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THE IDENTIFICATION OF FIVE *PHYTOPHTHORA* SPECIES ON THE BASIS OF DNA MARKERS OBTAINED *VIA* THE PCR TECHNIQUE WITH NON-SPECIFIC PRIMERS

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Abstract

We investigated the usefulness of 90 ISSR and 28 RAPD non-specific primers in PCR reactions to establish markers enabling the identification of five *Phytophthora* species (*P. cactorum*, *P. cinnamomi*, *P. citricola*, *P. citrophthora* and *P. palmivora*) which are pathogens of nursery plants. Two RAPD and four ISSR primers were selected; all initiated the amplification of qualitatively distinct DNA banding patterns for each of the five pathogen species. For each primer and species, band markers were selected. Two primers – C85 (RAPD) and 890 (ISSR), are recommended for identification of the five *Phytophthora* species mentioned.

Key words: DNA markers, ISSR, non-specific primers, RAPD, *Phytophthora* spp.

Introduction

Phytophthora species are primary soil pathogens with a high pathogenicity against host plants (Ristaino and Gumpertz 2000, Brasier et al. 1999). There are currently 106 known species in the *Phytophthora* genus (CABI bioscience database 2004). 11 were discovered in the last five years, thanks to improvements in analytical technology, and indeed the genus has come under greater scrutiny as there has been an increase in the incidence and severity of the diseases it causes (Atkins and Clark 2004, Lievens et al. 2006, Martin et al. 2000, Schaad et al. 2003, Wiejacha et al. 2004). With the increase of nursery production in Poland and the intensification of international trade, new *Phytophthora* pathogens are constantly being introduced to Poland in plant material (Orlikowski 2006). Controlling *Phytophthora* spp. introduction and restricting their spread relies to some extent on rapid and effica-

Phytopathol. Pol. 41: 27–37
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ISSN 1230-0462

cious methods of their detection and identification. Classical, microbiological identification, which still dominates in practice, is based on morphological and physiological traits of pathogens. The high variability of these traits and the overlapping of quantitative characteristics between species mean mistakes are easy to make in taxonomical analysis. In addition, the method is time-consuming and requires both theoretical and practical knowledge of the organisms. Therefore, 16 years ago, a shift in the methodology was noticed towards genome analyses (Goodwin et al. 1989, Forster et al. 1990). Due to rapid development of analytical techniques, new methods based on DNA amplification are often brought into practice, for example PCR, which is a relatively quick, cheap and easy method. Using non-specific RAPD and ISSR primers enabled amplification of DNA fragments of different size and the selection of markers, i.e. fragments characteristic of particular species. These techniques are characterized by a simple procedure and do not require previous knowledge about the DNA sequences to be amplified or a high quality or quantity of DNA.

The aim of this study was to select DNA markers generated by RAPD- and ISSR-PCR techniques, to be used to identify each of the five *Phytophthora* species, of which four were the most frequently isolated in ornamental nurseries from plants showing *Phytophthora* rot symptoms.

Materials and methods

We analyzed 46 isolates of *Phytophthora* obtained from different symptomatic host plants in Polish nurseries (39) and from reference isolates (7). The total number of isolates for each species was five of *P. cactorum* and *P. palmivora*, 10 of *P. cinnamomi*, 14 of *P. citricola* and 12 of *P. citrophthora* (Table 1). All the isolates were previously identified microbiologically (mycelium characteristics and spore morphology). The results were confirmed with species-specific PCR as follows: ADF1/ADR for *P. cactorum* and DC9/DC5 for *P. cinnamomi* (Boersma et al. 2000), CITR1/CITR2 for *P. citricola* (Schubert et al. 1999), and Ctph1/Ctph2 for *P. citrophthora* (Érsek et al. 1994). The confirmation for *P. palmivora* was made with the primers ITS4/ITS6 followed by digestion with the restriction enzymes *Alu*I, *Msp*I, *Tag*I (Cooke et al. 2000). The isolates were maintained on potato dextrose agar (PDA, Duchefa) at 4°C for less than one year. The DNA was extracted from cultures grown for three–seven days, depending on species, at room temperature in liquid organic medium (Molnar 2005) using the method of Aljanabi and Martinez (1997), modified by Wiejacha et al. (2002). The 28 non-specific RAPD primers belonged to the series OPC, OPQ, OPX, OPY and OPZ (Operon Technologies, USA), and also C85 and C92, described by Lee et al. (1996) were used. The 90 ISSR primers belonged to the series 800 (University of British Columbia, USA). For both RAPD and ISSR, the PCR reactions were set up in a 20-µl volume containing 20 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.5 u *Taq* DNA polymerase (Sigma-Aldrich), 100 µM of each dNTP, and 1.54 µM of the studied primer. Amplification

