SSU rDNA sequence support for a close relationship between the Elaphomycetales and the Eurotiales and Onygenales

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Based on parsimony analyses of eight new SSU rDNA sequences and 24 homologous sequences retrieved from the DNA databases, we suggest a possible phylogenetic relationship of Elaphomycetales with Eurotiales and Onygenales. Our three included Elaphomyces sequences strongly cluster together (bootstrap value 100%) within a monophyletic group (100%) of Elaphomycetales, Eurotiales, and Onygenales. Earlier reports that another cleistothecial lineage (Erysipe) is related to Leotiales, are supported by our discovery that also another cleistothecial species, Amylocarpus encephaloides, shows affinity to Leotiales. Ascosphaeraceae and Eremascales are possibly better accommodated in Onygenales. We describe a new DNA extraction method in which sonication is used to disrupt thick-walled spores. It is useful for both fresh and dried fungal material.

Key Words—cleistothecial ascomycetes; DNA extraction; Elaphomycetales; Plectomycetes; SSU rDNA phylogeny.

Species of the genus Elaphomyces Nees have hypogeous globose fruit-bodies, about 0.5-4 cm wide. When mature, they are filled with small, scattered, globose ascii containing thick-walled, ornamented spores. The genus is cosmopolitan and possibly of large economic importance, as its species form mycorrhiza with trees. Morphological studies of Elaphomyces have not given any definite indications of its phylogenetic relationships within Ascomycota. There is a superficial resemblance of Elaphomyces fruit-bodies to those of truffles. Because of this, the Elaphomycetaceae have been referred to the Tuberales (Paoletti in Saccardo, 1889; Martin in Ainsworth, 1961). Other authors have agreed with Dodge (1929) who transferred the Elaphomycetaceae to the Plectomycetes ("Plectascales") due to similar internal morphological characters of the ascomata. Korf (1973) chose to include the Elaphomycetaceae together with the other hypogeous ascomycetes in the Tuberales for practical reasons, but stated that "their true relationship may possibly be with the Eurotiales". Trappe (1979) transferred most of the hypogeous species from "Tuberales" to Pezizales. He saw, however, no close relationship of the Elaphomycetaceae with this or any other order. Therefore, he established a separate order, Elaphomycetales, for the family.

The "Plectomycetes" has traditionally included taxa possessing a cleistothecial type of ascoma with a centrum containing more or less scattered ascii. Such ascomata are, for instance, found in the orders Eurotiales, Onygenales, and Elaphomycetales. Cladistic analyses of Small Subunit ribosomal DNA sequences (SSU rDNA) have indicated that the cleistothecial type of fruit-body has evolved independently in different lineages. Saenz et al. (1994) presented a cladogram in which Blumeria graminis (DC.) Speer (Erysipehes: cleistothecia with one layer of elongated ascii) appeared as a sister group to an apothecium-producing ascomyctete, Sclerotinia sclerotiorum (Lib.) de Bary (Leotiales). Also the order Ophiostomatales has been referred to the Plectomycetes (Nannfeldt, 1932; Luttrell, 1951; Benny and Kimbrough, 1980) due to scattered ascii, but this has been rejected (Berbee and Taylor, 1992), and the group has been shown to have a polyphyletic origin with relationships with other perithecial ascomycetes (Spatafora and Blackwell, 1994). Further, SSU rDNA analyses have shown that Eurotiales and Onygenales are closely related and together form a sister group to the untexticate pyrenomycetes (Berbee and Taylor, 1992).

In a paper presented at the First International Workshop on Ascomycete Systematics in Paris 1993 (Hawksworth, 1994), SSU rDNA sequences of Elaphomyces were preliminarily reported to cluster with homologous sequences of Pezizales (Landvik and Eriksson, 1994a), and, therefore, did not appear to be closely related to any other group of cleistothecial ascomycetes. The conclusions were, however, most probably based on a PCR contamination, as described in a later paper (Landvik and Eriksson, 1994b). The present paper presents new molecular evidence for the relationships of Elaphomyces to the other cleistothecial and non-cleistothecial ascomycetes. We report three new SSU rDNA Elaphomyces sequences. Further sequences from other ascomycetes reinforced information about the relationships of Onygena equina (Willd.) Pers. (the type species...
of Onygenales), Amylocarpus encephaloides Cury (a cleistothecial ascomycete of uncertain relationships), and Microglossum viride (Pers.) Gillet, cf. Neobulgaria premnophila Roll-Hansen & H. Roll-Hansen, and Neobulgaria pura (Fr.) Petrak var. foliacea (Bres.) Dennis & Gammundl (three members of Leotiales).

