RESEARCH ARTICLE

Roselliniella revealed as an overlooked genus of *Hypocreales*, with the description of a second species on parmelioid lichens

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Abstract   Based on newly obtained 28S rDNA sequences from *Roselliniella atlantica* and *R. euparmeliicola* sp. nov., the genus *Roselliniella* has to be placed in *Hypocreales* and not in *Sordariales*; however, the family placement could not be resolved from the sequences obtained. The mature ascospores are single-celled and brown, but young ascospores are hyaline and sometimes have a median septum. The new species occurs on a *Parmelia* s.str. species in China, and differs in 24 nucleotide substitution positions in the nu-LSU rDNA region and ascospore size from *R. atlantica*. In this case, small variations in ascospore sizes and shape prove to be phylogenetically and taxonomically informative. The two species occur in the same clade with 95 % jack-knife support. *Roselliniella atlantica* occurs on *Xanthoparmelia* and *Melanohalea* species in Europe, whereas *R. euparmeliicola* was found on the species of *Parmelia* s.str. DNA was successfully recovered from a dried specimen of *R. atlantica* collected in 1992. Two unidentified fungi were also recovered from the Chinese specimen, and these belong to *Sordariomycetidae* and *Dothideomycetes*; whether these two are additional fungi living endolichenically in the lichen host, saprobes, or contaminants could not be ascertained.

INTRODUCTION

The generic name *Roselliniella* was introduced by Vainio (1921: 214) for pyrenocarpous fungi growing on lichens with single-celled brown ascospores and persistent filamentous interascal filaments. Vainio did not mention any included species, but referred to Saccardo (1882: 175) where three species were mentioned as comprising *Rosellinia* sect. *Lichenicolae* Sacc. 1882. The genus was generally treated as a later synonym of *Adelococcus* Theiss. & Syd. 1918, until resurrected first by Hafellner (1985). However, Hafellner based his concept of the genus on *Endococcus haplospora* Th.Fr. & Almq. 1867 which has thick-walled polyspored asci and paraphysoids, and this was not one of the original species mentioned by Saccardo. Santesson (in Eriksson & Hawksworth 1986: 310) drew attention to this misapplication, and introduced the new generic name *Rosellinula* for *E. haplospora* (syn. *Muellerella thallophila* Arnold 1888).

Santesson (in Eriksson & Hawksworth 1986) noted that lectotypification of Vainio's generic name should await a revision of the genera involved. This was done by Matzer & Hafellner (1990: 53) who designated *R. nephromatis* (P. Crouan) Matzer & Hafellner 1990 as the type species, and recognised eight additional species, all growing on lichens, and mostly occurring in the Southern Hemisphere. Since that time, six further species have been added to the genus, and we describe an additional one as new here.

The systematic position of *Roselliniella* has not been resolved by molecular phylogenetic methods. The thin-walled function-

ally unitunicate I- asci, with no specialised apical apparatus, filamentous interascal filaments, and in some species the brown single-celled ascospores becoming delicately ornamented, led Hoffmann & Hafellner (2000: 91) to place the genus in the order *Sordariales*, but not to any family within the order. This classification has been followed in subsequent editions of the 'Outline of *Ascomycota*' (e.g. Eriksson 2006: 62, Lumbsch & Huhndorf 2007). However, it was not referred to an order, but only to the subclass *Sordariomycetidae* in Kirk et al. (2008), but that was only a *lapsus* that has now been changed in the database from which that was prepared (P.M. Kirk, pers. comm.).

The objectives of this study were to use molecular approaches to: 1) determine the placement of the genus in the *Ascomycota* more precisely; and 2) confirm that the modest differences in ascospore size between the new species and one already described from different parmelioid lichens were a valid taxonomic character.

MATERIALS AND METHODS

Specimens examined

In addition to the type collection of the new species, the following specimens of *Roselliniella atlantica* Matzer & Hafellner 1990 were studied for comparison.