Table 1

List of the *Phytophthora* spp. isolates used in the study
(reference isolates are in bold)

Phytophthora species	Host plant	Isolate number	Source
1	2	3	4
P. cactorum	*Acer saccharinum*	702	Poland
	Chamaecyparis lawsoniana	100	Poland
	Fagus sylvatica	108	Poland
	Rhododendron sp.	138	Poland
	Sorbus aucuparia*	**372**	**Poland**
P. cinnamomi	*Calluna vulgaris*	66	Poland
	Chamaecyparis lawsoniana	15	Poland
	Cinnamomium burmanii**	**75**	**Indonesia**
	Empetrum nigrum	24	Poland
	Ledum palustre	65	Poland
	Microbiota decussata	62	Poland
	Pinus sylvestris	155	Poland
	Rhododendron sp.	187	Poland
	Taxus baccata	25	Poland
	Quercus* sp.	**74**	**France**
P. citricola	*Abies concolor*	49	Poland
	Alnus glutinosa	1 178	Poland
	Rhododendron sp.	225	Poland
	Calluna vulgaris	104	Poland
	Chamaecyparis lawsoniana	41	Poland
	Citrus sinensis**	**76**	**?**
	Fagus sylvatica	56	Poland
	Fraxinus excelsior	109	Poland
	Picea omorica	60	Poland
	Pinus sylvestris	1 294	Poland
	Rhododendron sp.	53	Poland
	Taxus baccata*	**71**	**The Netherlands**
	Thuja occidentalis	44	Poland
	Vaccinium vitis-ideae	150	Poland
P. citrophthora	*Abies concolor*	245	Poland
	Rhododendron* sp.	**373**	**France**
	Ilex aquifolium	248	Poland
	Kalanchoë blossfeldiana	612	Poland
	Photinia × fraseri	411	Poland
	Picea excelsa	246	Poland
	Pieris japonica	422	Poland

Table 1 – cont.

1	2	3	4
	Pinus sylvestris	249	Poland
	Podocarpus alpinus	242	Poland
	Rhododendron sp.	240	Poland
	Skimia japonica	158	Poland
	Syringa vulgaris	241	Poland
P. palmivora	***Areca catechu*****	**78**	**India**
	Cymbidium sp.	1 340	?
	Epidendrum sp.	1 338	?
	Hedera helix	237	Poland
	Phalaenopsis sp.	1 339	?

*Isolates obtained from State Plant Health and Seed Inspection Service, Toruń, Poland.
**Isolates obtained from Fungal Biodiversity Center CBS, KNAW, Utrecht, The Netherlands.

with RAPD primers was performed in a Gene*Amp* PCR System 9700 (Applied Biosystems) and for ISSR primers in a Mastercycler 533 (Eppendorf). The thermal profile for RAPD amplification was: an initial denaturation at 94°C for 30 s, followed by 45 cycles of 94°C for 15 s, 36°C for 30 s and 72°C for 74 s, followed by a final extension at 72°C for 5 min. The programme for ISSR was: an initial denaturation at 95°C for 5 min, followed by 43 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s, followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed in a 1.4% agarose gel (Sigma-Aldrich) at 4 V/cm of gel. Bands were visualized by ethidium bromide. Two independent PCR reactions were carried out for each primer × species. The band profiles were scored with the Biocapt software, version 1103 (Vilbert-Lourmat). Only bands between 400 and 2500 bp were considered.

Results

Of the 118 primers tested two RAPD and four ISSR were selected as they produced the most distinct, polymorphic and reproducible DNA bands usable to identify each of the five species of *Phytophthora* (Table 2). Selecting bands common to all the isolates within the species was the first step in this study. The investigated isolates of one species produced almost identical or markedly different banding patterns (Phots 1, 2). Isolates of *P. cactorum*, *P. palmivora*, *P. cinnamomi* and *P. citrophthora* obtained from different plant species showed lower band polymorphism within species (Phots 1 A, B, 2 A, B, and 1 C, D, 2 C, D) than isolates of *P. citricola* (Phots 1 E and 2 E). The number of marker bands – from one to three – depended on the level of polymorphism (Table 3).

