GEOGRAPHIC AND GENETIC DIVERSIFICATION OF *LIPOCHAETA* SECTION *APHANOPAPPUS* (ASTERACEAE) IN THE HAWAIIAN ISLANDS

by

STACY MARIE JORGENSEN

(Under the Direction of Kathleen C. Parker)

ABSTRACT

The Hawaiian Islands are well known for the spectacular examples of adaptive radiation among the native flora and fauna. The conveyor-belt like movement of the Pacific tectonic plate has created a series of chronologically arranged islands, and it is the constant creation of new, isolated habitat due to active volcanism that is thought to promote speciation in the islands. In this study, I examine the diversification of the native Hawaiian plant taxa *Lipochaeta* section *Aphanopappus* (Asteraceae: Heliantheae), a small group of mostly single-island endemics, many known from a single locality; one species, *L. integrifolia*, is known from coastal habitats on all eight of the main islands. To examine patterns of speciation in the group, I used amplified fragment length polymorphism (AFLP) markers, which are ideal for analyzing a large number of loci sampled from throughout the genome.

Ninety-five percent of the AFLP loci identified exhibited variation within or among species; surprisingly, few fixed differences among species were identified. Instead, diversification within *Lipochaeta* was largely attributed to variation in the frequency of fragments among species. Genetic variability, measured by the percentage

of polymorphic loci and heterozygosity, varied little among species. Notable exceptions were the O‘ahu endemics *L. remyi* and *L. tenuis*, both of which had substantially less variability than other species; unlike many species in the group, however, neither are protected with threatened or endangered status. While the relationships among species occurring on the oldest islands were obscured due to contemporary hybridization, a close relationship was found among *L. integrifolia*, *L. remyi*, and *L. venosa* of Hawai‘i, with the latter two species most closely related. In fact, *L. remyi* appears to have been derived from *L. venosa*, opposite of the general trend of species on older islands being parental to species on younger islands. Diversification among populations of *L. integrifolia* was qualitatively similar to patterns of diversification among the species of *L. integrifolia*, *L. remyi*, and *L. venosa*, although quantitative differences were seen in species vs. population differentiation. These data support gradual models of speciation and are generally inconsistent with the predications of founder-flush models of speciation.

INDEX WORDS: Adaptive radiation, AFLP, Conservation Genetics, Evolutionary Biogeography, Hawaiian Islands, *Lipochaeta*, Speciation

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B.S., The University of Montana, 1992

M.S., The University of Georgia, 1996

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

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December 2002

ACKNOWLEDGMENTS

I could not have successfully completed this project without the help of many people, including those in Georgia and Hawai‘i. I am especially grateful for the encouragement and support of Kathy Parker, who was generous in allowing me to attempt this project, understanding when it wasn't going well, and provided invaluable comments during the writing of this dissertation. I thank my committee members Mike Arnold, Steve Holloway, Al Parker, Bob Price, and Vern Meentemeyer for supporting this project and slogging through the dissertation. Rodney Mauricio deserves special recognition for generously allowing me to work in his laboratory for many, many months without once asking what I was still doing there and even let me borrow his laptop computer; I simply could not have conducted this project without his help. My laboratory work also benefited greatly from discussions with Gina Baucom, Amy Bouck, Brian Dunphy and others who have navigated the netherworld of PCR and AFLP markers. Randi Rotjan provided indispensable help with DNA extractions. In Hawai‘i, David Lorence and Steve Perlman of the National Tropical Botanical Garden generously shared their time to discuss where I might find populations of *Lipochaeta*, and Steve helped me to sample a population I would have never found on my own. Kapua Kawelo and Jobraith Rohrer of the O‘ahu Army Environmental Program collected samples for me and also brought me out in the field to find a population. My research was supported by a National Science Foundation dissertation improvement grant (BCS 9901022), as well as a small grant from the Biogeography Specialty Group of the Association for American

Geographers. Lastly, but certainly not least, I would like to recognized the unfailing support and love of my parents, Phyllis and John. *Mahalo*.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Both modern biogeography and evolutionary biology can trace their beginnings to the study of island biotas, particularly those of the Pacific Basin (Carson, 1996). Circumscribed by volcanic activity at the edges of tectonic plates, the region covers virtually half the globe. Located within the area are numerous isolated island groups, which attracted the attention of a small group of naturalist-explorers in the mid-19th century. These naturalists, Charles Darwin and Alfred Russel Wallace notable among them, sought to document the flora and fauna they encountered; of particular interest was the curious distribution of the terrestrial biota. While there were numerous distinctive, yet obviously closely related species within an island group, individual species were often confined to a single island; furthermore, many of these species had obvious affinities with continental biota (Briggs, 1984). At that time, species were considered immutable forms of creation, so the naturalists were simply catalogers of the biota they encountered; therefore, recording the pattern of the distribution of species was the only objective. Darwin (1859) and Wallace (1880) independently came to the conclusion that creation was not a plausible explanation for these patterns, and they hypothesized that these numerous singular (i.e., confined to one or a few islands), but obviously related species, were descended from a common ancestor. Thus, descent with modification was the process to account for these patterns of species distribution.

Nearly a century and a half after Darwin's *Origin of Species* (Darwin 1859), the study of island biotas remains relevant. Islands are of immediate interest because of the disproportionate impact of human influence on island species. Because of the restricted land area and isolation of oceanic archipelagos, island species are much more susceptible to extinction than continental species (Rieseberg and Swenson, 1994). For example, more than one-third of all threatened and endangered plant species in the United States are found in Hawai'i (Center for Plant Conservation, 1988; Royte, 1995), a state comprising less than 0.2% of the country's total land area. Reasons for the destruction of the native Hawaiian flora include clearing land for agriculture, the introduction of alien plants and animals, and the loss of pollinators (Morden *et al*., 1996). Aside from their intrinsic values, the loss of island biotas is also disturbing because of the major role they have played and continue to play in the understanding of evolutionary processes (Carson, 1987; Rieseberg and Swenson, 1994), particularly with regard to the process of speciation.

The traditional view of speciation is one of a gradual process, whereby two populations gradually diverge from one another over time (Fisher, 1930). Presumably, natural selection is the major force behind the acquisition of differences, the implication being that some element of the habitats of the diverging populations is different. Implicit in this view is the notion that the process of evolution within species can gradually lead to the formation of new species (Provine, 1989). This remains very similar to Darwin's (1859) original concept of the speciation process; the divergence of two populations into species requires no special mechanisms.

In contrast, Ernst Mayr did not view speciation as simply an extension of the evolutionary processes within species. In his view, geographic isolation is a necessary component of species formation (Mayr, 1942); geographic isolation is necessary to prevent gene flow between populations. Why the cessation of gene flow is so important largely hinges on Mayr's view of the genome. Mayr strongly believed in the 'cohesiveness' of the genome (Mayr, 1963). An organism's genome is not simply a collection of various genes on different chromosomes, but a highly integrated system in which the effects of a particular gene are not only dependent on the allele (i.e., form of a gene) at that locus but also on the alleles at other loci. Because he believed the entire genome to be involved in gene-gene interactions, Mayr saw the genome as a single 'coadapted gene complex' (Mayr, 1963). As a consequence of the integration within the system, most gene mutations will be deleterious and selected against. Thus, gene complexes serve not only to reinforce the generally slow process of natural selection, but they can actually be an impediment to divergence. Only with the cessation of gene exchange is divergence possible.

In Mayr's (1942) classic model of geographic, or allopatric, speciation, a population is split by a geographic barrier, and differences in the isolated populations gradually accumulate due to natural selection. The allopatric model of speciation is different from Darwin's because the cessation of gene flow is necessary for species formation. While the allopatric model is clearly applicable to continental species formation, it may be inadequate to explain speciation on islands. Mayr largely came to this conclusion after observing the distribution of bird species in the islands of the southwest Pacific. He was struck by the fact that while there was a single species of

kingfisher (*Tanysiptera*) on the island of New Guinea (occurring over wide variety of habitats), many of the smaller islands around New Guinea had their own endemic species, despite very similar habitats (Mayr, 1954, 1982). To explain the diversification by natural selection did not seem plausible by the similarity of habitat; some other mechanism was likely at play.

These observations led Mayr (1954) to propose a new model of speciation, later termed 'peripatric' speciation (Mayr, 1982), which involved drastic speciation in peripheral founder populations. He reasoned that if a small number of individuals founded the populations on the isolated islands, a breakup of the gene complexes of the parental population could occur. Because of a relaxed selection regime in the new habitat (because of fewer individuals and, presumably, less competition), new combinations of alleles may appear; over time, this could lead to a new combination of gene complexes in the founding population. Mayr termed this process a 'genetic revolution' (Mayr 1954). Since Mayr's original model of peripatric speciation via founder effects, other researchers have proposed similar models (e.g., Carson, 1971; Templeton, 1980). Despite minor differences, all have the same essential components: the assumption of the genome as an integrated system; the disruption of the system with founding (disorganization); population growth in the new habitat with gene combinations released from selection; and, with population growth, selection for a new set of coadapted gene complexes within the population (reorganization).

Speciation via the founder effect is not without controversy. Support for the theory largely comes from observations of biogeographic patterns of species' distributions; as such, the support is tautological. As noted by Barton and Charlesworth

(1984), present patterns cannot be taken as evidence for the *process* of speciation responsible. They further concluded that founder events do not need to be invoked over phyletic gradualism as a mechanism of speciation in many cases of diversification that had been attributed to founder events. Experimental support for the theory has come from studies focusing on the mating behavior of Hawaiian *Drosophila* (Templeton, 1979, 1989). The assumption is that mating behavior is a complex, multi-genic trait determined by many loci (a complex, if you will). Although the assumption is generally reasonable, others have demonstrated that mating behavior in insects can be controlled by a small number of loci (reviewed in Tauber and Tauber, 1989).

Molecular genetic evidence in support of a founder event model also has not been forthcoming. DNA sequence data overwhelmingly indicates little divergence among those Hawaiian species that have been studied. However, this is really no surprise. Sequence divergence indicates time of divergence, not overall changes in the genome, and data from a single locus cannot be extrapolated to genome-wide changes in polygenic balances. Hodges and Arnold (1994) found very little divergence among species of *Aquilegia* occurring over a very large (i.e., global) area despite strong morphological differences among species. Allozyme data, the workhorse of population geneticists, also has failed to support predictions of founder effect speciation models. A typical allozyme survey may examine only a small number of loci (e.g., 10-20), however, which makes the detection of complexes difficult. Lastly, traditional statistics of genetic diversity are used to describe population level patterns, not specific relationships among loci within individuals. For example, if a minority of loci are involved in gene complexes, overall levels of genetic divergence among populations would not be expected to be large.

My objectives for this project were to reexamine speciation via the founder effect with the newest molecular genetic techniques. Recently developed methods have made it possible to survey many loci across the genome quite easily, increasing the power of genetic assays by an order of magnitude over previously common techniques. In particular, amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) markers avoid the major problems of allozyme (not enough loci) and sequence (not representative of the entire genome) data. A typical AFLP analysis can generate greater than one hundred polymorphic loci sampled from throughout the genome, making the technique ideal for studying the relationships within a native Hawaiian plant species complex believed to have radiated *in situ*.

Lipochaeta DC (Asteraceae: Heliantheae) is an endemic Hawaiian genus of 20 species, which are placed in two morphologically, biochemically and genetically distinctive sections (Wagner and Robinson, 2001). Section *Lipochaeta* is characterized by four-petaled disk flowers, the presence of flavonols and flavones and a chromosome number $n = 26$, while members of section *Aphanopappus* have five-petaled disk flowers, possess only flavonols and $n = 15$ chromosomes. *Lipochaeta* is believed be derived from *Wollastonia biflora* (L.) DC[1] ($n = 15$), a widely distributed coastal species of the Pacific and Indian Oceans (but absent from Hawai‘i)(Wagner and Robinson, 2001); the relationship between the two sections of *Lipochaeta*, however, remains unclear. Gardner (1979) suggested that section *Lipochaeta* may have arisen by hybridization between two $n = 15$ individuals of *Lipochaeta* followed by aneuploid reduction in chromosome

[1] For many years *Wollastonia biflora* was incorrectly placed in the genus *Wedelia*, and in many older articles, the species is identified as *Wedelia biflora*.

number. Other studies suggest that two sections arose independently, with section *Lipochaeta* derived from a hybridization event between *Wollastonia biflora* and a yet to be identified $n = 11$ species, possibly of the genus *Wedelia* Jacq. (Rabakonandrianina, 1980; Rabakonandrianina and Carr, 1981). Because of the confusion surrounding the relationship between the two groups within *Lipochaeta*, members of section *Aphanopappus* were transferred to the genus *Wollastonia* in the 1999 (revised) edition of the *Manual of the flowering plants of Hawai'i* (Wagner *et al*., 1999a). Subsequent to an anlaysis of the subtribe Ecliptinae, in which *Lipochaeta*, *Wollastonia*, and the widely distributed genus *Melanthera* Rohr (also $2n = 30$) were determined to be closely related (Panero *et al*., 1999), Wagner and Robinson (2001) moved all the members of *L.* section *Aphanopappus* and the monotypic *W. biflora* to the genus *Melanthera*. An even more recent anlaysis suggested that the two sections may form a monophyletic group (Chumley *et al*., 2000), making one wonder if another transfer of the members of section *Aphanopappus* may occur. Because the analysis of Wagner and Robinson (2001) was conducted solely on the basis of morphological variations, without the incorporation of molecular data, I retain the traditional placement of section *Aphanopappus* within *Lipochaeta*; however, I do follow the specific designations of Wagner and Robinson (2001), which is slightly different from the older treatment in Wagner *et al*. (1990).

The distributional patterns of species in the two sections vary dramatically; while all six members of *L.* sect. *Lipochaeta* are known from multiple islands, 12 of 14 species of *L.* sect. *Aphanopappus* are single-island endemics, often being confined to a single locality. Because of a desire to examine speciation within a single, diploid lineage with a pattern of single-island endemism, only members of section *Aphanopappus* were

examined for this project. Objectives were to: (1) determine evolutionary relationships among the species within *L.* section *Aphanopappus*, (2) quantify genetic diversity within and among species, and (3) examine patterns of genetic diversity for changes in gene complexes associated with speciation events.

CHAPTER 2

EVOLUTIONARY BIOGEOGRAPHY OF THE HAWAIIAN ENDEMIC *LIPOCHAETA* SECTION *APHANOPAPPUS* (ASTERACEAE: HELIANTHAEA) USING AFLP MARKERS[2]

The study of Pacific island biotas has been central to the development of modern evolutionary biology and biogeography (Carson, 1996), and research on islands remains an important focus. The hallmark of remote chains is the dramatic adaptive radiations in the native flora and fauna. Radiations represent a "microcosm of the evolutionary process" (Emerson, 2002 p. 951), and are natural choices to study the process of speciation (Carson, 1987). Islands are also of interest because of the disproportionate impact of human influence on island species, which are more susceptible to extinction than continental counterparts (Rieseberg and Swenson, 1994).

No chain embodies the characteristics of remote islands more than Hawai'i, the most isolated chain in the world (Clague and Dalrymple, 1987). The Hawaiian chain has formed as the Pacific tectonic plate has moved over a fixed hot spot, resulting in a chronological arrangement to the islands (Carson and Clague, 1995). The eight major islands constitute the end of a chain stretching hundreds of kilometers and range in age from 5.1 million years (my) for Kaua'i to less than 0.5 my for Hawai'i, which is still volcanically active. Volcanism in the chain is constantly creating the new, empty, and,

[2] Jorgensen, S., K. C. Parker, and R. Mauricio. To be submitted to *Evolution*.

often, geographically isolated habitats that are thought to promote speciation (Mayr, 1954, 1982).

