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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 (*Erysiphe*) is related to Leotiales, are supported by our discovery that also another cleistothecial species, *Amylocarpus encephaloides*, shows affinity to Leotiales. Ascosphaeraceae and Eremascaceae 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 asci containing thick-walled, ornamented spores. The genus is cosmopolitian 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 asci. 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 (Erysiphales: cleistothecia with one layer of elongated asci) appeared as a sister group to an apothecium-producing ascomycete, *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 asci, 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 unitunicate 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* Currey (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 & Gamundí (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 teliospores 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 NaI 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 NaI 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. 8) 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 operculate 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'TGGTTGATCCTGCCAGTA and **NS 8** 5' TCCGCAGGTTCACCTACGGA (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'AGGCGCGCAAATTACCCAAT, **SL 334** 5'GAATAGGACGTGTGGTTCTA and **SL 344** 5' GGTCGCAAGGCTGAAACTTA), 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. *Neolecta 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).

Collections included in this study *Elaphomyces aculeatus* Vittad., Sweden, Sollentuna par., Järvafältet, 30. vii. 1982, leg. J. Nitare, **UME** 30049 (GenBank/EMBL Data Library accession number U45439); *E. leveillei* Tul., Sweden, Åre par., Storlien, 20. viii. 1983, leg. J. Nitare, **UME** 30086 (acc. nr. U45441); *E. maculatus* Vittad., Spånga par., Hansta, 24. ix. 1995, leg. L-E Kers, **UME** 31162 (acc. nr. U45440); *Onygena equina*, Sweden, Bergsjö par., Åkern, 5. xii. 1991, leg. S. Landvik, **UME** 29222 (acc. nr. U45442); *Amylocarpus encephaloides*, Sweden, Umeå par., near Holmön, alt. c. −15 m, 5. ix. 1993, leg. M. and L. Eriksson, **UME** 29765 (acc. nr. U45438); *Microglossum viride*, Sweden, Vännäs par., Harrsele, 1. ix. 1994, leg. S. Landvik, **UME** 30190 (acc.

