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Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Phytophthora cinnamomi* in Taiwan

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Abstract. Taiwanese isolates of *Phytophthora cinnamomi* were examined for genetic diversity by the random amplified polymorphic DNA assay. The amount of genetic variation was evaluated by polymerase chain reaction amplification with a set of nine random 10-mer primers. All amplifications revealed scorable polymorphisms among the isolates, and a total of 217 band positions was scored for the nine primers tested. Genetic distances between each of the isolates were calculated and cluster analysis was used to generate a dendrogram showing relationships between them. Isolates did not cluster into groups corresponding to their mating types. Primer OPS-13 did produce one 2.7 kb band for all isolates of A1 mating type, but not for A2 isolates. Moreover, primer OPE-06 amplified one 1.3 kb band for all A1 mating type isolates, while that was absent in A2 isolates except for three isolates obtained from avocado roots. Genetic differentiation of ten isolates obtained from the same location between two mating types was significantly high, indicating that no hybridization occurred between A1 and A2 mating types in that situation. Primer OPE-4 amplified a 680 bp fragment which was only observed in three isolates obtained from avocado roots. Also, genetic differentiation between isolates from avocado and those from other sources was significantly strong (distance between 0.262 and 0.412). These results suggest host specified races might occur in *P. cinnamomi* and the RAPD technique could be used to differentiate races within *P. cinnamomi*.

Keywords: *Phytophthora cinnamomi*; RAPD; Taiwan.

Introduction

Phytophthora cinnamomi Rands is an important soilborne plant pathogen in agricultural, horticultural, and forest ecosystems with a world-wide distribution (Zentmyer, 1980). *P. cinnamomi* is widely distributed in natural forests on the island of Taiwan (Ann, 1984; Ann and Ko, 1985; Ko et al., 1978). Meanwhile, the fungus appears to coexist with many indigenous plant species and causes no disease to those plants (Ko et al., 1978). But, *P. cinnamomi* has brought disease to many cultivated fruit trees and plantations of two *Cinnamomum* species. (Ann, 1984; Chang, 1993)

In Taiwan, the native flora in the natural forest which was inhabitated by both A1 and A2 mating types of *P. cinnamomi* remained healthy. Moreover, considerable variation in biological characteristics was noted among isolates of both A1 and A2 mating types of *P. cinnamomi* isolated from Taiwan. These observations led to the proposition that Taiwan was an Asian origin of *P. cinnamomi* (Ko et al., 1978) in accord with Vavilov's concept of centre of origin (Vavilov, 1951). However, genetic variation has not been used to assess this proposition or the differentiation between A1 and A2 mating types of *P. cinnamomi* isolated from Taiwan.

The random amplified polymorphic DNA (RAPD) markers generated with single primers of arbitrary nucleotide sequence have been shown useful in detecting intraspecific polymorphisms among fungi (Assigbetse et al., 1994; Crowhurst et al., 1991; Zimand et al., 1994). This technique can generate specific DNA fragments used for genome mapping, identification of isolates, and applications in molecular ecology (Hadrys et al., 1992). For plant pathogenic fungi, RAPD analysis can provide markers to differentiate races of *F. solani* f. sp. *cucurbitae* Snyder & Hansen (Crowhurst et al., 1991), *F. oysporum* f. sp. *pisi* (Schlecht.) Snyder & Hansen (Grajal-Martin et al., 1993), *F. o.* f. sp. *vasinfectum* (Assigbetse et al., 1994), aggressive and nonaggressive isolates of *Phoma lingam* (Tode ex Fr.) Desm. (Goodwin and Annis, 1991; Schäfer and Wöstemeyer, 1992), isolates with different geographic origins of *Collectotrichum graminicola* (Ces.) Wils. (Guthrie et al., 1992) and isolates of *Discula umbrinella* Sacc. from different hosts (Haemmerli et al., 1992). This RAPD techique has led us to investigate the genetic diversity within *P. cinnamomi*. The objective of this study was to examine the relationships among 26 isolates of both mating types from Taiwan.

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Table 1. Isolates of *Phytophthora cinnamomi* from Taiwan used in random amplified polymorphic DNA marker analysis.

