Molecular characterization of *Boeremia strasseri* the causal agent of black stems and rhizomes rot of peppermint

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Abstract

The molecular characterization of *Boeremia strasseri* (Moesz) Gruyter et Verkley (basionym *Phoma strasseri* Moesz) isolates from peppermint crops grown in south-eastern and central Poland was studied using the Random Amplified Polymorphic DNA (RAPD) -PCR technique. Tests were performed using randomly selected primers. DNA profiles obtained using five primers proved to be useful in determining the genetic variability among *B. strasseri* genotypes. Molecular analysis of four loci: (i) rDNA internal transcribed spacer region (ITS1, 5.8S, ITS2); (ii) LSU (partial large subunit DNA 28S); (iii) *tub 2* (gene region of β -tubulin); (iv) *act* (gene region of gamma-actin) proved that the actin gene is the most suitable DNA barcode for the accurate and rapid identification *B. strasseri* species.

Keywords *Phoma sensu lato* · *Mentha piperita* · Genetic relationship · Taxonomy

Introduction

The genus *Phoma* Sacc. emend. Boerema et G.J. Bollen (*Pleosporales*) is a cosmopolitan taxon of coelomycetous fungi containing numerous plant pathogenic, saprobic and endophytic species associated with a wide range of hosts (Aveskamp et al. 2008). This phytopathologically significant fungal genus has always been considered one of the most difficult to identify (Aveskamp et al. 2008; Zhang et al. 2009). Initially, it comprised more than 3000 species (Monte et al. 1991). Such a large number of taxa described within *Phoma* was related to the use of nomenclature mainly based on the characteristics of the host plant and marginalization of micro-morphological properties (Aveskamp et al. 2008). Intensive work carried out by Dutch mycologists, who had

studied the morphology of these fungi in artificial cultures since 1992 led to their division into nine sections (Boerema 1997). The results of 40 years of taxonomic research based on the properties of *Phoma* species were presented in an article on their identification, and, consequently, the number of species was reduced to 223 (Boerema 1997; Boerema et al. 2004). However, the developed taxonomic system seemed a little artificial to the above authors, thus they began molecular DNA analyses of the species within individual sections. These studies revealed the phylogenetic heterogeneity of the species in the *Phoma* sections, disproved the existing division of the genus into sections and caused the necessity to re-classify (de Gruyter et al. 2009, 2010, 2012; Aveskamp et al. 2010). The genus *Phoma* was divided into clades, raised to the rank of genera, that comprised species with similar levels of relationships (Aveskamp et al. 2010).

Boeremia strasseri (Moesz) Gruyter et Verkley (basionym *Phoma strasseri* Moesz), family Didymellaceae (Chen et al. 2015), has been reported as the causal agent of black stems and rhizome rot of peppermint (*Mentha piperita* L.) in the USA (Horner 1971), Japan (de Gruyter et al. 2002), India (Kalra et al. 2004), Hungary (Paizs and Naggy 1975), and is present in Poland since 2004 (Zimowska and Machowicz-Stefaniak 2005; Zimowska 2007). The disease causes yield losses up to 90% due to the fast degradation of stem and rhizome tissues. A very high activity of β -glucosidase allows *B. strasseri* to penetrate the host cell wall and tissues, followed

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Table 1 Origin and genetic similarity of *Boeremia* isolates

