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Genetic and Phenotypic characterization of *Phytophthora colocasiae* in Taro Growing Areas of India

Vishnu Sukumari Nath*, Shyni Basheer, Muthulekshmi Lajapathy Jeeva and Syamala Swayamvaran Veena

Division of Crop Protection, Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala, India

Abstract

Phenotypic and molecular methods were used for characterizing 40 *Phytophthora colocasiae* isolates obtained from Andhra Pradesh, Assam, Kerala, and Odisha regions of India over a period of five years. Phenotypic parameters such as virulence, colony morphology and mating type varied among isolates collected from different regions over the years. No correlation was observed between phenotypic parameters of the isolates and their geographical origins. Considerable inter and intra specific variation were detected by random amplified microsatellites (RAMS) analysis with 100% polymorphism among the isolates. Dendrogram constructed based on RAMS data using the unweighted pair group method with arithmetic mean (UPGMA) grouped the *P. colocasiae* isolates into two major clusters. No relationship was obtained between RAMS groups of the isolates and phenotypic characters/geographical origin. Population genetic analysis showed that *P. colocasiae* isolates were highly diverse among different regions. Analysis of molecular variance (AMOVA) showed that most of the genetic variability in *P. colocasiae* was confined to within a population (93.21%). These results indicate that *P. colocasiae* populations in India are highly diverse and care should be taken in developing disease management programmes or in breeding resistant cultivars.

Keywords: Disease management; Molecular markers; Pathogen characterization; Population structure; Taro leaf blight

Introduction

Taro (*Colocasia esculenta* (L.) Schott) which belongs to Araceae family is a major root crop with wide distribution in the tropics. Taro leaf blight caused by *Phytophthora colocasiae* is a major bottleneck for taro production worldwide, including India causing yield loss of up to 50% [1-4]. *Phytophthora colocasiae* can infect at any stage of the plant resulting in extensive damage of the foliage. Initial symptoms appear as small, water-soaked circular spots on the edges of the leaves. As the disease progresses, these spots enlarge, coalesce, and become dark brown in color with yellow margins and finally the entire leaf is destroyed. Epidemics are common in temperatures close to 20°C to 25°C with a relative humidity of 90% to 100% [2,4,5]. The disease is more severe in northern and eastern parts of the country which are major areas of taro production. While, in South India, the disease appears periodically in serious proportions [3].

At present, metalaxyl based fungicides are used to manage taro leaf blight. However, the presence of waxy coating on leaf lamina and the occurrence of disease during rainy season make this approach ineffective leading to rapid epidemics and crop loss. Besides this development of resistance to fungicide is another major concern. Build up of resistance to metalaxyl has already been demonstrated in field isolates of *P. colocasiae* [6]. Few cultivars are resistant to leaf blight and resistance breeding offers great potential, but the durability of resistance is largely challenged by the emergence of new virulence strains of pathogen [7].

P. colocasiae is usually diploid and requires the presence of A1 and A2 mating types for production of sexual oospores. The oospore not only serves as the overwintering propagules in the soil and as a source of initial inoculum for subsequent crops, it also contributes to genetic variability through potential new gene combination [8]. In the absence of sexual spores, the pathogen survives as asexual clones in infected plant or in harvested tubers. Mycelium from such infections produces numerous sporangia that are disseminated by wind or rain-splashes to a new host where they germinate directly or release multiple motile zoospores and thus the cycle continues. The abundant production of sporangia or zoospores, and ability to infect and colonize host tissue combined with the efficient dissemination makes *P. colocasiae* a devastating plant pathogen [4].

Effective management of taro leaf blight is only possible by understanding the characteristics of pathogen population. Molecular markers provide useful tools to track individual genotypes and also to study population diversity. *P. colocasiae* genetic diversity has been assessed using diverse genetic markers. Random amplified polymorphic DNA (RAPD) and Isozyme markers were employed to analyze the genetic diversity in *P. colocasiae* isolates from Southeast Asia and Pacific regions [9] and as well as from India [4]. Recently, amplified fragment length polymorphism (AFLP) and RAPD have proved effective in projecting genetic diversity in *P. colocasiae* isolates [10,11]. However, till date no studies have been reported concerning genetic diversity analysis of *P. colocasiae* using random amplified microsatellite markers (RAMS). RAMS technique was originally described by Zietkiewicz et al. [12] in which the DNA between the distal ends of two closely located microsatellites is amplified and the resulting PCR products are separated electrophoretically. RAMS can be performed easily with sufficient reproducibility and thereby offers advantages over RAPD and laborious techniques like AFLP.

