Population genetics of *Delissea waianaeensis* Lammers (Campanulaceae) on U.S. Army lands on O‘ahu, Hawai‘i

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Cover image:
Delissea wainaeensis Lammers
Photographer: Lauren A. Weisenberger

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Introduction

The endemic Hawaiian genus *Delissea* (Campanulaceae) is comprised of 15 single-island endemic species, four of which are listed as endangered, with the other 11 species being presumed extinct (OANRP 2009). *D. waianaeensis* Lammers (*haha* or *ohawai*) is found along, and is endemic to, the Wai‘anae Mountain Range. The species was federally listed as endangered in 1996 (Russell and Bruegman 1996; Lammers 2005). The O‘ahu Army Natural Resource Program (OANRP) is responsible for maintaining the stability of *Delissea waianaeensis* on O‘ahu, and is actively reintroducing populations of *D. waianaeensis* in areas that historically contained the species. A previous study of the genetics of the species using RAPDs analysis (James 2009) indicated that the management population units were largely genetically and geographically distinct, emphasizing the need to preserve each population unit. However, the limited number of samples in analysis was problematic in fully assessing the genetic variation in the species. The goal of this project is to further quantify the amount of genetic variation within and between locations and managed population units of *D. waianaeensis*, and delineate natural genetic population units for the species, thereby confirming or refuting the current management population units as defined by the OANRP.

Inter-Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) is a RAPDs-like approach for characterizing genetic relatedness among populations. The method accesses variation in the numerous microsatellite regions dispersed throughout the genome circumventing the need to characterize individual loci that other molecular approaches require. Microsatellites are very short, usually 10-20 base-pair, stretches of DNA characterized by mono-, di- or trinucleotide repeats consisting of 4-10 repeat units that are expressed as different variants within populations. ISSR-PCR primers include one of these highly variable microsatellite sequences and an arbitrary pair of bases at the 3’ end. Where the primer during polymerase chain reaction locates two microsatellite regions within an amplifiable distance on the DNA strands of the sample, the PCR reaction will

generate a band of a particular size, or molecular weight, for that locus representing the intervening region of DNA between the microsatellites. Usually many paired microsatellite areas exist in a particular sample resulting in the generation of multiple bands for each primer. Studies of ISSR-PCR locus heritability have demonstrated a close approximation to classic Mendelian ratios (Tsumura et al. 1997). ISSR-PCR analyses have demonstrated a greater robustness in repeatability experiments and reduced modifications to band patterns with changes in constituent or DNA template concentrations, making them superior to other marker systems, such as RAPDs. ISSR-PCR analyses have been recently used in many genetic conservation assessments for both plants and animals (e.g. Liu et al., 2013).

Materials and Methods

Leaf material was collected by O‘ahu Army Natural Resource Program staff from 32 shade-house-grown individuals representing the seven distinct wild population units, or management units, of *Delissea waianaeensis*: Kapuna, Pahole, ‘Ēkahanui, Mohiākea, Palikea Gulch, Kalua‘ā, Keālia, and Palawai (Table 1, Figure 1). Leaf tissues were rapidly dehydrated in silica gel and frozen at -80 °C for future molecular analyses. Genomic DNA was extracted from 6-10 mg dried plant material using a DNeasy Plant Mini Kit (QIAGEN Inc.) following the recommended protocol. Extracted DNA has been stored for long-term preservation at -80 °C. Tissues and extracted DNA were accessioned into Bishop Museum collections (BPBM accession numbers 2008.174 and 2012.139).

Eleven ISSR-PCR primers (Table 2) were tested, resulting in repeatable bands. ISSR-PCR was performed in 10 µL reaction volumes as follows: 8 µL Taq PCR Master Mix Kit (Qiagen Inc.), 0.8 µM primer, and 0.5-1 µL DNA. The PCR program was set at 94 °C for 2 min, followed by 35 cycles each of 94 °C for 30 s, 44 °C for 45 s, and 72 °C for 1 min, and a final extension of 72 °C for 20 min. Negative controls, where all reagents but DNA were added to the reaction mix, were run with each experiment to assess contamination. PCR reactions were visualized and scored on 1.5% agarose gels in 1x TBE buffer by loading the entire reaction volume into prepared wells in a Maxi Gel

