Rhynchostomatoid fungi occurring on Proteaceae

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Abstract: A new ascomycete fungus, with long-necked perithecia having central ostioles and striate ascospores, was isolated from flowerheads of Protea burchellii and P. laurifolia in South Africa and is described here as Rhynchostoma proteae sp. nov. Sequence data obtained from the small-subunit ribosomal DNA (SSU nrDNA) place this fungus with 100% bootstrap support in a clade containing the type species of Rhynchostoma, R. minutum. A similar fungus with verruculose ascospores also was observed on a member of the Proteaceae from Australia, Lomatia polymorpha, which is described here as Rhynchomeliola lomatiae sp. nov. These two species are illustrated and contrasted with a third species from Proteaceae, Rhynchomeliola australiensis, known from Grevillea in Australia.

Key words: Ascomycetes, Chaetothyriomycetes, Fynbos, South Africa

INTRODUCTION

The Proteaceae, which existed before Gondwanaland began to break up 140 million years ago (Rebelo 1995), is known to be one of the most prominent flowering plant groups in the Southern Hemisphere, with the majority of genera found in South Africa and Australia. The family is divided into more than 60 genera containing about 1400 species (Rebelo 1995). Protea L. has flowerheads (or inflorescences) comprising many flowers with bracts, which often are mistaken for flowers. Some are open only a few weeks and then close and remain intact for at least a few years (serotinous) (Rebelo 1995). Studies on the mycota of flowerheads (Wingfield and van Wyk 1993, Marais and Wingfield 1994) revealed the presence of unique ophiostomatoid fungi and proved flowerheads to be a potentially rich niche for saprobic fungi.

While studying saprobic fungi in flowerheads of Proteaceae as part of a fungal biodiversity program, an unusual ascomycete with a long perithecial neck was discovered among ophiostomatoid fungi. The fungus was identified as a new member of Rhynchostoma P. Karst. A similar fungus coincidentally was found on a herbarium specimen of Lomatia polymorpha R. Br. (Proteaceae), which was identified as a new member of Rhynchomeliola Speg.

The genera Rhynchomeliola and Rhynchostoma were introduced separately to accommodate fungi with perithecia having prominent ostiolar necks, persistent unitunicate asci without apical apparatus and two-celled, brown ascospores. In Rhynchostoma, ascomata are immersed to becoming erumpent in a stroma. Rhynchomeliola ascomata are superficial on a subiculum, or with a semi-immersed base. The hierarchical placement of these two genera varied from the Dietrypeaceae (Müller and von Arx 1962), Sphaeriaceae (Müller and von Arx 1973), Bolineaceae (Barr 1990), Trichosphaeriaceae (Eriksson 1984, Hawksworth et al 1995) to the Rhynchostomataceae (Winka and Eriksson 2000, Eriksson et al 2001) and remains uncertain. In an attempt to clarify their morphology these fungi were studied along with the third species, Rhynchomeliola australiensis (Petr.) E. Müll., known from Grevilla. Furthermore, to elucidate phylogenetic relationship of the new species of Rhynchostoma from Protea with other genera, a phylogenetic analysis was done based on the SSU nrDNA sequence data.

MATERIALS AND METHODS

Isolates.—One- to 2-year-old flowerheads of serotinous Proteaceae were collected from Stellenbosch Mountains in South Africa’s western Cape Province. The samples were inspected immediately for fungal structures and air-dried for further study. Air-dried samples were incubated in mois-

