ELSEVIER

FEMS Microbiology Letters 210 (2002) 149–155

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

Rocío González-Lamothe [a], Rosa Segura [b], Antonio Trapero [b], Luciana Baldoni [c], Miguel A. Botella [a], Victoriano Valpuesta [a,*]

[a] *Departamento de Biología Molecular y Bioquímica, Universidad de Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain*
[b] *Departamento de Agronomía, ETSIAM, Universidad de Córdoba, 14080 Córdoba, Spain*
[c] *IR Miglioramiento Genetico Piante Foraggere CNR, Via Madonna Alta, 130, 06128 Peruggia, Italy*

Received 11 February 2002; received in revised form 4 March 2002; accepted 6 March 2002

First published online 12 April 2002

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.

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.

* Corresponding author.
Tel.: +34 (95) 213 1932; Fax: +34 (95) 213 1932.
E-mail address: valpuesta@uma.es (V. Valpuesta).

0378-1097/02/$22.00
PII: S0378-1097(02)00598-0

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 de Ricerche sulla Olivicoltora' 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 N_2. 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′-GCTTGTCTCAAAGATTAAGCC-3′) and 18SR (5′-CCTTGTTACGACGACTTTTACTTCC-3′) primers were designed according to the conserved regions deduced from an alignment of 18S rDNA sequences from the following fungi: *Colletotrichum gloeosporoides* (M55640), *Trichoderma viride* (AF218788), *Neurospora crassa* (X04971), *Aspergillus fumigatus* (M55626), *Candida vinaria* (AB018135), and *Agaricus bisporus* (L36658). Fifty nanograms of genomic DNA from the *S. oleagina* 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 used according to the manufacturer's specifications (Roche Molecular Biochemicals). The final concentrations were 0.2 μM for 18SF and 18SR primers, 2 mM $MgSO_4$, 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 55°C and 2 min for primer extension at 72°C, plus a final extension step at 72°C for 10 min. Ten microlitres of the PCR product were visualized in a 1% agarose ethidium bromide stained gel, and a 1.8-kb fragment was identified. The remaining 90 μl of PCR product were precipitated and cloned into a pBSKII vector digested with *Eco*RV. The cloning was made from two independent PCR reactions, and both clones were sequenced in the 'Centro de Investigaciones Biologicas-CSIC' (Madrid, Spain).

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

Primers d123F (5′-AGCTCAAATTTGAAATC-3′) and d123R (5′-GATTGTTCACCATCTTT) were designed according to the conserved regions deduced from an 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 Biologicas-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 used 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 Biologicas-CSIC' (Madrid, Spain).

2.6.1. GenBank accession numbers

The GenBank accession numbers of the *S. oleagina* sequences 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), *Leptosphaeria maculans* (U04238), *Westerdykella dispersa* (U42488), *Sporomia lig-*

nicola (U42478), *Dothidea insculpta* (U42474), *Aureobasidium pullulans* (M55639), *Endothia gyrosa* (L42443), *Ophiostoma stenoceras* (M85054), *Podospora anserina* (X54864), *N. crassa* (X04971), *Hypocrea lutea* (D14407), *Pseudallescheria boydii* (M89782), *C. gloeosporoides* (M55640), *Sarcinomyces petricola* (Y18702), *Exophiala calicioides* (AB007655), *Capronia mansonii* (X79318), *Phaeococcomyces 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, 'overhanging' nucleotides had to be cut off for some species. The phylogenetic tree of the alignments was done using the neighbour-joining method with 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 africanum* (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 Mamenashi 12A1-3 (Af065846), *V. pyrina* Mamenashi 12A1-1 (AF065845), *V. pyrina* strain Il-11 (AF065843), *V. inaequalis* strain 685BF (AF065837), *V. inaequalis* strain 890F (AF064836), *Venturia cerasi* (AF065851), *Cladosporium cariygenum* strain LCF (AF065851), *C. cariygenum* JD (AF065850), *Venturia carpophila* (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 *Eco*RI-A/*Mse*I-C and those for the PCR selective amplification were *Eco*RI-AGC/*Mse*I-CAC and *Eco*RI-AGC/*Mse*I-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*

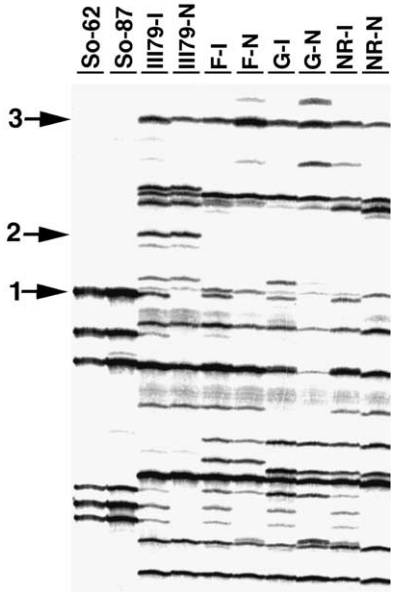

Fig. 1. Identity of *S. oleagina* in vitro culture: detail of an AFLP fingerprinting from two *S. oleagina* isolates, So-62 and So-87, four olive varieties, III-79, Frantoio (F), Giarrafa (G) and Nostrali di Rigale (NR), showing symptoms of olive leaf peacock disease (I) and symptomless (N). The arrows indicate DNA fragments of fungal origin (No. 1), olive polymorphic bands (No. 2) and olive monomorphic bands (No. 3).