This research was conducted to assess phenotypic and genotypic diversity of the *P. colocasiae* isolates collected from Andhra Pradesh,

***Corresponding author:** Vishnu Sukumari Nath, Division of Crop Protection, Central Tuber Crops Research Institute, Thiruvananthapuram 695017, Kerala, India, Tel: +919400368701; Fax: +914712590063; E-mail: vishnu4you007@gmail.com

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Assam, Kerala, and Odisha regions of India over a period of five years (2007-2012).

Materials and Methods

Isolates

A total of 40 *Phytophthora colocasiae* isolates were obtained from leaf blight infected samples collected primarily from Andhra Pradesh, Assam, Kerala, and Odisha regions from 2007-2012 which represents the major taro growing regions of India. Isolation and maintenance of the isolates were carried out according to Nath et al. [10]. All isolates were confirmed to the species level using species-specific PCR assay as described earlier [11]. Details of the isolates used in this study are given in Table 1.

Colony morphology

Colony morphology was studied on potato dextrose agar medium (PDA; 250 g/L potato, 20 g/L dextrose and 20 g/L agar). A 5 mm disc excised from the periphery of actively growing colony of *P. colocasiae* was placed at the centre of petri dishes containing PDA. Plates were incubated at 28°C in the dark for two weeks. Following incubation, morphology of *P. colocasiae* was characterized based on the colony texture. Three replicates were used for each isolate to confirm the colony characteristics at same incubation conditions mentioned above.

Virulence assay

All *P. colocasiae* isolates were assessed for their virulence using a floating leaf disc method. Five leaf discs (5 × 5 cm) of taro (cv Sree

Isolate code	Location	District/sampling site	Year of collection	Colony on PDA medium*	Mating type#	Lesion diameter (cm)#
P1	Kerala	Block 2, CTCRI field	2010	Group H	A1	1.26 ± 0.05^{c}
P3	Kerala	Block 1, CTCRI field	2010	Group H	A1	2.20 ± 0.10^{f}
P21	Kerala	Farm, CTCRI	2011	Group H	A1	3.20 ± 0.10^{i}
P42	Kerala	Thiruvananthapuram	2012	Group E	A1	3.30 ± 0.20^{i}
P9	Kerala	Thiruvananthapuram	2008	Group E	A1	0.00 ± 0.00^{a}
P4	Kerala	Aleppy	2011	Group F	A1	3.86 ± 0.05^{j}
P7	Kerala	Pathanamthitta	2011	Group F	A1	3.10 ± 0.10^{h}
P35	Kerala	Pathanamthitta	2011	Group F	A1	3.00 ± 0.10^{h}
P6	Kerala	Kottayam	2011	Group F	A1	3.86 ± 0.05^{j}
P23	Kerala	Kollam	2010	Group E	A1	1.46 ± 0.05^{d}
P15	Kerala	Haripad	2012	Group F	A1	4.53 ± 0.05^{k}
P28	Kerala	Idukki	2010	Group I	A1	1.26 ± 0.05^{c}
P11	Kerala	Calicut	2007	Group B	A2	0.00 ± 0.00^{a}
P32	Kerala	Block 2, CTCRI	2012	Group D	A1	3.30 ± 0.20^{i}
P33	Kerala	Block 2, CTCRI	2012	Group D	A1	3.28 ± 0.10^{i}
P22	Kerala	Calicut	2008	Group D	A1	0.00 ± 0.00^{a}
P16	Andhra Pradesh	Veerwada	2010	Group D	A1	1.26 ± 0.05^{c}
P34	Andhra Pradesh	Veerwada	2010	Group D	A2	1.24 ± 0.05^{c}
P26	Andhra Pradesh	East Godawari	2011	Group D	A1	2.60 ± 0.10^{g}
P29	Andhra Pradesh	Parudin pallam	2010	Group D	A1	1.76 ± 0.05^{e}
P36	Andhra Pradesh	Parudin pallam	2011	Group D	A1	2.50 ± 0.10^{g}
P27	Andhra Pradesh	Veerwada	2011	Group D	A1	2.53 ± 0.05^{g}
P5	Odisha	Nayagarh	2007	Group A	A1	0.00 ± 0.00^{a}
P12	Odisha	Khandapara	2007	Group A	A1	0.00 ± 0.00^{a}
P2	Odisha	RC, CTCRI	2008	Group C	A1	0.00 ± 0.00^{a}
P13	Odisha	Salepur	2008	Group A	A1	0.83 ± 0.05^{b}
P24	Odisha	Puri	2007	Group A	A1	0.00 ± 0.00^{a}
P25	Odisha	Puri	2007	Group A	A1	0.00 ± 0.00^{a}
P14	Uttar Pradesh	Malikpur	2007	Group A	A1	0.00 ± 0.00^{a}
P39	Uttar Pradesh	Malikpur	2008	Group A	A1	0.80 ± 0.05^{b}
P43	Uttar Pradesh	Malikpur	2009	Group A	A1	1.24 ± 0.05^{c}
P17	Delhi	New Delhi	2010	Group G	A1	0.83 ± 0.05^{b}
P19	Assam	Nellie Road	2007	Group B	A1	0.00 ± 0.00^{a}
P46	Assam	Nellie Road	2007	Group C	A2	0.00 ± 0.00^{a}
P30	Assam	Nellie Road	2010	Group B	A2	1.26 ± 0.05^{c}
P8	Meghalaya	Ribhoi	2009	Group C	A1	0.83 ± 0.05^{b}
P20	Meghalaya	Nongpoh	2010	Group G	A1	1.46 ± 0.05^{d}
P10	West Bengal	Nadia	2009	Group A	A1	1.76 ± 0.05^{e}
P18	Tripura	West Tripura	2010	Group A	A1	2.20 ± 0.10^{f}
P38	Tripura	West Tripura	2010	Group A	A1	2.00 ± 0.00^{f}

