Development and characterization of polymorphic microsatellite markers in taro (*Colocasia esculenta*)

Emma S. Mace and Ian D. Godwin

Abstract: Microsatellite-containing sequences were isolated from enriched genomic libraries of taro (*Colocasia esculenta* (L.) Schott). The sequencing of 269 clones yielded 77 inserts containing repeat motifs. The majority of these (81.7%) were dinucleotide or trinucleotide repeats. The GT/CA repeat motif was the most common, accounting for 42% of all repeat types. From a total of 43 primer pairs designed, 41 produced markers within the expected size range. Sixteen (39%) were polymorphic when screened against a restricted set of taro genotypes from Southeast Asia and Oceania, with an average of 3.2 alleles detected on each locus. These markers represent a useful resource for taro germplasm management, genome mapping, and marker-assisted selection.

Key words: *Colocasia esculenta*, microsatellite-enriched genomic library, simple sequence repeats, germplasm characterization.

Résumé : Des séquences contenant des microsatellites ont été isolées de banques génomiques enrichies du taro (*Colocasia esculenta*). Le séquençage de 269 clones a permis d'identifier 77 clones contenant des motifs répétés. La majorité de ceux-ci (81,7 %) étaient de nature di- ou trinucléotidique. Le motif GT/CA était le plus commun puisqu'il représentait 42 % des tous les motifs répétés. D'un ensemble de 43 paires d'amorces synthétisées, 41 ont produit des marqueurs de la taille attendue. Seize de ceux-ci (39 %) étaient polymorphes au sein d'une collection limitée de génotypes du taro provenant du Sud-est asiatique et de l'Océanie. En moyenne, 3,2 allèles ont été détectés pour chaque locus. Ces marqueurs constituent une ressource utile pour la gestion des ressources génétiques ainsi que pour des travaux de cartographie génétique et de sélection assistée chez le taro.

Mots clés : *Colocasia esculenta*, banque génomique enrichie en séquences répétées, microsatellites, caractérisation des ressources génétiques.

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Introduction

Taro (*Colocasia esculenta* (L.) Schott.), a member of the Araceae family, is a traditional root crop of the tropics grown for its edible corms and leaves, and is believed to be one of the earliest cultivated root crops in the world (Plucknett 1976; Kuruvilla and Singh 1981). Worldwide production is on the increase, with Food and Agriculture Organization (FAO) records indicating that taro production has doubled over the past decade (FAOSTAT 2000), and taro is now the fifth most-consumed root vegetable worldwide. Cultivated types are mostly diploid ($2n = 2x = 28$), although some triploids are also found ($2n = 3x = 42$). There are two major taxonomic varieties; one is the dasheen type (*Colocasia esculenta* var. *esculenta*), which has a large central corm with suckers and stolons; and the second is the eddoe type (*Colocasia esculenta* var. *antiquorom*), which has a small central corm and a large number of smaller cormels (Purseglove 1972).

Taro is the major food crop for Melanesian and Polynesian people, and is grown for both domestic consumption and export. The Pacific taros are, almost exclusively, of the dasheen type. Like most other root and tuber crops, taro is vegetatively propagated, although seed production is possible. There are growing concerns over the narrow genetic base of taro cultivars in the Pacific islands, particularly with the recent outbreak of taro leaf blight (*Phytophthera colocasiae*) in Samoa and American Samoa in 1993–1994. This has led to the initiation of several breeding programs with the aim of broadening the genetic base of breeding populations, in addition to selection for resistance to taro leaf blight. Recent studies using molecular techniques, specifically isozymes (Lebot and Aradhya 1991) and RAPDs (Irwin et al. 1998), have indicated that the Oceanian cultivars, particularly the Polynesian cultivars, show very little diversity and have stressed the importance of broadening the base of existing breeding programs.

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The development of molecular markers as a tool for taro germplasm characterization and early progeny selection is highly desirable for developing an efficient breeding program to speed the integration of new genetic material into elite germplasm. In addition, taro germplasm characterization using molecular markers will contribute to knowledge of the genetic relationships between accessions of the wild and cultivated genepool, and hence facilitate the breeding of taro cultivars to satisfy market needs and to respond to diverse biotic (e.g., taro leaf blight) and abiotic (e.g., drought) challenges.

Significant progress has been made in recent years in the application of molecular markers to plant genetic resources characterization and evaluation (Gupta and Varshney 2000). Microsatellites, also known as SSRs (simple sequence repeats), STRs (short tandem repeat), STMSs (sequence tagged microsatellite site), and VNTRs (variable number of tandem repeats), are short (1–8 bp long) tandemly repeated DNA sequences. Conservation of the flanking sequence of each microsatellite locus allows the design of primers for PCR amplification, and the amplification products are separated by electrophoresis to detect the polymorphism in repeat length. Microsatellites have emerged as the marker of choice for plant genetic resources applications owing to their codominant nature, their high levels of polymorphism, their abundant and uniform distribution throughout the genome, the simplicity of detection through PCR, and their ease of transferability and reproducibility. Microsatellites have proven to be particularly useful for inbreeding crops with low levels of intraspecific diversity (Roder et al. 1995) and are increasingly useful for root crops that are frequently vegetatively propagated such as cassava (Chavarriaga-Aguirre et al. 1998; Roa et al. 2000), sweet potato (Buteler et al. 1999), and yam (Terauchi and Konuma 1994).

