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Isozyme and RAPD variation among *Phytophthora colocasiae* isolates from South-east Asia and the Pacific

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Isozyme variation was studied in 94 isolates of *Phytophthora colocasiae* originating from Indonesia, Papua New Guinea, the Philippines, Thailand and Vietnam. Eight polymorphic enzyme systems (HK, PGM, PGI, Gluco, MDH, ICD, 6PDH, ME) revealed 52 isozyme patterns (zymotypes), each uniquely characterized by the presence or absence of 60 electromorphs. A core sample of 20 isolates was subsequently analysed with RAPD markers. Seven primers were used successfully and all profiles were reproducible. Clear bands were revealed and, in some cases, allowed differentiation between isolates exhibiting identical zymotypes. Results indicate that throughout this vast geographic region, taro leaf blight is caused by numerous and distinct strains that are genetically variable. Variation occurs within and between countries. The geographical distribution of zymotypes shows that none is common to two different countries. Although the differences in pathogenicity are not yet established, different *P. colocasiae* genotypes are likely to recombine and evolve rapidly as this species is heterothallic. From these results, a long-term breeding strategy is recommended for taro (*Colocasia esculenta*) based on recurrent selection using a wide genetic base composed of carefully selected parents from diverse geographic origins to maximize multigenic resistance in progenies.

Keywords: *Colocasia esculenta* isozymes, *Phytophthora colocasiae*, RAPD, taro leaf blight

Introduction

Taro (*Colocasia esculenta*) is grown over more than 1·8 million hectares annually in the world (FAO, 2000) and is an important food crop in many parts of the humid tropics. Several thousand cultivars exist in South-east Asia, where the crop is thought to have originated. *Phytophthora colocasiae* is the causal pathogen of taro leaf blight and one of the major constraints to the development of this crop. In all countries, most cultivars are susceptible while others are resistant or immune to leaf blight. However, little is known about the genetic diversity of the pathogen. When attempting to compare the performance of cultivars, it is somewhat difficult to assess accurately what is due to the genetic variability of *C. esculenta* and what is due to the possible variation of different strains of *P. colocasiae* existing in different countries.

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Phytophthora colocasiae was first described in Java causing taro leaf blight (Raciborski, 1900). The oomycetous pathogen is postulated to have moved from Java to Taiwan (Butler & Kulkarn, 1913) and subsequently from Taiwan to Japan and then Hawaii as early as 1920 (Trujillo, 1967). Taro leaf blight is now distributed throughout tropical Asia from India to China. The most recent dispersal of the pathogen has been into the South Pacific where taro is a major staple food and an important cash crop. It has spread to Australia, New Guinea and the Solomon Islands (Parham, 1949) and was introduced in 1993 to Samoa where it had disastrous effects on the national economy and the welfare of farmers. Thus far, the pathogen has not been reported in Fiji, French Polynesia, New Caledonia, Tonga or Vanuatu.

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. The epidemiology is characterized by rapid outbreaks in new areas resulting from the introduction of infected propagules. Endemicity occurs even in the absence of rainfall, but in most countries weather conditions

favour infections. Once infected, a leaf can be killed in a few days, the lower leaves often being more severely infected. Yield losses vary from 30 to 50% due to severe defoliation (Jackson, 1977; Vasquez, 1990). The pathogen has a limited host range and the sporangia are the main dispersal units, but *P. colocasiae* survives in corms and cormels and produces chlamydospores, and occasionally oospores (Erwin & Ribeiro, 1996; Misra & Chowdhury, 1997). Conditions for sporulation are optimum around 20–22°C with 100% relative humidity (RH) and sporulation occurs in less than 3 h. Sporangia are spread in leaf exudate, rain splash or wind blown rain, within and between plants. Survival of the pathogen in the soil is rather short, from a few days to 3 months (Jackson, 1977, 1980).

Isozyme analysis has been shown to be useful for the study of genetic variation existing between and within fungal species (Micales *et al*., 1986). The assessment of genetic distances between isolates within species has proven to be useful in *Phytophthora* systematics and diversity studies (Blaha, 1992). More recently, molecular (DNA) markers have also been shown to provide useful tools for accurately differentiating isolates of *Mycosphaerella fijiensis* (Carlier *et al*., 1996) and *P. infestans* (Mahuku *et al*., 2000). The objective of this study was to assess the extent of genetic variation existing within *P. colocasiae* in South-east Asia and Oceania using isozymes and to evaluate the potential of RAPD analysis for isolate fingerprinting. This work may provide the basis for future studies on the population structure of the taro leaf blight pathogen and the development of cultivars with improved resistance to *P. colocasiae*.

Materials and methods

Germplasm

More than 1780 accessions of taro maintained in national germplasm collections and representing most of the cultivar diversity existing within each country surveyed – Indonesia (ID), Papua New Guinea (PG), the Philippines (PH), Thailand (TH) and Vietnam (VN) – were evaluated for their susceptibility to taro leaf blight caused by *P. colocasiae*. Using the protocol described by Ivancic & Lebot (2000), plants were scored (immune, resistant, tolerant, susceptible or very susceptible). Evaluation was conducted during two consecutive growing seasons (1998–99 and 1999–2000). All plants were local cultivars.