Specimens examined. FRANCE, Campénéac, Morbihan, on *Xanthoparmelia mougeotii* on rock escarpment, 2 Apr. 1970, *B.J. Coppins*, E 00235005, paratype; Finistère, Chaos de St Herbot, on *X. mougeotii*, 8 June 1970, *A.R. Pentecost*, E 00235006, paratype. – UNITED KINGDOM, Outer Hebrides, Lewis, Port Geiraha, on *X. mougeotii* on rock, Aug. 1959, *S.A. Manning*, IMI 211909, paratype; Westerness, NE of Strontian, Ariundle Wood National Nature Reserve, on *Melanohalea exasperata*, 18 June 1992, *A.M. O'Dare* (Coppins 15370), E 00235007.

Morphological study

Specimens were studied using a Nikon zoom stereomicroscope and an Olympus BH-2 research microscope fitted with Nomarski differential interference contrast optics and a drawing tube.

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Freezing microtome sections were made on a Bright Starlet Cryostat, and both sections and squash preparations were used for microscopic examination. Preparations and measurements were made in water, lactofuchsin, and lactophenol cotton blue, the last two after warming.

Choice of additional taxa and outgroup

In addition to *Roselliniella* specimens we included representatives of other groups in *Sordariomycetes* and *Dothideomycetes*. Particularly, one species of *Xylariales*, five species of *Dothideomycetes*, 18 species of *Hypocreomycetidae* and 14 species of *Sordariomycetidae*, the two later respectively representing the four and six orders considered by Hibbett et al. (2007) for these two subclasses. Two specimens of *Peziza* (*Pezizales*, *Pezizomycetes*) were used as outgroup.

DNA extraction

DNA was extracted directly from dried specimens. Perithecia growing on the host thallus were carefully separated to avoid as much as possible obtaining tissue from the host with the point of a scalpel blade. Total DNA was extracted using the Qiagen DNeasy Plant MiniKit, according to the manufacturer's instructions from perithecia of *Roselliniella atlantica* and *R. euparmeliicola*.

Amplification and sequencing

A fragment of c. 1 000 bp in the nuLSU was amplified using the primers LR0R (Vilgalys, www.biology.duke.edu/fungi/mycolab/primers.htm) and LR5 (Vilgalys & Hester 1990).

PCR amplifications were performed using Illustra™ Hot Start PCR beads, according to the manufacturer's instructions, with the following settings: initial denaturalization of 95 °C for 3 min, four cycles (95 °C for 40 s, 56 °C for 40 s and 72 °C for 90 s), four cycles (95 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s) and finally 32 cycles (95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s) with a final extension of 72 °C for 6 min.

When cloning was necessary, PCR products were ligated to a PCR 2.1-TOPO plasmid vector and cloned using TOPO TA Cloning kit according to the instructions of the manufacturer's. Colonies were screened for the presence of the desired product, using the same primers and PCR programs described above. Clones with inserts of the appropriate size were sequenced.

Before sequencing, the PCR products were purified using the Viogene PCR-M Clean-up System or the enzymatic method Exo-sap-IT©.

Sequence alignment

Sequences were aligned using the software MAFFT v6.611 (Katoh et al. 2002, Katoh & Toh 2008) using the same procedures described in Wedin et al. (2009). This multiple alignment software has been frequently mentioned as one of the most accurate ones currently available (Niun et al. 2006, Carroll et al. 2007, Golubchik et al. 2007, Wedin et al. 2009). The ambiguous regions in the alignment were identified and eliminated using Gblocks v0.91b (Castresana 2000) with the following parameters: minimum number of sequences for a conserved position '20', minimum number of sequences for a flank position '20', maximum number of contiguous non-conserved positions '10', minimum length of a block '5' and allowed gap positions 'with half'.

Parsimony and parsimony jack-knifing analyses

The analyses were performed using PAUP v4.0b10 (Swofford 2002) with the following settings: gaps are treated as 'missing data', 1 000 random addition sequence replicates, TBR branch swapping, steepest descent off, collapse branches if minimum length is 0, and MulTrees on.

Jack-knife for identification of well-supported monophyletic groups (Farris et al. 1997) was performed in PAUP, with the following settings: heuristic search settings identical with the above analysis but with 20 random addition replicates; jack-knife settings: 1 000 jack-knife replicates with 'JAC'- emulation, nominal deletion of characters 37 %, full heuristic search, retain groups with frequency > 50 %.

