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Characterisation of *Phytophthora capsici* isolates from black pepper in Vietnam

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ABSTRACT

Phytophthora foot rot of black pepper caused by *Phytophthora capsici* is a major disease of black pepper (*Piper nigrum*) throughout Vietnam. To understand the population structure of *P. capsici*, a large collection of *P. capsici* isolates from black pepper was studied on the basis of mating type, random amplified microsatellites (RAMS) and repetitive extragenic palindromic (REP) fingerprinting. Two mating types A1 and A2 were detected in four provinces in two climatic regions, with A1:A2 ratios ranging from 1:3 to 1:5. In several instances A1 and A2 mating types were found to co-exist in the same farm or black pepper pole, suggesting the potential for sexual reproduction of *P. capsici* in the field in Vietnam although its contribution to disease epidemics is uncertain. RAMS and REP DNA fingerprinting analysis of 118 isolates of *P. capsici* from black pepper showed that the population was genetically more diverse where two mating types were found, although the overall genetic diversity was low with most of the isolates belonging to one clonal group. The implication of these findings is discussed. The low diversity among isolates suggests that the *P. capsici* population may have originated from a single source. There was no genetic differentiation of isolates from different climatic regions. In addition to the large clonal group, several isolates with unique RAMS/REP phenotypes were also detected. Most of these unique phenotypes belonged to the minority A1 mating type. This may have significant implications for a gradual increase in overall genetic diversity.

Introduction

Phytophthora foot rot, caused by *Phytophthora capsici*, is one of the most serious threats to the production of black pepper (*Piper nigrum*) throughout black pepper growing regions worldwide, including Vietnam. The pathogen infects the roots, stems, leaves and fruit at any stage of plant growth. *P. capsici* is heterothallic and requires both A1 and A2 mating types for sexual reproduction, mating type A1 being more pathogenic than A2 (Manohara *et al.* 2004b). These two mating types coexist in several areas of black pepper cultivation in Indonesia (Manohara *et al.* 2004a), Malaysia (Kueh & Sim 1988) and India (Sarma *et al.* 1988). The epidemic development of black pepper foot rot depends on environmental conditions, drainage, soil moisture, soil fertility, cultivar and cultural practices (Anandaraj 2000). In Vietnam, the disease is found in almost all black pepper growing areas (Truong *et al.* 2008).

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Owing to the high variability and overlaps in morphology within and between species of *Phytophthora* (Erwin 1983; Erwin & Ribeiro 1996), identification of some *Phytophthora* species based on morphological criteria is often difficult, unreliable and time-consuming (Förster & Coffey 1991; Ristaino *et al.* 1998; Drenth *et al.* 2006). *P. capsici*, being a cryptic species with high levels of intraspecific genetic diversity, exemplifies the controversy in *Phytophthora* taxonomy. The species was first described by Leonian (1922) as the causal agent of chilli pepper blight (*Capsicum annuum* L.) and considered to be host specific. However, *P. capsici sensu lato* is now known to have a broad host range, causing diseases on plant species from both temperate and tropical regions (Erwin & Ribeiro 1996). Currently, the definition of this species includes strains associated with many different plant hosts and exhibits the morphological features described by Leonian (1922) and amended by Al-Hedaithy & Tsao (1979) and Tsao (1988). Taxonomically, isolates from black pepper were previously classified as *Phytophthora palmivora* based solely on morphological characters (Holliday & Mowat 1963; Alconero *et al.* 1972; Turner 1973). These were later placed into one of four morphological groups of *P. palmivora*, *viz. P. palmivora* MF4 (Zentmyer *et al.* 1977), which was eventually reclassified as *P. capsici* on the basis of morphological characters (Tsao 1988).

The limited success in disease management in many situations is due to knowledge gaps in understanding the genetic structure of pathogen populations (Martin & English 1997). The importance of genetic analysis of pathogen populations is well accepted in understanding disease epidemiology, host-pathogen interactions, resistant breeding, and prediction of fungicide resistance (McDonald *et al.* 1989; McDonald & McDermott 1993; Milgroom & Fry 1997; McDonald & Linde 2002; Milgroom & Peever 2003). Many studies on genetic variation of *P. capsici* populations, which cause crown, root and fruit rot of *Capsicum* spp. in temperate regions, have been reported (Hwang *et al.* 1991; Lamour & Hausbeck 2001; Islam *et al.* 2005). Few investigations, however, have been conducted on the genetic diversity of this species associated with black pepper.

Variation among *P. capsici* isolates has been studied using protein profiles, isozymes, RAPD and mtDNA analysis (Erselius & Shaw 1984; Förster *et al.* 1990; Oudemans & Coffey 1991; Luz *et al.* 2003). Variation in protein profiles of *P. capsici* detected few differences among isolates in France (Erselius & Shaw 1984). However, a high degree of mtDNA diversity was found among isolates from various geographic locations and host plants (Förster *et al.* 1990). Based on isozyme analysis, *P. capsici* was found to be one of the most genetically complex species of *Phytophthora* examined. Isolates of *P. capsici* from black pepper, identified as *P. palmivora* MF4 were first distributed into two groups, CAP1 and CAP2 (Oudemans & Coffey 1991), which were later resolved into CapA and CapB, respectively (Mchau & Coffey 1995).

