Phylogeny of the fungus Spilocaea oleagina, the causal agent of peacock leaf spot in olive

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Abstract

The fungus Spilocaea oleagina causes peacock leaf spot in olive. Virtually nothing is known about S. oleagina despite the loss of crop yield caused by this fungus. In order to get insight, an in vitro culture of the fungus has been established and its identity confirmed by amplified fragment length polymorphism analysis. Using this in vitro culture, we have cloned and analysed the DNA sequences of the 18S and 28S ribosomal RNA genes (rDNA) as well as the internal transcribed spacers (ITS) and 5.8S rDNA region of S. oleagina. Sequence analysis and comparison to other fungi determined that this fungus belongs to the Dothideomycetes class. We have also determined that S. oleagina is an anamorphic phase of a yet unidentified Venturia species based on phylogenetic analysis using the 28S rDNA and ITS sequences. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: rDNA; Peacock leaf spot; Internal transcribed spacer; Molecular classification; Olea europaea; Spilocaea oleagina

1. Introduction

Peacock leaf spot, caused by the fungus Spilocaea oleagina, is among the most common fungal diseases in olive. It causes leaf abscission and, eventually, whole tree weakness, resulting in a subsequent loss in crop yield [1]. The S. oleagina mycelium normally develops under the cuticle of the leaf, but in severe cases may infect the petiole, the peduncle of the fruit, and the fruit itself [1]. The sexual phase of S. oleagina is unknown, and therefore it has been considered an imperfect fungus and classified within the Deuteromycetes class. However, the classification of Deuteromycetes as ascomycetes or basidiomycetes is now possible using molecular techniques [2]. These techniques can reveal evolutionary relationships that cannot be determined using classical phenotypic criteria [3].

Analysis of the DNA sequences of the rRNA genes (rDNA) and the internal transcribed spacer (ITS) regions has been used to study the phylogenetic relationships and the evolution of many organisms [4]. The sequences encoding the nuclear rRNA repeat units have evolved relatively slowly and are useful to compare distantly related organisms. The 18S and 28S rDNA sequences are considered powerful taxonomic indicators because they are universally found in living cells and are highly conserved [5]. The presence of highly variable stretches in the 28S rDNA permits the evaluation of the divergence between closely related species and eventually among varieties within a species [6]. The non-coding ITS sequences evolve faster than the coding sequences, and may vary among species and populations [7].

Since S. oleagina in vitro cultures do not sporulate we confirmed the identity of the isolates using molecular markers. Several DNA based markers have been used to evaluate genetic diversity, and even within fungal species, to identify particular races and pathotypes [8,9]. Among these, amplified fragment length polymorphism (AFLP) fingerprinting is becoming the most powerful and reliable technique for the characterization of genotype [9]. In this study we report the classification of S. oleagina using the 18S rDNA, 28S rDNA and ITS1–5.8S rDNA–ITS2 sequences.

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2. Materials and methods

2.1. Fungal isolates

The *S. oleagina* isolate So-91 was isolated from the infected olive cultivar Hojiblanca. The infected leaves were collected from trees cultivated in Spain, and the fungal isolate came from a single conidium. The fungal isolate was cultured in a 25-ml petri dish containing 25 ml Czapek-Dox broth (Difco Laboratories, Detroit, MI, USA) medium for 2 months at 15°C in darkness. The isolate belongs to the fungal collection of the ‘Departamento de Agronomía’ from the ETSIAM in Córdoba (Spain).

2.2. Plant material

Four different olive varieties were chosen for the AFLP experiment with the fungus and they included both infected and non-infected tissue. The olive varieties used were III-79, Giarrafa, Frantoio, and Nostrali di Rigale and came from the ‘Istituto di Ricerche sulla Olivicoltura’ in Peruggia (Italy).

2.3. Fungal and plant DNA extraction

Both olive leaves and mycelium were collected and ground to a fine power in liquid N₂. DNA extraction was made using procedures described earlier [10] including one additional extraction with phenol before the phenol: chloroform extraction step.