Table 1
Fungal species whose 18S rDNA sequences deposited in GenBank have the highest identity to the *S. oleagina* 18S rDNA sequence

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

Classification according to Eriksson et al. [14] and the classical morphological classification are shown for every sequence.

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-

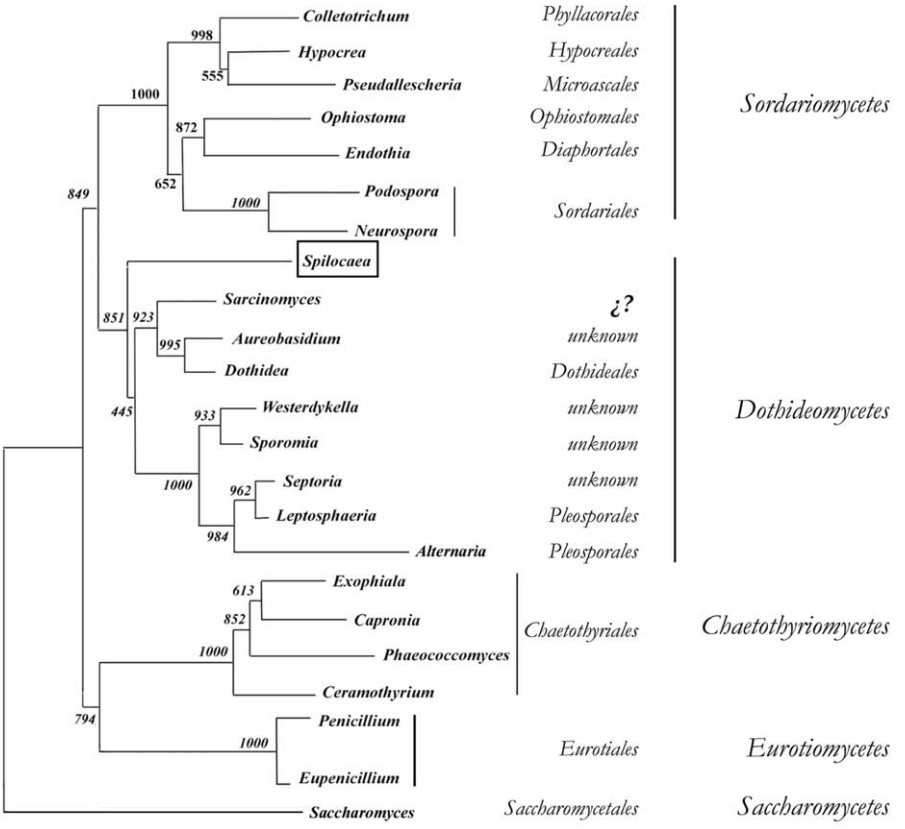

Fig. 2. Phylogenetic tree from 18S rDNA sequences: the tree was constructed with the neighbour-joining method and using *S. cerevisiae* as outgroup. Four classes of the subphylum Pezizomycotina are represented, and different orders are included for every class. Bootstrap values (1000 replicates) are indicated in the nodes.

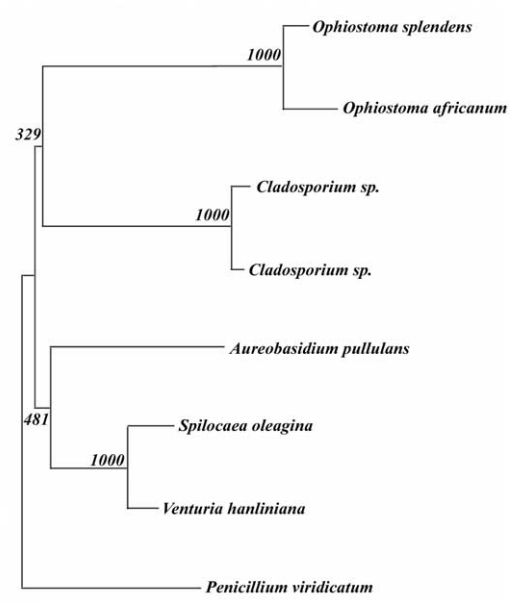

Fig. 3. Phylogenetic tree from 28S rDNA partial sequences: the tree was constructed using the neighbour-joining method. Bootstrap values (1000 replicates) are indicated above the nodes. Branch lengths are proportional to the genetic distance between the taxons.

sponds to the old class of Loculoascomycetes (Table 1). The presence of a species from the Leotiomycetes class (*B. inquinans*) is not surprising since this class is considered paraphyletic [14].