Mayr's peripatric model of speciation, created specifically to describe the patterns of species diversity found on remote islands, regards an organism's genome as a cohesive unit or 'coadapted gene complex' that resists change (Mayr, 1963). The dispersal of a small number of founders to isolated, empty habitats can allow new complexes to evolve. Speciation, therefore, is a process fundamentally different from the divergence of populations within a species. In fact, many groups of species in Hawai'i have a pattern of dispersal and divergence from older to newer islands (Funk and Wagner, 1995), which is consistent with peripatric models. Genetic evidence in support of peripatric models, however, has not been forthcoming. Both allozyme and DNA sequence data indicate little divergence among related Hawaiian taxa (*Bidens*, Helenurm and Guries 1985; *Dubautia*, Witter and Carr, 1988; *Clermontia*, Lammers, 1995; *Tetramolopium*, Lowery and Crawford, 1985). Neither type of data is ideal for studying peripatric speciation, however; sequence data do not indicate overall changes in the genome, and allozyme studies typically involve too few loci (e.g., <20) to detect gene complexes. New molecular techniques provide methods that allow the analysis of many loci sampled from throughout the genome. For example, amplified fragment length polymorphism (AFLP) markers (Vos *et al*., 1995) solve many of the problems of sequence and allozyme data; in a typical AFLP analysis well over 100 loci, distributed throughout the genome, can be examined relatively easily. Thus, they are ideal markers for studying the diversification among taxa in an adaptive radiation.

The endemic genus *Lipochaeta* (Asteraceae: Helianthaea) is an ideal group in which to explore patterns of speciation. Two sections, defined by morphological and genetic characteristics, are recognized, *Lipochaeta* (*n* = 26, four-petaled disk florets) and *Aphanopappus* (*n* = 15, five-petaled disk florets). Because of a desire to explore diversification in a single, diploid lineage, only members of *L.* section *Aphanopappus* were investigated in this study. Containing approximately two-thirds of the species within the genus, *L.* section *Aphanopappus* consists largely of single-island endemics, many of which are limited to a localized area (Fig. 2.1). The coastal species *L. integrifolia*, which is known from all of the major and several of the leeward islands, is a notable exception. The oldest of the larger main islands, Kaua‘i and O‘ahu, have the most endemic species, and for this reason one of them is believed to be the first island colonized by ancestors of *Lipochaeta*. As they have spread across islands and habitats, members of *L.* section *Aphanopappus* have diverged in growth habit and vegetative and floral morphology (Table 2.1). For example, habits range from upright to prostrate to scandent, and leaf shapes vary from entire to so dissected that the leaves appear compound. Three species, *L. bryanni*, *L. perdita*, and *L. populifolia*, are presumed extinct (Wagner *et al*., 1999b), and another six are federally protected as threatened or endangered, *L. fauriei*, *L. kamolensis*, *L. micrantha*, *L. tenuifolia*, *L. venosa*, and *L. waimeaensis*.

I examined patterns of genetic diversity and divergence in *L.* section *Aphanopappus* using AFLP markers. Goals of this study were to: (1) determine relationships among species in the group; (2) examine biogeographical patterns of

speciation; (3) assess whether divergence is correlated with fixed genetic differences; and, (4) determine levels of genetic variability at the species and section level.

Materials and Methods

Leaf tissue samples were collected from individuals in naturally occurring populations of *Lipochaeta*, except for *L. waimeaensis*, which was collected from specimens growing at the National Tropical Botanical Garden, Kaua‘i. Immediately after harvesting, leaves were placed into sealed plastic sample bags with desiccating silica gel for storage until DNA extraction. Total genomic DNA was extracted via standard phenol/chloroform procedure. Briefly, about two leaves per individual were crushed by vortexing in a 15 mL polyethylene tube with 2.4 and 4 mm ball bearings (Colosi and Schaal, 1993); because the leaves were already brittle due to desiccation, no liquid nitrogen was used in the initial crush. The crushed tissue was agitated for 60 minutes at 37° C in 600 μL of urea lysis buffer. Following phenol extraction, DNA was ethanol precipitated and resuspended in deionized water. DNA concentrations were estimated via electrophoresis by comparing sample concentrations to samples of λ phage of known concentrations in an ethidium bromide stained agarose gel. Samples were diluted with additional deionized water, if necessary, to concentrations of approximately 50 ng/μL for AFLP analysis.

AFLP analysis was performed with commercially available kits from Applied Biosystems (ABI) for fluorescent marker detection. A restriction-ligation reaction using the enzymes *Eco*RI and *Mse*I and kit-supplied ligators was conducted according to ABI protocols with few modifications, as were the subsequent rounds of preselective and selective amplification. In the preselective amplification the *Eco*RI and *Mse*I primers had

a single, additional nuceotide added at the 3' ends; the preselective amplification was diluted to serve as the template for the selective amplifications. During selective amplification, two more nucleotides were added; in the selective reactions the *Eco*RI primers were fluorescently labeled. In total, six *Eco*RI–*Mse*I primer pair combinations were used: (listed according to the additional nucleotides from the preselective and selective amplifications): ACA–CAT; ACA–CTT; ACG–CTG; ACT–CTG; ACC–CAT; AGG–CTT. All PCR reactions were conducted with MJ Research's DNA Engine using a total volume of 10 μL, and recommended kit dilutions were followed. AFLP fragments were separated by electrophoresis on an ABI Prism 377 DNA Sequencer using 6% polyacrylamide gels. To facilitate fragment size determination, a ROX-500 fluorescently labeled size standard was loaded with each sample during electrophoresis. Gels were read by laser, and fragment detection and sizing were conducted with Genescan 3.1; the program automatically determines fragment size by interpolation to the ROX-500 standard present in each lane. Only fragments between 70–450 bp were analyzed in this study. Fragments were scored as either present (1) or absent (0) in each individual analyzed, and for all analyses, each fragment was considered a single, unique gene locus with dominant expression. While this assumption is likely violated, the resulting bias is small (Parsons and Shaw, 2001). The calculation of standard measures of genetic variability within and among species required the additional assumption of Hardy-Weinberg proportions within species (Travis *et al*. 1996). Although *Lipochaeta* possesses the sunflower-like inflorescenses of Helianthaea, which are normally associated with outcrossing species, individuals of *Lipochaeta* are self-compatible (Rabakonandrianina, 1980); also, populations are generally small, which could lead to biparental inbreeding. It

is likely, then, that some species may exhibit non-Hardy-Weinberg proportions; however, the degree to which values of inbreeding may be nonzero is unknown.

Genetic variability in *Lipochaeta* section *Aphanopappus* was assessed at the species and section levels by the percentage of polymorphic loci (*P*) and heterozygosity (*H*). A locus was considered polymorphic if the associated fragment was present in at least one, but not all individuals. Heterozygosity was calculated at each locus from the equation $H = 1- [(1-q)^2 + q^2]$ where q^2 is the frequency of individuals without the fragment; total heterozygosity was calculated as the average heterozygosity across loci. Genetic structure among species was determined by Nei's (1973) measures of genetic diversity, which include total genetic diversity (i.e. heterozygosity) at a polymorphic locus (H_T), mean genetic diversity within species (H_S), and the proportion of genetic diversity occurring among species, $G_{ST} = (H_T - H_S)/H_T$. A procedure analogous to the calculation of *H* was used to estimate H_T and H_S, except that only polymorphic loci were used in the calculations. In addition to the section level, G_{ST} values were calculated between pairs of species. At each locus, significant heterogeneity at the section level and between species pairs was determined with a χ^2 test. All genetic diversity parameters and statistical determinations were conducted using the program POPGENE (Yeh *et al*., 1999). Because of small sample sizes, *L. fauriei*, *L. kamolensis*, and *L. waimeaensis* were excluded from the genetic diversity and structure analyses.

Phylogenetic relationships among the various species of *Lipochaeta* section *Aphanopappus* were estimated from a neighbor joining analysis. Dice's (1945) coefficient of similarity was calculated between each pair of individuals: $S_{ij} = 2a/(2a + b + c)$, where *a* is the number of fragments shared between individuals, *b* is the number of

fragments present in individual *i* only, and *c* is the number of fragments present in individual *j* only. The Dice coefficient was chosen because shared absences, which are more likely to be nonhomologous (Wolfe and Liston, 1998), are excluded from the calculation. All similarities were calculated with the program SPSS (SPSS, 1997). A phenogram of the similarity values was created using the neighbor joining method (Saitou and Nei, 1987) with midpoint rooting; tree generation was conducted with PAUP 4.0 (Swofford, 1998). With the exception of the two individuals of *L. fauriei*, for which only four primer-pair reactions were resolved, only individuals for which all six primer-pair reactions were successfully resolved were included in the neighbor joining analysis. Although the data are not presented here, other standard similarity measures, including measures that do include shared absences, and clustering techniques produced nearly identical patterns.

Results

The number of AFLP fragments detected per primer-pair reaction ranged from 13 to 39 (Table 2.2). The actual number of fragments identified within each species varied from a low of 49 for *L. fauriei*, for which only four primer-pair reactions totaling 107 fragments across all samples were resolved, to 131 fragments in *L. integrifolia*, which also had the largest sample size. Of the 146 fragments detected among all species, 95% (*n* = 139) displayed variation within or among species. Ten of the polymorphic bands were restricted to individuals of a single species (i.e., were 'private alleles'); these fragments generally occurred at low frequencies, and none was fixed within species. The multi-island species of *L. integrifolia* and *L. lavarum* had four and three unique fragments,

respectively; two unique fragments were identified in the Hawai‘i island endemic *L. venosa*, while *L. tenuifolia* of O‘ahu possessed a single, unique fragment. The remaining 129 fragments were found in more than one species at varying degrees of fixation. Overall, there were very few fixed differences between species, and no species-specific diagnostic markers (i.e., fixed present among all individuals of a single species) were identified. Thus, most of the variation among species of *Lipochaeta* sect. *Aphanopappus* can be attributed to be variations in the frequency of bands rather than fixed differences.

The percentage of polymorphic loci within species varied from a low of 27% for *L. remyi*, to a high of 61% for *L. tenuifolia* (Table 2.3). Genetic diversity (*H*) ranged from 0.087 in *L. remyi* to 0.219 in *L. tenuifolia*, and was 0.302 at the section level. Because of generally small population sizes and difficulties with DNA extraction and amplification, sample sizes varied among species. This makes direct comparisons problematic, as variation, expressed either as the percentage of polymorphic loci or heterozygosity, would be expected to increase commensurately with sample size. In general, however, there did not appear to be a strong relationship between study sample sizes and levels of genetic variability (Fig. 2.2), although *L. tenuis*, represented by only three individuals, did have low levels of genetic variability. Despite having one of the largest sample sizes, the annual species *L. remyi* possessed the least polymorphic loci and lowest levels of heterozygosity among all species included in this study. The endangered species *L. tenuifolia*, in contrast, had the highest percentage of polymorphic loci and heterozygosity.

Approximately 45% of the genetic diversity in *Lipochaeta* section *Aphanopappus* is distributed among species (Table 2.4). Pairwise estimates of genetic structure varied from a low of $G_{ST} = 0.159$ between *L. lavarum* and *L. tenuifolia* to 0.543 between the

O‘ahu endemics *L. remyi* and *L. tenuis* (Table 2.5). Among all species, significant heterogeneity was detected at 92 loci. Among species pairs, the number of loci exhibiting significant heterogeneity varied from 15 between *L. tenuifolia* and *L.lavarum* to 61 between *L. remyi* and *L. tenuifolia*, both of which are endemic to the Wai‘anae range on O‘ahu; the latter comparision suggests that at least two separate colonization events are responsible for generating the three O‘ahu endemics.

Average similarities calculated within species ranged from a low of 0.619 in *L. tenuis* to 0.890 in *L. lavarum*. In general, the multi-island species of *L. integrifolia* and *L. lavarum* averaged greater similarites than the single island endemic species; an exception was *L. remyi*, which had the lowest levels of genetic diversity of any species. *Lipochaeta venosa* was the only species for which the average similarity calculated within the species (0.771) was less than that calculated between individuals of *L. venosa* and another species, *L. remyi* (0.778). This is probably a function of the reduced genetic diversity of *L. remyi*, which is likely derived from *L. venosa*.

Several groups were evident in the neighbor joining analysis (Fig. 2.3). In one clade were *L. integrifolia*, the single individual of *L. waimeaensis*, *L. remyi*, and *L. venosa*, with the latter two species clustering together. *Lipochaeta kamolensis*, endemic to east Maui, appeared most closely related to *L. lavarum*, a species whose distribution ranges from the islands of Maui Nui to Hawai‘i. Single island endemics occurring on two of the oldest main islands, *L. fauriei* from Kaua‘i, and *L. tenuifolia* and *L. tenuis* from O‘ahu, formed the final clade. Individuals of these three species did not cluster by species; instead, some individuals of *L. tenuifolia* grouped with *L. fauriei* and some grouped with *L. tenuis*.

Discussion

While not popularly recognized as one of the classic examples of diversification within the Hawaiian Islands, species of *Lipocheata* section *Aphanopappus* are characterized by prominent differences in floral and vegetative morphology, and a high degree of AFLP polymorphism was observed in the group. Virtually all of the loci analyzed exhibited variation within and among species (95%), although the actual percentage of polymorphic loci varied substantially among species (27 - 61%). Estimates of the percentage of polymorphic loci for several species in the silversword alliance (*Argyroxiphium-Dubautia-Wilkesia*, Asteraceae) analyzed with RAPD markers, which, like AFLP markers, are dominant, ranged from 12.2% for *Argyroxiphium sandwicensis* ssp. *sandwicensis* (Friar *et al*., 1996) to 70% in *Dubautia ciliolata* (Caraway *et al*., 2001). Somewhat surprisingly, no invariant species-specific markers were found. Of the small number of fragments identified from a single species, none was fixed within the species in which they occurred and all but one were observed at low frequencies. Instead, virtually all of the diversification in *L*. section *Aphanopappus* can be attributed to variation in the frequency of fragments among species. The high levels of genetic diversity and lack of diversification among species of *Lipochaeta* echo those found with allozyme markers in other Hawaiian Asteraceae, such as *Bidens* (Helenurm and Ganders, 1985), *Tetramolopium* (Lowrey and Crawford, 1985), and the silversword alliance (Witter and Carr, 1988). Lowrey and Crawford (1985) failed to detect any unique, section-delimiting alleles among the three sections of *Tetramolopium*; they concluded that the allozyme data suggested a recent, rapid differentiation in the group and suspected that few genes were likely responsible for speciation (defined by morphological

divergence) within the group. Despite exhibiting high levels of genetic diversity overall, very low levels of divergence were seen among species of Hawaiian *Bidens*, a close relative of *Lipochaeta*, and levels of allozyme divergence did not generally correspond with morphological variation (Helenurm and Ganders, 1985). Genetic identities observed in the Hawaiian silversword alliance members analyzed by Witter and Carr (1988) did correspond to the biogeographic distribution and morphological distinctiveness of species within the group. In stark contrast to diversification in *Lipochaeta* and other Hawaiian Asteraceae, much of the AFLP variation detected in several species of crickets in the genus *Laupala* (Gryllidae: Trigonidiinae) sampled from the island of Hawai‘i was attributed to fixed differences among species (Parsons and Shaw 2001). While these data would seem to suggest that the process of diversification among plant and animals is fundamentally different, many more analyses of Hawaiian taxa with AFLP markers are needed before conclusions can be drawn.

The relationships detected among species using AFLP data deviated somewhat from those relationships hypothesized by Gardner and LaDuke (1978), although this is not surprising given that continuous morphological traits were used in their phylogenetic analysis. Although *L. remyi* and *L. venosa* were identified to share a similar floral morphology (Gardner, 1979), the close relationship between these two species and the widely distributed *L. integrifolia* was largely unexpected. Results of the neighbor joining analysis, coupled with the patterns of overall genetic variability seen within the species, indicate that *L. venosa* of Hawai‘i is parental to *L. remyi* of O‘ahu, suggesting that, in at least one instance, migration and divergence in the group has occurred from a younger to older island, opposite of the general trend seen among many groups of Hawaiian plants

and animals. It is also interesting to note that the Kauaʻi endemic *L. waimeaensis* may also have been derived from a species on a younger island. Unfortunately, only a single individual in this study represents *L. waimeaensis*, so conclusions about the species' place in the section are highly tentative. *Lipochaeta kamolensis* of Maui is also represented by a single individual; it clusters with individuals of *L. lavarum*, which is known largely from the islands of Maui Nui, which comprises Maui, Lanaʻi, Kahoʻolawe, and Molokaʻi. The relationships among species from the islands of Oʻahu and Kauaʻi were unclear, as individuals of *L. tenuifolia* clustered with both *L. fauriei* and *L. tenuis*. Of these species, *L. tenuifolia* has higher levels of genetic variability; in fact, *L. tenuifolia* had the highest percentage of polymorphic loci and heterozygosity among species included in this study, despite a small sample size. The degree to which hybridization may obscure relationships among species from the older islands is unclear. While species of *L*. section *Aphanopappus* do not occur in sympatry, making natural hybridization rare, species of *L*. section *Aphanopappus* will hybridize readily under laboratory conditions, although some decrease in fertility is seen (Rabakonanandriana, 1980). A naturally occurring, localized apparent hybrid population between *L. tenuifolia* and *L. tenuis* is known from the northern Waiʻanae Mountains, and individuals in the hybrid population exhibit a variety of leaf morphologies intermediate to those seen in the purported parental species; the age of the hybrid zone is unknown, but has been known for over 20 years (J. Lau, *pers. comm.*).