RESULTS

Taxonomy

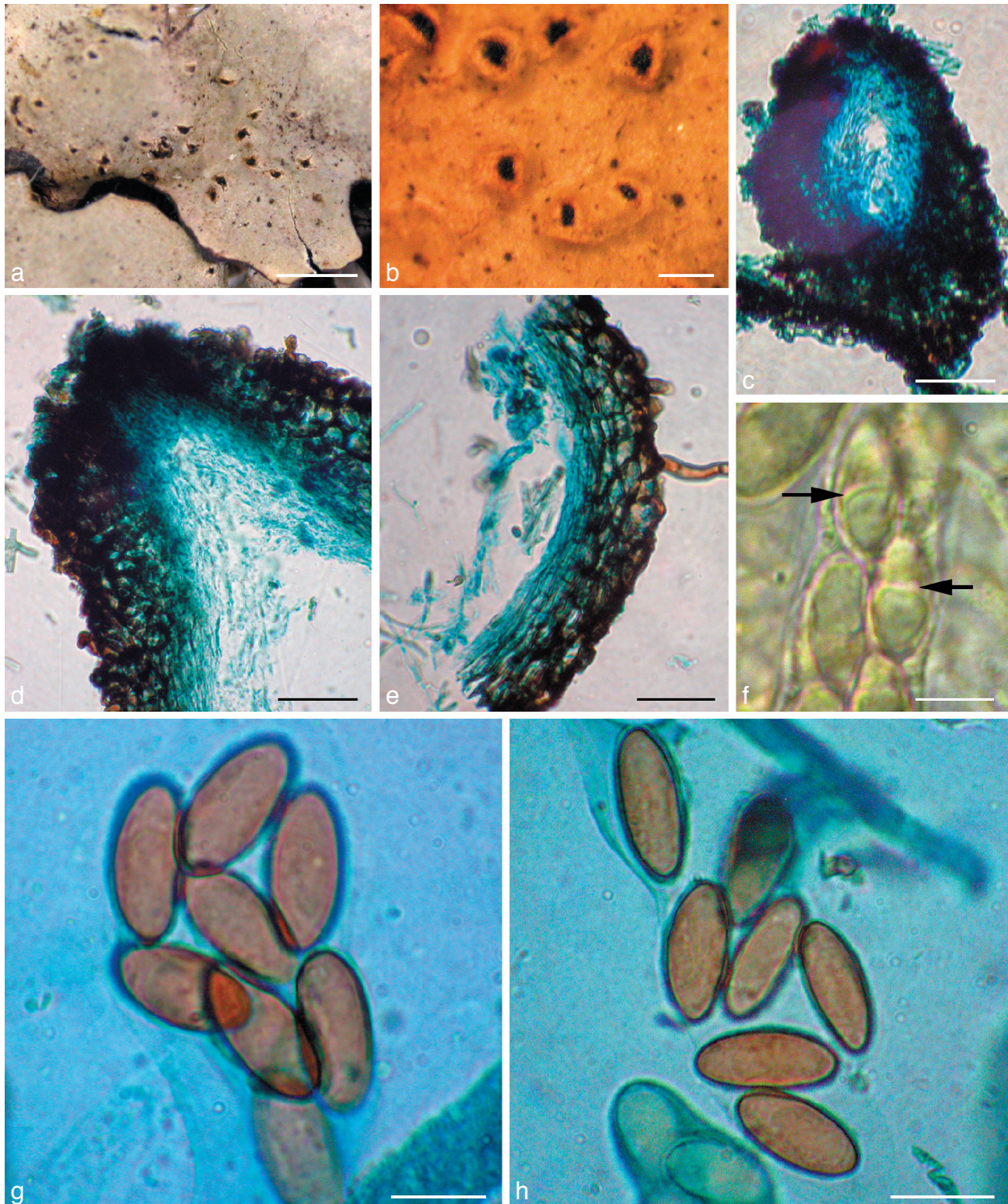**Roselliniella euparmeliicola** Millanes & D. Hawksw., *sp. nov.* – MycoBank MB515195; Fig. 1, 2

Similis *Roselliniella atlantica*, sed ascosporiis minoribus, 17–20(–22) × 8–9.5(–12) µm.

