Development and characterization of microsatellite markers for analysis of population differentiation in the tree legume *Acacia koa* (Fabaceae: Mimosoideae) in the Hawaiian Islands

Rudolph Fredua-Agyeman, Daniel Adamski, Richard Junfu Liao, Clifford Morden, and Dulal Borthakur

Abstract: The aim of this research was to develop and use microsatellite markers to characterize the high-value timber tree *Acacia koa* (koa), which is endemic to the Hawaiian Islands. Genomic DNA fragments of 300–1000 bp were cloned and sequenced following enrichment for microsatellite motifs by PCR using 7 oligonucleotide repeat primers in separate reactions. Among 96 sequences analyzed, 63 contained unique microsatellite motifs flanked by variable sequences. A dual PCR method involving a primer walking step was used to develop 15 primer pairs. Another 16 primer pairs were developed directly from the variable sequences on both sides of the microsatellite motifs. These 31 primer pairs were tested on 172 koa plants representing 11 populations collected from 4 of the major Hawaiian Islands. Nine of the primers that identified polymorphic microsatellite loci and 3 that detected unique alleles exclusively in some populations were used for genetic diversity studies of koa. Cluster analysis and multidimensional scaling of the allelic phenotype data revealed that koa from Kauai formed a distinct group separate from koa of the neighboring islands of Oahu, Maui, and Hawaii. The oldest of the four islands, Kauai, also had the most diverse populations of koa.

Key words: *Acacia koa*, dual-suppression PCR, walking sequence and direct sequence strategies, genetic diversity, population differentiation.

Résumé : Le but de ce travail était de mettre au point des marqueurs microsatellites pour caractériser l'*Acacia koa* (koa), une espèce forestière de grande valeur qui est endémique des îles hawaïennes. Des fragments d'ADN génomique de 300–1 000 pb ont été clonés et séquencés après enrichissement par PCR pour des microsatellites au moyen de 7 amorces à répétitions oligomériques dans des réactions séparées. Parmi les 96 séquences analysées, 63 contenaient des microsatellites uniques et étaient bordés de séquences variables. Une méthode de PCR double incluant une étape de marche chromosomique a été employée pour développer 15 paires d'amorces. Seize autres paires d'amorces ont été développées directement à partir des séquences variables bordant les deux côtés des microsatellites. Ces 31 paires d'amorces ont été testées sur 172 plants de koa représentant onze populations échantillonnées sur quatre des principales îles d'Hawaï. Neuf paires d'amorces qui amplifiaient des locus microsatellites polymorphes et trois paires d'amorces qui amplifiaient des allèles uniques exclusivement chez certaines populations ont été employées pour des études de diversité génétique chez le koa. Des analyses de groupement et d'échelonnement multidimensionnel des données alléliques phénotypiques ont révélé que le koa de Kauai forme un groupe nettement distinct de ceux des îles voisines d'Oahu, Maui et Hawaï. Kauai, la plus ancienne des quatre îles, avaient les populations de koa les plus diverses.

Mots-clés : *Acacia koa*, PCR à suppression double, stratégies de séquence marchée et de séquence directe, diversité génétique et différenciation des populations.

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Introduction

Genetic markers are specific DNA sequences in the genome of an organism that can be used to track inheritance and distribution of parental genotypes among a segregating progeny, and to determine the extent of genetic variation in a population (Schlötterer 2004). Closely related individuals within a species can have only a limited number of phenotypic variations, which may also be greatly influenced by changes in the environment (Yadav et al. 2007). On the con-

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R. Fredua-Agyeman, D. Adamski, R.J. Liao, and D. Borthakur.[1] Department of Molecular Biosciences & Bioengineering, University of Hawaii at Manoa, 1955 East-West Road, Agricultural Science 218, Honolulu, HI 96822, USA.
C. Morden. Department of Botany, University of Hawaii at Manoa, Honolulu, HI 96822, USA.

[1]Corresponding author (e-mail: dulal@hawaii.edu).

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trary, genetic markers based on DNA sequences can be innumerable and are generally not subject to environmental influences (Manifesto et al. 2001). However, because of the vastness of genome sequences, analyses of random gene sequences between individuals may not always lead to detectable differences. There are certain highly variable regions in the genomes of organisms where differences in sequences among individuals are more prevalent (Ellegren 2004; Weber 1990). Numerous methods of easy determination of differences within these hotspots of genetic variation, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP), and DNA repeat variation analyses, which include microsatellites, have been already described in other literature (Skolnick and White 1982; Tautz 1989; Williams et al. 1990; Vos et al. 1995; Weising et al. 1995; Brookes 1999; Toth et al. 2000). Molecular markers identified from these variable regions have wide application in a variety of fields including phylogeny, taxonomy, ecology, genetics, and plant and animal breeding (McCouch et al. 1997; Ouborg et al. 1999; Luo et al. 2001; Bussell et al. 2005; Wang et al. 2006). These markers can also provide information about allelic variation at a given locus that may be useful for understanding microevolution of populations at varying geographical and environmental locations (Zwettler et al. 2002).

Microsatellites, or simple sequence repeats (SSR), are presently among the most widely used DNA markers for detection of genetic variation in different organisms (Selkoe and Toonen 2006). Microsatellite markers, first developed for use in genetic fingerprinting of humans, are composed of tandem repeats of 1–6 nucleotides and are dispersed throughout the genome of an organism (Tautz and Renz 1984; Litt and Luty 1989). Microsatellite markers have been successfully applied in population genetics studies of polyploid trees including *Acacia* species (Butcher et al. 2000; Otero-Arnaiz et al. 2005; Millar and Byrne 2007; Ruiz-Guajardo et al. 2007). *Acacia koa* (koa) is a member of the subgenus *Phyllodineae* (Fabaceae: Mimosoideae) and is a tetraploid ($2n = 52$) species that is endemic to the Hawaiian Islands (Atchison 1948; Carr 1978). It is an ecosystem-dominant tree in the forests of Hawaii and is a source for high-value timber. It is also considered to be an important canopy tree that provides watershed and habitat for other endemic species of Hawaii (St. John 1979; Whitesell 1990). In natural forests, large phenotypic differences among island types of koa are observed in seed size and shape, pubescence, phyllode width and curvature, floral morphology and pigmentation, retention of juvenile leaves, and branch bark color (Sun et al. 1996; Daehler et al. 1999). Microsatellite markers for studying genetic diversity in koa had not yet been developed. Here, microsatellite markers were developed and used for the first time to characterize distinct morphological forms of koa among four major Hawaiian Islands.

Materials and methods

Plant materials and DNA extraction

For microsatellite isolation, total genomic DNA was extracted from a koa leaf sample obtained from Island of Hawaii using the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturer's instructions. The quality of the DNA was evaluated by spectrometry using the 260/280 nm absorbance ratio method and the DNA concentration was estimated at 260 nm with an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, Delaware, USA). For testing microsatellite primers, total genomic DNA from 172 koa trees representing 11 populations from the islands of Kauai, Oahu, Maui, and Hawaii (Table 1) was obtained from the Hawaiian Plant DNA Library (Morden et al. 1996; Randell and Morden 1999).

