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Using a multigene phylogenetic analysis to assess generic delineation and character evolution in *Verrucariaceae* (*Verrucariales*, *Ascomycota*)

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ABSTRACT

Verrucariaceae are a family of mostly crustose lichenized ascomycetes colonizing various habitats ranging from marine and fresh water to arid environments. Phylogenetic relationships among members of the *Verrucariaceae* are mostly unknown and the current morphology-based classification has never been confronted to molecular data. A multilocus phylogeny (nuLSU, nuSSU and *RPB1*) was reconstructed for 83 taxa representing all main genera of this family to provide a molecular phylogenetic framework necessary to assess the current morphology-based classification. Four main well-supported monophyletic groups were recovered, one of which contains seven robust monophyletic subgroups. Most genera, as traditionally delimited, were not monophyletic. A few taxonomic changes are proposed here to reconcile the morphology-based classification with the molecular phylogeny (*Endocarpon diffractellum* comb. nov., *Heteroplacidium fusculum* comb. nov., and *Bagliettoa marmorea* comb. nov.). Ancestral state reconstructions show that the most recent common ancestor of the *Verrucariaceae* was most likely crustose with a weakly differentiated upper cortex, simple ascospores, and hymenium free of algae. As shown in this study, the use of symplesiomorphic traits to define *Verrucaria*, the largest and type genus for the *Verrucariaceae*, as well as the non monophyly of the genera *Polyblastia*, *Staurothele* and *Thelidium*, explain most of the discrepancies between the current classification based on morphological similarity and a classification using monophyly as a grouping criterion.

Introduction

Verrucariaceae Zenker is a group of mainly lichenized ascomycetes comprising widely diverse habits. The structure of the thallus varies greatly in this family, with sizes ranging from a few millimeters to more than 10 cm diam, and shapes from granulose or crustose for the smallest thalli to squamulose or foliose umbilicate for the largest ones. Although vegetatively quite variable, members of *Verrucariaceae* are easy to recognize as their ascomata present good diagnostic features for the family. The perithecial ascomata are characterized by the presence of an apical ostiole and of short pseudoparaphyses (or periphysoids, but see Roux and Triebel 1994) bordering the upper part of the perithecial cavity and hanging into this cavity without or only barely reaching the hymenium (Janex-Favre 1970, 1975; Wagner 1987). Asci are typically bitunicate (Janex-Favre 1970, 1975; Wagner 1987), and their dehiscence was shown in some species to occur by a gelification of

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the apical part of the outer wall (Grube 1999). The lack, at least at maturity, of long, interascal, sterile hyphae and the positive reaction of the hymenial gel to potassium–iodine are also typical of this family (Henssen & Jahns 1973). The perithecium of some species of *Verrucariaceae* has been the subject of anatomical and ontogenetical studies (Doppelbauer 1959; Janex-Favre 1970, 1975; Wagner 1987). Because the ascoma development is quite variable within this family, the recognition of *Verrucariaceae* as either ascohymenial or ascolocular fungi has long been debated (Janex-Favre 1970, 1975).

Species classified within *Verrucariaceae* grow mainly on rocks, either epilithically or endolithically within the superficial layer of the rock. Members of this family can also colonize other types of substrates: soils (Breuss 1996), wood or bark (Breuss 1993a, 1994a, 1998a; Orange 1989), mosses (Döbbeler 1997; Döbbeler & Triebel 1985), and other lichens (Zehetleitner 1978). Saxicolous species of this family grow mostly in dry environments, but some species are also found in aquatic habitats, such as boulders located in rivers (Keller 1995, 2000; Thüs 2002), or marine intertidal and supralittoral zones of rocky shores (Brodo & Santesson 1997; Flenniken & Gibson 2003; Harada 2004; Sanders *et al.* 2004). Although saxicolous members of *Verrucariaceae* are particularly diverse on calcareous substrates, they can also colonize siliceous rocks, especially in aquatic or semi-aquatic conditions. Members of this family are found worldwide, from polar regions to the tropics (Aptroot 1998, 2002; Aptroot & Seaward 1999; Aptroot & Sipman 2001; Breuss 1993b, 1994b, 1998b; Brodo *et al.* 2001; Clauzade & Roux 1985; Harada 1993a,b; McCarthy 2001; Thomson 1997; Vězda 1973).

Eschweiler (1824) first attempted to classify genera of *Verrucariaceae*. For these taxa, he created two 'cohors' *Verrucariae* and *Dermatocarpeae* (Table 1), based mainly on the structure of the thallus. He attributed the crustose taxa to the *Verrucariae* and the squamulose and foliose taxa to the *Dermatocarpeae*. Unfortunately, Eschweiler (1824) also included many non-related genera within these two groups (Table 1). In 1827, Zenker validated the family *Verrucariaceae* (at the time called *Verrucariae*), in which he included the genus *Verrucaria* and some other unrelated crustose taxa (Table 1). This author also separated the squamulose and foliose taxa from the crustose *Verrucariae*, and placed them within the *Endocarpa*. It was only almost one century later that the first extensive monographic work on the family *Verrucariaceae* was published (Zschacke 1913, 1914, 1918, 1921, 1924, 1927). In 1933–1934, Zschacke recognized the two families *Verrucariaceae* and *Dermatocarpaceae* (Eschw.) Stizenb., as did Zahlbruckner (1921–22) in an earlier publication. Servít worked on the *Verrucariaceae* in the late 1940s and 1950s (Servít 1946, 1950a,b, 1952, 1953, 1954), and published a classification for the entire group in 1955. In this work, he also considered the family *Dermatocarpaceae* as a separate taxon, and recognized four additional families (*Staurothelaceae Servít*, *Microglaenaceae Servít*, *Pyrenidiaceae Zahlbr.*, and *Bagliettoaceae Servít*). However, two of these families are currently recognized in other orders as synonyms (*Microglaenaceae* is a synonym of *Thelenellaceae H. Mayrhofer*, and *Pyrenidiaceae* is a synonym of *Dacampiaceae Körb.*) and the validity of the two other families has always been questioned (Hale 1961; Henssen & Jahns 1973; Poelt 1973), and excluded from subsequent classifications (Eriksson 1983; Henssen & Jahns 1973; Poelt 1973). Currently, almost all genera belonging to the order *Verrucariales* are included in the family *Verrucariaceae* (Eriksson 2006). Two other genera, also included in *Verrucariales* and characterized by their long and persistent interascal elements (paraphysoides according to Triebel 1993) in addition to their short pseudoparaphyses, and by their lichenicolous habits, are classified in the second family of this order, the *Adelococcaceae* Triebel. Today, the family *Verrucariaceae* includes 45 genera (Eriksson 2006) and approximately 750 species (Hawksworth *et al.* 1995).

In the classifications of Servít (1955), Zahlbruckner (1921–22), and Zschacke (1933–34), taxa within *Verrucariales* were mainly circumscribed based on thallus structure, ascospore morphology, and the presence or absence of hymenial algae. However, the phylogenetic value of these characters was thought to be doubtful and in need of further investigation. Although Servít (1955) accepted the separation of the families *Verrucariaceae* and *Dermatocarpaceae* based on thallus morphology, early on he suggested the limitations of this character (1946: 49): 'In my opinion the distinction made between these two families on the basis of the degrees of the development of the thallus cannot be maintained, if we want to replace the present artificial system by one which is at least a little more natural.' Recently, molecular studies showed that, in other groups of ascomycetes (Miller & Huhndorf 2004, for the genus *Lasiosphaeria*; Staiger 2002, for the family *Graphidaceae*), ascospore septation was not always a reliable character to delineate monophyletic groups at the genus and higher taxonomical ranks. Because the generic delimitation within *Verrucariaceae* is mainly based on ascospore septation and thallus structure, molecular data are needed to assess the current morphology-based classification. To date, only a few molecular studies have been carried out on members of this family, either at the infrageneric level (Amtoft 2006; Amtoft *et al.* 2008; Heiömarsson 2003, on *Dermatocarpon*) or in the context of large-scale molecular phylogenies (Del Prado *et al.* 2006; Geiser *et al.* 2006; James *et al.* 2006; Liu & Hall 2004; Lumbsch *et al.* 2002, 2004, 2005; Lutzoni *et al.* 2001, 2004; Spatafora *et al.* 2006). However, the family *Verrucariaceae* was never the focus of a phylogenetic study and, before this study, only a few DNA sequences were available in GenBank for this family. This study aims to provide a multigene phylogeny for the main genera of *Verrucariaceae* in order to confront the current morphology-based classification with molecular data. Selected morphological traits and ecological aspects were studied to characterize inferred monophyletic groups. Generic delineation is discussed based on both molecular and morphological data, and a few taxonomic changes were undertaken. Ancestral state reconstructions were carried out to better understand: (1) the discrepancy between a morpho-similarity based classification and a classification based on a monophyletic grouping criterion, as well as, (2) the evolutionary history of the *Verrucariaceae*.

Materials and methods

Taxon sampling

Because of the relatively high number of taxa in the family *Verrucariaceae* and the difficulty in obtaining material for

Table 1 – Past and current classifications of *Verrucariales*

Eschweiler (1824)	Zenker (1827)	Zahlbruckner (1922)	Zschacke (1934)	Servít (1955)	Eriksson (2006)
Dermatocarpeae	*Cryolichenes*	*Dermatocarpaceae*	*Dermatocarpaceae*	*Bagliettoaceae*	*Adelococcaceae*
Solorina	*Verrucariae*	*Normandina*	*Normandina*	**Protobagliettoa**	*Adelococcus*
Dermatocarpon*	***Verrucaria****	*Anapyrenium*	***Trimmatothelopsis***	***Bagliettoa****	*Sagediopsis*
Gyrophora	*Stigmatidium*	***Psoroglaena***	***Dermatocarpon****	*Dermatocarpaceae*	*Verrucariaceae*
Endocarpon*	*Porophora*	***Dermatocarpon****	sect. *Catapyrenium*	***Dermatocarpon****	***Agonimia***
Capitularia	*Ocellularia*	***Placidiopsis****	sect. *Endopyrenium*	***Involucrocarpon***	***Anthracocarpon***
Peltidea	*Antrocarpon*	***Heterocarpon***	sect. *Entosthelia*	***Placidiopsis****	***Awasthiella***
Verrucariae	*Phyllolichenes*	***Endocarpon****	sect. *Polyrhizion*	*Microglaenaceae*	***Bagliettoa****
Variolaria	*Endocarpa*	***Agonimia***	***Placidiopsis****	*Thrombium*	***Bellemerella***
Porina	***Endocarpon****	*Verrucariaceae*	***Agonimia***	***Paraphysothele***	***?Bogoriella***
Thelotrema		*Sarcopyrenia*	***Endocarpon****	***Thelidiopsis***	***Catapyrenium****
Verrucaria*		***Verrucaria****	*Verrucariaceae*	*Pyrenidiaceae*	***Clauzadella***
Pyrenula		sect. *Amphoridium*	*Sarcopyrenia*	*Pseudoarthopyrenia*	***Clavascidium****
Pyrenastrum		sect. *Euverrucaria*	***Verrucaria****	*Staurothelaceae*	***Dermatocarpella***
Limboria		sect. *Lithoicea*	subgen. *Amphoridium*	***Staurothele****	***Dermatocarpon****
Urceolaria		***Lesdainea***	subgen. *Euverrucaria*	***Endocarpon****	***Diederimyces***
Lecidea		***Trimmatothele***	subgen. *Lithoicea*	*Verrucariaceae*	***Endocarpon****
Biatora		*Cocciscia*	***Trimmatothele***	***Amphoridium***	***?Glomerilla***
		Thelidium*	***Thelidium****	***Thelidium****	***?Haleomyces***
		Polyblastia*	***Polyblastia****	***Amphoriblastia***	***Henrica***
		Staurothele*	subgen. *Coccospora*	***Involucrothele***	***Heterocarpon***
		sect. *Eustaurothele*	subgen. *Halospora*	***Polyblastia****	***Heteroplacidium****
		sect. *Willeya*	subgen. *Thelidiopsis*	***Verrucaria****	***Involucropyrenium***
		Thelenidia	subgen. *Polyblastidea*	subgen. *Euverrucaria*	***Lauderlindsaya***
		Thrombium	subgen. *Sporodictyon*	subgen. *Lithoicea*	***Leucocarpia***
		Gongylia	subgen. *Bispora*	subgen. *Hydroverrucaria*	***Merismatium***
		Geisleria	***Staurothele****		***Muellerella***
		Microglaena	sect. *Sphaeromphale*		***Mycophyscias***
		Aspidopyrenium	sect. *Polyblastioides*		***Neocatapyrenium****
			Thelenidia		***Norrlinia***
			Thrombium		***?Phaeospora***
			sect. *Euthrombium*		***Placidiopsis****
			sect. *Bagliettoa*		***Placidium****
			Paraphysothele		***Placocarpus****
			Geisleria		***Placopyrenium****
			Gongylia		***Placothelium***
			sect. *Eugongylia*		***?Plurisperma***
			sect. *Beloniella*		***Polyblastia****
			Microglaena		***Psoroglaena***
			sect. Eumicroglaena		***Rhabdopsora***
			sect. Weitenwebera		***Scleropyrenium***
			Henrica		***?Spheconisca***
					Staurothele*
					Telogalla
					Thelidiopsis
					Thelidium*
					Trimmatothele
					?Trimmatothelopsis
					Verrucaria*

Question marks indicate taxa for which the position in the classification is uncertain. Asterisks indicate genera that are represented in this study. Genera that include or included taxa now belonging to *Verrucariaceae* (Eriksson 2006) are indicated in bold.

some of the rare or exotic species, the sampling for this project was restricted to 15 of the 45 genera included in this family (Eriksson 2006). This selection of genera represents *Verrucariaceae* well since its inception (Table 1) and includes all of its most species-rich genera. In total, 83 specimens of *Verrucariaceae* were included, with at least two representative species per genus, when possible (Supplementary Material Appendix 1). The most species-rich genera were subjected to a more intensive sampling: for example, the genus *Verrucaria*, including about 250 species, was represented by 29 taxa chosen to encompass the morphological and ecological diversity of this genus. Previous studies (Lumbsch *et al.* 2004, 2005; Lutzoni *et al.* 2001, 2004) showed that *Verrucariales* was sister to the non-lichenized order *Chaetothyriales*. As no molecular data were available for the second family of the order *Verrucariales*, two outgroup taxa were chosen from the genus *Capronia* (*Herpotrichiellaceae*, *Chaetothyriales*) to root the phylogeny of *Verrucariaceae*. For the most part, nomenclature

follows Clauzade & Roux (1985) and Nimis (1993), but also Breuss (1996) for the catapyrenioid *Verrucariaceae*, Navarro-Rosinés *et al.* (2007) for the genus *Verrucula*, and Brodo *et al.* (2001) for the North-American specimens. Author citations can be found in Supplementary Material Appendix 1.