some of the results have been previously published [10,11]. *Group A: Cottony; Group B: Stellate; Group C: Cottony with concentric rings; Group D: Plain with irregular concentric rings; Group E: Irregular pattern; Group F: Plain; Group G: Uniform with concentric rings; Group H: Uniform without pattern; Group I: Flat with concentric rings

Table 1: Characteristics of *Phytophthora colocasiae* isolates used in this study.

Kiran, leaf blight susceptible) were floated in sterile distilled water in 200 mm glass petri plates and inoculated with a mycelial disc excised from the margins of actively growing cultures of *P. colocasiae*. Leaf discs with sterile agar plugs served as control experiment. Following inoculation, the leaf discs were incubated at 25°C in dark and daily examined for disease symptoms. The lesion diameter was recorded 4 days after inoculation (d.a.i.). Isolations were established from resulting lesions in order to confirm the association of the pathogen with the observed symptom. There were five replicates for each isolate and the assay was repeated twice.

Mating type determination

The mating type of isolates was determined by paring each unknown isolate with the isolate of a known A1 (98-111) and A2 (98-35a) mating type on carrot agar (CA) medium at 5 cm apart. After incubation at 28°C in darkness for 4 weeks, agar blocks were examined microscopically. The presence of oospores at the interface between colonies indicated opposite mating type while absence of oospores indicated the same mating type. The single culture of each isolate was paired to examine for oospore formation as a control. The positive control was a cross between two tester isolates of opposite mating types. Three replicates were used for each isolate.

DNA isolation

For DNA isolation, *P. colocasiae* isolates were grown in potato dextrose broth medium (PDB; 250 g l^{-1} potato, 20 g l^{-1} dextrose) at 28°C with 50 rpm. After achieving sufficient growth, DNA was extracted from mycelium using a Genomic DNA purification kit (Fermentas, EU) according to manufacturer's protocol. The nucleic acid obtained was dissolved in TE buffer (100 µl; pH=8.0). The quality and integrity of DNA were assessed by agarose gel electrophoresis and stored at -20°C until further use.