In recent years, molecular markers based on the polymerase chain reaction (PCR) have been widely applied in the studies of *Phytophthora* species (McDonald 1997; Duncan & Cooke 2002; Cooke & Lees 2004). Random amplified microsatellites (RAMS), also known as simple sequence inter-repeats, originally described by Zietkiewicz *et al.* (1994) and developed by Hantula *et al.* (1996) for the detection of interspecific and intraspecific DNA-polymorphisms, combine the characters of RAPD and microsatellite analysis. RAMS marker has been considered for the assessment of genetic variation within the fungi (Hantula *et al.* 1996; Vainio & Hantula 1999; Elbakali *et al.* 2003; van der Waals *et al.* 2004) and the Oomycetes, including *Phytophthora* species (Hantula *et al.* 1997; Hantula *et al.* 2000; Chee & Jee 2001; Cohen *et al.* 2003; De Merlier *et al.* 2005). A considerable amount of genetic variation among isolates of *Phytophthora cactorum* was revealed using RAMS markers (Hantula *et al.* 1997; Hantula *et al.* 2000) and differentiation between the strawberry *P. cactorum* populations of North American and Europe was established. Similarly, Chee & Jee (2001) detected variation in Korean *P. capsici* isolates from pepper, tomato, squash, watermelon and melon based on RAMS analysis, although the variation was neither associated with host species nor geographic origin. More recently, an investigation on the intraspecific variation of *Phytophthora citrophthora* using RAMS showed that four patterns were produced by an anchored CGA-primer (Cohen *et al.* 2003).

REP-PCR was developed by Versalovic *et al.* (1991) for studying genetic variation of prokaryotic and eukaryotic microorganisms using repetitive sequences based on the PCR. The REP-PCR primers were designed to be complementary to interspersed palindromic repetitive sequences, 15–18 bases in length (Versalovic *et al.* 1991; Versalovic *et al.* 1994). The PCR amplifies differently sized DNA fragments consisting of unique sequences lying between these palindromic repeats. This technique has been used successfully to analyse fungal species to reveal diversity at the intraspecific levels (Edel *et al.* 1997; Jedryczka *et al.* 1999; Hierro *et al.* 2004). A recent study showed that REP-PCR fingerprints were very reproducible and detected variation between *Phytophthora infestans* populations (Bouws & Finckh 2007).

Since the recent establishment of *P. capsici* as the causal agent of black pepper foot rot in Vietnam (Truong *et al.* 2008), the population structure of this pathogen has not been studied. The role of genetic diversity and geographic structuring of *P. capsici* foot rot epidemics of black pepper is not clear. It is assumed that environmental effects, host susceptibility and cultivation practices facilitate selection pressures, which in turn affects the changes in the pathogen population structure, and subsequently the pattern of disease incidence. In order to make decisions regarding the direction of disease management strategies, the population structure of this pathogen needs to be explored. We begin to address these issues by testing two hypotheses. The first is that only one mating type exists in the *P. capsici* population from black pepper in Vietnam. The second is that the *P. capsici* population is genetically undifferentiated in two different climatic regions. The approach chosen to test these hypotheses involved collecting *P. capsici* isolates from two geographically different black pepper growing regions in Vietnam based on a hierarchical sampling strategy, characterising these isolates to determine their mating type, and analysing their DNA fingerprints based on RAMS and REP-PCR.

Materials and methods

Isolate origin

P. capsici was isolated from black pepper soil and diseased plant samples obtained from three provinces in the Southeast

(SE) region and one province in the North Central Coast (NCC) region of Vietnam (Fig 1) (Truong et al. 2008). Sampling strategy and pathogen isolation procedures are detailed in Truong et al. 2008. Only one province was included to represent NCC due to the relatively large growing area in that province. Details of sampling locations and isolates used in this study are given in Table 1.

The SE region (Fig 1) is characterized by a monsoon tropical climate with a rainy season from May to October and a dry season from November to April (General Statistics Office 2007; Wikipedia 2008). The annual average temperature is c. 27 °C. The average maximum temperature is c. 34 °C in April while the average minimum temperature is c. 21 °C in January. The annual rainfall is from 1500 to 1900 mm. The south of the NCC region is characterized by more severe weather conditions (Quang Tri Statistical Office 2005). Unlike the SE region, the weather is very hot with dry westerly winds during the dry and hot season (March–August) but typhoon and heavy rain often occurs during the rainy season (September–February). The average temperature varies from 20 to 25 °C, with an average maximum temperature of up to 40 °C in July (summer) and the average minimum temperature gets down to 8 °C in January (winter). The annual rainfall is from 2000 to 2700 mm, with more than 75 % received during the rainy season.