Molecular data

Genomic DNA was extracted from dried material or cultures using a protocol modified from Zolan & Pukkila (1986) with 2 % sodium dodecyl sulphate (SDS) as the extraction buffer. After precipitating the genomic DNA using isopropanol, pellets were washed once in 70 % ethanol, dried with a speedvac, and resuspended in 60 µl water to which 1 µl bovine serum albumin (BSA; 10 mg ml^{-1}) was added. Four gene regions [nuLSU and nuSSU, and the RNA polymerase II largest subunit (*RPB1*) regions A–D and D–G] were amplified using published, as well as newly designed, primers (Table 2, Fig 1). In some cases the amplification and sequencing of the intron-rich ribosomal loci (nuLSU and nuSSU) required the primers to be species specific. These primers are not listed here but are available on the website of the Lutzoni lab (http://www.lutzonilab.net/). One microlitre of a 1/10 or 1/100 dilution of genomic DNA was added to the following PCR mix: 2.5 µl PCR buffer (buffer IV with 15 mM $MgCl_2$, Abgene, Rochester, NY), 2.5 µl dNTP (2 mM), 2.5 µl BSA (10 mg ml^{-1}), 2 µl primers (10 µM), 0.15 µl Taq polymerase (5 U µl^{-1}, Denville, South Plainfield, NJ), and water to a total volume of 25 µl. PCR was performed on a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). For ribosomal genes, one initial cycle of 60 s at 95 °C preceded 35 cycles of the following steps: 45 s at 95 °C, 40 s at 52 °C and 2 min at 72 °C (elongation time up to 4 min for longer fragments). For *RPB1*, one initial cycle of 3 min at 95 °C preceded 35 cycles of the following steps: 45 s at 95 °C, 90 s at 52 °C, and 90 s (for region A–D) or 120 s (for region D–G) at 72 °C. All amplifications ended with a final cycle at 72 °C for 10 min. After examination with gel electrophoresis, PCR products were purified using the Microcon PCR cleaning kit (Millipore, Billerica, MA). Alternatively, cloning was conducted on weak PCR products, PCR products presenting multiple bands and most of the PCR products of *RPB1*, using the Topo TA cloning kit (Invitrogen, Carlsbad, CA). Sequencing was carried out in 10 µl reactions using: 1 µM primer, 3 µl purified PCR product, 1 µl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, CA), 3 µl Big Dye buffer, and 2 µl double-distilled water. Automated reaction clean up and visualization was performed at the Duke Center for Evolutionary Genomics, using Big Dye on an ABI 3730xl DNA analyser (Applied Biosystems).

Alignments and phylogenetic analyses

Sequences were assembled and edited using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI). A manual alignment was performed using MacClade 4.06 (Maddison & Maddison 2003), with the help of amino acid sequences for protein-coding loci and, for ribosomal loci, the help of the secondary structure of ribosomal genes from *Saccharomyces cerevisiae* (Cannone *et al.* 2002) following a method described in Kjer (1995). Ambiguous regions (*sensu* Lutzoni *et al.* 2000) and introns were delimited manually and excluded from the alignment. The four amplified regions nuLSU (1.4 kb), nuSSU (1.6 kb), *RPB1* region A–D (1.2 kb) and *RPB1* region D–G (1.8 kb) were tested for congruence using a 70 % reciprocal

Table 2 – List of primers and primer sequences (if novel) for the four loci used in this study (nuLSU, nuSSU, *RPB1* region A–D, *RPB1* region D–G)

	PCR primers	Sequencing primers
nuLSU	LR0R[a], LR7[b]	LR3, LR3R, LR5, LR5R, LR6, LR6R[b]
nuSSU	nssu131[c], NS24[d]	nssu1088, nssu1088R, nssu897R, nssu634[c], SR11R[e], NS23, NS22[d], SR7R, SR7, SR10R[f], vNS7 (5′-GGCTCAAGCCAATGGAAGTA-3′), vNS6 (5′-GCCTCGTACTTCCATTGGCTT-3′)
RPB1 region A-D	RPB1-AF[g], RPB1-6R1asc[h], RPB1-AbF (5′-GTRCCTGTYTAYCAYTAYGGT-3′)	
RPB1 region D-G	RPB1-DF1asc[h,i], RPB1-G2R[g], RPB1-G2bR (5′-GCAAGRACNCCCACCATYTC-3′), RPB1-G2cR (5′-GCNAGGACRCCNACCATTTC-3′)	RPB1-CG1R (5′-RAYNCCDATRCTRAADCG-3′), RPB1-CG2F (5′-TAYGGNGARGAYGGNYTNGAY-3′), RPB1-CG2R (5′-RTCNARNCCRTCYTCNCCRTA-3′)

As an intron often occurs in the genomic region corresponding to the primer sequences NS6 and NS7 (White *et al.* 1990), the new primers vNS6 and vNS7 were designed in a region adjacent to these primers. *RPB1*-AbF was designed to anneal a few basepairs upstream from *RPB1*-AF, as an alternative 5′ amplification primer. Similarly, *RPB1*-G2bR and *RPB1*-G2cR were designed to anneal a few basepairs downstream from *RPB1*-G2R, as alternative 3′ amplification primers.

a Rehner & Samuels (1994).
b Vilgalys & Hester (1990).
c Kauff & Lutzoni (2002).
d Gargas & Taylor (1992).
e Spatafora *et al.* (1995).
f Vilgalys (unpubl.).
g Hall (unpubl.).
h Hofstetter *et al.* (2007).
i James *et al.* (2006).

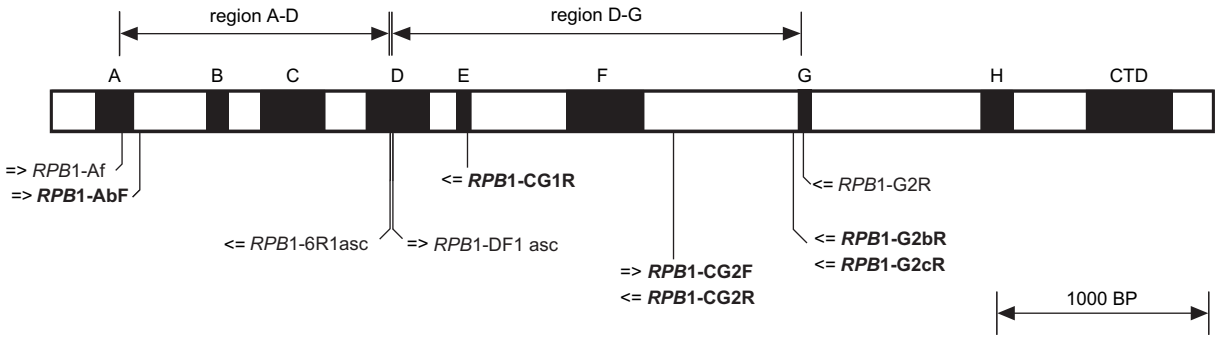


Fig 1 – RPB1 amplification and sequencing primers used in this study for the two regions A–D and D–G. Diagram modified from Matheny *et al.* (2002). Black boxes represent regions where amino acid sequences are conserved among eukaryotes. New primers designed for this study are shown in bold.

Neighbor-Joining bootstrap with Maximum Likelihood distances (NJ-ML BS; Mason-Gamer & Kellogg 1996; Reeb *et al.* 2004). For this congruence test, models of molecular evolution 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 10K replicates. Conflicts among partitions were eliminated by pruning out problematic sequences or taxa. Phylogenetic relationships and confidence were inferred using a Bayesian approach based on a combined nuLSU + nuSSU + *RPB1* dataset. Additional support values were estimated using weighted Maximum Parsimony (wMP) and ML BS. For the Bayesian approach, the AIC in Modeltest 3.7 was used to estimate the model of molecular evolution. A GTR + I + G model was used for the five partitions (nuLSU, nuSSU, *RPB1* first, second and third codon positions). Eight analyses of four chains were run for 5M generations using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), and trees were sampled every 500 generations. All runs converged on the same average likelihood score and topology. A burn-in sample of 5K trees was discarded for each run. The remaining 40K trees were used to estimate branch lengths with the sumt command in MrBayes, and Posterior Probabilities (PPs) with the majority rule consensus tree command in PAUP* version 4.0b10 (Swofford 1999). The wMP BS analysis was conducted in PAUP*. 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/). A tree search was carried out using 500 random addition sequences (RAS). The same most parsimonious tree was recovered for all 500 RAS. A BS analysis of 500 replicates and two RAS was then conducted using PAUP*. The program RAxML-VI-HPC (Stamatakis *et al.* 2005) was used for the ML BS analysis with 1K BS replicates and a GTRMIX model of molecular evolution.

Morphology

A subsample of about 30 taxa was selected to represent each well-supported group recovered with molecular phylogenetic analyses. The morphological study of these 30 taxa was based on the same specimens used for the molecular systematic study. Additional material was studied when the feature of interest was not present on the specimen used in the molecular study. A total of 70 ecological and morphological characters were investigated. Cross-sections were made by hand or using a Miles Cryostat Tissue-Tek II freezing microtome. Pycnidia were stained using Cotton blue in lactophenol, and other stains were tried on different structures (e.g., Sudan IV on oil cells, calcofluor on asci and potassium/iodine on hymenia). The different types of plectenchymas were defined as in Yoshimura & Shimada (1980). Drawings were made using a Leica DMLB microscope equipped with a camera lucida. Pictures were taken using a Zeiss Axiovision microscope with a SPOT Insight colour camera using the software SPOT version 4.0 (Diagnostic Instruments, Sterling Heights, MI). The conserved character states in the family *Verrucariaceae* and characters too variable to provide synapomorphies that could define generic entities, as well as characters for which the observation was problematic, were not further studied. Thirty-one characters were selected using these criteria and their microscopic observations were extended to all 83 taxa. These characters were then manually mapped on the most likely tree. On this basis, the most interesting characters were selected for ancestral state reconstructions. These characters were chosen because they were traditionally used to delimitate genera in the *Verrucariaceae* or because they provided synapomorphies that could be used to delineate new generic entities.

Ancestral state reconstruction

The program SIMMAP was used to reconstruct ancestral states (Bollback 2006). This program uses a Bayesian approach to estimate the PPs of ancestral states (Huelsenbeck & Bollback 2001), and takes into account both phylogenetic and mapping uncertainties (Ronquist 2004). Phylogenetic uncertainty was integrated into the analysis by sampling topologies and branch lenghts from a pool of 10K trees obtained from our Bayesian tree reconstruction. To account for mapping uncertainty, SIMMAP allows the rate of evolution of the investigated morphological characters to vary, and estimates their PPs. A Markov k class model (Mk class model, where k refers to the number of states observed) was used to define the evolution of these morphological characters (Lewis 2002), and the character states were set as unordered. The estimation of the PP was carried out using two priors for morphological characters, an overall evolutionary rate and a bias prior on two-state

