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 Polystigma 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 Masanhat, 1997; Cimen 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 perithecia 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-
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_TM-plus DNA extraction solution (Cinaclic) (Mostowfizadeh-Ghalamfarsa and Misroaineimani, 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 MgCl₂, 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 NL1 and 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 LSU dataset. For the LSU dataset, the GTR+G model with unequal base frequencies (A = 0.2262, C = 0.2477, G = 0.2262, T = 0.3000) and gamma distributed rates (shape parameter 0.6960) were selected.
Table 1. Fungal species included in phylogenetic analysis of this study.

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* 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 searchs 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.

**Phylogenic 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*
Phylogenetic analysis of Polystigma and its relationship to 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, Magnaportheles, Boliniales, Chaetosphaeriales, Meliolales, and Phyllachorales (Phyllachora graminis (Pers.) Fuckel and Coccodilla spp.). The third clade contained the Hypocreales, Microascales, Lusworthiales, Glomerelles 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 Coccodilla 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 Phyllachoraceae (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 Glomerella), Phyllachora graminis (Pers.) Fuckel, Physalospora corni Ellis & Everh (now Hyponectraceae, 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 Xylariomycetidae 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

A. Habibi et al.
paraphyses (Zhang et al., 2007), characteristics also present in P. amygda
dinum.

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

It is likely that morphological similarities among
P. amygda
dinum and some other species previously as
signed to the Phyllachorales have arisen convergen
tly. Classifications using only few taxonomic characters
easily create polyphyletic taxa that superficially re
semble each other (Hausner et al., 1993; Spatafora
and Blackwell, 1994).

The position of various taxa in the order Phylla
chorales remains unresolved. However, based on our
results, P. amygda
dinum and P. rubrum should in any
case be placed in the Xylariomycetidae of the Sordari
omy
cetes.

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