No.	Isolate	Origin of isolate			Mean genetic similarity of isolates
		Localization	Plant part	Host	
1.	F 126	51.0957 N, 22.9634E	stems	<i>Mentha piperita</i> L.	0.777
2.	F 289	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.841
3.	F 365	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.851
4.	F 742	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.881
5.	F 743	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.863
6.	F 937	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.830
7.	F 1023	51.0957 N, 22.9634E	stems	<i>M. piperita</i>	0.863
8.	ML 125	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.877
9.	ML 134	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.882
10.	ML 149	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.882
11.	ML 159	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.877
12.	ML 175	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.856
13.	ML 178	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.877
14.	ML 179	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.882
15.	ML 189	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.844
16.	ML 293	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.881
17.	ML 382	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.867
18.	ML 423	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.882
19.	ML 455	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.866
20.	ML 738	52.3724 N, 20.2231E	rhizomes	<i>M. piperita</i>	0.786
21.	K 922	50.4500 N, 20.4333E	stems	<i>M. piperita</i>	0.877
22.	K 1211	50.4500 N, 20.4333E	stems	<i>M. piperita</i>	0.874
23.	Lc 722	51.0957 N, 22.9634E	stems	<i>Leonurus cardiaca</i> L.	0.459
24.	M 1532	51.0957 N, 22.9634E	stems	<i>Melisa officinalis</i> L.	0.508
25.	CBS 261.92	USA Oregon		<i>M. piperita</i>	0.804
Mean similarity of all isolates 0.804					

F, M, Lc- Fajslawice- south-eastern region of Poland; ML- Płońsk- central region of Poland; K- Kołków- south-eastern region of Poland

by the action of other pectinolytic enzymes that break down pectin to low molecular weight compounds, which are the primary source of carbon for the pathogen (Zimowska 2012; Zimowska and Targoński 2015).

In this study we present the molecular characterization of *B. strasseri* isolates obtained from peppermint cultivated in various regions of Poland. We also attempted to evaluate the taxonomy of two isolates recovered from *Melissa officianatis*

Table 2 Characteristics of banding patterns obtained from selected primers

Primer	Products of RAPD				Size of products (bp)	
	Polymorphic	Monomorphic	Specific	Total	Min.	Max.
OPR-15	11	1	2	14	469	2126
OPV-10	9	0	1	10	604	2100
OPL-07	8	3	3	14	324	2300
OPU-05	7	2	0	9	511	2207
OPA-13	8	1	2	11	538	2124
Total	43	7	8	58	–	–
Average/primer	8.6	1.4	1.4	11.6	–	–
Percent (%)	74.14	12.07	12.07	–	–	–

Table 3 Primer used for PCR and sequencing

Locus	Primer	Primer sequence 5' to 3'	Melting temperature (°C)	Orientation	References
ITS	ITS1	TCCGTAGGTGAACCTGCGG	59	Forward	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC	59	Reverse	White et al. 1990
LSU	LROR	ACCCGCTGAACTTAAGC	60	Forward	Vilgalys and Hester 1990
	LR5	TCCTGAGGGAACTTCG	60	Reverse	Vilgalys and Hester 1990
β -tubulin	Bt ₂ a	GGTAACCAAATCGGTGCTGCTTTC	60	Forward	Glass and Donaldson 1995
	Bt ₂ b	ACCCTCAGTGTAGTGACCCTTGGC	60	Reverse	Glass and Donaldson 1995
Actin	ACT-512F	ATGTGCAAGGCCGTTTCGC	55	Forward	Carbone and Kohn 1999
	ACT-783R	TACGAGTCCTCTGGCCCAT	55	Reverse	Carbone and Kohn 1999

(lemon balm) and *Leonurus. cardiaca* (motherwort) using barcoding sequences. Many phylogenetic studies were based on internally transcribed spacers (ITS). However, in the case of *Phoma sensu lato*, ITS sequence analyses did not show significant nucleotide differences to infer relationships (Balmas et al. 2005). Considering the above, we have addressed other gene loci, i.e. LSU, β -tubulin and actin in data sets to enhance the phylogenetic analysis.