System (PerfectBlue Wide Gel System, Peqlab). Gels were run until the bromophenol blue markers ran 10 cm (5 h at 85V, 100 mA) and stained with ethidium bromide. ISSR-PCR bands were visualized on a UV transilluminator, digitized using a Bio-Rad GelDoc XR system, and analysed using Bio-Rad Quantity One software. Fragment sizes were estimated using a 1-kb ladder size standard (Promega). ISSR-PCR markers are inherited in a dominant or co-dominant Mendelian fashion, and are interpreted as dominant markers, scored as diallelic with band present (=1) or band absent (=0) (Wolfe *et al.* 1998).

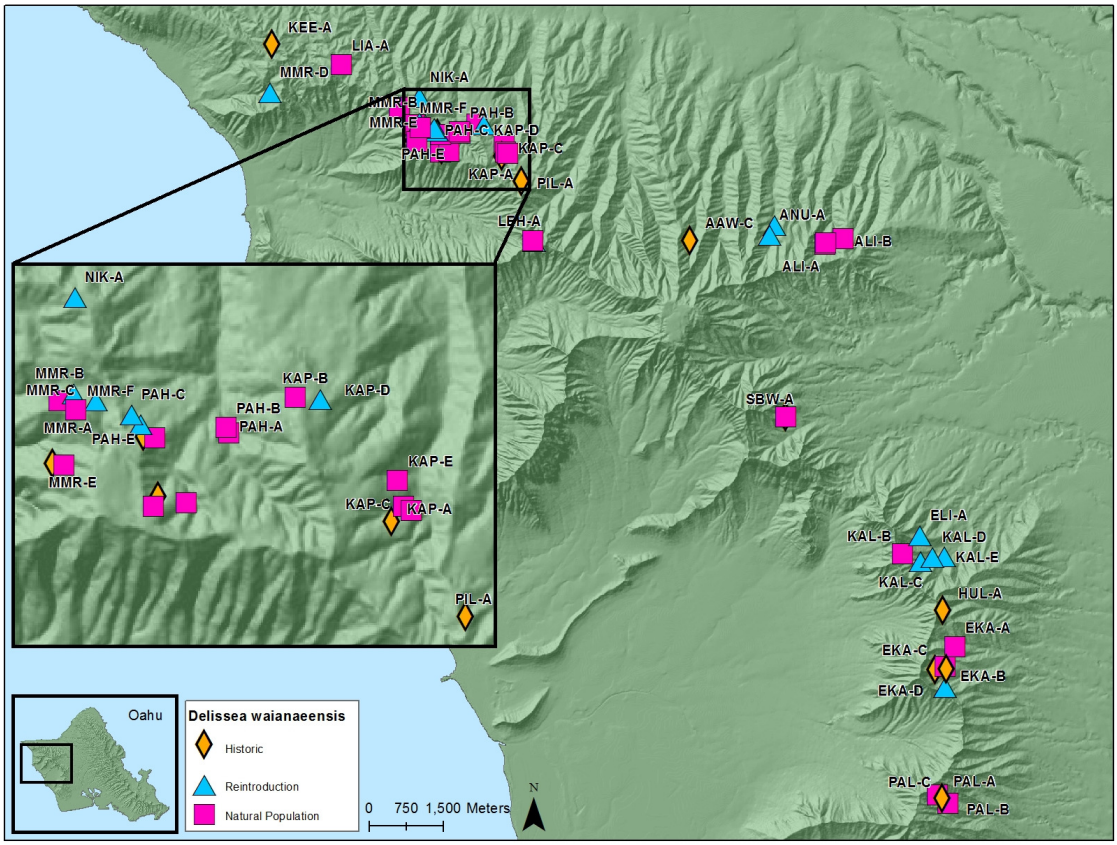

Figure 1: Distribution of population units of *Delissea waianaeensis* on O‘ahu. Map provided by OANRP.

Table 1: *Delissea waianaeensis* specimens included in analyses, with source plants ordered from north to south geographic localities of the wild founder in the Wai‘anae Mountains, O‘ahu. Sample Name is the US Army PopRefSiteID; PCMB number is the collection number of the tissues and DNA aliquots stored at the Bishop Museum's Pacific Center for Molecular Biodiversity.