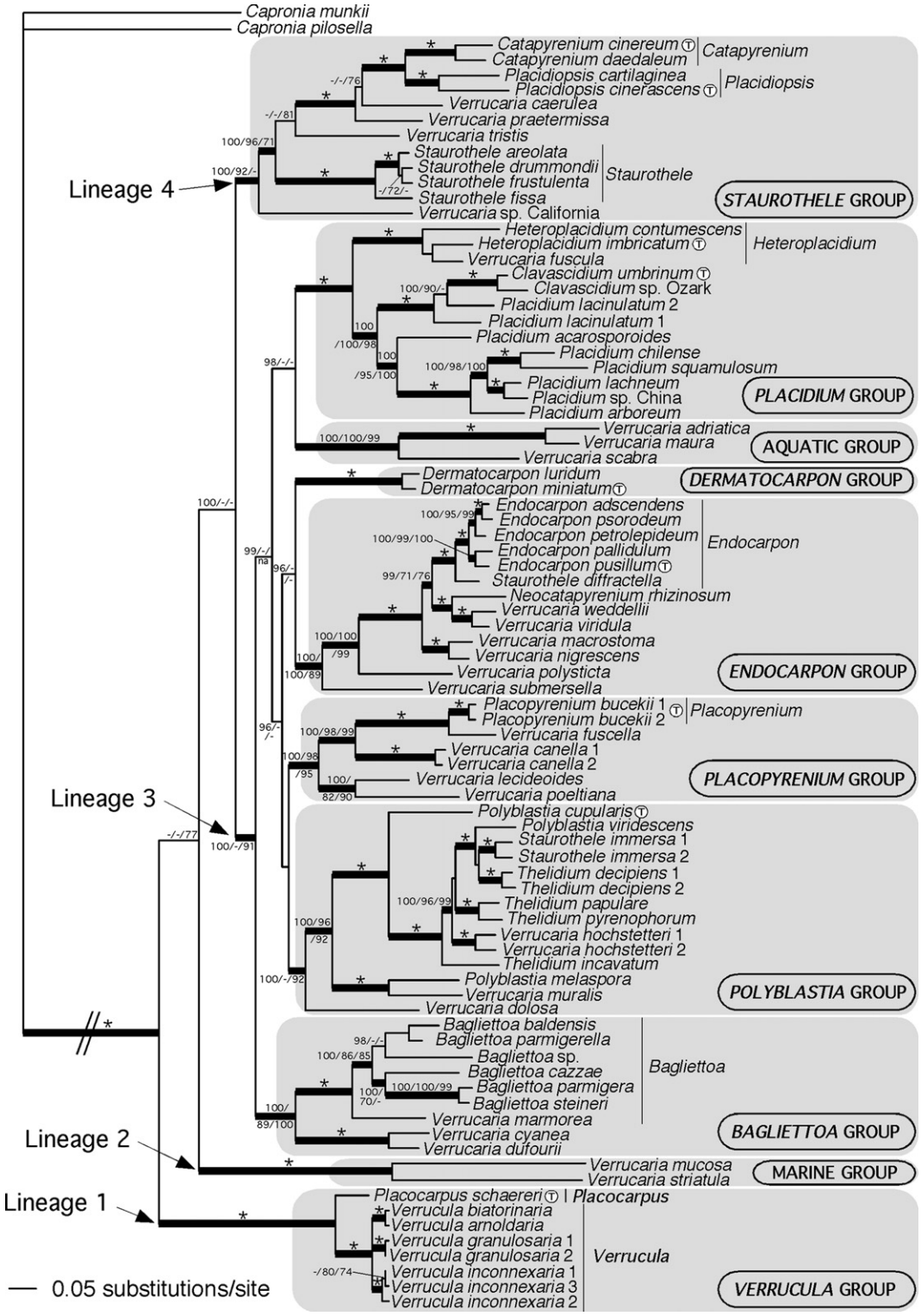


Fig 2 – Phylogenetic relationships among 83 members of *Verrucariaceae* based on a Bayesian analysis of the combined nuLSU, nuSSU, and RPB1 dataset. The tree was rooted using two species from *Chaetothyriales* (*Capronia* spp.). Bold branches correspond to a PP $\geq$ 95 % and either a ML or a wMP BS $\geq$ 70 %. An asterisk over a bold branch indicates that this node had a support value of 100 % for all three methods. Otherwise, the support values are specified in the following order: PP/ML BS/wMP BS. Values below 70 % for ML and wMP BSs and below 95 % for PP were replaced by a dash. If a node of the Bayesian tree was not recovered with either wMP or ML BS, the support value obtained with either of these two methods was marked as non-applicable (na). The letter T follows the names of the type species. For better readibility, the size of the branch leading to the outgroup was divided by a factor of six. Four main lineages were recovered with high phylogenetic confidence in the family *Verrucariaceae*, including ten well-supported monophyletic subgroups highlighted here by shaded boxes.

characters (Schultz & Churchill 1999). For binary and multistate characters, a gamma prior was chosen for the overall evolutionary rate, with the following parameters: $\alpha = 3.0$, $\beta = 2.0$ and $k = 60$. For binary characters, a symmetrical β prior was used with $\alpha = 1$ and $k = 19$. For each Bayesian tree sampled with SIMMAP during the ancestral state reconstruction, 100 draws were carried out from the prior distributions for the rate of evolution of morphological characters.

Results

Molecular data

Sequences of all three loci were recovered (no missing data) for 70 of the 83 members of *Verrucariaceae* selected for this study. Individuals with missing sequences included two taxa missing nuLSU, ten taxa missing nuSSU, and two taxa missing *RPB1* (Appendix 1). Individuals with incomplete sequences included five taxa for nuSSU and 13 taxa for *RPB1*. Nevertheless, taxa with missing or incomplete sequences were included in the phylogenetic analyses. The congruence analysis detected one conflict between the two regions of *RPB1* for the placement of *Verrucaria nigrescens*. Because this conflict could not be resolved without pruning out *V. nigrescens*, and because the support values for this conflicting relationship were not very high (NJ–ML BS of 76 % and 72 %), it was ignored and all the partitions were combined. The combined data matrix included 5818 characters after exclusion of ambiguous regions and introns (1330 included characters for nuLSU, 1569 for nuSSU and 2919 for *RPB1*). Both ribosomal loci had introns (group I and spliceosomal introns). The nuSSU was particularly rich in group I introns, with an alignment reaching 13,194 sites before all introns and ambiguously aligned regions were removed. Of the 5818 unambiguously aligned sites, 3522 were excluded from the wMP analysis because they were constant. Of the remaining 2296 sites, 1854 were parsimony-informative (250 for nuLSU, 181 for nuSSU and 1423 for *RPB1*). Constant sites were included for the ML and Bayesian analyses.

Phylogenetic inference

The Bayesian consensus tree is presented in Fig 2 with branch lengths and support values. Except for a few (mostly deep) internodes, most internodes received high support values from all three phylogenetic methods. The genus *Verrucaria* is highly polyphyletic, with many species forming a grade at the base of well-delimited groups, such as for the *Endocarpon* group (Fig 2). *Catapyrenium* and *Placidiopsis* appear to be sister genera (100 % PP, ML BS and wMP BS). The genus *Staurothele* is polyphyletic, with *S. diffractella* sister to the genus *Endocarpon* (100 % PP, NL BS and wMP BS) and *S. immersa* nested within the *Polyblastia* group (100 % PP, ML BS and wMP BS). As expected, the two genera *Polyblastia* and *Thelidium* are closely related and not monophyletic. However, their close relationship to *Staurothele immersa*, *Verrucaria hochstetteri*, *V. dolosa* and the type species *V. muralis*, together forming a monophyletic group (*Polyblastia* group), was unexpected. Two species of *Clavascidium* are nested within the genus *Placidium*. The genus *Heteroplacidium* includes the species *Verrucaria fuscula* and is sister to a group including *Placidium* and *Clavascidium* (100 % PP, ML BS and wMP BS), forming the *Placidium* group. The marine *Verrucaria* species are derived from at least two independent origins: one group including *V. adriatica* and *V. maura*, together with the freshwater species *V. scabra* (aquatic group; 100 % PP, ML BS and 99 % wMP BS) is nested within the speciose lineage 3, and the second group including *V. mucosa* and *V. striatula* (marine group; 100 % PP, ML BS and wMP BS) corresponds to lineage 2. The species *Placopyrenium bucekii* is nested within a group of *Verrucaria* species that are mostly lichen parasites, such as *Verrucaria canella* and *V. lecideoides* (100 % PP, 98 % ML BS and 95 % wMP BS). Another group of parasitic species comprises the two genera *Placocarpus* and *Verrucula* (lineage 1; 100 % PP, ML BS and wMP BS). Finally, the endolithic species *Verrucaria marmorea* is sister to the genus *Bagliettoa* (100 % PP, ML BS and wMP BS). Together with *Verrucaria cyanea* and *V. dufourii*, they form a monophyletic group (*Bagliettoa* group) within lineage 3 (Fig 2).

Morphology

Five morphological characters were selected for ancestral state reconstructions either because of their traditional use in generic delimitation (structure of the thallus, ascospore septation, and presence or absence of hymenial alga) or because they might represent key innovations associated with specific monophyletic groups (structure of the upper cortex and structure of the pycnidium). Because of the large amount of missing data (pycnidia have not been found for several species within *Verrucariales*), the ancestral state reconstruction of the character 'structure of the pycnidium' failed, but the taxonomic importance of this character was explored. The three characters 'thallus structure', 'ascospore septation', and 'upper cortex structure' have more than two states, whereas the character 'hymenial alga' is binary (Table 3). In order to minimize the number of states for a character as complex as 'upper cortex structure', the following four categories were created (Fig 3): (1) absent: the upper cortex was considered to be absent when no layer of fungal hyphae could be observed above the algal layer (Fig 3A), or when some fungal hyphae

Table 3 – Characters and character states for ancestral state reconstructions

Character	Character states
Thallus structure	0 = crustose and entirely endolithic; 1 = all other crustose thallus type; including semi-endolithic (medulla endolithic but algal layer and upper cortex epilithic), epilithic and placodioid thalli; 2 = squamulose; 3 = foliose umbilicate; 4 = not applicable
Upper cortex structure	0 = absent; 1 = pseudocortex; 2 = eucortex; 3 = lithocortex
Ascospore septation	0 = simple or mostly simple; 1 = transversally uniseptate or mostly uniseptate; 2 = transversally multiseptate; 3 = submuriform to muriform
Hymenial alga	0 = absent; 1 = present

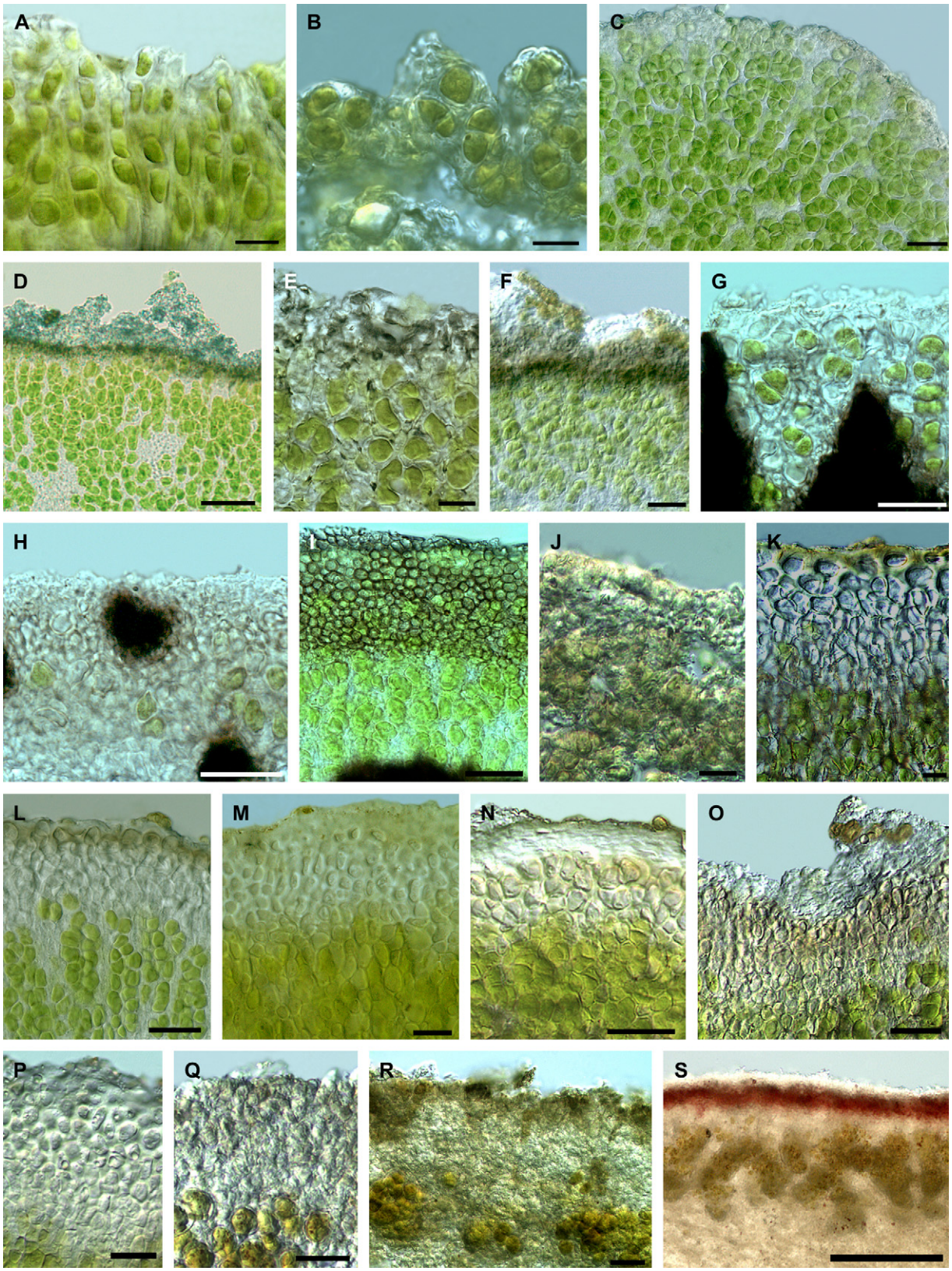


Fig 3 – Light micrographs showing the structure of the upper cortex of various species of *Verrucariaceae*. Bars = 20 μm (unless specified). (A–B) Cortex absent. (A) In *Verrucaria striatula* (bar = 10 μm). (B) In *Thelidium pyrenophorum* (bar = 10 μm). (C–J) Pseudocortex. (C) In *Staurothele frustulenta*, with a hyaline upper cortical layer. (D) In *Verrucula inconnexaria*, with a pigmented upper cortical layer (bar = 40 μm). (E) *Cinereum*-type cortex in *Catapyrenium cinereum* (bar = 10 μm). (F) Upper cortex with a thick epinecral layer in *Placidiopsis cartilaginea*. (G) *Maura*-type cortex in *V. maura*. (H) In *V. adriatica*. (I) Squash