Pathogen isolates

Overall, 94 isolates of *P. colocasiae* were collected from mature leaves showing typical symptoms of taro blight, including susceptible and tolerant cultivars. Twenty-one isolates originated from Indonesia, eight from Papua New Guinea, 15 from the Philippines, 45 from Thailand and five from Vietnam. The geographical origins of the isolates are presented in Table 1. Collection was spread over a period of 18 months, during 1998 and 1999. Isolates were sent from SE Asia to CIRAD (Centre International de Recherche Agronomique pour le Développement) laboratories in Montpellier, France. All isolates received were purified successfully. *Phytophthora colocasiae* was confirmed in all 94 isolates by comparing their morphology with several other accessions of *P. colocasiae* maintained in CIRAD collections. Typically *P. colocasiae* is characterized by the production of ovoid, ellipsoid or fusiform semipapillate sporangia that are caducous and with a medium pedicel (3·5–10 μm); chlamydospores are abundant in some isolates. Isolation of samples was done by tissue transplanting on selective media which consisted of benomyl 10 μg mL^{-1}, nystatin 25 μg mL^{-1}, pentachloronitrobenzene (PCNB) 25 μg mL^{-1}, rifampicin 10 μg mL^{-1}, ampicillin 10 μg mL^{-1} and hymexasol 10 μg mL^{-1} as a stock solution and adding 2 mL of this stock solution to 200 mL of potato dextrose agar. After isolation, the spores were grown on the same agar but without hymexasol and thereafter mycelial cultures were routinely maintained at 25°C on V8 juice agar medium (200 mL of V8 juice, 2·5 g of $CaCO_3$, 15 g of agar and 800 mL of distilled water) without illumination. Mycelial plugs from actively growing colonies were inoculated on V8 agar medium in Petri dishes with cellophane overlays and grown for 7 days in the dark at 25°C. The mycelial mats were collected and transferred into sterilized Eppendorf tubes with 1 mL of cold citrate buffer (pH 7·0). Triton X–100 at 1:10 of the volume of the buffer was added and each isolate was sonicated for 30 s at 4°C to break the cell walls and then centrifuged at 10 000 ***g*** for 15 min at 4°C. Supernatants were collected and stored at –20°C.

Isozymes

Protein extracts were obtained and subjected to electrophoresis. Protocols for isolate preparation, specific protein extraction, gel electrophoresis and enzyme-staining recipes have been described in detail elsewhere (Blaha, 1992; Ortiz-Garcia *et al*., 1993). Eight enzyme assay systems (hexokinase, HK; phosphoglucomutase, PGM; phosphoglucoisomerase, PGI; glucosidase, Gluco; malate dehydrogenase, MDH; isocitrate dehydrogenase, ICD; glucose-6-phosphate dehydrogenase, G6PDH; malic enzyme, ME) were successfully used and clear banding patterns were revealed. Isolate banding patterns (zymograms) were scored for each enzyme system and compared with those obtained from four different *Phytophthora* species – *P. megakarya* (two isolates), *P. capsici* (two isolates), *P. palmivora* (two isolates) and *P. citrophthora* (one isolate) – in order to determine if misidentifications might have occurred. Overall, 101 distinct isolates were studied for their isozyme variation. The stained gels were scored for the presence (1) or absence (0) of 60 different electromorphs, including 11 electromorphs for HK, five for PGM, seven for PGI, seven for Gluco, 12 for MDH, five for ICD, three for G6PDH, and 10 for ME. No interpretation of the genetic significance of the banding patterns was attempted. Polymorphic zymograms observed for each enzyme system were identified by different letters. Distinct zymotypes were therefore determined by

Table 1 Geographical origin of the *Phytophthora colocasiae* isolates collected in South-east Asia and analysed for isozyme variation

Country	Locality	Collector no.	CIRAD no.
Indonesia	Cugenang, West Java	INA 61	ID1*
Indonesia	Bogor, West Java	INA 90	ID2*
Indonesia	Sukabumi, West Java	INA 95	ID3*
Indonesia	Sukabumi, West Java	INA 101	ID4
Indonesia	Pandegelang, West Java	INA 136	ID16
Indonesia	Pandegelang, West Java	INA 142	ID5
Indonesia	Lebak, West Java	INA 161	ID6
Indonesia	Lebak, West Java	INA 169	ID7
Indonesia	Malang, East Java	INA 185	ID17
Indonesia	Malang, East Java	INA 226	ID18
Indonesia	Yogyakarta, East Java	INA 272	ID8
Indonesia	Yogyakarta, East Java	INA 324	ID19
Indonesia	Bali	INA 342	ID9
Indonesia	Kalimantan	INA 403	ID10
Indonesia	Lampung	INA 568	ID20
Indonesia	Lampung	INA 574	ID11
Indonesia	Mentawai Islands	INA 590	ID12
Indonesia	Mentawai Islands	INA 595	ID13
Indonesia	Mentawai Islands	INA 600	ID14
Indonesia	Mentawai Islands	INA 628	ID15
Indonesia	Mentawai Islands	INA 625	ID21*
Papua New Guinea	Ato, Madang	531	PG1*
Papua New Guinea	Bubia, Morobe	561	PG2*
Papua New Guinea	Bubia, Morobe	562	PG3*
Papua New Guinea	Passi, Sandaun	607	PG4*
Papua New Guinea	Gona, Oro	569	PG5
Papua New Guinea	Kwaliliau, Milne Bay	572	PG6
Papua New Guinea	Woganluhu, Morobe	508	PG7
Papua New Guinea	Wau, Morobe	527	PG8
Philippines	Visayas, Leyte Island	VISGA1	PH1
Philippines	Visayas, Leyte Island	VISGA2	PH2
Philippines	Visayas, Leyte Island	VISGA3	PH3*
Philippines	Visayas, Leyte Island	VISGA4	PH4*
Philippines	Visayas, Leyte Island	VISGA5	PH5*
Philippines	Visayas, Leyte Island	VISGA6	PH6
Philippines	Visayas, Leyte Island	VISGA8	PH8
Philippines	Luzon	GS1	PH9
Philippines	Luzon	GS2	PH10
Philippines	Basey, Luzon	Basey2	PH12*
Philippines	Luzon	PS1	PH13
Philippines	Luzon	PS2	PH14
Philippines	Dulag, Luzon	Dulag2	PH16
Philippines	Luzon	AB21	PH21
Philippines	Luzon	SB25	PH25
Thailand	North	DOA1152	TH1
Thailand	North	DOA1153	TH2
Thailand	North	DOA1154a	TH3*
Thailand	North	DOA1154b	TH4
Thailand	North	DOA1155	TH5
Thailand	North	DOA1156	TH6
Thailand	North	DOA1157	TH7
Thailand	North	DOA1158	TH8
Thailand	North	DOA1159	TH9
Thailand	North	DOA1160	TH10
Thailand	North	DOA1161	TH11*
Thailand	North	DOA1162	TH12*
Thailand	North	DOA1164	TH14*
Thailand	North	DOA1165	TH15
Thailand	North	DOA1167	TH17
Thailand	North	DOA1169	TH19
Thailand	North-east	DOA1170	TH20

