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Analysis of Genetic Diversity in *Phytophthora colocasiae* using RAPD Markers

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ABSTRACT

Leaf blight disease caused by *Phytophthora colocasiae* is one of the major production constraints in taro (*Colocasia esculenta*). A total of 12 *P. colocasiae* isolates isolated from blighted taro foliage from different locations of India were subjected to RAPD (random amplified polymorphic DNA) analysis using eight random primers to assess the genetic diversity present in them. A total of 112 distinct DNA fragments ranging in size from 200 to 1800 bp were amplified, of which 106 (94.6%) were polymorphic. The bands produced by the primers were distinct and reproducible. Considerable genetic diversity was revealed by genetic similarity coefficient (GSC) values that ranged from 0.20 to 0.87. Analysis of molecular variance confirmed that most of the genetic variability was within populations (63.1%). Cluster analysis with UPGMA using genetic distances did not reveal any spatial clustering of the isolates collected from the different geographic regions. The possible mechanisms and implications of this genetic variation are discussed.

Keywords: disease management, genetic relationship, random primer, taro leaf blight

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 (Lebot *et al.* 2000). In terms of production, it ranks 14th among staple crops, and 5th among root crops, behind potato, cassava, sweet potato and yams. In India, it is grown throughout the country occupying an area of 0.2 Mha with an annual production of 2.0 MT. The corms, leaves and petioles are used as a vegetable. The taro plant is a rich source of carbohydrates, proteins, minerals and vitamins and has medicinal properties to reduce tuberculosis, ulcers, pulmonary congestion and fungal infection (Misra and Sriram 2002; Brown *et al*. 2004; Sharma *et al*. 2008). Besides their medicinal properties, taro corms are utilized in various industries for the preparation of high fructose syrup and alcohols (Misra *et al*. 2008). These prospects make taro a useful and important tuber crop.

Taro leaf blight caused by *Phytophthora colocasiae* is the most destructive disease of taro. It was first reported from Java in 1900 (Raciborski 1900). Leaf blight has become a limiting factor in all taro-growing countries, including India, causing yield loss of up to 50% (Gollifer and Brown 1974; Misra and Chowdhury 1997). In India, this disease is more prominent in Northern and Eastern parts, which are potential areas for taro production. In South India, this disease appears occasionally but in serious proportions (Misra and Chowdhury 1997). The disease is characterized by the formation of brownish water-soaked circular spots on young and mature leaves. As the infection progresses, the spots enlarge to form patches and, as the disease spreads, the whole leaf rots (Lebot *et al.* 2003). In addition, *P. colocasiae* also causes serious post-harvest decay of corms (Jackson and Gollifer 1980). Several strategies are being advocated to combat the disease. Cultural practices like crop rotation are a simple and promising strategy, but may not be practicable because of the longevity of the pathogen in infested soil. Application of metalaxyl based fungicides is another alternative but the presence of a waxy coating on the leaf lamina makes it ineffective (Misra 1999), making it economically unfeasible because large quantities of fungicides and repeated applications are needed. Also, the effect of fungicide treatment is not always consistent and development of resistance to fungicides is another major threat (Cohen and Coffey 1986).

Genetic analysis of plant pathogen populations is fundamental to the understanding of epidemiology, host-pathogen coevolution, and resistance management (Milgroom and Fary 1997). The knowledge of pathogenic composition of populations is essential for efficient management of taro leaf blight and for initiating suitable breeding programs for the development of resistant cultivars of taro. Despite the huge economic loss associated with taro leaf blight, little attention has been paid to the biology of Indian *P. colocasiae* isolates. Specific studies to evaluate inter- and intraspecific genetic variability and to establish the possible pathways by which the pathogen has been introduced and distributed to new areas are quite limited. Variation among *P. colocasiae* isolates in phenotypic characters such as growth rate, colony morphology, metalaxyl resistance and virulence were recognized in an old population (Misra *et al.* 2011). Significant genetic diversity in *P. colocasiae* isolates from Asia and South pacific regions has been previously described through random amplified polymorphic DNA (RAPD) and isozyme markers (Lebot *et al.* 2003). We have observed that the severity of taro blight differed considerably across various taro-growing fields in India. This observation triggered us to examine the genetic makeup of this pathogen. Molecular markers are useful tools in the analysis of genetic variation in populations of phytopathogenic fungi. The advent of the random amplified polymorphic DNA-polymerase chain reaction (RADP-PCR), which generates profiles of amplified DNA products using random decamer oligonucleotide primers, has permitted the study of

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Table 1 Details of *Phytophthora colocasiae* isolates used in the study.