A phylogenetic tree was generated from the 18S rDNA of 23 species, including *S. oleagina* and using *S. cerevisiae* as the external group (Fig. 2), with the neighbour-joining method of the CLUSTAL X program. The sequences were selected by their presence in different classes, especially including the Dothideomycetes and the Chaetothyriomycetes. Bootstrap values of 1000 repetitions are indicated in each node. Four groups with high confidence level can be distinguished and they correspond to four ascomycete classes: Sordariomycetes with bootstrap 1000, Dothideomycetes with bootstrap 851, Chaetothyriomycetes with bootstrap 1000, and Eurotiomycetes with bootstrap 1000 (Fig. 2). *S. oleagina* is included (bootstrap 851) in a group that we have considered as Dothideomycetes since it includes three species classified as Dothideomycetes, one as Chaetothyriomycetes, and four as Dothideomycetes et Chaetothyriomycetes incertae sedis, i.e. species belonging to one of these two classes but still unclassified. *S. petricola* deserves specific attention. This species has been previously classified as Chaetothyriomycetes; however, it does not group with the other four members of this class in the present tree. The group of Chaetothyriomycetes, with a bootstrap of 1000, does not include *S. petricola* (Fig. 2). Furthermore, *S. petricola* appears among the 11 sequences with highest identity to *S. oleagina* (Table 1). Therefore we speculate that *S. petricola* must be classified as Dothideomycetes. A close analysis of the phylogeny within the Dothideomycetes class shows that *Spilocaea* does not group with species belonging to two distinct orders, Dothideales and Pleosporales, suggesting that *S. oleagina* may belong to a different order within the Dothideomycetes class not represented in the tree.

3.3. The 28S rDNA and the ITS sequences

In order to gain further insight into the classification of *S. oleagina*, a 0.7-kb fragment of 28S rDNA from *S. oleagina* was cloned and sequenced. The fragment contained the three hypervariable domains, D1, D2 and D3 [15]. The GenBank sequence with the highest identity was from *V. hanliniana* (97.8%) (Table 2). Second was a sequence from *Ochrolechia parella* (AF274097) with an identity of 89%. The identity values between two species of the same genus of either *Cladosporium*, *Ophiostoma* or *Penicillium* were very close to the value obtained between *Spilocaea* and *Venturia* (Table 2). The closest phylogeny of *S. oleagina* was also studied using the 28S rDNA partial sequence and ITS1–5.8 rDNA–ITS2 sequences. Thus, 28S rDNA sequences from several Dothideomycetes et Chaetothyriomycetes incertae sedis (*Cladosporium*, *Aureobasidium* and *Venturia*) and from Sordariomycetes (*Ophiostoma*), were selected, and *Penicillium* (Eurotiomycetes) was used as outgroup. The tree generated using the neighbour-joining method of the CLUSTAL X program is shown in Fig. 3. The species from the same genus, including *Spilocaea* and *Venturia*, are grouped with bootstrap values of 1000. The ITS1–5.8S rDNA–ITS2 sequence from *S. oleagina* was cloned, and the most homologous sequences in the GenBank were from *Venturia* species. The sequences (*S. oleagina* and different *Venturia* species) were compared by phylogeny (Fig. 4) and two *Cladosporium* sequences were used as an outgroup as before [11]. The observed distances between *S. oleagina* and the *Venturia* species analysed were in a similar range to that observed within the genus *Venturia*, indicating that the sexual phase of *S. oleagina* places this species within the *Venturia* genus.

Table 2
Percentage identity between the 28S rDNA sequences of *S. oleagina*, *V. hanliniana* and *O. parella*, separately

Accession No.	Organism	% identity
AF050264	*Cladosporium* sp.	98
AF050266	*Cladosporium* sp.	
AF221015	*O. africanum*	98.7
AF221013	*O. splendens*	
AF003359	*Penicillium expansum*	97.8
AF003358	*P. viridicatum*	
AF338397	*S. oleagina*	97.8
AF050239	*V. hanliniana*	
AF338397	*S. oleagina*	89
AF274097	*O. parella*	

Percent identity of 28S rDNA sequences between two species of the genera *Cladosporium*, *Ophiostoma*, and *Penicillium* are also shown.