From the first description of microsatellite markers in plants by Condit and Hubbell (1991), they have since been described and used extensively in numerous plant species and applied to a range of studies such as plant parentage and gene flow (Bowers et al. 1999), genetic diversity (e.g., Macaulay et al. 2001; Prasad et al. 2000), germplasm conservation (Lopes et al. 1999), marker-assisted breeding (Weising et al. 1998), and genome mapping (Mba et al. 2001; Taramino and Tingey 1996).

Microsatellite-containing sequences, i.e., non-coding sequences, are known to evolve at a faster rate as compared with coding sequences (Davierwala et al. 2000). Consequently, a larger number of nucleotide variations can be detected per microsatellite assay than with other techniques that do not target rapidly evolving sequences and only sample a small proportion of the genome (e.g., RAPDs and RFLPs), although it should be noted that RAPDs can amplify microsatellite sequences (e.g., RAHM (random amplified hybridization microsatellites), Cifarelli et al. 1995). The development of microsatellite markers, however, requires cloning and sequencing and hence is very cost and labour intensive, compared with PCR arbitrary priming techniques. Once developed, however, the microsatellite approach becomes cost effective. Microsatellites have also been shown to be relatively rare in plant genomes, as compared with animal species; recent figures indicated that there is a 10-fold decrease in the number of dinucleotide repeats detected in plant sequences as compared with humans (Powell et al. 1996). To avoid these potential limitations in the development of microsatellites, protocols for creating microsatellite-enriched genomic libraries have been developed, with selection either before (e.g., Edwards et al. 1996; Karagyozov et al. 1993; Kijas et al. 1994) or after genomic library construction (e.g., Ito et al. 1992). Pre-cloning enrichment methods involve the fragmentation of genomic DNA by sonication or digestion with restriction enzymes and the creation of DNA fragments with defined sequences at both ends (Powell et al. 1996). This is achieved either by ligation of adaptors or through the use of degenerate primers and PCR analysis. Microsatellite-containing fragments are enriched by hybridization either to a short length of biotinylated microsatellites and subsequent selection on streptavidin-coated magnetic beads, or to microsatellite-containing probes that have been covalently attached to a nylon membrane, with subsequent elution of hybridizing fragments.

The pre-cloning enrichment technique as described by Edwards et al. (1996) aims to maximize the efficiency of microsatellite enrichment through the creation of multiplex libraries and in addition the procedure is quick and relatively inexpensive in that it does not require the biotinylation of the oligonucleotides used to collect the microsatellites. This precloning enrichment technique has been successfully applied to numerous plant species, e.g., *Acacia* (Butcher et al. 2000); tea tree (*Melaleuca alternifolia*) (Rossetto et al. 1999), coconut (*Cocos nucifera*) (Rivera et al. 1999), and *Avicennia* (Maguire et al. 2000), with enrichment rates ranging from 40 to 85.5%.

We have produced a microsatellite-enriched genomic library following the Edwards et al. (1996) pre-cloning technique for *C. esculenta*. The developed microsatellite markers were then tested on a representative set of taro accessions from the Pacific island region to determine their level of polymorphism. Finally, the potential application of microsatellite markers in the conservation of taro genetic resources and taro breeding programs is discussed.

Materials and methods

Plant material and genomic DNA isolation

Plant material from one accession, 'Vutokoto' from Fiji (Ce097, the Secretariat of the Pacific Community (SPC), Fiji), was used for the enriched microsatellite library construction. Thirteen individuals of *C. esculenta* var. *esculenta* (dasheen type) accessions were used to investigate the level of polymorphism detected by the microsatellites. These accessions were selected based on country of origin, passport data, and, where available, isozyme electrophoretic patterns (TANSAO 1999), to be representative of the genetic diversity in the Pacific island region and South East Asia (Table 1). One accession of *C. esculenta* var. *antiquorum* (eddoe type) and one *Xanthosoma* species (a related aroid crop), both from Fiji, were also included for comparative purposes.

Single-plant samples of leaf tissue (0.1 g) were ground and genomic DNA was extracted and purified using Nucleon PhytoPure Plant DNA Extraction Kit (Amersham, Piscataway, N.J.), with the addition of β-mercaptoethanol in kit-provided Reagent One and Two. DNA concentration was measured

Table 1. List of *Colocasia esculenta* var. *esculenta* accessions assayed.

Accession No.	Origin	Variety name	Source
E122	Vanuatu	Akasten	SPC, Fiji
E348	Hawaii	White Moi	SPC, Fiji
E641	Samoa	82002–56	SPC, Fiji
E21	Fiji	Toakula	SPC, Fiji
E368	Niue	Maga Tea	SPC, Fiji
TE04	Tonga		SPC, Fiji
CIMG01	Cook Islands		SPC, Fiji
NC102	New Caledonia		CTT, New Caledonia
P3	Palau		SPC, Fiji
BC814	PNG (MP, Finschafen)	Balengho	NARI-Bubia, Papua New Guinea
J7	Japan	JP-S-35	Kyoto University, Japan
J27	China	Y-17	Kyoto University, Japan
J34	Vietnam	V-104	Kyoto University, Japan

Note: SPC, Secretariat of the Pacific Commission; CTT, Centre des Tubercules Tropicaux; NARI, National Agricultural Research Institute.

both on a fluorometer (Hoefer TKP 100, Hoefer Scientific Instruments, San Francisco, Calif.) following the manufacturer's instructions, and by agarose gel (0.8%) electrophoresis.

