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Phylogenetic study of *Catapyrenium s. str.* (Verrucariaceae, lichen-forming Ascomycota) and related genus *Placidiopsis*

María Prieto[1]
Isabel Martínez
Gregorio Aragón
Mónica A.G. Otálora
Área de Biodiversidad y Conservación, Universidad Rey Juan Carlos, C/ Tulipán s/n, 28933 Móstoles, Madrid, Spain

François Lutzoni
Department of Biology, Duke University, Box 90338, Durham, North Carolina 27708

Abstract: The current classification of what used to be called *Catapyrenium* comprises eight genera belonging to distinct lineages in the *Verrucariaceae*. Previous phylogenetic studies have shown that the redefined genus *Catapyrenium* (*Catapyrenium s. str.*) is monophyletic and sister of *Placidiopsis* within the *Staurothele* group, but this relationship was based on only two species from each genus. We conducted a phylogenetic study of *Catapyrenium* and *Placidiopsis* as currently delimited to evaluate the monophyly of each genus and infer infrageneric relationships. An initial family level phylogenetic analysis based on the nuLSU locus and implementing a backbone constraint tree (with both weighted maximum parsimony and bootstrap maximum likelihood approaches) was performed to infer phylogenetic placements of *Catapyrenium* and *Placidiopsis* taxa not included in previous molecular systematic studies. The results of this analysis were used to define the ingroup for a second phylogenetic analysis based on nuITS and nuLSU and centered on *Catapyrenium s. str.* and *Placidiopsis*. *Placidiopsis* was found to be monophyletic, whereas *Catapyrenium s. str.* was not. *Catapyrenium dactylinum* was found to be closely related to *Placopyrenium caeruleopulvinum* and *Placopyrenium stanfordii*, all of which were closely related to *Placocarpus schaereri* and *Verrucula*. In addition we found genus *Placopyrenium* to be polyphyletic. The resulting trees confirmed that *Catapyrenium s. str.* (excluding *C. dactylinum*) and *Placidiopsis* constitute two sister monophyletic entities. The data do not support *Placidiopsis cinerascens* and *P. tenella* as two distinct species because no characters can be used to distinguish them. Thus *P. tenella* is here reduced to synonymy with *P. cinerascens*.

Key words: ascospore septation, backbone constraint tree, *Catapyrenium s. str.*, lichens, phylogeny, *Placidiopsis*, *Placopyrenium s. str.*, synonymy, Verrucariaceae

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[1] Corresponding author. E-mail: maria.prieto@urjc.es

INTRODUCTION

Catapyrenium Flotow is a squamulose genus with perithecial ascomata belonging to the Verrucariaceae (Verrucariales, Ascomycota). The genus was split into eight genera (Harada 1993, Breuss 1996a), based on combinations of characters such as the type of pycnidium, ascus shape and arrangement of the ascospores, thallus anatomy and morphology (structure of the upper cortex and type of anchoring organs) and presence or absence of an involucrellum. Under this classification the eight genera of *Catapyrenium s.l.* are *Anthracocarpon* Breuss, *Catapyrenium* Flot. (*Catapyrenium s. str.*), *Clavascidium* Breuss, *Heteroplacidium* Breuss, *Involucropyrenium* Breuss, *Neocatapyrenium* H. Harada, *Placidium* A. Massal. and *Scleropyrenium* H. Harada.

Catapyrenium s. str. comprises eight squamulose species with a thin paraplectenchymatous upper cortex poorly delimited from the algal layer (called pseudocortex in Gueidan et al. 2007), perithecial ascomata, clavate asci with biseriate ascospores and *Dermatocarpon*-type pycnidia (the latter based on *Catapyrenium dactylinum*, a recently described species) (Breuss 1996a, 2000, 2002a). They inhabit soil, detritus, mosses and bark (Breuss 2001, 2002a) and are distributed worldwide in arid, semi-arid and arctic-alpine regions.

