MOLECULAR PHYLOGENETICS AND DIVERSITY OF THE ACACIA KOA
COMPLEX BASED ON DNA SEQUENCES AND MICROSATELITE MARKERS

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ABSTRACT

The *Acacia koa* complex, commonly referred to as koa, is a group of nitrogen-fixing legumes native to the Hawaiian Islands. Within the complex, *A. koa* and *A. koaia* are accepted nomenclature. Intermediate forms of koa, described in this study as ‘group three’, include individuals with the tree structure of *A. koa* and seed pod structure of *A. koaia*, or vice versa, which is similar to populations described as *A. kauaiensis* Hillebrand (St. John, 1979; Wagner *et al.*, 1999). These three groups of the koa complex, *A. koa, A. koaia*, and ‘group three’, were characterized using molecular techniques. DNA sequencing from nuclear (ITS1 / 5.8S / ITS2) and chloroplast (trnK / matK) regions placed koa within the subgenus *Phyllodineae*, and showed a close relationship with *Acacia melanoxylon*. Differentiation among groups of koa based on sequencing results was inconclusive. A total of 106 alleles were detected among koa groups using 12 different microsatellite markers, with an average of 8.8 alleles per polymorphic locus (AP). ‘Group three’ had the highest genetic diversity (*H* = 1.578), average alleles per locus (AP = 7.9), and number of unique alleles (21). The *A. koaia* group showed the lowest diversity (*H* = 0.960), and was differentiated from *A. koa* and ‘group three’ in analysis using an unweighted pair group method with arithmetic mean (UPGMA) dendogram and principle coordinate analysis. An analysis of molecular variance (AMOVA) found genetic variation partitioned within populations (72.2%) rather than among populations (27.8%). Clustering in the UPGMA dendogram, principle coordinate analysis, and genetic differentiation revealed three distinct groups among the populations. Koa-specific microsatellite markers showed little success in cross-amplification of non-native acacias found in Hawaii. This study provides molecular evidence distinguishing three groups of the *A. koa* complex; *A. koa, A. koaia*, and ‘group three’, found only on Kauai and similar to *A. kauaiensis*. 
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A  
(Acacia)

AFLP  
(amplified fragment length polymorphism)

Ak  
(Acacia koa)

Am  
(Acacia mangium)

AMOVA  
(analysis of molecular variance)

AN  
(Acacia angustissima)

AP  
(average alleles per polymorphic locus)

AR  
(Acacia auriculiformis)

bp  
(basepair)

C  
(degree celsius)

CON  
(Acacia confusa)

cpDNA  
(chloroplast deoxyribonucleic acid)

DBH  
(diameter at breast height)

DNA  
(deoxyribonucleic acid)

Dst  
(average diversity among populations of each group)

ETS  
(external transcribed spacer)

FAM  
(fluorescent dye color)

FDASH  
(statistics software)

F'  
(FDASH genetic differentiation)

F  
(FDASH average diversity within populations of each group)

Fst  
(FDASH total genetic diversity)

GBIF  
(Global Biodiversity Information Facility)

'group three'

GTHR  
(International Union for the Conservation of Nature and Natural Resources)

I  
(gram)

IGS  
(intergenic spacer)

INUC  
(Integrated Taxonomic Information System)

ITS  
(internal transcribed spacer)

KANA  
(Anahola)

KHAN  
(Hanalei)

KKA  
(Awa'awapuhi Trail)

KMC  
(Kokee-Mohihi)

KNT  
(Nounou Ridge)

KOAA  
(Hakalau Wildlife Refuge)

KOAB  
(Pua Akala)

KOAA  
(Acacia koa group)

KOAB  
(Acacia koaia group)

KTSA  
(Koaia Tree Sanctuary)

KTSB  
(Kohala Road)
KV (Kahana Valley)
L (liter)
LSU (large subunit)
M (Mimoseae)
m (meter)
MAC-PR (microsatellite allele counting-peak ratios)
matK (maturase encoding gene)
MG (Acacia mangium)
min (minute)
ML (Acacia melanoxylon)
mmol (millimole)
MR (Acacia mearnsii)
N (negative control)
NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool)
CED (fluorescent dye color)
gen (nanogram)
P (percent polymorphic locus)
PCR (polymerase chain reaction)
PET (fluorescent dye color)
mmol (picomole)
RAPD (random amplification of polymorphic DNA)
rbcL (large subunit of ribulose bisphosphate carboxylase/oxygenase)
rDNA (ribosomal deoxyribonucleic acid)
RFLP (restriction fragment length polymorphism)
RFU (relative fluorescent units)
SG (Waimea)
SSLP (simple sequence length polymorphism)
SSR (simple sequence repeat)
SSU (small subunit)
STR (simple tandem repeat)
trnK (transfer RNA gene for lysine)
uL (microliter)
UPGMA (unweighted pair grouping of arithmetic average)
VIC (fluorescent dye color)
CHAPTER 1
INTRODUCTION

The *Acacia koa* complex (koa) is a group of both shrubby and arboreal nitrogen-fixing tree-legumes native to the Hawaiian Islands (Pedley, 1975; Wagner *et al*., 1999). Koa thrives on well-drained, volcanic soils at elevations predominantly between 800-1600 m, although populations can be found as low as 100 m and up to 2300 m (Whitesell, 1990; Sun, 1996a; Elevitch, 2006). In addition to providing high value timber for Hawaii’s $29 million/annum forest industry, koa plays a vital role in Hawaii’s watershed ecosystem, serving as habitat for endangered birds and epiphytic plants (Whitesell, 1990, Elevitch, 2006; HFIA, 2007). Koa is a polyploid species with an outcrossed breeding system, and is thought to be self-incompatible because of unsuccessful self-pollination studies (Sun, 1996a). However, this classification is speculative and only the tetraploid nature of koa has been supported by ploidy analysis (Shi, 2003; Hipkins, 2004).

Morphologically, koa is similar to species from the largely Australian subgenus *Phylloidineae* (Miller *et al*., 2003). At lower taxonomic levels, koa is classified into many different types based on island origin and morphological characteristics, but only two types, *A. koa* A. Gray and *A. koaia* Hillebrand, are formally accepted (GBIF Data Portal, 2007). There are also descriptions of groups that are argued to be distinct subspecies or varieties, although they are not formally recognized (St. John, 1979, Wagner *et al*., 1999, GBIF Data Portal, 2007; ITIS, 2007). These include: *A. kauaiensis* Hillebrand, *A. hawaiiensis* Rock, *A. lanaiensis* Rock, *A. latifolia* Bentham, *A. waianaeensis* St. John, and variant populations exhibiting hybrid morphological characteristics. Differences
among groups are most evident in tree size, seed alignment within the pod, and phyllode size and shape.

Common garden studies have shown that families from Hawaii, when compared to those from Oahu and Kauai, have significant differences in seed width and mass, as well as phyllode length and width (Daehler et al., 1999). Variations in isozyme loci have been shown for koa populations on different islands in the State of Hawaii (Brewbaker, 1977; Conkle, 1996). Koa from Maui, Oahu, and Kauai are closely related, and their isozyme allele profiles differ greatly from koa on Hawaii (Conkle, 1996).

Distinguishing characteristics of *A. koalae* include shrubby form, tree height up to 5 m, longitudinally arranged seeds, and various phyllode shapes which are usually short and narrow. *Acacia koa*, on the other hand, has a large tree height reaching up to 35 m, transversely arranged seeds, and phyllodes that are generally wide and long (Wagner et al., 1999).

Among the groups of informal taxa are populations that have morphological characteristics that are a mixture of those found in *A. koa* and *A. koalae*, that will be referred to as 'group three'. These morphologically variable populations are influenced by environmental conditions, animal grazing, and genetics (Harrington et al., 1997; Daehler, et al., 1999; Elevitch, 2006). Koa found on Kauai are especially difficult to classify based on morphological characteristics because different ecotypes are thought to
exist due to extreme changes in elevation, rainfall, and soil types over short geographical distances (St. John, 1979; Smith, S., personal communication).

In addition to koa, other non-native acacias are found in Hawaii that have been planted over the past 100 years as windbreaks, biomass for energy, and for scientific research (Friday, J., personal communication). Many of these non-native acacias have become naturalized and include: *A. angustissima, A. auriculiformis, A. confusa, A. mangium, A. mearnsii,* and *A. melanoxylon.*

Koa forests have been reduced over the past 100 years due to land clearing for agriculture, ranching, and a devastating koa wilt disease caused by the fungal pathogen *Fusarium oxysporum f. sp. koae* (Anderson et al., 2002). However, *A. koa* is still commonly found throughout Hawaii. On the other hand, *A. koaia* is on *The World List of Threatened Trees* (Oldfield et al., 1998) and the International Union for Conservation of Nature and Natural Resources (IUCN) *Red List of Threatened Species* (Bruegmann et al., 2003). Small population size accompanied with a low total number of trees makes *A. koaia* vulnerable to extinction if it is not protected from disease and habitat degradation (Bruegmann et al., 2003).

As koa are morphologically variable within populations that comprise each described group, it is difficult to differentiate populations as common (*A. koa*), rare (*A. koaia*), or unknown ('group three'). For conservation of rare taxa, the genetic and evolutionary distinctiveness of populations are essential priorities. Genetic studies are
needed to determine distinctness, phylogenetic status, and diversity in koa, especially in small and fragmented populations. Molecular tools such as DNA sequencing and microsatellite markers are useful for conservation and population genetic studies, as they resolve differences at the genus and species levels (Murphy et al., 2003; Doyle and Luckow, 2003).

The objectives of this investigation are to determine the phylogenetic placement of koa within the *Acacia* genus using DNA sequencing (5.8s rDNA and ITS regions, and chloroplast *trnK/matK* regions). The utility of microsatellite markers for analyzing koa groups will also be assessed, and polymorphic microsatellite loci will be used to determine population distinctiveness and genetic diversity between three groups of koa; *A. koa, A. koaia,* and 'group three'. Finally, microsatellite markers will be tested for cross-species amplification of DNA from non-native *Acacia* species found in the Hawaii Islands. I expect to find highest levels of genetic diversity in the *A. koa* group, as it is most common and widely distributed in the Hawaiian Islands. The distinctiveness between populations is expected to be greatest between populations in the *A. koa* group from Hawaii and 'group three' populations from Kauai as they are geographically isolated and morphologically variable. The success of cross-species amplification is expected to be low, based on amplification of DNA from Australian *Acacia* species using microsatellite markers from *A. mangium* (Butcher et al., 2000).
CHAPTER 2
LITERATURE REVIEW

Acacia

The genus *Acacia* (Fabaceae:Mimosoideae) is non-monophyletic, having several lineages among the species (Wagner *et al.*, 1999; Clarke *et al.*, 2000; Miller and Bayer, 2001, 2003). Encompassing over 1380 species found in Asia, Africa, North and South America, and Australia, *Acacia* is classified into three subgenera: *Acacia*, *Aculeiferum*, and *Phylloideae* (*Heterophyllum*) (Maslin, 2003; Murphey *et al.*, 2003). Koa, morphologically classified in the largely Australian subgenus *Phylloideae*, is native to the Hawaiian Islands and includes *A. koa* and *A. koaia* (Pedley, 1975; Wagner *et al.*, 1999). Traditionally, Hawaiians carved seafaring canoes from the bole of large koa trees, and presently koa is used to produce high value furniture, crafts, and instruments because of its distinct grain and wood color (Cuddihy and Stone, 1990; Whitesell, 1990; Dachler, *et al.*, 1999). Koa also plays a vital role in Hawaii's watershed ecosystem, and is important habitat for epiphytic plants and birds, some of which are endangered (Rock, 1913; Whitesell, 1990; Elevitch, 2006).

