Molecular phylogeny and systematics of *Polyblastia* (*Verrucariaceae, Eurotiomycetes*) and allied genera

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ABSTRACT

Phylogenetic relationships of the lichen genus *Polyblastia* and closely related taxa in the family *Verrucariaceae* (*Verrucariales, Chaetothyriomycetidae*) were studied. A total of 130 sets of sequences (nuLSU rDNA, nuITS rDNA and *RPB1* region A–D), including 129 newly generated sequences, were analysed. Phylogenetic relationships were inferred using a Bayesian approach based on two datasets. A first analysis of a larger, two-locus dataset (nuLSU and *RPB1*) for 128 members of the *Verrucariaceae*, confirmed the polyphyly of *Polyblastia, Thelidium, Staurothele,* and *Verrucaria,* as currently construed. The second analysis focused on 56 *Polyblastia* and allied taxa, but using an additional locus (nuITS rDNA) and two closely related outgroup taxa. The latter analysis revealed strongly supported groups, such as *Polyblastia s. str.,* the *Thelidium* group (a mixture of *Polyblastia, Thelidium, Staurothele* and *Verrucaria* species). The genus *Sporodictyon,* which is here accepted, also accommodates *Sporodictyon terrestre* comb. nov. Morphological features traditionally used for characterizing *Polyblastia, Thelidium, Staurothele* and *Verrucaria,* such as spore septation and colour, occurrence of hymenial photobiont, involucrellum structure, and substrate preference, were found to be only partially consistent within the strongly supported clades, and thus are not always reliable features for characterizing natural groups.

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Introduction

Polyblastia is a crustose genus of lichenized fungi belonging to the *Verrucariaceae* (*Chaetothyriomycetidae, Eurotiomycetes, Ascomycota*) (Lutzoni *et al.* 2004; Geiser *et al.* 2006; Gueidan *et al.* 2007). Species within this genus are rarely encountered and difficult to identify. Consequently, a consensus had not been reached as to how many species this genus may include. Kirk *et al.* (2001) reported *ca* 80 *Polyblastia* species worldwide. Most of the genera in *Verrucariaceae* are crustose; these include, e.g. *Polyblastia, Agonimia, Staurothele, Thelidium,* and *Verrucaria.* A few, rather small genera are reasonably well

known, such as the foliose genus *Dermatocarpon* (Heiðmarsson 2000, 2003; Amtoft 2006; Amtoft *et al.* 2008), and the squamulose genera *Catapyrenium* and *Placidiopsis* (Breuss 1985, 1990a, b, 1996). However, only a few molecular studies have been performed on the *Verrucariales*; at the infrageneric level (Amtoft 2006; Amtoft *et al.* 2008; Heiðmarsson 2003, on *Dermatocarpon*) and in the context of large-scale molecular phylogenies (Lutzoni *et al.* 2001, 2004; Lumbsch *et al.* 2002, 2004, 2005; Liu & Hall 2004; Del Prado *et al.* 2006; Geiser *et al.* 2006; James *et al.* 2006; Reeb *et al.* 2004; Spatafora *et al.* 2007).

Preliminary results from molecular studies indicated that *Polyblastia* (Savić 2004) and the main genera within the

Verrucariales (Gueidan *et al.* 2004) did not appear to be monophyletic. The most recent phylogenetic study on *Verrucariaceae* (Gueidan *et al.* 2007) presented a multigene phylogeny for the 15 main genera of *Verrucariaceae*, and compared the current morphology-based classification with a molecular-based phylogeny. It showed that *Polyblastia* was not monophyletic, but was part of one of ten monophyletic species groups recognized by these authors and referred to as the 'Polyblastia group'. In addition to three species of *Polyblastia* (including the type *P. cupularis*), this group also included four species of *Thelidium*, *Staurothele immersa*, and three species of *Verrucaria* (including the type *V. rupestris*). The study further demonstrated that *Verrucaria*, *Thelidium*, and *Staurothele* are polyphyletic, and that hymenial algae have evolved independently in at least three distinct lineages of the *Verrucariaceae*. The phylogeny presented in Gueidan *et al.* (2007) provided a framework for our in-depth investigation of *Polyblastia* and allied genera.

Traditional circumscription of the genera Polyblastia, Thelidium and Staurothele

Traditionally, *Polyblastia* has been characterised as having asci without well-differentiated apical apparatus; paraphyses that early dissolve into a gelatinous mass; pseudoparaphyses forming a cushion below the ostiole; muriform ascospores; a trebouxioid or other non-trentepohlioid, green photobiont; no algal cells in the hymenium; and lack of chemical substances in the thallus. This circumscription of *Polyblastia* was largely canonized by Zahlbruckner (1907, 1926) in a classification that heavily rested on cardinal characters (Tibell 1998), such as photobiont association, ascoma structure, and ascospore morphology. Thus, *Verrucariaceae* species with muriform ascospores and a hamathecium without hymenial algae were referred to as *Polyblastia*, although *Agonimia* and *Henrica* have been recognised as separate genera. The latter two genera have been distinguished from *Polyblastia* by the structure of their perithecium wall, and they also have a different thallus structure; e.g. *Agonimia* is characterized by a non-umbilicate thallus with papillate cortical cells and *Henrica* as having umbilicate thalline squamules (Zahlbruckner 1909; de Lesdain 1921).

The only challenge to this view was offered by Servít (1954), who both introduced several new families in the *Verrucariales* and a more narrow circumscription of the *Verrucariaceae*. Servít used involucrellum structure rather than ascospore septation as the primary cardinal character for defining genera within this order.

Thelidium has usually been considered closely related to *Polyblastia*, but in the 'sporological' tradition it has been characterized by exclusively having transversal septa, normally one to three in number, whereas *Polyblastia* also has longitudinal septa and often more numerous transversal septa. Otherwise, there is a similar range in morphological variation in thallus and ascoma structure and ecology, except for the fact that no pigmented spores occur in *Thelidium*. One problem has consistently plagued this classification, the term 'submuriform' ascospores (i.e. ascospores with few transversal and rare longitudinal septa). These are often only a minority of the mature spores observed in an ascoma, and have

sometimes been considered anomalies, such as in *T. papulare* and *T. incavatum* (Purvis *et al.* 1992), and in a few other species.

Staurothele is another genus considered to be closely related to *Polyblastia*. In many ways it parallels *Polyblastia* and *Thelidium* as traditionally conceived in being vaguely characterized and quite variable with respect to thallus and ascoma morphology and ecology. The main diagnostic characteristic for this genus has been the occurrence of symbiotic green algae in the hymenium. Ascospores are muriform, hyaline to dark brown, and in many species there are fewer than eight per ascus.