Construction of enriched microsatellite library and sequencing of clones

A microsatellite-enriched library was constructed by hybridization to synthetic repeats and based on that described by Edwards et al. (1996) with some modifications. Two approaches were compared: the first based on a combination of multiple oligonucleotides ($(GA)_{15}$, $(CA)_{20}$, $(ACC)_{10}$, $(CAT)_{10}$, $(GAC)_{10}$, $(AGC)_{10}$, $(TAA)_{10}$, $(ACT)_{10}$) attached to a single nylon membrane, the second based on different pools of synthetic repeats. In this case, three separate enrichments were done using different pools as follows: pool 1, $(GA)_{15}$, $(CA)_{20}$, $(GT)_{15}$, and $(CT)_{15}$; pool 2, $(ACC)_{10}$, $(CAT)_{10}$, $(TAA)_{10}$, and $(ACT)_{10}$; and pool 3, $(GAC)_{10}$, $(AGC)_{10}$, $(GATA)_8$, $(GAG)_{10}$, $(GAA)_{10}$, and $(AGA)_{12}$. For the dinucleotide repeats a separate pool was formed because they are very abundant in plants.

Ten micrograms of each microsatellite was added to 1 mL of 3× SSC (45 mM sodium citrate (pH 7.0) and 450 mM NaCl), pipetted onto a 10-cm piece of Hybond N+ membrane (Amersham), and air dried for 1 h. The membrane was placed at 65°C for 1 h and then irradiated with ultraviolet light for 30 s using a 260-nm Cross Linker (Ultra-Lum, Claremont, Calif.). The membrane was cut into 0.5-cm^2 pieces and washed twice in hybridization buffer (50% formamide, 3× SSC, 25 mM sodium phosphate (pH 7.0), and 2.5% SDS) at 45°C to remove weakly bound oligonucleotides. Genomic DNA was digested with *Rsa*I and ligated with an *Mlu*I adaptor (consisting of a 21mer (5′-CTCTTGCTTACGCGTGGACTA-3′) and a phosphorylated 25mer (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′), as in Edwards et al. 1996), which served as a priming site for the subsequent PCR, where 1 µL of the ligation product was mixed with 200 ng of the 21mer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1× reaction buffer, and 2.5 U *Taq* polymerase (Promega) to a total volume of 50 µL. The PCR program consisted of 20 cycles of 94°C for 15 s, 60°C for 1 min, and 72°C for 3 min. After the PCR, three replicates of each sample were pooled and cleaned with Wizard® PCR Preps DNA Purification System (Promega, Madison, Wis.). The resulting products were hybridized to the prepared membrane-bound microsatellites for 24 h at 65°C. Non-hybridizing genomic DNA was washed away in a series of high-stringency washes; 5 times in 2× SSC – 0.01% SDS (5 min/wash), followed by 3 washes in 0.5× SSC – 0.01% SDS (5 min/wash) (Godwin et al. 2001). Bound, microsatellite-enriched DNA was then eluted from the membrane and amplified in a second PCR, under the same conditions as previously stated, with the exception that the PCR program cycled 25 times. The amplification products were digested with *Mlu*I, and after the removal of the adaptors with QIAquick PCR purification column (Qiagen, Valencia, Calif.) were ligated into 10 ng of pJV1 (a modified pUC19 vector, Edwards et al. 1996). Transformed cells were plated onto LB-agar plates containing 100 µg ampicillin/mL and 50 µg X-galactosidase/mL. The putatively positive clones were identified by plating out the genomic library, making colony lifts with positively charged Nylon Membranes (Roche Diagnostics) following Boehringer Mannheim's protocol (Boehringer Mannheim 1995), and hybridizing the membranes with digoxigenin-labeled probes containing the microsatellite motifs being searched. The putatively positive clones were then grown overnight in 5 mL of Luria–Bertani (LB) broth with 100 µg ampicillin/mL. Plasmid DNA was extracted using Promega's Wizard® Plus SV Minipreps DNA Purification System. Sequencing was performed by the dideoxynucleotide chain termination method using an M13 forward 24mer. DNA sequencing was carried out on an ABI377 sequencer (Applied Biosystems, Foster City, Calif.) at a commercial sequencing centre.

Primer design

Primers were designed, using dedicated primer design software (PRIME; GCG), from within the regions flanking the repeat motifs for mononucleotides greater than 15 bp in length, dinucleotides greater than 14 bp, trinucleotides greater than 15 bp, tetranucleotides greater than 16 bp, and penta-, sexta-, and octanucleotides greater than 20 bp (Cardle et al. 2000). Primer selection criteria were based on size, GC content, melting temperature (T_m) curve, and lack of secondary structure.

