Genetic variability within and identification markers for Hawaiian kalo varieties (*Colocasia esculenta* (L.) Schott - Araceae) using ISSR-PCR.

Hawaii Biological Survey

Final Report

September 2012

Cover image:
Taro leaf
Photographer: Shelley A. James

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Introduction

Taro (*Colocasia esculenta* (L.) Schott - Araceae) is a major staple in the diets of people around the Pacific, including Hawai‘i. Taro (*kalo* in Hawaiian language) was thought to have been introduced to Hawai‘i by Polynesian immigrants (Handy and Handy 1972). While only a few cultivars are though to have been brought initially, about 300 have been documented in Hawai‘i at one time as a result of subsequent crossing, selection, and propagation (MacCaughey and Emerson 1914), but experimental investigations in 1939 documented only 84 unique, extant varieties (Whitney *et al.* 1939). Subsequently, other varieties of taro have been introduced to the Hawaiian Islands, and the breeding of new varieties for commercial crops has taken place (Cho *et al*. 2007).

Studies have used different molecular techniques, such as isozyme, DNA hybridisation, RAPDs (Randomly Amplified Polymorphic DNAs) analyses, and microsatellites, to determine genetic diversity of Hawaiian, Pacific and Asian taro varieties (Irwin *et al.* 1998; Lebot and Aradhya 1991; Matthews and Terauchi 1994; Mace and Godwin 2002). Isozyme analyses conducted by Lebot and Aradhya (1991) found no variation between 343 Hawaiian accessions, despite distinct morphological differences. RAPDs analysis of taro varieties by Irwin *et al.* (1998) divided 23 Hawaiian accessions into three major groups, with the majority of Hawaiian accessions being closely related with about 80% similarity. These included the commercially important varieties. However, while RAPDs is an inexpensive method for determining genetic diversity, the technique suffers from repeatability issues (see Semagn *et al*. 2006 for review), and the analysis of newly available kalo varieties can be fraught with difficulties. Recent work (USDA 2012) evaluated the microsatellite markers developed by Mace and Godwin (2002) that can differentiate taro varieties collected from different continents. Microsatellites are very short pieces of DNA that are expressed as different variants within populations and among different species, characterized by nucleotide units, e.g., AA, AG, or CAG, that are repeated 4-10 times side-by-side. Using microsatellites, Palauan varieties were

distinguishable from Hawaiian varieties, and unique patterns were found for 11 Hawaiian cultivars, but the other 49 were too closely related to individually resolve, or resolve into groups, using this molecular technique.

This pilot study aims to identify molecular identification markers and determine the genetic diversity of extant varieties of taro found in collections in the Hawaiian Islands using a marker system called Inter-Simple Sequence Repeats (ISSRs) (Bornet and Branchard 2001). ISSR markers are based on single-primer polymerase chain reactions (PCR) where the primer sequence is derived from di- and trinucleotide (microsatellite) repeats (Wolfe *et al.* 1998). This is a RAPDs-like approach that assesses variation in the numerous microsatellite regions dispersed throughout the genome, and eliminates the requirement of characterizing individual loci needed for other molecular approaches, such as microsatellites and DNA sequencing. ISSR primers used to analyze variation in a given DNA sample consist of a variable microsatellite sequence and an arbitrary pair of bases at one end. Where the primer successfully locates two microsatellite regions within an amplifiable distance on the DNA strands of the sample, PCR will generate a band of a particular size for that locus and individual sample. Because several to many such paired microsatellite areas exist in any particular DNA sample, many representative bands, or markers, are generated for that sample, allowing the genetic diversity to be evaluated.

Methodology

85 taro (*kalo* - *Colocasia esculenta* (L.) Schott) varieties or cultivars were sampled from five living collections housed in the State of Hawai‘i (Amy Greenwell Ethnobotanical Gardens, Captain Cook, Hawai‘i; Maui Nui Botanic Gardens, Kahului, Maui; Waimea Valley Botanical Gardens, O‘ahu; and private collections of Penny Levin, Kahului, Maui and Anthony Deluze, Pearlridge, O‘ahu) between 2004-2005 and 2012. The Japanese variety *Zuiki* (PCMB 410), which is the species *Colocasia gigantea* (Blume) Hookf., was also included to evaluate the extent to which the ISSR technique can identify different *Colocasia* species. Pieces of immature leaves were harvested and rapidly dehydrated by immersing in silica gel. Genomic DNA was extracted from 6-10 mg dried plant material using a DNeasy Plant Mini Kit (QIAGEN Inc.) following the recommended protocol.

Tissues and extracted genomic DNA were accessioned within the collections of the Pacific Center for Molecular Biodiversity, Bishop Museum (Appendix 1), and tissues and extracted DNA were stored at -80C for long-term preservation.

Fifteen ISSR primers (Table 1) were tested against seven randomly chosen taro varieties from different varietal groups (*Mana keokeo*, *Piko uliuli*, *Ulaula moana*, *Papapueo*, *Lehua palaii*, *Lauloa eleele ula*, and *Kumu eleele*). A control (negative) sample was run for each primer to confirm lack of contamination. From these, six polymorphic primers (814, 844A, 17898B, 17899A, HB11, HB12) were chosen for use in final analyses.

Table 1: ISSR primers tested; primers used in final analysis are indicated in bold.

