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Genetic variability within *Septoria carvi* Syd.- a pathogen of caraway *Carum carvi* L

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Abstract Genetic variability within *Septoria carvi* isolates obtained from various organs of caraway cultivated in south-eastern and central Poland was studied using the RAPD-PCR technique. The tests were performed using randomly selected primers. The DNA profiles obtained using four primers proved useful in determining genetic variability among the genotypes of *Septoria carvi* isolates. The present study characterized the differences in the nucleotide sequence within the internal transcribed spacer region of rDNA (ITS1, 5.8S, ITS2) of selected *S. carvi* isolates and reference strains of *Septoria* spp. Moreover, eight isolates were sequenced for three loci: actin, calmodulin and translation elongation factor 1-alpha, and the obtained sequences were compared with the sequences of *Septoria* reference strains affecting other plants of the family *Apiaceae.* Phylogenetic analysis showed distinct differences of the tested isolates, which allowed to treat them *Septoria carvi* species affecting the above-ground organs of caraway *Carum carvi* L. This study is the first report on the genetic characteristics of the species *S. carvi.*

Keywords *Septoria carvi* · Caraway · Genetic variability · ITS region of rDNA · Taxonomy

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Introduction

Fungi of the genus *Septoria* (teleomorph *Mycosphaerella*) belong to the widespread pathogens of crops and wild plants in all regions of the world (Farr et al. 1995; Verkley et al. 2004, 2013; Marcinkowska 2012; Zalewska et al. 2015). *Septoria* spp. cause spots and symptoms on the leaves, leaf petioles, stems and fruits. On the basis of the morphological features observed in the host plant, almost 3000 species have been determined to date. Ongoing research in this important group of pathogens is aimed to revise the classification of this genus (Verkley et al. 2004, 2013; Marcinkowska 2012; Quaedvlieg et al. 2013). *Septoria cytisi* Desm. is a typical species of the genus *Septoria.* This species causes septoriose of *Laburnum anagyroides* and some other species of plants from the family *Fabaceae* (Farr et al. 1995; Muthumary 1999; Waterwereld 2009). The taxonomy of *Septoria* species is still confusing and depends mainly on the host plant. Anamorphs are very difficult to distinguish based only on morphological features. Currently, *Septoria* is identified mainly by pycnidial conidiomata, holoblastic conidiogenesis, hyaline, smooth-walled conidiogenous cells with

sympodial proliferation as well as filiform, hyaline and smooth-walled multi-septate conidia (Sutton 1980; Constantinescu 1984; Farr 1992; Verkley et al. 2004). On the other hand, species with apparently non-proliferating, phialidic conidiogenous cells and of acervuloid to pycnidial structures bearing spores are also included in this taxa (Sutton 1980; Verkley et al. 2004). Among *Septoria* spp., *S. cari* (I.E. Brezhnev), *S. umbelliferarum* Kalchbr. and *S. carvi* Syd. are recognized as the cause of dying leaves and umbels of caraway cultivated in European countries (Sydow 1932; Pidopličko 1978; Ondřej 1983; Teterevnikova-Babayan 1987; Farr et al. 1995; Odstrčilovà et al. 2002; Machowicz-Stefaniak and Zalewska 2004, 2008; Mazur and Nawrocki 2004; Bedlan 2005). In 2004, the species *S. carvi* was considered to be the major causal agent of caraway yield reduction in Austria. This fungus has been present in Poland since 2001 and its occurrence increases during periods of warm and humid weather (Machowicz-Stefaniak and Zalewska 2008; Zalewska 2012, 2013).

Knowledge of the phylogenetic relationship of the genus *Septoria* is still fragmentary. There are no reference isolates of *S. carvi* in the available databases. Sexual status of this species is unknown and only a small number of species produce teleomorphs, which are classified to the genus *Mycosphaerella* Johanson (Dothideales) (Verkley et al. 2004). Mycological studies carried out at the Department of Plant Protection indicated variation in the morphological features and growth conditions of *Septoria carvi* compared to other *Septoria* species colonizing various plants of the family *Apiaceae* (Machowicz-Stefaniak et al. 2008; Zalewska 2008, 2012, 2013; Verkley et al. 2013; Quaedvlieg et al. 2013). Based on our preliminary research, it is difficult to determine the affiliation of the studied isolates, especially by the analysis of growth, development and morphological structures of *S. carvi* in different culture conditions (Zalewska 2012). Thus it is necessary to apply different molecular tools.

The aim of the present study was to determine the genetic variability of *Septoria carvi* isolates obtained from caraway cultivated in various regions of Poland using RAPD-PCR (Random Amplified Polymorphic DNA), and to compare *S. carvi* rDNA sequences of four various loci with other *Septoria* spp. colonizing plants of the family *Apiaceae* available in the database. The purpose of our study was also to investigate whether *S. carvi* is a new pathogenic species of caraway or perhaps it is one of the described species colonizing other plants of the family *Apiaceae*. Moreover, we intended to submit the sequences of the studied isolates to the database.

Materials and methods

Sampling and isolation of *S. carvi* isolates

The material used for the study consisted of 54 isolates of *S. carvi,* obtained as a result of earlier research on the health of caraway in south-eastern and central regions of Poland. Among these isolates, 39 originated from the leaves and leaf petioles of caraway cultivated in Motycz near Lublin 51°14′21″N 22°22′46″E, while 15 other isolates from the same organs and additionally from the umbels of caraway cultivated in Trębanów near Kielce 50°51′06″N 21°29′10″E (Machowicz-Stefaniak and Zalewska 2008; Zalewska et al. 2015). Origin of isolates is shown in Table 1. Some of the aforementioned isolates were previously used to study morphological features and pathogenicity of this fungus towards different organs of caraway (Zalewska 2012, 2013). Single spore cultures of these isolates were grown on Difco™ Potato Dextrose Agar (PDA) medium in Petri dishes in a thermostat at a temperature 24 °C without access to light. The cultures were incubated for 2 weeks and 4 replications were made for each isolate (Table 2).

