used as houseplants, patio or bedding plants.

The tropical varieties popular as ornamentals are believed to be derived primarily from *H. rosa-sinensis*, often called the Chinese hibiscus or Shoe-black Plant, a "species" of unknown origin. *Hibiscus rosa-sinensis* is known only from cultivated sources, and includes numerous varieties (Gast 1980, Beers and Howie 1987, Schlueter 2003, Staples and Herbst 2005, Mabberley 2008). Because of its probable hybrid origin, Gast recommended that a more correct name would be *H.* × *rosa-sinensis* (Schlueter 2003). The first collections of *H. rosa-sinensis* by Europeans were of a double flowered form. A description and illustration of a double hibiscus were published in 1678 by Van Reede, and a double flowered form and other forms labeled as *H. javanica* were in the collection at the Chelsea Physic Garden in London in 1731 (Beers and Howie 1987, Schlueter 2003). Before Europeans reached tropical Asia, *H. rosa-sinensis* had apparently already been propagated by people for some time and distributed throughout the region (Staples and Herbst 2005). When Captain James Cook first arrived in Tahiti in 1769, his botanist discovered a double-flowered hibiscus and subsequently named it *H. floraplena* (Gast 1980, Beers and Howie 1987). This form was also reportedly observed by other European explorers in the Marquesas and Tonga, and was considered at that time an ornamental form rather than a distinct species. In Polynesia, the red hibiscus was sacred according to an old account that tells of a man being clubbed to death for walking in front of a temple wearing a flower on his ear (Neal 1965). Other tropical species in section

Lilibiscus that are believed to be closely related to *H. rosa-sinensis* have been used in crosses with this species. This includes some species endemic to Hawaii (*H. arnottianus, H. kahilii, H. kokio, H. kokio* subsp. *saintjohnianus* and *H. waimeae*), Africa (*H. schizopetalus*), islands of the Indian Ocean (*H. liliiflorus, H. boryanus,* and *H. fragilis*), Fiji (*H. storckii*), and others of unknown origin (*H. denisonii,* possibly from Australia) (Wilcox and Holt 1913, Neal 1965, Gast 1980, Nakasone and Rauch 1980, Schlueter 2003).

Chromosome Variation in Hibiscus

A wide range in chromosome numbers has been reported for the large and diverse genus *Hibiscus.* Kachecheba (1972) concluded that the various species he studied from 10 different sections represent a polyploid series ranging from $2n = 28$ to $2n = 170$ with the basic number $x = 21$ for the probable haploid number of the diploid ancestor of the section *Lilibiscus*. Research in section *Lilibiscus* has shown that all of the Hawaiian species studied have approximately the same chromosome number: $2n = 80$, 82 or 84 (Niimoto 1966, Kachecheba 1972). However, chromosome numbers were $2n = 170$ have been reported for *H. rosa-sinensis*, and $2n = 42$ for *H. schizopetalus* (Kachecheba 1972).

The Hibiscus Breeder's Manual (Fister 1985) attributes the ease of crossing between the various species and hybrids in section *Lilibiscus* to polyploidy. Paun *et al*. (2007) reported that polyploidy and hybridization are "major phenomena" in plant evolution as sources for more genetic variation, possibly resulting in reproductive isolation from parent plants which then functions as a starting point for speciation and adaptive radiations.

Hibiscus *in Hawaii*

Today the major centers for breeding hibiscus in section *Lilibiscus* are Florida, California and Australia, but this multi-million dollar industry had its roots in Hawaii in the early 1900's (Wilcox and Holt 1913, Neal 1965, Gast 1980, Nakasone and Rauch 1980, Schlueter 2003). The first record of breeding hibiscus in Hawaii is from the 1870's when then Governor Archibald Cleghorn created 12 new varieties (Roe 1961, Nakasone and Rauch1980). By the early 1900's, Hawaii became a center for hibiscus hybridization partly because of the availability of attractive native species as well as introduced species and varieties that could be crossed with *H. rosa-sinensis.* In 1911, the Hawaiian Hibiscus Society was formed (Wilcox and Holt 1913, Roe 1961, Bates 1965, Neal 1965, Gast 1980, Nakasone and Rauch 1980, Beers and Howie 1987, Schlueter 2003). The popularity of these flowers in Hawaii is evident in the choice of a native hibiscus as the official flower of the Territory of Hawaii in 1923, initially *H. kokio* but now *H. brackenridgei* (Degener and Degener 1977, Beers and Howie 1987).

Horticulturists are still using Hawaii's native species in hybrid hibiscus breeding programs and there is a growing demand for native species in the nursery industry as consumers learn more about the environment and the dangers associated with introduced ornamental plants that can become weeds. Hawaii's native *Hibiscus* species are currently quite popular and some local nurseries and botanical gardens have active selection and breeding programs. One outstanding example is *Hibiscus arnottianus* subsp. *arnottianus* 'Kanani Kea,' wild collected by John Obata on Tantalus, Oahu and named by Bob Hirano at Lyon Arboretum (Figure 1.1). This selection has large white petals with better than

FIGURE 1.1. Variation in petal size in wild collected individuals of *H. arnottianus* subsp. *arnottianus* from Oahu. The flower on the left is *H. arnottianus* subsp. *arnottianus* 'Kanai Kea,' from Manoa Cliffs, Koolau Mountains. This cultivar was selected for horticultural use because of the exceptionally wide petals. The flower on the right was collected from Palikea, Waianae Mountains, and was previously considered *H. arnottianus* f. *parviflora* Skottsb.

average overlap as well as very good fragrance. The flower lasts for 2 days, as is the case for all but one of the native white hibiscus, *H. arnottianus* subsp. *immaculatus*.

Fosberg (1948) estimated that the Hawaiian *Hibiscus* species were derived from four separate colonization events: one for the endemic red species (*H. clayi, H. kahilii* and *H. kokio*) and white (*H. arnottianus* and *H. waimeae*), one for the endemic *H. brackenridgei*, and one each for the 2 indigenous species, *H. tiliaceus* and *H. furcellatus* (Bates 1990). *Hibiscus* sect. *Lilibiscus* appears to follows the "progression rule" (Wagner and Funk 1995), a pattern of speciation observed in many endemic species in Hawaii where species descended from a single ancestor on the older islands with subsequent colonization of younger islands as they appeared. A pattern associated with this is the occurrence of more species in the radiation on the older islands than the younger islands, as is the case also with *Hibiscus*. Using Bates' circumscription (1990), there are five taxa on Kauai, three on Oahu, two on Molokai, and one each on Maui and Hawaii. Among the red and white species, there is quite a bit of variability in terms of flower color, size (Figure 1.2), shape, leaf size and shape, plant growth habits and drought tolerance. Flowers are red, orange, yellow or white and the white-flowered species are fragrant. Some species have miniature flowers less than 7 cm across (*H. clayi*) while others may have blooms more than 15 cm in diameter (*H. arnottianus* subsp. *punaluuensis*).

There is great potential for more selection from wild individuals with desirable horticultural characteristics. However, for more than 100 years there has been confusion over the names and the exact number of *Hibiscus* species endemic to Hawaii, especially with regard to the red-flowered species. Insufficient collection information and differing opinions of various botanists have led to many name changes of species and subspecies

FIGURE 1.2. Endemic Hawaiian *Hibiscus* sect. *Lilibiscus* flowers, left to right *H. waimeae* subsp. *waimeae, H. waimeae* subsp. *hannerae, H. arnottianus* subsp. *punaluuensis, H. arnottianus* subsp. *arnottianus, H. arnottianus* subsp. *immaculatus, H. clayi, H. kokio* subsp. *kokio, H. kokio* subsp. *saintjohnianus,* and two *H. kahilii* (*H. kokio* subsp. *kokio*). Some petals have been removed to show the staminal columns and calyces.

delineations (Forbes 1912, Degener and Degener 1959, Roe 1961, Bates 1965, Stone 1967, St. John 1972, Degener and Degener 1977, Bates 1990). Two complete treatments of endemic Hawaiian *Hibiscus* have been done to date (summarized in Table 1.1), both based on morphological characteristics such as calyx length, number of involucral bracts (epicalyx bracts), location of filaments on the staminal column, length of staminal column, position of style branches, leaf shape and vestiture. The first comprehensive treatment was by Roe (1961) and provides a detailed account of the confusing botanical history of the genus in Hawai'i since the first specimen, named *H. Youngianus* (*H. furcellatus*) by Gaudichaud in 1826*,* was collected by the Freycinet Expedition in 1819. Roe recognized 9 species, 5 varieties and one form as indigenous to the Hawaiian Islands. Three of these species were first described by Roe in 1961: *H. saintjohnianus, H. newhousei* (*H. clayi*) and *H. immaculatus.* She apparently was not aware of the existence of *Hibiscus waimeae* var. *hannerae,* a Kauai endemic collected in 1913 by Lydgate and known as "Lydgate's white" and was described by Otto Degener in 1957 (Degener and Degener 1962, Bates 1965).

A more recent treatment was done by Bates in 1990 (revised in 1999 but with no changes to *Hibiscus* species names) who modified Roe's work somewhat (Table 1.1) and recognized five species and nine subspecies as endemic to Hawaii, one indigenous species, *H. furcellatus,* and one possible indigenous species (*H. tiliaceus*).

The primary area of disagreement among the various treatments of the Hawaiian species involves the non-fragrant red-flowered species, most of which are found only on Kauai the oldest large island in the chain. *Hibiscus kokio* occurs on Kauai, Oahu, Molokai, Maui and the Hawaii Island. *Hibiscus clayi*, *H. kahilii*, and *H. kokio* subsp.

Table 1.1. Endemic and indigenous Hawaiian *Hibiscus* species delineations by Roe (1961) and Bates (1990).

Roe	**Bates**
H. arnottianus	*H. arnottianus* subsp. *arnottianus*
H. arnottianus f. *parviflorus*	*H. arnottianus* subsp. *arnottianus*
H. arnottianus var. *punaluuensis*	*H. arnottianus* subsp. *punaluuensis*
H. immaculatus	*H. arnottianus* subsp. *immaculatus*
H. newhousei	*H. clayi*
H. kahilii	*H. kokio* subsp. *kokio*
H. kokio	*H. kokio* subsp. *kokio*
H. saintjohnianus	*H. kokio* subsp. *saintjohnianus*
not described	*H. waimeae* subsp. *hannerae*
H. waimeae	*H. waimeae* subsp. *waimeae*
H. brackenridgei (Maui and Lanai)	*H. brackenridgei* subsp. *brackenridgei*
H. brackenridgei var. *molokaiana* (Molokai)	*H. brackenridgei* subsp. *brackenridgei*
H. brackenridgei var. *kauaiana* (Kauai)	*H. brackenridgei* subsp. *mokuleianus*
H. brackenridgei var. *mokuleiana* (Oahu)	*H. brackenridgei subsp. mokuleianus*
H. youngianus (endemic)	*H. furcellatus* (indigenous)
H. tiliaceus (Polynesian introduction)	*H. tiliaceus* (indigenous ?)

saintjohnianus occur only on Kauai. Bates (1990) and Stone (1967) considered *H. clayi,* which was first described by Degener in 1959 and now includes Roe's *H. newhousei*, a close relative of *H. kokio*, but still gave it species status. However, Bates concluded that *H. saintjohnianus* was a subspecies of *H. kokio* and he placed *H. kahilii*, first described by Forbes in 1912*,* in *H. kokio* with no subspecies or varietal status. Though *H. kahilii* (*sensu* Roe 1961) has flowers similar to *H. kokio*, it is composed exclusively of wet forest trees found on Kauai only whereas *H. kokio* are scandent shrubs found in mesic to wet forests on Kauai, Oahu, Molokai, Maui and Hawaii Island. All the earlier treatments give *H. kahilii* and *H. saintjohnianus* species status and some gave *H. newhousei* species status (Roe 1961). In Hawaii today, many professional horticulturists and field botanists consider *H. kahilii* a true species and refer to it as such following the treatment of Roe (1961). Table 1.2 compares morphological characteristics of the red and orange flowered taxa.

There is more agreement in the classification of the fragrant white-flowered species. One exception was in the placement of the Molokai form, *H. immaculatus*. Roe (1961) described it as a distinct species. However, Bates (1990) placed it in *H. arnottianus* as subsp. *immaculatus*. Table 1.3 compares morphological characteristics of the white-flowered taxa.

Uncertainty in the classification of these species causes confusion at the nursery level because these plants are popular with the public and where selection for superior cultivars from wild populations and controlled breeding programs are underway. Plants of the same species are being propagated and dispersed to the public with different names. Additionally, because two of these species (*H. brackenridgei* and *H. clayi*) and two

TABLE 1.2. Morphological characteristics of red-flowered endemic Hawaiian *Hibiscus* section *Lilibiscus* from Roe (1961) and Bates (1990).

Taxon	*H. kahilii*	*H. kokio* subsp. *kokio*	*H. kokio* subsp. *saintjohnianus*	*H. clayi*
Petal Color	red or pink	red	orange or yellow	red
Flower Length	7-8 cm	7-8 cm	6.5 cm	5 cm
Calyx Shape	bulb shaped	straight or slight bulb	straight	straight or slight bulb
Involucral Bracts	11-12 mm long	10-15 mm long	5-8 mm long	7-8 mm long
Leaves	scabrous, ovate-elliptic, serrate-entire	smooth, elliptic-ovate, sinuately crenate	smooth, raised veins, elliptic-oblong, serrate	glabrous, elliptic, margins mostly entire
Growth Habit	tree	scandent shrub	shrub or tree	tree
Habitat Preference	wet forest	dry to wet forest	dry to mesic forest	dry forest

TABLE 1.3. Morphological characteristics of white-flowered endemic Hawaiian *Hibiscus* section *Lilibiscus* from Roe (1961) and Bates (1990).

Taxon	*H. arnottianus* subsp. *arnottianus*	*H. arnottianus* subsp. *immaculatus*	*H. arnottianus* subsp. *punaluuensis*	*H. waimeae* subsp. *hannerae*	*H. waimeae* subsp. *waimeae*
Petal Color	white	white	white	white	white
Staminal Column Color	red	white	red	red	red
Flower Length	19 cm	19 cm	20 cm	6 cm	18 cm
Involucral Bracts	5-8 mm long, reflexed	5-8 mm long, horizontal	10-25 mm long, horizontal-upright	8 mm long, horizontal	1.5-2.5 cm long, reflexed
Leaves	coriaceous, ovate-elliptic, elliptic, glabrous, margin crenate to entire	glabrous, ovate-obovate, denticulate margin	puberlent-pilose, ovate, base subcordate, apex acuminate	coarsely pubescent, ovate-elliptic	velvety pubescent, obovate-orbicular, serrate margin
Growth Habit	Tree	tree	tree	tree	tree

Habitat Preference	mesic or wet forest	wet forest	wet forest	wet forest	mesic forest

subspecies (*H. arnottianus* subsp. *immaculatus* and *H. waimeae* subsp. *hannerae*) are federally listed as endangered species and some populations of other species are small, scattered and possibly in decline (*H. kahlii, H. kokio* subsp. *kokio, H. kokio* subsp. *saintjohnianus*), conservation and germplasm preservation issues are important to federal and state managers who are trying to preserve these plants. An issue particular to *H. kokio* is that the plants are sprawling shrubs that branch infrequently and tend to grow vertically straight if there is nearby vegetation to support them. However, if there is nothing to support them, they fall over and may root where they touch the ground (Figure 1.3) thus making it difficult to know how many individuals there are in a population.

Clarification of species boundries is important for conservation of these unique plants, as well as in the ornamental horticulture industry. In an effort to clarify the confusion over species delineations and evolutionary history in endemic Hawaiian *Hibiscus,* random amplified polymorphic DNA (RAPD) markers were used as a comparison to previous morphological studies and to assess the variation within and among populations of endemic Hawaiian *Hibiscus.* RAPD markers have been useful in clarifying population and species relationships as well as evolutionary history in various plants (Randell *et al*., 2004, Grant and Miller 2001, Reed, Joung and Roh 2002). This technique has helped to answer questions related to genetic diversity at both population and species levels in various endemic Hawaiian plants in recent years: *Haplostachys* (Lamiaceae; Morden and Loeffler 1999), *Cibotium* (Dicksoniaceae; Motley and Morden 2001), *Colubrina* and *Alphitonia* (Rhamnaceae; Kwon and Morden 2002), *Touchardia* (Urticaceae; Loeffler and Morden 2003), *Rubus* (Rosaceae; Randell *et al.*2004),

FIGURE 1.3. *Hibiscus kokio* subsp. *kokio* is a sprawling shrub with that requires the support of plants around it to grow vertically. If a stem becomes too tall to support its own weight it will fall to the ground and root. Arrows point to adventitious roots forming on a horizontal stem just above the soil surface.

Chamaesyce (Euphorbiaceae; Morden and Gregoritza 2005), *Dubautia* (Asteraceae; Caraway *et al.* 2005), *Delissea* (Campanulaceae; James 2009) and *Hesperomannia* (Asteraceae; Morden and Harbin 2013). Using the polymerase chain reaction (PCR) to amplify the DNA fragments, using RAPD markers is a relatively fast, inexpensive technique that requires only a small quantity of DNA. RAPD markers are abundant in the genome and randomly distributed. However, drawbacks of using RAPD markers are that they are dominant markers only and there can be issues with the reproducibility of results (Skoric *et al.* 2012). However, this can be overcome by carefully following standardized laboratory procedures in order to avoid variability in reaction conditions and repetition of experiments to verify consistency of results.

Objectives

1. a) Assess the genetic similarities among the endemic Hawaiian red and orange-flowered *Hibiscus* species using random amplified polymorphic DNA (RAPD) markers in order to resolve earlier conflicting taxonomic treatments of this group based on morphological characters. Species to be studied: *Hibiscus kokio* subsp. *kokio* including *H. kahilii, H. kokio* subsp. *saintjohnianus* and *H. clayi.* **b)** Assess the genetic similarities between the endemic Hawaiian white-flowered *Hibiscus* species using RAPD markers to clarify species boundaries also. Species to be studied: *H. arnottianus* subsp. *arnottianus, H. arnottianus* subsp. *immaculatus, H. arnottianus* subsp. *punaluuensis* and *H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.*

2. Assess the genetic relationship of the endemic Hawaiian red-flowered to the white-flowered *Hibiscus* species listed above using RAPD markers.

Materials and Methods

Population Sampling and DNA Extraction

Fresh leaf tissue of Hawaiian species of *Hibiscus* sect. *Lilibiscus* was collected from wild plants on the islands of Kauai, Oahu, Molokai, Maui or Hawaii. Plant material was also collected from accessions at Lyon Arboretum (Oahu), Waimea Arboretum (Oahu), and the National Tropical Botanical Garden (Kauai).

Because *H. kokio* subsp. *kokio* forms roots where it touches the ground, extra care was taken when collecting leaf samples. To avoid collecting from the same individual twice, leaves were collected from branches that were as far apart as was feasible at each collection site.

The number of individuals sampled, island and locations of source material are listed in Table 1.4. Total cellular DNA was extracted and purified from 0.5-1.0 g of fresh plant material that was kept refrigerated until DNA was extracted. DNA was extracted using the CTAB method of Doyle and Doyle (1987) with some modifications by Morden *et al.* (1996). DNA samples were purified by cesium chloride density-gradient ultracentrifugation (Sambrook *et al.* 1989). Ethidium bromide was removed using water-saturated butanol and DNA was precipitated using isopropanol to remove the cesium then washed once with 70% ethanol. All purified DNA samples were accessioned in the Hawaiian Plant DNA Library (Morden *et al.* 1996, Randell and Morden 1999).

TABLE 1.4. Hawaiian *Hibiscus* sect. *Lilibiscus* accessions used for genetic analysis (RAPD) and diagnostic floral characteristics.

Species/subspecies	Island	Location	HPDL[a]	N[b]	Diagnostic characters
Hibiscus arnottianus* subsp. *arnottianus (A. Gray) D. Bates; *H. waimeae* A. Heller var. *hookeri* Hochr.	Oahu	Koolau Mts.	6395-6405	10	white, fragrant flowers, red staminal column
H. a.* subsp. *arnottianus (Skottsb.) D. Bates; *H. a.* f. *parviflorus* Skottsb.	Oahu	Waianae Mts.	6392-6394	3	white, fragrant flowers, red staminal column
H. a.* subsp. *immaculatus (*H. immaculatus)* M. Roe	Molokai	Wailau, Waihanau	6416-6418	3	white, fragrant flowers, white staminal column
H. arnottianus* subsp. *punaluuensis (Skottsb.) D. Bates; *H. punaluuensis* (Skottsb.) Degener & I. Degener	Oahu	Manoa Cliffs, Koolau Mts.	7042-7048	7	white, fragrant flowers, red staminal column
H. arnottianus* subsp. *punaluuensis	Oahu	Pali, Koolau Mts.	6410, 6415, 6944-6949	8	white, fragrant flowers, red staminal column
H. clayi (Roe) D. Bates; *H. newhousei* M. Roe	Kauai	Nounou Mt.	6497-6499, 6501, 6503	5	red, no fragrance
H. clayi/H. newhousei	Kauai	Moloaa	6504	1	red, no fragrance
H. kahilii Forbes; *H. kokio* subsp. *kokio* (Forbes) D. Bates	Kauai	Mt. Kahili	6439-6443	5	pink/red, no fragrance

***H. kokio* subsp. *kokio* (**Hillebr.) D. Bates; *H. arnottianus* A. Gray var. *kokio* (Hillebr.) Hochr.; *H. k.* var. *pukoonis* Caum; *H. oahuensis* Degener & I. Degener; *H. ula* Degener & I. Degene**r**	Hawaii	Muliwai	6481	1	red, no fragrance
	Hawaii	Honokaninui	6482	1	
	Kauai	Mt. Haupu	6444-6448	5	red, no fragrance
	Maui	Honokohau	6463-6467	5	red, no fragrance
	Maui	Honolua	6469-6473	5	red, no fragrance
	Maui	Iao	6940	1	
	Molokai	Halawa	6950	1	
	Molokai	Wailau	6480, 6951	2	red, no fragrance
	Oahu	Kawaihapai	6449-6451	3	red, no fragrance
	Oahu	Kawai Iki	6939	1	
	Oahu	Malaekahana	6456	1	red, no fragrance
	Oahu	Pupukea	6452-6455	4	red, no fragrance
H. kokio* subsp. *saintjohnianus (M. Roe) D. Bates; *H. roetae* St. John; *H. saintjohnianus* M. Roe	Kauai	Awaawapuhi Trail	6491-6495	5	orange, no fragrance
H. k.* subsp. *saintjohnianus	Kauai	Hanakapiai	6485-6489	5	orange/yellow no fragrance

H. waimeae* subsp. *hannerae (Degener & I. Degener) D. Bates; *H. w.* var. *hannerae* Degener & I. Degener	Kauai	Limahuli	6432-6436	5	white, fragrant flowers, red staminal column
H. w.* subsp. *waimeae (Heller) D. Bates; *H. w.* var. *helleri* Hochr.	Kauai	Kokee	6427-6429	3	white, fragrant flowers, red staminal column
	Kauai	Waimea Canyon	6419, 6426	2	white, fragrant flowers, red staminal column

[a] Accession in the Hawaiian plant DNA library (Morden et al. 1996; Randell and Morden 1999).
[b] Number of plants sampled in population.

