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IDENTIFICATION OF ONE-SEPTATE *CYLINDROCLADIUM* SPECIES AFFECTING *SPATHIPHYLLUM* AND *RUMOHRA ADIANTIFORMIS* (LEATHERLEAF FERN) IN FLORIDA

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Abstract. **Single-septate conidial isolates of *Cylindrocladium* were collected from spathiphyllum and leatherleaf fern. To differentiate isolates into species *C. spathiphylli* and *C. pteridis,* isolates were compared using standard morphological characteristics, host pathogenicity, and molecular genomics. The molecular genomic comparison was completed using both random and rep-PCR primers. The isolates from leatherleaf fern produced significantly ($P = 0.05$) larger conidia (72×4.9 µm) than those produced by spathiphyllum isolates (65×5.5 µm). Vesicle shape of most leatherleaf fern isolates was clavate while those of spathiphyllum were globose. Pathogenicity on host species was examined at a standard conidial concentration. All spathiphyllum isolates produced leaf spots on leatherleaf fern similar to those produced by leatherleaf fern isolates. However, average number of lesions formed on fern fronds was 36% higher with leatherleaf fern isolates. All spathiphyllum and 13% of the leatherleaf fern isolates produced root rot on spathiphyllum. Morphological characteristics and pathogenicity are the key factors normally utilized in species identification. However, when identifying individual isolates, variation within these parameters may make it impossible to separate isolates to species level. Using multiple sets of RAPD or rep-PCR primers it is possible to separate *C. pteridis* and *C. spathiphylli* into two populations.**

Leaf spot of leatherleaf fern (*Rumohra adiantiformis* [Forst.] Ching) caused by *Cylindrocladium pteridis* Wolf was described in 1926 (Wolf, 1926). *Cylindrocladium pteridis* produces reddish brown to dark brown lesions on fronds of leatherleaf fern making infected fronds unmarketable as a cut foliage crop. Other studies have demonstrated that *C. pteridis* also attacks fern rhizomes and could be found infecting a number of other ornamental plant species in Florida (Sobers, 1968; Sobers and Alfieri, 1972).

Two other species of *Cylindrocladium, C. theae* and *C. heptaseptatum,* are occasionally found infecting leatherleaf (Uchida and Kadooka, 1997a, b). *Cylindrocladium theae* and *C. heptaseptatum* produce multiseptate conidia with an average of 3 and 7 septa, respectively (Peerally, 1991). However, *Cylindrocladium pteridis* produces single-septate conidia that easily distinguishes it from these two species, when other morphological features, such as size of conidia and vesicle shape, are also used for identification purposes.

During warm summer months root and petiole rot of spathiphyllum, caused by *Cylindrocladium spathiphylli,* can severely limit production. *Cylindrocladium spathiphylli* was originally described as *C. floridanum* (Schoulties and El-Gholl, 1980) and later given species status (Schoulties et al., 1982). *C. spathiphylli* is morphologically similar to *C. pteridis,* as it also produces single-septate conidia. Working with two *C. spathiphylli* isolates and one *C. pteridis* isolate, Schoulties and El-Gholl (1983) noted that the *C. spathiphylli* isolates produced leaf symptoms on leatherleaf fern; however, the *C. pteridis* isolate did not produce symptoms on spathiphyllum. The hosts of both these species are rarely grown by the same grower; leatherleaf fern is a field grown crop and spathiphyllum a potted greenhouse crop. However, both plants may be grown in the same area of Florida. The objective of this study was to collect single-septate *Cylindrocladium* isolates from leatherleaf fern and from spathiphyllum and to compare them using morphological characteristics, host range, and DNA analysis using PCR.

Material and Methods

Fifteen *Cylindrocladium* isolates from spathiphyllum and thirteen isolates from leatherleaf fern were collected from various locations in Florida. Single spore culture of each isolate was done to assure purity.