Primer name	Primer sequence	Polymorphic?
814	$(CT)_8TG$	**Y**
844A	$(CT)_8AC$	**Y**
844B	$(CT)_8GC$	N
17898A	$(CA)_6AC$	N
17898B	$(CA)_6GT$	**Y**
17899A	$(CA)_6AG$	**Y**
17899B	$(CA)_6GG$	N
HB8	$(GA)_6GG$	N
HB10	$(GA)_6CC$	Y
HB9	$(GT)_6GG$	N
HB11	$(GT)_6CC$	**Y**
HB12	$(CAC)_3GC$	**Y**
HB13	$(GAG)_3GC$	N
HB14	$(CTC)_3GC$	N
HB15	$(GTG)_3GC$	N

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

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 - see Appendix 2g). Bands were scored as present (=1) or absent (=0). Twelve of the specimens failed to amplify successfully with at least one primer during this study, and were removed from further analyses (Appendix 1). The taro variety *Zuiki* (PCMB 410) was also significantly different in banding patterns, and was also removed from further analysis.

ISSR markers are inherited in a dominant or co-dominant Mendelian fashion, and are interpreted as dominant markers, scored as diallelic with 'band present' or 'band absent' (Wolfe *et al.* 1998). Principal co-ordinates analysis and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was undertaken using the software MVSP 3.2.1 (Kovach Computing Services) using the Gower General Similarity Coefficient (Gower 1966).

Results

Similarity between the Hawaiian taro (*Colocasia esculenta*) varieties was high, and ranged from 0.58 (PCMB 373 - *Kai uliuli* & PCMB 6481 - *Apowale*) to 0.989 (PCMB 6480 - *Lehua eleele* & PCMB 6481 - *Apowale*) for the six ISSR primers. The proportion of polymorphic loci for the six ISSR primers was 80.7%. The Japanese variety *Zuiki* (PCMB 410) had a significantly different banding pattern for all primers than the other taro varieties (Appendix 2), expected given that this belongs to the species *Colocasia gigantea*, not *C. esculenta*. Principal co-ordinates analysis (PCO) indicated a high degree of similarity between the Hawaiian taro varieties, with only 12.9% of variation being expressed on the first axis, 10.65% in the second axis, and 6.99% on the third axis (Figure 1). The PCO analysis of ISSR banding patterns did not clearly distinguish the Hawaiian taro varieties into groupings as indicated by the naming system of Whitney *et al*. (1939). Cluster analysis, however, separated the taro varieties into five main groups, with an overall similarity of about 80% (Figure 2).

For both the PCO (Figure 1) and cluster analyses (Figure 2), *Lehua maoli*, "Hanks", KB-85, and *Piialii* were tightly clustered, indicating high genetic similarity of these varieties. Most taro varieties did not exhibit distinctive banding patterns for any of the six ISSR primers that would allow for easy identification. However, *Pololu* (PCMB 285) and *Nawao* (PCMB 387) consistently displayed unique markers for each of the ISSR primers. Two other varieties (*Lauloa eleeleula* - PCMB 289 and *Hinapuaa* - PCMB 288) also showed distinct banding patterns for each of the primers. Similarity between these varietal pairs was also reflected in the cluster analyses (Figure 2). For primer HB12, one distinct unique band of about 1,000 kb was observed for most of the *Manini*, *Lauloa* taro varieties, some of the *Mana* varieties, *Nihopuu*, *Kumu eleele*, *Niue ulaula*, *Pololu*, *Piko ulaula*, *Lenalena*, *Kai kea*, *Akuugawai*, "Hanks", PT *Ula*, and *Aweu* varieties (Appendix 2f).

Mana and *Manini* taro varieties tended to group together within the cluster analysis (Group IV - Figure 2). The two *Elepaio* varieties did not have high genetic similarity, and were separated on the cluster dendrogram. Varieties believed to have been introduced from the 'south seas' (e.g., *Bun-long*, *Niue ulaula*, *Tahitian*, *Akuugawai*) are scattered throughout the cluster analysis with Hawaiian or 'native' varieties (e.g., Whitney *et al*. 1939).