DNA extraction

Mycelium of *S. carvi* was sampled from the surface of the cultures grown on PDA medium and then transferred to an Eppendorf tube for DNA extraction. DNA isolation was performed using the CTAB method (Doyle and Doyle 1987) with some own modification. The obtained mycelium was transferred to an Eppendorf tube with a volume of 1.5 ml, and then frozen in liquid nitrogen. The frozen material was homogenized with a sterile pestle (SIGMA-Adrich). Then 600 ml of CTAB lysis buffer was added to each tube and incubated at a temperature of 65 °C for 2 h and subsequently centrifuged (10.000 rpm for 10 min). In the next step, 1.0 ml phenol/chloroform/alcohol mixture in the volume ratio of 25:24:1 was added to the supernatant and centrifuged (10.000 rpm for 8 min). DNA was precipitated with 40 μl of sodium acetate (5 M) and 400 μl of isopropanol. The obtained precipitate was washed with

Table 1 Origin and genetic similarity of *Septoria carvi* isolates used in the study

No.	Isolate	Origin of isolate		Mean genetic similarity of isolates
		Localization	Plant part	
1.	KML 74	south-eastern	leaves	0.281
2.	KML 74R	south-eastern	leaves	0.265
3.	KML 75	south-eastern	leaves	0.507
4.	KML 76	south-eastern	leaves	0.504
5.	KML 77	south-eastern	leaves	0.522
6.	KML 78	south-eastern	leaves	0.513
7.	KML 80	south-eastern	leaves	0.493
8.	KML 82	south-eastern	leaves	0.497
9.	KML 83	south-eastern	leaves	0.360
10.	KML 86	south-eastern	leaves	0.495
11.	KML 87	south-eastern	leaves	0.583
12.	KML 89	south-eastern	leaves	0.519
13.	KML 90	south-eastern	leaves	0.607
14.	KML91	south-eastern	leaves	0.284
15.	KML 92	south-eastern	leaves	0.462
16.	KML 93	south-eastern	leaves	0.573
17.	KML93R	south-eastern	leaves	0.481
18.	KML 94	south-eastern	leaves	0.499
19.	KML 95	south-eastern	leaves	0.583
20.	KML 98	south-eastern	leaves	0.613
21.	KTL 132	central	leaves	0.453
22.	KTO 140	central	leaves petioles	0.366
23.	KTL 162	central	leaves	0.431
24.	KTL 174	central	leaves	0.487
25.	KTL 175	central	leaves	0.542
26.	KTL 179	central	leaves	0.254
27.	KTL 180	central	leaves	0.539
28.	KTL 181	central	leaves	0.530
29.	KTL 182	central	leaves	0.333
30.	KTL 189	central	leaves	0.451
31.	KTL 193	central	leaves	0.510
32.	KTL 194	central	leaves	0.467
33.	KTL 195	central	leaves	0.361
34.	KTL 188	central	leaves	0.356
35.	KTB 303	central	umbels	0.522
36.	KML 330	south-eastern	leaves	0.602
37.	KML 334	south-eastern	leaves	0.517
38.	KML359	south-eastern	leaves	0.503
39.	KML 363	south-eastern	leaves	0.584
40.	KML 367	south-eastern	leaves	0.597
41.	KML 368	south-eastern	leaves	0.553
42.	KMO 372	south-eastern	leaves petioles	0.586
43.	KML 385	south-eastern	leaves	0.454
44.	KML 387	south-eastern	leaves	0.571
45.	KMO 388	south-eastern	leaves petioles	0.582
46.	KMO 389	south-eastern	leaves petioles	0.564
47.	KMO 391	south-eastern	leaves petioles	0.562
48.	KML 1806	south-eastern	leaves	0.601
49.	KML 1813	south-eastern	leaves	0.471
50.	KML 1816	south-eastern	leaves	0.474
51.	KML 1819	south-eastern	leaves	0.467
52.	KML1833	south-eastern	leaves	0.344
53.	KML1860	south-eastern	leaves	0.363
54.	KMO 2082	south-eastern	leaves petioles	0.503
Mean similarity of all isolates				0.480

K- caraway, M – Motycz - south-eastern region of Poland, T – Trębanów – central region of Poland, L – leaves, O – leaves petioles, B - umbels

70% ethanol, and then centrifuged at 14.000 rpm for 15 min and dried. Next, the supernatant was suspended in 50 μl of water for PCR (SIGMA-Adrich) supplemented with *RNase A,* and then incubated for 24 h at a temperature of 4 °C, 1 h at 37 °C, and 10 min at 65 °C. DNA concentration was first estimated on 1.5% agarose gel and compared with GeneRuler 100 bp DNA Ladder Plus (Thermo Scientific), and then estimated using a UV-Vis NanoDrop 2000c/2000 spectrophotometer (Thermo Scientific). DNA samples were diluted to a final concentration of 20 ng/μl and stored at -20 °C for further analysis.