RAMS analysis

The list of primers used for the study is presented in Table 2. To optimize the method, a preliminary RAMS assay was performed using different reagent concentrations and PCR reaction conditions on a random sample of 5 isolates representing different geographical origins (data not shown). Each 25 µl of PCR reaction consisted of 50 ng of template DNA, 100 µM each dNTPs, 20 ng of primer (Integrated DNA Technologies, Coralville, USA), 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 (Merck Genei, India). The samples were denatured by 10 min incubation at 95°C after which 35 (CGA- and GT-primers) or 37 (CCA-primer) cycles of amplification were carried out (30 s denaturation at 95°C, 45 s annealing at a temperature depending on the primer, 2 min primer extension at 72°C). The annealing temperatures for the primers were as follows: CCA-primer 55°C, CGA-primer 59°C, and GT-primer 46.6°C. After the cycles, the reaction was ended with a 7 min extension at 72°C. Amplified products were separated on a 1.8% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and visualized under UV light. Gel photographs were acquired using a Gel Doc System (Alpha Imager, Alpha Innotech, California, USA). The size of the amplification products was estimated by comparison with 1 kb plus DNA ladder (Fermentas). The assay was repeated two times with template DNA from two different DNA extractions to ensure the consistency of each band.

*Primer code	Sequence#	Total no. of bands	No. of polymorphic bands	Mean no. of bands
CGA	5'DHB$(CGA)_5$	11	11	1.60
GT	5'YHY$(GT)_7$G	10	10	3.0
CCA	5'DDB$(CCA)_5$	10	10	0.97
*Source Hantula et al. [28]. #The following designations are used for degenerate sites: B (G, T, or C), D (G, A, or T), H (A, T, or C), and Y (A, C or G).				

Table 2: Summary statistics of RAMS analysis of *Phytophthora colocasiae*.

Data analysis

All clearly detectable and distinct RAMS bands were scored for their presence (1) or absence (0) by visual inspection. Based on the scores, a genetic similarity matrix was constructed and a dendrogram was deduced to display relationships between isolates using the Nei and Li distance [13] according to the unweighted pair group mean algorithm using the TREECON software package version 1.3 [14]. The reliability of the clustering was assessed by bootstrap analysis (2000 replicates). A cophenetic correlation coefficient was calculated to assess the statistical support for the dendrogram obtained, and Mantel's test [15] was performed to check the goodness-of-fit of the cluster analysis (1000 permutations). The data within a cluster are most likely to be highly reliable when the value of cophenetic correlation coefficient was ≥0.8 [16].

The similarity matrix generated was also used for the analysis of molecular variance (AMOVA) [17] by using FAMD Software version 1.25 [18]. This analysis helps to understand how the genetic diversity is partitioned among populations of *P. colocasiae* (called Phi statistics).

Population genetic parameters such as percentage of polymorphic loci (*P*), observed number of alleles (N_A), effective number of alleles (N_E), Nei's gene diversity (*H*), and Shannon index (*I*) of *P. colocasiae* isolates based on RAMS markers were analyzed using the computer program POPGENE 32 [19]. Loci were considered polymorphic if more than one allele was detected.

Results

Isolation of pathogen

A total of 40 isolates of *P. colocasiae* were isolated from leaf blight infected samples collected from Andhra Pradesh, Assam, Kerala, and Odisha regions of India (Table 1). All isolates were confirmed as *P. colocasiae* using species specific PCR assay and produced 206 bp amplicon when amplified using PCSP RL-F and PCSP RL-R primers [11].

Colony morphology

The isolates exhibited diverse colony morphology when grown on PDA medium (Table 1 and Figure 1). There was no general trend for the morphology exhibited by the isolates from different geographical regions. The isolates collected from the same location but different sampling sites had different morphology. Also, isolates collected from same sampling site in consecutive years showed difference in their morphology (e.g. Block 2, CTCRI field). Based on the colony morphology the isolates were classified into different morphology groups as described earlier [10].

Virulence assay

The majority of the isolates was able to infect leaf disc and produced typical symptoms of leaf blight. A few isolates that were collected before 2008 failed to initiate disease symptoms. The isolates initiated lesion development 2 d.a.i. Lesions appeared yellow in the beginning, which turned dark brown with progression of the disease. There was a significant difference in the lesion diameter among the isolates collected from different regions with recently obtained isolate (P15) from kerala state being more aggressive (4.53 ± 0.05; P ≤ 0.5). The *P.*

