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Rationalization of taro germplasm collections in the Pacific Island region using simple sequence repeat (SSR) markers

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Abstract

A regional (Oceania) core collection for taro germplasm has been developed based on phenotypic and molecular characterization. In total, 2199 accessions of taro germplasm have been collected by *TaroGen* (Taro Genetic Resources: Conservation and Utilisation) from 10 countries in Oceania: Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, Fiji, Palau, Niue, Tonga, Cook Islands and Samoa. Our objective was to select 10% from each country to contribute to a regional core. The larger collections from Papua New Guinea, Vanuatu and New Caledonia were analysed based on phenotypic characters, and a diverse subset representing 20% of these collections was fingerprinted. A diverse 20% subsample was also taken from the Solomon Islands. All accessions from the other six countries were fingerprinted. In total, 515 accessions were genotyped (23.4% overall) using taro specific simple sequence repeat (SSR) markers. DNA fingerprint data showed that great allelic diversity existed in Papua New Guinea and the Solomon Islands. Interestingly, rare alleles were identified in taros from the Solomon Islands province of Choiseul which were not observed in any of the other collections. Overall, 211 accessions were recommended for inclusion in the final regional core collection based on the phenotypic and molecular characterization.

Keywords: genetic diversity; Pacific Island region; SSRs; taro

Introduction

Taro, *Colocasia esculenta* (L.) Schott, is one of the most important staple foods of Pacific Island countries, where it plays an important role both as a root crop and a leafy vegetable. World-wide it is the fifth most consumed root vegetable (FAOSTAT, 2005) with over 25% produced in Oceania and South-East Asia. The importance of the crop goes beyond its contribution to nutrition and income; in many Pacific Island countries, taro plays an important cultural role as it forms an integral part of customs and traditions. Taro is one of the oldest crops in this region, which spread eastwards into the Pacific, probably reaching the Polynesian islands 2000 years ago. There is now evidence to suggest that most cultivars found throughout the Pacific were not brought by the first settlers from the Indo-Malayan region as previously suggested (Plucknett *et al.*, 1970; Léon, 1977; Kuruvilla and Singh, 1981), but were

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domesticated from wild sources existing in Melanesian, resulting in two separate gene pools for cultivated taro (Matthews, 1990, 1991, 1995; Yen, 1991a, b, 1993; Lebot, 1992; Kreike *et al.*, 2004; Lebot *et al.*, 2005). From there, cultivars were taken eastwards to Polynesia during prehistoric migrations, with a progressive decline in their number and diversity (Yen and Wheeler, 1968; Yen, 1993; Lebot, 1992).

Two principal botanical varieties of taro are recognized: *C. esculenta* var. *esculenta*, commonly known as dasheen, and *C. esculenta* var. *antiquorum*, commonly known as eddoe. Dasheen varieties have large central corms, with suckers and/or stolons, whereas eddoes have a relatively small central corm and a large number of smaller cormels (Purseglove, 1972). The genetic diversity of the crop has been characterized to date largely by morphological and cytological variation (Yen and Wheeler, 1968; Kuruvilla and Singh, 1981; Tanimoto and Matsumoto, 1986; Coates *et al.*, 1988). Recent molecular marker studies (Kreike *et al.*, 2004) have suggested two centres of diversity, one in South-East Asia and the other in Papua New Guinea and surrounding islands. It has been observed that Polynesian cultivars are highly morphologically variable in contrast to the phenotypic homogeneity of the wild populations of Melanesia. It is thought that the high level of phenotypic variation is due to a high rate of vegetative propagation and, consequently, of somatic mutations. This would suggest that the majority of the cultivars in Polynesia are clones of a common source, and recent studies using isozymes (Lebot and Aradhya, 1991; Lebot *et al.*, 2004) indicated that there was very little genetic variation among the Polynesian cultivars, in contrast to the Melanesian and Asian cultivars. The results from a molecular study of taro genetic diversity, using randomly amplified polymorphic DNA (RAPD; Irwin *et al.*, 1998), confirmed that although the cultivars in the Pacific region exhibit remarkable morphological variation, the genetic base appears to be very narrow. Such a limited genetic base leaves the crop very vulnerable to disease epidemics and insect damage and provides a limited opportunity for genetic improvement of important traits by recombination. Consequently, germplasm collections from around the region have been undertaken to augment existing national collections and to safe-guard threatened and useful germplasm for use in regional breeding programmes.