Isolate number	Mating type	Habit association[a]	Collection location
PC 1	A1	Co	Wulai
PC 3	A2	Co	Wulai
PC 4	A1	Co	Wulai
PC 8	A1	Co	Wulai
PC 9	A2	Co	Wulai
PC 10	A2	Co	Wulai
PC 26	A1	Co	Chumpo
PC 33	A1	Soil	LiuKuei
PC 42	A1	Soil	Yuechih
PC 48	A1	Soil	Yuechih
PC 51	A2	Co	Lienhuachih
PC 52	A2	Co	Lienhuachih
PC 53	A2	Co	Lienhuachih
PC 54	A2	Co	Lienhuachih
PC 81	A1	Co	Chunpo
PC 95	A1	Co	Chunpo
PC 98	A1	Co	Chunpo
PC 101	A1	Co	Wulai
PC 102	A1	Co	Wulai
PC 103	A1	Co	Wulai
PC 119	A2	Co	Wulai
PC 123	A2	Soil	Yuanshan
PC 124	A2	Soil	Yuanshan
PC 131	A2	Av	Chiasien
PC 132	A2	Av	Chunpo
PC 133	A2	Av	Chunpo

[a] Co: *Cinnamomum osmophloeum* Keneh.; Av: avocado.

Materials and Methods

Isolates of Phytophthora cinnamomi

Twenty-six isolates of *P. cinnamomi* were examined for RAPD patterns (Table 1). Eighteen and three isolates were obtained from roots of *Cinnamomum osmophloeum* Keneh. and avocado, respectively. The remaining isolates were isolated from forest soils. The methods of Chang (1993) were used for isolation of *P. cinnamomi* from diseased tissues and soils.

DNA Isolation

Two pieces (ca. 3 × 3 × 3 mm) of culture blocks of *P. cinnamomi* were transferred to 25 ml of V-8 broth (10% V-8 juice, 0.02% $CaCO_3$) in a 250-ml flask. After incubation for 7-10 days at 25°C in darkness, the mycelial mass was removed from the liquid medium by vacuum filtering using Whatman No. 1 filter paper placed on a Buchner funnel and washed with distilled water. DNA was extracted by a method modified from Murray and Thompson (1980) using cetyltrimethylammonium bromide (CTAB) preparation. About 0.5 g vacuum dried mycelia were frozen in liquid nitrogen then ground to fine powder and suspended in 9 ml extraction buffer (50 mM Tris-HCl, pH 8.0, 350 mM sorbitol, 5 mM sodium EDTA, 10% Polyethylene glycol 3350, 0.1% bovine serum albumin (BSA), 0.1% spermine, 0.1% spermidine and 0.1% 2-mercaptoethanol). The extract was filtrated through Miracloth and centrifuged at 13,000 *g* for 15 min in a Kontron H-401 centrifuge. Pellet was resuspended in 350 μl resuspension buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 350 mM sorbitol, and 0.1% 2-mercaptoethanol). Nuclei and organelles in the suspension were lysed by adding 25 μl 20% sarkosyl (N-lauroyl sarcosinate) and incubated at room temperature for 15 min. After adding 70 μl 5M NaCl and 55 μl 8.6% CTAB and heating at 60°C for 10 min, the homogenate was extracted with 600 μl chloroform:isoamyl alcohol (24:1) and centrifuged in a Kubota KM-15200 microcentrifuge (Kubota, Tokyo, Japan) at 5,000 *g* for 10 min. The nucleic acid was precipitated from the aquaeous phase by adding 400 μl isopropanol and pelleted by centrifugation at 12,000 *g* for 10 min. The pellet was resuspended in 100 μl TE buffer (Tris-EDTA, pH 7.6) containing 20 μg/ml RNase and stored at -20°C. DNA concentration was determined using a TKO-100 fluorometer (Hoeffer, San Francisco, CA) and standardized to 10 ng/μl for use in the PCR reaction.

