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Genetic Analyses of Captive ʻAlalā (*Corvus hawaiiensis*) Using AFLP Analyses

By Susan I. Jarvi and Kiara R. Bianchi

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Genetic Analyses of Captive ‘Alalā (*Corvus hawaiiensis*) Using AFLP Analysis

By Susan I. Jarvi[1] and Kiara Bianchi[1]

Introduction

Population level studies of genetic diversity can provide information about population structure, individual genetic distinctiveness and former population size. They are especially important for rare and threatened species like the ‘Alalā, where they can be used to assess extinction risks and evolutionary potential. In an ideal situation multiple methods should be used to detect variation, and these methods should be comparable across studies. In this report, we discuss AFLP (Amplified Fragment Length Polymorphism) as a genetic approach for detecting variation in the ‘Alalā , describe our findings, and discuss these in relation to mtDNA and microsatellite data reported elsewhere in this same population (Fleisher, 2003).

AFLP is a technique for DNA fingerprinting that has wide applications (Vos et al., 1995). Because little or no prior knowledge of the particular species is required to carry out this method of analysis, AFLP can be used universally across varied taxonomic groups. Within individuals, estimates of diversity or heterozygosity across genomes may be complex because levels of diversity differ between and among genes (Sachidanandam et al., 2001; Bensch and Akesson, 2005). One of the more traditional methods of estimating diversity employs the use of codominant markers such as microsatellites. Codominant markers detect each allele at a locus independently. Hence, one can readily distinguish heterozygotes from homozygotes, directly assess allele frequencies and calculate other population level statistics. Dominant markers (*e.g.*, AFLP) are scored as either present or absent (null) so heterozygotes cannot be directly distinguished from homozygotes. However, the presence or absence data can be converted to expected heterozygosity estimates which are comparable to those determined by codominant markers. High allelic diversity and heterozygosity inherent in microsatellites make them excellent tools for studies of wild populations and they have been used extensively. One limitation to the use of microsatellites is that heterozygosity estimates are affected by the mutation rate at microsatellite loci, thus introducing a bias. Also, the number of loci that can be studied is frequently limited to fewer than 10. This theoretically represents a maximum of one marker for each of 10 chromosomes. Dominant markers like AFLP allow a larger fraction of the genome to be screened. Large numbers of loci can be screened by AFLP to resolve very small individual differences that can be used for identification of individuals, estimates of pairwise relatedness and, in some cases, for parentage analyses. Since AFLP is a dominant marker (can not distinguish between +/+ homozygote versus +/- heterozygote), it has limitations for parentage analyses. Only when both parents are homozygous for the absence of alleles (-/-) and offspring show a presence (+/+ or +/-) can the parents be excluded. In this case, microsatellites become preferable as they have the potential to

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exclude individual parents when the other parent is unknown. Another limitation of AFLP is that the loci are generally less polymorphic (only two alleles/locus) than microsatellite loci (often >10 alleles/locus). While generally fewer than 10 highly polymorphic microsatellite loci are enough to exclude and assign parentage, it might require up to 100 or more AFLP loci (reviewed in Bensch and Akesson, 2005). While there are pros and cons to different methodologies, the total number of loci evaluated by AFLP generally offsets the limitations imposed due to the dominant nature of this approach and end results between methods are generally comparable.

Overall objectives of this study were to evaluate the level of genetic diversity in the captive population of ʻAlalā, to compare genetic data with currently available pedigree information, and to determine the extent of relatedness of mating pairs and among founding individuals.

Methods

DNA samples and extractions: Tissue samples were provided by the Zoological Society of San Diego, the Maui Bird Conservation Center, and the Keauhou Bird Conservation Center. Tissues were stored in screw-capped tubes containing 500 µL of DNA lysis buffer (0.1M Tris-HCl, pH 8.0, 0.1M sodium EDTA, 2% SDS) until extracted. Extractions were completed between 2001 and 2003 by either standard organic methods using phenol/chloroform or by column using the Qiagen DNeasy Kit. Those that were initially extracted by organic methods were subsequently re-extracted using the column method for consistency. Several of the samples were also concentrated using "Zymo" columns to increase DNA concentration. All samples originated from blood or muscle except two that were from a yellowish, granular substance provided as necropsy samples. A total of 49 samples were extracted (Table 1).

Table 1. Blood samples that were extracted and used in final analyses (X).

Sample	Studbook #	DNA Log #	Name	Extraction Date	Final Analysis
1	7	8353	`Umi	18-Jun-02	X
2	13	9614/9760	Lu`ukia		X
3	17	9613/9759	Mana		
4	25	6933	Kolohe	21-Nov-01	X
5	26	8352	Keli`i	18-Jun-02	X
6	27	6943	Kalani	21-Nov-01	X
7	28	6934	Keawe	21-Nov-01	X
8	30	6935	Wa`alani	21-Nov-01	X
9	31	6936	Ho`okū	21-Nov-01	X
10	32	6937	Hō`ikeikapo	21-Nov-01	X
11	33	6938	Kīnohi	21-Nov-01	X
12	35	6939	Lanakila	21-Nov-01	X
13	36	7155	Hiwahiwa	11-Feb-02	X
	36	1377-9	Hiwahiwa	05-Mar-97	
14	37	7154	Hoapili	11-Feb-02	X
	37	1374-6	Hoapili	05-Mar-97	

15	38	6944	Lōkāhi	21-Nov-01	X
	38	7171	Lōkāhi	11-Feb-02	
16	39	7156	Mālama	11-Feb-02	X
	39	1380-2	Mālama	05-Mar-97	
17	40	7172	Hulali	11-Feb-02	X
18	41	6945	Kēhau	21-Nov-01	X
	41	7157	Kēhau	11-Feb-02	
	41	9940	Kēhau	25-Mar-03	
	41	1383-5	Kēhau	05-Mar-97	
19	54	6946	Pōmaika`i	21-Nov-01	X
20	56	6947	Leinani	21-Nov-01	X
21	57	6948	`Ula	21-Nov-01	X
22	58	7152	Noe	11-Feb-02	
	58	1398-1407	Noe	12-Apr-97	
	58	4116-18	Noe	07-Mar-98	
23	61	7160	Hilu	11-Feb-02	X
	61	1408-1417	Hilu	12-Apr-97	
	61	4119-21	Hilu	07-Mar-98	
24	66	6940	Nīele	21-Nov-01	X
25	67	6942	`Oli	21-Nov-01	X
	67	9939	`Oli	25-Mar-03	
26	68	7158	Uila	11-Feb-02	X
	68	4080-2	Uila	07-Mar-98	
	68	1386-8	Uila	05-Mar-97	
27	69	7170	`Ele`ele	11-Feb-02	X
	69	1392-5	`Ele`ele	05-Mar-97	
	69	4086-8	`Ele`ele	07-Mar-98	
28	70	7164	Makuni	11-Feb-02	
	70	4083-5	Makuni	07-Mar-98	
29	71	7159	Konakū	11-Feb-02	
	71	1395-7	Konakū	05-Mar-97	
	71	4089-91	Konakū	07-Mar-98	
30	73	6949	Kekoa	21-Nov-01	X
31	74	7153	Hiapo	11-Feb-02	X
	74	9938	Hiapo	25-Mar-03	
	74	4128-33	Hiapo	07-Mar-98	
32	75	6950	Laha	21-Nov-01	X
33	76	7163	Makoa	11-Feb-02	X
	76	4074-9	Makoa	07-Mar-98	
34	77	7169	Puanani	11-Feb-02	X
	77	4125-27	Puanani	07-Mar-98	

35	78	7168	Ao	11-Feb-02	X
	78	9941	Ao	25-Mar-03	
	78	4113-15	Ao	07-Mar-98	
36	79	7167	Kahuli	11-Feb-02	X
	79	4122-24	Kahuli	07-Mar-98	
37	80	7166	Hulu	11-Feb-02	X
	80	4107-9	Hulu	07-Mar-98	
38	81	7165	Lehua	11-Feb-02	
	81	4104-6	Lehua	07-Mar-98	
39	82	6941	Kauila	21-Nov-01	X
	82	7151	Kauila	11-Feb-02	
	82	4110-12	Kauila	07-Mar-98	
40	86	6951	Kilakila	21-Nov-01	X
41	87	6952	Hōkūlele	21-Nov-01	X
42	88	6953	`Oni`oni	21-Nov-01	X
43	89	6954	Pīkoi	21-Nov-01	X
44	90	6955	Akamai	21-Nov-01	X
45	91	6956	Mai`aokea	21-Nov-01	X
46	92	6957	Pīkaka	21-Nov-01	X
47		7161	12474(#4)	11-Feb-02	
		4053-5	212474	07-Mar-98	
		5428-30	12474	29-Sep-98	
48		7162	12475(#1)	11-Feb-02	
		4056-8	412475-1	07-Mar-98	
49		1389-91	NB	05-Mar-97	

AFLP: AFLP consists of four basic steps: (1) restriction enzyme digest of genomic DNA, (2) attachment of short DNA segments (adapters) to the ends of all digested fragments, (3) amplification of fragments using selective primers, of which one is labeled for later visualization, and (4) separation and visualization of amplified fragments by gel electrophoresis. While the protocol for AFLP appears quite straightforward, optimization is required to obtain high quality, consistent and replicable fingerprint patterns. Of particular importance is consistency in the quality and quantity of starting genomic DNA and the success of the ligation (attachment) reaction, which provides the priming sites for selective amplification. Steps (1), (2), and (3) (above) were completed at the University of Hawaii, Hilo, and step 4 (separation and visualization of products) was completed on an ABI genotyping system at the Biotech facility at the University of Hawaii, Manoa. For samples used in this study, genomic DNA was digested with restriction enzymes *Eco*R1 and *Mse*1 which cleave at 6 base pair sites (Step 1). Adapters were ligated to the ends of all fragments (Step 2). Selective amplification was then performed to amplify subsets of this genomic "soup" depending on the 2 or 3 base pair sequence at the end of each primer (Step 3, Appendix A). For each set of primers, only one was labeled with a fluorescent dye (either TET, HEX or FAM) which fluoresces at distinct wavelengths. PCR products from these reactions, in addition to a

known marker labeled with another dye (ROX) were run in polyacrylamide gels and bands were separated, identified by size and computerized (Step 4). We then evaluated the data in these files for repeatability and overall consistency. Criteria for selecting bands and individuals to include in final analyses were based on repeatability and consistency and are provided in stepwise fashion in Appendix A. We initially identified the bands or band classes to include in the analysis for each primer set and then decided (Step 2 in Appendix) whether to keep or remove individuals based on repeatability and consistency of runs within individual primer sets. We next determined which bands to use in the final analysis (Step 3). Finally, we compiled data over multiple primer sets for the final analysis (Step 4). This step is integral with Step 2. All AFLP data analyses were completed using the program Bionumerics (Applied Maths), with the exception of heterozygosity estimates which were completed using the program Hickory (v.1.0.3).