Some 2199 accessions were collected by *TaroGen* (Taro Genetic Resources: Conservation and Utilisation), a regional project funded by the Australian Agency for International Development (AusAID), which aimed to establish a core collection maintained *in vitro* at the Regional Germplasm Centre, Secretariat of the Pacific Community, Fiji, representative of the genetic diversity within Pacific Island countries. This required accessions to be well characterized in order to minimize genotypic redundancy and to identify gaps in the collections and hence maximize the genetic diversity conserved. To date, studies directed at the identification of redundant germplasm in the Pacific Island national collections have utilized biogeographic, agronomic and phenotypic characterization. However, although such data have been used traditionally for the construction of core collections (Ford-Lloyd, 2001), using this information to make meaningful comparisons between taro collections grown in different Pacific Island countries has proved to be difficult (Jackson and Firman, 1987), and less subjective methods are required. Increasingly, the characterization of germplasm collections also utilizes molecular techniques (e.g. Hokanson *et al.*, 1998; Teulat *et al.*, 2000; van Treuren *et al.*, 2001). We report here on the use of molecular markers, combined with phenotypic characterization for the finalization of a regional core collection for Pacific taro, building on the strategy detailed in Mace *et al.* (2006).

Simple sequence repeat (SSR) markers have previously been developed for taro (Mace and Godwin, 2002) and a set of polymorphic markers identified through screening with a limited range of genotypes from the Pacific Island region (Godwin *et al.*, 2001). Here, we report on the use of seven of the polymorphic SSR markers to evaluate genetic diversity and subsequently rationalize 10 national collections from the Pacific Islands, namely Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, Fiji, Palau, Niue, Tonga, Samoa and Cook Islands (Fig. 1).

Materials and methods

Plant material and DNA extraction

The national taro collections included in this study comprise, in total, 2199 accessions (see Table 1). From this entire collection, 515 accessions were fingerprinted (23.4% overall). The entire national collections of the Polynesian countries (Samoa, Tonga, Niue and Cook Islands), Fiji and Palau were included in the fingerprinting study, with the small discrepancies between the collection size and the fingerprinting subset being due to either samples being destroyed in transit to the University of Queensland, Australia or samples missing or too small at the time of collection. The larger national collections of Papua New Guinea, Vanuatu and New Caledonia were first analysed using passport and morphological data to select 20% of the most diverse accessions within each group to be further analysed using molecular markers. The Solomon Islands collection had to be re-collected following the loss of the original collection during the political instability caused

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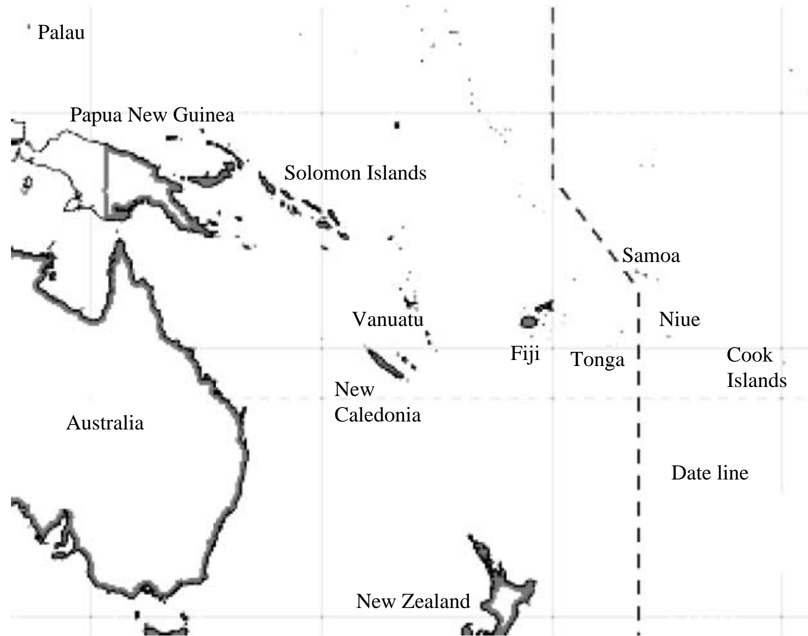

Fig. 1. Map of the Pacific Island region, with countries involved in the *TaroGen* network highlighted. Map modified from *National Geographic* website, http://www.nationalgeographic.com/xpeditions/atlas.

by the coup in 2000. A random sample of the total collection, representing 20% overall, was sent to the University of Queensland for fingerprinting.

From each of the 515 accessions, 50 mg of leaf material were collected and immediately frozen in liquid nitrogen. DNA was extracted using DNeasy® 96 Plant Kit (Qiagen). DNA was eluted in 2 × 50 μl sterile distilled water and stored at 4°C. DNA concentration was measured both on a fluorometer (Hoefer TKP 100) following the manufacturer's instructions, and by agarose gel (0.8%) electrophoresis.