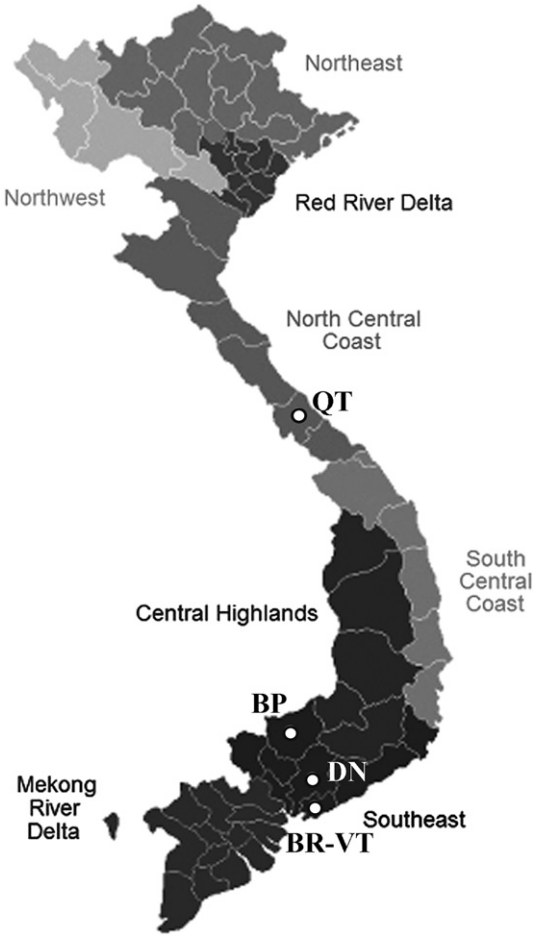

Fig 1 – Map of Vietnam indicating the two different climatic regions, SE region and NCC, from which black pepper *Phytophthora capsici* isolates were collected in 2004 and 2005. Provinces sampled are also indicated: BP = Binh Phuoc; BR-VT = Ba Ria-Vung Tau; DN = Dong Nai; and QT = Quang Tri.

Mating type analysis

Each isolate was paired on V8 Agar with known A1 and A2 testers. An isolate was considered to belong to mating type A1 if oospores were present when paired with a known A2 tester and vice-versa. Test isolates producing oospores with both A1 and A2 were scored as A1A2. The test was replicated three times.

DNA protocols

DNA extraction

Isolates were grown in liquid medium described by Zhang et al. (2004) and incubated at 25 °C in the dark for 5–6 d until the mycelium covered the surface of the medium. Mycelia were then harvested and dried on filter paper. Genomic DNA was extracted using the FastPrep DNA Kit (Qbiogene Inc., USA) according to the manufacturer's instructions. The resulting DNA in TE was electrophoresed at 80 V for 2 h on a 2 % agarose gel in Tris–Borate–EDTA (TBE) buffer (45 mM Tris–borate and 1 mM EDTA) to estimate concentration and assess integrity (Sambrook et al. 1989).

RAMS protocol

PCR amplification was carried out in a 20 μl reaction volume containing PCR buffer (Promega), 0.5 mM of $MgCl_2$ (Sigma), 1 unit of *Taq* DNA polymerase (Bioline), 0.05 mM each of dATP, dCTP, dGTP and dTTP (Promega), 0.2 μM of primer and approximately 20 ng of DNA. Six RAMS primers were used in the study. Thermocycling reactions were performed in a Corbett DNA thermocycler according to the following temperature profiles: an initial denaturation of 10 min at 95 °C, followed by 35 (primers ACA, AG, CGA, GT, TG) or 37 (primer CCA) cycles of amplification, i.e. denaturation for 30 s at 95 °C, annealing for 45 s at 49 °C (AG), 47 °C (ACA), 60 °C (CGA), or 61 °C (CCA), 58 °C (GT), 53 °C (TG), extension for 2 min at 72 °C, and a final extension step for 7 min at 72 °C (Hantula et al. 1996; Hantula et al. 1997).

REP-PCR protocol

PCR amplification using REP-primers was carried out in a 25 μl reaction volume containing PCR buffer (Promega), 0.5 mM of $MgCl_2$ (Sigma), 1 unit of *Taq* DNA polymerase (Bioline), 0.05 mM each of dATP, dCTP, dGTP and dTTP (Promega), 0.05 μM primer rep-1R (5′-III ICG ICG ICA TCI GGC-3′), 0.05 μM primer rep-2I (5′-ICG ICT TAT CIG GCC TAC-3′) and approximately 20 ng of DNA. Thermocycling was according to the following temperature profile: an initial denaturation of 10 min at 94 °C, 36 cycles of denaturation at 94 °C for 30 s, annealing at 38 °C for 45 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min (Versalovic et al. 1994; Rademaker & de Bruijn 1997).

Table 1 – Isolates of *Phytophthora capsici* from black pepper used in the study and their mating types.