Etymology. The species epithet indicates that the fungus lives on *Parmelia* s.str. species.

Ascomata perithecia, erumpent through the cortex of the host lichen, the cortex raised into wart-like convex to urceolate swellings, splitting apically or linearly or into teeth-like projections to expose the upper part of the perithecium, black, the area around the ostiole somewhat shiny, broadly pyriform, ostiole well-developed, (145–)170–410(–430) µm in surface view; exciple dark red-brown in section, intensifying in 10 % KOH, composed of 3–6 layers of pseudoparenchymatous cells, wall mainly 12.5–21 µm thick, but to 38 µm thick at the base and to 50 µm thick below the ostiole, with the innermost 5–7 µm layers of cells hyaline; cells irregularly polyhedral and somewhat angular, 7.5–10(–11) µm diam in surface view, smooth-walled, walls to 4–5 µm thick, radially compressed in vertical section and elongated vertically around the ostiole, cells in the innermost hyaline layers especially so; hyphae extending out from the lower parts and sides of the ascomata, pale brown to red-brown, flexuose, branched, smooth-walled, repeatedly septate and tending to be somewhat inflated between the septa, 3–4.5 µm thick. *Hamathecium* of periphyses and interascal filaments; periphyses abundant in the ostiolar canal, filamentous, septate, 1.5–2 µm thick; interascal filaments well-developed, persistent, filamentous, unbranched, septate, 1.5–2 µm thick; centrum I- (Lugol's solution after pre-treatment in 10 % KOH). *Asci* elongate-clavate to subcylindrical, stalked, unitunicate in structure, walls ± equal in thickness and without any distinct apical thickening or internal apical structures, (52–)77–85(–95) × (12.5–)14–17 µm, 8-spored. *Ascospores* overlapping and irregularly biseriate in the ascus, non-septate, ellipsoid, rounded at the apices, lacking germ-pores, hyaline at first and occasionally with a median septum while still in the ascus, becoming pale to dark brown before release from the ascus, smooth-walled, the wall 0.5–1 µm thick, 17–20(–22) × 8–9.5(–12) µm, length/width ratio 1.8–2.6 (av. = 2.1), with a
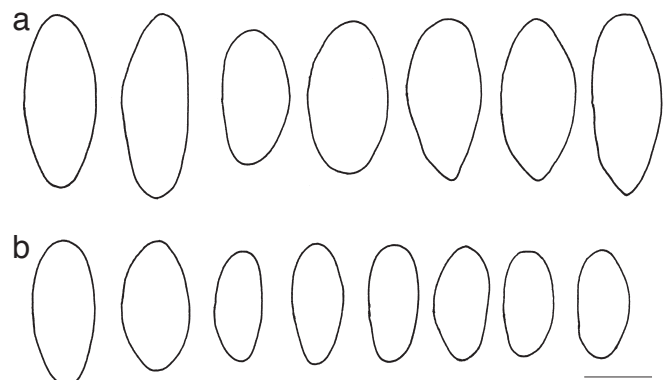
Fig. 1 Ascospore outlines of *Roselliniella* species. a. *R. atlantica* (IMI 211909 paratype); b. *R. euparmeliicola* (BM holotype). – Scale bar = 10 µm.

Fig. 2   *Roselliniella euparmeliicola* (BM holotype). a. Infected thallus of *Parmelia meiophora*; b. urceolate warts with perithecia; c. vertical section of perithecium; d. vertical section of ostiolar region of perithecium; e. vertical section of perithecial wall; f. asci containing young ascospores, some with a medium septum (arrows); g, h. mature ascospores. — Scale bars: a = 2 mm; b = 250 µm; c = 100 µm; d, e = 50 µm; f–h = 10 µm.

thin hyaline perispore c. 1.5 µm thick, not or slightly swelling in 10 % KOH (excluded from the measurements).

