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Population genetic structure of two rare tree species (*Colubrina oppositifolia* and *Alphitonia ponderosa*, Rhamnaceae) from Hawaiian dry and mesic forests using random amplified polymorphic DNA markers

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Abstract

Hawaiian dry and mesic forests contain an increasingly rare assemblage of species due to habitat destruction, invasive alien weeds and exotic pests. Two rare Rhamnaceae species in these ecosystems, *Colubrina oppositifolia* and *Alphitonia ponderosa*, were examined using random amplified polymorphic DNA (RAPD) markers to determine the genetic structure of the populations and the amount of variation relative to other native Hawaiian species. Relative variation is lower than with other Hawaiian species, although this is probably not a consequence of genetic bottleneck. Larger populations of both species contain the highest levels of genetic diversity and smaller populations generally the least as determined by number of polymorphic loci, estimated heterozygosity, and Shannon's index of genetic diversity. Populations on separate islands were readily discernible for both species as were two populations of *C. oppositifolia* on Hawai'i island (North and South Kona populations). Substructure among Kaua'i subpopulations of *A. ponderosa* that were ecologically separated was also evident. Although population diversity is thought to have remained at pre-disturbance levels, population size continues to decline as recruitment is either absent or does not keep pace with senescence of mature plants. Recovery efforts must focus on control of alien species if these and other endemic dry and mesic forest species are to persist.

Keywords: endangered plants, Hawai'i, population genetics, RAPD, Rhamnaceae

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Introduction

Dry forests are regarded as the most endangered major tropical ecosystem (Janzen 1988), and mesic forests have been impacted similarly in some regions (Gagné & Cuddihy 1999). Habitat loss to urban development and agriculture in this ecosystem has resulted in fragmentation of once extensive populations, and this coupled with low population densities has created small effective population sizes for dry and mesic forest organisms worldwide (Murphy & Lugo 1986; Janzen 1988; Hamrick & Murawski 1991). The genetic consequences of small population size has received the attention of conservation biologists in their attempts to characterize the prognosis of recovery for many rare and endangered species (Franklin 1980; Barrett & Kohn 1991; Ellstrand & Elam 1993). The concern for these species is their ability to persist in the face of reduced genetic variation and increased population differentiation associated with small population size (Bijlsma *et al*. 1994; Godt *et al*. 1996; Fischer & Matthies 1998). These problems are often exacerbated in island systems. For example, the threat of fire, alien plant invasion, ungulate and insect plant damage and human disturbance have aided in the loss of over 90% of Hawaiian dry forests (DLNR 1992). The magnitude of this disturbance has resulted in a mosaic of suitable habitat for Hawaiian dry and mesic forest species (Mehrhoff 1996) as well as species populations with age structures skewed toward older individuals due to limited regeneration (Medeiros *et al*. 1986).

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Island species have often been considered a model for the study of rarity due to their limited geographical range, habitat specificity, small population sizes and high level of endemism (Carlquist 1980; Crawford *et al*. 1987b). They have also been characterized as being genetically depauperate due to the recency of the founding event, isolation from source population, occurrence of stochastic processes and populations limited by size and distribution within the island environment (Carlquist 1980; Crawford *et al*. 1987a, 1988, 1990; Brauner *et al*. 1992; Elisens 1992). In particular, Pacific Island species represent an even more fragile resource because different island populations are scattered widely and overall population sizes are small (Sheely & Meagher 1996). Further, lowland habitat destruction in Hawai'i by Polynesians (Athens 1997) and in historic times (Gagné & Cuddihy 1999) has drastically altered the vegetation present, and Hawai'i is now referred to as the 'endangered species capitol of the nation' (Royte 1995), with nearly one-half of the species of native flora considered either endangered, threatened or species of concern (US Fish and Wildlife Service, Honolulu office, personal communication).

Colubrina oppositifolia Brogn. ex Mann and *Alphitonia ponderosa* Hillebr. (Rhamnaceae) are two forest tree species endemic to the Hawaiian Islands. Overlapping ecological ranges and similar growth form, wood and floral characters led native Hawaiians to name both species as *kauila* (Porter 1972; Abbott 1992). Populations of *C. oppositifolia* are found primarily in dry forests along the Kona Coast of Hawai'i and the Wai'anae Mountains of O'ahu, whereas *A. ponderosa* has its largest populations in dry and mesic forests of Kaua'i and smaller populations on other islands. *Kauila* was treasured for its extremely hard and beautiful wood (cf. Lamberton 1955) that allowed it to assume a role in the Hawaiian economy similar to that of metal in other civilizations (Wagner *et al*. 1999), and at least 24 uses of the wood are cited in the early literature (see Abbott 1992 and Krauss 1993 for reviews).

Both species of *kauila* are rare. The long-term survival of each is threatened by invasive alien weeds, insect damage, limited recruitment and human impacts (USFWS 1996). Currently, eight of the nine localities of *C. oppositifolia* (a federally listed endangered species) contain less than 50 individuals (USFWS 1996). Although not listed federally as endangered, *Alphitonia ponderosa* has been recognized recently as a 'species of concern' with fewer than 50 individuals on every island except Kaua'i (Hawai'i Heritage Program 1988; US Fish and Wildlife Service, Honolulu Office, personal communication).