Koa Origin

Koa is thought to be a descendent of seed that floated into the Indian and Pacific Oceans (Carlquist, 1965). *Acacia heterophylla*, endemic to Reunion and the Mascarene Islands in the Indian Ocean, is morphologically similar to koa (Whitesell, 1990). Differences between the two include larger pods and seeds for *A. koa*, and also racemes
with more heads, and distinct petals (Vassal, 1969; Pedley, 1975; and Wagner et al., 1999). It is also suggested that both A. koa and A. heterophylla share a common ancestor in A. melanoxylon (Coulaud et al., 1995, Wagner et al., 1999).

Ploidy / cytology

The genus Acacia has a base chromosome number x=13, and counts 2n=26, 2n=52, 2n=78, and 2n=104 have been reported (Atchison, 1948; Darlington and Wylie, 1955; Carr, 1978; Bennett and Leitch, 1995). Different ploidy levels (diploids to octaploids) can be found within species in Acacia, as is the case with A. tortilis, which has both tetraploid and octaploid individuals (Bukhari, 1997). Blakesley et al. (2002) reported naturally occurring triploid and tetraploid genotypes of A. dealbata, but no tetraploids of A. mangium. It has been suggested that koa is an allotetraploid from species hybridization (Brewbaker, 1996; Shi, 2003). A past study suggests A. heterophylla, which is a koa relative, may be an autotetraploid of A. melanoxylon (Couland et al., 1995), though this relationship is based on morphological similarities, not chromosome experiments. While not studied in depth, koa is known to be tetraploid (2n=4x=52) (Atchison, 1948; Pedley, 1975; Carr, 1978; Conkle, 1996; Shi, 2003). Ploidy analysis of A. koa seed from the island of Hawaii and Kauai showed no variation in ploidy levels, although one sample appeared to have a variable chromosome number (Hipkins, 2004). In plant biology, variable chromosome number can be caused by chromosome fragmentation, imbalance, fusion, or aneuploidy (Briggs and Walters, 1997). Shi (2003) also showed individuals from A. koa, A. koaia, and intermediate ('group three') populations having 2n=52 chromosomes and no variation in chromosome
number. It has been proposed that koa is an allotetraploid due to the fact that it is self-sterile, and because secondary constrictions are found in some pairs of chromosomes (Shi, 2003). This characterization is based on chromosome studies by Shi (2003), and no further studies have been undertaken to confirm this origin.

**Classification / Morphology**

Hawaiian koa is generally separated into three groups, that constitute either distinct species or subspecies/varieties. These groups include *A. koa* Gray, *A. koaia* Hillebrand, and *A. kauaiensis* Hillebrand. Additionally, *A. hawaiiensis* Rock, *A. lanaiensis* Rock, *A. latifolia* Bentham, and *A. waianaeensis* St. John have been used to characterize varieties of koa (St. John, 1979, Gardner, 1980, and Wagner et al., 1999). Only *A. koa* and *A. koaia* are currently accepted as taxonomic classifications for Hawaiian koa (The PLANTS Database, 2000; NatureServe. 2006, GBIF Data Portal, 2007). Therefore, morphological descriptions will be concentrated on these two types, along with a brief description of *A. kauaiensis*.

Differences are most evident in tree size, seed alignment within the pod, and phyllode size and shape. *Acacia koaia* is a small tree with a domed canopy (Figure 1), gnarled habit (Figure 2), and reaches heights up to 5 m (Wagner et al., 1999, Elevitch, 2006). Seed pods are narrow with longitudinally arranged seeds (Figure 3), that are smaller than those produced by *A. koa*. Additional distinctions include phyllodes that are shorter and narrower than *A. koa* (Figure 4), although this characteristic varies widely by island and habitat. Furthermore, *A. koaia* has denser wood, and usually grows in drier
Figure 1. *A. koaia* tree with domed canopy.

Figure 2. *A. koaia* tree displaying gnarled habit.
Figure 3. Narrow seed pods and longitudinally arranged seeds found on *A. koaia*.

Figure 4. *A. koaia* phyllode shape, generally long and narrow.
and more open habitats than *A. koa*, although a few populations have been found in mesic habitats (Wagner et al., 1999).

*A. koa* is a large tree (Figure 5) reaching heights up to 35 m, with tree form including both high (Figure 6) and low branching patterns (Wagner et al., 1999, Elevitch, 2006). Seed pods are wider than those of *A. koaia* and have the distinction of transversely arranged seeds (Figure 7). Phyllode shape varies greatly, but generally is long, wide, and curved (Figure 8). *Acacia koa* is found in a variety of habitats, including both wet and dry forests (Wagner et al., 1999). Koa wood color varies widely, spanning colors from blond to red to dark brown (Figure 9), and will rarely display an economically valuable curl, known as fiddleback. Both *A. koa* and *A. koaia* have expansive lateral root systems that are exposed on the soil surface, that makes them susceptible to suckering when damaged or stressed (Elevitch, 2006).

Figure 5. Large tree size of *A. koa*.

Figure 6. High branching pattern.
Figure 7. Wide seed pods and transversely arranged seeds of *A. koa*.

Figure 8. Wide phyllodes of *A. koa*.

Figure 9. Variation of wood color found in *koa*. 
Acacia kauaiensis (similar to ‘group three’) is distinct to Kauai above elevations of 1,000 m, has a short habit, and pods like those of A. koa. However, populations are also found on Kauai with the habit and phyllodes associated with A. koa, but seed pods characteristic of A. koaia (Wagner et al., 1999). Additional populations found below 1,000 m on Kauai and Oahu have similar variations in morphological traits. ‘Group three’ consists of koa that cannot be accurately characterized based on morphological characteristics due to wide variation in tree form (Figures 10), phyllodes (Figure 11), and seed pods (Figure 12). While A. kauaiensis is not officially recognized, evidence suggests further investigation could reveal species divergence or hybridization, especially in populations fitting the morphological profile of A. kauaiensis.

Figure 10. Variation in koa tree form on Kauai and Oahu for ‘group three’.
Figure 11. Phyllode variation in 'group three'.

Figure 12. Seed pod variation in 'group three'.
Distribution

Koa grows on a variety of volcanic soils, being most productive on those that are well drained (Whitesell, 1990, Elevitch, 2006). The distribution of koa is influenced by rainfall and elevation. Koa thrives at elevations ranging 800-1600 m and receiving between 1,900 and 5,100 mm of annual rainfall (Whitesell, 1990; Sun, 1996a). *Acacia koa* is common, and found on the Hawaiian Islands of Hawaii, Molokai, Maui, Lanai, Oahu, and Kauai at elevations ranging from 100-2300 m. *Acacia koaia* is rare and usually found as fragmented populations on the islands of Hawaii, Molokai, Maui, Lanai, and Kauai (Elevitch, 2006). In addition, *A. koaia* generally grows at lower elevations in open, drier habitats than *A. koa* (Wagner et al., 1999).

Conservation status

*Acacia koa* has been negatively impacted by ranching, but is still widely distributed throughout the major Hawaii Islands. Its conservation status is presently secure.

*Acacia koaia* was categorized as vulnerable on *The World List of Threatened Trees* (Oldfield et al., 1998) and the INUC (International Union for Conservation of Nature and Natural Resources) also included *A. koaia* on the *Red List of Threatened Species* (Bruegmann et. al., 2003). It currently has a global status ranking of G2, which is described as imperiled (NatureServe, 2006). Specific characteristics of these rankings include rare species with limited habitats that are often fragmented. Threatened trees are
also vulnerable to hybridization, inbreeding, and various pathogens. Small population size accompanied with a low total number of trees leaves *A. koaia* vulnerable to extinction if not protected and reforestation efforts implemented (Oldfield *et al*., 1998; Bruegmann *et al*., 2003). One preservation area is the Koaia Tree Sanctuary on the island of Hawaii, an enclosed area for preservation of *A. koaia* approximately 70 acres in size.

**Island Characteristics / Genetic Variability**

*Acaea koa* has wide variation in phenotypic characteristics across the Hawaiian Islands. Differences in phyllode width and shape, seed size, branch color, and nectary characters are most pronounced (St. John, 1979; Daehler *et al*., 1999). These differences are proposed to be a result of environmental conditions, genetic profile, or a combination of the two (Lamoureux, 1971; Sun *et al*., 1996b; Daehler *et al*., 1999). Sun (1996a) showed significant differences in tree height, DBH (Diameter at Breast Height), and phyllode development among koa using progeny tests. Common garden studies have shown that families from the island of Hawaii, when compared to those from Oahu and Kauai, have significant differences in seed width and mass, as well as phyllode length and width. These differences include longer, wider phyllodes with greater area, and seeds that have a greater mass and width for Hawaii families. Additionally, nectary diameter and branch color were also significantly different when compared to Oahu and Kauai families (St. John, 1979; Daehler *et al*., 1999). These results suggest a genetic basis in phenotypic variation between island families, and that Hawaii families are distinguishable from those of Oahu and Kauai (Daehler *et al*., 1999).
Variations in isozyme loci have been shown for koa populations on different islands in the state of Hawaii (Brewbaker, 1977; Conkle, 1996). Conkle (1996) showed the number of alleles per gene in samples of koa populations ranged from three to seven alleles for six polymorphic genes analyzed. Koa from Maui, Oahu, and Kauai are closely related, and their enzyme allele profiles differ greatly from those of Hawaii (Conkle, 1996). These results also support the conclusions of Daehler et al. (1999) that koa from Hawaii is genetically distinct from koa growing on Oahu and Kauai.

**Koa Decline and Disease**

The decline of koa can be attributed to a number of factors: land clearing for agriculture, fire, insects, fungi, and animal grazing (Scowcroft and Hobdy, 1987, Brewbaker, 1996; Daehler et al., 1999; Anderson et al., 2002; Shi, 2003). Most recently, fungal pathogens and the black twig borer (*Xylosandrus compactus*) have been the main cause of damage to koa trees (Anderson et al., 2002; Elevitch, 2006; Dudley et al., 2007).

In Hawaii Volcanoes National Park, Gardner (1980) observed wilt disease occurring in established stands of older koa trees. Root and stem tissues from wilted *A. koa, A. koaia, and A. confusa* trees were used as sample material and the fungal pathogen *Fusarium oxysporum f. sp. koae* was isolated. The fungus was determined to be seed born and the probable cause of premature decline in koa (Gardner, 1980), although other *Fusarium* species may also play a role (Elevitch, 2006; Dudley et al., 2007). Recently, Anderson et al. (2002) reported that koa ranging in size from small saplings to mature trees are affected by this fungus which causes a vascular wilt infection. Trees less than
fifteen years old and occurring at elevations lower than 760 m are most frequently affected, although koa wilt occurs at elevations up to 1650 m (Elevitch, 2006).

Various symptoms are associated with koa wilt disease, beginning with yellowing of phyllodes, followed by thinning of the tree's crown (Figure 13). Eventually the entire crown displays a chlorotic condition; necrosis of phyllodes ensues, leading to complete defoliation of the tree (Figure 14) (Anderson et al., 2002). Accompanying symptoms may include the development of cankers on bark, sap bleeding, and dark staining of sapwood (Figure 15). The fungus appears to originate in the roots and spreads upward, blocking vascular vessels, and causing canopy loss and tree death (Elevitch, 2006). Although infection throughout the entire tree is common, sometimes only one or a few branches are affected. Trees can survive with dead crowns for variable periods of time (Anderson et al., 2002; Elevitch, 2006). As a response to crown death, trees produce epicormic shoots on remaining healthy sections of the stem. A cycle of epicormic shoot production and eventual shoot death progresses downward along the stem until no healthy parts of the stem remains, and the infection kills the entire tree (Anderson et al., 2002).