were present, forming a thin, irregularly developed and prosoplectenchymatous net surrounding clusters of algae but not differentiating into a cortex (Fig 3B). (2) Pseudocortex: this term was used to define poorly differentiated types of cortex (Fig 3C–J), i.e., when thin, usually less than 30 μm high, not well delimited from the algal layer (with algal cells often present up to the uppermost layers of cells), paraplectenchymatous, formed by small cells of (2–)4–8(–9.5) μm diam and with relatively thin walls and large lumina. The uppermost layers of cortical cells can be hyaline (Fig 3C) or more generally pigmented (usually brown). An epinecral layer is often present (Fig 3D). This type includes the *cinereum*-type cortex (Fig 3E–F) described by Breuss (1990) for species in the genera *Catapyrenium* and *Placidiopsis*. In *Verrucaria maura* (Fig 3G), the cortex is weakly differentiated as the size and shape of the fungal cells differ between the upper cortex (small rounded cells, 2–4 μm diam, sometime pigmented) and the algal layer (larger cells, 6–8 μm long). This *maura*-type pseudocortex is also found in *Verrucaria adriatica* (Fig 3H) and *V. scabra* (Fig 3I). A pseudocortex can also be observed in some endolithic species (*Verrucaria dufourii* and *Verrucaria cyanea*), in which it is formed by a thin prosoplectenchyma intermingled with microcrystals, sometimes with the presence of one layer of slightly pigmented cortical cells at the top (Fig 3J). The term 'Scheinrinde' (which can translate to pseudocortex) has been used in the past (Poelt 1958: 418) to define the cortex of some *Lecanora* species. This type of upper cortex is formed of more or less vertical hyphae, which are not differentiated from the ones in the medulla and the algal layer. The 'Scheinrinde' develops from a zone located in the algal layer, and accumulates in its upward growth dead fungal and algal cells. The presence of dead algal cells in the upper cortex has been subsequently used to discriminate this type of cortex (presence of dead algal cells) from differentiated cortices ('Berindeter' or 'Normal' type in Poelt 1958; absence of dead algal cells) (Timdal 1984). It is likely that what is called pseudocortex in our study corresponds to the 'Scheinrinde' from Poelt (1958), but the presence or absence of dead algae was not investigated. (3) Eucortex: this term was used for a well-differentiated cortex, clearly delimited from the algal layer, paraplectenchymatous to scleroplectenchymatous, usually greater than 30 μm high (sometimes thinner in the *nigrescens*-type) (Fig 3K–P). The uppermost layers of cortical cells are in general pigmented (usually brown), and an epinecral layer is often present. Eucortex includes the *lachneum*-type cortex of *Placidium* and *Clavascidium* described by Breuss (1990) (Fig 3K). The *lachneum*-type cortex is formed by large cells of 5–14(–20) μm diam and with relatively thin walls and large lumina. In this type, the border between the algal layer and upper cortex is almost rectilinear. The eucortex is also present in the genus *Endocarpon* and other related squamulose species, but the border between the algal layer and the upper cortex is often irregular and sinuous due to the palisade structure of the algal layer (Fig 3L). In *Verrucaria nigrescens*, the cortex is somewhat thinner (sometimes less than 30 μm high) and formed by smaller cells (3.6–6.5 μm diam) (Fig 3M). However, the upper cortex constitutes a well-defined layer, well delimited from the algal layer and without any algal cells. This *nigrescens*-type eucortex is also present in *Verrucaria viridula* (Fig 3N) and *V. macrostoma* (Fig 3O). The upper cortex is usually paraplectenchymatous, but can be scleroplectenchymatous for some species (e.g. *Neocatapyrenium rhizinosum* in Fig 3P). (4) Lithocortex (Pinna *et al.* 1998, Gueidan and Roux 2007): this term was used to define the cortex of some endolithic species (Fig 3Q–S). Their upper cortex is about 30 μm thick, densely prosoplectenchymatous, and formed by conglutinated hyphae intermingled with microcrystals dissolving in chloridric acid (calcium carbonate). The top cortical layers of some species can contain amorphous pigments [pink to purple for *Verrucaria marmorea* (Fig 3S) or *Bagliettoa cazzae*, and greyish blue–green for *Bagliettoa parmigerella*]. This type of cortex could be similar to the lithocortex *s. str.* described by Bungartz *et al.* (2004) for the species *Verrucaria rubrocincta*. The cortex of this species is constituted of a layer of micrite reaching up to 50 μm in thickness including only sparse fungal hyphae.

The type of pycnidia also appears to be phylogenetically important. These structures were found and studied in 36 taxa (Figs 4 and 5). Observations were also made for *Verrucaria cyanea*, *V. dufourii*, *V. marmorea*, *V. maura* and *V. scabra*, but as they were incomplete, the pycnidial type could not be clearly interpreted. Additional anatomical studies will be conducted in order to define the types of pycnidia in these species. Two previously described types of pycnidia were observed in most of the species studied here (Fig 5): the *Dermatocarpon*-type and the *Endocarpon*-type (Janex-Favre & Wagner 1986). In the *Dermatocarpon*-type (Fig 4A–B), each pycnidium has several late-forming cavities that remain separated by a paraplectenchyma. The conidia are produced by cells bordering the cavities. The number of cavities varies significantly according to the genera, from many in the large pycnidia of *Dermatocarpon* to few in the small pycnidia of some species of *Verrucula* (two to six cavities visible in cross-section). These pycnidia are delimited by a thin wall and do not have well-differentiated ostioles. The conidia are released at maturity through fissures in the upper cortex. The *Endocarpon*-type pycnidium has a rudimentary wall and sometimes a sinuous outline (Fig 4C–E). They have a single early-forming cavity and radially oriented and differentiated hyphae bearing conidia cells (or conidiophores). Conidia are released through a narrow ostiole that is barely visible at the surface of the thallus (Fig 4C). A third type of pycnidium was observed in *Verrucaria mucosa* (Fig 4F). These *mucosa*-type pycnidia have a single cavity. The conidiogenous cells are elongated and directly border a rudimentary pycnidium wall. Nearby pycnidia can sometimes be laterally connected through their cavities, but each pycnidium always has its own well-differentiated ostiole.

mount of a thick section seen at an oblique angle showing a pigmented uppermost layer of cortical cells in *V. scabra*. (J) A thin and weakly differentiated endolithic cortex in *V. cyanea*. (K–P) Eucortex. (K) *Lachneum*-type cortex in *Placidium lachneum*. (L) With a palisade algal layer in *Endocarpon adscendens*. (M) In *V. nigrescens* (bar = 10 μm). (N) In *V. viridula*. (O) In *V. macrostoma*. (P) Scleroplectenchymatous in *Neocatapyrenium rhizinosum*. (Q–S) Lithocortex. (Q) In *Bagliettoa parmigerella*. (R) In *Bagliettoa* sp. (S) In *B. marmorea*, with purple pigmentation of the upper cortical layer (bar = 100 μm).

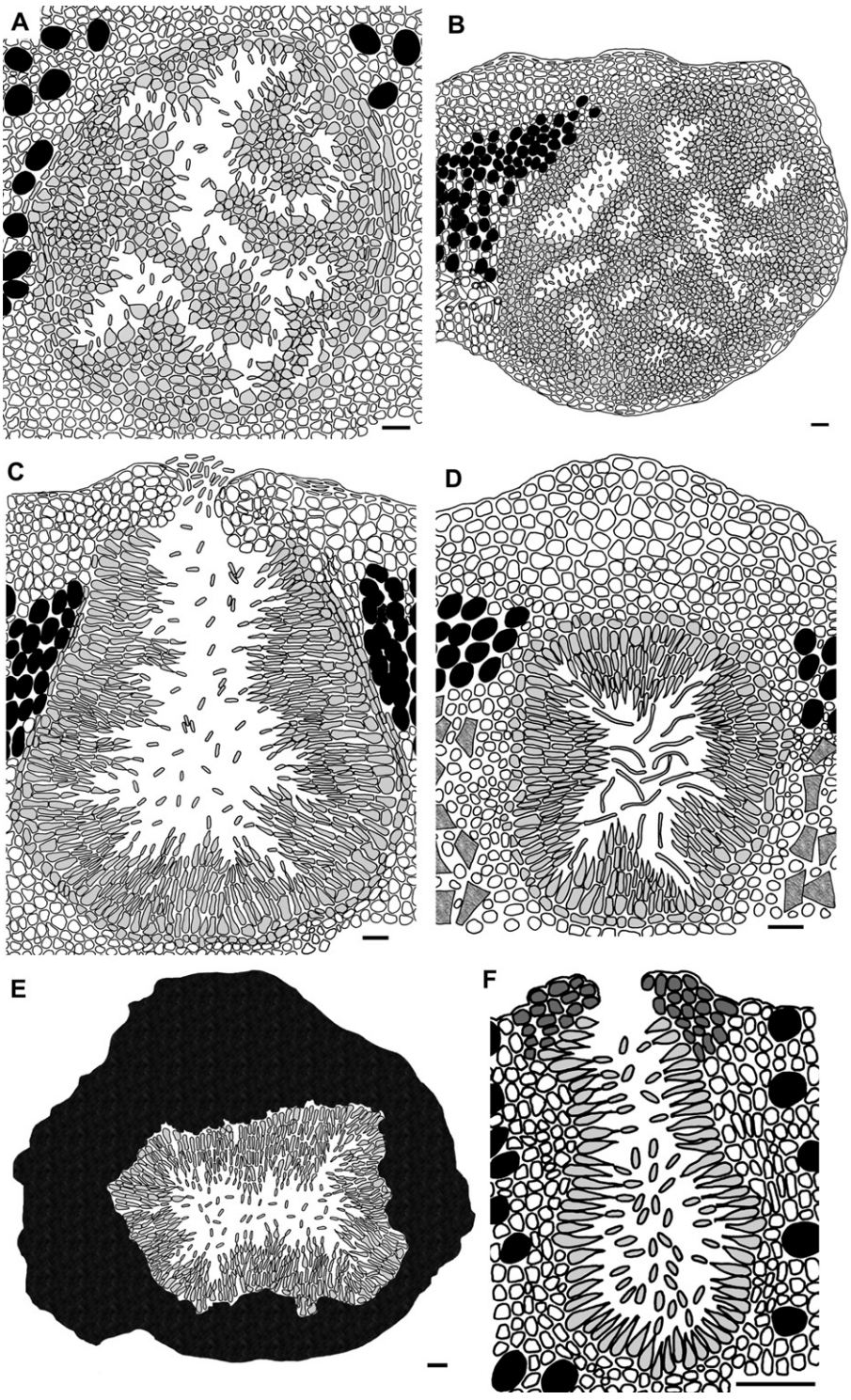


Fig 4 – Types of pycnidia observed in *Verrucariaceae*. Pycnidia are represented in light grey and algal cells in black. (A–B) Transversal sections of the thallus showing *Dermatocarpon*-type pycnidia, characterized by the presence of several cavities separated by a paraplectenchyma. (A) Laminal pycnidium in *Heteroplacidium fusculum*. (B) Marginal pycnidium in-*Placidium arboreum*. (C–E) *Endocarpon*-type pycnidia, characterized by the presence of conidiophores and one single cavity. (C) Longitudinal radial section of pycnidium with a narrow ostiole in *Endocarpon adscendens*. (D) Longitudinal tangential section of a pycnidium showing long and slightly curved conidiospores in *Verrucaria weddellii* (ostiole not visible). Angular grey shapes represent calcium carbonate crystals. (E) Longitudinal tangential section of a pycnidium embedded in a carbonaceous layer (in black) in *V. nigrescens*. (F) Longitudinal radial section of pycnidium in *V. mucosa*, with ostiole surrounded by black pigmented cells (in dark grey), and without conidiophores. Bars = 10 μm.

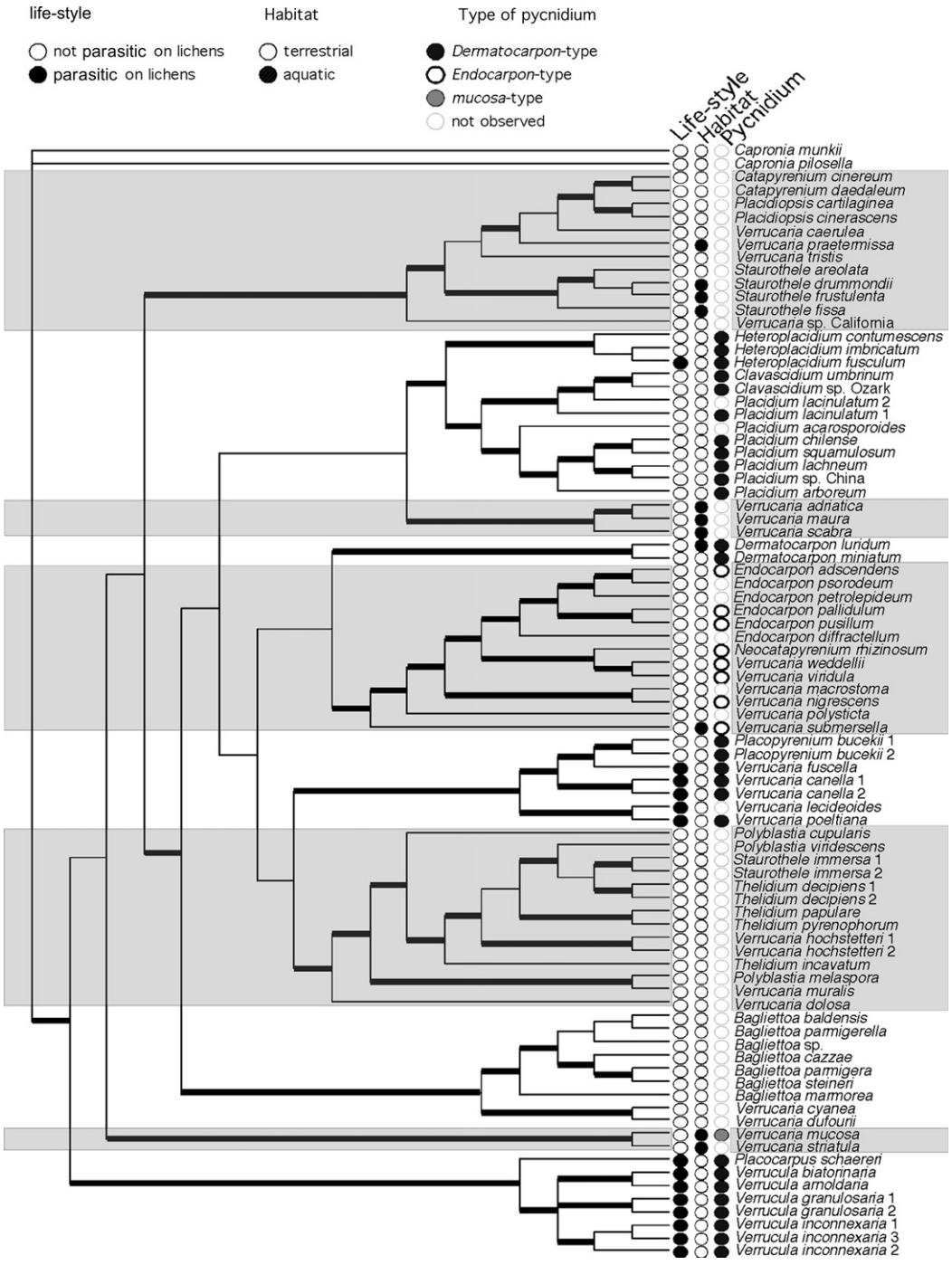


Fig 5 – Phylogenetic distribution of parasitic and aquatic species within the *Verrucariaceae*, and assessment of the importance of pycnidial structure as a phylogenetic character. Character states were reported on our most likely tree for each of the taxa. Thick branches represent well-supported nodes as defined in Fig 2. The alternating shaded pattern highlights the ten main monophyletic groups presented in Fig 2.

