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GENOTYPIC DIFFERENCES BETWEEN ISOLATES OF *PHYTOPHTHORA CINNAMOMI* RANDS AND *P. CITRICOLA* SAWADA OBTAINED FROM TWELVE NURSERY PLANT SPECIES

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Abstract: Genotypic differentiation among 10 isolates of *Phytophthora cinnamomi* Rands and 24 isolates of *Phytophthora citricola* Sawada from 12 different plant species grown in Polish ornamental nurseries was determined. DNA was extracted from pure pathogen cultures and amplified by the PCR technique using ISSR and RAPD primers. 9 primers were used to amplify *P. cinnamomi* and 8 to amplify *P. citricola* DNA. The analyzed amplification products were between 300 and 2300 bp. The genotypical differentiation was from 17 to 35% in *P. cinnamomi* and from 10 to 60% in *P. citricola*. Isolates from host plants of the same family showed, with some exceptions, similar levels of differentiation.

Key words: *Phytophthora cinnamomi* Rands, *Phytophthora citricola* Sawada, genetic distance, RAPD-PCR, ISSR-PCR

INTRODUCTION

Phytophthora cinnamomi Rands and *Phytophthora citricola* Sawada are multi-host, soil-borne pathogens posing an increasing threat to trees and shrubs (Erwin and Ribeiro 1996). Their harmfulness to horticultural and forest nurseries as well as to the natural environment has increased together with the broad introduction of industrial technology to nursery production and with the growth of an unrestricted cross-border market. Several *Phytophthora* species have been isolated in Polish nurseries (Orlikowski and Szkuta 2002) from trees and shrubs belonging to different families, and *P. cinnamomi* and *P. citricola* have been obtained most often. Therefore, a question arises concerning variability and specialization of isolates to different hosts.

The first step towards understanding of this problem is to determine the level of genomic diversity and relatedness between isolates on the basis of polymorphism in DNA profiles. In this work DNA profiles were generated by two types of Polymerase Chain Reaction: Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR), for 10 isolates of *P. cinnamomi* and 24 isolates of *P. citricola* obtained from different plant species in the years 1995–2002.

MATERIAL AND METHODS

Isolates of *Phytophthora* spp. were obtained from symptomatic plants of the following botanical families: *Cupressaceae* – *Chamaecyparis lawsoniana* (Murr.) Parl, *Microbiota decussata* Kom., *Thuja occidentalis* L., and *Thuja plicata* Donn ex D. Don; *Empetraceae* – *Empetrum nigrum* L.; *Ericaceae* – *Calluna vulgaris* (L.) Hull, *Rhododendron catawbiense* L., *Ledum palustre* L.; *Fagaceae* – *Fagus sylvatica* L.; *Pinaceae* – *Abies concolor* (Gord. et Glend.) and *Picea omorica* (Pancic.); *Taxaceae* – *Taxus baccata* L. The standard isolates of *P. cinnamomi* were from *Cinnamomum burmanii* Nees – *Lauraceae* and *Quercus* sp. L. – *Fagaceae* and of *P. citricola* from *Citrus sinensis* (L.) Osbeck – *Rutaceae* and *Taxus baccata* L. – *Taxaceae*. All the isolates (Tab. 1) were collected as pure cultures and DNA was extracted using the method of Aljanabi and Martinez (1997), modified by Wiejacha et al. (2002). After the preliminary screening, the primers which produced rich profiles containing polymorphic and reproducible bands were chosen (Tab. 2). PCR reactions on DNA of *P. cinnamomi* were carried out with 9 primers: C92 (Lee et al. 1996), OPC-02 (Operon Technologies, Alameda, CA, USA) and 827, 834, 842, 848, 850, 855, 860 (University of British Columbia – UBC). For profiling of *P. citricola* the following 8 primers were used: C92, AL4, AL6, AL8 (Lisek 2002), 808, 842, 889, 890 (UBC). Amplifications were performed by Gene*Amp* PCR System 9700 (PE Applied Biosystems) in 13 μl volumes. The thermal profile for RAPD amplifications was performed in 45 cycles: template denaturation at 94°C for 15 s, primer annealing at 36°C for 30 s, primer extension at 72°C for 74 s. The thermal profile for ISSR was performed in 43 cycles: template denatur-