The presence of *Fusarium* pathogens in soil does not automatically cause disease unless conditions for infection are favorable (Elevitch, 2006). Anderson *et al.* (2002) observed koa stands with healthy trees in close proximity, 50-100 m, to stands with wilt symptoms. Localized infections spread in a radial pattern from a central infection area,
Figure 13. Crown thinning in koa tree.

Figure 14. Complete defoliation of koa tree.

Figure 15. Koa wilt symptoms of cankers (left) and sap bleeding (right).
but are usually contained to defined stands of trees. Favorable conditions of high humidity, heavy rain, and low elevation seem to enhance fungal infection and symptoms of wilt disease. These characteristics make it difficult to determine if healthy-looking trees are indeed disease free.

Reproduction

The dichogamous nature of koa, where the anthers mature before the stigma on a single flower, favors an entirely outcrossed breeding system (Brewbaker, 1977). This system is enhanced by insects cross-pollinating koa (Sakai, 1995). Some polyploid acacias are able to reproduce by self-pollination, however, pollination studies have indicated koa is not likely one of them (Brewbaker, 1996; Sun, 1996a). Koa is also able to regenerate from seed banks and vegetatively from root suckers and stump sprouts, but tall grass may inhibit growth. (Spatz and Mueller-Dombois, 1973; Hatfield et al., 1996; Elevitch, 2006). Recently, Nelson (2006) reported intraspecific grafting of A. koa, as well as grafting onto rootstocks of A. mangium and A. confusa with success rates ranging from 20-70%. This data suggests koa may be compatible with a variety of Acacia species for grafting.

Plant Development

Koa is fast-growing during the first five years, potentially averaging more than 1.5 m/year, after which growth slows. Growth rates, especially stem diameter, are influenced by soil type, adequate rainfall, elevation, and stand density (Whitesell, 1990; Elevitch, 2006). As koa begins to grow it initially forms true leaves that consist of 12-15
bipinnately compound leaflets (Figure 16) (Whitesell, 1990; Wagner, 1999). Plants transition from producing true leaves to sickle-shaped phyllodes (Figure 17), usually before the sapling reaches 2 m (Whitesell, 1990). Hansen (1986) suggested that true leaves are better adapted to early plant growth when moisture is sufficient, while phyllodes provide better protection against drought once koa is established. This is a reasonable presumption considering phyllodes close their stomata in $\frac{1}{4}$ the time as true leaves during darkness, as well as transpiring only about 20 percent as much as true leaves during moisture stress (Walters et al., 1984). While mature trees usually display only phyllodes, true leaves may appear on the trunk, or protruding from roots, following injury or infection (Whitesell, 1990).

Figure 16. Bipinnately compound tree leaves characteristic of koa seedlings.
Field Experiments

Variation of quantitative traits in tree populations is often studied using the common-garden approach. These studies focus on traits that are economically important such as tree growth, survival, and disease tolerance (Krutovsky and Neale, 2005). Common-garden studies can identify families that are adapted to either a specific environment or broad range of environments (Krutovsky and Neale, 2005). This method has been used in Hawaii to evaluate phenotypic variation in koa from different islands. Significant differences were shown in seed, phyllode, and nectary characteristics among families from Oahu, Kauai, and Hawaii, suggesting phenotypic variation in koa is under genetic control (Daehler et al., 1999). While common garden approaches are useful for determining variation in measurable traits, they have many disadvantages. Field
experiments are very time consuming, dependent on consistent seedling establishment, and constrained by the long reproductive cycles of tree species (Nehra et al., 2005). Additionally, these studies are relatively expensive and based solely on phenotypes (Gonzalez-Martinez et al., 2006). Finally, the common-garden approach cannot explain how much phenotypic variation can be accounted for by genetic diversity and population structure.

Phylogenetics

Phylogenetics is the evolutionary relatedness of groups, and treats species as a group of lineage connected individuals over time (Doyle and Luckow, 2003). There are many approaches to reveal relationships among different species, most notably DNA sequences and molecular markers. Both nuclear and chloroplast DNA sequences have been used to explain phylogenetic relationships, while molecular markers such as isozymes, restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and microsatellite markers have been used to determine diversity and distinctiveness for Acacia species (Butcher et al., 2000; Broadhurst and Coates, 2002; Miller and Bayer, 2003; Murphey et al., 2003; Hemeida et al., 2004; Nanda et al., 2004; Millar and Byrne; 2007). The effectiveness of a particular marker depends on its polymorphism within and among species. Among molecular markers, microsatellites have broad applications in the field of genetics because it is a co-dominant marker and is highly variable (Rakoczy-Trojanowska and Bolibok, 2004).
Molecular Genetic Markers

Genetic markers are useful tools to estimate genetic diversity, phylogenetic relationships, population structure, and kinship (Krutovsky and Neale, 2005; Varshney et al., 2005; Selkoe and Toonen, 2006). As mentioned previously, the most common markers include: isozymes, RFLPs, RAPDs, AFLPs, chloroplast and nuclear DNA sequences, and microsatellite markers. Determining genetic variation within natural populations is essential for effective conservation and tree improvement programs, and molecular markers have proven useful for these applications (Mohammadi and Prasanna, 2003). Each type of marker is useful for different studies, with advantages and disadvantages as listed below.

ISOZYMES

Isozymes are co-dominant (although sometimes markers exist as present and null), genic markers where polymorphism is revealed by variations in a few amino acid differences. Isozyme markers are inexpensive to develop and have high reproducibility, however large amounts of fresh sample tissue are required compared to AFLPs and microsatellite markers, and some enzymes are tissue-specific (Krutovsky and Neale, 2005). An isozyme study on koa using six polymorphic genes revealed differences in koa populations from Hawaii, but was unable to differentiate populations from Maui, Oahu, and Kauai (Conkle, 1996). Isozyme studies have been widely used in the past for population genetic studies, but presently more powerful and versatile DNA-based...
markers such as AFLPs and simple sequence repeats (SSRs) are commonly used (Gonzalez-Martinez et al., 2006; Selkoe et al., 2006).

AFLPs

AFLPs are dominant, non-coding markers where genomic DNA is digested by restriction endonucleases, followed by selective amplification of DNA (Vos et al., 1995; Gonzalez-Martinez et al., 2006). Compared to isozyme markers, AFLPs are generally more expensive and difficult to assay. However, less tissue is needed per sample and multiplexing is possible (Krutovsky and Neale, 2005). AFLPs have been used to identify different Acacia species, and also combined with RAPD markers for higher resolution of relationships among species (Hemeida et al., 2004).

RAPDs

RAPDs are dominant, non-coding markers that utilize a single 10-mer oligonucleotide to amplify DNA (Phillips and Vasil, 2001). These markers are easy and inexpensive to develop, useful for multiplexing, and the amount of DNA required for each sample is low compared to other molecular marker techniques. RAPDs have been used successfully to differentiate Acacia species, both independently and in combination with AFLP markers (Hemeida et al., 2004; Nanda et al., 2004). The major disadvantage of RAPDs is the reproducibility of results is not as robust as RFLPs, AFLPs, and SSRs (Krutovsky and Neale, 2005).
RFLPs

RFLPs are co-dominant, non-coding markers where endonucleases cut pieces of DNA into different length restriction fragments. There are two types of RFLPs based on either complementary DNA or genomic DNA (Gonzalez-Martinez et al., 2006). RFLPs have very high reproducibility and are usually transferable across genera, however, they are difficult to develop, are not suitable for multiplexing, and are expensive to assay compared to other markers (Krutovsky and Neale, 2005).

Microsatellite Markers

Microsatellite markers are co-dominant, non-coding markers found throughout the nuclear genome, often referred to as simple sequence repeats (SSRs), short tandem repeats (STRs), or simple sequence length polymorphism (SSLP) (Rakoczy-Trojanowska and Bolibok, 2004). They consist of tandem repeats of 1-6 nucleotides, with the number of repeats varying from five to 100, although repeats between 10 and 40 are most common (Selkoe and Toonen, 2006). The effectiveness of microsatellites in determining complex relationships across taxa depends on the variability of a given locus, and the level of polymorphism in microsatellite loci has been shown to be positively related to the overall length of the repeating sequence (i.e. number of repeats, not repeat length such as dinucleotide and trinucleotide) (Dayanandan et al., 1997; Butcher et al., 2000).

The variation in repeat length for microsatellites is due to frequent mutations (between $10^{-2}$ and $10^{-6}$ mutations per locus per generation) during DNA replication.
caused by slippage of DNA polymerase and slipped-strand mispairing, and also proofreading errors (Bruvo et al., 2004; Rakoczy-Trojanowska and Bolibok, 2004; Selkoe and Toonen, 2006). These high mutation rates make microsatellites effective markers for genetic studies as they reveal high levels of allelic diversity among plants (Schlotterer, 2000). The flanking region surrounding a microsatellite locus consists of conserved sequences that are identical for individuals of the same or closely related species (Selkoe and Toonen, 2006). This allows some microsatellites developed for a specific species to cross-amplify in another, although the efficiency of cross-amplification decreases with increasing genetic distance between species (Dayanandan et al., 1997; Butcher et al., 2000; Selkoe and Toonen, 2006).

The most common microsatellites used for molecular genetic studies include dinucleotide, trinucleotide, and tetranucleotide repeats (Rakoczy-Trojanowska and Bolibok, 2004). Mononucleotide repeats are generally avoided due to their poor amplification reliability and lack of long repeats (Li et al., 2002). Dinucleotide repeats are generally most variable and account for the majority of microsatellites, but sometimes produce stutter bands due to slippage in DNA polymerase during amplification (Figure 18), which make allelic scoring difficult (Butcher et al., 2000; Holton, 2001; Li et al., 2002; Lacape et al., 2007). Trinucleotide and tetranucleotide repeats eliminate this problem, but have been shown to be less variable (Holton, 2001).
In *Acacia*, microsatellites have been shown to be less transferable among species than AFLP markers, and are relatively expensive and difficult to develop (Butcher *et al.*, 2000; Krutovsky and Neale, 2005). However, the advantages of microsatellites compensate for the difficult and expensive process of developing these markers.

Microsatellite markers are advantageous compared to other molecular markers because they are highly polymorphic, polymerase chain reaction (PCR)-based, provide reproducible results, require small amounts of sample DNA, and are relatively abundant throughout the genome (Dayanandan *et al.*, 1997; Butcher *et al.*, 2000; He *et al.*, 2003; Otero-Arnaiz *et al.*, 2005; Varshney *et al.*, 2005; Selkoe and Toonen, 2006). Previous studies in *Acacia* species found that genetic diversity detected using just five microsatellite loci, was three times higher than that found using 58 RFLP loci (Butcher *et al.*, 2000). Microsatellites are DNA-based and use PCR to amplify the marker with a
very small amount of sample tissue. Additionally, DNA is more stable than working with enzymes, can be easily preserved for future use, and microsatellites can often be amplified by PCR even after some amount of DNA degradation (Taberlet et al., 1999; Selkoe and Toonen, 2006).

DNA Sequencing

DNA sequencing has been used to infer phylogenetic relationships for many different plant taxa. One major advantage of DNA sequencing as a molecular tool is that it is easy to expand datasets to include additional plant species and provide phylogenetic resolution (Maslin et al., 2003). The utility of a specific gene region used for sequencing depends upon the rate of evolution of the gene relative to the taxonomic group being investigated (Doyle and Luckow, 2003). Nuclear and chloroplast are both useful, but they reveal relationships at different levels among taxa.

