Molecular phylogeny of two coelomycetous fungal genera with stellate conidia, Prosthemium and Asterosporium, on Fagales trees

Kazuaki Tanaka, Vadim A. Mel’nik, Maasa Kamiyama, Kazuyuki Hirayama, and Takashi Shirouzu

Abstract: Prosthemium (teleomorph Pleomassaria) and Asterosporium (teleomorph unknown) are coelomycetous genera with stellate conidia on Fagales trees. Their morphological resemblance suggests their close relationship, but phylogenetic relatedness remains unknown. They have been distinguished on the basis of either conidiomatal morphology (pycnidia in Prosthemium and acervuli in Asterosporium) or their differing conidial septation (euseptate in Prosthemium and distoseptate in Asterosporium). To reveal their phylogenetic affinities and clarify reliable distinguishing phenotypical characters, five species of Prosthemium and two species of Asterosporium were investigated using sequences of the small subunit, large subunit, and internal transcribed spacer region of nuclear ribosomal DNA and β-tubulin gene from 43 isolates of these species. The analyses revealed the following: (i) Asterosporium typified by Asterosporium asterospermum on Fagus is a member of the Sordariomycetes and is distinct from Prosthemium belonging to the Dothideomycetes; (ii) Asterosporium betulinum on Betula species should be excluded from Asterosporium and transferred to Prosthemium as Prosthemium neobetulinum nom. nov.; (iii) conidial septation does not seem to have a diagnostic value, whereas conidiomatal morphology is useful in distinguishing both genera; (iv) the number and length of conidial arms are useful criteria in distinguishing Prosthemium species. A new species, Prosthemium intermedium sp. nov., found on both Betula ermanii and Betula maximowicziana, is described and illustrated.

Key words: anamorphic fungi, Ascomycetes, Diaporthales, Pleomassaria, Pelosporales.

Introduction

During our recent studies of coelomycetous fungi in Japan and Russia (Endo et al. 2008; Hatakeyama et al. 2008; Sato et al. 2008; Shabunin et al. 2008; Yonezawa and Tanaka 2008; Kamiyama et al. 2009), a number of anamorphic species with star-shaped conidia were encountered on twigs of Fagales trees such as Alnus, Betula, and Fagus. Morphological studies of these fungi suggested that they are members of either Prosthemium or Asterosporium. These genera share...
several morphological and ecological features, but their phylogenetic relatedness is unknown. *Prosthemium* species have *Pleomassaria* tele morphs belonging to the Pleosporales (Dothideomycetes) (Sivanesan 1984; Hantula et al. 1998; Paavolainen et al. 2000; Tanaka et al. 2005), and their familial affiliations have been discussed by several authors (Liew et al. 2000; Lumbsch et al. 2000; Lumbsch and Lindemuth 2001; Schoch et al. 2006). In contrast, no tele morph information or DNA sequence data exists to suggest the phylogenetic placement of *Asterosporium* species.

Traditionally, these two genera have been differentiated by their conidiomatal morphology, with pycnidia in *Prosthemium* and acervuli in *Asterosporium* (Saccardo 1884; Morgan-Jones and Kendrick 1972). Sutton (1980), however, regarded the conidiomata of *Prosthemium* as acervuli or eu stromata and preferred to use conidial septation as the distinguishing feature between the two genera, where *Asterosporium* is distoseptate and *Prosthemium* is euseptate. These discrepancies in morphological circumscription were probably sources of taxonomic confusion. In fact, one species of *Asterosporium*, *Asterosporium orientale*, originally described from twigs of *Betula ermanii* in Russia (Mel’nik 1988), has been recently transferred to *Prosthemium* on the basis of its similarities to the type species of *Prosthemium* (*Prosthemium betulinum*) rather than the type of *Asterosporium*, *Asterosporium asterospermum* (Kamiyama et al. 2009).