Table 1. Isolates of *P. cinnamomi* and *P. citricola* taken to analysis: reference strains (71, 74, 75, 76) and those obtained from ornamental nurseries in Poland

Phytophthora species	No. of isolates	Host plant	
		Species	Family
P. cinnamomi	74	*Quercus* sp. L.	*Fagaceae*
	75	*Cinnamomum burmanii* Nees	*Lauraceae*
	24	*Empetrum nigrum* L.	*Empetraceae*
	66	*Calluna vulgaris* (L.) Hull	*Ericaceae*
	25	*Taxus baccata* L.	*Taxaceae*
	62	*Microbiota decussata* Kom.	*Cupressaceae*
	65	*Ledum palustre* L.	*Ericaceae*
	30	*Chamaecyparis lawsoniana* (Murr.) Parl	*Cupressaceae*
	15	ibidem	
	61	ibidem	

Phytophthora species	No. of isolates	Host plant	
		Species	Family
P. citricola	71	*Taxus bacctaca* L.	*Taxaceae*
	76	*Citrus sinensis* (L) Osbeck	*Rutaceae*
	41	*Chamaecyparis lawsoniana* (Murr.) Parl	*Cupressaceae*
	51	ibidem	
	57	ibidem	
	58	ibidem	
	63	ibidem	
	64	*Rhododendron* sp. L.	*Ericaceae*
	43	ibidem	
	45	ibidem	
	47	ibidem	
	52	ibidem	
	53	ibidem	
	54	ibidem	
	55	ibidem	
	42	*Thuja occidentalis* L.	*Cupressaceae*
	44	ibidem	
	48	ibidem	
	50	ibidem	
	46	*Thuja plicata Donn* ex D.Don	*Cupressaceae*
	59	*Abies concolor* (Gord. et Glend.)	*Pinaceae*
	49	ibidem	
	56	*Fagus sylvatica* L.	*Fagaceae*
	60	*Picea omorica* (Pancic.)	*Pinaceae*

ation at 95°C for 30 s, primer annealing at 55°C for 30 s, primer extension at 72°C for 90 s. Reaction mixtures were those given by Wiejacha et al. (2002). The PCR products were electrophoresed in 1.4% agarose gel at 4 V/gel cm and were stained with ethidium bromide. Only the bands with sizes from 300 to 2300 bp were analyzed. Each of PCR reactions was carried out at least twice. For a statistical analysis, the bands were coded in the binary form, 1 for the presence or 0 for the absence, for each isolate and primer. Genetic similarities were calculated using the Dice's estimate of the similarity coefficient (Nei and Li 1979) for each pair of isolates according to the formula $S = 2b_{ij}/(b_i+b_j)$, where b_{ij} is the number of amplicons shared by two isolates i and j, and b_i and b_j are the total number of amplicons found in the isolates i and j, respectively. The average similarity values are listed in tables 3 and 4. These values were used to group the isolates in clusters and to construct dendrograms by the unweighted paired group method with arithmetic averages (UPGMA) (Statistica v. 5).

RESULTS

Growth patterns of cultures on PDA – the Potato Dextrose Agar (Fig. 1, 2) did not show any remarkable differences between the isolates of either species. The