RAPD PCR and Data Analysis

Approximately 1µl (20 ng) of DNA was amplified in 15µl reactions via the polymerase chain reaction (PCR) under the following conditions: 0.2 µM random 10-mer oligonucleotide primers (Operon Technologies), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1x *Taq* polymerase PCR Buffer, 1.5 mM $MgCl_2$, 0.1% bovine serum albumin, and ca. 1 unit *Taq* polymerase (Promega, Madison, Wisconsin, USA). Thirty-six primers were screened (kits OPC-OPD; QIAGEN Operon, Almeda, CA, USA) using RAPD analysis of the PCR to evaluate each primer for use on all individuals. Primers that yielded consistent number and intensity of markers were then used for amplification for all individuals. Amplifications were performed in either an MJ Research PTC-200 or PTC-225 Thermocycler under the following conditions: 94 °C for 2 minutes, 94 °C for 45 seconds, 35 °C for 45 seconds, ramp to 35 °C at 0.5 °C/second, 72 °C for 2 minutes, ramp to 72 °C at 0.5 °C/second followed by 44 cycles of 94 °C for 45 seconds, 35 °C for 45 seconds, ramp to 35 °C at 0.5 °C/second, 72 °C for 2 minutes, ramp to 72 °C at 0.5 °C/second with a final incubation at 72 °C for 5 minutes. PCR amplification products were visualized on 1.5% agarose gels in 0.5x TBE (tris-borate- EDTA) buffer, and gel images were digitally recorded with a UVP BioImaging Systems Gel HR Camera (UVP LLC, Upland CA, USA). Negative control (i.e., no DNA) reactions were run for all PCR amplifications to ensure reaction components were uncontaminated. Size of amplification products was estimated by comparison to fragments in a 100 kb ladder (Promega, Madison, WI, USA) or to a pBS plasmid (Stratagene, La Jolla, CA, USA) digested with restriction enzymes to produce fragments in a size range of 0.448- 2.96 kb. Molecular markers were identified by the primer used to generate them and their

approximate size (kb). Gel scoring was performed independently by the author and lab technicians to produce unbiased and unambiguous analysis of the RAPD amplifications.

Each RAPD marker generated was assumed to represent a separate genetic locus in a two allele system consisting of the marker (amplified product present) and the null allele (amplified product absent) with the marker being dominant to the null allele as described by Lynch and Milligan (1994). A RAPD marker was determined to be polymorphic when found in less than 95% of the sampled individuals (i.e. not present in 3 or more individuals). Absence of a marker within a population, though present in other populations, was assumed to indicate that all the individuals in the population were null/null homozygotes rather than indicating that there was a loss of the locus. Percent polymorphic loci was calculated at the population and species level using MS Excel. Genetic similarity indices were estimated using both Gower (1971) and Nei and Li (1979) similarity coefficients for populations and species using MVSP Plus ver. 3.1 (Kovach 2007). Pairwise similarity was averaged for individuals within and among populations. Expected heterozygosity was calculated for each population (H_s) and species (H_t) for each locus as follows:

$$H = 1 - (p^2 + q^2)$$

where p is the frequency of the dominant allele (i.e., the visible marker) and q is the frequency of the null allele (i.e., the absent marker). Allele frequencies were estimated from the number of null/null homozygotes present in the population (Hartl and Clark 1989, Morden and Loeffler 1999). UPGMA cluster analysis from similarity coefficients and principle coordinate analysis (PCO) using Gower general similarity coefficients were

calculated using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1987-1998).

Results

Thirteen primers were examined for 92 individuals resulting in 217 different genetic loci that were scored from these primers (Table 1.5). Primers ranged from 11 to 24 (average of 16.7) loci per primer. Levels of polymorphism were calculated for each population (range from 14.3 to 43.3%)(Table 1.6). At the species level, the percent polymorphism was highest in the white species, *H. arnottianus* subsp. *arnottianus* (43.3%), which is found in a wide diversity of habitats of mesic and wet forests from 120-790 m elevation in both the Waianae and Koolau Mountains of Oahu. Second highest polymorphism was found in the western Kauai endemic, *H. kokio* subsp. *saintjohnianus* (37.8%), that occurs in dry to mesic forests along the Napali Coast from 150-890 m elevation. The percent polymorphism was 32.7% in *H. kokio* subsp. *kokio* (not including *H. kahilii*) even though *H. kokio* is the only species that occurs on all the main islands. The level of polymorphism was lowest in *H. arnottianus* subsp. *immaculatus* (14.3%), but this could be due to the small sample size of only 3 individuals available.

Populations were compared for genetic similarities based on the Nei and Li (1979) genetic identity (I) with a value range from zero (no markers in common) to 1.0 (complete genetic identity). Genetic similarity was higher within populations than among populations (Table 1.7). Similarity was highest among the white-flowered species *H.*

TABLE 1.5. Random amplified polymorphic DNA (RAPD) primers used on all individuals of *Hibiscus* sect. *Lilibiscus* and the corresponding number of markers scored with each primer.

Primer	Primer Sequence	# Scored Markers
OPC-05	GATGACCGCC	16
OPC-06	GAACGGACTC	21
OPC-07	GTCCCGACGA	12
OPC-10	TGTCTGGGTG	24
OPC-11	AAAGCTGCGG	19
OPC-12	TGTCATCCCC	13
OPD-02	GGACCCAACC	15
OPD-08	GTGTGCCCCA	18
OPD-09	CTCTGGAGAC	21
OPD-13	GGGGTGACGA	17
OPD-14	CTTCCCCAAG	12
OPD-15	CATCCGTGCT	18
OPD-18	GAGAGCCAAC	11
	Sum	217
	Average	16.7

TABLE 1.6. Variation in populations of Hawaiian species of *Hibiscus* sect. *Lilibiscus* based on RAPD data: *H. arnottianus* subsp. *arnottianus*, *H. arnottianus* subsp. *immaculatus, H. arnottianus* subsp. *punaluuensis, H. clayi. H. kahilii, H. kokio* subsp. *kokio, H. kokio* subsp. *saintjohnianus, H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.*

Taxon	Sample size	Total # of markers scored	# of polymorphic markers	# of unique markers found	# of markers found in all individuals	% polymorphism	Estimated Heterozygosity (H)
All	92	217	199	n/a	18	91.7	0.239
arnot arnot	13	217	94	3	67	43.3	0.133
arnot immac	3	217	31	1	91	14.3	0.056
arnot pun Pali	8	217	34	3	71	15.7	0.055
arnot pun Manoa	7	217	68	5	98	31.3	0.094
clayi	6	217	46	1	81	21.2	0.074
kahilii	5	217	33	1	95	15.2	0.047
kokio kokio	30	217	71	1	77	31.3	0.104
kokio saintjohn	10	217	82	2	61	37.8	0.132
waim hannerae	5	217	55	0	82	25.3	0.097
waim waimeae	5	217	53	1	80	24.4	0.097

TABLE 1.7. Levels of genetic similarity within and among Hawaiian species of *Hibiscus* sect. *Lilibiscus* based on Nei and Li (1979) coefficient (a value of 1 indicates complete genetic identity): *H. arnottianus* subsp. *arnottianus* (*arnottianus*), *H. arnottianus* subsp. *immaculatus* (*immaculatus*), *H. arnottianus* subsp. *punaluuensis* (*punaluuensis*) from the Pali (P) and Manoa (M) populations, *H. clayi, H. kahilii, H. kokio* subsp. *kokio* (*kokio*), *H. kokio* subsp. *saintjohnianus* (*saintjohnianus*), *H. waimeae* subsp. *hannerae* (*hannerae*) and *H. waimeae* subsp. *waimeae* (*waimeae*).

	Taxon	1	2	3	4	5	6	7	8	9	10
1	*arnottianus*	0.863									
2	*immaculatus*	0.816	0.905								
3	*punaluuensis* P	0.731	0.707	0.943							
4	*punaluuensis* M	0.721	0.707	0.663	0.910						
5	*clayi*	0.750	0.717	0.710	0.715	0.901					
6	*kahilii*	0.762	0.757	0.731	0.704	0.770	0.925				
7	*kokio*	0.759	0.757	0.731	0.696	0.774	0.892	0.916			
8	*saintjohnianus*	0.757	0.730	0.719	0.702	0.842	0.770	0.778	0.855		
9	*hannerae*	0.812	0.810	0.706	0.699	0.734	0.753	0.751	0.752	0.870	
10	*waimeae*	0.813	0.854	0.698	0.694	0.728	0.745	0.743	0.749	0.823	0.887

arnottianus subsp. *arnottianus, H. arnottianus* subsp. *immaculatus, H. waimeae* subsp. *hannerae,* and *H. waimeae* subsp. *waimeae* (0.862-0.812). Though *H. arnottianus* subsp. *punaluuensis* has white flowers also, both populations were unexpectedly equally similar to the other white-flowered species (values ranging from 0.698-0.731 for the Pali plants and 0.694-0.721 for the Manoa plants) and the red-flowered species (values ranging from 0.710-0.731 for the Pali plants and 0.696-0.715).

All populations of the endemic Hawaiian *Hibiscus* species and subspecies [according to Bates (1990) with the exception of *H. kahilii*] sampled were compared using principal coordinate analysis (PCO) resulting in a plot with four distinct groupings (Figure 1.4). The first (horizontal) PCO axis accounts for the distinction between *H. kokio* subsp. *kokio* (including *H. kahilii*; Group 1) and all the other species. The second axis distinguishes the Pali, Oahu population of *H. arnottianus* subsp. *punaluuensis* (Group 2) from all the other white-flowered populations (Group 3) as well as *H. clayi* and *H. kokio* subsp. *saintjohnianus* (Group 4). These data suggest that *H. kokio* subsp. *saintjohnianus* is more similar to *H. clayi* and most of the white-flowered species than to *H. kokio* subsp. *kokio*. The data also clearly differentiate the Pali, Oahu population of *H. arnottianus* subsp. *punaluuensis* from all the other populations of *H. arnottianus* (including subsp. *arnottianus,* subsp., *immaculatus*, and the Manoa Cliffs population of subsp. *punaluuensis*).

To examine these four distinct clusters further, separate PCO analyses were conducted for each group. Further analysis of Group 1 demonstrates that *H. kahilii* is well differentiated from all the populations of *H. kokio* subsp. *kokio* (Figure 1.5). There is also evidence of grouping by island with the Kauai and Oahu populations and the

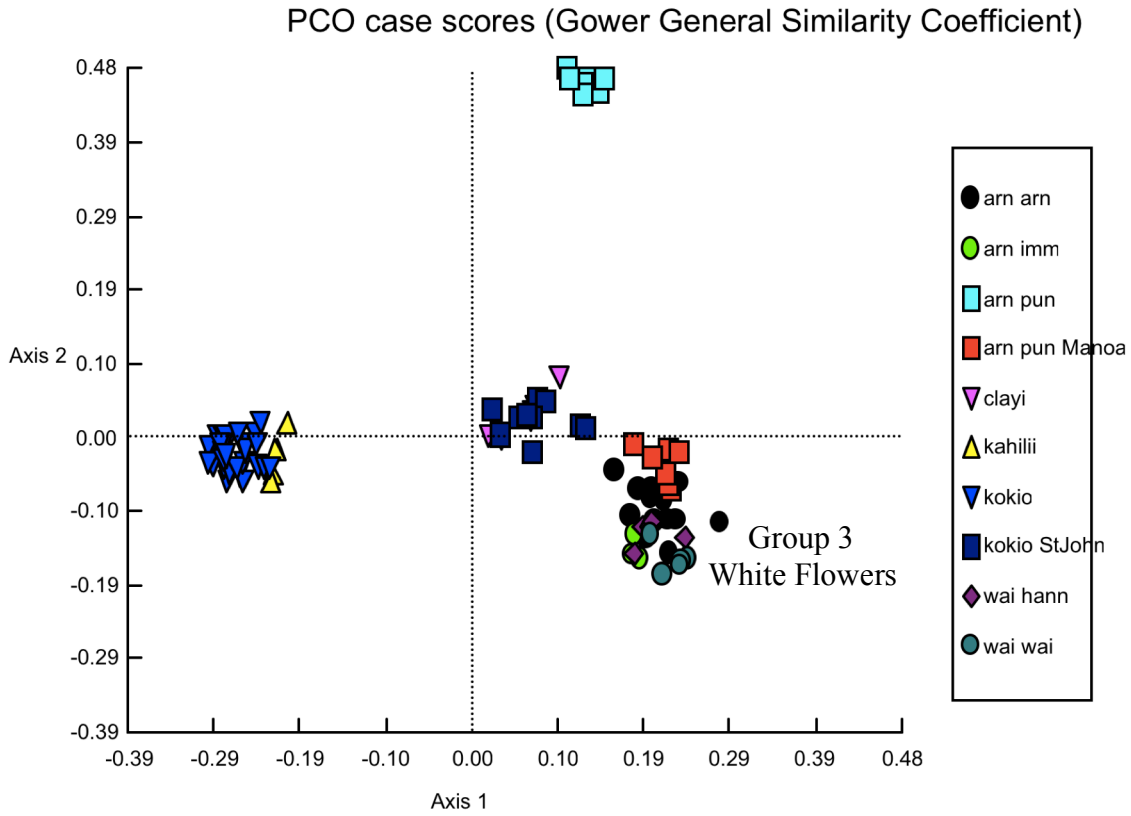


FIGURE 1.4. Principal coordinates analysis of all populations of endemic Hawaiian *Hibiscus* sect. *Lilibiscus* sampled. Group 1 (red-flowered species): *H. kokio* subsp. *kokio* and *H. kahilii.* Group 2 (white-flowered species): *H. arnottianus* subsp. *punaluuensis* from the Pali, Oahu. Group 3 (red- or orange-flowered species): *H. clayi* and *H. kokio* subsp. *saintjohnianus.* Group 4 (white-flowered species): *H. arnottianus* subsp. *arnottianus, H. arnottianus* subsp. *immaculatus, H. arnottianus* subsp. *punaluuensis* (from Manoa Cliffs, Oahu), *H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.* The first (horizontal) axis represents 19% of the total variation and the second (vertical) axis represents 10% of the variation.

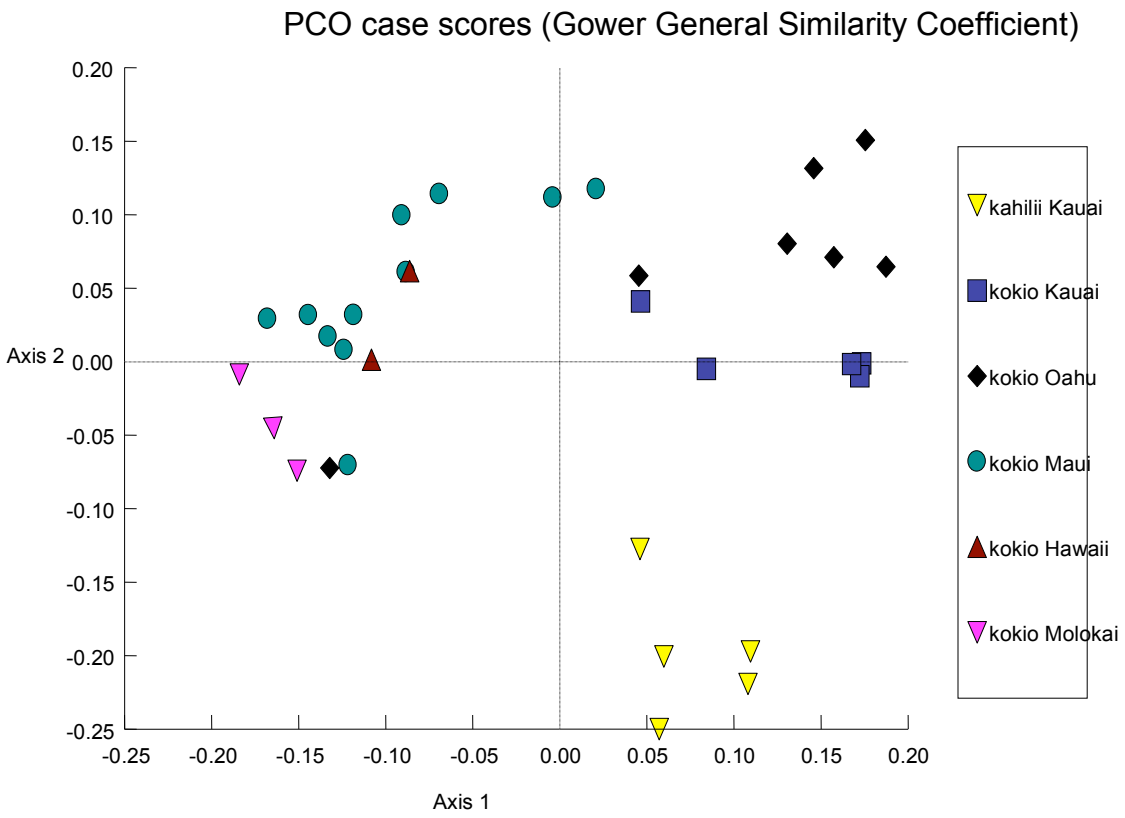


FIGURE 1.5. Principal coordinates analysis of Group 1 including all individuals of *H. kokio* subsp. *kokio* from all islands and *H. kahilii.* The first (horizontal) axis represents 17% of the total variation and the second (vertical) axis represents 11% of the variation.

Hawaii, Maui and Molokai populations clustering separate from one another. One exception is the Oahu plant from the Kawai Iki irrigation ditch trail that is clustering with plants from Molokai and Iao Valley on Maui.

Further analysis of Groups 3 and 4 (Figure 1.6) indicates further distinctions among these taxa. The first axis separates the Kauai *H. kokio* subsp. *saintjohnianus* and *H. clayi,* and the Manoa Cliffs, Oahu population of *H. arnottianus* subsp. *punaluuensis* from all populations of the other white-flowered species. The Manoa *punaluuensis* population is further differentiated from the other taxa along the second axis such that it is equally distinct from both species clusters as indicated by the similarity values.

A PCO analysis was conducted for the group consisting of *H. clayi* and *H. kokio* subsp. *saintjohnianus* (Figure 1.7). The first axis separated *H. clayi* from *H. kokio* subsp. *saintjohnianus.* The second axis distinguishes the two populations of *H. kokio* subsp. *saintjohnianus,* one from the Awaawapuhi Trail and one from the cliffs near Hanakapiai, both on Kauai's Napali coast.

A PCO analysis was conducted for all the white-flowered species excluding both populations of *H. arnottianus* subsp. *punaluuensis* (Figure 1.8). Here, the first axis clearly distinguishes *H. arnottianus* subsp. *immaculatus* from those of subsp. *arnottianus* and these individuals (subsp. *immaculatus*) are more closely affiliated with the Kauai plants, *H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.* The second axis accounts for the clear distinction of *H. waimeae* subsp. *hannerae* from *H. waimeae* subsp. *waimeae*. The Oahu *H. arnottianus* individuals from the Waianae Mountains (recognized as *H. arnottianus* f. *parviflorus* Skottsb. by Roe) were indistinguishable from the Koolau Mountain *H. arnottianus* populations and were not further examined.