Table 1 *continued*

Country	Locality	Collector no.	CIRAD no.
Thailand	North	DOA1171	TH21
Thailand	North	DOA1172	TH22
Thailand	North	DOA1173	TH23
Thailand	North-east	DOA1174	TH24
Thailand	North-east	DOA1175	TH25
Thailand	North-east	DOA1176	TH26
Thailand	North-east	DOA1177	TH27
Thailand	North-east	DOA1178	TH28
Thailand	North-east	DOA1180	TH30
Thailand	North-east	DOA1181	TH31
Thailand	North-east	DOA1183	TH33
Thailand	North-east	DOA1184	TH34
Thailand	North-east	DOA1185	TH35
Thailand	North-east	DOA1186	TH36
Thailand	North-east	DOA1187	TH37*
Thailand	North-east	DOA1188	TH38
Thailand	East	DOA1189	TH39
Thailand	East	DOA1190	TH40
Thailand	East	DOA1191	TH41
Thailand	East	DOA1192	TH42
Thailand	Central	DOA1193	TH43
Thailand	Central	DOA1195	TH45
Thailand	Central	DOA1197	TH47
Thailand	Central	DOA1198	TH48
Thailand	Central	DOA1199	TH49
Thailand	Central	DOA1202	TH52
Thailand	Central	DOA1203	TH53
Thailand	Central	DOA1204	TH54
Vietnam	Van Dien, Hanoi	VD1	VN1*
Vietnam	Dong Trieu, Quang Ninh	DT3	VN2*
Vietnam	Van Giang, Hung Yen	VG4	VN3
Vietnam	Vinh Tuong, Vin Phuc	VT7	VN4*
Vietnam	An Khanh, Ha Tay	AK5	VN5

*Isolates analysed with RAPD.

eight coded zymograms, each corresponding to an isozyme pattern. If two isolates were different for at least one electromorph (present or absent), they were considered to exhibit two different zymotypes.

Randomly amplified polymorphic DNA patterns (RAPD)

Randomly amplified polymorphic DNA pattern markers were used to study the variation existing at the DNA level between 20 *P. colocasiae* isolates. These isolates were selected on their geographical origin (four isolates from Indonesia, four from Papua New Guinea, four from the Philippines, four from Thailand, and three from Vietnam), but isolates with identical zymotypes in each country were also chosen so that the discriminating performance of RAPD markers could be assessed. Twenty *P. colocasiae* isolates exhibiting 10 distinct zymotypes were compared with one isolate of *P. citrophthora* because this species has been found to be closely related to *P. colocasiae*. Isolates were grown on a nutrient agar containing yeast extract (2 g mL^{-1}), glucose (10 g mL^{-1}), KNO_3 (3 g mL^{-1}), KH_2PO_4 (2 g mL^{-1}), adjusted to pH 6·0 using NaOH 5 M, sterilized at 120°C for 20 min and antibiotics were added (rifampicin 10 mg mL^{-1}, piramicin 10 mg mL^{-1}, ampicillin 250 mg mL^{-1}). DNA extraction was conducted using New Glucanex (Novo Nordisk Ferment Ltd, Bern, Switzerland – 18 mL H_2O, 42 mL NaCL 1 M, 1·8 g New Glucanex adjusted to pH 6·0). Digestion of cell walls was conducted in Eppendorf tubes (2 mL) for 2 h at room temperature, and precipitation was done using isopropanol and centrifugation for 7 min. Precipitates were washed with 70% ethanol. RNase treatment was done in 100 μL of TE with 1 μg mL^{-1} of RNase incubated at 37°C for 60 min. DNA concentration was evaluated on 0·8% agarose gels (1 μL DNA, 1 μL of 1% methyl blue, and 9 μL sterile H_2O). DNA extracts were diluted to 1 ng mL^{-1}. The solution for PCR amplification was composed of 5 ng of DNA extract, 2·5 μL of buffer (tris-HCl, pH 8·8, 67 mM NH_4, 16·6 nM $2SO_4$), 2 μL DNTP, 1 μL $MgCl_2$ (50 mM), 5 μL of primer, 9·2 μL H_2O, 0·3 μL H_2O, 0·3 μL of TAQ polymerase (Eurobio, Montpellier, France). The PCR programme consisted of 45 cycles of denaturation (95°C, 1 min), hybridization

Table 2 Primers used for RAPD analysis

Primers[a]	5′ 3′	Pur	Pyr	*T* °C	OD	Weight	Dry	Moles	Bands
M4	GGCGGTTGTC	5	5	34	4·9	3075·06	168·2	54·72	1
M6	CTGGGCAACT	5	5	32	5·9	3028·02	188·3	62·2	6
M7	CCGTGACTCA	4	6	32	6·5	2988·02	213·6	71·51	4
M17	TCAGTCCGGG	5	5	32	5·5	3044·05	182·9	60·09	5
R4	CCCGTAGCAC	4	6	34	6·5	2973·01	215·8	72·59	1
R6	GTCTACGGCA	5	5	32	5·0	3028·05	159·6	52·71	3
R11	GTAGCCGTCT	4	6	32	5·9	3019·03	200·3	66·35	11

[a]Oligo express: oligo@genomex.com.
Pur, no. of purine; Pyr, no. of pyrimidine; *T*, melting point; OD, optical density; Weight, molecular weight; Dry, dry weight; Bands, number of polymorphic bands observed.

