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USE OF RAPD-PCR AND ITS MARKERS FOR IDENTIFICATION OF *Diaporthe*/*Phomopsis* FROM FRUIT TREES IN SOUTH-EASTERN POLAND

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Abstract. In recent years, it has been reported the increasing harmfulness of *Diaporthe/Phomopsis* spp. in the orchards and nurseries worldwide. They cause nonspecific symptoms which in combination with the morphological similarity of the cultures and difficult sporulation creates problems with their correct identification using classical methods. This implies the need to integrate advanced molecular techniques to identify the strain on the species level. The aim of this research was to determine the identity and genetically characterize forty isolates of *Diaporthe/Phomopsis* obtained in 2010–2012 from the shoots of selected fruit trees grown in the south-eastern Poland. Using the RAPD-PCR technique the genetic similarity within studied population has been demonstrated irrespectively of the origin and type of the host plant from which the isolates derived. Based on the comparative analysis of the non-coding sequence of the ITS regions (ITS1, 5.8S rDNA, ITS2) with the sequences available in NCBI database the studied *Diaporthe* isolates were identified as *Diaporthe eres* species complex not described previously on fruit trees under Polish conditions.

Key words: *Diaporthe eres* species complex, molecular identification, orchard plants, fungi

INTRODUCTION

Recently, *Diaporthe* and their *Phomopsis* anamorphic stage are becoming increasingly important both in Poland and worldwide [Farr et al. 1999, Mostert et al. 2001,

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Król and Kowalik 2011, Udayanga et al. 2011, 2014 a, b, Gomes et al. 2013]. Among over 800 species of *Phomopsis* the pathogenic abilities has been demonstrated for about 60 representatives of this kind [Hewitt and Pearson 1988, Rehner and Uecker 1994, de Guido et al. 2003, Król 2007, Hyde et al. 2011].

The species most commonly found on fruit plants are: *Phomopsis viticola* Sacc., *P. perniciosa* Grove, *P. velata* (Sacc,) Traverso (syn. *P. ambigua* (Sacc.) Trav.), *P. amygdali* (Del.) Tuset et Portilla comb. nov., *P. prunorum* (Cooke) Grove (syn. *P. mali* Roberts), *P. juglandina* (Sacc.) Höhn, *P. ampelina* (Berk. et Curt.) Grove., *P. velata* (Sacc,) Traverso (syn. *P. oblonga* (Desm.) Traverso) and *P. vaccinii* Shear. [Sutton 1980, Uecker 1988, Machowicz-Stefaniak 1993, Farr et al. 1999, Mostert et al. 2001, Karaoglanidis and Bardas 2006, Król 2006, 2007, Kirk et al. 2008, Udayanga et al. 2011].

In recent years, it has been reported their increasing harmfulness in the orchards and nurseries worldwide [Uecker 1988, Uddin et al. 1997, 1998, Adaskaveg et al. 1999, Farr et al. 1999, 2002, Mostert et al. 2001, Michailides and Thomidis 2006, Santos and Philips 2009, Król and Kowalik 2010, Udayanga et al. 2011, 2014 a, b].

Species assignation in *Phomopsis* was initially based on morphological and cultural characteristics, spores dimensions and host affiliation [Uecker 1988]. More recent studies have demonstrated that several species of *Phomopsis* have wide host ranges [Uddin et al 1997, Mostert et al. 2001, van Niekerk et al 2005, Santos and Phillips 2009, Diogo et al. 2010, Gomes et al. 2013, Udayanga et al. 2014]. Moreover, strains of *Phomopsis* isolated from one host species are not necessarily closely related and may represent more than one taxon [Rehner and Uecker 1994, Farr et al. 2002]. The use of anamorph/teleomorph relationships in identifying *Phomopis* species is also insufficient since *Diaporthe* teleomorphs have been described for only approximately 20% of the *Phomopsis* species known to date [Uecker 1988, van Niekerk et al 2005]. Recently, the redefinition of *Phomopsis/Diaporthe* species has been ongoing and the name *Diaporthe* has been adopted over *Phomopsis* under the current convention of "one fungus one name" [Udayanga et al. 2011, 2014 a, b, Gomes et al. 2013].

Species of *Diaporthe* cause nonspecific symptoms such as: cankers, diebacks, fruit rots, leaf spots, blights, decay or wilts on wide range of plant hosts [Sutton 1980, Uecker 1988, Farr et al. 1999, 2002, Król 2002, Król and Kowalik 2010, 2011, Udayanga et al. 2011, 2014 a, b].