Primer screens. Four individuals (`Ele`ele, Kauila, Noe, Hiapo), were screened initially in 2001 in triplicate with 6 *Eco/Taq* primer sets (A, B, C, D, E, F). These samples were all extracted using organic methods. In 2002, we assayed 42 samples on 3 primer sets (M3, M5, M9), trying a new enzyme set, *Eco/Mse*. Low, but detectable, levels of polymorphism were observed. We determined that all samples required re-extraction by column DNA isolation. We subsequently screened 36 new primers on samples from Keawe, Wa`alani, `Oli, Pōmaika`i, and Laha which were digested with *Eco/Mse*. Of these 45 primer sets, 7 were selected for further population analyses.

Results

While samples from 49 individuals were available, we report results from 41 individuals (as indicated in Table 1). The primer sets used in the final analyses, along with the size ranges of fragments and polymorphic bands are listed in Table 2.

Table 2. Total number of bands scored and frequency of polymorphic bands in the final analyses of individual primer sets.

Eco/*Mse* primer set	Total # bands scored	Size Range (bp)	Total # polymorphic bands	Frequency of polymorphic bands
M3	35	63-203	1	0.029
M5	63	59-256	3	0.048
M55	38	67-223	2	0.053
M57	35	55-250	5	0.143
M60	19	51-313	6	0.316
M61	50	54-192	3	0.06
M62	15	61-323	5	0.333
Total	255		25	0.098

bp = base pairs

Four individuals that were evaluated for the initial primer screen in 2001 with 4 of the 6 primer sets (ABCD) were selected based on quantity and quality of available DNA and familial information provided by the U. S. Fish and Wildlife Service in the 2001 pedigree flow chart and studbook. According to the pedigree, Noe is a descendant of the Keālia pair, Ele'ele and Kauila are descendants of the founders `Umi and Lu`ukia, and Hiapo is an offspring of `Ula (Kalāhiki pair) and Kalani (`Umi and Lu`ukia). While these four primer sets clearly differentiated these four individuals with diversity ranging from 82%-91% (data not shown), we had concerns with repeatability and reliability in this trial. Also of concern was the fact that the Taq enzyme we used is methylation sensitive, and could possible influence the results. These data were removed from further analyses.

The second screen was completed with three primer sets M3, M5 and M9 on 42 samples using the *Eco*/*Mse* enzyme digest. We detected low levels of polymorphism. However, we decided to re-extract some of the samples to provide uniformity of methods of all samples. The information from this screening was useful in establishing the overall diversity detected by these primers, and two of them (M3, M5) were selected for further analyses (described below). We did not use this scoring data in the final analyses, however, due to lack of reproducibility.

In the screening done with 36 *Eco*/*Mse* primer sets, initial evaluation of the banding patterns among replicates revealed that several were unscorable (faint or unclear bands) or too difficult to score reliably (bands present but do not distinct peaks *i.e*., have a "shoulder"), and we selected 6 sets to score. The resulting relationships among 5 individuals with these 6 selected primer sets (M55, M57, M59, M60, M61, M62) are presented in Figure 1. Results from these sets of primers are illustrated in Figure 2.

Figure 1. Five individuals were selected for screening 36 sets of primers. The phylogenetic relationships among these five individuals based on six sets of primers are shown using all bands (upper, including monomorphic and polymorphic) and with only polymorphic (lower) with presence (shaded) or absence (non-shaded) of bands as indicated. Phylogenetic relationships were estimated by Dice methods and depicted by UPGMA (Unweighted Pair Group Method with Arithrimetic Mean). Primer/band class key (lower) is available upon request.

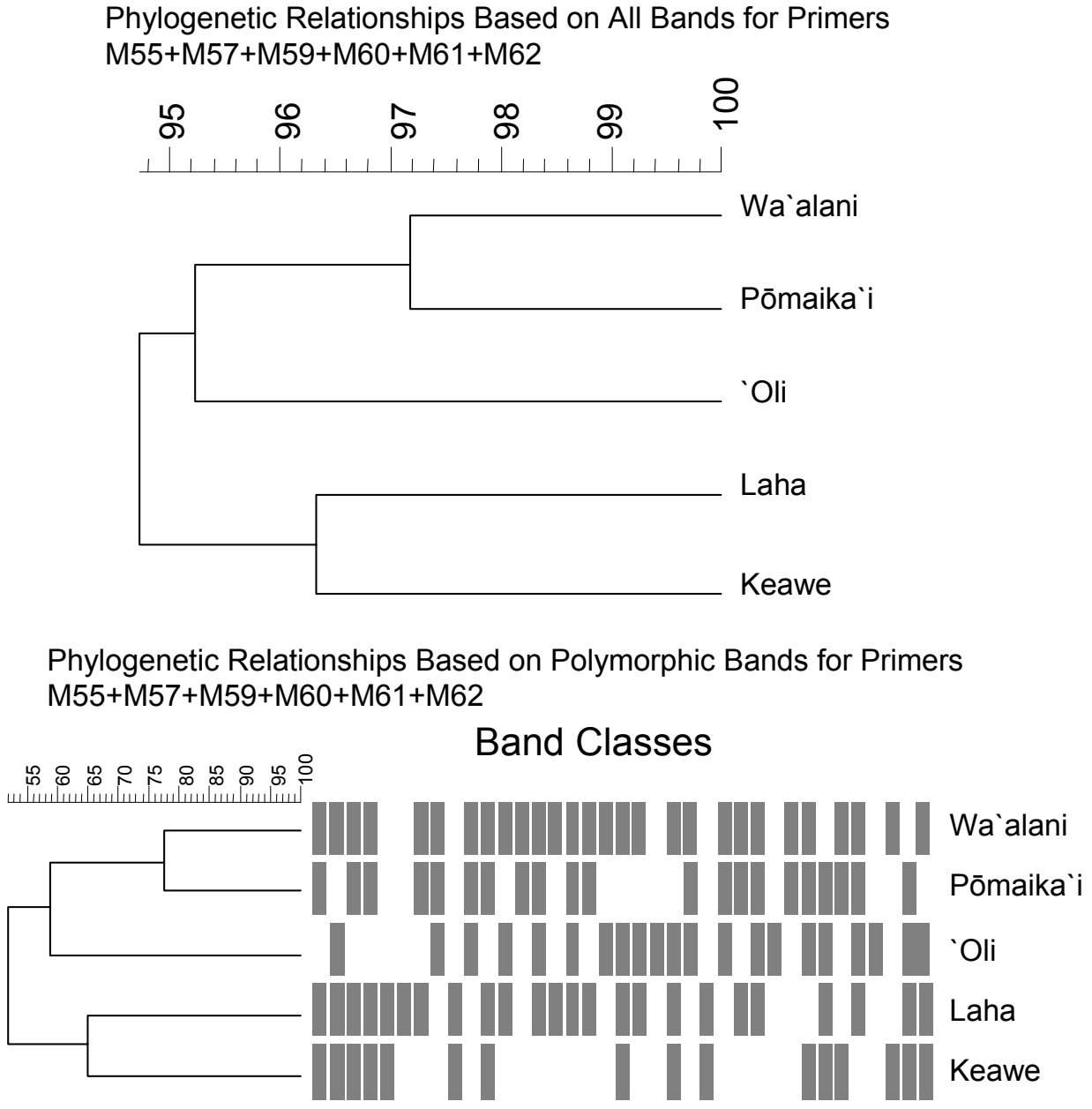
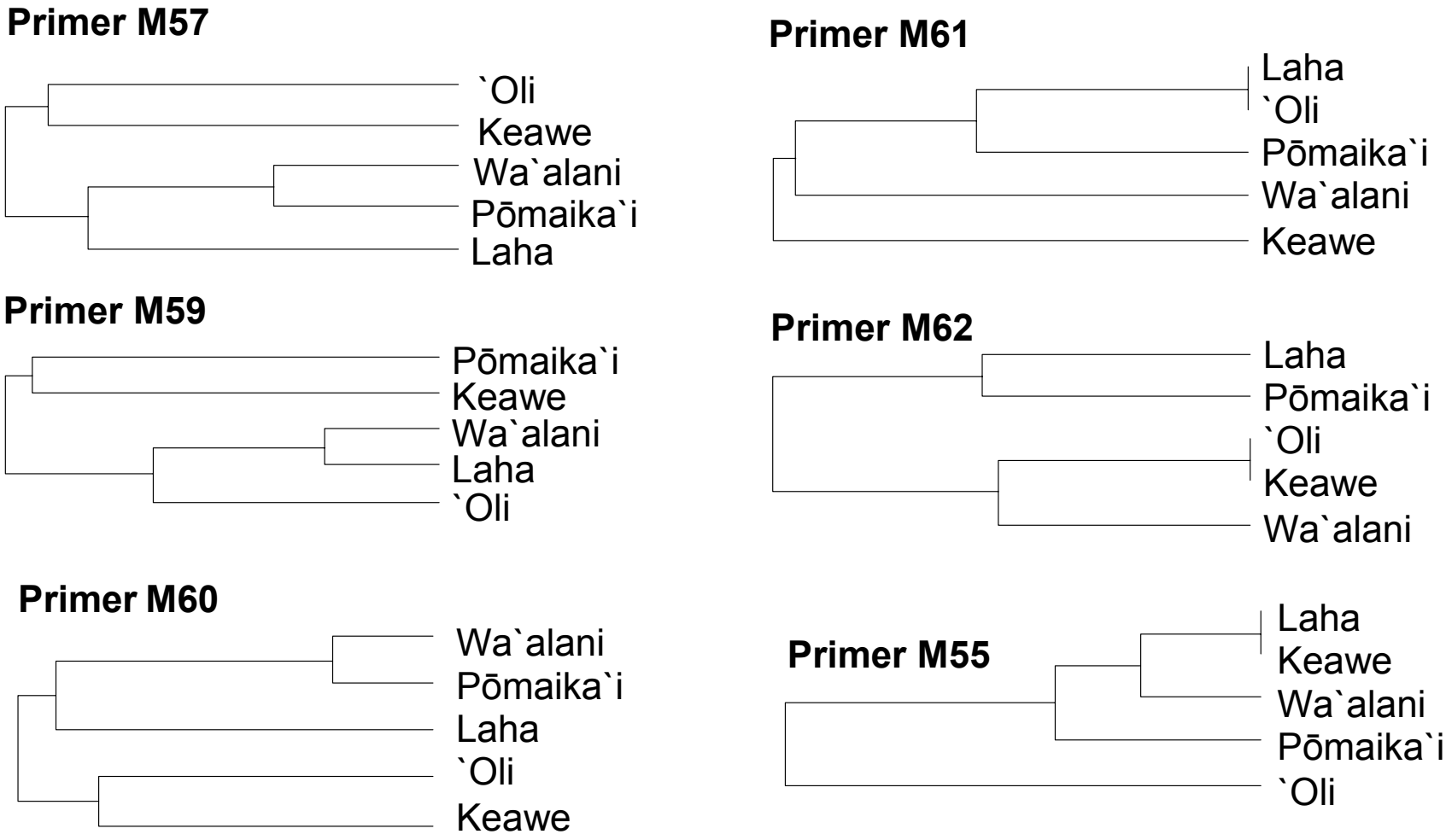


Figure 2. Phylogenetic relationships of 5 individuals involved in primer screens by individual primer sets. Primer sets are: M57, M59, M60, M61, M62, and M55. Distance estimates (Dice, Bionumerics) were used to depict phylogenetic relationships among these individuals in a UPGMA tree.



