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RELATIONSHIPS OF THE GENUS NEOLECTA (NEOLECTALES ORDO NOV., ASCOMYCOTINA), INFERRED FROM 18S rDNA SEQUENCES

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Abstract

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Partial 18S rDNA sequences from *Neolecta vitellina* were compared with corresponding sequences from three species of *Leotiales*, four of Pezizales (*Ascomycotina*), and three species tested as outgroups *Neurospora crassa* (*Sordariales*), and *Saccharomyces cerevisiae* (*Saccharomycetales*) and *Schizosaccharomyces pombe* (*Schizosaccharomycetales*). With *Saccharomyces* and *Schizosaccharomyces* as outgroups, *Neurospora* becomes an ingroup among the discomycete taxa used in this cladistic analysis. The analysis, with a statement for its decay, indicated that the leotialean and pezizalean taxa are separate groups, except for the genus *Peziza*, which was unresolved, and that *Neolecta* (*Neolectaceae*) branched off earlier than the other filamentous ascomycetes. Both morphological and molecular data support *Neolecta* being accommodated in a separate order, *Neolectales* Landvik et al., ordo nov. *Neolecta* may be a good outgroup for further phylogenetic studies of "higher" ascomycetes.

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Introduction

A majority of the non-lichenised discomycetes belong to the orders *Leotiales* ("inoperculate discomycetes") and *Pezizales* ("operculate discomycetes"). Most of these have more or less cup-shaped ascomata (apothecia) with a hymenium of paraphyses and spore-producing asci. Even "discomycetes" lacking such fruiting bodies have paraphyses, e.g. *Ascocorticium* Schröter (Jülich & de Vries 1982: 408) and *Karstenella* Harmaja (1969: 20). The only exceptions among taxa with ascomata are truffles (*Pezizales*) and two other unrelated genera, *Aparaphysaria* Speg. and *Neolecta* Speg., with cup-shaped and club-shaped ascomata, respectively. The lack of sterile elements (hamathecium) in the hymenium is a remarkable feature and there are probably fundamental ontogenetic differences between *Aparaphysaria* and *Neolecta* and other discomycetes. Therefore, the position of these two genera is difficult to determine on morphological data only. Korf (1990: 21) proposed that molecular methods might resolve problems of this kind.

We have sequenced the 1723 bp of the 18S rRNA gene from a *Neolecta* species and compared it with homologous sequences from some members of the three orders to which the genus has been assigned, viz. *Leotiales* (e.g. Korf 1973: 285), *Lecanorales* (Redhead 1977: 301), and *Pezizales* (e.g. Eriksson 1981: 188, 1982: 209). The included species are listed in Table 1.

The genus *Neolecta* was described for a species from South America by Spegazzini in 1881, but currently includes two additional species from the Northern Hemisphere (Redhead 1977). One of them, *Neolecta vitellina* (Bres.)

Discomycetes analysed
- Leotiales
  - *Leotia lubrica*, Leotiaceae
  - *Cudonia confusa*, Geoglossaceae?
  - *Spathularia flavida*, Geoglossaceae?
- Pezizales
  - *Peziza badia*, Pezizaceae
  - *Gyromitra esculenta*, Helvellaceae
  - *Inermisia aggregata*, Humariaceae
  - *Plectania nigrella*, Sarcosomataceae
- Uncertain position
  - *Neolecta vitellina*, Neolectaceae

Outgroups tested
- Sordariales
  - *Neurospora crassa*, Sordariaceae
- Saccharomycetales
  - *Saccharomyces cerevisiae*, Saccharomycetaceae
- Schizosaccharomycetales
  - *Schizosaccharomyces pombe*, Schizosaccharomycetaceae

Table 1. Species included in the study. Taxonomy according to Eriksson & Hawksworth 1991 and as modified by Eriksson et al. 1993.

Korf & Rogers, occurs in Sweden and has been used in this study. Its fruiting bodies are bright yellow, clavate and stipitate, and up to c. 3,5 cm in length. These fruiting bodies resemble basidiomata of some *Clavulinopsis* species, e.g. *C. helvola* (Fr.) Corner, but the hymenium asci instead of basidia. The ascogenous hyphae lack croziers and there is no tissue to produce paraphyses. The terminal parts of the hyphae form palm-like cells, from each of which develop finger-like cells ending in a new palm-like cell, which produce "fingers" that develop into asci. The asci are thin-walled, usually distinctly truncate at the apex, and open by a pore. The wall is KOH/I+ blue. The uniseriate ascospores are non-septate and hyaline.