No general trends in levels of genetic diversity were apparent; species on older islands did not have more polymorphic loci or higher levels of heterozygosity. The most notable aberration was the low level of genetic variability seen in *L. remyi*. *Lipochaeta*

remyi is the only annual species in the group, and annuals generally have less genetic diversity than perennials (Hamrick and Godt, 1989). If the species has recently arisen from *L. venosa*, reduced genetic variability could also be explained by the founder effect. Which of these hypotheses is correct is difficult to determine, and, in fact, both may be responsible for the low levels of variability in *L. remyi*. Unlike many of the other single island endemics within the genus, *L. remyi* is not currently federally recognized as threatened or endangered with extinction, although IUCN and Hawaii Natural Heritage ratings both consider the species endangered (Wagner *et al*., 1999b). The genetic data here suggest that federal protection is warranted.

Mayr's peripatric model of speciation suggests that older habitats are too stable to allow for genome reorganization, a necessary prelude for speciation to occur; and, indeed, in many groups of Hawaiian plants, species on the oldest islands are ancestral to species on the youngest islands. Diversification in *L.* section *Aphanopappus*, however, has deviated from this regular pattern in at least one (*L. remyi*), and likely in more instances. In fact, there are dramatic examples of groups in Hawai‘i that have radiated from newer to older islands. *Clermontia* (Campanulaceae: Lobelioideae) has spread among almost all of the eight main islands from its origin on the youngest island of Hawai‘i (Lammers, 1995). Unlike other Hawaiian lobeliods and other examples of dramatic radiations, species of *Clermontia* primarily occupy lowland to lower montane habitats. Species of *Lipochaeta* are also largely confined to lowland habitats; furthermore, a number of species are known from strongly denudational, or erosional, habitats. For example, *L. waimeaensis* is known from a single locality on the steep side of the lower reaches of Waimea Canyon; *L. fauriei* occurs in several valleys on the Na Pali

coast; *L. kamolensis* is known in and around two adjacent gulches. Other species are known from habitats that naturally have high levels of disturbance, such as the coastal areas occupied by *L. integrifolia*. Clearly, the formation of new species can and does occur on older islands. The regular pattern of diversification among Hawaiian plants may reflect the specific habitat preferences of taxa more than a general need for new, empty habitat. Taxa restricted to high elevations would necessarily be limited to either a pattern of diversification from older to new islands, because as the older islands erode they lose high elevation habitats, or a distribution restricted to Hawai‘i and Maui, the only islands with extensive high elevation habitats.

The apparent hybrid population of *L. tenuifolia* and *L. tenuis* not withstanding, natural hybridization among the species of *L.* section *Aphanopappus* is rare in nature because of the generally allopatric distributions among taxa. However, artificial hybridization among species is easily induced between species of *L.* section *Aphanopappus* (Rabakonandrianina, 1980). In fact, fertile hybrids can also be produced between individuals of the two sections of *Lipochaeta* and between individuals of *Lipochaeta* and *Wollastonia biflora* (Rabakonandrianina and Carr, 1981), the group's widely presumed progenitor (Wagner *et al*., 1990). A general absence of natural hybridization due to allopatry among species and ease of artificial hybridization is common among many Hawaiian plant taxa (Mayer, 1991). It is unclear whether the absence of contemporary hybridization is indicative of an absence of past hybridization. In the silversword alliance, past hybridization among species and genera can account for the lack of concordance among nuclear and chloroplast DNA markers and the production of polytomies in phylogenetic trees produced from chloroplast markers (Baldwin *et al*.,

1990); members of the silverswords are also known to hybridize freely where they come into contact (Caraway *et al*., 2001). While the distributions of most species in *Lipochaeta* have likely never been large, the current distributions can, nonetheless, be considered only fragments of populations that were present during pre-European and likely pre-Polynesian settlement of the islands; most species of *Lipochaeta* occur at lower elevations, where human activity has been greatest. Thus, opportunities for hybridization, particularly via pollen flow, were likely greater at times in the past. Hybridization would be expected to homogenize species and obscure relationships among taxa, and this is evident in the relationships of the species known to hybridize naturally, despite a narrowly distributed (restricted) hybrid population.

If the evolutionary process includes both the divergence in gene frequencies shared due to common ancestry and the acquisition of new mutations (Witter and Carr, 1988), *Lipochaeta* is clearly in the early stages of divergence. It has become standard to assume that the age of a group dates to the age of the island the group first colonized. For example, assuming that *Lipochaeta* first colonized O‘ahu then spread to other islands (Gardner, 1979), which is not inconsistent with the results of this study, one would conclude the age of the group to be 3.8 million years. However, he ease with which hybridization can occur across taxa, the lack of fixed differences among species, and the occurrence of speciation on older islands all argue for a more recent radiation of *Lipochaeta*.

Table 2.1. Morphological characteristics of known extant species of *Lipochaeta* sect. *Aphanopappus*; descriptions are from Wagner *et al*. (1990).

Species	Growth habit	Leaf Shape
Single island endemics		
Kauaʻi		
L. fauriei	suffruticose, weakly erect to scandent	deltate, entire
L. micrantha	suffruticose, decumbent	dissected
L. waimeaensis	suffruticose, decumbent	entire
Oʻahu		
L. remyi	annual, with erect stems	ovate with dissected margins, from deft to deeply lobed
L. tenuifolia	suffruticose, decumbent	highly dissected
L. tenuis	suffruticose, decumbent	deltate, entire
Maui		
L. kamolensis	decumbent to scandent	dissected
Hawaiʻi		
L. subcordata	suffruticose, erect to ascending stems	narrowly deltate to deltate
L. venosa	suffruticose, with arcuate-spreading stems	deltate, with two basal lobes
Multi-island species		
L. integrifolia	suffruticose, stems prostrate	ovoid, succulent
L. lavarum	suffruticose, stems erect	entire

Table 2.1, extended.

Elevational range	Ecological habitat
480-900 m	diverse mesic forest
300-400 m	diverse mesic forest
350-400m	diverse mesic forest
30-180 m	shrubland
700-900 m	diverse mesic forest
700-900 m	diverse mesic forest
250m	remnant dry forest
550-1800 m	dry forest
730-915m	dry shrubland
coastal	coastal habitats
lav0-550m	coastal habitats and dry forest

Table 2.2. Number of AFLP fragments detected per primer pair reaction.

*Eco*RI-*Mse*I primer pair	Number of bands
ACA-CAT	39
ACA-CTT	25
ACG-CTG	13
ACT-CTG	30
AGG-CAT	20
AGG-CTT	19

Table 2.3. Estimates of genetic variability for six species of *Lipochaeta* section *Aphanopappus* determined from 146 AFLP loci.

Species	*N*	Fragments[a]	*P*	*H* (SD)
Single island endemics				
L. remyi	11	95	26.7	0.087 (0.163)
L. tenuifolia	6	107	61.0	0.219 (0.200)
L. tenuis	3	94	32.2	0.119 (0.180)
L. venosa	7	117	53.4	0.206 (0.213)
Multi-island species				
L. integrifolia	12	131	57.5	0.193 (0.200)
L. lavarum	10	107	55.5	0.194 (0.202)
Section	49	146	95.2	0.302 (0.169)

[a]Number of fragments that occurred as 'present' in at least one individual.

Table 2.4. Nei's (1987) measures of genetic diversity and structure determined from 139 polymorphic AFLP loci sampled in six species of *Lipochaeta* section *Aphanopappus*.

H_T (SD)	H_S (SD)	G_{ST}
0.310 (0.029)	0.170 (0.011)	0.452

Table 2.5. Paired G_{ST} values and the number of loci with significant heterogeneity between pairs of species of *Lipochaeta* section *Aphanopappus*. The number of loci with statistically significant heterogeneity ($p < 0.05$) between species are given in the upper triangle in the table, and paired G_{ST} values are in the lower triangle.

	1	2	3	4	5	6
1 *L. integrifolia*	–	46	50	49	34	39
2 *L.lavarum*	0.279	–	57	15	30	31
3 *L. remyi*	0.354	0.427	–	61	44	39
4 *L. tenuifolia*	0.305	0.159	0.431	–	18	30
5 *L. tenuis*	0.384	0.389	0.543	0.299	–	25
6 *L. venosa*	0.230	0.238	0.286	0.270	0.395	–

Table 2.6 Dice's average similarity within and among species of *Lipochaeta* section *Aphanopappus*. Average similarities between individuals within species are shown in boldface along the diagonal, and average similarities among individuals of different species are given in the lower triangle.

	1	2	3	4	5	6
1 *L. integrifolia*	**0.835**					
2 *L.lavarum*	0.676	**0.890**				
3 *L. remyi*	0.714	0.639	**0.874**			
4 *L. tenuifolia*	0.591	0.600	0.571	**0.619**		
5 *L. tenuis*	0.566	0.555	0.579	0.595	**0.673**	
6 *L. venosa*	0.733	0.664	0.778	0.596	0.572	**0.771**

Figure 2.1. Distribution of *Lipochaeta* section *Apanopappus* in the Hawaiian Islands, with single island endemics in bold face. Species and distributions are after Wagner and Robinson (2001). Because they have been variously connected by land bridges when sea levels were lower (Carson and Clague, 1995), the islands of Maui, Molokaʻi, Lanaʻi, and Kahoʻolawe are often collectively referred to as Maui Nui. Collection locations: *L. fauriei*, Haeleele Valley; *L. waimeaensis*, individuals collected as seeds from Waimea Canyon growing at the National Tropical Botanical Garden; *L. remyi*, Kealia Trail; *L. tenuifolia*, Makua Valley; *L. tenuis*, near Kolekole Pass; *L. kamolensis*, Kepuni Gulch; *L. venosa*, Heihei Puʻu; *L. integrifolia*, Kaʻena and Makapuʻu Points (Oʻahu), Moʻomomi (Molokaʻi); *L. lavarum*, Lualailua (east Maui), Manele (Lanaʻi), Hualalai (Hawaiʻi).

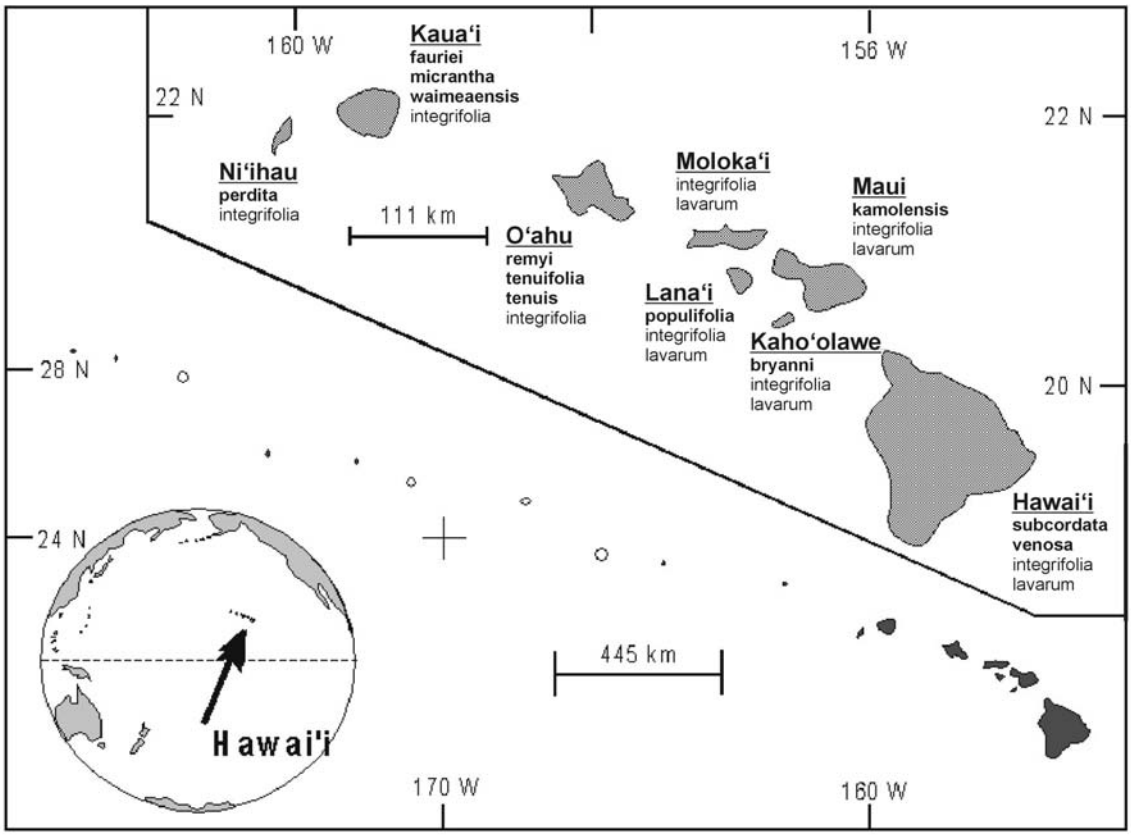

160 W
156 W
22 N
22 N
28 N
20 N
24 N
170 W
160 W
Kaua'i
fauriei
micrantha
waimeaensis
integrifolia
Ni'ihau
perdita
integrifolia
111 km
O'ahu
remyi
tenuifolia
tenuis
integrifolia
Moloka'i
integrifolia
lavarum
Maui
kamolensis
integrifolia
lavarum
Lana'i
populifolia
integrifolia
lavarum
Kaho'olawe
bryanni
integrifolia
lavarum
Hawai'i
subcordata
venosa
integrifolia
lavarum
445 km
Hawai'i

Figure 2.2. The percentage of polymorphic loci and heterozygosity in six species of *Lipochaeta* determined from 146 AFLP loci.