SSR analysis

Seven SSR primers were selected for use (Table 2), based on preliminary assays of amplification and product length polymorphism in taro genotypes (Mace and Godwin, 2002). SSR marker names have been modified from Mace and Godwin (2002) to accord with recent proposals by de Vicente *et al.* (2004) on standardizing marker nomenclature. The canonical name approach has been adopted here consisting of [Function][Lab designator][Species][marker type][serial number of clone]. PCR amplification was performed at the University of Queensland (Brisbane, Australia) in 0.2 ml 96-well plates (Abgene, Thermo-Fast ®LP) using a MJ-Research PTC-100™ Thermal Cycler. The PCR reaction mixture (10 μl) contained 10 ng template DNA, 0.2 μM forward primer, 0.2 μM reverse primer, 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, 0.5 unit *Taq* DNA polymerase (Promega) and 1 μCi [α-^{33}P]dATP. The PCR regime consisted of an initial denaturation (94°C for 5 min), 35 cycles each consisting of 30 s denaturation (94°C), 1 min annealing (ranging from 62 to 66°C; see Table 2) and 2 min elongation (72°C). Finally, an extension period of 10 min at 72°C was included. For detection via electrophoresis, the amplification products were first mixed with 5 μl stop solution [98% formamide, 10 mM EDTA, 0.05% (w/v) xylene cyanol, 0.05% bromophenol blue] and denatured at 94°C for 3 min. A 2 μl aliquot was loaded on to denaturing polyacrylamide gels (10% 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 for approximately 20 h.

Data analysis

Banding patterns observed at a particular locus were recorded as a presence/absence matrix. Similarity matrices were calculated from these data based on different measures; Nei and Li's (1979) definition of similarity: $Sij = 2a/(2a + b + c)$, where Sij is the similarity between two individuals, i and j, a is the number of bands present in both i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i; this is also known as the Dice coefficient (1945); Jaccard's coefficient (Jaccard, 1908):

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Table 1. Size of national taro germplasm collections in 10 Pacific Island countries, as established through the *TaroGen* network, together with details of number of accessions DNA fingerprinted per collection

Country	Collection size	DNA fingerprinting subset
Papua New Guinea	859	163
Vanuatu	452	89
New Caledonia	81	18
Solomon Islands	650	99
Fiji	72	71
Palau	16	11
Niue	24	24
Tonga	12	12
Cook Islands	18	15
Samoa	15	13

$Sij = a/a + b + c$; the simple matching (SM) coefficient (Sokal and Michener, 1958): $Sij = a + d/a + b + c + d$, where d is the number of bands absent in both i and j. Cluster analyses were performed on the similarity matrices using the unweighted pair-group method with arithmetic averages (UPGMA) and dendrograms constructed from these analyses. Cophenetic correlation values were calculated to evaluate the robustness of the resulting tree topologies. All analyses were conducted using the NTSYS-pc software, version 2.02i (Rohlf, 1999).

An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was used to partition genetic variability between and within country collections using Arlequin software, version 2.0 (Schneider *et al.*, 2000), and significance values assigned to variance components based on the random permutation (10,000 times) of individuals assuming no genetic structure.

Gene diversity values for each locus and averages across all loci for each country collection were calculated using Nei's (1987) unbiased estimate $\hat{H}$:

$$\hat{H} = n\left(1 - \Sigma P_i^2\right)/n - 1$$

where n is the number of individuals sampled and P_i is the frequency of the ith allele in the examined genotypes.

Results

In total, 38 alleles were amplified from the seven SSR loci across the 515 taro genotypes included in this study. No locus was monomorphic across the entire collection, however two alleles (5% of total) were found to be monomorphic, and 36 (95%) were found to be polymorphic. An example of SSR polymorphism is shown in Fig. 2. Table 2 lists the total number of alleles per locus, the allele size ranges and the locus diversity values obtained. An average of 5.4 alleles per locus was observed and the gene diversity

Table 2. List of SSR primers used: repeat motif, oligonucleotide primer sequences, PCR annealing temperatures, expected PCR product size, number of alleles and polymorphism information content (PIC) scores