The order *Leotiales* contains 11 families (Eriksson & Hawksworth 1991: 46). Most of the members have small cup-shaped ascomata, but three species resembl*e* *Neolecta* in having large, stiped ascomata, were included in this study. *Leotia lubrica* Pers. and *Cudonia confusa* Bres. have ascomata that are elastic when wet and a somewhat slimy surface. *Spathularia flavida* Pers.: Fr. has pale yellow, dryer and more fragile ascomata when wet, [illegible] as *Neolecta*. but differ in being flattened and spade-like. All three species, in contrast to *Neolecta*, have numerous paraphyses in their hymenia. Their ascogenous hyphae are more slender and have croziers. The asci are not truncate and do not stain KOH/I+ blue, but they open by a pore as in *Neolecta*. The spores are longer and have several septa. Thus, there are many morphological differences between the three genera and *Neolecta*.

Eriksson (1981: 188, 1982: 209) tentatively assigned *Neolecta* to *Pezizales* on the basis of the shape and structure of the ascus tip, and the anatomy and texture of the ascogenous hyphae. In the most recent outline of the ascomycetes, *Neolectaceae* was one of 18 families referred to the order, although with a "?" (Eriksson & Hawksworth 1991: 48). Typical members of *Pezizales* have comparatively large fruiting bodies and usually operculate asci (opening with a lid), but the order also includes some ascomycetes with quite different morphology, e.g. the true truffles. The spores are often hyaline and non-septate, very similar to those of *Neolecta*. The four species included in this study represent different families (Table 1). *Gyromitra esculenta* (Pers.) Fr. is a morel with stalked ascomata, *Inermisia aggregata* (Berk. & Broome) Svrcek has orange, small, densely aggregated, disc-like ascomata, *Peziza badia* Pers. has brown ascomata, large, sessile, cup-shaped, and, finally, *Plectania nigrella* (Pers.: Fr.) P. Karst. has black, large, stalked, cup-shaped ascomata. For more detailed morphological descriptions of the studied taxa, see Dennis (1978).

Redhead (1977) placed *Neolecta* in *Lecanorales*, in a new family, *Neolectaceae* Redh. However, the asci, hymenium and anatomy of the ascomata are very different from all other members of *Lecanorales*, most of which are lichens. We have not included any member of *Lecanorales* in our cladograms, as it was quite clear from previous analyses (Gargas 1992), that it would have appeared as an ingroup, close to the inoperculate discomycetes in our cladogram (see Results and Discussion).

Materials and methods

Materials. DNA was extracted and further processed by S. Landvik (except *Leotia* and *Peziza* by A. Gargas) from the following collections: *Cudonia confusa* Brcs., Sweden, Västerbotten, Umeå, Klabböle, 16.viii.1990, leg. S. Landvik & O.E. Eriksson (UME 29217). *Gyromitra esculenta* (Pers.) Fr., Sweden, Västerbotten, Tegsnäset 4.vi.1991, leg. N. Högberg (UME 29221). *Inermisia aggregata* (Berk. & Broome) Svrcek, Sweden, Västerbotten, Umeå, Ökberget, 7.vi.1991, leg. S. Landvik & O.E. Eriksson (UME 29218). *Leotia lubrica* Pers., Sweden, Västerbotten, Umeå, Brännland, 15.ix.1990, leg. S. Landvik & O.E. Eriksson (UME 29199). *Neolecta vitellina* (Brcs.) Korf & J.K. Rogers, Sweden, Västerbotten, Vännäs, Harrsele, 16.viii.1990, leg. O.E. Eriksson (UME 29192). *Neolecta vitellina*, Sweden, Västerbotten, Vännäs, Harrsele, 28.viii.1991, leg. O.E. Eriksson (UME 29232), only partially sequenced. *Peziza badia* Pers., Sweden, Västerbotten, Umeå, Mjösjön, W side. 14.x.1967, leg. B. Eriksson (UME 26389). *Plectania nigrella* (Pers.: Fr.) P. Karst., Sweden, Västerbotten, Umeå, forest at the University Campus 3.vi.1991, leg. S. Landvik (UME 29220). *Spathularia flavida* Pers., Sweden, Västerbotten, Vännäs, Harrsele 16.viii.1990, leg. S. Landvik & O.E. Eriksson (UME 29216).