Scope and aims of this study

Our work on *Polyblastia* primarily aims at a revision of the genus in Northern Europe, but at an early stage it also became evident that we had to deal with some supposedly closely related genera, such as *Thelidum* and *Staurothele*, to be able to recognize natural groups and reconcile the generic classification with the evolution of the *Verrucariaceae*.

The goals of this phylogenetic study were to: (1) reveal the position of *Polyblastia* s. *lat.* in relation to allegedly closely related taxa, and ten monophyletic 'species groups' of *Verrucariaceae* (*sensu* Gueidan *et al.* 2007); (2) infer phylogenetic relationships of *Polyblastia* s. *lat.* and allied genera based on Bayesian (BMCMC), ML, and MP analyses; and (3) investigate features traditionally used for generic circumscriptions of taxa included in the latter analyses.

Materials and methods

Taxon sampling

Material was collected in northern and central Europe, and voucher material is referred to in Appendix 1 (Supplementary Material). nuLSU, the largest subunit of the RNA polymerase II (*RPB1*, region A–D) (Stiller & Hall 1997), and the ITS regions of the nuclear encoded rDNA (ITS1–5.8S–ITS2 or nuITS) were sequenced for 44 specimens of *Polyblastia* and related genera. In total, 129 new sequences were produced for this study (nuITS, nuLSU and *RPB1* for 41 specimens; and nuITS and nuLSU for the remaining three taxa).

For the first dataset, i.e. the large dataset (LD), only the nuLSU and the *RPB1* loci were included. This dataset comprised the 44 species of *Polyblastia* and related genera selected for this study, and 84 taxa covering the morphological and ecological diversity within the *Verrucariaceae* for which the same loci were sequenced by Gueidan *et al.* (2007). In all, the LD included 128 specimens of *Verrucariaceae*, plus two species belonging to the *Chaetothyriales* that served as outgroup (*Capronia pilosella* and *C. munkii*).

The second dataset, the small dataset (SD), corresponds to the 'Polyblastia group' (Fig 1, clade A). NuLSU and *RPB1* sequences were combined with sequences from the nucITS region to form 58 sets of sequences representing 42 species. With this sampling restricted to closely related species, the nuITS region, which is otherwise difficult to align across the family, could be added. Based on the results of LD analyses,

Endocarpon pallidulum and *Verrucaria viridula* were chosen as outgroup species for the analyses of the SD (Fig 2).

Morphological study

Longitudinal sections, 12–18 μm thick, were obtained from thalli and perithecia using a freezing microtome. The samples were first hydrated with deionised water, and then mounted in diluted Gum Arabic and frozen at −20 °C. The sections were observed and measured in water, and then stained by lactophenol/Cotton Blue. Asci and ascospores were studied in squash preparations of perithecia.

Molecular study

Total DNA was extracted from freshly collected material, from dried material kept at −20 °C for a few months (up to two years), and from a few herbarium specimens (no older then four years) using the DNeasy Plant Mini Kit (Quiagen, Hilden, Germany), following the manufacturer's instructions. Perithecia were carefully cleaned from adjacent material to avoid contaminants. Diluted (10^{-1}–10^{-3}) or undiluted DNA was used for PCR amplifications. The nuITS region, the 5′ end of the nuLSU, and region A–D of the *RPB1* were amplified. Primers used were: (a) for the nuITS: ITS1f (Gardes & Bruns 1993), ITS 4, ITS5 (White *et al.* 1990); (b) for the nuLSU: LR0R, LR7 and LR6 (Vilgalys & Hester 1990), LR3 and LR5 (Vigalys RJ, http://www.biology.duke.edu/fungi/mycolab/primers.htm); and (c) for the *RPB1* region A–D: RPB1-Af (Stiller & Hall 1997), RPB1-6R1asc (Hofstetter *et al.* 2007).

For PCR amplification of the nuITS and nuLSU, we used the AccuPower® PCR PreMix (Bioneer, Daejeon, Korea), adding 3 μl diluted or undiluted DNA, 1.5 μl of each primer (10 μM), and water to a total volume of 20 μl. For nuITS and nuLSU the PCR thermal cycling parameters were: initial denaturation for 4 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 45 s. at 72 °C, and final elongation for 5 min at 72 °C. Amplification and thermal cycling parameters for PCR of the *RPB1* followed Gueidan *et al.* (2007). Amplification products were visualized on 0.5 % agarose gels stained with ethidium bromide and the PCR product was purified using Millipore plates (MultiScreen™ PCR, Danvers, Massachusetts, USA). Cloning was conducted for weak PCR products (approximately half of the PCR products of *RPB1*) and a few PCR products with multiple bands using the Topo TA cloning kit (Invitrogen, Carlsbad CA, USA), following the manufacturer's instructions. Sequencing, automated reaction clean up, and visualization were carried out as described by Macrogen. (www.macrogen.com).

Alignments and phylogenetic analyses

Sequences were assembled and edited using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Manual alignments were performed using MacClade 4.06 (Maddison & Maddison 2003), with the help of amino acid sequences for the protein-coding locus *RPB1*, and with the help of the secondary structure of the nuLSU from *Saccharomyces cerevisiae* (Cannone *et al.* 2002) following a method described in Kjer (1995). Ambiguously aligned regions (*sensu* Lutzoni *et al.* 2000)

and introns were delimited manually and excluded from phylogenetic analyses. Congruence among single locus phylogenies was explored using a 70 % reciprocal NJ BS with ML distances (Mason-Gamer & Kellogg 1996; Reeb *et al.* 2004). Models of molecular evolution to compute ML distances were estimated for each separate genomic region using the Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada & Crandall 1998) and the BS analyses were run for 10 K replicates.

Phylogenetic relationships and confidence were inferred using a Bayesian approach. Additional support values were estimated using ML BS. Using the AIC in Modeltest 3.7, the Bayesian analysis employed the GTR + I + γ model for all partitions, except for the third codon position of *RPB1*, for which the model GTR + γ was selected.

Using MrBayes 3.1.1 (Phylogeny of *Polyblastia* and allied genera www.mrbayes.net) two analyses of two parallel runs were carried out for 5 M generations. Each run included four chains, and trees were sampled every 500 generations. All runs converged on the same average likelihood score and topology. A burnin sample of 5 K trees was discarded from each run. The remaining 10 K (2 × 5 K) trees were used to estimate PPs with the majority rule consensus tree command in PAUP 4.0b10 (Swofford 1999). The branch lengths were obtained on a subsample of 5 K trees with the 'sumt' command in MrBayes. The program RAxML-VI-HPC (Stamatakis *et al.* 2005) was used for the ML BS analysis with 500 BS replicates and a single model of molecular evolution (GTRMIX).