SSR ID	SSR repeat motif	Primer sequence (5′–3′)	Temperature (°C)	Allele size range (bp)	No. of alleles	PIC score
Xuqtem84	(CT)18	Fwd: aggacaaaatagcatcagcac Rvs: cccattggagagatagagagac	65.0	197–217	7	0.4563
Xuqtem110	(TGA)6(TGGA)4	Fwd: agccacgacactcaactatc Rvs: gcccagtatatcttgcatctcc	66.0	250–287	8	0.3138
Xuqtem73	(CT)15	Fwd: atgccaatggaggatggcag Rvs: cgtctagcttaggacaacatgc	66.0	146–164	6	0.5695
Xuqtem55	(CAC)5	Fwd: cttttgtgacatttgtggagc Rvs: caataatggtggtggaagtgg	65.0	112–136	3	0.0726
Xuqtem88	(CAT)9	Fwd: cacacatacccacatacacg Rvs: ccaggctctaatgatgatgatg	62.0	94–108	6	0.4659
Xuqtem97	(CA)8	Fwd: gtaatctattcaacccccccttc Rvs: tcaaccttctccatcagtcc	66.0	248–256	4	0.1547
Xuqtem91	(TG)6(GA)4	Fwd: gtccagtgtagagaaaaaccag Rvs: cacaaccaaacatacggaaac	65.0	258–262	3	0.332 ± 0.026

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values ($\hat{H}$) ranged from 0.265 ± 0.014 for Xuqtem55 to 0.836 ± 0.006 for Xuqtem73.

The SSRs were informative in revealing genetic differences within and among the different countries. The AMOVA (Table 3) showed that the majority of variation detected could be ascribed to differences within country, with only 12.2% of the variation being due to differences between samples from different countries. Table 4 details the genetic variation observed within each country; the Solomon Islands collection revealed the highest proportion of total number of alleles observed (0.974) and the highest average number of alleles per locus (5.3). In contrast, the lowest average number of alleles per locus was 3.86, observed in the collections from Palau, the Cook Islands and Tonga, which also had the lowest proportion of total number of alleles observed (0.711). A general trend between the highest proportion of total number of alleles observed and the highest gene diversity values can also be observed, with the marked exception of Palau, which accounted for the lowest proportion of total number of alleles observed (0.711) and the highest gene diversity value (0.658 with a large standard error of ±0.105). Figure 3 shows the allele frequency variation across all loci and across countries, which clearly reveals a number of rare alleles (frequency ≤ 0.05) present in the germplasm collections, e.g. Xuqtem84 at 217 bp, Xuqtem110 at 279 bp, Xuqtem110 at 281 bp, Xuqtem88 at 102 bp and Xuqtem97 at 246 bp. In particular the latter allele, Xuqtem97-246, was only present in five genotypes from the Choiseul province of the Solomon Islands.

Overall, there were very few accessions that were indistinguishable based on the SSR data set; it should be noted that the percentage of unique fingerprints observed across the entire region was much lower (66.7%) than within country collections, indicating a level of redundancy across countries. It was therefore considered very important to take this redundancy, or duplication between countries, into account, quantified as 12.8% in the AMOVA, when selecting samples for inclusion in the core collection within each country.

Cluster analyses (UPGMA) were performed using the similarity matrices with the highest correlation coefficient (Jaccard's similarity coefficient for all countries excepting Papua New Guinea and Tonga, for which the SM coefficient revealed the highest value) based on the proportion of shared alleles across the seven SSR loci. The cluster analyses were carried out on SSR data sets for individual countries and additionally on a combined data set, across all countries, in order to ensure that between-country duplicates were not included in the final core set. Figure 4 gives an example of the level of duplication existing within and between the Polynesian countries, which again reflects the results of the AMOVA (Table 3) and highlights the importance of considering the collection as a whole when selecting accessions to include in the core collection. For the collections from Papua New Guinea, Vanuatu, New Caledonia and the Solomon Islands, 50% of the total number of accessions fingerprinted were selected for inclusion in the suggested final core. For the remaining six countries (Fiji, Palau, Niue, Tonga, Cook Islands, Samoa), 10% overall of the total number fingerprinted were selected for inclusion in the suggested final core (Fig. 5). In both cases, this was achieved by subdividing the dendrogram into sub-clusters, and selecting one or more accessions from each cluster, based on the level of diversity within each country and also the cluster analysis of the entire data set. Care was also taken to select the accessions containing the rare alleles identified.

Discussion

This study has contributed critical knowledge about the distribution of genetic variation within and among the taro germplasm collections of the Pacific Island countries, which has resulted in the use of molecular markers for the successful development of a core collection of taro germplasm for the Pacific Island region. The selection of accessions to be included was based primarily on cluster analysis of the molecular data sets for the 10 countries. However, the final selection took two other factors into account: first, comparison of selections with the entire data set cluster analyses, to ensure that duplicates were not included in the core; and second, the morphological analyses for New Caledonia, Papua New Guinea and Vanuatu. At least one representative accession from each morphological data grouping was selected. The strategy undertaken enabled us to maximize country

Fig. 2. Example of level of polymorphism observed, with SSR primer pair Xuqtem84, among Polynesian accessions. M, DNA marker, N, negative control.