Isolate	Province	District	Collection date	Mating type[a]
SE region				
VN58-1	Binh Phuoc	Loc Ninh	Sep. 2004	A2
VN58-2	//	Phuoc Long	//	A2
VN58-3	//	//	//	A2
VN58-4	//	//	//	A2
VN58-5	//	//	//	A2
VN58-6	//	Binh Long	//	A2
VN43-1	Ba Ria-Vung Tau	Chau Duc	Oct. 2004	A2
VN43-2	//	//	//	A2
VN43-3	//	//	//	A2
VN43-4	//	Xuyen Moc	//	A2
VN43-5	//	//	//	A2
VN43-6	//	//	//	A2
VN43-7	//	//	//	A2
VN43-8	//	//	//	–
VN43-9	//	//	//	–
VN43-10	//	//	//	–
VN43-11	//	Chau Duc	//	A2
VN43-12	//	Xuyen Moc	//	A2
VN43-13	//	Chau Duc	//	A2
VN43-14	//	//	//	A2
VN43-15	//	Xuyen Moc	//	A2
VN43-16	//	//	//	A2
VN43-17	//	Chau Duc	//	A2
VN43-18	//	//	//	A2
VN39-1	Dong Nai	Cam My	Nov. 2004	A1
VN39-2	//	//	//	A1
VN39-3	//	Xuan Loc	//	A2
VN39-4	//	//	//	A1
VN39-5	//	Cam My	//	A2
VN39-6	//	//	//	A2
VN39-7	//	Xuan Loc	//	A2
VN39-8	//	//	//	–
VN39-9	//	Long Khanh	//	A2
VN39-10	//	//	//	A2
VN39-11	//	Xuan Loc	1998	A2
VN39-12	//	//	Nov. 2004	A2
VN39-13	//	//	//	A1
VN39-14	//	//	//	A1
VN39-15	//	//	//	A1
VN39-16	//	//	//	A1
VN39-17	//	//	//	A2
VN39-18	//	//	//	A2
VN39-19	//	Long Khanh	//	A1
VN39-20	//	Cam My	//	–
VN39-21	//	Cam My	//	A2
VN39-22	Dong Nai	Xuan Loc	Nov. 2004	A1
VN39-23	//	//	//	A1
VN39-24	//	Dinh Quan	//	A2
VN39-25	//	Cam My	//	A2
VN39-26	//	//	//	A2
VN39-27	//	Dinh Quan	//	–
VN39-28	//	//	//	A2
VN39-29	//	//	//	–
VN39-30	//	//	//	A1
VN39-31	//	Xuan Loc	//	A2
VN39-32	//	Long Khanh	//	–
VN39-33	//	Cam My	//	–
NCC region				
VN25-1	Quang Tri	Huong Hoa	Dec. 2004	A2
VN25-4	//	//	//	A2
VN25-6	//	//	//	A2
VN25-8	//	//	//	A2

(*continued on next page*)

Table 1 – (*continued*)

Isolate	Province	District	Collection date	Mating type[a]
VN25-9	//	//	//	A2
VN25-10	//	//	//	A2
VN25-12	//	//	//	A2
VN25-15	//	//	//	A2
VN25-17	//	//	//	A2
VN25-19	//	//	//	A2
VN25-20	//	//	//	A2
VN25-28	//	//	//	A2
VN25-32	//	//	//	A1
VN25-37	//	//	//	A1
VN25-38	//	//	//	A1
VN25-39	//	//	//	A2
VN25-40	//	//	//	A1
VN25-41	//	Cam Lo	//	A2
VN25-45	//	Huong Hoa	//	A2
VN25-49	//	//	//	A2
VN25-51	//	//	//	A2
VN25-53	//	Cam Lo	//	A2
VN25-58	//	//	//	A2
VN25-59	//	//	//	A1
VN25-60	//	Huong Hoa	//	A2
VN25-61	//	//	//	A2
VN25-62	//	//	//	A2
VN25-63	//	Cam Lo	//	A2
VN25-64	//	Cam Lo	//	A1
VN25-71	//	Huong Hoa	//	A2
VN25-74	//	//	//	A2
VN25-75	//	//	//	A2
VN25-78	//	//	//	A1
VN25-81	Quang Tri	Cam Lo	Dec. 2004	A2
VN25-82	//	Huong Hoa	//	A2
VN25-84	//	//	//	A2
VN25-92	//	//	//	A2
VN25-93	//	//	//	A2
VN25-94	//	//	//	A2
VN25-95	//	//	//	A2
VN25-96	//	//	//	A2
VN25-97	//	//	//	A2
VN25-98	//	//	//	A2
VN25-99	//	//	//	A2
VN25-100	//	//	//	A2
VN25-101	//	//	//	A2
VN25-102	//	//	//	A2
VN25-103	//	//	//	A2
VN25-104	//	//	//	A2
VN25-107	//	//	//	A2
VN25-109	//	//	//	A2
VN25-110	//	//	//	A2
VN25-111	//	//	//	A2
VN25-113	//	//	//	A2
VN25-114	//	//	//	A1
VN25-120	//	//	//	A2
VN25-122	//	//	//	A1
VN25-136	//	//	//	A1A2
VN25-132	//	//	//	A2
VN25-138	//	//	//	A1A2
VN25-147	//	Vinh Linh	//	A1

a Mating type was not tested.

