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Genetic Relatedness of *Colocasia esculenta* as Revealed by RAPDs

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ABSTRACT

Ten taro (*Colocasia esculenta* (L.) Schott) accessions collected from different parts of India were subjected to RAPD (Random Amplified Polymorphic DNA) analysis using eight random primers to assess the genetic diversity prevalent in them. Band sizes obtained ranged from 0.4 to 2 kb and the number of scorable bands per primer ranged from 1 to 13 with an average of 9.75 bands per primer. One quarter of the primers analyzed showed 100% polymorphism. The bands produced by the primers were distinct and reproducible. High genetic diversity was revealed by similarity coefficient values that ranged from 0.62 to 0.98. No two accessions analyzed in the present study showed a similarity coefficient value of one thereby indicating their distinctness and presence of high genetic diversity in Indian taro. A dendrogram obtained from UPGMA analysis grouped 10 accessions in two clusters. Clustering did not show any strict relationship with geographical distribution, morphotype classification and genotypic diversity. Forty-three loci or 84.31% were polymorphic. The presence of new recombination events by random and natural processes of mutation may have explained the high genetic diversity.

Keywords: germplasm characterization, phylogenetic relationships, RAPD, taro

INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott) is a member of the Araceae family and an important tropical tuber crop, used as a staple food or subsistence food by millions of people in developing countries in Asia, Africa and Central America (Irwin *et al*. 1998). The corms, leaves and petioles are used as a vegetable and considered as a rich source of carbohydrates, proteins, minerals and vitamins (Kuruvilla and Singh 1981; Coates *et al*. 1988; Anigbogu 1996). Taro corms and leaves are also accredited to have medicinal values. It is claimed to reduce tuberculoses, ulcers, pulmonary congestion and fungal infection (Misra and Sriram 2002). The possibility of using taro in the production of biodegradable plastics was documented by Wang (1983). The Food and Agriculture Organization estimates that 9.1 million Mt of corms are produced annually on a surface of 2 million ha, but this largely underestimates production as few countries keep reliable figures (http://faostat.fao.org/). Taro exhibits great variability in India with cultivars adapted to such varied conditions as tropical wet rain forests, dry up lands and the foothills of the Himalayas (Ghosh *et al*. 1988). The cultivars of taro are generally distinguished based on the colour of their corms, cormels, lamina, veins, and petioles and the acridity of the tubers and leaves. Taro has three main colour groups viz. green, purple and intermediate (Onwueme 1978). Chromosome numbers of 2n = 22, 26, 28, 29, 38, and 42 have been reported in taro from various locations and it is therefore quite evident that new types of taro originate quite frequently in nature or under cultivation (Onwueme 1978).

Taro leaf blight caused by *Phytophthora colocasiae* Raciborski, is the most destructive disease of taro and has become a limiting factor for taro production in all taro-growing countries including India causing yield loss of 25-50% (Misra and Chowdhury 1997; Jackson 1999). It was first reported from Java in 1900 and this disease alone brought a decline of 30-50% taro cultivation and production in Solomon Islands, Paupa, New Guinea, Philippines, Indonesia, China, Malaysia, Japan, India and countries of Africa and Caribbean. In India this disease is most prominent in Northern and Eastern parts, which are also potential areas of taro production in the country (Misra and Chowdhury 1997). The disease appears with the onset of monsoon and continues until the rainy season. During this period it affects most of taro fields. This disease has been reported to have destroyed the taro plantations of Papua New Guinea (Packard 1975). In addition *P. colocasiae* causes a serious post harvest decay of corms (Jackson and Gollifer 1975). Several approaches have been advocated to control this disease. From India, some Colocasia varieties e.g. Sakina V from Ranchi and Poonam pat from Madras have been claimed to be resistant. Lack of flowering, shy flowering, self and cross-incompatibilities are some limiting factors for breeding programmes to develop high-yielding varieties resistant to leaf blight. Metalaxyl- and mancozeb-based fungicides have proved effective in controlling the disease but waxy leaf surface and occurrence of the disease during rainy season makes fungicidal spray ineffective (Misra 1999). Furthermore, fungicide sprays are too costly for marginal farmers, and soil microorganisms rapidly degrade metalaxyl which is released into the water and soil system and development of resistance against the fungicides is another major threat (Cohen and Coffey 1986). Thus there is an urgent need to develop integrated management strategies to combat this disease using natural and environmentally friendly mechanism.