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Genomic DNA was isolated respectively from mycelia and spore masses of *Rhynchostoma proteae*, following the method described by Crous et al. (2000). Three areas of the rDNA gene operon, namely part of the small subunit, part of the large subunit and the internal transcribed spacers along with the 5.8S rDNA gene, were selected for polymerase chain reaction (PCR) amplification. The small-subunit gene was amplified with primers NS1 and NS4 (White et al. 1990) and a part of the large-subunit gene using primers ITS1 (White et al. 1990) and LR5 (Vilgalys and Hester 1990). The primers ITS1 and ITS4 (White et al. 1990) were used to amplify the 3′ end of the 18S (small-subunit) nrDNA gene (SSU nrDNA), the first internal transcribed spacer (ITS1), the 5.8S rDNA gene, the second ITS (ITS2) region and the 5′ end of the 28S (large-subunit) nrDNA gene. PCR amplifications were conducted in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Foster City, California, U.S.A.) using standard protocols in a final volume of 25 µL. PCR products were purified according to the manufacturer’s instructions with a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Europe GmbH, Germany). Sequencing reactions were carried out with PCR primers and the ABI PRISM Big Dye Terminator Cycle version 3.0 Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer’s recommendations. The reaction was analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Sequence and phylogenetic analyses.—Sequence data were analyzed with EdiView 1.0.1 (http://www.appliedbiosystems.com). BLAST searches (Altschul et al. 1997) were performed with the analyzed sequences to identify similar sequences for determination and confirmation of the isolate’s taxonomic placement. The ITS, small-subunit and large-subunit sequences were deposited in GenBank (AY230152 and AY230151, respectively). To determine the phylogenetic placement of *Rhynchostoma proteae*, 30 small-subunit sequences were added from GenBank and manually aligned with the corresponding sequence from *R. proteae* by inserting gaps with Sequence Alignment Editor version 2.0 (Rambaut 2002).

Phylogenetic analyses, including maximum parsimony with 1000 random-addition replicates and neighbor joining with uncorrected “p”, Jukes-Cantor and Kimura-2-parameter models, were done with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swoford 2000). *Saccharomycyes cerevisiae* (V01335) and *Taphrina deformans* (U00971) were used as outgroups. For parsimony analysis, gaps were treated as a fifth character. Gapped regions caused by major insertions were excluded from the analysis. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis and Bull 1993). Other indices calculated for parsimony topology included tree length, consistency index (CI), retention index (RI) and rescaled consistency index (RC). Resulting trees were printed with TreeView version 1.6.6 (Page 1996).

RESULTS

Taxonomy.—Three species of the Chaetothyriomycetes collected from Proteaceae in South Africa and Australia, namely *Rhynchomeliola australiensis*, a new species of *Rhynchomeliola* from *Lonatia*, and a new species of *Rhynchostoma* from *Protea*, are treated below.

*Rhynchostoma proteae* S. Lee et Crous, sp. nov.

FIGS. 1–17

Stromata maxime reducta. Ascomata stromaticia, perithecioidea, immersa in textura hospitali, distincta, gregaria, in sectione verticali globosa vel subglobosa, usque ad 185 µm alta, usque ad 155 µm lata, collo ostiolato. Collum ostiolatum plerumque centrale, solum, obliquum, peripheribus, cylindricum, contractum ad apicem, usque ad 970 µm altum, usque ad 115 µm latum base, usque ad 50 µm apice. Peridium constans ex stratis duobus, 22.5–30 µm diam, carbonaceum. Paraphyses hyalinae, non ramosae, filamento-sae, flexuosae, septatae, abundae, 1–2 µm latae. Ascis circa septum peritheciale enascentes, unituniciati, octospori, fusiformes, apedicellati, comparate persistentes, (27–)29–35 × 5–7 µm, nullus apparatus apicalis observatus. Ascosporea biconcavae, elliptoideae vel fusiformes, constrictae septo, septum leniter submedium, extremum omne contractum vel extremum unum leniter apiculatum, brunnescentes maturitate, oblique striatae trans paginam sporiae in direcione una, porus germinis inconspicuus, (9–)9.5–10.5(–11.5) × 3(–4) µm.

