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RESEARCH PAPERS

Phylogenetic analysis of *Polystigma* and its relationship to *Phyllachorales*

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Summary. *Polystigma amygdalinum,* which causes red leaf blotch of almond, is one of the few fungal plant pathogens to remain a taxonomic enigma, primarily because it has resisted cultivation and causes almond leaf blotch only in restricted regions of the world. To place this species in the evolutionary tree of life, we amplified its ribosomal DNA internal transcribed spacer region (ITS), 18S small-subunit of ribosomal DNA (SSU rDNA) and 28S large-subunit of ribosomal DNA (LSU rDNA). Our phylogenetic analyses indicate that *P. amygdalinum* does not group with *Phyllachora* species (*Phyllachorales*) which have been thought to be its close relative. *Polystigma amygdalinum*is here shown to be a relative of *Trichosphaeriales* and *Xylariales* and placed in the *Xylariomycetidae*.

Key words: *Polystigma amygdalinum*, almond red leaf blotch, plum red leaf spot, ITS, SSU, LSU.

Introduction

Polystigma amygdalinum P.F. Cannon, the causal agent of red leaf blotch disease of almonds, has been reported to occur in many countries (Khan, 1961; Ghazanfari and Banihashemi, 1976; Saad and Masannat, 1997; Cımen and Ertugrul, 2007). This fungus is a serious leaf pathogen of almonds in almond growing areas of Iran (Ashkan and Asadi, 1974; Banihashemi, 1990) and the pathogen often causes premature defoliation of host trees (Suzuki *et al.*, 2008). Based on morphology, *P. amygdalinum* has been assumed to be a member of the order *Phyllachorales* and is considered to be a close relative of the genus *Phyllachora* (Cannon, 1996; Lumbsch and Huhndorf, 2007).

The *Phyllachorales* is an order of leaf-inhabiting, mostly tropical, perithecial ascomycetes (pyrenomycetes), and is the only member of the family *Phyllachoraceae* Theiss. & P. Syd. (with the unchallenged synonym *Polystigmataceae* Höhn. ex Nannf.) (Eriksson and Hawksworth, 1993; Cannon, 1997; Kirk *et al.*, 2008). The family *Phyllachoraceae* has had a controversial taxonomic position (Silva-Hanlin and Hanlin, 1998), and has been placed in several orders including the *Sphaeriales* (Miller, 1949), *Phyllachorales* (Barr, 1983), *Xylariales* (Barr, 1990), *Polystigmatales* (Hawksworth *et al.*, 1983) and *Diaporthales* (Cannon, 1988). This family might be artificial due to the lack of enough reliable morphological characters that clearly delimit the group, and also because of the emphasis that is placed only on a few characters, such as ascospore shape, colour, and septation, as well as on the extent of stromatic tissue (Cannon, 1991, Wanderlei-Silva *et al.*, 2003). Wehmeyer (1975) was aware that the *Phyllachoraceae* at that time was an artificial group, and included genera not closely related to each other.

Members of the *Phyllachoraceae* generally have perithecial ascomata which are strongly melanized at least near the perithecium ostioles, and are surrounded by black clypeal or stromatic tissue. Thin-walled paraphyses are usually present. The asci each have a small apical iodine-negative ring. Ascospores are mostly hyaline and aseptate. Anamorphs are inconspicuous, spermatial in function or with a dis-

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seminative role (Cannon, 1997). Which species were to be included in the family according to this description has been the cause of controversy.

There are few sequences available for taxa placed in *Phyllachorales*. The inability of some species to grow in culture is the main difficulty in working with molecular systematics of the species in this order. One of the few molecular systematics studies on *Phyllachorales* was by Wanderlei-Silva *et al*. (2003), in which *Polystigma* was not included.

Despite several decades of taxonomic investigation of ascomycetes, the relationship of *Polystigma* to other *Sordariomycetes* has remained elusive. Is *P. amygdalinum* related to genera in the *Phyllachorales*? If not, what are the closest relatives of *P. amygdalinum*? The main purpose of the present study was to investigate the relationships of *P. amygdalinum* to members of the *Phyllachorales* and other *Sordariomycetes* using 18S small-subunit of ribosomal DNA (SSU rDNA), 28S large-subunit of ribosomal DNA (LSU rDNA) and rDNA internal transcribed spacer regions (ITS), obtained from fresh and preserved herbarium specimens as well as GenBank sequences.