Individuals selected for inclusion were `Oli from the Hookena pair, Pōmaika`i from the Kalāhiki pair, Keawe from `Umi and Lu`ukia, founder Wa`alani and Laha from `Ula and Kalani. Of the total number of bands scored, 37 were polymorphic bands. A summary of the similarity matrix depicting relationships is shown in Table 3. Based on these similarity values, Pōmaika`i (McCandless) appears most similar to Wa`alani (Hu'alalāi descendent) and the least similar pair is Keawe (`Umi and Lu`ukia offspring, Hu'alalāi) and `Oli (Hookena pair, McCandless). Five of these primer pairs were selected for inclusion in the final analyses (M55, M57, M60, M61, M62). The similarity matrix depicting the relationships among these 5 individuals is shown in Table 3.

Table 3. Similarity matrix depicting relationships among 5 individuals used in primer screen (M55, M57, M59, M60, M61, M62). Numbers shown are based on all bands / polymorphic bands only. The highest (most similar) and lowest (least similar) values are underlined.

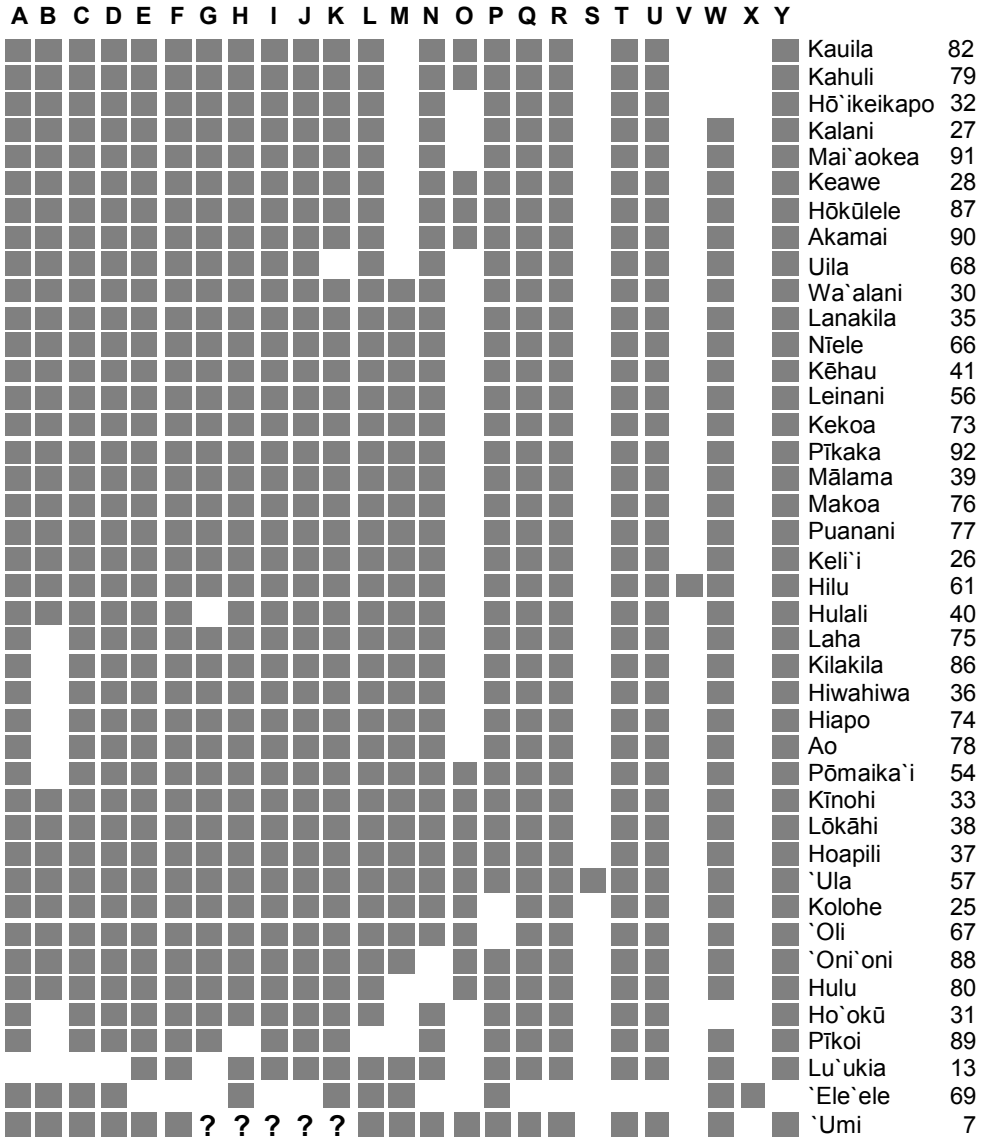
	Wa`alani	Pōmaika`i	`Oli	Laha	Keawe
Wa`alani	100 / 100				
Pōmaika`i	<u>97.19 / 77.55</u>	100 / 100			
`Oli	97.19 / 65.31	94.79 / 52.38	100 / 100		
Laha	95.94 / 69.23	95.09 / 57.78	94.57 / 53.33	100 / 100	
Keawe	94.3 / 50.00	94.46 / 43.24	<u>93.93 / 37.84</u>	96.33 / 65.	100 / 100

A total of seven primer sets were used for further analyses (M3, M5, M55, M57, M60, M61, M62). Of the 49 total samples available, 41 were included in the final analyses (indicated in Table 1). Bands produced by these 7 sets of primers are scored as either present (1) or absent (0). We evaluated a total of 255 loci for polymorphism and out of these 9.8% were polymorphic (25 band classes using all 7 sets of primers, but only 20 band classes if primer set M57 is excluded see Table 2).

A total of 19 distinct banding patterns were distinguished among these 41 individuals. The polymorphic bands distinguishing these genetic patterns are summarized as a binary table (Table 4) and as presence or absence in Figure 3. Individuals of identical pattern are color-coded in Table 4, with 12 of the 41 individuals evaluated showing unique patterns. Of note is that founding individuals Wa`alani, `Umi and Lu`ukia each appear distinct from one another.

Pattern 1 is the predominant pattern and includes individuals originating from several families and lineages (Table 5). Many of the remaining individuals are distinct from pattern one at only one locus and fall into Patterns 2, 3, 4, 5, 14 and 15. Individuals in Patterns 7, 9, 10, 11, 12, and 13 are distinct from the predominant pattern at two loci, Patterns 6, 8, 16 and 17 by three loci, Pattern 18 (Lu`ukia) at 5 loci and Pattern 19 (`Ele`ele) at 12 loci.

Figure 3. Polymorphic banding patterns detected in 40 `Alala. Presence of bands are indicated by box shading, and absence by none. Band classes (A-Y) are defined in the key*. Studbook number follows each name.



*Band Class Key for Figure 3 and all following Figures and Tables.

A = M3.118	H = M57.79	O = M60.306	V = M62.68
B = M5.59	I = M57.144	P = M60.307	W = M62.124
C = M5.118	J = M57.194	Q = M60.313	X = M62.146
D = M5.249	K = M57.225	R = M61.57	Y = M62.313
E = M55.115	L = M60.90	S = M61.81	
F = M55.131	M = M60.103	T = M61.99	
G = M57.58	N = M60.104	U = M62.61	

Table 4. Summary of pattern designation for 41 `Alala (including `Umi) based on presence (1) or absence (0) of polymorphic bands. Band classes (A-Y) as in Figure 3. Identical individuals are coded with the same color. Samples needing further analyses are indicated by "?".

Individual	Pattern	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Wa`alani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Lanakila	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Nīele	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kēhau	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Leinani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kekoa	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Pīkaka	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Mālama	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Makoa	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Puanani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Keli`i	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Laha	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kilakila	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Hiwahiwa	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Hiapo	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Ao	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kīnohi	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Lōkāhi	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Hoapili	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
`Umi	3?	1	1	1	1	1	1	?	?	?	?	?	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Keawe	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Hōkūlele	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Akamai	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Kalani	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1
Mai`aokea	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1

Kauila	6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1
Kahuli	6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1
Kolohe	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1
`Oli	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1
Hula	8	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	1	0	1
Hō`ikeikapo	9	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Uila	10	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	1
`Oni`oni	11	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0	1	0	1
`Ula	12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
Pōmaika`i	13	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Hilu	14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1
Hulali	15	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Ho`okū	16	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Pīkoi	17	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	0	1	0	1
Lu`ukia	18	0	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
`Ele`ele	19	1	1	1	1	0	0	0	1	0	0	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0

Table 5. Banding patterns for 41 captive `Alala summarized within families. Sire and Dam are the first two lines of each family group. Patterns are indicated and identical individuals are color coded as in Table 4. *Indicates possible pedigree discrepancy.

	Pattern	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Family 1																										
`Umi	3?	1	1	1	1	1	1	?	?	?	?	?	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Lu`ukia	18	0	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Keli`i	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kalani	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1
Keawe	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Family 2																										
Kalani	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1
Lu`ukia	18	0	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Ho`okū	16	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Hō`ikeikapo	9	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Family 3																										
Kalani	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1
`Ula	12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
Laha	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Hiapo	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Family 4																										
Ho`okū	16	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Keawe	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Kahuli	6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1
Kauila	6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1
Hokule	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
`Oni`oni	11	1	1	1	1	1	1	1	1	1	1	1	1	1*	0	1	1	1	1	0	1	1	0	1	0	1

Pīkoi	17	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	0	1	0	1
Akamai	4	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
Family 5																											
Hō`ikeikapo	9	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1
Wa`alani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Lanakila	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Nīele	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kekoa	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Makoa	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Puanani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Pīkaka	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Mai`aokea	5	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	1
Ao	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kilakila	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Uila	10	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	1
`Ele`ele	19	1	1	1	1	0	0	0	1	0	0	1	1	1	0	0	1	0	0	0	0	0	0	1	1*	0
Family 6 Kalāhiki pair																											
Hiwahiwa	2	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Lōkāhi	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Hoapili	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Lōkāhi	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
Pōmaika`i	13	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1
`Ula	12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1*	1	1	0	1	0	1
Leinani	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Family 7 Keālia pair																											
Mālama	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Kēhau	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1
Hulali	15	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	0	1

Evaluation of family relationships can be addressed using AFLP data when both parents are homozygous for the absence of alleles (0/0) and offspring show a presence of alleles (+/+ or +/0). This occurred in three instances (indicated by "*" in Table 5). One is the detection of a band at locus M60.103 in progeny of `Oni`oni, when parents Ho`okū and Keawe are both homozygous 0/0. The second is at locus M62.146, where `Ele`ele possesses this band, while neither parent does (Hō`ikeikapo and Wa`alani), and third at locus m61.81 where `Ula possesses this band and both parents (Hiwahiwa and Lōkāhi) do not. The corresponding microsatellite data submitted by Rob Fleischer do not validate or refute these data. The microsatellite data indicate other possible discrepancies (Kīnohi, Keli`i, and Uila) that the AFLP data neither validate nor refute. The microsatellite data also suggests that extra-pair fertilization resulted in the observance of 5 alleles at one locus MJG1 in the Keālia pair. The AFLP data show no abnormalities in that family (although the parents are not available for testing). Additional analyses are required to definitively resolve these discrepancies.