PCR Conditions

Polymerase chain reaction (PCR) amplifications of DNA fragments were performed with each of nine oligonucleotide primers (Table 2), 10 bases in length, obtained from Operon Technologies, Inc. (Alameda, CA). The following PCR condition was optimized for RAPD reactions with an Idaho Air Thermal Cycler. Each 20 μl of PCR reaction mixture in 50 mM Tris-HCl buffer (pH 8.5) containing 20 mM KCl, 2.5 mM $MgCl_2$, 0.5 mg/ml BSA, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.4 μM of a

single 10-base primer, 60 ng of template DNA and 1.7 unit of Taq DNA polymerase (Boeringer Mannheim Biochemica) was heat-sealed in a 25 μl glass capillary. The amplification conditions included a total of 45 cycles with template denaturation at 94°C for 60 sec, primer annealing at 37°C for 7 sec, and primer extension at 72°C for 70 sec during the first two cycles. Time for template denaturation was reduced to one sec for the remaining 43 cycles. The reactions were further incubated at 72°C for four min and the capillaries were stored at 4°C before amplification products were analyzed by gel electrophoresis. The experiment was conducted twice.

Analysis of PCR Products

The PCR products were resolved in a 1.5% agarose gel (Nusieve agarose 3:1; FMC Bioproducts) in 1X TBE buffer (pH 8.3) at 150V for 3 h with a Hoefer-HE100. The gel was stained with ethidium bromide, and visualized with uv light (302 nm). RAPD patterns were produced for at least two replicated PCR amplifications for each individual isolate. Data were collected only from replicated RAPD bands between fragment sizes 0.1 and 2.6 kb. The genetic data of each RAPD fragment was based on two possible character states: a value of 1 for band presence and 0 for its absence. The character state of each RAPD band was scored for each individual isolate of *P. cinnamomi*. Genetic distance, calculated using Nei's estimate of similarity coefficients (Nei and Li, 1979), was computed between all pairs of isolates. The genetic distance between two individuals is equivalent to their total number of observed band differences. A table of genetic distance (Table 2) was presented in order to show genetic variation among isolates of *P. cinnamomi*. A dendrogram (Figure 1) was constructed from the genetic distance by the unweighted paired group method of arithmetic averages (UPGMA).

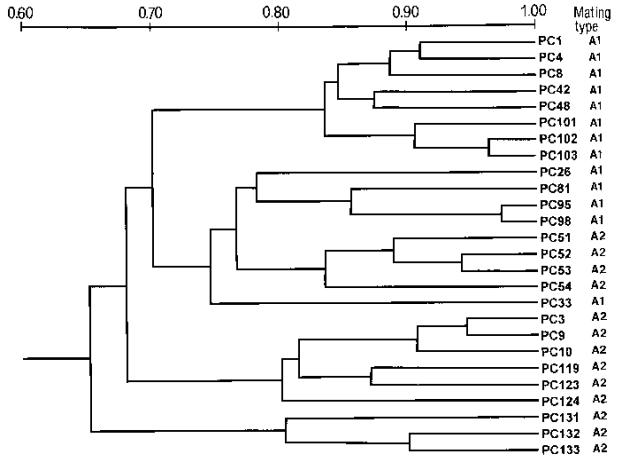

Figure 1. Dendrographic relationships among phenotypically unique random amplified polymorphic DNA patterns of 26 isolates of *Phytophthora cinnamomi*. Branch length is proportional to the gentic distance among isolates. The top scale is the percentage of similarity by Nei's similarity coefficient. Isolates are as given in Table 1.

Results and Discussion

The nine oligonucleotide primers produced a total of 217 bands among the 26 *P. cinnamomi* isolates. Among them, 174 RAPD markers showed polymorphism (80.2%) (Table 3).

Average similarity coefficients for A1 mating type, A2 mating type, and both mating type populations were 0.695, 0.63, and 0.63, respectively (Figure 1; Table 2). These results indicated that the A1 population had less variation than the A2 population. Genetic differentiation between the A1 and A2 populations was not significant because the average similarity coefficient of the A2 population was the same as that of both mating type populations indicating that RAPD technique could not be used to differentiate A1 from A2 in terms of a similarity coefficient.

Primer OPS-13 produced one 2.7 kb band for all A1 mating type isolates but not for A2 isolates (Figure 2, only some isolates were present in the figure). However, when primer OPS-13 was used for detecting mating types of other species of *Phytophthora*, it did not show the same result (unpublished data). Also, primer OPE-06 amplified one 1.36 kb band for all A1 mating type isolates, while that was absent in A2 isolates except for three isolates obtained from avocado roots (Figure 3, most of isolates were present in the figure). These results indicated that RAPD markers have potential as a means of identifying mating types of *P. cinnamomi* isolates. RAPD bands have been used to differentiate races, strains, and pathotypes of fungi of the same species (Assigbetse et al., 1994; Goodwin and Annis, 1991; Crowhurst et al., 1991; Zimand et al., 1994).