Gel electrophoresis

Amplification products were separated by electrophoresis in gels containing 0.8 % SynerGel (Diversified Biotech) and 0.8 % agarose (Amresco, Inc.). The electrophoresis was run in TBE buffer at room temperature at 70 V for 2.5 h. After staining in TBE buffer containing ethidium bromide (10 mg l^{-1}) for 30–45 min, the gels were visualized on a uv transilluminator and photographed using a digital camera. The size of

amplification products was estimated by comparison with a 100 bp ladder (Promega).

DNA sequence analysis

The ITS region of the ribosomal DNA was sequenced and analysed for four selected *P. capsici* isolates (VN39-2, VN39-22, VN39-23 and VN43-17) to represent the whole population for validation of species identification. The amplification reactions were as previously described for *P. capsici* (Truong *et al.* 2008). The resulting PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions and electrophoresed to confirm product size and to estimate the concentration. The PCR amplicons were then sent to the Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC) and the sequence of the DNA determined using an ABI PRISM® 3700 DNA Analyser (Applied Biosystems Inc., Foster City, California, USA) using the same primers as for the PCR amplifications. Both the forward and reverse strands were sequenced and aligned for each isolate using the multiple alignment program ClustalX (Version 1.81) (Thompson *et al.* 1997) in order to minimise the presence of ambiguous nucleotides. Cleaned sequences were compared with GenBank sequences using the BLAST algorithm for species identification. The ITS sequences obtained for VN39-2, VN39-22, VN39-23 and VN43-17 were submitted to GenBank, assigned with accession numbers GQ844981, GQ844982, GQ844983 and GQ844984, respectively.

Analysis of DNA fingerprints

RAMS and REP generated bands were scored as either present (1) or absent (0); only strong and reproducible bands were scored. Number and percent of polymorphic loci and Shannon diversity index (Shannon & Weaver 1949) were calculated using POPGENE version 1.32 (Yeh *et al.* 1997). A binary matrix combining the data from all six primers was constructed. Cluster analysis was performed using the DICE similarity coefficient and Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) agglomeration in the software NTSYSpc Version 2.1q (Applied Biostatistics, Inc., Setauket, NY, USA). The DICE similarity coefficient between each pair of isolates was calculated according to the following formula:

$$\frac{2a}{2a+b+c}$$

where a = the number of common bands between isolates, b = the number of bands found in only one isolate in each pairwise combination and c = the number of common loci with bands absent between isolates (but present in other isolates) (Dice 1945).

Table 2 – The distribution of A1 and A2 mating types of *Phytophthora capsici* from black pepper in Vietnam.

Region	A1	A2	A1A2	A1:A2 ratio[a]
SE				
Binh Phuoc	0	6	0	–
Ba Ria-Vung Tau	0	15	0	–
Dong Nai	11	16	0	1:1.4
Subtotal	11	37	0	1:3.6
NCC				
Quang Tri	21	109	2	1:5.2
Total	32	146	2	1:4.6

a Ratio was only calculated for isolates determined as either A1 or A2 (not A1A2).

Results

Validation of species identification

The four ITS sequences obtained were compared with GenBank sequences based on the BLAST analysis and were found to match sequences belonging to *P. capsici* in the GenBank database, validating the species identification, and confirming previous identification of *P. capsici* isolates from black pepper in Vietnam based on morphology and ITS-RFLP patterns (Truong *et al.* 2008).

Mating type analysis

Mating types were determined successfully for 175 isolates of *P. capsici*. The number of A1, A2 and A1A2 isolates was 32, 146 and 2, respectively (Tables 1 and 2). Both mating types A1 and A2 were detected in two provinces, Dong Nai and Quang Tri, but only A2 was found in Binh Phuoc and Ba Ria-Vung Tau provinces. The A1:A2 ratio in SE and NCC regions was 1:3.6 and 1:5.2, respectively, and for both regions in total, 1:4.6. Both A1 and A2 mating types were found to co-exist within the same farm in 13 cases in Dong Nai and Quang Tri provinces. In addition, A1 and A2 mating types were also observed

Table 3 – Summary statistics of 118 *Phytophthora capsici* isolates from black pepper based on molecular markers and geography regions.

	No. of isolates	Total no. of loci	No. of phenotypes	No. of clonal phenotypes	No. of unique phenotypes	No. of polymorphic loci (% of total)	Shannon diversity index
Molecular marker							
RAMS	118	48	7	112	6	40 (83.3)	0.19
REP	118	5	5	109	2	4 (80.0)	0.25
Combined RAMS and REP	118	53	10	108	8	44 (83.2)	0.20
Geography region							
SE	57	53	7	52	5	42 (79.2)	0.20
NCC	61	53	4	58	3	32 (60.4)	0.17

to co-exist on the same plant in one case in Quang Tri province.