Estimation of genetic variability of *Septoria carvi* by RAPD-PCR

The analysis of genetic diversity of *S. carvi* isolates were carried out based on the RAPD-PCR results. DNA amplification was performed according to the method of Williams et al. (1990) with some modifications. The reaction volume used was 20 μl and the reaction solution containing: 1 x buffer *Taq* (750 mM Tris HCl pH 8.8; 200 mM $(NH_4)_2SO_4$; 0.1% Tween 20) (Thermo Scientific), 2.5 mM x $MgCl_2$, 0,1 mM dNTP, 1.0 U polymerase *Taq* (Thermo Scientific), primer 0.2 μM, 20 ng/μl of genomic DNA and distilled water. Amplification was performed using a DNA Engine

Table 2 Characteristic of PCR product obtained from selected primers

No.	Primer RAPD	Sequence 5′-3’	Number of bands			Size of PCR product (bp)	
			Polymorphic	Specific	Total	Min.	Max.
1.	OPR-15	GGACAACGAG	19	3	22	280	2950
2.	OPAD-12	AAGAGGGCGT	12	1	13	380	1950
3.	OPN-09	TGCCGGCTTG	13	0	13	230	1900
4.	OPL-07	AGGCGGGAAC	16	3	19	360	2490
Sum			60	7	67	230	2950
Mean/primer			15	1,7	16,75	-	-
Percent			89,6	10,4	-	-	-

Dyad Thermal Cycler (Biorad), with the following program: initial denaturation for 3 min at 95 °C followed by 45 cycles consisting of 45 s of denaturation at 94 °C, 45 s of annealing at 37 °C, 45 s extension at 72 °C and a final extension at 72 °C for 10 min. Of 30 primers tested with five isolates of *Septoria carvi*, four that generated reproducible and detectable amplification products were selected for further experiments (Tab. 2). The amplification products were separated by electrophoresis using 1.5% agarose gel at 80 V for 1.5 h in 1 x TBE buffer containing 0.01% EtBr (bromidium ethidine). The products obtained were visualised under UV light and photographed.

The data were analysed and presented as a matrix on the basis of the presence or absence of bands – specific product - that were treated as a single feature. Genetic similarity between all isolates was estimated according to the formula by Nei and Li (1979). Matrix of genetic similarity index (SI index) was created based on the polymorphism of RAPD products: $SI = 2N_{xy}/(N_x + N_y)$ where N_{xy} is the number of shared fragments for two genotypes X,Y, while N_x and N_y are the numbers of fragments obtained in X,Y genotypes respectively.

The obtained data were used to construct dendrograms using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method implemented in a free online program (Garcia-Valle et al. 1999; Loncaric et al. 2009; http://genomes.urv.cat/UPGMA/).

Sequence analysis of ITS region, actin, calmodulin, translation elongation factor

Based on the RAPD-PCR results, eight isolates of *S. carvi*, representing various subgroups of genetic similarity, originated from plants cultivated in both regions of Poland, i.e. KML 93, KTL 188, KTB 355, KLM 359, KMO 391, KML 1806 KML 1833 and KML 1860, were selected for further study. These isolates were very similar in terms of morphological characteristics and showed very high aggressiveness towards various organs of caraway (Zalewska 2012, 2013). Phylogenetic analyses were based on the differences in nucleotide sequences of the PCR-amplified fragments of the rDNA ITS region (ITS1, 5.8 rDNA gene, ITS2), and three loci: actin (Act), calmodulin (Cal) and translation elongation factor 1-alpha (EF 1-α). Two universal primers were used for each reaction: ITS1 and ITS4, ACT–512F and ACT2Rd, CAL-235F and CAL-2Rd and EF1-728F and EF2, respectively (Table 3). The amplification reactions volume was 25 μl and contained: 20 ng/μl of template DNA, 1 x Buffer *Taq* (750 mM Tris HCl pH 8.8; 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 60 μM $MgCl_2$, 100 μM dNTP mix, 0.2 μM of each primer and 1.0 U polymerase (Thermo Scientific). Amplification was performed using a DNA Engine Dyad Thermal Cycler (Biorad), with the following program: an initial denaturation for 5 min at 95 °C, followed by 40 cycles at a denaturation temperature of 95 °C for 45 s, primer annealing at a temperature of 52 °C for 50 s, primer extension at 72 °C for 90 s and a final extension step at 72 °C for 5 min. PCR products with the addition of fluorescent dye were separated electrophoretically in 1.5% agarose gel at 80 V for 1.5 h in 1 x TBE buffer containing 0.01% EtBr and visualized under UV light. After checking and determining the size of the resulting PCR products, they were subjected to purification using an agarose gel of low melting point temperature (Sambrook and Russel 2001). The resulting fragments were sequenced using PCR primers and a Big Dye® Terminator Cycle Sequencing Kit V. 3.1 of Applied

Table 3 Primers used for PCR and sequencing

Locus	Primer	Primer sequence 5′ to 3’	Annealing temperature (°C)	Orientation	References
ITS	ITS - 1	TCCCAGGTGAACCTGCGG	55	Forward	White et al. (1990)
	ITS - 4	TCCTCCGCTTATTGATATGC	55	Reverse	White et al. (1990)
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	55	Forward	Carbone and Kohn (1999)
	ACT2Rd	ARRTCRCGDCCRGCCATGTC	55	Reverse	Groenewald et al. (2013)
Calmodulin	CAL-235F	TTCAAGGAGGCCTTCTCCCTCTT	55	Forward	Quaedvlieg et al. (2012)
	CAL2Rd	TGRTCNGCCTCDCGGATCATCTC	55	Reverse	Groenewald et al. (2013)
Translation elongation factor1-α	EF1-728F	CATCGAGAAGTTCGAGAAGG	55	Forward	Carbone and Kohn (1999)
	EF-2	GGARGTACCAGTSATCATGTT	55	Reverse	O’Donnell et al. (1998)

Biosystems (Life Technologies). Sequencing reaction products were separated by a capillary sequencer (3730XL DNA Analyzer). The products of the PCR reaction were sequenced by the company Genomed S.A. Poland. The obtained nucleotide sequences of *Septoria carvi* isolates were compared with reference strains of other *Septoria* spp. inhabiting plants from the family *Apiaceae*, downloaded from the GenBank at the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) (Table 4) (Verkley et al. 2013). The nucleotide sequences were analysed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) software and compared with sequences collected in the NCBI GenBank databases using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/; (Altschul et al. 1997).

