Technical Report HCSU-0036

POPULATION GENETIC STRUCTURE OF RARE AND ENDANGERED PLANTS USING MOLECULAR MARKERS

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ABSTRACT

This study was initiated to assess the levels of genetic diversity and differentiation in the remaining populations of *Phyllostegia stachyoides* and *Melicope zahlbruckneri* in Hawai`i Volcanoes National Park and determine the extent of gene flow to identify genetically distinct individuals or groups for conservation purposes. Thirty-six Amplified Fragment Length Polymorphic (AFLP) primer combinations generated a total of 3,242 polymorphic deoxyribonucleic acid (DNA) fragments in the *P. stachyoides* population with a percentage of polymorphic bands (PPB) ranging from 39.3 to 65.7% and 2,780 for the *M. zahlbruckneri* population with a PPB of 18.8 to 64.6%. Population differentiation (*Fst*) of AFLP loci between subpopulations of *P. stachyoides* was low (0.043) across populations. Analysis of molecular variance of *P. stachyoides* showed that 4% of the observed genetic differentiation occurred between populations in different kīpuka and 96% when individuals were pooled from all kīpuka. Moderate genetic diversity was detected within the *M. zahlbruckneri* population. Bayesian and multivariate analyses both classified the *P. stachyoides* and *M. zahlbruckneri* populations into genetic groups with considerable sub-structuring detected in the *P. stachyoides* population. The proportion of genetic differentiation among populations explained by geographical distance was estimated by Mantel tests. No spatial correlation was found between genetic and geographic distances in both populations. Finally, a moderate but significant gene flow that could be attributed to insect or bird-mediated dispersal of pollen across the different kīpuka was observed. The results of this study highlight the utility of a multi-allelic DNA-based marker in screening a large number of polymorphic loci in small and closely related endangered populations and revealed the presence of genetically unique groups of individuals in both *M. zahlbruckneri* and *P. stachyoides* populations. Based on these findings, approaches that can assist conservation efforts of these species are proposed.

INTRODUCTION

Rare and endangered species often have small populations and are therefore vulnerable to environmental and genetic risks (Bauert *et al.* 1998). They tend to lose genetic variability through random genetic drift faster than populations that are larger and stable or populations that are connected with active gene flow. The extent of genetic variability of a population determines the ability of that population to adapt to its environment through natural selection. When genetic variability is reduced, the possible combinations of genes that can confer fitness and vigor in response to critical changes in the environmental conditions are also considerably reduced (Reed and Frankham 2003).

Melicope zahlbruckneri is endemic to the island of Hawai`i, and *Phyllostegia stachyoides* is known from Hawai`i, Maui, and Moloka`i (Wagner *et al.* 1999) with limited and declining populations. *M. zahlbruckneri* belongs to the Rutaceae family and is listed along with many other *Melicope* species as federally endangered (U.S. Fish and Wildlife Service [USFWS] 1997). *M. zahlbruckneri* occurs as a single population with fewer than 30 individuals left on Hawai`i Island (Pratt *et al*. 2010). Species in this family generally have flowers that divide into four or five parts. In terms of size, they range from herbs to shrubs and small trees. *M. zahlbruckneri* grows to 10–12 m tall with mature leaves that are 6 to 24 cm long and 4 to 12.5 cm wide with well-defined lateral veins (Wagner *et al.* 1999). *P. stachyoides* is currently listed as a species of concern due to limited population availability. It is found mostly on higher elevations (from 1,625 m to just below 1,800 m elevation) on Hawai`i Island with dry to mesic forest habitat.

There is currently no information available on the genetics of either species, also, very little is known on their historic distribution. Thus, it is important to determine the current level of genetic variability, gene flow, and genetic structure in these populations in order to make informed recommendations about their conservation.

The application of molecular tools as an essential component in the conservation of rare and endangered species is becoming routine practice (Zawko *et al.* 2001, Luan *et al.* 2006) with wide application in both plant and animal populations. Molecular markers in combination with spatial statistical tools have contributed immensely to the understanding of the distribution of genetic diversity and differentiation, gene flow, and population size implications for a wide range of plant species (Cruzan 2001, Wang and Ge 2006, Raji *et al.* 2009, Yuan *et al.* 2012). Due to their neutral nature, these markers have been particularly useful for studies involving intra- and inter-species genotypic variation (Robinson *et al.* 1999) in relatively small populations. Molecular random markers are able to provide a more precise understanding of genetic diversity through the identification of genomic segments that differentiate individuals or populations without the need for genetic information about the genome (Young *et al*. 1996, Montgomery *et al.* 2000).

Several markers are available for the determination of population genetic variation with the choice of marker system often determined by the availability of information for the species, the population type and size, and available resources. Microsatellites and Single Nucleotide Polymorphic (SNP) markers are recognized as the most efficient at revealing genetic diversity within and between species and with the ability to distinguish homozygous and heterozygous individuals. However, Amplified Fragment Length Polymorphism (AFLP) is robust, and proficient at revealing population diversity and estimating genetic distance between samples and populations. Furthermore, AFLP has the potential to screen a large number of genetic loci in a single experiment and does not require prior information about the genome of the species under investigation. A number of conservation genetic studies have been conducted using AFLP markers to evaluate genetic diversity and differentiation in endangered plant species (e.g., AFLP analysis was used to describe patterns of genetic variation and population structure in seven extant populations of *Isoetes sinensis* [Kang *et al.* 2005], wild populations of *Sinopodophyllum hexandrum* [Xiao *et al.* 2006], and apricot [*Prunus armeniaca* L; Yuan *et al.* 2007]). Here we report an AFLP-based molecular assessment to evaluate the genetic structure of *M. zahlbruckneri* and *P. stachyoides* populations actively managed at Hawai`i Volcanoes National Park.

METHODS

Sample Collection

Populations were sampled by collecting leaf tissue from individual plants; a small-sized healthy leaf was chosen and stored in a sealed plastic bag and taken to the laboratory for immediate processing or stored at -20^{o}C until DNA (deoxyribonucleic acid) extraction. Sampling of the population was done from sites where both *M. zahlbruckneri* and *P. stachyoides* species are known to naturally occur on the island of Hawai`i. *M. zahlbruckneri* was collected from Kīpuka Puaulu, the only remaining site where the species is known to be extant (other than a previously discovered individual at Laupahoehoe [USFWS 1997]). *P. stachyoides* was collected from the Kīpuka Mauna`iu at Mauna Loa where three spatially separated clusters of plants were sampled in the upper, lower, and middle kīpuka—referred to as “populations” throughout this

study. Nine samples were collected from the upper kīpuka, 11 from the middle kīpuka, and 20 from the lower kīpuka (Table 1).

Table 1. List of *P. stachyoides* plant samples and kīpuka population analyzed

Lab ID	Field ID	Field tag	Kīpuka pop.	Location
P1	L_4	4_40	Lower	Kīpuka Mauna`iu (Mauna Loa)
P2	L_8	4_26	Lower	Kīpuka Mauna`iu (Mauna Loa)
P3	L_2	4_41	Lower	Kīpuka Mauna`iu (Mauna Loa)
P4	L_17	4_10	Lower	Kīpuka Mauna`iu (Mauna Loa)
P5	L_16	No tag	Lower	Kīpuka Mauna`iu (Mauna Loa)
P6	L_15	4_2	Lower	Kīpuka Mauna`iu (Mauna Loa)
P7	L_12	4_32	Lower	Kīpuka Mauna`iu (Mauna Loa)
P8	L_10	4_29	Lower	Kīpuka Mauna`iu (Mauna Loa)
P9	L_3	4_37	Lower	Kīpuka Mauna`iu (Mauna Loa)
P10	M_21	3_2	Middle	Kīpuka Mauna`iu (Mauna Loa)
P11	M_25	3_12	Middle	Kīpuka Mauna`iu (Mauna Loa)
P12	M_22	3_1	Middle	Kīpuka Mauna`iu (Mauna Loa)
P13	M_26	3_9	Middle	Kīpuka Mauna`iu (Mauna Loa)
P14	M_27	3_15	Middle	Kīpuka Mauna`iu (Mauna Loa)
P15	M_31	3_5	Middle	Kīpuka Mauna`iu (Mauna Loa)
P16	M_30	3_4	Middle	Kīpuka Mauna`iu (Mauna Loa)
P17	M_24	3_13	Middle	Kīpuka Mauna`iu (Mauna Loa)
P18	M_23	3_14	Middle	Kīpuka Mauna`iu (Mauna Loa)
P19	M_28	3_10	Middle	Kīpuka Mauna`iu (Mauna Loa)
P20	M_29	3_8	Middle	Kīpuka Mauna`iu (Mauna Loa)
P21	U_42	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P22	U_35	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P23	U_33	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P24	U_36	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P25	U_41	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P26	U_32	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P27	U_39	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P28	U_40	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)
P29	L_5	4_19	Lower	Kīpuka Mauna`iu (Mauna Loa)
P30	L_7	4_23	Lower	Kīpuka Mauna`iu (Mauna Loa)
P31	L_6	4_15	Lower	Kīpuka Mauna`iu (Mauna Loa)
P32	L_20	328	Lower	Kīpuka Mauna`iu (Mauna Loa)
P33	L_9	4_27	Lower	Kīpuka Mauna`iu (Mauna Loa)
P34	L_1	329	Lower	Kīpuka Mauna`iu (Mauna Loa)
P35	L_13	4_28	Lower	Kīpuka Mauna`iu (Mauna Loa)
P36	L_14	No tag	Lower	Kīpuka Mauna`iu (Mauna Loa)
P37	L_18	4_38	Lower	Kīpuka Mauna`iu (Mauna Loa)
P38	L_19	4_29	Lower	Kīpuka Mauna`iu (Mauna Loa)
P39	L_11	4_31	Lower	Kīpuka Mauna`iu (Mauna Loa)
P40	U_37	No tag	Upper	Kīpuka Mauna`iu (Mauna Loa)

Sampling of plants was done systematically throughout the population so that sampling intensity reflected plant density in each kīpuka. All remaining plants of *M. zahlbruckneri* and several *Melicope* trees of uncertain identity adjacent to known *M. zahlbruckneri* were sampled (Table 2).

Table 2. List of *M. zahlbruckneri* plant samples analyzed

Lab ID	Field ID	Location
MZ01	MZ-13	Kīpuka Puaulu
MZ02	MSP-40	Kīpuka Puaulu
MZ03	MSP-45	Kīpuka Puaulu
MZ04	MZ-3	Kīpuka Puaulu
MZ05	MZ-26	Kīpuka Puaulu
MZ06	MZ-9	Kīpuka Puaulu
MZ07	MZ-27	Kīpuka Puaulu
MZ08	MZ-28	Kīpuka Puaulu
MZ09	MZ-21	Kīpuka Puaulu
MZ10	MZ-14	Kīpuka Puaulu
MZ11	MZ-20	Kīpuka Puaulu
MZ12	MSP-43	Kīpuka Puaulu
MZ13	MZ-6	Kīpuka Puaulu
MZ14	MZ-23	Kīpuka Puaulu
MZ15	MZ-15	Kīpuka Puaulu
MZ16	MZ-16	Kīpuka Puaulu
MZ17	MZ-17	Kīpuka Puaulu
MZ18	MSP-1	Kīpuka Puaulu
MZ19	MZ-8	Kīpuka Puaulu
MZ20	MSP-46	Kīpuka Puaulu
MZ21	MSP-38	Kīpuka Puaulu
MZ22	MZ-22	Kīpuka Puaulu
MZ23	MZ-19	Kīpuka Puaulu
MZ24	MZ-29	Kīpuka Puaulu
MZ25	MSP-31	Kīpuka Puaulu
MZ26	MZ-36	Kīpuka Puaulu
MZ27	MSP-39	Kīpuka Puaulu
MZ28	MSP-44	Kīpuka Puaulu

MZ: *Melicope zahlbruckneri*

DNA Isolation and Quantitation

The isolation of good quality and high-molecular-weight genomic DNA is essential for many molecular biology applications, including polymerase chain reaction (PCR) and the endonuclease restriction digestion required for successful AFLP procedures. Several methods and commercial kits are available for the extraction of DNA from plant material. However, standard protocols and some commercially available DNA kits that we tested did not produce adequate yields and quality of DNA needed for the AFLP analysis. We modified and optimized a DNA protocol developed by Dellaporta *et al.* (1983) to produce consistently high yields of good quality and

amplifiable DNA for all samples. Key modifications of the protocol include the use of hexadecyltrimethylammonium bromide (CTAB), a cationic detergent for the effective denaturation of proteins and solubilization of cellular membranes, and the use of polyvinylpyrrolidone (PVP) to reduce the effects of polyphenols, quinines, and tannins which tend to be more abundant in leaves of tree species. Incubation of samples after the addition of extraction buffer was also extended by 10 min at 65^{o}C. Finally, an additional chloroform-isoamyl alcohol (24:1) extraction step was included in the protocol and a final DNA clean-up step using the Dneasy plant DNA column (Qiagen Inc., Valencia, CA) was added. The purified DNA from both fresh and frozen leaf tissue showed excellent spectral qualities suitable for our downstream application needs. This method is cost-effective and efficient in the removal of phenolic compounds that can interfere with downstream analysis of the DNA and also produces high molecular weight DNA. The concentration and purity of DNA was measured using the nanodrop spectrophotometer, and quality was assessed on agarose gel electrophoresis.

Molecular Marker Screening and PCR Optimization

Microsatellite markers

Simple sequence repeats (SSRs) have had limited use in studies involving natural plant populations because they have to be developed specifically for each plant species. This process is often time consuming and expensive. However, several studies have demonstrated the successful transferability of gene-based microsatellites across related genera (Varshney *et al.* 2005; Raji *et al.* 2009; De Bang *et al.* 2011). Among the advantages of microsatellite markers are their high reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance, and good genome coverage, plus, only a small amount of DNA template is required for their analysis. Since there are currently no SSR markers developed for *P. stachyoides* and *M. zahlbruckneri*, we searched the National Center for Biotechnology Information database and literature for available genomic or gene-based microsatellites that have been developed for their close taxa to explore the prospects of transferability of such markers to fingerprint the populations.

Phyllostegia stachyoides — Twenty-four Expressed Sequence Tags (ESTs) based microsatellite markers (Lindqvist *et al.* 2006), developed for *Stenogyne* (one of the members of the three genera that make up the Hawaiian mints including the *Phyllostegia* species), were screened. Of these, 20 primer sequences were selected and synthesized (Integrated DNA Technologies, Coralville, IA). We optimized PCR reaction components and cycling parameters for each primer pair and tested them for successful amplification and polymorphism of *P. stachyoides* DNA samples.

Melicope zahlbruckneri — Seven compound microsatellite loci developed for *Melicope quadrilocularis* (Katoh *et al.* 2007) were tested for amplification and polymorphism on *M. zahlbruckneri.*

PCR reactions for SSR marker assay were performed in a Biorad DNA Engine (Peltier Thermal Cycler). PCR products were separated on 1.5% Tris-acetate-ethylenediaminetetraacetic acid (TAE) agarose gels and visualized under ultraviolet light (UV) after ethidium bromide staining.

AFLP Genotyping

Restriction digestion and adapter ligation

The AFLP procedure followed the original method described by Vos *et al.* (1995) with a few modifications, and unless otherwise indicated, Life Technologies (Invitrogen and Applied Biosystems) reagents were used in all reaction procedures. Total genomic DNA (500 ng) was

digested with 4.0 units of Eco RI and Mse I restriction enzymes at 37°C for 4 h in a 25 µl reaction volume that included 5X reaction buffer and 1 mg/ml bovine serum albumin (BSA). After digestion, 5 µl of digested DNA from each sample was run on TAE 1% agarose gel to check for a complete and uniform digestion.

The restriction digestion was followed by adapter ligation. Ligation reaction was performed in a 40 µl solution and contained 20 µl of the digested DNA, 5 pmol of the EcoRI adapter, 50 pmol of the Mse I adapter, 1 unit of T4 DNA ligase, 0.5 mg/ml BSA, and 1X DNA ligase buffer. The reaction was left to incubate for 3 h at 20°C.