2.4. Cloning and sequencing of 18S rDNA gene

18SF (5’-GCTTGCTCTAAAGATAAGCC-3’) and 18SR (5’-CTTTGTTACGACGTTCACCTCC-3’) primers were designed according to the conserved regions deduced from an alignment of 18S rDNA sequences from the following fungi: *Colletotrichum gloeosporioides* (M55640), *Trichoderma viride* (AF218788), *Neurospora crassa* (X04971), *Aspergillus fumigatus* (M55626), *Candida vinaria* (AB018135), and *Agaricus bisporus* (L36658). Fifty nanograms of genomic DNA from fungal isolate So-91 were used for PCR amplification using primers 18SF and 18SR in a 100-µl final reaction volume. PCR reagents and the proofreading *Pwo* polymerase were according to the manufacturer’s specifications (Roche Molecular Biochemicals). The final concentrations were 0.2 µM for 18SF and 18SR primers, 2 mM MgSO₄, 100 µM for each deoxynucleoside triphosphate (dNTP), and 5 U of *Pwo* polymerase. The PCR program was 3 min at 95°C followed by 30 cycles of 30 s for template denaturation at 95°C, 30 s for primer annealing at 50°C and 1 min and 30 s for primer extension at 72°C, plus a final extension step at 72°C for 10 min. The PCR product were precipitated and cloned into a pBSKII vector digested with *EcoRV*. The cloning was made from two independent PCR reactions, and both clones were sequenced in the ‘Centro de Investigaciones Biológicas-CSIC’ (Madrid, Spain).

2.5. Cloning and sequencing of a partial 28S rDNA clone

Primers d123F (5’-AGCTCAATTTGAACAC-3’) and d123R (5’-GATTTGCCACCATCTTT) were designed according to the conserved regions from alignment of 28S rDNA from the following fungi: *Saccharomyces cerevisiae* (J01355), *Cryptococcus neoformans* (L14068), *Tricholoma matsutake* (U62964), *Phytophthora megasperma* (X75631), and the plant *Acorus gramineus* (AF036490). PCR conditions and cloning were the same as for the 18S rDNA gene except for the PCR program: 3 min at 95°C followed by 30 cycles of 30 s for template denaturation at 95°C, 30 s for primer annealing at 50°C and 1 min and 30 s for primer extension at 72°C, plus a final extension step at 72°C for 10 min. Three different clones were sequenced coming from three independent PCR reactions. Both strands of the clones were sequenced in the ‘Centro de Investigaciones Biológicas-CSIC’ (Madrid, Spain).

2.6. Cloning and sequencing of the ITS1–5.8S–ITS2 DNA sequence

The *S. oleagina* ITS1–5.8S–ITS2 DNA sequence was cloned using ITS1 and ITS4 primers [11]. Fifty nanograms of genomic DNA from fungal isolate So-91 were for PCR amplification using the primers ITS1 and ITS4 [11] in a 100-µl final reaction volume. PCR conditions and cloning were the same as for 18 rDNA except for the PCR program: 3 min at 95°C followed by 30 cycles of 30 s for template denaturation at 95°C, 30 s for primer annealing at 50°C and 1 min for primer extension at 72°C, plus a final extension step at 72°C for 7 min. Three different clones were sequenced coming from three independent PCR reactions. Both strands of the clones were sequenced in the ‘Centro de Investigaciones Biológicas-CSIC’ (Madrid, Spain).

2.6.1. GenBank accession numbers

The GenBank accession numbers of the *S. oleagina* sequences in GenBank are AF338403 (18S rDNA), AF338397 (28S rDNA) and AF338403 (ITS1–5.8S rDNA–ITS2).