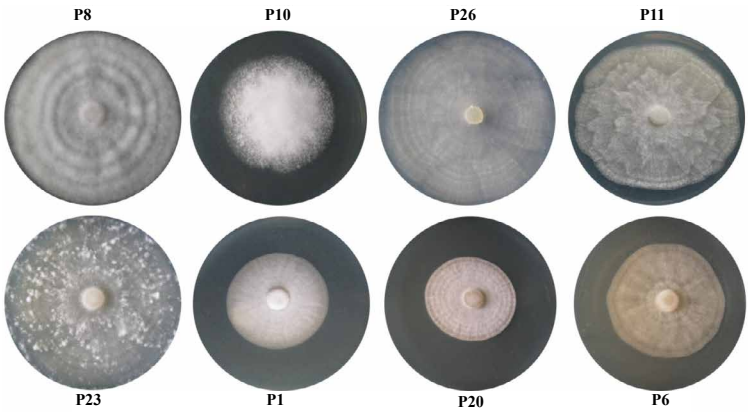

Figure 1: Colony morphology exhibited by *P. colocasiae* isolates collected from different regions of India on PDA medium.

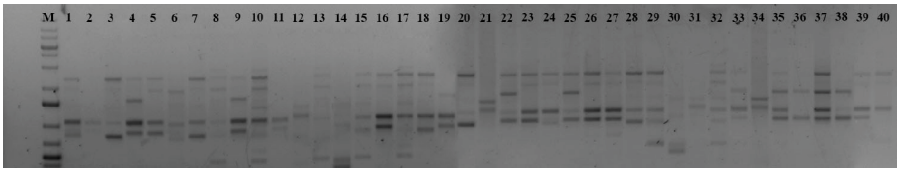

Figure 2: Example of RAMS amplification of *P. colocasiae* isolates by GT primer.

colocasiae isolates collected earlier (2009-2010) were found to be less virulent (0.83 ± 0.05 to 2.20 ± 0.10; P ≤ 0.5) (Table 1). No lesions were produced on the control leaf discs. The pathogen was successfully re-isolated from the lesions completing Koch's postulate.

Mating type determination

All the isolates were heterothallic and produced oospores when paired with known A1 or A2 mating types on carrot agar. Out of the 40 isolates analyzed, 34 (90%) isolates were of A1 mating type while the remaining 4 (10%) isolates were of A2 mating type (Table 1). Isolates collected from different regions showed A1 and A2 mating types. No isolate produced homothallic oospores (A0, or A1/A2).

RAMS analysis

Forty isolates of *P. colocasiae* from various geographical origins of India were analyzed to estimate the level of genetic diversity by using RAMS markers. The bands were distinct, reproducible and easy to score. To ensure reliability in scoring, all markers were scored at least twice. The three primers amplified 31 reproducible fragments ranging in size from 500 to 2000 bp, of which 31 (100%) were polymorphic. The highest number of amplification products (11) was obtained with the CGA primer, while GT and CCA primer had 10 bands each. A summary of the RAMS data is presented in Table 2. Examples of RAMS DNA fingerprints are shown in Figure 2.

The UPGMA dendrogram grouped the isolates into two major clusters (Figure 3) with high bootstrap values. Cluster I had 45 isolates and formed the major group, while cluster II had only 5 isolates. The clustering of isolates in the dendrogram was not correlated with geographical origin or phenotypic characters. The cophenetic correlation coefficient between dendrogram and the original similarity matrix was significant for RAMS marker (r=0.898).

Analysis of genetic diversity

Genetic parameters varied among populations with the percentage of polymorphic bands (*PPB*) values ranging from 45.45% (Kerala) to 72.73% (Andhra Pradesh), with an average of 61.365%. The average Nei's gene diversity (*H*) was estimated to be 0.10 within populations and 0.11 for the pooled populations. The observed number of alleles (*NA*) and the effective number of alleles (*NE*) varied among populations (Table 3).

Analysis of molecular variance (AMOVA) revealed that a high percentage (93.62%) of the *P. colocasiae* genetic diversity in this study was distributed within populations and only 6.37% among populations (Table 4). The coefficient of genetic differentiation among populations (*GST*) was 0.049, which supports the AMOVA analysis indicating only limited genetic diversity among populations and high diversity within populations. The estimate of gene flow (*Nm*) among populations was 9.68 migrants per generation, obtained from the GST value.