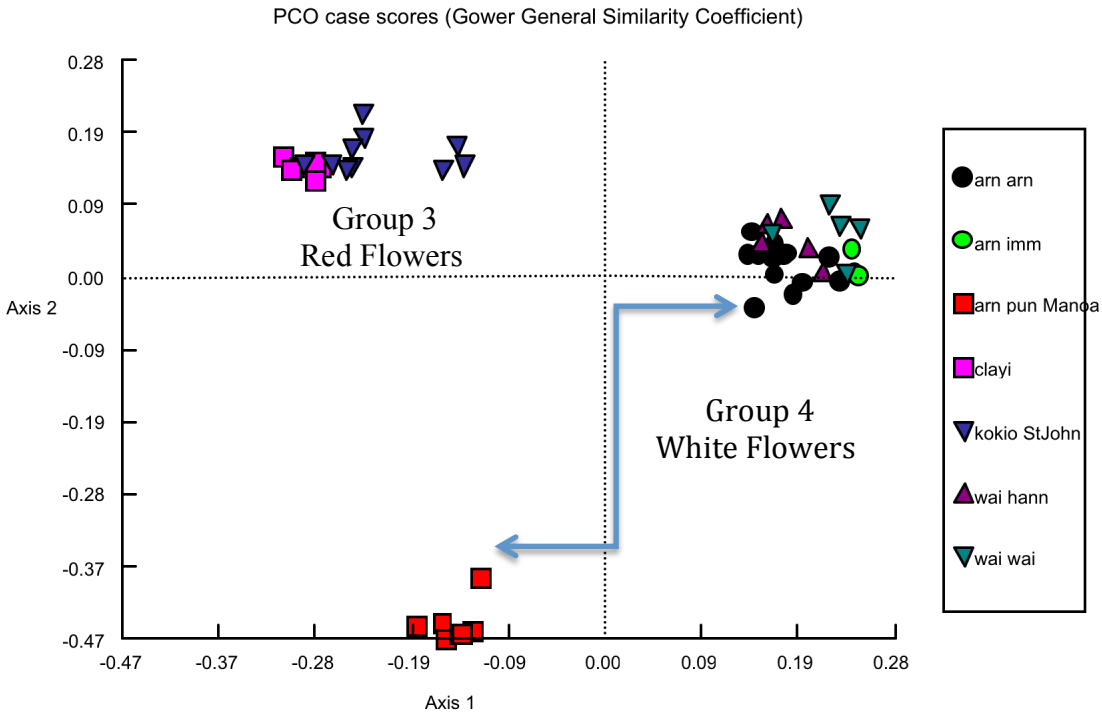


FIGURE 1.6. Principal coordinates analysis of Groups 3 and 4 including *H. clayi, H. kokio* subsp. *saintjohnianus, H. arnottianus* subsp. *arnottianus, H. arnottianus* subsp. immaculatus, *H. arnottianus* subsp. *punaluuensis* (Manoa Cliffs), *H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.* The first (horizontal) axis represents 20% of the total variation and the second (vertical) axis represents 17% of the variation.

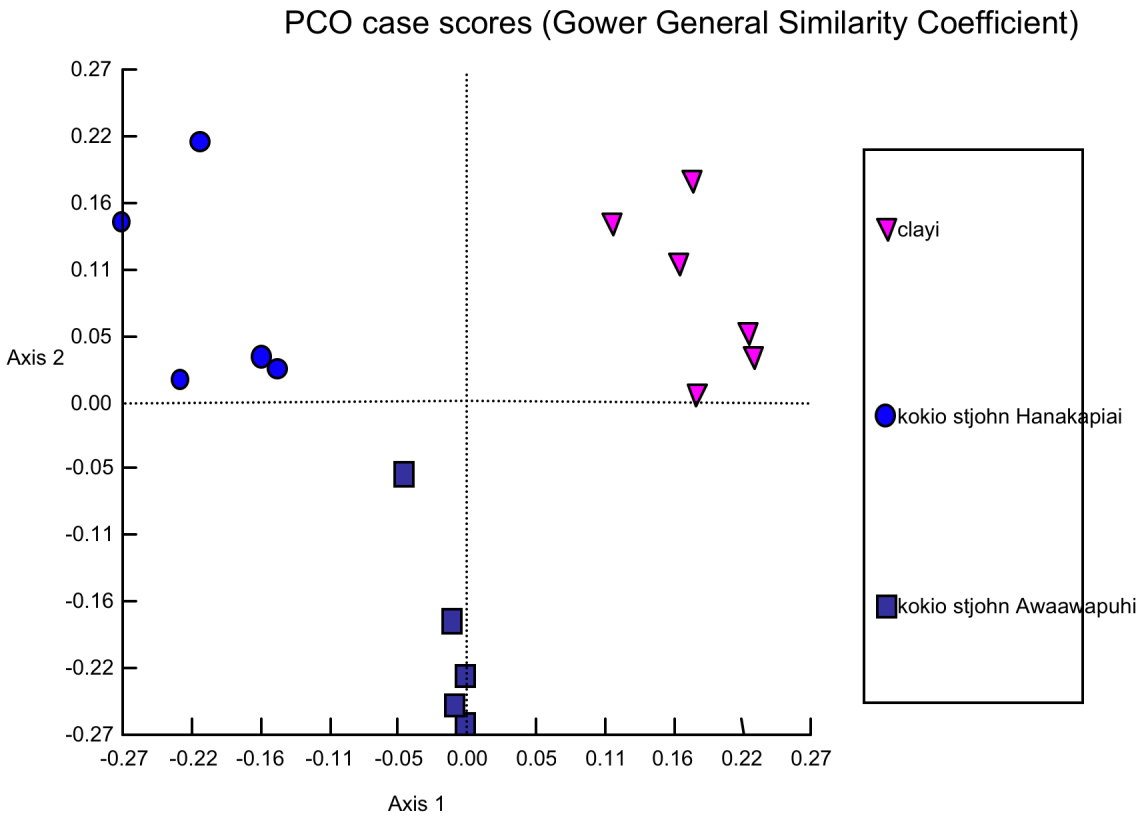


FIGURE 1.7. Principal coordinates analysis of Group 3 including *H. clayi* and two populations of *H. kokio* subsp. *saintjohnianus* (Awaawapuhi Trail and Hanakapiai). The first (horizontal) axis represents 19% of the total variation and the second (vertical) axis represents 16% of the variation.

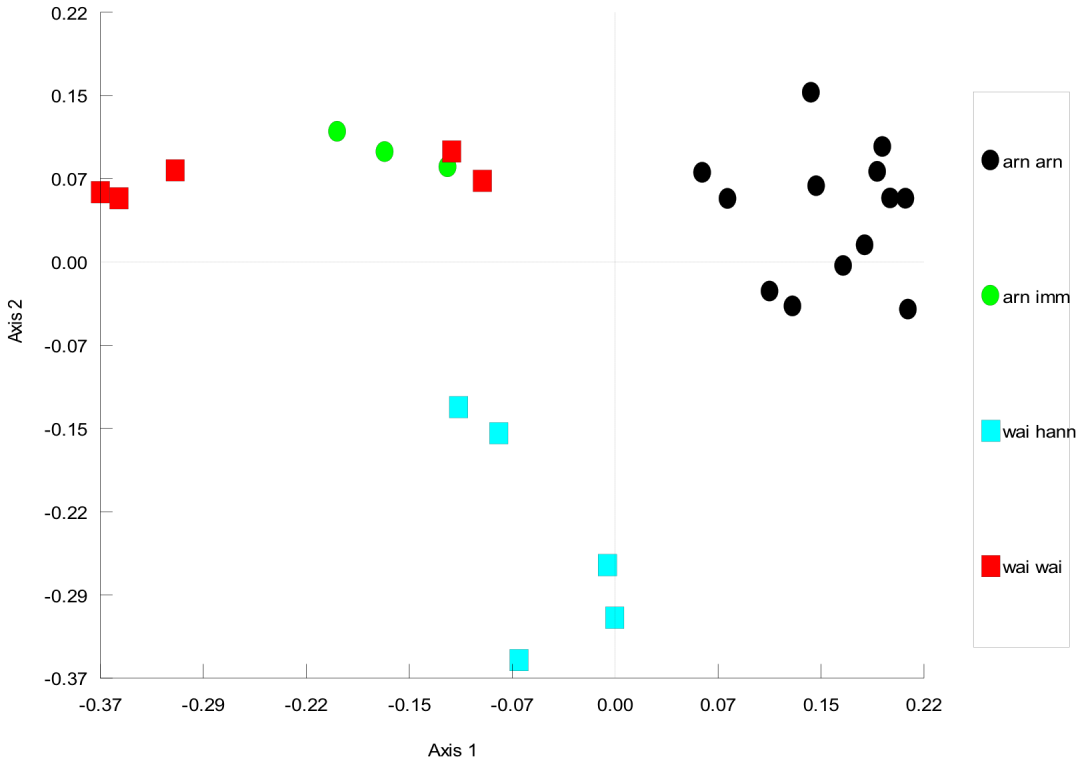


FIGURE 1.8. Principal coordinates analysis of all white-flowered *Hibiscus* species except *H. arnottianus* subsp. *punaluuensis*. Species included are *H. arnottianus* subsp. *arnottianus, H. arnottianus* subsp. *immaculatus, H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae*. The first (horizontal) axis represents 17% of the total variation and the second (vertical) axis represents 12% of the variation.

The relationships of the two Kauai white subspecies, *H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae,* and the Molokai white, *H. arnottianus* subsp. *immaculatus,* were further analyzed (Figure 1.9). The first axis shows a clear distinction between *H. waimeae* subsp. *hannerae* and the other two taxa, *H. waimeae* subsp. *waimeae* and *H. arnottianus subsp. immaculatus*. Here, *H. waimeae* subsp. *waimeae* is more closely associated with *H. arnottianus* subsp. *immaculatus* than with *H. waimeae* subsp. *hannerae.*

Discussion

The RAPD data indicate that the red- and white-flowered endemic Hawaiian *Hibiscus* species are genetically similar indicative of their close relationship as a monophyletic lineage in Hawaii. However, clear genetic distinctions are evident that correspond to morphological differences among all nine taxa that were examined. The RAPD results are generally more in agreement with the circumscription of the species by Roe (1961) rather than that of Bates (1990).

Red-Flowered Hibiscus

Within the red-flowered species, four distinct species are recognizable based on differences in genetic profiles and morphology. *Hibiscus kahilii* (*sensu* Roe 1961) is more closely associated with *H. kokio* subsp. *kokio* whereas *H. kokio* subsp. *saintjohnianus* was unexpectedly more similar to *H. clayi* than to *H. kokio* subsp. *kokio* in the RAPD analysis, although there are no apparent morphological traits that clearly indicate that *H. kokio* subsp. *saintjohnianus* is more closely related to *H. clayi* than to *H.*

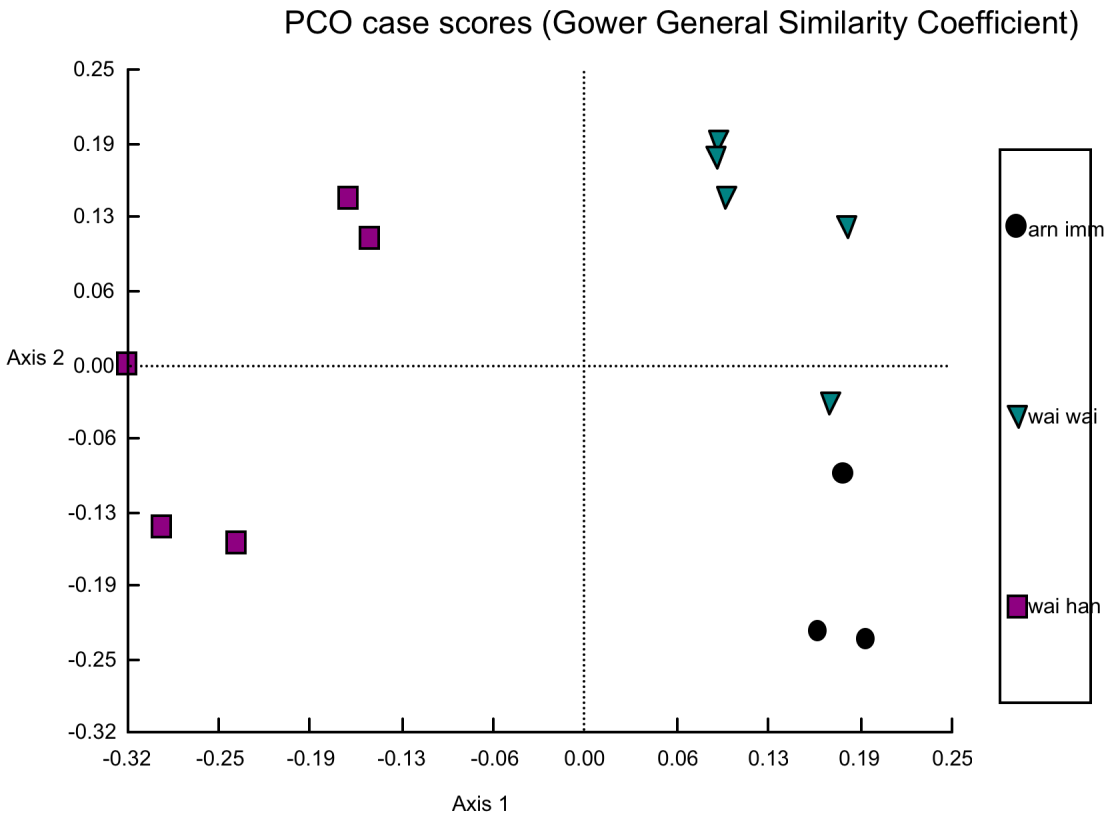


FIGURE 1.9. Principal coordinates analysis of *H. arnottianus* subsp. *immaculatus, H. waimeae* subsp. *hannerae* and *H. waimeae* subsp. *waimeae.* The first (horizontal) axis represents 26% of the total variation and the second (vertical) axis represents 16% of the variation.

kokio subsp. *kokio* or *H. kahilii* (Figure 1.10). *Hibiscus kahilii* is clearly genetically distinct from all populations of *H. kokio* subsp. *kokio* and not synonymous with it in contradiction to the classification of Bates (1990). The flowers of *H. kahilii* and *H. kokio* subsp. *kokio* are similar, but are distinguishable. Flowers of *H. kahilii* have short involucral bracts, petals that fold downward when fully mature and a bulbous calyx compared to the much longer involucral bracts, horizontal petals and straight calyx of *H. kokio* subsp. *kokio* (Table 1.2). The leaves and the growth habits are also quite different. *Hibiscus kokio* subsp. *kokio* are sprawling shrubs found on Kauai, Oahu, Molokai, Maui and Hawaii Island in ecologically diverse habitat ranging from mesic to wet forests with leaves that are elliptical-ovate and smooth with sinuately crenate margins and an acute apex. In contrast, *H. kahilii,* endemic to wet forests of Kauai, are multi-branched trees with ovate-elliptic leaves that are scabrous on both sides and are serrate only on the upper section with a more rounded apex. Where subsp. *kokio* is fast growing and roots readily from cuttings growing well in a wide variety of conditions in cultivation*, H. kahilii* plants are fairly difficult to root from cuttings and often do not survive well when grown in drier, low elevation conditions.

There is evidence of grouping by island in *H. kokio* subsp. *kokio* with the plants from the older islands, Kauai and Oahu, clustering together, and those from the younger islands, Hawaii, Maui and Molokai, in another cluster. One exception is the Oahu plant from the Kawai Iki irrigation ditch trail that is associated with plants from Molokai and Iao Valley on Maui. Otto Degener communicated to colleague and field botanist John Obata that the Kawai Iki plant was brought by Edward Caum of the Hawaiian Sugar Planter's Association to Oahu from another island because they were reported to be good

FIGURE 1.10. Red-flowered taxa of Hawaiian *Hibiscus* sect. *Lilibiscus* showing variation in involucral bracts, calyx shape, flower color and size. Left to right: *H. kokio* subsp. *kokio, H. kahilii, H. kokio* subsp. *saintjohnianus,* and *H. clayi.*

plants to grow near irrigation ditches (personal communication, 2007). Its position in the graph is consistent with this in being closely affiliated with the plants from Molokai and Maui.

Hibiscus kokio subsp. *saintjohnianus* and *H. clayi* are genetically well differentiated from *H. kokio* subsp. *kokio* and *H. kahilii* and morphological traits readily distinguish each taxon (Table 1.2). Hibiscus kahilii has leaves with scabrous surfaces compared to the smooth and glabrous dark green surfaces of the others. Both *H. clayi* and subsp. *saintjohnianus* have further differentiated; margins of *H. clayi* are entire and the veins of subsp. *saintjohnianus* are distinctly raised in comparison to the sinuately crenate margins and smooth leaf surface of *H. kokio* subsp. *kokio*. Flowers among these species are also distinct. Those of subsp. *saintjohnianus* are orange or yellow-orange with the petals ribbed whereas the flowers of *H. clayi* are red, similar to that of *H. kokio* subsp. *kokio*, but are conspicuously shorter, up to 5 cm long compared to flowers being 6.5 cm long or greater in the other species.

The results of the genetic analyses correlate with the morphological variation among the red-flowered *Hibiscus* and is consistent with the recognition of four species without subspecific classification. This classification is consistent with the interpretation based solely on morphological variation found by Roe (1961). Red-flowered *Hibiscus* species recognized herein are *H. clayi, H. kahilii, H. kokio* and *H. saintjohnianus.*

White-Flowered Hibiscus

The white-flowered taxa separate into five distinct groups in the PCO analyses that are inconsistent with the present taxonomic classifications. Two unexpected outcomes

were the divergent relationship of the two populations of *H. arnottianus* subsp. *punaluuensis* from the remainder of the white-flowered species and the close relationship of *H. arnottianus* subsp. *immaculatus* (from Molokai) to both *H. waimeae* subspecies (from Kauai) rather than to *H. arnottianus* subsp. *arnottianus* (from Oahu).

The Oahu subspecies *H. arnottianus* subsp. *punaluuensis* is geographically closely affiliated to *H. arnottianus* subsp. *arnottianus*, yet are widely divergent genetically. Subsp. *arnottianus* is found in the eastern Koolau Mountains from Wahiawa to Niu Valley and in the Waianae Mountains, where subsp. *punaluuensis* is present from Kaipapau to Waiahole in the Koolau Mountains (Bates 1990). The populations are intermixed along the Manoa Cliff's trail and intermediate morphology is evident in some plants. Collection of plants from this region was targeted toward plants that appeared to have the distinctive characteristics of each subspecies; apparent hybrid individuals were avoided. The separate population near the Pali Lookout was also compared. Results indicate that plants of the Pali population were very distinct from all the other Hawaiian *Hibiscus* sect. *Lilibiscus,* whereas plants from the Manoa Cliffs population subsp. *punaluuensis* were more closely aligned with, although still distinct from, subsp. *arnottianus* plants. Both populations of subsp. *punaluuensis* have unique markers that differentiate them from the remainder of section *Lilibiscus* (Table 1.6). Given the intermediate nature of the Manoa Cliffs population, it is likely that hybridization has occurred between the two subspecies with possible greater genetic influence within the population from subsp. *arnottianus*. This relationship had been suggested previously by field botanists Joel Lau and Mashuri Waite (personal communication 2011). A more

extensive sampling of the two taxa at Manoa Cliffs is needed to further understand the population dynamics at this location.

Morphologically, *H. arnottianus* subsp. *arnottianus* and subsp. *punaluuensis* are distinct (Table 1.3). The involucral bracts of subsp. *arnottianus* are small (less than 1 cm) and often reflexed compared to the much longer (2 cm or greater) and broader involucral bracts that are horizontal or curved upward in subsp. *punaluuensis* (Figure 1.11). Calyces of subsp. *punaluuensis* are also puberlent to pilose compared to the glabrous calyces of subsp. *arnottianus*. Vegetative distinctions among these taxa include the glabrous stems, leaves, and pedicels and smaller leaves (4-10 cm long) in subsp. *arnottianus* compared to the moderately to coarsely pubescent stems, leaves, and pedicels and much larger leaves (10 to 20 cm long) of subsp. *punaluuensis* (Bates 1990; personal observation).

More support for the distinctness of *H. arnottianus* subsp. *punaluuensis* from *H. arnottianus* subsp. *arnottianus* is the association of an endemic mealybug species, *Clavicoccus tribulus,* with some individuals in the Manoa Cliffs area on Oahu. The mealybugs have only been found on plants with leaves that are slightly tomentose on the with glabrous leaves, subsp. *arnottianus* (Beardsley 1959, personal communication M. Waite 2011). Based on the genetic and morphological differences that are evident, *H. arnottianus* subsp. *punaluuensis* is recognized as a distinct species, *H. punaluuensis* (Skottsb.) O. Degener & I. Degener. It is evident that the two species intermix forming an apparent hybrid swarm in the Manoa Cliffs area.

The RAPD data clearly align *H. arnottianus* subsp. *immaculatus* more closely with *H. waimeae* subsp. *waimeae* than with *H. arnottianus* subsp. *arnottianus.* In fact *H.*

FIGURE 1.11. White-flowered taxa of Hawaiian *Hibiscus* sect. *Lilibiscus* showing variation in flower size, and staminal column color. Clockwise from bottom left: *H. waimeae* subsp. *waimeae, H. waimeae* subsp. *hannerae, H. arnottianus* subsp. *arnottianus* (Waianae Mts., Oahu), *H. arnottianus* subsp. *arnottianus* (Manoa Cliffs, Oahu), *H. arnottianus* subsp. *punaluuensis* (Pali, Oahu), and *H. arnottianus* subsp. *immaculatus* (white staminal column).

waimeae subsp. *waimeae* is genetically more similar to *H. arnottianus* subsp. *immaculatus* than to *H. waimeae* subsp. *hannerae*. Each of these four taxa is genetically and morphologically distinct. The most obvious difference is the small flower size (less than 6 cm long) separating *H. waimeae* subsp. *hannerae* from the other three (greater than 15 cm long; see Fig. 1.2, Table 1.3, Figure 1.11). The vestiture of leaves and calyx lobes in *H. waimeae* subsp. *waimeae* is velvety pubescent and coarsely pubescent in *H. waimeae* subsp. *hannerae*, but glabrous in both *H. arnottianus* subsp. *arnottianus* and subsp. *immaculatus*. The staminal column is typically white in subsp. *immaculatus* (from which its name is derived) compared to the usually reddish (varying from pink to magenta) staminal columns in the other three taxa (Fig. 1.2). Because of these genetic and morphological distinctions, each of these four taxa is being recognized as a distinct species: *H. arnottianus* A. Gray, *H. immaculatus* M. Roe, *H. hannerae* (O. Degener & I. Degener) Huppman (new combination), and *H. waimeae* A. Heller.