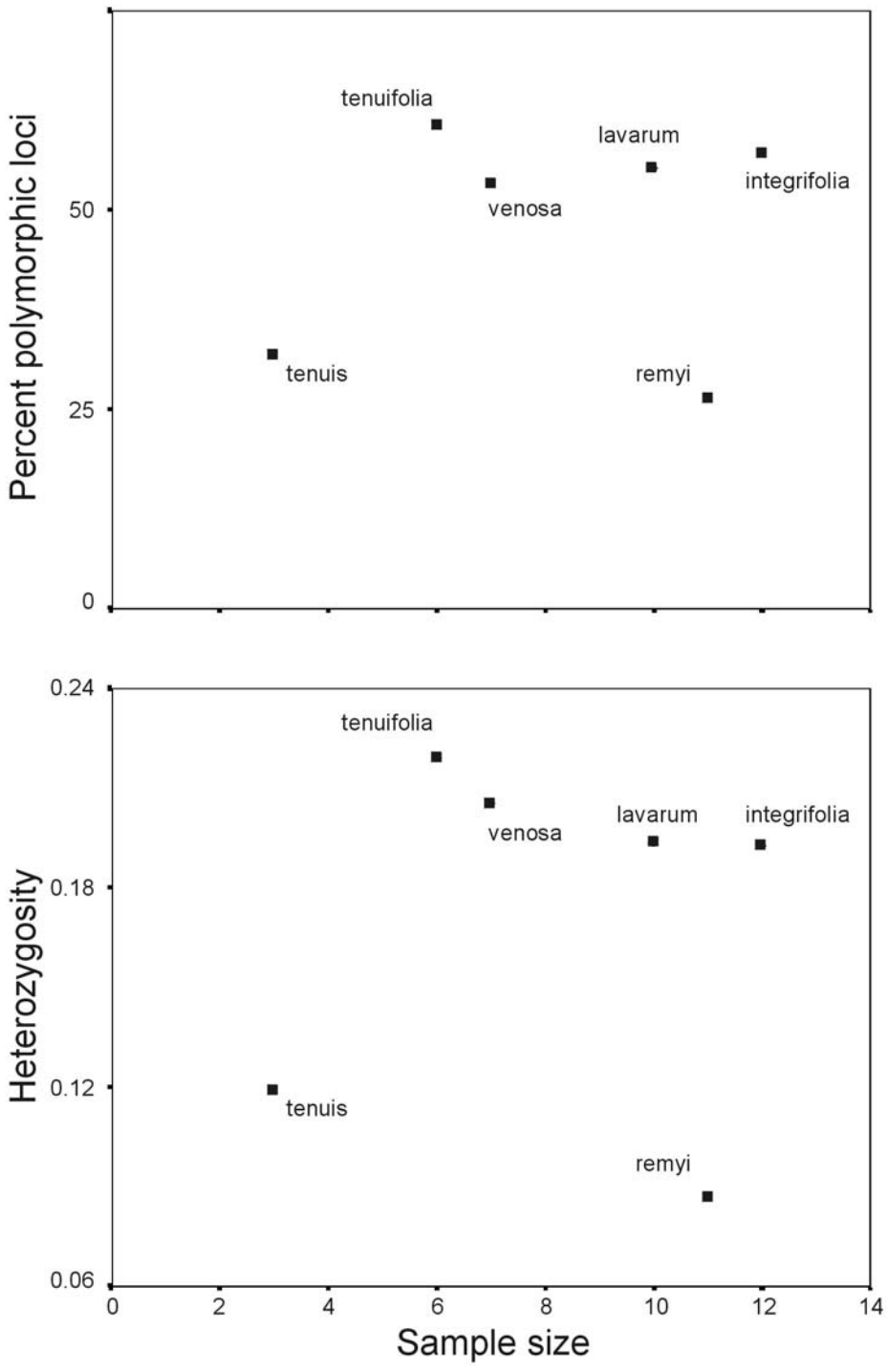
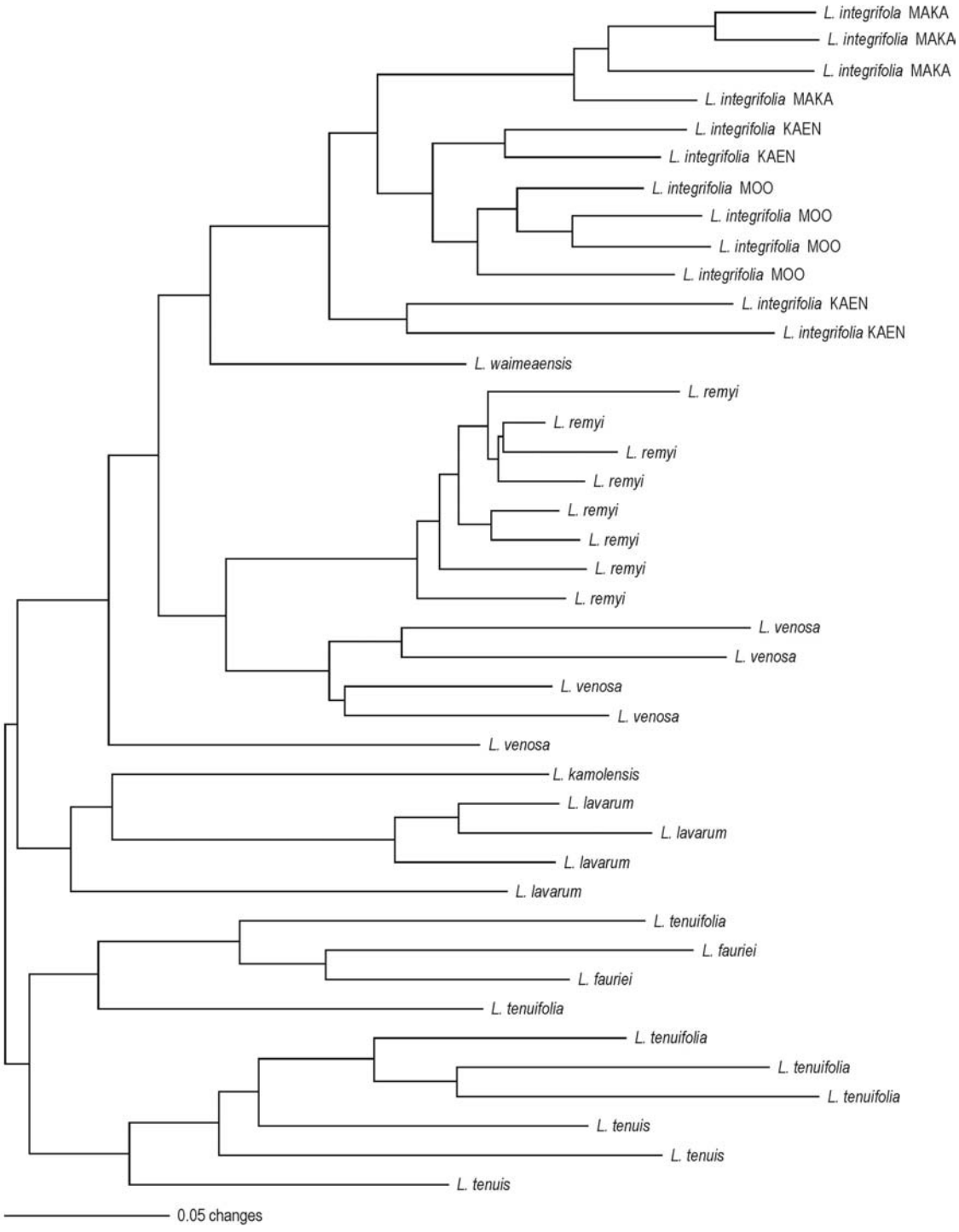

Percent polymorphic loci
50
25
0
tenuifolia
lavarum
integrifolia
venosa
tenuis
remyi
Heterozygosity
0.24
0.18
0.12
0.06
tenuifolia
venosa
lavarum
integrifolia
tenuis
remyi
0
2
4
6
8
10
12
14
Sample size

Figure 2.3. Neighbor-joining tree from Dice's similarity between individuals determined from 146 AFLP loci; only those individuals for which all six primer pair reactions were resolved were included. Individuals of *L. integrifolia* are indicated by sample location: KAEN: Ka ʻena Point, O ʻahu; MAKA: Makapuʻu, O ʻahu; MOO: Moʻomomi, Molokaʻi. All individuals of *L. lavarum* included in the neighbor joining analysis were from the population at Manele, Lanaʻi.


L. integrifola MAKA
L. integrifolia MAKA
L. integrifolia MAKA
L. integrifolia MAKA
L. integrifolia KAEN
L. integrifolia KAEN
L. integrifolia MOO
L. integrifolia MOO
L. integrifolia MOO
L. integrifolia MOO
L. integrifolia KAEN
L. integrifolia KAEN
L. waimeaensis
L. remyi
L. remyi
L. remyi
L. remyi
L. remyi
L. remyi
L. remyi
L. remyi
L. venosa
L. venosa
L. venosa
L. venosa
L. venosa
L. kamolensis
L. lavarum
L. lavarum
L. lavarum
L. lavarum
L. tenuifolia
L. fauriei
L. fauriei
L. tenuifolia
L. tenuifolia
L. tenuifolia
L. tenuifolia
L. tenuis
L. tenuis
L. tenuis
0.05 changes

CHAPTER 3

DIVERSIFICATION OF THE HAWAIIAN COASTAL ENDEMIC *LIPOCHAETA INTEGRIFOLIA* (ASTERACEAE): INTRA- AND INTER-SPECIFIC COMPARISONS[3]

Remote island chains are virtually synonymous with the evolutionary phenomenon of adaptive radiation, and nowhere are the examples more dramatic than in the Hawaiian Islands. Some of the most famous and largest radiations include the Hawaiian Drosophilidae, comprising a monophyletic group of some 400 described species, and the 98 fleshy-fruited lobeliods (Campanulaceae) derived from a single common ancestor. In large radiations, the species often have localized and limited distributions. It is the unique geographic and geologic characteristics of the Hawaiian Islands that are thought to have promoted speciation within the chain. The most isolated island group in the world, the islands were formed as the Pacific Tectonic Plate moved over the immobile Hawaiian hot spot (Clague and Dalrymple, 1989), resulting in a chronological arrangement to the islands. Among the eight main islands, ages range from less than 0.5 million years for the youngest island, Hawai‘i, to approximately 5.1 million years for Kaua‘i. Because of continued, active volcanism in the islands, new, empty, habitat is constantly being created.

Vacant habitat is the key to Mayr’s model of ‘peripatric’ speciation (1954, 1982), which was specifically designed to explain patterns of diversification within remote

[3] Jorgensen, S. and R. Mauricio. To be submitted to *The American Naturalist*

island chains. The theory was based on his belief that the genome was an integrated system, or 'coadapted gene complex,' which resisted change; it was only with disturbance and geographic isolation that speciation could occur. Mayr's model, as well as similar theories proposed by Carson (1971) and Templeton (1980), consisted of several essential components: the assumption of the genome as an integrated system; the disruption of the system with founding (disorganization); population growth in new, empty habitat with gene combinations released from selection; and, with population growth, selection for a new set of coadapted gene complexes within the population (reorganization).

The patterns of diversification within many groups in the Hawaiian Islands do appear consistent with the peripatric model. For example, there is a general trend for radiations to progress from older to younger islands (Funk and Wagner, 1995), suggesting that new, empty habitat does promote speciation. Among taxa, however, there are considerable differences in the degree of diversification. For example, the common forest trees *Acacia koa* (Fabaceae) and *Metrosideros polymorpha* (Myrtaceae) apparently speciated on arrival to the Hawaiian Islands but have not diversified further; a single species is present across multiple islands and habitats. If Mayr's peripatric model is correct, and speciation is a fundamentally different process than diversification of populations within species, quantitative and qualitative genetic differences should differentiate these processes.

Making the leap from patterns to actual processes, however, has proved difficult, and no solid experimental or molecular genetic data have supported founder-mediated models of speciation (Charlesworth, 1997). DNA sequence data overwhelmingly indicate

little divergence among those Hawaiian species that have been studied. It is not surprising that molecular data have been generally unsupportive of peripatric models, however. Sequence divergence indicates time of divergence, not overall changes in the genome, and data from a single locus cannot be extrapolated to genome-wide changes in polygenic balances. Allozyme data, the workhorse of population geneticists, also have failed to support predictions of founder effect speciation models. A typical allozyme survey may examine only a small number of loci (e.g., 10-20), however, which would make the detection of complexes difficult. Amplified fragment length polymorphism (AFLP) markers (Vos *et al*., 1995) have advantages over allozymes and sequence data that make them ideal for examining diversification in island species. The AFLP technique can easily generate many times the number of loci typically analyzed in allozyme studies. While sequencing can also produce hundreds of loci, the loci are all sampled from the same portion of the genome. In contrast, with AFLP markers a large number of loci from across the genome can be identified and analyzed.

Lipochaeta DC (Asteraceae: Helianthaea) is an ideal group to analyze whether speciation is different from population-level diversification. Section *Aphanopappus* is composed almost exclusively of narrowly distributed single-island endemics, a pattern typical of adaptive radiations. A single species, *L. integrifolia*, however, is widely distributed, being found across all eight of the main Hawaiian Islands. Despite its relatively large geographic range, however, the species has very localized distributions on the islands where it occurs. For example, on O‘ahu the species is known from the extreme northwestern and southeastern points, but not in between. Thus, within a single taxonomic group, there are numerous examples of both speciation and differentiation

among populations, affording a unique opportunity to test Mayr's peripatric model of speciation.

We examined AFLP diversity in *L. integrifolia* to answer the following questions: (1) How do levels of genetic diversity compare across populations in the species?, (2) What is the distribution of genetic diversity among populations?, (3) What are the relationships among populations?, (4) How does the divergence among populations of *L. integrifolia* compare to the divergence among species of *Lipochaeta*?, (5) Are the data supportive of peripatric or gradual models of speciation?

Materials and Methods

Field sampling and laboratory analyses

Leaf tissue samples were collected from individuals in six naturally occurring populations of *Lipochaeta integrifolia* sampled from the islands of Kaua'i, O'ahu, Moloka'i, and Hawai'i (Fig. 3.1). In addition, individuals were sampled from a single population each of the single island endemics *L. remyi* from O'ahu and *L. venosa* from Hawai'i. Final sample sizes among the populations of *L. integrifolia* varied from two to nine, while *L. remyi* and *L. venosa* were represented by 9 and 6 individuals, respectively; samples sizes differed among the populations and species because of variations in population sizes and the ability to extract DNA. Immediately after harvest, leaves were placed in plastic sample bags with silica gel for storage until DNA extraction. DNA was extracted from the dried samples, which were crushed by vortexing in a polyethylene tube with ball bearings, after the procedure of Colosi and Schaal (1993). Total genomic

DNA was extracted by a standard phenol/chloroform procedure; following extraction and ethanol precipitation, DNA was resuspended in deionized water to an approximate concentration of 50 ng/mL. Final concentrations were determined by comparing 1 μL of the sample to known concentrations of λ phage, and samples were further diluted, if necessary, for AFLP analysis.

AFLP analysis was performed using kits and equipment available from Applied Biosystems for fluorescent marker detection; kit protocols were followed, with few modifications. Digestion and ligation were conducted simultaneously using the enzymes *Eco*RI and *Mse*I and kit-supplied enzyme-specific adapters. Two rounds of PCR are required for AFLP analysis, with the adaptors as the sites for primer annealing. During the preselective amplification, a single, additional nucleotide is added to the 3' ends of the primers. A single preselective product is diluted and serves as the template for the subsequent selective amplifications, during which two additional nucleotides are added. The *Eco*RI-specific primers used in the selective amplifications are fluorescently labeled to permit fragment detection. Seven *Eco*RI-*Mse*I primer-pair combinations were used (listed according to the additional nucleotides from the preselective and selective amplifications): ACA-CAT; ACA-CTT; ACG-CTG; ACT-CTC; ACT-CTG; AGG-CAT; and, AGG-CTT. Recommended kit dilutions and a total volume of 10 μL were used for both the preselective and selective PCR reactions. PCR products were separated by electrophoresis using 6% polyacrylamide gels; an ABI Prism 377 automated sequencer, equipped with a laser to detect fragments, was used to conduct electrophoresis. A ROX-500 fluorescently labeled size standard was loaded with each sample to permit size determination for fragments between 70-450 bp. Fragment detection and sizing were

conducted with Genescan 3.1. Each of the variously sized fragments was treated as corresponding to a single gene locus with dominant expression, and fragments were scored as either present (1) or absent (0) in each individual analyzed.

Genetic diversity of Lipochaeta integrifolia

With a dominant marker, the calculation of standard measures of genetic variability requires either an estimate of the inbreeding present within populations or the assumption of Hardy-Weinberg proportions within populations (Travis *et al*., 1996); because no data were available regarding inbreeding in *L. integrifolia*, Hardy-Weinberg proportions were assumed in all populations. Genetic variability in *L. integrifolia* was assessed at the population and species levels by the percentage of polymorphic loci (*P*) and heterozygosity (*H*). A locus was considered polymorphic if a fragment occurred in at least one, but not all individuals. Heterozygosity was calculated at each locus from the equation $H = 1- [(1-q)^2 + q^2]$ where q^2 is the frequency of individuals with the fragment absent; total heterozygosity was calculated as the average heterozygosity across loci. The distribution of genetic diversity among populations was determined using Nei's (1973) measures, which include total genetic diversity at a polymorphic locus (H_T), mean genetic diversity with populations (H_S), and the proportion of genetic diversity occurring among populations, $G_{ST} = (H_T - H_S)/H_T$. A procedure analogous to that for determining heterozygosity was used to estimate H_S and H_T, except that calculations were conducted only on polymorphic loci. Because of small sample sizes, the populations from Kaua'i were not included in the calculation of Nei's measures.