Stromata reduced around the venter of the ascomata, up to 15 µm wide, comprising dark, thick-walled cells (Fig. 6). Ascomata stromatic, perithecioidei, im-mersed in host tissue, scarcely becoming superficial, separate, gregarious, in vertical section globose to subglobose, up to 185 µm high and 155 µm wide, with an ostiolar neck (Figs. 1–4). Ostiolar neck most-
ly central, single or rarely two, oblique, lined with periphyses, cylindrical, tapering off to the apex, consisting of longitudinally angular cells, up to 970 μm high, 115 μm wide at the base and 50 μm wide at the apex, with an outer amorphous crust (Figs. 5, 7–9). Peridium consisting of two layers, 22.5–30 μm diam, carbonaceous, cells of outer layer angular, moderately thick-walled, brown, becoming paler toward the inside, cells of inner layer hyaline, angular, compressed (Figs. 10, 11). Paraphyses hyaline, unbranched, filamentous, flexuose, septate, 1–2 μm wide (Figs. 7, 12). Ascii lining the perithecial wall unitunicate, octosporous, fusiform, apedicellate, relatively persistent, indistinguishable when ascospores are fully developed, (27–)29–35 × 7–5 μm, no apical apparatus observed (Figs. 13, 14). Ascospores overlapping, multiseriate, bicellular, ellipsoid to fusiform, constricted at the septum, septum slightly submedian, ratio of upper to lower cell 1–1.3:1, each end tapered or one end slightly apiculated, hyaline to pale brown when young, becoming medium brown at maturity, obliquely striated all over spore surface in one direction, striations already visible when immature, germ pore inconspicuous, (9–)9.5–10.5(–11.5) × 3(–4) μm (av. 9.9 ± 3 μm) (Figs. 15–17).

Colonies sterile, circular with entire margins, convex, lavender gray (43″′f) to fuscous black (7″″k), reverse fuscous black (7″″k) with olivaceous buff margin (21″″d). Dense, velvety superficial mycelium; colonies 4.5 mm diam on MEA after 35 days at 25°C in the dark.

Etymology. In reference to the host genus, "Protea."


Notes. "Rhynchostoma proteae" can be distinguished easily from "Rhynchostoma" species known to date. The type of "Rhynchostoma, R. minutum" P. Karst., has asci of similar dimensions (30 × 8 μm) but smaller ascospores (6–8 × 3–4 μm) (Saccardo 1882, Yue and Eriksson 1986, Winka and Eriksson 2000). Furthermore, "R. minutum" has a remarkably strong red pigment at the apex of the neck, which is absent in "R. proteae." In contrast, "R. exasperans" P. Karst. has comparable ascospore dimensions (9–12 × 4–5 μm) but much larger ascus (65 × 6 μm) (Saccardo 1882). Ascospores of "R. cornigerum" P. Karst. (8–10 × 3–4 μm) are similar to those of "R. proteae," but the overall size of its ascomata, including the ostiolar neck (about 600 μm), is smaller than that of "R. proteae" (Saccardo 1882).


Ascomata nonstromatic, perithecioid, superficial among trichomes, with the base immersed, separate, gregarious, globose to subglobose, up to 80 μm high and 90 μm wide, with an ostiolar neck. Ostiolar neck central, single, cylindrical, tapering off toward the apex, consisting of longitudinally angular cells, up to 290 μm high and 30 μm wide at the base, and 23 μm wide at the apex (Fig. 18). Paraphyses hyaline, unbranched, filamentous, flexuose, septate, scantly, 1–2 μm wide. Ascii lining the perithecial wall, unitunicate, octosporous, fusiform, apedicellate, relatively persistent, indistinguishable when ascospores are fully developed, 50–60 × 6–8 μm, no apical apparatus observed (Fig. 19). Ascospores overlapping, multiserial, bicellular, ellipsoid to fusiform, constricted at the septum, septum slightly submedian, ratio of upper to lower cell 1–2.1:1, each end tapered, hyaline to pale brown when young, becoming medium brown at maturity, obliquely striated all over spore surface in one direction, striations visible even when immature, germ pore inconspicuous, (12–)12.5–13 (–14) × (3–)4(–4.5) μm (av. 12.9 ± 4 μm) (Figs. 20–22).

Specimen examined. AUSTRALIA, TASMANIA: Mount Franklin, on leaves of "Grevillea" sp. (Proteaceae), 20 Nov 1952 [HOLOTYPE, BPI618690].