Genetic variation among taro can be used to solve the potential effects of leaf blight of taro. Different varieties respond differentially to *P. colocasiae* with varying degrees of infection. This has led to several breeding programs being initiated with the aim of broadening the genetic base of breeding populations, in addition to selection for resistance to taro leaf blight. Germplasm characterization and the evolutionary process in viable populations are important links between the conservation and utilization of plant genetic resources. The development of molecular and biochemical techniques help researchers, not only to identify

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genotypes, but also to assess and exploit genetic variability (Whitkus *et al*. 1994). Insights into the relative genetic diversity among taro cultivars would be useful in plant breeding and *ex situ* conservation of plant genetic resources (Irwin *et al*. 1998). Among several efficient methods, random amplified DNA polymorphism-RAPD (Wolff and van Rijn 1993; Brummer *et al*. 1995; Wachira *et al*. 1995; Swoboda and Bhalla 1997) is the most widely applied technique for revealing genetic variability within and among plant populations. RAPD analysis produces reproducible, and often distinctive, sets of DNA fragments by subjecting genomic DNA to PCR primed by short (10-25 bases) oligonucleotide primers of arbitrary sequences (Welsh and McClelland 1990; Williams *et al*. 1990). The attractiveness of this method is that no knowledge of the sequence of the target organism is required and a very large number of arbitrary primers can be tested to identify those that might be suited to a particular application. In this study, 10 major growing taro cultivar collected from different region of India were used. We used RAPD to evaluate the levels of genetic variation among ten cultivars of taro that can be used to conserve taro genetic resources in a taro breeding program.

MATERIALS AND METHODS

Plant material

Ten major growing cultivars of *C. esculenta* var. *esculenta* were used to investigate the level of polymorphism detected by RAPD (**Table 1**). These accessions were selected based on their tolerance to taro leaf blight, corm/cormel yield, tolerance against drought and salinity.

DNA isolation

DNA was extracted from young leaves using the CTAB method as described by Sharma *et al*. (2008). 500 mg of fresh leaf material were washed in distilled water and rinsed with 80% ethanol. The surface sterilized leaves were ground in liquid nitrogen and extracted with 1.5 ml of CTAB extraction buffer. DNA was precipitated with isopropanol and washed with 76% ethanol washing solution and dissolved in TE buffer. DNA was quantified using spectrophotometer (U-2000, Hitachi, Japan) and diluted to 10 ng μl^{-1}.

Primer screening

Twenty primers, corresponding to RAPD kit S from Integrated DNA Technologies (Coralville, USA) were initially screened by using four cultivars to determine suitability of each primer for investigation. Primers were selected based on their ability to detect and resolve polymorphic amplified product within the *Colocasia* species. To ensure reproducibility, the primers generating no, weak or complex pattern were not selected for this study.

RAPD analysis

A set of eight screened random decamer oligonucleotides primers of RAPD kit S (Integrated DNA Technologies) were used for RAPD analysis (**Table 2**). Each 25 µl of PCR reaction consisted of 10 ng of template DNA, 100 µM of each deoxynucleotide triphosphate, 20 ng of decanucleotide primers (integrated DNA Technologies, Coralville, USA), 1.5 mM $MgCl_2$, 1 x *Taq* buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 1U of *Taq* DNA polymerase (Promega). Amplifications were performed in a Techne progene thermal cycler (Techne Cambridge Ltd.). The PCR reaction mixtures were heated at an initial step of 94°C for 2 min and then subjected to 35 cycles of the following programme: 94°C for 30 s, 37°C for 1 min, 72°C for 1 min 45 s. After the last cycle temperature was maintained at 72°C for 8 min. Amplified products were resolved on a 1.5% agarose gel containing 0.5 mg ml^{-1} ethidium bromide and visualized under UV light. Gel photographs were scanned through a Gel Doc System (Alpha imager, Alpha Innotech, USA). Clear bands were revealed and were scored for their presence (1) or absence (0). All profiles were reproducible and gave clear and easy to score bands.

Table 1 Taro (*Colocasia esculenta*) cultivars used in RAPD analysis along with their region of cultivation.