A characteristic feature of *Diaporthe/Phomopsis* spp. is the formation of perithecia, unitunicate, 8-spored asci with two-celled ascospores and pycnidia with two types of spores: alpha (α) and beta (β), but only one in four species are known to produce β-conidia [Rehner and Uecker 1994, Udayanga et al. 2014]. Sometimes a third type of conidia called gamma (γ) have been also observed [Cristescu 2007, Rodeva et al. 2009, Udayanga et al. 2011].

The morphological similarity of the cultures and difficult sporulation creates problems with their correct identification using classical methods [Sutton 1980, Uecker 1988, Farr et al. 1999, 2002, Król 2002, Król and Kowalik 2010, 2011, Udayanga et al. 2011, 2014 a, b, Gomes et al. 2013]. This implies the need to integrate advanced molecular techniques to identify the strain on the species level [Crous 2005, Ash et al. 2010].

The aim of this research was to determine the identity and genetically characterize the species of *Diaporthe/Phomopsis* that occur on fruit trees in the south-eastern Poland based on RAPD-PCR and ITS sequencing.

MATERIAL AND METHODS

Fungal isolates. Studies of genetic variability within the *Diaporthe* spp. population were performed using RAPD technique (Random Amplified Polymorphic DNA).

Isolates of *Diaporthe* spp. obtained from the shoots of selected fruit trees which were used in this study are listed in Table 1. Additionally, the reference strains i.e. *Phomopsis mali* NBRC 31031 = IFO 31031 received from Collection Service Culture Collection Division, Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Japan and *P. amygdali* CBS 126679 received from the Netherlands were included for comparative purposes.

In order to confirm the species affiliation of tested *Diaporthe* isolates eight out of forty two isolates were subjected to DNA sequence analyses using as a molecular marker a non-coding sequence of the ITS regions within the ribosomal DNA (ITS1, 5.8S rDNA ITS2).

DNA extractions. One spore cultures of tested isolates were grown on PDA medium at 25°C. After 14 days mycelia were aseptically transferred to Eppendorf tubes. Total genomic DNA was isolated from fresh mycelium using the Invisorb Spin Plant Mini Kit (Stratec Molecular, Germany) according to the protocol supplied by the manufacturer.

The concentration of the extracted genomic DNA was determined by running the samples on 1% agarose gel and comparing the electrophoretic bands to the standard MassRuler ™ DNA Ladder Mix (Thermo Scientific, USA) run in a separate lane on the same gel. All samples were adjusted to the same concentration of 20 ng/ul of DNA by preparing the appropriate dilutions.

The collected DNA served as a template for RAPD reaction and to amplify a region of ITS1, 5.8S rDNA, ITS2.

RAPD primers and amplification conditions. The RAPD reactions were done for the 42 fungal genomic DNA samples using 30 primers synthesized in Genomed Laboratory (Warsaw, Poland) which were used in other related works and whose numbers and sequences are shown in Table 2.

Initially, several RAPD-PCR test reactions were done using the conditions considered to be optimal in previous studies on *Phomopsis viticola* and other species by Król [2002]. Finally, DNA amplification was performed according to modified method of Williams et al. [1990].

RAPD-PCR was performed in 20 µl reaction volume containing 20ng of genomic DNA, 0.4 µl (5 pmol) of oligonucleotide primer, 1 µl dNTPs (2 mM), 2 µl of 10 × Taq Buffer (750 mM Tris HCl pH 8.8; 200 mM (NH4)2SO4; 0.1% Tween 20), 2 µl $MgCl_2$ (25 mM), 0.4 µl of 2U Taq polymerase (Thermo Scientific) and 12.2 µl of MQ H_2O.

The amplifications were performed on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA), using the following programme conditions: an initial denaturation step for 3 min. at 95°C, followed by 45 cycles (denaturation for 45 s at 94°C, annealing for 45s at 37°C, extension for 45 s at 72°C) and the final extension at 72°C for 10 minutes.

Reproducibility of the results was evaluated by repeating the PCR amplification conditions of all strains three times for each primer. In parallel with the test samples a negative control containing all reaction components except template DNA was used.

RAPD analysis. Among pre-tested 30 primers (Genomed Laboratory, Warsaw, Poland) which were used in other related works [Król 2002, unpublished] with 5 *Diaporthe* isolates only three i.e.: OPA-13- 5'CAGCACCCAC 3', OPL-07-5'AGGCGGGAAC 3' and OPR-15-5'GGACAACGAG 3' generated significant and reproducible amplification products and were selected to evaluate polymorphism within *Diaporthe* population.