The biogeographic distribution of these species does not follow the stepping stone pattern evident among many other plant and animal lineages from older to younger islands (Hennig 1966, Funk and Wagner 1995). The pattern of the relationship among *H. immaculatus* with *H. hannerae* and *H. waimeae* is distinct, but has been found in several other plant and animal lineages. Carr *et al.* (1989) found a similar dispersal pattern among species of *Dubautia*. Carson (1983), examining chromosomal inversions among picture-wing drosophilids, found several instances of dispersal from Kauai to Maui Nui (and the reverse) bypassing Oahu. The grass genus *Dichanthelium* has four species recognized in the islands, three on Kauai and islands of Maui Nui and one on Oahu (Wagner *et al*. 1990).

Conservation Issues in Hawaiian Hibiscus

Accurate taxonomic circumscription is important especially for the management of endangered species in order to avoid mistakes in propagation and out-planting of individuals from various populations and to preserve genetic diversity. Correct taxonomy is also important to plant breeders and nursery managers in the horticulture industry. This study clearly demonstrated that the current circumscription of the taxa does not accurately reflect the genetic relationships indicated in the RAPD results.

Several of the taxa examined here are federally listed as endangered species. However, the recovery objectives for these three taxa have not been met according to the USFWS (2008, 2010, 2011). These are *H. clayi* (USFWS 2008)*, H. hannerae* (USFWS 2010) and *H. immaculatus* (USFWS 2011. In recent years more individuals of both *H. clayi* (on Kauai) and *H. immaculatus* (on Molokai) have been discovered (S. Perlman, personal communication 2011), but they are still very rare species. Hurricanes Iwa (1982) and Iniki (1992) devastated Napali, the western coast of Kauai, damaging the native forest there, including the population of *H. saintjohnianus,* and opening the area to various invasive plant species that are spreading. Steve Perlman reported that the hibiscus there have never recovered and appear to be declining in numbers (S. Perlman, personal communication 2007).

The Hanakapiai population of *H. hannerae* was also seriously damaged by Hurricane Iniki. Before the storm there were about 50 plants at this location, but only 25 survived it. In 2010 a total of 80 to 85 individuals were reported from the three populations on Kauai (Hanakapiai, Limahuli and Pohakuao) (USFWS 2010). This plant grows close to the edges of streams in wet valleys and thus is vulnerable to flooding during large storms.

Unfortunately, most of the seeds on wild plants are eaten by insects (USFWS 2010). In cultivation, this species is difficult to grow from cuttings but easy to grow from seed. At Lyon Arboretum, chewing insects eat the leaves of individuals planted out on the grounds, and in the greenhouse it is susceptible to spider mites, white fly and mealybug.

The Kauai endemic *H. clayi* was listed as endangered in 1994. At the time of listing, only four wild plants were known from Nounou Mountain on Kauai. More have been outplanted there since 1994 (USFWS 2008) and in 2011 approximately 120 additional plants were found by Merlin Edmonds, National Tropical Botanical Garden's Restoration Collector, in Anahola, Moloaa Forest Reserve (S. Perlman and K. Winter, personal communication 2011, M. Edmonds and N. Tangalin, personal communication 2013) where they had been found by earlier collectors (Roe 1961, Bates 1990).

All of the species in this study are relatively long-lived trees or shrubs, yet all their habitats, not only those of the endangered species, are threatened today by invasive plants, various arthropods including seed eating insects, rodents, pigs that destroy forest understory, the probable extinction of at least some of their pollinators, and stochastic events (USFWS 2008, 2010, 2011). Most of their populations are relatively small and isolated from other populations of the same taxon. They are found in pockets of remnant native forest. Natural recruitment was rare in all the wild populations I visited while collecting plant material for this research. Very few seedlings or saplings were observed. Conservation measures to preserve these various populations should be considered before they all reach critically low numbers and loss of genetic diversity.

Chapter II

Flower Maturation and Nectar Sugar Composition in Endemic Hawaiian *Hibiscus* section *Lilibiscus* (Malvaceae)

Introduction

Flower nectar and pollen are the two primary rewards for pollinators in angiosperms and coevolution of plants and their pollinators is thought to be an important factor in speciation for these two groups (Darwin 1862, Grant 1992, Hapeman and Inoue 1997, Wilbert *et al*. 1997, Schemske and Bradshaw 1999, Temeles and Kress 2003, Johnson 2007, Martins and Johnson 2007, Fleischer *et al*. 2008, Chase *et al*. 2009, Micheneau *et al*. 2009). Many studies have been conducted to examine possible correlations between floral morphology and nectar sugar content as they relate to pollinator type (Faegri and van der Pijl 1979, Baker and Baker 1983, Heyneman 1983, Lammers and Freeman 1986, Grant 1992, Bruneau 1997, Nicolson 1998, Nicolson 2007, Schemske and Bradshaw 1999, Tian *et al*. 2004, Wilson *et al.* 2006, Martins and Johnson 2007, Micheneau *et al*. 2009). Other nectar constituents that have not been as extensively studied as sugars but may be important as rewards, preservatives or deterrents to flower visitors are amino acids, lipids, proteins, phenols and alkaloids (Baker and Baker 1983, Heyneman 1983, Nicolson and Thornburg 2007, Pacini and Nicolson 2007). Nicolson (1998) found that *Protea* and *Faurea* were unique in being the only genera with xylose in their nectar, in addition to the other sugars common in nectars. Amino acids may be important in influencing nectar taste (Heyneman 1983, Nicolson and Thornburg 2007) and Bob Hobdy (Hawaii State Forester, DOFAW) reported that the nectar in the flowers of the endangered Hawaiian *Hibiscadelphus* and *Kokia* (Malvaceae) species contained high

amounts of amino acids (Hobdy 1984, personal communication 2012). Yeasts (Herrera *et al*. 2008, Herrera *et al*. 2009) and bacteria (Fridman *et al*. 2012) have been detected in floral nectar and in some cases have been shown to alter sugar content in some plant species and to degrade nectar quality. Microorganisms in nectar may change nectar chemistry significantly enough to affect its attractiveness to pollinators or nectar robbers (Herrera *et al*. 2009, Fridman *et al*. 2012). Evolution of floral traits, pollinator preferences and pollination systems as well as avoidance of floral herbivores and nectar robbers can be important factors in reproductive isolation, adaptation and speciation (Carlquist 1980, Lammers and Freeman 1986, Grant 1992, Weller *et al.* 1998, Schemske and Bradshaw 1999, Ziegler 2002, Wilson 2006, Bernardello 2007, Johnson and Nicolson 2007, Kay 2007).

Adaptation to a particular pollinator can result in the flowers of distantly related species sharing convergent traits associated with a particular pollinator (Bruneau 1997, Wilson *et al*. 2006, Chase 2009), or the opposite may be true, where close relatives may have evolved different floral traits that represent shifts favoring pollination by different pollinating mechanisms (Carlquist 1980, Grant 1992, Hapeman and Inoue 1997, Weller *et al.* 1998, Schemske and Bradshaw 1999, Ziegler 2002, Wilson 2006, Johnson 2007, Kay 2007, Micheneau *et al*. 2009). Flower color, size, texture, orientation of petals, presence or absence of fragrance, location and length of anthers, perianth shape, length and width of the floral tube, and nectar sugar content and volume are factors that can be important in pollination syndromes (Faegri and van der Pijl 1979, Baker and Baker 1983, Grant 1992, Bruneau 1997, Luyt and Johnson 2001, Wilson 2006, Johnson and Nicolson 2007, Nicolson 2007).

Historically, plant systematics has been based primarily on floral morphology, but recent genetic studies of closely related species based on molecular data are revealing multiple cases of convergent evolution in a single genus. These studies have necessitated changes in the systematics of some groups such as the fringed orchid genus, *Platanthera* (Hapeman and Inoue 1997), and the tropical American orchid genus *Oncidium* (Chase *et al*. 2009). In both genera what were thought to be closely related species because of similarities in flower color and morphology were actually genetically distinct, but did share a similar primary pollinator. These investigations offer a more complete understanding of the importance of pollinator partitioning as a driving force in speciation, influencing flower color and morphology.

Other genera that have been studied where sympatric species have adapted to different pollinators such as birds, hawkmoths or bees are *Aquilegia* (Ranunculaceae; Grant 1992, Puzey *et al*. 2012), *Ipomopsis* (Polemoniaceae; Grant 1992), *Mimulus* (Scrophulariaceae; Wilbert *et al*. 1997, Schemske and Bradshaw 1999), *Silene* (Caryophyllaceae; Reynolds *et al*. 2009, Dudash *et al*. 2011) in north America, Heliconia (Heliconiaceae) in the Caribbean (Temeles and Kress 2003), Angraecoid orchids (Orchidaceae) in Africa and Reunion Island respectively (Martins and Johnson 2007, Micheneau *et al*. 2009), the southern African orchid genus *Disa* (Johnson *et al*. 1998) and Proteaceae in South Africa and Australia (Nicolson 1998). *Schiedea* (Caryophyllaceae; Weller *et al*. 1998) includes some probable bird pollinated species and other species that are wind pollinated and sexually dimorphic.

A detailed study of four species of columbine (*Aquilegia*) was conducted to determine the mechanisms that control petal spur length development in this diverse genus that has

rapidly radiated into many species (Puzey *et al*. 2012). Flower petal spur length varies from approximately 1-15 cm and spur lengths are associated with the primary pollinators for each species. In this study the major associated pollinators were bees for the species with short, curled spurs, hummingbirds for the short straight spurs, and hawk moths for the flowers with long narrow spurs. The authors demonstrated that 99% of spur elongation was due to anisotropic cell expansion (controlled directional cell elongation) not cell division, and that the number of days cell expansion took place was correlated with the length of the spur - 10 days for the species with the shortest spurs compared to 16 days for the species with the longest spurs. They concluded that rapid speciation in *Aquilegia* was directly related to the genes that control cell expansion in the petal spurs.

Fosberg (1948) speculated that the endemic Hawaiian Hibiscus species in section *Lilibiscus* were all derived from the same colonizing ancestor (Bates 1990). This group consists of the smaller flowered (approximately 5 cm - 7 cm long) endemic red and orange species, *H. clayi, H. kahilii, H. kokio*, and *H. saintjohnianus*, and the fragrant, mostly much larger (13 cm -19 cm long), species with white petals, *H. arnottianus, H. immaculatus, H. punaluuensis, H. hannerae* and *H. waimeae.* The endemic Hawaiian *Hibiscus* taxa are shown in Figure 2.1. *Hibiscus hannerae* is the exception in the white, fragrant species because it is approximately the same size (6 cm long) as the red species, much smaller than the other whites. Interestingly, most individuals in the white species, except for *H. immaculatus,* have magenta or pink staminal columns, rarely are they completely white, and some have a pink or yellowish tinge on the petals (Figure 2.2),

FIGURE 2.1. Endemic Hawaiian *Hibiscus* sect. *Lilibiscus* flowers (left to right): *H. waimeae, H. hannerae, H. punaluuensis, H. arnottianus, H. immaculatus, H. clayi, H. kokio, H. saintjohnianus,* and two *H. kahilii*. Some petals have been removed to show the calyces and staminal columns.

FIGURE 2.2. *H. arnottianus* showing the pink and pale yellow colors on the petals that are sometimes observed in the white-flowered species of Hawaiian endemic *Hibiscus*.

whereas the staminal column of *H. immaculatus* is always white (Bates 1990). If Hawaii's red and white *Hibiscus* species share the same ancestor as Fosberg suggested, shifts in pollinators could have been a significant factor in speciation for this group.

The typical passerine (perching) bird pollinated flower is red, with a tubular shaped perianth, no fragrance and is diurnal with dilute nectar that is predominately hexose (glucose and fructose). Red flowers have been reported as being difficult for insects to see (Raven 1972, Schemske and Bradshaw 1999). The typical moth pollinated flower is white and fragrant, with a longer, more narrow perianth than in bird pollinated flowers, opens at night and produces a high sucrose, dilute nectar (Raven 1972, Faegri and van der Pijl 1979, Baker and Baker 1983, Scogin 1983, Grant 1992, Josens and Farina 2001, Luyt and Johnson 2001, Johnson and Nicolson 2007, Nicolson 2007, Nicolson and Thornburg 2007). Birds and hawkmoths have high-energy requirements due to the costs of flying and hovering (Heyneman 1983, Josens and Farina 2001, Kelber 2003, Nicolson 2007) so a concentrated nectar with high energy content might be expected in flowers pollinated by these animals. However, though nutrient content is less per volume, dilute nectars are ingested more easily and faster than more concentrated solutions, so feeding time is more efficient and exposure to predators is reduced (Heyneman 1983, Josens and Farina 2001). Since hawkmoths must suck nectar through a long, narrow proboscis, viscosity of nectar is especially important (Josens and Farina 2001) and Heyneman (1983) reported and an average sugar concentration of 19% in flowers visited by hawkmoths.

Grant (1992) concluded that the mouthparts of the pollinator and the length and width of the floral tube are indicative of the pollination system for a particular plant. Though there are exceptions to this general rule and animals other than the primary

pollinator might visit flowers in one of these classes, many studies have concluded that the most efficient transfer of pollen is accomplished by the best adapted animal (Faegri and van der Pijl 1979, Grant 1992, Hapeman and Inoue 1997, Luyt and Johnson 2001, Wilson *et al.* 2006). However, Sahli and Conner (2006) concluded that for most plants, pollinator importance was more accurately predicted by visitation rates than by pollinator effectiveness except in flowers with specialized pollen removal or deposition systems.

Extreme specialization of flowers to one pollinator type can be dangerous. The likely pollinators of many now endangered Hawaiian lobeliads (Lobelioidae, Campanulaceae) were the extinct or endangered Hawaiian honeycreepers (Drepanidinae) and Hawaiian honeyeaters (Mohoidae; Lammers and Freeman 1986, Givnish *et al*.1995, Lammers 1995, Corbet 1997, Cox and Elmqvist 2000, Ziegler 2002, Fleischer *et al*. 2008). These birds were probably the pollinators of the red and orange native Hawaiian *Hibiscus* as well, though I have found no documentation for this. Figure 2.3 is a photograph of *Cyanea koolauensis* and *H. kokio*. These hibiscus species exhibit the floral traits characteristic of passerine pollinated flowers.

The evolution of Hawaiian honeycreepers, descended from a single ancestral finch species, into at least 47 species, many of which were nectar-feeding specialists, is considered one of the most spectacular examples of adaptive radiation (Carlquist 1965, Banko and Banko 1976, Tarr and Fleisher 1995, Eggert *et al*. 2008). Many of these birds are now extinct because of introduced avian diseases carried by non-native birds, predators (such as rats and mongoose) and habitat destruction. Most of those that remain live at elevations of 900 m or more, higher than most Hawaiian *Hibiscus,* out of the range of the vector mosquito (*Culex quinquefasciatus*), the carrier of avian malaria and avian

FIGURE 2.3. *Cyanea koolauensis* (left) and *Hibiscus kokio* (right) both from Oahu, and both probably pollinated by Hawaiian honeycreepers and honeyeaters. Nectar is visible at the bottom of the calyx in the hibiscus.

pox (Carlquist 1980, Pratt *et al*. 1987, Bates 1990, Ziegler 2002, Eggert *et al*. 2008, Krend 2011). The extant honeycreepers that feed on flower nectar are Iiwi (*Vestiaria coccinea*) on all the main Hawaiian Islands but now very rare on Oahu (J. Rohrer personal communication 2012), Apapane (*Hematione sanguiea*) on all the main islands, Akohekohe or Crested Honeycreeper (*Palmeria dolei*) on Maui, the 3 species of Amakihi (*Hemignathus kauaiensis* on Kauai, *H. flavus* on Oahu, and *H. virens virens* on Hawaii), and Anianiau (*Hemignathus* parvus) on Kauai (Pratt 1987). The latter two are probably too small to effectively pollinate even the smallest Hawaiian hibiscus, *H. clayi*, but I have observed Oahu Amakihi stealing nectar from the calyx below the petals of the native white species *H. arnottianus* (Figure 2.4) and the yellow indigenous *H. tiliaceus*. Either they make a small incision at the base of the calyx or they insert their beaks between the top of the calyx and the base of the petals, thus avoiding the anthers and stigmatic lobes completely. I have also observed non-native passerine birds taking nectar from both the white and the red- and orange-flowered native *Hibiscus* in the same way the amakihi do. These non-native species include Japanese White-eye (*Zosterops japonicus*), Red-whiskered Bulbul (*Pycnonotus cafer*), and Red-vented Bulbul (*Pycnonotus jocosus*).

In recent years several studies have concluded that the Oahu Amakihi and some lower elevation populations of amakihi on Hawaii Island have developed resistance to avian malaria and the numbers of amakihi are increasing (Eggert *et al*. 2008, Krend 2011) which is very encouraging news, especially since many of the trends for Hawaiian endemics are negative. In 2012 the Pacific Island Ecosystems Research Center and the Hakalau Forest National Wildlife Refuge reported that three of the rarest birds on Hawaii Island, already federally listed as endangered, were seen or heard at lower elevations than

FIGURE 2.4. Oahu Amakihi (*Hemignathus flavus*) taking nectar from *H. arnottianus.* Even a larger bird entering from the front of the flower would probably not come into contact with the stigmatic lobes at the end of the staminal column.

they had been seen in 30 years (American Bird Conservancy 2012). The scientists who conducted this study hope that the observations of these three species, the Akaipolaau (*Hemignathus munroi*), the Hawaiian Akepa (*Loxops coccineus*) and the Hawaii Creeper (*Oreomystis mana*), might be evidence that these birds were also developing resistance to mosquito borne diseases. There has been concern that as temperatures rise globally, mosquitoes will successfully invade higher elevation forests that have been refuges for what is left of the endemic Hawaiian forest birds.

The impact of introduced mammalian predators was studied on the main North Island of New Zealand (Anderson *et al*. 2011). These predators have either reduced or driven to extinction several species of bird pollinators. The authors reported that pollination, seed set and numbers of an endemic forest shrub, *Rhabdothamnus solandri* (Gesneriaceae), were greatly reduced compared to the populations of the same plant on offshore islands where the native bird pollinators were still present (Anderson *et al*. 2011). New recruitment of this long-lived plant species on the main island is much less than on the outer islands, therefore long-term survival of this species on the main island without human intervention does not look promising.

Many Hawaiian plants may be facing a similar situation. Several of the Hawaiian *Hibiscus* examined here are already federally listed as endangered species. These include *H. immaculatus* (USFWS 2011), *H. clayi* (USFWS 2008), and *H. hannerae* (USFWS 2010). In recent years more individuals of both *H. clayi* (on Kauai) and *H. immaculatus* (on Molokai) have been discovered (S. Perlman, personal communication 2011) but they are still very rare species. According to the latest 5-Year Review for *H. waimeae* subsp. *hannerae* (USFWS) the number of individuals in the wild has decreased from 75 - 125

individuals in 1996 to 80 to 85 in 2008. Some of the decrease is attributed to damage done by Hurricane Iniki but other threats are loss of pollinators, seed eating insects and rodents, invasive weeds and goats. Similar threats are affecting all hibiscus species in Hawaii.

The ancestors of endemic Hawaiian insects were more successful at colonizing the islands than birds and are important components of native ecosystems including as pollinators (Gagne 1982, Howarth and Mull 1992, Cox and Elmqvist 2001). Two large endemic hawk moths (Sphingidae), Blackburn's Sphinx Moth (*Manduca blackburni*), Hawaii's largest native insect (Rubinoff and San Jose 2010, Rubinoff *et al*. 2012), and the Fabulous Green Sphinx Moth (*Tinostoma smaragditis*) are very rare today with limited ranges. *Hyles* is a third, less rare and smaller, hawk moth genus with two endemic Hawaiian species, *H. calida* and *H.perkinsi* (Gagne 1982, Jamieson and Denny 2001, Rubinoff and San Jose 2010). These may have been important pollinators of the fragrant white *Hibiscus* species, *H. arnottianus, H. immaculatus, H. punaluuensis, H. hannerae*, *and H. waimeae.* The white Hawaiian *Hibiscus* exhibit the floral traits characteristic of moth pollination mentioned earlier (white flowers, fragrance etc.). It is possible that these moths were more numerous and widespread in the past and that there were even more, large moth species, now extinct, that were never recorded. Blackburn's Sphinx Moth, a relative of the Tomato Hornworm from North and South America (*Manduca quinquemaculatus*), was found on all the main Hawaiian Islands in the past, but now is found only on Maui, Hawaii and Kahoolawe (Jamieson and Denny 2001, Rubinoff and San Jose 2010). It seems likely that with five hibiscus tree taxa with large, showy, fragrant white flowers in Hawaiian forests, that large moths must have been

present in sufficient numbers to favor the evolution of this suite of floral characteristics. There are no other known *Hibiscus* in section *Lilibiscus* with white fragrant flowers. Unfortunately, habitat destruction, loss of host plant species, and introduced parasites and predators have negatively impacted native lepidopterans (Gagne 1982, Rubinoff and San Jose 2010). Several species of introduced hawkmoths might be large enough to successfully pollinate these *Hibiscus* species today. However, I have found no reports of their ranges overlapping with native *Hibiscus* in their natural habitats. These species are the Sweet Potato Hornworm (*Agrius cingulata*), the Gray Hawkmoth (*Psilogramma memephron*), the Oleander Hawkmoth (*Deilephila nerii*), and the Yam Sphinx Moth (*Theretra nessus*) (Jamieson and Denny 2001). Host plant availability is always a limiting factor with Lepidoptera.