Phylogenetic relationships were estimated with a neighbor joining analysis (Saitou and Nei, 1987) of similarity values. Dice's (1945) coefficient of similarity was calculated between each pair of individuals $S_{ij} = 2a/(2a + b + c)$ where a is the number of fragments shared between individuals, b is the number of fragments present only in individual i, and c is the number of fragments present only in individual j; shared absences, which are more likely to be nonhomologous (Wolfe and Liston, 1998), are not used in the calculation. Similarity values were calculated with SPSS (SPSS, 1998), and the neighbor joining tree, with midpoint rooting, was generated with PAUP 4.0 (Swofford 1998).

Interspecific comparisons

For the interspecific analyses, the four largest populations of *L. integrifolia* were compared to populations of *L. remyi* and *L. venosa*. These species were chosen for comparison because they are closely related to one another and to *L. integrifolia* (Chapter 2). Because neither *L. remyi* nor *L. venosa* were analyzed using the primer-pair combination ACT-CTC, only data from six of the primer pair reactions, comprising 143 loci, were used in the interspecific comparisions. The gross levels of divergence among species and populations were determined by calculating G_{ST} values and the number of loci exhibiting significant heterogeneity. Calculations were made at the species level, by pooling data from the four populations of *L. integrifolia*, and at the population level, by calculating values of divergence between each pair of *L. integrifolia* populations. The number of loci exhibiting significant heterogeneity between the groups being compared was determined with a χ^2 test.

An essential part of Mayr's theory is the presence of coadapted gene complexes; that is, associations among alleles at gene loci. The peripatric model predicts that speciation occurs with a change in gene complexes; it can, therefore, be concluded that population-level differentiation does not involve drastic changes in gene complexes. Principal components analysis (PCA) was used to asses the degree to which gene complexes may exist within populations and species; this analysis was chosen because it will reflect associations among variables, which in this study are the individual AFLP fragments. PCA was also desirable because it does not require *a priori* placement of individuals into groups. While PCA is not normally conducted on binary data, it can be used as a supplemental analysis; in fact, the technique has been used to analyze random amplified polymorphic DNA (RAPD) markers, which, like AFLP data, are binary in nature (Demeke and Adams, 1994). Two separate analyses were conducted: one including all indviduals, and another with only individuals of *L. integrifolia*; all calculations were done with the program PC-ORD (McCune and Mefford, 1999).

Results

Genetic diversity and structure of Lipochaeta integrifolia

The seven primer-pair reactions yielded 186 bands; of these, 14 (7.5%) were fixed within the species and 172 (92.5%) were polymorphic either within or among populations. The population at Mahaulepu, Kaua‘i, which had small population and sample sizes, had the lowest percentage of polymorphic loci (13%; Table 3.1). The population with the most polymorphic loci (70%) was the population at Ka‘ena Point,

O‘ahu, which had one of the largest sample sizes and a large population size. Heterozygosity at the species level was 0.269, and varied from 0.086 for the population at Mahaulepu to a maximum of 0.246 for the population at Ka‘ena Point. Overall divergence among populations was G_{ST} = 0.176 (Table 3.2), indicating that approximately 18% of the diversity at the species level is distributed among populations.

The neighbor joining analysis revealed the populations on O‘ahu to be closely related, and they are, in turn, related to the individuals from Mo‘omomi (Fig. 3.2). Individuals did not cluster exclusively by population, however; some individuals from the population at Ka‘ena Point also group with individuals from Mo‘omomi or Ka Lae.

Comparisons with L. remyi *and* L. venosa

Interestingly, the species-level G_{ST} values and the number of loci with significant differences told two slightly different stories. G_{ST} values indicated that *L. integrifolia* and *L. venosa* are most closely related (G_{ST} = 0.202), while *L. integrifolia* and *L. remyi* exhibited the greatest divergence (G_{ST} = 0.269). However, the smallest number of loci with significant heterogeneity was detected between *L. remyi* and *L. venosa* (Table 3.3), which corresponds with the results from a previous neighbor joining analysis (Chapter 2).

When the populations of *L. integrifolia* were analyzed separately, it was clear that populations were more similar to one another than to either of the other species. Intraspecific G_{ST} values ranged from 0.191 (Makapu‘u–Ka‘ena) to 0.256 (Moomomi-Ka Lae). In the interspecific comparisons, values for comparisons between populations of *L. integrifolia* and *L. remyi* were from 0.362 to 0.408, and values for the *L. venosa* comparisons were between 0.273 and 0.304. All populations of *L. integrifolia* were

genetically more similar to *L. venosa* than to *L. remyi* (Table 3.4), and there was no overlap in values when the two species were compared to populations of *L. integrifolia*. Patterns were similar for the number of loci exhibiting significant variation. From 6 to 21 loci were significantly heterogeneous among populations of *L. integrifolia*. When populations of *L. integrifolia* were compared to *L. remyi*, from 34 to 43 loci were significantly heterogeneous, while values ranged from 12 to 31 when the populations were compared to *L. venosa*. The divergence among *L. remyi* and *L. venosa* (G_{ST} = 0.246; 24 loci exhibiting significant heterogeneity) is comparable to the levels of divergence seen among populations of *L. integrifolia*, and some pairs of populations of *L. integrifolia* exhibit greater differentiation than the species of *L. remyi* and *L. venosa*.

The species-level PCA easily separated out the three taxa, which occupy unique positions along the first axis (Fig. 3.3); the first principal component accounted for approximately 26% of the total variation in the data set (Table 3.5). While individuals separate further on the second axis, which accounted for another 11% of the data's variation, a single axis is sufficient to describe divergence among the species. The cumulative variation accounted for by the first 10 components was 75%. Less variation (19%) was accounted for by the first axis in the population-level PCA (Table 3.6)(Fig. 3.4), indicative of fewer associations among loci within populations of *L. integrifolia* than found among species. The first two axes failed to completely segregate populations, although individuals from the populations at Ka‘ena, Makapu‘u and Mo‘omomi occupy relatively discrete areas along the combined first and second axes. The cumulative variance accounted for by the first 10 components in the population-level analysis was 81%, slightly more than that observed in the species-level analysis.

Discussion

Lipochaeta integrifolia is the most widely distributed member of the genus and the only member of *Lipochaeta* to occur across all eight of the main Hawaiian Islands. Levels of genetic diversity observed within populations of *L. integrifolia* are similar to the range of values observed among species of *Lipochaeta* (Chapter 2) and appeared to correlate somewhat with population size. Although *L. integrifolia* has a relatively large geographic range, it is very patchily distributed across islands and observed population sizes varied dramatically. At one extreme were the populations on the island of Kaua‘i, which were extremely small numerically and geographically. In contrast, the populations at Ka‘ena Point and Ka Lae stretched for a number of kilometers along the coast and numbered in the hundreds, if not thousands. Although the population at Mo‘omomi was observed to be quite small, genetic diversity in the population was relatively high. The high levels of genetic diversity in the populations at Makapu‘u and Mo‘omomi may indicate recent declines in population size, as populations that remain small lose genetic variability over time.

Diversity among populations of *L. integrifolia* was large. The G_{ST} of 0.176 was somewhat higher than typical values for plants with localized distributions surveyed at allozyme loci (Hamrick and Godt, 1989). The divergence among populations of *L. integrifolia* was substantially less than that observed in the native Hawaiian forest trees *Colubrina oppositifolia* and *Alphitonia ponderosa* (Rhamnaceae), which were surveyed with RAPD markers (Kwon and Morden, 2002). Because of the great distances involved, it is unlikely that substantial gene flow occurs among populations; thus, it is unlikely the

divergence among populations of *L. integrifolia* represents an equilibrium value. An increase in diversification among populations would be expected over time.

Individuals of *L. integrifolia* did not strictly cluster by populations in the neighbor joining analysis, although some patterns were evident. The populations surveyed from the opposite ends of O‘ahu do, indeed, appear to be sister to one another. A number of reasons for this relationship may be surmised: these now isolated populations may have been previously connected by a string of populations in other locations on the island; the populations may have been derived from the same unsampled or extinct source population; or, there may be gene flow between the two populations. Herbarium and published records (*cf*. Gardner 1979) show that *L. integrifolia* once had a more widespread distribution on the island than is currently found, especially near Makapu‘u. In 1999 and 2000, no evidence of the presence of *L. integrifolia* at many of these sites could be found, which suggests that populations of the species are in decline. The populations from O‘ahu are, in turn, most closely related to the population sampled from Mo‘omomi; an individual each from populations at Ka‘ena Point and Ka Lae form a group that is sister to most of the individuals from Mo‘omomi. Other relationships among populations and individuals are difficult to discern, with individuals from a number of populations clustering together in a clade that is sister to the Ka‘ena-Makapu‘u-Mo‘omomi clade.

Interestingly, the number of loci exhibiting significant heterozygosity is a better indicator of relationships among the species of *L. integrifolia*, *L. remyi*, and *L. venosa* than the gross measure of divergence, G_{ST}. The neighbor joining tree based on Dice’s similarity between individuals (Fig. 2.3) indicates that *L. reymi* and *L. venosa* are more

closely related to each other than either species is to *L. integrifolia*. The number of significantly different loci at the species level reveals the same relationships, whereas the G_{ST} values suggest that *L. venosa* is more closely related to *L. integrifolia* than to *L. remyi*. Values based on pairs of populations also indicate a closer relationship of *L. venosa* to *L. remyi* than to *L. integrifolia*. Each of the four populations of *L. integrifolia* was more similar to *L. venosa* than to *L. remyi*, as measured by both the number of loci with significant heterozygosity and G_{ST} values. Additionally, all of the G_{ST} values calculated for pairs of populations of *L. integrifolia* were less than those calculated for the *L. integrifolia*-*L. remyi* and *L. integrifolia*-*L. venosa* pairs. These patterns are generally supportive of differences between population-level divergence and speciation. Notably, however, the level of divergence between the species *L. remyi* and *L. venosa* is within the range of values observed between populations of *L. integrifolia*; furthermore, there is overlap between the *L. integrifolia* intraspecific comparisions and the *L. integrifolia*-*L. venosa* comparisons of the number of loci exhibiting significant heterogeneity.

The results of the principal components analyses were most surprising. While only a single component was necessary to separate individuals from the three species, populations of *L. integrifolia* did not occupy discrete areas along the first two components. While there were quantitative differences in the gross divergence of populations vs. species, as measured by G_{ST} values and the number of loci exhibiting significant heterogeneity, strong qualitative differences were seen in the association among loci between the population and species level analyses. These results are

consistent with Mayr's peripatric theory of speciation, which predicts that speciation involves changes in gene complexes.

Founder-mediated models of speciation predict that speciation is a process fundamentally different from the divergence of populations. Many of the patterns in *Lipochaeta* are consistent with the predictions of these models. Unfortunately, however, the patterns are not inconsistent with gradual divergence, either. Most of the variation among species and populations are the result of allele frequency differences. Very few fixed differences were observed among species of *Lipochaeta* (Chapter 2); in fact, no species-specific fixed markers were identified. The patterns in *Lipochaeta* contrast strongly with those observed in a recent study of Hawaiian crickets in the genus *Laupala* (Parsons and Shaw, 2001). In that study, fixed differences at AFLP loci accounted for most of the variation among species of *Laupala*. Speciation in animals (insects, at least) may be fundamentally different from speciation in plants. Experimental tests of founder-mediated species have focused exclusively on insects, typically species of *Drosophila*, and have focused on whether reproductive isolation can be induced by laboratory-created population bottlenecks; in general, these studies have not been able to produce reproductive isolation (Rice and Hostert, 1993). In fact, the degree to which Hawaiian insects are reproductively isolated is not very well understood, and under laboratory conditions reproductive isolation is often uni-directional. Hybridization among the species in many Hawaiian plant radiations can be readily induced in laboratory conditions, producing fertile hybrids. Additionally, cases of natural hybridization have been observed in groups including the silversword alliance (*Dubautia*) and *Lipochaeta*

(Chapter 4), although natural hybridization is generally rare because of the geographically isolated nature of the range of many plants.

It has become standard practice to accept that founder-mediated processes as responsible for the creation of adaptive radiations, even with a lack of supportive data. A typical example of this is evidence by the statement by Weller *et al*. (1996, p. 23):

> "Evolutionary patterns are often discerned more readily in remote island settings because infrequent colonization leads to reduced competition, the availability of more habitats, and consequently an increased likelihood of adaptive radiation."

Despite the authoritative tone of the statement, not a single study has shown that reduced competition plays any definitive role in the speciation process. If the Hawaiian flora is examined more closely, examples of adaptive radiation that do not follow the standard pattern of older-to-younger island diversification can be found. *Clermontia* (Campanulaceae) has radiated throughout the main islands from an origin on the island of Hawai‘i (Lammers, 1995); *L. venosa* of Hawai‘i appears parental to *L. remyi* of O‘ahu; and, some Hawai‘i island members of the silversword alliance are parental to species on Maui. Clearly, ‘empty’ or new habitat is not necessary for speciation to occur, and diversification can and does progress from younger to older islands. The general view that speciation occurs from older to younger islands may reflect the ecological requirements of the species being studied rather than the evolutionary process. For example, groups consisting of species that occupy high-elevation habitats cannot occur on any but the two youngest islands; if a single ancestor is found on an older island it may appear as though new habitat played a role in the speciation process when, in fact, it was simply geographic isolation.

More studies are clearly necessary to address whether founder mediated models of speciation are "defensible" (Charlesworth, 1997). Also, clear tests of the models are needed. In the typical adaptive radiation of remote island chains, species are described based on morphological characteristics, and species may not be reproductively isolated from one another. Thus, experimental studies may be focused on creating conditions that are not actually present in nature. Despite its liberal use, the invocations of founder-mediated processes are based on scant data. The term peripatric or founder-mediated speciation should be restricted to cases where gradual models of divergence have been falsified. As of yet, no such studies exist.

Table 3.1. Standard statistics of genetic diversity for seven populations of *Lipochaeta integrifolia*.

Population	Sample size	*P*	*H*
1 Ka Lae Amana, Kauaʻi	3	39.7	0.138 (.183)
2 Mahaulepu, Kauaʻi	2	12.7	0.086 (.169)
3 Kaʻena Point, Oʻahu	9	70.4	0.246 (.204)
4 Makapuʻu, Oʻahu	9	55.0	0.201 (.213)
5 Moʻomomi, Molokaʻi	7	57.7	0.206 (.207)
6 Ka Lae, Hawaiʻi	5	51.3	0.187 (.204)
Species	35	92.5	0.269 (.180)

Table 3.2. Nei's (1987) measures of genetic diversity and structure determined from 175 polymorphic AFLP loci sampled in four[a] populations of *Lipochaeta integrifolia*.

H_T (SD)	H_S (SD)	G_{ST}
0.255 (0.033)	0.210 (0.023)	0.176

[a]Because of very small population sizes, the populations from Ka Lae Amana and Mahaulepu, Kaua'i were excluded from this analysis.

Table 3.3. Divergence between *Lipochaeta integrifolia*, *L. remyi*, and *L. venosa* determined from 143 AFLP loci. The number of fragments unique to each species is listed in boldface along the diagonal; paired G_{ST} values are in the lower triangle; the number of loci with significant heterogeneity between species is in the upper triangle.

	1	2	3
1 *L. integrifolia*	**24**	47	30
2 *L. remyi*	0.269	**1**	24
3 *L. venosa*	0.202	0.246	**3**

Table 3.4. Divergence among populations of *Lipochaeta integrifolia*, *L. remyi*, and *L. venosa*. The number of fragments unique to each population or species is shown in boldface along the diagonal; paired G_{ST} values are in the lower triangle; the number of loci with significant heterogeneity between groups is in the upper triangle. Populations of *L. integrifolia* are listed by the numbers indicated in Table 3.1.

	L. integrifolia					
	3	4	5	6	*L. remyi*	*L. venosa*
3	**6**	21	13	13	43	27
4	0.191	**3**	18	13	44	31
5	0.225	0.203	**2**	6	40	21
6	0.219	0.235	0.256	**1**	34	12
L. remyi	0.372	0.362	0.408	0.382	**1**	24
L. venosa	0.296	0.273	0.330	0.304	0.246	**3**

Table 3.5. Pricipal components scores for multi-species comparison.