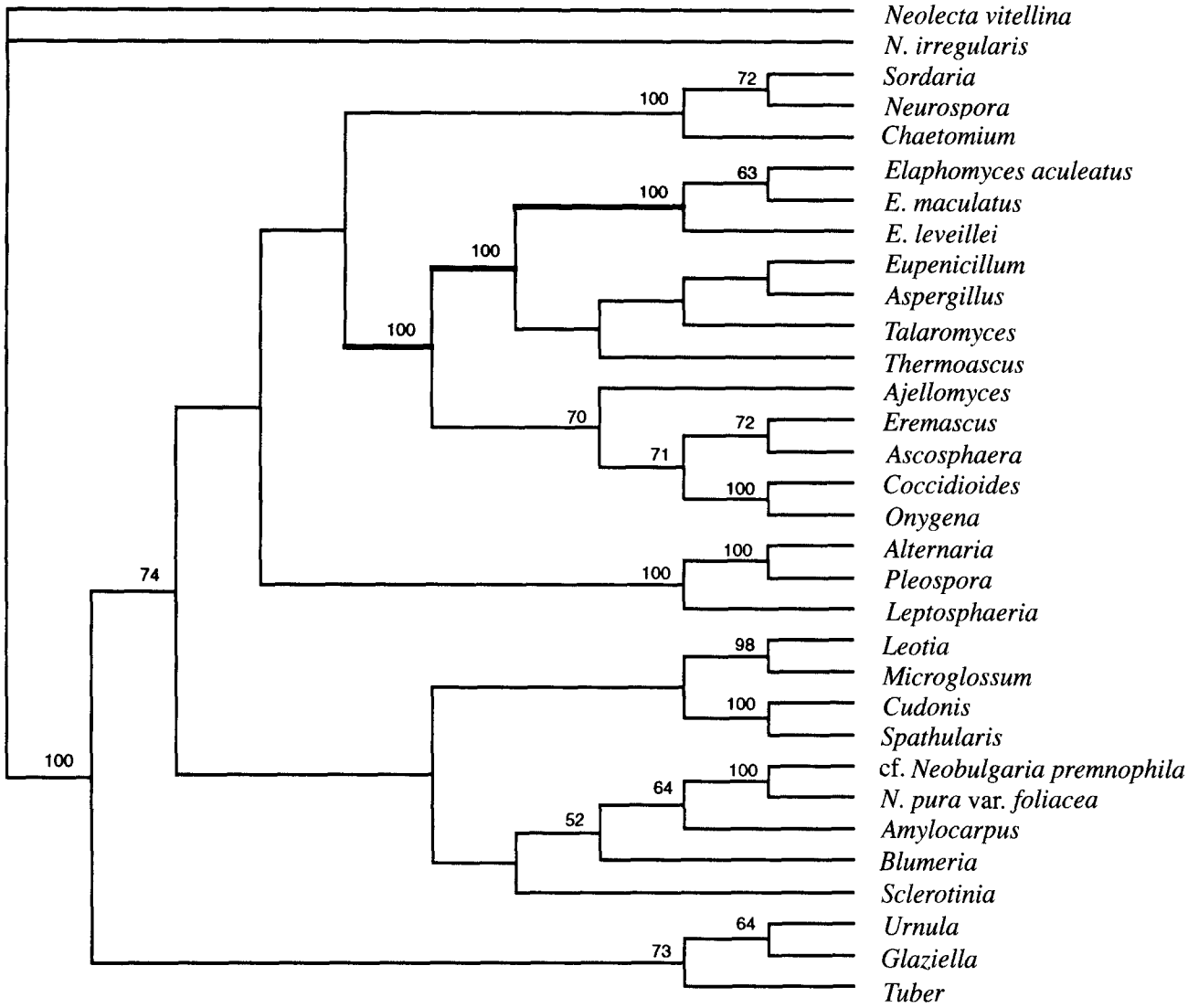

Fig. 1. A phylogram of the single most parsimonious tree from ten heuristic analyses of 388 variable characters among 32 SSU rDNA sequences (tree length=885, consistency index=0.58, retension index=0.77). Bootstrap values (1,000 replications) higher that 50% are shown above the branches. The three most important branches for this report are in bold face, and are all supported by a high bootstrap value (100%). These branches show that (1) the three *Elaphomyces* sequences reported in this paper cluster as one clade, (2) the *Elaphomyces* clade constitute a sister group to Eurotiales, and (3) Elaphomycetales, Eurotiales and Onygenales together form a monophyletic group. Cleistothecial types of fruit-bodies are also found among Leotiales, viz. in *Blumeria* and *Amylocarpus*.

nr. U46031); cf. *Neobulgaria premnophila*, Sweden, Bergsjö par., Åkern. 27. iix. 1995, leg. S. Landvik, **UME** 31148 (acc. nr. U45445); *Neobulgaria pura*, Sweden, Umeå, Klabböle, 13.x. 1990, O. E. Eriksson, **UME** 30277 (acc. nr. U45443 and U45444, partial SSU rDNA sequences).

The new SSU rDNA sequences were manually aligned with the following homologous sequences retrieved from GenBank/EMBL Data Library (with accession numbers): *Neolecta irregularis* (Z47721); *Neolecta vitellina* (Z27408); *Ajellomyces dermatitidis* McDonough & Lewis (X59420); *Alternaria brassicicola* (Schwein.) Wiltshire (U05197); *Ascosphaera apis* (Maasen ex Claussen) Olive & Spiltoir (X69849); *Aspergillus fumigatus* Fresen. (M60300); *Blumeria graminis* f. sp. *hordei* (L26253); *Chaetomium elatum* Kunze (M83257); *Coccidioides immitis* Rixford & Gilchrist (X58571); *Cudonia confusa* Bres. (Z30240); *Eremascus albus* Eidam (M83258); *Eupenicillium javanicum* (J. F. H. van Beyma) Stolk & D. B. Scott (U21298); *Glaziella aurantiaca* (Berk. & Curtis) Cooke (Z49753); *Leotia lubrica* Pers. (L37536); *Leptosphaeria doliolum* (Pers.) Ces. & De Not. (V04205); *Neurospora crassa* Shear & B. O. Dodge (M11033); *Pleospora herbarum* (Pers.) Rabenh. (V05201); *Sclerotinia sclerotiorum* (L37541); *Sordaria fimicola* Ces. & De Not. (X69851); *Spathularia flavida* Pers. (Z30239); *Talaromyces flavus* (Klöcker) Stolk & R. A. Samson (M83262); *Thermoascus crustaceus* (Apinis & Chester) Stolk (M83263); *Tuber* cf. *rapaeodorum* Tul. (Z49755); *Urnula hiemalis* Nannf. (Z49754).

Results and Discussion

Ten heurisic searches (with taxa added in a random order), based on 388 informative characters from 32 aligned SSU rDNA sequences (1828 basepairs), all resulted in one and the same single most parsimonious tree (tree length 885, consistency index=0.58, retension index=0.77, Fig. 1). The monophyletic clades (bootstrap values in parenthesis) correspond to higher groups as follows: (1) unitunicate pyrenomycetes (100%, represented by Sordariales), (2) plectomycetes (100%, Eurotiales, Elaphomycetales and Onygenales), (3) bitunicate pyrenomycetes (100%, Dothideales), (4) inoperculate discomycetes (<50%, Leotiales, *Blumeria* and *Amylocarpus*), and (5) operculate discomycetes (73%, Pezizales). An enforced constraint heuristic analysis, excluding *Elaphomyces* from Eurotiales and Onygenales, resulted in a cladogram 13 steps longer than the presented most parsimonious tree.

All new SSU rDNA sequences reported in this study cluster with either the "Plectomycetes" or the Leotiales. These groups are discussed below, with comments on the genera represented by new sequences in this study.