One of the main features distinguishing *Catapyrenium s. str.* from other genera formerly classified in *Catapyrenium s.l.* is the type of upper cortex (*cinereum*-type sensu Breuss 1990, 1996a). In this genus the upper cortex is thin (less than 30 μm), not distinctly differentiated from the medulla and with small cells. *Anthracocarpon*, *Clavascidium*, *Heteroplacidium*, *Neocatapyrenium* and *Placidium* develop a well defined eucortex, clearly differentiated from the medulla, and with larger cells (*lachneum*-type sensu Breuss 1990, 1996a). *Scleropyrenium* is the only genus that has a pachydermatous upper cortex (Harada 1993). Another important character that segregates this complex is a *Dermatocarpon*-type pycnidium,

present in *Catapyrenium s. str.* (based only on *Catapyrenium dactylinum*), *Clavascidium*, *Heteroplacidium* and *Placidium*, while *Anthracocarpon*, *Neocatapyrenium* and *Scleropyrenium* have *Endocarpon*-type pycnidia (for explanations and pictures of the pycnidia types see Janex-Favre and Wagner 1986, Ménard and Roux 1995, Gueidan et al. 2007, Prieto et al. 2008). Members of genus *Involucropyrenium* share the same type of upper cortex with *Catapyrenium*; however the former has perithecia situated between the squamules, which have an involucrellum. The type of pycnidia has been described as *Endocarpon*-type based on one recently described species of *Involucropyrenium* (Roux 2005).

Other species not closely related to the *Catapyrenium s.l.* complex have been described or combined as *Catapyrenium* (i.e. *Catapyrenium caeruleopulvinum* and *Dermatocarpon zahlbruckneri*) and later subsumed within *Placopyrenium* Breuss by Breuss (2002c, 2009). *Placopyrenium* comprises species with crustose areolate to placodioid or subsquamulose thalli, some of them being parasitic species. The ascospores are colorless, simple to uni- or triseptate with pycnidia of *Dermatocarpon*-type (Breuss 2002c, 2009). Unlike *Catapyrenium s. str.*, *Placopyrenium* includes placodioid thalli and areoles fixed to the substrate by constricted bases or stipe-like holdfasts (TABLE I).

While *Placidiopsis* Beltramini is very similar to *Catapyrenium s. str.*, sharing the same type of upper cortex, it differs in having septate ascospores and lacking pycnidia (TABLE I). This genus consists of 14 species worldwide (Breuss 1996b, 2002b), inhabiting soil, detritus, mosses and algal or lichen crusts. Most *Placidiopsis* species are easily identified, except for *P. cinerascens* and *P. tenella*, which are more difficult to distinguish from each other. Both species were described by Nylander in the same year (1853a, b) on the basis of different ascospore lengths. However more than a century later Breuss (1994, 1996b) and Nimis and Martellos (2004) showed that by including more specimens the range of measurements for this character overlapped with the result that the presence of a small lid-like involucrellum in *P. tenella* became the main trait used to distinguish it from *P. cinerascens*.

Although ascospore septation is the character used to distinguish *Placidiopsis* from *Catapyrenium s. str.*, molecular studies showed that in other groups of Ascomycota (Staiger 2002, Miller and Hundorf 2004) ascospore septation was not a reliable character for delineating monophyletic groups at genus and higher taxonomical ranks. Within the Verrucariaceae Savić et al. (2008) pointed out that the *Thelidium* group contained a mixture of species with nonseptate, transversely septate and muriform ascospores, due

TABLE I. Main diagnostic characters for distinguishing between genera included in *Catapyrenium s.l.*, *Placidiopsis* and *Placopyrenium s.l.*