*Prosthemium* species have been mainly reported as endo phytes or phelophytes from twigs of *Betula* and *Alnus* in Betulaceae (Kowalski and Kehr 1992, 1996). Although seven taxa have been previously recognized in the genus (Saccardo 1884, 1895, 1899, 1906; Kowalski and Holdeni ried 1996; Tanaka et al. 2005; Kamiyama et al. 2009), the boundaries between these species are not necessarily clearly defined. For example, *Prosthemium stellare* has conidia with 10–14 radiating arms equally developed (Sivanesan 1984), but *Prosthemium orientale*, characterized typically by 4-armed conidia, also rarely produces up to 10-armed conidia (Kamiyama et al. 2009). *Prosthemium canba* was introduced as a distinct species primarily based on its largest conidial arm (Tanaka et al. 2005), but later some reduced conidia resembling those of *P. betulinum* were observed in culture (K. Tanaka, personal observation). Furthermore, an unnamed *Prosthemium* sp. showing conidial morphology intermediate between *P. orientale* and *P. canba*, was also found in our recent survey. To evaluate the monophyly of *Prosthemium* taxa that possess a continuum of morphological characters, a molecular phylogenetic analysis was much needed.

This study was undertaken with three objectives: (i) to infer the taxonomic placement of *Asterosporium* species and to clarify their phylogenetic affinities to *Prosthemium* species using molecular data from the small and large subunit nuclear ribosomal DNA (SSU and LSU nrDNA, respectively); (ii) to determine reliable morphological characters as taxonomic criteria for the separation of these genera; and (iii) to evaluate the species validity of each *Prosthemium* taxon, including an unidentified *Prosthemium* sp., based on sequence analyses of the internal transcribed spacer 5.8S nrDNA (ITS) and the β-tubulin gene (*BT*).

**Materials and methods**

**Morphological studies and fungal isolates**

Collections of *Prosthemium* and *Asterosporium* were made from woody plants such as *Alnus, Betula*, and *Fagus*, primarily in Japan and Russia. Voucher specimens were deposited in the herbaria of Hirosaki University (HHUF) and Komarov Botanical Institute (LE) (Table 1). Methods of morphological observation used are described by Tanaka et al. (2009). Single-spore cultures were obtained according to the methods of Shearer et al. (2004). To validate isolations, the induction of conidiomatal formation was encouraged by placing a small piece of mycelial culture on rice straw agar (Tanaka and Harada 2003). Fungal cultures newly obtained in this study were deposited at the Japan Collection of Microorganisms (JCM); the Ministry of Agriculture, Forestry, and Fisheries, Japan (MAFF); and the Centraalbureau voor Schimmelcultures (CBS).

**DNA extraction and amplification**

A total of 43 isolates, including six strains obtained from CBS and one herbarium specimen, were used for DNA extraction (Table 1). DNA from mycelia was extracted using the ISOPLANT Kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer’s instructions. Partial SSU (17 isolates, ca. 1000–1300 bp of the 5′ end) and LSU nrDNA (43 isolates, ca. 1250 bp of the 5′ end) were determined to elucidate familial and generic positioning. The complete ITS region of nrDNA (ca. 500 bp), and exons 1–6 and the respective introns of the *BT* gene (ca. 600 bp) were sequenced (37 isolates) to confirm generic and species-level placements (Table 1). Four primer sets, NS1–NS4 (White et al. 1990), LR0R–LR7 (Rehner and Samuels 1994), ITS1–ITS4 (White et al. 1990), and T1–BT2B (Glass and Donaldson 1995; O’Donnell and Cigelnik 1997), were used for the amplification of SSU, LSU, ITS, and *BT*, respectively. DNA was amplified and sequenced according to the methods described by Tanaka et al. (2009). Newly obtained sequences were deposited in GenBank (Table 1).