Amplification of AFLP templates

A series of two amplifications are required to optimize the AFLP reactions; the pre-amplification reaction utilized AFLP primers with one selective nucleotide to enrich a subset of the AFLP template and to reduce background interference in amplified products during electrophoresis. The pre-amplification reaction was performed in a 30 µl volume using 1 µl of the ligation reaction as the template and combined with 21.5 ng each of the Eco RI+A and Mse I+A primers, 1X Amplitaq 360 DNA buffer (500 mM KCl), 15 mM $MgCl_2$, 10 mM deoxyribonucleotide triphosphates (dNTPs; each at 2.5 mM), and 1 unit of Taq DNA polymerase. The reaction was processed in a thermal cycler using the following cycling parameters: an initial denaturation step at 94°C for 60 s, 20 cycles of 94°C/30 s, 56°C/60 s, 72°C/60 s and a final extension at 72°C for 10 min.

Selective amplification was performed using primers with three selective nucleotides. Thirty-six primer pairs were used to fingerprint both populations. These primer pairs were chosen based on the number, clarity, and reproducibility of bands produced in a preliminary screening of 60 primer combinations. The selective amplification was performed in a 10-µl reaction volume containing 2.5 µl of diluted pre-amplification products (diluted 1:20 in 1× Tris–ethylenediaminetetraacetic acid buffer), 5 ng of EcoRI+3 and 15 ng of Mse I+3 selective primers, 1X Amplitaq 360 buffer, dNTPs (2.5 mM each), and 0.2 units of Amplitaq 360 DNA polymerase. All the EcoRI+3 selective primers were fluorescently labeled with FAM, NED, and JOE using the DS-30 Matrix standard (Applied Biosystems, Foster City, CA). Amplification was performed with a touch down cycling procedure as follows: an initial denaturation step at 94°C for 2 min, 1 cycle of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C; followed by 11 cycles in which the annealing temperature decreased 0.7°C per cycle and 22 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C; and a final extension step at 72°C for 5 min.

Polyacrylamide Gel Electrophoresis and Silver Staining

Polyacrylamide gel electrophoresis and silver staining procedures were done as a preparative technique to screen for polymorphism and optimize conditions for capillary electrophoresis. This technique enables the screening of markers in a cost-effective manner using unlabeled primers for fingerprinting and silver staining for the detection of DNA fragments. Prior to electrophoresis, 2 µL of selective amplification product was added to 2 µL loading buffer mix (98% formamide (v/v), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.25% xylene cyanol (w/v), 0.25% bromophenol blue (w/v)), heated at 95°C for 3 min and placed on ice. Three microliters of the mixture was loaded on a 6% denaturating polyacrylamide gel. Electrophoresis was performed at a constant voltage (1460 V) at 55°C for 2.5 h using a Gibco BRL vertical electrophoresis apparatus. Fragments were visualized by staining with 2% silver nitrate. Thirty-six primer pairs with polymorphic and clear scorable fragments were selected for fragment analysis of the *Melicope* and *Phyllostegia* populations.

Sample Genotyping

AFLP fragment analysis of all individuals was performed by capillary electrophoresis using an automated Genetic Analyzer ABI 3730 (University of Illinois sequencing services). AFLP fragment data was normalized based on standard height and intensities as well as prior information from gel images. Also, local southern size-call algorithm, peak saturation, baseline saturation, pull-up correction, and spike removal corrections were used for data normalization. Following normalization, allele sizing and call procedures were performed using the GENEMAPPER (ver. 4.1, Applied Biosystems) and GENEMARKER (ver. 2.2, SoftGenetics) software programs. ROX labeled size standard that range in size from 35–500 bp was used for fragment sizing. Individual peak was called on the basis of the total signal intensity, and the peak was scored only if the intensity exceeded a fixed threshold. Manual scoring from a standardized template was used for each primer combination to ensure that peak sizing and position were precise for all electropherograms.

Data Analysis

Allele sizing and calls were done with a pattern recognition algorithm of GeneMarker® Software (SoftGenetics, State College, PA) to eliminate or reduce false and shoulder peaks and to improve analysis consistency. Fragments were scored as either present or absent to create a binary matrix for each individual. The resulting binary matrix was used as input data for the SIMQUAL module in NTSYSpc ver. 2.20d (Rohlf 2005) to generate Jaccard's similarity coefficient (Sneath and Sokal 1973) and then transformed into a dissimilarity measure (1 – Jaccard's similarity). Pairwise genetic distance (Nei and Li 1979) was also computed for each population. The TREECON program (Y. Van de Peer, Antwerp, Belgium) was used to compute UPGMA (unweighted pair-group method with arithmetic averages) dendrograms and neighbor joining cladograms for all distance matrices. The tree branching pattern was evaluated by bootstrapping, and was based on 1000 replicates using the PHYLIP software package 3.66 (Felsenstein 2006). Genetic diversity measure for each population was calculated using the AFLP-SURV (Vekemans 2002) and MVSP (Ver. 3.21) programs. Hardy-Weinberg equilibrium (FIS = 0) was assumed for all populations where the frequency of an absent band or fragment is q^2 (q is the null allele frequency). The presence of a band indicates either a heterozygote or homozygote for the dominant allele; therefore, allele frequencies are calculated based on the frequency of the null allele. With this assumption, genetic diversity within each population was estimated as the percentage of within-population polymorphic loci relative to the overall polymorphic loci using the Nei's unbiased expected heterozygosity and the Lynch and Milligan's (1994) estimates where:

$$\text{Within Population Variation} = H_j(i) = 2q_j(i)[1 - q_j(i)] / 2\text{Var}[q_j(i)] \qquad \text{(Equation 1)}$$

$$\text{Between Population Variation} = \text{Var}[H_j(i)] = 4[1 - 2q_j(i)]^2 \text{Var}[q_j(i)] \qquad \text{(Equation 2)}$$

with H_j, Nei's gene diversity measure; q, the frequency of the null allele (estimated for sampled individuals) for the jth locus in population j; and Var, the sampling variance.

Total diversity within each population and between subpopulations of *P. stachyoides* was computed using the polymorphic fragment frequencies for each primer combination. Percent polymorphic fragment frequencies at each AFLP marker loci were computed using the PopGene program (Exeter Software, Setauket, NY).

The *P. stachyoides* individuals were analyzed in the first instance as a single population and later partitioned into separate groups corresponding to the different kīpuka where they were collected in order to estimate the extent of differentiation between the populations. Wright's fixation index (Fst; Hartl and Clark 1997) was calculated using the method of Lynch and Milligan (1994) for each population. Significance of the Fst values was tested by a permutation procedure of 1000 replicates, which randomly permutes individuals among the populations and then recalculates Fst for each permutation; the observed value of Fst was then compared to the distribution of randomized Fst values. Interpopulation genetic distances and analysis of molecular variance (AMOVA) were computed using the GenAlEx 6 (Peakall and Smouse 2006) program to partition genetic variation between *P. stachyoides* populations and among all individuals.

To further explore the genetic structure of each population, a model-based Bayesian analysis was performed using the program STRUCTURE ver. 2.4.1 (Pritchard *et al.* 2000, Falush *et al.* 2007; Hubisz *et al.* 2009). A non-uniform prior distribution of allelic frequencies was assumed with its parameters derived from the observed distribution of fragment frequencies among loci (Zhivotovky 1999). Estimates for the log likelihood were obtained using the admixture model and correlated allele frequencies options with a burn-in period of 80,000 and 100,000 Markov Chain Monte Carlo (MCMC) iterations after burn in. Likely genetic cluster (K) was set over a range from K = 1-6 with 10 replicate runs at each K. The posterior probability [P(X|K)] was estimated to give an indication of the true likely number of groups without prior information about the population. The CLUMPP program (Pritchard *et al.* 2000; Jakobsson and Rosenberg 2007) was used for permutations of the most likely observations among replicate runs for each K, and results were visualized in the DISTRUCT (Rosenberg 2004) program.

Genetic relatedness among individuals of each population was determined by principal coordinate analyses (PCoA) based on the modified Roger's distance matrices generated from the binary data for both populations. PCoA computations were performed with the MVSP (Ver. 3.21; Exeter Software, Setauket, NY) program. A two-dimensional plot of the population was generated such that the geometrical distances among samples in the plot reflect the genetic distances within the population.

For gene flow analysis, proportion of between population diversity (Gst) was estimated for individual populations of *P. stachyoides* using Wright's F statistics (Wright 1978) and expressed as:

$$Gst = HT - HS / HT \qquad \text{(Equation 3)}$$

where HT and HS represent total and partial tests, respectively, for heterozygosity for each subpopulation using a method that is less sensitive to sample size and suitable when a large number of polymorphic loci are examined (Nei 1973, 1978). Gene flow was estimated from Gst (GST) values and is expressed as:

$$(Nm) = 0.5(1 - GST)/GST \qquad \text{(Equation 4)}$$

(McDermott and McDonald 1993).

Since the genetic structure of populations can be influenced by both ecology and physical geographic distance and isolation, these effects were assessed in *P. stachyoides* populations at different kīpuka. The geographic distance between collecting sites was calculated from their

global positioning system (GPS) locations and combined with the genetic distance values into a pair-wise genetic and geographic distance matrix (GenAIEx). Geographic distance was calculated as a common Euclidean distance

$$Dij = [(xi - xj)2 + (yi - yj)2]1/2 \quad \text{(Equation 5)}$$

where *Dij* is the distance between points *i* and *j*. Otherwise, Universal Transverse Mercator (UTM) coordinates were transformed into geodesic coordinates. Genetic distances were generated using pair-wise population distance values of AFLP fragments between individuals of each population. Mantel statistics were used to perform a correlation test to assess spatial correlation between the geographic and genetic distance matrices for each population.

RESULTS

Microsatellites Marker Analysis

Twenty-eight plant samples from the *M. zahlbruckneri* population were genotyped using seven compound microsatellite primers developed for *Melicope quadrilocularis* (Katoh *et al.* 2007). PCR-detected loci were identical and monomorphic in all samples tested. The amplified alleles matched the expected fragment sizes for each primer pair as described for *M. quadrilocularis*. Two of the primers did not detect any amplifiable product.

Populations of *P. stachyoides* were analyzed using 20 EST-based microsatellite markers (Lindqvist *et al.* 2006). The EST microsatellites did not detect any polymorphism in the population. Since all primer pairs tested were unable to detect polymorphism in both the *P. stachyoides* and *M. zahlbruckneri* populations, the microsatellite marker genotyping was discontinued.

AFLP Pattern and Analysis

AFLP allele calls and sizing were done using the GeneMapper (ver. 4.1) and GeneMarker program (V2.2.0). Fragments were scored by converting sizes in bp to 0/1 matrix and used as input data for estimating genetic distances.

Thirty-six AFLP primer pairs generated 3242 fragments in the *P. stachyoides* population and 2780 polymorphic fragments in the *M. zahlbruckneri* population (Tables 3 and 4). The percentage of polymorphic fragments across all *P. stachyoides* ranged from 39.3 to 65.7% with an average of 51.4%. Polymorphic fragments ranged from 26.0 to 64.6% with an average of 54.9% in the *M. zahlbruckneri* population. Of the total fragments scored in the *P. stachyoides* population, 648 unique fragments (20%) were detected in a few plant samples across all populations, while the remaining fragments were common to the rest of the population.

Genetic distance and population genetic structure

Similarity matrices based on Nei and Li's similarity index (Nei and Li 1979) and Jaccard's coefficients were estimated for all samples using the binary AFLP data. Coefficients of similarity for all possible pair-wise comparisons ranged from 0.493 to 1.407 among *P. stachyoides* individuals (Appendix 1) and 0.621–1.171 in the *M. zahlbruckneri* population with an average of 0.688 (Appendix 2). Cluster analysis dendrograms based on the UPGMA of the AFLPSurv program and the FLEXI method in NTSYS are shown in (Figures 1 and 2). Hierarchical clusters with bootstrap values revealed low to moderate substructuring in both the M. zahlbruckneri and

Table 3. AFLP marker variation and polymorphism in selective amplification of P. stachyoides

Primer code	Selective sequence E/M pairs	Total number of fragments	Polymorphic fragments	Polymorphic fragments (%)	Size range (base pair)
EM-1	E-AAC/M-CTC	120	73	60.8	35–480
EM-2	E-ACT/M-CTT	150	59	39.3	50–500
EM-3	E-AAG/M-CAC	135	70	51.9	45–450
EM-4	E-ACA/M-CAC	160	88	55.0	50–500
EM-5	E-ACC/M-CAC	167	84	50.3	65–480
EM-6	E-ACC/M-CAT	190	92	48.4	55–500
EM-7	E-AGC/M-CTA	189	93	49.2	50–480
EM-8	E-AGC/M-CAC	140	64	45.7	50–500
EM-9	E-AGG/M-CTA	221	103	46.6	60–500
EM-10	E-AGG/M-CAG	152	73	48.0	50–500
EM-11	E-ACT/M-CAG	189	90	47.6	50–490
EM-12	E-ACG/M-CTC	175	86	49.1	50–475
EM-13	E-ACA/M-CTG	199	97	48.7	55–480
EM-14	E-AAC/M-CTT	102	53	52.0	50–500
EM-15	E-ACG/M-CTT	114	57	50.0	40–500
EM-16	E-ACT/M-CTC	121	57	47.1	50–500
EM-17	E-AAG/M-CTC	156	82	52.6	45–500
EM-18	E-AAC/M-CAG	192	94	49.0	40–500
EM-19	E-ACT/M-CTG	198	130	65.7	50–500
EM-20	E-ACA/M-CTC	174	86	49.4	60–500
EM-21	E-ACC/M-CAG	152	83	54.6	64–500
EM-22	E-AGG/M-CTT	181	80	44.2	50–500
EM-23	E-ACC/M-CTG	213	102	47.9	45–490
EM-24	E-AAG/M-CTT	165	88	53.3	40–500
EM-25	E-AGG/M-CTG	207	119	57.5	55–480
EM-26	E-ACA/M-CTT	195	90	46.2	50–500
EM-27	E-AAC/M-CAT	223	142	63.7	40–500
EM-28	E-AAC/M-CTA	242	121	50.0	40–500
EM-29	E-ACT/M-CAT	235	130	55.3	40–490
EM-30	E-ACA/M-CTA	161	82	50.9	50–500
EM-31	E-ACA/M-CAT	153	72	47.1	50–500
EM-32	E-AAC/M-CAC	129	78	60.5	50–500
EM-33	E-ACT/M-CTA	184	88	47.8	55–500
EM-34	E-ACG/M-CAT	176	88	50.0	50–500
EM-35	E-ACG/M-CAG	196	109	55.6	55–500
EM-36	E-AAG/M-CTA	228	139	61.0	55–500
Mean		174.56	90.06	51.44	

P. stachyoides populations. A low, but well-defined level of genetic variation was detected in *M. zahlbruckneri* populations as shown by the UPGMA cluster pattern (Figure 2).