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Table 3. Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) for 102 individuals from Papua New Guinea, 68 from Vanuatu, 18 from New Caledonia, 64 from Fiji, 11 from Palau, 16 from Niue, 11 from the Cook Islands, 10 from Tonga, 23 from Samoa and 81 from the Solomon Islands, based on 38 SSR markers

Source of variation	df	SSD	Variance components	% of total variation	*P* values
Between countries	9	216.323	0.53851	12.18	<0.001
Within countries	394	1529.348	3.88159	87.82	<0.001
Total	403	1745.671	4.42011		

Nested analysis was carried out on all populations. Degrees of freedom (df), sum of squared deviations (SSD) and the significance (*P*) of the variance components are shown.

representation and diversity in the final regional core collection, encompassed in the minimum number of accessions to provide regional farmers with efficient screening opportunities and to make best use of the limited resources available for germplasm preservation. Overall, the process of the taro regional core development can be summarized as below, following core development steps as defined by Brown and Spillane (1999):

(i) Data assembly: molecular data analyses presented in the results section considered in conjunction with all other available passport and characterization data.

(ii) Grouping, i.e. assigning the accessions to groups, the members of which are likely to be genetically similar, achieved through cluster analysis. Brown and Spillane (1999) states that the number of groups depends on the size of the collection, the intended size of the core and the dissimilarity of the groups at the lowest level of sorting, the latter point reflecting the issue that the level to which the division of the collection into smaller groups proceeds should depend upon their distinctiveness. In this case, the cluster analyses of the molecular data formed the basis of the stratified core sampling on an individual country approach. However, the clusters from the analyses of the combined data sets from the entire region were also referred to, in order to avoid duplication between countries, and in this sense it can be said that the clusters, rather than the distinct countries, formed the basis of the stratified core sampling.

(iii) Selection: having divided the collection into groups, the third step was to select entries from each group. If redundancy does not differ between groups, then the Kimura and Crow (1964) theoretical equilibrium model of selectively of neutral mutants implies representation should be in direct proportion to the size of each group. Consequently, the richness of diversity within each group was the major criterion for determining representation in the core, and this was achieved through the careful selection of a proportional number of unique accessions from each group, based on both within- and between-country analyses, which were cross-referenced to the morphological analyses in order to try to capture the broadest spectrum of morphological diversity in the regional core, in addition to genetic diversity. It was found in a number of cases that accessions with contrasting morphological characteristics clustered together, based on the results of the molecular analyses. This may be a reflection of the clonal nature of taro, and 'sports' type mutations (Lebot and Aradhya, 1991) and it was considered that in such cases, the levels of similarity based on the molecular analyses was a better indicator of genetic similarity than the phenotypic characteristics.

(iv) Handling: as a clonally propagated crop, the core collection is being maintained in tissue culture, under slow-growing conditions, in the regional facility, SPC (Secretariat of the Pacific Community). The core collection has also been duplicated and is being held at the University of the South Pacific, School of Agriculture and Food Technology, Alafua Campus, Samoa.

Table 4. Genetic variation within the 10 country collections analysed across seven SSR loci

Country	*n*	*A*	PA	Gene diversity
Palau	11	3.86	0.711	0.658 ± 0.105
Cook Islands	15	3.85	0.711	0.573 ± 0.107
Tonga	12	3.86	0.711	0.522 ± 0.104
Niue	24	4	0.737	0.566 ± 0.085
Vanuatu	89	4.14	0.763	0.539 ± 0.039
Fiji	71	4.29	0.789	0.512 ± 0.043
New Caledonia	18	4.43	0.816	0.553 ± 0.093
Samoa	26	4.43	0.816	0.579 ± 0.078
Papua New Guinea	163	4.86	0.895	0.589 ± 0.028
Solomon Islands	99	5.3	0.974	0.618 ± 0.031

n, sample size; *A*, average number of alleles per locus; PA, proportion of total number of alleles observed.

Due to resource limitations being a significant factor in the region, the need to reduce the size of the collections would