The purpose of this study was to investigate the genetic diversity that is present within and among populations of *kauila*, both *C. oppositifolia* and *A. ponderosa*, using random amplified polymorphic DNA (RAPD) markers. The principle objectives were to: (i) assess the level of genetic variation in both species; (ii) determine the degree of population subdivision and differentiation; (iii) observe population genetic structure; and (iv) determine if there is a relationship between population size and the level of variation. Genetic studies of tropical tree species are becoming more numerous, but the majority of them feature New World species with continental distributions (Hamrick & Loveless 1989; Hamrick & Murawski 1991; Eguiarte *et al*. 1992; Hall *et al*. 1994, 1996), often neglecting oceanic island species. The Hawaiian dry forests contain a diverse assemblage of species (Rock 1913; Gagné & Cuddihy 1999) that face similar challenges to those of *kauila*, and understanding the genetic structure of *kauila* populations may provide insight useful in establishing management practices to preserve this endangered ecosystem.

Materials and methods

Sample collections and DNA extraction

Leaf tissue of *Colubrina oppositifolia* was collected from three populations distributed on the islands of O'ahu and Hawai'i, and a single recently discovered individual on Maui. Leaf tissue of *Alphitonia ponderosa* was collected from one population on each of the islands of Kaua'i, O'ahu and Maui. Size of populations and number of individuals collected are provided in Table 1. Individuals of larger populations were sampled throughout the area with the provision that only mature trees were selected; all accessible plants of smaller populations were sampled. At least 10 trees per population were sampled except on Maui for the single plant of *C. oppositifolia* and on O'ahu where only five extant individuals of *A. ponderosa* are known and one was accessible (Table 1). Plants occur frequently on rocky and inaccessible mountain sides, and in some cases sampling was limited due to safety concerns. A single voucher specimen representative of the population was prepared for each locality and deposited at the B. P. Bishop Museum (BISH).

A. ponderosa is distributed broadly on Kaua'i and estimates have been made of up to 10 000 individuals on this island with 2000–3000 individuals in the Koke'e area [KR Wood (National Tropical Botanical Garden) and J Lau (Hawai'i Heritage Program), personal communication]. Five subpopulation (Table 1) were collected in a 6-km^2 region in the vicinity of Koke'e State Park (approximately 900–1100 m elevation) to test for substructure to this population. Three southerly subpopulations (Koke'e Road, Miloli'i Ridge, and Makaha Ridge) are separated from the two northerly subpopulations (Nualolo Trail and Awa'awapuhi Trail) by a wet forest in Mahanaloa Valley, *c*. 2–3 km broad. Distances among populations within these clusters was 1–1.5 km.

Leaf specimens were placed into sealed plastic bags and chilled until DNA was extracted. Total DNA was extracted

Table 1 Individuals of *Colubrina oppositifolia* and *Alphitonia ponderosa* sampled. Estimates of population sizes from US Fish and Wildlife Service, Honolulu Office

Island	Locality	*N*	N_s	HPDL	Voucher
Colubrina oppositifolia					
Hawai'i	Ka'ūpūlehu, North Kona	185–205	20	949–963, 1213–1231†	*Kwon sn.*
	Manukā, South Kona	30	10	134, 148–156	*Morden 1115*
Maui	Kapunakea Preserve	1	1	1235	*Kwon sn.*
O'ahu	North Wai'anae Mountains	94	13	949–963, 1232–1234†	*Kwon sn.*
Alphitonia ponderosa					
Kaua'i	Koke'e State Park*	2000–3000		1236–1284	*Kwon sn.*
	Awa'awapuhi Trail		4	1280–1284†	
	Koke'e Road		3	1266–1268	
	Makaha Ridge		11	1236–1260†	
	Miloli'i Ridge		5	1261–1265†	
	Nualolo Trail		11	1269–1279	
Maui	Auwahi-Kanaio	20–30	5	1285–1289	*Kwon sn.*
O'ahu	Makaleha Valley	5	1	1290	*Kwon sn.*

N, estimated population size; N_s, number of plants sampled; HPDL, accessions of each individual are deposited in the Hawaiian Plant DNA Library (Morden *et al*. 1996; Randell & Morden 1999).

*Estimates of population size of *A. ponderosa* in the Koke'e dry and mesic forests from J Lau (Hawai'i Heritage Program) and KR Wood (National Tropical Botanical Garden).

†Samples used in the analysis are an arbitrary subset of the total samples collected and extracted. Once selected, samples were used throughout the study.

from 0.5 to 1.0 g of fresh leaf material using the CTAB method of Doyle & Doyle (1987) with some minor modifications (Morden *et al*. 1996). All DNA samples were purified by caesium chloride density gradient ultracentrifugation. Water saturated butanol was used to remove the ethidium bromide, and the banded DNA dialysed to remove the caesium. DNA samples were accessioned into the Hawai'i Plant DNA Library (Morden *et al*. 1996; Randell & Morden 1999).