№	Cultivar	Region of cultivation
1	UL-85	Champawat,Uttaranchal
2	KH-23	Khandwa, Madhya Pradesh
3	UL-60	Dehradoon, Uttaranchal
4	BS-1	Barua Sagar, Uttar Pradesh
5	BHS-21	Lalitpur, Uttar Pradesh
6	Jhankari	Bhubeneswar, Orissa
7	469	Trivandrum, Kerala
8	BHS-36	24 Pargams, West Bengal
9	543	Cuttack, Orissa
10	BHS-34	Purulia, West Bengal

Genetic data analysis

Allelic frequencies for RAPD markers was used to estimate the percentage of polymorphic loci (P), mean number of alleles per locus (A), effective number of alleles (A_E), observed hetrozygosity (H_O), and expected mean hetrozygosity (H_E) (Hedrick 2004) using the computational program POPGENE 32 (Yeh and Yang 1999). Loci were considered polymorphic if more than one allele was detected. Fixation index (F) was calculated and out-crossing rate (t) was estimated using $t = (1- F)/(1 + F)$ (Weir 1996). The portioning of genetic diversity within and among the cultivar of taro were analyzed using F- statistics (Nei 1973) according to the equations of Weir and Cockerham (1984). Cluster analysis of the binary RAPD data was performed separately with the assistance of the SIMQUAL programme of NTSYS software, version 2.10 (Applied Biostatistics Inc. Setauket, NY, USA). Similarity matrices were generated using DICE and simple matching coefficients. An unweighted pair grouping by mathematical averaging (UPGMA) cluster analysis was produced from similarity matrices constructed for isozyme and RAPD data and resulting dendrograms were compared.

RESULTS

Analysis of RAPD data

The amplification products showed a distribution of amplified fragment unique for each primer. The eight primers OPA-5 OPA-6, OPA-7, OPA-8, OPA-9, OPA-17, OPA-19 and OPA-20 were employed to perform the amplification reactions. All primers generated distinct bands. Polymor-

Table 2 Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers in 10 cultivars of *Colocasia esculenta*.

Name of primer	Sequence of the primer (5′-3′)	Total № of amplification products	№ of polymorphic products
OPA-5	AGGGGTCTTG	08	05
OPA-6	GGTCCCTGAC	06	05
OPA-7	GAAACGGGTG	08	06
OPA-8	GTGACGTAGG	09	06
OPA-9	GGGTAACGCC	10	07
OPA-17	GACCGCTTGT	15	09
OPA-19	CAAACGTCGT	06	04
OPA-20	GTTGCGATCG	16	09

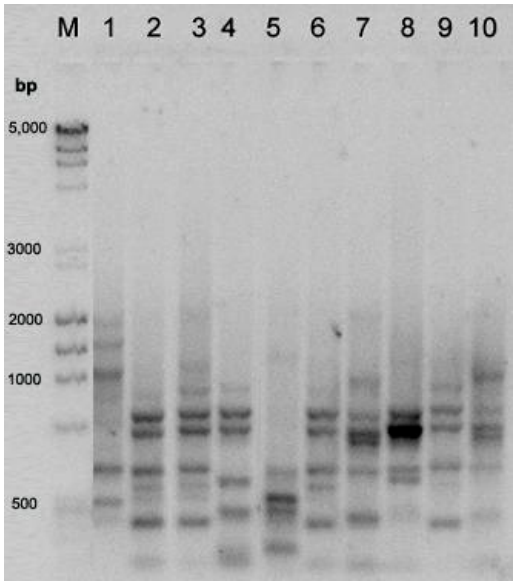

Fig. 1 RAPD patterns of 10 cultivars of taro generated by primer OPA-17. M = 2 Kbp molecular weight ladder.