Materials and methods

Fungal isolates The material used for the study consisted of 22 isolates of *B. strasserii*, obtained from symptomatic peppermint (*M. piperita*) stems and rhizomes from earlier research on the health of peppermint in the south-eastern and central regions of Poland, and two *Boeremia*-like isolates collected from different hosts (Zimowska 2007, 2008). For comparative purposes, the reference isolate CBS 261.92 was retrieved from the culture collection of the CBS KNAW Fungal Biodiversity Centre, Utrecht (The Netherlands). Origin of the isolates is shown in Table 1. The *Boeremia* isolates have previously been used to study their morphological features following the methods described by Boerema et al. (2004). Some of these isolates were also examined for pathogenicity to peppermint stems and rhizomes (Zimowska and Machowicz-Stefaniak 2005; Zimowska 2012). Single spore cultures of these isolates were grown on 2% malt extract agar plates (MEA; Difco Laboratories, USA). at 22 °C in the dark for two weeks. Four replicates of each isolate were performed.

For phylogenetic analysis, sequences of 20 additional isolates of *Boeremia* spp. were retrieved from GenBank.

DNA extraction and analysis Genomic DNA was extracted from fungal mycelium using the Invisorb Spin Plant Midi Kit (Strattec Molecular, Germany), according to the manufacturer's protocol. DNA concentration was first estimated on 1.5% agarose gel and compared with GeneRuler™ DNA Ladder Plus (Thermo Scientific, USA) then measured using

a UV-Vis NanoDrop 2000c/2000 spectrophotometer (Thermo Scientific). DNA samples were diluted to a concentration of 20 ng/μl and stored at 20 °C for future use.

Estimation of *Boeremia strasserii* genetic variability Genetic variability of the studied isolates was investigated using randomly selected Random Amplified Polymorphic DNA (RAPD) primers (Operon Technologies, USA). A set of 30 primers were tested in five *B. strasserii* isolates, and five primers generated reproducible and detectable amplification products and were selected for further study (Table 2).

RAPD-PCR analyses were performed according to Williams et al. (1990) with some modification. The reaction volume was 20 μl and the reaction solution contained 1 x Taq buffer [750 mM Tris HCl, pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% Tween 20] (Thermo Scientific), 2.5 mM x MgCl₂, 0.1 mM dNTP, 0.1 U polymerase Taq (Thermo Scientific), 0.2 μM primer, 20 ng/μl of genomic DNA and distilled water. The control reaction was run with water instead of DNA template. Amplification was performed using a DNA Engine Dyad Thermal Cycler (Biorad, USA), using 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. Amplification products were separated by electrophoresis using a 1.5% agarose gel at 80 V for 1.5 h in 1 x TBE buffer containing 0.01% ethidium bromide. The products obtained were visualized under UV light, photographed and analyzed using BioGene (UK) software.

RAPD products were scored on photographs as present (1) or absent (0). Only bright and reproducible products were scored. Genetic pairwise similarities (SI-similarity index) between genotypes were evaluated according to Dice's formula after Nei and Li (1979). Cluster analysis was conducted using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) distance method implemented in the PAST program (Hammer et al. 2001). Clustering was verified by bootstrapping. Principal component analysis (PCA) was performed using PAST software (Hammer et al. 2001).