Neighbor-joining (NJ) trees were constructed to further examine the population structure of the clusters. The NJ cladogram showed a similar cluster grouping to the dendrogram with a few

Table 4. AFLP marker variation and polymorphism in selective amplification of *M. zahlbruckneri*

Primer code	Selective sequence E/M pairs	Total number of fragments	Polymorphic fragments	Polymorphic fragments (%)	Size range (base pair)
EM-1	E-AAC/M-CTC	130	84	64.6	50–480
EM-2	E-ACT/M-CTT	189	114	60.3	50–500
EM-3	E-AAG/M-CAC	145	85	58.6	45–450
EM-4	E-ACA/M-CAC	172	96	55.8	50–500
EM-5	E-ACC/M-CAC	136	75	55.1	65–480
EM-6	E-ACC/M-CAT	130	80	61.5	55–500
EM-7	E-AGC/M-CTA	142	81	57.0	50–480
EM-8	E-AGC/M-CAC	106	66	62.3	50–500
EM-9	E-AGG/M-CTA	132	80	60.6	60–500
EM-10	E-AGG/M-CAG	135	73	54.0	50–500
EM-11	E-ACT/M-CAG	134	72	53.7	50–490
EM-12	E-ACG/M-CTC	167	95	56.8	50–475
EM-13	E-ACA/M-CTG	155	87	56.1	55–480
EM-14	E-AAC/M-CTT	102	59	57.8	50–500
EM-15	E-ACG/M-CTT	103	39	37.9	40–500
EM-16	E-ACT/M-CTC	150	85	56.6	50–500
EM-17	E-AAG/M-CTC	137	78	56.9	45–500
EM-18	E-AAC/M-CAG	140	67	47.9	40–500
EM-19	E-ACT/M-CTG	185	110	59.4	50–500
EM-20	E-ACA/M-CTC	157	92	58.5	60–500
EM-21	E-ACC/M-CAG	127	65	51.8	64–500
EM-22	E-AGG/M-CTT	101	40	39.6	50–500
EM-23	E-ACC/M-CTG	100	62	62.0	45–490
EM-24	E-AAG/M-CTT	85	16	18.8	40–500
EM-25	E-AGG/M-CTG	97	61	62.9	55–480
EM-26	E-ACA/M-CTT	96	25	26.0	50–500
EM-27	E-AAC/M-CAT	145	81	55.0	40–500
EM-28	E-AAC/M-CTA	135	77	57.0	40–500
EM-29	E-ACT/M-CAT	168	101	60.2	40–490
EM-30	E-ACA/M-CTA	167	102	61.0	50–500
EM-31	E-ACA/M-CAT	150	92	61.3	50–500
EM-32	E-AAC/M-CAC	120	64	53.3	50–500
EM-33	E-ACT/M-CTA	165	102	61.8	55–500
EM-34	E-ACG/M-CAT	156	93	59.6	50–500
EM-35	E-ACG/M-CAG	163	100	61.3	55–500
EM-36	E-AAG/M-CTA	145	81	55.9	55–500
Mean		137.97	77.2	54.9	

sample overlaps between clusters. The *P. stachyoides* population was separated into four genetic cluster groups (Figure 3): the first cluster comprised five closely grouped plant samples, all samples in this group are from the lower kīpuka population; the second cluster had eight plants from the lower and middle kīpuka; and the third cluster group contained a mixture of samples from all kīpuka. The final cluster group consisted of five plants from the lower and

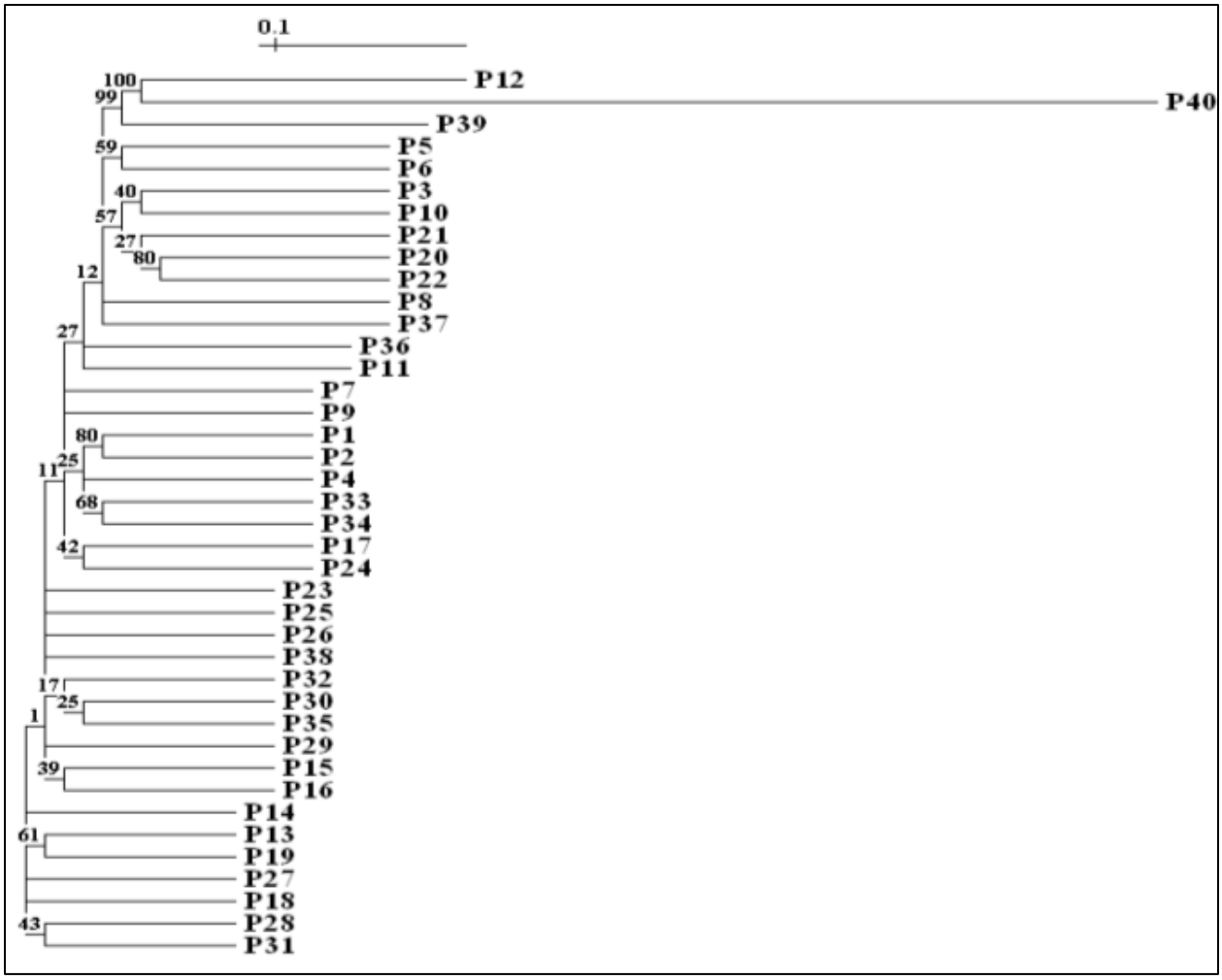


Figure 1. Hierarchical cluster analysis of *P. stachyoides* using UPGMA (Unweighted Pair Group Method with Arithmetic Mean). Bootstrap values are indicated at tree node.

upper kīpuka and a middle kīpuka plant that appeared to be genetically distinct from the rest of the population. This seemingly unique sample also accounted for more than 35% of the private alleles observed in the entire population. Other than the plants in the fourth cluster group, no shared allele was observed between this plant (P40) and the rest of the *P. stachyoides* population.

The NJ tree constructed for *M. zahlbruckneri* showed a less structured genetic variation; individuals appeared to be less differentiated than in the *P. stachyoides* population. However, a few plants (particularly MZ23 and MZ27) that accounted for many of the private alleles in the *M. zahlbruckneri* population were shown to display unique genetic profiles and considerable degrees of variation from the rest of the population (Figure 4).

Genetic Differentiation, Gene Flow, and Partitioning of Molecular Variance

Genetic differentiation among and within *P. stachyoides* populations is presented in Table 5. Low differentiation was observed between populations (kīpuka), supporting the possibility of an

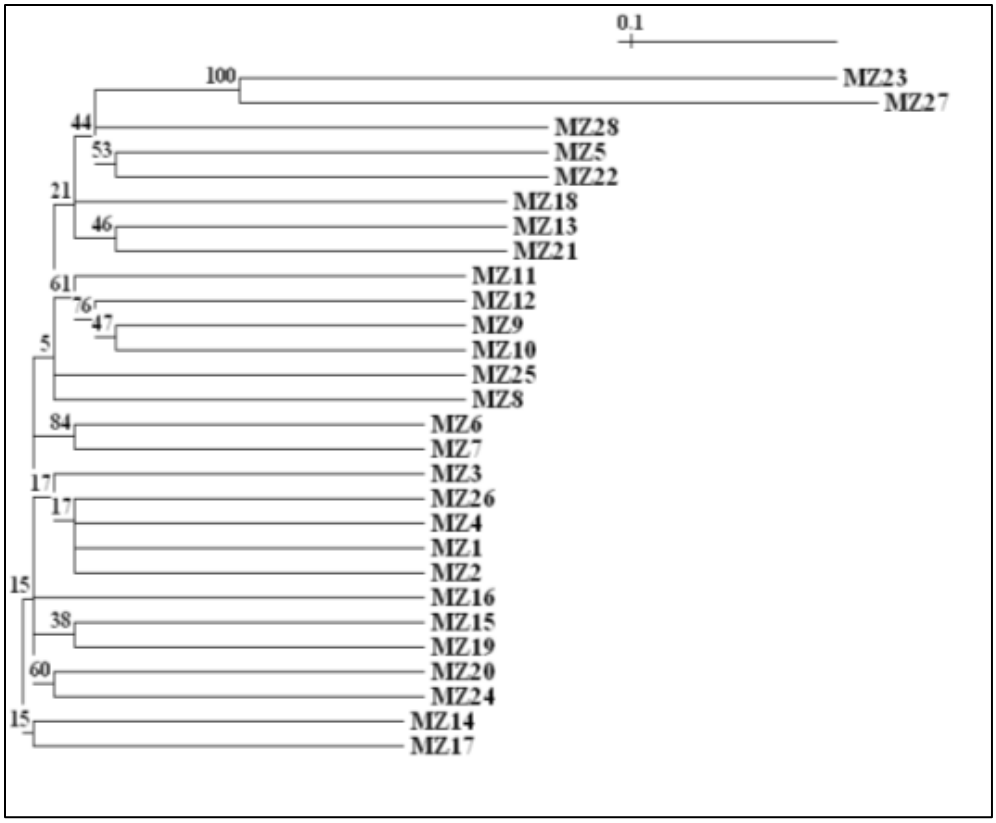


Figure 2. Hierarchical cluster analysis of *M. zahlbruckneri* using UPGMA (Unweighted Pair Group Method with Arithmetic Mean). Bootstrap values are indicated at tree node.

active gene flow across the kīpuka range of the population. However, differentiation among individuals regardless of their geographic designation was moderately high as shown by the neighbor-joining cladogram (Figure 3). Analysis of molecular variance (AMOVA) among the populations indicated that the majority of genetic variation (96%) occurred across populations while variation between populations (different kīpuka) only accounted for 4% of the overall observed variation (Table 6). Pairwise Fst (PhiPT) values averaged Fst = 0.043 ($P < 0.001$) between populations.

Gene flow was estimated between kīpuka populations of *P. stachyoides*. Allele frequencies at different loci were used to estimate Nm, which is the gene flow between populations or groups of individuals. The average gene flow estimate between populations per generation was moderate (Nm = 4.9; Table 6). Gene flow between *M. zahlbruckneri* and other populations of *Melicope* in close proximity was not assessed, but there are indications that possible hybridization may have occurred due to the considerable number of private alleles observed in the population as well as the presence of a few individuals with unique genetic profiles.

Bayesian analysis showed that four cluster partitions (K) were empirically determined for *P. stachyoides* and *M. zahlbruckneri* populations. The optimal convergence of the MCMC algorithm was achieved by using a burn-in period of 80,000 steps, followed by 100,000 steps of data collection and 10 replicated runs to ensure efficiency in clustering computation. The plot of

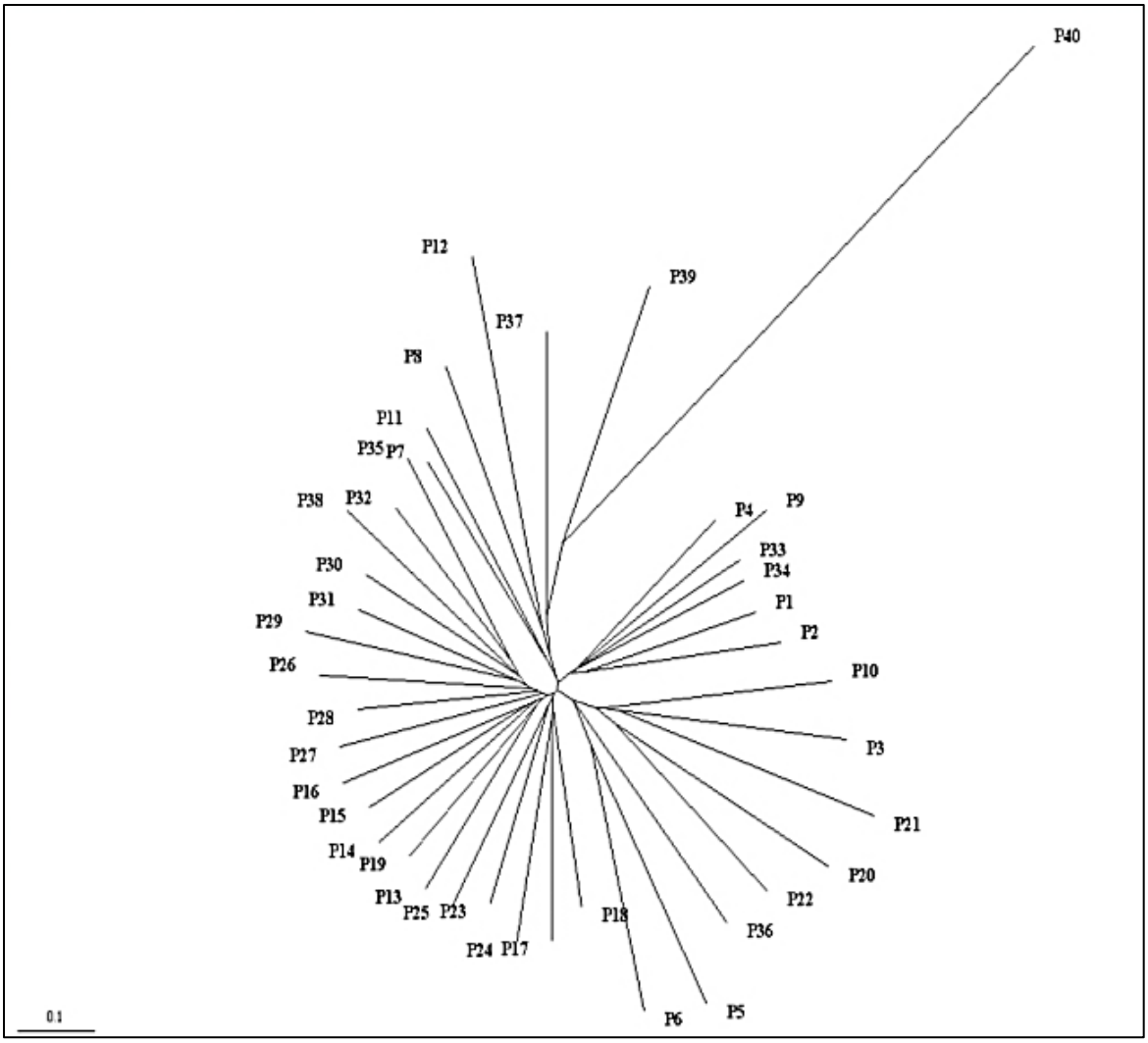


Figure 3. Neighbor-joining tree derived from Euclidean distance matrix generated for AFLP fragments of *P. stachyoides* populations

distribution of alleles showing the proportion of shared genomic segments as detected by AFLP markers are presented in Figures 5 and 6. As noted in the cluster analysis, MZ23, MZ27, and MZ28 showed different allelic patterns than the rest of the population. Similarly, nine individuals (MZ3, MZ8, MZ13, MZ14, MZ16, MZ24, MZ25, MZ26, and MZ28) were observed to show the occurrence of both the rare and common alleles. These individuals are likely hybrids with variable genomic contributions from MZ23 and MZ27.

The Fst values of allele distribution provided additional information on allele sharing and differentiation within individuals in each cluster identified (Appendices 5 and 6). The Fst of the rarest alleles represented in the *M. zahlbruckneri* population ranged from 0.87–0.96 and is restricted to only a few individuals (Appendix 5). The Fst distribution of alleles in the *P. stachyoides* population is shown in Appendix 6. The average distance between individuals in the same cluster and the mean value of Fst among clusters are presented in Appendix 7 for both *M. zahlbruckneri* and *P. stachyoides* populations.