Notes. Paraphyses were sparse in this specimen, but they might have disintegrated with time. Although not mentioned in the original description (Petrak 1954), ascospores were found to be striate.

"Rhynchomeliola lomatiae" S. Lee et Joanne E. Taylor, sp. nov.

Figs. 23–28

Ascomata nonstromatic, perithecioid, superficialia inter trichomata, base immersa, distincta, gregaria, globose vel subglobose, usuque ad 100 μm alta, usuque ad 95 μm lata, collo ostiato. Collum ostiolum centrale et solum, cylindricum, contractum ad apicem, usuque ad 400 μm altum,

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Ascomata nonstromatic, perithecioid, superficial among trichomes, with the base immersed, separate, gregarious, globose to subglobose, up to 100 μm high and 95 μm wide, with an ostiolar neck (Figs. 23, 24). Ostiolar neck central and single, cylindrical, tapering off toward the apex, consisting of longitudinally angular cells, up to 400 μm high, 30 μm wide at the base, and 20 μm wide at the apex (Fig. 25). Paraphyses hyaline, filamentous, flexuous, septate, sparse, 1–2 μm wide. Asci lining the perithecial wall, unitunicate, octosporous, fusiform, apedicellate, relatively persistent, indistinguishable when ascospores are fully developed, 45–50 × 7–8 μm, no apical apparatus observed. Ascospores overlapping multiseriate, bicellular, ellipsoidal, constricted at the septum, septum slightly submedian, ratio of upper and lower cell 1:1.2:1, each end tapered, hyaline to pale brown when young, becoming medium brown at maturity, verruculose, germ pore inconspicuous, (8–)
9–10(–11) × (2–)3(–4) μm (av. 9.5 × 3 μm) (Figs. 26–28).

Etymology. In reference to the host genus, Lomatia. Specimen examined. AUSTRALIA, TASMANIA: Mountfield National Park, on leaves of Lomatia polymorpha R. Br. (Proteaceae), 5 Mar 1951, type of Apogloeum concinnum and Rhynchomeliola lomatiae [HOLOTYPE, BP1110296].

Notes. Rhynchomeliola lomatiae differs from R. australiense in ascospore ornamentation, verruculose in the former and striate in the latter. Another species with verruculose ascospores is R. usterriana (Speg.) Arx & E. Müll., which is similar to R. lomatiae but has larger ascospores (13–18 × 3.5–5.5 μm) (Müller and von Arx 1962).

Sequence and phylogenetic analyses.—The small-subunit dataset contained 31 sequences, including two outgroups (TreeBASE study accession number S854). After the introduction of gaps, the alignment included 1375 nucleotide positions. Three areas with major insertions were excluded from the analysis. The first insertion (spanning characters 134–188), as well as the second insertion (characters 281–335), was present only in Rhynchostoma minutum AF242268. The third excluded region spanned characters 884 to 1296 and contained insertions present from Rhynchostoma proteae (263 nucleotides), Rhynchostoma minutum AF242268 (279 nucleotides) and Ascolacicolota australica AF242263 (371 nucleotides). Of the remaining 852 characters, 575 characters were constant, 119 were parsimony uninformative and 160 characters were parsimony informative.

Neighbor-joining analysis of the dataset, using uncorrected (‘p’), Jukes-Cantor (Fig. 29) and Kimura-2-parameter models, placed Rhynchostoma proteae and Rhynchostoma minutum with 100% bootstrap support as a sister group in the Chaetothyriales. Parsimony analysis of the small-subunit alignment yielded only two equally parsimonious trees (TL = 581 steps, CI = 0.623, RI = 0.761, RC = 0.474) and supported the placement of Rhynchostoma proteae as provided by neighbor-joining analysis (data not shown). The topology of the two parsimony trees were identical to the NJ tree, except for a polytomy involving Pullularia prototropha X91898 and Cataponia mansonii X79318. Similar bootstrap values were obtained for both parsimony and NJ analyses. BLAST results of the ITS, large- and small-subunit sequences also confirmed the association between Rhynchostoma proteae and the Chaetothyriales.