Axis	Eigenvalue	Percent of Variance	Cumulative % of Variance	Broken-stick Eigenvale
1	167.4	25.7	25.7	25.6
2	73.8	11.3	37.0	20.9
3	51.4	7.9	44.9	18.6
4	41.1	6.3	51.2	17.1
5	37.3	5.7	56.9	15.9
6	29.0	4.4	61.4	15.0
7	25.4	3.9	65.3	14.2
8	24.0	3.7	68.9	13.6
9	19.5	3.0	71.9	13.0
10	16.8	2.6	74.5	12.5

Table 3.6. Principal components scores for population comparisons.

Axis	Eigenvalue	Percent of Variance	Cumulative % of Variance	Broken-stick Eigenvale
1	58.2	18.8	18.8	12.2
2	39.3	12.7	31.5	10.0
3	32.5	10.5	42.0	8.9
4	27.5	8.9	50.8	8.1
5	23.2	7.5	58.3	7.6
6	19.5	6.3	64.6	7.1
7	15.0	4.8	69.4	6.8
8	13.0	4.2	73.6	6.4
9	11.8	3.8	77.4	6.2
10	10.2	3.3	80.7	5.9

Figure 3.1. Locations of populations of *Liopchaeta integrifolia*, *L. remyi*, and *L. venosa* sampled for this study. Populations of *L. integrifolia* are listed by the numbers used in Table 3.1., and populations of *L. remyi* and *L. venosa* sampled are noted by an 'r' and 'v', respectively. Collection locations for *L. integrifolia*: 1, Ka Lae Amana, Kauaʻi; 2, Mahaulepu, Kauaʻi; 3, Kaʻena Point, Oʻahu; 4, Makapuʻu, Oʻahu; 5, Moomomi, Molokaʻi; 6, Ka Lae, Hawaiʻi.

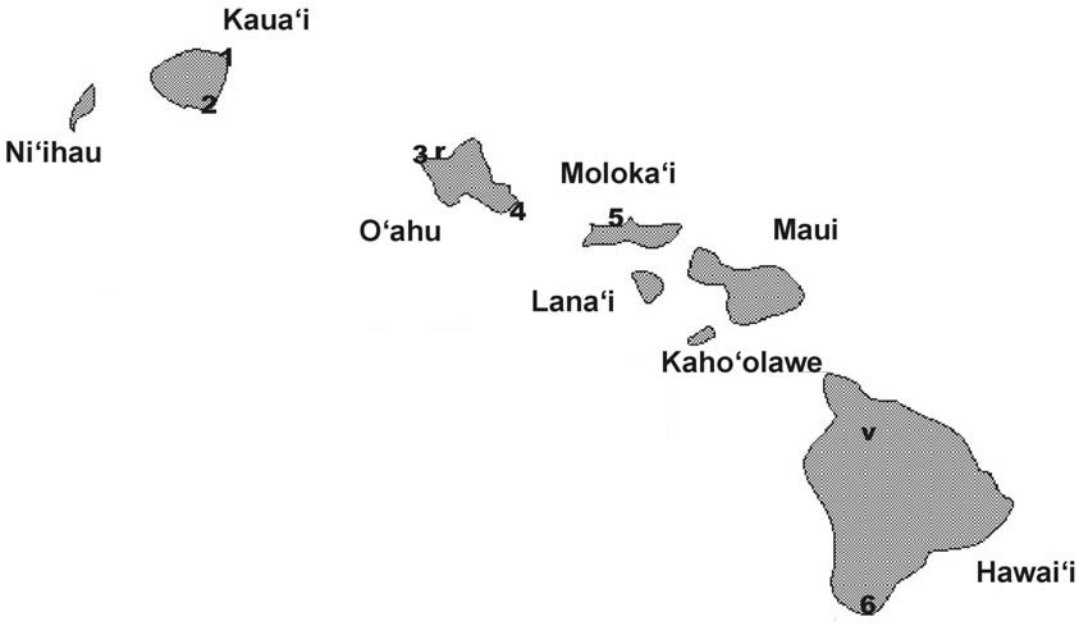

Kaua‘i
1
2
Ni‘ihau
3
Moloka‘i
4
O‘ahu
5
Maui
Lana‘i
Kaho‘olawe
v
Hawai‘i
6

Figure 3.2. Neighbor-joining tree of individuals sampled from six populations of *Lipochaeta integrifolia*. Only those individuals for which all seven primer pair reactions were resolved were included in the analysis.

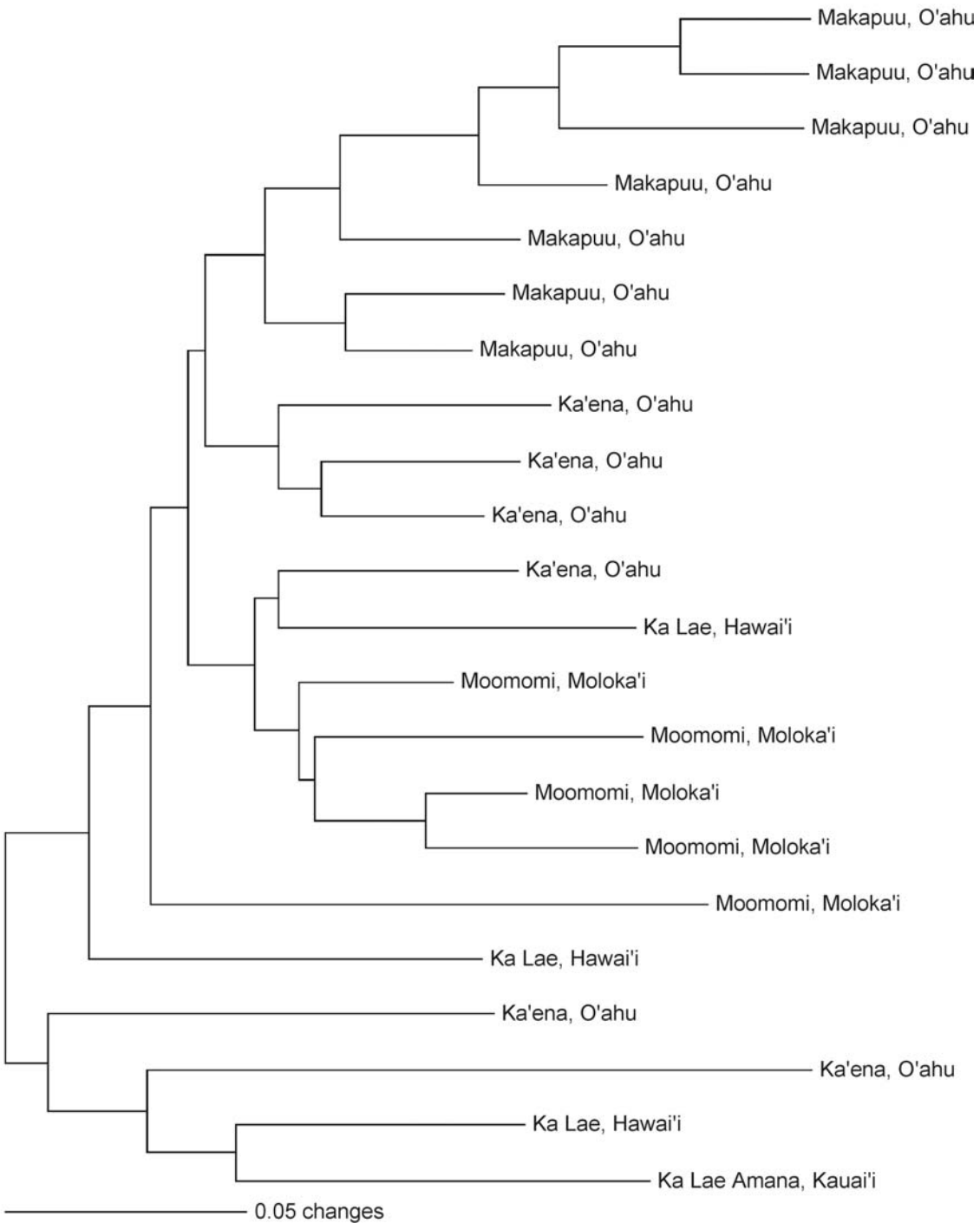

Makapuu, O'ahu
Makapuu, O'ahu
Makapuu, O'ahu
Makapuu, O'ahu
Makapuu, O'ahu
Makapuu, O'ahu
Makapuu, O'ahu
Ka'ena, O'ahu
Ka'ena, O'ahu
Ka'ena, O'ahu
Ka'ena, O'ahu
Ka Lae, Hawai'i
Moomomi, Moloka'i
Moomomi, Moloka'i
Moomomi, Moloka'i
Moomomi, Moloka'i
Moomomi, Moloka'i
Ka Lae, Hawai'i
Ka'ena, O'ahu
Ka'ena, O'ahu
Ka Lae, Hawai'i
Ka Lae Amana, Kauai'i
0.05 changes

Figure 3.3. Plot of scores for principal components analysis of individuals of *L. integrifolia*, *L. remyi*, and *L. venosa* based on AFLP loci.

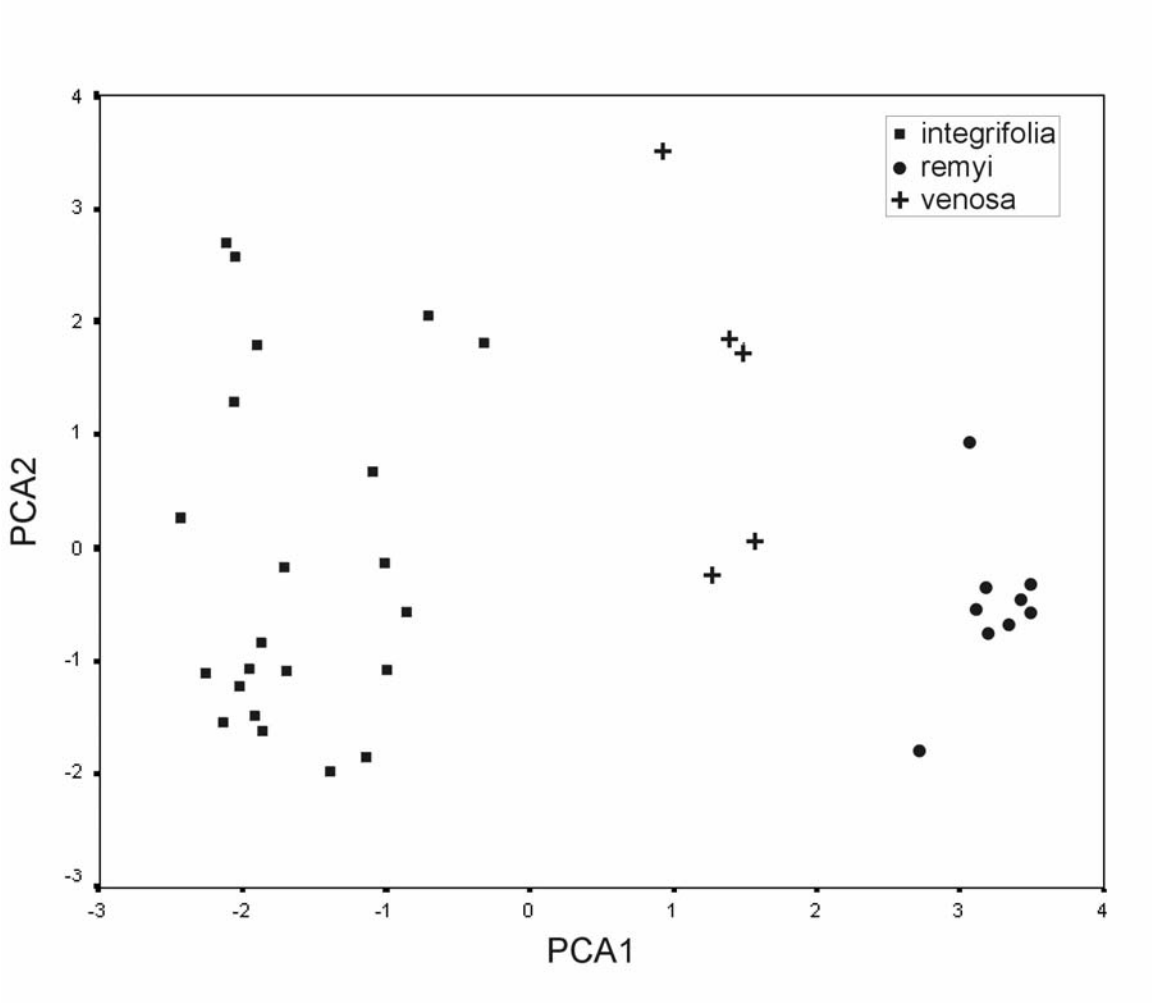
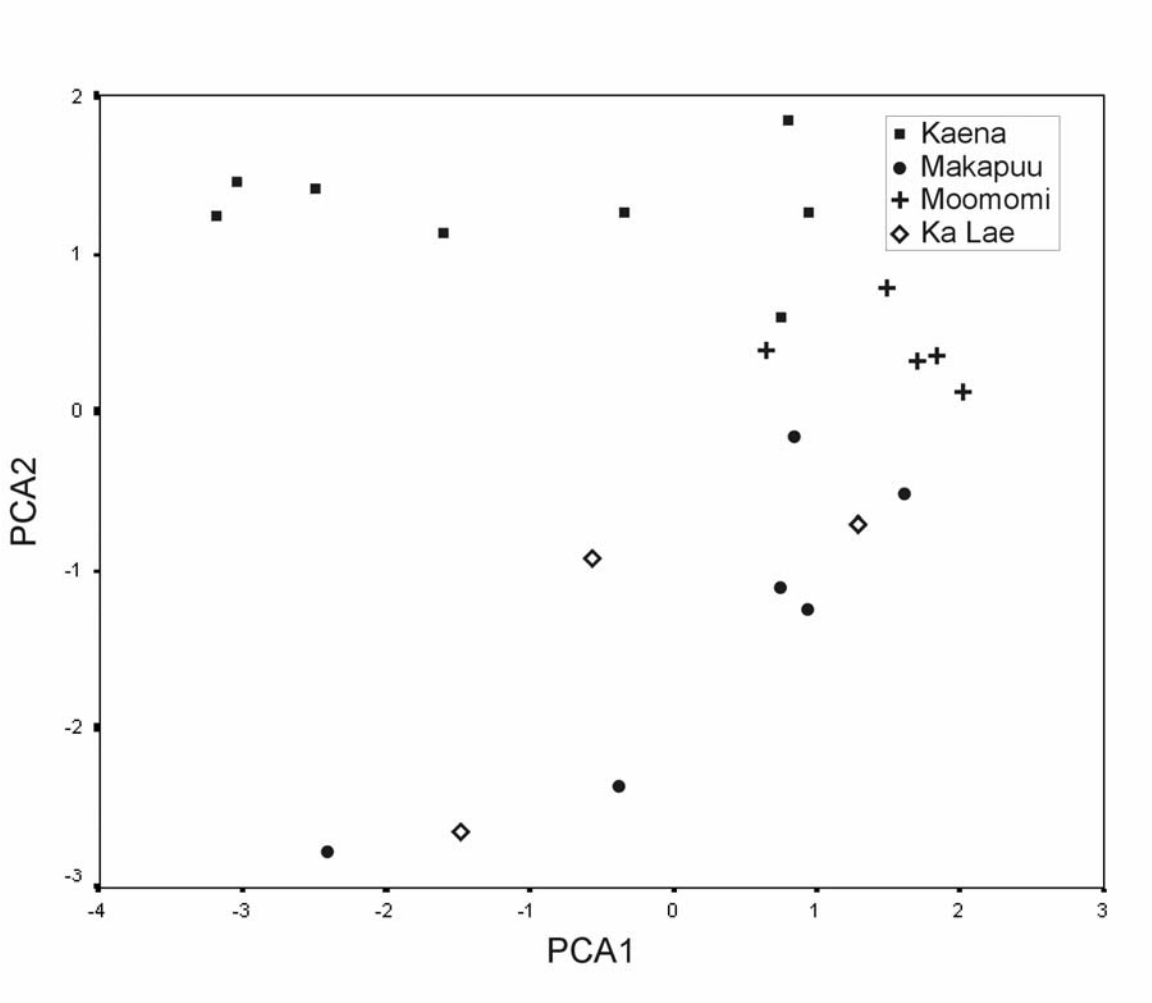

integrifolia
remyi
venosa
PCA2
PCA1
4
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
4

Figure 3.4. Plot of scores for principal components analysis of individuals of based on AFLP loci sampled among four populations of *L. integrifolia*.