For the SD the three amplified regions were tested for congruence, divided into five partitions (RPB1 first, second and third position, nuLSU and nuITS), and analysed with MrBayes and RAxML as described for the LD. For the SD, weighted MP bootstrapping, conducted with PAUP 4.0b10, was used in order to obtain additional support values. Step matrices were obtained for each of the five previously mentioned partitions by using StMatrix 4.2 (Lutzoni & Zoller, Duke University; www.lutzonilab.net/downloads/). All characters had equal weight; gaps were treated as a fifth character state. A tree search was carried out using 1 K random addition sequences (RAS). Nine equally most parsimonious trees were recovered, and a BS analysis of 500 replicates with five RAS per replicate was then conducted using PAUP 4.0b10. Bayesian PPs ≥ 95 % (Alfaro *et al.* 2003), ML BS and weighted MP bootstrapping ≥70 % were considered to be significant.

Results

LD

The congruence analysis between the two loci of the LD detected conflicts only within *Polyblastia melaspora* and within *Sporodictyon terrestre*. Because the detected incongruence was intraspecific, the two sets of sequences (nucLSU and *RPB1*) were concatenated. After exclusion of ambiguously aligned regions and introns, the concatenated data matrix contained 2030 unambiguously aligned sites. The two-locus Bayesian phylogeny is presented in Fig 1. All species in our sampling traditionally placed in *Polyblastia* (the ingroup for the SD analysis) were found to be nested within the '*Polyblastia* group' of Gueidan *et al.* (2007) with high support values. All sampled
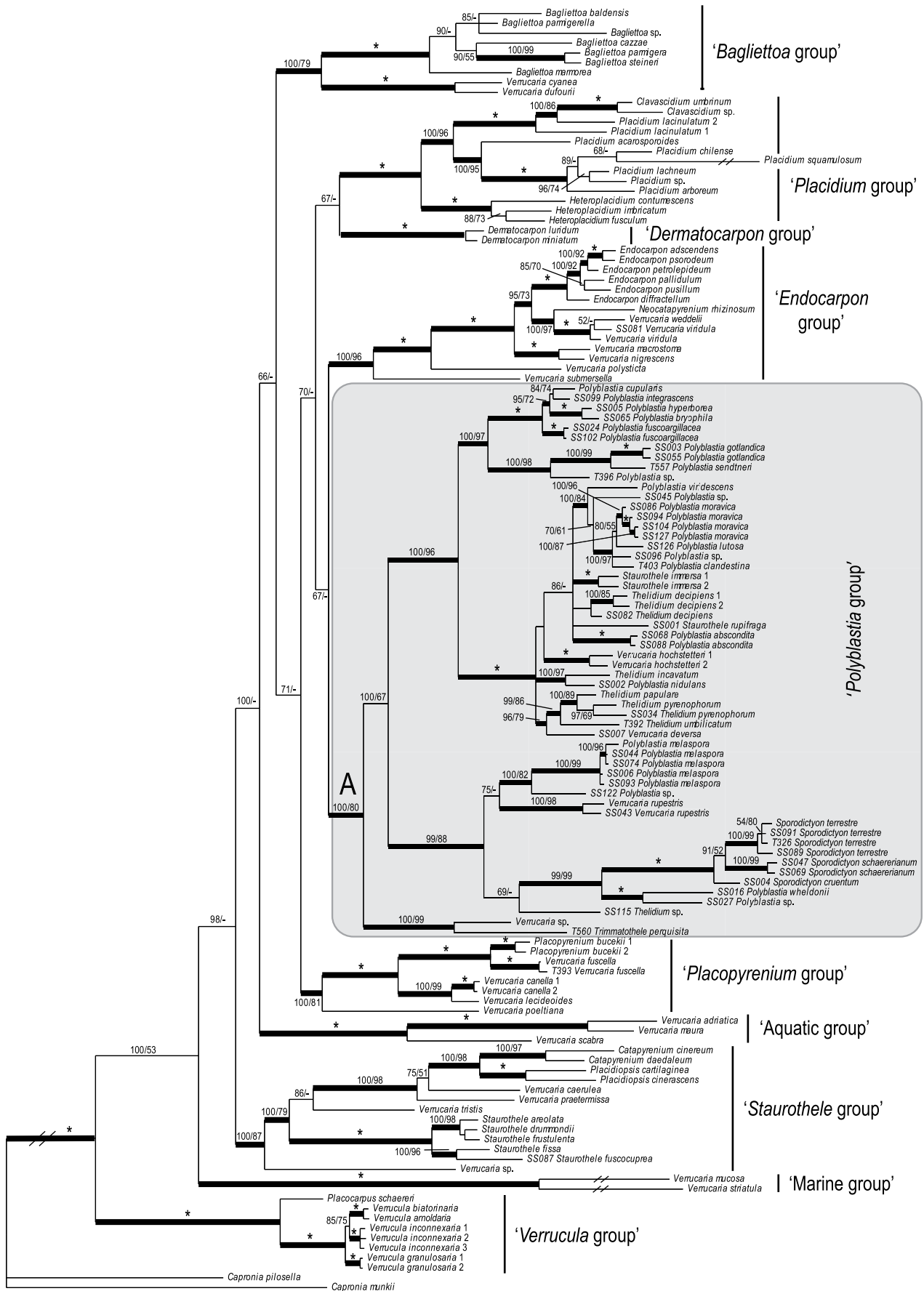
species traditionally placed in *Thelidium* also belong to this group. In addition to *Polyblastia* and *Thelidium*, this monophyletic group contains species currently classified within *Staurothele*, *Trimmatothele*, and *Verrucaria*. The 'Endocarpon group' of Gueidan *et al.* (2007) was found to be sister to the '*Polyblastia* group', although with virtually no support.

SD

The alignment of the nuITS resulted in the exclusion of about 60 % of the characters for this region. After exclusion of ambiguously aligned regions and introns, the concatenated data matrix contained 2289 unambiguously aligned sites. In the parsimony analysis 1588 characters were constant, while 141 variable characters were parsimony-uninformative, for a total of 560 parsimony-informative characters. The tree resulting from the Bayesian analysis is presented in Fig 2. *Trimmatothele* groups with *Verrucaria* sp. (clade C). The sister group to the *Trimmatothele*–*Verrucaria* sp. clade (clade B) is only weakly supported. The genera *Polyblastia* and *Thelidium* are not monophyletic. Clade D, a subclade within clade B, is very strongly supported, whereas clade E is significantly supported only by ML BS. In clade E, *Thelidium* sp. forms the sister group to the rest of the species. The strongly supported clade D consists of two likewise strongly supported subclades (L and M). Clade M contains species currently classified within *Polyblastia*, and clade L contains species currently referred to *Polyblastia*, *Staurothele*, *Thelidium*, and *Verrucaria*. The main subclade of clade L (clade N) is very strongly supported, and contains a mixture of *Polyblastia*, *Staurothele*, and *Thelidium* species. Another subclade of clade L, clade P, contains three *Thelidium* species, and is the sister group of *V. deversa*. The early divergences within clade L are poorly resolved, and the relationships within clade L are not well understood from this phylogeny. Within the ingroup (clade A), the SD analysis provided better support for approximately 25 % of all internodes compared with the LD analysis, and the results of both analyses are fully congruent for portions of the trees with PPs ≥ 95 % and/or BS values ≥70 %. Some of the features previously used as 'cardinal characters' for species recognition, *viz.* ascospore septation and pigmentation, as well as involucrellum development and the occurrence of a photobiont in the hamathecium, have been mapped onto Fig 2 along with the substrate on which these species are found.