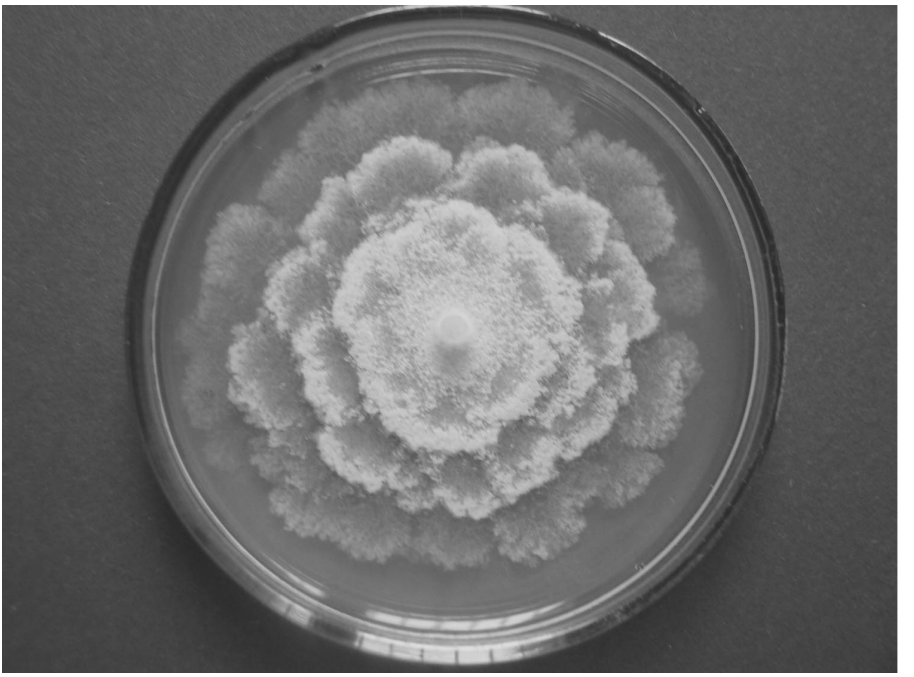

Fig. 1. Growth pattern of *P. cinnamomi* cultures characteristic for PDA medium after 8 days

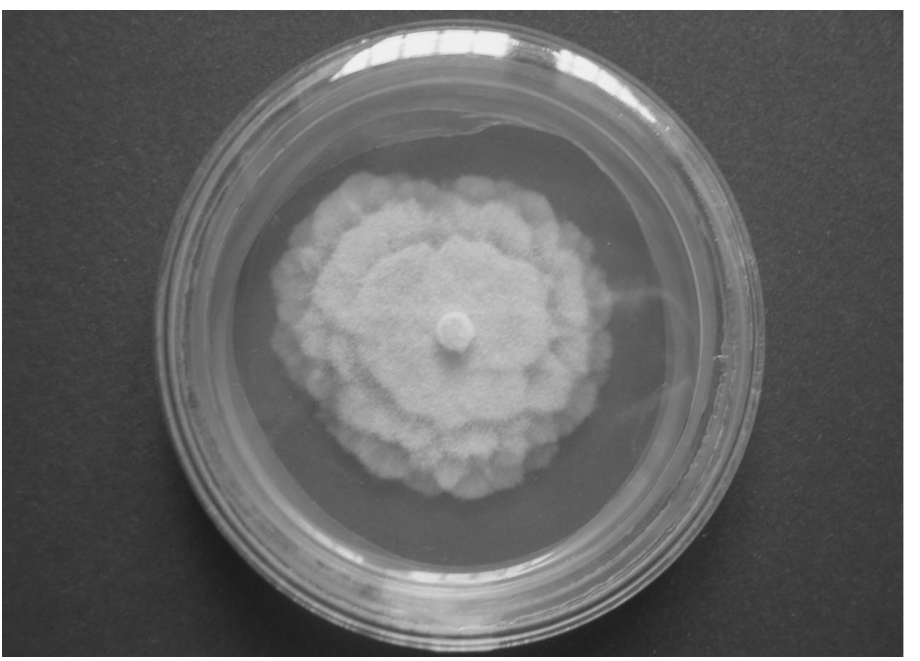

Fig. 2. Growth pattern of *P. citricola* cultures characteristic for PDA medium after 8 days

Table 2. Nucleotide sequences of primers, the number of scorable bands and the number and percentage of polymorphic bands obtained in PCR reactions with templates of two *Phytophthora* species

Primers		No. of scorable bands	No. of polymorphic bands	% of polymorphic bands
Code	Nucleotide sequence 5' to 3'			
		Phytophthora cinnamomi		
C92	AGG CAC CCT TCG	9	4	44
OPC-02	GTG AGG CGT C	7	4	57
827	AC(8) G	4	2	50
834	AG(8) YT	4	2	50
842	GA(7) G	12	3	25
848	CA(8) RG	7	3	42
850	GT(7) YC	6	3	50
855	AC(8) YT	8	5	45
860	TG(8) RA	5	2	40
	Total	62	28	
	Average	7	3	45
		Phytophthora citricola		
C92	AGG CAC CCT TCG	11	10	90
AL4	DBD GA(7)	8	8	100
AL6	AC(8) G	9	6	66
Al8	DBD AC (7)	7	5	71
808	AG(8) C	6	4	66
842	GA(7) G	7	6	85
889	DBD AC(7)	9	7	77
890	VHV GT(7)	6	3	50
	Total	63	49	
	Average	8	6	78