The phylogenetic analysis of the studied native isolates of *S. carvi* was carried out in several steps using the Phylogeny.fr program (http://www.phylogeny.fr/). Sequences were aligned to each other using the MUSCLE (v 3.7), program and the sequence regions containing gaps or mismatched ends with the Gblocks (v 0.91b) program. A phylogenetic tree was constructed by using the PhyML bootstrap (v 3.0) with an HKY85 algorithm of the maximum likelihood of substitution model and presented graphically using TreeDyn (v 198.3) gamma shape parameter 88.402.

Results

A total of 67 DNA fragments were obtained on the basis of the RAPD reaction. The number of amplicons generated using a single primer ranged from 13 to 22. On average, 16.75 bands were produced by a single primer (Table 2). As a result of the RAPD-PCR reaction, 60

Table 4 Accessions number of reference strains of *Septoria* spp. used in study

Species	Isolate no[1]	Host	Location	GenBank Accession no[2]			
				EF	ITS	Act	Cal
Septoria *aegopodina*	CBS 123740	*Aegopodium podagraria*	Czech Republic	KF253281	KF251334	KF253639	KF253986
	CBS 123741	*Aegopodium podagraria*	Czech Republic	KF253282	KF251335	KF253640	KF253987
Septoria *apiicola*	CBS 116465	*Apium graveolens*	Netherlands	KF253289	KF251342	KF253646	KF253994
	CBS 395.52	*Apium sp.*	Netherlands	KF253291	KF251344	KF253648	KF253996
Septoria *petroselini*	CBS 109521	–	Netherlands	KF253445	KF251496	KF253800	KF254149
	CBS 182.44	*Petroselinum sativum*	Netherlands	KF253446	KF251497	KF253801	KF254150
Septoria *sii*	CBS 102369	*Berula erecta*	Netherlands	KF253496	KF251548	KF253851	KF254200
	CBS 102370	*Berula erecta*	Netherlands	KF253497	KF251549	KF253852	KF254201
	CBS 118.96	*Berula erecta*	Netherlands	KF253498	KF251550	KF253853	KF254202

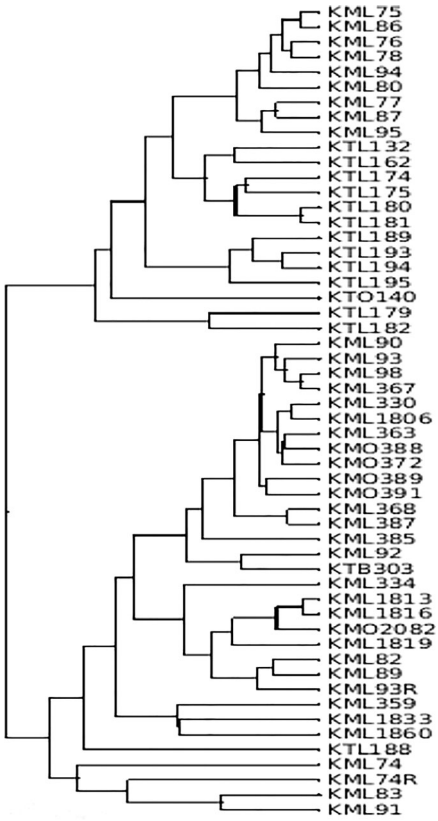

Fig. 1 Dendrogram of 54 *Septoria carvi* isolates constructed by the UPGMA method basing on RAPD-PCR

polymorphic products were obtained and the number of banding patterns scored for each primer ranged from 12 to 19. On average, 15 polymorphic banding patterns were generated by a single primer. Specific products were also obtained from three primers. In total, seven specific banding patterns were obtained and band number ranged from 1 to 3, depending on the isolate (Table 2). The matrix of Dice's indicated genetic similarity between the isolates ranging from 0.254 to 0.607, whereas the average similarity of all isolates was 0.480. Cluster analysis using the UPGMA method indicated the presence of two main groups of isolates (Table 1, Fig. 1). The first group consisted of isolates obtained from central and south-eastern regions of Poland, while the second group contained mainly isolates from the south-eastern region of Poland (Table 1).

One characteristic band of approximately 580 bp was obtained for all 8 native *S. carvi* isolates, as a result of PCR reaction with the ITS1 and ITS4 primer set (Fig. 2). The analysis of the nucleotide sequence of ITS1 and ITS2 regions of *S. carvi* isolates showed 100% identity over a length of 418 nucleotides, i.e. 85% of the total length. Single substitution for isolate KLM 1806 was observed only in the ITS2 region. For this reason, only one of these amplicons (KML 93) was used in further studies as a representative of 8 native isolates. Sequence comparison of these isolates showed 98–100% homology with DNA sequences of the reference cultures along 83% of the total length (in all cases). Of all the sequences used in phylogenetic analyses, *S. carvi* displayed the greatest homology with *S. petroselini* (100%). In the case of other species, a lower sequence identity was