When selecting a gene or spacer region for constructing phylogenies, gene function is not as important as characteristics such as copy number and rate of gene evolution. The gene region needs to have sufficient sequence variation to differentiate taxa-splitting events while still preserving relationships at higher taxonomic levels (Doyle and Luckow, 2003). The high copy number of chloroplast and ribosomal genes in the genome allows successful amplification from total DNA (Baldwin et al., 1995).

Chloroplast DNA sequences have frequently been used for plant molecular studies at different taxonomic levels (Shaw et al., 2005). To reveal relationships at the
family level, the slow evolving gene \textit{rbcL} (large subunit of ribulose bisphosphate carboxylase) is commonly used, while genera and species are differentiated by more rapidly evolving spacer regions (Doyle and Luckow, 2003). The large amount of data available for \textit{Acacia} and other genera for the chloroplast \textit{trnK} (transfer gene for lysine) intron spacer region make it useful for phylogenetic and comparative studies with other taxa (Miller and Bayer, 2001; Shaw \textit{et al.}, 2005). While this region is sufficient to resolve species relationships in some taxa, it is sometimes combined with other sequence data for better phylogenetic resolution at low taxonomic levels (Shaw \textit{et al.}, 2005).

The nuclear ribosomal cistron is also commonly used for genetic studies, with different portions used to reveal various relationships among taxa. The small subunit, 5.8S rDNA, and large subunit genes are highly conserved and utilized at high taxonomic levels. The internal transcribed spacer regions (ITS), on the other hand, are more variable non-coding regions that are used at the genus and species level (Murphy \textit{et al.}, 2003; Doyle and Luckow, 2003).

\textbf{Chloroplast trnK intron and matK coding sequence}

The use of multiple approaches to infer phylogeny, along with a measure of internal support such as bootstrap or jackknife, reveals more robust phylogenetic trees (Doyle and Gaut, 2000; Soltis and Soltis, 2003). Along with ribosomal ITS sequences, chloroplast DNA sequences are primarily used to infer phylogeny at the intergeneric and interspecific levels (Shaw \textit{et al.}, 2005). The chloroplast \textit{matK} (maturase encoding gene) sequence, along with its flanking \textit{trnK} intron region has been extensively used to resolve
discrepancies regarding the interrelationships of the three subgenera of the genus *Acacia* (Miller and Bayer, 2001; Miller *et al*., 2003). The *matK* evolves faster than *rbcL*, and is useful at the infrageneric level, while the *trnK* intron is noncoding and is used at lower taxonomic levels (Johnson and Soltis, 1994; Miller and Bayer, 2001). While little sequence variation is expected for this region within the koa complex, sequence variation between koa and non-native *Acacia* species found in Hawaii is expected. This chloroplast region will be most useful for determining the placement of koa within one of the three subgenera of *Acacia*.

*Internal transcribed spacer regions (ITS1 and ITS2) of nuclear ribosomal DNA*

Traditionally genetic relatedness has been inferred through morphological similarities at the genus and species levels (Coleman and Mai, 1997). However, a combination of molecular approaches, such as nuclear and chloroplast DNA sequencing, can answer the question of genetic relatedness among taxa that are difficult to distinguish morphologically, as is the case with koa. The internal transcribed spacer regions (ITS 1 and 2) separating the nuclear ribosomal genes have been shown to be highly variable in the *Leguminosae* family, and have been used to reconstruct plant phylogeny in the subgenus *Phyllodineae* (Murphey *et al*., 2003; Varela *et al*., 2004; Choi *et al*., 2006). While the internal transcribed spacer regions allow discrimination among different species within the subgenus, the 5.8S gene region separating these spacer regions is highly conserved within the subgenus with no length variation observed (Murphey *et al*., 2003). This allows for easy alignment of DNA sequences from previously uncharacterized samples. Sequencing near the 5' end of ITS 1 has been reported to be
problematic for some *Acacia* species, where only partial sequences are obtained (Murphey et al., 2003). However, the combined length of approximately 700 bp for the entire region allows for easy amplification and successful sequencing from only one pair of primers (Kress et al., 2005). Additionally, the ITS region is the most commonly used locus at the species level, which allows comparison with sequence data from many different taxa (Kress et al., 2005; Kress and Erickson, 2007).
CHAPTER 3
EXPERIMENTAL METHODS

Plant material sampling and DNA isolation

Mature koa phyllodes and seed pods were collected from Hawaii, Oahu, and Kauai. Sampling included representatives of various forms of koa based on morphological distinctions (Table 1). A total of 215 samples were collected, from populations comprising three groups characterized by morphological distinctions: *A. koa*, *A. koaia*, and populations with characteristics intermediate of *A. koa* and *A. koaia*, termed 'group three' (See Table 2, Figures 19 and 20). *Acacia* species non-native to the state of Hawaii were collected from Oahu and Hawaii for DNA isolation and included: *A. angustissima*, *A. auriculiformis*, *A. confusa*, *A. mangium*, *A. mearnsii*, and *A. melanoxylon*. Plant samples were transported on ice and frozen at -20°C until processed. Three to four phyllodes from each individual tree sample were pulverized in liquid nitrogen, and total DNA was isolated from 5 g samples using DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA). The DNeasy® Plant System purified total cellular DNA from plant tissue samples using the following isolation procedure according to the kit manual. First, tissue was lysed and RNase A treated while incubated at 65°C. Next, polysaccharides, detergents, and proteins were precipitated with depleting buffer during centrifugation. After, the supernatant was collected and centrifuged through a QIAshredder spin column to remove cell debris and precipitates. DNA was then precipitated with ethanol buffer and this solution was loaded onto the DNeasy spin column and centrifuged, leaving the DNA bound to a silica gel membrane. DNA bound
### Table 1. Variation in morphological characteristics among groups of koa.

<table>
<thead>
<tr>
<th></th>
<th>Habitat</th>
<th>Tree Height</th>
<th>Seed Pods</th>
<th>Seed Arrangement</th>
<th>Phyllodes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. koa</strong></td>
<td>Wet/Mesic forest</td>
<td>Large</td>
<td>Wide</td>
<td>Transversely</td>
<td>Wide variation generally long and wide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10-35 m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. koaia</strong></td>
<td>Dry, open woodland</td>
<td>Small</td>
<td>Narrow</td>
<td>Longitudinal</td>
<td>Wide variation generally short and narrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-5 m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'group three'</td>
<td>Wet/Mesic forest and</td>
<td>Variable</td>
<td>Wide</td>
<td>Transversely</td>
<td>Wide variation</td>
</tr>
<tr>
<td></td>
<td>open woodland</td>
<td>3-10 m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Narrow</td>
<td>Longitudinal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intermediate</td>
<td>Diagonal</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Population descriptions for koa samples.

<table>
<thead>
<tr>
<th>Population Label / Location</th>
<th>Island</th>
<th>Morphological Group</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>KANA- Anahola</td>
<td>Kauai</td>
<td>'group three'</td>
<td>20</td>
</tr>
<tr>
<td>SG- Waimea</td>
<td>Kauai</td>
<td>'group three'</td>
<td>15</td>
</tr>
<tr>
<td>KHAN- Hanalei</td>
<td>Kauai</td>
<td>'group three'</td>
<td>20</td>
</tr>
<tr>
<td>KV- Kahana Valley</td>
<td>Oahu</td>
<td>'group three'</td>
<td>20</td>
</tr>
<tr>
<td>KOAB- Pua Akala</td>
<td>Hawaii</td>
<td>A. koa</td>
<td>20</td>
</tr>
<tr>
<td>KOAA- Hakalau Wildlife Refuge</td>
<td>Hawaii</td>
<td>A. koa</td>
<td>20</td>
</tr>
<tr>
<td>KKA- Awa’awapuhi Trail</td>
<td>Kauai</td>
<td>A. koa</td>
<td>20</td>
</tr>
<tr>
<td>KMC- Kokee-Mohihi</td>
<td>Kauai</td>
<td>A. koa</td>
<td>20</td>
</tr>
<tr>
<td>KTSB- Kohala Road</td>
<td>Hawaii</td>
<td>A. koaia</td>
<td>20</td>
</tr>
<tr>
<td>KTSA- Koaia Tree Sanctuary</td>
<td>Hawaii</td>
<td>A. koaia</td>
<td>20</td>
</tr>
<tr>
<td>KNT- Nounou Ridge</td>
<td>Kauai</td>
<td>A. koaia</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 19. Map of sample populations on the islands of Kauai, Oahu, and Hawaii. Images copyright Google, 2007; DigitalGlobe, 2008.
Figure 20. Terminology used to describe koa.
to the membrane was washed and then eluted from the membrane using elution buffer. DNA concentration and purity was checked for each sample using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Sequence amplification and analysis

Chloroplast trnK intron and matK coding sequence

To determine phylogenetic relationships of koa within the broader context of Acacia subgenera, chloroplast and nuclear DNA sequences were investigated. A total of 33 samples were sequenced including 10 each from the groups A. koa, A. koaia, and 'group three'. Additional sequencing for the chloroplast region included non-native Acacia species A. auriculiformis and A. mearnsii, while A. melanoxylon was sequenced for both DNA regions. For chloroplast analysis the cpDNA intron spacer of trnK was sequenced. This region includes matK, a maturase encoding gene, in addition to flanking noncoding regions (Figure 21). The coding region of matK is approximately 1500 bp, while the entire intron is approximately 2300 bp (Miller and Bayer, 2001, 2003). Techniques for PCR amplification were adapted and modified from those previously published. Initial DNA amplification by PCR used primers trnK-3914 (GGG GTT GCT AAC TCA ACG G) and trnK-2R (AAC TAG TCG GAT GGA GTA G) described for Saxifragaceae (Johnson and Soltis, 1994). Subsequent reactions used Acacia specific primers internal to trnK- 2R including: Ac283R (CAC TGA CGG CAA GCC CCT CTG), Ac12F (GGT GCA (A/C)AA TCT AGG TTA TGA C), Ac1290R (AAT ACA AGA AAG CCG AAG), Ac1104F (CCT CTA ATT AGA TCA TTG GC), and Ac1707R.
Figure 21. Diagram (not to scale) of the chloroplast trnK intron region and matK coding sequence region (Miller and Bayer, 2001). Arrows represent primer direction.
(TGC ACA CGG CTT TCC CTA TG) (Miller and Bayer, 2001). Primers were synthesized by Integrated DNA Technologies, INC. (Coralville, IA, USA). The PCR reaction mixture consisted of: 4 µL of 25 mmol/L magnesium chloride solution; 10 µL of 5x Colorless GoTaq® Flexi Buffer; 1.0 unit of GoTaq® Flexi DNA polymerase (Promega Corp., Madison, WI); 25 pmol primer; 6-10 ng template DNA; and 1.0 µL of 25 mmol/L dNTP solution (Bioline USA Inc., Taunton, MA) in equimolar ratio. The total volume of each PCR reaction was 50 µL. Total DNA was amplified using a MJ Research PTC-200 Peltier Thermal Cycler (San Francisco, CA) with the following program: initial denaturation at 94°C for 5 minutes; followed by 30 cycles of denaturation (1 min. at 94°C), primer annealing (1 min. at 46-53°C), and extension (2 min. at 72°C). A final extension of 72°C for 10 minutes followed the 30th cycle and PCR products were stored at 4°C. Annealing temperatures for each primer pair were optimized using a temperature gradient between 42-60°C. PCR amplification was confirmed by electrophoresis using 15 µL of PCR product in 1.0% agarose gels for 2 h at approximately 65V. Double stranded PCR products were cleaned using the QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified PCR samples were sequenced on an Applied Biosystems 3730XL capillary-based DNA sequencer (Applied Biosystems, Foster City, CA) by University of Hawaii at Manoa ASGPB. Sequencing used 2 µL of a 10 µL solution consisting of 3.2 pmol of primer and 20 ng PCR product per 100 bp sequenced.
Internal transcribed spacer regions (ITS) of nuclear ribosomal DNA