Restriction fragmentation, size selection, and oligonucleotide repeat PCR

The koa genomic DNA was separately digested with 5 blunt-end-producing restriction enzymes (*Rsa*I, *Alu*I, *Sca*I, *Hae*III, and *Eco*RV), and fragments of 300–1000 bp were extracted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN Inc.). The DNA digests were pooled to create a library of overlapping fragments. Fragments that contained 2 or more microsatellite motifs were amplified from the DNA library using 7 oligonucleotide repeats ($[AC]_{10}$, $[AAC]_8$, $[AG]_{10}$, $[AAG]_8$, $[TG]_{10}$, $[AAT]_8$, and $[TAGA]_8$) in separate reactions. All oligonucleotide repeats for this study were obtained from Integrated DNA Technologies (San Diego, California, USA). PCR was performed in a total volume of 50 μL containing 3–10 ng of genomic DNA, 1× *Taq* buffer, 2.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 0.2 μmol/L primer, and 1.25 U of *Taq* polymerase (Promega Corp., Madison, Wisconsin, USA). The reactions were carried out in an MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA); amplifications consisted of an initial denaturation step of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 1 min 30 s at 72 °C, and a final extension of 30 min at 72 °C. Aliquots of the PCR products were electrophoresed on 2% agarose gels, stained for 30 min in ethidium bromide, and photographed under ultraviolet light for visualization of the amplified fragments.

Construction of genomic DNA libraries and sequencing of amplified fragments

The amplified fragments were directly ligated into the pGEM-T Easy Vector (Promega Corp.) and ligation reactions were used to transform competent *E. coli* DH5α cells. The cloned fragments from the libraries were evaluated by colony PCR using the M13 forward and reverse primers. For each library, 10 to 30 transformants containing different-sized fragments were grown overnight in liquid LB medium containing 100 μg/mL ampicillin. Plasmid DNA was extracted from positive clones using the QIAprep Spin Miniprep Kit (QIAGEN Inc.) and then used as template in the sequencing reactions. Sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and analyzed on an ABI PRISM 3730*xl* DNA analyzer (Applied Biosystems, Foster City, California, USA) at the Advanced Studies in Genomics, Proteomics and Bioinformatics sequencing facility at the University of Hawaii at Manoa. Clone sequences were extracted from the chroma-

Table 1. List of 172 *Acacia koa* trees analyzed by microsatellite markers.

Sample IDs	HPDL acc. Nos.	No. of samples	Island of origin	Population sampled
H1BPH–H16BPH	2005–2020	16	Hawaii	Bird Park, Hawaii Volcanoes National Park
H17LNR–H32LNR	2021–2036	16	Hawaii	Laupahoehoe Natural Area Reserve
M33MRW–M48MRW	2379–2394	16	Maui	Mahana Ridge West
M49MFR–M64MFR	2395–2410	16	Maui	Makawao Forest Reserve
M65KFR–M80KFR	2411–2426	16	Maui	Kapunakea Forest Reserve
K81NPC–K96NPC	1944–1959	16	Kauai	Na Pali Coast
K97MKR–K110MKR	1980–1993	14	Kauai	Makaha Ridge, Kokee
K111HAL–K126HAL	2979–2994	16	Kauai	Hanalei
O127KHV–O142KHV	1887–1902	16	Oahu	Kahana Valley
O143HLR–O157HLR	1871– ▽ 1886	15	Oahu	Hawaii Loa Ridge
O158WNK–O172WNK	1733– ▽ 1748	15	Oahu	Waianae Kai

Note: Source of DNA was the Hawaiian Plant DNA Library (HPDL). ▽ indicates numbering break in HPDL accessions.

tograms using the SeqVerter program (GeneStudio, Inc., Suwannee, Georgia, USA). To eliminate redundant clones, the retrieved sequences were aligned using the ClustalW package at the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/). The isolated sequences were compared against the GenBank database sequences available at the National Center for Biotechnology Information (NCBI) using the BLASTN search program (http://www.ncbi.nlm.nih.gov/blast). The sequences obtained in this study have been deposited in the GenBank database under the accession numbers EU574702–EU574732.

Genomic SSR analyses and primer development

Two approaches, namely, "walking sequence" and "direct sequence", were used for primer development (Fig. 1). For the "walking sequence" approach, genomic sequences that contained microsatellite motifs only at the ends were selected for amplification using two primers (Lian et al. 2001; Siebert et al. 1995). One of the primers, known as the "downstream primer", was developed from the variable sequence located adjacent to the 3′ end of the microsatellite within the cloned fragment, using the Primer3 program (Rozen and Skaletsky 2000). For developing the "upstream primer", which is based on variable sequence on the original genomic DNA fragment containing the same microsatellite motif, a multi-step approach was taken as follows. (*i*) Koa genomic DNA was digested with 5 blunt-end-producing restriction enzymes (*Rsa*I, *Alu*I, *Sca*I, *Hae*III, and *Eco*RV) separately and the resulting fragments were purified using the QIAquick PCR Purification Kit. (*ii*) The digested genomic DNA was ligated at both ends to the blunt end of an adapter obtained by annealing a 48 nucleotide (nt) primer with an 11 nt primer; this adapter contains an amino group at the 3′ end that blocks further extension, thereby leaving the longer adapter primer free (Table 2). The short primer was complementary to the 3′ end of the long primer, producing a blunt end and a 5′ overhang of 37 nt. (*iii*) The adapter-ligated DNA fragments were used as templates in PCR using the downstream primer and a primer containing a 27-mer direct sequence of the 5′ overhang of the adapter. The newly synthesized single-stranded DNA from the downstream primer serves as the template for annealing of the adapter primer. In this way, the microsatellite and adjacent regions were amplified. (*iv*) PCR products were cloned and sequenced. If multiple bands were obtained from the PCR amplification, a nested primer approach was applied. A second downstream primer was developed from sequences adjacent to the first downstream primer and used with the adapter primer to obtain a discrete PCR product, which was cloned and sequenced. (*v*) Upstream primers were developed from the non-repeated variable sequence adjacent to the adapter sequence. For the "direct sequence" primer development strategy, forward and reverse primers were developed from the sequenced fragments that contained centrally located microsatellite motifs in addition to variable sequences of at least 60 bp on both sides of the motifs.

Testing primers by PCR amplification of genomic DNA

PCR was performed in a total volume of 20 μL containing 3–10 ng of genomic DNA, 1× *Taq* buffer, 2.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 0.2 μmol/L primer, and 1.25 U of *Taq* polymerase (Promega Corp.). Each microsatellite marker was tested separately with genomic DNA from each of the 172 test plants. The reactions were carried out using the same PCR amplification protocol described above. Aliquots of the PCR products from the samples were electrophoresed on 2% agarose gels, stained for 30 min in ethidium bromide, and photographed under ultraviolet light for visualization to confirm the success of amplification reactions. One primer from each pair that positively amplified genomic DNA to produce a single band was fluorescently labeled with FAM, VIC, PET, or NED (Applied Biosystems).

Allele size determination and polymorphism detection

Aliquots (1 μL) of the amplified DNA fragments were size-separated by capillary electrophoresis on an ABI PRISM 3730*xl* DNA analyzer (Applied Biosystems) at the Advanced Studies in Genomics, Proteomics and Bioinformatics sequencing facility at the University of Hawaii at Manoa. The amplified DNA fragment lengths or allele sizes were determined by comparison with the standard size marker GS 500 ROX (Applied Biosystems) using the GeneMarker software program, version 1.60 (SoftGenetics, LLC, State College, Pennsylvania, USA). To confirm the reproducibility of peaks and the accuracy of size calling, PCR amplifications were repeated on about 10% of the samples and allele sizes for each microsatellite marker were rescored.

Fig. 1. (*a*) Steps in cloning microsatellite motifs: (1) digestion of genomic DNA to produce blunt ends, (2) isolation of fragments of 300–1000 bp, (3) some of the purified fragments may contain microsatellite motifs, (4) enrichment of microsatellite motifs by oligonucleotide repeat PCR, (5) fragments containing microsatellite motifs at the two ends were used for primer development using the walking sequence strategy, in which a downstream primer is made from the variable sequence in the middle. (*b*) Additional steps in the walking sequence strategy: (6) adapter is ligated to the blunt-ended genomic fragments, (7) microsatellite motifs with upstream variable regions are amplified by nested PCR using adapter-specific primers and downstream primers, and (8) the upstream primer, developed from the variable upstream region, is used with the downstream primer to amplify the microsatellite locus.