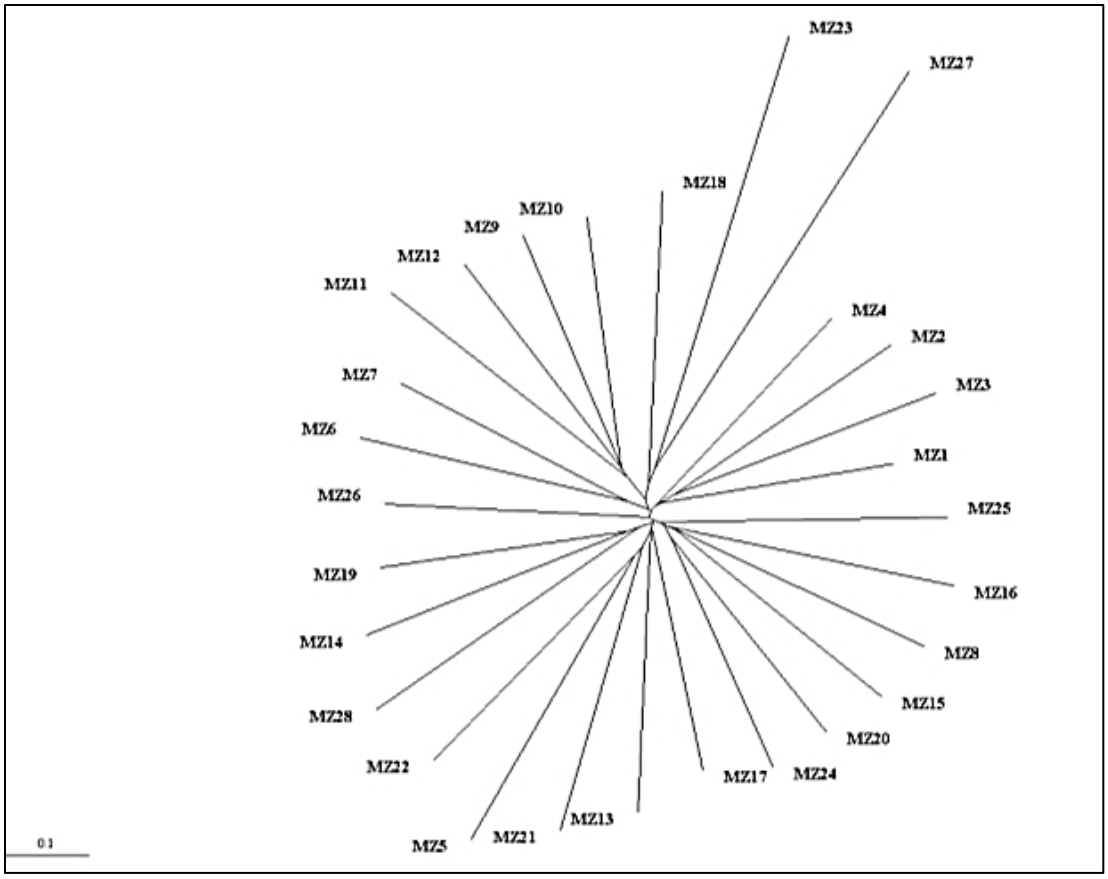


Figure 4. Neighbor-joining tree derived from the Euclidean distance matrix generated for AFLP fragments of *M. zahlbruckneri* population.

Principal coordinate analysis scores from the first three coordinates showed that the Eigen vectors accounted for 63.05% of the total variance in the *P. stachyoides* population and 65.82% in the *M. zahlbruckneri* population (Tables 5 and 7). Additional results from the PCoA analysis showing the population patterns from each kīpuka are presented in a two-dimensional representation of multidimensional genetic distances between individuals of each population. The *P. stachyoides* population showed a spread of individual samples along the PCO1 (Y axis) that is not indicative of geographical location (Figure 7). The first coordinate (x-axis) accounted for 28.064% of the variance in genetic distance among individuals, the second coordinate (y axis) accounted for 18.62%, and the third coordinate accounted for 16.37% of the variance in the *P. stachyoides* population (Table 5). Principal coordinates in the *M. zahlbruckneri* population showed a total of 65.82% cumulative variation estimated from scores of principal coordinates with the first coordinate accounting for the most variation at 29.9% (Table 7). The plot of genetic distances estimated from the coordinates showed genetic grouping and relationships between individual samples that is reflective of a high degree of common alleles in more than 50% of the individuals but also highlights the impact of unique alleles along the PCO2 grouping (Figure 8). Two individuals in the *M. zahlbruckneri* population (MZ23 and MZ27) were shown to be genetically distinct from the rest of the population. This result is consistent with the pattern of variation observed from Bayesian analysis, NJ cladogram, and UPGMA trees constructed for the population.

Table 5. Principal coordinate analysis and Eigen values of the first three coordinates with sample Eigen vectors of AFLP loci of *P. stachyoides*

Individual	PCO1	PCO2	PCO3
P01	-0.370	0.430	-2.007
P02	-0.431	-0.413	-3.048
P03	-0.248	3.321	-1.548
P04	-0.577	-0.925	-1.625
P05	-0.509	2.166	-1.211
P06	-0.040	0.826	-2.741
P07	-0.211	-0.264	-2.056
P08	0.667	-1.255	-2.844
P09	0.134	-1.359	-2.804
P10	-0.611	3.390	-0.675
P11	-0.468	-1.011	-1.193
P12	0.793	2.294	0.469
P13	-1.059	-0.342	1.030
P14	-0.984	-0.336	0.365
P15	-0.897	0.795	0.136
P16	-0.412	0.592	-1.479
P17	-0.007	0.250	-0.878
P18	-0.894	-0.947	0.243
P19	-1.092	-0.089	1.307
P20	-0.370	3.998	1.011
P21	-0.284	3.454	1.521
P22	-0.824	2.928	1.718
P23	-0.798	0.583	0.984
P24	-0.145	-0.324	-0.891
P25	-0.117	-0.269	0.178
P26	0.007	-1.551	1.339
P27	-1.007	-0.687	2.548
P28	-0.770	-1.146	0.647
P29	-0.542	-1.926	1.826
P30	-0.868	-1.489	1.671
P31	-0.905	-2.051	1.569
P32	-0.228	-1.880	0.180
P33	0.104	-1.921	-1.613
P34	-0.111	-1.612	-0.676
P35	-0.158	-2.422	0.944
P36	-0.557	0.933	1.969
P37	0.746	-0.268	1.574
P38	0.096	-1.599	1.529
P39	1.628	0.049	2.085
P40	12.320	0.079	0.444
% variation	28.06	18.62	16.37
Cum %	28.06	46.68	63.05

% variation is percentage of variation explained by each of the first three axes

Cum % is the cumulative percentage of all three axes

Table 6. Analysis of molecular variance between populations of *P. stachyoides* at three different kīpuka

Source	df	SS	MS	Est. var.	Percent (%)
Among Pops	2	940.445	470.223	13.547	4
Within Pops	37	11145.405	301.227	301.227	96
Total	39	12085.850		314.774	100

Stat	PhiPT(Fst)	P(rand >= data)	Nm
Value	0.043	0.010	4.9

SS: sums of squared observations
MS: mean of squared observations
Est. var.: estimated variance
PhiPT = AP/(WP + AP) = AP/TOT = proportion of the total genetic variance among individuals within populations
P(rand >= data), the probability of a random value greater than and equal to the observed data value, for PhiPT is based on permutation across the full data set
Nm: 0.5(1 – GST)/GST

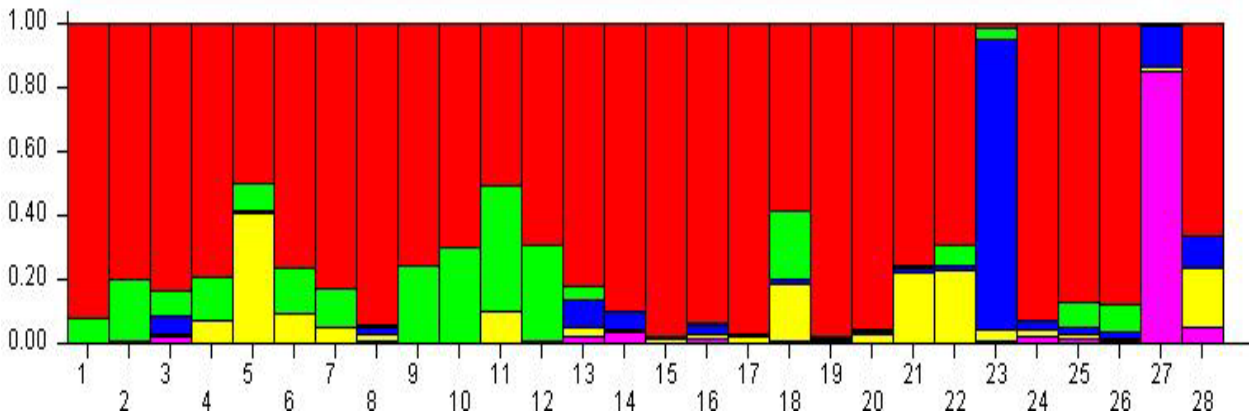


Figure 5. Bar plot showing a model-based clustering of 28 individuals of *M. zahlbruckneri* by the STRUCTURE program with K = 4. Color indicates the proportion of shared alleles or genomic segments derived from each cluster.

Analysis of genetic and geographic distances

Mantel statistics with 1000 random permutations indicated no significant evidence of correlation between genetic and geographic distance in the *M. zahlbruckneri* population (r = 0.03, $P < 0.001$; Figure 9). No significant association was detected in the *P. stachyoides* population from the lower and upper kīpuka (lower: r = 0.016, $P < 0.001$; upper: r = 0.012, $P < 0.001$) the middle kīpuka showed a low correlation (r = 0.664, $P < 0.05$) between genetic and geographic distances Mantel correlation probability and plots for *P. stachyoides* are presented in Table 8, and Figures 10 and 11.

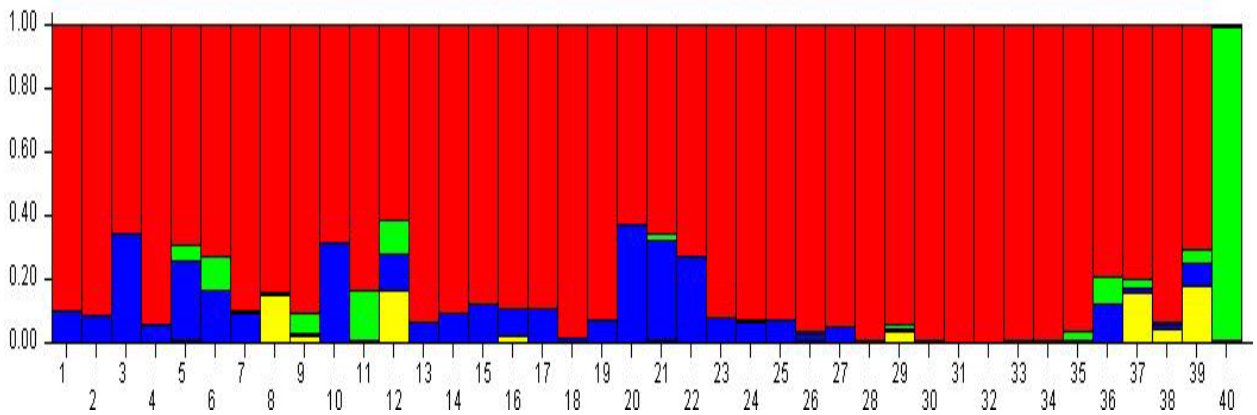


Figure 6. Bar plot showing a model-based clustering of 40 individuals of *P. stachyoides* by the STRUCTURE program with K = 4. Color indicates the proportion of shared alleles or genomic segments derived from each cluster.

DISCUSSION

This study investigated the population genetics of the endangered *Melicope zahlbruckneri* and the species of concern *Phyllostegia stachyoides* using AFLP markers. Microsatellite markers used in this study were developed for distantly related taxa of *P. stachyoides* and *M. zahlbruckneri*, and even though some conserved loci were amplified, no polymorphism was observed. The lack of polymorphism observed in the microsatellite markers for both *Phyllostegia* and *Melicope* can be attributed to the characteristic low transferability of microsatellite markers in most species (Peakall *et al.* 1998).

The number of polymorphic AFLP loci analyzed in this study provided informative estimates of population genetic structure and overall genetic diversity of both *M. zahlbruckneri* and *P. stachyoides*. The AFLP analysis offered a better representation of the genome of both *M. zahlbruckneri* and *P. stachyoides* since population genomics studies based on a few molecular marker loci tend to be biased (Mariette *et al.* 2002). In order to compensate for the inability of AFLP markers to differentiate between heterozygote and homozygote individuals, the presence and absence binary data were converted to expected heterozygosity through the assumption of Hardy-Weinberg equilibrium. This provided estimates that were almost as informative as those generated from co-dominant markers. Furthermore, the criteria described by Lynch and Milligan (1994) were applied to the data to obtain an unbiased estimate of heterozygosity.

Analyses of AFLP data revealed considerable variation among individuals of *P. stachyoides* and *M. zahlbruckneri* with most variation explained within each population. Since the distribution of genetic variability within or among populations of the same species can be affected by ecological and environmental factors and lead to genetic differentiation over relatively small geographic distances (Knowles 1984), we estimated levels of differentiation in the *P. stachyoides* population by partitioning the data and analyzing it as population groups based on geographic location in different kīpuka. Comparing the level of differentiation among the three populations, it was evident that the overall population grouping showed greater differentiation than individual kīpuka populations. This observation is consistent with the gene flow results and indicated an unrestricted gene exchange that could be attributed to reported dispersal by

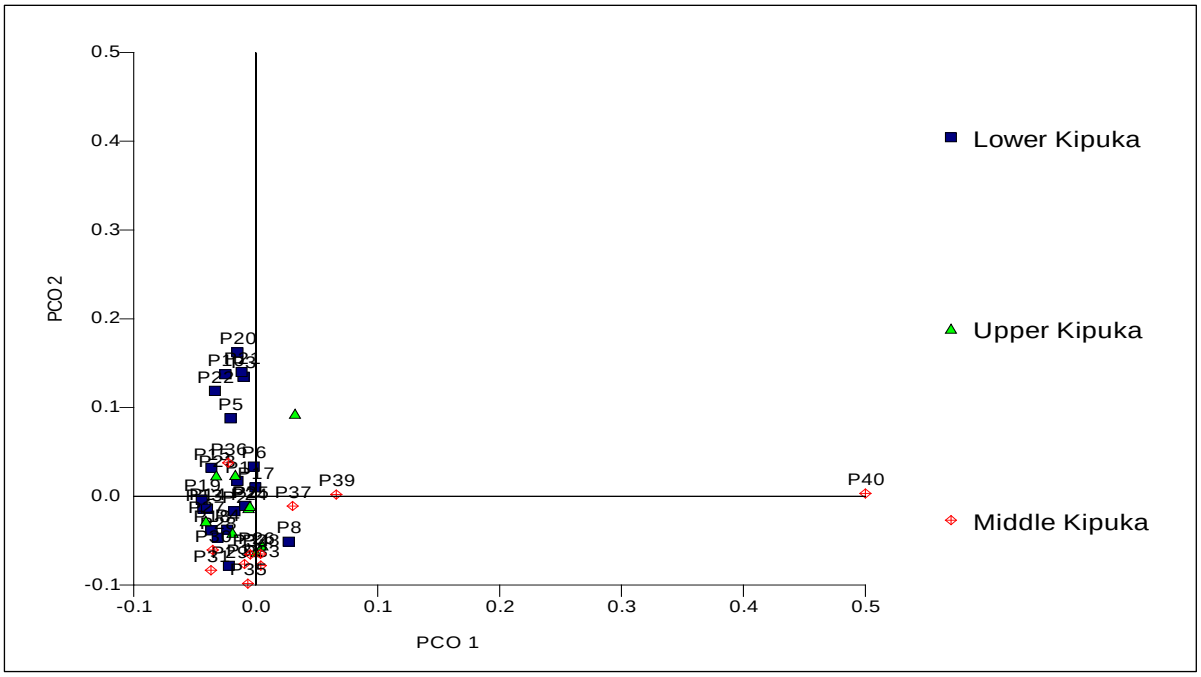
Table 7. Principal coordinate analysis and Eigen values of the first three coordinates with sample Eigen vectors of AFLP loci of *M. zahlbruckneri.*