Within-family analyses can shed light on individual level of heterozygosity. In the instances where at least one of the offspring are (0/0) at a particular locus, and either or both parents are (+), the (+) parent(s) can be defined as being heterozygous at that particular locus. This occurs in 2/25 loci in Family 1 (0.08), 3/25 in Families 2 and 3 (0.12), 5/25 in Family 4 (0.2), and 14/25 in Family 5 (0.56) (Table 5). Frequency of polymorphic loci and corresponding levels of heterozygocity among parents Hō`ikeikapo and Wa`alani in Family 5 appear to be greater than in other families that were evaluated. However, 11/14 of these polymorphic loci in Family 5 are due to the absence of a band in only one individual, `Ele`ele, where all other siblings were scored as present for these bands. `Ele`ele appears very distinct from siblings. We obtained similar results when the sample was repeated. Further work to confirm these differences would be useful.

It is not possible to directly distinguish heterozygotes (+/-) from homozygotes (+/+) at the population level because of the dominant nature of AFLP, but presence or absence data can be converted to expected heterozygosity estimates which are then comparable to those determined by codominant markers (*e.g.*, microsatellites). Most of these estimates are calculated using the assumptions of the standard random-mating model (Hardy-Weinberg equilibrium). The average expected heterozygosity (H_0 = 1-q2-(1-q)2) for each AFLP locus is presented in Table 6 (Hartl and Clark 1997). Two average values of H_0 are listed. One (inclusive) is the average H_0 for all loci regardless of the number of homozygous absence individuals (-/-) at each locus. There have been concerns regarding bias introduced when calculating small values of q (frequency of absence allele). This bias is nearly eliminated when only loci that have 4 or more individuals who are homozygous for the absence allele (-/-) are included in the analyses. Another method applies Bayesian statistics to estimate the frequency of the absence allele (q) that nearly eliminates all bias and does not assume that genotypes within populations are in Hardy-Weinberg equilibrium (Zhivotovsky 1999; Holsinger et al., 2002). We have included an average H_0 using only the loci with 4 or more (-/-) individuals as well as the Bayesian estimate generated in the program Hickory (v.1.0.3). Our H_0 estimate range of 0.229-0.265 (Table 6) based on 25 polymorphic loci is in the range of the estimates provided by the microsatellite data from 5 loci (0.248) (excluding the MJG1 locus) as reported by Fleischer (2003). It is much lower than the average H_0 estimate provided from 6 microsatellite loci (0.319) when the MJG1 locus is included. The statistic F_{IS} is the inbreeding coefficient of a group of inbred organisms relative to the subpopulation to which they belong. The statistic θ^B = Fst which is the reduction in heterozygosity due to increased inbreeding

relative to the total population. These low statistical estimates (Table 6) are indicative of a highly inbred population.

Table 6. Heterozygosity (H_0) at each locus

Locus	n	A	R	q	p	p2	q2	H0
M3.118	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M5.59	41	9	0.219	0.468	0.532	0.283	0.219	0.498
M5.118	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M5.249	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M55.115	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M55.131	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M60.90	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M60.103	41	12	0.293	0.541	0.459	0.211	0.293	0.497
M60.104	41	3	0.073	0.271	0.729	0.531	0.073	0.395
M60.306	41	26	0.634	0.796	0.204	0.042	0.634	0.325
M60.307	41	2	0.049	0.22	0.78	0.608	0.048	0.343
M60.313	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M61.57	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M61.81	41	40	0.976	0.987	0.013	0.00017	0.974	0.026
M61.99	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M62.61	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M62.68	41	40	0.976	0.987	0.013	0.00017	0.974	0.026
M62.124	41	4	0.096	0.312	0.688	0.473	0.097	0.429
M62.146	41	40	0.976	0.987	0.013	0.00017	0.974	0.026
M62.313	41	1	0.24	0.156	0.844	0.712	0.024	0.263
M57.58	40	3	0.075	0.274	0.726	0.527	0.075	0.398
M57.79	40	1	0.025	0.158	0.842	0.709	0.025	0.266
M57.145	40	1	0.025	0.158	0.842	0.709	0.025	0.266
M57.194	40	1	0.025	0.158	0.842	0.709	0.025	0.266
M57.250	40	1	0.025	0.158	0.842	0.709	0.025	0.266
Mean H_0								0.265
inclusive Mean H_0								0.229

A>3	
Mean H_0	0.2424
Bayesian	
fis	0.1352
θ^B	0.1297

n= number of individuals evaluated
A=number of individuals homozygous for absence alleles (-/-)
R= Frequency of homozygous recessives (A/n)
q= √R (frequency of absence allele)
H_0 = 1-q2-(1-q)2

fis= inbreeding coefficient of group of inbred organisms relative to the subpopulation to which they belong.
θ^B = fst, reduction in heterozygosity due to increased inbreeding relative to the total population.

Results of phylogenetic relationships are summarized using all 7 sets of primers (Figures 4 and 5, excluding founding individual `Umi) or using only 6 sets of primers (Figures 6 and 7, including `Umi). In Figures 6 and 7, primer set M57 is excluded because it produced inconclusive results for `Umi. Results are also presented as either all 255 polymorphic and monomorphic bands (Figures 4 and 6) or only the 35 polymorphic band classes (Figures 5 and 7). Relationships between individuals are depicted in UPGMA dendrograms derived from similarity values estimated by methods of Dice.

Results from analyses of all 7 sets of primers including both monomorphic and polymorphic bands are presented in Figure 4. `Umi is not included (but is inserted in parentheses where he would be expected). Two distinct main clusters were generated from the data. All individuals originating from McCandless Ranch fall within the larger of the two clusters. Similarity values range from 97.2 to 100. If monomorphic bands are removed from the analyses and only polymorphic bands are used, the same two basic clusters are generated based on similarity values, with `Oni`oni now placed outside the larger cluster (containing the McCandless birds) with sibling `Ula (from Ho`okū and Keawe, Figure 5). To include `Umi in phylogenetic analyses, we needed to remove one primer set (M57) in the analysis as DNA results from `Umi need to be re-run with this primer set. With only 6 sets of primers, the very distinct clustering is lost, but the relationship of `Umi is established (Figures 6 and 7).

Figure 4. Relationships of 40 individuals (`Umi excluded) based on seven primer sets are depicted in a UPGMA dendrogram using distance estimates (Dice) calculated in the program Bionumerics. The individuals originating from McCandless Ranch (boxes) are contained within one cluster. All monomorphic and polymorphic bands (n=255) are included. Studbook number follows each name.

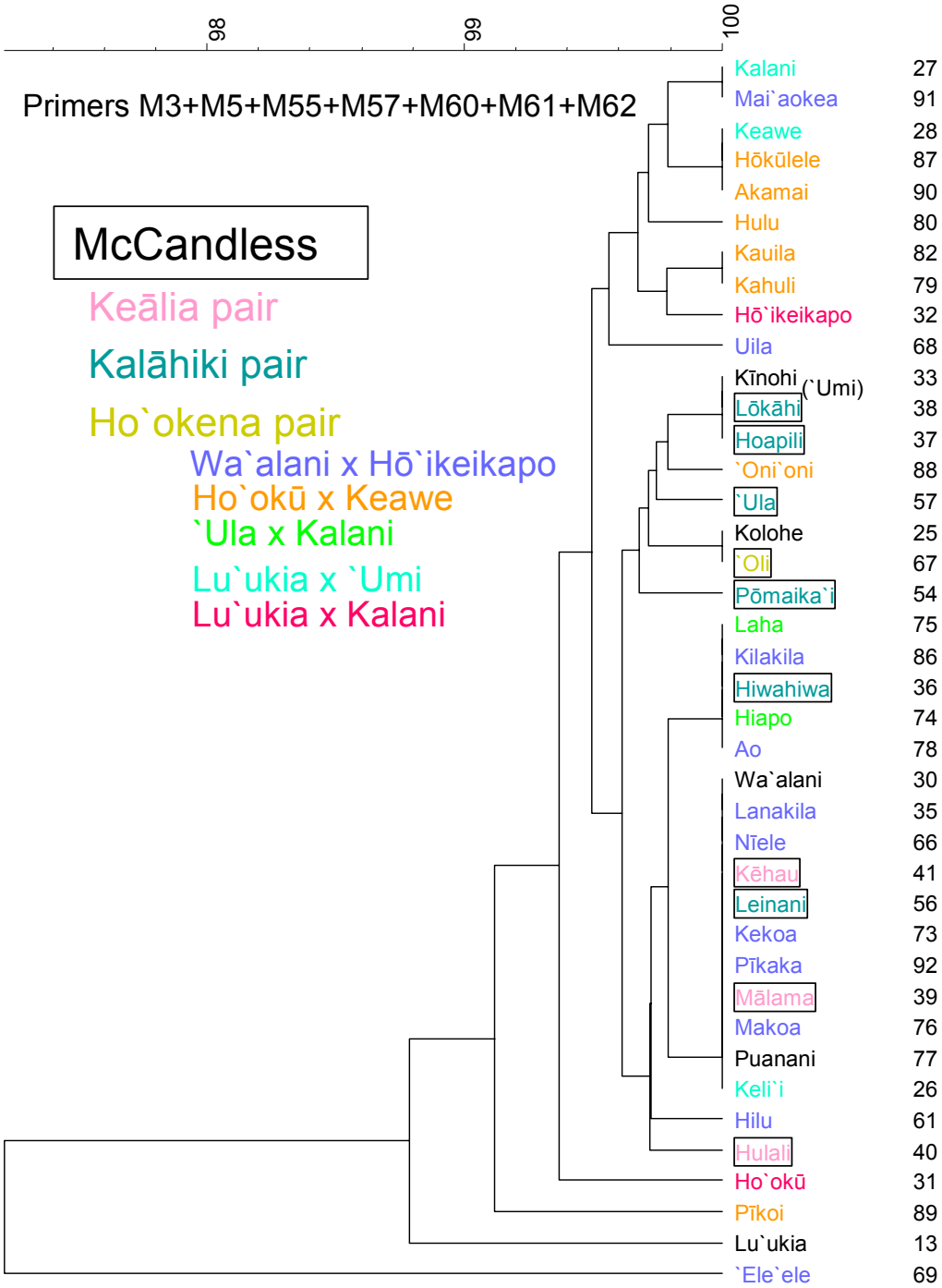


Figure 5. Relationships of 40 individuals based on seven primer sets (`Umi excluded) are depicted in a UPGMA dendrogram using distance estimates (Dice) calculated in the program Bionumerics. The individuals originating from McCandless Ranch are contained within one cluster. Only polymorphic bands (n=25) are included. Studbook number follows each name.