For nuclear ribosomal analysis the ITS regions and 5.8S rDNA was sequenced (Figure 22). This region includes ITS 1 [between the SSU (small-subunit rDNA) and 5.8S rDNA], the 5.8S rDNA gene, and ITS 2 [between the 5.8S rDNA and the LSU (large-subunit rDNA)]. The coding region of the 5.8S rDNA gene is 159 bp, while the entire rDNA cistron region is approximately 700 bp (Murphey et al., 2003). Techniques for PCR amplification were adapted from those previously published. DNA amplification by PCR used an *Acacia* specific primer, ACF (GGA GAA GTC GTA ACA AGG TTT CCG) (Murphey et al., 2003) and 26SE (TAG AAT TCC CCG GTT CGC TCG CCG TTA C) (Sun et al., 1994) for initial amplification. The ITS regions were further amplified using nested PCR with primers S3 (AAC CTG CGGA AGG ATC ATT G), S4 (TAG CCC CGC CTG ACC TGA GG), S5 (TTC GGG CGC AAC TTG CGT TC) and S6 (ATA TCT CGG CTC TTG CAT CG) (Käss and Wink, 1997). Primers were synthesized by Integrated DNA Technologies, INC. (Coralville, IA). The PCR reaction mixture consisted of: 4 µL of 25 mmol/L magnesium chloride solution; 10 µL of 5x Colorless GoTaq® Flexi Buffer; 1.0 unit of GoTaq® Flexi DNA polymerase (Promega Corp., Madison, WI); 25 pmol primer; 6-10 ng template DNA; and 1.0 µL of 25 mmol/L dNTP solution (Bioline USA Inc., Taunton, MA) in equimolar ratio. The total volume of each PCR reaction was 50 µL. Total DNA was amplified using a MJ Research PTC-200 Peltier Thermal Cycler (San Francisco, CA) with the following program: initial denaturation at 94°C for 3 minutes; followed by 30 cycles of denaturation (1 min. at 94°C), primer annealing (1 min. at 55°C), and extension (2 min. at 72°C). A final
Figure 22. Diagram (not to scale) of rDNA cistron for the ITS region in *Acacia*. SSU = small-subunit rDNA; ITS = internal transcribed spacer; LSU = large-subunit rDNA (Murphey et al., 2003). Arrows represent primer direction.
extension of 72°C for 7 minutes followed the 30th cycle and PCR products were stored at 4°C. Nested PCR reactions were carried out using the following program: initial denaturation at 95°C for 15 min; followed by 30 cycles of denaturation (30 sec. at 94°C), primer annealing (30 sec. at 63.8°C), and extension (20 sec. at 72°C). A final extension of 72°C for 5 minutes followed the 30th cycle and PCR products were stored at 4°C (Murfhey et al., 2003). PCR amplification was confirmed by electrophoresis using 15 µL of PCR product in 1.0% agarose gels for 2 h at approximately 65V. Double stranded PCR products were cleaned using the QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified PCR products were sequenced on an Applied Biosystems 3730XL capillary-based DNA sequencer (Applied Biosystems, Foster City, CA) by University of Hawaii at Manoa ASGPB. Sequencing used 2 µL of a 10 µL solution consisting of 3.2 pmol of primer and 20 ng PCR product per 100 bp sequenced.

**Microsatellite marker amplification and analysis**

A total of 20 microsatellite markers were tested, six that were *A. mangium*-specific, and 14 that were *A. koa*-specific. The six *A. mangium*-specific markers were chosen on the basis of cross-species amplification of species closely related to *A. koa* in the subg. *Phyllodineae*, section *Plurinerves* (Butcher et al., 2000). Phylogenetic trees and sequence analysis indicated high sequence similarity between *A. koa* and *A. melanoxylon*, indicating likely amplification of *A. koa* samples using microsatellite markers developed for *A. mangium*. The PCR reaction mixture followed the same protocol used in PCR reactions for sequencing analysis and the forward primer of each primer pair was fluorescently labeled with one of four fluorescent dyes; VIC, PET, FAM,
or NED (Applied Biosystems Custom Oligonucleotides, Foster City, CA, USA). PCR amplification was as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation (1 min. at 94°C), primer annealing (1 min. at 48-60°C), and extension (1 min. 30 sec. at 72°C). Annealing temperatures for each primer pair were optimized using a temperature gradient between 46-64°C. A final extension of 72°C for 30 minutes followed the 35th cycle and PCR products were stored at 4°C. Initially, samples were individually genotyped for allele size determination. Additional test samples were genotyped using multiplexes involving four microsatellite markers, each with a unique dye color. Multiplexes consisting of between five and eight microsatellite amplification products were evaluated, but resulted in poor separation of PCR products during capillary electrophoresis. This caused problems with allele scoring, so only four markers were multiplexed at a time. Amplification products were separated by capillary electrophoresis on an ABI Prism 377XL DNA sequencer. Fragment sizes were determined using GeneMarker V1.6 software (Softgenetics LLC, State College, PA).

Data analysis

*trnK / matK and ITS1 / 5.8S / ITS2 sequencing*

Boundaries of the chloroplast *trnK / matK* and the nuclear ITS1 / 5.8S / ITS2 regions were determined by comparison to those of published *Acacia* species (Miller and Bayer, 2001; Miller and Bayer, 2003; Miller et al., 2003; Murphey et al., 2003). A set of homologous *Acacia* sequences were assembled using NCBI BLAST (Basic Local Alignment Search Tool), and combined with koa sequence data for the *trnK / matK*
DNA sequences were initially aligned using the Clustal X package with all multiple alignment characters used at default settings (Thompson et al., 1997), and further edited by hand. Indels were scored as separate characters and manual alignments were with minimal gaps. Sequence alignments with missing data were excluded from analysis and all characters were un-weighted. For the \textit{trnK} / \textit{matK} region, 47 nucleotide positions were excluded from further analysis because of ambiguous alignments at these positions. A total aligned sequence length of 805 bp including the \textit{trnK} intron region and partial \textit{matK} coding region was used for further analysis. For the ITS spacer region, 85 nucleotide positions were excluded because of ambiguous alignments. A total aligned sequence length of 582 bp including partial ITS 1 region, complete 5.8S gene, and partial ITS 2 region was used for further analysis. Phylogenetic analysis was performed using PHYLIP (Phylogeny Inference Package) v3.6 phylogenetic programs (Felsenstein, 1989; 2005). Parsimony analysis was performed using DNAPENNY where the branch and bound algorithm was used to find most parsimonious trees. \textit{Mimosa tenuiflora} and \textit{Lysiloma divaricata} were used as outgroups for all phylogenetic analysis of the \textit{trnK} / \textit{matK} and ITS1 / 5.8S / ITS2 regions, respectively, based on their close morphological and genetic similarity to \textit{Acacia} (Miller and Bayer, 2001; Murphey et al., 2003). Multiple random data sets were generated using SEQBOOT for use in parsimony analysis to eliminate bias associated with the order of data entry. Bootstrap data sets and consensus trees were created using SEQBOOT and CONSENSE programs. A majority rules consensus tree was created with 100 bootstrap replicates. Phylogenetic trees were created using
Table 3. Species used for \textit{trnK} / \textit{matK} analysis. (Species with GenBank accession numbers are from Miller and Bayer, 2001; Miller and Bayer, 2003; Miller \textit{et al.}, 2003)

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Table 4. Species used for ITS 1/ 5.8S / ITS 2 analysis. (Sequences with GenBank accession numbers are from Murphey et al., 2003)

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KOA = A. koa; KOAIA = A. koaia; GTHR = 'group three'
DRAWGRAM, and TreeView (Page, 1996) was used to view the PHYLIP format tree data.

**Microsatellite marker analysis**

Twelve of the 20 microsatellite markers tested were utilized for data analysis (Table 5). The other eight markers were excluded due to poor quality of amplification, ambiguities in allele assignment, and stutter bands. Variation in allele sizes and frequency were scored for 12 microsatellite markers using GeneMarker V1.51 software. Four of these markers were developed for *A. mangium* (Am030, Am465, Am502, and Am770; Butcher *et al.*, 2000), and the other eight were developed for *A. koa* (Ak05, Ak37, Ak44, Ak84, Ak89, Ak180, Ak196, and Ak284; Fredua-Agyeman, unpublished). A data matrix of allele sizes was created in Microsoft Excel and used to calculate genetic parameters of populations and groups (subscripts *P* and *G*, respectively) for the average number of alleles per polymorphic locus (*AP*) and percentage of polymorphic loci (*P*, criterion 99%). At the population level, the average number of alleles per polymorphic locus (*AP*ₚ) was calculated by summing the alleles detected at polymorphic loci in a population and dividing by the number of polymorphic loci. At the group level, *AP*ₒ was calculated by summing the alleles detected at polymorphic loci in all populations representing a group, and dividing by the number of polymorphic loci. The same approach was used to determine percentage of polymorphic loci, where the number of loci polymorphic within a group/population was divided by the total number of loci.
Table 5. Microsatellite marker primer sequences, annealing temperature, and allele sizes.

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The polyploid nature of plants is believed to contribute advantages to their growth within an environment. It has been shown to increase their ability to survive in extreme habitats (Soltis and Soltis, 2001). Polyploid plants are not as well studied as diploids, especially using molecular markers such as allozymes and microsatellite markers (Esselink et al., 2004). Analysis of tetraploids using these two molecular tools is inherently difficult due to variation in allele dosage.

Tetraploid plants may have up to four alleles present at each locus, and allele scoring can be recorded as one copy of each allele (eg. A:B:C:D). However, in the case of two or three alleles being present at a specific locus, different genotypes can account for the same band pattern, (eg. A:A:A:B; A:B:B:B; A:A:B:C; A:B:B:C; A:B:C:C; A:B:C:null allele; etc.) referred to as ‘partial heterozygotes’ (Bruvo et al., 2004). To account for this potential genotypic variation, the MAC-PR (microsatellite allele counting-peak ratios) method can be used, which utilizes calculations of the area under each microsatellite allele peak to infer allele dosage and distribution (Esselink et al., 2004; Babaei et al., 2007). However, the success of the MAC-PR approach is dependent on having consistent allele amplification and limited stutter bands (Esselink et al., 2004). Considering all koa samples cannot be analyzed from the same PCR reaction, variation in allele intensity was to be expected, and it makes this approach impractical. Additionally, MAC-PR is not commonly applied to studies where genetic relationships are unknown (Esselink et al., 2004).
A more common approach to analyze molecular data is to score the presence or absence of each allele at a particular locus and infer diversity statistics from allele phenotype patterns (Hormaza, 2002; Wang *et al.*, 2004; Medini *et al.*, 2005; Segarra-Moragues *et al.*, 2005; Obbard *et al.*, 2006a; Obbard *et al.*, 2006b; Yifru *et al.*, 2006; Hamilton and Eckert, 2007). Phenotype frequency measures such as $H_{\text{Phen}}$ and $H_{\text{SW}}$ only take into account whether allele band patterns are identical or different. On the other hand, $H$, calculated using the software FDASH, takes into account the fact that phenotypes sharing a high number of bands are more similar, even though the band patterns are not identical (Obbard *et al.*, 2006b). Although this approach is intended for allotetraploids, which koa is thought to be, it also captures essential information from polyploids with alternative inheritance (e.g., polysomic polyploids) and when common ancestry may not relate to allele sharing (Obbard *et al.*, 2006b). These factors made FDASH the most reasonable approach for tetraploid data analysis.