Genus	Growth form	Life strategy	Upper cortex	Attaching organ	Ascospore	Pycnidia type
Anthracocarpon	squamulose	nonparasitic	eucortex (*lachneum*-type)	rhizohyphae and rhizines	simple	*Endocarpon*
Catapyrenium s.str.	squamulose	nonparasitic	pseudocortex (*cinereum*-type)	rhizohyphae	simple	*Dermatocarpon*[a]
Clavascidium	squamulose	nonparasitic	eucortex (*lachneum*-type)	rhizohyphae and rhizines	simple	*Dermatocarpon*
Heteroplacidium	crustose areolate to squamulose	nonparasitic	eucortex (*lachneum*-type)	rhizohyphae and rhizines	simple	*Dermatocarpon*
Involucropyrenium	squamulose	nonparasitic	pseudocortex (*cinereum*-type)	rhizohyphae	simple	*Endocarpon*
Neocatapyrenium	squamulose	nonparasitic	eucortex (*lachneum*-type)	rhizohyphae and rhizines	simple	*Endocarpon*
Placidiopsis	squamulose	nonparasitic	pseudocortex (*cinereum*-type)	rhizohyphae and rhizines	uniseptate	—
Placidium	squamulose	nonparasitic	eucortex (*lachneum*-type)	rhizohyphae and rhizines	simple	*Dermatocarpon*
Placopyrenium	crustose areolate to placodiod or subsquamulose	parasitic or not	pseudocortex	rhizines or stipes	simple to 1(–3)-septate	*Dermatocarpon*
Scleropyrenium	squamulose	nonparasitic	eucortex (pachydermatous)	rhizohyphae and rhizines	simple	*Endocarpon*

[a] Data for pycnidia type is based on *Catapyrenium dactylinum*.

to the homoplasious nature of these traits. There are also examples at the infrageneric level where ascospore septation has been shown to be strongly homoplasious (Ihlen and Ekman 2002, Naesborg et al. 2007). Otálora et al. (2010) revealed that ascospore septation is homoplasious even at the species level within the Collemataceae. Gueidan et al. (2007) showed nonseptate ascospores to be a symplesiomorphic character state retained in several distinct lineages across the Verrucariaceae, together with convergent and parallel evolution of various types of ascospore septation occurring at different rates among clades, thus rendering more difficult our understanding of the evolution of ascospore septation within this family.

Breuss (1983, 1996b) and Breuss and Hansen (1988) noted that, because the only character state separating *Catapyrenium s. str.* from *Placidiopsis* was the presence of a transverse septum in the ascospores of *Placidiopsis*, the separation of these two genera was in need of reappraisal. Specimens of *Catapyrenium s. str.* with uniseptate ascospores, as well as some *Placidiopsis* specimens with nonseptate ascospores, have been observed by the first author and were reported by Gueidan et al. (2007). Breuss and Hansen (1988) pointed out that ascospore septation in *Placidiopsis* (''true'' septa) was distinct from *Catapyrenium s. str.* (pseudosepta).

On the other hand the ascus apex of *Placidiopsis* has been reported to differ from that of *Catapyrenium s. str.* in having a slight thickening in the apical ascus wall (tholus) showing an ocular chamber (i.e. a dome-shaped indentation of the lower edge of the tholus) (Harris 1979, Breuss 2002b, Nimis and Martellos 2004). However this feature has not been observed in all species of *Placidiopsis*, being visible only at a certain developmental stage (Breuss pers comm).

The circumscription of the Verrucariaceae and the relationships among the main genera included in the family has been investigated by molecular methods (Gueidan et al. 2007, 2009; Savić et al. 2008). These studies showed that members of *Catapyrenium s.l.* were nested within different monophyletic groups. Gueidan et al. (2007, 2009) reported C*atapyrenium s. str.* as a sister group of *Placidiopsis* within the *Staurothele* group (=Lineage 4), a clade distantly related to the *Placidium* group nested within Lineage 3 and containing *Clavascidium*, *Neocatapyrenium*, *Placidium* and *Placopyrenium*. *Catapyrenium s. str.* and *Placidiopsis* formed a monophyletic group within the *Staurothele* group along with at least two putatively paraphyletic species of *Verrucaria*. Although both *Catapyrenium s. str.* and *Placidiopsis* seemed to be monophyletic, only two species of each genus were included in these analyses. Therefore the monophyly of both genera is still considered an open question necessitating more taxon sampling to clarify whether these current genera are well delineated.

The main goals for this phylogenetic study were to evaluate whether *Catapyrenium s. str.* is a monophyletic group separate from *Placidiopsis* and whether the gain of transversally uniseptate ascospores is a synapomorphy for the latter genus. We also investigated whether *P. cinerascens* and *P. tenella* are conspecific.