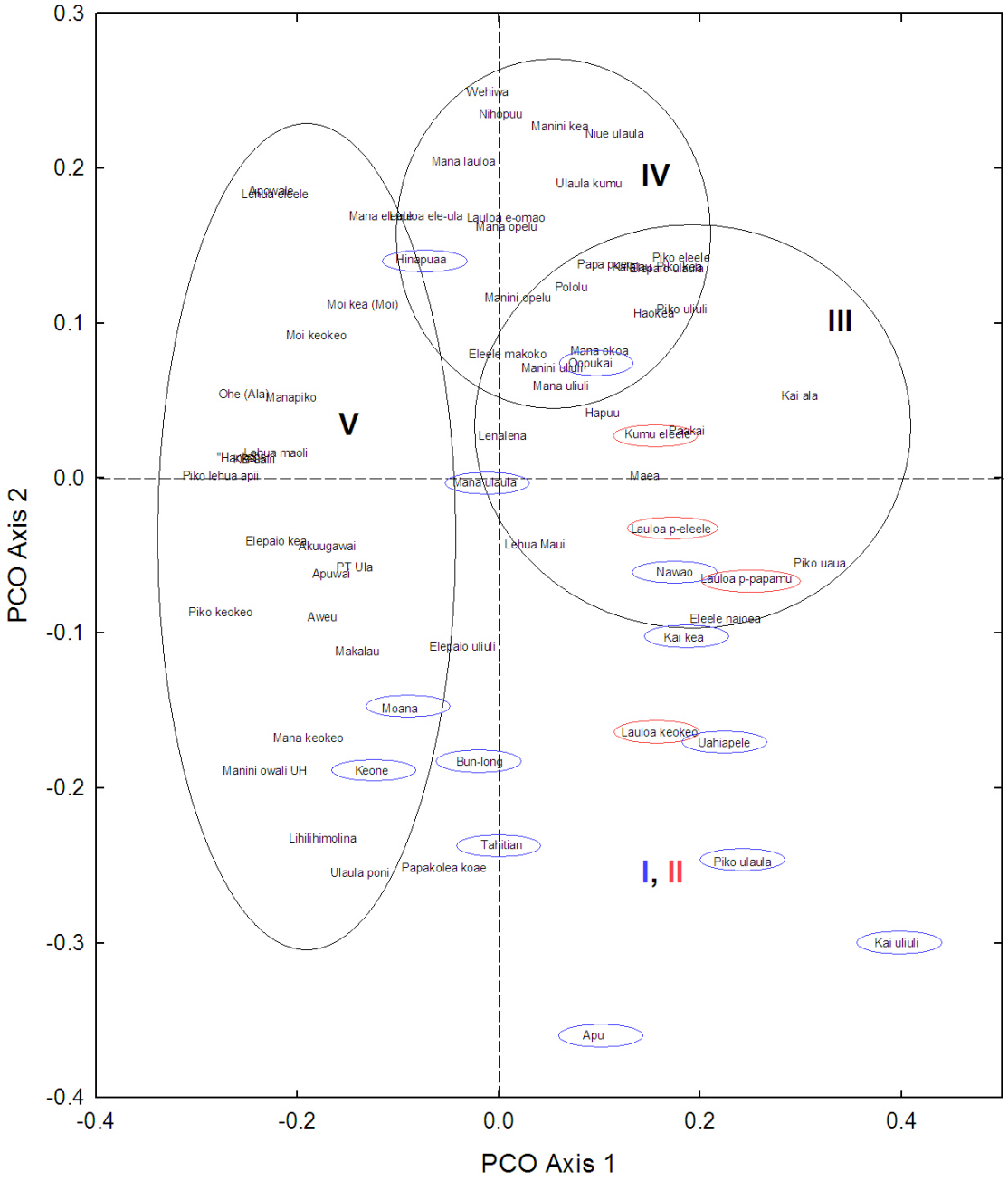

Figure 1. Principal co-ordinates analysis of ISSR data from 6 primers for 73 varieties of Hawaiian taro. Groupings as indicated by cluster analysis (Figure 2) have been outlined.