Materials and methods

Specimens used and DNA extraction

DNA extraction, PCR and sequencing were attempted from various fresh and dried specimens representing *P. amygdalinum* and *Polystigma rubrum* (Pers.) DC. The specimens were from various parts of Iran including many regions of Fars, Yasooj, Hamedan, Kohgiluyeh and Boyer-Ahmad and East Azerbaijan Provinces.

Superficial pseudostroma composed of plant and fungus tissue and infected plant material, was freeze-dried and stored at -20°C. Freeze-dried tissue was homogenized using sea sand (Fluka, Darmstadt, Germany) and plastic disposable pestles. Cells were lysed using CTAB solution and DNA was extracted using DNG™-plus DNA extraction solution (Cinaclon) (Mostowfizadeh-Ghalamfarsa and Mirsolaeimani, 2012). DNA concentrations were estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA). DNA extractions were each diluted to 20 ng·mL^{-1} in sterile distilled water for use as template DNA in PCR. In some cases, serial dilutions of DNA extractions were used to find the appropriate concentration for PCR, due to presumptive PCR inhibitors coming from environmental materials.

PCR and sequencing

Primers PyITS1 (Green *et al.*, 2004) and ITS4 (White *et al.*, 1990) were used to amplify internal transcribed spacer1, 5.8S rDNA and internal transcribed spacer 2 from all isolates. Twenty-five μL PCR reactions contained 1 × reaction buffer, 0.4 mM of each primer, 200 mM dNTPs, 2.5 mM $MgCl_2$, 20ng of DNA and 1 unit of *Taq* polymerase. PCR was carried out in a CG1-96 thermo cycler (Corbett Research) and cycling conditions consisted of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 5 min at 72°C. Small subunit regions were amplified by NS1 and NS4 primers (White *et al.*, 1990) and large subunit regions were amplified using NL1and NL4 primers (O'Donnell, 1993) with the same concentrations of PCR reagents and cycling conditions as described above for ITS. Sequencing was performed by Tech Dragon (Korea) and Elim Biopharmaceuticals, Inc. (USA). ITS, SSU and LSU sequences were deposited in GenBank (Table 1).

Phylogenetic analyses

Sequences were aligned by Geneious version 7 (Biomatters, USA). Phylogenetic analyses were performed using PAUP* 4.0a133 (Swofford, 2002) for parsimony, neighbour-joining and maximum likelihood analyses, and MrBayes v3.2.2 (Ronquist *et al.*, 2012) for Bayesian analyses of phylogeny. *Erysiphe friesii* (Lév.) U. Braun & S. Takam. was used as an outgroup.

Models of sequence evolution were evaluated for each dataset and model parameter estimates obtained with JModeltest v.2.1.4 (Posada, 2008) implemented in PAUP v. 4.0a133 (Swofford, 2002). The Akaike information criterion (AIC), Bayesian information criterion (BIC) and hierarchical likelihood ratio tests were used to select models. For the nuclear ribosomal internal transcribed spacer (ITS) dataset, the TIM2ef+I+G model with equal base frequencies, six substitution rate parameters (1.3892, 1.4482, 1.3892, 1.0000, 2.4398, 1.000) and gamma distributed rates (shape parameter 0.6960) were selected. The TIM+G model with equal base frequencies, six substitution rate parameters (11.0000, 2.1366, 1.0000, 1.0000, 4.4032, 1.000) and gamma distributed rates (shape parameter 0.7130) were chosen for the SSU dataset. For the LSU dataset, the GTR+G model with unequal base frequencies (A = 0.2262, C = 0.2477, G

Table 1. Fungal species included in phylogenetic analysis of this study.