Cells bordering the ostiole have dark pigmentation. The type of conidiogenesis was not investigated in this study but should be examined using the terminologies of Vobis (1987).

Ancestral state reconstruction

Ancestral character states were reconstructed for about 30 critical nodes, depending on the character being analysed (Fig 6). The most recent common ancestor for *Verrucariaceae* was crustose and epilithic (Fig 6A), with a pseudocortex (Fig 6B), simple ascospores (Fig 6C) and without algae in its hymenium (Fig 6D). More complex squamulose thalli evolved at least four times in this family (all within lineages 3 and 4): in the ancestral lineage of the genus *Endocarpon*, of the *Placidium* group, of the species *Neocatapyrenium rhizinosum* and of the group including *Catapyrenium* and *Placidiopsis*. Two

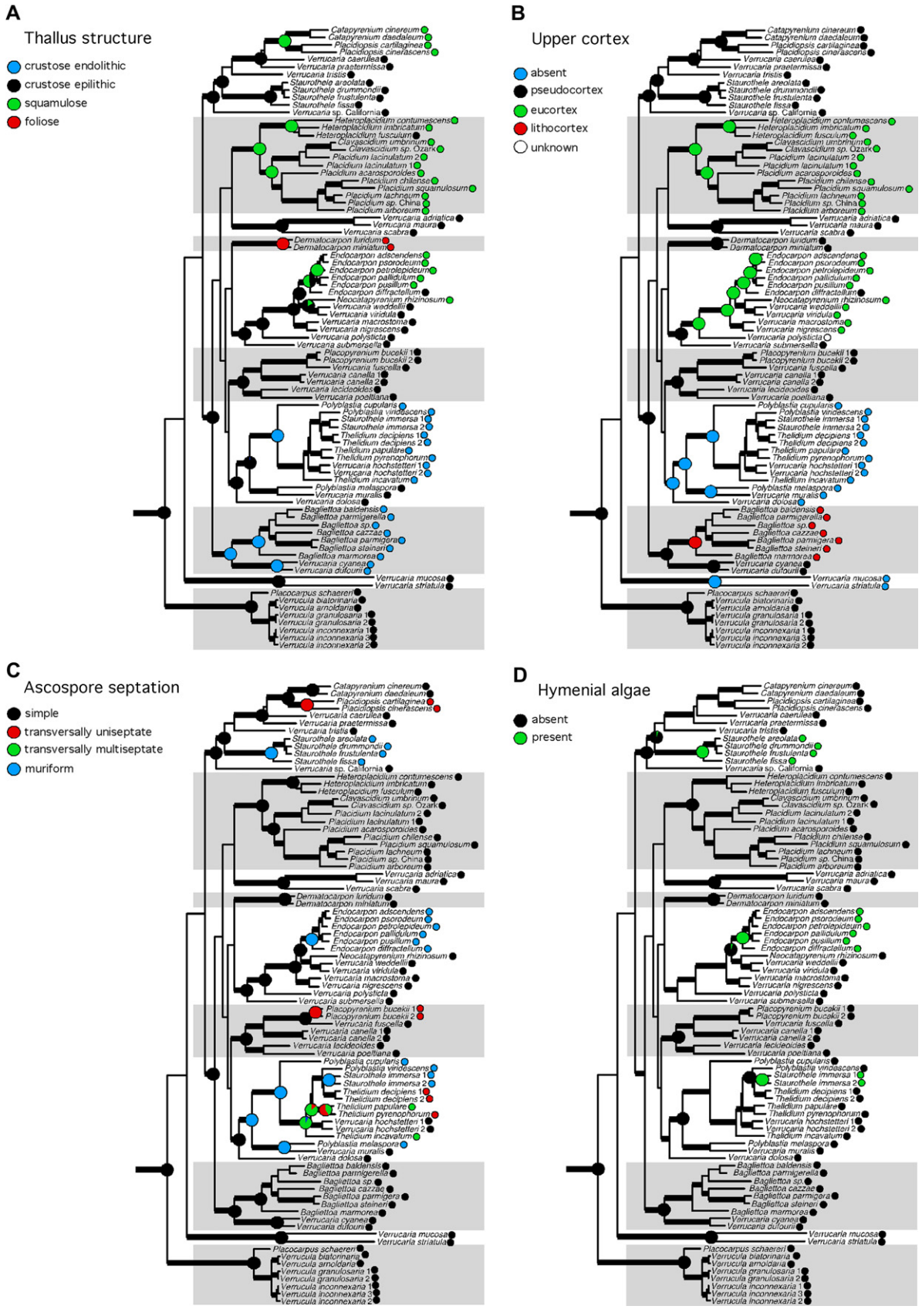


Fig 6 – Ancestral state reconstructions of the characters 'thallus structure' (A) 'upper cortex structure' (B) 'ascospore septation' (C) and 'hymenial algae' (D) in *Verrucariaceae* using a Bayesian approach. Estimates obtained with this method take into account phylogenetic and mapping uncertainties. Reconstructions are shown on each corresponding node by large coloured pie charts. Character states of each taxon are indicated by small coloured circles. The two outgroup species are not shown on the tree due to the lack of space, but were coded as follows: 'absent' for the thallus structure, the upper cortex structure and hymenial algae, and 'muriform' for ascospore septation. The alternating shaded pattern highlights the ten main monophyletic groups presented in Fig 2.

independent transitions to endolithism were detected in lineage 3 (in the *Polyblastia* group and at the base of the *Bagliettoa* group; Figs 2 and 6A), both derived from a crustose epilithic ancestor. An upper pseudocortex (ancestral state for this family) was lost independently in the ancestor of the marine group and the ancestor of the *Polyblastia* group. A eucortex evolved from an ancestor with a pseudocortex, at least twice in the family *Verrucariaceae*, once in the ancestral lineage of the *Placidium* group, and once during the early evolution of the *Endocarpon* group, followed by a reversal in the latter group (Figs 2 and 6B). A lithocortex evolved from an ancestor to the genus *Bagliettoa* with a pseudocortex, as what seems to be an adaptation to an earlier transition to endolithism in the ancestral lineage of the *Bagliettoa* group (Fig 6A–B).

Muriform ascospores have evolved three times independently from an ancestral simple ascospore state (in *Staurothele, Endocarpon,* and the *Polyblastia* group). Transversally uniseptate ascospores evolved independently twice, also from an ancestral simple ascospore state (in *Placidiopsis* and *Placopyrenium*). In the *Polyblastia* group, transversally uniseptate ascospores and transversally multiseptate ascospores might have evolved twice each. In this group, simple ascospores and transversally uniseptate and multiseptate ascospores seem to have evolved from an ancestral muriform character state. The presence of algae in the hymenium seems to have evolved independently at least three times without reversals (in the ancestral lineage of *Staurothele s.str.*, *Endocarpon,* and *Staurothele immersa*; Fig 6D).

Taxonomy

Endocarpon diffractellum (Nyl.) Gueidan & Cl. Roux, **comb. nov.**
MycoBank no.: MB511193
Basionym: *Verrucaria difractella* Nyl., *Mém. Soc. Acad. Maine-et-Loire* **4**: 33 (1858).
Synonym: *Staurothele diffractella* (Nyl.) Tuck., *Gen. Lich.*: 258 (1872).

Type: Nova Anglia, ad schistes micaceas, *Frost* 44, ex Tuckerman 134 (H-NYL 3645 – ***lectotypus hic designatus***!).

Remarks: For a description of this species and additional taxonomic information, see Thomson (1991). This species, until now included in *Staurothele*, is transferred to the genus *Endocarpon* based on molecular and morphological data. Although *E. diffractellum* differs from other species of *Endocarpon* by being crustose, this species shares homologous morphological character states typical of this genus, such as muriform ascospores (Fig 6C) and the presence of algae in the hymenium (Fig 6D; character states also shared with *Staurothele*), and a palisade algal layer (Fig 3L; character state most commonly found in *Endocarpon*).

Specimens examined: **USA**: *Missouri*: Perry County, Seventy-Six Conservation Area, 37°42′58″ N, 89°36′59″ W, alt. 125–150 m, on calcareous rocks, 13 Oct. 2003, *C. Gueidan* 585 (NY). *Vermont*: [Windham County:] Brattlebough, on damp rocks, *Frost* 44, 1856, (H-NYL 3645, lectotype; FH-Tuck 4131, H-NYL PM6906, isolectotypes); *sine loc.*, *C. C. Frost s. n.*, 1866 (FH, MICH).

Heteroplacidium fusculum (Nyl.) Gueidan & Cl. Roux, **comb. nov.**
MycoBank no.: MB511194
Basionym: *Verrucaria fuscula* Nyl., *Bot. Not.* 161 (1853).
Synonyms: *Encliopyrenia fuscula* (Nyl.) Trevis., *Conspect. Verruc*: 19 (1860).
Placidium insulare A. Massal., *Lotos* **6**: 78 (1856).
Verrucaria insularis (A. Massal.) Jatta, *Syll. Lich. Ital.*: 502 (1900).
Endopyrenium insulare (A. Massal.) Dalla Torre & Sarnth., *Flora v. Tirol* **4**: 509 (1902).
Physalospora insularis (A. Massal.) Sacc. & D. Sacc., *Syll. fung.* **17**: 586 (1905).
Laestadia insularis (A. Massal.) Vouaux, *Bull. Soc. Mycol. Fr.* **28**: 218 (1912).
Guignardia insularis (A. Massal.) Keissl., *Rabenh. Kryptfl. Deutschl.* **8**: 343 (1930).
Dermatocarpon insulare (A. Massal.) Migula, *Kryptfl. Deutschl.* **4**: 578 (1931).
Placidium iranicum Szat., *Ann. Mus. Nat. Hung., n. ser.,* **5**: 131 (1954).

Type: Ad calcem Jurassicam prope Cambouse (H-NYL).

Remarks: Both molecular and morphological evidence supports the inclusion of this taxon within the genus *Heteroplacidium* (Figs 2 and 6). As for *Heteroplacidium*, the structure of the thallus of *Verrucaria fuscula* is entirely paraplectenchymatous (synapomorphy for the genus *Heteroplacidium*), and as for all members of the *Placidium* group studied so far, pycnidia are of the *Dermatocarpon*-type (Figs 4A and 5). The inclusion of *Verrucaria fuscula* within *Heteroplacidium* is in agreement with Breuss (1990) who reported morphological similarities between *V. fuscula* and *Heteroplacidium contumescens*. The genus *Heteroplacidium*, first described as including species with mostly small squamulose thalli (Breuss 1996), is here shown to also comprise species with crustose-areolate thalli.

Specimens examined: **France**: *Bouches-du-Rhône*: Vauvenargues, Pic des Mouches, alt. 1000 m, on calcareous rocks, 19 May 2003, *C. Gueidan 582* (DUKE); Le Destet, Les Alpilles, alt. *ca.* 150 m, on calcareous rocks, 22 Jan. 2002, *C. Gueidan 463* (MARSSJ). *Haute-Savoie*: La Muraz, le Grand Salève, alt. 1250 m, on calcareous rocks, 22 Aug. 2001, *C. Gueidan* 555 (MARSSJ).