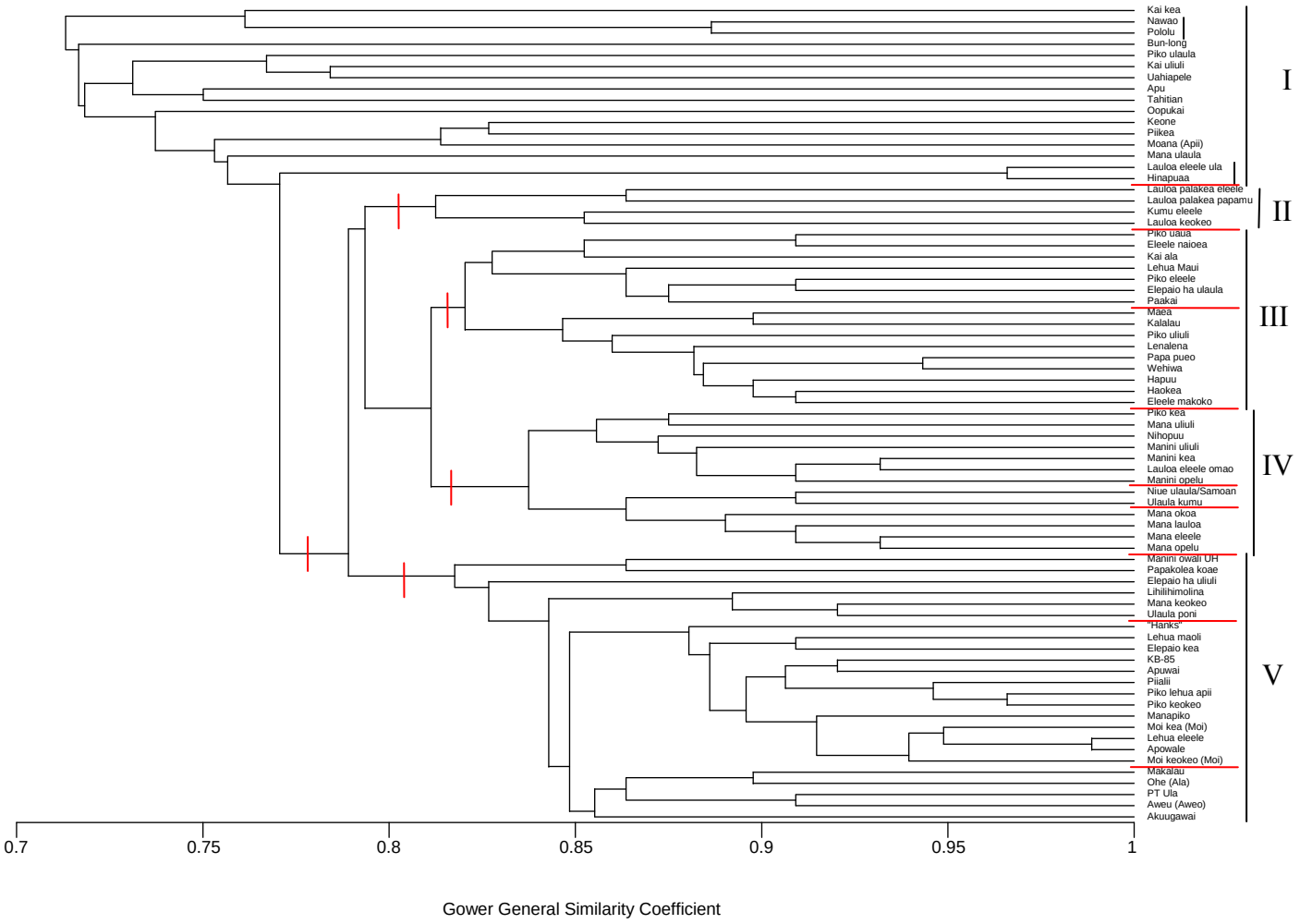

Figure 2: Dendrogram illustrating genetic similarity among 73 Hawaiian *Colocasia esculenta* varieties using UPGMA cluster analysis using Gower General Similarity Coefficient calculated from 6 ISSR markers. Roman numerals on the right and red lines indicate taro subgroups.

Discussion

Although taro cultivars in the Pacific and Polynesian region exhibit extreme morphological variation, high levels of genetic similarity and low level of polymorphism in *Colocasia esculenta* is thought to be due to the species being a vegetatively propagated root crop with clonal and/or varietal selection, arising from a common source. It is generally accepted that taro originated in China, its center of diversity (Lu *et al.* 2011) As such, taro populations from southwestern China show greater variation in genetic variability (Lu *et al.* 2011) than other Asian populations, and Kreike *et al.* (2004), using AFLP analysis of 255 taro from South East Asia and the Pacific, found genetic diversity of taro decreases geographically from east to west. The pilot study reported here is further indication of the high genetic similarity of the many extant Hawaiian taro varieties. Values of similarity using the ISSR markers were similar to that found using RAPD makers (Irwin *et al.* 1998). Irwin *et al.* (1998), using RAPDs analysis, found 23 Hawaiian varieties were closely related with about 80% similarity, and separated into three main phylogenetic branches, with one branch comprising the majority of the varieties. The ISSR analysis in this study separated the 73 taro varieties into five main groupings, some of which corresponded to clusters within the Irwin *et al.* (1998) study. While Mace and Godwin (2002) found the level of polymorphism in microsatellite loci of Asian taro to be relatively low (39%), the six ISSR markers used in this current study showed relatively high polymorphism. The development and screening of other ISSR primers may prove worthwhile in further elucidating the genetic relationship and determining unique markers for identification of between Hawaiian taro varieties due to the relative ease and repeatability of the ISSR technique.

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Appendix 1: *Colocasia esculenta* varieties analysed, and specimen details.
AGG= Amy Greenwell Ethnobotanical Gardens; MNBG = Maui Nui Botanical Garden, Kahului; WAIM= Waimea Valley Botanical Gardens, O‘ahu. PCMB Accessions labelled with * were not successfully amplified with at least one primer, and were removed from further analyses.

PCMBAcc	Variety	Collection Location
6476	"Hanks"	MNBG
6472	Akuugawai	MNBG
6481	Apowale	MNBG
408	Apu	AGG
6474	Apuwai	MNBG
6499	Aweu (Aweo)	WAIM
6496	Bun-long	Anthony Deluze loi, Pearlridge
388	Eleele makoko	AGG
390	Eleele naioea	AGG
318	Elepaio ha ulaula	Bishop Museum Science Garden loi patch
6486	Elepaio ha uliuli	MNBG
6651	Elepaio kea	WAIM
399	Haokea	AGG
395	Hapuu	AGG
288	Hinapuaa	AGG
*401	Iliuaua	AGG
372	Kai ala	AGG
11006	Kai KBS	Waimanalo Agricultural Station
397	Kai kea	AGG
373	Kai uliuli	AGG
281	Kalalau	AGG
*6484	Kauai 7	MNBG
11005	Keone	Waimanalo Agricultural Station
276	Kumu eleele	AGG
*386	Kuoho	AGG
383	Lauloa eleele omao	AGG
289	Lauloa eleele ula	AGG
384	Lauloa keokeo	AGG
380	Lauloa palakea eleele	AGG
*6498	Lauloa palakea keokeo	Anthony Deluze loi, Pearlridge
381	Lauloa palakea papamu	AGG
*6478	Lauloa palakea ula	MNBG
6480	Lehua eleele	MNBG
6655	Lehua maoli	WAIM
263	Lehua Maui	AGG
*403	Lehua palaii	AGG
389	Lenalena	AGG
6488	Lihilihimolina	Penny Levin kalo loi, Waihee
396	Maea	AGG
6485	Makalau	MNBG
266	Mana eleele	AGG
405	Mana keokeo	AGG