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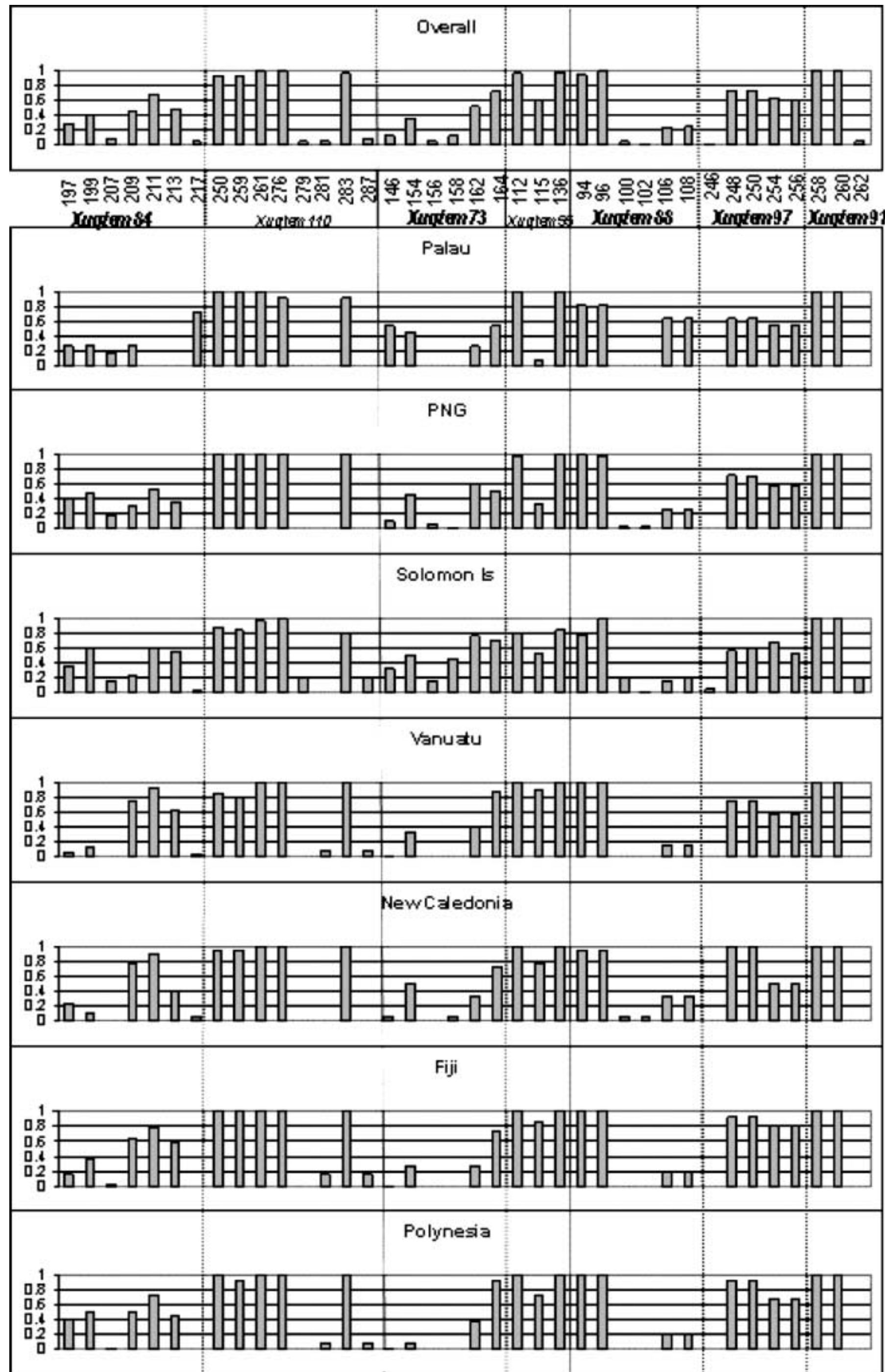

Fig. 3. Frequency of alleles for seven SSR markers within and between the taro germplasm collections from Palau, Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, Fiji and Polynesia (Tonga, Samoa, Niue, Cook Islands).

need to addressed, whether or not a core has been set up. This is particularly true for a clonally propagated crop such as taro, as field genebanks are expensive to run and are vulnerable to damage from poor management, inappropriate environments, pathogens, herbivores, theft and loss of support (Morales *et al.*, 1995). Size of field genebanks is an increasingly pressing problem as national programmes assume responsibility for their indigenous germplasm. Clearly, the elimination of duplicates is an obvious and important first step in rationalizing clonal collections, but frequently such reduction alone may be insufficient to meet the restrictions due to resources.