Table 1. Isolates of *Diaporthe* spp. used in this study

Isolates	Host plant	Origin	Isolates	Host plant	Origin
260J		Wojciechów	398orz		Wojciechów
264J		Wojciechów	401orz		Wojciechów
269J	Apple	Wojciechów	404orz	Walnut	Wojciechów
270J		Wojciechów	405orz		Wojciechów
1430J		Bełżyce	1544orz		Bełżyce
292G		Wojciechów	372L		Wojciechów
1472G		Bełżyce	381L		Wojciechów
1473G	Pear	Bełżyce	386L	Hazel	Wojciechów
1475G		Bełżyce	1569L		Bełżyce
1477G		Bełżyce	1789L		Wojciechów
322W		Wojciechów	3300B		Sandomierz
336W		Wojciechów	3301B		Sandomierz
1328W	Cherry	Felin	3302B	Peach	Sandomierz
1652W		Wojciechów	3319B		Sandomierz
1940W		Felin	3320B		Sandomierz
352S		Wojciechów	437CZ		Wojciechów
353S		Wojciechów	3122CZ		Sandomierz
359S	Plum	Wojciechów	3151CZ	Wild cherry	Sandomierz
1419S		Felin	3127CZ		Sandomierz
1521S		Bełżyce	3124CZ		Sandomierz

PCR products were resolved by gel electrophoresis on 1.5% agarose gel with TBE buffer (1%) containing ethidium bromide, photographed under UV light using the Gel Doc software package. The size of the bands was determined by comparing to the GeneRuler 100 bp DNA Ladder Plus marker (Thermo Scientific, USA). The banding

patterns were analyzed using the Bio-Profil-BIO-1D++ (Vilber-Lourmat 2009) software package.

Genetic similarity between all isolates was estimated according to the Dice formula [Nei and Li 1979]. The resulting matrix were used to construct dendrograms by the software available from the website http://genomes.urv.cat/UPGMA/ [Garcia-Vallve et al. 1999] using unweighted pair group method with arithmetic means (UPGMA).

Table 2. Sequences of the RAPD primers tested in this study

PRIMERS	SEQUENCES 5'–3'	PRIMERS	SEQUENCES 5'–3'
OPA-13	CAGCACCCAC	OPP-11	AACGCGTCGG
OPB-08	GTCCACACGG	OPQ-17	GAAGCCCTTG
OPC-19	GTTGCCAGCC	OPR-15	GGACAACGAG
OPD-16	AGGGCGTAAG	OPS-19	GAGTCAGCAG
OPE-02	GGTGCGGGAA	OPT-03	TCCACTCCTG
OPF-14	TGCTGCAGGT	OPU-05	TTGGCGGCCT
OPG-06	GTGCCTAACC	OPV-10	GGACCTGCTG
OPH-20	GGGAGACATC	OPW-12	TGGGCAGAAG
OPI-01	ACCTGGACAC	OPX-04	CCGCTACCGA
OPJ-20	AAGCGGCCTC	OPY-18	GTGGAGTCAG
OPK-04	CCGCCCAAAC	OPZ-01	TCTGTGCCAC
OPL-07	AGGCGGGAAC	OPAA-16	GGAACCCACA
OPM-02	ACAACGCCTC	OPAB-20	CTTCTCGGAC
OPN-09	TGCCGGCTTG	OPAC-06	CCAGAACGGA
OPO-05	CCCAGTCACT	OPAD-12	AAGAGGGCGT

PCR amplification and sequencing of ITS regions. Eight out of forty two *Diaporthe* isolates (264J, 292G, 322W, 352S, 398orz, 381L, 437Cz, 3300B) from different types of host plants (apple, pear, cherry, plum, walnut, hazel, wild cherry and peach respectively) were selected as representative species for further analysis. The criteria used for considering the selected isolates as representatives were mainly: 1) belonging to the same main cluster which reference strain *P. mali* IF031031, 2) the origin of the different species of host plants (apple, pear, cherry, plum, cherry, peach, walnut and hazelnut) and 3) their application in a previous pathogenicity test in relation to selected fruit plants.

The ITS 1, 5.8S and ITS 2 regions of the fungal rDNA were amplified using the universal primers ITS 1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') [White et al. 1990]. The PCR reaction was performed in 25 µl reaction volume containing: 20 ng of genomic DNA, 0.5 µl of primers ITS 1 and ITS 4, 0.5 µl of dNTPs (10mM), 2.5 µl of 10 × Taq Buffer (750 mM Tris HCl pH 8.8; 200 mM (NH4)2SO4; 0.1% Tween 20), 2.5 µl of MgCl2 (25 mM), 0.5 µl of 2U Taq polymerase (Thermo Scientific) and 16 µl of MQ H_2O.