Neurospora crassa Shear & B.O. Dodge (*Sordariales*), *Saccharomyces cerevisiae* Hansen (*Saccharomycetales*), and *Schizosaccharomyces pombe* Lindner (*Schizosaccharomycetales*) were tested as outgroups. These sequences were retrieved from the EMBL DNA Sequences Data Library.

DNA extraction. DNA was extracted as described by Lee *et al.* (1988) with some modifications. Less than 50 mg of dry weight material, or 150 mg of fresh material in eppendorf tubes was submerged in liquid nitrogen for 1 min. Dry material was first rehydrated in 400 µl of TE-buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) for 1 hour. The frozen tissue was ground with the aid of a pellet mixer (Kontes Glass Comp., U.S.A.). The samples were incubated at 65°C for 1 hour in 750 µl lysis buffer (50 mM Tris-HCl, 50mM EDTA, 3% 2-mercaptoethanol). After extraction with chloroform: phenol (1:1, 700 µl) and chloroform:isoamyl alcohol (24:1, 700 µl), the DNA was precipitated once with 80 µl of 4M $NaNH_4$ and 700 µl of isopropanol for 10 min or at -20°C. The pellet was resuspended in 100 µl of TE (0.1-10 ng DNA/100 µl). It was usually necessary to dilute the sample 100-fold before PCR-processing.

Amplification and sequencing The DNA was sequenced in either of the following two ways:

1) *Direct sequencing of asymmetric PCR-products*. Single-stranded fragments of 18S rDNA were obtained by asymmetric PCR (Gyllensten & Erlich 1988) using the following primer pairs: NS1/2, NS2/1, NS6/5 and NS8/7 (primers described in White *et al.* 1990) in ratios of 50:4 pmol for each pair. 1-50 ng of DNA were amplified in a total volume of 100 µl including 2 units of AmpliTaq Polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA), 200 µM of each of the four dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.8 mM $MgCl_2$, and 0.1 mg/ml gelatine. The amplifications were denaturated at 95°C for 3 min, followed by 37 cycles of 1 min at 95°C, annealed 1 min at 53°C, and extended 1 min at 72°C, with a final extension for 10 min, in a Perkin Elmer Cetus DNA Thermal Cycler (model TC-1). The products were purified in 100

000 NMWL Millipore filters (Millipore Products Division, Bedford, Mass., USA) by centrifugation for 8 min at 5000 rpm. The retentate was diluted in 100 µl of TE-buffer and the DNA precipitated by isopropanol. PCR products were resuspended in 15 µl of TE and 5 µl was annealed with 0.5 pmol of the limiting primer and sequenced using the Sequenase kit (United State Biochemical Corp., Cleveland, Ohio, U.S.A.).

2) *Cycle sequencing of dsPCR fragments*. DsPCR fragments were obtained by a first amplification using the end-primers NS1-NS8 under the same conditions as above except for a lower annealing temperature (48°C). The products were purified by Magic PCR Preps DNA Purification System (Promega Corp., Madison, Wisc., U.S.A.). 1-100 ng of the double-stranded products was run in a cycle sequencing PCR (2 min at 95°C, then 30 cycles of 95°C /30 sec, 48°C /30 sec and 70°C /1 min; fmol DNA Sequencing System, Promega). NS1 to NS8, and the following new primers (S. Landvik) were used:

SL12 5'GTTCATTCAAATTTCTGCCC
SL34 5'TTGTCAGAGGTGAAATTCTTGGATTTA
SL56 5'GGTGGAGTGATTTGTCTGCTTAATTGCG
SL65 5'CGCAATTAAGCAGACAAATCACTCCACC
SL43 5'GAACCACACGTCCTATTC

The internal primer locations are marked in Appendix 1. The major parts of the gene has been sequenced in both directions. For methods used in the sequencing of *Leotia lubrica* and *Peziza badia*, see Gargas 1992.

Sequence alignment. Sequences were aligned visually and adjusted to preserve their secondary structures alignments which were calculated by HIBIO MacDNASIS Pro, (Hitachi Software Engineering Co., Ltd's San Bruno, California, U.S.A). Sequence gaps were marked "-" and unresolved nucleotides or unknown sequences were indicated by "?". Variable sites were printed in boldface and those used as characters in the cladistic analyses were marked by "!". Bases that could not be unambiguously aligned within variable regions (not printed in boldface) were excluded from the analyses. See Appendix 1. *Spathularia flavida* appeared to have one insertion, approximately 300 bases long, close to the NS5 primer region (at position 1153). The insertion was not included in the data set. Part of the NS1-2 area (positions 1-310) of *Neolecta vitellina* (UME 29232), was compared to the sequence from another collection of that species (UME 29192) with no dissimilarities.