RAMS and REP analysis

RAMS analysis

One hundred and eighteen isolates of *P. capsici* from black pepper were analysed using RAMS markers. The genetic analysis revealed that 40 of the 48 loci (83.3 %) were polymorphic (Table 3). Seven RAMS phenotypes were identified, of which six were unique and one clonal. Overall, the population of *P. capsici* from black pepper was highly clonal (Shannon diversity index = 0.19) with 112 of the isolates belonging to one clonal group (94.9 %). UPGMA cluster analysis indicated that all of the isolates were clustered into two groups differentiated by DICE similarity of 54 % (data not shown).

REP-PCR analysis

A summary of REP-PCR data is presented in Table 3. Five REP phenotypes among the 118 isolates were revealed, of which two were unique. Four out of five loci were polymorphic (80 %). The UPGMA dendrogram generated from the REP data showed that the 118 *P. capsici* isolates were grouped into two clusters and differentiated by DICE similarity of 40 % (data not shown). One of these clusters comprised most of the isolates including one large clonal group of 109 isolates and two small clonal groups, consisting of two and five isolates, respectively.

Combined RAMS and REP analysis

A summary of the combined RAMS and REP data is presented in Table 3. Fifty-three loci were identified, of which 44 were polymorphic (83.2 %). Ten phenotypes were detected, of which eight were unique and two clonal groups. One of the clonal types comprised 108 isolates, while the other contained only two isolates. Comparing the isolates from the two geographical regions, *P. capsici* isolates from the SE region were more diverse than those from the NCC region, based on phenotypes, polymorphic loci and Shannon diversity index.

In order to assess the overall genetic diversity of the whole population and relationship between the two regional

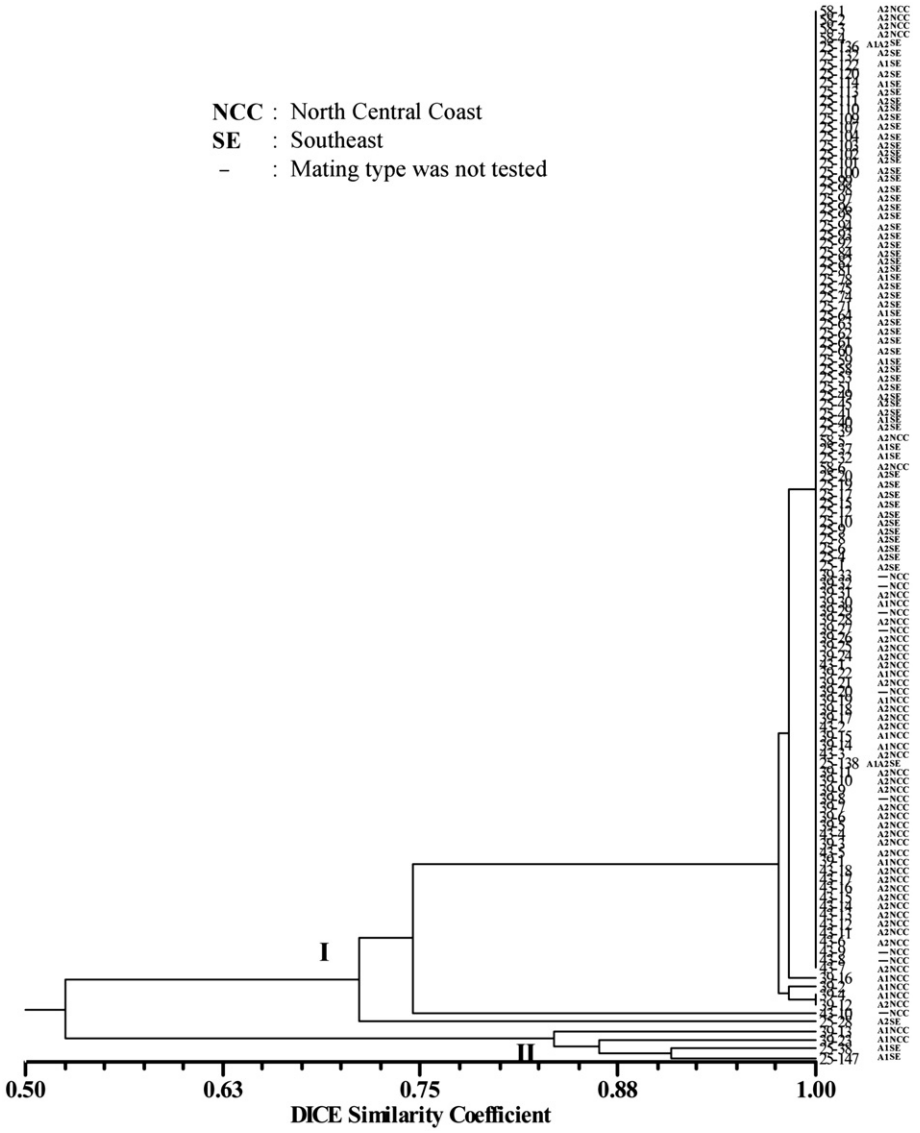

Fig 2 – UPGMA dendrogram of 118 *Phytophthora capsici* isolates based on the combined RAMS and REP data.

Table 4 – Summary statistics of RAMS and REP phenotypes of 118 *Phytophthora capsici* isolates from black pepper based on provinces and mating types.