Individual	PCO1	PCO2	PCO3
MZ-01	-21.889	-1.535	7.740
MZ-02	-15.016	-19.112	6.796
MZ-03	-13.051	-12.038	24.693
MZ-04	-30.710	-16.271	-26.390
MZ-05	6.176	14.428	-73.684
MZ-06	-22.781	13.364	-34.619
MZ-07	-22.615	8.103	-19.866
MZ-08	-32.652	7.645	18.911
MZ-09	-25.912	-4.160	6.132
MZ-10	-17.833	-13.031	0.513
MZ-11	0.118	-14.202	-56.733
MZ-12	-18.998	-1.571	-16.943
MZ-13	-13.837	-2.233	18.519
MZ-14	-19.925	7.103	36.328
MZ-15	-33.882	8.084	26.441
MZ-16	-22.140	6.449	32.324
MZ-17	-27.565	12.423	13.447
MZ-18	10.352	-4.206	-41.973
MZ-19	-24.581	2.790	23.286
MZ-20	-26.529	16.953	19.931
MZ-21	-4.943	-6.048	1.905
MZ-22	-4.350	7.861	-30.056
MZ-23	94.356	-133.951	15.355
MZ-24	-23.055	13.991	15.972
MZ-25	-17.621	7.407	15.687
MZ-26	-23.157	-4.757	6.271
MZ-27	150.001	92.604	20.527
MZ-28	-5.872	29.001	-18.616
% variation	29.90	21.11	14.81
Cum %	29.90	51.01	65.82

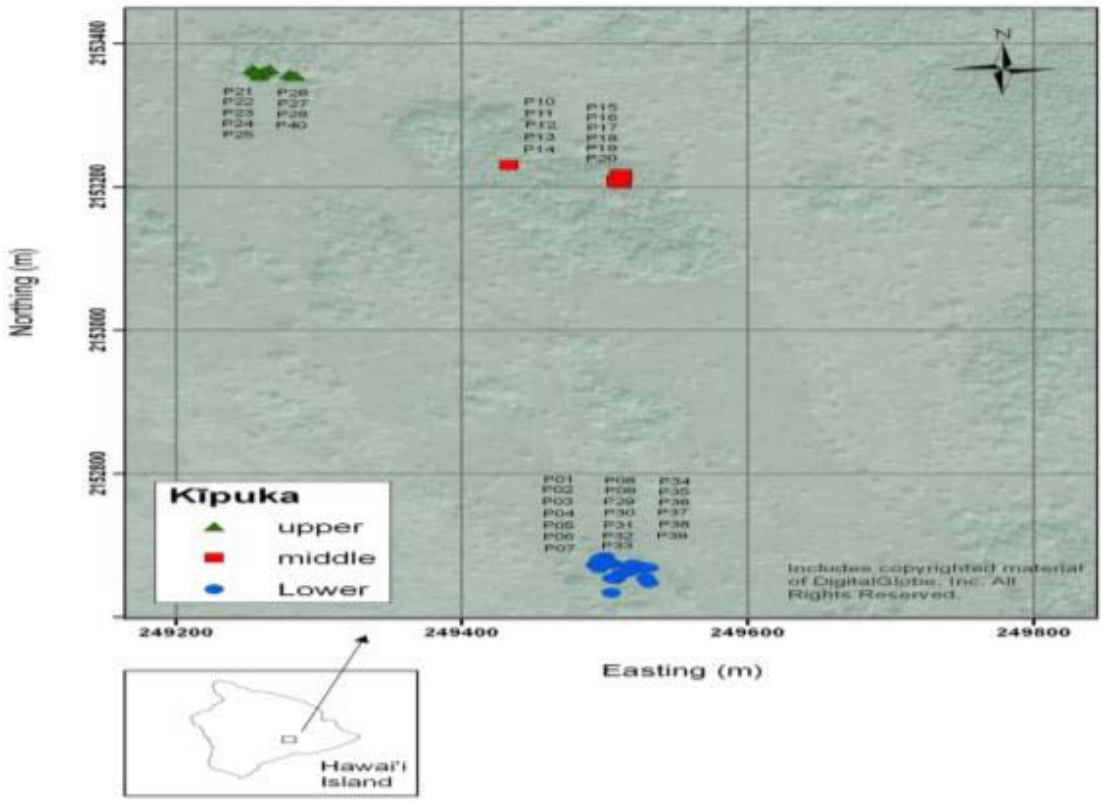
% variation is the percentage of variation explained by each of the first three axes

Cum % is the cumulative percentage of all three axes

insects and birds moving between populations of *P. stachyoides* in different kīpuka (Pratt *et al.* 2012). Furthermore, partitioning of the genetic variability by *P. stachyoides* populations by AMOVA did not indicate any significant form of genetic delineation between kīpuka. Observed genetic differentiation was distributed among individuals across kīpuka.

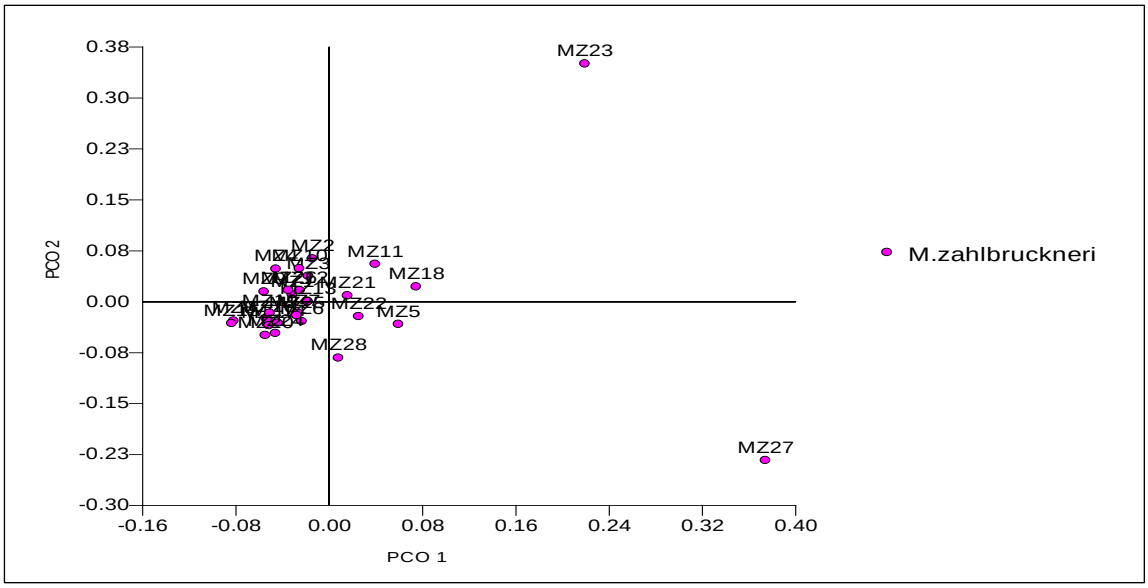


a)

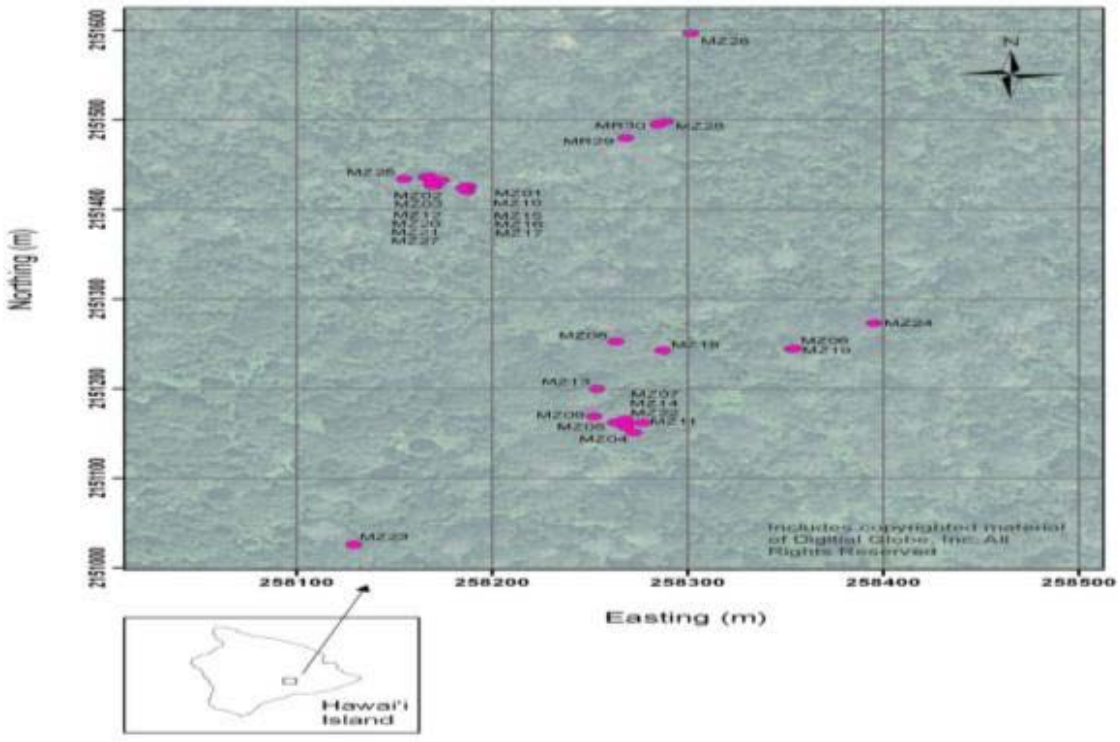


b)

Figure 7. Population structure of *P. stachyoides* showing (a) principal coordinate analysis based on genetic distances of AFLP markers and (b) distribution based on geographic distances of plants according to their location in different kīpuka.



a)



b)

Figure 8. Population structure of *M. zahlbruckneri* showing (a) principal coordinate analysis based on genetic distances of AFLP markers and (b) distribution based on geographic distances of plants according to their location.

These results support the lack of association observed between geographical and genetic distances as revealed by Mantel test and demonstrate that there is no genetic isolation between the *P. stachyoides* populations in different kīpuka. Identical patterns of genetic clustering of populations were obtained from both the UPGMA and NJ cladograms. Many of the samples were

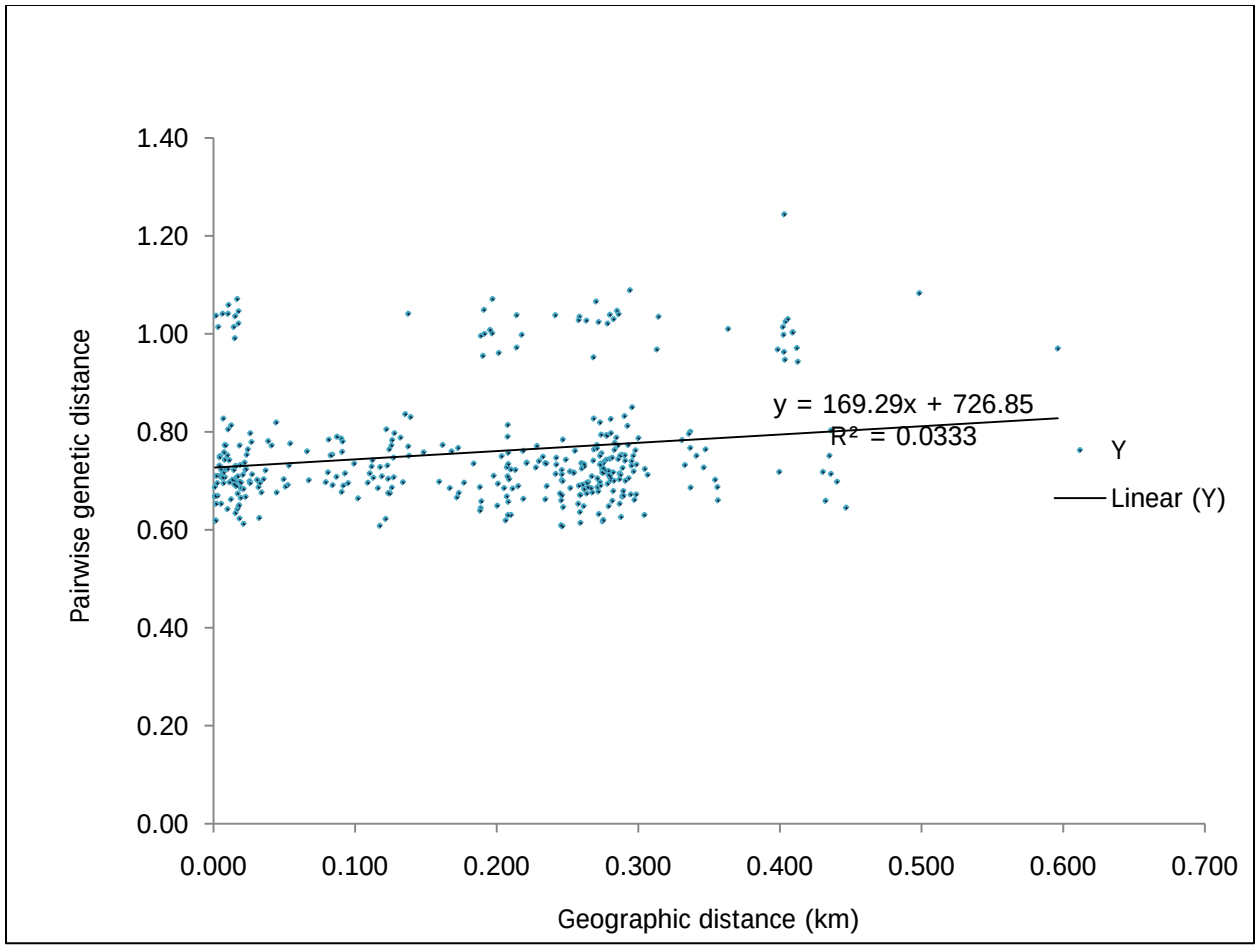


Figure 9. Mantel correlation of *M. zahlbruckneri* population based on matrices of pairwise genetic distances (Nei and Li 1979) and the natural logarithm of pairwise geographic distances

Table 8. Mantel test for correlation between genetic and geographical distances in the *P. stachyoides* populations from three kīpuka

Population	Pop size	SSX*	SSY†	SPXY‡	RXY§	Probability
Upper kīpuka	n=9	0.001	3760977.889	13.777	0.012	0.540
Middle kīpuka	n=11	0.008	322520.545	33.156	0.664	0.011
Lower kīpuka	n=20	0.004	922777.453	4.032	0.016	0.503

* Sum of products of the X matrix (genetic distance) elements
† Sum of products of the Y matrix (geographical distance) elements
‡ Sum of cross products of corresponding elements of the X and Y matrices
§ Mantel correlation coefficient

consistently grouped together by both methods with only a minor overlap of samples between clusters. The genetic clustering patterns in both populations were mostly substantiated by the bootstrap analysis with assigned values indicated at each node. Genetic diversity analysis of the *P. stachyoides* individuals showed clear evidence of different gene pool clusters by identifying distinct genetic cluster groups.