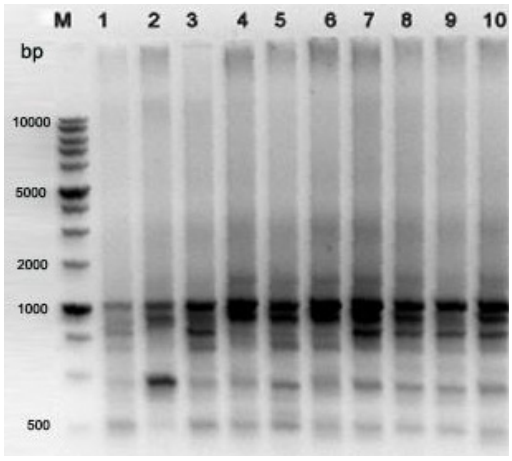

Fig. 2 RAPD patterns of 10 cultivars of taro generated by primer OPA-6. M = 2 Kbp molecular weight ladder.

phic DNA bands were scored as present (1), absent (0) or ambiguous (9) for each accession by visual inspection (**Table 2**). To ensure accurate scoring, all markers were scored at least twice. RAPD primers used in DNA amplifications gave scorable PCR bands or loci (**Fig. 1**). A total of 78 bands were scored. Band sizes ranged from 0.4 to 2 kb and the number of scorable bands per primer ranged from 1 to 13 with an average of 9.75 bands per primer. Primers utilized were 'highly informative' because they either amplified more than 5 polymorphic or monomorphic bands which could differentiate between specific taro accessions. The RAPD fragments observed in the 10 accessions showed a high degree of polymorphism within the populations. The population specific bands could be discerned from the fragment patterns generated. The pattern of amplification by OPA-9 was relatively similar in all the taro accessions while that of OPA-17 was highly polymorphic and informative. Amplification by primers OPA-6 gave common loci of amplification which depicted the presence of similar genetic characteristics in the taro accessions collected from different sites (**Fig. 2**). Each band produced by the primers was distinct and reproducible. The polymorphic bands produced were efficient in assessing the genetic diversity among the cultivars. Cluster analysis of the genetic similarity values was performed to generate a dendrogram illustrating the overall genetic relationships between the species studied and the accessions and individuals within those species. Based on RAPD bands amplified by 8 primers, genetic distances among the 10 taro accessions were calculated and a dendrogram was constructed by UPGMA method (**Fig. 3**). The UPGMA dendrogram based on genetic distance indicated the segregation of the taro populations collected from ten different sites into two clusters in which common characteristics such as degree of tolerance against *Phytophthora* leaf blight and morphological characteristics among members were not separated. Each cluster contained 5 accessions of taro from different sites. The accessions from the same location clustered together in same sub-group, while those collected from distant region were clustered separately. This was because the accessions from the same sites were genetically identical because there may not be active pollination or sexual hybridization and thus shared some common characteristics. This further supports the view that natural selection plays a major role in producing genetic variability among the different cultivars of taro collected from different sites. The accessions collected from same state of India were clustered in to the same group while those collected from other states were grouped separately. Similarity matrices were generated based on SAHN program (**Table 3**).

Population genetic analysis

Population genetic analyses in different taro accessions were done using POPGENE software. The populations were shown to differ in genetic variability of their representatives,

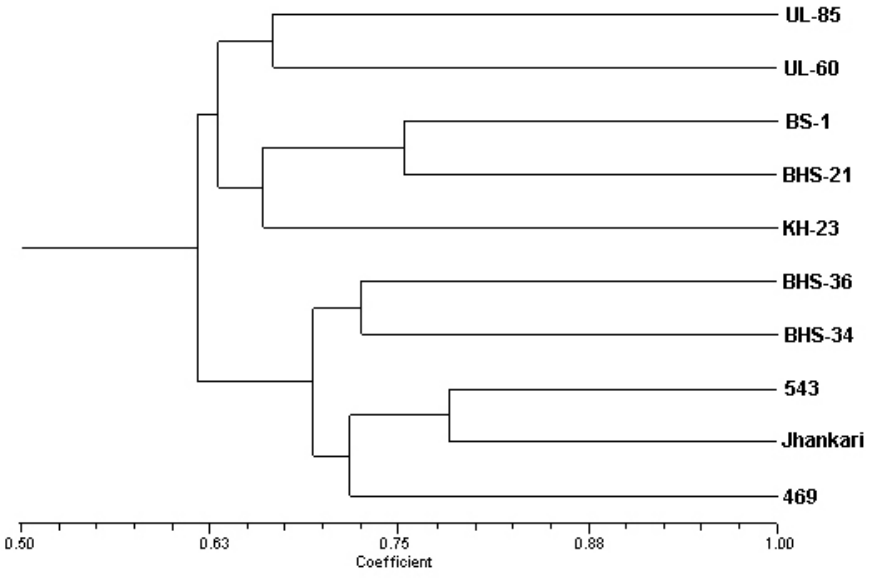

Fig. 3 Dendrogram based on UPGMA analysis of genetic diversity obtained from RAPD data, showing relationship among 10 cultivars of taro.