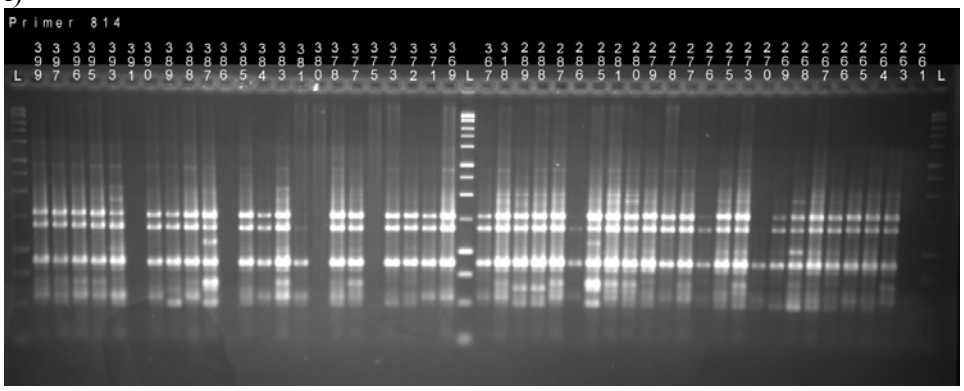
Cont..

PCMBAcc	Variety	Location
268	Mana lauloa	AGG
269	Mana okoa	AGG
267	Mana opelu	AGG
264	Mana ulaula	AGG
270	Mana uliuli	AGG
*265	Mana ulu	AGG
6470	Manapiko	MNBG
377	Manini kea	AGG
385	Manini opelu	AGG
*375	Manini owali	AGG
*6493	Manini owali Kanewai	Penny Levin kalo loi, Waihee
6491	Manini owali UH	Penny Levin kalo loi, Waihee
*6652	Manini toretore	WAIM
378	Manini uliuli	AGG
6473	Moana (Apii)	MNBG
6482	Moi kea (Moi)	MNBG
6654	Moi keokeo (Moi)	WAIM
*261	Moi ulaula	AGG
387	Nawao	AGG
273	Nihopuu	AGG
279	Niue ulaula/Samoan	AGG
6479	Ohe (Ala)	MNBG
393	Oopukai	AGG
278	Paakai	AGG
286	Papa pueo	AGG
400	Papakolea koae	AGG
6471	Piialii	MNBG
6490	Piikea	Penny Levin kalo loi, Waihee
277	Piko eleele	AGG
369	Piko kea	AGG
6653	Piko keokeo	WAIM
6469	Piko lehua apii	MNBG
367	Piko uaua	AGG
371	Piko ulaula	AGG
287	Piko uliuli	AGG
285	Pololu	AGG
6483	PT Ula	MNBG
411	Tahitian	AGG
391	Uahiapele	AGG
275	Ulaula kumu	AGG
*6487	Ulaula moano	Penny Levin kalo loi, Waihee
407	Ulaula poni	AGG
280	Wehiwa	AGG
*410	Zuiki (*Colocasia gigantea*)	AGG

Appendix 2: Images of the two gels (i) and (ii) for each of the primers used for amplification, and the DNA ladder used as reference. a) 814, b) 844A, c) 17898B, d) 17899A, e) HB11, f) HB12; g) Promega 1kB DNA ladder as a standard ("L"). Each lane represents a sample, labelled with the PCMB collection number; blank lanes are either a lack of amplification (re-run at a later date) or negative control ("C") to ensure lack of contamination in the reaction process.

a) Primer 814

i)



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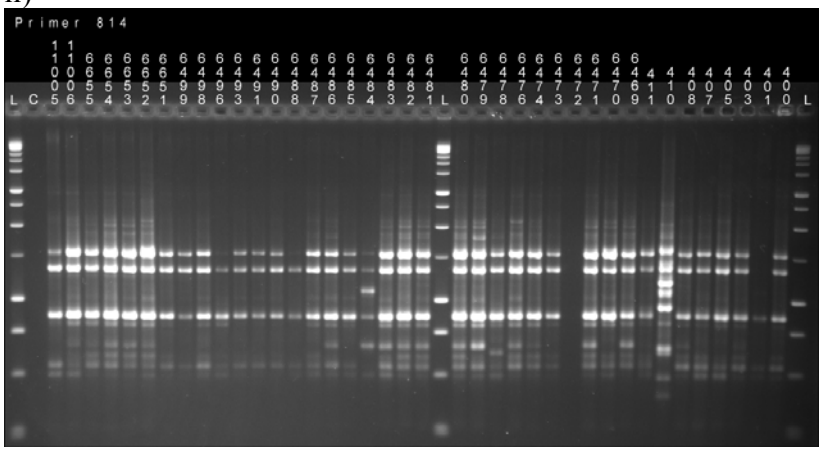
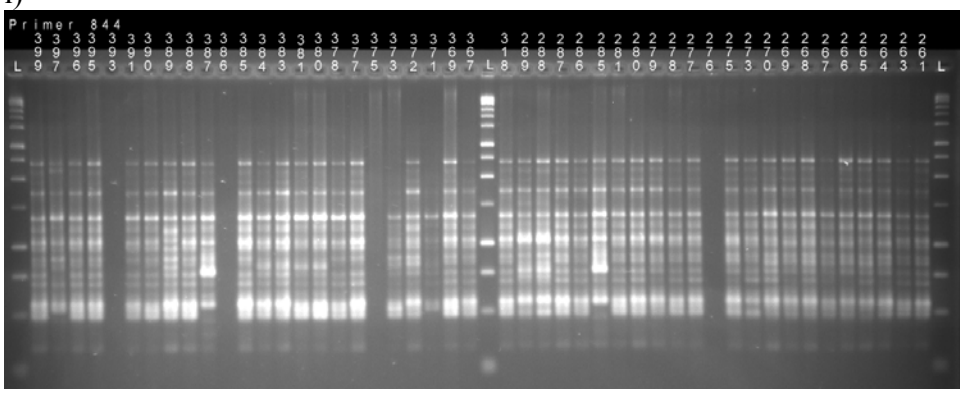