(35°C, 1 min) and elongation (72°C, 8 min). Amplified extracts underwent electrophoresis on agarose gels (1·4% in TBE 1X) using constant voltage (100 V) for 3 h. Coloration was done by soaking the gel in an ethidium bromide solution for 115 min, followed by rinsing in water (10 min), observation and photography under UV. Seven primers were used (Table 2). 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. Overall, 81 electromorphs were identified and 21 were found to be polymorphic.

Data analysis

Cluster analysis of the binary isozyme data was performed 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 and the resulting dendrograms were compared:

$$\text{DICE} = 2a/(2a + b + c)$$

where a is the number of shared bands, b is the number of bands present only in isolate 1, and c is the number of bands present only in isolate 2. This SIMQUAL programme was also used to calculate the simple matching coefficient (SM) as a measure of genetic diversity for each pair of isolates:

$$\text{SM} = (a + d)/(a + b + c + d)$$

where d is the total number of bands absent in isolates 1 and 2. For each coefficient, the similarity matrix was used to construct dendrograms with the help of the unweighted pair grouping by mathematical averaging (UPGMA) methods, using the SAHN and TREE programmes in NTSYS. Principal component analysis was also conducted using the NTSYS software.

Results

In all countries surveyed, infection was notable during the wet and rainy season and isolates were successfully collected during that period on mature leaves. In most cases, it was observed that the most agronomically desirable cultivars were susceptible while the most resistant ones exhibited some undesirable traits (i.e. stolons, acridity, low yield, poor palatability). In Papua New Guinea, however, all accessions, including both recommended and poor cultivars, were found to be susceptible. In both the Philippines and Vietnam, approximately three-quarters of the accessions were found to be tolerant and resistant, compared with a third in Indonesia and approximately 5% in Thailand (Table 3).

Table 3 Geographical distribution of the resistance against leaf blight caused by *Phytophthora colocasiae* (percentages of accessions). The analysis was conducted during 1998–2000 in national germplasm collections. PNG, Papua New Guinea

	Philippines	Vietnam	Thailand	Indonesia	PNG
No. accessions	172	350	300	685	278
Reaction to infection					
Very susceptible	4·1	–	–	–	–
Susceptible	21·5	1·7	94·7	65·4	100
Tolerant	73·8	34·9	0·3	32·3	–
Resistant	0·6	41·1	5·0	2·3	–
Immune	–	22·3	–	–	–

Isozymes

The zymograms obtained for the eight enzyme systems and for the five distinct species of *Phytophthora* are shown in Fig. 1. The most polymorphic enzyme system was hexokinase (HK), with 11 different zymograms. PGM exhibited seven distinct zymograms, PGI exhibited five, Gluco exibited six, MDH exibited eight, ICD exibited six, G6PDH exibited four, ME exibited eight.

The zymotypes obtained for related *Phytophthora* species are presented in Table 4. Several zymograms were found to be species-specific and allowed isolates to be distinguished unambiguously. *Phytophthora citrophthora* exhibited only one species-specific zymogram at hexokinase (HK) while *P. megakarya* was found to be the most distinct species, exhibiting 10 species-specific zymograms (Table 4). Overall, 58 distinct zymotypes were identified for the five distinct *Phytophthora* species: 52 for *P. colocasiae* (Table 5) and six for the others (Table 4).

Zymograms and zymotypes obtained for the 94 *P. colocasiae* isolates, using the eight enzyme systems, are presented in Table 5. Two zymotypes were identified in

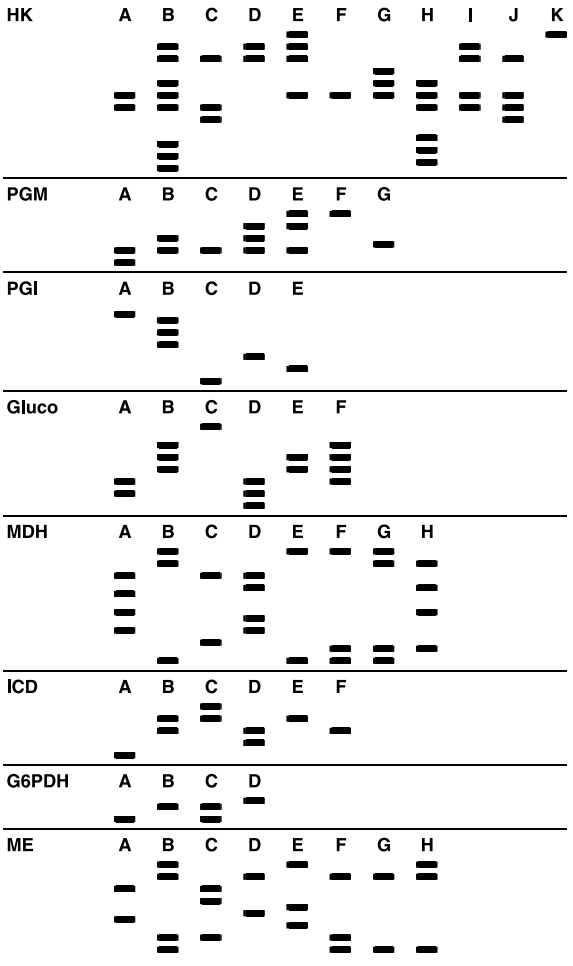

Figure 1 Zymograms (denoted alphabetically by upper case letters) observed for each enzyme system. HK, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; Gluco, glucosidase; MDH, malate dehydrogenase; ICD, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme.