Province	No. of isolates	No. of phenotypes	No. of unique phenotypes	No. of polymorphic loci (% of total)	Shannon diversity index
Single MT[a] detected					
Binh Phuoc	6	1	–	6 (0.0)	0.00
Ba Ria-Vung Tau	18	2	1	16 (30.2)	0.09
Multi-MT detected					
Dong Nai	33	4	4	33 (62.3)	0.21
Quang Tri	61	4	3	34 (64.1)	0.18
Mating type					
A1	21	8	6	37 (69.8)	0.31
A2	86	2	2	18 (34.0)	0.06

a Mating type.

subpopulations, the combined data from RAMS and REP analyses were used to construct an UPGMA dendrogram (Fig 2). The *P. capsici* isolates from black pepper are distributed in two main groups, I and II, which are differentiated at DICE similarity of 53 %. Group I comprises 114 isolates with 108 belonging to one large clonal group, two isolates in a small clonal group and four with unique phenotypes. Group II comprises four isolates, with unique phenotypes. The genetic similarity analysis showed that more than 91 % of all isolates were genetically identical and the whole population was nearly homogeneous. The clustering of isolates in the dendrogram does not correlate with geographic origin. The large clonal group consists of isolates obtained from all provinces in both regions. Isolates of both mating types are also found within this single clonal lineage. The results also indicate that isolates are not genetically correlated with mating type.

Comparison between mating type and genetic diversity

As indicated in the combined RAMS and REP data, *P. capsici* isolates appeared to be genetically more diverse in provinces where two mating types were detected (Table 4). The number of phenotypes, polymorphic loci and Shannon diversity index of isolates from Dong Nai and Quang Tri provinces with two mating types detected were all higher than those of isolates from Binh Phuoc and Ba Ria-Vung Tau provinces where only the A2 mating type was detected. It could be argued that the results may be skewed by the larger number of isolates analysed for Dong Nai and Quang Tri provinces. However, sample size bias does not appear to be a factor here since neither a larger number of phenotypes nor a higher Shannon diversity index was observed for Quang Tri compared to Dong Nai, the former province having almost twice the number of isolates analysed.

The number of phenotypes, polymorphic loci and Shannon diversity index of A1 isolates were all much higher than those of A2 isolates (Table 4). This higher level of genetic diversity for the A1 mating type was detected despite the relative lower number of isolates. Of particular note is that the A1 isolates account for six of the eight unique phenotypes and eight out of ten total phenotypes detected in the whole population, despite the low ratio of this mating type (<20 %) in the entire population.

Discussion

The current study represents the first intensive study of mating type and genetic diversity of *P. capsici* isolates associated with black pepper collected from various growing regions in Vietnam. The findings contribute to a wider understanding of the population structure of *P. capsici* that causes black pepper foot rot. The analysis of *P. capsici* isolates revealed the presence of both mating types in two different climatic regions, with the A2 type detected at higher frequency than the A1 type. As a result, the first hypothesis that only one mating type exists in the *P. capsici* population from black pepper is rejected. This finding is in contrast to reports on the distribution of *P. capsici* mating types in India and Indonesia where only mating type A1 was detected (Chowdappa & Chandramohanan 1997; Purwantara *et al.* 2004). The presence of both mating types from a single field or plant indicates that the sexual stage of the disease cycle can potentially occur under favourable conditions. However, sexual reproduction may not be playing a direct role in disease epidemics of black pepper foot rot in Vietnam. One of the conditions which support random sexual reproduction of *Phytophthora* species is the presence of both compatibility types in an even ratio (Brasier 1992; McDonald & McDermott 1993; Goodwin 1997; Lamour & Hausbeck 2000). In the current study, the ratio of A1:A2 differed greatly from 1:1 ratio.

According to Drenth & Goodwin (1999), the coexistence of both mating types does not always imply the occurrence of sexual reproduction in *Phytophthora*. Although sexual reproduction of *P. capsici* from black pepper in Vietnam is yet to be established, it is speculated that sexual recombination may still be a contributor to the small amount of genetic diversity observed among the *P. capsici* isolates. Genetic analysis from the current study showed that the population was generally clonal throughout the different growing regions, but exhibits a higher level of diversity in provinces where two mating types were found. Although the results may be biased by the larger sample size in Dong Nai and Quang Tri provinces where both mating types are present, this is still an indication of a correlation between the potential for sexual reproduction and higher level of genetic diversity. Furthermore, due to the predominantly clonal nature of the population, a larger

sample size does not necessarily detect higher levels of diversity as indicated by the lower diversity in Quang Tri compared to that of Dong Nai with nearly twice the sample size. These findings imply that sexual reproduction may still occur in the field, perhaps at a very low frequency. Further analysis to reveal allelic recombination among isolates would provide more insight on the sexual reproduction of this pathogen.