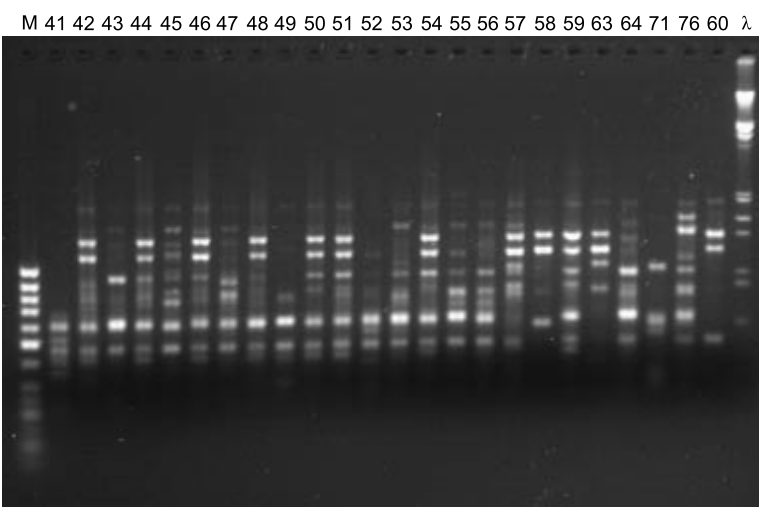

Fig. 3. ISSR-PCR products obtained by means of AL4 primer. Lanes 41–60: *P. citricola* isolates from Polish nurseries, 71 and 76 – reference strains. M: DNA ladder: 50bp GeneRuler (Fermentas), λ: DNA length marker – λ phage DNA digested by *Eco*R I and *Hin*d III enzymes

Table 3. Genetic similarity coefficients of *P. cinnamomi* isolated from Polish ornamental nurseries and reference strains calculated from polymorphism in DNA profiles obtained in PCR reactions

	P74	P75	P15	P24	P25	P30	P61	P62	P65	P66
P74	1									
P75	0.67	1								
P15	0.68	0.66	1							
P24	0.63	0.62	0.76	1						
P25	0.75	0.67	0.67	0.68	1					
P30	0.75	0.69	0.70	0.63	0.83	1				
P61	0.67	0.68	0.75	0.70	0.65	0.61	1			
P62	0.70	0.71	0.78	0.68	0.66	0.64	0.72	1		
P65	0.69	0.71	0.78	0.76	0.62	0.64	0.73	0.74	1	
P66	0.64	0.65	0.73	0.68	0.57	0.59	0.60	0.69	0.69	1

number of reproducible bands analyzed for *P. cinnamomi* was 479, and for *P. citricola* – 912. Separate primers produced from 4 to 12 scorable amplicons on *P. cinnamomi* templates, and from 6 to 11 on *P. citricola* templates (Tab. 2). Polymorphic bands constituted from 25 to 50% for *P. cinnamomi*, and from 50 to 100% for *P. citricola* (Tab. 2, Fig. 3). The range of the genetic similarity coefficients calculated for *P. cinnamomi* was from 0.83 (P 25 – P 30) to 0.57 (P 25 – P 66) (Tab. 3), and for