The staminal columns of some Hawaiian *Hibiscus* species may significantly impact what may pollinate their flowers. The staminal column of *H. arnottianus* is approximately 19 cm long when fully extended (Bates 1990) and *H. punaluuensis*, the largest Hawaiian hibiscus, can have an even longer staminal column reaching more than 20 cm in length. The long staminal column and long, flexible stamens of the white species block easy access to the small openings between the bases of the petals behind which is the nectar at the bottom of the calyx. Figure 2.1 shows all Hawaiian *Hibiscus* sect. *Lilibiscus* and the variation in lengths of staminal columns and stamens. Figure 2.5 is a photograph taken from the apex of the staminal column of *H. arnottianus*. *Hibiscu*s do not have long tubular flowers like *Brighamia*, an endemic Lobelioid, but the long staminal column of the white flowered species may serve to facilitate pollination by a

FIGURE 2.5. *H. punaluuensis* looking down through the stigmatic lobes and stamens toward the base of the staminal column and the openings between the petals that lead to the calyx.

hovering moth with a long tongue (proboscis) (Figure 2.6 *Brighamia* and *H. arnottianus*). Either the pollinator would have to land on the petals to avoid the numerous stamens or hover outside or very close to the staminal column and access the nectar with a long proboscis, the latter a typical feeding behavior typical of long-tongued hawk moths (Grant 1992, Martins and Johnson 2007).

The flowers of the red and white Hawaiian Hibiscus species open at different times of the day. The red flowers are fully open at sunrise and the anthers are entirely dehisced. The petals begin to fade later in the day, though the stigmatic lobes may retain turgor into the next day. In contrast, the whites, except for *H. immaculatus,* slowly begin to open the first morning but the anthers don't dehisce until the second day (Figure 2.7) and during the early part of the first day the nectar is not always available because the calyx is usually constricting the base of the petals too tightly. During the first night the petals extend fully from the calyx (Figure 2.8) and there are spaces between the bases of the petals that lead to the calyx where the nectar is located (Figure 2.9). The anthers dehisce during the second day in all the white species except *H. immaculatus* whose anthers are fully dehisced in the morning of the first day and the flower has faded noticeably by the next morning. Another unusual condition sometimes observed in *H. immaculatus* is the inability of the stigmatic lobes to extend beyond the apex of the staminal column (they are not visible but they are present), thus making the flowers effectively male only (Figure 2.10). On the third day the petals on the other white species begin to fold forward, sometimes twirling tightly around the staminal column like a pinwheel (Figure 2.11), and then the petals drop off.

FIGURE 2.6. *Hibiscus arnottianus* and *Brighamia insignis* flowers showing differences in flower morphology of these two fragrant and probable moth pollinated Hawaiian endemics.

FIGURE 2.7. Flowers of *H. arnottianus*: first day (in background) and second day (foreground) showing elongation of the staminal column and expansion of the petals as the flowers age. The pollen is dehisced only in the second day flower. Photo taken at 6:00 p.m..

FIGURE 2.8. Close up of calyces of *H. arnottianus* Day 1 and Day 2 flowers (white petals), and *H. saintjohnianus* (orange petals) with some petals removed to expose the staminal columns. The fused base of the petals in *H. arnottianus* is approximately 1 cm longer in the Day 2 flower (left) than on Day 1. The petals of *H. saintjohnianus* form a wide tube. Also visible is the darker base of the petals in *H. saintjohnianus,* a characteristic seen in *Hibiscus* species from the Mascarene Islands but not in any other Hawaiian *Hibiscus* in sect. *Lilibiscus.*

FIGURE 2.9. Variation in petal size in *H. arnottianus* from Oahu showing the openings at the base of the petals that permit access to nectar at the base of the calyx. The flower on the left is from Manoa Cliffs, Koolau Mountains. The flower on the right is from Palikea, Waianae Mountains.

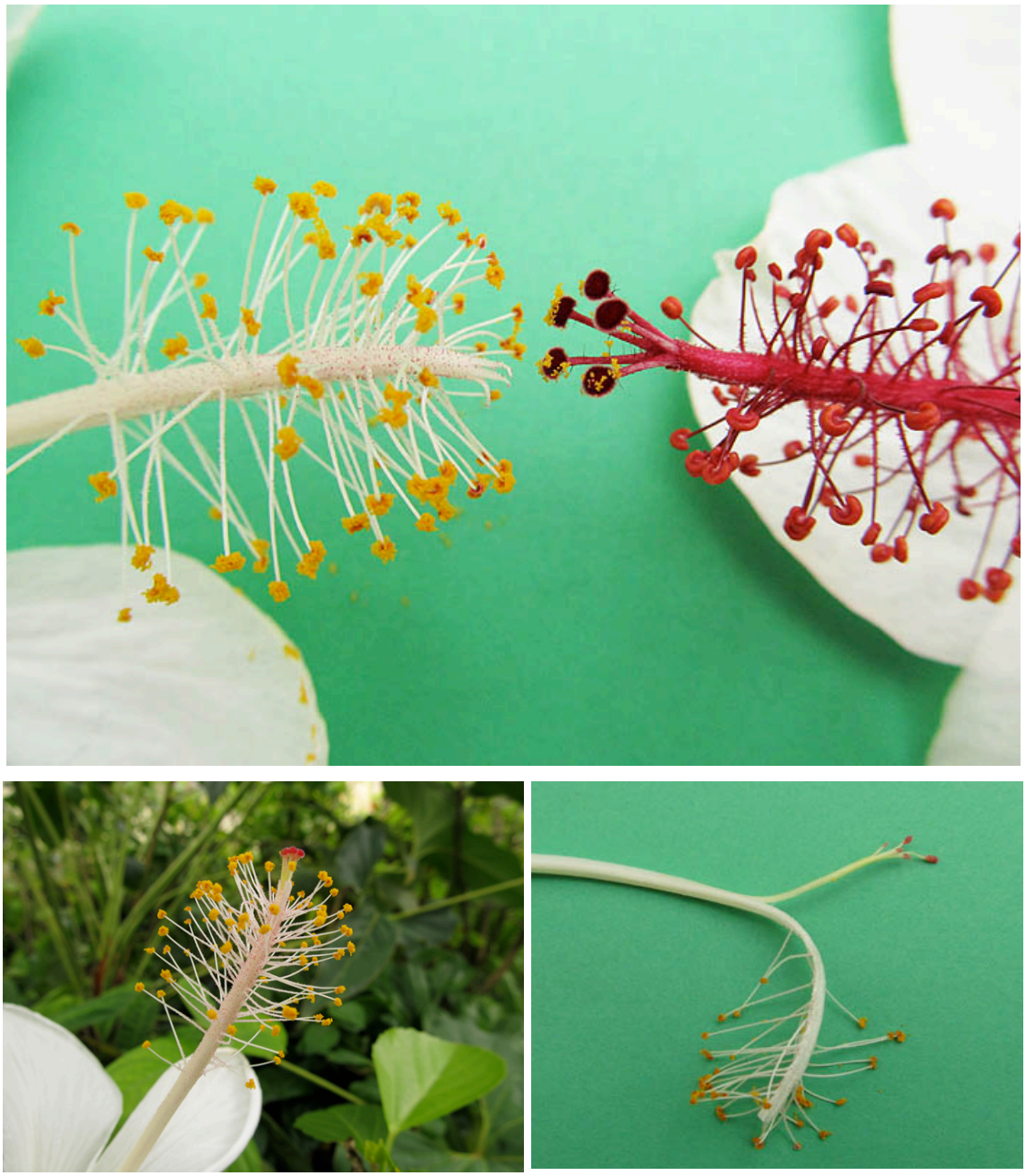


FIGURE 2.10. Abnormal development of stigmatic lobes in *H. immaculatus*. *H. immaculatus* with stigmatic lobes still within staminal column (top left) and normally developed *H. arnottianus* (top right). Normal development in the same accession of *H. immaculatus* (bottom left) on a different day, and excised staminal column with abnormal staminal lobes (bottom right).

FIGURE 2.11. Flower of *H. punaluuensis* in the morning of the third day with petals turned back forming a pinwheel, a condition observed in some plants of *H. punaluuensis* and *H. arnottianus.*

In addition to flower morphology, the relative percentages of the three primary sugars in floral nectar (sucrose, glucose and fructose) have been correlated with specific pollinators in many plant species and interesting comparisons of nectar content have been made between closely related species in the same plant genus (Baker and Baker 1983, Nicolson 1998, Wilson *et al.* 2006, Nicolson and Thornburg 2007). Baker and Baker (1983) reported that flowers with glucose and fructose dominant nectars were generally associated with pollination by perching birds, New World bats, short-tongued bees, or flies. They found that plants with nectar that is predominately sucrose were primarily pollinated by insects (long-tongued bees, butterflies, settling moths, and hawk moths) and hummingbirds. There are examples of closely related species in the same genus having some species that are predominantly pollinated by hummingbirds that have flower nectar with a higher percentage of sucrose in the nectar than hexose, and other species in the same genus with hexose rich nectar that are mostly passerine pollinated such as in *Penstemon* (Scrophulariaceae) and *Erythrina* (Fabaceae) (Baker and Baker 1983, Nicolson and Thornburg 2007).

As more studies have been conducted comparing nectar sugars and associated pollinators it has been reported that in many cases plant phylogeny may be a more important factor in determining nectar content than pollinator preference. Some plant families appear to be "phylogenetically constrained." For example the dominant sugars in nectar of Asteraceae, Fabaceae, Solanaceae and Verbenaceae are hexose regardless of the type of pollinator (Nicolson and Thornburg 2007).

Lammers and Freeman (1986) analyzed the floral nectars of endemic Hawaiian Lobelioids (Campanulaceae), which, as mentioned earlier, were believed to be

predominantly pollinated by the Hawaiian honeycreepers (Drepanidinae) and honeyeaters (Mohoidae). This study was undertaken to test the hypothesis that the nectar sugars would consist primarily of glucose and fructose (hexose) as would be expected for passerine pollinated flowers since, unfortunately, most of these birds are now extinct and their behaviors cannot be observed. Using high-performance liquid chromatography to analyze the nectar of 10 species in three genera they found that the average percentages of the three sugars were 43.5 % fructose, 54.5% glucose, and 2% sucrose, supporting the hypothesis.

Evidence of probable pollinator shifts from insects to birds has been reported among several Hawaiian plant lineages. These include *Bidens* (Asteraceae; Ganders and Nagata 1983), *Geranium* (Geraniaceae; Carlquist 1980, Ziegler 2002), the endemic mints, *Haplostachys, Phyllostegia* and *Stenogyne* (Lamiaceae; Lindqvist and Albert 2002) and *Polyscias* (Araliaceae; Costello and Motley 2007). In Hawaiian *Geranium* and the mints, there are examples of genera with closely related species, some of which have white, fragrant, cup-shaped flowers, characters associated with pollination by moths or other insects, while other species with red, tubular flowers and no scent, have characters associated with bird pollination. The floral traits of Hawaii's white and red *Hibiscus* species most likely indicate whether the flowers were predominantly bird or insect adapted. It is possible that the two hibiscus groups evolved to rely on different pollinators and that a shift in pollinator preference could have led to divergence in this group before or after arriving in Hawaii.

Objectives for Nectar Analysis

The purpose of this study was to compare the flower nectar sugar contents of the both red- and white-flowered Hawaiian *Hibiscus* taxa to determine if they are consistent with pollination syndromes for these species. Nectar from four non-Hawaiian *Hibiscus* species sect. *Lilibiscus* (Figure 2.12), and *H. tiliaceus* were also tested. Species of Hawaiian lobeliads (in the genera *Brighamia, Cyanea, Delissea* and *Lobelia*), unrelated to *Hibiscus*, were also sampled for comparative purposes (Figure 2.13). Like *Hibiscus*, several species of this radiation are presumed to be bird or moth pollinated and, in some cases, have habitats that overlap in range with those of *Hibiscus*. Also sampled is the non-native white, fragrant *Brunfelsia americana* (Solanaceae), similar in flower structure to that of *Brighamia*, and two Malvaceae species, *Hibiscadelphus distans* (conjectured to be bird pollinated) and *Malvaviscus arboreus* var. *drummondii* (a known hummingbird-pollinated species; George 1980)(Figure 2.12). Sugar analysis will be conducted using high-performance liquid chromatography and refractometry to determine the concentration and relative percentages of sucrose, glucose, and fructose in these taxa in order to provide an indication of the nature of the pollinator.

Materials and Methods

Ninety-two samples of nectar were collected from the flowers of 14 *Hibiscus* taxa including all nine endemic Hawaiian species in sect. *Lilibiscus,* four from the Mascarene Islands also in sect. *Lilibiscus,* and one indigenous Hawaiian species, *H. tiliaceus.* These and other non-section *Lilibiscus* taxa are listed in (Table 2.1). Plants were grown in the

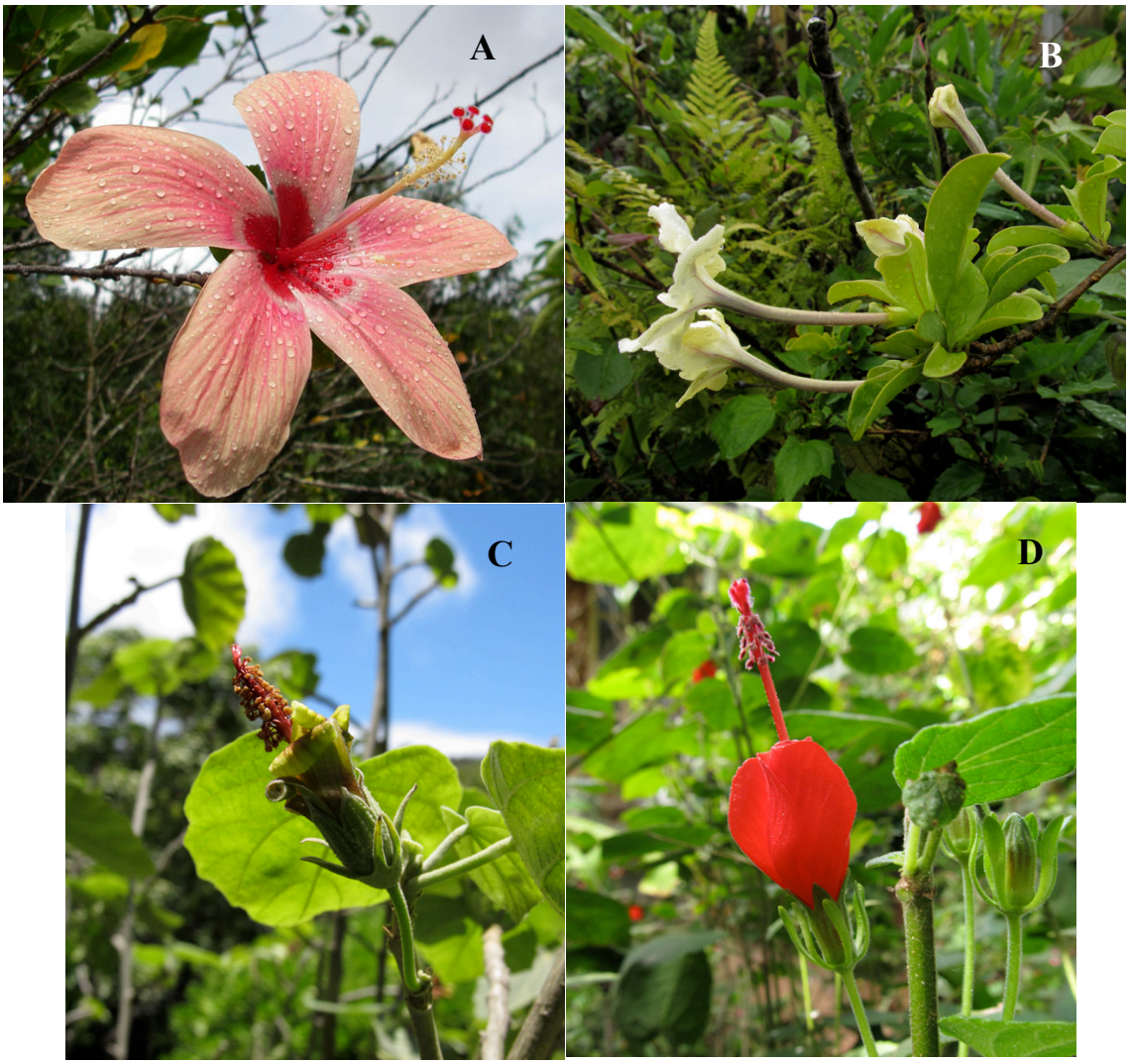


FIGURE 2.12. Outgroups (Group 1) included in nectar analysis: (A) *Hibiscus genevii* sect. *Lilibiscus* from Mauritius, (B) *Brunfelsia americana* (Solanaceae) from the West Indies, (C) *Hibiscadelphus distans* (Malvaceae) from Kauai and (D) *Malvaviscus arboreus* var. *drummondii* (Malvaceae) from Texas.

FIGURE 2.13. Outgroups (Group 2). Tubular flowers of Hawaiian Lobelioids sampled for nectar analysis: (A) *Cyanea koolauensis*, Oahu, (B) *C. lanceolata,* Oahu, (C) *Delissea rhytidosperma,* Kauai, (D) *D. waianaeensis,* Oahu (E) *Lobelia grayana,* Maui, and (F) *L. niihauensis*, Niihau, Kauai and Oahu

TABLE 2.1. The species studied, locality, flower color, number of individuals sampled (N) and average percentages (± 1 S.D.) of fructose (F), glucose (G) and sucrose (S) in floral nectar.

Taxon	Island	Flower Color	N	% F	% G	% S
Hibiscus. clayi Degener & I. Degener [*H. newhousei* M. Roe]	Kauai	red	11	43.55 ± 1.05	56.34 ± 1.06	0.11 ± 0.30
H. kahilii Forbes*; H. kokio* subsp. *kokio* (Forbes) D. Bates	Kauai	red or pink	7	42.90 ± 0.71	57.06 ± 0.66	0.03 ± 0.06
H. kokio Hillebr.; *H. kokio* subsp. *kokio* (Hillebr.) D. Bates	Oahu, Molokai, Maui, Hawaii	red	16	42.91 ± 1.02	57.06 ± 0.99	0.04 ± 0.06
H. saintjohnianus M. Roe; *H. kokio* subsp. *saintjohnianus* (M. Roe) D. Bates; *H. roetae* St. John; *H. saintjohnianus* M. Roe	Kauai	orange or yellow	10	42.88 ± 4.75	56.94 ± 5.03	0.18 ± 0.55
H. arnottianus A. Gray; *H. arnottianus* subsp. *arnottianus* (A. Gray) D. Bates; *H. waimeae* A. Heller var. *hookeri* Hochr.	Oahu	white	12	44.53 ± 2.18	55.37 ± 2.14	0.10 ± 0.20
H. immaculatus M. Roe; *H. arnottianus* subs*p. immaculatus* (M. Roe) D. Bates; *H. immaculatus* M. Roe	Molokai	white	3	43.48 ± 0.20	56.51 ± 0.19	0.01 ± 0.01

H. arnottianus subsp. *punaluuensis* (Skottsb.) D. Bates; *H. punaluuensis* (Skottsb.) Degener & I. Degener	Oahu	white	5	43.46 ± 0.62	56.31 ± 0.72	0.23 ± 0.52
H. hannerae (D & D) Huppman; *H. waimeae* subsp. *hannerae* (Degener & I. Degener) D. Bates; *H. w*. var. *hannerae* Degener & I. Degener	Kauai	white	2	43.82 ±0.08	56.03 ± 0.01	0.16 ± 0.09
H. waimeae A. Heller*; H. waimeae* subsp. *waimeae* (Heller) D. Bates; *H. w.* var. *helleri* Hochr.	Kauai	white	5	43.84 ± 1.04	56.55 ± 0.45	0.01 ± 0.03
Outgroups						
H. boryanus Hook. & Arnot.	Mauritius, Reunion	orange or pink	3	46.19 ± 3.40	52.54 ± 3.90	1.27 ± 1.79
H. denisonii Hort. Ex Flor.	unknown	white + pink	1	44.49	54.50	1.01
H. fragilis DC.	Mauritius, Rodrigues	dark pink	1	47.70	52.30	0
H. genevii Bojer	Mauritius	light pink+dark eye	1	42.75	57.25	0
H. tiliaceus L.	Oahu	yellow + dark eye	1	49.61	50.39	0

Hibiscadelphus distans L. Bishop & Herbst	Kauai	green/red	3	43.97 ± 3.54	55.99 ± 3.5	0.04 ± 0.05
Malvaviscus arboreus var. *drummondii* (Torr. & A. Gray) Schery	Texas	red	1	43.17	31.30	25.53
Brighamia insignis A. Gray	Kauai	yellow	1	16.42	2.86	80.72
Cyanea koolauensis Lammers, Givnish & Systma	Oahu	magenta	1	43.56	56.39	0.05
C. lanceolata (Gaud) Lammers, Givnish & Systma	Oahu	white + purple	2	43.45 ± 0.83	53.74 ± 0.51	2.81 ± 0.33
Delissea rhytidosperma H. Mann	Kauai	green	2	46.88 ± 3.18	52.25 ± 4.41	0.87 ± .23
D. waianaeensis Lammers	Oahu	green + white	1	45.45	53.9	0.65
Lobelia grayana F. Wimmer	Maui	blue	1	41.08	58.46	0.45
L. niihauensis St. John	Kauai	pink	1	46.52	52.94	0.54
Brunfelsia americana L.	West Indies	white	1	49.9	49.92	0.18

greenhouse, outside nursery, or on the grounds of Lyon Arboretum, Honolulu Hawaii. Some additional samples were obtained from plant accessions at Waimea Arboretum, the Manoa Heritage Center, and Hui Ku Maoli Ola Native Plant Nursery on Oahu. Nectar samples were taken from open flowers using a 10 µl microcapillary tube and stored frozen at -20 °C until ready for processing.

Sugar Separation HPLC

Forty microliters of nectar were diluted in 560 µl of deionized water. Sucrose, fructose, and glucose were separated and quantified by high-performance liquid chromatography (HPLC) using a 20 µl sample. The Shimadzu Model 20 HPLC with a CBM-20A controller, LC-20AT pump, SIL-20A automatic injector, CTO-20A column oven and an ELSD-LT-II Evaporative Light Scattering Detector was used. The analysis column (Fast Carbohydrate Analysis Column 100 x 7.8 mm with a precolumn, Bio-Rad Laboratories, Hercules, CA) was run at 1 $ml \cdot min^{-1}$ and 80°C with degassed deionized water. The ELSD was at 40°C. Retention times of the sugars were compared with that of pure standards: glucose 10 mg/ml, fructose 10 mg/ml and sucrose 10 mg/ml. Sugar concentration was calculated on the height of the individual sugar peaks compared to pure standards. Relative percentages of fructose, glucose and sucrose were calculated using the response peak heights of the sugars present in each sample.