Taxonomy

Sporodictyon terrestre (Th. Fr.) S. Savić & Tibell, **comb. nov.**
Basionym: *Polyblastia terrestris* Th. Fr., *Lichenes Arctoi*: 365 (1860)
MycoBank no.: MB 512264.

Type: 'Øst-Finmark, Varanger, Mortensnes, 28/8 57. Th. M. Fries' (UPS — *lectotypus hic designatus*).

Sporodictyon terrestre is a morphologically very variable species growing among mosses on gravelly soil and more often on rocks. The thallus is sometimes thick with almost completely immersed perithecia enclosed in a thick, verrucose thalline cover, but on exposed rocks it is often thin, and then the thalline cover of the sessile perithecia is thin and smooth.
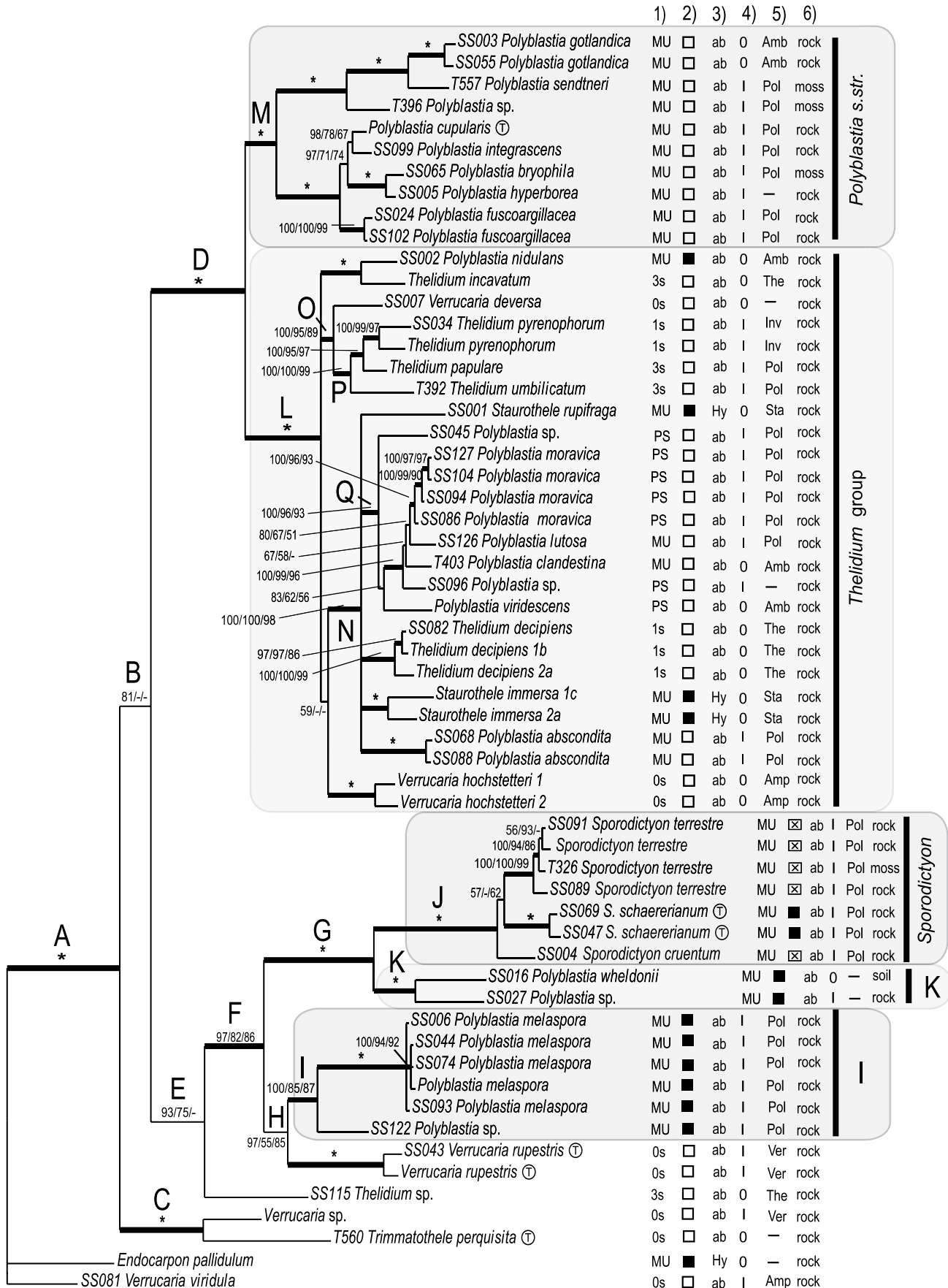
Discussion

Phylogenetic position of *Polyblastia and allied genera within* Verrucariaceae

In the LD analysis (Fig 1), the '*Polyblastia* group' forms a strongly supported monophyletic group (PP 100 %, ML BS 80 %) nested within the *Verrucariaceae*. Gueidan *et al.* (2007) found the '*Placopyrenium* group', containing *Placopyrenium bucekii* and four *Verrucaria* species (*V. fuscella*, *V. canella*, *V. lecideoides* and *V. poeltiana*; all parasitic species) to be the sister group of the '*Polyblastia* group', although with virtually no support. In our analysis the '*Endocarpon* group' (Fig 1) *sensu* Gueidan *et al.* (2007), also with nearly no support, was found to be sister to the '*Polyblastia* group'. However, taking into consideration the weak support for the deep internodes within the current *Verrucariaceae* phylogeny, relationships among the main groups (including the '*Polyblastia* group') are not yet known.