**Sequence analysis**

SSU and LSU sequences of *Prosthemium* and *Asterosporium* were aligned along with those of other related species obtained from GenBank. To clarify their validity at the species level, the alignments of ITS and *BT* sequences were also generated. A combined data set of ITS + *BT* was used for the analysis, because the phylogenetic resolution from each ITS and *BT* data set was relatively low. Preliminary multiple alignments of sequences were generated using MAFFT version 6 (Katoh and Toh 2008; mafft.cbrc.jp/ alignment/software). Final alignments were manually adjusted using BioEdit version 7.0.8 (Hall 1999). Alignment gaps and ambiguous positions were excluded from the analysis. All alignments used in this study were deposited in TreeBASE (www.treebase.org). Maximum parsimony (MP) analyses were carried out using PAUP version 4.0b10 (Swofford 2003). MP analyses with the heuristic search option using the tree bisection-reconnection (TBR) algorithm with 1000 random sequence additions were performed to find the global optimum tree. All sites were treated as unordered and unweighted. Neighbor-joining (NJ) analyses based
on the Kimura two-parameter substitution model were carried out using MEGA version 4 (Tamura et al. 2007). Characters were weighted equally. Bootstrap support (BS) values for nodes were computed from 1000 replicates for both the MP and NJ analyses. Bayesian analyses were done using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). MrModeltest version 2.3 (Nylander 2004), in conjunction with PAUP version 4.0b10 (Swofford 2003), was used to select substitution models for Bayesian analyses. On the basis of the Akaike information criterion of MrModeltest version 2.3, a general time-reversible, invariant, \( \gamma \)-distributed (GTR+I +G) model was applied to the LSU data sets of Prosthemium and Asterosporium. In the ITS + BT data sets of Prosthemium species, the symmetrical invariant (SYM + I) and Hasewaga–Kishino–Yano invariant (HKY + I) models were applied to ITS and BT sequences, respectively. Two runs with 10 chains of Markov chain Monte Carlo (MCMC) iterations were performed for 4.5 million generations of the LSU data set of Prosthemium, 1.3 million generations of the LSU data set of Asterosporium, and 3 million generations of the ITS + BT data sets of Prosthemium species, keeping one tree every 100 generations. The runs were deemed to have converged if the mean standard deviation of split frequencies became less than 0.01. The first 3, 0.3, and 2 million generations of the LSU of Prosthemium, the LSU of Asterosporium and ITS + BT of Prosthemium species were discarded as burn-in, and the remaining 20 002 trees were used to calculate 50% majority rule trees and to determine the posterior probabilities (PP) for the individual branches.

Results

Phylogenetic analyses

SSU phylogeny

An SSU alignment consisting of 18 sequences of Prosthemium/Asterosporium and 40 sequences retrieved from GenBank, after excluding insertions in Lecanora hybocarpa (DQ782883; 257–361) and Geoglossum nigritum (AY544694; 485–877), resulted in a 953 character data set with 325 (34.1%) variable sites. The NJ tree generated from this alignment showed that all Prosthemium species and one species of Asterosporium, Asterosporium betulinum (not \( \text{betulinum} \)) formed a strongly supported monophyletic group (0.99 PP and 100% BS) and grouped within the Diaporthales. The clade of \( \text{Asterosporium} \) was sister to the main diaporthalean families, except for the Togniniaceae, and this relationship received strong support (1.00 PP, 98%–99% BS) (Fig. 2).

ITS + BT phylogeny

To clarify the species validity and boundaries of Prosthemium species, a combined alignment of ITS + BT from 37 taxa of Prosthemium species and \( \text{A. betulinum} \) was generated. A Pleosporales sp. (HC27033), closely related to Prosthemium (Fig. 1), was used for the outgroup. Out of 1014 characters, 177 (17.5%) and 83 (8.2%) were variable and parsimony-informative, respectively. An MP analysis resulted in three equally parsimonious trees with a length of 212 steps (consistency index = 0.9245, retention index = 0.9497) (Fig. 3). The trees obtained from NJ and Bayesian analyses had a topology identical to that of the MP tree. All species formed distinct monophyletic lineages with strong or moderate statistical support (>0.95 PP and 81%–100% BS) in all analyses, with the exception of the Prosthemium sp.; this was without PP support (<0.95). Prosthemiumstellare, a parasite on Alnus, was sister to the clade consisting of species mainly occurring on Betula. Asterosporium betulinum was in the basal lineage of a large group containing \( \text{P. canba}, \text{P. orientale}, \text{P. betulinum} \), and the Prosthemium sp. (Fig. 3).

Taxonomy

Conidiomata of Prosthemium and Asterosporium were sectioned with a freezing microtome and their morphology compared (Figs. 4–9). As expected, all conidiomata of Prosthemium species were globose to subglobose pycnidia with a circular ostiule (Figs. 6–9). The type species of Asterosporium (\( \text{A. asterospermum} \)) had typical flattened acervuli with a wide opening (Fig. 4), as was noted in several previous reports (Morgan-Jones and Kendrick 1972; Sutton 1980; Kobayashi and Kubono 1986; Prěšil and Rěblová 1995). Conidiomata of \( \text{A. betulinum} \) were pycnidial with a wide ostiule of more than 100 \( \mu \text{m} \) diameter (Fig. 5). Conidial septation was considered euseptate in all species (Figs. 10–15). A distinct central cell connecting each conidial arm was found in all Prosthemium species as well as in \( \text{A. betulinum} \), whereas the central cell of \( \text{A. asterospermum} \) was indistinct.
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<td>B. ermanii</td>
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<td>AB553777</td>
<td>AB554107</td>
<td>AB554144</td>
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<tr>
<td><strong>Pleosporales sp.</strong></td>
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<td>—</td>
<td>HHUF 27033</td>
<td>F. crenata</td>
<td>Japan</td>
<td>AB553783</td>
<td>AB554113</td>
<td>AB554150</td>
<td>A</td>
</tr>
</tbody>
</table>