Comparison of morphological features. When comparing morphological features of isolates, it is important that all environmental and nutritional factors remain constant. Research has demonstrated that morphological features of *Cylindrocladium*, such as conidia size, are influenced by type of media utilized (Crous et al., 1992). In order to keep abiotic factors constant, all isolates were handled using the following procedures. First, a conidial suspension of each isolate was made in water to approximately 1×10^6 conidia/mL. A 0.5 ml aliquot of the spore suspensions were spread onto a 9 cm diam petri plates containing 20 mL of Difco Potato Dextrose Agar (PDA). Isolates were subsequently cultured for 36 h at 25 ± 1 °C under cool white fluorescent lights on a 12 h day/night cycle. Slide mounts of the conidia and vesicles were then made. Width and length of 30 conidia were measured for each isolate, and measurements were statistically compared between isolates. Shape of 30 vesicles was also recorded using vesicle the shape key of Peerally (1991). Type cultures of *C. spathiphylli* (ATCC 44730) and *C. floridanum* (ATCC 18882) were included for comparison. Two additional isolates (*C. heptaseptatum*, ATCC 38228; *C. quinqueseptatum* ATCC 16550, previously submitted to ATCC as *C. pteridis*) were utilized in the molecular comparison; however, neither strain could be induced to produce conidia.

Comparison of host range. For comparison of host range, conidia of each isolate were separately cultured, harvested, and adjusted to a concentration 1×10^6 conidia/mL water.

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For each isolate, a conidial suspension was sprayed to run-off onto all fronds of three 6 inch pots of leatherleaf fern (~50 fronds per plant). To maintain high humidity and thus aid infection, fern plants were placed into polyethylene bags for 24 h. Five-ml aliquots of the same spore suspension were also poured onto the soil surface of three 4 inch pots containing 6 inch tall spathiphyllum plants (cv. 'Petite'). After two weeks in the green house, number of lesions was counted on each leatherleaf fern up to a cut-off level of 500 lesions. Spathiphyllum root damage was evaluated after 4 weeks using a pretransformed rating scale (Little and Hills, 1978). Reisolations were made for each isolate from each host plant species to confirm pathogenicity. Noninoculated water controls were also used in each experiment. Results were statistically compared using ANOVA and Tukey's LSD.

Molecular genomics. A molecular genomic comparison of the isolates was done using two RAPD primers OPE 2 and OPE 3 (Overmeyer et al., 1996) and two rep-PCR primers BOX and REP (Louws et al. 1995; Opgenorth et al., 1996). Isolates were cultured as outlined previously, conidia harvested, and DNA extracted from conidia using FastPrep Cell Disruptor (FP101), FastDNA kit, and protocol (BIO 101, Vista, Calif.). PCR were done using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech, Piscataway, N.J) in a PTC-100 thermalcycler (M. J. Research, Inc., Watertown, Mass.). Cycling programs suggested by Overmeyer et al., (1996) were utilized for the OPE primers. A modified version of the PCR cycling profile used by Amersham Pharmacia and Rademaker and de Bruijn (1997) was used with the rep-PCR primers. The following profile was used for the Box primer: 1 cycle at 95 °C for 5 min, followed by 45 cycles of: 95 °C for 1 min; 53 °C for 1 min; 72 °C for 2 min; and hold at 4 °C when cycling is completed. For the REP primer, the above cycling profile was modified by replacing the 53 °C temperature with 40 °C. Banding patterns were recorded under UV light using a Kodak Digital Documentation System 120 (Rochester, N.Y.). Pearson Correlation and UPGMA cluster analysis were performed using the BioNumerics program ver. 1.0 (Applied Maths, Kortrijk, Belgium). All isolates with unique banding patterns were regrown, DNA extracted, and PCR redone. Using the BioNumerics program, comparmisons were made of banding patterns from RAPD alone, rep-PCR alone, and total combined patterns of all four primers. Cluster cut-off values were also calculated in each comparison. The previously mentioned ATCC isolates along with three additional *C. heptaseptatum* isolates from leatherleaf fern were used as controls.

Results and Discussion

Cylindrocladium isolates from leatherleaf fern produced conidia that were significantly longer than those produced from spathiphyllum isolates (Table 1). The conidia measurements for these two populations were within the ranges given by Peerally (1991) for *C. pteridis* and *C. spathiphylli.* In that study, length and width of conidia were 61-118 µm × 5 µm for *C. pteridis* and 45-101 µm × 5-7 µm for *C. spathiphylli.* Though conidial size and host of origin are good indicators of species identification, individual isolates may not fall within standard parameters for *C. pteridis* or *C. spathiphylli* (Table 1). Vesicle shape within populations was also variable with fern isolates having clavate to globose vesicles and with only globose vesicles being observed from spathiphyllum isolates.