Table 3 Allelic frequencies of polymorphic loci studied in 10 cultivars of *C. esculenta*, sample size (n = 10).

Locus	Allele	Allelic frequency	Locus	Allele	Allelic frequency	Locus	Allele	Allelic frequency
R1	0	0.8944	R18	0	****	R35	0	0.6325
	1	0.1056		1	1.0000		1	0.3675
R2	0	0.7746	R19	0	0. 8367	R36	0	0.8367
	1	0.2254		1	0.1633		1	0.1633
R3	0	0.8367	R20	0	0.3162	R37	0	0.3162
	1	0.1633		1	0.6838		1	0.6838
R4	0	0.8367	R21	0	0.8944	R38	0	0.4472
	1	0.1633		1	0.1056		1	0.5528
R5	0	0.9487	R22	0	0.5477	R39	0	0.3162
	1	0.0513		1	0.4523		1	0.6838
R6	0	0.6325	R23	0	****	R40	0	0.5477
	1	0.3675		1	1.0000		1	0.4523
R7	0	****	R24	0	****	R41	0	0.8367
	1	1.0000		1	1.0000		1	0.1633
R8	0	0.5477	R25	0	0.8367	R42	0	0. 8944
	1	0.4523		1	0.1633		1	0.1056
R9	0	0.5477	R26	0	0.4472	R43	0	****
	1	0.4523		1	0.5528		1	1.0000
R10	0	0.4472	R27	0	0.9487	R44	0	0.8367
	1	0.5528		1	0.0513		1	0.1633
R11	0	0.8944	R28	0	0.9487	R45	0	****
	1	0.1056		1	0.0513		1	1.0000
R12	0	0.9487	R29	0	0.8367	R46	0	0.4472
	1	0.0513		1	0.1633		1	0.5528
R13	0	0.5477	R30	0	0.5477	R47	0	0.6325
	1	0.4523		1	0.4523		1	0.3675
R14	0	0.7746	R31	0	0.3162	R48	0	0.5477
	1	0.2254		1	0.6838		1	0.4523
R15	0	0.4472	R32	0	****	R49	0	****
	1	0.5528		1	1.0000		1	1.0000
R16	0	0.7746	R33	0	0.8367	R50	0	0.7746
	1	0.2254		1	0.1633		1	0.2254
R17	0	0.9487	R34	0	0.4472	R51	0	0.7746
	1	0.0513		1	0.5528		1	0.2254

which showed not only in the presence of polymorphous loci in the DNA of different accessions, but in varied intensity of homologous fragments in DNA amplification profiles in various accessions. Each band produced was treated as a locus and variations among the alleles were calculated. The RAPD markers used in the study were able to differentiate between the homozygote and the heterozygote in the taro accessions. The genetic diversity of taro was revealed by percentage of polymorphic loci (P), mean number of alleles per locus (A), effective number of alleles (A_E), observed heterozygosity (H_O), and expected mean heterozygosity (H_E). Each band obtained by RAPD procedure was treated as a gene locus and the homozygosity and heterozygosity for each locus was determined (**Table 3**). The genetic analysis in taro accessions revealed that a large percentage of heterozygosity is present in the different accessions keeping aside the homozygous gene locus viz. R7, R18, R23, R24, R32, R43, R45, R49. These loci are homozygous in nature and express only one of the allele at a time. The number of polymorphic loci and percentage of polymorphic loci was 43 and the 84.31% respectively. The observed mean number of alleles (A), effective number of alleles (A_E), observed heterozygosity (H_O), expected mean heterozygosity (H_E) was 1.8431, 1.5085, 0.2945 and 0.3370, respectively (**Table 4**). The Shannon's Information index for gene diversity was found to be 0.4402. The above data shows that new alleles are formed in taro population by the random and natural process of mutation, and the frequency of occurrence of an allele changes regularly as a result of mutation, genetic drift, and selection.

DISCUSSION

The use of RAPD markers to genetically fingerprint plants which are morphologically similar or indistinguishable has been established as a reliable, efficient and very informative tool as RAPD markers tend to reside in regions with many repeated sequences and therefore in noncoding regions which are more susceptible to mutations can also be amplified (Irwin *et al*. 1998).