Discussion

Taro leaf blight caused by *P. colocasiae* leads to significant economic loss in taro cultivation globally. A better understanding of *P. colocasiae* population dynamics will contribute to more durable disease management strategies. Here, we analyzed *P. colocasiae* isolates collected over a period of five years (2007-2012), to understand the overall population structure with respect to phenotypic and genotypic diversity. Our results indicate that considerable intra specific diversity exists among *P. colocasiae* isolates used in this study.

Analysis of colony morphology on PDA revealed that *P. colocasiae* isolates have highly diverse morphology with isolates collected from different regions showing different morphologies. Our results were consistent with a previous study by Misra et al. [20], however the present study revealed more morphological groups, probably due to the greater number of isolates used in this study, which provides a better coverage of different geographical regions of India. One of the striking results of the study was the finding that *P. colocasiae* isolates collected from the same sampling site in different years had different

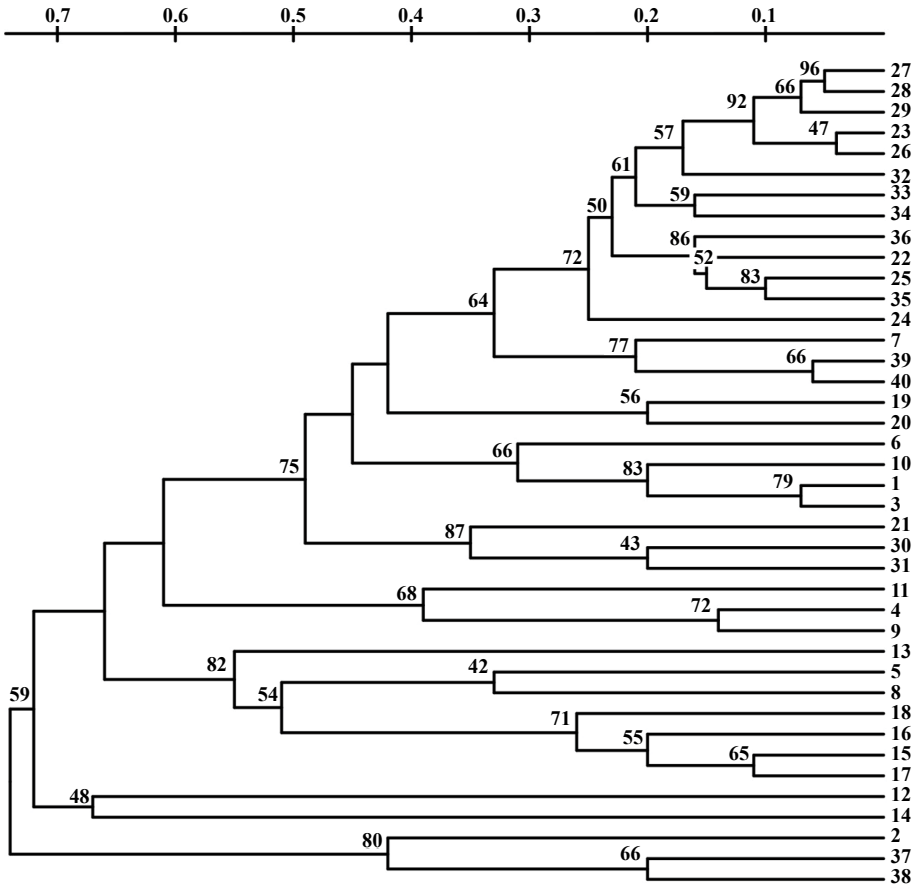

Figure 3: UPGMA dendrogram of *Phytophthora colocasiae* isolates based on RAMS analysis. Numbers at nodes indicate bootstrap values (2000 replications).

Marker	Population Code	Polymorphic bands	PPB[a](%)	N_A[b]	N_E[c]	H[d]	I[e]
RAMS	Kerala	15	45.45	1.45 ± 0.50	1.14 ± 0.19	0.10 ± 0.13	0.17 ± 0.21
	Andhra Pradesh	24	72.73	1.72 ± 0.45	1.14 ± 0.14	0.11 ± 0.10	0.21 ± 0.16
	Assam	19	57.58	1.57 ± 0.50	1.14 ± 0.18	0.10 ± 0.12	0.18 ± 0.19
	Odisha	23	69.70	1.69 ± 0.46	1.12 ± 0.10	0.10 ± 0.08	0.18 ± 0.14
	Total	33	100	2.00 ± 0.00	1.13 ± 0.09	0.11 ± 0.07	0.21 ± 0.11

[b] Observed number of alleles (NA), [c] Effective number of alleles (NE); [d] Nei's gene diversity (H); [e] Shannon's information index (I)

Table 3: Population genetic analysis of *Phytophthora colocasiae* isolates.