b) Primer 844A

i)



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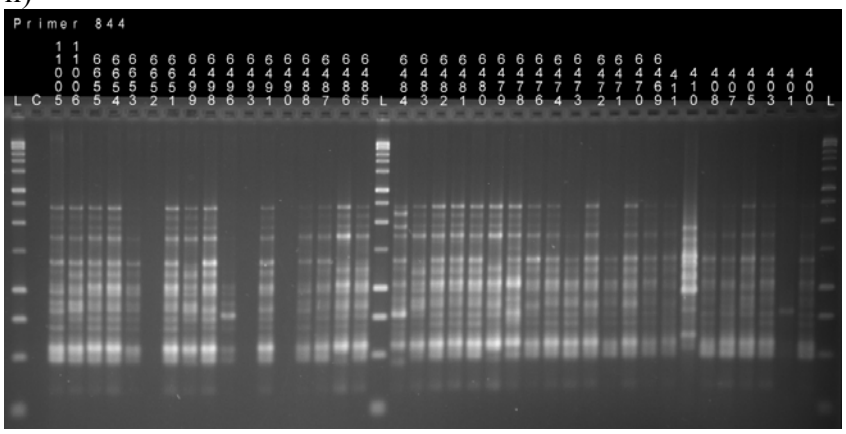
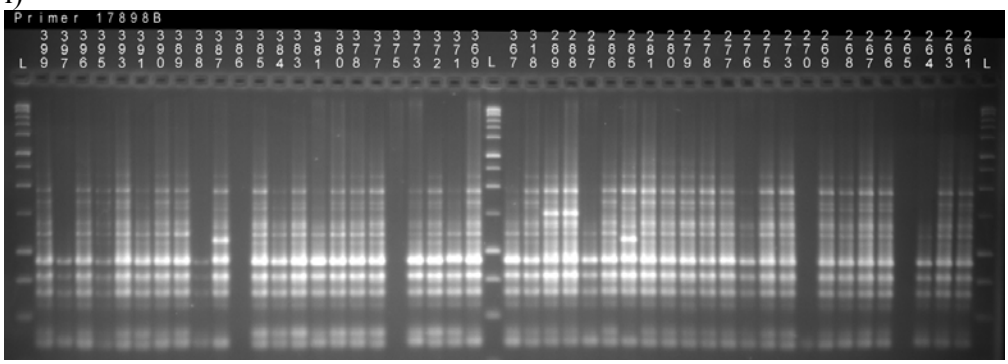
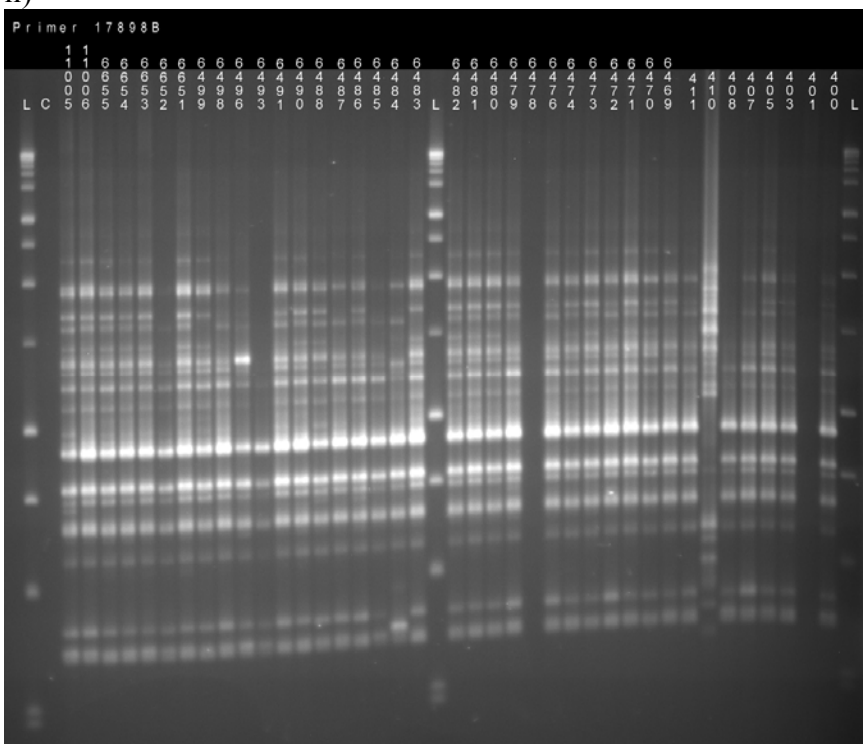


c) Primer 17898B

i)