Kaena
Makapuu
Moomomi
Ka Lae
PCA2
PCA1
2
1
0
-1
-2
-3
-4
-3
-2
-1
0
1
2
3

CHAPTER 4

CONFIRMATION OF HYBRIDIZATION BETWEEN *LIPOCHAETA TENUIFOLIA* AND *L. TENUIS* (ASTERACEAE) IN THE WAI‘ANAE MOUNTAINS, O‘AHU[4]

The Hawaiian Islands are well known for their spectacular examples of adaptive radiation within the native flora and fauna. Among plants, some of the most dramatic examples include the silversword alliance (Asteraceae) and the fleshy-fruited lobeliods (Campanulaceae), groups in which striking morphological differences are seen across species. Morphological differences are not indicative of reproductive isolation, however; a high rate of fertility is often observed in artificially induced interspecific and intergeneric hybrids. Examples include a number of groups within the Asteraceae: *Bidens* (Gillet and Lim, 1970), *Tetramolopium* (Lowrey, 1986), and *Argyroxiphium-Dubautia-Wilkesia* (Carr and Kyhos, 1981). Excepting the silversword alliance, which is known to hybridize freely when species co-occur (Caraway *et al*., 2001), natural hybridization appears to be rare in most Hawaiian plant genera, presumably because the allopatric distribution of species prevents pollen flow (Mayer, 1991).

Lipochaeta (Asteraceae) is an endemic Hawaiian genus of about 20 species of primarily suffruticose perennials; two sections, based on morphology and cytology (*Lipochaeta*, $n = 26$, four-petaled disk florets; *Aphanopappus*, $n = 15$, five-petaled disk florets), are recognized within the genus. Artifical hybrids can be induced in crosses

[4] Jorgensen, S. and R. Mauricio. To be submitted to *Evolution*

within and between sections (Rabakonandrianina, 1980), and between *Lipochaeta* and *Wollastonia biflora* (*n* =15), which is the widely presumed progenitor of *Lipochaeta* (Rabakonandrianina and Carr, 1979). Natural hybridization within the group is uncommon (Gardner, 1979) but not unknown (Wagner *et al*., 1990). However, the documentation of natural hybridization within *Lipochaeta* consists of morphological descriptions of intermediacy instead of biosystematic criteria.

One presumed case of natural hybridization in *Lipochaeta* occurs where the ranges of *L. tenuifolia* and *L. tenuis* (both *n* = 15) meet in the northern Wai‘anae mountains of O‘ahu (Fig. 4.1), to which the species are restricted. *Lipochaeta tenuifolia* and *L. tenuis* are characterized by very different leaf morphologies; leaves of *L. tenuifolia* are dissected to a degree that the leaves appear compound, while leaves of *L. tenuis* are deltate and entire. In the hybrid population at Pu‘u Kawiwi, individuals with a variety of intermediate leaf morphologies are found. While individuals with the typical leaf morphology of *L. tenuis* occur in the population, no individuals with the extremely dissected leaf morphology of *L. tenuifolia* were seen and have ever been seen (Joel Lau, Hawai‘i Natural Heritage Program, *pers. comm.*).

In this study, I use amplified fragment length polymorphism (AFLP) markers to examine the relationship of individuals found at the population at Pu‘u Kawiwi to their purported parental species. The main objectives of this study were to determine whether the population was of hybrid origin and, if hybridization is confirmed, to assess whether the population consists of early or later generation hybrids.

Materials and Methods

Individuals were sampled from naturally growing populations of *Lipochaeta tenuifolia*, *L. tenuis*, and a purported hybrid population between the two species; sample sizes were five, three, and 13, respectively. Approximately two leaves were collected per individual and placed in plastic bags with desiccating silica gel. In the hybrid population, leaf morphologies included types that were typical of *L. tenuis* but not the highly dissected form of *L. tenuifolia*. Each individual collected in the hybrid population was assigned to a morphological class: 1, *L. tenuis*-typical, deltate and entire; 2, *L. tenuis*-alternate, deltate, with small basal lobes; 3, deltate with several distinctive lobes; 4, deltate with numerous lobes and some further dissection of lobes; 5, very highly dissected with numerous lobes and sub-lobes, but less dissected than the parental species *L. tenuifolia*.

Leaves were crushed via vortexing with ball bearings (Colosi and Schaal, 1993), and total genomic DNA was extracted according to a standard phenol-chloroform procedure. Following phenol extraction, DNA was precipitated with ethanol alcohol and resuspended in deionized water to an approximate concentration of 50 ng/μL. AFLP analysis (Vos *et al*., 1995) was conducted with kits available from Applied Biosystems (ABI) for fluorescent marker detection. A restriction-ligation was conducted with the enzymes *Eco*RI and *Mse*I and enzyme-specific ligators from the preselective amplification kit. Following ligation, two rounds of PCR were conducted. During preselective amplification, a single nucleotide was added to the 3' end of the primers. The preselective amplification was diluted to serve as the template for the subsequent rounds of selective amplification. During selective amplification, two additional

nucleotides were added to the primers, and the *Eco*RI primer was fluorescently labeled to permit fragment detection. Six *Eco*RI-*Mse*I primer-pair combinations were used for selective amplification (listed by the additional nucleotides added): ACA-CAT, ACA-CTT, ACG-CTG, ACT-CTG, ACC-CAT, and, AGG-CTT.

Fragments were separated by electrophoresis using 6% polyacrylamide gels on an ABI 377 sequencer, which automatically read the gels via laser. A ROX-500 fluorescently labeled size standard was loaded with each sample during electrophoresis to permit fragment-size determination. Genescan 3.1 was used to visualize the gels and determine fragment size by interpolating to the ROX-500 standard loaded with each sample; the standard permitted the analysis of fragments between 70–450 bp. Each differentially sized fragment was considered a single gene locus, and individuals were scored by the presence (1) or absence (0) of the indicated fragment. The calculation of standard measures of genetic diversity and structure required the additional assumption of Hardy-Weinberg proportions within populations.

Genetic diversity within each of the three taxa was assessed by the percentage of polymorphic loci (*P*) and heterozygosity (*H*). A locus was considered polymorphic if any variation in expression was found within a species. Heterozygosity at each locus was estimated from the equation

$$H = 1 - [(1 - q)^2 + q^2]$$

where q^2 is the frequency of individuals in which a fragment was absent; total heterozygosity was calculated as the mean heterozygosity among loci. To determine

relatedness among individuals within and among species and the hybrids, Dice's (1945) coefficient of similarity was calculated between each pair of individuals

$$S_{ij} = 2a/(2a + b + c)$$

where a is the number of fragments shared between individuals, b is the number of fragments present only in individual i, and c is the number of fragments present only in individual j; shared absences, which are more likely to be nonhomologous (Wolfe and Liston, 1998), are not used in the calculation. Principal components analysis (PCA), using a variance-covariance matrix, was used to assess the relationship among individuals within the hybrid population to the parental taxa without *a priori* divisions into groups (Caraway *et al*., 2001; Wiley, 1981); all loci were used for the PCA analysis. Only those samples for which all six primer-pair combinations were resolved were included in the similarity and PCA analyses.

Results and Discussion

The six primer pairs yielded 133 AFLP fragments among all individuals. Well over half (61%) of the fragments were shared by the parental species (Table 4.1). Four unique fragments (i.e., also absent from hybrids) were detected each in *L. tenuifolia* and *L. tenuis*. Fixed differences between the parental species were detected at only two loci; in both cases, the fragments were fixed for presence in *L. tenuis* and were absent in *L. tenuifolia*. Twenty-two fragments were detected in only *L. tenuifolia* and the hybrids, and nine were shared by only *L. tenuis* and the hybrids. A single fragment was detected in

both parental species but was absent in the hybrids. In contrast, 13 fragments detected in the hybrids were absent from both parental species. Of the hybrid-only fragments, all but one fragment, which was found in a single hybrid individual, were observed in other species of *Lipochaeta*.

The number of polymorphic markers varied substantially between the parental species. Ninety (84%) of the fragments detected in *L. tenuifolia* were polymorphic, while only 47 (50%) polymorphic fragments occurred in *L. tenuis*. Not surprisingly, the hybrids possessed the greatest number (109) and percentage (88%) of polymorphic fragments. Heterozygosity, as averaged across all 133 loci, also was greatest in the hybrid population ($H = 0.30$). Heterozygosity in *L. tenuifolia* was, at $H = 0.24$, almost twice the level observed in *L. tenuis* ($H = 0.13$) (Table 4.2). Ironically, *L. tenuis* is one of the few single island endemics within *Lipochaeta* that is not federally recognized as threatened or endangered. Both the IUCN and Hawaii Natural Heritage Program do consider *L. tenuis* endangered (Wagner *et al*. 1999b). The data presented here suggest that federal protection of the species is likely warranted; at the least, further study of *L. tenuis* is needed. Normally, endangered species are considered susceptible to extinction through gene flow from widespread congeners (Ellstrand, 1992; Levin *et al*. 1996). Except for the coastal species *L. integrifolia*, no species of *Lipochaeta* are widespread, and even *L. integrifolia* has restricted, localized distributions on the islands where it occurs. In this case, the hybrid population may actually serve as a reservoir of genetic diversity (Arnold, 1997).

In the two parental species, mean similarity was greatest between individuals of *L. tenuis* (Table 4.3). In the comparisons of the parental taxa to the hybrids, comparable

similarities were observed, although hybrids were slightly more similiar to individuals of *L. tenuis* than to individuals of *L. tenuifolia* (mean similarities of 0.650 vs. 0.633). Surprisingly, the mean similarity between individuals of *L. tenuifolia* and the hybrids was greater than the mean similarity calculated between individuals of *L. tenuifolia*, although the difference was slight. As would be expected, among all comparisons the lowest similarities were observed between individuals of the two parental taxa. When hybrids were broken down into the various leaf type categories, no clear patterns were seen in similarity values when compared to the parental species (Fig. 4.2). Individuals with leaves that were categorized as leaf type 1, which were *L. tenuis*-like, were only slightly more similar to individuals of *L. tenuis* than to individuals of *L. tenuifolia*. Although hybrid individuals with leaf type 5 were observed to be the most *L. tenuifolia*-like morphologically, they were much more similar to *L. tenuis* genetically.

In the principal components analysis (Fig. 4.3), individuals failed to segregate clearly by species along the first two axes, which accounted for 27% and 18% of the variance observed in the total data set (Table 4.4). Despite the lack of discrete clusters, the first principal component did largely segregate the two parental species from the hybrids; the two parental species were separated from each other by the second principal component. As would be expected given the observed levels of genetic similarity, hybrid individuals of leaf type 5 clustered with the individuals of *L. tenuis*.

The AFLP data presented here clearly demonstrate a *L. tenuifolia* × *L. tenuis* hybrid origin of the population of Pu‘u Kawiwi. Virtually all the markers detected in the parental species were also found in the purported hybrid population, and the high degree of polymorphism, as measured by both the number of polymorphic loci and

heterozygosity, were consistent with a hybrid origin hypothesis. Approximately 10% (13) of the fragments detected in the hybrids were absent in both parental species. The failure to detect these fragments in the parental species likely represents sampling error rather than differentiation from the parental species, as all but one of the fragments were detected in other species of *Lipochaeta*. However, none of the other species occurred nearby.

The degree and direction of hybridity in the population are more difficult to ascertain, primarily because of the paucity of fixed differences between the parental species. Fixed differences between *Dubautia ciliolata* and *D. scabra* allowed Caraway *et al*. (2001) to conclude that many individuals in a hybrid represented later generation backcrosses to *D. ciliolata*. Genetically, the hybrid individuals are equally similar to both *L. tenuifolia* and *L. tenuis*, which would seem to argue for a large occurrence of F1 individuals. However, various leaf morphologies intermediate to that found in the parental species occur in the population; furthermore, there is variation in the degree of relatedness to the parental species when leaf morphologies are considered. Unexpectedly, those individuals with leaf morphologies most similar to *L. tenuifolia* were genetically much more closely related to *L. tenuis*; the high degree of similarity may indicate backcrossing to *L. tenuis* individuals among the hybrids. Populations of *L. tenuifolia* and *L. tenuis* have been identified around Pu‘u Kawiwi, although the status of these populations as representative of ‘pure’ populations is questionable (J. Lau, *pers. comm*). Selection among the hybrids may further complicate patterns, because different classes of hybrids may have varying levels of fitness (Arnold and Hodges, 1995). The lack of clear patterns of relatedness with respect to morphology, combined with the lack of fixed

differences among the parental species, makes it impossible to determine whether individuals in the population at Pu‘u Kawiwi represent later hybrid or backcross generations.

DNA markers are powerful tools for the confirmation of hybridization within plant species, and, in fact, are necessary to assess the contribution of each parental taxa to the hybrid population. The leaf morphologies of *L. tenuifolia* and *L. tenuis* represent the ends of a continuum found within the genus, and hybrids between the two species yield individuals with a variety of intermediate morphologies. In fact, the variety of leaf morphologies found in the *L. tenuifolia* X *L. tenuis* hybrid population at Puu Kawiwi is indicative of later generation hybrids or backcrosses. The genetic composition of the hybrid individuals could not be determined simply by their vegetative morphology. Further studies of this hybrid population should include controlled crosses between the parental taxa; these crosses could yield important information about the number of genes controlling leaf morphology and whether epistatic interactions among loci may affect leaf morphology.

Table 4.1 Summary of AFLP markers analyzed in *Lipochaeta tenuifolia*, *L. tenuis*, and their hybrid. One hundred thirty-three markers were detected among all individuals sampled.

AFLP markers	*L. tenuifolia*	*L. tenuis*	*L. tenuifolia* × *L. tenuis*
Total number	107	94	124
Constant markers	17	47	15
Polymorphic markers	90	47	109
Shared by both parental species	81	81	—
Constant in both parental species	12	12	—
Shared by parent and hybrid	102	89	—
Absent in other parent	22	9	—
Unique to species or hybrid	4	4	13

Table 4.2. Sample sizes, percent polymorphic loci, and heterozygosity calculated in *L. tenuifolia*, *L. tenuis*, and their hybrids determined from 133 AFLP loci. The percentage of polymorphic loci was calculated using all fragments (*P*) and only those fragments actually occurring within each taxa (*P*').

Species	*N*	*P*	*P*'	H_e (SD)
L. tenuifolia	5	67.7	84.1	0.238 (0.195)
L. tenuis	3	35.3	50.0	0.131 (0.185)
Hybrids	10	82.0	87.9	0.300 (0.192)

Table 4.3. Genetic similarity of individuals compared within and between *L. tenuifolia*, *L. tenuis*, and their hybrids calculated with the association coefficient of Dice (1945).

Species	Range	Mean
L. tenuifolia	0.451–0.757	0.621
L. tenuis	0.701–0.756	0.729
L. tenuifolia vs. *L. tenuis*	0.541–0.662	0.586
L. tenuifolia vs. hybrids	0.420–0.784	0.633
L. tenuis vs. hybrids	0.483–0.850	0.650

Table 4.4. Summary of the principal components analysis conducted on individuals of *L. tenuifolia*, *L. tenuis*, and their hybrids.