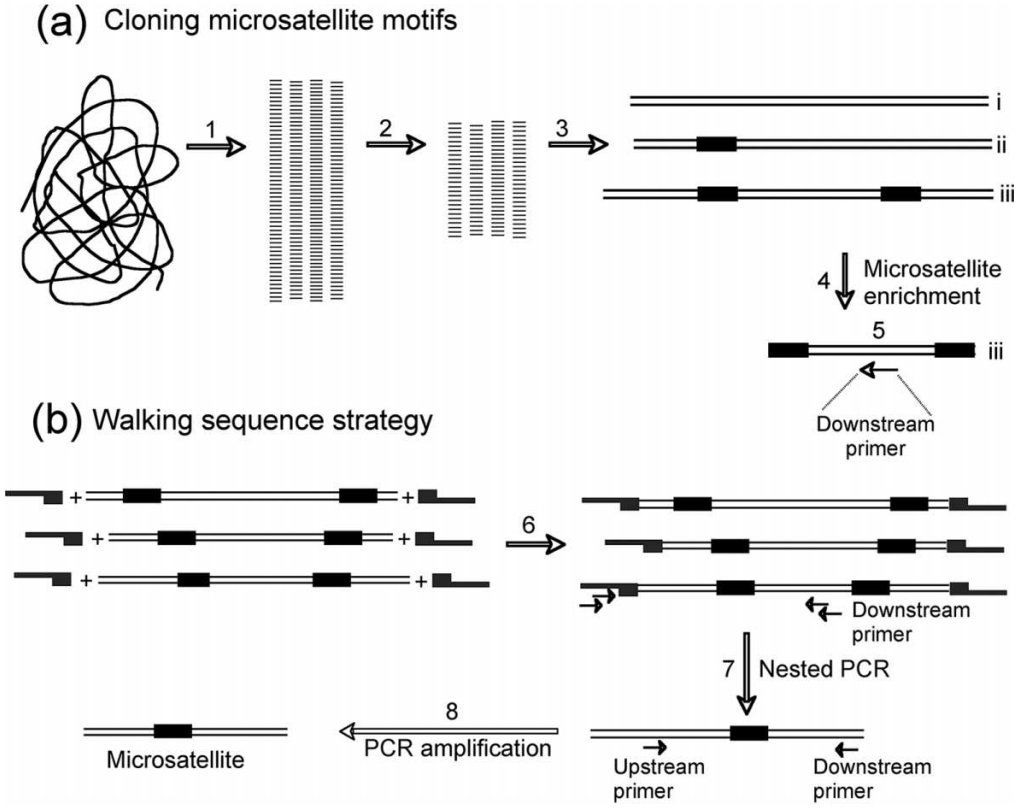


Table 2. Adapter and primer sequences used for the development of microsatellite markers by the dual-suppression PCR method.

Oligonucleotide	Sequence
Single-stranded adapter sequences	
48 nt	5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′
11 nt	5′-ACCAGCCCGGG-NH_2-3′
Overhang adapter derived primer sequences used for nested PCR	
27 nt	5′-CCATCGTAATACGACTCACTATAGGGC-3′
18 nt	5′-CTATAGGGCACGCGTGGT-3′

Microsatellite allele counting using peaks of electropherograms was used for the determination of the presence of an allele at a locus. The number of alleles per locus was determined by counting the total number of alleles for each locus following genotyping of 172 koa plants with the corresponding set of primers. The polymorphic information content (PIC) of the primers was calculated according to the equation of Anderson et al. (1993):

$$[1] \qquad \mathrm{PIC} = 1 - \sum_{i=1}^{n} P_i^2$$

where n is the total number of alleles detected for each primer pair and P_i is the frequency of the ith allele detected. Markers that identify polymorphic microsatellite loci were then used for studying genetic diversity of koa.

Microsatellite data analysis

As has been previously found with polyploids (De Silva et al. 2005), complexity of inheritance patterns and the presence of multiple alleles in koa makes it difficult to assign allele dosage or the copy number of each allele at a specific locus. A molecular binary phenotype was therefore used to assign microsatellite DNA genotypes for individual plants, in which amplified fragments were analyzed as dominant–recessive markers. The presence of an allele was scored as "1" and its absence as "0". Alleles were scored for 12 markers (see Results) following capillary electrophoresis of

the 172 koa samples. The data, organized in a spreadsheet, were then used for further analysis to determine genetic similarities between different island types of koa.

Cluster analysis and multidimensional scaling of allelic phenotype data

Allelic phenotype data for the 172 plants representing 11 populations of koa were analyzed using the SIMQUAL (similarity for qualitative data) module to compute pairwise similarity coefficients (Jaccard 1908; Dice 1945). The similarity estimates were used to draw UPGMA (unweighted pair group method with arithmetic means) dendrograms with the SAHN (sequential, agglomerative, hierarchical and nested clustering) module of the NTSYSpc 2.2 program (Exeter Software Co., Setauket, New York, USA). Bootstrap analysis was performed with 1000 repetitive samplings of the data and a consensus tree was constructed. The cophenetic correlation coefficient was calculated to test the goodness of fit between the original similarity matrices and the cophenetic matrices using a Mantel test (Mantel 1967) and the COPH and MXCOMP modules of the NTSYSpc 2.2 program. High values indicate that the phenograms represent the relationships among the populations. The principal coordinate analyses (PCO) were also performed on the same similarity indices using the NTSYSpc 2.2 program to provide a pictorial representation of relationships among populations. The first 3 principal axes were used to plot scatter diagrams that show the relationships among the different island types.

Measuring genetic diversity and differentiation statistics

Cytogenetic investigation of koa revealed a maximum of one putative pair of chromosomes with secondary constrictions, which is consistent with the ploidy level of an allotetraploid species (Langer and Koul 1983; Coulaud et al. 1995; Shi 2003). Therefore, in addition to the above analysis, we estimated phenotype-based genetic diversity and differentiation statistics with the FDASH program developed for use in allopolyploids by Obbard et al. (2006*b*). The phenotype-based statistics performed extremely well under computer simulations and were recently used in population genetics studies of the allohexaploid herb *Geum triflorum* (Hamilton and Eckert 2007) and hexaploid populations of *Mercurialis annua* (Obbard et al. 2006*a*).

The overall genetic diversity across all populations was determined by estimating H'_T, a parameter that is based on the average number of unshared alleles between pairs of individuals taken from all populations (Obbard et al. 2006*b*):

$$[2] \qquad H'_T = \frac{1}{n(n-1)} \sum_{i=1}^{n} \sum_{j>1}^{n} \sum_{k \in \{\text{alleles}\}} x_{ijk}$$

where H'_T is the average number of unshared alleles between pairs of individuals across all populations, n is the total number of individuals, and $x_{ijk} = 1$ if only one of the individuals i and j carry allele k, otherwise $x_{ijk} = 0$. For measuring genetic diversity within a population, H'_S was calculated based on the average number of unshared alleles between pairs of individuals taken from the same population.

The genetic differentiation among populations was investigated by estimating F'_{ST}, a parameter that is based on the proportion of total diversity found between populations (Obbard et al. 2006*b*):

$$[3] \qquad F'_{ST} = \frac{H'_T - \overline{H'_S}}{H'_T}$$

where F'_{ST} is the genetic variance among populations relative to the total variation and $\overline{H'_S}$ is the mean within-population diversity. The statistical significance of H'_T, H'_S, and F'_{ST} was tested after 1000 bootstraps with 95% nominal confidence intervals.