Isolate code	Place of collection	Year of collection
PC1	Veerwada, Andhra Pradesh	2011
PC2	Godawari, Andhra Pradesh	2011
PC3	Varanasi, Uttar Pradesh	2010
PC4	West Tripura, Tripura	2010
PC5	CTCRI field, Trivandrum	2011
PC6	Kottayam, Kerala	2011
PC7	Haripad, Kerala	2011
PC8	Bubaneshwar, Odisha	2009
PC9	Puri, Odisha	2009
PC10	Delhi, New Delhi	2010
PC11	Ribhoi , Meghalaya	2010
PC12	Nellie Road, Assam	2009

the population structure of many organisms that have fewer discernible morphological characters or are otherwise difficult to characterize using more traditional markers (Williams *et al.* 1990; Demeke *et al.* 1992; Lynch and Milligan 1994). RAPD-PCR has been used in fungi to study the genetic variation at the level of genus, species and/or subspecies (Gherbawy 1999, 2001; Gherbawy and Abdelzaher 2002). RAPD has been successfully used to estimate genetic diversity of several *Phytophthora* species (Whisson *et al.* 1994; Chang *et al.* 1996; Meng *et al.* 1999; Mahuku *et al.* 2000; Wang *et al.* 2003; Peters *et al.* 2005) to detect interspecific hybrids between *P. sojae* and *P. vignae* (May *et al.* 2003). 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.

This investigation represents an initial survey of genetic diversity among geographically diverse groups of *P. colocasiae* isolates. The goal of the present study was (1) to isolate *P. colocasaie* from various geographical boundaries of India (2) to estimate genetic distance among these isolates (3) to assess how this genetic diversity is distributed among *P. colocasiae* isolates (4) to examine the genetic differentiation by cluster analysis based on allele frequencies of gene loci.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade and purchased from Sigma-Aldrich, St. Louis, MI, USA

Biological materials

Isolates of *P. colocasiae* used in this study were obtained from mature taro leaf showing typical symptoms of leaf blight from different geographical origins within India (**Table 1**). Samples were collected from 2009 to 2011. Regions representing high disease incidence were given preference in the study.

Isolation of pathogen

For isolation, leaf tissue segments (1–2 cm) from leaf blight-infected area were excised. The segments were sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water, and placed onto *Phytophthora*-selective media (rye agar amended with 20 mg/L rifamycin, 200 mg/L vancomycin, 200 mg/L ampicillin, 68 mg/L pentachloronitrobenzene, and 50 mg/L 50% benlate). Segments were incubated in Petri dishes for 4–5 days at 25ºC, and mycelia were then transferred and maintained on potato dextrose agar medium (PDA; 250 g/L potato, 20 g/L dextrose and 20 g/L agar). For long-term storage mycelial discs were maintained in sterile 50% glycerol stock.

Genomic DNA isolation

P. colocasiae isolates were grown on potato dextrose broth medium (PDB; 250 g/L potato, 20 g/L dextrose). For DNA isolation, small blocks (1 cm) of actively growing cultures were used to inoculate Erlenmeyer flasks (250 mL) containing 100 mL of autoclaved PDB. The cultures were placed on a rotary shaker (Innova-4230, New Jersey, USA) at 50 rpm and incubated at 28 ± 2°C. After 5–10 days, depending on the growth of each isolate, mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels, and used immediately for DNA isolation. DNA was isolated using a genomic DNA purification kit (Fermentas, EU) according to manufacturer's instructions. The integrity and quality of the DNA isolated were evaluated by electrophoresis on 0.8% agarose gel using a 1-kb DNA ladder (Fermentas) as DNA size marker. The DNA concentration was adjusted to 20 ng/µl for amplification by PCR.

Primer screening

In total, 30 primers, corresponding to RAPD primer kits T, A and G from Integrated DNA Technologies (Coralville, USA), were initially screened to determine the suitability of each primer for investigation. Primers were selected based on their ability to detect and resolve polymorphic amplified products. To ensure reproducibility, the primers generating no, weak or complex patterns were not selected for this study.