Vietnam, nine were identified in the Philippines, 34 in Thailand, three in Indonesia and four in Papua New Guinea. Several isolates within each country were found to be identical for all eight enzyme systems. In Indonesia, for example, 13 isolates exhibited zymotype 53. The geographical distribution of the *P. colocasiae* zymotypes, however, indicates that none of these zymotypes is common to two different countries. At the isozyme level, the species *P. colocasiae* appears to be represented by different genotypes in the five different countries surveyed (Table 5).

The binary data obtained for all the zymotypes of the five *Phytophthora* species constituted a matrix of 58 zymotypes × 60 electromorphs that was subjected to multivariate analysis. The projection on axes 1 and 2 clearly differentiates the four species from the group of related *P. colocasiae* zymotypes (Fig. 2). Zymotypes 1 and 2 represent *P. megakarya*, with zymotypes 3 and 4 representing *P. palmivora* and *P. citrophthora*, respectively, and 5 and 6 both representing *P. capsici*. Zymotypes 11, 12, 13 (all *P. colocasiae* isolates from the Philippines) and 8 (a *P. colocasiae* isolate from Vietnam) appear to be genetically distant from the group of 46 distinct but closely related *P. colocasiae* zymotypes.

The binary data obtained for *P. colocasiae* zymotypes constituted a matrix of 52 zymotypes × 60 electromorphs that were subjected to cluster analysis. The three distances computed using the NTSYS software (Jaccard, DICE and Simple Matching) gave very similar results and the simple matching (SM) coefficient was retained for the final analysis (Fig. 3). Distances between zymotypes were significant, ranging from 0·63 to 0·98 on the SM coefficient scale. Zymotypes 8 (Vietnam), 7 (Vietnam), 53 (Indonesia), 11, 12 and 13 (the Philippines) clustered far apart from the major group of zymotypes. It was impossible to observe any geographical groupings on the dendrogram (Fig. 3). All zymotypes from Papua New Guinea (55, 56, 57, 58) were closely related and appeared to cluster with zymotypes 54 from Indonesia and 40, 41, 42 and 43 from Thailand. Zymotypes of isolates 11, 12 and 13 collected in the Philippines appeared to be significantly distant from

Table 4 Zymotypes of related *Phytophthora* spp. and species-specific zymograms

		Zymograms*								
Isolates	Species	HK	PGM	PGI	Gluco	MDH	ICD	6PDH	ME	Zymotype
1	*P. megakarya*	A	A	A	A	A	A	A	A	1
1	*P. megakarya*	A	A	A	E	H	A	A	A	2
2	*P. palmivora*	D	A	B	D	D	D	A	D	3
1	*P. citrophthora*	E	D	D	E	E	E	A	A	4
1	*P. capsici*	F	E	E	E	E	E	D	A	5
1	*P. capsici*	G	E	E	E	C	E	D	E	6
	Species-specific:									
	P. megakarya	A	A	A	A	A, H	A	A	A	
	P. palmivora	D	–	–	D	D	D	–	D	
	P. capsici	F, G	–	E	E	C	–	D	–	
	P. citrophthora	E	–	–	–	–	–	–	–	

*HK, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; Gluco, glucosidase; MDH, malate dehydrogenase; ICD, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme.

Table 5 Geographical distribution of *Phytophthora colocasiae* zymotypes (isolate numbers refer to Table 1)

Country	Isolate numbers	Zymograms*								Zymotypes
		HK	PGM	PGI	Gluco	MDH	ICD	6PDH	ME	
Vietnam	1, 2, 4, 5	B	B	B	B	B	B	B	F	7
	3	C	C	C	C	C	C	C	C	8
Philippines	9, 10	C	C	B	E	F	E	B	B	9
	13, 14	C	F	B	E	F	E	B	B	10
	12	I	C	D	B	F	E	B	B	11
	16	K	E	B	E	B	E	B	B	12
	25	C	F	B	E	F	E	B	E	13
	8	C	C	B	E	F	E	C	B	14
	21	J	F	B	E	F	E	C	B	15
	1, 2	C	E	B	E	F	B	C	G	16
	3, 4, 5, 6	C	E	B	E	F	E	C	G	17
Thailand	1	C	B	B	B	B	B	B	B	18
	2	C	B	B	F	B	B	B	F	19
	3	C	B	B	F	E	E	C	F	20
	4, 5, 11, 12, 14	C	E	B	E	B	E	B	B	21
	6	C	E	B	E	B	E	C	B	22
	7, 8	C	E	B	E	F	B	C	B	23
	9, 10	C	E	B	E	F	B	C	F	24
	15	C	E	B	E	F	E	B	B	25
	17	C	E	B	E	F	E	C	B	26
	19	C	F	B	E	F	B	C	B	27
	20	C	F	B	E	F	B	C	H	28
	21, 22	C	F	B	E	F	E	C	B	29
	23	C	F	B	F	B	B	B	F	30
	24, 25	C	F	B	F	E	E	B	F	31
	26	C	C	B	B	B	B	B	F	32
	27	C	C	B	B	E	E	C	G	33
	28	C	C	B	E	B	B	C	B	34
	30, 31	C	C	B	E	B	B	C	F	35
	33	C	C	B	E	F	B	C	F	36
	34	C	G	B	E	F	E	B	B	37
	35	C	G	B	F	E	E	B	B	38
	36	C	G	B	F	E	E	B	F	39
	37	C	G	B	F	E	E	C	B	40
	38	I	B	B	B	E	E	C	G	41
	39	I	B	B	F	E	E	C	B	42
	40	I	B	B	F	E	E	C	F	43
	41	I	C	B	E	E	E	B	B	44
	42, 43	J	E	B	E	B	B	C	B	45
	45	J	E	B	E	F	B	C	B	46
	47	J	E	B	E	F	E	C	G	47
	48	J	F	B	E	B	B	C	B	48
	49, 52	J	F	B	E	F	B	C	B	49
	53	J	G	B	E	F	B	C	B	50
	54	J	G	B	E	F	B	C	H	51
Indonesia	9, 12, 13, 14, 15, 21	I	E	B	E	B	B	C	F	52
	1, 2, 3, 4, 5, 6, 7, 8, 10, 16, 17, 18, 19	I	E	B	E	G	B	C	F	53
	15, 21	H	D	B	B	B	B	B	F	54
Papua New Guinea	1, 2, 3, 7, 8	I	B	B	E	E	E	C	G	55
	5	I	B	B	E	F	B	C	G	56
	6	I	B	B	E	F	E	C	G	57
	4	I	B	B	E	F	F	C	G	58