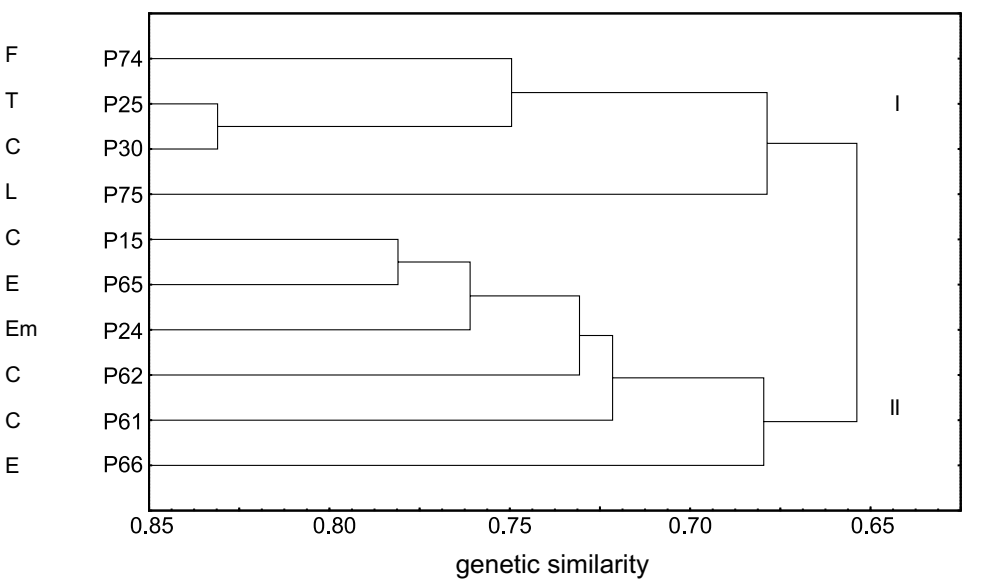

Fig. 4. Dendrograms of genomic relationships between 10 isolates of *P. cinnamomi*. Letters indicate a family of host plant: C – *Cupressaceae*, E – *Ericaceae*, Em – *Empetraceae*, F – *Fagaceae*, L – *Lauraceae*, T – *Taxaceae*

P. citricola it was from 0.71 (P 71 – P 76) to 0.1 (P 44 – P 46) (Tab. 4). The mean genetic similarity value between all the isolates of *P. cinnamomi* was 0.69 and between the isolates of *P. citricola* – 0.38. The high genomic variation in isolates of *P. citricola* is shown on figure 3, where no identical DNA profiles, obtained in PCR reaction with starter AL4, were found.

The isolates of both *Phytophthora* species were distributed within two clusters. In cluster I of *P. cinnamomi* there were four isolates obtained from plants belonging to four different botanical families – two reference isolates from *Quercus* sp. (*Fagaceae*) and *Cinnamomum burmanii* (*Lauraceae*), and isolates from *Chamaecyparis lawsoniana* (*Cupressaceae*) and *Taxus baccata* (*Taxaceae*) (Fig. 4). In cluster II, isolates from plants of the families *Cupressaceae* (*Chamaecyparis lawsoniana* and *Microbiota decussata*), *Empetraceae* (*Empetrum nigrum*) and *Ericaceae* (*Calluna vulgaris* and *Ledum palustre*) were grouped. In cluster I of *P. citricola* there were nine isolates obtained from *Cupressaceae* (*Chamaecyparis lawsoniana, Thuja occidentalis* and *Thuja plicata*) as well as single isolates from *Pinaceae* (*Picea omorica* and *Abies concolor*) and *Rhododendron* sp. plants (Fig. 5). In the second cluster there were seven isolates from plants of *Ericaceae* family and single isolates from *Fagus sylvatica* and *Abies concolor* plants. The isolate P41 from *C. lawsoniana* and the reference isolates P71 and P76 from *Citrus* sp. and *Taxus* sp. were genomically different from the rest.