Species	Order	GenBank Accession No.		
		ITS rDNA	18S rDNA	28S rDNA
Camarops ustulinoides	*Boliniales*	AY908991	DQ470989	DQ470941
Chaetosphaeria curvispora	*Chaetosphaeriales*		AY502933	GU180636
Diaporthe phaseolorum	*Diaporthales*	KC343180	AY779278	AY346279
Valsella melostoma	*Diaporthales*	AF191184		
Valsella salicis	*Diaporthales*		DQ862057	AF408389
Erysiphe friesii	*Erysiphales*	AB000939	AB033478	AB022382
Colletotrichum gloeosporioides	*Glomerellales*	DQ084498	JN940370	
Glomerella miyabeana	*Glomerellales*			JN939929
Glomerella cingulata	*Glomerellales*	GQ373209	AY083798	GCU48428
Hypomyces chrysospermus	*Hypocreales*	HQ604858	AB027339	AF160233
Kohlmeyeriella tubulata	*Lulworthiales*		AY878998	AF491265
Lindra thalassiae	*Lulworthiales*		DQ470994	DQ470947
Lulworthia fucicola	*Lulworthiales*		AY879007	AY878965
Lulworthia lignoarenaria	*Lulworthiales*			AY878968
Haloguignardia irritans	*Lulworthiales*	AY581943	AY566252	
Magnaporthe salvinii	*Magnaporthales*	JF414838	DQ341477	JF414887
Meliola centellae	*Meliolales*	KC252606		
Meliola niessleana	*Meliolales*		AF021794	KC833049
Microascus cirrosus	*Microascales*	JQ906771	M89994	JQ434680
Coccodiella melastomatum	*Phyllachorales*		U78543	
Coccodiella toledoi	*Phyllachorales*		CTU78544	
Phyllachora graminis	*Phyllachorales*		AF064051	
Sphaerodothis acrocomiae	*Phyllachorales*		SAU76340	
Ophiodothella vaccinii	-		OVU78777	
Polystigma amygdalinum	-	KC756360[a]	KM111539[a]	KM111540[a]
Polystigma amygdalinum	-	KC756362[a]		
Polystigma rubrum	-	KC966927[a]		
Cercophora caudata	*Sordariales*	AY999135	DQ368659	AY999113
Chaetomium elatum	*Sordariales*	HF548695	M83257	DQ368628
Chaetomium globosum	*Sordariales*	AY429056	JN939003	
Farrowia longicollea	*Sordariales*		AF207685	
Gelasinospora tetrasperma	*Sordariales*	AY681178	DQ471032	AY681144
Lasiosphaeria ovina	*Sordariales*	GQ922528	AY083799	AY436413
Neurospora crassa	*Sordariales*	AY681193	X04971	AY681158
Sordaria fimicola	*Sordariales*	FN392318	AY545724	AY681160
Nigrospora oryzae	*Trichosphaeriales*	JN198503	FJ176838	FJ176892
Xylaria acuta	*Xylariales*	JQ862676	JQ419764	JQ862637
Xylaria hypoxylon	*Xylariales*	DQ491487	NG_013136	

[a] Sequences generated in this study

Empty spaces mean the sequences were not available.

= 0.3234, T = 0.2027), six substitution rate parameters (0.5868, 1.7561, 1.1475, 0.6368, 5.4597, 1.000) and gamma-distributed rates (shape parameter 0.3320) were selected.

Maximum likelihood and Parsimony phylogenies were estimated independently for each data partition (ITS, SSU, LSU) using heuristic searches in PAUP v. 4.0a133 (Swofford, 2002). Data were analyzed using Bayesian inference based on a Markov chain Monte Carlo (MCMC) approach in the software package MrBayes v3.2.2 (Ronquist *et al.*, 2012). For Bayesian analyses, a general time-reversible model of evolution was used. Rate heterogeneity across sites was modeled with a gamma distribution. Four chains starting with a random tree were run for 10,000,000 generations, retaining each 1000th tree and the first 25% of each analysis were discarded as burn-in.

Results

PCR and DNA sequencing

Polystigma spp. produce a pseudostroma composed both of plant and fungus tissue. This problem is solved for the ITS regions either by using the plant-excluding primer pair PyITS1/ITS4 (Green *et al.*, 2004) or by extracting the fungal amplicon from the agarose gel. In some cases, however, special care was taken to separate only the centrum with fungus materials, placed in a micro centrifuge tube for DNA extraction.