Results

Oligonucleotide repeat PCR analyses of genomic fragments

Restriction digestion with each of the 5 blunt-end-producing restriction enzymes resulted in smeared banding patterns, indicating generation of numerous fragments of sizes less than 3.0 kb. When 7 oligonucleotide repeats were used separately as PCR primers for amplification of the gel-extracted genomic DNA fragments, only 4 primers resulted in numerous amplified bands. Positive amplifications by these primers indicated that genomic DNA fragments containing AC, AG, TG, and AAG repeats were selectively amplified (Fig. 2). These oligonucleotide primers were expected to amplify internal segments of the digested DNA fragments between two microsatellite motifs. The resulting PCR products did not contain sequences beyond the two motifs on the ends of the template. Four clone libraries were constructed with the amplified genomic DNA fragments containing the repeated sequences and these were designated as AC, AG, TG, and AAG libraries. Several hundred colonies were obtained for each library. Representative clones from the four libraries showed cloned fragments in the size range of 300 to 1000 bp (Fig. 3).

Sequence analyses of cloned AC-, AG-, TG-, and AAG-rich fragments

Among 511 randomly selected colonies from the four libraries that were screened by colony PCR using M13 primers for amplified fragments of different sizes, a total of 96 clones were selected as representatives of inserts of different sizes for sequence analyses (Table 3). Among these, 63 clones (66%) had unique microsatellite sequences. The remaining 33 clones displayed the same sequences present in the selected unique clones. Because single oligonucleotide repeat primers were used for selective amplification of microsatellite-rich DNA fragments, all clones contained reverse complementary repeated motifs at the two ends. The clones containing AC, AG, AAG, and TG motifs at the 5′ ends carried GT, CT, CTT, and CA motifs, respectively, at the 3′ ends. The average size of the inserts obtained from the walking sequence method was 232 bp, compared with 199 bp inserts obtained from the direct sequence method. Among the selected clones, 43% contained centrally located microsatellite motifs in addition to those at the ends. Fifteen different repeats were identified in the central motifs, among which 2 were similar to the terminal motifs identified by the walking sequence strategy. The central motifs were also

Fig. 2. Electrophoretic profiles showing amplified PCR products obtained from four of the seven oligonucleotide repeat primers. Lanes for oligonucleotides $(AC)_{10}$, $(AG)_{10}$, $(TG)_{10}$, and $(AAG)_8$ showed PCR products, while those for $(AAC)_8$, $(AAT)_8$, and $(TAGA)_8$ showed faint or no products. M, DNA size marker.

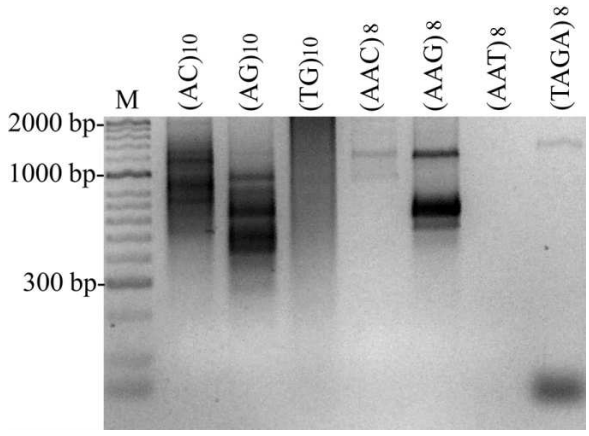
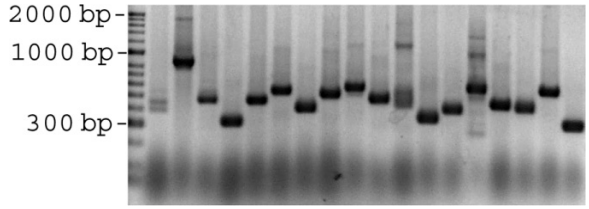


Fig. 3. PCR amplification of plasmid DNA of positive clones with M13 primers indicated that fragments in the size range of 300–1000 bp were amplified. The first lane shows the DNA size marker.



shorter than the motifs at the two ends. Eighty percent of the microsatellites, terminal or central, contained perfect dinucleotide or trinucleotide repeats. About 13% of the microsatellites contained two adjacent distinctive repeats, while about 7% had 2–4 bp intervening sequences within the motifs. As expected, almost all clones present in the $(AC)_{10}$ library were also present in the complementary $(TG)_{10}$ library.

Primer development

Primer development was not possible for 26 of the 63 selected sequences because the flanking regions contained similar microsatellite-like sequences or were too short for primer development. A total of 37 sequences from the four libraries were found to be suitable for primer development. Twenty-one sequences that contained microsatellite motifs at the ends were selected for primer development using the "walking sequence" strategy. The remaining 16 sequences that contained microsatellite motifs in the middle and had variable flanking regions of at least 60 bp on both sides were selected for "direct sequence" primer development.

With the "walking sequence" strategy, 16 microsatellite-containing regions were successfully amplified, appearing as discrete single bands on agarose gels. The amplified products from the other 5 sets of primers showed multiple bands or smears and were not used for further analyses. The PCR products from the 16 reactions that showed discrete single bands were cloned and sequenced. The sequence data showed that the amplified fragments contained microsatellite loci and also the adjacent 3′ regions beyond the loci. From the variable sequence between the adapter sequence and the microsatellites, 16 upstream primers were developed that had the same orientation as the adapter primers and opposite directionality from the downstream primers. Although these 16 upstream primers and their corresponding downstream primers (Table 4) were expected to amplify 16 different microsatellite loci, only 15 of these primer pairs successfully amplified DNA fragments from test populations under optimized reaction conditions.

Table 3. Summary of the clone library constructed with seven oligonucleotide repeats.

	Number of clones		
Oligonucleotide repeat	Screened by colony PCR	Cultured for plasmid DNA	Containing unique microsatellite motif
$(AC)_{10}$	133	30	19
$(AG)_{10}$	255	30	27
$(TG)_{10}$	16	16	3
$(AAG)_8$	107	20	14
$(AAC)_8$	3	—	—
$(AAT)_8$	2	—	—
$(TAGA)_8$	4	—	—
Total	520	96	63

An additional 16 primer pairs were developed by using the "direct sequence" primer development strategy from the sequenced fragments that contained centrally located microsatellite motifs in addition to variable sequences of at least 60 bp on both sides of the motifs. The variable regions on both sides of the microsatellite motifs were used for developing a pair of forward and reverse primers for each of the 16 sequences. These 16 primer pairs could amplify the expected microsatellite loci of the test populations under optimized reaction conditions (Table 5).

Polymorphism analyses

All 31 primer pairs could amplify microsatellite loci of the 172 koa test plants. Characterization of each microsatellite locus was based on the number of alleles detected on electropherograms obtained from capillary electrophoresis of the PCR products (Fig. 4). The number of alleles per locus and the polymorphic information content (PIC) of the primers, based on peak counts in the electropherograms for the test population, are presented in Tables 4 and 5. The highest number of alleles per locus was obtained for Ak08, followed by Ak284 and Ak99. None of the microsatellite loci was found to be monomorphic. The microsatellite loci amplified by 4 of the primer pairs were dimorphic, while those amplified by 4 other primer pairs were trimorphic. The remaining 23 pairs of primers amplified microsatellite loci that had 4 or more alleles. The PIC values ranged from 0.13 to 0.89 and did not correlate with the number of alleles detected at a particular locus.