Specimen examined. CHINA, Yunnan Province, Jade Dragon Mountain, White River Stop, on *Parmelia meiophora*, 5 Oct. 2005, *M.A. Allen & B.H. Hilton 405-8-2(c)*, holotype BM 000920346.

Host — In the thallus of *Parmelia meiophora*, apparently commensalistic as the infected areas of the thallus are not discoloured and there was no sign of necrosis.

Distribution — China (Yunnan Province); known only from the holotype collection.

Notes — This new species on *Parmelia* s.str. is very similar to *Roselliniella atlantica* Matzer & Hafellner 1990, a taxon first referred to by Hawksworth (1978: 181) as '*Adelococcus* cfr. *groedensis* (Zopf) Keissl. 1930' on the basis of the similarly sized ascospores as reported in the literature. However, Matzer & Hafellner (1990) showed that *A. groedensis* had ascospores with apical pores, and when mature a septum near each apex, and occurred on *Pertusaria lactea*; they described the new genus *Roselliniopsis* Matzer & Hafellner 1990 to accommodate that fungus and a related tropical species.

The ascospores in the specimens of *Roselliniella atlantica* we examined for comparison in this study were (18−)22−26(−29) × (11.5−)12−14(−14.5) μm, thus being longer and broader than in the new species (Fig. 1); the shortest (from 17 μm) and narrowest (from 9 μm) ends of the range measurements in Hawksworth (1978) were based on somewhat immature spores. Also, according to Ihlen & Wedin (2008: 315) they can reach 30 μm in length. Matzer & Hafellner (1990: 57, fig. 5c) illustrate one spore with a septum in the lower third. We saw no septa in the slides we made of this species, either in hyaline immature or brown mature spores. In addition to the differences in size, the ascospores of *R. euparmeliicola* tend to be somewhat more pointed at one or both ends.

Roselliniella atlantica occurs on two species of a different parmelioid lichen genus from the new species: *Xanthoparmelia* (incl. *Neofuscelia*), notably the yellow-green *X. mougeotii*, and the brown *X. verruculifera*, and is also reported here on *Melanohalea exasperata*. That fungus is only known from Europe, where it occurs in France, Sweden, and the UK. In the specimen IMI 211909, it may be significant that the perithecia were not developed on two other parmelioid lichens growing mixed with *X. mougeotii* on the piece of rock: *Melanelixia fuliginosa* and *Parmelia omphalodes*. The specimen on *Melanohalea exasperata*, with ascospores we measured as 23.5−26.5 × (10.5−)12−13 μm, agrees with *R. atlantica* in all the features we could study on the rather sparse material. The ascomata of *R. atlantica* also differ from those of the new species in the way they originate on the different host lichens, emerging erumpently from the thallus but without producing raised wart-like structures with splits where the cortex has been forced apart. The species may also be more pathogenic than *R. euparmeliicola* as infected lobes of *X. mougeotii* become discoloured and friable, so that infected scraps are often all that is found.

Phylogenetic analysis

We generated five new sequences of the 28S rDNA (Table 1) which were aligned together with sequences already available in GenBank. Three of the five new sequences which were amplified during cloning experiments corresponded to other fungi in the sample, and were different from those of the host genus *Parmelia* s.str.

The two *Roselliniella* sequences differed in 24 nucleotide positions, and formed a single clade with 95 % jack-knife support.

The matrix analysed contained 1 057 aligned characters. After the exclusion of ambiguously aligned and uninformative sites, 376 parsimony informative sites were used in the analyses. Our parsimony analysis resulted in six equally most parsimonious trees (1 739 steps, CI = 0.389, RI = 0.657 and RC = 0.256).

The species in *Hypocreales* we included in the analysis formed a monophyletic clade with 89 % jack-knife support, and both *Roselliniella* species were included within it. The two *Roselliniella* species joined with 95 % jack-knife support and were a sister group to a clade including species in *Nectriaceae* (Fig. 3), though without significant support.