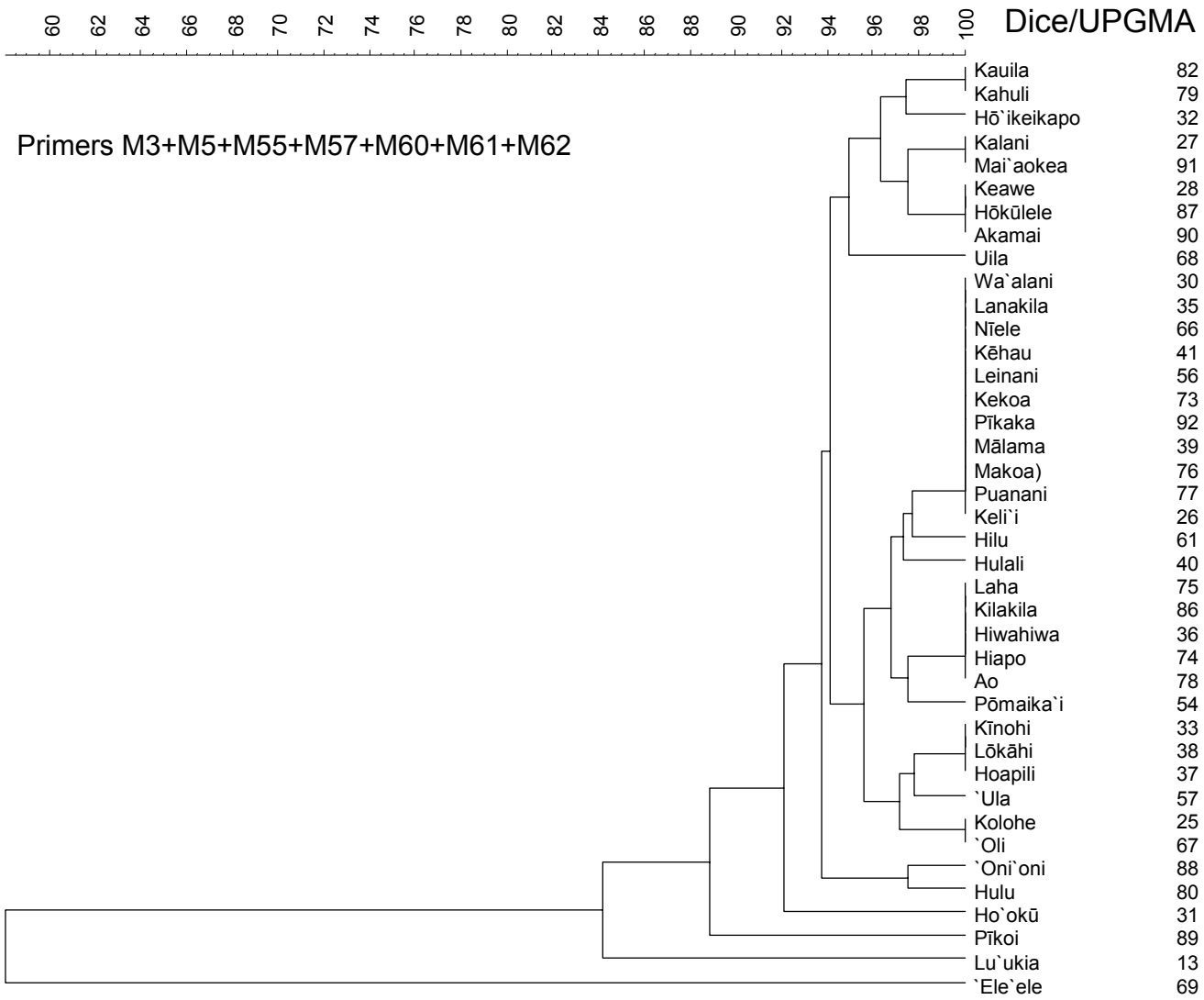


Figure 6. Relationships of 41 individuals (`Umi included) based on six primer sets are depicted in a UPGMA dendrogram using distance estimates (Dice) calculated in the program Bionumerics. Birds originating from McCandless Ranch are designated by boxes. All monomorphic and polymorphic bands (n=255) are included. Studbook number follows each name.

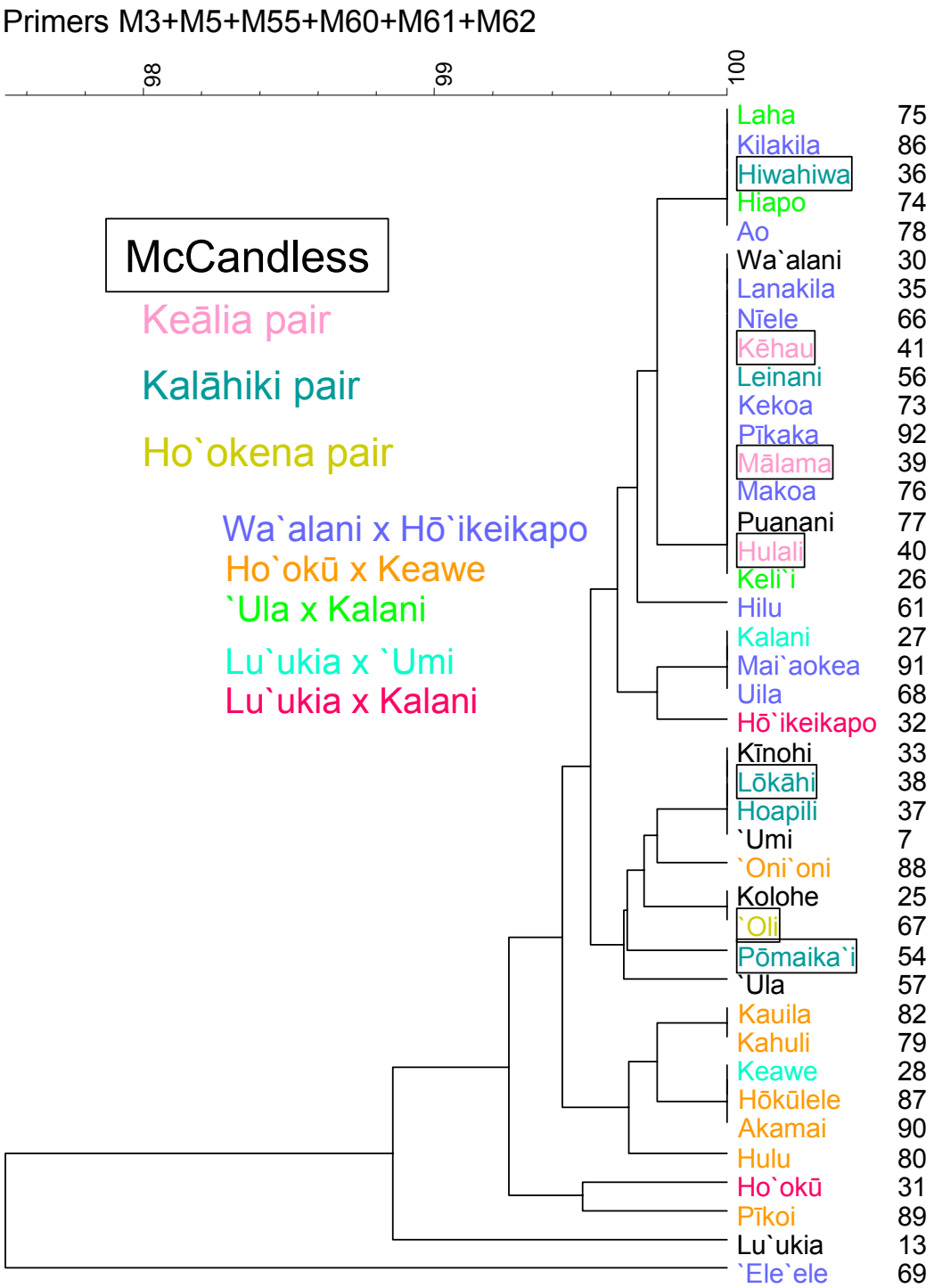
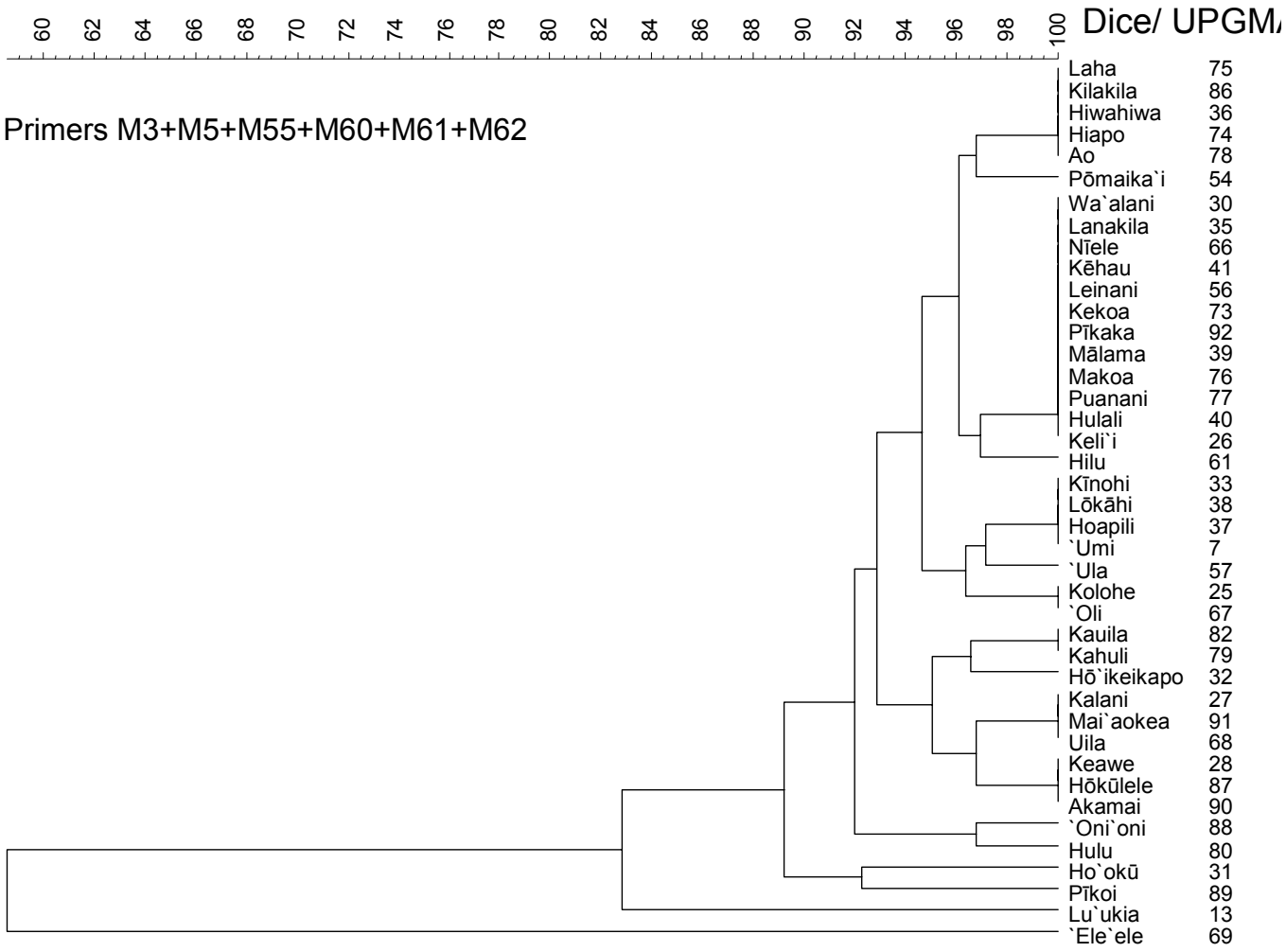


Figure 7. Relationships of 41 individuals (`Umi included) based on six primer sets are depicted in a UPGMA dendrogram using distance estimates (Dice) calculated in the program Bionumerics. Only polymorphic bands (n=25) are included in calculating distance estimates. Studbook number follows each name.