Microsatellite marker selection and allele frequency determination

Nine highly variable koa-specific microsatellite markers that were part of the initial set of 31 markers were selected for genetic diversity studies. Three additional primers that

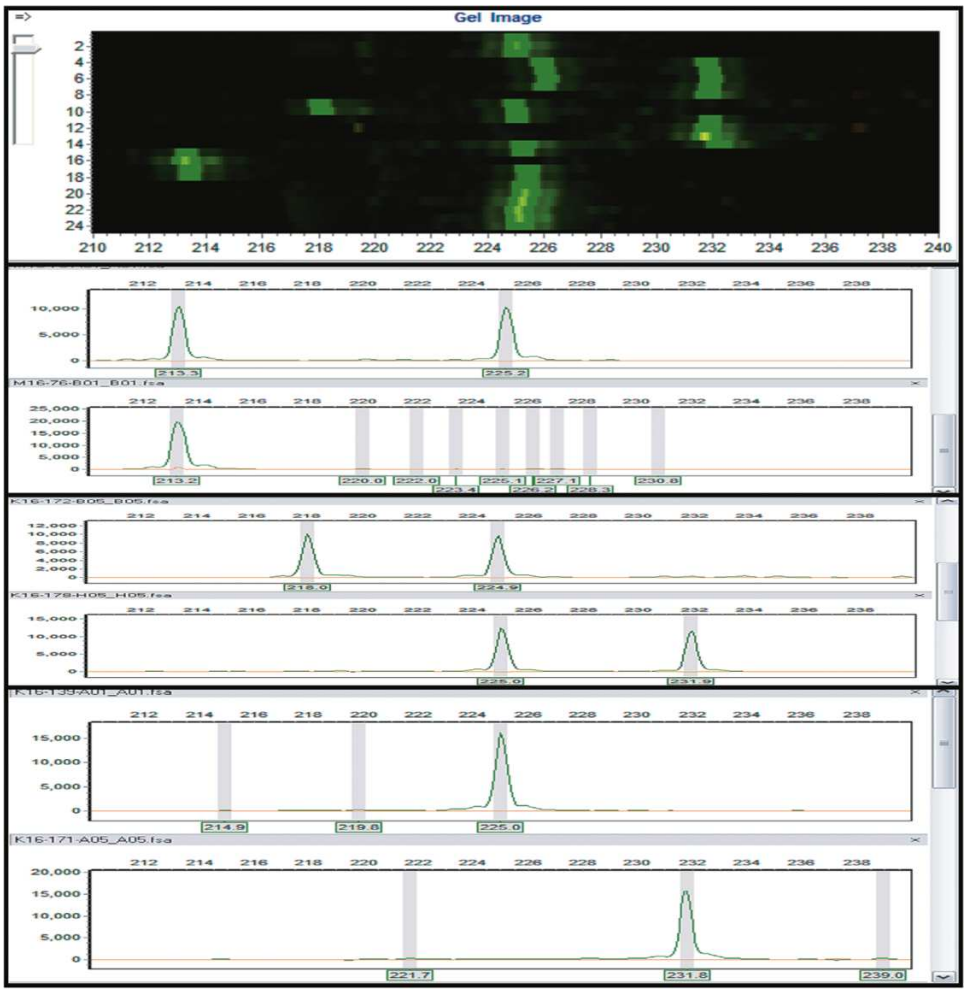
Table 4. Primer sequences and allele characteristics of 15 "downstream" and "upstream" microsatellite markers isolated from *Acacia koa* by the "walking sequence" strategy.

No.	Locus	Library	Downstream primer (5′–3′) Upstream primer (5′–3′)	Repeat motif	T_a (°C)	Dye	Allele size (bp)	No. of alleles	PIC	GenBank acc. No.
1	Ak06	AAG	AGGTTGATGAAAAGGCATGG TCTCAGGTTTGGTGGGTTTT	$(AAG)_6$	55	NED	234	7	0.48	EU574702
2	Ak08	TG	ACAGTTCCACCTCACCGTTC CGACCCTATCACCTTCTTGC	$(TACA)_8$	56	PET	230	17	0.76	EU574703
3	Ak10	AC	AATGCTGCCACCCTATATCA TTGTGAGTGAATTTGAAGAATGTAA	$(GT)_9(GA)_{11}$	56	VIC	223	8	0.65	EU574704
4	Ak14	TG	AGAAGCCTGGTCCCTCTTGT GTCTAGCCGGACCGTAACAG	$(TG)_{12}$	53	PET	224	5	0.49	EU574705
5	Ak31	AC	TTGAACATGACTGGCGAAAG AAAGCAATAAAGAAGTTTATGTCTGC	$(CA)_{10}$	53	NED	236	5	0.19	EU574706
6	Ak36	AC	GCAGGACTTGACGAACCTTT TTGGCTCCATCTTTTCCTTG	$(CA)_9$	52	VIC	224	8	0.65	EU574707
7	Ak37	AG	TCCTCATCAGCGTCTCACAC GAGCCGAGATGCTGAGAGTT	$(CTC)_4$	60	FAM	210	4	0.51	EU574708
8	Ak41	AAG	CCCATGCTTGACATCACTTG TCATCGTCGTCTAGATCCCTTT	$(GAA)_5$	56	FAM	211	8	0.66	EU574709
9	Ak50	AAG	AGGTTGATGAAAAGGCATGG TCTCAGGTTTGGTGGGTTTT	$(AAG)_8$	55	VIC	240	6	0.49	EU574710
10	Ak63	AG	AGTTGCGCTCGTCAAGTTTC AACACCGTTTCTTCTCTCTTCG	$(TC)_{14}$	54	PET	236	3	0.18	EU574711
11	Ak99	AG	GCTGGTCCAACGTAGAAGGA CCAGCATGAGAACGAACAAT	$(CT)_{10}$	54	NED	226	14	0.77	EU574712
12	Ak107	AG	TTGGTAATGCCGTTTGAGTT CACCACAAAAGCAACAGCAG	$(GA)_5$	56	FAM	158	2	0.50	EU574713
13	Ak141	AC	TGCACTCAAGCAACATAACAA GAGGAATGGAATAAAAAGAAGCA	$(AC)_4AAT(AC)_{13}$	55	PET	152	2	0.50	EU574714
14	Ak180	AAC	GAGCACACATTCCAGGTTCA ATCGTCCTCGTCAGACTGCT	$(GTT)_4$	53	NED	189	6	0.54	EU574715
15	AK219	AAG	AACAAATACCAAGGCGACAAA GGGTACTGGAAGAGCAGGTG	$(GAA)_4$	55	NED	190	3	0.27	EU574716

Table 5. Primer sequences and allele characteristics of 16 forward and reverse microsatellite markers isolated from *Acacia koa* by the "direct sequence" strategy.