RAPD analysis

A set of 8 screened random decamer oligonucleotides primers were used for RAPD analysis (**Table 2**). Each 25 µl of PCR reaction consisted of 50 ng of template DNA, 100 µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers (Integrated DNA Technologies), 1.5 mM $MgCl_2$, 2.5 µl *Taq* buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. Amplifications were performed in a Biorad C1000 thermal cycler (Biorad, Singapore). The PCR reaction mixtures were heated at an initial step of 95°C for 2 min and then subjected to 35 cycles of the following program: 95°C for 30 s, 35°C for 1 min, 72°C for 1 min 45 s. After the last cycle, temperature was maintained at 72°C for 10 min. Amplified products were resolved on a 1.8% agarose gel containing 0.5 mg/ml ethidium bromide and visualized under UV light. Gel photographs were scanned by a Gel Doc System (Alpha imager, Alpha Innotech, CA, USA). At least two replicates of the amplification assay were performed with template DNA to ensure the consistency of each band.

Data analysis

Differences in amplification patterns between isolates were

Table 2 Attributes of RAPD primers used in the study.

Primer code	Sequence (5'-3')	Total no. of bands	No. of polymorphic bands	Mean no. of bands	% of polymorphism
OPA3	AGTCAGCCAC	15	13	5.8	86.6
OPA17	GACCGCTTGT	13	13	3.9	100
OPG1	CTACGGAGGA	18	18	3.5	100
OPG8	TCACGTCCAC	14	14	3.6	100
OPG13	CTCTCCGCCA	15	15	4.6	100
OPT9	CACCCCTGAG	15	15	3.9	100
OPT11	TTCCCCGCCA	12	12	3.2	100
OPT15	GGATGCGACT	10	6	6.8	60

assessed visually. All clearly detectable RAPD bands were scored for their presence (1) or absence (0). In order to ensure credibility only reproducible and well defined bands were scored. Fragments were scored as putative loci with two alleles (presence and absence). Dice matching coefficients were computed using the SIMQUAL module. A dendrogram was constructed using genetic similarity matrices to display relationships between isolates using the Nei and Li distance (1979) according to an unweighted pair group mean algorithm (UPGMA; NTSYS-pc, version 2.02) in the SAHN module (Rohlf 2000). The cophenetic correlation coefficient was calculated to provide statistical support for the dendrograms obtained, and Mantel's test (Mantel 1967) was performed to check the goodness-of-fit of the cluster analysis to the matrix on which it was based. The similarity matrix was also used to perform a hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) by using FAMD Software version 1.25 (Schluter and Harris 2006). This analysis enables partitioning of the total RAPD variation into within and among geographical region variation components, and provides a measure of inter-region genetic distances as the proportion of the total RAPD variation residing between *P. colocasiae* of any two regions (called Phi statistics; Excoffier *et al.* 1992). Allelic frequencies of RAPD markers were used separately to estimate the percentage of polymorphic loci (P), mean number of alleles per locus (A), effective number of alleles (AE), observed heterozygosity (HO), expected mean heterozygosity (HE) and Shannon's information index (I) with respect to Hardy–Weinberg equilibrium (Hedrick 2000) using the computational program POPGENE 32 (Yeh and Yang 1999). Loci were considered polymorphic if more than one allele was detected.

RESULTS

Isolation of *P. colocasiae*

A total of 12 isolates of *P. colocasiae* was successfully isolated from several leaf blight-infected samples collected across different geographical regions of India which covers all major taro-growing areas of the country (**Fig. 1**). Isolation of the pathogen was not successful from decayed or rotten leaf samples. All isolates were confirmed as *P. colocasaie* by comparing the morphology with authentic cultures maintained in Central Tuber Crops Research Institute, CTCRI, Sreekariyam, India.

RAPD analysis

The amplification products showed a distribution of amplification fragment unique for each primer. To ensure credibility in scoring, all markers were scored at least twice. All the bands produced were distinct and reproducible. Polymorphic and monomorphic bands were determined for each primer, however only polymorphic bands were taken for further analysis. The 8 primer combinations amplified 112 reproducible fragments ranging in size from 200 to 1800 bp, of which 106 (94.6%) were polymorphic (**Table 2**). When fingerprints of these isolates were compared, some bands common to the majority of isolates were observed, while others were unique to one or a few isolates. The highest number of amplification products was obtained with the primer OPG1 (18), while lowest with OPT15 (10); the average number of bands among total 8 primers was 11.5. The number of polymorphic fragments detected by each primer varied from 6 to 18, with an average of 13.25 bands/primer. The highest number of polymorphic bands (18) was produced by the primers OPG1 whereas primer OPT15 generated the lowest number of polymorphic bands (6). The RAPD profile of primer OPG1 is shown in **Fig. 2**.