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*a* Generic names of host plants: *A.* *Alnus;* *B.* *Betula;* *F.* *Fagus;* *P.* *Pterocarya,* *S.*, *Salix.*

*b* GenBank accession No. in parentheses indicates a sequence obtained from a previous work (Schoch et al. 2006).

*c* Origin of isolates: A, single ascospore; C, single conidium.
Morphologically, the *Prosthemium* sp. and *A. betulinum* were close to the generic type of *Prosthemium*. Our phylogenetic analyses revealed that these constitute a separate lineage within the genus. Therefore, these two taxa are described below as new *Prosthemium* species. Some morphological and phylogenetic characters of existing species in *Prosthemium* are also noted below.

*Prosthemium intermedium* Kaz. Tanaka & Melnik, sp. nov. (Figs. 16–29)

**Mycobank accession no.** MB 518629.

**Teleomorph:** *Pleomassaria* sp.

**Etymology:** In reference to the intermediate morphology of conidia between *P. canba* and *P. orientale*.

Conidiomata pycnidioidea, 490–560 μm alta, 920–1180 μm diametro, unilocularia, immersa, sparsa vel gregaria, depresso-globosa vel globosa. Paries conidiomatis "textura angularis," 20–30(–45) μm crassus ad latus et basim. Conidiophora ad 300 μm longa, 2–4 μm lata, simplicia, septata, hyalina. Cellulæ conidioeæ holoblasticae. Conidia stellata, ex 4 brachis obclavatis composita, echinulata, brunnea, ad cellulam apicales pallidiora; brachium conidia stellata, ex 4 brachiis obclavatis composita, echinulata, brunnea, ad cellulam apicales pallidiora; brachium longissimum 57–73 μm × 16.5–23 μm (mean = 63.4 μm × 19.6 μm, n = 60), 4–5-eusep'tatum; brachium altitud (37.5–) 40–55.5(–60) μm × (11–)13–19.5 μm (mean = 46.3 μm × 16.0 μm, n = 60), 3–4-eusep'tata.

**Description:** Conidiomata pycnidial, 200–280 μm alta, 750 μm diametro, unilocularia, immersa, scatellata, euseptata, hyalina. Cellulæ conidioeæ holoblasticae. Conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium longissimum 57–73 μm × 16.5–23 μm (mean = 63.4 μm × 19.6 μm, n = 60), 4–5-eusep'tatum; brachium altitud (37.5–) 40–55.5(–60) μm × (11–)13–19.5 μm (mean = 46.3 μm × 16.0 μm, n = 60), 3–4-eusep'tata.

**Description:** Conidiomata pycnidial, 490–560 μm alta, 920–1180 μm diametro, unilocularia, immersa, scatellata, eusep'tata, hyalina. Cellulæ conidioeæ holoblasticae. Conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium longissimum 57–73 μm × 16.5–23 μm (mean = 63.4 μm × 19.6 μm, n = 60), 4–5-eusep'tatum; brachium altitud (37.5–) 40–55.5(–60) μm × (11–)13–19.5 μm (mean = 46.3 μm × 16.0 μm, n = 60), 3–4-eusep'tata.

**Mycobank accession no.** MB 518630.

**Teleomorph:** Unknown

**Description:** Conidiomata pycnidial, 200–280 μm alta, 580–750 μm diametro, unilocularia, immersa, scatellata, eusep'tata, hyalina. Cellulæ conidioeæ holoblasticae. Conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium conidia stellata, hyalina, ad cellulam apicales pallidiora; brachium longissimum 57–73 μm × 16.5–23 μm (mean = 63.4 μm × 19.6 μm, n = 60), 4–5-eusep'tatum; brachium altitud (37.5–) 40–55.5(–60) μm × (11–)13–19.5 μm (mean = 46.3 μm × 16.0 μm, n = 60), 3–4-eusep'tata.