DISCUSSION

Six species have been included in the genus Rhynchomeliola since its introduction. They are all foliicolous and distributed in the Southern Hemisphere, whereas the approximate 27 species of Rhynchostoma are mostly lignonicolous or corticolous and have a worldwide distribution. Twenty of these species have been included in lists of Saccardo and Petrak, with three having been allocated to other genera.

Müller and von Arx (1962) placed Rhynchostoma in the Diatrypaceae (Sphaeriales) and Rhynchomeliola in the Sphaeriaceae (Sphaeriales), a family to which both genera later were allocated (Müller and von Arx 1973). The segregation between Rhynchostoma and Rhynchomeliola was based respectively on the presence or absence of stromatic tissue. Stromatic structures of Rhynchostoma had not been recognized before the treatment of Müller and von Arx (1962), who recognized immersed ascomata growing out of a stroma in the substratum. Species of Rhynchostoma vary from having immersed to superficial perithecia, while species of Rhynchomeliola form their ascomata superficially on a subiculum or on the leaf surface among trichomes, having a partly immersed base. Ascomata of Rhynchomeliola lomatiae and R. australiense are formed superficially among the trichomes, with the base slightly immersed (Figs. 18, 23–25), while those of Rhynchostoma proteae mostly are found deeply imbedded between subepidermal cells (Figs. 1–4). This feature is variable, however, because a few ascomata of R. proteae also were found to occur superficially on host tissue (Fig. 2).

Barr (1990) generally accepted the concept of Müller and von Arx (1962) and classified Rhynchostoma in the Bolineaceae (Xylariales), while Rhynchomeliola was omitted from her monograph of Hymenoascomycetes. Barr (1990) characterized Rhynchostoma as having ascomata immersed in stroma, forming numerous paraphyses and 1–2-celled ascospores with a minute germ pore at one end. The stroma of Rhynchostoma were described as poorly developed and reduced to a narrow, gelatinized outer layer over brownish, interwoven hyphae, which covered the widely erumpent and beaked ascomata (Barr 1990). Although the stromatic tissue around the venter of ascomata of Rhynchostoma proteae was not clearly visible, vertical sections through these perithecia revealed the stroma to have separated from the peridium, yet the peridium appeared to be complete (Fig. 6).

Hawksworth et al (1995) tentatively placed both Rhynchostoma and Rhynchomeliola in the Trichosphaeriaceae (Trichosphaeriales), of which the genera are characterized by nonstromatic perithecial ascomata with a periphysate ostiole, presence of paraphyses and cylindrical asci with a nonamyloid apical ring and hyaline to brown, sometimes septate ascospores. Eriksson (1984, 1999) placed both genera in the Trichosphaeriae (Trichosphaeriales) but questioned the placement of Rhynchostoma in this family.
Fig. 29. Phylogenetic placement of Rhynchostoma proteae (in bold) inferred from SSU nrDNA sequence data. The distance tree was generated using neighbor-joining analysis with Jukes-Cantor distance correction. The numbers at the branch nodes indicate the confidence value (values higher than 50%) obtained from bootstrap analysis using 1000 replications. Vertical bars indicate morphological affinity.
Both *Rhynchomeliola* and *Rhynchostoma* share one or more characteristics that fit into the hitherto mentioned families. However, they do not exactly correspond to them in stromatic structure, ascal shape and the presence of an ascospore germ pore.

Analyses of sequence data of SSU nrDNA of *Rhynchostoma proteae* revealed an unexpected result, which placed this species as a sister clade to the Chaetothyriales (Chaetothyriomycetes), along with *R. minutum*, with 100% bootstrap support (Fig. 29). The taxon is distant from any pyrenomycetous clades in which *Rhynchostoma* is supposed to belong in the sense of Müller and von Arx (1962, 1973), Barr (1990) and Hawksworth et al (1995). Sequence data of large-subunit and ITS regions confirmed the relatedness of *Rhynchostoma* to the Chaetothyriales. This result confirms a similar finding based on a phylogenetic analysis of SSU nrDNA sequence data of the type species, *R. minutum*, which clustered within the Chaetothyriomycetes clade, providing the foundation for the new family Rhynchostomataceae Winka & O.E. Erikss. (Winka and Eriksson 2000).