The lengths of the *Polystigma* spp. DNA sequences obtained were approximately 560bp for ITS, 555bp for LSU, and 954bp for SSU. All efforts to amplify SSU and LSU of *P. rubrum* failed due to lack of fresh specimens. Evidence that the DNA sequences generated for *P. amygdalinum* were not from contaminants is as follows: No identical matches were found in GenBank when either SSU, ITS, or LSU sequences of *P. amygdalinum* were blasted. All searches yielded closest matches to taxa in the *Pezizomycotina*, between 87–93% DNA sequence identities in the aligned regions (data not shown). Additionally, DNA from *P. amygdalinum* was extracted more than once, and yielded identical sequences (data not shown). Phylogenetic analyses showed that the *P. amygdalinum* SSU and LSU sequences were divergent from all other taxa in the analyses, including the closest BLAST search matches.

Phylogenetic analyses

SSU rDNA

To determine the position of *P. amygdalinum* among the *Sordariomycetes*, an SSU rDNA sequence alignment was created. Preliminary analyses with representative taxa from all classes of ascomycetes showed that *P. amygdalinum* grouped with representatives of the *Sordariomycetes* (*Pezizomycotina*) (data not shown). To facilitate phylogenetic analyses, all taxa outside of the *Sordariomycetes* were excluded from subsequent analyses except for *Erysiphe friesii* as an outgroup. Trees obtained using different analyses of the SSU data resembled each other, and only one tree (Figure 1) is presented. The resulting SSU rDNA alignment comprised 34 taxa and 833 characters. The nodes relevant for this study were supported by all analyses (Figure 1).

ITS

The resulting ITS alignment comprised 22 taxa and 658 characters. Isolates sampled from almond in various locations and assigned to *P. amygdalinum* generally had identical ITS haplotypes. The only exception was an isolate MA4 from the Maharlou region (Fars, Iran), which differed from other isolates at one nucleotide position. *Polystigma rubrum* also appeared as the sister taxon of *P. amygdalinum* and both species were related to the *Xylariales* and *Trichosphaeriales* (Figure 2).

LSU rDNA

The LSU dataset contained fewer taxa than the SSU dataset. It included representatives of the orders of the *Sordariomycetes* available from GenBank (Table 1); however no DNA sequences were available for other *Phyllachorales*. The resulting LSU rDNA alignment comprised 26 taxa and 576 characters.

Comparison of topologies between the most parsimonious trees, the Bayesian consensus tree, and the neighbour-joining tree showed that they shared the branches of importance to this study, supporting the relatedness of *P. amygdalinum* to the *Trichosphaeriales* and *Xylariales* (Figure 3).

Discussion

The main goal of this study was to examine whether placing *P. amygdalinum* in the *Phyllachorales*