Allele scoring revealed individuals with one, two, three, and four alleles present. Multilocus scoring was done where the presence or absence of each allele was scored for each microsatellite locus (Hormaza, 2002; Wang *et al.*, 2004; Medini *et al.*, 2005; Segarra-Moragues *et al.*, 2005; Yifru *et al.*, 2006). To measure population diversity and differentiation, the software FDASH was used, which allows analysis of polyploid data by comparing allelic phenotypes. The diversity measure calculated by FDASH is similar to Nei's gene diversity ($H$), and the Shannon-Weaver diversity index of phenotypes ($H_{\text{SW}}$), and is termed $H$. $H$ measures diversity by accounting for different numbers of unshared alleles between individuals, calculated by the following formula:
\[ H' = \frac{1}{n(n-1)} \sum_{i=1}^{n} \sum_{j=1}^{n} x_{ijk} \]

where \( n \) is the number of individuals sampled and \( x_{ijk} \) is a variable that is equal to one if allele \( k \) it carried by individual \( i \) or individual \( j \), but not both, and otherwise equal to zero (Obbard et al., 2006). While some information is lost compared to analysis using allele dosage frequencies, this method includes more information than other phenotypic diversity measures because it takes into account band sharing between different phenotypes instead of treating each phenotype separately (Obbard et al., 2006b). Significant differences in diversity between groups were tested using a randomization test (1000 permutations) in FDASH, and differences in \( H' \) between groups was considered significant if it was greater than 95% of all randomized differences.

Based on diversity calculated by \( H' \), genetic differentiation, \( F_{ST} \), was also calculated using FDASH by the following formula:

\[ F_{ST} = \frac{(H'_{T} - H'_{S})}{H'_{T}} \]

where \( H'_{S} \) is the average diversity \( (H') \) within populations and \( H'_{T} \) is the total genetic diversity \( (H) \) for both populations pooled (Obbard et al., 2006b).
The relatedness of populations was evaluated by an unweighted pair grouping of arithmetic average (UPGMA) cluster method based on pairwise genetic distances between populations, calculated as $F'_{ST}$ in FDASH. The dendogram of these relationships as well as principle coordinate analysis (PCORDA) of populations was computed using the software NTSYSpc version 2.2 (Rohlf, 2007). PCORDA was also carried out for individuals to assess their inclusion in each group.

Regression analysis was used to assess the correlation between population differentiation, measured as $F'_{ST}$, and geographic distance between populations to determine isolation by distance effects. The significance of this effect was measured using Mantel tests implemented in NTSYSpc version 2.2 (Rohlf, 2007).

To describe the partitioning of genetic variation within and among populations, an analysis of molecular variance (AMOVA) was performed using Arlequin 2.0 (Schneider et al., 2000), based on the same matrix of presence (1) or absence (0) of alleles used for the diversity statistics. For this binary data, AMOVA is based on allelic differences between individuals defined as Euclidean distance (Mengoni and Bazzicalupo, 2002; Excoffier et al., 1992, 2005).

**Cross-species amplification**

To assess the utility of microsatellite markers for cross-species amplification, three individuals from the following acacias were tested for positive amplification using PCR: *A. angustissima*, *A. auriculiformis*, *A. confusa*, *A. mangium*, *A. mearnsii*, and *A.*
*melanoxylon*. PCR amplification cycles were the same as those used for koa samples. Markers were considered successful to cross-amplify if all three samples showed sharp PCR products.
CHAPTER 4
RESULTS

Chloroplast trnK intron and matK coding sequence

The aligned length of the trnK / matK region was 805 bp, of which 29% of the sequence was variable. The region contained 82 potentially informative base substitutions, however, only four informative substitutions were observed among the three koa groups. The occurrence of relatively small sequence variation within species for the trnK / matK region is the reason for its application at the genus and subgenus levels (Miller et al., 2003). The two non-native acacias that were also sequenced, A. auriculiformis and A. melanoxylon, were more variable from individuals in the koa groups, as expected. Ten individuals representing each koa group, a total of 30 individuals, were sequenced. However, no more than 1 bp was variable within each group. Therefore, one consensus sequence was created from the 10 individuals of each group and used for further phylogenetic analysis.

A majority rules consensus tree was created from 10,000 most parsimonious trees to reveal the placement of each koa group within the Acacia genus (Figure 23). The koa groups were closely related to species in the Australian subgenus Phyllodineae, especially A. melanoxylon. Sequence comparison revealed that A. koa had over 98% sequence homology with A. melanoxylon. The two non-native Acacia species sampled from Hawaii (A. auriculiformis and A. melanoxylon) were also clustered within Phyllodineae (P). Bootstrap values based on 100 replicates showed strong support for relationships among species within each subgenus, in the phylogenetic tree. Within
Figure 23. Majority rule consensus tree of 10,000 most parsimonious trees from \(trnK/matK\) sequence analysis with a length of 341 steps. Bold numbers above lines are bootstrap support values. The second column indicates subgenus classification within *Acacia*: A= Acacia; L=Aculeiferum; P= Phyllodineae; Outgroup represented by M= Mimoseae. The third column represents each species native origin (Miller and Bayer, 2001). Italicized numbers represent the number of steps for each branch.
subgenus *Aculeiferum* (L), bootstrap support was strongest for *A. modesta* and *A. senegal*, the two species with Asia/Africa origin, although relationships of all species in the subgenus were strongly supported based on bootstrap analysis.

**Internal transcribed spacer regions (ITS) of nuclear ribosomal DNA**

The aligned length of the ITS region was 582 bp, of which 37% of the sequence was variable. The region contained 88 potentially informative base substitutions, including nine among the three koa groups. Five individuals from each group, including at least one from each of the sample populations, were included in the phylogentic analysis. A majority rules consensus tree was created from 10,000 most parsimonious trees to determine the relationship of koa groups within the subgenus *Phyllodineae* (Figure 24). Based on *trnK/matK* sequence analysis, koa groups should be closely related to species in the section *Plurinerves*. Bootstrap replicates (100) were used to analyze the strength of relationships determined from parsimony analysis.

While sequencing from the ITS region revealed more than twice as many informative sites among koa groups as the *trnK/matK* region, no conclusive relationships among koa were supported by the parsimony analysis. All koa samples used for analysis formed a single clade with *A. melanoxylon* as the closest related sister taxon (consistent with results from analysis of the *trnK/matK* region). A total of 17 characters were variable among individuals within this clade, but there were no patterns of association evident among samples of koa populations. Individuals from the three koa groups were most related to *A. melanoxylon* of the section *Plurinerves*. However,
Figure 24. Majority rule consensus tree of 10,000 most parsimonious trees from ITS1 / 5.8S / ITS2 sequence analysis. Bold numbers above lines are bootstrap support values. Classifications into sections (right side in bold) for taxa are after Vassal and Pedley (Murphey et al., 2003). KOA = A. koa; KOAIA = A. koaia; GTHR = ‘group three’. Italicized numbers represent the number of steps for each branch.
A. translucens, also classified in the section Plurinerves, was clustered with species from other sections in subgenus Phyllodineae. Bootstrap values were highest between species in both the Pulchellae and Lycopodiifoliae sections, which is consistent with reported data (Murphey et al., 2003).

**Multiplexing of microsatellite markers**

Theoretically, any four fluorescent-labeled microsatellite markers could have been multiplexed, as long as the markers were each labeled with a different dye, such as VIC, PET, FAM, or NED. Markers of the same dye color could also have been analyzed together if the expected base pair sizes are known for each marker, and they do not overlap. Groups were designed to keep the A. mangium-specific markers and the koa-specific markers in separate groups. Multiplexing with more than four markers proved to be problematic, as amplified products were not as intense and allele scoring was difficult. Therefore multiple combinations using four markers were tested as multiplex groups. Analysis with these multiplex groups produced easily identifiable allele patterns for all samples. The multiplex groups used for all microsatellite allele scoring were as follows: Group 1; Am030, Am465, Am502, and Am770; Group 2; Ak05, Ak44, Ak84, and Ak284; and Group 3; Ak37, Ak89, Ak180, and Ak196.

**Microsatellite markers**

All 12 microsatellite loci were polymorphic for each group, and resulted in a total of 106 alleles that were detected across all populations. Allelic variation for each locus
ranged from three (Ak180, Am770) to 17 (Ak44), with an average of 8.8 alleles per locus (AP) for all samples combined (Table 6). Of all alleles detected, 29% (31 of 106) were unique to a particular group ('Group three', 21 unique alleles; A. koa, 7; and A. koaia, 3). Although all loci were polymorphic, there were wide variations in the APo for each group of the koa complex. Acacia koaia had the lowest APo with 4.3, while 'group three' had the highest APo with 7.9. This group also had the most unique alleles, 21, and contained a combination of alleles found exclusively in the A. koa or A. koaia groups (Figure 25). Results for APp were consistent with those seen for each group, as KANA ('group three'), had the highest APp with 5.7, while two populations of A. koaia (KTSA and KNT) had the lowest APp (2.7 and 3.1, respectively).

Genetic diversity measures for each group, based on allele phenotypes, were not significantly different and were correlated with the total number of alleles per group (Figure 26). Diversity was strongly partitioned within populations of each group (H'g) rather than among (D'st), which explains the low levels of differentiation between groups (F'st) (Table 7). Diversity and differentiation statistics over all loci showed the A. koaia group as the least diverse (Table 7). At the population level, total diversity (H'T) showed an A. koaia population on Kauai (KNT) was the least diverse (Table 8). Population statistics were consistent with those for groups, as the highest levels of diversity were found in 'group three' populations. The two most diverse populations were KANA and SG, but surprisingly, 'group three' also contained one of the least diverse populations, KV (Table 8). An AMOVA for the microsatellite data reaffirmed differentiation
<table>
<thead>
<tr>
<th>Marker</th>
<th>Total Alleles</th>
<th><em>A. koa</em> alleles</th>
<th><em>A. koaia</em> alleles</th>
<th>'group three' Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am030</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>6**</td>
</tr>
<tr>
<td>Am465</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>7*</td>
</tr>
<tr>
<td>Am502</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>10****</td>
</tr>
<tr>
<td>Am770</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3*</td>
</tr>
<tr>
<td>Ak05</td>
<td>8</td>
<td>6</td>
<td>7*</td>
<td>6</td>
</tr>
<tr>
<td>Ak37</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4*</td>
</tr>
<tr>
<td>Ak44</td>
<td>17</td>
<td>13***</td>
<td>4</td>
<td>14****</td>
</tr>
<tr>
<td>Ak84</td>
<td>9</td>
<td>6***</td>
<td>4*</td>
<td>5**</td>
</tr>
<tr>
<td>Ak89</td>
<td>13</td>
<td>11</td>
<td>6</td>
<td>13**</td>
</tr>
<tr>
<td>Ak180</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ak196</td>
<td>10</td>
<td>6*</td>
<td>2*</td>
<td>8**</td>
</tr>
<tr>
<td>Ak284</td>
<td>16</td>
<td>14</td>
<td>9</td>
<td>16**</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>80</td>
<td>52</td>
<td>95</td>
</tr>
<tr>
<td><em>AP</em></td>
<td>8.8</td>
<td>6.7</td>
<td>4.3</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*Unique Allele
Figure 25. Electropherogram of allelic scoring for *A. koa*, *A. koaia*, and 'group three' for primer Am502. Numbers below peaks represent allele sizes (bp); y axis measurement is relative fluorescent units (RFU). 'Group three' has: allele 115, only found in *A. koa*; allele 118, only found in *A. koaia*; as well as alleles 105, 109, 127, and 133, unique to 'group three'.