Fig. 1. Majority rule consensus tree of Prosthemium species within the Pleosporales (Dothideomycetes) from Bayesian analysis based on LSU nrDNA sequences (1220 bp). Bayesian posterior probabilities (PP) above 0.90 and maximum parsimony (MP) and neighbor-joining (NJ) bootstrap values greater than 70% are indicated at the nodes as PP/MPBS/NJBS. A hyphen (‘‘-’’) indicates values lower than 0.90 (PP) or 70% bootstrap support (BS), and a node not present in an analysis is shown with ‘‘x’’. A thick line is used for a clade with high statistical support (more than 1.00 PP and 90% BS). Either GenBank accession Nos. or the original isolate numbers are noted after the species names. The tree was rooted to Lophium mytilinum (Mytilinidiales) and Rhytidhysteron rufulum (Hysteriales).
2 October 2008, VM 20081002 (LE 230931 = HHUF 30040; monoconidial isolate CBS 126960).

NOTES: This species is characterized by its relatively small conidia (40.4 μm mean diameter) that consistently have 4 arms. It appears in the most basal position within the clade of species that occur on *Betula*. Since its discovery (Peck 1880), it has been treated as a species of *Asterosporium* owing to the misinterpretation of its conidiomata as acervuli (Sutton 1980). Our analyses of sequences from SSU, LSU, ITS, and the BT gene, obtained from two isolates, clearly indicate that this species belongs within *Prosthemium*.

*Prosthemium betulinum* Kunze, Mykologische Hefte (Leipzig) 1:18, 1817. (Figs. 6 and 12)


NOTES: The unusual conidial morphology of *P. betulinum*, with its 4–5 unequally developed arms (the largest arm is 40–54 μm × 14–20 μm; Hantula et al. 1998), is easily recognized on twigs of *Betula* (Kunze 1817; Clements and Shear 1931; Morgan-Jones and Kendrick 1972). However, the species might sometimes have been confused with *P. orientale*, as illustrated by Truskowska and Chlebicki (1983), mainly on account of sharing the same host genus and having a similar length of the longer conidial arm. Five isolates of this species clustered in a distinct and well-supported clade in the combined tree (99%–100% BS and 1.00 PP; Fig. 3), as well as in the analyses of individual data sets of ITS (85%–89% BS) and BT (97%–98% BS) (data not shown).
Prosthemium canba Kaz. Tanaka, Y. Harada & M.E. Barr, Mycoscience 46: 253, 2005 (Figs. 7 and 13)

TELEOMORPH: Pleomassaria sp.

NOTES: This species was originally described from B. ermanii solely based on morphological features such as conidia with their longer arm (73.2 μm × 16.9 μm), and an additional 2 or 3 arms unequally developed (Tanaka et al. 2005). In culture, abnormal, reduced conidia recalling those of P. betulinum were sometimes observed. However, in our phylogeny, P. canba was distinct from P. betulinum (Fig. 3). Although the monophyly of P. canba was weakly supported in all analyses using ITS or BT sequences (less than 70% BS), it received moderate support in the ITS + BT tree (81%–87% BS and 0.97 PP; Fig. 3).

NOTES: Morphologically, this species is similar to P. betulinum, but differs in that the conidia have 4 equally developed arms (Kamiyama et al. 2009). This fungus was previously known as A. orientale (Mel’nik 1988; Mel’nik et al. 2001)
Figs. 16–29. Prosthemium intermedium. Figs. 16 and 17. Conidiomata on host surface. Fig. 18. Exuded conidia from conidioma. Fig. 19. Pycnidium in longitudinal section (arrows indicate clypeus-like structure around ostiole). Fig. 20. Wall of pycnidium with brown hyphae. Figs. 21–23. Conidia with 4 arms (arrows indicate central connecting cell of conidia). Fig. 24. Abnormal conidium with 8 arms. Fig. 25. Developing conidium. Fig. 26. Surface of conidium with verrucose ornamentation. Fig. 27. Euseptate conidium bleached by 5% sodium hypochlorite solution. Fig. 28. Germinating conidium. Fig. 29. Ascus and ascospores. Figs. 16–28. From HHUF 30063 (holotype); Fig. 29 From HHUF 30062. Scale bars = 1 cm (Fig. 16); 1 mm (Fig. 17); 100 μm (Figs. 18 and 19); 10 μm (Figs. 20 and 25–27); 20 μm (Figs. 21–24, 28, and 29).
or *Prosthemium asterosporum* (Kowalski and Holdenrieder 1996; Barengo et al. 2000; Paavolainen et al. 2001) in Europe. It formed a robust clade (100% BS and 1.00 PP) in our analyses, based on six isolates, including an ex-type (CBS 431.96) of *P. asterosporum* (Fig. 3).