The amplifications were performed on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA) using the following program an initial denaturation step for 5 min. at 95°C, followed by 40 cycles (denaturation for 30 s at 95°C, annealing for 50 s at 55°C, extension for 1 min. at 72°C) and the final extension at 72°C for 10 minutes.

The amplification products were separated on 1.5% agarose gel with TBE buffer (1%) containing ethidium bromide in analogy to the products obtained by RAPD-PCR.

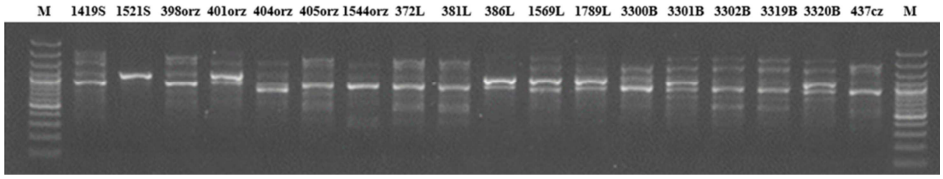
The PCR products were send for sequencing to the Genomed Laboratory in Warsaw, Poland. The analysis of sequence data were carried out using the BioEdit software packages. New sequences generated in this study were deposited in NCBIs GenBank nucleotide database under following accession numbers: 264J_KX505997, 292G_KX505998, 322W_KX505999, 352S_KX506000, 398orz_KX506001, 381L_KX506002, 437cz_KX506003, 3300B_KX506004 [www.ncbi.nlm.nih.gov]. To ascertain the phylogeny of these organisms sequences were aligned and compared with a selected *Phomopsis* and *Diaporthe* species from GenBank (NCBI) using the BLAST software on the NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ [Altschul et al. 1990]. The selected species are listed in Table 3. The average length of the correct reading sequence was about 550 bp. To construct neighbour-joining phylogenetic tree the program from the website http://phylogeny.lirmm.fr/phylo_cgi/index.cgi, were used [Dereeper et al. 2008].

Table 3. The selected species of fungi from NCBI database which sequences were used for comparative analysis

Fungi	Host plant	Origin	Genbank number
Alternaria alternata	*Betula pendula*	Lithuania	AY354228
Diaporthe ambigua	*Vitis vinifera*	Canada	KF017912
Diaporthe eres	*Corylus avellana*	Austria	JQ807460
Diaporthe perniciosa	*Fruit trees*	Japan	AB302254
Diaporthe rudis	*Fruit trees*	Japan	AB302251
Phomopsis amygdale CBS 126679	*Prunus dulcis*	Portugal	KC343022
Phomopsis juglandina	*Juglans sp.*	China	KC242236
Phomopsis mali IFO31031	*Malus pumila* var. *domestica*	Japan	AB665315
Phomopsis viticola	*Vitis sp.*	USA: Connecticut	JN214593
Diaporthe eres	*Pyrus*	Korea	JQ807433
Diaporthe eres	*Malus pumila*	Korea	JQ807441
Diaporthe eres	*Malus sylvestris*	Unknown	KC343088
Diaporthe eres	*Juglans regia*	Italy	KC343074

RESULTS

RAPD analysis. Genetic variability between 42 isolates of *Diaporthe* was determined using the data generated by three RAPD primers (phot. 1).



Phot. 1. RAPD fingerprints obtained with the arbitrarily selected primer OPL-07, separated on a 1.5% agarose gel with ethidium bromide (M-ladder)

Twenty nine RAPD markers were obtained in total (tab. 4). The size of the PCR products ranged from 0.4 to 3.1 kb. The number of polymorphic fragments varied from 8 to 12 per primer. The RAPD-PCR gave in a total of 24 polymorphic bands (82.75%), one monomorphic and four specific (tab. 4).

Based on a binary matrix data the cluster analysis was made and the dendrogram of the 42 *Diaporthe* spp. isolates was drawn using UPGMA (fig. 1). Clustering analysis resolved two main groups. In one of them, the reference isolate of *P. mali* IFO31031 was placed next to almost all isolates, regardless of origin. Only 2 isolates, one from cherry and one from plum located at the second main group. The reference strain *P. amygdali* CBS126679 was the only one outside the main groups of clusters (fig. 1).

Table 4. Results of banding patterns obtained with selected primers

Rapd primers	Number of bands				Size of the products (bp)	
	polymorphic	monomorphic	specific	total	min.	max.
OPR-15	8	0	0	8	607	2112
OPL-07	7	0	2	9	400	2519
OPA-13	9	1	2	12	612	3024
Total	24	1	4	29	–	–
%	82.75	3.44	13.79	–	–	–

Based on the RAPD results the genetic similarity was calculated based on the equation of Nei and Li [1979]: GSij = 2Nij/(Ni + Nj), where Nij = number of bands common to both the i and the j isolates, Ni = total number of bands appearing in i isolate, Nj = total number of bands appearing in j isolate.