RAPD analysis

Approximately 25 ng of DNA was amplified via the polymerase chain reaction in 25 μL volume under the following conditions: *c*. 0.2 μM random 10-mer oligonucleotide primers and 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1× Taq polymerase PCR buffer, 1.5 mM $MgCl_2$, and *c*. 1 unit of Taq polymerase (Promega, Madison, WI, USA). Each reaction was overlaid with two drops of mineral oil. Amplifications were performed in a Hybaid Omni Gene thermocycler programmed for one cycle at 94 °C for 3 min, 35 °C for 30 s, and 72 °C for 2 min, followed by 43 cycles at 95 °C for 45 s, 35 °C for 30 s, and 72 °C for 2 min, and a final cycle at 94 °C for 45 s, 35 °C for 30 s, and 72 °C for 6 min Amplification products were mixed with loading dye (20 mM EDTA, 10% glycerol, 1% sarcosyl with bromophenol blue and xylene cyanol) and separated in 1.5% agarose in 0.5 × TBE (tris-borate-EDTA) buffer with 125 ng ethidium bromide per litre. Sizes of the amplification products were estimated by using a pBS plasmid (Stratagene, La Jolla, CA, USA) digested with restriction enzymes to produce fragments in a size range of 0.448–2.96 kb. RAPD primers (Operon Technology, CA, USA; kits OPE-OPI) were screened for amplification of *kauila* DNA and selected primers were then used for amplification of all individuals. Molecular markers were identified by the primer used to generate them and their approximate size as estimated from the pBS marker. Gel scoring was performed independently by both authors to produce unbiased and unambiguous analysis of RAPD amplifications.

Data analysis

Assumptions associated with RAPD marker analysis were made as described by Lynch & Milligan (1994). A RAPD marker was determined to be polymorphic when found in less than 95% of the individuals sampled (i.e. absent in three or more individuals). Absence of a marker within a population, although present in others, was assumed to indicate that all individuals of the population were null/null homozygotes rather than there being a loss of the locus. Expected heterozygosity was calculated for each population (H_s) and species (H_t) for each locus as follows:

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

where p is the frequency of the dominant allele and q is the frequency of the null allele. Allele frequencies were estimated from the number of null/null homozygotes present in the population (Hartl & Clark 1989, also see

Morden & Loeffler 1999). Genetic relationships within and among populations were estimated using similarity coefficients and UPGMA cluster analysis with the NTSYS-pc computer program (Rohlf 1993). Genetic similarity between individuals was estimated using the similarity coefficient of Dice (1945). Similarity coefficients from the triangular data matrix were grouped by population in order to calculate an average similarity value within and among populations. Principal coordinate analysis (PCO) with Gower general similarity coefficient was calculated using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1986–1999). Summary statistics of average similarity measures (means, standard errors and *t*-test) were calculated using MINITAB (1996). Distribution of genetic variation within and among populations was estimated using Shannon's information measure:

$$H_o = -\Sigma\, p_i \log_2 p_i$$

where p_i is the frequency of a given RAPD phenotype within a population or species group (Lewontin 1973; King & Schaal 1989). A RAPD phenoptype consists of the collection of presence/absence data over all scored markers for a given RAPD primer. Analysis of molecular variation (AMOVA; Excoffier *et al*. 1992) was used to estimate variance components and to test the significance of partitioning of RAPD variation within and among populations.

Results

RAPD profiles

Of the 100 RAPD primers screened, 18 produced repeatable amplification products in *Colubrina oppositifolia* that were scored for band presence/absence and yielded 155 scorable RAPD markers (Table 2). Of these, 82 (53%) were present for all 44 individuals and 73 (47%) markers

Table 2 Primer name, nucleotide sequence, number of scored amplification products, and partitioning of genetic diversity within and among populations using Shannon's Index for *Colubrina oppositifolia* (18 random oligonucleotide primers) and *Alphitonia ponderosa* (20 random oligonucleotide primers)

		C. oppositifolia					*A. ponderosa*				
Primer	Primer sequence	Scored products	H_{POP}	H_{SP}	D_{WP}	D_{AP}	Scored products	H_{POP}	H_{SP}	D_{WP}	D_{AP}
OPE-01	CCCAAGGTCC	13	3.579	5.471	0.654	0.346	14	1.515	2.298	0.659	0.341
OPE-03	CCAGATGCAC	5	0.604	0.994	0.607	0.393	—	—	—	—	—
OPE-18	GGACTGCAGA	7	1.597	2.139	0.746	0.254	6	0.935	1.811	0.516	0.484
OPE-19	ACGGCGTATG	6	0.414	1.347	0.307	0.693	—	—	—	—	—
OPE-20	AACGGTGACC	7	0.569	1.877	0.303	0.694	8	1.184	1.428	0.829	0.171
OPF-03	CCTGATCACC	—	—	—	—	—	6	1.862	2.897	0.643	0.357
OPF-04	CCTGATCACC	6	1.171	1.410	0.831	0.169	—	—	—	—	—
OPF-05	CCGAATTCCC	5	0.497	1.375	0.361	0.639	—	—	—	—	—
OPF-09	CCAAGCTTCC	4	0.861	1.068	0.806	1.194	4	0.454	0.984	0.462	0.538
OPF-12	ACGGTACCAG	11	2.157	3.653	0.591	0.409	6	0.458	0.831	0.551	0.449
OPG-09	CTGACGTCAC	10	1.752	30.76	0.570	0.430	—	—	—	—	—
OPG-11	TGCCCGTCGT	10	2.245	2.922	0.768	0.232	4	0.945	2.113	0.447	0.553
OPG-13	CTCTCCGCCA	13	2.866	4.471	0.641	0.359	5	1.934	2.434	0.795	0.205
OPG-14	GGATGAGACC	—	—	—	—	—	6	1.356	2.753	0.493	0.507
OPG-16	AGCGTCCTCC	—	—	—	—	—	6	0.454	0.971	0.468	0.532
OPG-17	ACGACCGACA	—	—	—	—	—	9	2.111	3.185	0.663	0.337
OPG-18	GGCTCATGTG	8	0.000	0.000	—	—	—	—	—	—	—
OPG-19	GTCAGGGCAA	—	—	—	—	—	10	2.364	3.333	0.709	0.291
OPH-05	AGTCGTCCCC	—	—	—	—	—	9	0.917	1.710	0.536	0.464
OPH-06	ACGCATCGCA	—	—	—	—	—	7	1.187	1.670	0.711	0.289
OPH-07	CTGCATCGTG	3	0.156	0.267	0.586	0.414	5	1.339	2.488	0.538	0.462
OPH-08	GAAACACCCC	15	2.855	4.315	0.669	0.331	9	1.099	1.633	0.673	0.327
OPH-09	TGTAGCTGGG	14	2.946	4.164	0.707	0.293	6	0.458	0.941	0.486	0.514
OPI-12	AGAGGGCACA	11	1.022	2.394	0.428	0.572	5	0.161	0.286	0.563	0.437
OPI-14	TGACGGCGGT	7	1.108	1.369	0.810	0.190	6	1.266	1.720	0.736	0.264
OPI-16	TCTCCGCCCT	—	—	—	—	—	7	1.050	1.168	0.899	0.101
Ave.		8.6	1.468	2.351	0.625	0.375	6.7	1.152	1.833	0.619	0.381