Gueidan *et al.* (2007) revealed *Staurothele* to be polyphyletic, with four epilithic species nested within the '*Staurothele* group' and endolithic *S. immersa* nested within the '*Polyblastia* group'. In our analysis the placement of the crustose epilithic *S. fuscocuprea* within the '*Staurothele* group' (*Staurothele s. str.*), and the endolithic *S. rupifraga* in the '*Polyblastia* group' is not surprising (Fig 1). However, the monophyly of the two endolithic species of *Staurothele*, belonging to the '*Polyblastia* group' (clade L, Fig 2), cannot be rejected from our analysis. *Verrucaria* is highly polyphyletic as revealed by Gueidan *et al.* (2007), and this is also evident from our analyses (Fig 1), which include four additional specimens: *V. deversa* ('*Polyblastia* group'), *V. fuscella* ('*Placopyrenium* group'), *V. rupestris* ('*Polyblastia* group'), and *V. viridula* ('*Endocarpon* group').

V. rupestris (the type of *Verrucaria*), a species not closely related to any other *Verrucaria* species in this study, is a sister to *Polyblastia melaspora* and *Polyblastia* sp. (Fig 2, clade I). The placement of *V. rupestris*, although moderately supported, emphasizes the taxonomic complexity of *Verrucaria*, which will require extensive nomenclatural changes to reflect the

Fig 1 – Phylogenetic relationships among 128 specimens (Appendix 1) representing 103 species of *Verrucariaceae* based on a Bayesian analysis of a concatenated nuLSU and RPB1 dataset. The tree was rooted using two species from the *Chaetothyriales* (*Capronia* spp.). The two support values associated with each internal branch correspond to PPs and ML BS proportions, respectively. Branches in bold indicate a support of PP ≥ 95 % and a ML BS ≥ 70 %. An asterisk on a bold branch indicates that this node has a support of 100 % for both support estimates. A dash instead of a ML BS value indicates that the node of the Bayesian tree was not recovered by ML bootstrapping. The branches with double-slash are shortened. Species groups (within annotation marks) are in accordance with Gueidan *et al.* (2007). *Polyblastia* and allied taxa (the '*Polyblastia* group', which is the focus of Fig 2) are highlighted by a shaded box.

	1)	2)	3)	4)	5)	6)
SS003 *Polyblastia gotlandica*	MU	□	ab	0	Amb	rock
SS055 *Polyblastia gotlandica*	MU	□	ab	0	Amb	rock
T557 *Polyblastia sendtneri*	MU	□	ab	I	Pol	moss
T396 *Polyblastia* sp.	MU	□	ab	I	Pol	moss
Polyblastia cupularis ⊤	MU	□	ab	I	Pol	rock
SS099 *Polyblastia integrascens*	MU	□	ab	I	Pol	rock
SS065 *Polyblastia bryophila*	MU	□	ab	I	Pol	moss
SS005 *Polyblastia hyperborea*	MU	□	ab	I	—	rock
SS024 *Polyblastia fuscoargillacea*	MU	□	ab	I	Pol	rock
SS102 *Polyblastia fuscoargillacea*	MU	□	ab	I	Pol	rock
SS002 *Polyblastia nidulans*	MU	■	ab	0	Amb	rock
Thelidium incavatum	3s	□	ab	0	The	rock
SS007 *Verrucaria deversa*	0s	□	ab	0	—	rock
SS034 *Thelidium pyrenophorum*	1s	□	ab	I	Inv	rock
Thelidium pyrenophorum	1s	□	ab	I	Inv	rock
Thelidium papulare	3s	□	ab	I	Pol	rock
T392 *Thelidium umbilicatum*	3s	□	ab	I	Pol	rock
SS001 *Staurothele rupifraga*	MU	■	Hy	0	Sta	rock
SS045 *Polyblastia* sp.	PS	□	ab	I	Pol	rock
SS127 *Polyblastia moravica*	PS	□	ab	I	Pol	rock
SS104 *Polyblastia moravica*	PS	□	ab	I	Pol	rock
SS094 *Polyblastia moravica*	PS	□	ab	I	Pol	rock
SS086 *Polyblastia moravica*	PS	□	ab	I	Pol	rock
SS126 *Polyblastia lutosa*	MU	□	ab	I	Pol	rock
T403 *Polyblastia clandestina*	MU	□	ab	0	Amb	rock
SS096 *Polyblastia* sp.	PS	□	ab	I	—	rock
Polyblastia viridescens	PS	□	ab	0	Amb	rock
SS082 *Thelidium decipiens*	1s	□	ab	0	The	rock
Thelidium decipiens 1b	1s	□	ab	0	The	rock
Thelidium decipiens 2a	1s	□	ab	0	The	rock
Staurothele immersa 1c	MU	■	Hy	0	Sta	rock
Staurothele immersa 2a	MU	■	Hy	0	Sta	rock
SS068 *Polyblastia abscondita*	MU	□	ab	I	Pol	rock
SS088 *Polyblastia abscondita*	MU	□	ab	I	Pol	rock
Verrucaria hochstetteri 1	0s	□	ab	0	Amp	rock
Verrucaria hochstetteri 2	0s	□	ab	0	Amp	rock
SS091 *Sporodictyon terrestre*	MU	⊠	ab	I	Pol	rock
Sporodictyon terrestre	MU	⊠	ab	I	Pol	rock
T326 *Sporodictyon terrestre*	MU	⊠	ab	I	Pol	moss
SS089 *Sporodictyon terrestre*	MU	⊠	ab	I	Pol	rock
SS069 *S. schaererianum* ⊤	MU	■	ab	I	Pol	rock
SS047 *S. schaererianum* ⊤	MU	■	ab	I	Pol	rock
SS004 *Sporodictyon cruentum*	MU	⊠	ab	I	Pol	rock
SS016 *Polyblastia wheldonii*	MU	■	ab	0	—	soil
SS027 *Polyblastia* sp.	MU	■	ab	I	—	rock
SS006 *Polyblastia melaspora*	MU	■	ab	I	Pol	rock
SS044 *Polyblastia melaspora*	MU	■	ab	I	Pol	rock
SS074 *Polyblastia melaspora*	MU	■	ab	I	Pol	rock
Polyblastia melaspora	MU	■	ab	I	Pol	rock
SS093 *Polyblastia melaspora*	MU	■	ab	I	Pol	rock
SS122 *Polyblastia* sp.	MU	■	ab	I	Pol	rock
SS043 *Verrucaria rupestris* ⊤	0s	□	ab	I	Ver	rock
Verrucaria rupestris ⊤	0s	□	ab	I	Ver	rock
SS115 *Thelidium* sp.	3s	□	ab	0	The	rock
Verrucaria sp.	0s	□	ab	I	Ver	rock
T560 *Trimmatothele perquisita* ⊤	0s	□	ab	0	—	rock
Endocarpon pallidulum	MU	■	Hy	0	—	rock
SS081 *Verrucaria viridula*	0s	□	ab	I	Amp	rock

0.1

Phylogenetic relationships within the 'Polyblastia group'

Traditionally, the ingroup species of the SD have been classified in the genera *Polyblastia*, *Staurothele*, *Thelidium*, *Trimmatothele*, and *Verrucaria* (Fig 2; for Servít's classification, see below and column 5 of Fig 2). In the study by Gueidan *et al.* (2007), *Verrucaria* sp. was derived from the first divergence within the '*Polyblastia* group'. Here we show this species to form a strongly supported group with *Trimmatothele perquisita* (clade C; PP, ML BS and weighted MP BS = 100 %), a rarely collected species. Recently, Ertz & Diederich (2004) stated that *T. perquisita*, the type of *Trimmatothele*, only differs from *Verrucaria* in having polysporous asci and, therefore, they treated *Trimmatothele* as a synonym of *Verrucaria* (see also Eriksson 2005). *Trimmatothele* is here, for the first time, placed in a molecular phylogeny.