This work described here is part of a research project on the systematics and phylogeny of 10 major cultivated taro cultivars of India to reflect their geographical variation. With respect to geographical origin of the taro accessions considered here, the accessions investigated were collected from distant regions. Levels of RAPD variation found within the population of taro were high whether measured in terms of percentage of polymorphic loci (84.31%) or observed hetrozygosity H_0 (0.2945). The 10 accessions were clustered into two major groups and sub clustered into hierarchy subgroups on the basis of the UPGMA tree of similarity coefficients. Relationships at the regional level may be conveniently viewed in the dendrograms. Genetic dissimilarity coefficients for pair-wise comparisons of accession comprising group A and B ranged from 0.01 to 0.37 and 0.01 to 0.28 respectively. The grouping of these accessions may reflect their having a common geographic origin, though we were unable to determine the exact origin of all. The cophenetic correlation coefficient (r) comparing the relationship of the cophenetic value matrix with the dissimilarity matrix was high, with an r value of 0.92, indicating a

Table 4 Genetic variation parameters of *Colocasia esculenta* based on RAPD data.

	P	A_0	A_E	H_0	H_E	F	t
RAPD	84.31%	1.8431	1.5085	0.2945	0.3370	0.1262	0.7758

P = percentage of polymorphic loci; A_0 = Mean number of allele per locus; A_E = Mean effective number of alleles; H_0 = Mean observed heterozygosity; H_E = Mean expected heterozygosity; F = Wright's fixation index; t = out crossing rate

very good fit of the dendrogram (**Fig. 3**). The results pointed out the substantial variation within *C. esculenta* but the extent to which the measured variation is representative of the natural range of variation within the species is unknown. A mean fixation index (F) was 0.1262, indicating an overall conformance to Hardy-Weinberg equilibriums. F value was significantly greater than zero and positive, indicating excess of homozygotes. Outcrossing rates (t) based on fixation indices was 0.7758. Genetic changes by mutation are by far slower and much restricted than those by genetic recombination through sexual propagation (Ochiai *et al*. 2001). The population genetic analysis data further provides ample evidence for the fact that recombination events have occurred in the taro accessions due to the phenomenon of natural selection. Out crossing within taro as well as possible genetic introgression from other wild, weedy and cultivated forms of taro could account for the variation (Lakhanpaul *et al*. 2003). The increase in the observed number of alleles than the effective number of alleles in the population analysis data further supports this view. Moreover the crossover frequencies of the majority of taro accessions were above the minimum value required for the successful crossing over (Kreike *et al*. 2004). The study of population genetics is increasingly important as we struggle to maintain healthy wild and domestic populations and ecosystems and thus the information can be used for our benefit. Moreover, information on the population's effective population size, heterozygosity levels, and inbreeding coefficients for particular individuals can be used to design relocation or captive breeding programs which will help to maximize the genetic variation in successive generations. The highly informative primers identified in our fingerprinting studies will be useful in future genetic analysis to establish evolutionary and phylogenetic relationships. Genetic differentiation was evident in the taro accessions collected from different sites indicating genetic diversification of taros existing in these areas. This further supports the evidence for the fact that recombination events have occurred in the taro accessions due to the phenomenon of natural selection. Moreover, the knowledge about the genetic diversity of taro accessions has a great potential in mitigating the leaf blight of taro. Different varieties respond differentially against the taro leaf blight disease caused by *P. colocasiae*. This is mainly due to the genetic make up of taro which may sometimes favour the growth and spread of the pathogen or may resist and eliminate the spread of the pathogen (Lebot *et al*. 2003). Seeking out the resistant varieties from the taro accessions may help in eradicating the harmful effects of the disease. Besides diversity information derived from molecular data, a good knowledge of breed characteristics and values, the risk status of breeds, availability and cost efficiency of possible conservation programs, among others, need to be understood and specified. The current study provides a data base for taro breeders to make informed choices in selection of parental accessions to use in a breeding program based upon genetic diversity and information on the population's effective population size, heterozygosity levels, and inbreeding coefficients for particular individuals can be used to design relocation or captive breeding programs which will maximize the genetic variation in successive generations.

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