As the next step in marker design, the banding patterns generated with one primer for the five species were compared in order to select markers differentiating them (Table 3, Phot. 3). Although all six primers generated markers usable to dif-

Table 2

List of the primers recommended for identification of five *Phytophthora* spp. – *P. cactorum, P. cinnamomi, P. citricola, P. citrophthora, P. palmivora*

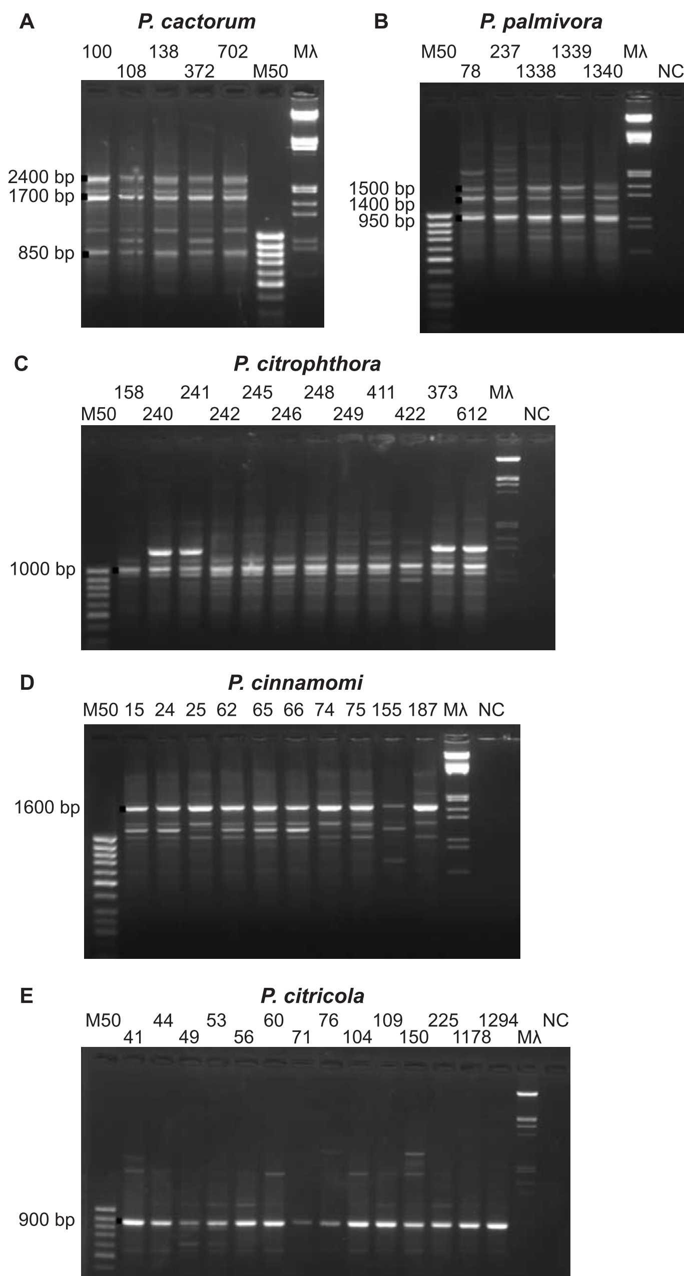
Primer	Nucleotide sequence 5' to 3'
C85	ATG GCT ACT GGC
C92	AGG CAC CCT TCG
808	$(AG)_8C$
827	$(AC)_8G$
889	DBD$(AC)_7$ D = (A,G,T); B = (C,G,T)
890	VHV$(GT)_7$ V = (A,C,G); H = (A,C,T)

ferentiate between species, the most reliable ones were produced with RAPD C85 and ISSR 890. Both generated clearly different DNA profiles for species, and had single marker bands that were distinctly visually different. C85 produced three marker bands with the DNA of *P. cactorum*, of sizes 2400, 1700 and 850 bp, and with the DNA of *P. palmivora*, of sizes 1500, 1400 and 950 bp (Phot. 1 A, B). For each of the remaining taxa, the primer generated single markers of different sizes: 1000 bp for *P. citrophthora* (Phot. 1 C), 1600 bp for *P. cinnamomi* (Phot. 1 D) and 900 bp for *P. citricola* (Phot. 1 E). ISSR 890 generated two marker bands of 900 and 700 bp with the DNA of *P. cactorum* (Phot. 2 A), two of 1700 and 800 bp with *P. citrophthora* (Phot. 2 C), two of 1400 and 550 bp with *P. cinnamomi* (Phot. 2 D), one of 1000 bp with *P. citricola* (Phot. 2 E) and one of 750 bp with *P. palmivora* (Phot. 2 B). In Photograph 3, proposals for each of the markers for primer × *Phytophthora* species are showed.