Isolates 1, 3, 4, 8, 9, 10, 101, 102, 103, and 119 of *P. cinnamomi* were isolated from the same location within about one m^2 area. Isolates 3, 9, 10, and 119 were A2, while others were A1. Similarity within each mating type

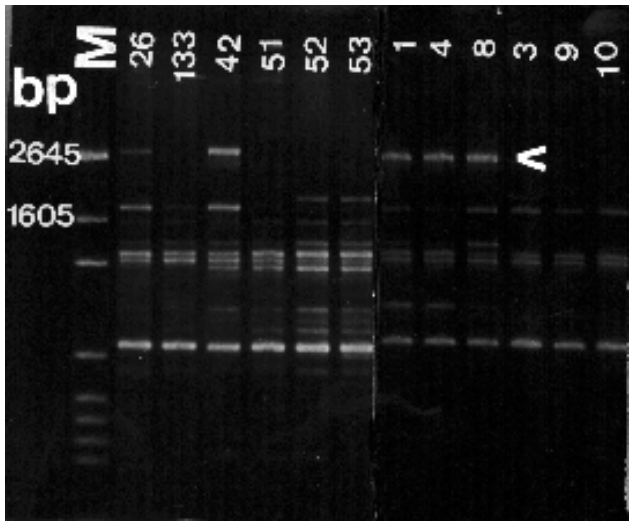

Figure 2. Random amplified polymorphic DNA products for the primer OPS-13. The arrow indicates a 2.7 kb band present in all A1 mating type isolates. Only some representive isolates were present in the figure.

Table 2. Genetic distance of *Phytophthora cinnamomi* isolated from Taiwan used in random amplified polymorphic DNA anaylsis.

Isolate	Isolate number (PC-)																											
number (PC-)	1	4	8	42	48	101	102	103	26	81	95	98	51	52	53	54	33	3	9	10	119	123	124	131	132	133		
1	0																											
4	0.090	0																										
8	0.117	0.111	0																									
42	0.158	0.141	0.169	0																								
48	0.159	0.134	0.160	0.125	0																							
101	0.188	0.181	0.217	0.194	0.192	0																						
102	0.153	0.119	0.173	0.167	0.131	0.093	0																					
103	0.156	0.131	0.176	0.103	0.134	0.095	0.037	0																				
26	0.290	0.300	0.352	0.327	0.273	0.296	0.285	0.290	0																			
81	0.294	0.256	0.311	0.303	0.248	0.291	0.251	0.256	0.248	0																		
95	0.277	0.241	0.284	0.275	0.205	0.302	0.264	0.269	0.187	0.139	0																	
98	0.289	0.252	0.296	0.268	0.198	0.295	0.257	0.262	0.217	0.150	0.150	0																
51	0.294	0.275	0.330	0.283	0.295	0.350	0.308	0.295	0.267	0.200	0.244	0.246	0															
52	0.315	0.296	0.312	0.295	0.279	0.358	0.309	0.315	0.260	0.225	0.211	0.213	0.091	0														
53	0.336	0.309	0.324	0.308	0.273	0.352	0.303	0.309	0.273	0.238	0.224	0.226	0.133	0.059	0													
54	0.305	0.305	0.358	0.352	0.324	0.359	0.309	0.333	0.231	0.232	0.228	0.230	0.146	0.181	0.167	0												
33	0.363	0.294	0.369	0.323	0.304	0.340	0.298	0.294	0.256	0.259	0.282	0.284	0.208	0.233	0.246	0.271	0											
3	0.314	0.333	0.368	0.313	0.305	0.350	0.327	0.324	0.305	0.310	0.282	0.266	0.280	0.282	0.286	0.252	0.371	0										
9	0.340	0.301	0.356	0.299	0.301	0.347	0.324	0.320	0.340	0.307	0.288	0.272	0.296	0.269	0.273	0.307	0.347	0.055	0									
10	0.303	0.324	0.388	0.374	0.353	0.370	0.337	0.343	0.324	0.320	0.320	0.314	0.279	0.320	0.314	0.241	0.371	0.076	0.112	0								
119	0.346	0.336	0.397	0.346	0.327	0.362	0.312	0.327	0.336	0.286	0.297	0.272	0.295	0.306	0.291	0.269	0.353	0.162	0.187	0.169	0							
123	0.339	0.313	0.345	0.330	0.296	0.390	0.333	0.357	0.339	0.300	0.258	0.260	0.291	0.266	0.261	0.239	0.364	0.200	0.205	0.198	0.130	0						
124	0.327	0.327	0.352	0.337	0.308	0.382	0.358	0.337	0.393	0.333	0.352	0.346	0.294	0.305	0.308	0.304	0.404	0.184	0.180	0.212	0.232	0.186	0					
131	0.365	0.346	0.389	0.346	0.336	0.305	0.284	0.262	0.346	0.343	0.370	0.364	0.343	0.370	0.382	0.390	0.353	0.411	0.417	0.412	0.401	0.392	0.385	0				
132	0.315	0.324	0.312	0.333	0.297	0.321	0.274	0.288	0.369	0.340	0.294	0.306	0.330	0.321	0.324	0.349	0.388	0.368	0.374	0.368	0.378	0.328	0.352	0.196	0			
133	0.352	0.360	0.348	0.343	0.316	0.321	0.292	0.306	0.369	0.340	0.330	0.324	0.377	0.364	0.351	0.367	0.387	0.367	0.374	0.396	0.378	0.353	0.354	0.198	0.101	0		