The similarity indices ranged from 0.376 to 0.738, and the average value was 0.654. The highest similarity of all tested isolates (0.738) showed: 260J, 264J, 269J, 270J, 322W, 336W, 352S and 437Cz while the lowest similarity was found for the reference strain *P. amygdali* CBS126679. Moreover, *P. mali* IFO 31031 was similar to the other isolates in approximately 62% (fig. 1).

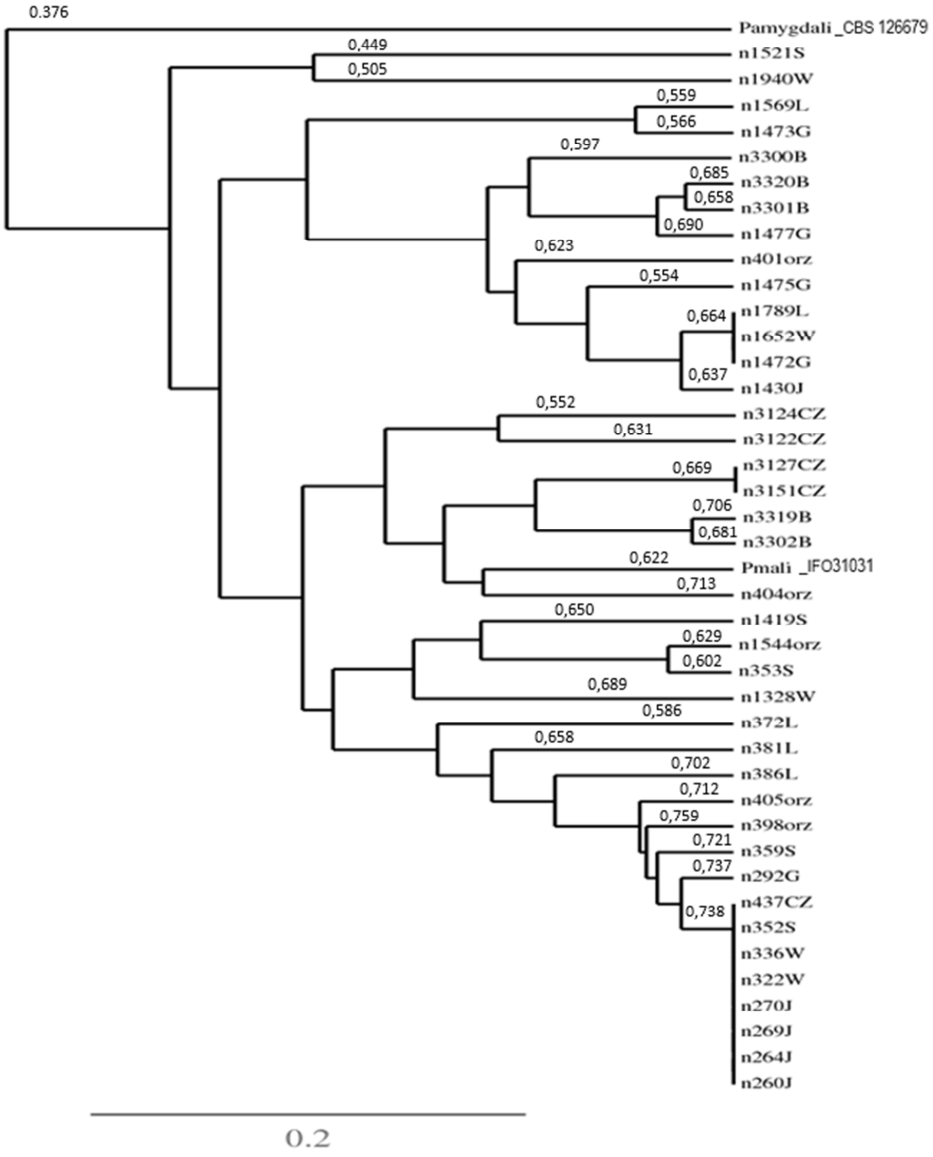
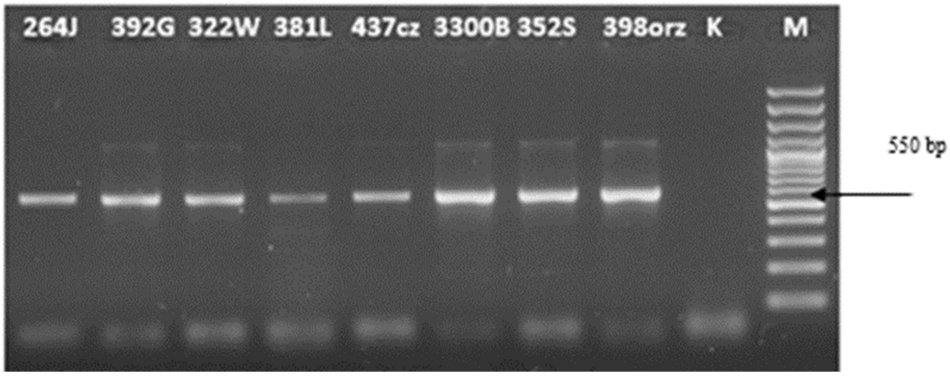