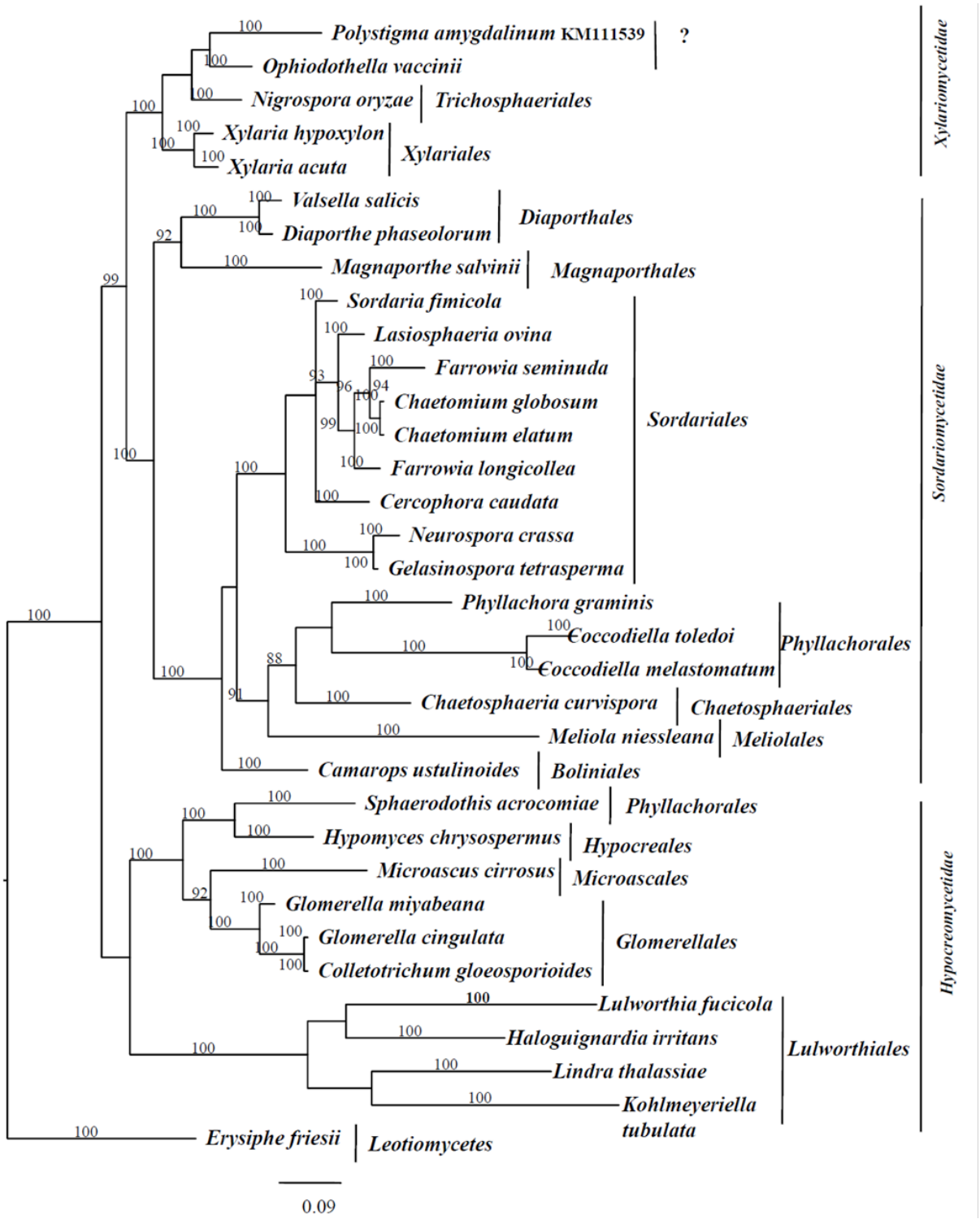

Figure 1. Bayesian phylogenetic tree of species belonging to different orders of *Sordariomycetes* based on SSU rDNA sequences. Bayesian posterior probabilities (probability %) are shown next to the branch points. The scale bar represents the number of changes per sites.