H_{POP}, average diversity within populations; H_{SP}, diversity within species; D_{WP}, proportion of diversity within populations as measured by H_{POP}/H_{SP}; D_{AP}, proportion of diversity among populations as measured by $(H_{SP}–H_{POP})/H_{SP}$.

Table 3 Analysis of molecular variance (AMOVA) for 44 individuals in four populations of *C. oppositifolia* and 40 individuals in three populations of *A. ponderosa*

Source of variation	d.f.	SSD	MSD	Variance component	% Total	*P*-value
Colubrina oppositifolia						
Among populations	3	241.03	80.45	7.46	45.73	< 0.002
Within populations	40	354.39	8.86	8.85	54.27	< 0.002
Alphitonia ponderosa						
Among populations	2	58.60	29.30	3.84	29.35	< 0.002
populations	37	341.99	9.34	9.24	70.65	< 0.002

d.f., degrees of freedom; SSD, sum of squared deviation; MSD, mean squared deviation;% Total, percentage of total variance contributed by each component; *P*-value, probability of obtaining a more extreme component by chance alone.

were polymorphic. Similarly, 20 RAPD primers were used to produce repeatable amplification products in *Alphitonia ponderosa*. These 20 primers yielded a total of 138 scorable RAPD markers, of which 81 (59%) were present in all 40 individuals and 57 (41%) polymorphic.

Levels of variation in *C. oppositifolia*, measured by the number of polymorphic markers, exhibited slight differences among populations and displayed no apparent relationship to the number of individuals sampled in each population. The O'ahu population was most variable (50 polymorphic markers), although the estimated population size (Table 1) is only half that of North Kona (45 polymorphic markers). The smallest estimated population, South Kona, also had the fewest (40) polymorphic markers. (Data from the single Maui individual were not appropriate for this statistic.) A total of 144 (93%) RAPD markers were shared by more than one population, while the remaining 11 markers (7%) were confined to, and diagnostic of, single populations (seven to O'ahu, three to North Kona and one to Maui). Five markers were unique to and shared among the two Hawai'i populations, and five additional markers were shared among individuals from Maui and Hawai'i.

In contrast, the amount of population variation found in *A. ponderosa* was clearly related to the size of the population. Of these, the number of polymorphic markers was more than twice as high in the Kaua'i population (52 markers) than within the Maui population (23 markers). (Data from the single O'ahu individual were not appropriate for this statistic.) A total of 112 RAPD markers are shared by more than one population, while the remaining 26 were confined to either the Kaua'i (22 markers) or Maui (four markers) populations; the O'ahu plant had no unique markers.

Genetic diversity estimates

Shannon's Index was used to examine genetic diversity partitioned into within and among population components for each of the 18 primers used with *C. oppositifolia* and 20 primers used with *A. ponderosa* (Table 2). Diversity within *C. oppositifolia* populations, H_{POP}, ranged from 0.000 (no variation within population) to 3.579 with a mean of 1.468, whereas diversity of the entire species, H_{SP}, ranges from 0.000 (no variation present) to 5.471 with a mean of 2.351. Examination of the diversity present within and among populations indicates that, on average, most of the diversity (63%) is found within *C. oppositifolia* populations, although considerable variation among populations (37%) also exists. These results are similar to those from AMOVA (Table 3) where slightly more variability is attributed to individuals within populations (54%) rather than among populations (46%).

Genetic diversity partitioned within and among *A. ponderosa* populations showed a pattern similar to that of *C. oppositifolia* (Table 2). H_{POP} ranged from 0.161 to 2.364 with a mean of 1.152, whereas H_{SP} ranged from 0.286 to 3.333 with a mean of 1.833. Examination of the diversity present within and among populations is very similar to that of *C. oppositifolia*; most of the diversity (62%) found within *A. ponderosa* populations yet considerable variation (38%) still evident among populations. This too is consistent with results of AMOVA (Table 3) that show more variation within populations (71%) rather than among populations (29%).