Primer evaluation

Primers were screened against the core set of *Colocasia esculenta* accessions described, including the original genotype from which the library was constructed (as a positive size control) using a two-stage strategy. PCR was initially carried out in a total volume of 25 μL under the following conditions: 10 ng of template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1 U *Taq* polymerase (Promega). PCRs were run on an MJ-Research PTC-100™ (Waltham, Mass.). The cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, optimal annealing temperature (ranging from 55 to 67°C) for 1 min, 72°C for 2 min, and a single extension at 72°C for 10 min. Amplification products were resolved using polyacrylamide gel electrophoresis (PAGE), using BIORAD's Mini PROTEAN® 3 system. Ten percent polyacrylamide gels (8.3 × 7.3 cm) were run at 300 V for 35 min in 1× TBE and visualized by silver staining. The polymorphic primers were further evaluated through a second PCR as follows: 10 ng of DNA, 0.2 μM of each primer, 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Promega), and 1 μCi [α-^{33}P]dATP in a total volume of 10 μL. These amplification products were resolved using the larger polyacrylamide gels (34.5 × 50.0 cm) of the BIORAD SequiGen (Hercules, Calif.) system. PCR products were first mixed with 5 μL stop solution (98% formamide, 10 mM EDTA, 0.05% w/v xylene cyanol, 0.05% w/v bromophenol blue), and denatured at 94°C for 3 min. A 2-μL aliquot was loaded onto denaturing polyacrylamide gels (10% v/v acrylamide–bisacylamide 19:1) and electrophoresed at 100 W constant power for 2 h 10 min. The gels were transferred to 3-mm paper and dried in a Bio-Rad Gel Drier, model 583, at 80°C for 1 h. Once dried, the gel was exposed to Kodak Biomax MR film (Kodak, Rochester, N.Y.) for approximately 20 h.

The final quality of the patterns was assessed according to a quality rating modified from Smulders et al. (1997), where a rating between 1 and 3 indicates a good electrophoretic pattern; a score of 1 indicates weak stutter bands only giving unambiguous product; 2 indicates stutter bands relatively strong, but product still scorable; 3 indicates appearance of bands of unexpected size, but product still scorable; 4 indicates appearance of bands of unexpected size and product not scorable; 5 indicates ladders of bands of unequal intensity, but product still scorable; 6 indicates ladders of bands of equal intensity and product not scorable; and 7 indicates very weak bands or no amplification.

Results

Enrichment

In total, 269 putatively positive clones were sequenced based on the results of hybridization and detection with digoxigenin-labeled probes containing the microsatellite motifs being searched. The library created from pool 1, the dinucleotide repeats only, gave the highest enrichment rate, with 35.7% (10 out of 28) of the clones sequenced containing a microsatellite. The combined approach was the second most successful, with 31% (60 out of 193) of the clones sequenced containing a microsatellite. The success rate from pools 2 and 3 was much lower, with only 13.8 and 15.8%, respectively, of clones sequenced containing a microsatellite. Across all libraries, the insert size ranged from 88 to 690 bp with an average of 303 bp.

Microsatellite description

Of the 269 clones sequenced, 77 (28.6%) contained microsatellites. Fifty percent of these were dinucleotide repeats, 31.7% were trinucleotide repeats, 7.9% were mononucleotide repeats, and the remaining 10.4% consisted equally of one of each of tetra-, penta-, sexta-, and octanucleotide repeats (see Table 2). Overall, 26.3% of the microsatellites sequenced were perfect, 36.8% were imperfect, and 36.8% were compound, following Weber's (1990) definitions that state that perfect repeats have no interruptions, imperfect repeats are interrupted by non-repeat bases, and compound repeats are two or more repeat runs adjacent to each other. The level of redundant microsatellites was very low, with 75 of the 77 sequences being unique.

The most common repeat motifs were GT/CA (42% of all repeat types) and GA/CT (27.3%). Ten different trinucleotide repeat types were isolated, the most common being GAA/CTT (14.3%) and CAT/GTA (9.1%). Interestingly, 22% of clones contained two or more different microsatellite sequences.

Primer design and polymorphism

Overall, based on repeat size, 48 (62.4%) microsatellite sequences were considered useful; however, 5 of the 48 (7.8%) did not have sufficient flanking regions for primer design. Therefore, primer pairs were designed for 43 microsatellites (Table 3), 41 of which produced fragments of the expected size.

Of the 41 primers designed that produced fragments of the expected size, the majority (59%) were derived from dinucleotide repeats, followed by 17% from trinucleotide repeats, and 12.2% from mononucleotide repeats. The remaining primers were derived from tetra- (2.5%), penta- (2.5%), and sextanucleotide (6.3%) repeats.

All 41 primers were screened for polymorphism among 14 taro genotypes from the Pacific island region and Southeast Asia, together with one *Xanthosoma* sample, using the initial, rapid, small-scale PAGE plus silver-staining screening strategy. The polymorphic primers were then subjected to the second, higher-resolution screening strategy of larger-scale PAGE plus radio-labelled PCR product detection. Examples of polymorphisms detected by the second screening strategy in the 14 samples are shown in Fig. 1.

A total of 137 alleles were detected from all microsatellite loci across the 14 *Colocasia* accessions. However, each of the microsatellite primers tested failed to amplify within *Xanthosoma*. The number of alleles per locus amplified within *C. esculenta* ranged from one to eight, with an average of 3.2 alleles/locus. Sixteen (39%) of the primer pairs revealed polymorphism. However, there did not appear to be any correlation between either the size of the repeat unit or the number of repeats and the level of polymorphism observed.