Fig. 1. Dendrogram of 42 analyzed strains of *Diaporthe* spp. revealed by their RAPD markers

Analysis of ITS1, 5.8S rDNA, ITS2. The universal primers ITS1/ITS4 [White et al. 1990] amplified the fragment of the ITS1, 5.8S, ITS2 region of the ribosomal DNA of approximately 550 bp length for all tested *Diaporthe/Phomopsis* isolates (phot. 2).

The results of the BLASTn analysis revealed that the sequences of the tested isolates were identical in 99% to the sequence of the reference strain – *P. mali* IFO31031, except from the 352S were the homology was equal to 98% (fig. 2). For comparison with the sequence of the reference strain of *P. amygdali* CBS126679 the degree of homology was 92%, except from the 353S for which was equivalent to 91%. Within the sequences of *Diaporthe* spp./*Phomopsis* spp. isolates obtained from the tested fruit trees compared with the reference strain of *P. mali* IFO31031 some diversity was found in the form of deletions, substitutions and insertions mainly in the ITS1 region (fig. 2).



Phot. 2. The amplification products obtained with the universal primers ITS1/ITS4 (M – ladder, K – control)

Sequences of isolate 352S from plum tree differed from the reference strain by 4 deletions, the isolates: 264J, 392G, 322W, 381L, 437cz and 3300B – by 2 deletions while isolate 398orz walnut – by 1 deletion. Additionally, isolate 381L from the hazel tree contained two deletions within the sequence of ITS2 region. Furthermore, there was one substitution (conversion of thymine to cytosine) in a sequence of isolates: 264J, 392G, 322W, 352S, 398orz, 437cz and 3300B in the ITS1 region, and one substitution (conversion of adenine to guanine) in a sequence of the same isolates but in the ITS2 region. Only one isolate 352S differed from the reference strain by occurrence of insertion (additional nitrogen – containing nucleobase – adenine) in a sequence of ITS1 region. In addition, comparative analysis showed that isolates from apple (264J), pear (292G), cherry (322W) and peach (3300B) trees were identical (fig. 2). Sequence analysis of ITS regions confirmed in 99% that the tested isolates are closely related and may belong to the one species.

In the present study the nucleotide sequences of the ITS1 and ITS2 region of rDNA gene of tested isolates were compared with the sequences deposited in the NCBI database. The results are shown as a dendrogram on which *Diaporthe* isolates (264J_KX505997, 292G_KX505998, 322W_KX505999, 352S_KX506000, 398orz_KX506001, 381L_KX506002, 437cz_KX506003, 3300B_KX506004) derived from the tested fruit plants, together with *D. perniciosa*_AB302254, *D. eres*_JQ807460, JQ807433, JQ807441, KC343088, KC343074, *D. rudis*_AB302251 and the reference

strain – *P. mali*_ IFO31031_AB665315 formed a vast main group showing 100% homology (fig. 3). Whereas, *P. juglandina*_KC242236, *D. ambigua*_KF017912, *P. viticola* JN214593, and the reference strain – *P. amygdali* CBS 126679_ KC343022 formed the subgroup. The out-group was represented by *A. alternata*_AY354228 (fig. 3).