**Prosthemium stellare** Riess, Bot. Ztg. 11: 130, 1853 (Figs. 9 and 15)

**Teleomorph:** *Pleomassaria holoschista* (Berk. & Broome) Sacc.

**Notes:** This quite distinctive species has relatively fewer pig-

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**Key to the species of Prosthemium**

1. a Conidia mostly 9–14 armed, occurring on *Alnus* .................................................. *P. stellare*
   1. b Conidia mostly 4–5 armed, occurring on *Betula* .................................................. 2
2. a Conidia almost less than 60 μm diameter (between widest points of conidial arms) ................. *P. neobetulinum*
   2. b Conidia more than 60 μm diameter ................................................................. 3
3. a Conidia with 4 equally developed arms ........................................................................... *P. orientale*
   3. b Conidia with unequally developed arms ..................................................................... 4
4. a Longer conidial arm is less than 55 μm long .................................................................. *P. betulinum*
   4. b Longer conidial arm is more than 55 μm long .......................................................... 5
5. a Conidia with a longer arm (L/W ca. 4.4) and 2 or 3 unequally developed arms .................... *P. canba*
   5. b Conidia with a longer arm (L/W ca. 3.3) and 3 equally developed arms ....................... *P. intermedium*

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**Discussion**

**Phylogenetic placements of Prosthemium and Asterosporium**

The type species of *Prosthemium* (*P. betulinum*; Kunze 1817) has been known to possess the pleosporalean ascomatal state of *Pleomassaria* (Tonolo 1956), a genus previously placed in the Pleomassariaceae (type *Pleomassaria siparia*; Barr 1982; Tanaka et al. 2005; Kirk et al. 2008). Sequence data from several genes (e.g., SSU, LSU, *RPB2*, *TEF1*) from one strain of *Pleomassaria siparia* (CBS 279.74) have been used for phylogenetic analyses within the Dothideomycetes (Liew et al. 2000; Lumbsch et al. 2000; Lumbsch and Lindemuth 2001; Schoch et al. 2006). In our analysis of LSU nrDNA data, all *Prosthemium* species formed a strongly supported monophyletic clade within the Pleosporales (Dothideomycetes) and nested within the Melanommataceae (Fig. 1). This familial placement of *Prosthemium* species is in agreement with the recent reassessment of the family by Mugambi and Huhndorf (2009) based on the LSU and *TEF1* gene. In this study, *Pleomassaria siparia* was basal to other members of Melanommataceae but had relatively low bootstrap support (Mugambi and Huhndorf 2009). Similar topology was obtained in the phylogeny based on the SSU, LSU, *RPB2*, and *TEF1* provided by Zhang et al. (2009), who synonymized the Pleomassariaceae with the Melanommataceae. On morphological grounds, however, *Pleomassaria* species possessing *Prosthemium* anamorphs are rather unique within the Melanommataceae because of their large-sized ascomata immersed in host tissue, large-sized muriform ascospores, and star-shaped conidia (Barr 1982; Tanaka et al. 2005). Further investigation based on additional molecular data using additional *Pleomassaria* taxa will be needed to resolve phylogenetic relationships within the Melanommataceae and to evaluate the validity of the Pleomassariaceae as a family for *Pleomassaria* species with *Prosthemium* anamorphs.