Marker	Source	df	SSD	Φ statistics	Variance components	Proportion of variation components (%)
RAMS	Among Populations	3	2.25	0.067	0.032	6.78
	Within populations	36	16.07		0.446	93.21
	Total	40	18.33		0.479	

Table 4: AMOVA analysis of *Phytophthora colocasiae* isolates.

morphology. This observation clearly suggests that *P. colocasiae* populations are constantly evolving in nature.

P. colocasiae is a soil borne pathogen and a heterothallic species, requiring the presence of A1 and A2 mating types for sexual reproduction. In our study, the presence of both mating types was detected, with the A1 type occurring at a higher frequency than the A2 type. However, the A1 and A2 type were not found in close proximity (e.g. in a single field), which facilitates sexual reproduction under favourable conditions. Similar results were reported by Misra et al. [20], who also observed the lack of compatible mating types (A1 and A2) in India. Recently, a study by Tyson and Fullerton [21] found only one mating type of *P. colocasiae* (A2) throughout the Pacific region, including Guam, Hawaii, Indonesia, the Philippines, Papua New Guinea, and Samoa. Therefore, from the results it can be commented that sexual recombination may not be playing a major role in the high diversity seen among the Indian *P. colocasiae* isolates.

The results of the virulence tests showed a significant difference in the mean lesion diameter of all the isolates studied. Majority of the isolates was highly virulent and were able to cause serious infection on taro leaf discs irrespective of the geographical origin. The variation in aggressiveness of the isolates projects their high genetic diversity. It is also possible that the variation could be the outcome of the differences

in resistance levels of various cultivars from which these isolates were obtained. Difference in virulence property as observed in this study has also been reported in other *Phytophthora* spp. [22,23] and in other plant pathogens [24,25]. No correlation could be elucidated from the phenotypic or genotypic characteristics and geographical origin of the isolates.

This is the first time that RAMS markers have been used for assessing the genetic diversity of Indian *P. colocasiae* isolates. In our study, these markers revealed an intraspecific diversity of *P. colocasiae* isolates from different regions. Profound genetic diversity was evident with 100% polymorphism among the isolates. UPGMA dendrogram grouped the isolates into two clusters with high bootstrap values. Nei's gene diversity examination revealed that the *P. colocasiae* were highly different within a close population, which was further confirmed by AMOVA analysis. The isolates were not grouped according to their geographical origin or phenotypic characters, which was in agreement with previous reports on genetic diversity analysis in *P. colocasiae* [4,7,9,10]. This observation reinforces the fact that *P. colocasiae* frequently move within the country, contributing to the increased genetic diversity of the pathogen.

Several reasons could be attributed to the high intrazonal diversity detected in the present investigation. It is an accepted fact that sexual recombination increases genotype diversity in populations by creating novel recombinants. Mechanisms such as mutation, translocations, chromosomal deletions and duplications are common in *Phytophthora* species [26], which may also be responsible for the genetic variation observed in the *P. colocasiae* populations. According to Goodwin [26], mutation is regarded as the primary source of genetic variation in oomycetes. These mutations in most cases can be neutral and may not cause any observable changes in phenotype, but it is possible that at least a part of the genotype variation might have been the result of spontaneous mutation [27,28]. In *Phytophthora sojae,* mitotic gene conversion was observed to occur at remarkably high frequencies leading to rapid generation of variation.

Conclusion

This study represents a comparative analysis of phenotypic and genotypic diversities present among isolates of *P. colocasiae* from India. Although the number of isolates used is limited, the study nevertheless projects the extend of genetic diversity among *P. colocasiae* isolates from different regions of India. The presence of high phenotypic and genetic variation within the *P. colocasiae* population in India may prove detrimental to the development of sustainable management strategies to curb the disease. More precise conclusions regarding gene flow and genetic recombination would require comprehensive studies on the *P. colocasiae* population with more representative isolates using codominant DNA markers.

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