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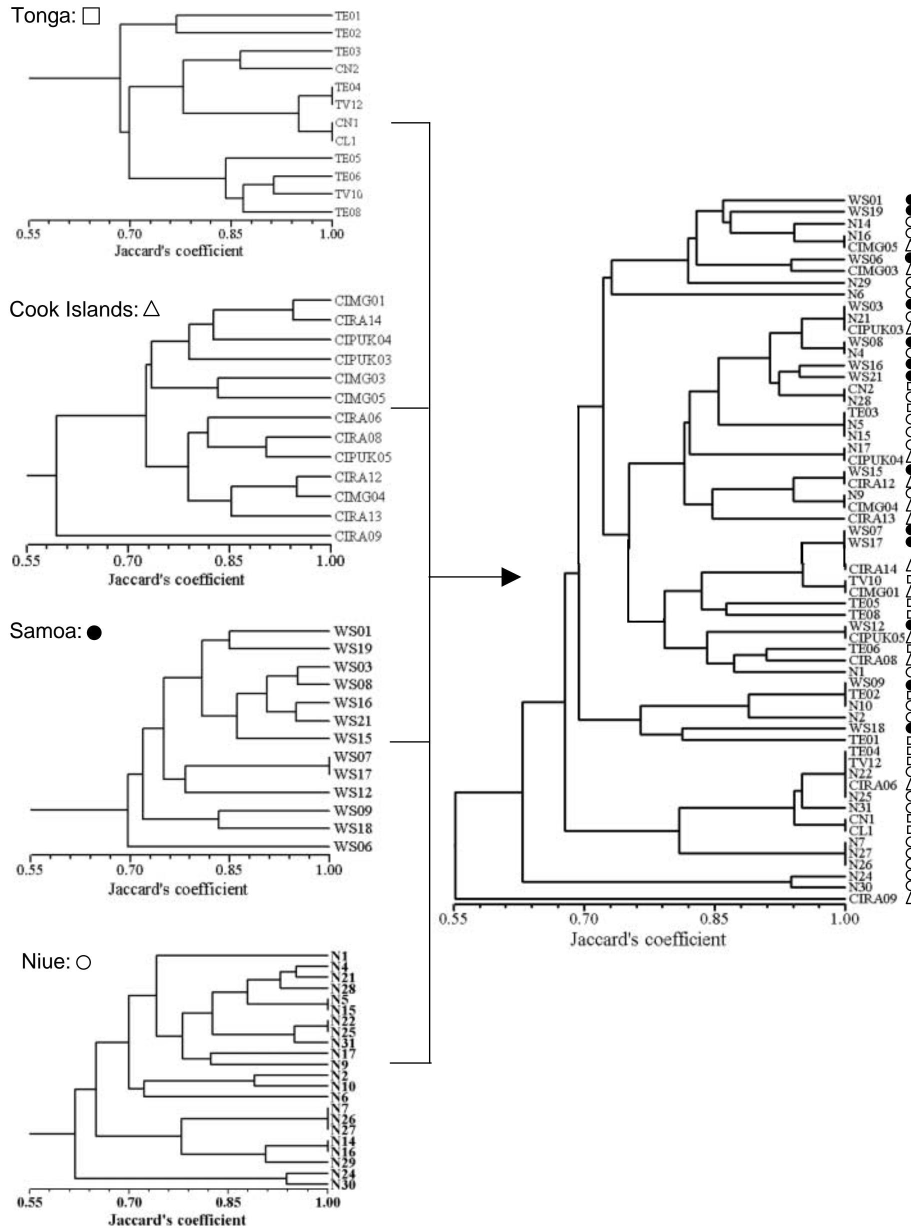

Fig. 4. Cluster analysis, based on Jaccard's similarity coefficient and UPGMA, for the four Polynesian countries separately and combined.

The cluster analysis of the entire data set revealed that there were numerous accessions in Pacific Island countries that were identical to Papua New Guinea accessions. This indicates that originally the cultivars could have been introduced to the region from Papua New Guinea. It also suggests that regional breeding programmes using taro leaf blight-resistant lines from Papua New Guinea could be highly successful when breeding for this trait, as there should be no barriers to breeding, as they originally came from a common source. The fact that many lines from the Pacific Island countries can be traced back to Papua New Guinea also raises the issue of whether that country is part of a second centre of diversity for taro. In particular, accessions from Melanesia, especially Papua New Guinea and the Solomon Islands, had the largest number of different alleles across SSR loci, in addition to high gene diversity values. This supports recent evidence suggesting that there are two separate gene pools for taro, both in Asia, the Indo-Malayan region, and in the Pacific, focused on Melanesia (Lebot, 1992; Kreike *et al.*, 2004). The highest gene diversity value was observed from the Palau collection, reflecting the presence of rare alleles in this collection, such as