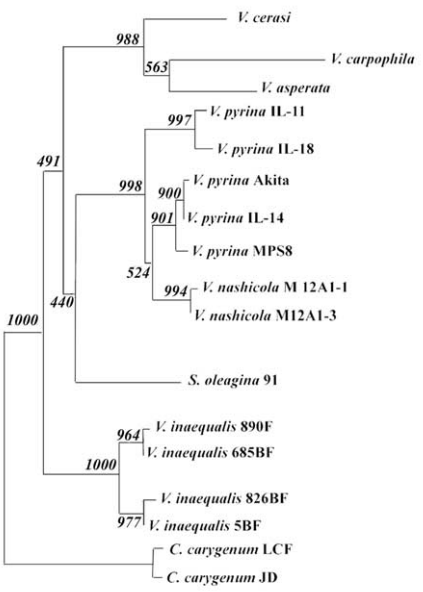

Fig. 4. Phylogenetic tree from the ITS1–5.8S rDNA–ITS2 sequences: the tree was constructed using the neighbour-joining method. Bootstrap values (1000 replicates) are indicated above the nodes. Branch lengths are proportional to the genetic distance between the taxons.

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 taxons [3]. The ascomycetes present two stages in their life cycle, sexual or telemorphic, 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 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 (Capnodiales, Myriangales 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.

Acknowledgements

This work was supported by the D6ICYT (Spain), Grant No. OLI96-2185-CO4-03. We thank Dr. Joshua Hylne for critical reading of the manuscript.

References

[1] Millers, H.N. (1949) Development of the leaf spot fungus in the olive leaf. Phytopathology 39, 403–414.
[2] Messner, R., Schweigkofler, W., Ibl, M., Berg, G. and Prillinger, H. (1996) Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18S rRNA-gene. J. Phytopathol. 144, 347–354.
[3] Taylor, J.W. (1995) Molecular phylogenetic classification of fungi. Arch. Med. Res. 26, 307–314.
[4] Varga, J., Tóth, B., Rigó, K., Téren, J., Hoekstra, R.F. and Kozakiewicz, Z. (2000) Phylogenetic analysis of *Aspergillus* section Circumdati based on sequences of the internal transcribed spacer regions and the 5.8 S rRNA gene. Fungal Genet. Biol. 30, 71–80.
[5] Bruns, T.D., White, T.J. and Taylor, J.W. (1991) Fungal molecular systematics. Annu. Rev. Ecol. Syst. 22, 525–564.
[6] Lachance, M.A., Starmer, W.T., Bowles, J.M., Phaff, H.J. and Rosa, C.A. (2000) Ribosomal DNA, species structure, and biogeography of the cactophilic yeast *Clavispora opuntiae*. Can. J. Microbiol. 46, 195–210.
[7] Lee, S.B. and Taylor, J.W. (1990) Isolation of DNA from fungal mycelia and single spores. In: PCR Protocols: a Guide to Methods and Applications, pp. 282–287. Academic Press, New York.
[8] Dunkle, L.D. and Levy, M. (2000) Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90, 486–490.
[9] Arenal, F., Platas, G., Martín, J., Salazar, O. and Peláez, F. (1999) Evaluation of different PCR-based DNA fingerprinting techniques for assessing the genetic variability of isolates of the fungus *Epicoccum nigrum*. J. Appl. Microbiol. 87, 898–906.

[10] Mueller, U.G. and Wolfenbarger, L.L. (1999) AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14, 389–394.
[11] Schnabel, G., Schnabel, E.L. and Jones, A.L. (1998) Characterization of ribosomal DNA from *Venturia inaequalis* and its phylogenetic relationship to rDNA from other tree-fruit *Venturia* species. Phytopathology 89, 100–108.
[12] Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.
[13] Angiolillo, A., Mencuccini, M. and Baldoni, L. (1999) Olive genetic diversity assessed using amplified fragment length polymorphisms. Theor. Appl. Genet. 8, 411–421.
[14] Eriksson, O.E., Baral, H.O., Currah, R.S., Hansen, K., Kurtzman, C.P., Rambold, G. and Laessoe, T. (2001) Outline of Ascomycota 2001. Myconet 7, 1–88.
[15] Voigt, K., Cigelnik, E. and O'Donnell, K. (1999) Phylogeny and PCR identification of clinically important Zygomycetes based on nuclear ribosomal-DNA sequence data. J. Clin. Microbiol. 37, 3957–3964.
[16] Trapero, A. (1994) El repilo del olivo. Agricultura 746, 788–790.
[17] Berbee, M.L. (1996) Loculoascomycete origins and evolution of filamentous ascomycete morphology based on 18S rRNA gene sequence data. Mol. Biol. Evol. 13, 462–470.
[18] Valente, P., Ramos, J.P. and Leoncini, O. (1999) Sequencing as a tool in yeast molecular taxonomy. Can. J. Microbiol. 45, 949–958.