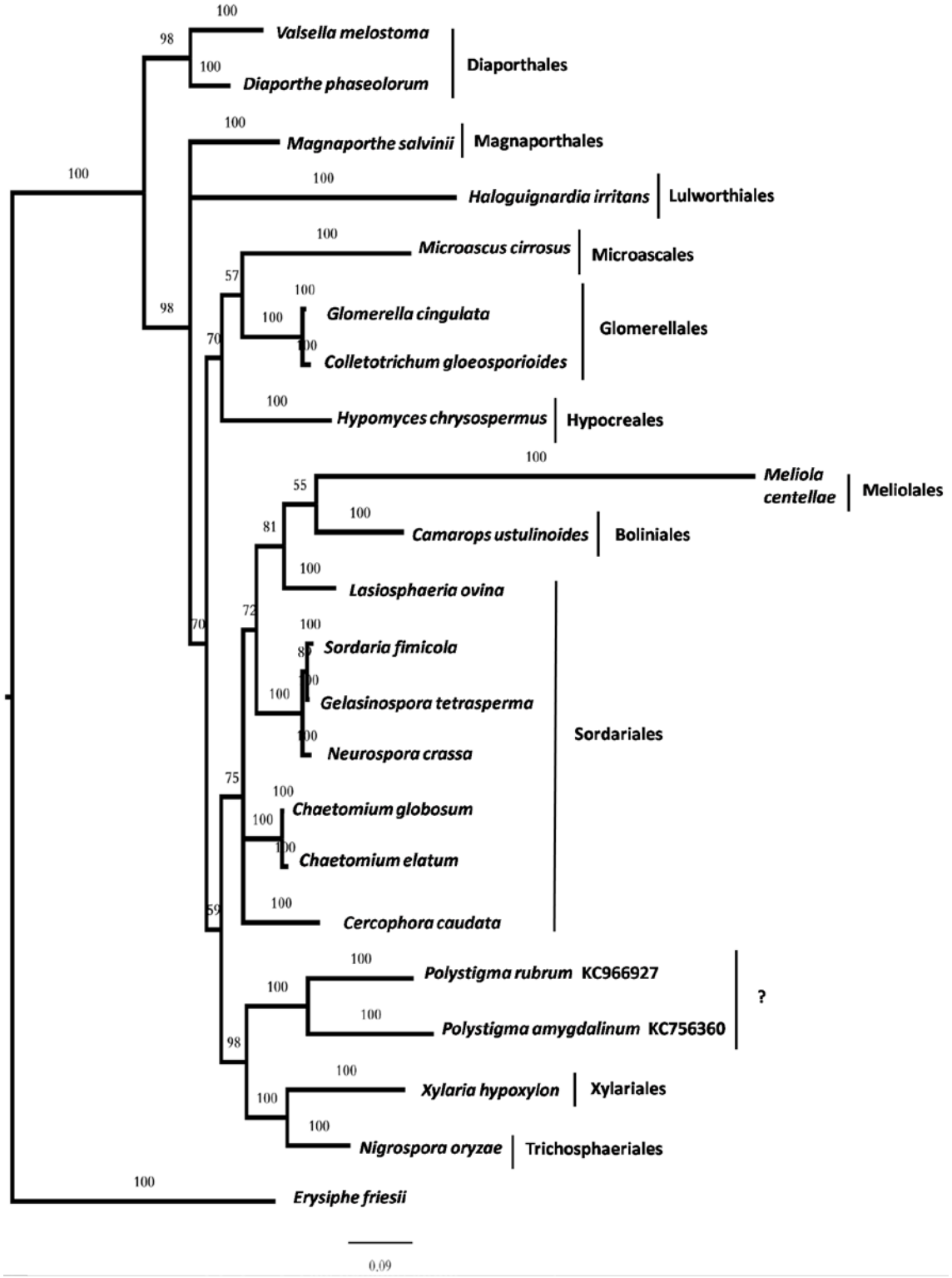

Figure 2. Bayesian phylogenetic tree of species belonging to different orders of *Sordariomycetes* based on ITS sequences. Bayesian posterior probabilities (probability %) are shown next to the branch points. The scale bar represents the number of changes per sites.