In contrast, there was no prior information about the ordinal or familial affiliations of *Asterosporium*. It has been suspected that the type species of the genus (*A. asterospermum*; Kunze 1819; Hughes 1958) has a teleomorph belonging to the Massariaceae (Wehmeyer 1926) or to *Asteromassaria macrospora* (Spooner and Kirk 1982), but no teleomorph has been found. In our study, SSU and LSU sequences from seven strains of two *Asterosporum* taxa were analyzed to clarify their affinities. The results clearly reveal that *Asterosporum* is polyphyletic. One species of *Asterosporum* on *Betula*, *A. betulinum*, is in a different clade from the type of *Asterosporum* and groups with *Prosthemium* with high statistical support (1.00 PP and more than 96% BS; Fig. 1). Therefore, *A. betulinum* is transferred to *Prosthemium* and given a new name, *P. neobetulinum*, to avoid creating a later homonym of *P. betulinum*. While, *A. asterospermum* is located in a clade within the Diaporthales (Sordariomycetes) (Fig. 2). A BLAST search using the LSU sequence of *A. asterospermum* suggested that the species is close to members of the Diaporthaceae, in particular *Diaportha pus- tulata* (AF408358), *Diaporthe padi* (AF408354), and *Diaporthe perjuncta* (AF408356). However, in the phylo-

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der are generally known to have non- or one-septate conidia, produced from phialidic or annellidic conidiogenous cells (Rossman et al. 2007).

**Morphological delimitation of Prosthemium and Asterosporium**

Traditionally, *Prosthemium* and *Asterosporium* have been distinguished on the basis of conidiomatal morphology and have been placed in artificial anamorphic groups known as the Sphaeroideae and the Melanconieae, respectively (Saccardo 1884; Lindau 1922; Clements and Shear 1931). The delimitation of these genera was accepted and their conidiomata regarded as pycnidia in *Prosthemium* and as acervuli in *Asterosporium* (Morgan-Jones and Kendrick 1972; Kendrick and Nag Raj 1979). However, Sutton (1973, 1980) interpreted the conidiomata of *Prosthemium* as acervular to eustromatic, without an ostiole but dehiscing by the irregular rupture of the overlying tissues. In a dichotomous key to genera of acervular fungi, Sutton (1973) used the presence (in *Prosthemium*) or absence (in *Asterosporium*) of a distinct basal cell connecting each conidial arm to distinguish these genera. Later, Sutton (1980) adopted conidial septation as a key character distinguishing the euseptate *Prosthemium* from the distoseptate *Asterosporium*.

We examined the morphological characters previously used for circumscribing *Prosthemium* and *Asterosporium*. In longitudinal section the conidiomata of *A. asterospermum* were obviously acervuli with flattened bases (Fig. 4), as illustrated by several authors (Morgan-Jones and Kendrick 1972; Sutton 1980; Kobayashi and Kubono 1986; Prašil and Rěbolvá 1995). All species in the *Prosthemium* lineage illustrated in our LSU tree (Fig. 1), including *A. betulinum* (= *P. neobetulinum*), had globose to subglobose pycnidia with circular ostioles (Figs. 5–9). In contrast to Sutton’s (1980) idea, our result clearly reveals that conidiomatal morphology has taxonomic significance for the delimitation of these genera. The pycnidia of *A. betulinum* (= *P. neobetulinum*; Fig. 5) had a tendency to become somewhat incomplete because of the collapse of the upper wall layer surrounding the ostiole. These might be misinterpreted as acervuli, as illustrated by Sutton (1980).

We do not believe that conidial septation has diagnostic value, as we consider all species treated here to have euseptate conidia, at least at the light microscope level. A similar opinion has been noted for these fungi by Kowalski and Holdenrieder (1996). However, the presence or absence of a distinct basal cell connecting the conidial arms might have taxonomic significance in distinguishing between *Prosthemium* and *Asterosporium*. All species in *Prosthemium* have conidia with a central cell connecting several conidial arms (Figs. 11–15), but this was not found in *A. asterospermum* (Fig. 10). This result supports Sutton’s (1973) circumscription of both genera, although later he did not use this character (Sutton 1980).

It appears that these genera can also be distinguished by host differences. Species of *Asterosporium* are restricted to *Fagus* (Fagaceae). With some exceptions, *Prosthemium* species are associated with *Alnus* and *Betula* (Betulaceae). *Prosthemium orientale* usually occurs on twigs of *Betula*. It has been recorded occasionally from other plant leaves such as those of *Carex* and *Salix*, but these examples are consid-

In our study, *P. betulinum*, a common fungus on *Betula* in Europe (Hantula et al. 1998; Paavolainen et al. 2000), was found on twigs of *Pterocarya rhoifolia* ( Juglandaceae) (Table 1), and formation of typical pycnidia was confirmed on this substrate. Whether *P. betulinum* occurs on *Pterocarya* as a natural (not occasional) host remains unknown, because the specimens were collected from *Pterocarya* neighboring *Betula* trees in a botanical garden; more collecting surveys are needed to confirm the host range.