No.	Locus	Library	Forward primer (5′–3′) Reverse primer (5′–3′)	Repeat motif	T_a (°C)	Dye	Allele size (bp)	No. of alleles	PIC	GenBank acc. No.
16	Ak02	AC	TGTATTCCAAGCGGACAAAA TGACAAATGGCACATGGTCT	$(AC)_{10}$	59	NED	237	3	0.13	EU574717
17	Ak05	AC	GACTGATTGAATAATAGGCAC CAAGCAAAAATTGTATACC	$(AC)_5(AT)_5$	52	PET	185	6	0.64	EU574718
18	Ak15	AG	CACCCCCACGTTATCTTACA GACTGGCGAAAGAGTCGAA	$(TAT)_5$	55	VIC	297	4	0.56	EU574719
19	Ak16	AAG	GCTCCTTGTCATGCTCTTTCA GCTGGCAGCTGCTGTAGTTT	$(A_4G)_2AA(A_4G)_2$	54	VIC	227	4	0.38	EU574720
20	Ak21	AC	CCTGCACGCTCATTTGAGTA TCCGGTGCTCCAAACATAAT	$(ATT)_6$	56	FAM	217	3	0.28	EU574721
21	Ak26	AAC	TCTCCCGTCCCTACCCTACT ATGCAAAGGCGAAGGTTATG	$(GGA)_4$	58	PET	244	4	0.21	EU574722
22	Ak28	AC	ACTGGTGCAGTGTCTGTTGC ACGCAGGTCAATTGTGTTGA	$(ATTA)_4$	54	NED	197	4	0.18	EU574723
23	Ak39	AC	AGCAAACTTGGCCTTCAAGA CAACTGCTCCTGTTGGTGAA	$(GTGC)_3$	56	FAM	225	7	0.42	EU574724
24	Ak43	AAG	AGAGGAAAAGAGGGCAGCAT TCCCTTTATTCGGCCTTATG	$(TA)_{16}$	56	FAM	235	2	0.23	EU574725
25	Ak44	AG	TTCTGTGTTCACCGTCGTTC CTGCATCCAACCTTTGACCT	$(CT)_{13}$	60	NED	174	13	0.73	EU574726
26	Ak52	AG	ACGCAGGTCAATTGTGTTGA ACTGGTGCAGTGTCTGTTGC	$(AATT)_7$	57	PET	209	4	0.18	EU574727
27	Ak84	AAC	AACCAATGAGAGCCTTTCTTC CATCTCCTACAAACCGCCC	$(TTTA)_4$	59	FAM	256	6	0.47	EU574728
28	Ak89	AC	AGGGGAAGGACGAAAGTTGT GCAAGAGGAGCTTCAAGTGG	$(AC)_7$	56	PET	158	11	0.65	EU574729
29	Ak176	AG	CAGACTTCTCCCCACAGGTC GTTCATGCTCGATCCGTCTC	$(GGA)_4(AGA)_2$	55	FAM	104	2	0.50	EU574730
30	Ak196	AG	TGACCGCCATTGATGTTAAA TCAGTGACCAAGATGTAGCC	$(TA)_5$	55	VIC	288	7	0.31	EU574731
31	Ak284	AG	ACAACCATCGGCAACTTAGC CCCAACGAAGAAGCTAGACG	$(AG)_3A(AG)_{11}$	59	FAM	242	16	0.89	EU574732

Fig. 4. Electropherogram showing all 4 alleles detected at locus Ak16. The *x*-axis shows fragment size (bp) and the *y*-axis shows the peak height.



were observed to detect unique alleles exclusively in some populations were also included in the analysis. A total of 116 distinct alleles were identified after 172 koa trees were genotyped with the 12 selected markers. The average number of alleles per locus was 9.7. A summary of the loci analyzed and the number of alleles detected in each population is presented in Table 6. The highest number of alleles (62) was detected in the 16 samples from the Hanalei population (HAL) on Kauai, while the lowest number of alleles (39) was detected in 16 samples from the Bird Park (BPH) population on Island of Hawaii.

Genetic relationships among populations based on similarities and principal coordinate analyses

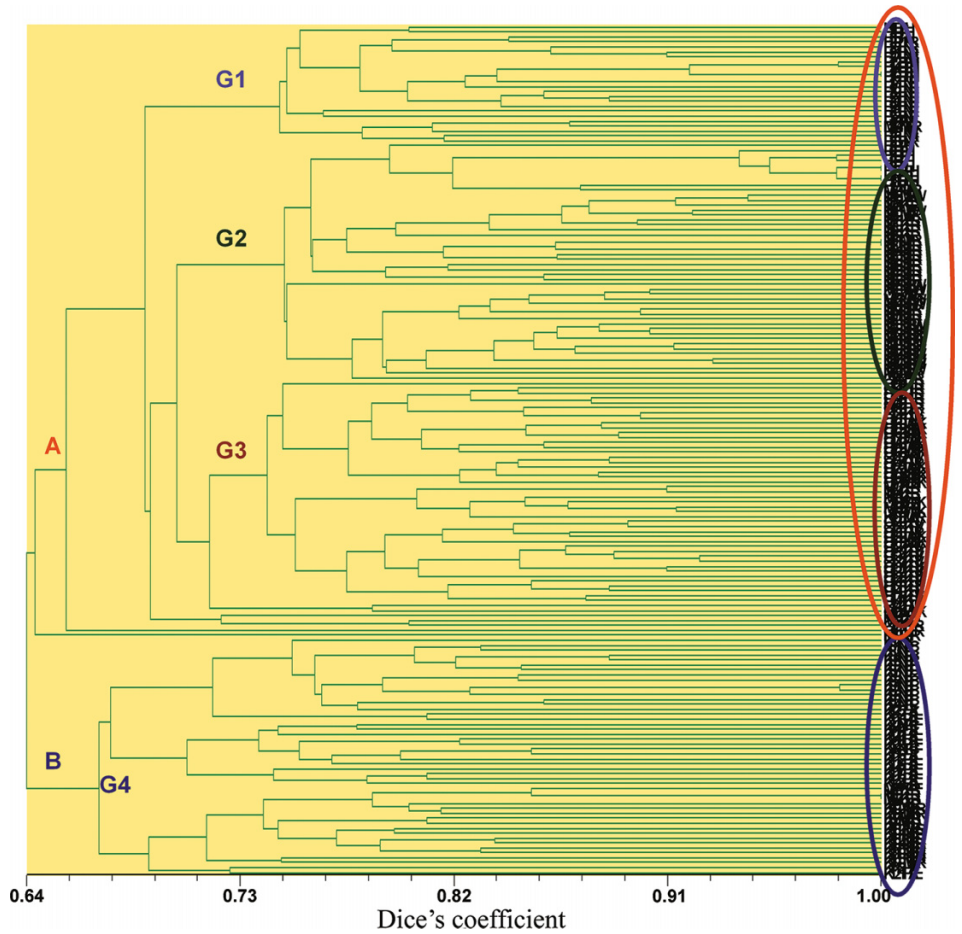
The binary data scores obtained from genotyping 172 koa trees with the 12 selected markers were used to generate Dice's and Jaccard's coefficients. Both Dice's and Jaccard's coefficients avoid shared absences, although Dice's coefficient gives more weight to matches, resulting in higher similarity values compared with those from Jaccard's coefficient. Regardless of which similarity coefficient was employed, the UPGMA cluster analysis of the 172 koa trees readily separated the populations into two major clusters, A and B (Fig. 5), with minor differences in relationships within the major clusters (Figs. S1, S2, and S3).[2] Cluster A can be subdivided into 3 minor subgroups, G1, G2, and G3, at the demarcation having 64% and 47% similarities for Dice's and Jaccard's coefficients, respectively. In both UPGMA dendrograms, subgroup G1 contained 75% of the koa populations from Island of Hawaii, of which 62.5% and 37.5% were LNR and BPH genotypes, respectively. Only 3% of the samples in this subgroup were from another island, Maui. This represented 2% of koa populations from Maui. Subgroup G2 contained the remaining 25% of the koa populations from Island of Hawaii, 77% of koa populations from Maui, and 2.6% of koa populations from Oahu. The Island of Hawaii genotypes in this subgroup were 6.3% LNR and 93.7% BPH. The Maui genotypes were 40.6% MFR, 43.2% MRW, and 16.2% KFR. Subgroup G_3 contained 14.6% of the remaining 21% of the koa populations

[2] Supplementary data for this article are available on the journal Web site (http://genome.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3840. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/cms/unpub_e.html.