P. cupularis, the type of *Polyblastia*, and seven other named species currently classified within *Polyblastia*, are strongly supported as monophyletic (clade M). Therefore, we recognize this clade as *Polyblastia s. str.* (for more details see below, under 'Phylogeny and the classification of *Polyblastia*'). In our analysis the phylogeny of the *Thelidium* group (clade L) is not completely resolved. It is very likely that the group contains the type of *Thelidium* (*T. amylaceum*). For a more comprehensive understanding of the phylogeny of the *Thelidium* group a much better taxon sampling and/or more phylogenetic information from other loci is needed.

Morphology and cardinal characters

The concept of *Polyblastia* as a distinct genus was part of the new paradigm entitling ascospore morphology to be of major importance for generic recognition. This was made possible by LM studies, and the paradigm had an important role in the generic classification of lichens. During the 1850s, a school heralded by De Notaris (1846) and followed by Massalongo (who described *Polyblastia*), increased the number of

genera recognized by about 100 (Rambold & Triebel 1999). The artificial system of Zahlbruckner (1907, 1926) also strongly rested on a few cardinal characters for generic circumscriptions, e.g. thallus morphology, ascospore morphology and pigmentation, gross ascoma morphology, and photobiont association. In *Verrucariaceae* this led to a 'sporology' centred generic classification, which primarily considered thallus growth form and then ascospore morphology as criteria for defining genera in a tradition where little or no consideration was given to the importance of evolutionary relationships among taxa. A kind of peripety of this tradition was the comprehensive treatment of *Verrucariaceae* and *Dermatocarpaceae* by Zschacke (1933–1934). Thallus structure is rather uniform in our ingroup, but it was shown by Gueidan *et al.* (2007) that septate ascospore, hymenial photobiont and squamulose/foliose thallus have evolved repeatedly in *Verrucariaceae* (including *Dermatocarpaceae*). In the following, a brief review of some of these 'cardinal characters' will be given with respect to their distribution in our ingroup phylogeny (Fig 2).

Ascospore morphology and pigmentation

Non-septate ascospores are plesiomorphic in *Verrucariaceae* and transversally septate and muriform ascospores have evolved repeatedly in different lineages (Gueidan *et al.* 2007; see Fig 2, columns 1–2). Our results show that a dark ascospore pigmentation is also homoplasious (Fig 2). With respect to our SD phylogeny, ascospores in clade C (*Trimmatothele perquisita*, *Verrucaria* sp.) are non-septate and non-pigmented. The *Thelidium* group (clade L) contains species with both non-septate, transversely septate, and muriform ascospores (Fig 2). Clade G and clade I only contain species with muriform ascospores, just like *Polyblastia* (clade M) which, however, exclusively has hyaline ascospores. Only a few species have strongly pigmented, dark brown to blackish ascospores (see Fig 2, column 2). Such ascospores are found in clade G, which, in addition, has species with colourless ascospores that turn straw-coloured at maturity. Moreover, species in clade I, and *Staurothele rupifraga*,