Heterozygosity estimates

Total species heterozygosity within *C. oppositifolia* varied from 0.023 to 0.500 among the 73 polymorphic markers with a mean estimated heterozygosity (H_P) of 0.389 (Table 4). Of the three populations, North Kona plants displayed the highest level of mean estimated heterozygosity (0.310 over polymorphic loci), whereas South Kona plants displayed considerably less variation (0.286) and the O'ahu population was lowest (0.271). Heterozygosity estimates using all markers (H_A) showed a pattern similar to those of the polymorphic markers although much lower (Table 3). Estimates of average genetic diversity based on Shannon's Index (H_O) within the three populations were similar, and North Kona again had the highest level of diversity (1.692), followed by O'ahu (1.463) and South Kona (1.249).

Table 4 Genetic variability among populations of *Colubrina oppositifolia* and *Alphitonia ponderosa*

Population	P	H_P	H_A	H_O
Colubrina oppositifolia				
South Kona	40 (26%)	0.286	0.134	1.249
North Kona	45 (29%)	0.310	0.146	1.693
O'ahu	50 (32%)	0.271	0.128	1.463
All individuals*	73 (47%)	0.389	0.183	2.351
Alphitonia ponderosa				
Kaua'i	52 (37%)	0.325	0.116	1.762
Maui	23 (17%)	0.148	0.053	0.543
All individuals*	57 (41%)	0.331	0.118	1.833

P, number (and percentage) of polymorphic markers; H_P, estimated mean heterozygosity over polymorphic markers; H_A, estimated mean heterozygosity over all markers; H_O, mean genetic diversity estimated by Shannon's Index.

Total estimated mean heterozygosity (H_P) in *A. ponderosa* varied from 0.025 to 0.500 with a mean of 0.331 for polymorphic loci (Table 4). Mean estimated heterozygosity was highest for plants on Kaua'i (0.325), whereas heterozygosity among individuals on Maui was considerably lower at (0.148). Estimates of average genetic diversity based on Shannon's Index (H_O) displayed similar disparity in the relative amounts of variation among the two populations with the Kaua'i plants (1.762) possessing a much greater diversity than Maui plants (0.543).

Genetic similarity indices

Genetic similarity within and among populations was calculated using the similarity coefficient of Dice (1945) where the coefficient ranges between 0 and 1 with the former indicative of complete genetic dissociation and the latter genetic identity. Overall, the *C. oppositifolia* individuals sampled exhibited a high degree of genetic similarity (0.888). As expected, individuals were most similar to members of their own population. Plants from South Kona shared the highest similarity (0.938); the North Kona and O'ahu populations only slightly lower (0.929 and 0.921, respectively). For interpopulation comparisons, North and South Kona populations were most similar to each other (0.920), and most distant from the O'ahu population (0.843 and 0.840, respectively). The single Maui tree was about equally similar to each of the other three populations (0.863 to North Kona, 0.859 to South Kona, and 0.849 to O'ahu), thus the Maui plant is regarded as a remnant of a distinct and previously unknown population rather than a recent introduction or colonization from a neighbour island. Relationships among population and the relative levels of within population variation are clearly reflected in the UPGMA cluster analysis (Fig. 1) and PCO

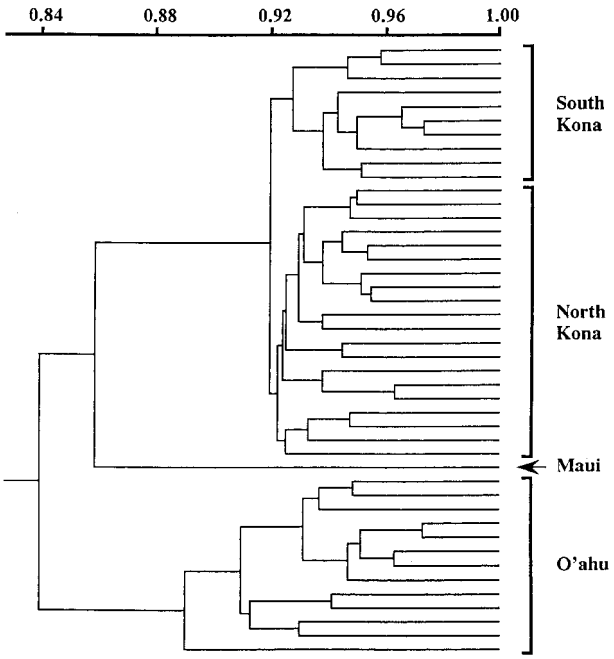

Fig. 1 UPGMA cluster analysis of *Colubrina oppositifolia* individuals sampled. Bar scale represents coefficient of genetic similarity.

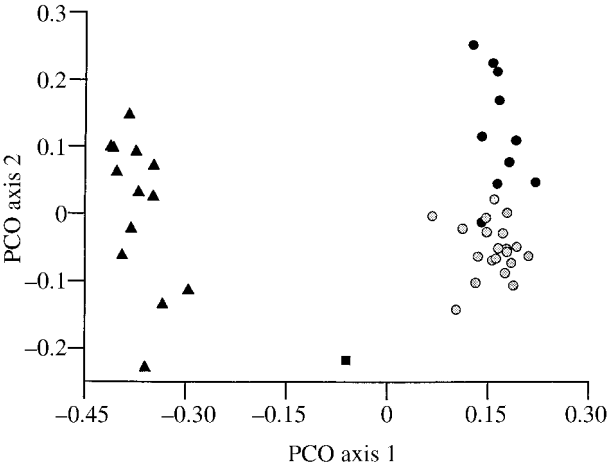

Fig. 2 Principal coordinate analysis of RAPD data using all scored markers for *Colubrina oppositifolia*. PCO axis 1 and 2 accounted for 40.2% of the overall variation. Population symbols: circle, Hawai'i (solid, South Kona; shaded, North Kona); square, Maui; triangle, O'ahu.