2.6.2. Sequence analysis

The 18S sequences of the following fungi were selected for an alignment of the 18S rDNA sequence from *S. oleagina*: *Eupenicillium javanicum* (U21298), *Penicillium chrysogenum* (M55628), *Alternaria alternata* (U05194), *Septoria nodorum* (U04236), *Leptothesphaeria maculans* (U04238), *Westerdykella dispersa* (U42488), *Sporomia lig-
nicola (U42478), Dothidea insulcpta (U42474), Aureobasidium pullulans (M55639), Endothia gyroa (L42443), Ophiostoma snecneras (M58054), Podospora anserina (X54864), N. cussa (X04971), Hypocrea lutea (D14407), Pseudallescheria boydii (M89782), C. gloeosporoides (M55640), Sacimonyces petricola (Y18702), Exophiala calicoides (AB007655), Capronia mansoni (X79318), Phaeococcomycex exophialae (X80709), Ceramothyrium linnaeae (AF022715) and S. cerevisiae (J01353). The sequences were aligned using the CLUSTAL X program. Because of the slightly different gene lengths, ‘overhang’ nucleotides had to be cut off for some species. The phylogenetec tree of the alignments was done using the CLUSTAL X program. Bootstrap confidence values were calculated from 1000 repeats.

For the 28S rDNA sequences, the following sequences were chosen for the alignment and construction of the phylogenetic tree: Ophiostoma splendens (af221013), Ophiostoma africamum (af221015), Cladosporium sp. (af050265), Cladosporium sp. (af050264), A. pullulans (af050239), Venturia hanliniana (af050290), and one from Penicillium viridicatum (af003358) as an outgroup. The programs used to study the phylogeny from the 28S rDNA sequence were as those for the 18S rDNA sequence. The percentage of identity among sequences of species from the same genus or from different genera was calculated using the ‘gap’ program of the University of Wisconsin Genetics Computer Group (UWGCG) package program.

The sequences used for the ITS1–5.8S rDNA–ITS2 tree were the same as those used previously by Schnabel et al. [11]: Venturia inaequalis strain 5BF (AF065838), V. inaequalis strain 826BF (AF065839), Venturia pyrina strain Il-4 (AF065842), V. pyrina strain MPS8 (AF065840), V. pyrina strain Akita FB-1 (AF065841), V. pyrina strain Il-18 (AF065844), V. pyrina strain Mamensashi 12A1-3 (AF065846), V. pyrina Mamensasi 12A1-1 (AF065845), V. pyrina strain II-11 (AF065843), V. inaequalis strain 685BF (AF065837), V. inaequalis strain 890F (AF064836), Venturia cerasi (AF065851), Cladosporium cariymenum strain LCF (AF065851), C. cariymenum JD (AF065850), Venturia carphoila (AF065849) and Venturia asperata (AF065848). The programs used to study the phylogeny from ITS sequences were the same as for the 18S and 28S rDNA sequences.

2.7. Olive AFLP

The technique used for the AFLP with two fungal isolates, infected olive and non-infected olive, was as described previously [12]. Experimental conditions were the same as described earlier [13]. The primers used for PCR preamplification were EcoRI-A/MseI-C and those for the PCR selective amplification were EcoRI-AGC/MseI-CAC and EcoRI-AGC/MseI-CTG.

3. Results

3.1. The fungus cultured in vitro is the agent causing peacock leaf spot in olive

*S. oleagina* is a recalcitrant fungus to manipulate due to the difficulty in culturing it, and its slow growth rate. Because *S. oleagina* cultured in vitro does not sporulate, we determined whether the fungus grown in vitro was indeed the same as that which produced the peacock leaf spot disease in olive. AFLP profiles of the fungus grown in vitro, from diseased and healthy olive leaves, are shown in Fig. 1. Two fungal isolates and four olive cultivars were used for the analysis. Two primer combinations with three selective nucleotides were employed as described previously [13]. Bands from the fungal isolates So-62 and So-87 were also found in the AFLP profile of olive leaves that showed the typical disease symptoms but were absent in the AFLP profile of healthy olive leaves (Fig. 1). This result demonstrates that the fungus cultured in vitro is the one causing peacock leaf spot disease in olive.