Sequences of three additional fungal taxa were isolated from the *Parmelia meiophora* thalli during cloning experiments. One of them is placed within the *Chaetosphaeriales* clade, whereas the remaining two group with *Dothideomycetes* representatives in a strongly supported clade (100 % jack-knife value). None had matches in GenBank and remain unidentified.

DISCUSSION

In our phylogenetic analyses, the two species of *Roselliniella* were conclusively placed in the *Hypocreales* clade, though

the family placement within the order was not recovered with phylogenetic support. There can consequently be no doubt that *Roselliniella* belongs to the order *Hypocreales* in class *Sordariomycetes*. This was a somewhat surprising result as the ascomata of *Roselliniella* species are erumpent and not superficial on a stroma, appear dark walled and have persistent interascal filaments, the asci lack an apical ring, and at maturity the ascospores are brown and single-celled rather than colourless or pale two- or more-celled. However, while ascospores that are single-celled from the first are known in some *Hypocreales*, for instance *Nigrosabulum globosum* in *Bionectriaceae* (Plishka et al. 2009), we found that immature hyaline spores of *R. euparmeliicola*, while inside the ascus, sometimes had a medium septum (Fig. 2f). The ascomatal walls are rather robust and dark coloured for *Hypocreales*, but the layered wall, with the cells somewhat swollen and rounded in surface view, does recall many members of the order. More fundamentally, while interascal filaments are characteristically absent in *Hypocreales*, 'apical paraphyses' resembling those of the *Roselliniella* species are described and illustrated in the type species of *Nectria*, *N. cinnabarina*, and also in *N. lamyi*, by Rossman et al. (1999: 143, pl. 30b, h). We were unable to determine the origins of the filaments in the Chinese specimen, and so could not resolve whether they were 'apical' or 'true' paraphyses; if the latter, thus would be most unusual for the family and merit an emendation of its features.

Table 1 Sequences newly produced (**bold**) or downloaded from GenBank. Specimen numbers are given for newly produced sequences.

Taxon	Specimen	GenBank accesion number (n-LSU)
Bionectria ochroleuca		AY686634
Bionectria pityrodes		AY489728
Botryosphaeria ribis		AY004336
Camarops petersii		AY346265
Chaetosphaerella phaeostroma		AY346274
Chaetosphaeria lateriphiala		AF466072
Chaetosphaeria pygmaea		AF466077
Coniochaeta sp.		AY346275
Coniochaetidium savoryi		AY346276
Coniothyrium obiones		DQ678054
Cordyceps tuberculata		AF327384
Cordyceps militaris		AF327374
Cosmospora coccinea		AY489734
Cosmospora vilior		AY015626
Cylindrocarpon lichenicola		AY097324
Diaporthe phaseolorum		AY346279
Hypocrea rufa		AY489726
Lasiosphaeriella nitida		AY346289
Leptosphaeria maculans		AY849946
Leptosporella gregaria		AY346290
Melanospora singaporensis		AY015629
Melanospora zamiae		AY046579
Microascus trigonosporus		DQ470958
Mycopepon smithii		AF279400
Nectriopsis sporangiicola		AF210661
Neonectria fuckeliana		AY283551
Neonectria radicicola		U17415
Neurospora crassa		AF286411
Nitschkia grevillei		AY346294
Ophiostoma piceae		AF234837
Peziza proteana		AY544659
Peziza vesiculosa		AY500552
Poroconiochaeta discoidea		AY346297
Roselliniella atlantica	E-235007	**GQ888763**
Roselliniella euparmeliicola	BM-920346	**GQ888764**
Schizoparme botrytidis		AF408383
Sordaria macrospora		AY346301
Sphaerodes fimicola		AY015628
Unknown *Dothideomycetes*-a	BM-920346	**GQ888765**
Unknown *Dothideomycetes*-b	BM-920346	**GQ888766**
Unknown *Sordariomycetidae*	BM-920346	**GQ888767**
Valsa ceratosperma		AF408387
Vittatispora coorgii		DQ017375
Xylaria hypoxylon		AY544648