Population Sample	Sample number	Sample name	PCMB Number
Keālia (LIA)	1	LIA-A2	4405
Unknown parentage	2	MMR-C1	4415
Pahole (PAH)	3	PAH-A1	4406
	4	PAH-B2	4408
	5	PAH-B3	4409
	6	PAH-E1	4410
Kapuna (KAP)	7	KAP-A1	4403
	8	KAP-A3	11115
	9	KAP-A5	4404
	10	KAP-C1	11117
	11	KAP-C2	4402
	12	KAP-C4	1119
Palikea (ALI)	13	ALI-A2	4392
	14	ALI-A3	11114
	15	ALI-B1	11112
	16	ALI-B3	4395
	17	ALI-B4	11113
	18	ALI-B5	4393
	19	ALI-B6	4394
South Mohiākea	20	SBW-A1	4413
(SBW)	21	SBW-A3	4411
	22	SBW-A4	4412
Kalua‘ā (KAL)	23	KAL-B1	4401
	24	KAL-B3	11118
	25	KAL-B11	11116
‘Ēkahanui (EKA)	26	EKA-A1	4396
	27	EKA-A4	4397
	28	EKA-A7	4398
	29	EKA-A10	4399
	30	EKA-B1	4400
Palawai (PAL)	31	PAL-A1	4414
	32	PAL-C8	11120

Table 2: ISSR-PCR primers tested, the number of fragments for each primer, and percentage polymorphism displayed by each and for the species as a whole.

Primer name	Primer sequence	Total number fragments	Number polymorphic fragments	Polymorphism %
814	$(CT)_8TG$	6	0	0
844A	$(CT)_8AC$	7	3	43
844B	$(CT)_8GC$	12	5	42
17898A	$(CA)_6AC$	5	2	40
17898B	$(CA)_6GT$	12	4	33
17899A	$(CA)_6AG$	5	4	80
HB10	$(GA)_6CC$	11	4	36
HB11	$(GT)_6CC$	8	3	37
HB12	$(CAC)_3GC$	2	0	0
HB13	$(GAG)_3GC$	14	2	14
HB14	$(CTC)_3GC$	2	0	0
		84	**27**	**32%**

A Bayesian analysis of the microsatellite data with STRUCTURE 2.3.3 was used to assign individuals to putative genetic groups (Hubisz *et al.* 2009). Because only a single individual of LIA and MMR was available, these were both grouped with the PAH population in analysis due to geographic proximity (Figure 1). An admixture ancestry model was assumed, and 10 replicates for *K* from 1 to 9 (number of managed populations + 2) was analysed using a burn-in period of 10,000, 50,000 and 100,000, and a Markov chain Monte Carlo (MCMC) simulation of 10,000, 50,000 or 100,000 iterations, respectively. Results for all three models were similar, therefore only the 10,000 iteration analysis is reported here. The number of genetic clusters in the dataset was estimated by examining the posterior probabilities (ln Pr(*X*|*K*)) for the varying values of *K*.

Expected (He) heterozygosities and percent polymorphic loci (%P) were estimated using GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Heterozygosity values range from 0 to 1, with 0 inferring that all individuals are genetically identical. Percent polymorphic loci for all individuals combined and for each population grouping were determined as an estimate of the amount of genetic variation within the populations. A hierarchical AMOVA analysis was performed using the software GenAlEX 6.5 (Peakall and Smouse

2006, 2012) to examine the distribution of variation and differential connectivity among populations (pairwise population PhiPT - an analogue of F_{st}, i.e., genetic diversity among populations). Estimates of gene flow (*Nm*, number of migrants per generation = 0.25[1/PhiPT-1]) were calculated for each population grouping. Indirect estimation of *Nm* from PhiPT involves numerous assumptions such a constant population size, random migration, and no selection, mutation or spatial structure, but can provide useful information about the approximate magnitude of gene flow (Wood and Gardner 2007). Principal Coordinate Analysis (PCoA) was undertaken using GenAlEx 6.5.