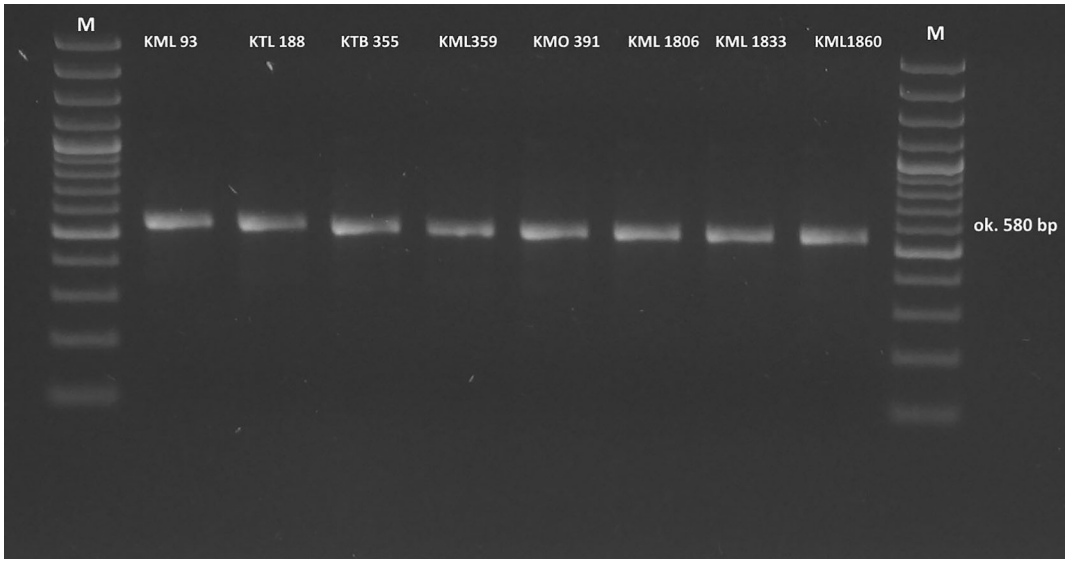

Fig. 2 PCR products amplified with ITS1 and ITS4 primers set: M – marker Mass

Table 5 Query and identity of *S. carvi* sequence in comparison to the sequence of various species of *Septoria* affecting various plants from *Apiaceae* for studied loci

Species of fungi, No of strain	Number of GenBank Accesion of barcodes,											
	ITS			Actin			Calmodulin			Translation elongation factor (EF 1-α)		
	No	Query %	Identity %	No	Query %	Identity %	No	Query %	Identity %	No	Query %	Identity %
Septoria aegopodina												
CBS 123740	KF251334	83	98	KF253639	22	93	KF253986	77	94	KF253281	80	82
CBS 123741	KF251335	83	98	KF253640	22	93	KF253987	77	94	KF253282	80	82
Septoria apiicola												
CBS116465	KF251342	83	99	KF253646	22	93	KF253994	77	99	KF253289	74	90
CBS 395.52	KF251344	83	99	KF253648	22	93	KF253996	77	99	KF253292	74	90
Septoria petroselini												
CBS 109521	KF251496	83	100	KF253800	22	93	KF254149	73	94	KF253445	75	89
CBS 182.44	KF251497	83	100	KF253801	22	93	KF254150	73	94	KF253446	75	89
Septoria sii												
CBS 102369	KF251548	83	99	KF253851	24	97	KF254200	77	94	KF253496	81	99
CBS 102370	KF251549	83	99	KF253852	24	97	KF254201	77	93	KF253497	81	99
CBS 118.96	KF251550	83	99	KF253853	24	97	KF254202	77	94	KF253498	81	99

observed: 99% to *S. apiicola* and *S. sii,* and 98% to *S. aegopodina* (Table 5). Some mutations were observed in the sequence of the ITS region in these isolates, namely these were: one transversion and 15 transitions in seven reference isolates representing *S. apiicola*, *S. sii* and *S. aegopodina* species. ITS dendrogram, based on the genetic distance using the Phylogeny program indicated segregation of 17 *Septoria* sequences in two main clusters: strains of *S. aegopodina* and *S. sii* were grouped into cluster 1. Isolates of *S. carvi* and reference strains of *S. apiicola* and *S. petroselini* were grouped in cluster 2 (Fig. 3). Two reference strains of *S. apiicola* constituted a distinct subgroup in this cluster. Isolates of *S. carvi* and *S. petroselini* were grouped in one subgroup (Fig. 3).

Electrophoresis of PCR products of native 8 isolates of *S. carvi* with actin primers showed the presence of a characteristic band of 650 bp in each isolate (Fig. 4a). The identity of isolate sequences was 97% in 24% of the sequence of the reference strains of *S. sii* and 93% in 22% of the sequence of *S. aegopodina*, *S. apiicola* and *S. petroselini* strains (Table 5). A comparison of *S. carvi* isolates and *S. aegopodina* actin sequences showed differences for four of eight isolates tested, i.e. isolates KML 93, KML 355, KMO 391 and KML 1860. The insertion of thymine was observed at position 11of the sequence of aforementioned isolates. Thymine insertion was also observed for the isolates KML 359 and KLM 1806 at position 15. Furthermore, in the sequence of isolates KTL 188 and KML 1833, thymine insertion was observed at position 5, cytosine also at position 5, adenine at position 3 and guanine at position 4 of the sequence compared to the sequence of *S. aegopodina* reference strains. In the sequence of *S. carvi,* the lack of a nucleotide at position 75 was observed. In addition, the occurrence of 13 transitions and 19 transversions was detected. A phylogenetic tree, based on the actin sequence of eight isolates of *S. carvi* and other selected *Septoria* reference strains generated using the Phylogeny.fr analysis, indicated the segregation of all isolates into two main clusters. The first cluster was divided into two sub-clusters, the first one grouped reference strains of *S. sii* and *S. aegopodina,* while the second sub-claster included strains of *S. apiicola* in an individual sub-group, and strains of *S. petroselini,* which were grouped in a separate subgroup and isolates of *S. carvi* in the second sub-group (Fig. 5a). Isolates of *S. carvi* in the two sub-groups and isolates KTL188 and KML1806 constituted two single separate sub-groups (Fig. 5a).