Fig. 3 One of the 6 most parsimonious trees based on the analysis of 376 informative sites of 44 nucLSU sequences; length 1 739, CI = 0.389, RI = 0.657 and RC = 0.256. Support measured by jack-knifing (≥ 70 %) is represented with the numerical values written above the corresponding branches. Branch lengths are proportional to the number of nucleotide differences. Sequences new generated are in **bold**, and a shaded box states the clade including both species of *Roselliniella* sequenced in this study. Selected class, subclass, order and family designations following Hibbet et al. (2007) are given in the right side. Two specimens of *Peziza* (*Pezizales*, *Pezizomycetes*) were used as outgroup.
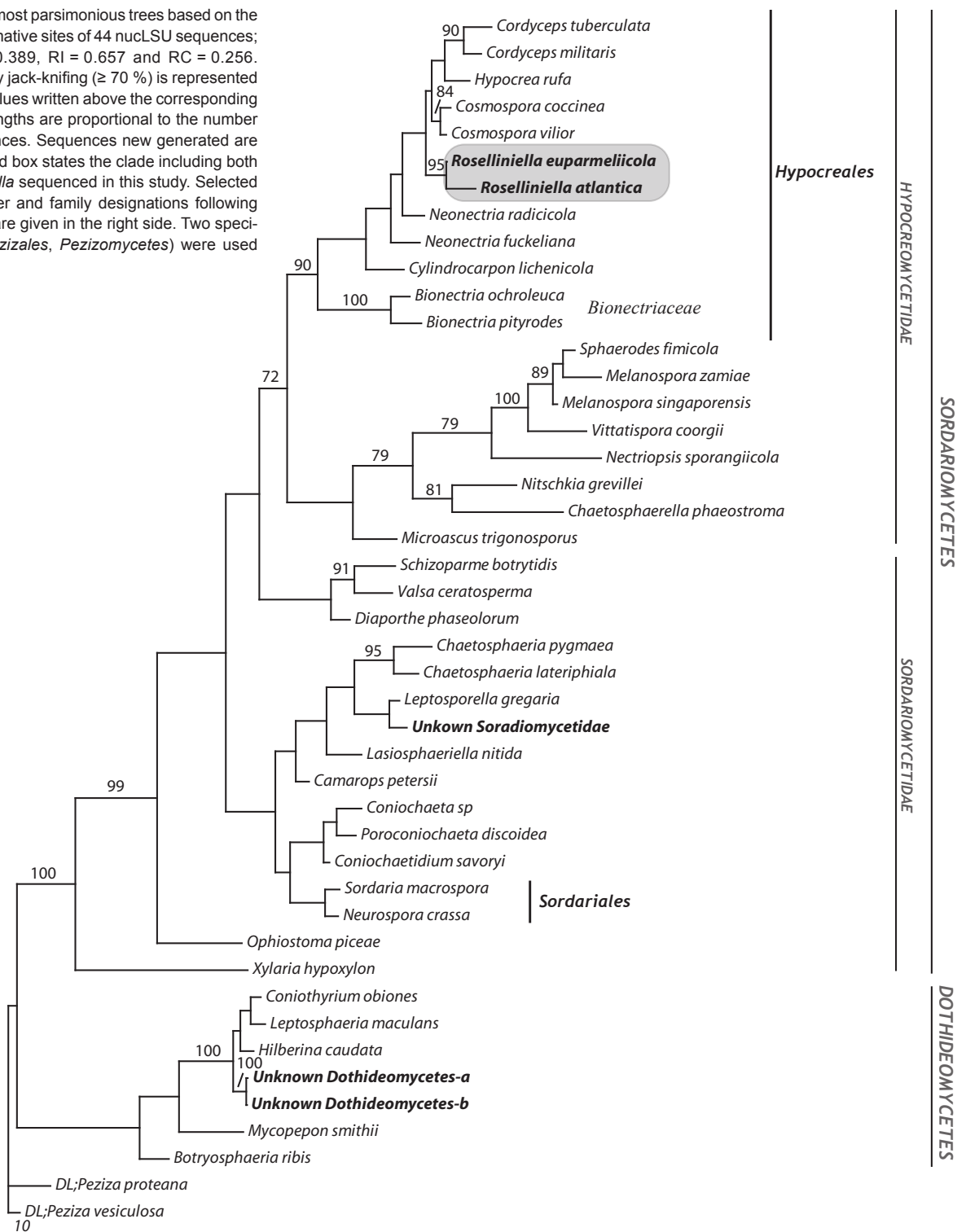
Biologically, it is interesting to see that *Roselliniella* appears in the hypocrealean clade that includes many fungicolous and also some lichenicolous fungi. *Cosmospora* species in particular mainly occur on perithecioid ascomycetes, though a few are entomogenous or found directly on plant material (Rossman et al. 1999). However, most lichenicolous hypocrealean genera (e.g. *Nectriopsis*, *Paranectria*, *Pronectria*, *Trichonectria*), are placed in *Bionectriaceae*, although some are in *Nectriaceae* (e.g. *Xenonectriella*). *Cylindrocarpon lichenicola* (syn. *Fusarium lichenicola*) was also placed in *Hypocreales* out of the *Bionectriaceae* clade in our tree, but we stress that this is not obligately lichenicolous but a primarily saprobic fungus known from diverse habitats (Hawksworth 1979).