Materials and Methods

DNA extraction Extraction and PCR-amplification of fungal DNA is sometimes problematic. Many of the problems are caused by polymerase-inhibitory polysaccharides, which occur in large amounts especially in lichenized fungi. Efforts to remove the polysaccharides often also result in a loss of DNA, because of the similar chemical properties of the macromolecules. In the case of herbarium material, other problems arise. Inappropriate desiccation or storage of the collections may cause degradation of the DNA, or the DNA from the desiccated material does not get into the solution. In collections of fully mature Elaphomyces, the spore-mass is the most suitable part for the extraction. The spores, however, are extremely tough and thick-walled, and do not easily disrupt by standard lysis buffers or by mechanical grinding. Gang and Weber (1995) described a method for extraction of DNA from thick-walled teleospores suitable for RAPD analyses. For PCR reactions, however, the demands for highly purified genomic DNA is lower, and a simpler extraction method is preferred.

Extraction of DNA from thick-walled spores is often successful when these are first disrupted by ultrasonic waves (sonication) prior to further processing. With sonication, the buffer soaking the spores should not contain any detergents (the ultrasonic waves are impeded by the foam that would be formed).

Our DNA-extraction method is based on Boom et al. (1990), but differs in the inclusion of a sonication step, the addition of Nal in the DNA binding step, and in modifications in experimental procedures, see below. The L6-buffer and the Silica coarse (Silicon dioxide, Sigma Chemical Co.) are prepared as described in that paper, but with the exclusion of triton-x from the L6-buffer. A similar method, but with no sonication step, has been described for isolating DNA from lichen ascomata (Grube et al., 1995).

DNA extraction from thick-walled spores 1) In a 1.5 ml eppendorf tube, fill to less than 3 mm depth with spore mass. Add 200 µl of L6-buffer (without triton-x), mix. Disrupt the spore walls by sonication (Branson Sonifier B15, output 7, duty cycles 50%) for up to 1 min, or until the solution appears somewhat granular and viscous (the amount of disruption can be observed microscopically). 2) Add 3 µl of triton-x to each tube and incubate the samples at 56°C for half an hour. 3) Centrifuge the tube for 3 min at 14,000 rpm to pellet the cell-debris. Transfer the supernatant to a new tube. 4) To the supernatant, add at least twice the volume of the supernatant of 8.2 M Nal and 20 µl of silica to bind the DNA for 5 min at room temperature, or, up to 56°C (which may increase the yield of bound DNA even further (Smith et al., 1995)). 5) Pellet the silica by momentary centrifugation, and wash the pellet three times with 70% ethanol. 6) After the last wash, allow the pellet to dry before eluting the DNA at 56°C for 5 min in 50 µl of 1 M TE-buffer. Pellet the silica again and transfer the DNA-containing supernatant to a new tube.

The same protocol can be used also for other fresh or dried fungal material. The sonification step can then be excluded, and the triton-x can be added directly to the L6-buffer. Fresh material should be incubated in the L6-buffer at 56°C for 5 min. Desiccated material should be incubated in the buffer for at least half an hour prior to further extraction procedures.

The reported DNA extraction method is potentially widely applicable. Because it does not involve any ethanol precipitation steps, and because of the high DNA-binding capacity of the silica, minute amounts of fungal material can be successfully extracted. On two occasions, DNA from a single fruit-body of Amylocarpus encephaloides (less than 2 mm wide in diam) was successfully extracted and sequenced in this study. It was possible to extract DNA from a spore print on a cover glass from the thick-walled ocellate species Ascodesmis sphaerospora Obrist. The obtained Ascodesmis sequence was compared and found to be identical to a sequence from a cultivated Ascodesmis sample provided by K. Egger, Prince George, Canada.

PCR, sequencing and phylogenetic analyses 1–10 µl of the extracted DNA solution and the flanking primers SL 1 5'TGGTTGTATCTCGCCAGTATA and NS 8 5'TCCCGAGTTTCACCTACGGA (White et al., 1990) were used for the PCR amplification, following standard PCR procedures. As described in earlier papers (Landvik et al., 1993; Landvik and Eriksson, 1994b, with the additions of the new primers SL 122 5'AGGCAGGAATTACCCAAT, SL 334 5'GAATAGGACGTGTGGTTCTA and SL 344 5'GTCGCAAGGCGCTAACTTA), the samples were manually cycle sequenced and aligned. The matrix, excluding the gaps, was analysed by the PAUP 3.0.s. package (Swofford, 1991). The heuristic option, using ten random taxon addition replicates, were utilized. The resulting tree was tested by a bootstrap analysis based on 1,000 replicates. Neoleptia vitellina (Bres.) Korf & J. K. Rogers and N. irregularis (Peck) Korf & J. K. Rogers were used as outgroups based on the results presented in Landvik et al. (1993) and Landvik (1996).