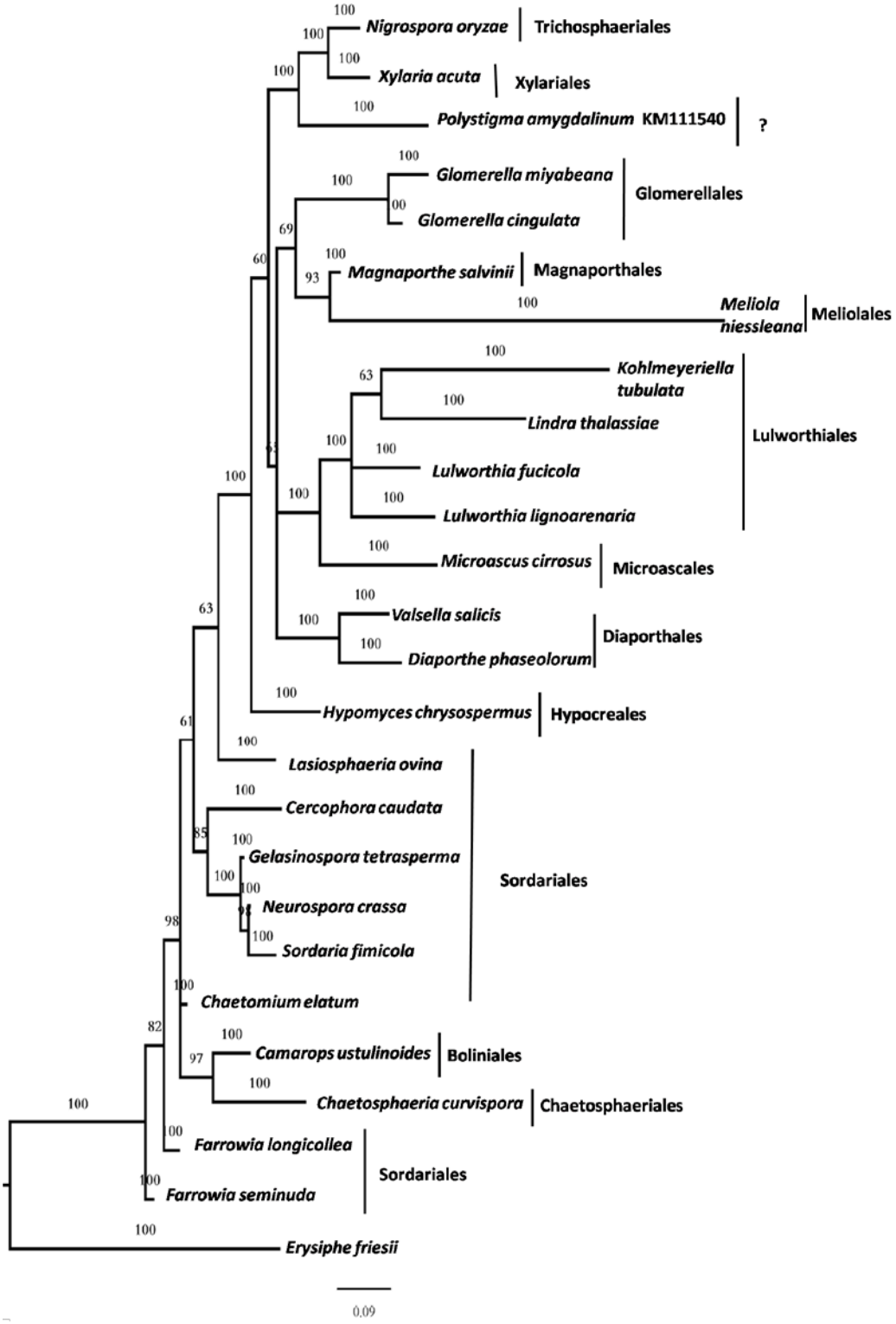

Figure 3. Bayesian phylogenetic tree of species belonged to different orders of *Sordariomycetes* based on LSU rDNA sequences. Bayesian posterior probabilities (probabilities %) are shown next to the branch points. The scale bar represents the number of changes per sites.

based on morphology would be supported by molecular data, and illuminate the position of this species in the fungal tree of life. Our phylogenetic analyses based on ITS, SSU and LSU DNA sequences show that the *Polystigma* spp. are related to the *Trichosphaeriales* and the *Xylariales*. There was no evidence of any relationship to the *Phyllachorales* in spite of the morphological resemblance (Cannon, 1996).

In the SSU rDNA tree, three clades were observed (Figure 1). The first was represented by the orders *Xylariales* and *Trichosphaeriales*, and two species *Ophiodothella vaccinii* Boyd and *P. amygdalinum*. The second clade held the orders *Sordariales*, *Diaporthales*, *Magnaporthales*, *Boliniales*, *Chaetosphaeriales*, *Meliolales*, and *Phyllachorales* (*Phyllachora graminis* (Pers.) Fuckel and *Coccodiella* spp.). The third clade contained the *Hypocreales*, *Microascales*, *Lulworthiales*, *Glomerellales* and part of the *Phyllachorales* (*Sphaerodothis acrocomiae* Chardón & R.E.D. Bakerand).

Polystigma amygdalinum and *Ophiodothella vaccinii* form a clade in sister position to the *Trichosphaeriales* (*Nigrospora oryzae* (Berk. & Broome) Petch) and together they seem to be related to the *Xylariales*. On the other hand, the two species of *Coccodiella* and *Phyllachora graminis* form a clade that was a sister group to the *Chaetosphaeriales* and they were related to the *Meliolales* and the *Sordariales*. The SSU dataset analysis shows that *P. amygdalinum* is not closely related to the morphologically similar fungus *Phyllachora*, but groups with high support in all analyses in a separate clade in the *Sordariomycetes*, closer to the *Xylariales*.