(Fig. 2). Cluster analysis resulted in three distinct clusters with members of each population grouped exclusively together; South Kona and North Kona individuals were associated most closely, the Maui plant associated more closely with the Hawai'i plants and the O'ahu population most distant genetically. In contrast, only two major groups, separating O'ahu and Hawai'i populations, were evident in the initial PCO; North and South Kona populations segregate along a continuum of variation in

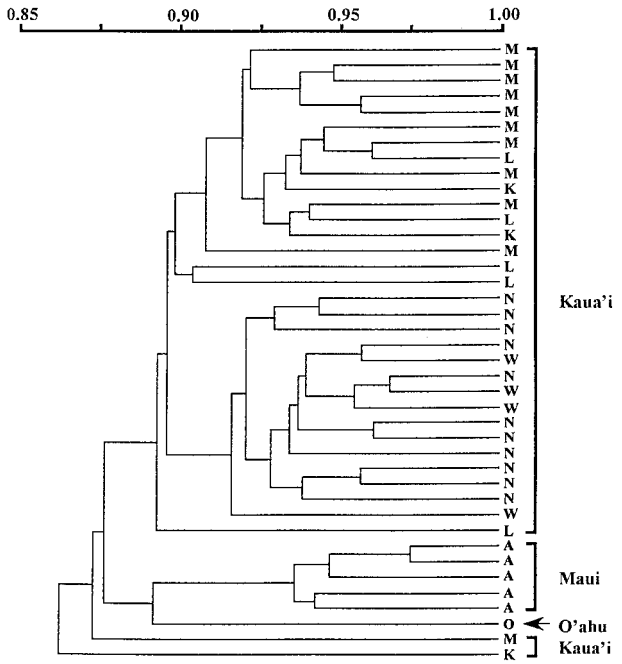

Fig. 3 UPGMA cluster analysis of *Alphitonia ponderosa* individuals sampled. Subpopulation sources of Kaua'i plants: K, Koke'e Road; L, Miloli'i Ridge; M, Makaha Ridge; N, Nualolo Trail; W, Awa'awapuhi Trail. Bar scale represents coefficient of genetic similarity.

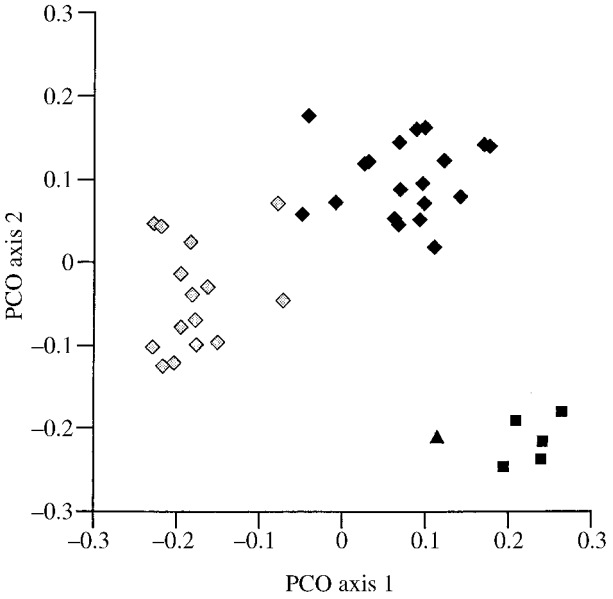

Fig. 4 Principal coordinates analysis of RAPD data using all scored markers for *Alphitonia ponderosa*. PCO axis 1 and 2 accounted for 26.9% of the overall variation. Population symbols: diamond, Kaua'i (shaded, Nualolo and Awa'awapuhi Trail subpopulations; solid, Koke'e Road, Miloli'i Ridge, and Makaha Ridge subpopulations); square, Maui; triangle, O'ahu.

PCO axis 2. A subsequent analysis with samples from only North and South Kona populations did indicate the populations were clearly distinct (data not shown). As expected, the single Maui individual was intermediate to the O'ahu and Hawai'i populations.

Overall genetic similarity among *A. ponderosa* individuals was higher (0.906) than that found in *C. oppositifolia* (0.888) although similarity was lower within specific populations. Maui plants shared a higher level of similarity (0.948) than was found for Kaua'i plants (0.912). As in *C. oppositifolia*, each individual was most similar to members of its own population, the average genetic similarity between Maui and Kaua'i individuals being considerably lower (0.889) than found within the populations. These relationships among individuals and populations are depicted graphically with cluster analysis (Fig. 3) and PCO (Fig. 4). Cluster analysis revealed a large group that included all but two of the Kaua'i individuals and a second group that contained the five individuals from Maui and the single O'ahu plant. Each of the three island groups were also identified in PCO, although separation of island populations was not as clear as for *C. oppositifolia*; plants from Maui and O'ahu were clearly separated from those of Kaua'i although not as clearly separated from each other. Each subpopulation from Kaua'i was not readily distinguishable from one another in the analysis, yet there is clear evidence of distinct subgrouping such that individuals from Nualolo and Awa'awapuhi Trails were separate from the three subpopulations to the South.