Electrophoresis of PCR products (calmodulin primers) of 8 native isolates of *S. carvi* showed the presence of a characteristic band of 450 bp size (Fig. 4b). The comparison of isolate sequences with sequences of the reference strains demonstrated 99% identity of 77% of *S. appicola* sequence and 94% identity also of 77% of the sequence for *S. aegopodina* and *S. sii* reference strains. In contrast, 94% sequence identity of *S. carvi* isolates was observed in 73% of the length of *S. petroselini* sequence (Table 5). As regards the sequence differences between the isolates and *S. apiicola,* two transitions and two transversions were recorded while compared to the sequence of *S. aegopodina,* 13 transitions and five transversions occurred. Three additional tranversions were detected with respect to the CBS 102370 strain of *S. sii*. Three gaps, 10 transversions and five transitions were observed in the sequence of *S. carvi* isolates when compared to the corresponding sequence of *S. petroselini*. Phylogenetic tree of 8 isolates of *S. carvi* and other selected reference strains of *Septoria* generated using the

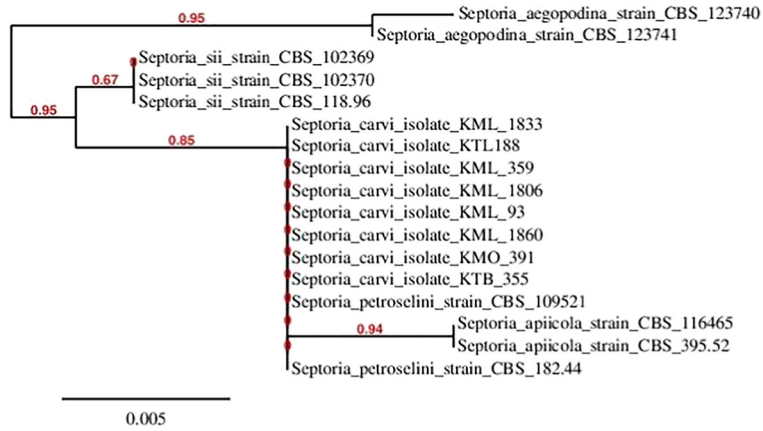

Fig. 3 Phylogenetic tree of 8 *S. carvi* isolates and other selected reference strains of *Septoria* generated from Phylogeny.fr analysis of the ITS

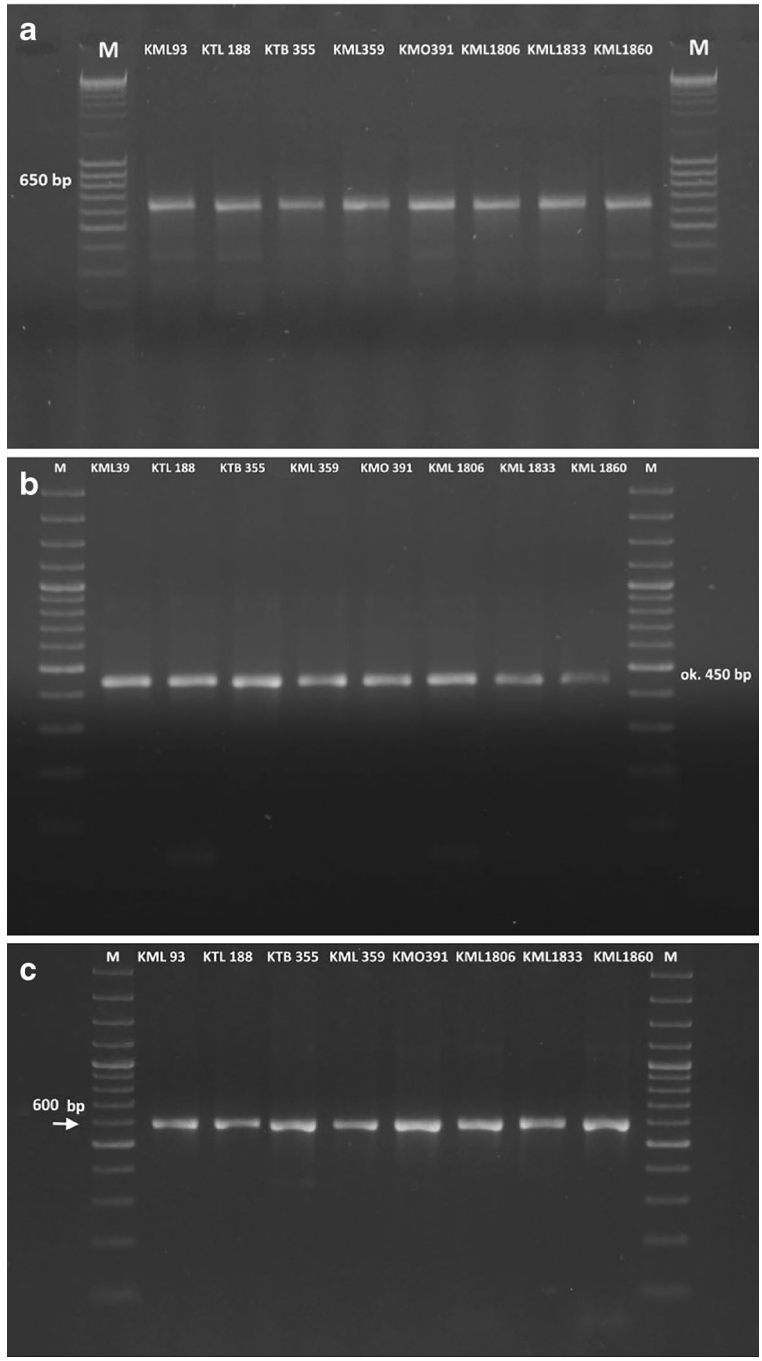

Fig. 4 PCR products amplified with: ACT-512F and ACT2Rd primers set (**a**), CAL-235F and CAL 2Rd primers set (**b**) and EF1-728F and EF 2 primers set (**c**), M – marker Mass

Phylogeny.fr analysis of calmoduline sequence indicated segregation of all isolates into two main clusters (Fig. 5b). The first cluster contained strains of *S. sii* and *S. aegopodina*, which were divided into a single distinct sub-cluster (Fig. 5b). The second main cluster included all studied isolates of *S. carvi* divided into single sub-groups and a second sub-group with reference strains of *S. petroselinii* – and *S. apiicola* clustered into a separate single sub-groups (Fig. 5b).