Discussion

We have successfully isolated microsatellites from an enriched library of *C. esculenta* using the precloning enrich-

ment technique of Edwards et al. (1996), in which 28.6% of clones sequenced contained a microsatellite sequence. Primers for PCR amplification were designed for 55% of the cloned sequences. Seventy percent (30/43) of these primer pairs yielded unambiguously scorable PCR products, i.e., achieved a quality score rating of 3 or above. Just under two fifths (39%) of the primer pairs also revealed polymorphism among the different accessions of *C. esculenta* from the Pacific island region and Southeast Asia. However, the microsatellite markers were found not to be transferable across genera to *Xanthosoma*. The two-stage primer pair screening strategy allowed for initial rapid and low-cost screening of high numbers of microsatellite loci, together with the more detailed investigation of a smaller number of loci using the higher resolution and more reliable second-stage strategy involving larger polyacrylamide gels and radio-labeled PCR product detection.

Although approximately 30% of all clones hybridized with the multi-microsatellite probe, only 77 out of the 269 putatively positive clones sequenced contained the expected microsatellite sequence. Such high frequencies of false positives have been recorded previously, e.g., Rallo et al. (2000) and Chavarriaga-Aguire et al. (1998), and may be reduced by increasing the stringency of both the hybridization conditions and the post-hybridization washes. The overall enrichment rate of 8.5%, although low compared with values obtained for many crops, is still within the range observed for similar enriched libraries, e.g., 6.6% enrichment rate obtained for an olive microsatellite enriched genomic library (Rallo et al. 2000).

The rate of redundant SSR-containing clones was found to be very low (2/77; 2.5%), in contrast to other studies utilizing enriched libraries, e.g., redundancy rates ranging from 16.6 to 24.3% as found by Rallo et al. (2000) and Fisher et al. (1998), respectively. However, rather than suggesting that the abundance of multiple copies of some microsatellites in the taro genome may be quite low in comparison with other species, this may be a reflection of the overall low rate of enrichment. The low number of alleles detected by each microsatellite marker, on average only 3.2/locus, could also suggest that there is only a low degree of SSR locus duplication in the taro genome, particularly in contrast to other recent studies on microsatellite diversity in cassava (Chavarriaga-Aguirre et al. 1998) and poplar (van der Schoot et al. 2000), which revealed on average 7.5 and 13 alleles/locus, respectively.

The most abundant repeat motif in *C. esculenta* was AC/GT. This contrasts with previous surveys carried out on microsatellite abundance in plant genomes, where AT repeat types were found to be the most predominant, followed by AG/TC repeats, and finally AC/GT repeats (Powell et al. 1996; Morgante and Olivieri 1993). However, the finding that the AC/GT repeat motif is the most abundant in the taro genome may be due in part to the microsatellite enrichment procedure used, in addition to the fact that the (AT) repeat is self-complementary and difficult to screen for by colony hybridization and is therefore frequently not included in oligonucleotide enrichment mixtures. It has been noted that enrichment procedures may increase the probability of detecting AC/GT repeats, e.g., the number of GT repeats relative to GA repeats has been found to be as high as 20 and 14 times more, respectively, in recent studies by van der Schoot et al. (2000) and Butcher et al. (2000), with both studies using libraries with an equal mix of GA and GT synthetic oligonucleotides. The enrichment procedures used involve several PCR steps and a selective hybridiztion step, and in such cases the use of mixtures of different repeat motifs can affect the frequency of the repeats clones (van der Schoot et al. 2000). The use of separate GA and GT filters would probably avoid this problem, and associated potential problems with GT repeats. GT/AC repeats have been found to be less polymorphic in maize (Taramino and Tingey 1996) and also less perfect in *Elymus* (Sun et al. 1998), and interruptions in the microsatellite repeat structure have been shown to lead to lower levels of polymorphism. However, GT repeats cloned in poplar (van der Schoot et al. 2000) and barley (Struss and Plieske 1998) were found to be mostly perfect and highly polymorphic. The issue of the compound nature of microsatellites isolated from enriched genomic libraries is also worth mentioning. Van de Wiel et al. (1999) noted that the use of oligonucleotide mixtures for enrichment could, for some types of microsatellites, lead to selective accumulation of compound repeats. Butcher et al. (2000) found that 66.6% of microsatellite sequences from an enriched library were compound, whereas only 16.6% of microsatellite sequences from a standard genomic library were compound. For taro, only 26.3% of microsatellite se-

Table 2. Results of microsatellite cloning and sequencing.