Results

For the ISSR-PCR analysis of the 32 *Delissea waianeensis* individuals, the total number of repeatable bands for the 11 primers was 84, with band sizes ranging from 200 bp to 3,000 bp (Table 2). Expected heterozygosity (He) for the seven management population units combined was 0.089 ± 0.017 and percentage polymorphism was 32.1%. Individual OANRP management population units had expected heterozygosities and %P ranging from 0.019 and 4.76% (SBW) to 0.067 and 20.24% (LIA/MMR/PAH) (Table 3).

STRUCTURE bayesian analyses estimated the number of natural genetic groups as K=4 (Table 3). The EKA-A and ALI-B populations were consistently identified as distinct genetic groups, as also indicated by the Principal Co-ordinates Analysis (Figure 4). The first three axes of the PCoA explained 37% of the variation in the ISSR-PCR data. For the four genetic population groups, expected heterozygosity and % polymorphism ranged from 0.01 and 2.38% for the EKA-A and ALI-B population groups, to 0.07 and 25% for population group 4, consisting of several management population groups (Table 4).

Percentage of molecular variance among and within populations for the seven management population units was 45% and 55%, respectively with PhiPT estimated at 0.453 ($p<0.001$). However, for the four populations units predicted by STRUCTURE, variation among populations was 53% and within population variation was 47%, with

PhiPT estimated at 0.532 ($p<0.001$). Estimates of gene flow (*Nm*) were 0.30 and 0.22 for the seven management populations and four genetic populations, respectively.

Table 3. Mean ± SE expected heterozygosity (*He*) and percentage polymorphism (%P) for the nine management populations (arranged geographically from north to south combining LIA and MMR with PAH populations due to geographical proximity).

Population	**Number individuals**	***He***	**%P**
1 - LIA/MMR/PAH	6	0.067 ± 0.016	20.24%
2 - KAP	6	0.044 ± 0.014	13.10%
3 - ALI	7	0.054 ± 0.016	14.29%
4 - SBW	3	0.019 ± 0.009	4.76%
5 - KAL	3	0.029 ± 0.011	8.33%
6 - EKA	5	0.058 ± 0.017	14.29%
7 - PAL	2	0.044 ± 0.014	10.71%
Mean	32	0.045 ± 0.005	12.24 ± 1.87

Table 4. Mean ± SE expected heterozygosity (*He*) and percentage polymorphism (%P) for the proposed four genetic populations as determined by STRUCTURE analysis.

Population	**Number individuals**	***He***	**%P**
1 - EKA-A	4	0.011 ± 0.008	2.38%
2 - ALI-B	5	0.012 ±.0.013	2.38%
3 - SBW/KAL/ALI-A/PAH-B3	9	0.050 ± 0.014	15.48%
4 - PAH/KAP/PAL/EKA-B/ LIA/MMR	14	0.070 ± 0.016	25.00%
Mean	32	0.036 ± 0.006	11.31 ± 5.51