Sequence analysis of PCR reaction of *S. carvi* isolates with selected EF 1-α primers showed the presence of a characteristic 600-bp band present in all isolates

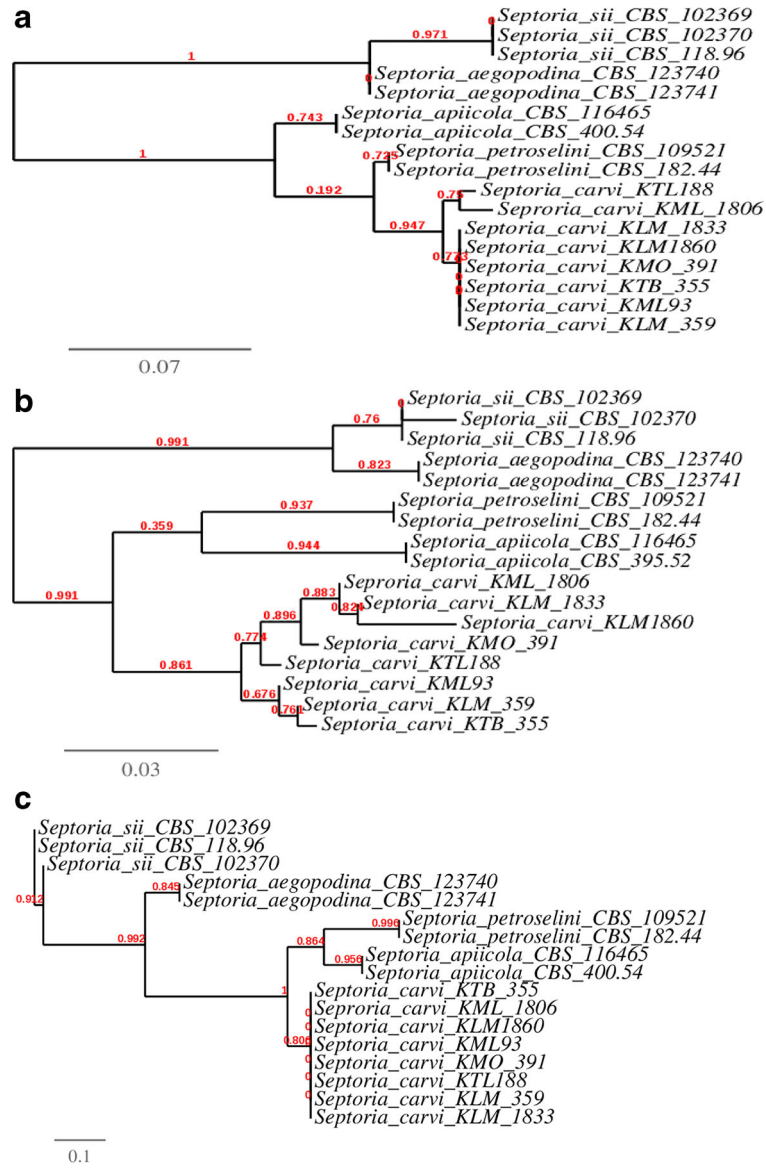

Fig. 5 Phylogenetic tree of 8 *S. carvi* isolates and other selected reference strains of *Septoria* generated from Phylogeny.fr analysis of the actin (**a**), calmoduline (**b**) and translation elongation factor EF 1-α (**c**)

(Fig. 4c). A comparison of the sequence of our native isolates of *S. carvi* with the sequence of selected reference strains of other *Septoria* spp. demonstrated 99% identity along 81% of *S. sii* sequence length, 90% along 74% of *S. apiicola* sequence length and 89% identity of 75% of the *S. petroselini* sequence. Regarding the reference strains of *S. aegopodina,* the identity of *S. carvi* isolates was 82% in 80% of the sequence (Table 5). The comparison of the *S. carvi* isolate sequences to the sequence of *S. sii* reference strains showed the presence of two transitions and sequence containing adenine and guanine at position 120 in the CBS 1023 70 strain as well as adenine and guanine at position 145 in the reference strain sequences. The comparison of nucleotide sequences of *S. carvi* isolates with the sequences of *S. apiicola* strains demonstrated the presence of 12 transitions and 11 transversions. Moreover, the lack of purines or pyrimidines at positions 76, 77 and 110 was observed in the isolates` sequences, while insertions were observed at positions 88, 97 and 99. The comparison of *S. petroselinii* and *S. carvi* sequences with the sequences of the isolates revealed the presence of 16 transitions and 11 transversions. A phylogenetic

tree of eight *S. carvi* isolates and other selected *Septoria* reference strains generated using the Phylogeny.fr analysis of the elongation factor EF 1-α sequence indicated segregation of all studied isolates into two clusters (Fig. 5c). One cluster included only two strains of *S. sii,* and the third strain of *S. sii* and other studied isolates were grouped into the second cluster. This cluster contained studied isolates of *S. carvi* - grouped into a single sub-group (Fig. 5c).

The obtained sequences of *S. carvi* isolates of have been submitted to the GenBank database (Table 6).

Discussion

Phylogenetic relationships of fungi of the genus *Septoria* have been the subject of an ongoing research of some Dutch researches (Quaedvlieg et al. 2013; Verkley et al. 2013). Mycologists constantly ask the following questions: "What are *Septoria*?" and "Which fungi belong to the genus *Septoria*?" The studies conducted by the above-mentioned authors showed that we can not clearly define the genus based solely on its morphology or host plants infected by these fungi. Investigating nucleotide sequence obtained in PCR reactions with various primers, allowed us to correctly identify fungal species and its affiliation to the genera. The study by Quaedvlieg et al. (2013) divided, the genus *Mycosphaerella* into 47 clades. *Septoria* is the first clade of the anamorphic stage of these fungi.