Overall the level of genetic diversity detected among the *P. capsici* isolates from black pepper was relatively low. One hundred and eight isolates were found to be identical in their RAMS and REP phenotypes. The genetic pattern of the *P. capsici* population was not found to be associated with geographic origin. Previous studies which found a high level of genetic diversity among isolates of *P. capsici* from capsicum did not reveal any clear correlation between DNA pattern and geographic origin either (Förster *et al.* 1990; Hwang *et al.* 1991). The second hypothesis for this study that *P. capsici* isolates from black pepper from two different climatic regions in Vietnam are genetically undifferentiated is accepted. This finding is similar to that of a study documented for *P. capsici* showing that all isolates from cocoa were identical and formed a single cluster (Chowdappa & Mohanan 1995). This reflects a typical disease epidemic scenario in which the rapid and ferocious spread of the pathogen is generated by asexual or clonal reproduction. According to Chee & Jee (2001), the low level of genetic diversity in oomycetous pathogens is due to inbreeding or the dominance of asexual reproduction with little activity of oospores in the life cycle. It is reasonable to propose that Phytophthora foot rot epidemics of black pepper in Vietnam are the direct result of a single clone. In addition to taking into account the longer term disease cycle involving sexual reproduction as mentioned earlier, the rapid widespread clonal dispersal of *P. capsici* throughout growing regions must be considered seriously in developing disease management strategies. The clonal nature of black pepper *P. capsici* is to some extent akin to *P. infestans* in that a single clonal genotype is believed to have caused the Irish potato famine (Goodwin *et al.* 1994). Similarly, Hantula *et al.* (1997) found that *P. cactorum* population, causing strawberry crown rot in Europe, was also of a single clonal lineage. The findings in the current study agrees with the theory that the population of a *Phytophthora* species outside its centre of origin is likely be greatly reduced in genetic diversity and contains only a subset of the diversity found in the primary centre of origin (Goodwin 1997).

RAMS and REP fingerprinting analysis indicated that the group II *P. capsici* isolates were clearly separate from the large clonal group I in the current study. Other than sexual recombination, these different genetic groups could be explained by either multiple genotype introductions, mutation or interspecific hybridisation. According to Goodwin (1997), clonal reproduction is predominant in populations of many *Phytophthora* species. Any variation within a lineage must arise from mutation or possibly mitotic recombination. Our finding is consistent with the observations of Hwang *et al.* (1991) who proposed that the significant variations in the mtDNA of *P. capsici* isolates from capsicum were due to both length mutations and alteration of base sequences. Mitotic recombination, mutation and gene conversion have all been considered to be sources of variation for *Phytophthora* species that spread as large clonal lineages such as *P. infestans*, *Phytophthora cinnamomi*, and *Phytophthora ramorum* (Goodwin *et al.* 1994; Dobrowolski *et al.* 2003; Prospero *et al.* 2007). In Spain and Peru, the variation of *P. capsici* isolates was also reported to be a product of mutation or mitotic recombination rather than sexual recombination (Silvar *et al.* 2006; Hurtado-Gonzales *et al.* 2008).

Another potential source of genetic variation could have arisen from hybridisation between individuals from other *Phytophthora* species. The phenomenon of outcrossing between *Phytophthora* species has been demonstrated in the laboratory (Man in 't Veld *et al.* 2007). Although interspecific hybridisation in fungi is very rare, potential evolutionary developments ranging from the acquisition of new host specificities to emergence of entirely new *Phytophthora* taxa has been documented (Brasier *et al.* 1999). The species hybrids created from *in vitro* fusion of zoospores of *P. capsici* and *Phytophthora nicotianae* were new hybrid genotypes that could contribute to the genetic diversity of heterothallic species of *Phytophthora* (English *et al.* 1999). In a previous study, two other species of *Phytophthora*, *P. nicotianae* and *P. cinnamomi* were also isolated from black pepper tissue and field soil (Truong *et al.* 2008). The potential for inter-specific hybridisation is uncertain but it would be of interest to test the hypothesis of possible outcrossing between *P. capsici* and these *Phytophthora* species in future studies.

Regardless of how the small extent of genetic diversity detected came about, the presence of it, i.e. the unique genotypes, in black pepper farms together with the presence of both mating types, suggests that the pathogen has an enhanced ability to adapt to and overcome environmental changes as a result of disease management measures. The spread of contaminated plant or soil material between growing regions should therefore be controlled to prevent further distribution of isolates representing different mating types and genotypes. The spatial and temporal dynamics of this pathogen should also be monitored.

Although RAMS and REP markers are useful in analysing the genetic diversity of *Phytophthora* species (Hantula *et al.* 1997, 2000; Bouws & Finckh 2007), these markers are generally limited in distinguishing between different mating types and other physiological variations. In this study, both A1 and A2 mating types were shown to be distributed in the same clonal group, which indicates that these markers were not sufficiently powerful to discriminate fine variations between genomes. Furthermore, RAMS and REP markers behave as dominant markers; heterozygous loci are not always differentiated from homozygous loci (Burnett 2003). Further work with a larger number of codominant markers (e.g. RFLPs and microsatellites) would be required to identify and determine with greater confidence the population dynamics of *P. capsici* in Vietnam.

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