Discussion

Relative genetic variation

Both *Colubrina oppositifolia* and *Alphitonia ponderosa* exhibited low levels of genetic diversity in comparison to other Hawaiian plant taxa that have also been examined using RAPD markers. The percentage of polymorphic loci in these species (47% and 41%, respectively) across their entire distribution is lower than that found in *Dubautia ciliolata* (70%; Caraway *et al.* 2001), *D. scabra* (59%; Caraway *et al.* 2001) and *Haplostachys haplostachya* (51%; Morden & Loeffler 1999) in just a single population, but more than was found in two populations of silversword (*Argyroxiphium sandwicensis* ssp. *sandwicensis*, 12.2%, and *A.* s. ssp. *macrocephalum*, 14.6%; Friar *et al.* 1996), a severely bottlenecked endangered species. Other species with interisland distributions that have been examined show much greater levels of polymorphism than found in *kauila* (75% in *Touchardia latifolia*, Loeffler 1997; > 80% within *Labordia* spp., Motley 1996). Genetic similarity within and among populations showed a similar trend of reduced variability relative to these other Hawaiian species.

Both *kauila* species are long-lived woody perennials, whereas most other Hawaiian taxa that have been examined

for RAPD variation to date are short-lived herbaceous (Morden & Loeffler 1999), woody perennials (Motley 1996; Loeffler 1997; Caraway *et al*. 2001) or monocarpic plants (Friar *et al*. 1996). One would expect populations of *kauila* to harbour higher levels of genetic variation than found in other species (Hamrick & Loveless 1989; Hamrick & Godt 1990), yet this is not the case. However, it is likely that genetic diversity now extant is representative of the diversity present prior to European contact over 200 years ago. Most extant plants are older mature trees in populations that have probably experienced minimal impact from genetic drift (very little regeneration is occurring and many plants may actually predate European contact), and the large population of *A. ponderosa* on Kaua'i has levels of genetic diversity similar to that found in populations of *C. oppositifolia*. As such, differences found between *kauila* species and other Hawaiian taxa investigated are probably a consequence of factors unrelated to population disturbance. Overall, both species demonstrate a high level of similarity among populations suggesting that they have experienced recent gene flow, have recently diverged and/or were each founded by individuals harbouring similar genotypes (Gemmill *et al*. 1998), the latter explanation being the most probable.

Population size and diversity

Genetic diversity within populations was not related closely to estimated population size in *C. oppositifolia*, but was strongly so in *A. ponderosa*. There are about twice as many plants of *C. oppositifolia* in North Kona as there are on O'ahu and six times as many than in South Kona. As predicted, estimated heterozygosity was highest in the North Kona population, but was next highest in the South Kona population. In contrast, polymorphism and estimated genetic diversity was higher among the O'ahu plants than either Kona population. These data suggest that the South Kona and O'ahu populations were at one time much larger, and reduction in population sizes have been recent with little or no loss in genetic variation.

Trends in population variation for *A. ponderosa* were as predicted. The Kaua'i population is much larger and distributed over a wider geographical area compared to the Maui population, and similarly had higher levels of polymorphism, estimated heterozygosity and genetic diversity. Although this relationship is probably related to population size, it is difficult to establish with certainty because of the unbalanced number of individuals sampled from each island (34 from Kaua'i compared to only five plants found on Maui). However, there are few extant individuals believed to still exist on Maui (Medeiros *et al*. 1986), and as many as 25% of these were examined in this study. Thus, it is unlikely that sampling more of the Maui individuals would significantly alter these results. Overall, data for these species indicate that populations encompassing a larger geographical area are retaining more genetic diversity than smaller or more isolated ones.

Distribution of variation

The majority of the variation in both *kauila* species was found within rather than among populations as estimated by both Shannon's index and AMOVA analysis. This is attributed to the majority of markers being present in all populations. Only 18% of the *A. ponderosa* polymorphic markers and 14% of those in *C. oppositifolia* are found exclusively in a single population. Long-lived, woody, late-successional organisms such as these typically harbour a greater percentage of their variation within populations (Hamrick & Loveless 1989; Hamrick & Godt 1990). Although both *kauila* species have low genetic diversity relative to other Hawaiian species (discussed above), it is possible that the handful of alleles unique to each population are signs of differentiation among the populations following selection or drift from the ancestral condition. Alternatively, these alleles may represent new mutations that have arisen within populations following their initial dispersal.

It is interesting to note that the Maui plants of *A. ponderosa* are more similar to those of the Kaua'i population than is the single O'ahu tree suggesting a possibility other than that of a stepping stone colonization on the three islands. Most biogeographical patterns that have been discerned for Hawaiian species involve a stepping stone colonization (or the 'progression rule'; Funk & Wagner 1995) from older to younger islands (i.e. Kaua'i to O'ahu to Maui in this example). *Colubrina* populations reflect this type of distribution, but notable exceptions have been found among other taxa (Carson 1983; Carr *et al*. 1989). The possibility exists with *A. ponderosa* that there was a colonization from Kaua'i to Maui with a back-dispersal to O'ahu. Alternatively, if the original founder of *Alphitonia* was on Maui, there could have been separate dispersal events to both O'ahu and Kaua'i. However, this latter possibility is unlikely (Barrett & Husband 1990) given the species broad distribution on Kaua'i, the oldest of the high Hawaiian Islands (Carson & Clague 1995), and its limited distribution and variation on Maui and O'ahu. Because there were few samples available for study here and the limitations of RAPD markers, this should be further explored by other means such as microsatellite markers.