The study of genetic diversity of *S. carvi* isolates collected from the south-eastern and central regions of Poland using the RAPD-PCR method showed a high genetic diversity among them, despite very similar macro- and micro-morphological features. This fact could raise some doubts whether the studied isolates belonged to the same species. However, some authors have also observed a high variation within the populations of different fungal species. Zhou et al. (2005) found a wide variation of *Phoma macrostoma* isolates used for the biological control of weeds, similarly as did Tiwari et al. (2013) within the population of *Phoma tropica* isolates originated from different host plants growing under various environmental conditions. Likewise, Król (2002) observed genetic differentiation between the isolates of *Phomopsis* originated from different species of fruit trees, including few closely related genera from the same plant family. On the other hand, Baturo et al. (2004) showed little intraspecific variability among the isolates of *Bipolaris sorokiniana* from different regions of Brazil. Moreover, Balmas et al. (2005) found that *Phoma tracheiphila* isolates from Italy were genetically similar and created identical banding patterns with selected RAPD primers. Generally, it is believed that the RAPD-PCR technique is useful for detecting variability within many fungal species (Baturo et al. 2004; Sagar et al. 2011). Furthermore, the data obtained from the RAPD analysis may form the basis for further research using other molecular techniques (Baturo et al. 2004; Salem et al. 2007). The analyses of ITS1 and ITS2 regions of *S. carvi* isolates revealed their high similarity. Therefore, our data suggest that these isolates belong to one fungal species. The comparison of ITS1 and ITS2 sequences of native isolates of *Septoria* species to the sequences of various species (reference strains) occurring on different plants from the *Apiaceae* family showed 98, 99 or even 100% similarity. However, other study indicated differences

Table 6 Accessions number of submited *Septoria carvi* sequences in GeneBank

Species	Host	Location	GenBank Accession no			
			EF	ITS	Act	Cal
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822111	KX443416	KX822103	KX822095
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822112	KX443417	KX822104	KX822096
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822113	KX453683	KX822105	KX822097
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822114	KX453684	KX822106	KX822098
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822115	KX453685	KX822107	KX822099
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822116	KX453686	KX822108	KX822100
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822117	KX453687	KX822109	KX822101
Septoria carvi Syd.	*Carum carvi* L.	Poland	KX822118	KX453688	KX822110	KX822102

between the tested isolates and the species *S. petroselini* (Verkley et al. 2013). Nonetheless, some authors have noted that *Septoria* material collected even from the same location and host species, but under different environmental conditions or at different times in the same season, can differ considerably in the average conidial sizes, particularly the length (Verkley et al. 2013). Simultaneously, a molecular phylogenetic study of the same authors on *Septoria* species infecting *Asteraceae* and woody perennials demonstrated that species capable of infecting hosts of the same plant family did not always cluster in monophyletic groups. Furthermore, Verkley et al. (2013) reported that the ITS sequence of *Septoria anthrisci* was distinct from that of *S. apiicola,* but identical to that of *S. petroselini*, which suggested insufficient diversity within the rDNA regions and the need to use different molecular tools.

In the most recent phylogenetic studies of the genus *Septoria,* there was no information about the phylogenetic relationships of *Septoria carvi,* although this species is recognized as a dangerous pathogen of caraway grown in the Czech Republic, Poland, Austria, Bulgaria and Lithuania (Ondřej 1983; Odstrčilovà et al. 2002; Machowicz-Stefaniak and Zalewska 2004, 2008; Mazur and Nawrocki 2004; Bedlan 2005; Mačkinaitė 2012).

It seems that the difficulties in arriving at a clear definition of the relationship between native isolates of *Septoria* result mainly from the lack of the reference isolate of *S. carvi* and its nucleotide sequence, and perhaps also from little variation in the sequences of the ITS region of the genus *Septoria.* This fact makes the comparison of the ITS sequence impossible (Quaedvlieg et al. 2013). Similar relationships were found in species of the genus *Phoma.* Badillo-Vargas et al. (2008) observed that the ITS region sequences of the isolates of *Phoma putaminum* and *P. macrostoma var. incolorata* were similar to the sequences of closely related species. In another study on *Phoma exigua* species variants, it was impossible to distinguish the variants based on the nucleotide sequence of the ITS regions, despite clear differences in the appearance of the cultures (Abeln et al. 2002). The conducted research using typical differentiating proteins for *Septoria* genera showed too low sequence similarity to the sequences of reference strains infesting plants of the family *Apiaceae.* This means that the fungus tested can be considered a separate species *Septoria carvi* Syd. affecting *Carum carvi* L. plants. The results confirmed the usefulness of ITS barcodes to extract the genus of *Septoria.*

The obtained results in the phylogenetic analysis of actin, calmodulin and translation elongation factor 1-α sequences showed the distinctness of the eight studied isolates of *S. carvi* from the sequences of other species of the genus *Septoria* infecting plants of the family *Apiceae.* All translation elongation factor (EF 1-α) sequences of *S. carvi* isolates were located on one branch of the phylogenetic tree. They did not differ between each other, as opposed to the nucleotide sequences of actin and calmodulin. Actin gene variation was found between isolates KTL 188 and KLM 1806 compared to other *S. carvi* isolates. Calmodulin sequences showed differences in all isolates tested. All sequences of *S. carvi* formed one common group with *S. pertoselinii* and *S. apiicola.* This fact indicated close relationship of these species, which was demonstrated by the sequence analysis of ITS regions. The obtained results showed differences between the studied barcodes and indicated that translation elongation factor 1-α constituted the best primer to identify *S. carvi.* The results of our study are consistent with a previous study conducted by Verkley et al. (2013).

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