The similarity values among mating pairs are summarized in Table 7. Genetic distances between `Umi and Lu`ukia and Kalani and Lu`ukia appear greater then others with lower similarity values of 82.76 and 83.33, respectively. Among founders (data not shown) based on only polymorphic bands `Umi appears more similar to Wa`alani with a similarity value 96.97 than to mate Lu`ukia (similarity value 82.76). Wa`alani and Lu`ukia have a similarity value of 85.71.

Table 7. Summary of similarity values among mating pairs. All bands/polymorphic bands only. All based on matrix generated using 7 sets of primers, except `Umi x Lu`ukia which was based on 6 sets (excluding M57). Studbook numbers follow each name.

	Hō`ikeikapo (32)	Keawe (28)	Kalani (27)	`Umi (7)
Wa`alani (30)	99.58/95.00			
Ho`okū (31)		99.37/92.31		
`Ula (57)			99.37/93.02	
Lu`ukia (13)			98.73/83.33	98.79/82.76

When comparing results from the 6 (20 polymorphic bands) *versus* 7 sets (25 polymorphic bands), the inclusion of primer set M57 provides 25% overall increased resolution. While much valuable information was obtained by these analyses, further additional analyses are needed to: (1) resolve discrepancies found by AFLP and microsatellite data and further clarify the pedigree, (2) repeat AFLP analyses to include all individuals available (including 2002-present), and (3) include at least 12 sets of primers to further resolve genetic distinctions among individuals. Based on numbers of polymorphic bands observed in other (non-inbred) species, we would estimate that 9-12 sets of primers should be included for a full analysis of this population.

Summary

AFLP (amplified fragment length polymorphism) is a universal technique for DNA fingerprinting. This study was undertaken to evaluate the level of genetic diversity in the captive population of `Alalā by AFLP, to compare genetic data with currently available pedigree information, and to determine the extent of relatedness of mating pairs and among founding individuals. A total of seven primer sets and 41 individuals were used for final analyses. Bands produced by these 7 sets of primers are designated as either present (1) or absent (0). We evaluated a total of 255 loci for polymorphism and of these 9.8% (25) were polymorphic. Nineteen (19) distinct banding patterns were distinguished among these 41 individuals with Pattern 1 being the predominant pattern. This pattern includes individuals from several families and lineages. Evaluation of family relationships can be addressed using AFLP data when both parents are homozygous for the absence of alleles (0/0) and offspring show a presence (+/+ or +/0). This phenomenon occurred in three instances, however, parallel microsatellite data neither validate nor refute these observations. Heterozygosity values (H_0) were estimated based on the 25 polymorphic loci. Included are average H_0 using only the loci with 4 or more (-/-) individuals, and the Bayesian estimate generated in the program Hickory (v.1.0.3). Our H_0 estimate range of 0.229-0.265 based on 25 polymorphic loci is in the range of estimates provided by microsatellite data

from 5 loci (0.248) (excluding the MJG1 locus) as reported by Fleischer (2003). It is much lower than the average H_0 estimate provided from 6 microsatellite loci (0.319) when the MJG1 locus is included. The value of the use of additional primer sets is evident when comparing results using 6 (20 polymorphic bands) vs 7 sets (25 polymorphic bands). Inclusion of one additional primer set provides 25% overall increased resolution.

With all 7 sets of primers (Figure 3), two main clusters include 36 of the 40 (excluding `Umi) birds analyzed, with all of the McCandless Ranch birds contained in one cluster. While overall diversity is quite low, it is recommended that when the option for selecting breeding pairs exists, managers should avoid selecting individuals with similar or identical banding patterns. No data are currently available on more recently hatched captive birds. While valuable information was obtained by these analyses, additional analyses are needed to: (1) resolve discrepancies found by AFLP and microsatellite data and further clarify the pedigree, (2) repeat AFLP analyses to attempt to include all individuals currently available, and (3) include additional sets of primers to further resolve genetic distinctions among individuals. Based on numbers of polymorphic bands observed in this species compared with other (non-inbred) species, at least 9-12 sets of primers should be included for a full analysis of this captive population.

Acknowledgments

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Appendix

Details of AFLP procedures

Reference Codes for *Eco* x *Mse* primer combinations

Primer Set	*Eco*	*Mse*
M3	ER1.P2 (ATA)	*Mse*.P45 (ATG)
M5	ER1.P4 (ACA)	*Mse*.P35 (ACA)
M55	ER1.P59 (CTA)	*Mse*.P51 (CCA)
M57	ER1.P59 (CTA)	*Mse*.P56 (CGC)
M60	ER1.P55 (CGA)	*Mse*.P56 (CGC)
M61	ER1.P49 (CAG)	*Mse*.P51 (CCA)
M62	ER1.P49 (CAG)	*Mse*.P53 (CCG)

STEP #1 – Band Classes and Scoring

Scoring procedure for each primer set is as follows (after all gel input and normalizing is complete).

1. Scan gel, observe both data lanes and ROX500 lanes to determine any problems with lanes, such as poor loading or poor reaction.
2. Using previously determined bands from primer screen gel, make an Excel spreadsheet to note which bands will be used or not, the range of bp (to 1 decimal pt) for each class, and if not used, why (i.e., shoulder, abnormal). Note the number of bands in original list from primer screen gel.
3. Score each gel for that primer set as described next, making any necessary changes to band classes used. It is most consistent to do the entire gel at one time, but within the gel one band at a time for all individuals on a gel will suffice.
4. Open comparison window with all individuals. Select the primer set and have the bands show. Stretch out the view area and turn off band class and turn it back on. Use same settings always used for band classes (optimization 1%, position tolerance 0.16 %, others default 0). Proof each band class against Excel sheet from Step 1. Note # bands and # polymorphic bands.
5. Compare reruns of same individual (4 repeats, the gel reruns and 2 samples from extraction rerun) using band class table (green squares). Make notes in column 3 of Excel sheet as to which samples have inconsistencies for which band. (Note that repeats are labeled as 6933.13 for 6933 rpt 13). Re-examine each sample with inconsistencies. Highlight cell in column 3 pink if deciding not to use and make changes to gel and band class in comparison window. Decide which bands not to use based on comparing bands in lanes of reruns. Note new total # bands and # polymorphic bands.

6. In comparison window, compare bands across family groups. Note which bands are not inherited
7. After all primer sets are scored for the first 4 Steps, remove individuals that should be rerun and recount the # of bands.

Primer set #1 – M3

Bad lanes:
Gel A10_03 (A10.01-A10.42):
Gel A10_04: On full exposure there is pull up on all samples, appears to be overflow from neighboring lanes from loading, throughout gel.
Quality of reaction: A10.09 is a bit light but usable
In preamp: The following lanes had very light reactions: A10.11, A10.12, A10.15, A10.16, A10.34, A10.40, and A10.41
Gel A15_03 (A15.15-A15.21):
Gel A15_04: unknown bands until after 250, OK
Quality of reaction: A15.18 is blank, unusable
In preamp: the following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_03 (A16.21-A16.30):
Gel A16_04: Lanes A16.22 and A16.24 have some pull up after 250, but light
Quality of reaction: All look good
In preamp: The following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_03 (A17.25-A17.36):
Gel A17_04: A17.29+35 have pull-up at 185bp and A17.32+34 at 175bp and A17.29 at 235bp
Quality of reaction: A17.26 looks questionable
In preamp: A17.13-A17.15, A17.21-A17.23 all had light reactions, with A17.14, A17.15, and A17.23 being the worst.
bands: 36
polymorphic bands: 19

Primer set #2 – M5

Bad lanes:
Gel A10_02 (A10.01-A10.42):
Gel A10_04: On full exposure there is pull up on all samples, appears to be overflow from neighboring lanes from loading, throughout gel.
Quality of reaction: A10.09 is a bit light but usable
In preamp: The following lanes had very light reactions: A10.11, A10.12, A10.15, A10.16, A10.34, A10.40, and A10.41
Gel A15_02 (A15.15-A15.21):
Gel A15_04: Unknown bands until after 250, OK
Quality of reaction: A15.18 is blank, unusable
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_02 (A16.21-A16.30):

Gel A16_04: Lanes A16.22 and A16.24 have some pull-up after 250, but light
Quality of reaction: All look good
In preamp: The following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_02 (A17.25-A17.36):
Gel A17_04: A17.29+35 have pull-up at 185bp and A17.32+34 at 175bp and A17.29 at 235bp
Quality of reaction: A17.26 looks questionable
In preamp: A17.13-A17.15, A17.21-A17.23 all had light reactions, with A17.14, A17.15, and A17.23 being the worst.
bands: 36
polymorphic bands: 19

Primer set #3 – M55

Bad lanes:
Gel A13_03 (A13.01-A13.42):
Gel A13_04: on full exposure there is pull-up on many at 62, 164, 315, 410 and a few pull-ups for a single sample, but they are all faint and not patterned to be an overflow from a neighboring lane.
Quality of reaction: A13.27 is blank, A13.33 is faint, A13.37 gets faint after 100bp, A13.35 and A13.36 are present and look strong but missing a few of the more monomorphic bands.
In preamp: The following lanes had very light reactions: A13.27+A13.28, A13.35-A13.38.
Gel A15_03 (A15.01-A15.07):
Gel A15_04: On full exposure there is likely overflow on lanes A15.02 (*i.e.*, approx. 155bp, 165, 175, 220, 265, 315, etc) , A15.04 (ie. 220 and 265, etc), and A15.06 (not until after 450bp).
Quality of reaction: All look good.
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_03 (A16.01-A16.10):
Gel A16_04: On full exposure there is much pull up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: many at 165, and a large streak on lanes A16.05 and A16.10 at about 58-72bp.
Quality of reaction: A16.03 is missing few of the monomorphic bands even though other bands are very dark. A16.08 seems faint. When rescaled A16.09 is missing many bands.
In preamp: The following lane had a very light reaction: A16.09, A16.19, A16.29.
Gel A17_03 (A17.01-A17.12):
Gel A17_04: On full exposure there is much pull up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: few at 68, A17.09 has a few dark bands at around 85,
Quality of reaction: A17.02 +A17.11 is missing a few of the monomorphic bands even though other bands are very dark.
In preamp: A17.01-A17.03, A17.09-A17.11 all had light reactions, with A17.02, A17.03 and A17.11 being the worst.
Note: Didn't call all missing bands for 7151.16 and 7151.17 because both were bad runs and were missing lots of bands.
bands: 40
polymorphic bands: 38