Nectar Concentrations

Nectar samples were taken from open flowers using a 10 µl microcapillary tube and stored frozen. Nectar concentrations were determined using a pocket refractometer

(Bellingham and Stanley, BS Eclipse 45-03, made in U. K.). Undiluted 10 µl samples were used to measure sugar concentration in degrees Brix (1% Brix = 1g sucrose in 100g water).

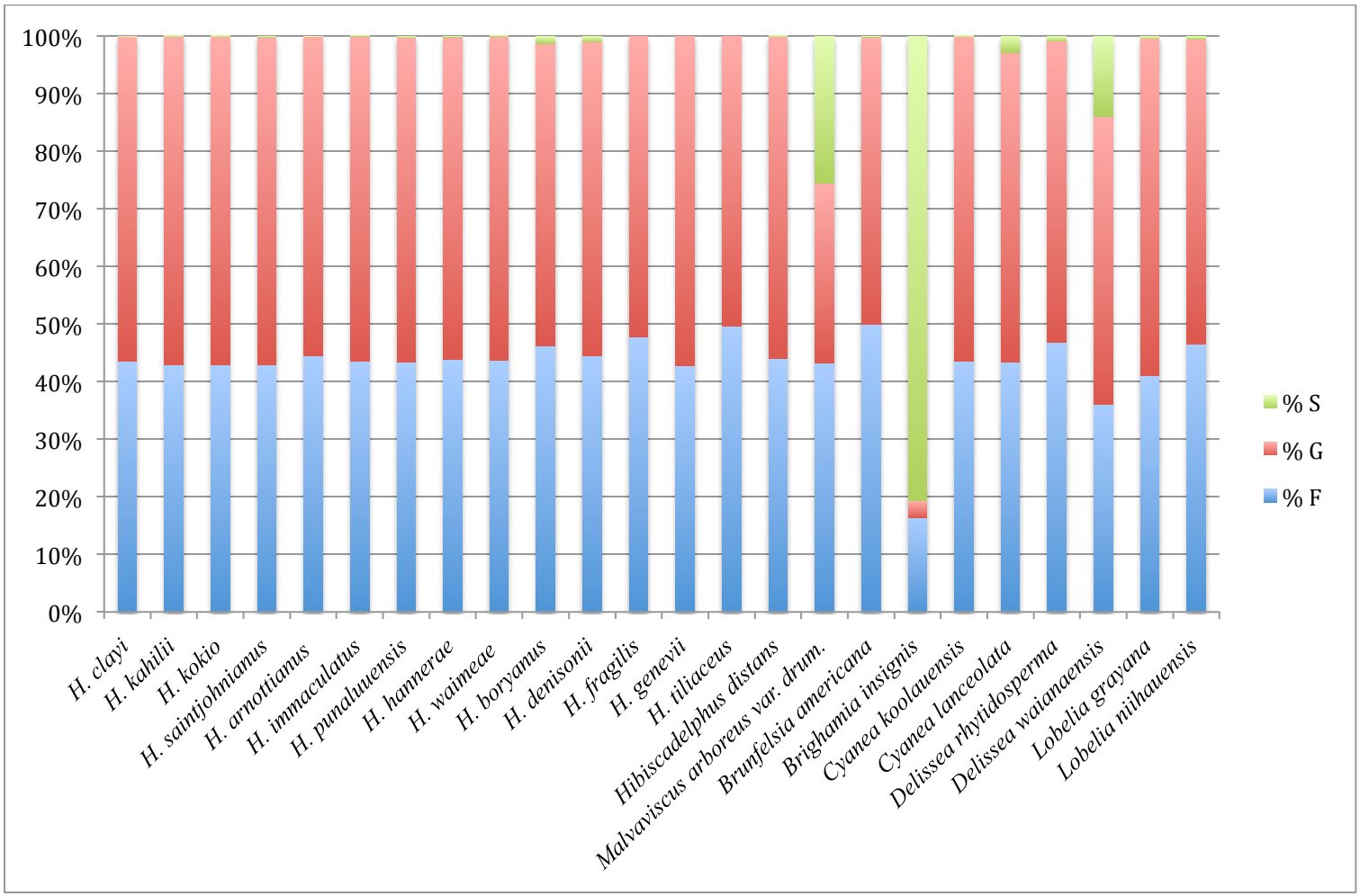
Results

Relative Percentages of Fructose, Glucose and Sucrose

The flower nectars of all of the endemic Hawaiian *Hibiscus* species examined were primarily glucose and fructose with little or no sucrose detected, and were very consistent within and among species (Table 2.1 and Figure 2.14). The relative percentages of the three sugars in the red endemic Hawaiian *Hibiscus* were very similar to those observed in the white-flowered species. The ranges of the relative percentages of the three sugars in the four red-flowered Hawaiian *Hibiscus* taxa (± 1 S.D.) were: fructose 42.88 ± 4.75% to 43.55 ± 1.05%, glucose 56.34 ± 1.06% to 57.06 ± 0.99%, and sucrose 0.03 ± 0.06% to 0.18 ± 0.55%. The ranges for the three sugars in the white-flowered species were: fructose 43.46 ± 0.62% to 44.53 ± 2.18%, glucose 55.37 ± 2.14% to 56.55 ± 0.45%, and sucrose 0.01 ± 0.01% to 0.23 ± 0.52%. The results for the three *Hibiscus* from the Mascarene Islands were very similar to the ranges for the Hawaiian *Hibiscus* in the same section *Lilibiscus*: fructose 42.75% to 47.70%, glucose 52.30% to 57.25%, and sucrose 0.0% to 1.27%.

Of the two other Malvaceae sampled, *Hibiscadelphus distans* was very similar to all the hibiscus species: fructose 43.97 ± 3.54, glucose 55.99 ± 3.5, and sucrose 0.04 ± 0.05. The proportion of the three sugars in the nectar of *Malvaviscus arboreus* var. *drummondii*

FIGURE 2.14. Relative percentages of 3 floral nectar sugars, sucrose (S), glucose (G) and fructose (F), in *Hibiscus, Hibiscadelphus, Malvaviscus,* Hawaiian lobeliads and *Brunfelsia americana*.



was notably different from the other Malvaceae. There were more equal amounts of the three sugars: fructose (43.17%), followed by glucose (31.30%) and sucrose (25.53%).

The results for most of the lobelioid (Campanulaceae) species in this study were very similar to an earlier examination of other Hawaiian lobeliads (Lammers *et al*. 1989) and to the *Hibiscus* percentages found here. The range in the two *Cyanea* species was: fructose 43.45% – 43.56%, glucose 53.74 – 56.39%, and sucrose 0.05% – 2.81%. The two *Delissea* species had slightly higher percentages of fructose and sucrose than the *Cyanea* tested: fructose 45.45% – 46.88%, glucose 52.25% – 53.90%, and sucrose 0.65% - 0.87%. The blue-flowered *Lobelia grayana* had the highest value for glucose at 58.46%, fructose was 41.08% and sucrose was very low (0.45%). *Lobelia niihauensis* had less glucose (52.94%) and slightly more fructose (46.52%) but also was very low in sucrose (0.54%).

The notable exception in the present study was the nectar of *Brighamia insignis.* The nectar was predominately sucrose (80.72%), followed by fructose (16.42%), but was very low in glucose (2.86%).

The proportions of fructose and glucose in the fragrant, white, tubular-flowered *Brunfelsia americana* (Solanaceae) were close to 50% each with almost no sucrose detected.

Nectar Sugar Concentration

The percent soluble sugar concentrations were similar in the red and white-flowered species of Hawaiian *Hibiscus* (Table 2.2). The overall average percent soluble sugar concentrations were 15.24 ± 5.67% in the reds and 16.90 ± 6.87% in the whites. The

TABLE 2.2 Concentrations of soluble sugars in floral nectar of Hawaiian *Hibiscus* sect. *Lilibiscus* (± 1 S.D.).

White-Flowered	N[a]	% Soluble Sugars	Red-Flowered	N[a]	% Soluble Sugars
H. arnottianus	9	17.94 ± 4.86	*H. clayi*	5	13.60 ± 4.39
H. immaculatus	5	13.40 ± 6.35	*H. kahilii*	6	16.25 ± 6.05
H. punaluuensis	11	19.68 ± 7.16	*H. kokio*	12	18.04 ± 6.50
H. hannerae	2	21.50 ± 2.12	*H. saintjohnianus*	10	12.10 ± 2.56
H. waimeae	8	12.93 ± 7.70			
Average		16.90 ± 6.87	Average		15.24 ± 5.67

[a] **N** number of individual plants sampled per taxon

concentrations for the red species ranged from 12.10 ± 2.56% in *H. saintjohnianus* to 18.04 ± 6.05% in *H. kokio.* The whites ranged from 12.93 ± 7.70% in *H. waimeae* to 21.50 ± 2.12% in *H. hannerae.* The variation in sugar concentrations between individuals within taxa was much larger in some cases, particularly in *H. waimeae, H. hannerae, H. kokio* and *H. saintjohnianus*. The degree of variation in the percent sugar values within taxa was larger than the amount of variation in relative amounts of sucrose, fructose and glucose within taxa.

Flower Maturation

Flowers of the red- and white-flowered Hawaiian *Hibiscus* species open at different times of the day and are open for different lengths of time. Flower maturation is very consistent among all four of the red-flowered species. Red flowers are fully open by sunrise of Day 1 with nectar available and the anthers entirely dehisced by this time; petals begin to wilt later that day and are completely wilted by Day 2. Stigmatic lobes of red flowers may retain turgor into Day 2 although it is not known if they are still receptive at this stage.

In contrast, white flowers (except for *H. immaculatus*) slowly begin to open the morning of Day 1, but the petal margins remain tightly overlapping and the calyx is usually constricted at the base of the petals preventing potential pollinators from gaining access to the nectar. Flower petals and anthers continue to elongate over the course of Day 1 and into the afternoon of the Day 2 (Figure 2.15). By late afternoon of Day 2, petals are fully elongated with space between their adjacent petal near the base allowing access to nectaries (Figure 2.9), and anthers are fully elongated and begin to dehisce

FIGURE 2.15. Comparison of *H. arnottianus* (white petals) Day 1 (short staminal column) and Day 2 (long staminal column) with flowers of *H. clayi* (center, red petals), and *H. saintjohnianus* (bottom, orange petals). The flowers of the red and orange species last one day only.

(Figure 2.7). On Day 3, the petals of some white flowers may fold forward and then fall off later that day, or in others the petals may twirl tightly around the staminal column like a pinwheel before the petals drop off (Figure 2.11).

Hibiscus immaculatus is the lone exception to the two-day flowering cycle among the white-flowered species. Anthers are fully dehisced in the morning of Day 1 (similar to what occurs in red flowers) and the flower is noticeably wilted by the morning of Day 2. It had been previously noted that stigma lobes of some flowers of *H. immaculatus* are absent (D. Orr, personal communication 2007). Investigation into this here has shown that stigmatic lobes are present, but do not extend beyond the apex of the staminal column. These stigmas remain small and non-functional (deformed) rendering these flowers functionally male (Figure 2.10). This occurrence is variable within and among plants, sometimes found in all flowers on one plant at the same time and later in none of the flowers on that same plant. This has not been correlated as yet with climatic or seasonal conditions and should be looked into further.

Discussion

Flower nectar sugars in the red and white flowered endemic Hawaiian *Hibiscus* sect. *Lilibiscus* are predominantly hexose containing slightly more glucose (56-57%) than fructose (43-44%) and less than 1% sucrose. Though it was expected that the red and orange flowered *Hibiscus* species and *Hibiscadelphus distans* would have nectar dominated by glucose and fructose, as is characteristic of the pollination syndrome for passerine pollinated flowers, there was no evidence of a transition to predominantly

sucrose rich nectar in any of the white flowered *Hibiscus* species even though they are fragrant and the floral morphology is typical of hawk moth pollination. There is no clear evidence that the relative amounts of the nectar sugars tested played an important role in speciation in this group. The only Malvaceae species examined that had very different relative sugar percentages (lower glucose and higher sucrose levels) was the known hummingbird pollinated *Malvaviscus arboreus* var. *drummondii* from the southern U.S. (Texas to Florida) (George 1980).

A frequent corollary with relative concentration of the three sugar types is the total concentration of all sugars in nectar. Bird pollination (as hypothesized for the red-flowered *Hibiscus*) is often correlated with more dilute sugar concentrations below 45% (Heyneman 1983, Nicolson 2007, Nicolson and Thornburg 2007). Hawkmoth pollination (hypothesized for the white-flowered species) is also correlated with more dilute nectar (19-34%) than what has been reported for most other types of insect pollinated flowers especially those pollinated by bees (35-60%) (Heyneman 1983, Luyt and Johnson 2001, Nicolson 2007). Nectar concentration in *Hibiscus* examined here was equally dilute in both the red- and white-flowered species (15.24-16.90%) and more dilute than nectar concentrations Heyneman (1983) and Nicolson (2007) reported for birds and hawkmoths in reviews that examined optimal nectar concentrations for various animal pollinators.

It is possible that the genes controlling nectar sugar content in members of *Hibiscus* sect. *Lilibiscus* (including those from the Mascarene Islands in the Indian Ocean) are much less variable or subject to selection pressure than those controlling flower color, and timing in flower development and size. There may be a mechanism in *Hibiscus* similar to that in *Aquilegia* (Puzey *et al*. 2012) where the genetic control of anisotropy is

involved in the differences in overall length of flowers in the red and white Hawaiian species, especially pertaining to elongation of the staminal column and filaments. The red flowers are fully developed the first day but the petals, staminal column and filaments continue to elongate for two days in the majority of white-flowered species.

Floral herbivory can influence flower evolution. Unidentified caterpillars have been observed eating both red and white flowers, and katydids (Orthoptera) appear to only feed on the white flowered native *Hibiscus* in the nursery at Lyon Arboretum. I have observed no preference for the nectar of either the red or white-flowered hibiscus in the birds (endemic or not) that rob nectar from the flowers on a daily basis at the arboretum. These birds are relatively long-lived species that have learned to recognize hibiscus flowers and the potential nectar reward regardless of petal color. Hawkmoth behavior is more difficult to observe because they are smaller and mostly nocturnal. Also, the Hawaiian white *Hibiscus* are trees (up to 10 m tall) and the flowers may be difficult to observe closely in the dark. Only once have I observed a large moth hovering in front of *H. arnottianus*.

Ants are occasionally seen in association with mealybugs, aphids and scale insects on *Hibiscus* at Lyon Arboretum in the nursery. They are often found in the calyces of Hawaiian *Hibiscus* stealing nectar both in the nursery and out on the grounds sometimes in very large numbers (Figure 2.16). Currently the most numerous ant species I have observed on Hawaiian *Hibiscus* at Lyon Arboretum is the White-footed ant (*Technomyrmex difficilis*). Invasive ants are a serious threat to native pollination systems.

Most of the Hawaiian lobeliads sampled, like the *Hibiscus*, had hexose dominant

FIGURE 2.16. *H. immaculatus* (left) and *H. punaluuensis* (right) with White-footed ants (*Technomyrmex difficilis*) that are frequently observed in the calyces of hibiscus flowers at Lyon Arboretum feeding on nectar. The calyx of *H. immaculatus* has a tear made by nectar robbing birds.

nectar regardless of flower color (ranging from blue and magenta in *Lobelia,* magenta and white with magenta streaks in *Cyanea*, to green and white in *Delissea*). Corolla length also varied from 22-32 mm in *Lobelia*, 14-60 mm in *Delissea* and 5-8 cm long in the two *Cyanea* species, but they all have more narrow tubular corollas than the red hibiscus. This data is very similar to Lammers *et al*. (1989) reported in an earlier examination of other Hawaiian lobelioids (*Clermontia* and *Lobelia*).

Brighamia insignis, the critically endangered and most unusual member of the Hawaiian lobeliads sampled, has all the characteristics of a plant adapted for hawk moth pollination: a long, narrow tubular corolla, pale yellow to white fragrant flowers and sucrose dominant nectar. *Brunfelsia americana* is fragrant especially at night and has narrow, white tubular flowers similar to *Brighamia* and characteristic of a hawk moth pollinated flowers yet the nectar was predominantly fructose and glucose as reported for other species in Solanaceae (Nicholson and Thornburg 2007).

In conclusion, there is no evidence that nectar sugar content greatly influenced the evolutionary history of the Hawaiian red and white species of *Hibiscus,* though the flower morphology is considerably different. Further assessments of nectar constituents in the plants sampled could shed light on factors other than sugars that may have been important in evolution and diversification of closely related *Hibiscus* species in Hawaii. Endemic and introduced bird species I observed drinking *Hibiscus* nectar did not appear to prefer red flowers over white flowers. In a study comparing hummingbird and bee preferences in *Mimulus,* Schemske and Bradshaw (1999) reported that higher levels of petal carotenoids led to a significant decrease in bee visitation, though hummingbirds showed only a weak preference for red flowers over pink. This supports Raven's (1972)

hypothesis that red flowers are not necessarily preferred by birds but have a negative effect on bee visitation (Schemske and Bradshaw 1999). However, considering the long staminal columns of the white *Hibiscus* it is unlikely that even a large bird would effectively cross-pollinate these flowers. Flower structure would prevent cross-pollination between the red- and white-flowered *Hibiscus* even if birds visited both.

Pollination webs are complex systems. Similarities in the relative percentages of sucrose, fructose, and glucose in unrelated taxa may represent convergent evolution in some cases, and in other cases genetic history appears to be a more important factor. In a review of studies on nectar chemistry in a variety of plant families, Nicolson and Thornburg (2007) concluded that phylogenetic history was more important than pollinator preferences in influencing nectar constituents. Pollinators may not always be as particular about nectar content as some earlier studies have reported (Baker and Baker 1983) or they may not have a variety of flower types to choose from, especially on isolated oceanic islands like the Hawaiian chain.

It is difficult to piece together the evolutionary history of Hawaiian flora and fauna when many of the ecosystems in Hawaii have been severely impacted by the activities of people and alien species. In many cases we can only speculate as to what the pollinators were and what their relative importance was in various habitats throughout the Hawaiian Islands. The extinction or rarity of effective pollinators for Hawaiian *Hibiscus* threatens the long-term future for these plants. Preservation now of intact ecosystems that support a diversity of native organisms, including the food plants for the caterpillar stages of moths, is important (and many of these are not known). It will be too late to act once these species are determined to be endangered. Field studies of Hawaiian *Hibiscus*, their

pollinators and their reproductive status are recommended to gain a better understanding of what the current and long term prospects are for these plants and the communities in which they live.

Chapter III

Population Variation in *Hibiscus brackenridgei* section *Furcaria* (Malvaceae) Based on RAPD Markers

Introduction

Hawaii's state flower, *Hibiscus brackenridgei* A. Gray, known in Hawaiian as *mao hau hele*, is a federally listed endangered species that is found in dry forests and shrublands (130 - 800 m elevation) in small, scattered populations on Oahu, Lanai, Maui and Hawaii (Bates 1990, USFWS 2009). *Hibiscus brackenridgei* is included in the large, mostly tropical *Hibiscus* section *Furcaria* that also includes the pink flowered *H. furcellatu*s Desr. (=*H. younginanus* Gaud. ex Hook. & Arn.), indigenous in Hawaii but also occurs in Central and South America, Mexico, the Caribbean and Florida (Menzel and Wilson 1969, Bates 1990, Wilson 1993). Other important species in section *Furcaria* are *H. cannabinus* L. (kenaf) a fiber crop originally from Africa, *H. sabdariffa* L. (roselle) from Africa grown for fiber and juice, and the pantropical *H. diversifolius* Jacq. (2*n* = 144 and 180), suggested by Bates (Wilson 1993) as a possible ancestor of *H. brackenridgei* as was *H. divaricatus* Jacq., another widespread species (Bates 1965, Bates 1990, Wilson 1993). Wilson (1993) determined that *H. brackenridgei* was morphologically more similar to other species from Australia than to *H. divericatus* and *H. diversifolius.*

Characters that distinguish members of section *Furcaria* from other *Hibiscus* are the unique venation of the calyx with marginal ribs and a thickened midrib. Some species have involucral bracts forked at the apex (*H. furcellatus*), which is the source of the name *Furcaria*, and some have a nectary on the midrib of the calyx. Some subspecies of *H.*

brackenridgei have a nectary at the base of the leaves on the abaxial surface (*H. brackenridgei* subsp. *brackenridgei*) (Menzel and Wilson 1969, Wilson 1993). The chromosome numbers for *H. brackenridgei* are $n = 70$ or 72 (Niimoto 1966, Wilson 1993).

Hibiscus brackenridgei is a variable species differing in growth habit from shrubs to erect trees up to 10 m tall, leaves that are slightly to deeply lobed and may or may not have leaf nectaries, and stems varying from glabrous to densely stellate pubescent to aculeate (Roe 1961, Bates 1965, Bates 1990, Wilson 1993). The flowers are bright yellow, often with maroon spots at the base of the petals, and the petals are usually wide with good overlap. They open in the late afternoon and close late the following morning. *Hibiscus brackenridgei* is fast growing under favorable conditions, drought tolerant, and a prolific seed producer but the plants appear to be short lived compared to the endemic Hawaiian *Hibiscus* in section *Lilibiscus* (Staples and Herbst 2005). Blooming season is more restricted than in the endemic Hawaiian *Hibiscus* species in sect. *Lilibiscus*. These species bloom on and off throughout the year whereas *H. brackenridgei* flowers from November to May or June. In seasonally dry locations growth slows down considerably during the drier summer months and resumes when the rains return.