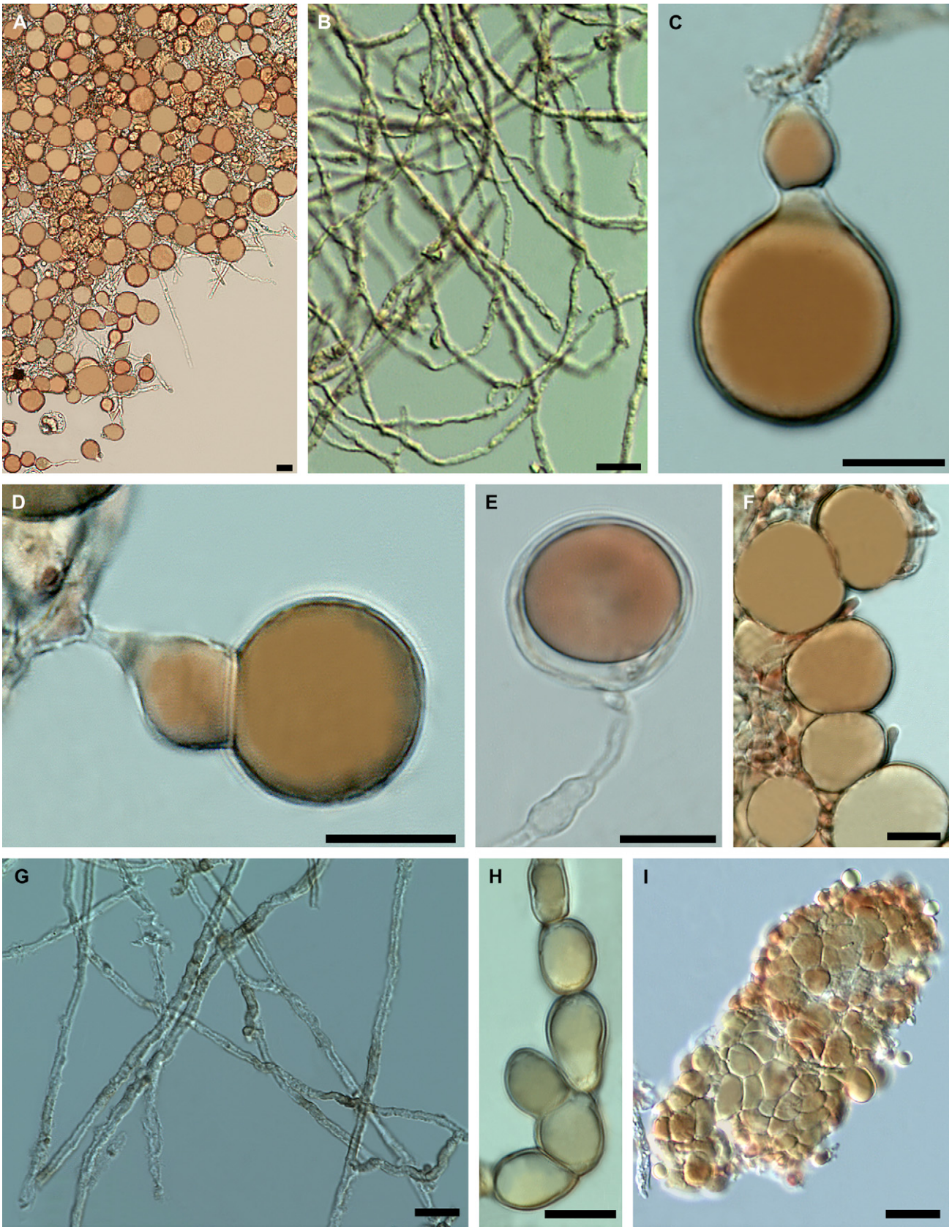
Bagliettoa marmorea (Scop.) Gueidan & Cl. Roux, **comb. nov.**
MycoBank no.: MB511195
Basionym: *Lichen marmoreus* Scop., *Fl. Carniol.* **2**: 367 (1772).
Synonyms: *Verrucaria marmorea* (Scop.) Arnold, Verh. *Zool. Bot. Ges. Wien* **32**: 147 (1882).
Amphoridium marmoreum (Scop.) Baroni, *Nuov. Giorn. Bot. Ital.* **23**: 445 (1891).
Verrucaria purpurascens Hoffm., *Descr. Adumbr. Pl. Lich.* **1**: 74 (1790).
Amphoridium purpurascens (Hoffm.) Massal., *Mem. Lich.*: 145 (1853).
Verrucaria calciseda var. *decipiens* Trevis., *Conspect. Verruc.*: 8 (1860).

Type: [**Slovenia**:] montes Julijske Alpe, in declivibus vallis 'dolina Triglavskih jezer' supra Veliko jezero, secus viam, 1830 m, *J. Halda, S. Haldová,* 2000/06/23 (PRM-900619 – neotype; Halda 2003).

Remarks: The presence of an involucrellum with a star-shaped aperture was a diagnostic character in the original description of the genus *Bagliettoa* (Massalongo 1853). However, our molecular and morphological study shows that this genus also includes species without this characteristic aperture of the involucrellum, such as *Bagliettoa* sp. and *Verrucaria marmorea* (Fig 2). The generic concept for *Bagliettoa* is, therefore, slightly broadened to include species with the following characters: (1) endolithic thallus (Fig 6A), (2) immersed perithecia, (3) when present, oil cells branching laterally or terminally (Fig 7A–F), and (4) lithocortex (Figs 3Q–S and 6B). Because the upper cortex is differentiated into a lithocortex, the outline of the thallus is usually well visible on the rock surface. Although *Verrucaria marmorea* lacks both an involucrellum with a star-shaped aperture and oil cells in the

medulla, molecular data and other morphological characters (endolithic habit, immersed perithecia and lithocortex; Figs 3Q–S, and 6A–B) support its placement within the genus *Baglietoa*.

Specimens examined: **France**: *Lozère*: Freyssinet-de-Fourques, Nîmes-le-Vieux, alt. *ca*. 1000 m, on calcareous rocks, 26 Aug. 2005, *C. Gueidan 739* (DUKE). *Ardèche*: Vallon-Pont-d'Arc, Le Planas, alt. 150 m, 28 Jul. 2001, *C. Gueidan 368* (DUKE). *Var*: Plan-d'Aups



Sainte-Baume, vallon de Castelette, alt. 680 m, on calcareous rocks, 13 May 2001, *C. Gueidan 15* (MARSSJ).

Discussion

Genera within the family *Verrucariaceae* were traditionally defined by the morphology of their thallus, their ascospore septation, and the presence or absence of algae in the hymenium (Servít 1955; Zahlbruckner 1921–22); Zschacke 1933–34. The morphological heterogeneity within *Verrucaria* was acknowledged to be problematic by Halda (2003) and Poelt & Hinteregger (1993), but molecular data were not available at that time, or insufficient, to use monophyly as a grouping criterion and to decipher which morphological characters provided synapomorphies for these recognized monophyletic entities. Our molecular phylogenetic study revealed that the four most speciose genera of this family (*Verrucaria*, *Staurothele*, *Thelidium,* and *Polyblastia*) are polyphyletic and that the genus *Verrucaria* was defined using sympleisiomorphic character states (such as crustose thallus and simple ascospores) rendering the taxonomic disentanglement of this family virtually impossible without a broad and comprehensive molecular phylogenetic survey of this family (Fig 6). The complexity of this taxonomic problem is best exemplified by *Verrucaria* species being found in eight of ten main monophyletic groups of the *Verrucariaceae*, as highlighted in Fig 2. For *Staurothele*, *Thelidium,* and *Polyblastia*, polyphyly resulted from the use of homoplastic traits, such as ascospore septation and presence/absence of hymenial algae (Fig 6C–D). Our study brings a novel insight into the generic delineation and character evolution within the family *Verrucariaceae* by combining molecular and morphological data. First, the traditional generic circumscription within *Verrucariaceae* is compared to the monophyletic groups inferred using molecular data. Second, the evolution of some selected morphological characters is discussed based on ancestral state reconstructions.

Traditional generic circumscriptions within Verrucariaceae *versus monophyly*

Squamulose taxa

In previous taxonomical treatments (Breuss 1990, Clauzade & Roux 1985, Nimis 1993), the squamulose taxa within *Verrucariaceae* were mainly included in two different genera: *Catapyrenium s. lat.* (simple ascospores and absence of algae in the hymenium) and *Endocarpon* (muriform ascospores and presence of algae in the hymenium). The genus *Catapyrenium s. lat.* has recently been divided into eight genera (Breuss 1996, Harada 1993b): *Anthracocarpon* Breuss 1996, *Clavascidium* Breuss 1996, *Catapyrenium* Flot. 1850, *Placidium* A. Massal. 1855, *Heteroplacidium* Breuss 1996, *Scleropyrenium* H. Harada 1993, *Involucropyrenium* Breuss 1996, and *Neocatapyrenium* H. Harada 1993, which will be further referred to as catapyrenioid genera. The splitting of *Catapyrenium s. lat.* was based on a combination of characters such as structure of the pycnidium, structure of the upper cortex, ascus shape, and arrangement of the ascospores in the ascus (Breuss 1996; Harada 1993b). As suggested by Breuss (1983, 1996; Breuss & Hansen 1988), *Catapyrenium s. str.* is part of a lineage distinct from other catapyrenioid genera and are more closely related to the genus *Placidiopsis* (Fig 2). *Catapyrenium s. str.* and *Placidiopsis* have the same thin, small-celled, and not clearly delimited type of upper cortex (pseudocortex), also called *cinereum*-type (Fig 3E–F), which greatly differs from the upper cortex of the other catapyrenioid genera *Placidium*, *Clavascidium,* or *Heteroplacidium* (Figs 3K and 6B).

The main monophyletic group of catapyrenioid species (*Placidium* group) comprises the three genera *Placidium*, *Clavascidium,* and *Heteroplacidium* (Fig 2). Both *Placidium* and *Clavascidium* have a well-delimited and large-celled upper cortex (eucortex of the *lachneum*-type) and *Dermatocarpon*-type pycnidia (Figs 5 and 6B), but differ from each other by their ascus structure and ascospore arrangement. *Clavascidium* has clavate asci with ascospores in a biseriate order, whereas *Placidium* has asci which are, at least at the beginning of their development, cylindrical and with ascospores in a uniseriate order (Breuss 1996). As shown in Fig 2, *Clavascidium* is nested within *Placidium,* questioning the value of these two linked characters to recognize *Clavascidium* and *Placidium* as distinct genera. The simplest solution to maintain the monophyly of *Placidium* would be to subsume *Clavascidium* within *Placidium s. lat.* especially, if no other character can explain the phylogenetic structure revealed here within *Placidium s. lat.* (Fig 2). Taxonomic changes will be proposed in a later study after obtaining molecular data from the type species *Placidium michelii* and from more *Clavascidium* species. The genus *Heteroplacidium* (now including *H. fusculum*) is also characterized by *Dermatocarpon*-type pycnidia (Fig 5), but differs from the genera *Placidium* and *Clavascidium* by having all thallic layers (i.e., upper cortex, algal layer, medulla, and lower cortex) paraplectenchymatous.

The genus *Neocatapyrenium* constitutes a third lineage of catapyrenioid species in our phylogeny. It is closely related to the genus *Endocarpon* and forms a monophyletic group with this genus when *V. viridula* and *V. weddellii* are included (Fig 2). This close relationship between *Neocatapyrenium* and *Endocarpon* is supported by two morphological character states: (1) palisade algal layer (algal cells arranged in columns; Fig 3L), and (2) *Endocarpon*-type pycnidium (Figs 4C–E and 5).

Fig 7 – Light micrographs showing the structure of the pseudomedulla of some endolithic members of the *Verrucariaceae*. Stained for lipids with Sudan IV. (A–F) In *Bagliettoa*. (A) Presence of numerous oil cells in the pseudomedulla of *B. parmigera*. (B) Oil cells absent in the pseudomedulla of *B. marmorea*. (C–F) In *B. parmigera*. (C–D) Oil cells can be branched laterally to the hypha and have a basal swelling. (E) Oil cells can also be formed at the extremity of the hypha and can lack the basal swelling. (F) When numerous and dense, the oil cells look like if they were forming chains. (G–I) In the *Polyblastia* group. (G) Oil cells absent in *Polyblastia cupularis*. (H) Chain of oil cells in *Thelidium pyrenophorum*, formed by the swelling of consecutive hyphal cells. (I) Large cluster of aggregated oil cells in *V. muralis*. Bars = 10 μm.

The palisade algal layer is also present in some of the related species, such as *V. nigrescens*, but was never observed in the catapyrenioid genera *Placidium*, *Clavascidium*, *Heteroplacidium,* and *Catapyrenium s. str.* (Breuss 1990). The use of this character state as a diagnostic feature for the *Endocarpon* group has to be done with caution because it is absent in some *Endocarpon*-related species (*V. viridula* and *V. weddellii*), and present in non-related taxa (e.g., *Placopyrenium bucekii* and *V. maura*). A typical *Endocarpon*-type pycnidium has been observed in *Neocatapyrenium rhizinosum* and is likely to be a synapomorphy for the *Endocarpon* group (Fig 5), whereas other catapyrenioid taxa (*Placidium*, *Clavascidium,* and *Heteroplacidium*) are characterized by *Dermatocarpon*-type pycnidia (Breuss 1990).

The genus *Endocarpon* traditionally includes squamulose species with muriform ascospores and hymenial algae, whereas crustose species with muriform ascospores and hymenial algae were placed in the genus *Staurothele*. In our study, the crustose species *S. diffractella* is sister to the genus *Endocarpon* and is distantly related to the core of the genus *Staurothele* found in lineage 4 (Fig 2). *Staurothele diffractella* differs from other species of *Staurothele* by having a palisade algal layer, a character commonly present amongst species of *Endocarpon*. This character, as well as our molecular results, justified the new combination *Endocarpon diffractellum* (see Taxonomy section).