To summarize the diagnostic features of these genera, *Prosthemium* is characterized by pycnidial conidiomata with circular or widely open ostioles, conidia with a central cell connecting several arms, and a host usually within the Betulaceae. *Asterosporium* is characterized by acervular conidiomata with flattened bases, conidia without an obvious central cell, and a *Fagus* host.

**Reconsideration of Prosthemium species**

Our LSU tree of *Prosthemium* strongly confirmed the monophyly of the genus (Fig. 1). However, species boundaries within the genus could not be clarified because of the low sequence variability. Similar results were obtained from the ITS analyses, and the BS values for each node were relatively low (data not shown). The analyses of the *BT* gene showed most species as distinct monophyletic lineages with higher bootstrap support, but *P. canba* received low support (less than 70% NJ and MPBS). Consequently, we analyzed the combined data set of ITS and *BT* sequences to determine species boundaries within the genus. The ITS + *BT* tree revealed six significantly supported monophyletic species within *Prosthemium* (Fig. 3). These were divided into two groups correlated with their host and conidial morphology. The most basal clade thus consisted of *P. stellare* (mostly 9- to 14-armed conidia and an *Alnus* host), and the remaining clade consisted of other species, mostly with 4- to 5-armed conidia and a *Betula* host. Among the clade of *Betula* parasites, species recognized by both number and length of conidial arms were shown to be separate phylogenetically. Therefore, these morphological characters are considered to be reliable taxonomic indicators in *Prosthemium*, although they are to some degree overlapping among these species.

**The challenge for holomorphic names**

All *Prosthemium* species except for *P. neobetulinum* form teleomorphs belonging to *Pleomassaria* in nature, but we have used their anamorphic names because the application of the holomorph names was somewhat complicated, particularly among pathogens of *Betula*. *Prosthemium stellare* on *Alnus* has the distinct teleomorph *Pleomassaria holo- schista*. This can be separated from other species in *Pleomassaria* by its relatively small ascospores (35–48 μm × 11–13 μm; Sivanesan 1984). *Prosthemium betulinum* and *Prosthemium orientale* both have teleomorphs within the *Pleomassaria siparia* species complex that cannot be distinguished by their ascospore morphologies (Paavolainen et al. 2000). Consequently, the teleomorphs have been provisionally referred to as *Pleomassaria siparia* type A (anamorph *P. orientale*) and type B (anamorph *P. betulinum*) without
formal nomenclature (Paavolainen et al. 2000; Tanaka et al. 2005). A similar situation was observed between *P. canba* and *P. intermedium*. We obtained several isolates of these species from their teleomorphs (Table 1, Fig. 29), but we did not establish their holomorphic identity, mainly because of a lack of plentiful specimens of the teleomorph. However, even if we could obtain teleomorphic specimens in good condition, it may remain difficult to distinguish the teleomorphs of *P. canba* and *P. intermedium*. The ascospores of these species tend to be somewhat larger (ca. 61–89 μm × 14–22 μm) than those of *P. betulinum* and *P. orientale* (the *Pleomassaria siparia* complex; 51–74 μm × 15–26 μm; Hantula et al. 1998), but those of *P. canba* and *P. intermedium* are almost identical. In addition to their teleomorphic similarity, *P. canba* and *P. intermedium* occur sympatrically on the same host (*B. ermanii*), and frequently even on the same twigs. Thus, we cannot decide whether a *Pleomassaria* species on a specimen represents a single species, unless we observe anamorphic states from each *Pleomassaria* fungus on the specimen. To characterize teleomorphs, it would be effective to observe ascostatal states induced by different mating pairs in culture. Apparently, all *Prosthemium* species used in this study are heterothallic, and their abilities to form ascomata in culture are unknown. Mating experiments have been successful within other dothideomycetous genera such as *Didymella* (Chilvers et al. 2009), *Leptosphaeria* (Shoemaker and Brun 2001), and *Mycosphaerella* (Mondal et al. 2004). Results from mating experiments could provide some taxonomic implications for the differentiation of teleomorphs among *Prosthemium* species.

**Acknowledgments**

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