Phylogenetic tree construction. Cladistic analyses were performed with the PAUP software package (Swofford 1991, version 3). *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* were used as outgroups.

Results and Discussion

The genus *Neolecta* Spegazzini has been placed in three different orders – *Lecanorales*, *Leotiales*, and *Pezizales*, but it deviates morphologically from each of these orders. We assumed that the position of the genus might be inferred from molecular data, so we performed a cladistic analysis of 18S rDNA sequences from *Neolecta* and 10 other ascomycetes.

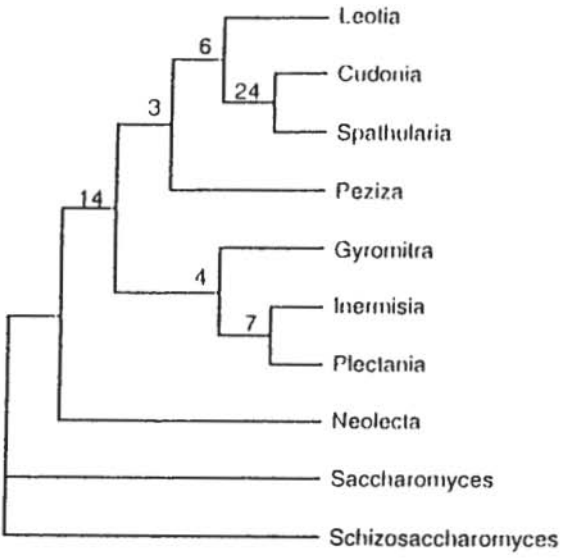

Fig. 1. A PAUP branch-and-bound analysis (PAUP, Swofford 1991) of 18S rDNA sequence using *Saccharomyces* and *Schizosaccharomyces* as outgroups produced two most parsimonious trees (length=358; CI=0.61; RI=0.54; all values the same in both trees). *Neurospora* was placed among the discomycetes, either as a sister group to the leotialean genera or together with *Cudonia*/*Spathularia* as a sister group to *Leotia*.

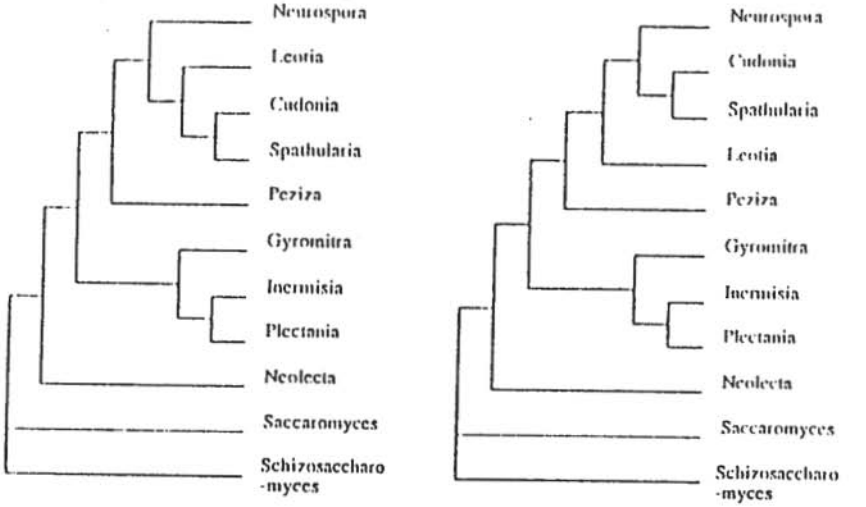

Fig. 2. The most parsimonious tree (PAUP, Swofford 1991) from an exhaustive analysis of 18S rDNA sequences based on 144 informative characters (tree length=299; CI=0.64 ; RI=0.59; g1=-1.08). *Saccharomyces* and *Schizosaccharomyces* were used as outgroups. The decay number, shown above the branches (Donoghue et al. 1992), indicate that *Neolecta* branched off earlier than the other represented discomycetes.

Of the 1776 characters, 159 were informative, but 7 of them could not be unambiguously aligned, so they were excluded from the analysis. If secondary structures were not considered, and sites with one or more questions were excluded, 86 characters remained unambiguous. Analyses with these characters gave results comparable to those below (data not shown).