Our results are consistent with many authors who questioned the monophyly of *Phyllachorales* (Wehmeyer, 1975; Jensen, 1985; Wanderlei-Silva *et al.*, 2003). In the SSUrDNA analyses, different members of the *Phyllachorales* show relationships to the *Xylariales*, *Hypocreales* and *Chaetosphaeriales* and are placed in different clades, in agreement with Wanderlei-Silva *et al*. (2003). Yet, the topology of our gene tree provides support for three major clades in the *Sordariomycetes* corresponding to three clades shown by Zhang *et al.* (2007), the *Xylariomycetidae*, *Hypocreomycetidae* and *Sordariomycetidae*.

The phylogenetic position of *P. amygdalinum* and *Ophiodothella vaccinii* in the same clade (Figure 1), based on molecular characters, agrees with similar morphology and both could be placed in one family. The conidia of *O. vaccinii* show marked resemblance to the spermatia of *P. rubrum*, which are curved filiform, and the perithecial primordia arise in the center of mesophyll and each originate from one or several coiled archicarps which at this stage are seen in a triangle of vegetative threads, as in *P. rubrum* (Boyd, 1934). Also in some instances, the stromata of *Polystigma* species accumulate starch (Cannon, 1996), which is common with *Ophiodothella* spp., where the ascus tips stain blue in iodine (Wanderlei-Silva *et al.*, 2003). Their similar disease cycle, symptoms and development (Hanlin, 2003; Bezerra *et al.*, 2006) also support their relatedness.

Polystigma amygdalinum has traditionally been classified in the *Phyllachorales* because of its morphological similarities to *Phyllachora*. There are morphological differences between *Phyllachora* spp. and *P. amygdalinum* such as stromatal pigmentation and sympodial proliferation of conidia in *P. amygdalinum* rather than percurrent proliferation as is usual in the *Phyllachoracae* (Cannon, 1996). Additionally in some instances, the stromata of *Polystigma* species accumulate starch (Cannon, 1991), atypical of the *Phyllachorales*. This feature and the *Xylaria*-type centrum both are characteristics of the *Xylariales*. Wanderlei-Silva *et al.*, (2003) showed that *O.vaccinii* grouped with the *Xylariales*.

Among former studies, Jensen (1985) examined the morphology of peridia of certain pyrenomycetes and found that four species of *Phyllachorales*, viz. *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (now *Glomerellales*), *Phyllachora graminis* (Pers.) Fuckel, *Physalospora corni* Ellis & Everh (now *Hyponectriaceae, Xylariales*) and *Polystigma ochraceum* Pers. ex DC. (now *P. amygdalinum*) have highly varied peridial constructions and suggested further studies to determine if this family was a natural group. Whether *Phyllachora* spp. and *P. rubrum* could be placed in the same family was also questioned by Miller (1949).

Polystigma amygdalinum did not group with other former members of the *Phyllachorales*; *Sphaerodothis acrocomiae* is not related to this order (Wanderlei-Silva *et al.*, 2003) and was placed in the *Hypocreales* based on SSU sequences.

According to ITS, SSU and LSU datasets, *P. amygdalinum* is located in the *Xylariomycetideae* subclass of the *Sordariomycetes*, in agreement with their morphological similarities. This subclass contains a single order, *Xylariales*, which is characterized by well-developed stromata, dark colored perithecia, and persistent asci, often with amyloid apical rings and true

paraphyses (Zhang *et al.*, 2007), characteristics also present in *P. amygdalinum*.

Polystigma amygdalinum appears to be related to *Nigrospora oryzae* which is in agreement with morphological similarities of the *Khuskia* teleomorph. Hudson (1963) placed *N. oryzae* in the *Polystigmataceae* based on morphological similarity with several members of this family.

It is likely that morphological similarities among *P. amygdalinum* and some other species previously assigned to the *Phyllachorales* have arisen convergently. Classifications using only few taxonomic characters easily create polyphyletic taxa that superficially resemble each other (Hausner *et al.*, 1993; Spatafora and Blackwell, 1994).

The position of various taxa in the order *Phyllachorales* remains unresolved. However, based on our results, *P. amygdalinum* and *P. rubrum* should in any case be placed in the *Xylariomycetidae* of the *Sordariomycetes*.

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