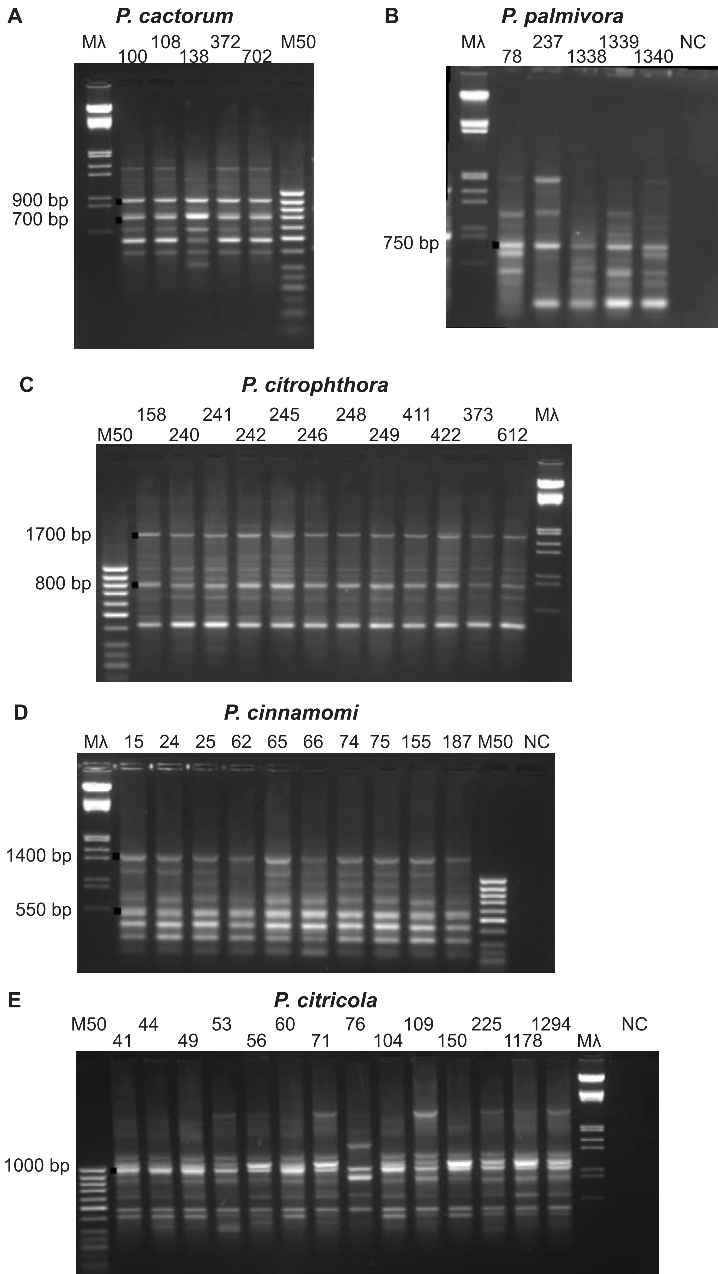
Discussion

The identification of *Phytophthora* species using microbiological techniques requires highly qualified personnel and is costly due to the labour involved. As it is partly based on quantitative characteristics, it can also be unreliable due to the high plasticity of microorganisms. Therefore, a range of supplementary protocols is at our disposal – from isoenzymatic to genotypic (Atkins and Clark 2004, Drenth et al. 2006). Those techniques are fast and efficient, but also costly because special equipment and licensed kits are needed. The PCR technique using non-specific primers that is reported on here is rather simple. The DNA to be amplified does not have to be of high purity and quantity. The disadvantage of the technique is that the DNA has to be isolated from pure pathogen cultures, which are only possible to obtain on selective media that will eliminate other microorganisms.