(*a*) Results of microsatellite cloning.	
Characteristics of clone	Ratio (%)
Lacking SSR	71.0
Lacking flanking sequence	7.8
Redundant sequences	0.3
(*b*) Results of microsatellite sequencing.	
Type of repeat	Ratio (%)
Monomer	7.9
Perfect	17
Imperfect	50
Compound	33
Dimer	50
Perfect	37
Imperfect	24
Compound	39
Trimer	31.7
Perfect	20.8
Imperfect	41.7
Compound	37.5
Tetramer	2.6
Imperfect	100
Pentamer	2.6
Imperfect	50
Compound	50
Sextamer	2.6
Imperfect	50
Compound	50
Octamer	2.6
Imperfect	100
Total sequencing reactions	269

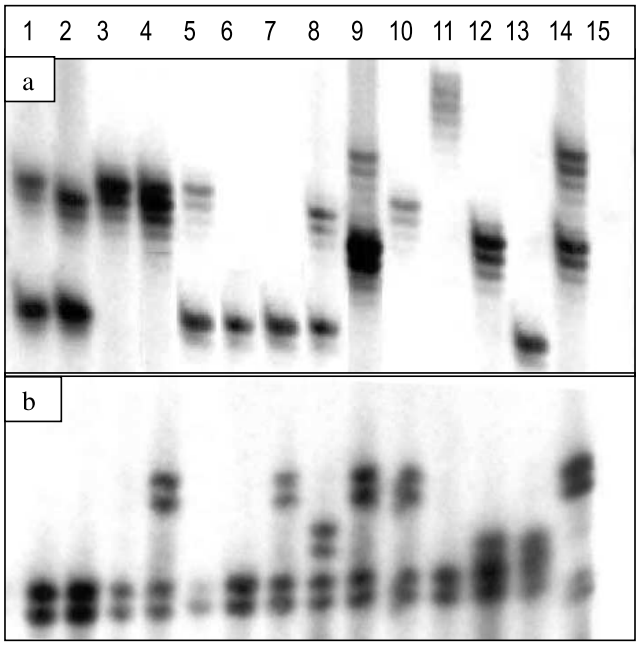
Table 3. Taro microsatellite primer sets.

SSR designation	Repeat type	Primer sequence (5′→3′)	T_m (°C)	Expected size (bp)	Quality of electrophoretic patterns	No. of alleles	Polymorphism
uq55-112	$(CAC)_5$	F: CTTTTGTGACATTTGTGGAGC R: CAATAATGGTGGTGGAAGTGG	65.0	112	1	3	y
uq56-191	$(GT)_7$	F: TGTCCCTTTTGATCTGTACAAG R: CTCAACGGCTCATACACAC	66.0	191	1	2	y
uq71-92	$(TG)_9$	F: ACAGACCCTAGCTGAGTCCTAC R: ACACGTCGCATCACAACAC	65.0	92	3	2	n
uq73-164	$(CT)_{15}$	F: ATGCCAATGGAGGATGGCAG R: CGTCTAGCTTAGGACAACATGC	66.0	164	1	6	y
uq72-194	$(CA)_8$	F: TCAGAACAACACACSCACG R: TCAACCTTCTCCATCAGTCC	65.0	194	3	2	n
uq88-151	$(CT)8(CA)_{14}$	F: GCGTGGACTAACAGACAGAAG R: ATTAAGAGAGAGGGGGCCAAG	61.0	151	6	4	n
uq84-207	$(CT)_{18}$	F: AGGACAAAATAGCATCAGCAC R: CCCATTGGAGAGATAGAGAGAC	65.0	207	1	7	y
uq88B-94	$(CAT)_9$	F: CACACATACCCACATACACG R: CCAGGCTCTAATGATGATGATG	62.0	94	1	6	y
uq75-100	$(GT)_{14}$	F: TTGGTCAGATCAAGGCTAGAG R: GACTAACATCACACACACACG	66.0	100	2	2	n
uq77-174	$(CA)_8$	F: GATCTCAAGCACAAGAGACG R: TCAACCTTCTCCATCAGTCC	65.0	174	3	3	n
uq82-117	$(GT)_8$	F: TCAAGCGTAGGGGAAAAAC R: CCACAACACAAAACTGTAAACC	67.0	187	1	2	n
uq90-102	$(GT)_8$	F: TGGTGCGTTGGTCAGATCAAGG R: ACAACACACACACGAGCACAC	66.0	102	4	4	n
uq91-262	$(TG)_6(GA)_4$	F:GTCCAGTGTAGAGAAAAACCAG R: CACAACCAAACATACGGAAAC	65.0	262	1	2	y
uq95-219	$(CA)_9$	F: ACAACTCGTGTATCCTACATCC R: TCAACTCTCAAACCCTTCCC	65.0	219	1	1	n
uq97-256	$(CA)_8$	F: GTAATCTATTCAACCCCCCTTC R: TCAACCTTCTCCATCAGTCC	66.0	256	1	4	y
uq98-294	$(TG)_{13}$	F: AGTCCAGAGCACTCAAGTCG R: CACAACAGTGTATCCTACGTCC	66.0	294	3	2	n
uq102-177	$(CA)_{10}$	F: CTAACAACACACACACGGG R: TGGACTAACGTGGTGTAGG	62.0	177	3	3	y
uq103-220	$(GT)_{10}$	F: GGATCTCTGGATTGGCTTCC R: ATGATGCACTCACACCCAC	62.0	220	3	2	n
uq105-267	$(AAT)_{10}$	F: CACCAAGGCATGGGAAAC R: CCTGAAAATGGCAAAATACTTTAC	65.0	267	3	2	n
uq110-283	$(TGA)_6(TGGA)_4$	F: AGCCACGACACTCAACTATC R: GCCCAGTATATCTTGCATCTCC	66.0	283	1	8	y
uq111-300	$(CA)_{10}$	F: AGTGTATCCTACGTCCACGG R: CAACCTTCTCCATCAGTCCAG	65.0	300	3	2	n