Table 6. Number of alleles detected at each of 12 microsatellite loci in 11 populations of *Acacia koa*.

	Number of alleles												
Population	Ak05	Ak08	Ak10	Ak15	Ak16	Ak36	Ak41	Ak44	Ak89	Ak99	Ak196	Ak284	Total
BPH	2	4	3	2	1	2	2	4	2	8	1	8	39
LNR	4	5	4	2	1	3	3	3	5	5	3	11	49
MRW	4	4	5	3	2	5	4	3	2	4	1	5	42
MFR	4	4	4	3	1	4	3	5	2	6	3	7	46
KFR	3	4	3	3	2	5	4	8	4	6	2	10	54
NPC	3	8	2	2	2	3	4	5	2	5	2	9	47
MKR	3	11	4	3	3	3	3	6	2	8	2	10	58
HAL	4	12	2	2	2	2	3	7	3	9	2	14	62
KHV	4	5	4	2	1	5	4	4	4	6	1	4	44
HLR	5	5	4	2	1	5	3	3	3	6	2	6	45
WNK	3	6	3	2	2	4	4	3	4	5	2	4	42
Total	6	17	8	4	4	8	8	13	11	14	7	16	116

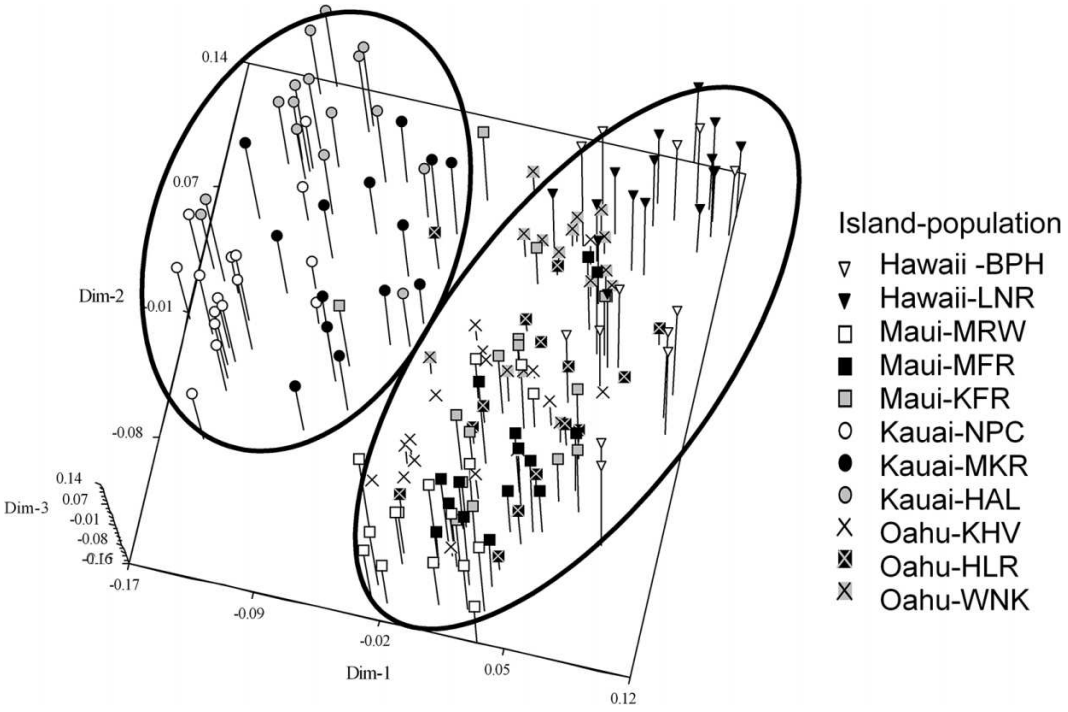
Fig. 5. UPGMA dendrogram derived from Dice's coefficient of similarity, demonstrating the genetic relationships among 172 individual trees of *Acacia koa* based on 12 microsatellite markers. The 11 populations were grouped into two major clusters, A and B (subgroup G4), with cluster A further divided into 3 subgroups (G1, G2, and G3). Cophenetic correlation coefficient = 0.92.



from Maui, 97.8% of koa populations from Oahu, and 2.2% of koa populations from Kauai. The Oahu genotypes were 31.1% HLR, 35.6% KHV, and 33.3% WNK. All the KHV and WNK samples analyzed were present in this subgroup. Also, 93.3% of HLR genotypes were present in this subgroup. Cluster B, or subgroup G4, contained the remaining 6.3% of koa populations from Maui and 97.8% of koa populations from Kauai. Thus, UPGMA cluster analysis of the 172 koa trees was able to segregate the 11 populations into 4 clusters, G1, G2, G3, and G4, which contained 75%, 77%, 97.8%, and 97.8% of the samples from Hawaii, Maui, Oahu, and Kauai, respectively.

The binary scores for the presence or absence of a peak in the electropherogram were also used for PCO by both

Fig. 6. Principal coordinate analysis of microsatellite loci showing relative positions of 172 individual trees of *Acacia koa* based on Dice's coefficient. The three axes explain 40.3% (principal coordinate 1), 8.2% (principal coordinate 2), and 5.6% (principal coordinate 3) of the genetic variation among the populations. The 11 populations were grouped into two major clusters, the Kauai populations forming one distinct group and the populations from Oahu, Maui, and Hawaii forming the other group.



Dice's and Jaccard's methods. To capture the maximum variation in our microsatellite diversity data, we used the first, second, and third principal axes, with a total variability of 54%. Plots derived from the first 3 principal coordinate axes using either method showed that koa populations from Kauai form a distinct group separate from koa populations on all the other neighboring islands (Fig. 6). Thus, PCO based on both Dice's and Jaccard's coefficients was able to divide koa populations from the four islands into two major groups.

Genetic diversity statistics

The overall genetic diversity (H'_T) estimated across all koa populations with the FDASH program was 1.001. The average within-population genetic diversity (H'_S) ranged from 0.566 to 0.988, indicating that the within-population genetic diversity accounts for a very high percentage of the overall diversity. This trend was due to significantly higher H'_S within populations from all four islands (randomization test, $P < 0.05$). Pairwise genetic differentiation, determined by F'_{ST}, ranged from 0.028 to 0.230 and averaged 0.093, 0.060, 0.065, and 0.070 for Kauai, Oahu, Maui, and Hawaii populations, respectively (Table 7). These averages were not significantly different from each other, suggesting that differentiation of koa populations within islands is very similar across the four islands sampled (t test, $P \geq 0.38$). Pairwise comparison of the 11 populations showed that koa from Maui Makawao Forest Reserve (MFR) were closely related to those from Kapunakea Forest Reserve (Table 7). Similarly, the Oahu Hawaii Loa Ridge (HLR) population was closely related to the Waianae Kai (WNK) population. On the other hand, the population from Mahana Ridge West (MRW) on Maui was most dissimilar to the population from Waianae Kai (WNK) on Oahu. Thus, the spatial separation of the islands also appeared to influence genetic differentiation of koa populations.