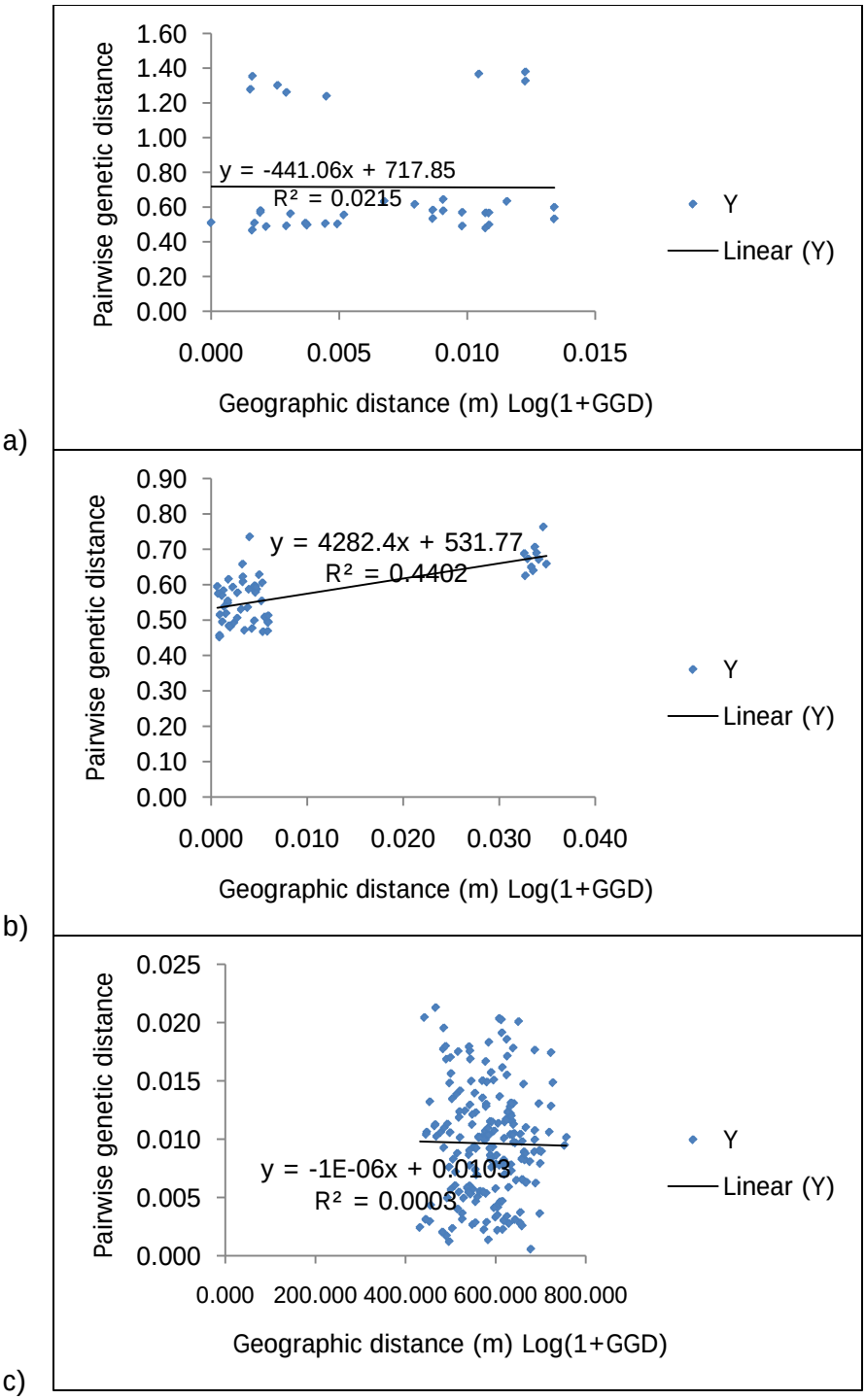


Figure 10. Mantel correlation within kīpuka population of *P. stachyoides* based on pairwise distance (Nei and Li 1979) of AFLP alleles vs. log 10 geographic distance for population from (a) upper, (b) middle, and (c) lower kīpuka

Genetic structure was less pronounced in the *M. zahlbruckneri* population. This pattern was expected given the small population size and the endangered status of the population.

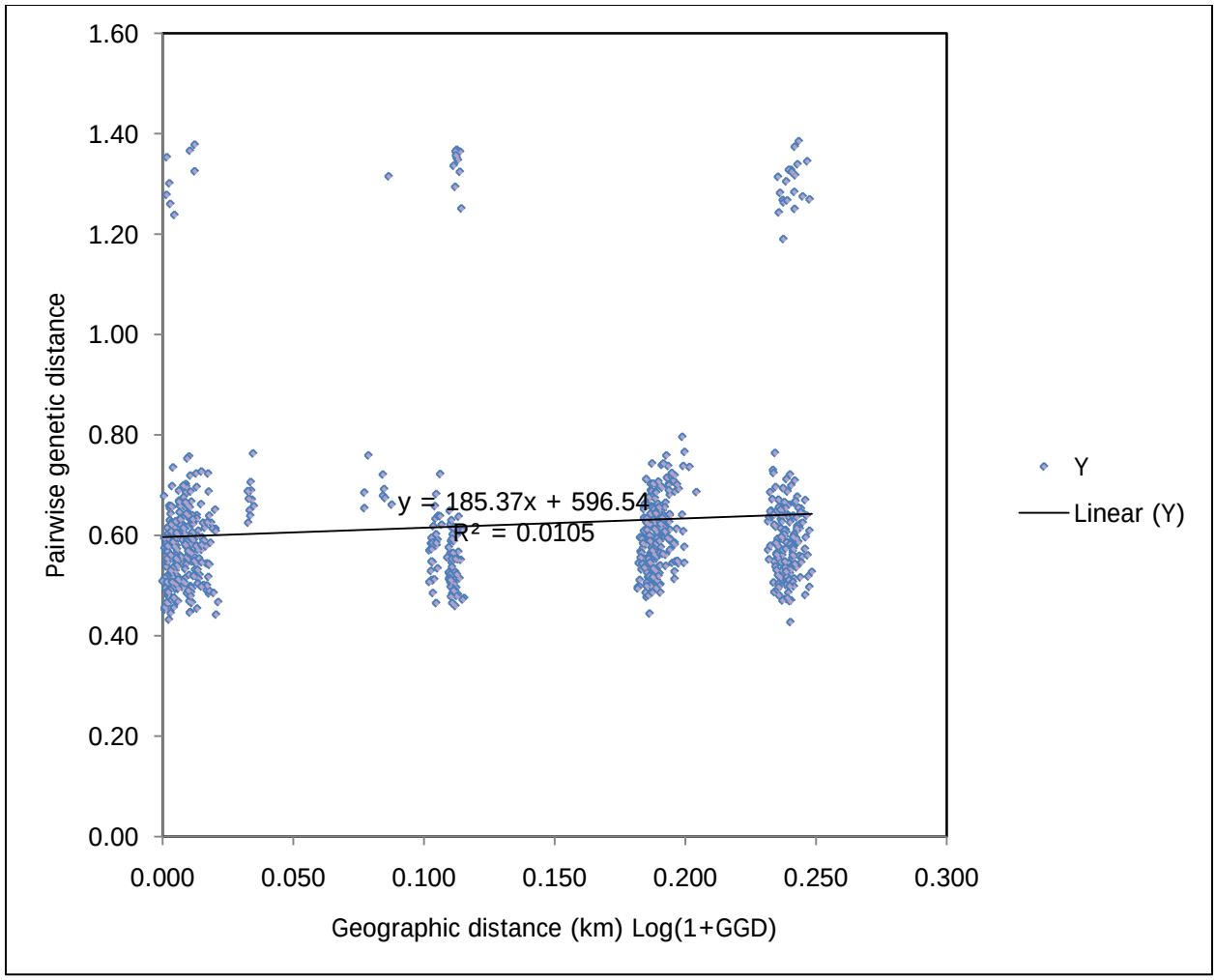


Figure 11. Mantel correlation of *P. stachyoides* population based on matrices of pairwise genetic distances (Nei and Li 1979) and the natural logarithm of pairwise geographic distances

However, unique alleles were observed in a few samples within the population, notably MZ23 and MZ27, with up to 100% bootstrap value. These samples and a few others showing the presence of unique alleles are largely responsible for the total genetic diversity observed in the population. The level of variability and the percentage of unique alleles detected in the population relative to the rest of the AFLP alleles can be attributed to a mixed species population and possible hybridization with other *Melicope* population species growing nearby, because even moderate levels of gene flow can help maintain genetic diversity.

Information from Bayesian analysis also validated the occurrence of the unique alleles and estimated their actual proportion in each of the genetic groups identified. The inferred population structure and proportion of individual membership in each cluster distribution further substantiate the presence of gene flow and an evidence of a mixed *M. zahlbruckneri* population.

The percent allele distribution suggests the likelihood that *M. zahlbruckneri* individuals MZ23 and MZ27 may essentially belong to either *M. radiata* or *M. pseudoanisata* species since these are the closest species populations to *M. zahlbruckneri*. Although the present study did not

examine *M. radiata* and *M. pseudoanisata* species due to limited funds, there are strong indications based on the allele frequency results that the extant population of *M. zahlbruckneri* is a mixed population. Furthermore, there was considerable evidence of gene flow with other *Melicope* species. It remains unknown how widespread the identified unique alleles are with respect to other *Melicope* species in Hawai`i. It would be worthwhile to obtain the nucleotide sequence, assess these unique alleles for fixation in other *Melicope* populations, and determine sample pedigree and the extent of gene flow across species populations.

The results presented here demonstrate that even within closely related genera, and with small populations of rare and endangered taxa, AFLP analyses in conjunction with appropriate statistical tools enable an unbiased assessment of genetic variability and provide useful information for conservation purposes. Similar observations of relatively high to moderate genetic diversity in small populations have been reported in other tropical species (e.g., *Swietenia humilis* [White *et al.* 1999] and *Eucalyptus albens* [Prober and Brown 1994]) and endangered populations (Breinholt *et al.* 2007, Duffy *et al.* 2011). Some of the authors attributed the observed level of genetic diversity mainly to the outcrossing mating system of the species, which enabled pollination across isolated and small populations for an effective gene flow (Hamrick *et al.* 1993). There are clearly some limitations with using AFLP markers for genetic studies. They are dominant markers and are unable to provide locus information content when compared with co-dominant markers (Dasmahapatra *et al.* 2008). However, owing to their ease of application across taxa, ability to detect small genetic differences, and genome-wide coverage, the use of AFLP markers is highly recommended in situations where resources are limited to develop co-dominant markers.

Given the current amount of genetic diversity and gene flow in *P. stachyoides*, and the relative amount of variability detected in the *M. zahlbruckneri* population, it may be safe to reason that the genetic risk for these species is relatively moderate at present. However, considering their small population sizes and their narrow geographic ranges, additional studies are needed to examine their sustainability, and how long-term ecological factors affect their distribution and survival. Future studies should endeavor to expand the genetics and gene flow studies at the species level by including other available *Melicope* and *Phyllostegia* species as well as closely related taxa. Furthermore, co-dominant marker systems such as SNP markers and microsatellites as well as DNA barcoding should be developed for these species to enable finer scale genomic studies and to provide better access to comparative genomics information across species. In addition, genome sizes and ploidy levels should be determined to get an insight into the structure, organization, and evolution of the species' genome. This information will better assist resource managers in planning conservation of these endangered species.

CONSERVATION IMPLICATIONS

Maintenance of a high amount of genetic diversity is critical for the conservation of endangered and threatened species. This study provides useful baseline information that will assist conservation efforts of endangered *M. zahlbruckneri* and *P. stachyoides* as follows:

- A summary of results illustrates that both *M. zahlbruckneri* and *P. stachyoides* currently maintain a moderate level of within population genetic diversity despite their small population sizes. However, due to the difficulty associated with germination of *M. zahlbruckneri* via conventional methods (Susan Dale, HAVO plant propagator, pers. comm.), management priority can possibly focus on alternative propagation methods

employing in-vitro seed and tissue regeneration measures using information from this study to select individuals from different genetic groups as parent samples to preserve as much of the existing genetic diversity as possible in subsequent generations.

- Further conservation of genetic variability can be maintained through the establishment of seed and in-vitro banks for all endangered *Melicope* species. Furthermore, future restoration efforts for *M. zahlbruckneri* should be focused on individuals that have been identified as true *M. zahlbruckneri* and their putative hybrids, and should exclude MZ23 and MZ27 pending confirmation of their species identity.

- Since there is no evidence of genetic isolation between populations of *P. stachyoides*, kīpuka should not be treated as genetic units in the selection of planting materials for restoration efforts, rather, seeds and other planting materials can be selected from genetic clusters identified in the present study to ensure clonal diversity and to minimize multiple sampling from identical individuals.

- A broader genetic survey of other existing populations of *Melicope* and *Phyllostegia* across the islands may be necessary to determine the level of available genetic variability across the species' ranges.

- Since genetic diversity is a critical factor to restoration success, all of the identified *M. zahlbruckneri* individuals, including their hybrids, should be represented in restorative planting at new sites. It is important to note that using only a few samples as founding individuals can considerably impact allelic diversity and result in higher genetic drift in the long run.

- The application of irradiation-induced mutation and in-vitro techniques may be considered in future conservation efforts of critically endangered species, particularly in populations with reduced germination and sterility issues. This technique has been used successfully in many tropical tree species to create favorable levels of genetic variability and safeguard a realistic potential in the recovery of endangered populations.

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APPENDIX 1.