Both types of primer are non-specific and can be equivalently used to establish fingerprints (Briard et al. 2001, Fernández et al. 2002). ISSR type primers are considered more advantageous as they have better reproducibility, produce more bands and possess higher specificity (Bornet and Branchard 2001, Moisan-Thiéry et al. 2001, Rakoczy-Trojanowska and Bolibok 2004). PCR with non-specific primers found its widest application in the identification of plant genotypes – species, cultivars, pure lines and hybrids (Nybom 2001, Law et al. 2001, Rakoczy-Trojanowska and Bolibok 2004).



Phot. 1. Banding patterns obtained with DNA from *Phytophthora cactorum* (A), *P. palmivora* (B), *P. citrophthora* (C), *P. cinnamomi* (D) and *P. citricola* (E) using the primer RAPD C85. M50 bp – GeneRuler™ 50 bp DNA Ladder, Mλ – Lambda DNA/*Eco*RI + *Hind*III Marker (Fermentas), NC – negative control. The black squares show the bands designated as markers for the species (photo by A. Trzewik)



Phot. 2. Banding patterns obtained with DNA from *Phytophthora cactorum* (A), *P. palmivora* (B), *P. citrophthora* (C), *P. cinnamomi* (D) and *P. citricola* (E) using the primer ISSR 890. M50 bp – GeneRuler™ 50 bp DNA Ladder, Mλ – Lambda DNA/*Eco*RI + *Hind*III Marker (Fermentas), NC – negative control. The black squares show the bands designated as markers for the species (photo by A. Trzewik)

Table 3

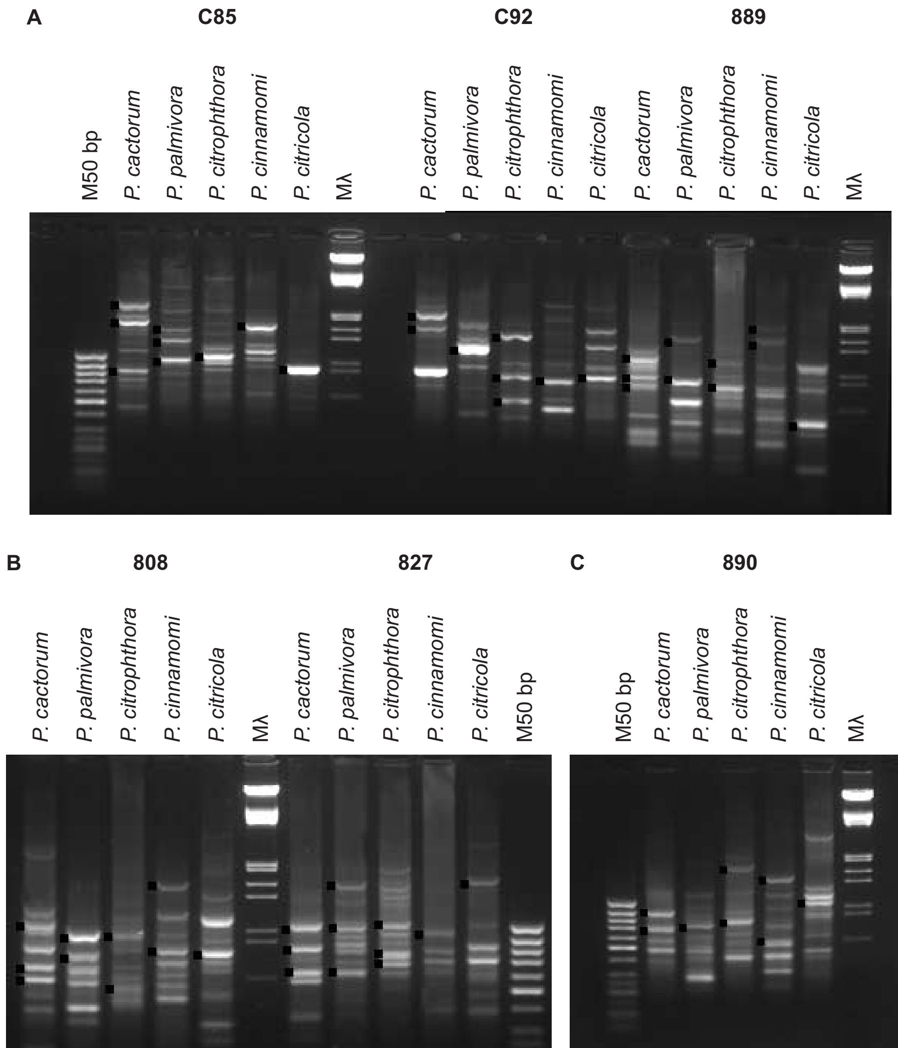
Size of DNA fragments nominated for markers identifying five species of *Phytophthora*