Table 1. Morphological comparison of single septate *Cylindrocladium* isolates from leatherleaf fern and spathiphyllum.

Isolation Source	Conidia Size				Vesicle Shape
	Length		Width		
	Mean	Range	Mean	Range	
Leatherleaf Fern N = 13	72 µm**	48-80 µm	4.9 µm**	4.3-5 µm	Clavate to globose[z]
Spathiphyllum N = 15	65 µm**	55-72 µm	5.5 µm**	5-7 µm	globose

**Data in columns is significantly different ($P = 0.05$).
[z]Most vesicles were clavate although certain isolates also produced some globose vesicles.

All isolates from spathiphyllum produced leaf spots on leatherleaf fern similar to isolates from fern. At a standard conidial concentration, number of lesions produced by fern isolates, on fern, were more than 36% greater than those produced by spathiphyllum isolates (Table 2). When inoculated onto spathiphyllum only two of the thirteen fern isolates (92-195, 97-149) produced root rot with a mean root damage of 28% and 33%, respectively. Even though significant differences exist in disease severity between isolate groups, the variability is too great to make this trait reliable in distinguishing between species.

For decades morphological features and host specificity have been the primary mechanism utilized to distinguish between species of fungi. In the past decade, however, new techniques have been utilized for identification and comparison such as protein profiles (Crous et al., 1992, 1993; El-Gholl et al., 1992), fatty acid analysis (Müller et al., 1994; Stahl and Klug, 1996) and RFLP (Crous et al., 1995, 1997; Jeng et al., 1997; Koenig et al., 1997). In the last few years internal transcribed spacers (ITS) of rDNA regions have been used extensively in both inter- and intraspecies studies (Brown et al., 1996; Cooke and Duncan, 1997; Poupard et al., 1993; White et al., 1990). In certain species of *Cylindrocladium,* ITS regions have been useful in delineating species (Crous et al., 1999). However, low polymorphism of the ITS regions in *C. pteridis* and *C. spathiphylli* has been observed, with greater than 97% similarity in sequence (Risède and Simoneau, 2001). Differences in ITS sequence between these two species were based on rare events such as insertion/deletion or substitution of a single nucleotide. Other sequences such as intergenic spacer regions and the ß-tublin gene have proven to have higher levels of polymorphism necessary for species differentiation (Crous et al., 1999; Risède and Simoneau, 2001).

Table 2. Comparison of pathogenicity of single, septate *Cylindrocladium* isolates from leatherleaf fern and spathiphyllum on both host species.

Isolate Source	Number lesions on Leatherleaf fern		Percent spathiphyllum root damage	
	Mean	Range	Mean	Range
Leatherleaf fern N = 13	386**	110-500	4.74**	0-33
Spathiphyllum N = 15	140**	5-500	27.5**	7-91

**Significantly different in columns ($P = 0.05$).

Overmeyer et al. (1996) found that RFLP analysis and five RAPD-PCR primers were useful in finding genetic variability among *Cylindrocladium scoparium* isolates (anamorph *Calonetria*). Using two of Overmeyer's five primers (OPE 02, OPE 03), it was possible to separate most of the spathiphyllum and fern isolates into two separate groups (Fig. 1) except for two isolates (spathiphyllum, 95-246; fern, 97-143). Using these two RAPD primers, single-septate isolates from spathiphyllum and fern were distinct from other species used as controls.