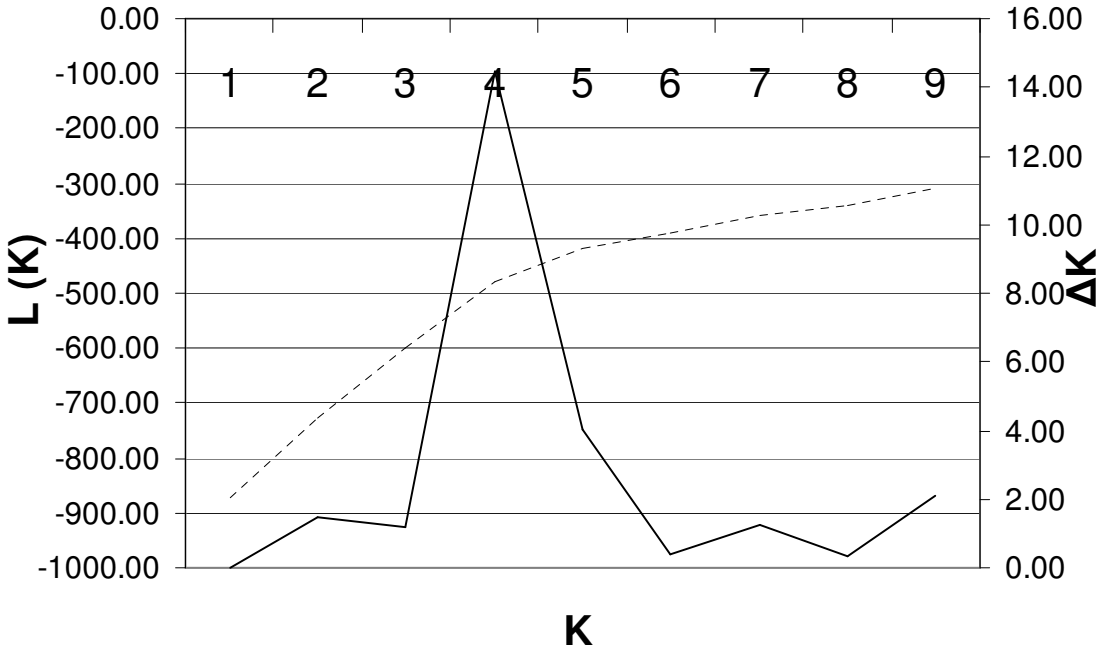

Figure 2: STRUCTURE analysis of 32 *Delissea waianaeensis* samples, 10,000 burn-in and MCMC simulation, suggesting the samples fall within four genetic groups (K).

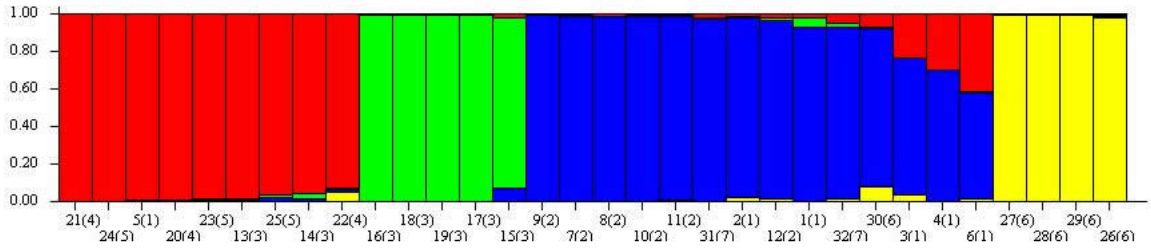

Figure 3: Representative STRUCTURE bar plot showing the four (K=4) genetic groups identified by the analysis. Numbers on the x-axis are the number of each individual as indicated in Table 1, and their respective purported management population in parentheses as indicated in Table 3.