uq114-342	$(A)_{10}(T)_3(A)_7$	F: CTATGCCGACAAAAGAAG R: TTAGGGTGGTAACACAAC	55.0	342	7	2	y
uq115-71	$(T)_6A(G)_6(A)_{12}$	F: CCCCTCTTTTGTAATAATCC R: GTTTAAATGACTTGTTCTGC	55.0	71	1	4	y
uq118-221	$(AATT)_3(CTT)_3(CAT)_5$	F: GACTAACCGTTATGCTGCC R: TAGATTGGAGCCCTTGGAC	65.0	221	1	1	n
uq119-367	$(GT)_8$	F:GGTCAAGCGTAGGGGAAAAAC R: AGCTAGGGAGCACCAAACAC	65.0	367	3	1	n
uq132-147	$(TTTGAA)_4$	F: ACCCCGAAAAAGCCAATG R: CTATCACTTGTTCCTCCTTCTC	55.0	147	3	5	y
uq192-245	$(ATTA)_3(CTT)_3$ $(CAT)_6$	F: GGACTAACCGTTATGCTGC R: CTATGACTCGCCGTCATTG	62.0	245	4	2	n
uq199-93	$(GCGA)_5(GA)_{18}$	F: GCATACAAAGTTGAGCAAG R: GTGGACTAACGTAGAATCC	55.0	93	7	5	n
uq201-302	$(C)_{15}$	F: CTAAGGAGAGGAGATCCGAAC R: CAAGACGATGCTGAACCAC	66.0	283	5	4	y
uq206-122	$(GAAGA)(CAACA)(CA)_9$	F: CGTTCAACACAGACCACTAC R: TCCTTTGGAAAGGAGGTCC	66.0	122	1	2	n
uq208-253	$(T)_9C(T)_6$	F: TAGAGGGTGGACAGGAG R: CTAGAGGCACTGATGTAAC	63.0	253	3	4	n
uq209-120	$(ACACATTT)2(ACACTT)4$	F: CTACTCTACTGCCATCTAC R: GTGAGTGAGAAAGTGAATG	60.0	120	5	2	n
uq211-202	$(CA)_9$	F: CTAACCACACACACATGAGCAC R: TACTGTCCTGCTTCATCCCTCC	66.0	202	6	3	y
uq220-211	$CA(C)_{13}TC$	F: CTAAGGAGAGGAGATCCGAAC R: CTGATACCACTTGTTGCCC	65.0	211	6	2	n
uq222-107	$(TAT)_3(GT)_6$	F: TCCCGGTTCTGCATAGTTAC R: TGACACATAGGCTGAACCAC	65.0	107	6	3	n
uq223-157	$(CTT)_9$	F: GAGATGGTGTGAGTAAAGGAAG R: TGGACTACTACTGAAGCAGAG	65.0	157	2	6	n
uq228-110	$(GAA)_5$	F: CCAGACTTCTCTCTACACCAAG R: GATCTGTTGAAGAGATCCGTTG	65.0	110	2	3	n
uq233-167	$(GATTT)_3(CATTT)_3$	F: TGCACAGTCAACAATGTCG R: ATCTCCAAGCCCAATCTCC	65.0	167	6	3	n
uq240-223	$(GA)_{15}$	F: ACTAACACGAGCACTCTCC R: ACCATTTCCTACCACCTCC	60.0	223	2	1	n
uq245-109	$(CT)_{18}$	F: GTGGACTAACTACGTGGCATACT R: CACCCAACAAACCCAAACAT	66.0	109	4	1	n
uq247-147	$(CT)_{10}$	F: ATATTGCCGTCCACTCCTG R: AAAGACTCTCTCCCCAGATTAC	66.0	142	3	4	y
uq249-155	$(AG)_{10}$	F: GACGGTCCAAATGTTAG R: CCAAGGAAGATATTACCAAG	56.5	249	7	4	y
uq252-275	$(GATA)_4$	F: GTGTCAACTCTCTGCAAGG R: CTTTTCTTCCGACTGTGTTCTG	60.0	275	3	4	n

Note: F, forward; R, reverse; y, yes; n, no. For definitions of quality scores, see Materials and methods.

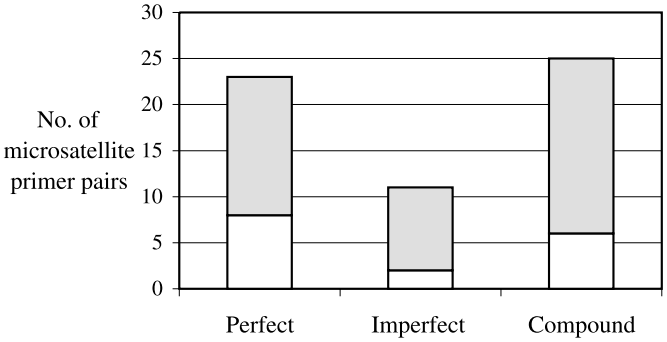
Fig. 1. Examples of microsatellite polymorphism detected among 14 taro genotype samples using SSR primers (*a*) uq84-207 and (*b*) uq88B-94. Genomic DNA was amplified from the 13 samples listed in Table 1, together with one sample of *C. esculenta* var. *antiquorum* and one *Xanthosoma* sample. 1, *C. esculenta* var. *esculenta* from Vanuatu; 2, from Hawaii; 3, from Samoa; 4, from Fiji; 5, from Niue; 6, from Tonga; 7, from Cook Islands; 8, from New Caledonia; 9, from Palau; 10, from Papua New Guinea; 11, from Japan; 12, from China; 13, from Vietnam; 14, *C. esculenta* var. *antiquorum*; 15, *Xanthosoma*.