Table 3. Nucleotide sequence of Operon primers used in the experiment and the number of scorable and polymorphic PCR products for *Phytophthora cinnamomi.*

Code	Nuclootide sequence 5' to 3'	No. of scorable PCR products	No. of polymorphic PCR products
OPE-01	CCCAAGGTCC	31	23
OPE-04	GTGACATGCC	15	13
OPE-06	AAGACCCCTC	18	13
OPE-10	CACCAGGTGA	32	30
OPS-13	GTCGTTCCTG	20	17
OPS-14	AAAGGGGTCC	17	16
OPY-10	CAAACGTGGG	29	24
OPY-15	AGTCGCCCTT	25	20
OPY-20	AGCCGTGGAA	30	18
Total		217	174
Average		24.1	19.3

was relatively high. Average similarity coefficient for isolates of A1 mating type and isolates of A2 mating type were 0.80 and 0.79, respectively. But, genetic differentiation between these two mating types was significantly high and greater than 0.1 between A1 and A2 mating type populations (similarity coefficient among A1 and A2 isolates=0.64). These results demonstrated that no hybridization occurred between A1 and A2 mating type in the fields. Isozyme variability among isolates of *P. cinnamomi* from Australia and Papua New Guinea showed that the lack of recombinants between A1 and A2 mating types suggested an absence of sexual reproduction in the fields (Old et al., 1988; Old et al., 1983).

Moreover, primer OPY-15 produced one 1.3 kb band for all A1 mating type isolates from Wulai but not in any A2 isolates from the same location (Figure 4). However, this band was not an unique band for all A1 mating type isolates tested. Some A2 isolates such as 51, 52, and 53, which were not obtained at Wulai, produced this 1.3 kb band, while some A1 isolates such as 131 and 132, which were isolated from avocado at Chiasien and Chunpo, did not produce this band.

Presumably, if exchange of genetic traits occurs between two mating types in the field by sexual means, it would be more readily detected at Wulai than at other sites in the study. The assumption is that where different mating types are found in such a small area, opportunities would exist in the field for sexual reproduction to occur. The generally low level of variable intra-mating types, but high level of variable inter-mating types with respect to these markers in samples of the fungus at Wulai, is confirmed by this work.

One possible explanation is that sexual reproduction does not exist in the diploid organism in the field at Wulai. If it did, the exchange of chromosomes would surely lead to the detection of high variability inter-mating types in the progeny. However, it is equally possible that other factors such as oospore abortion (Rutherford and Ward, 1985) or failure of the oospore to germinate (Chang and Ko, 1991) may have prevented the reproduction of diversified genotypes.

Primer OPE-04 amplified a 680 bp fragment which was only observed in isolates obtained from avocado roots (Figure 5, most of isolates were present in the figure).