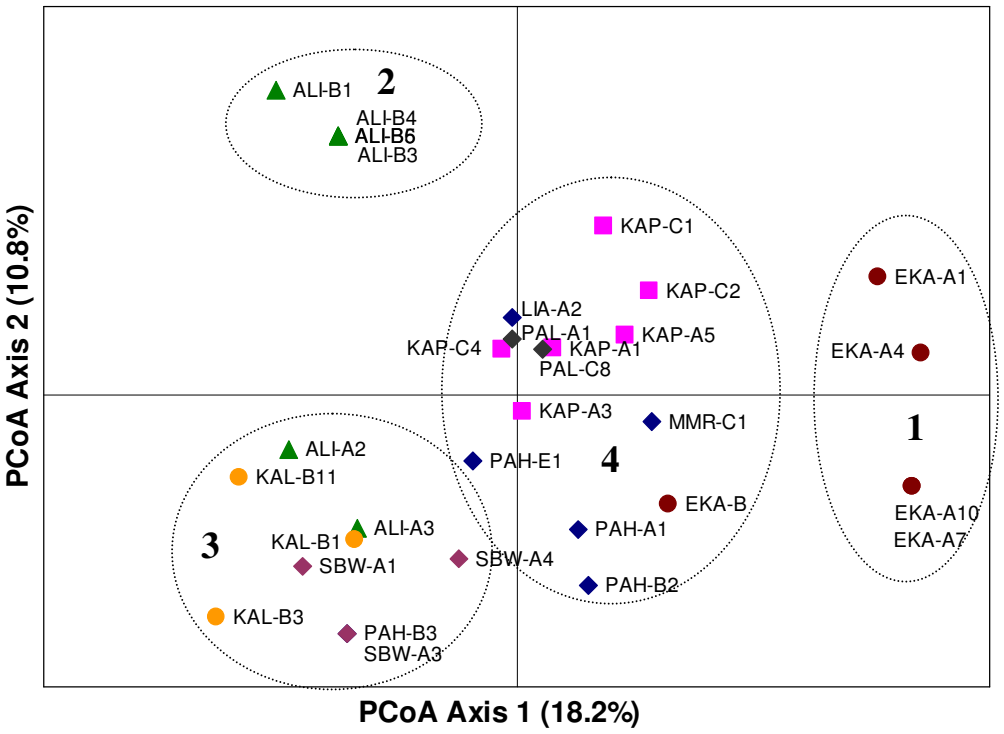

Figure 4: Principal Coordinate Analysis of ISSR-PCR data for 32 *Delissea waianaeensis* individuals. The four genetic groups as determined by STRUCTURE analysis have been indicated.

Discussion

ISSR-PCR analysis of *Delissea waianaeensis* from the Wai‘anae Range, O‘ahu, has indicated that the remaining founder individuals of the species fall within four natural genetic population groups, rather than the seven currently managed populations (Table 3, 4). The management populations in the centre of the geographic range of the species fall within three of the four population genetic groups as indicated by STRUCTURE analysis of the ISSR-PCR data, and suggest that the genetic groups ALI-B, EKA-A and ALI-A/SBW/KAL-B be managed separately from the remaining population groups currently being individually managed by OANRP. Previous RAPDs analysis of the species similarly indicated that the designated management population units of *Delissea*

waianaeensis were genetically distinct (James 2009), particularly the ALI-B, SBW and EKA-A population units. The EKA-A and ALI-B populations are smaller, and demonstrate lower genetic diversity than the other two genetic population groups which have a larger number of founder individuals, and cover a larger geographic area.

The ISSR-PCR analysis revealed a lower level of expected heterozygosity and percentage polymorphism than was expressed in a previous RAPDs analysis of the species (James 2009), but this may be due to the fewer markers used in this ISSR-PCR analysis. Estimates of gene flow between the populations, calculated from PhiPT, when considering either the current management populations or the natural groups as determined using STRUCTURE analysis, is low. The median genetic variation revealed by AMOVA (ca. 45 and 53% for 7 and 4 populations, respectively) within the *Delissea waianaeensis* populations points to the fact that the populations are heterogenous in nature, and the similar genetic variability (ca. 55 and 47%) among the populations indicates that the populations have largely not differentiated into separate genetic pools. This suggests that the different founder populations may not benefit from conserving gene pools and may benefit from intermixing during reintroduction activities.

Populations of *D. waianaeensis* are actively being reintroduced by OANRP in areas that historically contained the species. One reintroduction population in the Kahanahāiki area was started from individuals from the wild Kapuna population located approximately 1800 meters away, using KAP-A and KAP-B stock. The reintroduced plants have been documented as having flowered and fruited, and seeds have been collected from these individuals. In year 2000, a new recruit was found approximately 100 meters from the reintroduced plants. It was unclear whether this individual was an F_1 from a reintroduction parental source or from the seed bank of the historical population from the area, which could have genetics similar to the PAH (Pahole) populations about 850 m away. The ISSR-PCR data in this study has indicated that MMR-C1 individual was more closely related to the KAP (Kapuna) population unit than to the PAH (Pahole) population unit. This was also suggested in the previous RAPDs study (James 2009), indicating that

the parentage of new recruit is most likely to be the KAP outplanted individuals and can be managed as part of the KAP population.

Conclusions

Given the results of both this ISSR-PCR genetic study and the James (2009) RAPDs study, the endemic species *Delissea waianaeensis* could be delineated into natural genetic population units in three ways: maintaining the currently identified OANRP management populations; the four genetic groupings of median heterozygosity; or as a large, single heterozygous population. This current study, with its limited number of polymorphic ISSR-PCR markers available for analysis, suggests that managing the species as four populations (EKA-B, ALI-B, ALI-A/SBW/KAL complex, and the remaining populations) would be the best method for maintaining genetic diversity and the distinct genotypes of the geographic mid-range populations. If the management goal for the species is to maintain the highest level of genetic heterozygosity, the data suggest that managing the species as a single population may be the best method.

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