Analysis of genetic diversity

A similarity matrix was generated using the SAHN module of NTSYS software. GSC varied between 0.20 and 0.87 among the 12 *P. colocasiae* isolates. The lowest GSC (0.20) was between PC4 and PC9, which suggests that these were the least-related isolates, whereas the highest GSC was 0.87,

Fig. 1 Taro-growing regions of India. ● Sample collection spots.

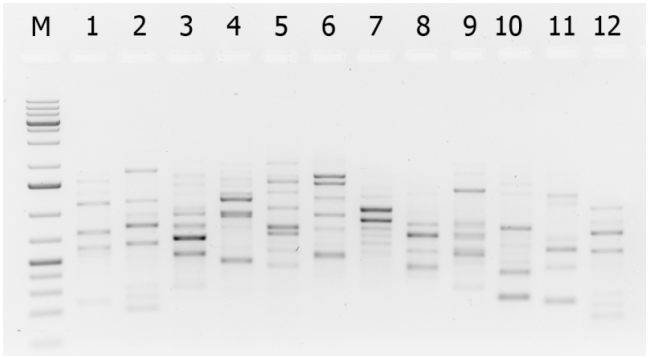

Fig. 2 Amplification profile of *Phytophthora colocasiae* with OPG 1 primer. M, 1Kb DNA size ladder, Lanes 1-12 *P. colocasaie* isolates from diverse geographical origin (as shown in **Table 1**).

detected between isolates PC5 and PC6, indicating a very close relationship between the two. Cluster analysis of the genetic similarity matrix was performed to generate a dendrogram depicting overall relationship of the *P. colocasiae* isolates. The dendrogram produced by the UPGMA analysis grouped the isolates into 2 major clusters (**Fig. 3**). Group A included 9 isolates (PC1, PC2, PC3, PC5, PC6, PC7, PC8, PC9 and PC10); Group B contained another 3 isolates (PC4, PC11 and PC 12). The cophenetic correlation coefficient between the dendrogram and the original distance matrix of the RAPD profiles was significant, with a high correlation value $r = 0.925$ (1 = best possible fit). The cluster analysis with the UPGMA using genetic distances failed to identify any spatial clustering among the different geographic regions.

The AMOVA analysis for the 12 individuals revealed that most of the molecular variability was attributable to differences within populations (63.11% of total variance). A comparatively less yet significant proportion of the variability was explained by differences between populations (36.88%) (**Table 3**). AMOVA confirmed the high level of genetic and genotypic variability of *P. colocasiae* isolates. It also confirmed the high level of genetic differentiation between clusters: FST = 0.368 ($P < 0.001$).