Table 4 Isolates of *Boeremia* used for DNA analyses

Species name	Strain number	Host	Locality	GenBank accession no.		
				ITS	LSU	act
<i>B. crinicola</i>	CBS 109.79	<i>Crinum powellii</i> L.	Netherlands	GU237737.1	GU237927.1	GU237489.1 KY484558.1
	CBS 118.93	<i>Crinum</i> sp.	Netherlands	GU237758.1	GU237928.1	GU237490.1 KY484559.1
<i>B. diversispora</i>	CBS 102.80	<i>Phaseolus vulgaris</i> L.	Kenya	GU237725.1	GU237930.1	GU237492.1 EU880861.1
	CBS 101214	<i>Phaseolus lunatus</i> L.	Zambia	KY484635.1	–	KY484738.1 EU880862.1
	CBS 531.86	<i>Vigna</i> sp.	Burundi	KY484636.1	–	EU880863.1
<i>B. exigua</i>	CBS 101194	<i>Phaseolus vulgaris</i> L.	Netherlands	GU237716.1	GU237929.1	GU237491.1 EU880864.1
	CBS 107.21	<i>Abelmoschus esculentus</i> (L.) Moench	–	KT389491.1	KT389708.1	KT389785.1 –
	CBS 118.38	<i>Cheiranthus cheiri</i> L.	Denmark	KT389489.1	KT389706.1	KT389783.1 –
	CBS 119.38	<i>Nicotiana tabacum</i> L.		KT389490.1	KT389707.1	KT389784.1 –
<i>B. exigua</i> var. <i>exigua</i>	CBS 101201	<i>Phlox</i> sp.	Netherlands	KY484641.1	–	KY484561.1
	CBS 101150	<i>Cichorium intybus</i> L.	Netherlands	GU237715.1	EU754182.1 GU237933.1	–
<i>B. exigua</i> var. <i>gilvescens</i>	CBS 101150	<i>C. intybus</i>	Netherlands	–	–	KY484562.1
<i>B. exigua</i> var. <i>inoxydabilis</i>	CBS 372.75	<i>Vinca minor</i> L.	Netherlands	KY484656.1	–	KY484565.1
<i>B. exigua</i> var. <i>heteromorfa</i>	CBS 119730	<i>Coffea arabica</i> L.	Brasil	–	–	KY484564.1
<i>B. exigua</i> var. <i>coffeae</i>	CBS 119730	<i>C. arabica</i>	Brasil	GU237759.1	GU237942.1	GU237504.1 –
	CBS 109183	<i>C. arabica</i>	Cameroon	GU237748.1	GU237943.1	GU237505.1 KY484560.1
<i>B. foveata</i>	CBS 341.67	<i>Solanum tuberosum</i> L.	Northern Ireland	GU237834.1	GU237947.1	GU237509.1 EU880894.1
	CBS 109176	<i>S. tuberosum</i>	Bulgaria	GU237742.1	GU237946.1	GU237508.1 EU880892.1
	CBS 200.37	<i>S. tuberosum</i>	United Kingdom	–	–	KY484761.1 EU880893.1
<i>B. strasserii</i>	CBS 126.93	<i>Mentha</i> sp.	Nederland	GU237773.1 NR_135985.1	GU237956.1	GU237518.1 EU880904.1
	CBS 261.92	<i>Mentha piperita</i> L.	USA	GU237813.1	GU237957.1	GU237519.1 EU880905.1
	F 126	<i>M. piperita</i>	Poland	MF113457	MF113433	MF121809 MF121785
	F 289	<i>M. piperita</i>	Poland	MF 113458	MF113434	MF121810 MF121786
	F 365	<i>M. piperita</i>	Poland	MF113459	MF113435	MF121811 MF121787
	F 742	<i>M. piperita</i>	Poland	MF113460	MF113436	MF121812 MF121788
	F 743	<i>M. piperita</i>	Poland	MF113461	MF113437	MF121813 MF121789
	F 937	<i>M. piperita</i>	Poland	MF113462	MF113438	MF121814 MF121790
	F 1023	<i>M. piperita</i>	Poland	MF113463	MF113439	MF121815 MF121791
	ML 125	<i>M. piperita</i>	Poland	MF113464	MF113440	MF121816 MF121792
	ML 134	<i>M. piperita</i>	Poland	MF113465	MF113441	MF121817 MF121793
	ML 149	<i>M. piperita</i>	Poland	MF113466	MF113442	MF121818 MF121794
	ML 159	<i>M. piperita</i>	Poland	MF113467	MF113443	MF121819 MF121795
	ML 175	<i>M. piperita</i>	Poland	MF113468	MF113444	MF121820 MF121796
	ML 178	<i>M. piperita</i>	Poland	MF113469	MF113445	MF121821 MF121797

Table 4 (continued)