In our first comparison of sequences with those in GenBank, *Melanospora fallax* U17404 and *M. zamiae* U17405 were in a clade with 100 % jack-knife support, and a sister group (but without support) to the two *Roselliniella* species. However, the LSU sequence from U17405 (Rehner & Samuels 1995) was found to be erroneous when the same isolate from the American Type Culture Collection (ATCC) was re-sequenced by Zhang & Blackwell (2002), and this must also be so for U17404 which those authors did not examine. Neither of these two sequences fall in the well-supported *Melanosporales* clade along with other species of *Melanospora*, *Sphaerodes* and *Vittatispora* (Chaudhary et al. 2006).

The extent to which small differences in ascospore sizes and shapes is phylogenetically informative in lichenicolous fungi is uncertain, especially where closely related hosts are involved as in the parmelioid lichens (Doré et al. 2006). In the case of the two *Roselliniella* species we investigated, the differences in 24

nucleotide positions show that *R. atlantica* and *R. euparmelii-cola* represent different phylogenetic species, and consequently the differences in ascospore size ranges are informative in this case. However, we recognise that further studies including more *Roselliniella* species are needed to test the phylogenetic value of ascospore size as a synapomorphic character in the genus as a whole. Further, this result should not be automatically extrapolated to other cases in which small differences in ascospore size in lichenicolous fungi occurring on different host lichens are found. It does show, however, that in such instances molecular data should be obtained to see whether or not the variation is phylogenetically informative.

That the two *Roselliniella* species distinguished here occur on different genera of parmelioid lichens is of special interest as it appears to provide another instance where lichenicolous fungi per se are taxonomically and phylogenetically informative in this group of lichens. However, such conclusions must remain tentative when few specimens have been available for study. Correlations between lichenicolous fungi and the genera now recognized in *Parmelia* s.l. (Blanco et al. 2006) have previously been demonstrated in, for example, species of *Abrothallus* (Ihlen & Wedin 2008) and *Homostegia* (Hawksworth et al. 2004).

It is not possible to state whether the unidentified *Chaetosphaeriales* and *Dothideomycetes* fungi from the Chinese specimen are contaminants or asymptomatic taxa growing inside the host lichen, i.e. endolichenic fungi. It does appear that fungi other than that making the lichen thallus can commonly be detected by isolation into culture (Petrini et al. 1990, Möller & Dreyfuss 1996, Suryanarayan et al. 2005, Li et al. 2007) or molecular sequencing (Miadlikowska et al. 2005). What is not resolved is whether these fungi are actually living in the lichen tissues or if they are present as captured spores fortuitously present.

Our success in obtaining sequence data from a dried specimen of *Roselliniella atlantica* collected 15 years earlier, however, demonstrates that molecular approaches to studies of phylogenetic relationships and species concepts amongst lichenicolous fungi can be feasible in the absence of very recently collected material. We believe that this is the oldest material of a lichenicolous ascomycete to have sequence data obtained from it to date.

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