Population genetic analysis was performed separately

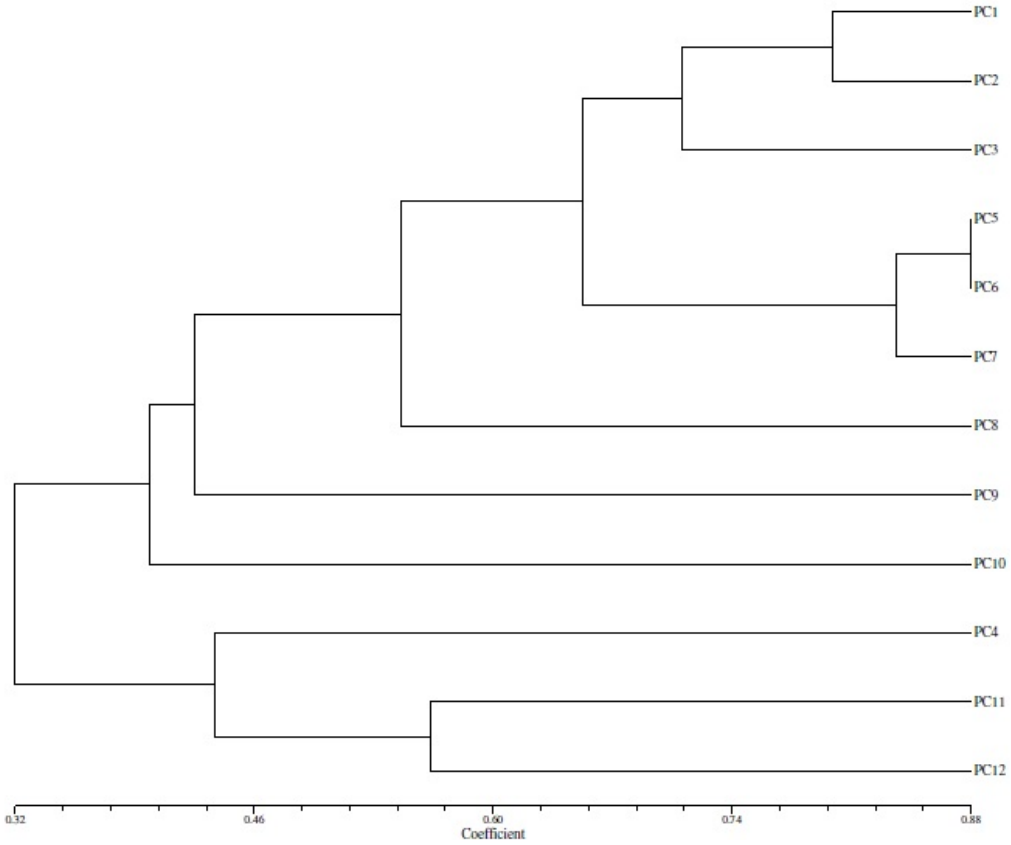

Fig. 3 Dendrogram based on UPGMA clustering depicting genetic relatedness of *P. colocasiae* isolates.

Table 3 Analysis of molecular variance of *Phytophthora colocasiae* isolates.

Source	df	SSD	Φ statistics	Variance components	Proportion of variation components (%)
Among populations	4	1.58	0.368	0.097	36.88
Within populations	7	1.16		0.166	63.11
Total	11	2.74		0.263	

df: degrees of freedom; SSD: sums of squared deviations

Table 4 Population genetic variation parameters of *Phytophthora colocasiae* based on RAPD data.

	P	A_o	A_E	H	I
RAPD	97.32%	1.9732	1.3457	0.2337	0.3808

P = percentage of polymorphic loci; Ao = mean number of allele per locus; AE = mean effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index

with the assistance of POPGENE software. 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 distinguish between homozygotes and heterozygotes in *P. colocasiae* isolates. The genetic analysis of *P. colocasiae* revealed the presence of a high percentage of heterozygosity except for the homozygous gene loci viz. R89, R92 and R96. The number of polymorphic loci was 109 and the percentage of polymorphic loci was 97.32%. The observed mean number of alleles per locus (A), effective number of alleles (AE), Nei's gene diversity (H) and Shannon's information index (I) were 1.9732, 1.3457, 0.2337, 0.3808 respectively (**Table 4**). The value of the observed frequency for crossing over was higher than the minimum required frequency suggesting that recombination events are common in populations of *P. colocasiae*.

DISCUSSION

Accurate and reliable characterization of variation is not always possible using morphological characters; even the identification of variations at the intraspecific level could be difficult. In the present investigations, RAPD has been employed to assess variability at the intraspecific level. RAPD has been successfully used to differentiate and identify fungi at the intraspecific level (Gutheric *et al.* 1992; Assigbetse *et al.* 1994; Nicholson and Reozanoor 1994) and at the interspecific level (Lehman *et al.* 1992). Differences between isolates from different areas are therefore more easily detected where the RAPD technique has been used to detect genetic variation among strains/isolates within a species (Cook *et al.* 1996; Boyd and Carris 1997).

The work described here is a part of research project aimed at investigating the overall population structure of *P. colocasaie* distributed throughout the Indian subcontinent, in an attempt to explore the possible mechanisms governing their genetic composition. Our results demonstrated the utility of RAPD markers to assess genetic diversity among isolates of *P. colocasiae*. The high proportion of polymorphic loci found in the isolates revealed profound variability. Amplification of *P. colocasiae* with arbitrary primers has proved to be successful for characterization of genetic diversity, as already established by Lebot *et al.* (2003). RAPD markers provide useful information because they detect length polymorphisms arising from base sequence changes, insertions, deletions and substitutions either at or between the priming sites (Cooke *et al*. 1996).