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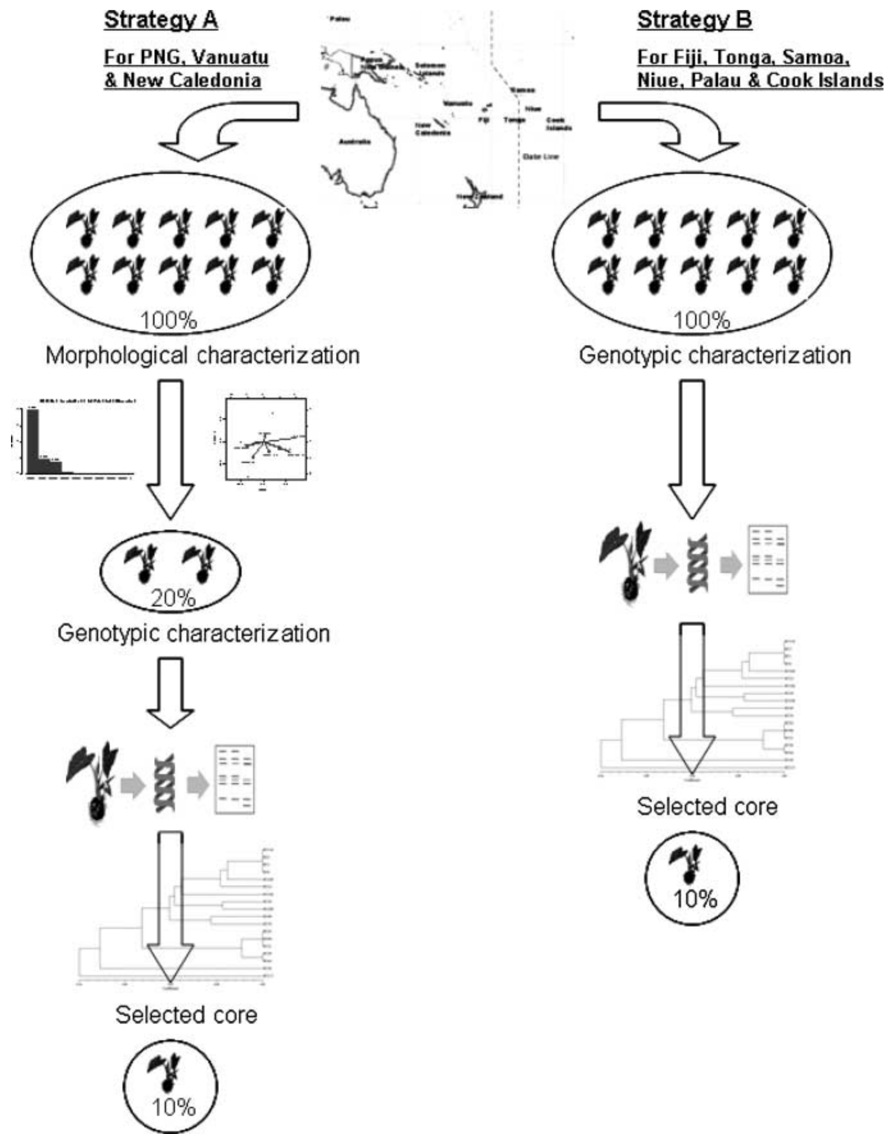

Fig. 5. Strategy for rationalization of taro collections in Oceania.

Xuqtem84 (217 bp) and Xuqtem73 (146 bp). The presence of these rare alleles together with the presence of rare alleles in the taro collections from Choiseul province of the Solomon Islands provides further evidence for independent domestication of taros from wild germplasm in the region, and possible gene flow from Melanesia to Polynesia. Further collections and genotypic characterization of taro germplasm from the eastern provinces of Papua New Guinea, in particular Bougainville, and comparisons with countries in South-East Asia would help clarify the issue of whether the allelic richness in Melanesia is due to independent domestication events or has arisen from gene flow from the previously accepted centre of diversity in South-East Asia, between Myanmar and Bangladesh (Plucknett, 1976), to the Pacific.

The SSR marker analysis described here has proved to be a powerful tool in identifying duplicates and assessing allelic diversity within the national collections of the Pacific Island countries, through identifying the proportion of shared alleles, in combination with UPGMA cluster analysis. This particular analysis approach has been proved to be very successful for managing germplasm collections of other clonally propagated crops, e.g. grapes (Savage Dangl *et al.*, 2001), as it does not make assumptions about the nature of the population under study. The use of SSR markers for DNA fingerprinting offers many advantages compared to other molecular marker techniques; SSRs are evenly distributed throughout the eukaryotic genome, show high allelic diversity, are relatively fast and easy to analyse compared to amplified fragment length polymorphisms (AFLPs) and restriction

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fragment length polymorphisms (RFLPs) (Powell *et al.*, 1996) and have also been shown to be more informative in revealing differences within populations compared to AFLPs, e.g. in coconut populations across the entire geographic range (Teulat *et al.*, 2000) The next task will be validate the core, which can be carried out in two ways; first, by comparing phenotypic characterization data of the core and with a randomly selected 10% of the Papua New Guinea collection; and second, by comparing the genotypic characterization of a random 10% sample from Papua New Guinea (81 accessions) with the level of diversity of the core. The assumption in both cases is that the core will contain more than 75% of the genetic diversity of the regional collections, a figure that could have been obtained by random selection alone.

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