Population differentiation

Despite the apparently high levels of similarity within a species, individuals of both *C. oppositifolia* and *A. ponderosa*

are most similar with other individuals of their own population. UPGMA cluster analysis and PCO shows distinct intrapopulation clustering of all members of both species with the exception of two *A. ponderosa* outlier plants on Kaua'i. There are noteworthy signs of genetic distinction between populations, and because of limited gene flow this population differentiation is likely to continue among both species. Isolation, created by geographical distance and fragmentation, has provided the initial means by which populations may diverge.

Pollination biology in these species has not been examined closely, but evidence suggests that pollen movement among populations is restricted geographically. Mature individuals of both species are relatively large, reaching up to 20 m in height with large spreading crowns containing hundreds of flowers. As with most members of the Rhamnaceae, ample nectaries in flowers attract insect pollinators, and insects have been observed visiting the flowers of these species (JA Kwon, personal observation). Hamrick & Loveless (1989) found high rates of gene flow via pollen movement among populations of trees in a wet tropical forest for distances of up to 2–3 km. Although the Kaua'i individuals of *A. ponderosa* examined share high similarity, genetic structuring is evident among the subpopulations suggesting gene flow is restricted and that localized inbreeding may be occurring. Although several potential factors may be important in limiting gene flow at this site (e.g. topography, available moisture and or flight range of pollinators), the separation of these subpopulations by a wet valley is undoubtedly the overriding factor by creating an ecological boundary that pollinators do not penetrate.

Little is also known about *kauila* seed dispersal or palatability, but it had been thought that gene flow among populations by this means was minimal beyond their initial colonization (cf. Guppy 1906; Carlquist 1980). Fruits of both species are woody capsules that encase seeds *c*. 6 mm long (terete in *C. oppositifolia*; flattened in *A. ponderosa*). Although fruits of *C. oppositifolia* are explosively dehiscent (Wagner *et al*. 1999), seeds of *A. ponderosa* remain enclosed within the capsule. No extant native birds have mandibles powerful enough to open these fruits, yet large species of crows and other birds now extinct (Olson & James 1982) may have been important dispersers of these species previously. Further, seeds of both species are buoyant in sea water (CW Morden, personal observation). Dry forests are typically associated with leeward coast regions of all islands, and ocean currents may have also played a role in dispersal of these species. Thus, seed dispersal and gene flow within and among island populations may have been considerably greater prior to Polynesian inhabitation and the large-scale destruction of low elevation forests (Athens 1997) and extinction of bird species (Olson & James 1982) that followed.

Conservation implications

Maintaining current population numbers may not be enough to ensure species survival for either species of *kauila* over the long-term. The impact of alien species has played a pivotal role in the erosion of plant diversity of Hawaiian dry and mesic forests. Recruitment of native species is reduced severely when seedlings are overcrowded by alien grass species, such as fountain grass (*Pennisetum setaceum*) (Mehrhoff 1996) and kikuyu grass (*Pennisetum clandestinum*) (A. Medeiros, Haleakala National Park, personal communication), or browsed by feral animals. Damage to *kauila* saplings and large portions of adult individuals caused by the black twig borer (*Xylosandrus compactus*) is also currently reducing population numbers (Hara & Beardsley 1979). Research on control methods for the black twig borer should be performed, and the results applied to the many affected hardwood species (i.e. *Fluggea neowawraea*, *Bobea* spp., and *Acacia koa*, among others) in dry and mesic forest areas. Similar efforts should be taken to control invasive alien plant species and to determine essential plant–animal interactions. This would aid land managers in preparing strategies to reverse the trend of decline among remnant adults in a once-thriving forest, as well as the juveniles from gradually dwindling seed reservoirs and rare species in the limited intact natural areas remaining (Janzen 1988).

Numerous studies have shown that loss of genetic variation has a harmful effect on fitness (O'Brien & Evermann 1988; Quattro & Vrijenhoek 1989; Ledig *et al*. 1997). Knowledge of effective population size, N_e, would allow prediction of the expected time when reduced genetic variation is likely to threaten continued existence of an isolated population (Gilpin & Soulé 1986). However, simulation of the percentage of genetic variation and heterozygosity remaining after a genetic bottleneck followed by multiple matings subject to genetic drift suggests that significant reductions occur primarily after repeated generations at small population sizes (Meffe & Carroll 1994). This study has revealed that there has probably been no significant reduction in overall genetic variation within populations of *C. oppositifolia* or *A. ponderosa* at this time, due probably to the recency of habitat disturbance (primarily since, and accelerated by, human occupation) and the long-lived habit of the species. Thus, overall genetic variation in *kauila* species may not be severely impacted in the immediate future if given a low level of population recruitment. However, recruitment was not evident within the current populations, probably because of predation by the black twig borer (Hara & Beardsley 1979) and/or competition with alien grasses (Stone *et al*. 1992). Further, Medeiros *et al*. (1986) have documented that seeds from Maui populations of *A. ponderosa* have very poor germination and survival rates, and that no known plants have survived in cultivation. Eventual

extinction of such populations may be anticipated. Research studying seed bank genetic variation such as that conducted by Cabin *et al*. (1998) would provide a comprehensive understanding of latent genetic diversity, and potentially the future direction of dry and mesic forest tree populations.

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