In a first analysis with the pyrenomycete genus *Neurospora* as outgroup, the yeast genera *Saccharomyces* and *Schizosaccharomyces* clustered as an ingroup among the discomycetes, a classification that we consider unacceptable, as data from various RNA and protein genes show that these yeasts are unrelated to each other (cf. Eriksson et al. 1993).

Using *Saccharomyces* and/or *Schizosaccharomyces* as outgroups, unambiguously placed *Neurospora* among the discomycetes, closely associated with *Leotiales* (this is in agreement with studies including several other pyrenomycete, plectomycete and discomycete taxa; unpublished data). The analysis produced 2 trees with a length of 358 steps, a consistency index of 0.61, and a retention index of 0.54, by using PAUP's branch-and-bound algorithm (Fig. 1). The two trees differ in the position of *Neurospora*. It may either be a sister group to the three discomycete genera *Cudonia/Spathularia* + *Leotia* or together with *Cudonia/Spathularia* be a sister group to *Leotia*. Morphological characteristics make the latter alternative less probable, which we assume will be demonstrated when further data become available.

In order to investigate the position of *Neolecta* more closely, we excluded *Neurospora* from our analysis and performed an exhaustive search on the remaining species, using the same outgroups; the number of informative characters was now 144. The search gave one most parsimonious tree with a length of 299 steps, a consistency index of 0.64, and a retention index of 0.59 (Fig. 2).

An indication of the information content in the data is provided by the skewness of the distribution of trees found during the search. The skewness of tree distribution can be measured by the g1 value (Hillis & Huelsenbeck 1992). A negative value indicates significant information content. In this case the tree distribution was highly skewed, and the g1 value was highly negative, -1.08.

An indication of the stability (support) of each node may be calculated by retaining longer trees during the analysis to find out at what tree length the different nodes break down, "decay" (Donoghue et al. 1992). The decay values for the different nodes (Fig. 2) show that *Neolecta* is very strongly supported as the sister group of the rest of the investigated taxa (14 steps); only the *Cudonia/Spathularia* branch is better supported (24 steps). Even with these two nodes constrained, the g1 value is -0.86, indicating that there is strong support also for the remaining nodes. Surprisingly, *Peziza* did not cluster with *Gyromitra*, *Inermisia* and *Plectania*, the other operculate discomycetes included. However, the position of *Peziza* is comparatively weakly supported (3 steps), and might change if further taxa are included.

The cladistic analysis based on the molecular data strongly indicates that *Neolecta* should not be placed in either *Pezizales* or *Leotiales*, and, based on other information it is unlikely that it belongs in *Lecanorales*. This is in agreement with morphological data. The absence of a hamathecium may represent a fundamental difference between *Neolecta* and other ascomycetes pro-

ducing ascomata, except *Aparaphysaria* and the truffles. Also the asci of *Neolecta* lack an operculum, but they are apically truncated as in many operculate ascomycetes. The entire ascus wall is KOH/I+ blue, and *Neolecta* differs in that respect from all other inoperculate discomycetes. Morphological and molecular differences, together, provide reasons for recognizing a separate order to accomodate the genus *Neolecta*.

Neolectales Landvik, O.E. Erikss., Gargas, & P. Gustafsson, ordo nov.

Ascomata clavata, stipitata, laete colorata. Hymenium sine hamathecio. Asci cylindrici vel leniter clavati, tenuiter tunicati, in iodo coerulescentes, octospori. Ascosporae ellipsoideae, non-septatae, hyalinae. Ad terram.

Typus: *Neolectaceae* Redhead, *Can. J. Bot.* 55: 305 (1977).

Ascomata clavate, stalked, light coloured. Hymenium without hamathecium. Asci cylindrical or somewhat clavate, thin-walled, KOH/I+ blue. Ascospores ellipsoid, non-septate, hyaline. Terricolous.

The single family, *Neolectaceae* Redh., contains one genus with three species. For detailed descriptions of the two species from the Northern Hemisphere, we refer to Redhead (1977). All species have club-shaped ascomata, but this is not a very important character. Club-shaped fruit-bodies have evolved several times in the *Ascomycotina* and *Basidiomycotina*. *Neolecta*, with its isolated position, may turn out to be a good choice for outgroup in many future studies of discomycete and pyrenomycete interrelationships.

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