In the present investigation maximum effort was made to incorporate isolates of *P. colocasaie* from all major taro-growing regions of India. The GSC obtained through RAPD analysis displayed profound genetic variation among isolates, 0.20 to 0.87, respectively. No two isolates depicted close relatedness with each other. The dendrogram reconstituted based on the genetic similarity coefficient distributed isolates evenly irrespective of their geographical origin. A

similar observation was reported in previous studies where authors failed to identify geographic grouping in *P. colocasiae* isolates revelaed by RAPD markers (Lebot *et al.* 2003; Mishra *et al.* 2010). No clear correlation ($r = 0.255$) could be made between geographical origin and their relative grouping in the dendrogram. Several studies have reported the lack of a correlation of geographical origin coupled with molecular marker data (Schilling *et al.* 1996; Day *et al.* 2004; Linzer *et al.* 2008; Cardenas *et al.* 2011). Even isolates obtained from the same geographical area viz. PC5, PC6 and PC7 have different RAPD patterns, indicating that many populations of this oomycete are made up of more than one genet and that few are derived clonally. It is becoming clear from recent molecular studies that fungi assumed to be exclusively clonal actually are capable of recombination in nature (Taylor *et al.* 2000), and this appears to be the case with *P. colocasiae* as well. The occurrence of homothallic isolates of *P. colocasiae* capable of self-fertilization has been previously described in Taiwan (Lin and Ko 2008). The presence of larger than expected RAPD variation in isolates of *P. colocasiae* suggests that genetic recombination (or less likely hybridization) is at least possible in these oomycetes.

The origin of this variation is not known. Mutation events are thought to be the primary source of new genetic variation in oomycetes (Goodwin 1997). Rapid rates of mutation events have been already discovered in *P. sojae* (Drenth *et al*. 1996) and occurrence of clonal lineages have been identified in *P. infestans* (Goodwin 1997). The existence of sexual reproduction in *P. colocasiae* has already been reported (Narula and Mehrotra 1981; Zhang *et al*. 1994). However, the occurrence of a compatible mating type in closer geographical boundaries is rather rare (Misra *et al*. 2011). Outcrossing can occur when two strains are mixed in culture (Drenth *et al*. 1995; Fry *et al*. 1995; Drenth *et al*. 1996) or infect the same plant (Fry and Goodwin 1995). Based on these observations, it can be suggested that high levels of variability are most likely due to random mutation, exposure of the pathogen to diverse climates, hosts, cropping patterns and movement of the pathogen within the country.

Another possible explanation of this extreme variation is the profound host (*Colocasia esculenta*) diversity (Lebot and Aradhya 1991; Lebot *et al*. 2000; Mishra *et al*. 2010). The vast amount of host diversity imposed tremendous selection pressure on *P. colocasiae* and may possibly be one of the reasons for the existence/formation of new strains of the pathogen. It is possible that different pathogen genotypes may have evolved to selectively parasitize an individual host range (Lebot *et al.* 2003). High levels of diversity in a fungal population enhance its ability to adapt to diverse conditions and overcome host resistance (Chamnanpunt *et al.* 2001). In many cultivars, there is a differential degree of response against the taro leaf blight disease caused by *P. colocasiae*. This is mainly due to the genetic makeup of taro which may favor the growth and spread of the pathogen or may resist and eliminate the spread of the pathogen (Lebot *et al.* 2003). With a huge number of propagules and strong selection imposed by host resistance genes and fungicides, a moderate mutation rate would be sufficient to generate all of the observed variation. The knowledge about the genetic diversity of pathogen has great potential in mitigating taro leaf blight.

In conclusion, the profound genetic diversity displayed among *P. colocasiae* isolates supports the assumption that recombination or migration events play a major role in the life cycle of the pathogen (Lebot *et al.* 2003; Lin and Ko 2008; Mishra *et al.* 2010). Wide molecular diversity reflects the highly dynamic nature of the pathogen indicating its constant evolution in nature. Continuous evolution of races indicates the complexity of pathogen which in turn warrants the scientists to breed new varieties against the most complex/evolved races. The finding that extensive genetic differences occur in *P. colocasiae* populations should be an important consideration in choosing breeding tactics to develop durable leaf blight resistance. Although the present study draws a preliminary conclusion on the population structure of *P. colocasaie*, the study can be further extended by including more isolates and identifying regions at the molecular level that are subjected to frequent mutation events which could be the next step of this research.

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