3.2. Phylogeny of *S. oleagina* based on the 18S rDNA sequence

A 1.8-kb DNA fragment of 18S rDNA from *S. oleagina* was amplified and sequenced. The 11 GenBank sequences with highest identity to the 18S rDNA from *S. oleagina* were identified using BLASTN analysis, and are shown in Table 1. Three of the sequences corresponded to the species *Botryosphaeria ribis*, *A. pullulans*, and *Mycosphaerella*.
mycopappi, classified as Dothideomycetes et Chaetothyriomycetes incertae sedis according to Eriksson et al. [14]. Two species, *D. insculpta* and *Coccodinium bartschii* are classified as Dothideomycetes. *S. petricola* is classified as Chaetothyriomycetes, and *Bulgaria inquinans* as Leotiomycetes. The four remaining sequences corresponded to fungi classified as Mitosporic Ascomycota although their taxonomy from genus to division is unknown. These results suggest that *S. oleagina* belongs to the Dothideomycetes et Chaetothyriomycetes incertae sedis group, which corre-

![Phylogenetic tree from 18S rDNA sequences: the tree was constructed with the neighbour-joining method and using *S. cerevisiae* as outgroup. Four classes of the subphylum Pezizomycotina are represented, and different orders are included for every class. Bootstrap values (1000 replicates) are indicated in the nodes.](image)

**Table 1**

Fungal species whose 18S rDNA sequences deposited in GenBank have the highest identity to the *S. oleagina* 18S rDNA sequence

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>GenBank accession No.</th>
<th>Eriksson 2001 Morphological classification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. ribis</em></td>
<td>U42477</td>
<td>Dothideomycetes et Chaetothyriomycetes incertae sedis/Loculoascomycetes</td>
</tr>
<tr>
<td><em>A. pullulans</em></td>
<td>AF258607</td>
<td>Dothideomycetes et Chaetothyriomycetes incertae sedis/Loculoascomycetes</td>
</tr>
<tr>
<td><em>M. mycopappi</em></td>
<td>U43463</td>
<td>Dothideomycetes et Chaetothyriomycetes incertae sedis/Loculoascomycetes</td>
</tr>
<tr>
<td><em>D. insculpta</em></td>
<td>U42474</td>
<td>Dothideomyces/Dothideales/Dothideomycetes incertae sedis/Loculoascomycetes</td>
</tr>
<tr>
<td><em>C. bartschii</em></td>
<td>U77668</td>
<td>Dothideomyces/Dothideales/Coccodinaceae/Loculoascomycetes</td>
</tr>
<tr>
<td><em>S. petricola</em></td>
<td>Y18702</td>
<td>Chaetothyriomycetes/Chaetothyriales/Mitosporic Chaetothyriales/Loculoascomycetes</td>
</tr>
<tr>
<td><em>B. inquinans</em></td>
<td>AJ224362</td>
<td>Leotiomycetes/Leotiales/Leotiaceae/Discomycetes</td>
</tr>
<tr>
<td><em>Scytalidium hyalinum</em></td>
<td>AF258607</td>
<td>Mitosporic Ascomycota/Deuteromycetes</td>
</tr>
<tr>
<td><em>Phaeosclera dematioides</em></td>
<td>Y11716</td>
<td>Mitosporic Ascomycota/Deuteromycetes</td>
</tr>
<tr>
<td><em>Lasioderma serricorne</em> yeast-like symbiont</td>
<td>D49656</td>
<td>Mitosporic Ascomycota/Deuteromycetes</td>
</tr>
<tr>
<td><em>Hortaea werneckii</em></td>
<td>Y18700</td>
<td>Mitosporic Ascomycota/Deuteromycetes</td>
</tr>
</tbody>
</table>

Classification according to Eriksson et al. [14] and the classical morphological classification are shown for every sequence.
From the image, it appears to be a page from a scientific journal article discussing phylogenetic analysis of fungi. The text includes a phylogenetic tree and a discussion of the classification of fungi, specifically from the Dothideomycetes class. The text mentions the use of 18S rDNA partial sequences to generate a phylogenetic tree and discusses the relationships between different fungal species. The article also includes a table comparing the percentage identity of 28S rDNA sequences between species of the genera Cladosporium, Ophiostoma, and Penicillium.