MATERIALS AND METHODS

Taxon sampling.—To infer phylogenetic relationships of specimens selected for this study within family Verrucariaceae we conducted a first set of analyses based on the nuLSU locus (Verrucariaceae dataset, VD). In total VD included 114 sequences of Verrucariaceae, of which 81 were obtained from GenBank and 33 were obtained by the first author during this study. Two sequences from GenBank belonging to the Chaetothyriales were used as outgroup.

For the second dataset (*Catapyrenium-Placidiopsis* dataset, C-PD) sequences of the nuITS and nuLSU rDNA were combined for a total of 26 specimens belonging to *Catapyrenium s. str.* and *Placidiopsis* genera. Two sequences of *Staurothele* were downloaded from GenBank and selected as outgroup based on previous phylogenies (Gueidan et al. 2007, 2009; Savić et al. 2008).

In summary a total of 59 sequences were produced in this study and 85 were downloaded from GenBank. Information on the material, area of collection, collector, location of voucher specimens and GenBank accession numbers is provided for newly obtained sequences (TABLE II). For sequences obtained from GenBank identification numbers are found after taxon names (FIGS. 1, 2). The classification of groups follows Gueidan et al. (2007) and taxon names follow Gueidan et al. (2009).

Morphological study.—Characters suitable for differentiating *Placidiopsis cinerascens* from *P. tenella* were identified in preliminary morphological and anatomical studies. Criteria for delimiting these two species followed Breuss (1996b). Longitudinal sections (14–16 μm thick) were obtained from thalli and perithecia with a freezing microtome. The sections were mounted and measured in water. Sixteen anatomical characters were analyzed in all specimens studied (thallus, epinecral layer, upper cortex, algal layer, medulla and lower cortex thickness, cell diameter in all layers, rhizohyphae, perithecium and exciple thickness and color, ascospore length and width). Ten measurements were made for each sample. All variables were normally distributed. We have provided results for the 12 characters with continuous variables (TABLE III).

We used generalized linear mixed models (GLMM; McCullagh and Nelder 1989, Breslow and Clayton 1993) that consider variation between specimens within groups. All specimens were separated into putative species groups based on the phylogenetic results (FIG. 2, groups A and B). We analyzed data with a two-level hierarchical approach

TABLE II. List of taxa for which DNA sequences were generated in this study (GenBank identification numbers of sequences obtained from GenBank are found after species names in FIG. 1), with countries and provinces/states of origin, collector names, herbarium names where specimens were deposited with herbarium accession numbers, and GenBank accession numbers