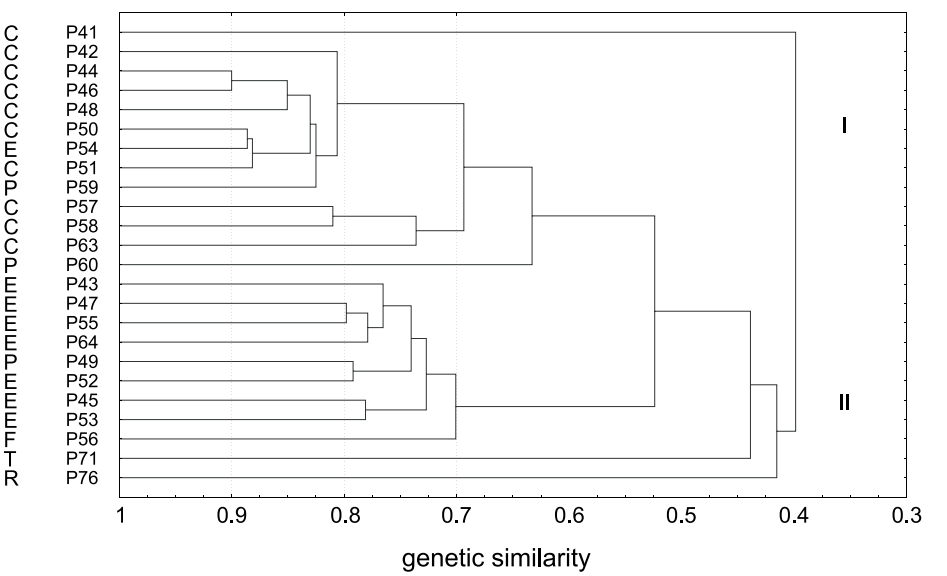

Fig. 5. Dendrograms of genomic relationships between 24 isolates of *P. citricola*. Letters indicate a family of host plant: C – *Cupressaceae*, E – *Ericaceae*, P – *Pinaceae*, R – *Rutaceae*, T – *Taxaceae*

Table 4. Genetic similarity coefficients of *P. citricola* isolated from Polish ornamental nurseries and reference strains calculated from polymorphism in DNA profiles obtained in PCR reactions

	P41	P42	P43	P44	P45	P46	P47	P48	P49	P50	P51	P52	P53	P54	P55	P56	P57	P58	P59	P60	P63	P64	P71	P76
P41	1																							
P42	0.53	1																						
P43	0.53	0.38	1																					
P44	0.58	0.16	0.40	1																				
P45	0.55	0.38	0.23	0.51	1																			
P46	0.57	0.17	0.44	0.10	0.50	1																		
P47	0.60	0.37	0.22	0.38	0.29	0.51	1																	
P48	0.57	0.20	0.39	0.18	0.46	0.12	0.40	1																
P49	0.60	0.40	0.28	0.49	0.31	0.46	0.27	0.46	1															
P50	0.64	0.22	0.42	0.16	0.51	0.13	0.42	0.14	0.43	1														
P51	0.65	0.22	0.40	0.17	0.54	0.18	0.37	0.16	0.45	0.12	1													
P52	0.58	0.37	0.27	0.49	0.23	0.52	0.29	0.46	0.21	0.49	0.49	1												
P53	0.65	0.47	0.26	0.50	0.22	0.54	0.26	0.48	0.29	0.49	0.46	0.35	1											
P54	0.59	0.16	0.35	0.19	0.50	0.20	0.41	0.21	0.51	0.11	0.12	0.45	0.44	1										
P55	0.57	0.35	0.24	0.44	0.26	0.53	0.20	0.43	0.24	0.40	0.41	0.23	0.31	0.48	1									
P56	0.60	0.48	0.34	0.46	0.31	0.50	0.29	0.49	0.30	0.46	0.45	0.30	0.24	0.42	0.27	1								
P57	0.61	0.27	0.48	0.30	0.57	0.29	0.51	0.32	0.56	0.22	0.25	0.55	0.52	0.31	0.50	0.56	1							
P58	0.54	0.26	0.50	0.38	0.50	0.31	0.51	0.30	0.52	0.22	0.24	0.49	0.47	0.29	0.52	0.58	0.19	1						
P59	0.64	0.23	0.40	0.17	0.53	0.18	0.45	0.17	0.47	0.16	0.14	0.47	0.54	0.23	0.46	0.51	0.22	0.27	1					
P60	0.62	0.35	0.53	0.39	0.57	0.33	0.53	0.34	0.54	0.35	0.36	0.48	0.55	0.47	0.50	0.63	0.28	0.34	0.39	1				
P63	0.70	0.38	0.52	0.33	0.56	0.37	0.52	0.40	0.58	0.36	0.36	0.50	0.44	0.36	0.55	0.65	0.23	0.30	0.36	0.44	1			
P64	0.58	0.42	0.25	0.45	0.27	0.45	0.23	0.46	0.24	0.37	0.43	0.26	0.24	0.41	0.21	0.35	0.50	0.46	0.41	0.54	0.56	1		
P71	0.59	0.59	0.48	0.61	0.61	0.62	0.52	0.62	0.51	0.56	0.56	0.51	0.57	0.54	0.48	0.58	0.60	0.56	0.62	0.62	0.57	0.46	1	
P76	0.77	0.61	0.60	0.65	0.55	0.58	0.57	0.61	0.63	0.58	0.58	0.62	0.54	0.56	0.52	0.51	0.56	0.56	0.51	0.64	0.60	0.57	0.71	1