*HK, hexokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; Gluco, glucosidase; MDH, malate dehydrogenase; ICD, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme.

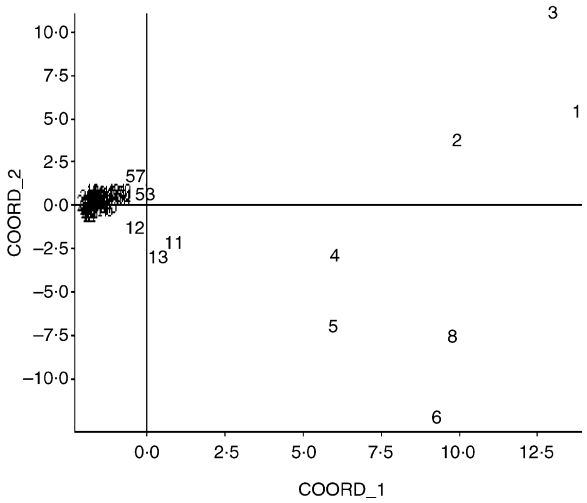

Figure 2 Distribution of 58 zymotypes from five *Phytophthora* species in plane (axes 1 = 25·1% and 2 = 13·7%) of a principal-component analysis of isozyme variation in 101 isolates (1, *P. megakarya*; 2, *P. megakarya*; 3, *P. palmivora*; 4, *P. citrophthora*; 5, *P. capsici*; 6, *P. capsici*; 7–58, *P. colocasi*).

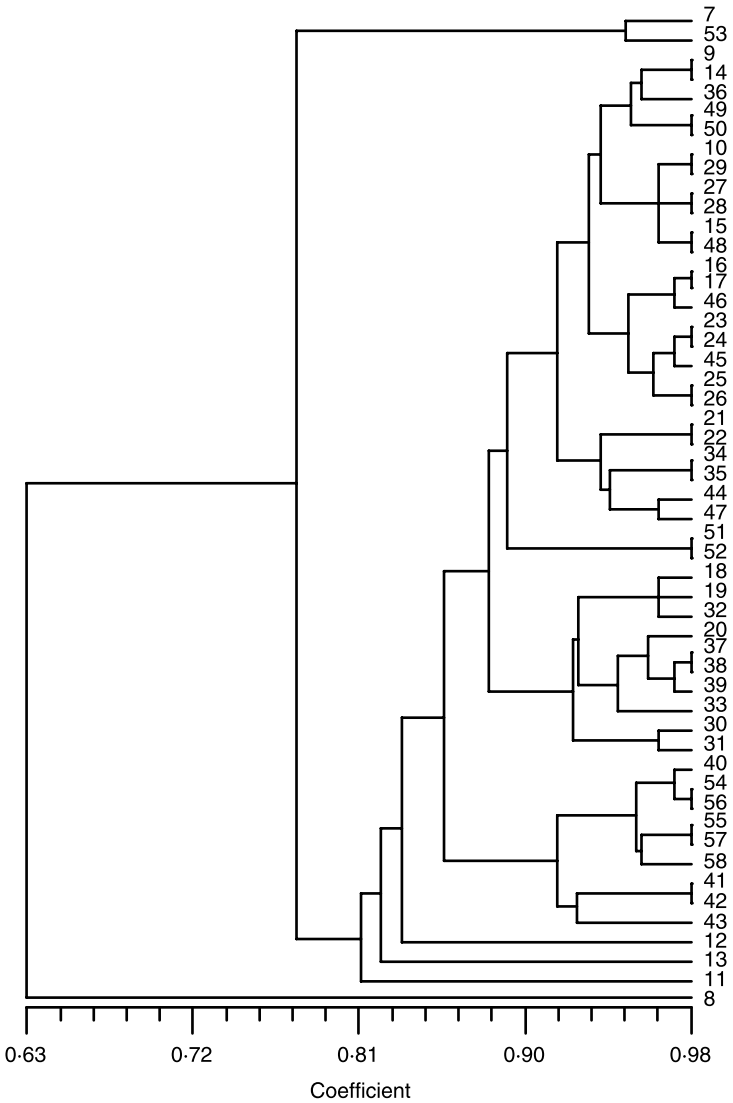

Figure 3 Cluster analysis using the unweighted pair group method (UPGMA) using the simple matching coefficient (SM) of 52 *Phytophthora colocasiae* zymotypes representing 94 isolates from South-east Asia (zymotype numbers refer to Table 5).