Primer	*P. cactorum*	*P. cinnamomi*	*P. citricola*	*P. citrophthora*	*P. palmivora*
C85	2400	1600	900	1000	1500
	1700				1400
	850				950
C92	2100	800	850	1500	1300
	1600			850	
				550	
808	1000	1500	750	950	900
	600	800		500	700
	550				
827	1000	950	1700	1050	1600
	850			750	1000
	600			650	600
889	1000	1700	400	950	1500
	900	1500		750	850
	750				
890	900	1400	1000	1700	750
	700	550		800	

In our investigation, two primers – C85 and C92, generated reproducible and informative profiles with the DNA of *Phytophthora* spp. The amplicons are distinct for their strength and different sizes. The high availability of RAPD primers for the identification of mating types of *P. cinnamomi* was reported by Chang et al. (1996). Migheli et al. (1998) also demonstrated the usefulness of RAPD primers for the identification of all the main races of *Fusarium oxysporum* f. sp. *dianthi*, isolated in Italy.

The primers differ in the number of amplicons; usually, the ISSR type primers produce more than the RAPD type. In our investigations, the highest number of bands was produced by primer ISSR 808 – 11 bands with the DNA of *P. citricola* and eight with that of *P. cinnamomi* – whereas the most effective RAPD primer, C85, produced only four bands with each of those species. That peculiarity makes the selection of a higher number of markers easier with ISSR type primers.

Each of the six primers selected here generates profiles that can independently differentiate between five *Phytophthora* species. However, the identification of an isolate would be more certain if confirmed in the second reaction with the use of another primer, especially in such a variable species as *P. citricola.* In the case of even more differentiated species, it should be more dependable to identify on the basis of markers generated by a pair of species-specific primers. Routine application of the latter technique is more expensive due to the two- to threefold higher concentration



Phot. 3. Banding patterns obtained with DNA from five species of *Phytophthora* using the primers C85, C92, 889 (A), 808, 827 (B) and 890 (C). M50 bp – GeneRuler™ 50 bp DNA Ladder, Mλ – Lambda DNA/*Eco*RI + *Hind*III Marker (Fermentas). The black squares show the bands designated as markers for the species (photo by A. Trzewik)

of DNA polymerase. Also, the recommended *Phytophthora* RFLP technique (Cooke et al. 2000) is expensive, as it usually requires the use of more than one restriction enzyme. According to Ristaino et al. (1998), one restriction enzyme, *Rsa*I, generated very similar profiles for eight *Phytophthora* species, and only with the use of the second enzyme, *Msp*I, did it enable differentiation between species.

In conclusion, of the non-specific RAPD and ISSR primers listed in Table 2, we can recommend the use of one or two for the identification of five *Phytophthora* spe-

cies (*P. cactorum, P. cinnamomi, P. citricola, P. citrophthora, P. palmivora*), which are the causal agents of diseases in perennials, trees and shrubs grown in nurseries.

Streszczenie

IDENTYFIKACJA PIĘCIU GATUNKÓW *PHYTOPHTHORA* NA PODSTAWIE MARKERÓW DNA OTRZYMANYCH TECHNIKĄ PCR Z NIESPECYFICZNYMI STARTERAMI

Część izolatów otrzymanych z chorych roślin szkółkarskich zidentyfikowano jako *Phytophthora cactorum, P. cinnamomi, P. citricola, P. citrophthora* i *P. palmivora*. Dla wymienionych gatunków opracowano zestaw markerów identyfikacyjnych wytwarzanych techniką PCR ze starterami niespecyficznymi typu RAPD i ISSR. Spośród 118 testowanych starterów dwa startery RAPD i cztery startery ISSR wytwarzały bogate profile z wyraźnymi prążkami. Startery C85 (RAPD) i 890 (ISSR) mogą być polecane do identyfikacji pięciu wymienionych gatunków *Phytophthora*.

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Accepted for publication: 25.09.2006