Wilson (1993) described *H. brackenridgei* as always being a rare species. A variety of threats have led to its decline. These include fire, grazing ungulates, invasive arthropods [Chinese rose beetle (*Adoretus sinicus*), the seed eating scentless plant bug (*Niesthrea louisianica*), and the hibiscus erineum mite (*Eriophyes hibisci*)], root-knot nematodes (*Meloidogynes* spp.), and invasive weeds (Munro 1960, Nakasone and Rauch 1980, Staples and Herbst 2005, USFWS 2009). In addition, there is probably reduced

fitness due to the presence of small, isolated populations (USFWS 2009). Rodents eating seeds might also be a problem. Decline of the population at Kanepuu, Lanai has been monitored since 1920 when its decline was first noted by George Munro (1960) as a consequence of being grazed by cattle, eventually being reduced to only a single plant by 1950. These threats continue to be serious obstacles in the path to recovery of this species, first listed as endangered by the U. S. Fish and Wildlife Service on November 10, 1994 (USFWS 2009).

Wildfires continue to be among the greatest threats to populations. In 2006, a fire almost reached the small Waikapu, West Maui exclosure of *H. brackenridgei* plants, but the fire stopped before reaching them. In 2007, a wildfire on the north side of the Waianae Mountains, Oahu burned about 2,288 hectares (5,655 acres). Serious damage was done to sections of native dry forest including one of the largest and most varied populations of *H. brackenridgei* at Waialua (USFWS 2009). The Oahu Army Natural Resources Program (OANRP) staff that had been monitoring the area before the fire reported that 97 percent of the plants in the area were critically damaged or killed (all 28 mature plants, 532 immature plants, and 58 seedlings). Fences containing livestock on nearby ranches were also burned releasing animals into protected areas. Matt Kerr (OANRP, personal communication 2011) has observed regeneration of *H. brackenridgei* seedlings from the seed bank, but the invasive grass *Panicum maximum* Jacq. is also regenerating quickly and it is unknown what the long-term prognosis will be for this population.

OANRP staff is also managing the *H. brackenridgei* population in Makua Valley on Oahu. They discovered that the introduced seed eating scentless plant bug was damaging

most of the seeds produced by wild plants. The following year, they were able to increase seed production by pruning, fertilizing, and spraying the plants with insecticide; these are intensive horticultural practices for wild plants.

Populations on each island are dwindling. There are approximately 211 individuals in seven populations of *H. brackenridgei* on Oahu as determined in the most recent 5-year review by the USFWS (2009) and field monitoring by OANRP (Matt Kerr, personal communication, 2012). These include populations at Kaumokunui, Kawaiu, Palikea, Kihakapu, Kaimuhole Gulch, Makua and Keaau. Only two small populations remain on Lanai (Kanepuu and Keomuku) and Maui (Kaonohua East Maui, and Keokea West Maui) (USFWS 2009). About 80 individuals remain on Hawaii Island in three populations (Puu Anahulu, Lalamilo and Puu Iwaiwa). No plants are extant on Molokai where it was last collected in 1920. Active conservation, propagation and reintroduction measures are being taken to preserve the remaining populations by OANRP staff, the statewide Plant Extinction Prevention Program, the Volcano Rare Plant Facility, Waimea Arboretum, Harold H. Lyon Arboretum and several other organizations. The total number of wild individuals estimated by the USFWS (2009) including reintroductions was approximately 245 individuals.

The first description of *H. brackenridgei* was of a specimen from west Maui described by Asa Gray in 1854 (Roe 1961). Since then there have been a number of taxonomic treatments of various forms of *H. brackenridgei* (Roe 1961, Bates 1990, Wilson 1993). A summary of the delineations by Roe (1961) and Bates (1990) are compared in Table 3.1. Roe recognized one species with four varieties: *H. brackenridgei* var. *brackenridgei* from Lanai and Maui, *H. brackenridgei* var. *molokaiana* Rock ex

Table 3.1. *Hibiscus brackenridgei* species delineations by Roe (1961) and Bates (1990).

Roe	**Bates**
H. brackenridgei (Maui and Lanai)	*H. brackenridgei* subsp. *brackenridgei*
H. brackenridgei var. *molokaiana* (Molokai)	*H. brackenridgei* subsp. *brackenridgei*
H. brackenridgei var. *kauaiana* (Kauai)	*H. brackenridgei* subsp. *mokuleianus*
H. brackenridgei var. *mokuleiana* (Oahu)	*H. brackenridgei* subsp. *mokuleianus*

ex Caum from Molokai, *H. brackenridgei* var. *kauaiana* Caum from Kauai and *H. brackenridgei* var. *mokuleiana* M. Roe from Oahu. Variety *molokaiana* was first collected in 1910 and last collected in 1920, but is now presumed extinct (USFWS 2009). Roe (1961) did not describe any collections from Hawaii Island. Bates (1990) reduced the number of taxa to two and recognized them as subspecies: *H. brackenridgei* subsp. *brackenridgei* (Figure 3.1) from Lanai, Maui, Molokai and Hawaii Island and *H. brackenridgei* subsp. *mokuleianus* (M. Roe) Bates (Figure 3.2) from Kauai and Oahu. In the most recent taxonomic treatment by Wilson (1993), three subspecies of *H. brackenridgei* are recognized: *H. brackenridgei* subsp. *brackenridgei* from Kahoolawe (now extinct), Lanai, Maui and Hawaii Island; *H. brackenridgei* subsp. *mokuleianus* on Oahu; and *H. brackenridgei* subsp. *molokaianus* from Molokai. Wilson (1993) concluded that the herbarium specimens he examined from Kauai (collected by Rock) were too distinct from *H. brackenridgei* subsp. *molokaianus* to be included in it and because there were no extant specimens available for study he did not give it nomenclatural recognition.

In 2000, a new population of *H. brackenridgei* was discovered in Makua (Figure 3.3) on Oahu (Joel Lau personal communication 2012) that was morphologically more similar to subsp. *brackenridgei* or subsp. *molokaianus* than to the other populations on Oahu. Subspecies *mokuleianus* is composed of generally taller trees armed with aculeate (spinose) stems, and leaves that are less deeply lobed than the Makua plants. The Makua plants are smaller with a distinctly shrubby growth habit that makes them more desirable in the horticulture industry. Questions have remained regarding the affinity of the Makua

FIGURE 3.1. *H. brackenridgei* subsp. *brackenridgei* present on Maui, Lanai and Hawaii Island. (A) Flower. (B) Flower bud, involucral bracts and stems covered with a fine stellate pubescence. (C) Abaxial leaf surface with leaf nectary at the base of the midrib (arrow).

FIGURE 3.2. *H. brackenridgei* subsp. *mokuleianus* from Oahu. (A) Flower. (B) Prickles on stems and pustular-based hairs on peduncles, involucral bracts and calyces. (C) Pustular based hairs on calyx and petals. (D) Abaxial leaf surface with no nectary on the midrib.

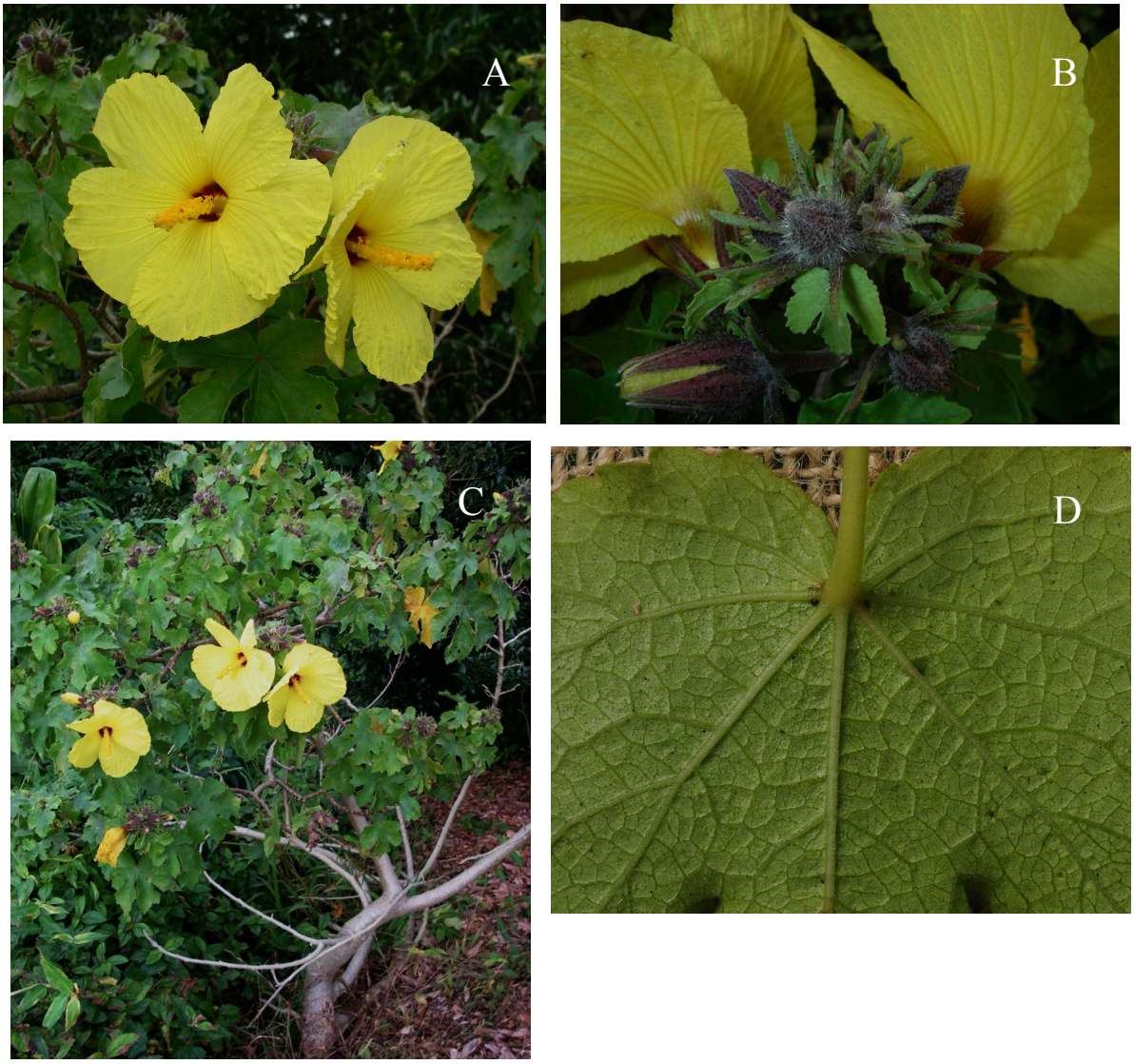


FIGURE 3.3. *H. brackenridgei* from Makua, Oahu. (A) Flowers. (B) Flower buds and involucral bracts with finer hairs than *H. brackenridgei* subsp. *mokuleianus*. Makua plants also lack prickles on stems and leaf nectaries. (C) Compact growth habit typical of Makua plants. (D) Abaxial leaf surface with no nectary on the midrib.

plants and whether they are more closely related to subsp. *brackenridgei* or subsp. *molokaianus* although it seems clear that they are morphologically distinct from subsp. *mokuleianus*.

Clarification of species boundries is important for conservation of these unique plants, as well as in the local ornamental horticulture industry, where *H. brackenridgei* is popular. It is one of the best known native plants in Hawaii. In an effort to clarify the confusion over subspecies delineations in *H. brackenridgei,* random amplified polymorphic DNA (RAPD) markers were used to compare to previous morphological studies and to assess the variation within and among populations from the different Hawaiian Islands. RAPD markers have been useful in clarifying population and species relationships as well as evolutionary history in various plants (Randell *et al*. 2004, Grant and Miller 2001, Reed, Joung and Roh 2002). This technique has helped to answer questions related to genetic diversity at both population and species levels in various endemic Hawaiian plants in recent years: *Haplostacys* (Lamiaceae; Morden and Loeffler 1999), *Cibotium* (Dicksoniaceae; Motley and Morden 2001), *Colubrina and Alphitonia* (Rhamnaceae; Kwon and Morden 2002), *Touchardia* (Urticaceae; Loeffler and Morden 2003), *Rubus* (Rosaceae; Randell *et al.* 2004), *Chamaesyce* (Euphorbiaceae; Morden and Gregoritza 2005), *Dubautia* (Asteraceae; Caraway *et al.* 2005), *Delissea* (Campanulaceae; James 2009) and *Hesperomania* (Asteraceae; Morden and Harbin 2013). Using the polymerase chain reaction (PCR) to amplify the DNA fragments, using RAPD markers is a relatively fast, easy, inexpensive technique, and requires only a small quantity of DNA. RAPD markers are abundant in the genome and randomly distributed. However, drawbacks of using RAPD markers are that they are dominant markers only

and there can be issues with the reproducibility of results (Skoric *et al.* 2012). However, this can be overcome by carefully following standardized laboratory procedures in order to avoid variability in reaction conditions and repetition of experiments to verify consistency of results.

Objectives

Assess the genetic distance between the various populations and population variation in the endemic *Hibiscus brackenridgei* (Section *Furcaria*) on Oahu, Maui, Lanai and Hawaii Island using random amplified polymorphic DNA (RAPD) markers to resolve earlier conflicting treatments of this endangered species based on morphological characters and to assist botanists who are managing the wild populations of this rare plant.

Materials and Methods

Population Sampling and DNA Extraction

Fresh leaf tissue of *H. brackenridgei* was collected from wild and cultivated plants representing populations of *H. brackenridgei* subsp. *mokuleianus* from of Oahu, and *H. brackenridgei* subsp. *brackenridgei* from Lanai, Maui and Hawaii Island. The presumed extinct subspecies, *H. brackenridgei* subsp. *molokaianus,* was not sampled. It was collected once by J. Rock in 1910 and again in 1920, and has not been seen since (Wilson 1993). Cultivated plant material was collected from accessions at Waimea Arboretum

(Oahu), the Army Natural Resources nursery on Oahu and the Volcano Rare Plant Facility on Hawaii Island. The number of individuals sampled from each population and the population locations are listed in Table 3.2.

Total cellular DNA was extracted and purified from 0.5-1.0 g of fresh plant material that was kept refrigerated until DNA was extracted. DNA was extracted using the CTAB method of Doyle and Doyle (1987) with some modifications by Morden *et al.* (1996). DNA samples were purified by cesium chloride density-gradient ultracentrifugation (Sambrook *et al.* 1989). Ethidium bromide was removed using water-saturated butanol and DNA was precipitated using isopropanol to remove the cesium then washed once with 70% ethanol. All purified DNA samples were accessioned in the Hawaiian Plant DNA Library (Morden *et al.* 1996, Randell and Morden 1999).

RAPD PCR and Data Analysis

Approximately 1µl (20 ng) of DNA was amplified in 15µl reactions via the polymerase chain reaction (PCR) under the following conditions: 0.2 µM random 10-mer oligonucleotide primers (Operon Technologies), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1x *Taq* polymerase PCR Buffer, 1.5 mM $MgCl_2$, 0.1% bovine serum albumin, and ca. 1 unit *Taq* polymerase (Promega, Madison, Wisconsin, USA). Thirty-six primers were screened (kits OPC-OPD; QIAGEN Operon, Almeda, CA, USA) using RAPD analysis of the PCR to evaluate each primer for use on all individuals. Primers that yielded consistent number and intensity of markers were then used for amplification for all individuals. Amplifications were performed in either an MJ Research PTC-200 or

TABLE 3.2. Accessions of *Hibiscus brackenridgei* used for genetic analysis (RAPD).

Taxon	Island	Location	# of Individuals
***H. brackenridgei* subsp. *brackenridgei* D. Bates**	Hawaii Island	Puu Anahulu	1
	Hawaii Island	Puu Huluhulu	1
	Hawaii Island	Puu Waawaa	2
	Hawaii Island	Waikaloa Stream	1
		Hawaii Island (all)	5
	Maui	Waikapu, West Maui	6
	Lanai	Kanepuu	5
	Lanai	Keomuku	8
		Lanai (all)	13
***H. brackenridgei* subsp. *mokuleianus* [*H. b.* var. *mokuleiana* (M. Roe) D. Bates**	Oahu	Oahu (Makua)	8
	Oahu	Oahu (Kealia/Kawaiu)	4
	Oahu	Oahu (Keeau)	5
	Oahu	Oahu (Waialua)	12

	Oahu (all)	29
Total	All Islands	55

PTC-225 Thermocyler under the following conditions: 94 °C for 2 minutes, 94 °C for 45 seconds, 35 °C for 45 seconds, ramp to 35 °C at 0.5 °C/second, 72 °C for 2 minutes, ramp to 72 °C at 0.5 °C/second followed by 44 cycles of 94 °C for 45 seconds, 35 °C for 45 seconds, ramp to 35 °C at 0.5 °C/second, 72 °C for 2 minutes, ramp to 72 °C at 0.5 °C/second with a final incubation at 72 °C for 5 minutes. PCR amplification products were visualized on 1.5% agarose gels in 0.5x TBE (tris-borate- EDTA) buffer, and gel images were digitally recorded with a UVP BioImaging Systems Gel HR Camera (UVP LLC, Upland CA, USA). Negative control (i.e., no DNA) reactions were run for all PCR amplifications to ensure reaction components were uncontaminated. Size of amplification products was estimated by comparison to fragments in a 100 kb ladder (Promega, Madison, WI, USA) or to a pBS plasmid (Stratagene, La Jolla, CA, USA) digested with restriction enzymes to produce fragments in a size range of 0.448- 2.96 kb. Molecular markers were identified by the primer used to generate them and their approximate size (kb). Gel scoring was performed independently by the author and lab technicians to produce unbiased and unambiguous analysis of the RAPD amplifications.

Each RAPD marker generated was assumed to represent a separate genetic locus in a two allele system consisting of the marker (amplified product present) and the null allele (amplified product absent) with the marker being dominant to the null allele as described by Lynch and Milligan (1994). A RAPD marker was determined to be polymorphic when found in less than 95% of the sampled individuals (i.e. not present in 3 or more individuals). Absence of a marker within a population, though present in other populations, was assumed to indicate that all the individuals in the population were null/null homozygotes rather than indicating that there was a loss of the locus. Percent

polymorphic loci was calculated at the population and species level using MS Excel. Genetic similarity indices were estimated using both Gower (1971) and Nei and Li (1979) similarity coefficients for populations and species using MVSP Plus ver. 3.1 (Kovach 2007). Pairwise similarity was averaged for individuals within and among populations. Expected heterozygosity was calculated for each population (H_s) and species (H_t) for each locus as follows:

$$H = 1 - (p^2 + q^2)$$

where p is the frequency of the dominant allele (i.e., the visible marker) and q is the frequency of the null allele (i.e., the absent marker). Allele frequencies were estimated from the number of null/null homozygotes present in the population (Hartl and Clark 1989, Morden and Loeffler 1999). UPGMA cluster analysis from similarity coefficients and principle coordinate analysis (PCO) using Gower general similarity coefficients were calculated using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1987-1998).

Results

RAPD Analyses

Twelve primers were examined (Table 3.3) for 55 individuals and 103 different genetic markers were scored from these primers (range of 8 – 35 markers identified for each primer with an average of 18) (Table 3.4). The percent polymorphism for the species was 69.9% with an average of 17.2% among the populations sampled. Levels of polymorphism were calculated for each population (range from 6.9 to 34.0) (Table 3.4).

TABLE 3.3. Random amplified polymorphic DNA (RAPD) primers used on all individuals of *H. brackenridgei* and the corresponding sequences.

Primer	Primer Sequence
OPC-5	GATGACCGCC
OPC-6	GAACGGACTC
OPC-7	GTCCCGACGA
OPC-10	TGTCTGGGTG
OPC-11	AAAGCTGCGG
OPD-8	GTGTGCCCCA
OPD-9	CTCTGGAGAC
OPD-12	CACCGTATCC
OPD-13	CTCTGGAGAC
OPD-14	CTTCCCCAAG
OPD-16	AGGGCGTAAG
OPD-18	GAGAGCCAAC

TABLE 3.4. *Hibiscus brackenridgei* population variation based on RAPD analysis.

Location	Sample Size	# of Unique markers	# of markers present in all individuals	# of Polymorphic Markers	% Polymorphism	Estimated Heterozygosity (H)
Hawaii Island (Puu Anahulu, Puu Huluhulu, Puu Waawaa, Waikaloa Stream)	5	1	76	14	13.6	0.054
Maui (Waikapu)	6	2	72	25	24.3	0.097
Lanai (Kanepuu)	5	1	81	8	6.9	0.031
Lanai (Keomuku)	8	1	77	16	15.5	0.062
Lanai (all populations)	13	n/a	74	22	21.4	0.078
Oahu (Makua)	8	0	73	18	17.5	0.072
Oahu (Kealia/Kawaiu)	4	0	67	20	19.4	0.082
Oahu (Keeau)	5	0	54	35	34.0	0.147

Oahu (Waialua)	12	2	66	24	23.3	0.082
Oahu (all)	29	n/a	41	56	54.4	0.188
Total	55	n/a	31	72	69.9	0.226

The lowest level of polymorphism was found at Kanepuu, Lanai (6.9%). The level of polymorphism was highest at Keeau, Oahu (34.0%) followed by Waikapu, Maui (24.3%) and Waialua, Oahu (23.3%). Variation within each population was relatively low. This could be attributed to the small sample sizes available for analysis (ranging from 4 to 12 individuals per population) although the populations with the highest and lowest levels of polymorphism both had only five individuals available for examination.