Parasitic taxa

Poelt was the first author to study the systematics and the biology of parasitic lichens (Poelt 1958, 1990, Poelt & Doppelbauer 1956). This author recognized different levels of parasitism, spanning from unspecialized facultative parasites, to specialized facultative parasites and specialized obligate parasites (Poelt 1990). Since then, very few studies have treated the systematic aspect of these parasitic lichens (Hertel 1970; Zehetleitner 1978). In *Verrucariaceae*, the first exhaustive study of parasitic lichens listed 11 taxa, mostly belonging to the genus *Verrucaria* (Zehetleitner 1978). In this study, Zehetleitner (1978) used the term parasitic lichens (or specialized parasites according to Poelt 1990) for species growing on other lichens, killing the fungal partner or both fungal and algal partners of their host and, at the end, always building their own lichen thallus. Some of these species only start the first stage of their development by growing on other lichens, and later become independent from their host. These 'juvenile' parasites can be facultative or obligate. Other species strictly depend on the presence of their lichen host for which they are often highly specific (Zehetleitner 1978). It is noteworthy that high host specificity is also generally found in lichenicolous fungi (non-lichenized fungi growing as commensals or parasites on other lichens), which are quite abundant in the order *Verrucariales*, in both families *Adelococcaceae* and *Verrucariaceae*. Many more parasitic lichens have now been described within *Verrucariaceae* (Clauzade & Roux 1985; Navarro-Rosinés *et al.* 2007). In our study, parasitic lichens were found to be present in three distinct lineages (Fig 5): (1) the *Verrucula* group, including *Verrucula* and *Placocarpus* (lineage 1; Fig 2), (2) the *Placopyrenium* group, including *Placopyrenium* and closely related *Verrucaria* species (in lineage 3), and (3) *Heteroplacidium fusculum*, a member of the *Placidium* group (in lineage 3).

Placocarpus schaereri is generally found, at least in its early stage of development, parasitizing *Protoparmeliopsis muralis* (Zehetleitner 1978). It is sister to some species mainly parasitic on a group of *Caloplaca* with anthraquinones and lobate or not, and on *Xanthoria elegans* (Fig 2). This group of parasitic species is monophyletic, except for *Verrucaria poeltiana* (parasitic on *Caloplaca aurantia*), which is nested within the *Placopyrenium* group. The monophyly of these parasitic taxa (excluding *V. poeltiana*) confirms their close relationship inferred by Navarro-Rosinés *et al.* (2007), and Zehetleitner (1978) based on morphological data. The genus name *Verrucula*, previously described by Steiner (1896) to accommodate a group of parasitic *Verrucaria,* but never subsequently used, is available for this group (Navarro-Rosinés *et al.* 2007). In *Verrucula*, specificity to the lichen host seems to be high, as in general each host species is colonized by a different species of *Verrucula* (Navarro-Rosinés *et al.* 2007, Zehetleitner 1978). However, it has also been suggested that, because of the low morphological variation in this group, all species parasitic on *Caloplaca* sec. *Gasparrinia* could in fact constitute a single species, and that specificity for a lichen host cannot be used to delimit these species (see Zehetleitner 1978: 703, McCarthy 1988). Our very preliminary sampling (Fig 2), supports, or at least, does not oppose, the recognition of these taxa (some with overlapping geographical ranges) as distinct *Verrucula* species. A complete revision of the genus *Verrucula* will be available in Navarro-Rosinés *et al.* (2007).

In the second parasitic lineage, the two parasitic lichens *Verrucaria canella* and *V. fuscella* are most closely related to *Placopyrenium bucekii*, a non-parasitic species. *Verrucaria canella* is a crustose lichen mainly found growing on *Aspicilia calcarea* (formely known as *V. aspiciliicola*, but synonymized with *V. canella* by Orange 2004) and was described as a facultative parasite by Zehetleitner (1978). *Verrucaria fuscella* is also crustose and often starts its development on *V. nigrescens*. *Placopyrenium bucekii* differs from these two species by several features such as early gelatinizing asci, a placodioid thallus with peripheral lobate areoles, and a black prothallus (Ménard & Roux 1995). The thalli of *V. fuscella* and *V. canella* are generally not lobate, the areoles are smaller, and the prothallus is absent. However, a few specimens of *V. canella* have been observed growing partially or entirely on rock, independently from their host. In these specimens, the thallus is slightly lobate, and, similarly to *P. bucekii*, a black prothallus can sometimes be observed. *Verrucaria lecideoides* and *V. poeltiana* also belong to the *Placopyrenium* group (Fig 2). All species included in this study that are part of this group have very similar perithecia: immersed, with colourless excipulum, becoming dark brown around the ostiole (with the exception of *V. lecideoides*, for which the excipulum becomes completely dark brown at maturity). *Placopyrenium bucekii* has simple or uniseptate ascospores. Zehetleiner's (1978) diagnosis, as well as our observations on diverse specimens of *V. canella,* showed that the ascospores of this species could be simple or uniseptate. *Verrucaria fuscella*, *V. lecideoides,* and *V. poeltiana* also occasionally have uniseptate ascospores, although always in small number. Both morphological and molecular evidence suggest that these parasitic species of *Verrucaria* are more closely related to the genus *Placopyrenium* than to other species of *Verrucaria* and, therefore, could be subsumed within *Placopyrenium*

if no morphological features correlate with the phylogenetic structure found within this group (Fig 2).

Heteroplacidium fusculum, a parasite on *Aspicilia calcarea*, is part of the third lineage that includes parasitic species (Fig 5). Based on morphology, two other parasitic lichens, *V. compacta* (A. Massal.) Jatta (found as a saxicolous or as an epiphyte on crustose or non-crustose allied lichens) and *Verrucaria zamenhofiana* Clauz. & Cl. Roux (a parasite on *Staurothele areolata*), might also belong to the genus *Heteroplacidium*.

Endolithic taxa

The strictly endolithic species (i.e. excluding species that can sometime have a partially endolithic thallus with a medulla anchored in the rock; e.g., *Verrucaria tristis*, *V. weddellii*), are restricted to the *Polyblastia* and the *Baglietto*a groups (Figs 2 and 6A). Massalongo (1853) included in the genus *Baglietto*a the calcicolous endolithic species of *Verrucariaceae* characterized by an involucrellum with a star-like aperture. This genus was subsequently accepted by some authors (Poelt & Vězda 1981; Santesson 1993; Santesson *et al.* 2004) but not by others (Clauzade & Roux 1985; Halda 2003). In his recent revision of this group, Halda (2003) concluded that the validity of this genus could not be confirmed without a global study of the involucrellum in all taxa of *Verrucaria*. Our results show that these calcicolous endolithic species having an involucrellum with a star-like aperture are indeed closely related and form a well-defined lineage within the *Verrucariaceae* (Fig 2). However, *Baglietto*a sp. (see Appendix 1), which lacks this characteristic involucrellum, also belongs to this group. Moreover, the species *V. marmorea*, which is sister to the genus *Baglietto*a, also lacks this typical involucrellum. Because this species shares three main diagnostic characteristics with other species of this genus (endolithic growth, immersed perithecia and lithocortex), *V. marmorea* is included within *Baglietto*a. Another particularity of some species in the genus *Baglietto*a is the frequent absence of ascospores. In these species, asci are abundant but stay sterile even in mature perithecia (Fig 8A). It is noteworthy to mention that this absence of ascospores is often correlated with the presence of well-developped short pseudoparaphyses that can easily fragment and could propagate through the ostiole of the perithecia (Fig 8B). These hyphae are present from the beginning of the formation of the primordium and cover the upper part of the perithecial cavity up to the inner part of the ostiole (Janex-Favre 1970). For three species, for which we did not observe any ascospores (*Baglietto*a *cazzae*, *B. parmigera* and *B. parmigerella*), and for one species, for which asci produced only deformed ascospores (*B. baldensis*), short pseudoparaphyses were particularly abundant, well developed, and the terminal segments of these sterile hyphae were slightly swollen and easily detachable (Fig 8B). Because sexual ascospores are absent in these species, and the layer of rock covering their thallus (endolithic thallus) prevents the propagation of typical asexual propagules, it is possible that these short pseudoparaphyses, or at least their apical segments, are adaptations by mycobionts to reproduce asexually when living endolithically. Two endolithic species, *V. dufourii* and *V. cyanea*, form a monophyletic group sister to *Baglietto*a (Fig 2). They differ from this genus by having half-emergent perithecia with a large involucrellum covering almost the entire perithecium. Moreover, their upper cortex is not as well differentiated as the lithocortex of *Baglietto*a (Fig 3J). Therefore, they were not included in this genus, and are in need of a new genus name, which we plan to publish in a subsequent article focusing on the establishment of a new classification for *Verrucariaceae*.

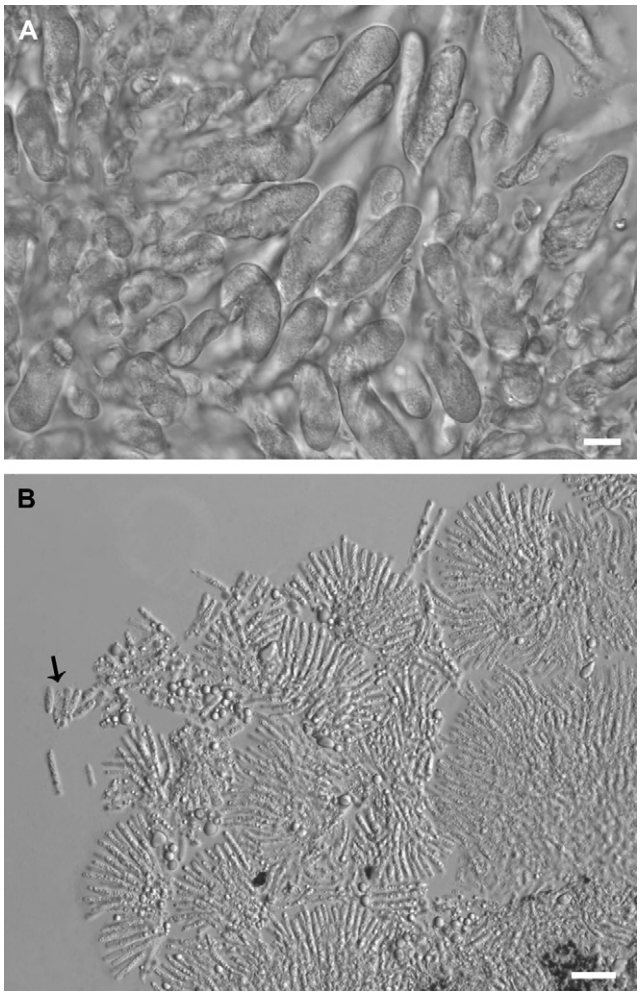


Fig 8 – Light micrographs of: (A) the sterile hymenium of *Baglietto*a *parmigerella*; (B) well-developped short pseudoparaphyses of *B. parmigera*, with detachable terminal segments (arrow). Bar = 10 μm.

The second monophyletic group of endolithic taxa is found in the *Polyblastia* group, and includes species currently classified across four genera: *Polyblastia*, *Staurothele*, *Thelidium,* and *Verrucaria* (Figs 2 and 6A). It is sister to a group of epilithic taxa represented by *Polyblastia melaspora* and *Verrucaria muralis*. This second group of endolithic taxa differs from the *Baglietto*a group by lacking an upper cortex (Figs 3B and 6B), and in the morphology of their oil cells (Fig 7), i.e. rounded cells containing lipids, located in the pseudomedulla of diverse endolithic species of lichens (macrospheroids according to Zukal 1886). Several morphological studies have been conducted on these enigmatic structures (Doppelbauer 1959; Fry 1922; Kushnir *et al.* 1978; Pinna *et al.* 1998) and, although their function is still unknown, it has been shown that the morphology of the oil cells is quite diverse. In the *Baglietto*a

group, some species (*B. baldensis*, *B. cazzae*, *B. parmigera*, *B. parmigerella* and *B. steineri*) have numerous large oil cells in the pseudomedulla (Fig 7A), whereas other species lack them (*Baglietoa* sp., *B. marmorea* Fig 7B, *V. cyanea* and *V. dufourii*). When present, these oil cells are either laterally branched on the hyphae (Fig 7D) or formed at their tip (Fig 7E), and can sometimes be so densely aggregated along hyphae that they resemble chains of cells (Fig 7F). In *Baglietoa*, rounded oil cells with a swollen base are typical (Fig 7C–D). In the endolithic clade within the *Polyblastia* group (Figs 2 and 6A), most taxa (*Polyblastia cupularis*, *P. viridescens*, *Thelidium decipiens*, *T. incavatum*, *Staurothele immersa*, *V. hochstetteri*) lack oil cells in their pseudomedulla (Fig 7G). However, oil cells have been observed for *Thelidium papulare* and *T. pyrenophorum* (two sister species). These oil cells differ from those observed in *Baglietoa*, by forming chains that resulted from the swelling of consecutive hyphal cells (Fig 7H). In *V. muralis*, oil cells are also present but form large clusters of strongly aggregated cells (more than 50 cells per cluster; Fig 7I). Therefore, we do not consider the oil cells found in members of the genus *Baglietoa* to be homologous to oil cells found in *T. papulare* and *T. pyrenophorum*. The *Polyblastia* group remains taxonomically problematic because it potentially includes the type species of the three genera *Polyblastia*, *Thelidium*, and *V. muralis*). Additional sampling and more detailed observation of morphological characters are necessary before carrying out any taxonomic changes in this group.