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Fig 2 – Phylogenetic relationships among 56 members of *Polyblastia* and allied taxa, based on a Bayesian analysis of concatenated nuITS, nuLSU, and RPB1 datasets. *Endocarpon pallidulum* and *Verrucaria viridula* were chosen as outgroup species based on results shown in Fig 1. PP, ML BS and weighted MP BS are represented with the first, second, and third numbers associated with branches. Branches in bold indicate a support of PP ≥ 95 %, and both ML and weighted MP BS ≥ 70 %. An asterisk on a bold branch indicates that this node has a support value of 100 % for all support estimates. A dash instead of a ML BS or weighted MP BS value indicates that the node of the Bayesian tree was not recovered by ML or weighted MP bootstrapping. Well-supported monophyletic groups, *Polyblastia s. str.*, the *Thelidium* group, *Sporodictyon*, and groups K and I, are highlighted by shaded boxes. The letter T follows the names of the type. Six features are depicted after the names of the species: (1) ascospore septation [0s = non-septate spores; 1s = 1-septate spores; 3s = 3-septate spores; PS = pauciseptate spores – transverse septa ≤4, longitudinal septa in the middle of the spore 1 per transversal cell row, without or with just occasional secondary transverse septa (Fig 3G); MU = muriform spores – transverse septa >4, longitudinal septa in the middle of the spore >1 per transversal cell row, with several to numerous secondary transverse septa (Fig 3F)]; (2) ascospore pigmentation (□ = spores at maturity colourless, ⊠ = spores at maturity pale or straw yellow, ■ = spores at maturity medium to blackish brown); (3) occurrence of photobiont in the hymenium (Hy = hymenial photobiont present, ab = hymenial photobiont absent); (4) involucrellum (I = involucrellum present, 0 = involucrellum absent); (5) generic assignment of the species by Servít (1954) (Amb = *Amphoroblastia*, Amp = *Amphoridium*, Inv = *Involucrothele*, Pol = *Polyblastia*, Sta = *Staurothele*, The = *Thelidium*, Ver = *Verrucaria*, – = not treated by Servít); and (6) substrate (rock, moss, soil).
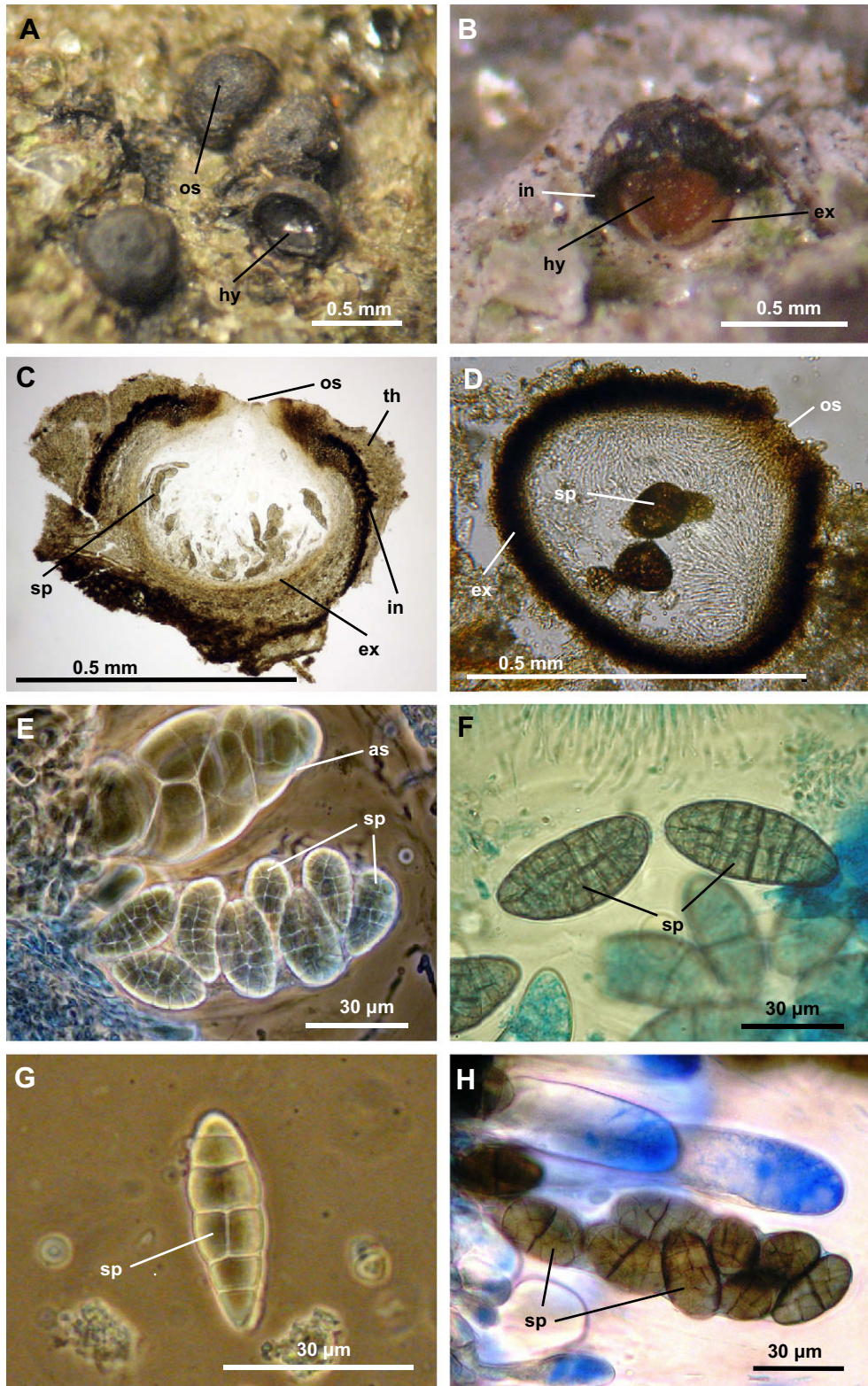
Fig 3 – Morphological and anatomical features of *Polyblastia* and related species: ostiolum (os), hymenium (hy), excipulum (ex), involucrellum (in), thalline tissue (th), ascospore (sp), ascus (as). For vouchers used in the molecular analyses (e.g. SS016), see Appendix 1. (A) *Sporodictyon terrestre* — perithecia on thin, slightly verrucose thallus; one perithecium sectioned vertically (Norway, Troms, Berg hd, N Senja, Mefjordbotn, 11 July 2006, *L. Tibell*, UPS). (B) *Thelidium pyrenophorum* — vertically sectioned perithecium with thick involucrellum and at the base pale excipulum (Norway, Nordland, Rana hd, 19 July 2006, *S. Savić*, UPS). (C) *Sporodictyon terrestre* — section of perithecium covered by thalline tissue almost to the ostiolum; involucrellum thickened apically and merged with the excipulum [Norway, Sör-Tröndelag, Dovre, Kongsvoll, 1931, *Vrang* (S)].

S. immersa and *Polyblastia nidulans* in the *Thelidium* group have pigmented ascospores.

Hymenial algae

Hymenial algae have evolved independently in three lineages, according to Gueidan *et al.* (2007); in the genus *Staurothele s. str.*, in the genus *Endocarpon*, and in *Staurothele immersa* in the 'Polyblastia group'. In our phylogeny, the *Thelidium* group includes two species with hymenial algae (*S. rupifraga* and *S. immersa*; Fig 2, column 3). However, the relationship between these two species is not resolved, and many species of endolithic *Staurothele* have not been sampled. Therefore, they might be one or more independent gains of hymenial algae in this group.

Involucrellum

Servít referred species without an involucrellum (Fig 3D) to: *Amphoridium* (non-septate ascospores; Servít 1954), *Thelidium* (transversely septate ascospores), and *Amphoroblastia* (muriform ascospores, Servít 1953). He recognized analogous genera with an involucrellum (Fig 3B) as: *Verrucaria* and *Sarcopyrenia* (non-septate ascospores), *Involucrothele* (transversely septate ascospores; Servít 1953), and *Polyblastia* (muriform ascospores); Servít also emphasised the importance of the morphology of the involucrellum. For comparisons with current recognition of the genera, as well as the results of this study, see Fig 2, column 4 and 5. In our experience, however, the development of the involucrellum may sometimes vary and depends on the development of the thallus, which in turn depends on ecological conditions, such as exposure and humidity (see also Thüs 2002). Halda (2003) directed some rather acrid criticism against Servít's classification in connection with a revision of *Bagliettoa*, which he referred to as a section of *Verrucaria* (*cf.* the '*Bagliettoa* Group' of Gueidan *et al.* 2007). His criticism was directed to the emphasis in Servít's classification on ascospore septation and involucrellum structure.

In clade F an involucrellum is missing in *P. wheldonii*; in *Polyblastia s. str.* (clade M) only *P. gotlandica* is lacking an involucrellum, and this may be interpreted as an evolutionary loss. In the poorly resolved *Thelidium* group (clade L), the relationships are complex and species both with and without an involucrellum occur. In several cases the involucrellum might have been lost in species with immersed perithecia, like *Staurothele rupifraga*, *P. clandestina* and *V. hochstetterii*. Species with immersed perithecia studied here usually do not have an involucrellum (*Bagliettoa* being an exception). However, it is very possible that the involucrellum has been lost secondarily

on a number of occasions in connection with the evolution of immersed perithecia. As it is often the case, 'cardinal characters' do not reflect the inferred evolutionary patterns of these characters, and the usefulness of the character of the involucrellum structure can only be evaluated *a posteriori* in relation to a specific phylogenetic hypothesis.