Species name	Origin	Collector	Voucher	GenBank accession number	
				nuITS	nuLSU
Catapyrenium cinereum 1 (Pers.) Körb.	Spain, Cantabria	G. Aragón, A. García & M. Prieto	MA 16299	GQ344599	GQ344586
Catapyrenium cinereum 2	Spain, Asturias	M. Prieto	MA 16301	GQ344598	GQ344587
Catapyrenium dactylinum Breuss	Mexico, Chihuahua	T.H. Nash	LI 412469	—	GQ344593
Catapyrenium daedaleum 1 (Kremp.) Stein	Spain, León	G. Amo & G. Aragón	MA 16297	GQ344596	GQ344582
Catapyrenium daedaleum 2	Spain, Cuenca	G. Aragón	MA 16296	GQ344597	GQ344583
Catapyrenium exaratum 1 Breuss	Argentina, Salta	G. Aragón & I. Martínez	BCRU 4920	GQ344600	GQ344589
Catapyrenium exaratum 2	Argentina, Salta	G. Aragón & I. Martínez	MA 16314	GQ344601	GQ344588
Catapyrenium psoromoides 1 (Borrer) R. Sant.	Spain, Toledo	G. Aragón & M. Prieto	MA 16298	GQ344594	GQ344584
Catapyrenium psoromoides 2	Portugal, Portagem	M.A.G. Otálora & M. Prieto	MA 16313	GQ344595	GQ344585
Heteroplacidium acervatum (Breuss) Breuss	Spain, Mallorca	O. Breuss	LI 271015	—	GQ344564
Placidiopsis cinerascens 1 (Nyl.) Breuss	Spain, Palencia	G. Aragón & M. Prieto	MA 16307	GQ344605	GQ344566
Placidiopsis cinerascens 2	Spain, Zaragoza	I. Martínez & M. Prieto	MA 16306	GQ344606	GQ344567
Placidiopsis cinerascens 3	Spain, Cáceres	M. Prieto	MA 16302	GQ344610	GQ344565
Placidiopsis cinerascens 4	Spain, Zaragoza	I. Martínez & M. Prieto	MA 16305	GQ344609	GQ344568
Placidiopsis cinerascens 5	Portugal, Alvados	M.A.G. Otálora & M. Prieto	MA 16309	GQ344607	GQ344569
Placidiopsis cinerascens 6	Spain, Mallorca	M. Prieto	MA 16304	GQ344614	GQ344571
Placidiopsis cinerascens 7	Spain, Madrid	M. Prieto	MA 16308	GQ344613	GQ344570
Placidiopsis custnani 1 (A. Massal.) Körb.	Spain, Huesca	M. Prieto	MA 16303	GQ344602	—
Placidiopsis custnani 2	Spain, La Rioja	I. Martínez & M. Prieto	MA 16310	GQ344604	GQ344578
Placidiopsis custnani 3	Portugal, Alvados	M.A.G. Otálora & M. Prieto	MA 16312	GQ344603	GQ344579
Placidiopsis pseudocinerea Breuss	Austria, Steiermark	O. Breuss & R. Türk	LI 281386	GQ344619	GQ344577
Placidiopsis tenella 1 (Nyl.) Zahlbr.	Spain, Castellón	M. Prieto	MA 16315	GQ344611	GQ344572
Placidiopsis tenella 2	Spain, Lérida	J. Pérez-Redondo	BCC 12680	GQ344608	GQ344574
Placidiopsis tenella 3	Turkey, Tekirdağ	A. Özdemir Türk	LI 281350	GQ344615	GQ344573
Placidiopsis tenella 4	Italy, Calabria	D. Puntillo	LI 281352	GQ344616	GQ344575
Placidiopsis tenella 5	Spain, Navarra	J. Etayo & O. Breuss	LI 136521	GQ344612	GQ344576
Placidiopsis tiroliensis 1 Breuss	Austria, Steiermark	E. Hörandl	LI 281351	GQ344617	GQ344580
Placidiopsis tiroliensis 2	Austria, Kärnten	R. Türk & O. Breuss	LI 281349	GQ344618	GQ344581
Placidium pseudorufescens Breuss	USA, New Mexico	R. D. Worthington	LI 566404	—	GQ344563
Placidium tenellum (Breuss) Breuss	Spain, Madrid	I. Martínez, M.A.G. Otálora & M. Prieto	MA 16300	—	GQ344562

TABLE II. Continued

Species name	Origin	Collector	Voucher	GenBank accession number	
				nuITS	nuLSU
Placopyrenium caeruleopulvinum 1 (Thomson) Breuss	USA, New Mexico	R. D. Worthington	COLO 465590	—	GQ344591
Placopyrenium caeruleopulvinum 2	USA, Arizona	T.H. Nash	DUKE 146691	—	GQ344592
Placopyrenium trachyticum (Hazsl.) Breuss	Belgium, Flemish Brabant	A. Aptroot	DUKE 139586	—	GQ344561
Placopyrenium stanfordii (Herre) K. Knudsen	USA, Arizona	K. Knudsen	LI 582724	—	GQ344590

with specimens nested within groups. All GLMM computations were performed with SAS Macro program GLIMMIX (Littel et al. 1996).