quences cloned were perfect, and 36.8% were compound. However, of the microsatellite sequences suitable for primer design that were selected owing to the presence of sufficient flanking sequences and the total length of the repeat unit, 44.2% were compound (19/43), 34.8% were perfect (15/43), and 20.9% were imperfect (9/43).

Significantly, the levels of polymorphism revealed by the compound and imperfect repeat types were much lower compared with the perfect repeat types (see Fig. 2), where 53.3% of perfect repeat types revealed polymorphism, compared with just 22.2 and 31.5% for imperfect and compound repeat types, respectively. In addition, the compound repeat types accounted for the highest proportion (53%) of poor-quality electrophoretic patterns, i.e., quality score of 4 and below. These factors might account for the relatively low level of polymorphism revealed overall, 39%, in comparison with other recent studies using microsatellites that revealed polymorphism rates as high as 93% for lettuce (van de Wiel et al. 1999) and coconut (Rivera et al. 1999). However, the level of polymorphism revealed between taro genotypes by the microsatellites generated in this study is comparable with a recent study carried out on potato, also a vegetatively propagated root crop, where microsatellites revealed a polymorphism rate of just 33.3% (Ashkenazi et al. 2001) and also in olive tree (Rallo et al. 2000) with a polymorphism rate of just 38.4%, which, like taro, has been traditionally bred through clonal and (or) varietal selection. Therefore,

Fig. 2. Comparison between the different levels of polymorphism revealed by perfect, imperfect, and compound repeat types, where the grey section of each column represents the total number of primer pairs for each repeat type and the white section of each column represents the number of polymorphic primer pairs for each repeat type.



the low levels of polymorphism revealed in this study may be a consequence of the prolonged period of clonal propagation that taro has undergone in Southeast Asia and Oceania, rather than a reflection of the nature of the repeat types isolated.

A recent study on microsatellites in cassava (Chavarriaga-Aguirre et al. 1998) noted a 27% increase in both the number of alleles observed and the level of polymorphism for a $(GA)_n$ microsatellite locus, when the number of accessions screened increased from 38 to over 500. In a similar study using microsatellites to assess genetic diversity in coconut (Rivera et al. 1999), it was found that the number of alleles per locus increased from 5.2 to 8 when the number of accessions screened was increased from 20 to 40. Consequently, the microsatellites isolated in this study are expected to be highly successful in revealing heterozygosity in taro genotypes from the Pacific island region, despite the relatively low levels of polymorphism revealed and the low numbers of alleles per locus. Although the taro cultivars in the Pacific region exhibit remarkable morphological variation, the genetic base appears to be very narrow and it is thought that the majority of cultivars existing in Polynesia are clones of a common source. An isozyme study on 1417 cultivars and wild forms of taro in Asia and Oceania revealed very low levels of variation among cultivars from Polynesia and Micronesia (Lebot and Aradhya 1991), whereas Melanesian germplasm showed slightly higher isozyme variation, most probably as a result of more active sexual reproduction and the presence of active insect pollinators in the region. Another more recent study using RAPDs (Irwin et al. 1998) also revealed that the Melanesian and Indonesian taros are far more diverse than the cultivars from Polynesia. Consequently, to differentiate between clonal varieties of taro, a highly discriminatory molecular marker technique is required. The microsatellites isolated in this study meet this requirement, because although they amplify fewer loci compared with other techniques such as RAPDs and AFLPs, they do have a higher information content, i.e., more than two alleles per locus. Therefore the 16 polymorphic microsatellite primers developed here have a very large resolving power with the ability to discriminate between clonal varieties, as revealed by this study.

The work undertaken here was part of a collaborative effort with the Taro Genetic Resources Network (TaroGen), which was designed to assist Pacific island countries in the collection and conservation of taro germplasm and in the use of the genetic resources in plant improvement programs. The molecular characterization of germplasm is a major factor in rationalizing the national collections and in establishing a core collection for the region, which will consist of a limited number of genotypes that are more accessible to regional breeding programs selected to represent the genetic diversity of the region. An additional factor is that taro germplasm characterization is often limited by budget constraints. Thus, unequivocal identification of each accession is essential to avoid duplicate sampling, which increases germplasm maintenance costs. Microsatellites have great potential in this respect, owing to their multi-allelic and codominant nature, which allows individuals to be uniquely genotyped. Therefore, the polymorphic set of microsatellite markers identified in this study will make an important contribution to both the evaluation of in situ and ex situ taro germplasm and the establishment of a core collection for the Pacific island region.

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