### Table 2

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Organism</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF050264</td>
<td>Cladosporium sp.</td>
<td>98</td>
</tr>
<tr>
<td>AF050266</td>
<td>Cladosporium sp.</td>
<td>98.7</td>
</tr>
<tr>
<td>AF221015</td>
<td>O. africamum</td>
<td>98.7</td>
</tr>
<tr>
<td>AF221013</td>
<td>O. splendens</td>
<td>97.8</td>
</tr>
<tr>
<td>AF003359</td>
<td>Penicillium expansum</td>
<td>97.8</td>
</tr>
<tr>
<td>AF003358</td>
<td>P. viridicatum</td>
<td>97.8</td>
</tr>
<tr>
<td>AF338397</td>
<td>S. oleagina</td>
<td>89</td>
</tr>
<tr>
<td>AF050239</td>
<td>V. hanliniana</td>
<td>89</td>
</tr>
<tr>
<td>AF338397</td>
<td>O. parella</td>
<td>89</td>
</tr>
</tbody>
</table>

The table compares the percentage identity of 28S rDNA sequences between two species of the genera Cladosporium, Ophiostoma, and Penicillium. The text mentions that the presence of a species from the Leotiomycetes class is not surprising since this class is considered paraphyletic. A phylogenetic tree was generated from the 18S rDNA sequences of 23 species, including S. oleagina and S. cerevisiae, and compared to the Neighbour-joining method of the CLUSTAL X program to gain further insight into the classification of S. oleagina. The species from the same genus were considered as belonging to the Dothideomycetes class, and the close analysis of the phylogeny within the Dothideomycetes class shows that S. oleagina may belong to a different order within the Dothideomycetes class not represented in the tree.
of a tree with sequences from the GenBank grouped S. oleagina with the sequences from the Dothideomycetes class. Since S. oleagina was not grouped with the two orders included in the tree (Dothideales and Pleosporales), we hypothesized that it belongs to one of the three orders not represented in the tree (Capnodiaceae, Myriangiales and Patellariales), or to a new order.

The study of evolutionary relationships among species of the same genus often requires information from sequences with a higher level of variability. This is the case for the 28S rDNA sequence, including the hypervariable regions (D1, D2, D3) [8]. Comparison of this hypervariable sequence from S. oleagina with other sequences from the GenBank showed that S. oleagina and V. hanliniana were significantly associated in a group, as other species of the same genus. Moreover, the use of more variable sequences like ITS1–5.8S rDNA–ITS2 as a tool to establish the phylogeny of S. oleagina confirmed that this species belongs to the Venturia genus. This is valuable information for studying the interaction between S. oleagina and olive since previous information known from species of the Venturia genus can be applied.

4. Discussion

The study of a plant–pathogen interaction requires a reliable classification of the organisms involved. The phylogenetic classification of any organism constitutes the basis to apply previous information of model organisms or related taxa [3]. The ascomycetes present two stages in their life cycle, sexual or teleomorphic, and asexual or anamorphic. Historically, every organism has received a binomial name. In the literature it is not uncommon to find an ascomycete of a sexual genus associated with several asexual species, and vice versa. In mycology there have been conflicting phylogenetic classifications based exclusively on morphology. This is particularly true for Deuteromycetes, making it difficult to compare related sexual and asexual pathogens [5]. Previous reports based on morphological analogy with other Spilocaea species have considered S. oleagina to be an asexual phase of the genus Venturia [16]. This genus consists mostly of plant-pathogenic species, many of them attacking the fruit and the foliage of important tree-fruit crops [11].

The classification of ascomycetes by morphological characteristics is complicated by being paraphyletic in different classes. This was evident for Loculoascomycetes where the morphological characteristics used to classify them have evolved into two parallel lines [17]. These problems may be overcome with the use of molecular markers. At present, the most reliable sequence used for this purpose is DNA encoding the small ribosomal subunit [18]. This sequence allows phylogenetic classification based on evolution. The cloning and sequencing of a 1.8-kb fragment of 18S rDNA from S. oleagina and the construction

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