Axis	Eigenvalue	Percent of Variance	Cumulative % of Variance	Broken-stick Eigenvale
1	88.9	26.7	26.7	13.7
2	60.8	18.3	45.0	11.2
3	29.0	8.7	53.7	9.9
4	22.2	6.7	60.4	9.1
5	21.3	6.4	66.8	8.5
6	18.6	5.6	72.3	8.0
7	16.1	4.8	77.2	7.6
8	15.5	4.7	81.8	7.2
9	13.2	4.0	85.8	6.9
10	11.0	3.3	89.1	6.6

Figure 4.1 Distributions of *Lipochaeta tenuifolia* and *L. tenuis* and the location of a purported hybrid population in the northern Waiʻanae Mountains, Oʻahu. The locations of the populations sampled from the parental taxa are indicated by a closed square for *L. tenuifolia* and a closed circle for *L. tenuis.*

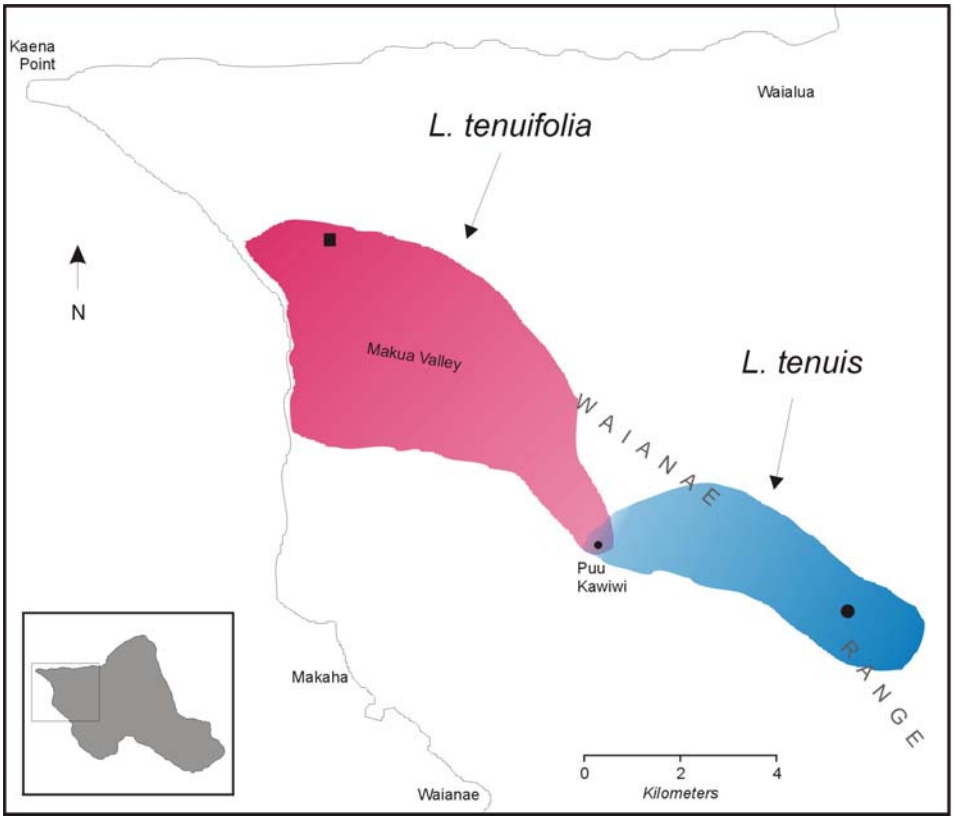

Kaena
Point
Waialua
L. tenuifolia
N
Makua Valley
L. tenuis
WAIANAE
RANGE
Puu
Kawiwi
Makaha
Waianae
0
2
4
Kilometers

Figure 4.2. Mean genetic similarity as calculated between individuals from the parental species *L. tenuifolia* and *L. tenuis* and their hybrids. Hybrid leaf categories ranged from 1 (*L. tenuis*-like, entire) to 5 (highly dissected).

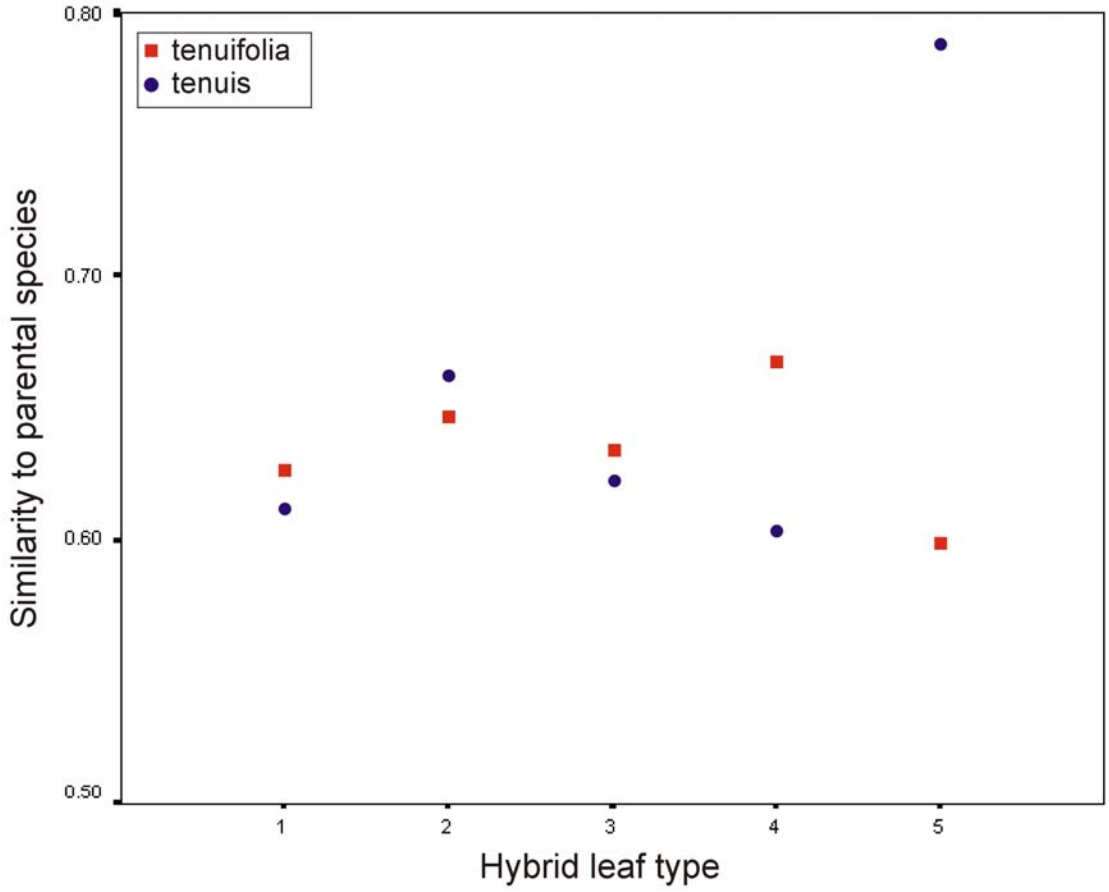
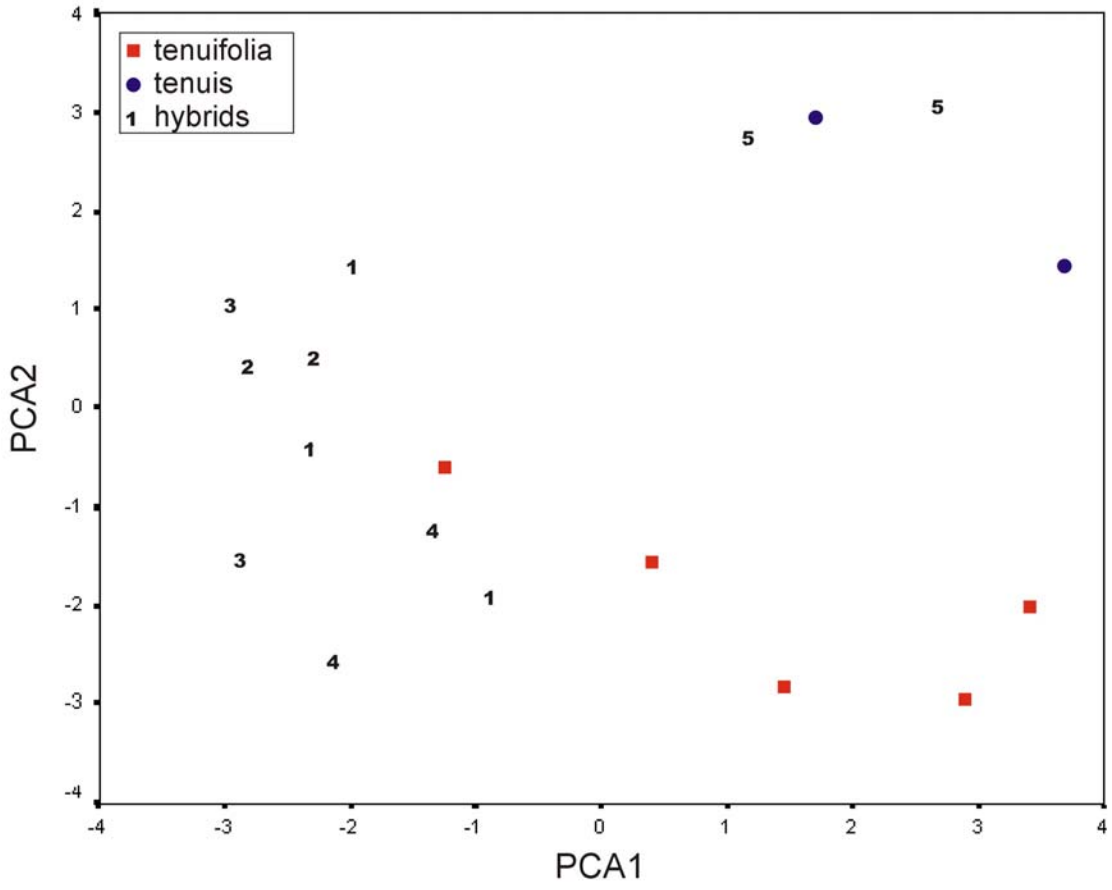

tenuifolia
tenuis
Similarity to parental species
0.80
0.70
0.60
0.50
1
2
3
4
5
Hybrid leaf type

Figure 4.3. Principal components analysis of AFLP data using all scored fragments. Hybrid leaf categories ranged from 1 (*L. tenuis*-like, entire) to 5 (highly dissected).


tenuifolia
tenuis
1 hybrids
PCA2
PCA1
4
3
2
1
0
-1
-2
-3
-4
-4
-3
-2
-1
0
1
2
3
4

CHAPTER 5

CONCLUSIONS

The study of remote island biotas, particularly those of the Pacific Basin, has been fundamental to the development of evolutionary biology and biogeography, and research on islands remains an important focus (Carson, 1996). The dramatic examples of adaptive radiation found on remote chains such as Hawai‘i represent a “microcosm of the evolutionary process” (Emerson, 2002 p. 951), and continue to attract the attention of researchers interested in the process of speciation. Islands are also of interest because of the imperiled status of many remote island biota, which are more susceptible to extinction than their continental counterparts (Rieseberg and Swenson, 1994). The Hawaiian Islands epitomize the characteristics of remote island chains. For example, a high degree of endemism is seen in the native flora, and the islands are home to the world’s most imperiled flora, with over 300 species recognized as threatened or endangered.

Lipochaeta sect. *Aphanopappus* is typical of the adaptive radiations for which remote island chains, such as Hawai‘i, have become famous. The group has diversified from a single common ancestor to numerous species spread throughout the eight main islands in the chain. Nearly all species of *Lipochaeta* sect. *Aphanopappus* are single island endemics with severely restricted ranges; the coastal species *L. integrifolia* is a notable exception. The species of *Lipochaeta* are characterized by differences in growth

habit and vegetative and floral morphologies, and they occur in habitats ranging from coastal scrub to mesic forest, although most of the species occupy habitats at relatively low elevations. Also, most species in the group are federally recognized as threatened or endangered.

I used amplified fragment length polymorphism (AFLP) markers to analyze the patterns of diversification within *L.* sect. *Aphanopappus*. I was particularly interested in determining how the group diversified across islands; in many radiations, the species on the oldest islands are parental to those on the youngest islands, a pattern consistent with Mayr's peripatric model of speciation, which was developed specifically to explain the development of morphologically distinctive species in remote island chains.

Almost all of the AFLP loci examined (95%) exhibited polymorphism within or among species of *Lipochaeta*, although no fixed differences were detected between species. Levels of genetic diversity varied substantially among species. The fewest polymorphic loci and the least heterozygosity were found in the O‘ahu endemic *L. remyi*, which is the only annual species in the group; *L. tenuifolia*, also of O‘ahu had the most polymorphic loci as well as the greatest heterozygosity. Neither *L. remyi* nor *L. tenuis*, also an O‘ahu endemic exhibiting low levels of genetic variability, are currently recognized as endangered. Because low levels of genetic diversity are associated with a greater probability of extinction, a protected status for *L. remyi* and *L. tenuis* may be warranted.

Diversification within *Lipochaeta* did not strictly follow an older-to-younger island colonization pattern. For example, *Lipochaeta venosa* of Hawai‘i is parental to *L. remyi* of O‘ahu. Not all relationships among species were clearly determined, however.

Individuals of *L. tenuifolia* clustered with individuals of both *L. fauriei* of Kauaʻi and *L. tenuis* of Oʻahu, with which *L. tenuifolia* was thought to hybridize. It seems most likely that the species of *L. tenuifolia* and *L. tenuis* resulted from two separate colonizations, and their relationships have been obscured by hybridization.

AFLP data clearly indicated that the population at Puu Kawiwi, where the ranges of *L. tenuifolia* and *L. tenuis* overlap, is of a hybrid origin. While *L. tenuifolia* possessed the highest levels of diversity among species of *Lipochaeta*, genetic variability was higher still in the purported hybrid population. Virtually all of the fragments identified in the two parental species were also found in the hybrid population; a small number of fragments detected in the hybrids were absent from both parents. The variety of intermediate leaf morphologies found in the hybrid population, along with the genetic data, suggest that later generation hybrids or backcrosses occur in the population; interestingly, the individuals that appeared morphologically most similar to *L. tenuifolia* were genetically most similar to *L. tenuis*. While the hybrid population is apparently stable and not spreading, the degree to which gene flow may have occurred between the hybrid population and pure populations of the parental species is unknown and worthy of future study.

The wide distribution of *L. integrifolia*, which occurs on all of the main islands, is unusual within sect. *Aphanopappus*, which otherwise generally consists of single-island endemics. Levels of genetic diversity within populations of *L. integrifolia* were similar to those seen within species of *Lipochaeta*. The amount of genetic diversity appeared to correlate with population size, although not all small populations had low levels of genetic diversity. For example, the population at Makapuʻu, Oʻahu had high levels of

genetic diversity despite a relatively small population size; herbarium records indicate that the distribution of *L. integrifolia* around Makapu‘u was more extensive than its current status. If so, levels of genetic diversity in this population may be expected to fall in the near future due to the effects of genetic drift.

Mayr (1954) believed speciation to be a process fundamentally different from population-level divergence. His peripatric model of speciation (Mayr, 1982), created specifically to describe the adaptive radiations common in remote island chains, proposed that speciation was accompanied by changes in ‘gene complexes.’ Founder-mediated speciation has been a controversial topic within evolutionary biology, however. The multi-island distribution of *L. integrifolia* allowed for a comparison between speciation and population differentiation while controlling for taxonomic history.

Diversification among populations of *L. integrifolia* was compared to the species-level divergence of *L. integrifolia*, *L. remyi* and *L. venosa*. Speciation was distinguished from population-level divergence by both quantitative and qualitative differences. As measured by gross divergence, all populations of *L. integrifolia* were more similar to one another than to either *L. remyi* or *L. venosa*; also, each population of *L. integrifolia* was more similar to *L. venosa* than *L. remyi*. Interestingly, however, the divergence between *L. remyi* and *L. venosa* was within the range of divergences observed among populations of *L. integrifolia*. The strongest support for the difference between population and species-level divergence came from a principal components analysis. Only a single component was necessary to differentiate the species of *L. integrifolia*, *L. remyi* and *L. venosa*, indicative of strong associations among loci within species. In contrast, populations of *L. integrifolia* failed to segregate when the first two components were

plotted. While these data are generally consistent with peripatric model, it is difficult to exclude gradual speciation as the cause of these patterns.

This brings up the important question of whether the peripatric model of speciation is workable. Peripatric speciation is a verbal not mathematical model. Another problem with founder-mediation models is that the very species complexes they were created to describe may be fundamentally different from continental groups. For example, species in remote island chains have traditionally been described based on morphological characteristics. Because species within an adaptive radiation are often geographically isolated from one another, the degree to which reproductive isolation, which is key to delineating continental species, occurs is unknown for most island taxa. In fact, hybridization among species within many radiations of Hawaiian plants can often readily be induced in laboratory conditions. Until explicit tests of founder-mediated models of speciation are determined, a gradual pattern of speciation should be assumed.

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