The "Plectomycetes" The three *Elaphomyces* species included in this study cluster as a well supported clade (100%). Together with species from the Trichocomaceae (Eurotiales), this group constitutes a sister taxon (100%) to the Onygenales and two other Eurotialean families: Eremascaceae and Ascosphaeraceae. Eurotiales as currently classified (Eriksson and Hawksworth, 1993) thus appears to be paraphyletic. However, there is a strong bootstrap support for the monophyly of a higher taxon (100%) consisting of Eurotiales, Elaphomycetales and Onygenales.

In the seventh edition of the Dictionary of the Fungi (Hawksworth et al., 1983), no taxa above the ordinal level were used for ascomycetes. Based on SSU rDNA sequence data, Berbee and Taylor (1992) proposed that the former classes Plectomycetes and Pyrenomycetes should be accepted in the classification of the ascomycetes. However, only a small number of species was included in that study, none of them being the type species of the orders of the Plectomycetes (as defined in the sixth edition of the Dictionary of the Fungi; Ainsworth et al., 1971), which currently are classified as Eurotiales, Onygenales, Erysiphales and Meliolales (Eriksson and Hawksworth, 1993; Hawksworth et al., 1995). Analyses by e.g. Wilmotte et al. (1993) of sequences from a larger set of taxa, and our analyses which include also sequences from *Onygena equina* and the *Elaphomyces* species, give further support for grouping certain cleistothecial taxa corresponding to the Plectomycetes sensu Berbee and Taylor (1992). However, ranking this group at the level of class is inconsistent with the proposal by Nishida and Sugiyama (1994) who divided the ascomycetes into the three classes Archiascomycetes, Hemiascomycetes and Euascomycetes. Further, the name Plectomycetes is not based on a generic type and may be interpreted differently by different authors. Thus, we need a discussion regarding the name, as well as the ranking, of the Elaphomycetales/Eurotiales/Onygenales group.

The large cleistothecial fruitbodies are not unique to *Elaphomyces* in this group. *Onygena equina* has stalked ascomata up to 1.5 cm long and *Shanorella spirotricha* R. K. Benj. (Onygenales) has up to three cm wide conglomerates of ascomata and vegetative hyphae (Currah, 1985). On the other hand, the thick and hard ascomal walls of the genus are not found elsewhere in the group, and this has been the main reason for some authors not to place it among the plectomycetes. The mycorrhizal life style of the genus is also remarkable, with no analogues within Eurotiales. The same refers to Onygenales, where parasitism on plants and animals and keratin saprophytism are the predominant life styles. *Onygena equina*, being found on hooves and horns of dead ungulates, well represents the keratinophilous life-style of most Onygenales. Currah (1985) suggested that it is disseminated by insects, as the fungus produces an odour that may attract insects which can carry spores between the decaying animals. He also pointed out that a reliance on animals for dispersal of the fungi may be found in all four families of Onygenales sensu Currah (1985). *Elaphomyces* are also dispersed by animals.

Leotiales As mentioned above, Saenz et al. (1994) reported the cluster of the cleistothecial *B. graminis* (Erysiphales), with species of apothecial Leotiales. In this paper, we report sequences from another species with cleistothecia, *A. encephaloides*, that also is nested within Leotiales, in our analyses. The up to 3 mm wide subglobose fruit-bodies of the monotypic genus *Amylocarpus* are solitary or gregarious on marine wood, possibly preferring "reduced saline conditions" (E. B. G. Jones in Crumlish and Curran, 1994). Kohlmeyer and Kohlmeyer (1979) referred this monotypic genus to the Eurotiaceae (i.e. Trichocomaceae). The ascomata are superficially similar to those typical of that family, but a deviating morphological feature is the short stalk of the ascus. This character is also found in *Blumeria* species, while the "Plectomycetes" possess globose asci. *Amylocarpus encephaloides*, together with *B. graminis*, clusters in a clade with the Leotialean genus *Neobulgaria*. The grouping of these species may change when more related taxa are incorporated in analyses, as long branches often may lead to unreliable results. Our reported sequences from *Neobulgaria*, *N. pura* var. *foliacea* and from material that most probably represents *N. premnophila*, differed by only 2 nucleotides in 1224 positions compared. Thus, they strongly support each other even though the sequence from the former species is not complete. The molecular data do not indicate that they are close to *Leotia*, Leotiaceae, the family in which they are currently included.

Pezizales, Rhytismatales and Cyttariales The present study shows that Elaphomycetales is not closely related to Pezizales, contrary to the results of earlier preliminary analyses (Landvik and Eriksson, 1994a). Molecular studies of Rhytismatales (*Rhytisma salicinum* (Pers.) Fr., *Colpoma quercinum* (Pers.) Wallroth and *Lophodermium pinastri* (Schrad.) Chev. and Cyttariales (*Cyttaria darwinii*

Berk. and *C. hariotii* Fisher) also exclude any close connections between these apothecioid orders with Elaphomycetales. Preliminary analyses group these species with Leotiales (data not shown).

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