Figure 26. Total alleles, unique alleles, and genetic diversity ($H'$) among three groups of *koa*.
Table 7. Diversity statistics over all loci for groups in the *A. koa* complex.

<table>
<thead>
<tr>
<th>Group</th>
<th>$H'_T$</th>
<th>$H'_G$</th>
<th>$D'_{ST}$</th>
<th>$F'_{ST}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. koa</em></td>
<td>1.206</td>
<td>1.065</td>
<td>0.141</td>
<td>0.098</td>
</tr>
<tr>
<td><em>A. koaia</em></td>
<td>0.960</td>
<td>.848</td>
<td>0.112</td>
<td>0.082</td>
</tr>
<tr>
<td>'group three'</td>
<td>1.578</td>
<td>1.360</td>
<td>0.218</td>
<td>0.141</td>
</tr>
</tbody>
</table>

$H'_T$, total diversity; $H'_G$, average diversity within populations of each group; $D'_{ST}$, average diversity among populations of each group; $F'_{ST}$, differentiation between populations of each group.

Table 8. Genetic diversity of 11 populations in the *A. koa* complex arranged according to morphological group.

<table>
<thead>
<tr>
<th>Population</th>
<th>Group</th>
<th>$H'_T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KANA</td>
<td>'group three'</td>
<td>1.682</td>
</tr>
<tr>
<td>SG</td>
<td>'group three'</td>
<td>1.662</td>
</tr>
<tr>
<td>KHAN</td>
<td>'group three'</td>
<td>1.455</td>
</tr>
<tr>
<td>· KV</td>
<td>'group three'</td>
<td>0.778</td>
</tr>
<tr>
<td>KOAB</td>
<td><em>A. koa</em></td>
<td>1.318</td>
</tr>
<tr>
<td>KOAA</td>
<td><em>A. koa</em></td>
<td>1.180</td>
</tr>
<tr>
<td>KKA</td>
<td><em>A. koa</em></td>
<td>0.939</td>
</tr>
<tr>
<td>KMC</td>
<td><em>A. koa</em></td>
<td>0.828</td>
</tr>
<tr>
<td>KTSB</td>
<td><em>A. koaia</em></td>
<td>0.977</td>
</tr>
<tr>
<td>KTSA</td>
<td><em>A. koaia</em></td>
<td>0.954</td>
</tr>
<tr>
<td>KNT</td>
<td><em>A. koaia</em></td>
<td>0.613</td>
</tr>
</tbody>
</table>
statistics, showing that most of the genetic variation, 72.2% was found within populations rather than among populations, 27.8%.

Pairwise differentiation ($F_{ST}$) between populations is shown in Table 9. Two populations from Hawaii representing the _A. koa_ group (KOAA and KOAB) were most closely related, with a $F_{ST}$ of 0.007. KTSA and KTSB, _A. koaia_ populations from Hawaii, were also very similar, with a $F_{ST}$ of 0.010. The most dissimilar populations were all from Kauai and in the groups _A. koa_ and 'group three'. KNT (_A. koaia_) and SG ('group three') were most differentiated ($F_{ST} = 0.207$), followed by KMC (_A. koa_) and KANA ('group three'), with a $F_{ST} = 0.190$. These results were a not expected, considering that these populations were all from the same island and in close proximity to one another. As the above results would suggest there was no correlation observed between genetic distance and geographic distance between populations as revealed by the Mantel test (Figure 27).

A dendogram based on the UPGMA cluster method (Figure 28), as well as a plot from principle coordinate analysis (Figure 29) were constructed using average pairwise differentiation ($F_{ST}$), to analyze the relatedness of populations. Three populations representing 'group three' (KANA, KHAN, SG) clustered together and were distinct from other populations in both the UPGMA dendogram and principle coordinate analysis. Another distinct cluster seen in both analyses was KTSA and KTSB, both from Hawaii and in the _A. koaia_ group. One unexpected placement in the cladogram was KV, a 'group
Table 9. Matrix of pairwise differentiation between 11 populations of koa, based on microsatellite allele scoring. Populations arranged according to morphological group.

<table>
<thead>
<tr>
<th></th>
<th>SG</th>
<th>KHAN</th>
<th>KV</th>
<th>KOAB</th>
<th>KOAA</th>
<th>KKA</th>
<th>KMC</th>
<th>KTSB</th>
<th>KTSA</th>
<th>KNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>KANA</td>
<td>0.076</td>
<td>0.047</td>
<td>0.154</td>
<td>0.122</td>
<td>0.134</td>
<td>0.119</td>
<td>0.190</td>
<td>0.143</td>
<td>0.128</td>
<td>0.156</td>
</tr>
<tr>
<td>SG</td>
<td></td>
<td>0.101</td>
<td>0.168</td>
<td>0.098</td>
<td>0.130</td>
<td>0.119</td>
<td>0.149</td>
<td>0.175</td>
<td>0.173</td>
<td>0.207</td>
</tr>
<tr>
<td>KHAN</td>
<td></td>
<td></td>
<td>0.176</td>
<td>0.094</td>
<td>0.119</td>
<td>0.092</td>
<td>0.122</td>
<td>0.152</td>
<td>0.136</td>
<td>0.131</td>
</tr>
<tr>
<td>KV</td>
<td></td>
<td></td>
<td></td>
<td>0.084</td>
<td>0.122</td>
<td>0.064</td>
<td>0.029</td>
<td>0.181</td>
<td>0.163</td>
<td>0.113</td>
</tr>
<tr>
<td>KOAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
<td>0.057</td>
<td>0.076</td>
<td>0.083</td>
<td>0.077</td>
<td>0.091</td>
</tr>
<tr>
<td>KOAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.082</td>
<td>0.112</td>
<td>0.114</td>
<td>0.091</td>
<td>0.117</td>
</tr>
<tr>
<td>KKA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.086</td>
<td>0.109</td>
<td>0.092</td>
<td>0.046</td>
</tr>
<tr>
<td>KMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.175</td>
<td>0.164</td>
<td>0.110</td>
</tr>
<tr>
<td>KTSB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.010</td>
<td>0.113</td>
</tr>
<tr>
<td>KTSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.097</td>
</tr>
<tr>
<td>KNT</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 27. Comparison of genetic differentiation and geographic distance between populations. The blue diamond symbol represents a pair on populations.
Figure 28. Dendogram of 11 populations of the *A. koa* complex revealed by UPGMA cluster analysis based on pairwise differentiation.
Figure 29. Principle coordinate analysis for microsatellite analysis of koa populations. The plot explains 59.5% of the total variability, 27.4% for the first principle coordinate axis, 18.3% for the second, and 13.8% for the third.
three' population from Oahu clustered with KMC, an *A. koa* population from Kauai. These two populations also displayed a close association in the principle coordinate analysis. While both *A. koa* populations from Hawaii were clustered together, they also displayed a close association with Kauai populations KKA (*A. koa*) and KNT (*A. koaia*). All three microsatellite analyses showed the close association of KNT and KV with populations from the *A. koa* group. Although KNT and KV were originally classified in different groups based on morphological characteristics, molecular results indicate they should correctly be classified as *A. koa* (Figures 28, and 29). The plot from principle coordinate analysis explained 59.5% of the total variability, 27.4% for the first principle coordinate axis, 18.3% for the second, and 13.8% for the third (Figure 29).

Principle coordinate analysis for individual samples showed clustering among individuals from each group, although overlapping of individuals (red circle) from some populations was observed (Figure 30). When individuals were categorized according to populations rather than groups, several trends in clustering were observed. Individuals from KMC and KV showed a close association (Figure 31, red circle). Additionally, KV individuals were also clustered closer to individuals from the *A. koa* groups from Hawaii, KOAA and KOAB, than they were to any of the 'group three' populations (Figure 31, pink circle). Based on pairwise differentiation, the two closest related populations to KV were also *A. koa* groups, KMC and KKA, while 'group three' populations SG, KANA, and KHAN were among the most distantly related (Table 9). The combination of these molecular data again show that KV, although morphologically classified in 'group three', is actually in the *A. koa* group. Molecular analysis also showed a similar relationship for
Figure 30. Principle coordinate analysis for individuals, categorized into groups according to morphological characteristics.
Figure 31. Principle coordinate analysis for individuals, labeled for each population.
population KNT. Principle coordinate analysis of individuals (Figure 31, blue circle) and the differentiation dendogram (Figure 28) showed KNT should be categorized in the *A. koa* group. While a few individuals from each population were still found intermixed, three distinct clusters were observed when the KV and KNT individuals were appropriately assigned to the *A. koa* group (Figure 32). The inclusion of populations into one of three groups was consistent with the UPGMA dendogram showing average differentiation between populations (Figure 28).

**Cross-species amplification**

Cross-species marker-amplification for *A. mangium* microsatellite markers was previously reported by Butcher et al. (2000), but was tested again using *Acacia* species collected from Hawaii. All *Acacia* samples cross-amplified using *A. mangium*-specific markers Am030, Am465, Am502, and Am770, as expected. This step was done to confirm that DNA extracted from non-native acacias was of sufficient quality for testing on koa-specific markers.

Five of the six non-native acacias amplified at least one koa-specific marker, with only *A. angustissima* failing to amplify any of the eight markers. Overall success was low, with *A. auriculiformis* and *A. mangium* amplifying only one marker, and *A. confusa, A. mearnsii*, and *A. melanoxylon* amplifying just two markers each (Table 10, Figures 33, and 34). DNA from all non-native acacias successfully amplified universal primer
Figure 32. Principle coordinate analysis for individuals, labeled according to population and group. All individuals colored green are from 'group three'; pink individuals are from the *A. koa* group and blue individuals are from the *A. koaia* group. Population KV and KNT are categorized in the *A. koa* group according to molecular data, resulting in three distinct groups.
Table 10. Cross species amplification of koa-specific markers by non-native acacias from Hawaii. Successful amplification denoted by X, unsuccessful amplification denoted by -.

<table>
<thead>
<tr>
<th>Marker</th>
<th>\textit{A.angustissima}</th>
<th>\textit{A.ariculiformis}</th>
<th>\textit{A.confusa}</th>
<th>\textit{A.mangium}</th>
<th>\textit{A.mearnsii}</th>
<th>\textit{A.melanoxyylon}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ak05</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ak37</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ak44</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Ak84</td>
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<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ak180</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ak196</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ak284</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 33. Cross-species amplification results from microsatellite marker Ak37, with expected fragment size of 210 bp. Successful amplification of two A. mangium individuals (third individual not shown). Codes for each species are as follows: M=Standard Marker; A= A. koa positive control; N=negative control; CON= A. confusa; AR= A. auriculiformis; AN= A. angustissima; ML= A. melanoxylon; MR= A. mearnsii; MG= A. mangium. Numbers below species codes represent an individual of each species.

Figure 34. Cross-species amplification results from microsatellite marker Ak284, with expected fragment size of 242 bp. No cross-amplification for any non-native Acacia. Codes for each species are as follows: M=Standard Marker; A= A. koa positive control; N=negative control; CON= A. confusa; AR= A. auriculiformis; AN= A. angustissima; ML= A. melanoxylon; MR= A. mearnsii; MG= A. mangium. Numbers below species codes represent an individual of each species.
ccSSR9 (Chung and Staub, 2003), confirming that failure to cross-amplify was not the result of poor DNA quality.
CHAPTER 5
DISCUSSION

Morphological variations among populations of the *A. koa* complex provide a basis for comparison between different groups. While some characteristics such as seed alignment within the pod and tree height are useful to distinguish different groups, other variables such as phyllode size and shape, seed size, and branching patterns are more variable and not effective at differentiating populations. Another problem with identification using seed pod or seed characteristics is that seed production occurs over a relatively short three month period usually between August and December, or whenever the tree is stressed. Wood density has been shown to be significantly different between *A. koa* and *A. koaia*, but destructive sampling is required to analyze this property, which is not practical for threatened plant taxa such as *A. koaia* (Oldfield *et al.*, 1998; Wagner *et al.*, 1999).