DISSIMILARITY MATRIX OF *PHYLLOSTEGIA STACHYOIDES* POPULATION USING JACCARD'S INDEX OF AFLP MARKERS

P1	0.000	0.494	0.615	0.523	0.659	0.631	0.547	0.625	0.552	0.600	0.616	0.788	0.566	0.536	0.543	0.527	0.541	0.538	0.569
P2	0.494	0.000	0.692	0.537	0.708	0.649	0.561	0.607	0.573	0.671	0.644	0.810	0.605	0.587	0.548	0.542	0.550	0.544	0.600
P3	0.615	0.692	0.000	0.650	0.666	0.669	0.680	0.799	0.712	0.627	0.786	0.867	0.705	0.681	0.654	0.662	0.689	0.708	0.668
P4	0.523	0.537	0.650	0.000	0.694	0.657	0.576	0.662	0.556	0.659	0.579	0.782	0.571	0.540	0.585	0.574	0.598	0.560	0.549
P5	0.659	0.708	0.666	0.694	0.000	0.652	0.671	0.851	0.793	0.706	0.709	0.876	0.701	0.717	0.679	0.688	0.729	0.707	0.720
P6	0.631	0.649	0.669	0.657	0.652	0.000	0.686	0.792	0.653	0.726	0.743	0.925	0.691	0.664	0.705	0.679	0.705	0.713	0.719
P7	0.547	0.561	0.680	0.576	0.671	0.686	0.000	0.633	0.594	0.629	0.587	0.806	0.604	0.586	0.563	0.545	0.592	0.590	0.595
P8	0.625	0.607	0.799	0.662	0.851	0.792	0.633	0.000	0.663	0.796	0.676	0.763	0.695	0.692	0.679	0.630	0.660	0.665	0.707
P9	0.552	0.573	0.712	0.556	0.793	0.653	0.594	0.663	0.000	0.718	0.654	0.879	0.665	0.639	0.652	0.636	0.631	0.623	0.625
P10	0.600	0.671	0.627	0.659	0.706	0.726	0.629	0.796	0.718	0.000	0.707	0.805	0.634	0.652	0.610	0.614	0.667	0.656	0.672
P11	0.616	0.644	0.786	0.579	0.709	0.743	0.587	0.676	0.654	0.707	0.000	0.856	0.624	0.651	0.619	0.621	0.626	0.548	0.591
P12	0.788	0.810	0.867	0.782	0.876	0.925	0.806	0.763	0.879	0.805	0.856	0.000	0.791	0.748	0.726	0.750	0.759	0.768	0.779
P13	0.566	0.605	0.705	0.571	0.701	0.691	0.604	0.695	0.665	0.634	0.624	0.791	0.000	0.518	0.510	0.584	0.570	0.541	0.499
P14	0.536	0.587	0.681	0.540	0.717	0.664	0.586	0.692	0.639	0.652	0.651	0.748	0.518	0.000	0.501	0.550	0.585	0.551	0.533
P15	0.543	0.548	0.654	0.585	0.679	0.705	0.563	0.679	0.652	0.610	0.619	0.726	0.510	0.501	0.000	0.501	0.522	0.508	0.509
P16	0.527	0.542	0.662	0.574	0.688	0.679	0.545	0.630	0.636	0.614	0.621	0.750	0.584	0.550	0.501	0.000	0.549	0.552	0.544
P17	0.541	0.550	0.689	0.598	0.729	0.705	0.592	0.660	0.631	0.667	0.626	0.759	0.570	0.585	0.522	0.549	0.000	0.493	0.533
P18	0.538	0.544	0.708	0.560	0.707	0.713	0.590	0.665	0.623	0.656	0.548	0.768	0.541	0.551	0.508	0.552	0.493	0.000	0.517
P19	0.569	0.600	0.668	0.549	0.720	0.719	0.595	0.707	0.625	0.672	0.591	0.779	0.499	0.533	0.509	0.544	0.533	0.517	0.000
P20	0.676	0.720	0.694	0.734	0.767	0.813	0.759	0.870	0.800	0.671	0.813	0.851	0.709	0.707	0.650	0.690	0.634	0.689	0.668
P21	0.702	0.791	0.664	0.781	0.844	0.846	0.775	0.898	0.832	0.681	0.819	0.921	0.735	0.737	0.717	0.767	0.732	0.736	0.691
P22	0.672	0.696	0.710	0.670	0.770	0.749	0.710	0.834	0.706	0.619	0.737	0.809	0.660	0.650	0.632	0.650	0.661	0.663	0.581
P23	0.534	0.606	0.650	0.543	0.711	0.692	0.589	0.702	0.617	0.637	0.601	0.744	0.580	0.540	0.536	0.562	0.568	0.489	0.517
P24	0.578	0.568	0.695	0.624	0.747	0.685	0.591	0.621	0.641	0.676	0.644	0.763	0.609	0.599	0.558	0.535	0.520	0.513	0.564
P25	0.557	0.557	0.695	0.570	0.754	0.703	0.628	0.655	0.644	0.674	0.647	0.781	0.604	0.598	0.559	0.563	0.540	0.528	0.583
P26	0.597	0.609	0.721	0.586	0.748	0.753	0.621	0.722	0.629	0.687	0.628	0.830	0.568	0.588	0.602	0.627	0.580	0.552	0.550
P27	0.597	0.635	0.751	0.594	0.730	0.745	0.645	0.733	0.708	0.685	0.633	0.781	0.527	0.540	0.550	0.601	0.598	0.524	0.539
P28	0.537	0.570	0.667	0.527	0.669	0.659	0.582	0.674	0.601	0.645	0.575	0.784	0.516	0.526	0.543	0.549	0.558	0.481	0.501
P29	0.643	0.664	0.771	0.585	0.746	0.738	0.600	0.733	0.670	0.727	0.655	0.820	0.572	0.568	0.614	0.644	0.657	0.571	0.598
P30	0.549	0.592	0.704	0.542	0.704	0.696	0.594	0.701	0.612	0.653	0.630	0.783	0.555	0.518	0.546	0.566	0.622	0.518	0.524
P31	0.557	0.605	0.718	0.558	0.708	0.726	0.580	0.682	0.647	0.670	0.606	0.812	0.524	0.523	0.534	0.561	0.572	0.473	0.527
P32	0.555	0.584	0.694	0.575	0.729	0.659	0.589	0.688	0.601	0.711	0.637	0.841	0.572	0.577	0.561	0.550	0.558	0.539	0.562
P33	0.549	0.551	0.686	0.525	0.723	0.628	0.587	0.637	0.545	0.674	0.626	0.845	0.591	0.610	0.602	0.568	0.548	0.553	0.583
P34	0.520	0.517	0.708	0.504	0.743	0.665	0.609	0.666	0.563	0.698	0.644	0.781	0.581	0.583	0.609	0.587	0.572	0.547	0.582
P35	0.606	0.640	0.737	0.631	0.720	0.695	0.626	0.729	0.599	0.753	0.708	0.866	0.597	0.604	0.616	0.646	0.628	0.583	0.594
P36	0.646	0.692	0.741	0.655	0.706	0.710	0.711	0.794	0.753	0.698	0.726	0.816	0.630	0.677	0.641	0.671	0.682	0.609	0.633
P37	0.689	0.751	0.812	0.730	0.762	0.827	0.678	0.812	0.761	0.806	0.727	0.853	0.707	0.718	0.666	0.639	0.713	0.684	0.652
P38	0.619	0.691	0.712	0.596	0.777	0.691	0.613	0.707	0.619	0.734	0.694	0.837	0.622	0.593	0.612	0.667	0.643	0.630	0.574
P39	0.754	0.823	0.876	0.805	0.915	0.897	0.778	0.812	0.861	0.819	0.810	0.931	0.710	0.783	0.738	0.765	0.752	0.768	0.747
P40	1.289	1.340	1.376	1.316	1.406	1.360	1.307	1.265	1.277	1.381	1.366	1.266	1.375	1.356	1.351	1.287	1.260	1.336	1.358

Appendix 1, continued

P1	0.676	0.702	0.672	0.534	0.578	0.557	0.597	0.597	0.537	0.643	0.549	0.557	0.555	0.549	0.520	0.606	0.646	0.689	0.619
P2	0.720	0.791	0.696	0.606	0.568	0.557	0.609	0.635	0.570	0.664	0.592	0.605	0.584	0.551	0.517	0.640	0.692	0.751	0.691
P3	0.694	0.664	0.710	0.650	0.695	0.695	0.721	0.751	0.667	0.771	0.704	0.718	0.694	0.686	0.708	0.737	0.741	0.812	0.712
P4	0.734	0.781	0.670	0.543	0.624	0.570	0.586	0.594	0.527	0.585	0.542	0.558	0.575	0.525	0.504	0.631	0.655	0.730	0.596
P5	0.767	0.844	0.770	0.711	0.747	0.754	0.748	0.730	0.669	0.746	0.704	0.708	0.729	0.723	0.743	0.720	0.706	0.762	0.777
P6	0.813	0.846	0.749	0.692	0.685	0.703	0.753	0.745	0.659	0.738	0.696	0.726	0.659	0.628	0.665	0.695	0.710	0.827	0.691
P7	0.759	0.775	0.710	0.589	0.591	0.628	0.621	0.645	0.582	0.600	0.594	0.580	0.589	0.587	0.609	0.626	0.711	0.678	0.613
P8	0.870	0.898	0.834	0.702	0.621	0.655	0.722	0.733	0.674	0.733	0.701	0.682	0.688	0.637	0.666	0.729	0.794	0.812	0.707
P9	0.800	0.832	0.706	0.617	0.641	0.644	0.629	0.708	0.601	0.670	0.612	0.647	0.601	0.545	0.563	0.599	0.753	0.761	0.619
P10	0.671	0.681	0.619	0.637	0.676	0.674	0.687	0.685	0.645	0.727	0.653	0.670	0.711	0.674	0.698	0.753	0.698	0.806	0.734
P11	0.813	0.819	0.737	0.601	0.644	0.647	0.628	0.633	0.575	0.655	0.630	0.606	0.637	0.626	0.644	0.708	0.726	0.727	0.694
P12	0.851	0.921	0.809	0.744	0.763	0.781	0.830	0.781	0.784	0.820	0.783	0.812	0.841	0.845	0.781	0.866	0.816	0.853	0.837
P13	0.709	0.735	0.660	0.580	0.609	0.604	0.568	0.527	0.516	0.572	0.555	0.524	0.572	0.591	0.581	0.597	0.630	0.707	0.622
P14	0.707	0.737	0.650	0.540	0.599	0.598	0.588	0.540	0.526	0.568	0.518	0.523	0.577	0.610	0.583	0.604	0.677	0.718	0.593
P15	0.650	0.717	0.632	0.536	0.558	0.559	0.602	0.550	0.543	0.614	0.546	0.534	0.561	0.602	0.609	0.616	0.641	0.666	0.612
P16	0.690	0.767	0.650	0.562	0.535	0.563	0.627	0.601	0.549	0.644	0.566	0.561	0.550	0.568	0.587	0.646	0.671	0.639	0.667
P17	0.634	0.732	0.661	0.568	0.520	0.540	0.580	0.598	0.558	0.657	0.622	0.572	0.558	0.548	0.572	0.628	0.682	0.713	0.643
P18	0.689	0.736	0.663	0.489	0.513	0.528	0.552	0.524	0.481	0.571	0.518	0.473	0.539	0.553	0.547	0.583	0.609	0.684	0.630
P19	0.668	0.691	0.581	0.517	0.564	0.583	0.550	0.539	0.501	0.598	0.524	0.527	0.562	0.583	0.582	0.594	0.633	0.652	0.574
P20	0.000	0.712	0.603	0.653	0.734	0.679	0.745	0.694	0.709	0.778	0.698	0.737	0.734	0.768	0.695	0.805	0.729	0.835	0.759
P21	0.712	0.000	0.682	0.679	0.732	0.671	0.745	0.745	0.714	0.803	0.727	0.751	0.723	0.753	0.741	0.790	0.746	0.855	0.765
P22	0.603	0.682	0.000	0.570	0.668	0.627	0.655	0.642	0.627	0.688	0.652	0.660	0.692	0.704	0.689	0.730	0.657	0.774	0.680
P23	0.653	0.679	0.570	0.000	0.563	0.504	0.571	0.534	0.509	0.579	0.510	0.527	0.576	0.578	0.538	0.619	0.631	0.674	0.595
P24	0.734	0.732	0.668	0.563	0.000	0.547	0.611	0.608	0.548	0.650	0.572	0.558	0.550	0.546	0.567	0.626	0.659	0.700	0.639
P25	0.679	0.671	0.627	0.504	0.547	0.000	0.551	0.554	0.522	0.632	0.551	0.557	0.542	0.528	0.515	0.595	0.630	0.706	0.615
P26	0.745	0.745	0.655	0.571	0.611	0.551	0.000	0.562	0.531	0.592	0.529	0.522	0.525	0.549	0.546	0.586	0.647	0.689	0.578
P27	0.694	0.745	0.642	0.534	0.608	0.554	0.562	0.000	0.514	0.572	0.526	0.515	0.579	0.609	0.602	0.591	0.610	0.663	0.596
P28	0.709	0.714	0.627	0.509	0.548	0.522	0.531	0.514	0.000	0.540	0.503	0.454	0.508	0.523	0.551	0.545	0.615	0.673	0.574
P29	0.778	0.803	0.688	0.579	0.650	0.632	0.592	0.572	0.540	0.000	0.532	0.505	0.588	0.602	0.590	0.586	0.663	0.684	0.580
P30	0.698	0.727	0.652	0.510	0.572	0.551	0.529	0.526	0.503	0.532	0.000	0.466	0.499	0.557	0.520	0.495	0.602	0.649	0.519
P31	0.737	0.751	0.660	0.527	0.558	0.557	0.522	0.515	0.454	0.505	0.466	0.000	0.495	0.537	0.531	0.540	0.618	0.677	0.536
P32	0.734	0.723	0.692	0.576	0.550	0.542	0.525	0.579	0.508	0.588	0.499	0.495	0.000	0.501	0.538	0.497	0.633	0.693	0.557
P33	0.768	0.753	0.704	0.578	0.546	0.528	0.549	0.609	0.523	0.602	0.557	0.537	0.501	0.000	0.478	0.554	0.669	0.705	0.557
P34	0.695	0.741	0.689	0.538	0.567	0.515	0.546	0.602	0.551	0.590	0.520	0.531	0.538	0.478	0.000	0.533	0.614	0.701	0.543
P35	0.805	0.790	0.730	0.619	0.626	0.595	0.586	0.591	0.545	0.586	0.495	0.540	0.497	0.554	0.533	0.000	0.652	0.679	0.562
P36	0.729	0.746	0.657	0.631	0.659	0.630	0.647	0.610	0.615	0.663	0.602	0.618	0.633	0.669	0.614	0.652	0.000	0.730	0.643
P37	0.835	0.855	0.774	0.674	0.700	0.706	0.689	0.663	0.673	0.684	0.649	0.677	0.693	0.705	0.701	0.679	0.730	0.000	0.669
P38	0.759	0.765	0.680	0.595	0.639	0.615	0.578	0.596	0.574	0.580	0.519	0.536	0.557	0.557	0.543	0.562	0.643	0.669	0.000
P39	0.851	0.875	0.867	0.748	0.777	0.777	0.763	0.768	0.738	0.763	0.728	0.751	0.774	0.762	0.750	0.790	0.794	0.772	0.715
P40	1.396	1.393	1.408	1.321	1.285	1.263	1.245	1.368	1.301	1.321	1.331	1.328	1.267	1.254	1.272	1.306	1.385	1.219	1.267

APPENDIX 2.

DISSIMILARITY MATRIX OF *MELICOPE ZAHLBRUCKNERI* POPULATION USING JACCARD'S INDEX OF AFLP MARKERS

MZ1	0.000	0.621	0.665	0.632	0.803	0.665	0.642	0.672	0.710	0.671	0.723	0.719	0.696	0.718
MZ2	0.621	0.000	0.667	0.650	0.887	0.705	0.688	0.752	0.722	0.728	0.775	0.751	0.738	0.774
MZ3	0.665	0.667	0.000	0.700	0.888	0.770	0.748	0.738	0.807	0.725	0.814	0.787	0.775	0.751
MZ4	0.632	0.650	0.700	0.000	0.749	0.696	0.651	0.686	0.732	0.709	0.698	0.698	0.707	0.765
MZ5	0.803	0.887	0.888	0.749	0.000	0.764	0.789	0.840	0.880	0.895	0.792	0.853	0.837	0.892
MZ6	0.665	0.705	0.770	0.696	0.764	0.000	0.643	0.718	0.738	0.768	0.748	0.725	0.743	0.765
MZ7	0.642	0.688	0.748	0.651	0.789	0.643	0.000	0.727	0.733	0.720	0.760	0.730	0.743	0.733
MZ8	0.672	0.752	0.738	0.686	0.840	0.718	0.727	0.000	0.723	0.772	0.813	0.728	0.757	0.727
MZ9	0.710	0.722	0.807	0.732	0.880	0.738	0.733	0.723	0.000	0.636	0.711	0.640	0.726	0.730
MZ10	0.671	0.728	0.725	0.709	0.895	0.768	0.720	0.772	0.636	0.000	0.723	0.647	0.769	0.759
MZ11	0.723	0.775	0.814	0.698	0.792	0.748	0.760	0.813	0.711	0.723	0.000	0.691	0.849	0.842
MZ12	0.719	0.751	0.787	0.698	0.853	0.725	0.730	0.728	0.640	0.647	0.691	0.000	0.736	0.770
MZ13	0.696	0.738	0.775	0.707	0.837	0.743	0.743	0.757	0.726	0.769	0.849	0.736	0.000	0.705
MZ14	0.718	0.774	0.751	0.765	0.892	0.765	0.733	0.727	0.730	0.759	0.842	0.770	0.705	0.000
MZ15	0.637	0.745	0.724	0.733	0.849	0.751	0.705	0.669	0.720	0.753	0.831	0.765	0.759	0.744
MZ16	0.707	0.764	0.741	0.727	0.883	0.747	0.738	0.715	0.735	0.793	0.837	0.741	0.769	0.748
MZ17	0.626	0.695	0.710	0.664	0.795	0.717	0.703	0.659	0.696	0.737	0.772	0.718	0.679	0.673
MZ18	0.727	0.755	0.804	0.742	0.813	0.738	0.732	0.827	0.821	0.794	0.765	0.782	0.806	0.826
MZ19	0.623	0.725	0.696	0.688	0.864	0.696	0.704	0.703	0.711	0.746	0.763	0.755	0.719	0.647
MZ20	0.656	0.713	0.724	0.680	0.826	0.751	0.683	0.682	0.719	0.744	0.787	0.736	0.699	0.702
MZ21	0.754	0.752	0.801	0.759	0.797	0.809	0.764	0.828	0.841	0.829	0.893	0.818	0.724	0.770
MZ22	0.747	0.804	0.809	0.726	0.773	0.753	0.768	0.790	0.809	0.816	0.825	0.791	0.814	0.794
MZ23	0.942	0.949	0.968	0.955	1.089	1.050	1.006	1.031	0.997	0.980	0.981	1.012	0.966	1.000
MZ24	0.679	0.761	0.766	0.689	0.820	0.725	0.706	0.719	0.725	0.750	0.808	0.760	0.720	0.691
MZ25	0.634	0.715	0.755	0.723	0.868	0.746	0.716	0.697	0.743	0.729	0.794	0.753	0.733	0.755
MZ26	0.622	0.670	0.709	0.651	0.852	0.701	0.672	0.736	0.733	0.653	0.777	0.734	0.729	0.711
MZ27	0.969	1.019	1.016	1.071	1.027	1.014	0.996	1.056	1.056	1.033	1.038	1.029	1.018	1.019
MZ28	0.770	0.843	0.842	0.771	0.841	0.751	0.745	0.794	0.801	0.821	0.825	0.819	0.797	0.778