Primer set #4 – M61

Bad lanes:
Gel A13_01 (A13.01-A13.42):
Gel A13_04: On full exposure there is pull up on many at 62, 164, 315, 410 and a few pull-ups for a single sample, but they are all faint and not patterned to be an overflow from a neighboring lane.
Quality of reaction: A13.27 is very light (almost absent) but when rescaled can see peaks, A13.33 is quite faint and A13.35 a bit faint.
In preamp: The following lanes had very light reactions: A13.27+A13.28, A13.35-A13.38.
Gel A15_01 (A15.01-A15.07):
Gel A15_04: On full exposure there is likely overflow on lanes A15.02 (*i.e,*. approx. 155bp, 165, 175, 220, 265, 315, etc) , A15.04 (*i.e*., 220 and 265, etc) and A15.06 (not until after 450bp).
Quality of reaction: A15.07 very faint (almost absent) but rescaled can see some peaks – not really usable, all others look good, though A15.01+A15.03 is bit lighter but not really faint.
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_01 (A16.01-A16.10):
Gel A16_04: On full exposure there is much pull-up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: many at 165, and a large streak on lanes A16.05 and A16.10 at about 58-72bp.
Quality of reaction: A16.08 a bit light but might be OK, A16.06 light but OK.
In preamp: The following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_01 (A17.01-A17.12):
Gel A17_04: On full exposure there is much pull-up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: few at 68, A17.09 has a few dark bands at around 85,
Quality of reaction: A17.02 +A17.11 are missing a few of the monomorphic bands even though other bands are very dark.
In preamp: A17.01-A17.03, A17.09-A17.11 all had light reactions, with A17.02, A17.03, and A17.11 being the worst.
bands used on primer screen gels: 59
bands: 54
polymorphic bands: 46

Primer set #5 – M57

Bad lanes:
Gel A14_03 (A14.01-A14.42):
Gel A14_04: On full exposure there is pull-up on many at 185, 225, 285, 330, 335, 390, 430, and 480, likely overflow from neighboring lanes, but it is faint. Also a few larger pull-ups for a single sample, but they are all faint and not patterned to be an overflow from a neighboring lane.
Quality of reaction: A14.27 is blank but when rescaled can see peaks (don't use), A14.02, A14.10 are of a little lighter intensity than others, but OK to use, A14.28+A14.35+A14.36+A14.40 are missing some monomorphic bands.

In preamp: The following lanes had very light reactions: A14.27+A14.28, A14.35-A14.38.
Gel A15_03 (A15.08-A15.14):
Gel A15_04: On full exposure there is likely overflow on lanes A15.14 (*i.e.*, approx. 265, 315, etc), A15.04 (*i.e.*, 220 and 265, etc).
Quality of reaction: A15.09 very faint (almost absent) but rescaled can see some peaks – not really usable, all others look good.
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_03 (A16.11-A16.20):
Gel A16_04: On full exposure there is little pull up, and it tends to be scattered
Quality of reaction: At around 180 bp, lane A16.19 fades out, but top looks great.
In preamp: the following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_03 (A17.13-A17.24):
Gel A17_04: On full exposure there is much pull up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: large patch between 50-60bp for A17.21-A17.23, and A17.14 has a few at 90 and 125-135bp.
Quality of reaction: A17.14 +A17.23 are missing a few of the monomorphic bands even though other bands are v. dark.
In preamp: A17.13-A17.15, A17.21-A17.23 all had light reactions, with A17.14, A17.15, and A17.23 being the worst.
bands used on primer screen gels: 42
bands: 44
polymorphic bands: 44

Primer set #6 – M60

Bad lanes:
Gel A14_02 (A14.01-A14.42):
Gel A14_04: On full exposure there is pull-up on many at 185, 225, 285, 330, 335, 390, 430, and 480, likely overflow from neighboring lanes, but it is faint. Also a few larger pull-ups for a single sample, but they are all faint and not patterned to be an overflow from a neighboring lane.
Quality of reaction: A14.27 is blank but when rescaled can see peaks (don't use), A14.36 (might not use)+A14.40 are missing many bands, A14.28+A14.35 are missing some monomorphic bands.
In preamp: the following lanes had very light reactions: A14.27+A14.28, A14.35-A14.38.
Gel A15_02 (A15.08-A15.14):
Gel A15_04: On full exposure there is likely overflow on lanes A15.14 (*i.e,.* approx. 265, 315, etc) , A15.04 (*i.e,.* 220 and 265, etc).
Quality of reaction: A15.13 has high background, all others look good.
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_02 (A16.11-A16.20):
Gel A16_04: On full exposure there is little pull-up, and it tends to be scattered
Quality of reaction: All look OK, but A16.19 is missing few bands, might be OK
In preamp: The following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_02 (A17.13-A17.24):

Gel A17_04: On full exposure there is much pull up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: large patch between 50-60bp for A17.21-A17.23, and A17.14 has a few at 90 and 125-135bp.
Quality of reaction: A17.23 is missing a few of the monomorphic bands even though other bands are very dark, probably OK, A17.15 missing lots of bands, might not want to use
In preamp: A17.13-A17.15, A17.21-A17.23 all had light reactions, with A17.14, A17.15, and A17.23 being the worst.
bands used on primer screen gels: 23
bands: 28
polymorphic bands: 28

Primer set #7 – M62

Bad lanes:
Gel A14_01 (A14.01-A14.42):
Gel A14_04: On full exposure there is pull-up on many at 185, 225, 285, 330, 335, 390, 430, and 480, likely overflow from neighboring lanes, but it is faint. Also a few larger pull-ups for a single sample, but they are all faint and not patterned to be a overflow from neighboring lane.
Quality of reaction: A14.27 is blank but when rescaled can see peaks (don't use)
In preamp: The following lanes had very light reactions: A14.27+A14.28, A14.35-A14.38.
Gel A15_01 (A15.08-A15.14):
Gel A15_04: On full exposure there is likely overflow on lanes A15.14 (i.e. approx. 265, 315, etc), A15.04 (i.e. 220 and 265, etc).
Quality of reaction: all good.
In preamp: The following lanes had very light reactions: A15.06, A15.13, A15.20.
Gel A16_01 (A16.11-A16.20):
Gel A16_04: On full exposure there is little pull up, and it tends to be scattered
Quality of reaction: All look OK
In preamp: The following lane had very light reaction: A16.09, A16.19, A16.29.
Gel A17_01 (A17.13-A17.24):
Gel A17_04: On full exposure there is much pull up, but they are not patterned to be an overflow from neighboring lanes, rather they might be a large peak in a different dye: large patch between 50-60bp for A17.21-A17.23, and A17.14 has a few at 90 and 125-135bp.
Quality of reaction: OK
In preamp: A17.13-A17.15, A17.21-A17.23 all had light reactions, with A17.14, A17.15, and A17.23 being the worst.
bands used on primer screen gels: 19
bands: 19
polymorphic bands: 19

STEP #2 – Removal of poor runs for analysis

5 August 2003 - Using Comparison "2003working"

1. Started by excluding primer set M59 because it was poor across all samples, with many ambiguous bands
2. Remove junk repeats -
   - 6933 – across all primer sets, 6933.17 is not usable, decided to exclude from our repeat list, and removed from comparison window
   - 6934 – across all primer sets, 6934.17 is not usable, decided to exclude from our repeat list, and removed from comparison window
   - 6935 – across all primer sets, 6935.17 is not usable, decided to exclude from our repeat list, and removed from comparison window
   - 7151 - across all primer sets, 7151.16 and 7151.17 are not usable, decided to exclude from our repeat list, and removed from comparison window, also 6941.17 (run 2) which is a second extraction of 7151 had a poor run and excluded it as well
   - 7163 – kept all 4 repeats for now, but 7163.15 is missing run for M61
   - 7153 and 9938 are 2 extractions of Hiapo. 7153 had poor preamp and missing lots of bands, while 9938 good preamp, decided to remove 7153 from comparison
   - 7160 ran 2x with same DNA extraction. Run 3 of 3 was poor so it was removed leaving 2 runs. 7159 – 2 runs with same DNA, neither good, removed both from analysis.
   - 7164 and 7165 - Removed from comparison because so many missing bands indicate bad run.
   - Once poor runs were removed, we examined each primer set looking for missing band discrepancies.

STEP #3 – Final decision of confusing bands – to keep or not use

Primer set M3

Band	Lane	Decision
63.8	A16.21 v 22	21absent, 22present
	A15.21 v other	Makoa, 15.21 absent but big nearby peak, masking?
75.8	A17.30	called present, shoulder but clear band in repeats
75.8	A10.18	no repeats, but shoulder, so called present
75.8	A15.19	called present, shoulder, but clear band in repeats
	A16.25 v 17.31	clearly missing in 16.25, and present in 17.31
84.4	A15.21	missing, probably masked by shoulder of previous band 91.9 miscellaneous bad band don't use
118.5	A15.20	missing but present in other repeats
118.5	A16.21 v 22	clearly absent in both
138.2	A10.13 present v 17.30 absent but possibly masked by big shoulder following	
144.2	A16.21 v 22	21 present, 22 absent

Primer set M5

Band	Lane	Decision
59.3	misc.	no discrepancies between repeats, clear in others
88.8	A10.02	called present because large masking peak immediately before it,
94.1	A16.21v22	shoulder in 22, called present
101.8	A17.36	absent, could be shoulder, called band present
106.9	A17.31	a bit different in size but present due to repeats
106.9	A15.21	absent even though present in other repeats
114.5	A17.36	shoulder but called present
116.9	A16.21v22	absent in 22, undecided
118.5	A16.21+22	only ones missing, called absent
125.9	A15.21	shoulder called present based on repeats
128.2	misc.	don't use class
130.1	A10.32	shoulder, called present because of repeats
130.1	A16.21	shoulder, called present because of repeat
147.6	A17.31	missing (but called present) *versus* 16.35 present
166.5	A16.21+22	very small but called present
166.5	A17.29	very small but called present based on repeat
185.2	misc.	don't use class
189.0	A10.26	very small but called present due to repeats
205.2	A16.21v22	very small in 21, called present
230.1	A16.21v22	absent in 22, undecided
249.4	A16.21v22	absent in both