In the case of Hawaii's diverse ecosystems where rainfall, topography, and soils are variable over short geographic distances, environmental conditions may allow for development of morphologically variable ecotypes (St. John, 1979). These factors make characterization based on phenotype difficult. The advantage of molecular analysis of DNA from individual samples is that it is at the genotypic level, not phenotypic analysis which may be influenced by environmental factors. Also, molecular analysis requires only a small amount of phyllode tissue per tree for DNA extraction, therefore it is a non-destructive sampling procedure. These characteristics of molecular methods make them valuable for studies of diversity and population genetics.
DNA sequencing

The molecular approaches used to assess the *A. koa* complex revealed general relationships regarding placement within the *Acacia* genera, as well as specific associations between populations from different islands and groups. Little variation was observed between groups using chloroplast DNA sequencing, although the *trnK / matK* region was still informative as it revealed the placement of koa groups within the *Acacia* genus.

Compared to the nuclear genome, the chloroplast genome is highly conserved and has a lower mutation rate (Johnson and Soltis, 1994). Generally the chloroplast genome has a uniparental (maternal) mode of transmission in plants, and may exhibit patterns of genetic variation different than that of the nuclear genome (Basu *et al.*, 2004). The possibility of genetic variation between the two genomes was the basis for utilizing both nuclear and chloroplast gene regions. The clustering of subgenera *Acacia* and *Aculeiferum*, and relative isolation of *Phyllodineae* in the phylogenetic tree (Figure 23), support the proposed independent evolution of *Phyllodineae* from *Acacia* (Maslin and Stirton, 1997).

Neither the *trnK / matK* region, nor the ITS region was able to clearly differentiate the three koa groups, although this comprehensive analysis using two gene regions had been shown to differentiate Australian *Acacia* species (Miller and Bayer, 2001; Miller and Bayer, 2003; Miller *et al.*, 2003; Murphey *et al.*, 2003). As the nuclear ITS region contains more variable internal spacers, it was expected to provide resolution.
below the genus level (Murphey et al., 2003). The ITS region contained more than twice as many potentially informative characters within koa groups as the \textit{trnK / matK} region; however, the characterization of distinct groups was inconclusive based on the sequencing results. Both sequencing analyses supported the close relationship between koa and \textit{A. melanoxylon} as seen in the phylogenetic trees based on parsimony analysis (Figures 23 and 24). The \textit{trnK / matK} sequencing confirmed a close association of koa to Australian acacias. However, the placement of koa within the sections of subgenus \textit{Phyllodineae} was not further revealed from the ITS region sequencing results.

**Molecular Studies**

Conkle (1996) showed high polymorphism in koa using isozymes and that allele profiles of koa from Hawaii were very different than those from Maui, Oahu, and Kauai. Koa from the latter islands were not differentiated. Other than this study, little information is known about molecular characteristics of koa populations in the Hawaiian Islands.

**Microsatellite markers / Diversity**

Due to its relatively restricted geographic range and distribution in fragmented habitats, it was expected that the \textit{A. koaia} group contained the fewest total alleles and showed the lowest level of diversity ($H'$) compared to the other two groups. Furthermore, the \textit{A. koaia} populations from Hawaii (KTSA and KTSB) were among the least differentiated ($F_{ST}$) among all population pairs. This was expected because the two
populations were very close geographically. However, no support for isolation by distance was observed for all populations, as there was no correlation between geographic and genetic distance (Figure 27).

*Acacia koa* was expected to have more alleles and higher diversity than *A. koaia*, and it was hypothesized that ‘group three’ would have values between the two. This is because ‘group three’ individuals were more common than *A. koaia*, but still not as widely distributed as *A. koa*. It is noteworthy that ‘group three’ had the most alleles, but more unexpected was the high number of unique alleles found in ‘group three’ as well. Both total diversity and differentiation between populations were highest for ‘group three’, and diversity of all groups was higher within populations than among populations. The diversity measures were confirmed by the AMOVA, which found 72.2% of genetic variation was within populations and only 27.8% was among populations of each group. This may be due to the high percentage of unique alleles present in each group.

**Microsatellite markers / Population differentiation**

The total genetic diversity of each population reflected the overall group diversity measures. The three most diverse populations were from ‘group three’ (KANA, KHAN, SG), and the three populations with the lowest diversity were all from the *A. koa* group (KMC, KNT, and KV). Genetic differentiation shown in the UPGMA dendogram and clustering in the principle coordinate analysis suggests that KV and KNT should be included in the *A. koa* group, although individuals from KV are morphologically similar to those in ‘group three’, and KNT individuals were morphologically similar to *A.
*koai*a. These results show that phenotypic variation among populations is not consistent with molecular differences. Differentiation among populations in each group was low (Table 7). It was similar to interpopulation differentiation reported for the *A. acuminata* complex in Australia (Broadhurst and Coates, 2002).

The range of 'group three' is restricted to populations on Kauai (KANA, KHAN, SG), but the diversity found in these populations was higher than *A. koa* populations found on different islands. Studies on Australian acacias have shown that genetic diversity of restricted or rare species is often close to that of widespread species, but generally is lower (Gitzendanner and Soltis, 2000; Broadhurst and Coates, 2002; Elliot et al., 2002). The genetic diversity of *A. koai*a populations is similar to that of populations in the widespread *A. koa* group. However, all populations of 'group three' have higher diversity than any of the *A. koa* populations.

Clustering in the UPGMA dendogram shows some relatedness between populations from the same island. However, the close association of *A. koa* populations from Kauai, Oahu, and Hawaii reveal clustering of populations according to groups rather than island of origin (Figure 28). The highest differentiation among all populations was between KNT (*A. koa*) and SG ('group three'), which are found in close proximity to one another on Kauai. This observation also supports clustering by groups rather than by island (Table 9).
Principle coordinate analysis based on variation in allele patterns between koa individuals showed that most populations clustered according to group with very few individuals overlapping (Figure 32). The separation of populations into three groups was similar to relationships shown in the UPGMA dendogram. Aside from the Kauai populations KMC and KNT (*A. koa*) which had a few individual outliers, individuals from each population clustered into one of three groups (Figures 32).

Genetic differentiation among populations of ‘group three’ (KHAN, KANA, and SG) and those of *A. koa* or *A. koaia*, was greater than differentiation among populations of *A. koa* and *A. koaia* (Table 9), which are recognized as distinct groups. These results were also confirmed by both the UPGMA dendogram and the principle coordinate analysis, suggesting that ‘group three’ Kauai populations form a group that is independent of *A. koa* and *A. koaia*.

The results of microsatellite marker analyses show that ‘group three’ is distinct from *A. koa* and *A. koaia*. This characterization is based on the large amount of unique alleles observed, as well as clear differentiation from *A. koa* and *A. koaia* in both the UPGMA dendogram and principle coordinate analysis. The results also show that the Oahu population (KV) and Kauai population (KNT) are classified in the *A. koa* group. ‘Group three’ is restricted to just three populations, but diversity levels were higher than those found in any population of *A. koa*. 
Cross-species amplification

The conservation of microsatellite markers across non-native acacias was relatively low, having at most 25% amplification success. This was similar to results from cross-species amplification of other acacias in the subgenus *Phyllodineae*, where one-third of markers were conserved (Butcher *et al.*, 2000). It was expected that more *A. koa*-specific markers would be amplified by *A. melanoxylon* than the other non-native *Acacia* because DNA sequencing results showed *A. melanoxylon* was most closely related to koa compared to non-native acacias in Hawaii. Since the efficiency of cross-amplification decreases with increasing genetic distance between species, limited cross-amplification was expected between koa-specific markers and the other non-native acacias. (Dayanandan *et al.*, 1997; Butcher *et al.*, 2000; Selkoe and Toonen, 2006). Therefore, it was unexpected that *A. confusa* and *A. mearnsii* amplified the same number of markers as *A. melanoxylon*. It is difficult to relate the success of cross-species amplification to genetic distance between these *Acacia* species with only eight markers tested. The eight koa-specific microsatellite markers would be of limited use for molecular studies of non-native acacias found in Hawaii, based on these results.

Koa groups

The classification of the *A. koa* complex often includes descriptions of koa based on island origin and have been argued to be distinct species based on variations in morphological characteristics. *Acacia koa* and *A. koaia* are accepted as distinct groups, but *A. kauaiensis* is often grouped together with either *A. koa* or *A. koaia* (St. John, 1979,
Wagner et al., 1999, GBIF Data Portal, 2007; ITIS, 2007). Phenotypic variation in the ‘group three’ populations included individuals with the tree structure of A. koa and seed pods of A. koaia, and also individuals with the tree structure of A. koaia and seed pods of A. koa. These are similar to characteristics of taxa described by Wagner et al. (1999) as A. kauaiensis Hillebrand, and it is proposed that these populations be classified as such. The phenotypic similarity of ‘group three’ populations to those described by Wagner et al. (1999), accompanied with molecular analysis from this study supports the inclusion of ‘group three’ (similar to A. kauaiensis) with A. koa and A. koaia, as distinct groups of koa. Whether this distinction is at the species, subspecies, or variety level remains unclear, due to inconclusive sequencing results. These results suggest that the genetic differentiation between ‘group three’ (similar to A. kauaiensis) and either A. koa or A. koaia, is equal to the differentiation between A. koa and A. koaia.

Conclusions / Applications

Several conclusions can be made based on the results of molecular analysis of the A. koa complex. The three koa groups are closely related to Acacia species found in Australia, rather than those native to Asia and the Americas. Within the subgenus Phyllodineae, koa is most similar to A. melanoxylon based on the high similarity of nuclear and chloroplast DNA sequences. Among the koa groups, populations comprising the A. koaia group have the lowest diversity and ‘group three’ has the highest diversity and most unique alleles based on microsatellite marker analysis. It can be concluded that three distinct groups exist in the koa complex, A. koa, A. koaia, and ‘group three’ (similar to A. kauaiensis).
The primary objectives of this study were to determine the placement of koa in the broader genus of *Acacia*, to reveal associations between groups of koa, and to analyze diversity in populations from common and rare species. Many koa populations have been described by island for morphological variations, but limited genetic diversity studies using molecular approaches have been done. DNA sequencing of koa populations using gene and spacer regions revealed a close association with *A. melanoxylon*. It did not provide conclusive evidence for differentiation of the three koa groups. Microsatellite marker analysis, on the other hand, showed evidence of three clearly distinct groups of koa, described here as *A. koa*, *A. koaia*, and 'group three'. Diversity and differentiation between uncharacterized populations are understood more clearly by utilizing microsatellite markers, as shown in this study. Among molecular markers, the variability of microsatellite markers allowed greater differentiation of groups of koa than studies utilizing isozymes (Conkle, 1996).

The degree of differentiation and diversity in populations provides insight for conservation and disease resistance questions by providing a genetic framework on which to base future studies. By determining the level and partitioning of diversity in koa, a greater understanding of conservation needs can be developed. This study provides a molecular determination of koa population diversity that can be utilized for conservation, and has application for selecting germplasm to be used in disease resistance and breeding studies.
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