Appendix 2, continued

MZ1	0.637	0.707	0.626	0.727	0.623	0.656	0.754	0.747	0.942	0.679	0.634	0.622	0.969	0.770
MZ2	0.745	0.764	0.695	0.755	0.725	0.713	0.752	0.804	0.949	0.761	0.715	0.670	1.019	0.843
MZ3	0.724	0.741	0.710	0.804	0.696	0.724	0.801	0.809	0.968	0.766	0.755	0.709	1.016	0.842
MZ4	0.733	0.727	0.664	0.742	0.688	0.680	0.759	0.726	0.955	0.689	0.723	0.651	1.071	0.771
MZ5	0.849	0.883	0.795	0.813	0.864	0.826	0.797	0.773	1.089	0.820	0.868	0.852	1.027	0.841
MZ6	0.751	0.747	0.717	0.738	0.696	0.751	0.809	0.753	1.050	0.725	0.746	0.701	1.014	0.751
MZ7	0.705	0.738	0.703	0.732	0.704	0.683	0.764	0.768	1.006	0.706	0.716	0.672	0.996	0.745
MZ8	0.669	0.715	0.659	0.827	0.703	0.682	0.828	0.790	1.031	0.719	0.697	0.736	1.056	0.794
MZ9	0.720	0.735	0.696	0.821	0.711	0.719	0.841	0.809	0.997	0.725	0.743	0.733	1.056	0.801
MZ10	0.753	0.793	0.737	0.794	0.746	0.744	0.829	0.816	0.980	0.750	0.729	0.653	1.033	0.821
MZ11	0.831	0.837	0.772	0.765	0.763	0.787	0.893	0.825	0.981	0.808	0.794	0.777	1.038	0.825
MZ12	0.765	0.741	0.718	0.782	0.755	0.736	0.818	0.791	1.012	0.760	0.753	0.734	1.029	0.819
MZ13	0.759	0.769	0.679	0.806	0.719	0.699	0.724	0.814	0.966	0.720	0.733	0.729	1.018	0.797
MZ14	0.744	0.748	0.673	0.826	0.647	0.702	0.770	0.794	1.000	0.691	0.755	0.711	1.019	0.778
MZ15	0.000	0.689	0.691	0.792	0.629	0.695	0.804	0.779	1.035	0.675	0.698	0.706	1.056	0.778
MZ16	0.689	0.000	0.689	0.854	0.687	0.680	0.806	0.777	1.007	0.718	0.722	0.691	1.026	0.807
MZ17	0.691	0.689	0.000	0.763	0.680	0.688	0.702	0.745	1.022	0.650	0.699	0.636	0.993	0.755
MZ18	0.792	0.854	0.763	0.000	0.787	0.774	0.823	0.797	0.964	0.772	0.774	0.724	0.998	0.778
MZ19	0.629	0.687	0.680	0.787	0.000	0.670	0.777	0.758	0.978	0.702	0.733	0.672	1.006	0.689
MZ20	0.695	0.680	0.688	0.774	0.670	0.000	0.755	0.777	1.031	0.634	0.710	0.732	0.988	0.761
MZ21	0.804	0.806	0.702	0.823	0.777	0.755	0.000	0.754	0.993	0.788	0.773	0.756	1.056	0.796
MZ22	0.779	0.777	0.745	0.797	0.758	0.777	0.754	0.000	1.015	0.792	0.761	0.740	1.033	0.787
MZ23	1.035	1.007	1.022	0.964	0.978	1.031	0.993	1.015	0.000	1.011	1.005	0.972	1.171	1.077
MZ24	0.675	0.718	0.650	0.772	0.702	0.634	0.788	0.792	1.011	0.000	0.696	0.696	0.991	0.759
MZ25	0.698	0.722	0.699	0.774	0.733	0.710	0.773	0.761	1.005	0.696	0.000	0.669	0.997	0.769
MZ26	0.706	0.691	0.636	0.724	0.672	0.732	0.756	0.740	0.972	0.696	0.669	0.000	1.013	0.736
MZ27	1.056	1.026	0.993	0.998	1.006	0.988	1.056	1.033	1.171	0.991	0.997	1.013	0.000	1.005
MZ28	0.778	0.807	0.755	0.778	0.689	0.761	0.796	0.787	1.077	0.759	0.769	0.736	1.005	0.000

APPENDIX 3. AFLP MARKER FREQUENCY AND ALLELIC VARIANCE ACROSS *P. STACHYOIDES* POPULATION PER MARKER LOCUS

Marker locus	Mean freq+all	Mean freq-all	Mean var-all	Polymorphic fragments
P01	0.733	0.267	0.002	73
P02	0.642	0.348	0.003	59
P03	0.781	0.219	0.001	70
P04	0.732	0.268	0.002	88
P05	0.561	0.439	0.003	84
P06	0.621	0.379	0.002	92
P07	0.686	0.314	0.002	93
P08	0.579	0.421	0.003	64
P09	0.600	0.400	0.003	103
P10	0.749	0.251	0.002	73
P11	0.808	0.192	0.002	90
P12	0.618	0.382	0.003	86
P13	0.752	0.248	0.003	97
P14	0.583	0.417	0.003	53
P15	0.550	0.450	0.003	57
P16	0.560	0.440	0.003	57
P17	0.703	0.297	0.002	82
P18	0.496	0.504	0.002	94
P19	0.723	0.277	0.002	130
P21	0.621	0.379	0.003	86
P22	0.571	0.429	0.002	83
P23	0.655	0.345	0.002	80
P24	0.742	0.258	0.002	102
P25	0.606	0.394	0.003	88
P26	0.729	0.271	0.002	119
P27	0.801	0.199	0.001	90
P29	0.644	0.356	0.002	142
P30	0.619	0.381	0.002	121
P31	0.553	0.447	0.001	130
P32	0.581	0.419	0.002	82
P34	0.586	0.414	0.003	72
P35	0.553	0.447	0.003	78
P37	0.572	0.428	0.003	88
P38	0.612	0.388	0.002	88
P39	0.567	0.433	0.002	109
P40	0.714	0.286	0.002	139
Mean	0.645	0.355	0.002	90.056

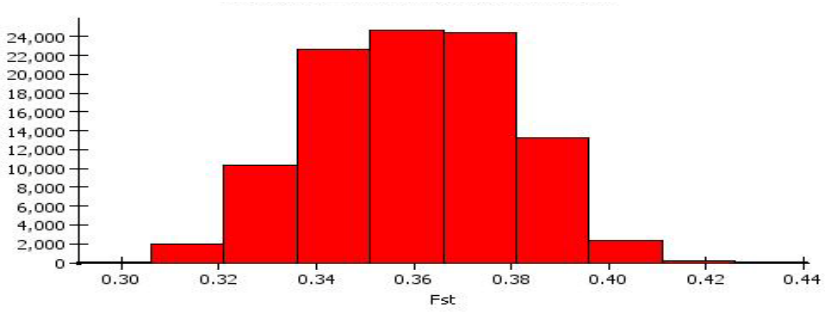
Mean freq+all: Mean allelic frequency across sampling loci
Mean var-all: Mean variance attributable to sampling individuals

APPENDIX 4. AFLP MARKER FREQUENCY AND ALLELIC VARIANCE ACROSS *M. ZAHLBRUCKNERI* POPULATION PER MARKER LOCUS

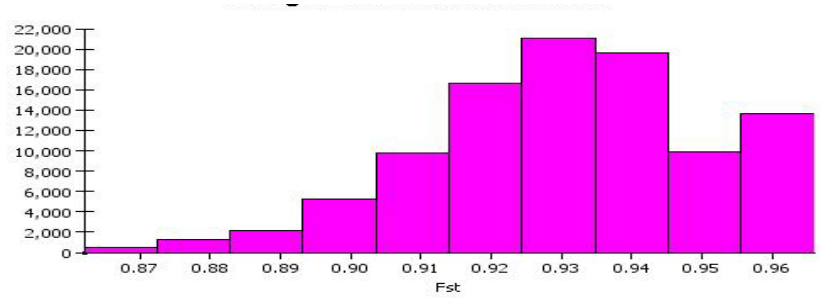
Marker locus	Mean freq+all	Mean freq-all	Mean var-all	Polymorphic fragments
P01	0.5	0.5	0.004	84
P02	0.627	0.373	0.003	114
P03	0.581	0.419	0.004	85
P04	0.651	0.349	0.004	96
P05	0.582	0.418	0.004	75
P06	0.655	0.345	0.003	80
P07	0.538	0.462	0.006	81
P08	0.491	0.509	0.006	66
P09	0.589	0.411	0.004	80
P10	0.518	0.482	0.005	73
P11	0.696	0.304	0.004	72
P12	0.576	0.424	0.005	95
P13	0.689	0.311	0.004	87
P14	0.439	0.561	0.006	59
P15	0.62	0.38	0.004	39
P16	0.623	0.377	0.004	85
P17	0.735	0.265	0.003	78
P18	0.659	0.341	0.004	67
P19	0.587	0.413	0.004	110
P20	0.597	0.403	0.004	92
P21	0.455	0.545	0.006	65
P22	0.662	0.338	0.004	40
P23	0.533	0.467	0.004	62
P24	0.344	0.656	0.004	16
P25	0.634	0.366	0.006	61
P26	0.564	0.436	0.005	25
P27	0.713	0.287	0.003	81
P28	0.658	0.342	0.004	77
P29	0.554	0.446	0.005	101
P30	0.683	0.317	0.004	102
P31	0.578	0.422	0.004	92
P32	0.423	0.577	0.005	64
P33	0.608	0.392	0.005	102
P34	0.557	0.443	0.006	93
P35	0.579	0.421	0.005	100
P36	0.614	0.386	0.004	81
Mean	0.586	0.414	0.004	77.2

Mean freq+all: Mean allelic frequency across sampling loci
Mean var-all: Mean variance attributable to sampling individuals

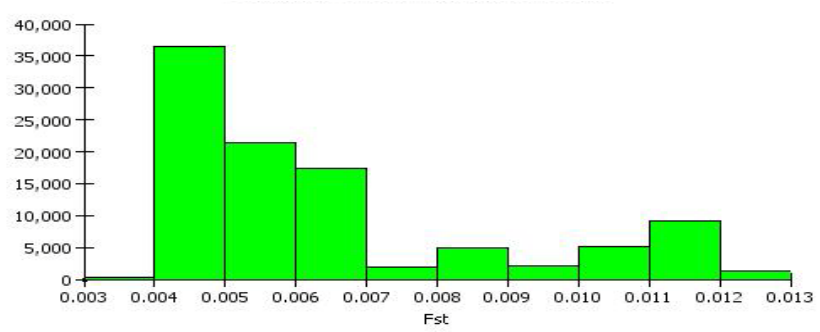
APPENDIX 5.
HISTOGRAM DISTRIBUTION SHOWING ESTIMATES OF THE POSTERIOR DISTRIBUTION OF FST IN CLUSTERS (A-D) OF *M. ZAHLBRUCKNERI*



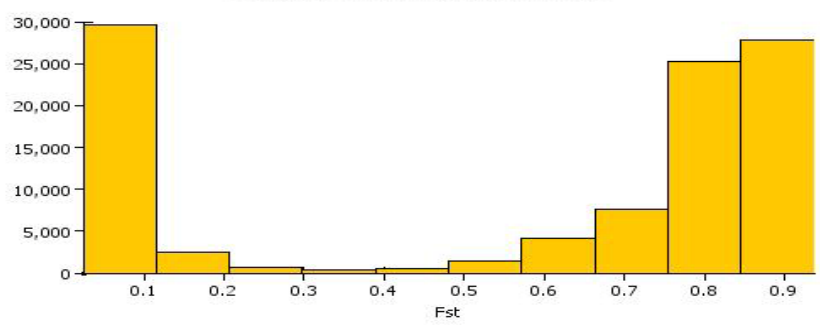
A



B

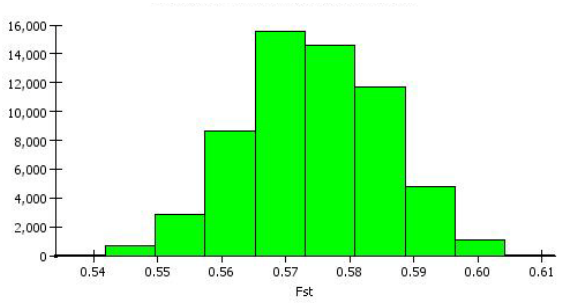


C

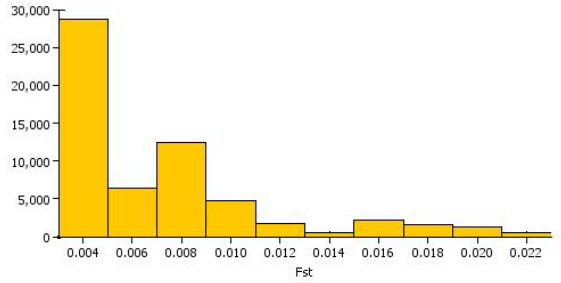


D

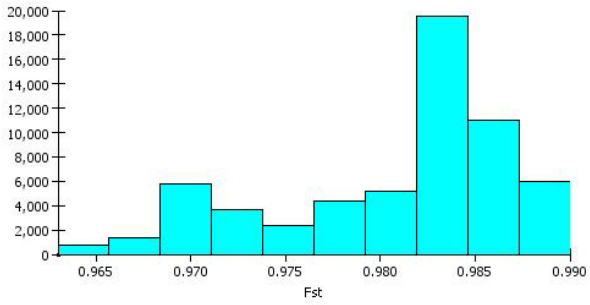
APPENDIX 6.
HISTOGRAM DISTRIBUTION SHOWING ESTIMATES OF THE POSTERIOR DISTRIBUTION OF FST IN CLUSTERS (A-D) OF *P. STACHYOIDES*



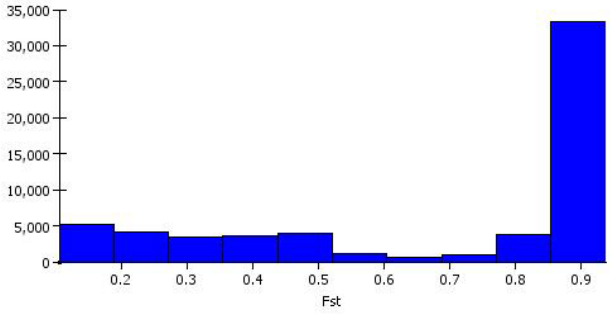
A



B



C



D

APPENDIX 7.

AVERAGE DISTANCES BETWEEN INDIVIDUALS AND ESTIMATED MEAN VALUE OF FST WITHIN EACH CLUSTER OF *M. ZAHLBRUCKNERI* AND *P. STACHYOIDES*

	M. zahlbruckneri		*P. stachyoides*	
Cluster	Average distance*	Mean Fst value	Average distance*	Mean Fst value
1	0.3762	0.0117	0.1526	0.5745
2	0.3661	0.0150	0.3959	0.0069
3	0.2271	0.3471	0.1569	0.9809
4	0.2317	0.9157	0.3398	0.6843
Parameter				
Estimated ln prob of data	-33461.6		-42081.7	
Mean value of ln likelihood	-31309.1		-36597.0	
Variance of ln likelihood	4305.0		10969.4	
Mean value of alpha	0.1453		0.1084	

* Average distance (between individuals within a cluster)

Ln likelihood: log likelihood of the data given values of P (estimated allele frequencies) and Q (the estimated membership coefficient for each individual in a cluster)

Estimated ln prob of data: current estimate of ln (P9X|K)), averaging over all iterations after burn in period

APPENDIX 8.
MELICOPE ZAHLBRUCKNERI SHOWING (A) PLANT AND (B) MATURE CUBIC FRUIT CAPSULES

A

B

APPENDIX 9.
***PHYLLOSTEGIA CF. STACHYOIDES* SHOWING (A) PLANT AND (B) FLOWER ARRANGEMENT AND LEAF SHAPE, SIZE, AND ANGLE**

A

B