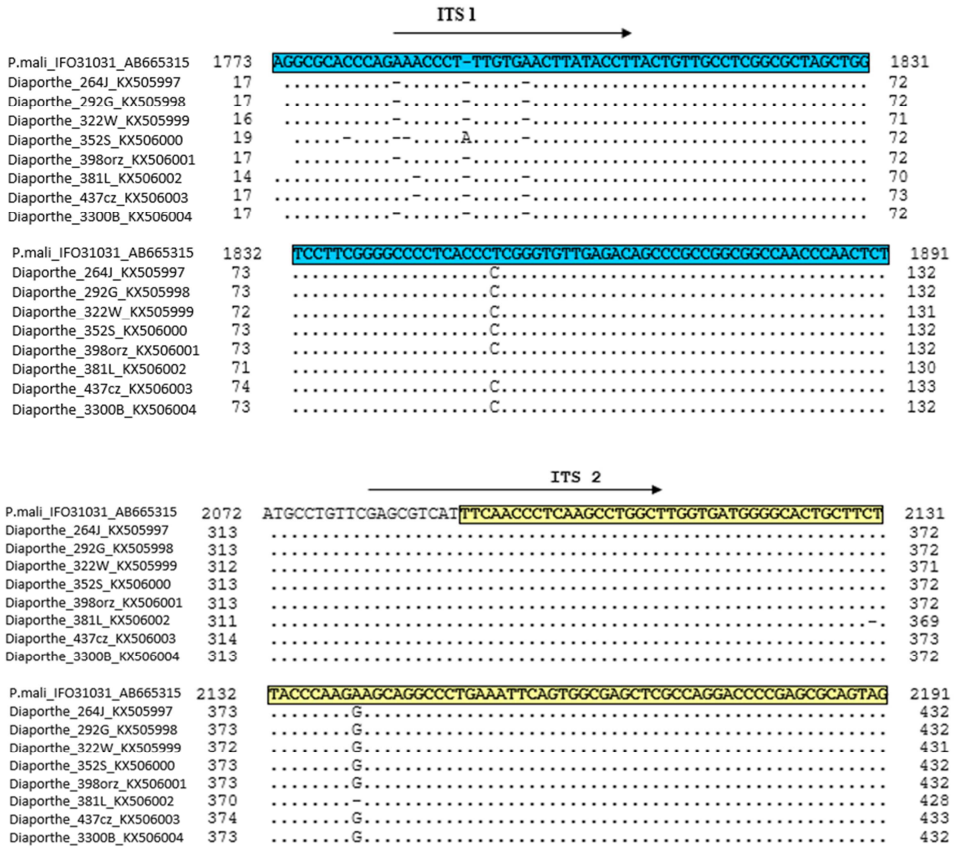


Fig. 2. Fragment of alignment of the sequences of ITS1–ITS2 rDNA regions of the tested isolates with the reference strain *P. mali*_IFO31031_AB665315. Sequences identical to the reference strain are indicated with dots, and the bars represent the lack of the sequence

The authors are aware that the number of the tested samples was too small to draw far-reaching conclusions about the *Diaporthe* spp. population in Poland. However, the conducted analysis can provide valuable access to further, extensive research on the molecular level using multi-gene and biochemical analysis.

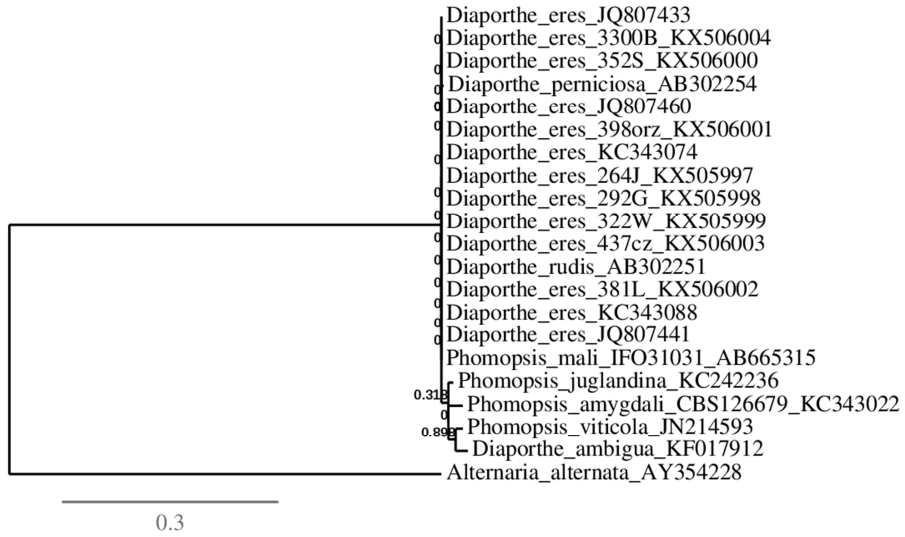


Fig. 3. Molecular Phylogenetic analysis by Maximum Likelihood method based on the Tamura-Nei model [Tamura and Nei 1993]. The tree was rooted to *Alternaria alternata* used as the out group isolate (the branch length is proportional to the number of substitutions per site)

DISCUSSION

According to the literature, morphological similarity of the fungi requires the molecular techniques in order to obtain reliable results for the identification of the microorganism on the species level [Rehner and Uecker 1994, Uddin et al. 1997, Chen et al. 2002 a, b, Król 2002 a, b, van Rensburg et al. 2006, Santos and Phillips 2009, Diogo et al. 2010, Udayanga et al. 2011, 2012 a, b, 2014 a, b, Gomes et al. 2013].