DISCUSSION

Molecular biology techniques, such as PCR, which reveals DNA polymorphism, scorable as band patterns, are used to study genomic variability in living organisms. The final results show genetic similarity between objects on the basis of the differences in DNA structure and depict groupings of objects with equal similarity levels on dendrograms. 10 isolates of *P. cinnamomi* and 24 isolates of *P. citricola* analyzed here, using 9 and 8 primers respectively, show genetic variation among the isolates within the species. The level of genomic variation between the isolates of *P. cinnamomi* was twice as low as that of *P. citricola*. *P. cinnamomi* is a highly destructive root pathogen infecting more than 1000 plant species (Erwin and Ribeiro 1996). It occurs in almost all climatic conditions and has well-known molecular uniformity (Brasier and Hansen 1992; Chang et al. 1996). This uniformity is connected with an almost exclusive vegetative mode of propagation since mostly the A2 mating type has been found in production environments (Chang et al. 1996). Hüberli et al. (2001) reported broad morphological and pathogenic variations found in one clonal lineage, accompanied by low microsatellite DNA polymorphism. The genetic diversity of the isolates of *P. cinnamomi* obtained in Polish nurseries from plants of four families was at a level similar to that found among the isolates obtained from diseased roots of *Cinnamomum osmophloeum* and avocado in Taiwan (Chang et al. 1996). The clustering mode presented by Chang et al. (1996) was not dependent on the territorial derivation of isolates. In contrast, isolates of *P. citricola* are highly diverse genomically, depending on the host plant and climatic zone, with similarity levels of 0.32–0.96, as determined by the mtDNA analysis (Förster and Coffey 1991). The high genetic dissimilarity between the isolates of this species was also found in our investigation. A possible explanation of such high variability is that the species was geographically dispersed in ancient times, forming isolated populations which later hybridized leading to new genetic combinations (Brasier and Hansen 1992). Such high variability in the DNA profiles obtained by the ISSR-PCR technique may be very helpful in finding molecular markers for the identification of single isolates with specific characteristics, especially those associated with virulence.

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POLISH SUMMARY

ZRÓŻNICOWANIE GENOTYPOWE IZOLATÓW *PHYTOPHTHORA CINNAMOMI* RANDS I *P. CITRICOLA* SAWADA WYOSOBNIONYCH Z 12 GATUNKÓW ROŚLIN

Analizowano zróżnicowanie genotypowe 10 izolatów *P. cinnamomi* i 24 izolatów *P. citricola*, wyosobnionych z porażonych roślin (cis, cyprysik, jodła, mikrobiota, świerk, żywotnik, bagno, bażyna, różanecznik, wrzos, buk) oraz izolatów referencyjnych dla gatunków. DNA izolowano z czystych kultur i prowadzono analizę produktów powielania metodą PCR (Polymerase Chain Reaction), przy zastosowaniu starterów typu ISSR (Inter Simple Sequence Repeat) i RAPD (Random Amplified Polymorphic DNA). Do analizy *P. cinnamomi* zastosowano 9 starterów, a do *P. citricola* 8 starterów. Analizowane produkty amplifikacji mieściły się w zakresie od 300 do 2 300 pz. Zróżnicowanie genotypowe pomiędzy izolatami *P. cinnamomi* było od 17 do 35% a pomiędzy *P. citricola* od 10 do 60%. Izolaty z roślin należących do jednej rodziny, wykazywały, z nielicznymi wyjątkami, podobny poziom zróżnicowania.