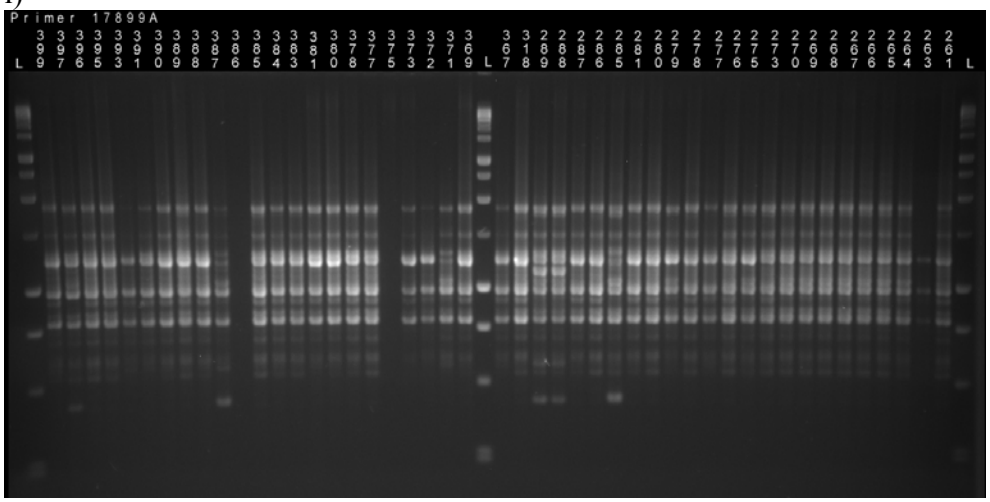


ii)



d) Primer 17899A

i)



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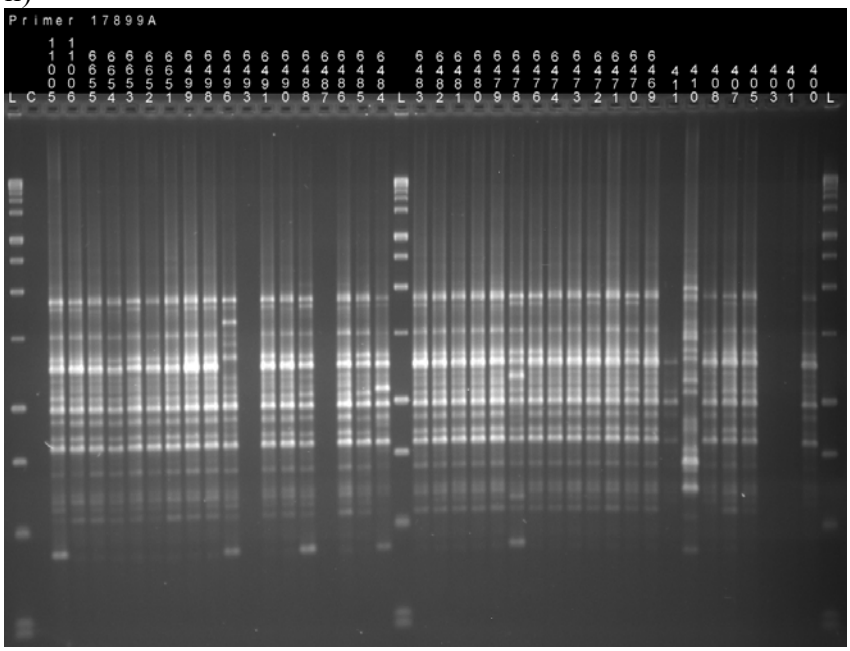
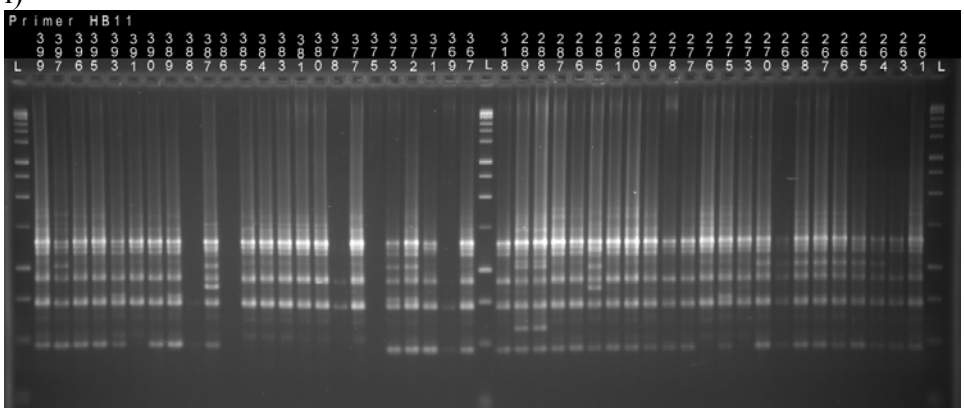


e) Primer HB11

i)