The phylogenetic status of *Rhynchomeliola* based on molecular data could not be investigated because both cultivation and direct DNA extraction from herbarium specimen were unsuccessful.

The order Chaetothyriales Bat. & Cif. ex M.E. Barr, traditionally known as a member of loculoascomycetous fungi, was restored to accommodate the fungi that occasionally form locules in a stroma and have a hamathecium composed of short periphysoids and bitunicate asci (Barr 1987). In contrast, perithecia of both *Rhynchostoma proteae* and *R. minutum* form in a rudimentary stromatic structure, have distinct paraphyses observed in an early stage of ascomatal development, which sometimes extend over the asci, and have thin-walled, unitunicate asci. The appearance of *Rhynchostoma* species with the Chaetothyriales reflects the sister-group relationship of the Chaetothyriomycetes and the Eurotiomycetes, which lacks any morphological support (Winka et al 1998, Liu et al 1999, Tehler et al 2000). Lindemuth et al (2001) suspected the possibility of long-branch attraction based on accelerated substitution rate in the sister-group relationship of the Chaetothyriomycetes and Eurotiomycetes, but statistical data rejected their hypothesis. They eventually concluded that additional morphological research was required in these two groups. To resolve the puzzle of *Rhynchostoma*, a multiple gene molecular study, combined with ultrastructural and developmental studies of perithecia in *Rhynchostoma* would be required to shed further light on the related position.

Eriksson et al (2001) maintained the Rhynchostomataceae in the Chaetothyriomycetes as a family of uncertain position, while leaving *Rhynchomeliola* as a member of the Trichophaeraceae in the Sordariomycetes. Kirk et al (2001) also mentioned the Chaetothyriales connection of *Rhynchostoma* and *Rhynchomeliola* in the Sordariomycetidae. In this paper, the genera are treated sensu Müller and von Arx (1962), with the separation of *Rhynchostoma* and *Rhynchomeliola* based on stromatic structures. The affinity between two genera based on molecular data could not be examined due to the lack of information on *Rhynchomeliola*. It is apparent, however, that the two genera are almost identical in morphology, having rostrate ascomata, thin-walled unitunicate asci, paraphyses and ascospores with surface ornamentation. The only difference thus lies in their habit, being imbedded in a stroma or on a subiculum. Fresh collections on *Rhynchomeliola* species with molecular data might help further investigation of its generic relationship with *Rhynchostoma* and familial placement.

Mature ascospores of *Rhynchostoma proteae* emerge through the ostiolar neck and gather at the tip or around the neck in an outer amorphous crust that acts like glue (Figs. 3, 5). This is accepted as a mechanism of insect-dispersed fungi, in which they make spores available for dissemination. Many insects are known to inhabit the inflorescences of *Protea* species (Coetzee and Giliomee 1987a, b, Coetzee 1989, Wright 1990, Visser 1992), but at this stage it still is unknown which insects might act as vectors. No red stain was seen at the apex of perithecial necks, as reported for *R. minutum* (Yue and Eriksson 1986). It is suspected that this pigment might play a role in insect attraction (O. E. Eriksson, pers comm). The availability of nutrients and the protective and moist environment, make flowerheads an ideal niche for flourishing fungal populations. Finding the insect vectors of these fungi will add greatly to our current meager knowledge of *Protea* flowerhead ecology and to the ecological processes within the fynbos biome as a whole. It is interesting to note that we could find *Rhynchostoma proteae* only in the Stellenbosch Mountains (Stellenbosch, South Africa) and in two *Protea* species, whereas ophiostomatoid fungi were found in several other, far distant, areas and in various proteas. The role of *Rhynchostoma proteae* in the relationship between fungal and insect communities within this niche and the microclimate within the inflorescences might be another exciting area for study.

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