Aquatic taxa

In his classification of the *Verrucariaceae*, Servít (1953, 1954) created the subgenus *Hydroverrucaria* (Table 1) for the species of *Verrucaria* with completely prosoplectenchymatous or paraplectenchymatous thallus and growing in freshwater aquatic habitats. Several revisions of these freshwater species (Keller 1995; McCarthy 1995; Swinscow 1968; Thüs 2002) revealed a particularly high level of infraspecific variability and the need for further studies on their relationships with non-aquatic taxa. The colonization of aquatic habitats seems to have evolved several times in the *Verrucariaceae* (Fig 5). However, a denser sampling of species with less phylogenetic uncertainty for deep relationships within this family is needed to reconstruct the evolutionary history of this trait. The genera *Staurothele* and *Dermatocarpon* include both aquatic and non-aquatic taxa (Fig 5). Aquatic species are also known to belong to the genera *Polyblastia* and *Thelidium* (Keller & Scheidegger 1994; Keller 2000; Orange 1991; Swinscow 1971), although none of these species were included in our study. The freshwater species *V. praetermissa* shares a most recent common ancestor with *Catapyrenium*, *Placidiopsis* and *V. caerulea* (Figs 2 and 5). Another freshwater species, *V. submersella*, is shown here to be sister to the rest of the members of the *Endocarpon* group that we sampled. The two other putative independent colonizations of aquatic habitats are associated with marine inhabiting species. *Verrucaria maura* and *V. adriatica* form a monophyletic group with the freshwater species *V. scabra* within lineage 3 (Fig 5). All three species are characterized by ascospores greater than 10 μm long. Moreover, Keller (1995) mentioned that *V. rheitrophila*, a species closely related to *V. scabra*, develops thalline black punctae (Swinscow 1968) similar to those found in the marine species *V. maura* and *V. adriatica*. These black punctae are also found in *V. scabra*. The second marine group (lineage 2), which includes *V. mucosa* and *V. striatula*, is characterized by smaller ascospores (Flenniken & Gibson 2003) and the lack of an upper cortex (Figs 3A and 5).

Evolution and taxonomic relevance of selected morphological traits for a phylogenetically based classification of Verrucariaceae

Pycnidia

Pycnidia and pycnidiospores are terms commonly used to refer to the generally flask-shape conidiomata and their conidia frequently found in lichen-forming fungi (Kirk *et al.* 2001). The role of the conidia as either spermatia or asexual propagules has long been controversial (Smith 1921), and empirical data are still lacking to clarify their biological role. Although their function is unknown, they have been the subject of morphological studies since the 1850s and recognized as important characters in lichen classification (Glück 1899; Lindsay 1859, 1872; Steiner 1901; Tulasne 1852). Unfortunately, they have been often neglected by systematists, probably because of their sporadic presence for many species and their apparent absence in some groups. More recently, some authors contributed to the understanding of pycnidia morphology by studying their ontogeny (Janex-Favre 1977, 1980, 1982; Letrouit-Galinou 1972, 1973, 1984; Letrouit-Galinou & Lallemant 1977; Vobis 1980), and several types of pycnidia were defined (Vobis 1980; Vobis & Hawksworth 1981).

Conidiomata in the family *Verrucariaceae* have also been the subject of morphological and ontogenetical studies (Janex-Favre & Wagner 1986). Since then, some authors working on members of *Verrucariaceae* have systematically included a description of the pycnidium in their taxonomic work (Breuss 1990; Harada 1992a, 1992b, 1993a; McCarthy 1991; Ménard & Roux 1995), and new genera were segregated based on pycnidial characters (Breuss 1996; Harada 1993b).

Two main types of pycnidia were described for *Verrucariaceae*: the *Dermatocarpon*-type and the *Endocarpon*-type (Janex-Favre & Wagner 1986). The *Dermatocarpon*-type (also known as the *Endocarpon*-type *sensu* Glück 1899 and *Xanthoria*-type *sensu* Vobis 1980) is characterized by its paraplectenchymatous net and its multiple cavities (Fig 4A–B). This pycnidium type has been reported, and systematically characterized, for four main groups within *Verrucariaceae*: (1) *Dermatocarpon*; (2) the *Verrucula* group; (3) the *Placopyrenium* group and; (4) the *Placidium* group (Figs 2 and 5). The *Endocarpon*-type is characterized by the presence of radially oriented and differentiated hyphae (conidiophores) bearing conidiogenous cells and a single cavity (Fig 4C–E), and has been observed only in members of the *Endocarpon* group (Fig 5). In the two sister species *V. weddellii* and *Verrucaria viridula*, the *Endocarpon*-type pycnidia are characterized by long and slightly curved conidiospores (Fig 4D). Illustrations of pycnidia from *V. nipponica* (Harada 1992b), *V. igii* (Harada 1996a), and *Staurothele iwatsukii* (Harada 1992a) show conidiophores, suggesting a close affinity to the *Endocarpon* group. Harada described the pycnidia of these three species as being of the *Staurothele*-type. He also considered pycnidia from *Neocatapyrenium*, *Endocarpon,* and *Scleropyrenium* to be of

the *Staurothele*-type (Harada 1993b). The use of the term *Staurothele*-type to define pycnidia in *Verrucariaceae* has previously been questioned (Ménard & Roux 1995). Pycnidia have never, or only very rarely, been observed in the *Staurothele* group (Fig 5). Moreover, as the term *Endocarpon*-type predates the term *Staurothele*-type, it is here retained to define pycnidia with conidiophores and a single cavity.

A third type of pycnidia has been reported for *Verrucariaceae*, unilocular and with conidiogenous cells directly bordering the pycnidium wall, i.e., without conidiophores (*Verrucaria mucosa*, Fig 4F). Illustrations of pycnidia for *Verrucaria maura* (Harada 1996b) show conidiogenous cells that seem to be directly bordering the pycnidium wall, similar to *V. mucosa*.

Based on the type of pycnidium, it is now possible to propose hypothetical affiliations for some genera or species of the family *Verrucariaceae* to specific monophyletic groups recovered here (Figs 2 and 5). The genera *Scleropyrenium* (Harada 1993b) and *Anthracocarpon* (Breuss 1996) previously belonged to *Catapyrenium s. lat.*, a genus including mostly species with *Dermatocarpon*-type pycnidia (Breuss 1990). Because these two genera have *Endocarpon*-type pycnidia, they are more likely to be closely related to the *Endocarpon* group (Fig 5).

Thallus structure

In five instances during the evolution of the *Verrucariaceae* the thallus evolved from a crustose to a squamulose or foliose growth form (Fig 6A). In the lineage leading to the genus *Dermatocarpon*, a transition to a foliose umbilicate thallus took place. The first divergence within the *Placidium* group seems associated with the evolution of large squamulose thalli in the ancestral lineage leading to *Clavascidium* and *Placidium* in contrast to smaller squamulose thalli in the sister lineage leading to *Heteroplacidium,* with probably a reversal to the crustose form for *H. fusculum* (a crustose parasitic species with a thallus ranging from areolate to squamulose-areolate). A squamulose thallus seems to have evolved in the most recent common ancestral lineage of the genera *Catapyrenium* and *Placidiopsis*, although they never reach large sizes as in other catapyrenioid taxa (e.g., *Placidium*). Many taxa with squamulose thalli such as *Anthracocarpon*, *Scleropyrenium,* or *Involucropyrenium* are suspected to belong to the *Endocarpon* group, but could not be included in this study because of lack of material. Therefore, in this group, the evolution of squamulose thalli might have been earlier than suggested here and might have evolved only once (Fig 6A). An evolutionary trend away from typical crustose thalli is also noticeable in the *Placopyrenium* group. Species from this genus have placodioid thalli, i.e. crustose thalli with radiating marginal lobes (Purvis *et al.* 1992), whereas the earlier diverging related species have small crustose non-lobate thalli.

Some groups of *Verrucariaceae* have adopted an endolithic growth form. This specialization seems to be a key innovation associated with the origin of the *Bagliettoa* group, as all species of this group are endolithic. For the *Polyblastia* group, endolithic species seem to form an exclusive monophyletic group. However, preliminary results from a phylogenetic study focusing on the genera *Polyblastia* and *Thelidium* revealed a more complex evolution of these traits, and that some epilithic species are nested within this apparently strictly endolithic group (Savić et al. in press).

Upper cortex

Similarly to the thallus structure, the upper cortex mostly evolved from a weakly differentiated to a more differentiated form. The evolution of a eucortex seems correlated with the evolution of a squamulose thallus in the *Placidium* and *Endocarpon* groups, where the evolution of the eucortex preceded the origin of the squamulose thallus in the later group (Fig 6A–B). The reversal to a pseudocortex in the *Endocarpon* group could be related to the development of a crustose thallus in *Endocarpon diffractellum*. The evolution of a lithocortex in the most recent common ancestral lineage to the genus *Bagliettoa*, was subsequent to the evolution of endolithism in the *Bagliettoa* group (Figs 2 and 6A–B). However, in the *Polyblastia* group, a loss of a pseudocortex preceeded the origin of endolithism. The second loss of a pseudocortex in the *Verrucariaceae* is associated with an adaptation to the marine environment (Marine group in Fig 6B).

Ascospore septation

In some lineages in *Verrucariaceae*, simple ascospores have evolved to become septate, mostly transversally uniseptate (in *Placidiopsis* and *Placopyrenium*) and muriform (in *Staurothele*, *Endocarpon,* and the *Polyblastia* group) (Fig 6C). In the *Polyblastia* group, where all four different types of ascospore can be observed (simple, transversally uniseptate, transversally multiseptate, and muriform), the evolution of ascospore septation appears to be complex. A more comprehensive taxon sampling for this group is necessary to better understand the evolution of ascospore septation.

The taxonomic practice of using primarily ascospore septation to circumscribe generic entities has been questioned by Fröberg (1989), Halda (2003), Nimis (1993: 685), and Poelt & Hinteregger (1993). One recurrent problem is that some specimens producing mostly transversally multiseptate ascospores, except for a few, rare ascospores with one longitudinal septum, could be attributed to either the genus *Thelidium* (transversally multiseptate ascospores) or to the genus *Polyblastia* (submuriform to muriform ascospores) (Halda 2003). In our study, these two genera are not monophyletic, and some *Thelidium* species are more closely related to *Polyblastia* species than to other *Thelidium* species. Based on the high frequency of polymorphism for this trait in the *Polyblastia* group, this character needs to be used with caution in future generic treatment of this group.

Hymenial algae

Hymenial algae are small rounded or cylindrical algal cells occurring in the hymenium of some species of lichen-forming fungi. Their occurrence has been reported in early taxonomic studies of lichens (Fuisting 1868; Nylander 1858), and was the taxonomic basis for the recognition of two genera in *Verrucariaceae* (*Staurothele* and *Endocarpon*), and one genus (*Thelenidia*) currently classified as *Dothideomycetes incertae sedis* (Eriksson 2006). The genus *Sporopodium* and a few species in *Lecidea* are also known to host algae in their hymenium (Lücking & Lumbsch 2001; Nylander 1858; Santesson 1952; Smith 1921; Vainio 1890).

Based on *in vitro* experiments, it was proposed that, in *Verrucariaceae*, these hymenial algae, although different in size and shape, belong to the same species as the algae present in the thallus (Stahl 1877; Geitler 1938; Bertsch & Butin 1967; Ahmadjian & Heikkilä 1970). In these experiments (Ahmadjian & Heikkilä 1970), large algal cells isolated from the thallus and grown in culture free from the mycobiont, became small rounded or cylindrical cells after a few division cycles. Moreover, the lichen resynthesis experiments carried out in this study using *Endocarpon pusillum* showed that these small rounded or cylindrical hymenial algae became morphologically identical to thalline algae when in contact (appressoria) with the mycobiont. Therefore, the increase in size of the photobiont cells in the thallus is believed to be induced by the mycobiont, and has been suggested to be associated with an increase in the number of chloroplasts (Ahmadjian & Heikkilä 1970). Suzanne Joneson (unpubl.) has found no difference between ITS sequences from the thalline and hymenial algae of *Endocarpon pallidulum*. The study of the development of the perithecium in some species of *Verrucariaceae* by Janex-Favre (1975) and Wagner (1987) are also in agreement with these results. Large thalline algal cells, present in or around the primordium, are trapped in this primordium at an early stage of the formation of the perithecium. In later stages, these large algal cells divide, become smaller and rounded or cylindrical, and fill the perithecial cavity. The presence of algae in the hymenium maximizes the likelihood of the mycobiont codispersing with the photobiont, which might increase the sexual reproductive success of the mycobiont. When a lichenized fungus without hymenial algae reproduces sexually, ascospores are dispersed and their probability of forming new lichen thalli depends on the chance of finding the appropriate photobiont. For mycobionts with hymenial algae, the likelihood of finding the appropriate photobiont is greatly increased because fungal ascospores can codisperse with photobiont cells. It is noteworthy that in all three lineages where this codispersal mechanism independently evolved (*Staurothele s. str.*, *Endocarpon*, and *Staurothele immersa*), it was associated with the presence of muriform ascospores (Fig 6C–D). These ascospores are usually quite large and present irregular walls that might help trapping and carrying algal cells when they are released through the ostiole.

Conclusions

This first attempt to evaluate the current morphology-based classification of *Verrucariaceae* with molecular data shows that the current generic delineations were not monophyletic, and that the use of traditional diagnostic morphological characters was problematic in defining natural taxonomic entities. In particular, character states that were used to define the genus *Verrucaria*, such as the crustose thallus, the absence of hymenial algae or the simple ascospore, are symplesiomorphic, and therefore were retained in several non-related lineages within *Verrucariaceae*. Convergence is another common evolutionary process in the *Verrucariaceae*. Many derived character states such as the squamulose thallus, the muriform ascospore or the presence of hymenial algae evolved independently (homoplastic) in several lineages.

The high polyphyly of the genus *Verrucaria* and the problems of plesiomorphy and homoplasy of morphological characters in the *Verrucariaceae* make changes in generic delimitation difficult but necessary. Two approaches are possible. A narrow generic concept, in which the well-characterized genera will stay untouched (e.g., *Endocarpon*, *Catapyrenium*, *Placidiopsis*) and new genera will be described for each of the basal paraphyletic lineages of *Verrucaria*, would lead to a large number of new taxa. A broader generic concept, which includes these basal paraphyletic taxa of *Verrucaria* with their closely related genera, would significantly enlarge the concept of some genera and their morphological circumscription. In any case, as shown in other studies (Miller & Huhndorf 2004, 2005), only a few genera can be defined by unique synapormorphies, and a combination of several characters, some of them being slightly homoplasious, might be the solution.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mycres.2007.08.010.

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