Unlike RAPD-PCR, rep-PCR is designed to complement interspaced repetitive sequences. This feature allows for the amplification of unique sequences lying between these repetitive elements. Rep-PCR primers BOX and REP have been used extensively for fingerprinting genomes for bacterial taxonomy (De Bruijn, 1992; Louws et al., 1995; Opgenorth et al., 1996; Rademaker and de Bruijn, 1997). However, rep-PCR has not been extensively used for fungal taxonomy. Using two rep-PCR primers, the *Cylindrocladium* isolates from spathiphyllum and fern were separated into two populations distinct from other *Cylindrocladium* species (Fig. 2). With the rep-primers there was one outlier from spathiphyllum (98-136) which was grouped with the fern isolates. Isolate 98-136 was isolated from a spathiphyllum production facility located in Lake County, Fla. Many leatherleaf fern producers are located in this county. Isolate 98-136 had the highest percentage

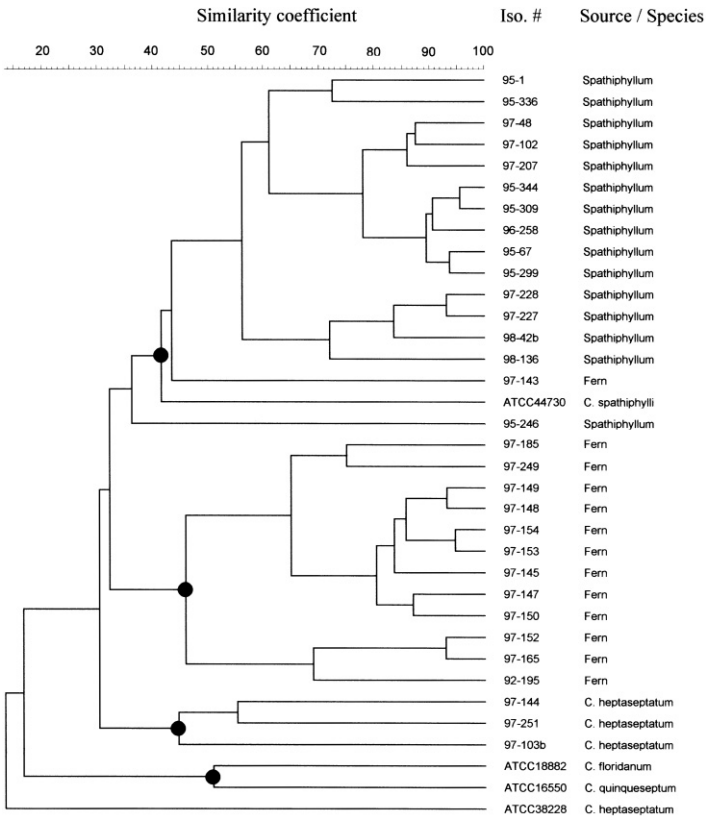

Fig. 1. Cluster analysis (UPGMA) of *Cylindrocladium* isolates from spathiphyllum and leatherleaf fern based on similarity of banding patterns produced by RAPID primers OPE 2 and OPE 3. Cluster cut-off values are indicated by black dots on dendrogram.