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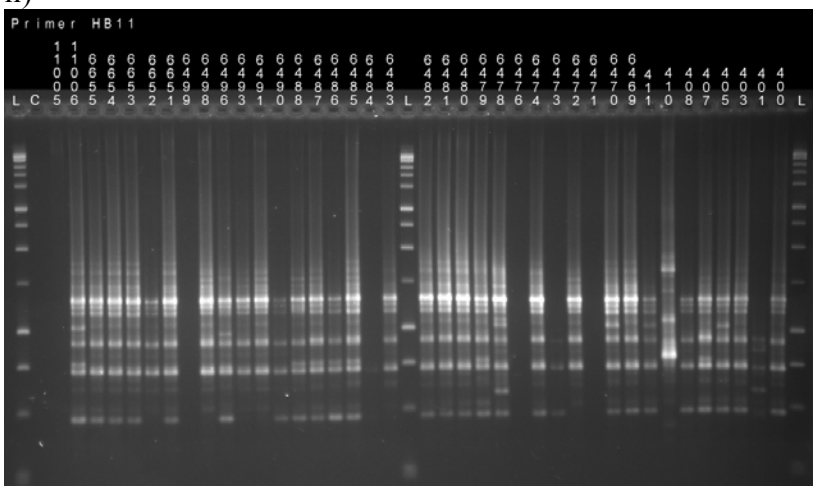
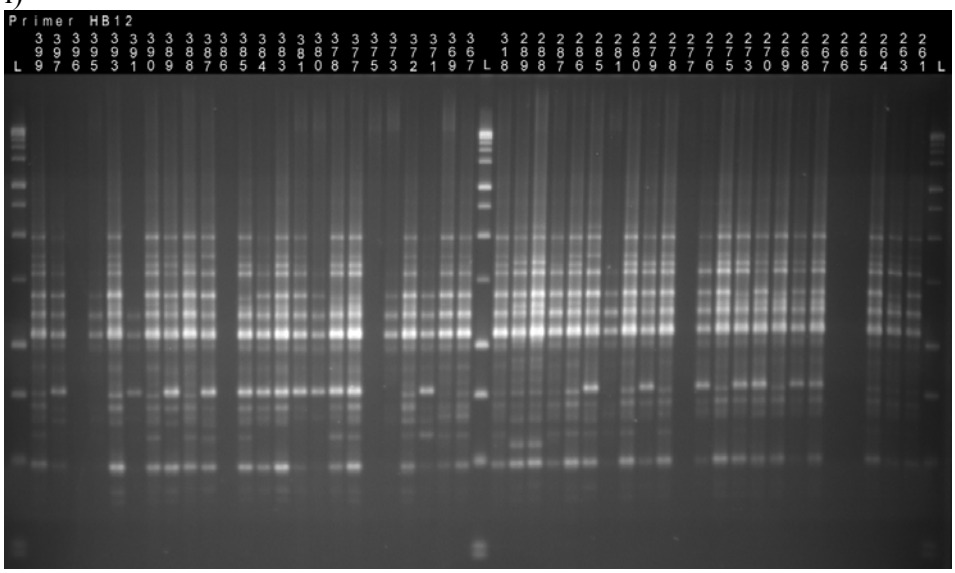
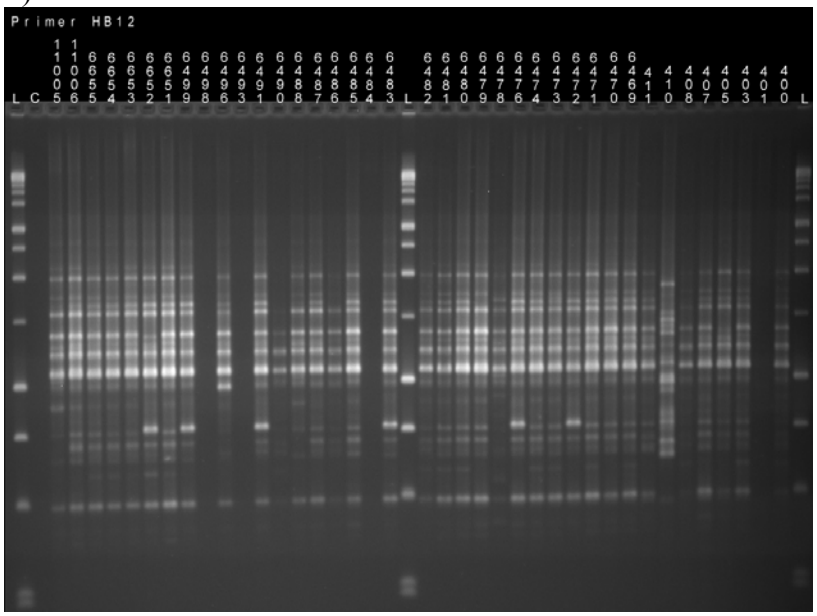


f) Primer HB12

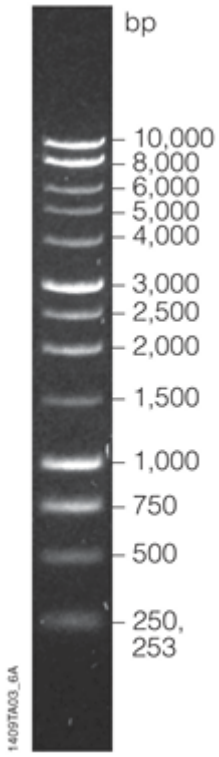
i)



ii)



g) 1kb Standard ladder for estimating DNA fragment sizes for comparison between samples and gels.



0.7% agarose