Species name	Strain number	Host	Locality	GenBank accession no.			
				ITS	LSU	tub2	act
<i>B. exigua</i> var., <i>exigua</i>	ML 179	<i>M. piperita</i>	Poland	MF113470	MF113446	MF121822	MF121798
	ML 189	<i>M. piperita</i>	Poland	MF113471	MF113447	MF121823	MF121799
	ML 293	<i>M. piperita</i>	Poland	MF113472	MF113448	MF121824	MF121800
	ML 382	<i>M. piperita</i>	Poland	MF113473	MF113449	MF121825	MF121801
	ML 423	<i>M. piperita</i>	Poland	MF113474	MF113450	MF121826	MF121802
	ML 455	<i>M. piperita</i>	Poland	MF113475	MF113451	MF121827	MF121803
	ML 738	<i>M. piperita</i>	Poland	MF113476	MF113452	MF121828	MF121804
	K 922	<i>M. piperita</i>	Poland	MF113477	MF113453	MF121829	MF121805
	K 1211	<i>M. piperita</i>	Poland	MF113478	MF113454	MF121830	MF121806
	Lc 722	<i>Leonurus cardiac</i> L.	Poland	MF113479	MF113455	MF121831	MF121807
	M 1532	<i>Melisa officinalis</i> L.	Poland	MF113480	MF113456	MF121832	MF121808

The GenBank accession numbers in bold have been obtained from our studies

Sequence analysis of ITS region, LSU, β -tubulin, actin
Phylogenetic analyses were based on differences in nucleotide sequences of PCR-amplified fragments of the rDNA ITS region (ITS1, 5.8 rDNA gene, ITS2) and three other loci: LSU, β -tubulin (*tub2*), and actin (*act*). Two universal primers were used for each reaction: ITS1 and ITS4, the LSU region with LR0R and LR5, the *tub2* region with Bt₂a and Bt₂b primers, and the *act* region with ACT-512F and ACT-783R, respectively (Table 3).

PCR reaction was performed in a total volume of 25 μ l according to Sambrook and Russel (2001). PCR products with fluorescent dye addition were separated electrophoretically in a 1.5% agarose gel at 80 V for 1.5 h in 1X TBE buffer containing 0.01% ethidium bromide and visualized under UV light. After checking and determining the size of the resulting PCR product, it was subjected to purification using a low melting point temperature agarose gel. Sequencing was conducted by the Genomed S.A. (Poland) using PCR primers and a Big Dye® Terminator Cycle Sequencing Kit V. 3.1 (Applied Biosystems, Life Technologies, USA) and separated on a 3730XL DNA Analyzer capillary sequencer. Sequences from each primer combination were used to obtain consensus sequences with BioEdit v. 7.2.5 (Hall 1999). Nucleotide sequences were analysed with sequences retrieved from GenBank using BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al. 1997). The sequence of native isolates were compared with reference strains of other *Boeremia* spp. downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/>) (Table 4). Phylogenetic analysis was carried out with the “one klik” tool 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 PhyloML bootstrap (v 3.0) with an HKY85 algorithm of the maximum likelihood of substitution model and presented grafically using TreeDyn (v 198.3) gamma shape parameter 88.402.

Results

Of the 30 RAPD primers only five, i.e. OPA-13, OPL-07, OPR-15, OPU-05 and OPV-10, were useful for genetic similarity determination among the studied isolates of *B. strasserii*.

RAPD analysis amplified 58 fragments based on five selected primers, 43 of which (74.14%) were polymorphic, while seven fragments were monomorphic. The number of amplified DNA fragments per primer ranged from nine (OPU-05) to 14 (OPR-15 and OPL-07) with sizes ranging between 324 and 2300 bp (Table 2). Of the 58 amplification products, eight were specific for single isolates. The highest number of specific products was identified in isolates Lc722

Fig. 1 Dendrogram of 24 *Boeremia* isolates constructed by the UPGMA method based on RAPD-PCR

and M1532 (three specific products each) recovered from *L. cardiaca* and *M. officinalis*, respectively.