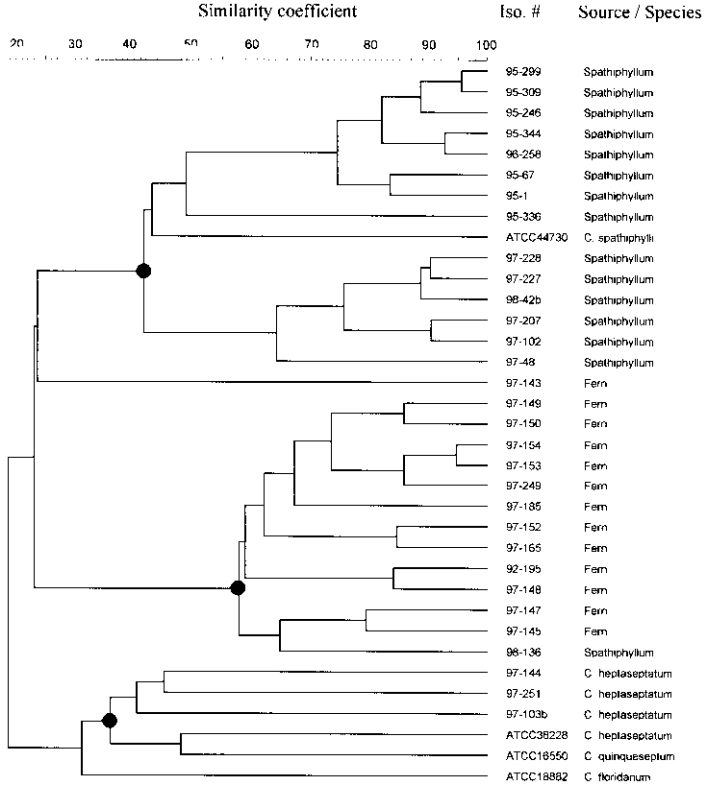

Fig. 2. Cluster analysis (UPGMA) of *Cylindrocladium* isolates from spathiphyllum and leatherleaf fern based on similarity of banding patterns produced by BOX and REP primers. Cluster cut-off values are indicated by black dots on dendrogram.

of root damage observed in this study (91%) and a moderately low mean of 68 lesions per fern plant. This would indicate that this isolate is most likely *C. spathiphylli* and not *C. pteridis*. Isolate 97-143 from leatherleaf fern had the lowest mean lesion count on fern observed in this study and was not pathogenic on Spathiphyllum, and thus may be a true outlier in *C. pteridis* species.

In Fig. 3 when all banding patterns from all the four primers are analyzed together no significant changes occur in genetic position of spathiphyllum and fern isolates from those produced only with the rep-PCR primers. However, *C. floridanum* and *C. quinqueseptatum* control cultures are now separated into a distinct cluster separate from other species and isolates.

It is not always reliable to use host of origin, level of virulence, or morphological features to distinguish isolates of *C. pteridis* from *C. spathiphylli*. Individuals within a species vary around mean morphological measurements used to describe the species, or may possess morphological features that closely resemble those of other species. In addition, certain isolates may have the ability to infect other traditional nonhosts if grown in proximity to a known host. Therefore, to compare isolates for taxonomic and epidemiological interests new molecular techniques are needed to separate populations. Genomic fingerprints produced by RAPD or rep-PCR were found to be useful in this study to distinguish these two species. As genomic comparisons become easier, a larger number of isolates should be compared from diverse geographic locations where these *Cylindrocladium* species can be found.

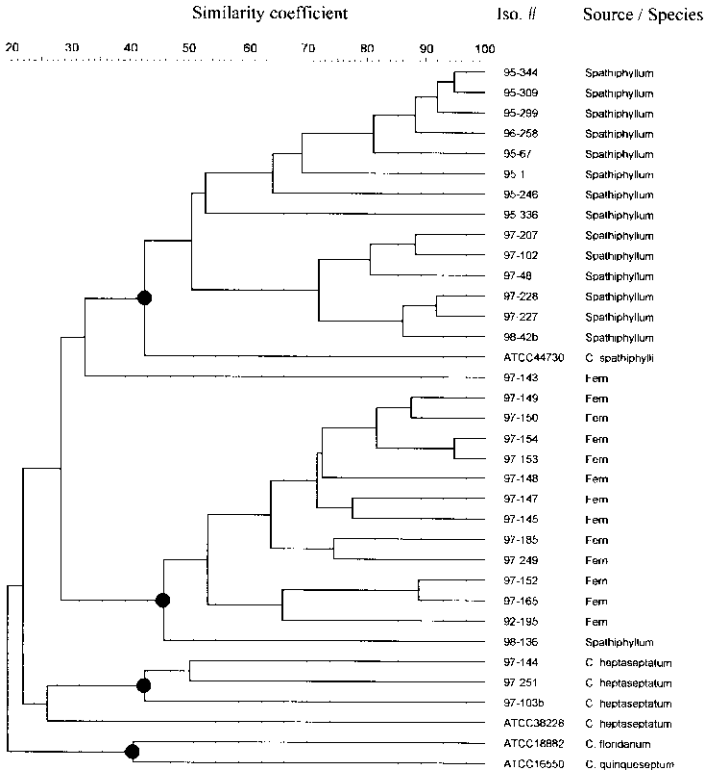

Fig. 3. Combined cluster analysis (UPGMA) of *Cylindrocladium* isolates from spathiphyllum and leatherleaf fern based on similarity of banding patterns produced by primers OPE 2, OPE 3, BOX and REP. Cluster cut-off values are indicated by black dots on dendrogram.

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