The present study confirmed the effectiveness of the RAPD method in conjunction with the ITS sequence analysis of the isolates in identification of the tested *Diaporthe* spp. We detected no differences and host specialization correlated with molecular analysis what suggested that the tested isolates were very similar.

Furthermore, due to the applied comparative analysis of the ITS fragments with the sequences available in the NCBI database the tested isolates were identified as *Diaporthe eres* species complex, not described previously on fruit trees in Polish conditions. *Diaporthe eres* Nitsche, the typical species of the genus, has been regarded as pathogen of woody plants in diverse families, including Rosaceae in various regions worldwide [Gomes et al. 2013, Udayanga et al. 2014]. Similarly, in our study *Diaporthe* isolates originated from various host plants: *Corylus*, *Pyrus*, *Prunus*, *Juglans*, *Malus*. Udayanga et al. [2014] reported that species occurring on wide range of hosts often show genetic diversity and are sometimes regarded as species complex.

According to the literature, other scientists confirmed the suitability of using RAPD method and ITS region analysis to diversify of *Phomopsis* isolates from different host plants [Rehner and Uecker 1994, Chen et al. 2002 a, b, Król 2002, 2005]. These studies

have initiated a series of experiments with the using of the similarities of ribosomal DNA (rDNA) within the ITS regions to differentiate fungal species, and to examine the evolutionary relationship i.a. among species of *Phomopsis*/*Diaporthe* [Zhang et al. 1998, van Niekerk et al. 2005, van Rensburg et al. 2006, Santos and Phillips 2009, Diogo et al. 2010].

The analyses of the variable ITS regions, were considered a universal marker for fungi and are commonly used in molecular studies. However, there are reports in the literature evidencing that they are not able to differentiate some fungi species. This indicates the need to perform phylogenetic analysis of the DNA sequence of the genes fragments [Taylor et al. 2000, Schoch et al. 2012, Gomes et al. 2013, Udayanga et al. 2014].

According to the concept of GCPSR (Genealogical Concordance Phylogenetic Species Recognition) developed by Taylor et al. [2000] in order to diagnose the species the analysis should include a minimum of three independent loci.

CONCLUSIONS

1. The RAPD-PCR technique is suitable for analyzing genetic variation in the *Diaporthe* population

2. The assignation of *Diaporthe* isolates to the different genetic similarity groups and subgroups does not depend on the origin of the isolates and the species of plants from which they derived.

3. The applied comparative analysis of the ITS fragments with the sequences available in the NCBI database allowed the identification of studied isolates as a *Diaporthe eres* species complex, not described previously on fruit plants under Polish conditions.

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WYKORZYSTANIE RAPD-PCR ORAZ MARKERÓW ITS DO IDENTYFIKACJI *Diaporthe/Phomopsis* WYIZOLOWANYCH Z DRZEW OWOCOWYCH W POŁUDNIOWO-WSCHODNIEJ POLSCE

Streszczenie. W ostatnich latach zanotowano rosnącą szkodliwość grzybów z rodzaju *Diaporthe/Phomopsis* w sadach i szkółkach na całym świecie. Grzyby te powodują niespecyficzne objawy chorobowe, co w połączeniu z podobieństwem morfologicznym kultur i utrudnionym zarodnikowaniem stwarza duże problemy z ich poprawną identyfikacją metodami klasycznymi. Oznacza to potrzebę włączania zaawansowanych technik molekularnych do identyfikacji szczepu na poziomie gatunku. Celem przeprowadzonych badań była identyfikacja oraz zróżnicowanie genetyczne czterdziestu izolatów *Diaporthe/Phomopsis* wyizolowanych w latach 2010–2012 z pędów wybranych drzew owocowych uprawianych w południowo-wschodniej Polsce. Za pomocą techniki RAPD-PCR wykazano podobieństwo genetyczne uzyskanych izolatów niezależnie od miejsca izolacji oraz gatunku rośliny gospodarza. Za pomocą analizy porównawczej niekodujących sekwencji regionów ITS w obrębie genu rDNA (ITS1, 5.8S rDNA, ITS2) z sekwencjami dostępnymi w bazie danych NCBI zidentyfikowano badane izolaty jako jeden gatunek należący do kompleksu *Diaporthe eres*, nieopisany wcześniej na roślinach sadowniczych w warunkach naszego kraju.

Słowa kluczowe: *Diaporthe eres* kompleks, identyfikacja molekularna, rośliny sadownicze, grzyby

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