Discussion

Microsatellite loci of plants and animals are known to be associated mostly with noncoding regions or regions flanking coding sequences of the genome, and are heritable as Mendelian traits (Hancock 1995; Toth et al. 2000; Ellegren 2004; Schlotterer 2004). These loci are used as markers in linkage studies, for construction of genetic maps, and for determining genetic diversity in populations. Isolation of microsatellite markers is especially useful in species for which sufficient genomic sequences are not available (Zane et al. 2002). We used the dual-suppression PCR protocol (Lian et al. 2001) for enrichment and isolation of adjacent microsatellite loci from genomic DNA of koa. As a result, nearly all the cloned fragments contained microsatellite sequences. Also, during the second PCR amplification using the downstream and adapter primers, we selected fragments of different sizes for cloning and sequencing. This resulted in the isolation of mostly unique microsatellite loci and only a few redundant sequences. Analyses of the sequences of the PCR fragments obtained from the initial step of this protocol showed that about 18% of the fragments contained microsatellite motifs in the middle, with flanking variable sequences on both sides of the motifs. Based on this observation, we developed the "direct sequence" strategy to design primer pairs from the variable sequences on both sides of the microsatellite motifs. Thus, we applied two strategies,

Table 7. Pairwise genetic differentiation (F'_{ST}) among 11 populations of *Acacia koa* based on 12 microsatellite loci.

	LNR	MRW	MFR	KFR	NPC	MKR	HAL	KHV	HLR	WNK
BPH	0.070	0.151	0.078	0.074	0.123	0.124	0.070	0.147	0.129	0.184
LNR	—	0.212	0.132	0.112	0.129	0.135	0.091	0.160	0.103	0.097
MRW		—	0.098	0.070	0.166	0.135	0.132	0.173	0.144	0.230†
MFR			—	0.028*	0.094	0.057	0.105	0.101	0.087	0.145
KFR				—	0.079	0.036	0.086	0.071	0.063	0.115
NPC					—	0.117	0.077	0.175	0.131	0.171
MKR						—	0.085	0.099	0.091	0.131
HAL							—	0.166	0.124	0.154
KHV								—	0.032	0.120
HLR									—	0.028*
WNK										—

*The lowest population differentiation detected among the populations analyzed.
†The highest population differentiation detected among the populations analyzed.

"walking sequence" and "direct sequence", for the isolation of microsatellite loci from koa. From our results, the walking sequence strategy produced more polymorphic loci than the direct sequence approach. The walking sequence strategy yielded an average of 6.5 alleles per locus, while the direct sequence strategy produced 6.0 alleles per locus. The average polymorphic information content (PIC) values for the walking sequence strategy and direct sequence strategy were 0.51 and 0.42, respectively. Also, the walking sequence strategy produced more primers with PIC values > 0.6. There were also differences in the types of repeats that were isolated by the two strategies. Thirteen of 15 central motifs were different from the oligonucleotide repeats used in the initial screening. This was because the initial PCR enrichment step selected fragments that contained microsatellite motifs at the two ends, while the central motifs were independent of this selection. Development of microsatellite markers by the direct sequence strategy is much easier and less expensive than by the walking sequence strategy. For developing microsatellite markers for a new species using the direct sequence strategy, we recommend screening at least 100 clones obtained from the initial PCR amplification using oligonucleotide repeat primers. We developed 16 markers from 96 clones that were obtained using 7 oligonucleotide repeat primers. Increasing the number of oligonucleotide repeat primers in the initial PCR step should further enhance the number of clones containing unique microsatellite motifs. We observed that complementary primers, such as $(AC)_{10}$ and $(TG)_{10}$, can lead to selection of the same clones.

Although there are more than 1300 *Acacia* species distributed in Australia, Africa, Asia, and North and South America (Maslin et al. 2003; Miller et al. 2003; Murphy et al. 2003), none of these have been characterized for genomic sequences. Microsatellite markers have so far been developed for *A. mangium*, *A. brevispica*, *A. saligna*, and *A. mellifera* (Butcher et al. 2000; Otero-Arnaiz et al. 2005; Millar and Byrne 2007; Ruiz-Guajardo et al. 2007). The number of microsatellite markers developed for the other acacias varies from 10 to 33. Millar and Byrne (2007) and Ruiz-Guajardo et al. (2007) isolated 10 and 11 microsatellite markers for *A. saligna* and *A. mellifera*, respectively, while Otero-Arnaiz et al. (2005) and Butcher et al. (2000) developed 24 and 33 markers to determine genetic diversity in *A. brevispica* and *A. mangium*, respectively. Microsatellite markers are largely species-specific: among 33 markers developed for *A. mangium*, only 4 could amplify the corresponding loci from *A. melanoxylon* and none could amplify corresponding loci from *A. boliviana* and *A. nilotica* (Butcher et al. 2000). Koa is distinct from all other acacias and is found only in the Hawaiian Islands. Koa is among the few tetraploid species of this genus ($2n = 52$); many other acacias, such *A. melanoxylon* and *A. mangium*, are diploid (Elevitch et al. 2006). There are considerable phenotypic variations among koa populations on different islands. The development of microsatellite markers for koa will therefore facilitate future population genetics studies and breeding programs for this tree.

Classification of koa based on morphological characters is greatly affected by the limited number of available phenotypic characters and also by the ambiguities in the scoring of the phenotypic characters (Sun et al. 1996; Daehler et al. 1999). Molecular markers have a unique power not possessed by any other taxonomic method in allowing the estimation of similarity between organisms that are extremely diverse (Kirst et al. 2005; Garcia-Martinez et al. 2006). Analyses of populations by molecular markers without the complementary morphological characters have been successfully carried out in other plants (Staub et al. 2000; Palombi and Damiano 2002; Yifru et al. 2006). Both the UPGMA cluster analysis and the PCO subdivided the populations into two major groups: the group with the populations from Kauai was distinct from the group comprising the populations of Oahu, Maui, and Hawaii. The UPGMA cluster analysis further divided one of the major groups into three additional subgroups. Thus, among the koa populations from the four major Hawaiian Islands, the Kauai populations had the highest level of genetic diversity and formed a distinct group. This was not surprising considering that Kauai is the oldest among the major Hawaiian Islands (Macdonald et al. 1983), permitting koa to evolve and differentiate into various ecological groups under a wide range of micro-environmental conditions. Also, Kauai possesses the most undisturbed forests among the islands sampled and hence its populations formed a distinct group. Although Hawaiian Islands are located far apart, the diversity in the micro-environments within each island appeared to play a

major role in the evolution of diversity in the koa population. If the environment within an island was homogeneous, the exchange of alleles between individual trees on the same island should have resulted in island ecotypes that were genetically more similar within islands than between islands. This phenomenon, referred to as the isolation-by-distance effect, has been observed for other species (Epperson 2007; Medina et al. 2007; Bessert and Orti 2008; Worheide et al. 2008). The calculated F'_{ST} statistics, used to compare the degree of heterozygosity, showed little evidence of population differentiation among koa populations from the different islands. Our findings that intra-island population diversities contributed more to the overall genetic diversity may have also resulted in part from anthropogenic activities such as planting of collected seeds during the last century.

The microsatellite markers developed in this study will be useful in koa breeding and improvement programs in the future. Many trees in koa plantations die owing to wilt disease within a few years of planting (Whitesell 1990; Wilkinson and Elvevitch 2003). In recent years, some koa provenances have been identified that appear to be resistant to wilt disease (N. Dudley, Hawaii Agriculture Research Center, Aiea, Hawaii, personal communication 2008). In some crops, microsatellite loci linked to disease resistance have been identified after analyzing a large number of samples from disease-resistant and susceptible plants (Adhikari et al. 2004; Minamiyama et al. 2007; Bernet et al. 2008; Guo et al. 2008; Yang et al. 2008; Zhao et al. 2008). Microsatellite loci linked to genes conferring resistance to wilt disease may be identified and used as selectable markers in future koa improvement programs. The markers developed in this study may also be used to determine whether the extent of genetic diversity in various koa plantations is similar to that found in natural koa forests. These markers may also be useful for distinguishing koa from other acacias.

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