Populations were compared for genetic similarities based on the Nei and Li coefficient (1979) where a value of 1.0 indicates complete genetic identity (Table 3.5). Genetic similarity was higher within populations than among populations and was highest among individuals in the Lanai populations (0.965 and 0.943 for Kanepuu and Keomuku, respectively). Similarly high values are also found among the Hawaii Island plants (0.934), and within Oahu populations at Waialua (0.936) and Makua (0.934). Two populations showed closer affinities to populations on other islands than to those on the same island. Plants at Kanepuu, Lanai were slightly more similar to plants from Hawaii Island (0.906) than to the other Lanai population at Keomuku (0.902). The Makua, Oahu population shows the highest similarity to plants Keomuku, Lanai (0.912) rather than other populations from Oahu (range 0.855 to 0.889).

All populations of *H. brackenridgei* sampled were compared using principal coordinates analysis (PCO) resulting in a plot with two distinct groupings (Figure 3.4). The first (horizontal) PCO axis accounts for the distinction between all the Oahu populations excluding Makua (Group 1), and the populations from Hawaii Island, Maui, Lanai, and Makua, Oahu (Group 2). The Makua plants are clearly aligned more closely with those from the other islands rather than those from Oahu. The second axis

TABLE 3.5. Levels of genetic similarity within and among populations of *Hibiscus brackenridgei* subsp. *brackenridgei* from Hawaii Island, Maui and Lanai, and *H. brackenridgei* subsp. *mokuleianus* from Oahu based on Nei and Li (1979) coefficient. A value of 1 indicates complete genetic identity.

Location	Hawaii	Maui	Lanai (Kanepuu)	Lanai (Keomuku)	Oahu (Makua)	Oahu (Kealia, Kawaiu)	Oahu (Keeau)	Oahu (Waialua)
Hawaii	0.934							
Maui (Waikapu)	0.858	0.879						
Lanai (Kanepuu)	0.906	0.891	0.965					
Lanai (Keomuku)	0.882	0.877	0.902	0.943				
Oahu (Makua)	0.874	0.877	0.887	0.912	0.934			
Oahu (Kealia, Kawaiu)	0.829	0.815	0.813	0.841	0.850	0.889		
Oahu (Keaau)	0.794	0.802	0.785	0.805	0.831	0.855	0.851	
Oahu (Waialua)	0.843	0.841	0.833	0.859	0.871	0.889	0.877	0.936

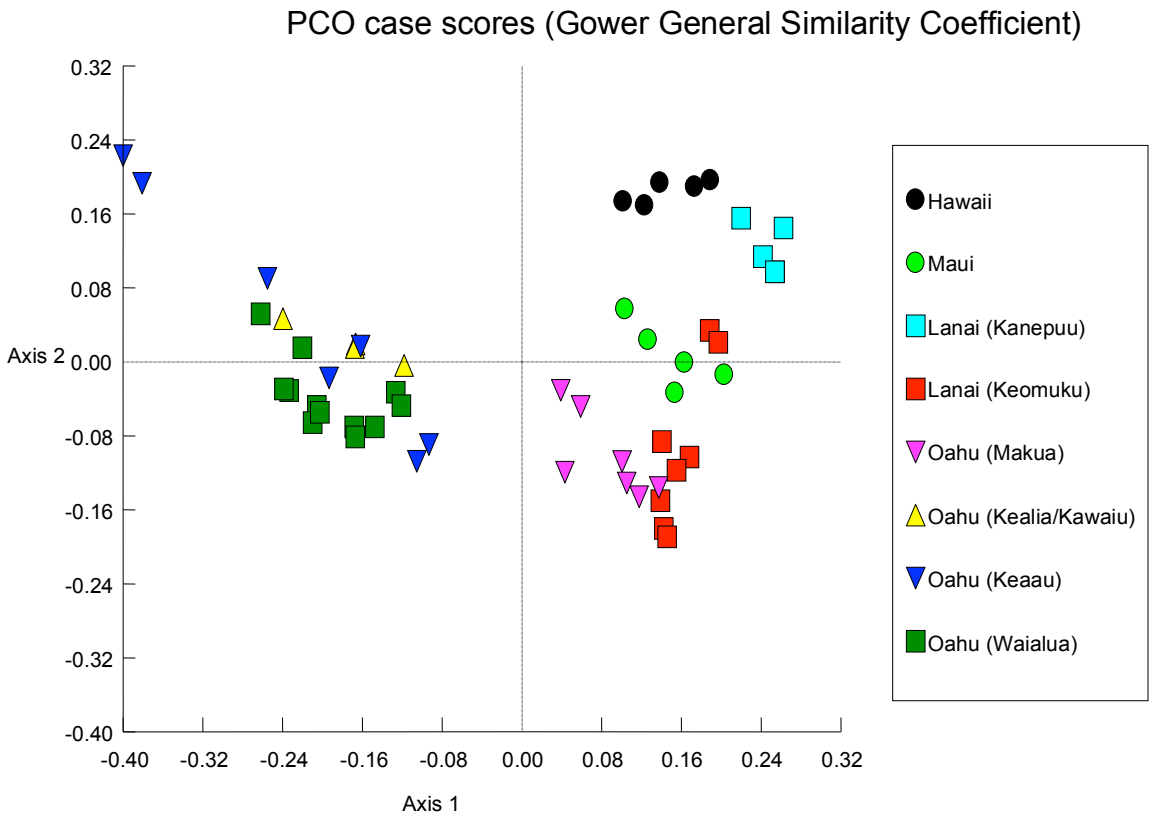


FIGURE 3.4. Principal coordinates analysis of all populations sampled of *Hibiscus brackenridgei*. Group 1: *H. brackenridgei* subsp. *mokuleianus* from Oahu and Group 2: *H. brackenridgei* subsp. *brackenridgei* from Lanai, Maui and Hawaii Island. The first (horizontal) axis represents 27% of the total variation and the second (vertical) axis represents 9% of the variation.

distinguishes the Makua, Oahu and most of the Keomuku, Lanai plants from the Kanepuu, Lanai, Maui and Hawaii Island plants.

To examine these two clusters further separate PCO analyses were conducted for each group separately. The PCO analysis of the Oahu populations of *H. brackenridgei* (Group 1; Figure 3.5) indicates nearly complete differentiation of the Waialua population from the Kealia/Kawaiu and Keaau populations. The latter two populations appear to be mixed showing no clear differentiation. The Keaau population is most variable reflective of its low within population similarity (Table 3.5).

To examine Group 2 further, a PCO analysis was conducted of the Hawaii Island, Maui, two Lanai populations and the Makua, Oahu population (Figure 3.6). Individuals aligned into four distinct clusters. With one exception, populations form distinct separate clusters. The exception is the Keomuku, Lanai and the Makua, Oahu populations that are completely overlapping. The Kanepuu, Lanai population is well separated from these along Axis 1.

Discussion

The RAPD data are mostly in agreement with the circumscription of extant *H. brackenridgei* subspecies by Bates (1990) and Wilson (1993): *H. brackenridgei* subsp. *brackenridgei* on Lanai, Maui and Hawaii Island, and *H. brackenridgei subsp. mokuleianus* on Oahu. The one exception is the Makua, Oahu population that clearly does not align closely with the three other Oahu populations sampled (Keaau, Kealia/Kawaiu, and Waialua). This population was discovered after Bates (1990) and

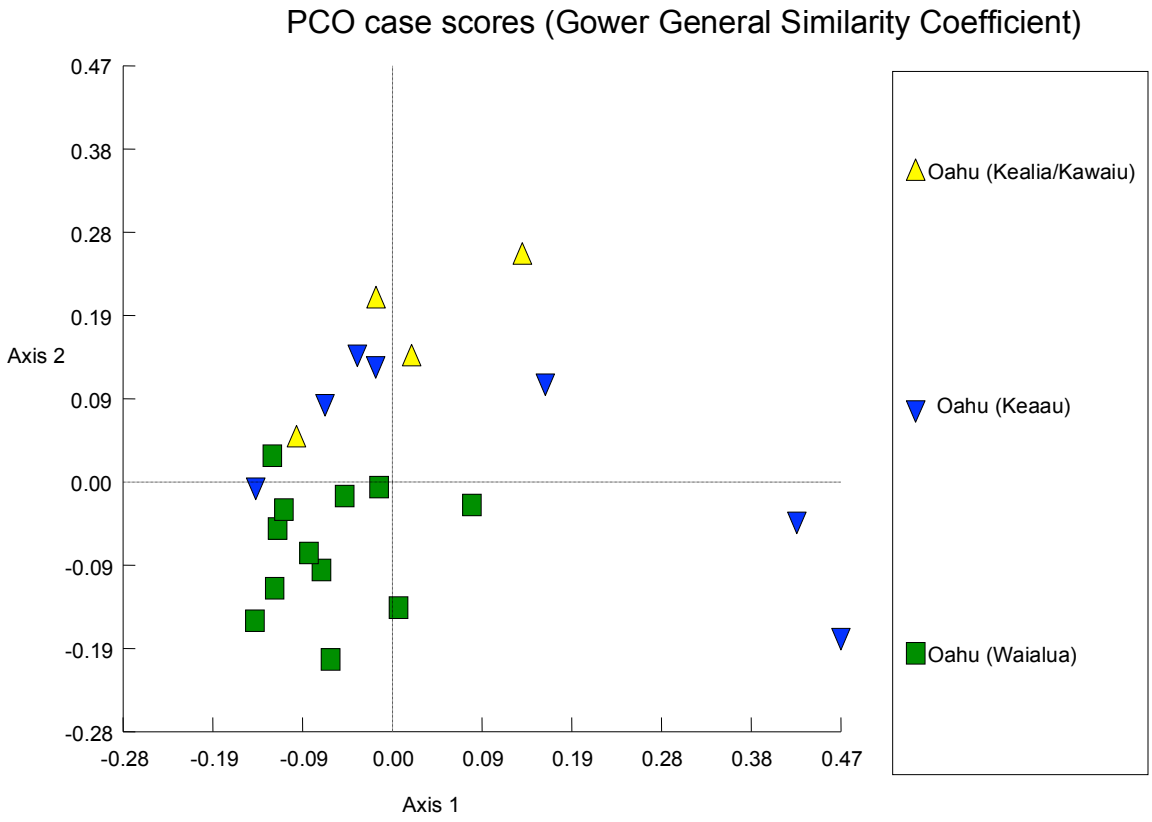


FIGURE 3.5. Principal coordinates analysis of Group 1: *H. brackenridgei* subsp. *mokuleianus* from populations sampled on Oahu. The first (horizontal) axis represents 25% of the total variation and the second (vertical) axis represents 13% of the variation.

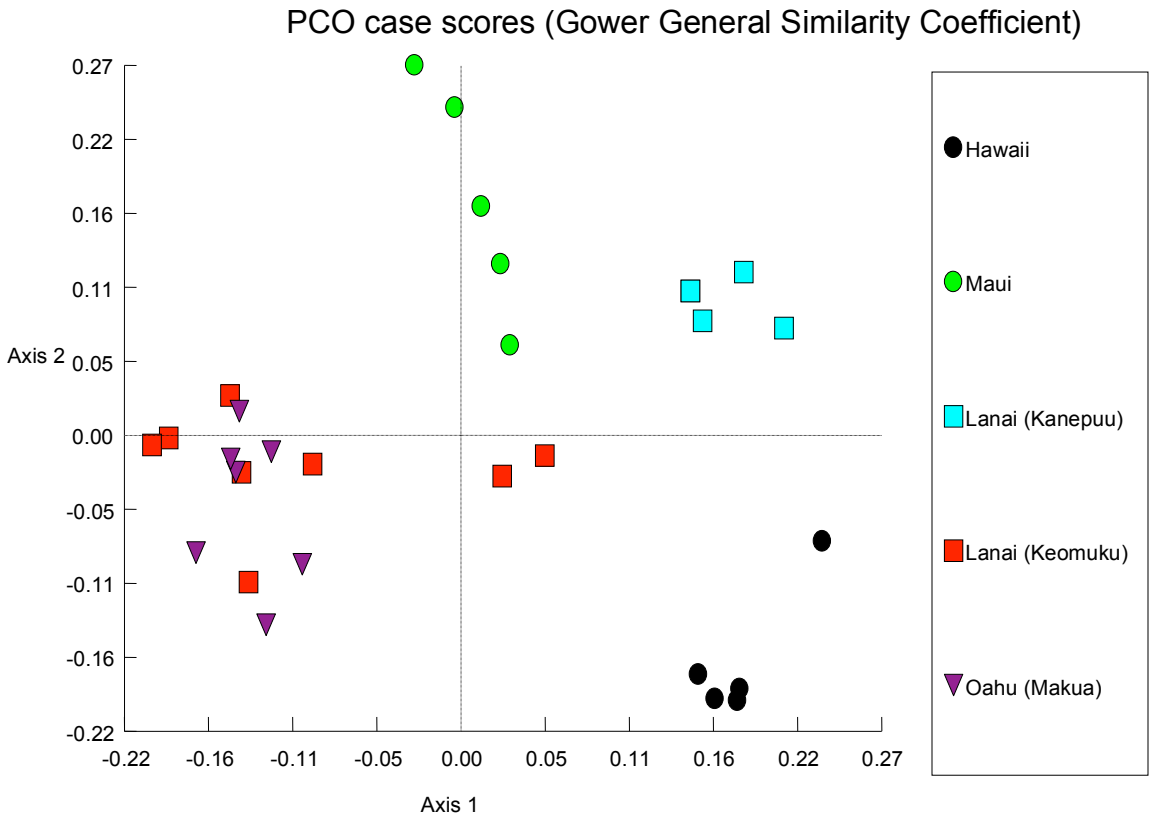


FIGURE 3.6. Principal coordinates analysis of Group 2: *H. brackenridgei* subsp. *brackenridgei* from Hawaii Island, Maui and Lanai and the Makua, Oahu plants. The first (horizontal) axis represents 23% of the total variation and the second (vertical) axis represents 18% of the variation.

Wilson (1993) had published their treatments of the species. Instead, the Makua individuals are genetically more similar to populations on the other islands (especially the Keomuku, Lanai population). The Makua individuals are shrubs rather than trees with smooth, unarmed branches, similar to subsp. *brackenridgei,* but the leaf nectary on the abaxial surface is absent in the Makua plants in contrast to subsp. *brackenridgei* plants. The *H. brackenridgei* subsp. *mokuleianus* plants do not have leaf nectaries either, but they are much more variable across their range than subsp. *brackenridgei* and the Makua plants. Subspecies *mokuleianus* varies from shrubs to upright trees and has smooth to aculeate (prickly) stems (USFWS 2009).

Wilson (1993) observed that the only herbarium specimen of the now extinct *H. brackenridgei* subsp. *molokaianus* did not have leaf nectaries in the upper or lower leaves but that they were present or absent in mid-level leaves. The Molokai subspecies were described as "straggling shrubs" which is similar to the growth habit of the Makua, Oahu plants. It was collected once by J. Rock in 1910 and 1920, and has not been seen since (Wilson 1993).

Field Biologists Joel Lau and Matt Kerr (OANRP, personal communication) have observed that the three other Oahu populations are variable morphologically throughout their range in the Waianae Mountains (personal communication 2012). The Keaau population (also newly discovered) is in the southern Waianae's as is Makua, and is located most closely to the Makua plants (See map of Oahu populations, Figure 3.7).

A more in depth study of the Makua plants is needed to determine if this population should be included in *H. brackenridgei* subsp. *brackenridgei* with the plants from Lanai, Maui and Hawaii Island or be placed in possibly another subspecies. The other three

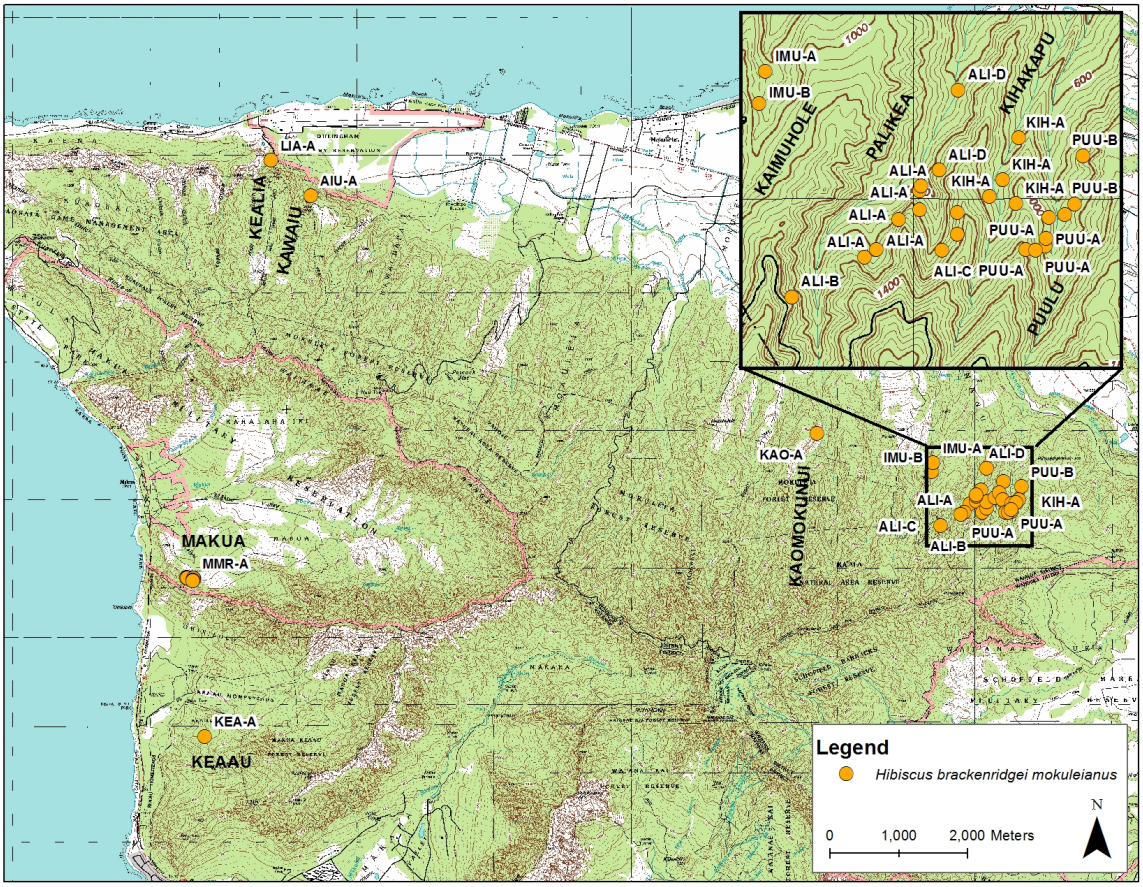


FIGURE 3.7. Oahu Army Natural Resources Program Map showing all the populations of *H. brackenridgei* subsp. *mokuleianus* on West Oahu and the proximity of the Makua population to the Keaau population.

Oahu populations are more genetically similar than any of the other populations are to each other, but the Waialua individuals did show some differentiation from the other two Oahu populations. Unfortunately, this is the population that was seriously burned in 2007. Before that it was the largest population of *H. brackenridgei* in the state (USFWS 2009).

The analysis of the relationship of the two Lanai populations indicates that, though they fall within *H. brackenridgei* subsp. *brackenridgei,* they are more closely related to populations on other islands than to each other: Kanepuu to Hawaii Island individuals, and Keomuku to Maui individuals. Hank Oppenheimer (Maui Nui Coordinator, Plant Extinction Prevention Program, personal communication 2008) observed that these two very small populations (8 or less) appeared to be morphologically different enough to keep them separate. This study confirms that the two groups are genetically distinct as well.

Conservation Implications

Hibiscus brackenridgei is a very vulnerable species subject to attack from multiple negative forces, some of them very difficult to control (fire and grazing ungulates for example) and it has been reduced to small, scattered populations with low genetic variability. It has been extinct on Kauai, Molokai and Kahoolawe for many years. Fortunately, it has some important positive characteristics: it is fast growing and drought tolerant, has good seed production (pollinators are apparently still available - native or non?), seed germination rates are good, and it is easy to propagate from cuttings (Staples and Herbst 2005, USFWS 2009). Also, regeneration from the seed bank can be substantial (Matt Kerr, personal communication 2012), more than has been reported for

any other endemic *Hibiscus* species. The difficulty is controlling the weeds around the hibiscus in the open shrublands where it prefers to grow.

Continued outplanting in suitable new locations or in areas where the plants were known to exist historically would help to avoid catastrophic losses due to fire such as the one in Waialua, Oahu in 2007. Effective fencing is required to prevent feral ungulates from accessing the small numbers of plants that still exist and regular weed management is needed to prevent excessive competition from weeds for water and light. Some of the wild populations have suffered severely from drought in recent years and it is possible that as the climate changes and some areas in Hawaii appear to be getting drier, it may be advisable to plant *H. brackenridgei* at locations that previously were considered too wet. Identifying the pollinators of these plants in the field would be useful for conservationists. The flowers open late in the afternoon and close late in the morning of the following day indicating that the pollinators could be crepuscular or nocturnal.

Quite a few organizations throughout the state of Hawaii have active programs working to conserve this species by storing seeds, keeping nursery stock, outplanting at botanical gardens, and outplanting at restoration sites (USFWS 2009). These organizations are the U.S. Army, the Hawaii Division of Forestry and Wildlife, Kauai District and Maui District, the Center for Conservation Research Training Seed Storage Laboratory, the Volcano Rare Plant Facility, the Amy B. H. Greenwell Ethnobotanical Garden, the Harold L. Lyon Arboretum, the Waimea Arboretum, the Honolulu Botanical Gardens, the Maui Nui Botanical Gardens, and the David T. Fleming Arboretum. The most recent USFWS 5-year Review Summary (2009) of *H. brackenridgei* reports that 181 individuals have been reintroduced on Hawaii Island and 134 on Oahu (315 total) in

addition to the naturally occurring 245 wild plants, 70% of which are seedlings. Even with these organizations working to preserve genetic diversity, all the populations of *H. brackenridgei* are still endangered.

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