Genetic similarity matrices were produced based on RAPD markers using the Sørensen-Dice coefficient. The genetic similarity between isolates ranged from 0.370 to 1.00. Isolates F 126 and F 1023, F 126 and ML 125, F 289 and ML 134, F 1023 and ML 125, F 1023 and ML 175, ML 125 and ML 175 as well as a group of three isolates (F 365, F 742 and F 743) showed 100% similarity. The average similarity of the

analyzed isolates was 0.820. Isolates collected from *L. cardiaca* and *M. officinalis* showed the lowest similarity to other isolates, with values of 0.459 and 0.508, respectively. Cluster analysis using the UPGMA method divided isolates into two main groups A and B (Fig. 1). Group A comprised isolates from peppermint that were distributed into three sub-groups, regardless of the plant organ and place of origin. Two isolates collected from lemon balm and motherwort were grouped in cluster B.

Fig. 2 RAPD-PCR results of the normalized component analysis (PCA) of 24 *Boeremia* isolates and reference strain

Fig. 3 Phylogenetic tree of 24 *Boeremia* isolates and reference strains generated from Phylogeny.fr analysis. a, ITS region; b, LSU region; c, *tub2* region and d, *act* region

The relationships between the 25 analyzed native isolates were determined based on principal component analysis (PCA) (Fig. 2), which gave results comparable to those obtained by UPGMA clustering. *Boeremia* spp. isolates formed two distinct groups, that corresponded to UPGMA clusters A and B. For the RAPD data, the first three principal components explained 70.5% of the total variation, with PC-1, PC-2 and PC-3 accounting for 40.8%, 16.4% and 13.3% of the total variation, respectively.

Sequence similarity to the reference *B. strasseri* isolate CBS 261.92 was 99% for the ITS region, 99% for the LSU

region. M 1532 isolate showed 98% similarity to the *tub2* region, Lc 722 isolate 97%, while the similarity for the *act* region was 96%.

The phylogenetic tree, based on the ITS sequences of 24 tested isolates and other selected *Boeremia* reference strains showed the clustering of all isolates into two main clades. The first clad was divided into two sub-clades. The first one included 22 native isolates of *B. strasseri*, and reference strains of *B. strasseri*, two reference strains of *Boeremia diversispora* (Bubák) Aveskamp, Gruyter et Verkley and *Boeremia crinicola* (Siemaszko) Aveskamp, Gruyter et Verkley,

Fig. 3 (continued)

respectively, while the reference strain of *B. diversispora* was in a separate branch. The second clad included one reference strain of *Boeremia exigua* (Desm.) Aveskamp, Gruyter et Verkley, *Boeremia foveata* (Foister) Aveskamp, Gruyter et Verkley and the native isolates M 1532 and Lc 722 (Fig. 3a). As a result of the phylogenetic analysis on the LSU region two clades were obtained, one of which grouped only native isolates, while the other included reference strains of *Boeremia* spp. (Fig. 3b).

The phylogenetic tree constructed with *tub2* sequences of 24 native isolates and reference strains of *Boeremia* spp. grouped these isolates into two main clades, the first of which was divided into five sub-clades, characterized by smaller or larger sequences variability. The one of them included two

reference strains of *B. crinicola*, while the second grouped 15 native isolates and two reference strains of *B. strasseri*. The second clad was divided on tree sub-clades. The first one grouped in the individual tree reference strains of *B. diversispora*, second included tree reference strains of *B. foveata*, while the third included two native isolates Lc 722 and M 1532 and reference *B. exigua* strains (Fig. 3c).