Habitat ecology

Polyblastia and allied taxa inhabit different types of habitats (Fig 2, column 6). Saxicolous species occur on a variety of substrates, such as limestone, mica-schist, chalk pebbles, mortar, bricks, and siliceous rocks; and in this study most specimens are from such habitats. *Polyblastia s. str.* (clade M) occur on rocks and mosses, and have their perithecia only partly immersed (¼, ½) in the substrate (with the exceptions of *P. gotlandica*, which has fully immersed perithecia), or the perithecia are partly covered by the thallus as in the case of bryophilous species (*P. bryophila*, *P. sendtneri*). In clade L, the *Thelidium* group, many representatives grow on rocks and are semi-endolithic or endolithic; the exceptions being clade O and clade Q, with representatives of both epilithic and semi-endolithic species. *Sporodictyon* species grow on rocks, particularly by streams and on lakeshores, and among mosses. Their perithecia are more or less covered by the thallus, a feature characteristic for this genus, and are not immersed.

Phylogeny and the classification of Polyblastia s. lat

Polyblastia s. str. – clade M

This clade is, along with clade G and clade I, the only monophyletic group that exclusively contains *Polyblastia* species in the traditional sense. In clade M, all species have colourless, muriform ascospores with rather numerous cells (Fig 3E), and all species have a well-developed involucrellum except for *P. gotlandica* — the only species in this clade, that has fully immersed perithecia. No hymenial algae are present, and the species occur both on rocks and on mosses. In this restricted sense *Polyblastia* is not only monophyletic in our three-locus phylogeny, but it is also morphologically rather uniform, with a few species that would risk to be erroneously placed in *Polyblastia*. Servít (1954) placed most of these species in *Polyblastia*, except for *P. gotlandica*, which he referred to *Amphoroblastia* as it lacks an involucrellum. Here we accept the following species in *Polyblastia s. str.*: *P. bryophila*, *P. cupularis*, *P. fuscoargillacea*, *P. gotlandica*, *P. hyperborea*, *P. integrascens*, and *P. sendtneri*. *Polyblastia* sp. (T396) grows on mosses, and is probably an undescribed species.

(D) *Polyblastia wheldonii* — vertical section of perithecium without involucrellum (SS016). (E) *Polyblastia bryophila* — hyaline, muriform spores (Fig 2/MU, □) in mature ascus; phase contrast illumination (Spitsbergen, Kings Bay, 18 August 1868, *Th. M. Fries*, UPS). (F) *Sporodictyon cruentum* — spores muriform with numerous lumina, here semi-mature and medium brown (Fig 2/ MU, ⊠) stained in lactophenol/Cotton Blue (SS004). (G) *Polyblastia moravica* — hyaline, pauciseptate spore, here with five transversal and only one longitudinal septum (Fig 2/PS, □) in phase contrast illumination; stained in lactophenol/Cotton blue (Norway, Troms, Gratangen hd, 17 July 2007, *S. Savic*, UPS). (H) *Polyblastia melaspora* — dark brown, muriform spores (Fig 2, MU, ■); stained in lactophenol/Cotton Blue (Norway, Nordland, Rana hd, 19 July 2006, *S. Savic & L. Tibell*, UPS).

The Thelidium *group – clade L*

This clade is strongly supported and contains species traditionally placed in *Polyblastia*, *Staurothele*, *Thelidium*, and *Verrucaria*. Ascospore septation varies dramatically in this clade, where we find non-septate, 1-septate, 3-septate, pauciseptate (Fig 3G) and muriform ascospores. Most species have colourless ascospores, but dark ascospores occur in at least two and maybe three different lineages. Both the occurrence of 3-septate, pauciseptate, and muriform ascospores seems homoplasious, as is the occurrence of dark ascospores. Hymenial algae occur in one or two different lineages. Many species have a well-developed involucrellum, but nearly as many do not. However, it is interesting to note that all the species occur on more or less basic/calcareous rocks.

Sporodictyon — clade J

Sporodictyon forms a strongly supported group in our analyses, and includes the type, S. *schaererianum* along with S. *cruentum*, and S. *terrestre*. *Sporodictyon*, in our opinion, is best treated as a distinct genus, which also is reasonably easy to characterise and recognise morphologically. The thallus is epilithic, more or less well developed, and often quite thick (Fig 3A). The young perithecia have an outer thalline cover, often reaching almost to the ostiole (Fig 3C). Perithecia are hence often described as immersed in thalline verrucae. The thalline cover of the perithecia is thick and uneven in some morphs of S. *terrestre*; very thick and irregularly lobate in S. *schaererianum*, but thin in some other species not included in this study. Perithecia are rather large, 0.5–1.2 mm diam, and have a well-developed involucrellum. Ascospores are large, usually 50–80 μm long, muriform with numerous longitudinal and transversal septa, often slightly asymmetric, with one narrower end that is slightly curved (Fig 3F). Ascospores are colourless when young and later turn yellowish to very dark brown. The photobiont is a green alga, but cyanobacteria occur in most species as superficial cephalodia. *Sporodictyon* species grow on rocks, soil and mosses. *Sporodictyon* species contain a very characteristic indel in the nucITS1: GGGGYGCSCCYYCGCGGGTTCCCCGMYAYCTCCCACCC. This has not been found in any other species of *Verrucariaceae* studied. Similarly, there is in the nuITS2 another indel unique to the genus: GGRAGTGTSDMRRCW. A new combination within *Sporodictyon* is proposed in the Taxonomy section above.

Clade K

This clade (Fig 2) is the sister group to *Sporodictyon* with a strong support. It only contains two species: *Polyblastia wheldonii* and an undescribed *Polyblastia* sp. (SS027). They are similar to *Sporodictyon* in having large, muriform ascospores, but differ by having a thin thallus and also in their possible association with cyanobacteria, which, however, do not form cephalodia. There is no generic name available for this clade.

Clade I

This clade (Fig 2) is strongly supported and only contains two species, *Polyblastia melaspora* and *Polyblastia* sp. (SS122). This clade is also supported by some morphological features. The thallus is well developed and it contains green algae as photobiont. Perithecia have a well-developed involucrellum. Asco-

spores are muriform and dark brown at maturity (Fig 3H), *ca* 30–70 μm broad and 20–30 μm wide. *Polyblastia* sp. (SS122) is, however, similar to *Sporodictyon*, and no morphological synapomorphy has yet been found to distinguish it unequivocally from that genus. There is no generic name available for this clade.

Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mycres.2008.05.002.

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