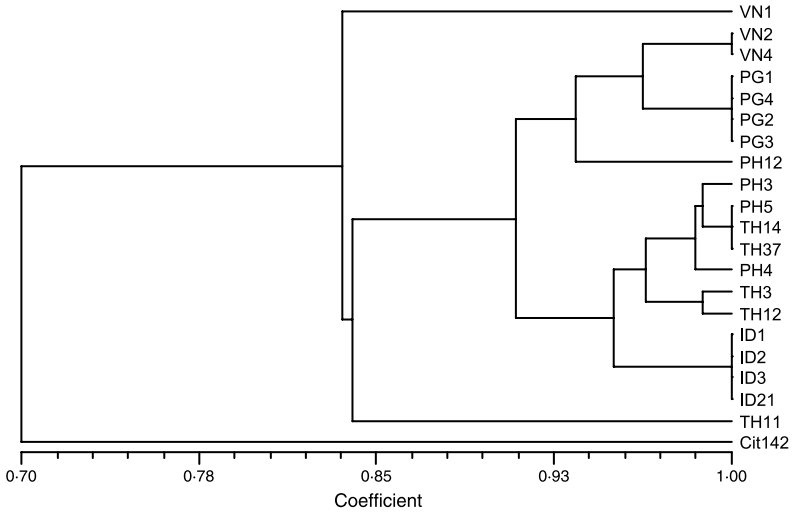

Figure 4 Cluster analysis of 20 *Phytophthora colocasiae* isolates (identification numbers refer to Table 1) and one *P. citrophthora* isolate (Cit142) using RAPD data generated with seven couples of primers.

others from the Philippines (9, 14, 15, 16, 17) which clustered with zymotypes from Thailand. This suggests that in the Philippines, *P. colocasiae* was introduced from various geographical origins. Because this clustering analysis indicated that zymotype 8 (Vietnam) was very distant from others, it was decided to examine its morphological attributes more closely. After double checking, it was confirmed that zymotype 8 was truly *P. colocasiae*.

RAPD markers

The 20 isolates of *P. colocasiae* selected for RAPD analysis are presented (marked with an asterisk) in Table 1. Cluster analysis was conducted on the matrix (20 *P. colocasiae* isolates and an isolate of *P. citrophthora* × 81 electromorphs) using the SM coefficient and the resulting dendrogram is presented in Fig. 4. Although *P. citrophthora* is a closely related species, it was clearly differentiated from all *P. colocasiae* isolates. RAPD markers permitted differentiation of isolates exhibiting identical zymotypes. Isolate VN1 (zymotype 7) was differentiated from isolates VN2 and VN 4 (both zymotype 7). Isolate PH5 (zymotype 17) was differentiated from isolates PH3 and PH4 (both zymotype 17), which were differentiated from each other. However, the dendrogram also revealed that, based on RAPD data, isolates PH5, TH14 and TH37 were identical although they exhibited distinct zymotypes (17, 21 and 41, respectively). Isolate ID21 was found to be identical to isolates ID1, ID2 and ID3 (zymotype 53) although it exhibited a distinct zymotype (54). Isolates PG1, PG2 and PG3 were found to be identical to PG4 although the latter exhibited a distinct zymotype (58). RAPD and isozyme data were not congruent. Cluster analysis, however, confirmed that there were significant genetic distances between *P. colocasiae* isolates, and two of them (VN1 and TH11) appeared to be very distant from other isolates. However, in this case also, it was impossible to detect geographic groupings in the variation revealed by the dendrogram.

Discussion

The objective of this study was to use isozyme markers to elucidate the population structure of *P. colocasiae* in five countries of South-east Asia and Oceania. It has already been shown that South-east Asia is probably the area of origin of *P. colocasiae* (Zhang *et al*., 1994). This study represents the first eco-geographical survey of the genetic variation existing within *P. colocasiae*, conducted on 94 isolates originating from this region. Both isozyme and RAPD markers appear to be useful to study the extent of molecular variation existing within this pathogen species. The use of eight enzyme systems revealed significant differences between isolates of *P. colocasiae*, while RAPD markers demonstrated that it was possible to distinguish further between isolates exhibiting identical zymotypes.

Isozymes and RAPD also revealed discordant pictures of the core sample analysed with both markers. Discordance among molecular marker sets is common and has been extensively reported (Wendel & Doyle, 1998) especially when they have different inheritance patterns, co-dominant in isozymes and dominant in RAPD. It is the first time that these markers have been used to study the intraspecific variation of *P. colocasiae*. RAPD markers detect variation that is distributed throughout the genome whereas zymotypes represent defined loci on the genome. These markers are likely to be fingerprinting different regions or sequences of the genome. 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 this study, isozymes and RAPD have shown that genetic diversity exists among strains of *P. colocasiae* originating from different countries. Both markers revealed that significant genetic variation also occurs within countries. In Thailand, for example, where 45 isolates were collected, not fewer than 34 distinct zymotypes were identified. RAPD analysis confirmed that strains were genetically variable in Thailand. In the Philippines, where 15 isolates

were collected, nine distinct zymotypes were identified. In Vietnam, Indonesia and Papua New Guinea, the isozyme variation was apparently lower but this is most likely due to the smaller size of the samples collected. Nevertheless, two, three and four distinct zymotypes, respectively, were identified. Throughout this geographic region, taro leaf blight is caused by numerous and distinct strains. Overall, no fewer than 52 distinct genotypes of *P. colocasiae* were identified and all correspond to isolates collected on mature leaves of plants exhibiting symptoms of taro leaf blight.

The origin of this variation is not known. Because *P. colocasiae* is diploid and heterothallic, different strains are likely to recombine and evolve rapidly depending, of course, on the frequency of A1 and A2 mating type in particular populations. It is possible that zymotype variants arose from recombination during sexual reproduction. The existence of sexual reproduction in *P. colocasiae* (Narula & Mehrotra, 1981; Zhang *et al*., 1994) has already been documented. The study reported here has shown that all zymotypes are unique to each country. This might be an indication of rapid evolution and genetic drift within isolated populations, i.e. *P. colocasiae* was introduced recently (*c.* 1940) to Papua New Guinea (Ivancic & Lebot, 2000) and is already variable.