The phylogenetic analysis of the *act* region produced four clades. The first one included 22 native isolates and two reference strains of *B. strasseri*, the second grouped two native isolates Lc 722 and M 1532 with *Boeremia exigua* var. *exigua* (Desm.) Aveskamp, Gruyter et Verkley and *B. foveata* reference strains, while *B. foveata* was included in the individual sub-clades. The third clad grouped reference strains of

Fig. 3 (continued)

B. crinicola, while the fourth included only reference strains of *B. diversispora* (Fig. 3d).

Discussion

The identification of *Phoma* species based on *in vitro* morphological characteristics is rather difficult and depends on the fungal strain, its place of origin and the host plants. Since this resulted in discrepancies and misidentifications, a multifaceted approach including rapid molecular tools, is desirable, to

enable accurate identification. PCR-based RAPD is one of the most common techniques to estimate genetic diversity among *Phoma* isolates (Zhuo et al. 2005; Rai and Tiwari 2014).

Balmas et al. (2005) used the RAPD method to characterize Italian isolates of *Phoma tracheiphila* (Petri) L.A. Kantsch. et Gikaschvili. They found that the analyzed isolates were genetically identical. On the other hand, Zhuo et al. (2005) found a significant genetic variation among *Didymella macrostoma* (Mont.) Q. Chen et L. Cai (Basionym *Phoma macrostoma* Mont.) isolates based on RAPD markers. Tiwari et al. (2013) showed that *Allophoma tropica* (R. Schneid. et

Fig. 3 (continued)

Boerema) Q. Chen et L. Cai (basionym *Phoma tropica* R. Schneid. et Boerema) isolates collected from various host plants and environmental conditions were genetically differentiated. Our study analyzed 22 isolates of *B. strasseri*, obtained from different parts of plants growing under different environmental conditions, as well as two *B. exigua* var. *exigua* isolates from various hosts. The results showed that the isolates from peppermint were highly similar and grouped together on the dendrogram. In contrast, the isolates from lemon balm and motherwort were genetically different and formed a separate cluster. Tiwari et al. (2013) suggested that the genetic diversity observed among *P. tropica* species might be due to difference in environmental conditions or different

host types from which the species were isolated. Our study confirmed that host differences significantly influenced the genetic diversity of the analyzed isolates.

Comparison of the ITS region sequence, which is widely used in taxonomy and molecular phylogeny (Balmas et al. 2005; Aveskamp et al. 2009; Rai and Tiwari 2014), showed insignificant nucleotide differences. For this reason, it is difficult to clearly confirm the affiliation of the tested isolates to any given species. Similar correlations were found by Badillo-Vargas et al. (2008) who studied *Phoma* species. They observed that nucleotide sequences in the ITS region of *Phoma putaminum* Speg. and *Didymella macrostoma* (Mont.) Q. Chen et L. Cai [basionym *Phoma macrostoma* var. *incolorata*

(A.S. Home) Boerema et Dorenb.)] matched the sequence of closely related species. In another study distinct morphological variants of *Phoma exigua* could not be distinguished from one another based on the molecular analysis of their ITS regions, (Abeln et al. 2002).

Our results confirmed the usefulness of the actin barcode in species determination. The same locus had previously been addressed while identification techniques were developed, proving highly reliable for deep-level phylogeny (Voigt et al. 2001; Aveskamp et al. 2009). and useful for discriminating closely related *Phoma* taxa (Aveskamp et al. 2009).

The present study, which is based on DNA phylogeny, showed that morphological characterization on standardized media is not reliable for identification at the species level. In fact, two fungal isolates from *L. cardiaca* and *M. officinalis* that were classified as *B. strasseri* based on their morphological characteristics, showed greater similarity to *B. exigua* var. *exigua* than to *B. strasseri* at the DNA level.

Concluding, to truly elucidate the taxonomy of *Phoma*-like genera, a concerted global effort is required not only for reclassifying previously described species, but also for providing GenBank with new sequences of isolates from countries that have been largely neglected or undersampled by mycologists and plant pathologists.

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