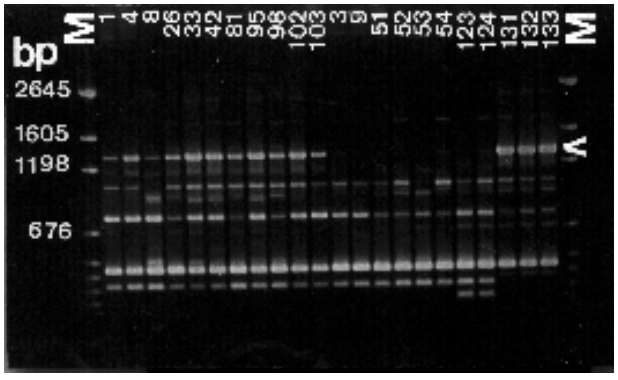

Figure 3. Random amplified polymorphic DNA products for the primer OPE-06. The arrow indicates a 1.36 kb band present in all A1 mating type isolates and three A2 isolates obtained from avocado. Most of isolates were present in the figure.

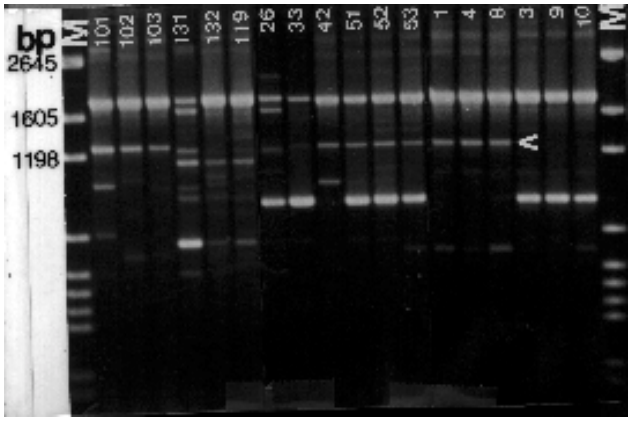

Figure 4. Random amplified polymorphic DNA products for the primer OPY-15. The arrow indicates a 1.3 kb band present in all A1 mating type isolates obtained from Wulai but not in any A2 isolate from the same location.

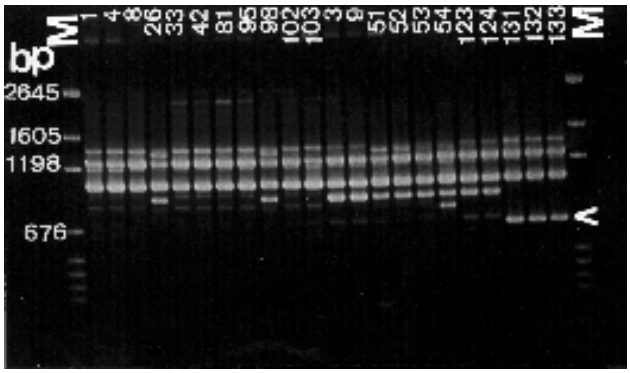

Figure 5. Random amplified polymorphic DNA products for the primer OPE-04. The arrow indicates a 680 bp band present in three A2 isolates obtained from avocado. Most of isolates were present in the figure.

These three isolates were obtained from three different locations more than 50 km away from each other. In addition to the unique RAPD fragment, they had a very low genetic distance (between 0.101 and 0.198) within three of the isolates but high genetic distance (between 0.262 and 0.412) with other isolates (Table 2). These results demonstrated that host-specified races might occur in *P. cinnamomi* and RAPD technique could be used to differentiate races within *P. cinnamomi*. RAPD techique has been used for differentiation of *Trichoderma* strains, *Fusarium oxysporum* f. sp. *vasinfectum* races and *F. solani* f. sp. *cucurbitae* races (Assigbetse et al., 1994; Crowhurst et al., 1991; Zimand et al., 1994).

Numerous methods are available for analyzing molecular data for use in genetic variation of fungi. All methods have inherent assumptions, but through the use of appropriate methods, genetic variation can be made more definitive. The UPGMA is based on the assumption that mutation rates among the different lineages is constant. Since it is currently used for analyses of genetic variation of fungi (e.g. Assigbetse et al., 1994; Folkertsma et al., 1994), it was employed in our results.

Although some unique bands were present in certain groups of isolates in *P. cinnamomi*, the absence of bands of common length did not mean there were no sequence similarities. For a better understanding of relationships, hybridization experiments should be performed between mating types with RAPD fragments or other specific probes.

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