The high level of genetic diversity revealed within country populations (e.g. Thailand) indicates that migration and sexual recombination probably play important roles in the population dynamics of *P. colocasiae* in South-east Asia. Taro is a clonally propagated species and cultivars share a narrow genetic base in most countries (Lebot & Aradhya, 1991; Lebot *et al*., 2000). Their susceptibility to *P. colocasiae* is variable (Table 3) but it is unknown if this represents genetic variation of the host or variable pathogenicity of genetically different isolates of the pathogen. Breeding taro cultivars for resistance to leaf blight in one country and distributing clonal material internationally might not be efficient in the long term. Results from this study indicate that if a cultivar is found to be resistant in one country, it is likely that it will be exposed to genetically different isolates of *P. colocasiae* in another country and thus its resistance will not be guaranteed. Consequently, it might be more appropriate to breed for resistance to local strains of *P. colocasiae* in all countries where the pathogen is present.

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References

Blaha G, 1992. *Variability and Specificity in the* Phytophthora *Genus: Review of Methods Using Morphocultural Characters and Enzymatic Banding Patterns. Proceedings of the Coconut Phytophthora Workshop Manado, Indonesia, 1992.* Montpellier, France: CIRAD, 79–84.

Butler EJ, Kulkarn GS, 1913. *Colocasia* blight caused by *Phytophthora colocasiae*. *Memoirs of the Department of Agriculture of India, Botanical Series* **5**, 233–59.

Carlier J, Lebrun MH, Zapater MF, Dubois C, Mourichon X, 1996. Genetic structure of the global populations of bananas black leaf streak fungus *Mycosphaerella fijiensis*. *Molecular Ecology* **5**, 499–510.

Cooke DEL, Kennedy DM, Guy DC, Russel J, Unkles SE, Duncan JM, 1996. Relatedness of Group I species of *Phytophthora* as assessed by randomly amplified polymorphic DNA (RAPD) and sequences of ribosomal DNA. *Mycological Research* **100**, 297–303.

Erwin DC, Ribeiro OK, 1996. *Phytophthora Diseases Worldwide*. St Paul, MN, USA: APS Press.

FAO, 2000. *On Line Statistical Data Base of the FAO* [www.fao.org]. Rome, Italy: FAO.

Ivancic A, Lebot V, 2000. *The Genetics and Breeding of Taro*. *Collection 'Repères' CIRAD*. Montpellier, France: CIRAD.

Jackson GVH, 1977. *Taro Leaf Blight*. *SPC Advisory Leaflet no. 3*. Nouméa, New Caledonia: South Pacific Commission.

Jackson GVH, 1980. *Diseases and Pests of Taro*. Nouméa, New Caledonia: South Pacific Commission.

Lebot V, Aradhya KM, 1991. Isozyme variation in taro (*Colocasia esculenta* (L.) Schott) from Asia and Oceania. *Euphytica* **56**, 55–66.

Lebot V, Hartati S, Hue NT, Viet NV, Nghia NH, Okpul T, Pardales J, Prana MS, Prana TK, Thongjiem M, Krieke CM, VanEck H, Yap TC, Ivancic A, 2000. Genetic variation in taro (*Colocasia esculenta*) in South East Asia and Oceania. *Potential of Root Crops for Food and Industrial Resources. Proceedings of the Twelfth Symposium of the ISTRC, 2000, Tsukuba, Japan.* Ibaraki, Japan: Coultio Corp, 524–33.

Mahuku G, Peters RD, Platt HW, Daay F, 2000. Random amplified polymorphic DNA (RAPD) analysis of *Phytophthora infestans* isolates collected in Canada during 1994–96. *Plant Pathology* **49**, 252–60.

Micales JA, Bonde MR, Peterson GL, 1986. The use of isozyme analysis in fungal taxonomy and genetics. *Mycotaxon* **27**, 405–49.

Misra RS, Chowdhury SR, 1997. Phytophthora *Leaf Blight Disease of Taro.* Technical Bulletin Series 21. Trivandrum, India: Central Tuber Crops Research Institute and St Joseph Press.

Narula KL, Mehrotra RS, 1981. Occurrence of A1 mating type in *Phytophthora colocasiae*. *Indian Phytopathology* **33**, 603–4.

Ortiz-Garcia C, Herail C, Blaha G, 1993. *Utilisation des Isozymes en Tant que Marqueurs pour l'Identification Spécifique des* Phytophthora *Responsables de la Pourriture Brune des Cabosses dans les Pays Producteurs de Cacao. Proceedings 11ème Conférence Internationale Sur la Recherche Cacoyère, 1993, Yamoussoukro, Côte d'Ivoire.* Montpellier, France: CIRAD, 135–43.

Parham BEV, 1949. Annual report of the economic botanist for the year 1948. *Fiji Legislative Council Paper* **24**, 31–5.

Raciborski M, 1900. Parasitic algae and fungi, Java. *Batavia Bulletin of the New York State Museum* **19**, 189.

Trujillo EE, 1967. *Diseases of the Genus Colocasia in the Pacific Area and Their Control. Proceedings of the*

Second International Symposium on Tropical Root and Tuber Crops, Honolulu, HI, USA. Honolulu, HI: University of Hawaii, 136–40.

Vasquez EA, 1990. Yield losses in taro due to *Phytophthora* leaf blight. *Journal of Root Crops* **16**, 48–50.

Wendel JF, Doyle JJ, 1998. Phylogenetic incogruence: window into the genome history and molecular evolution. In: Soltis DE, Soltis PS, Doyle JJ, eds. *Molecular Systematics of Plants. II. DNA Sequencing*. Boston, USA: Kluwer Academic Publishers, 265–96.

Zhang KM, Zheng FC, Li YD, Ko WH, 1994. Isolates of *Phytophthora colocasiae* from Hainan Island in China: evidence suggesting an Asian origin of this species. *Mycologia* **86**, 108–12.