Primer set M55

Band	Lane	Decision
72.5	A17.04	present but small, called as present
84.41	A13.37	repeat with presence elsewhere, called this small peak present
	A13.32	repeat with presence elsewhere, called this small peak present
99.77	A16.02	repeat with presence elsewhere, called this small peak present
110.67	A13.37	repeat with presence elsewhere, called this small peak present
115.0	A13.40	no peak present, but questionable run with no repeat
121.6	A16.07	repeat with presence elsewhere, called this small peak present
129.8	A13.41	peak present, but size shift, called present
131.41	A16.01	repeat with presence elsewhere, called this small peak present
131.40	A13.40	no peak present, but questionable run with no repeat
187.18	A16.01	repeat with presence elsewhere, bad run, did not call peak, probably just use A16.02 b/c better run for this primer set also decided not to use the following band classes due to shoulders 77.9, 91.5, 105.9, 125

Primer set M57

Band	Lane	Decision
56.2	misc.	shoulder, might use for pedigree, remove band class now
58.1	misc.	using for now
79.9	A14.22	only one not present, called absent
89.65	A16.01	one of Lu`ukia runs, called present because definite in other run

92.37	misc.	part doublet below, can't call and be sure, don't use
118.66	A17.24	repeat with presence elsewhere, called this small peak present
144.9	A14.40	`Ele`ele, questionable run, but not present
194.5	A14.40	`Ele`ele, questionable run, but not present
225.7	A14.33	does not appear present
		also decided not to use the following band classes due to shoulders
		56.2, 76.1, 92.4, 102.2, 110.0, 110.7, 111.3, 170.0, 171.1, 227.7
		for pedigree comparisons, might want to check these:
		56.2, 76.2

Primer set M60

Band	**Lane**	**Decision**
61.7	A14.33	shoulder, don't use
	A16.15 v A17.19	faint but present, call present
90.5	A14.22	strong run, but no peak present, call absent
100.7	A14.39	small, called present
103	misc	called present even if small, different from 102.7
104.4	misc.	shoulder, took out
105	misc.	kept, might be problem though
113	A14.40	small, called present
	A14.37	likely leakover, don't use
	A16.11vA16.12	likely leakover, don't use
185.5	A16.18v14.03v15.12	likely leakover, don't use
306	misc.	good polymorphism, consistent across repeats
307	misc.	good polymorphism, consistent across repeats
313	A14.40	definitely not there
		also decided not to use the following band classes due to shoulders
		61.7, 91.7, 159.5
		for pedigree comparisons, might want to check these:
		185.5

Primer set M61

Band	**Lane**	**Decision**
57.3	A13.40	not present
57.3	A16.05vA17.07	not present in A16.05 left uncalled, but present A17.07
	present in A13.09+13.26, not in 15.06, left uncalled	
61.8	A16.01v02	not in 02, left uncalled
65.4	misc.	shoulder but recalled and used for all
77.1	misc.	shoulder but recalled and used for all
81.4	A13.16	only lane with good polymorphism
84.6	misc.	small, but using, call present
85.7	misc.	shoulder, but recalled and used for all
86.4	misc.	shoulder, but recalled and used for all
99.3	misc.	shoulder, but recalled and used for all, except A13.40
100.8	A13.40	small but called present
102.6	misc.	shoulder, but recalled and used for all
105.9	A13.33	very small but called present

111.8	A13.40	small but called present
114.7	A15.05 has but 13.03+16.08 don't use	
127.7	A13.32 has but 13.13+17.06 don't use	
139.3	A13.40	small but called present
145.4	A16.01v.02	small in .02 but called present
145.4	A13.33	small but called present
145.4	A13.40	small but called present
153.1	A16.01v.02	present in .02, shoulder in .01 called present in both
153.1	A16.04	small and shoulder but called present
157.5	A16.01v02	not in 02, left uncalled
158.8	A16.04	small, but called present
183.1	A13.28	can be small, but is absent in this lane, didn't call

A16.05 v A17.07 present in 17.07, but not in 16.05, left 16.05 not called
also decided not to use the following band classes due to shoulders
114.7+127.7+200.4+315.0 (not shoulder but mixed presence in repeats)
for pedigree comparisons, might want to check these:

Primer set M62

Band	**Lane**	**Decision**
61.4	A14.40	clearly missing, didn't call
69.0	A16.14	clearly present, kept band
88.6	misc.	removed, not consistent, very small
107.5	A16.15 no band v A17.19 big band ????	
107.5	A16.11v12	called small band in 12 present
124.3	mult samples missing including Kauila repeats, keep as absent polymorphism	
146.2	A14.40	clearly present, kept called

gel A15# band very small, but almost all samples repeats, so called present even though extremely small

313.0	A14.40	not present
323	A14.34 vs other repeats, is vsmall called present	

also decided not to use the following band classes due to shoulders:
88.6 (very small)
for pedigree comparisons, might want to check these: N/A

STEP #4 – Consensus patterns for individuals with repeats

(Reduced total # in comparison from 58 to 41 individuals)
Kauila – kept 6941, removed 7151 samples
7151.13 – M57(1)
7151.15 – M3(1) M61(1)
6941 – none, kept
Keawe – all 3 identical except 6934.15
6934.13 - keep
6934.15 – M3 missing all

6934.16 – none
Kēhau – kept 6945
6945 – keep
7157 – none
9940 – M3(1) M57(1) M60(1)
Kolohe – all 3 identical, kept 6933.13 (removed 6933.15 and 6933.16)
Lōkāhi – kept 6944
6944 – kept
7171 – M57(1)
Makoa – kept 7163.13
7163.13 kept
7163.15 – M3(2) M5(1) M60(1) (missing M61)
7163.16 – none
7163.17 – M57(1) M60(1)
Wa`alani – all 3 same, kept 6935.13 (removed 6935.15 and 6935.16)
Ao – called present the M5 and M60 band in 9941 and then removed 7168
9941 – M5(147bp) M60(103bp)
7168 – M3(1) M61(2) M62(1)
Lu`ukia – called present 3 missing bands in run 1 and then remove run 2
Run 1 – M3(63.8) M55(187.1) M57(89)
Run 2 – M3(1) M5(1) M60(1) M61(2)
`Oli – use 9939 after calling the M60 band present
9939 – M60(105)
6942 – M57(1)

AFLP TIMELINE DETAILS

September-October 2001 –New digestion ligation of 4 individuals (4086 `Ele`ele, 4110 Kauila, 4116 Noe, 4131 Hiapo) repeated 3 times, using PCl extracted samples. Screened with 6 *Eco*/Taq primer sets (A,B,C,D,E,F). Ran on ABI gels A1# and A2#.

7-8 January 2002 – First run through of 31 samples, starting from new digestion/ligation for each (*Eco*/*Mse*). 25 samples were from Nov 2001 Qiagen column extraction and 7 were from PCl extractions (4 being repeats from above).
A1t-A4# - *Eco*/Taq digestion/ligation not proceeded with.
A1m-A1# - *Eco*/*Mse* was taken through to ABI gels, with 6 primer sets: M5, M9, M3, M6, M7, M1. Ran on ABI gels A3# and A4#.
A1m-A2# - repeats were with 4 PCl samples and not taken past digestion/ligation.
A1m-A3# - repeats were with 4 PCl samples and not taken past digestion/ligation.

27 January 2002 –Run 16 new samples, all are PCl extracted samples.
A1t-A5# - Not taken past digestion/ligation.
A1m-A4# - Not taken past pre-amplification because of poor smear.

4-7 February 2002 –Run same 16 PCl samples. Again discarded and decided to re-extract the samples from blood using Qiagen for similarity to other extracted samples.
A1t-A6# - Not taken past digestion/ligation.

A1m-A5# - Not taken past pre-amplification because of poor smear. Did retry pre-amplification with differing volumes of digestion/ligation to see if amount of d/l affected preamp, not successful.

12 February 2002 –Ran second set of Qiagen extracted and associated repeats. A1t-A7# and A1t-A8# - Not taken past digestion/ligation.A1m-A6# and A1m-A7# - *Eco/Mse* was taken through to ABI gels, with 6 primer sets: M5, M9, M3, M6, M7, M1. Ran on ABI gels A5# and A6#

4 April 2002 –Wanted to test effect of primer concentration at pre-amplification stage and looked at repeatability between 2 repeats of each individual. Tested range of final concentrations from 0.2 to 5 uM. Run on ABI gel A7#. Decided to go with 1 µM concentration (i.e. 1 µl of 20 µM stock) as compared to 5 µM for each as used prior to this.

? Month 2002 –Reran 26 (+3 repeats) and did pre-amplification with them, but not any further.

6 September 2002 –Tested a 4 individuals using both HPLC and non-HPLC purified pre-A primers to see if necessary. Run on ABI gel A8#. No major difference seen to justify more expensive purification.

31 October 2002 –Reran all 45 samples + 4 new samples + 5 repeats.
A1m-A12# - Some didn't pre-amp very well so did varied amounts of d/l template going into pre-amp. Did 3 primers: M5, M6, M9. Ran on ABI gels A10# (sent over A9#-A11# to test dilution for gel loading). On 25 April 2003, did 6 new selective amp primers.
A1m-A13# - Didn't go past pre-amplification, until 22 April 2003.

7 November 2002 –Run 8 more samples with 5 repeats. Not taken into pre-amplification, only digestion/ligation done, until 22 April 2003.

4 March 2003 – Tested 36 new (-C/-A or –C/-C) primer on samples 6943 Keawe, 6935 Wa`alani, 6942 `Oli, 6946 Pōmaika`i, and 6950 Laha. Ran on ABI gel A12#. Picked out 6 to work with: NED- M55 and M57, FAM- M59 and M60, HEX- M61 and M62. All can be run with a pre-amplification of –C/-C.

31 March 2003 – (A1m-A15#) Run 4 old samples that have had problem with preA and 4 new extractions of old samples that have been very different from other samples in past, possibly due to poor extractions (low concentration).

14 April 2003 – (A2m1-A15# and A2m2-A15#). Pre-amplification was unsuccessful. Repeated with same set up the next day.

15 April 2003 – (A2m1-A15b# and A2m2-A15b#). Pre-amplification was unsuccessful. Repeated one more time on 21 April 2003.

16 April 2003 – (A1m-A16#) Repeat of A15# samples. Possibly contaminated some of the samples during ligation set up. Will carry through samples to use to test pre-amplification and then discard these.

21 April 2003 –Even with all new reagents, pre-amplification was still unsuccessful. In this reaction, also ran the A1m-A16# samples to see if might be bad D/L rather than preA. Because A16# worked and not A15#, likely the problem was with the digestion/ligation. So need to start these samples over (can't use A16# because possible contamination).

22 April 2003 –Repeat of A15# samples.

22 April 2003 –Preamp of good reactions from past to prepare for 6 new selective amps.

24 April 2003 –Pre-amplification still didn't work. Redo digestion/ligation with all new reagents.

25 April 2003 –6 new primer sets run on A12#. Run on ABI gels A13# and A14#.

28 April–1 May 2003 –9 primer sets run on A13#. Run on ABI gel A15#.

29 April–1 May 2003 –#). 9 primer sets run on A14#. Run on ABI gel A16#.

29 April 2003 –Repeat of A15# samples with new reagents.
30 April 2003 –Pre-amp finally worked.
30 April–1 May 2003 –9 primer sets run on A18#. Run on ABI gel.