DNA isolation and sequencing.—Genomic DNA was obtained from fresh samples and herbarium specimens. Small samples were ground in liquid nitrogen. Total genomic DNA was extracted with DNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions, with slight modifications described by Crespo et al. (2001). Dilutions (1 : 10 and 1 : 100) of genomic DNA were used for PCR amplifications of the nuclear nuITS and nuLSU rDNA regions. Fungal nuITS rDNA was amplified with primers ITS5 and ITS4 (White et al. 1990), while nuLSU was amplified with primers LR0R (Rehner and Samuels 1994) and LR7 (Vilgalys and Hester 1990). Because these primer pairs also amplified the algal component we designed the specific primers PRI1 and PRI2 (5′-GTGTGAAGCTCCTTCGAC-3′ and 5′-AAAAATGGCCCACTAGTAACG-3′). We used combinations of these four primers for amplifications. Amplifications were performed in 25 μL volumes containing a reaction mixture of 7.5 μL QIAGEN Multiplex PCR Kit (HotStarTaq DNA Polymerase plus Multiplex PCR Buffer and dNTP mix), 2.5 μL Q-Solution, 2.5 μL primer mixture (10 uM), 10.5 μL dH2O and 2 μL diluted genomic DNA. Amplifications were carried out in a PTC-100 Peltier thermal cycler and performed with these programs: initial denaturation at 94 C for 15 min for nuITS or 95 C for nuLSU, followed by 35 cycles of 94 C for nuITS or 95 C for nuLSU for 1 min, 54 C for 1 min for nuITS and 52 C for 0.40 min for nuLSU rDNA, 72 C for 1.3 min for nuITS or 3 min for nuLSU, followed by a final extension at 72 C for 10 min. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's instructions. Purified PCR products were sequenced with the same amplification primers, as well as LR3R (Vilgalys and Hester 1990) for the nuLSU region. Both strands were sequenced with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) with these PCR settings: denaturation 3 min at 94 C, 25 cycles at 96 C for 10 s, 50 C for 5 s and 60 C for 4 min. Sequencing products were subjected to electrophoresis with a 3730 DNA analyser (Applied Biosystems).

Sequence alignment.—Sequences were subjected to BLAST analyses for a first verification of their entities. They subsequently were assembled and edited with SeqNavigator 1.0.1 (Applied Biosystems) and aligned manually with McClade 4.06 (Maddison and Maddison 2003) with the help of the secondary structure of the nuLSU from *Saccharomyces cerevisiae* (Cannone et al. 2002) following the method described in Kjer (1995).

A total of 116 sequences were aligned for the VD matrix. Fourteen ambiguously aligned regions were recoded and subjected to specific step matrices obtained with program INAASE 2.3b (Lutzoni et al. 2000, http://www.lutzonilab.net/downloads), incorporating the phylogenetic signal from these regions without violating positional homology. Three ambiguous regions that were more than 100 bp long or highly variable (i.e. more than 32 character states) were recoded into 23 characters with the aid of program arc 1.5 (Kauff et al. 2003, Miadlikowska et al. 2003) with the nucleotide option as outlined by Reeb et al. (2004). Each of the 23 characters obtained with program arc were subjected to a specific weight: 1 for character 1, 0.25 for characters 2–5, 0.10 for characters 6–15 and 0.50 for characters 16–23. Introns were delimited manually and excluded from the phylogenetic analyses.

A total of 28 specimens were aligned for C-PD. Only one taxon with missing nuLSU sequences was included in the analysis (TABLE II). Ambiguous regions (sensu Lutzoni et al. 2000) and introns were delimited manually and excluded from the phylogenetic analyses. The sequences alignments have been deposited in TreeBASE (accession number SN4626).

Phylogenetic analyses.—We performed two weighted maximum parsimony (wMP) analyses: a first wMP analysis with VD was executed with nuLSU, including coded (INAASE and arc) characters. The second search with C-PD included both loci nuITS and nuLSU and exclusively unambiguously aligned sites.

wMP analyses were performed with PAUP* 4.0b10 (Swofford 2002). In both analyses symmetric step matrices were created for unambiguous portions of each of the genomic regions as follows. The option ''character status/full detail/hide excluded characters'' from the data menu in PAUP* was implemented. From the resulting table the column states showing all nucleotide states and gaps found

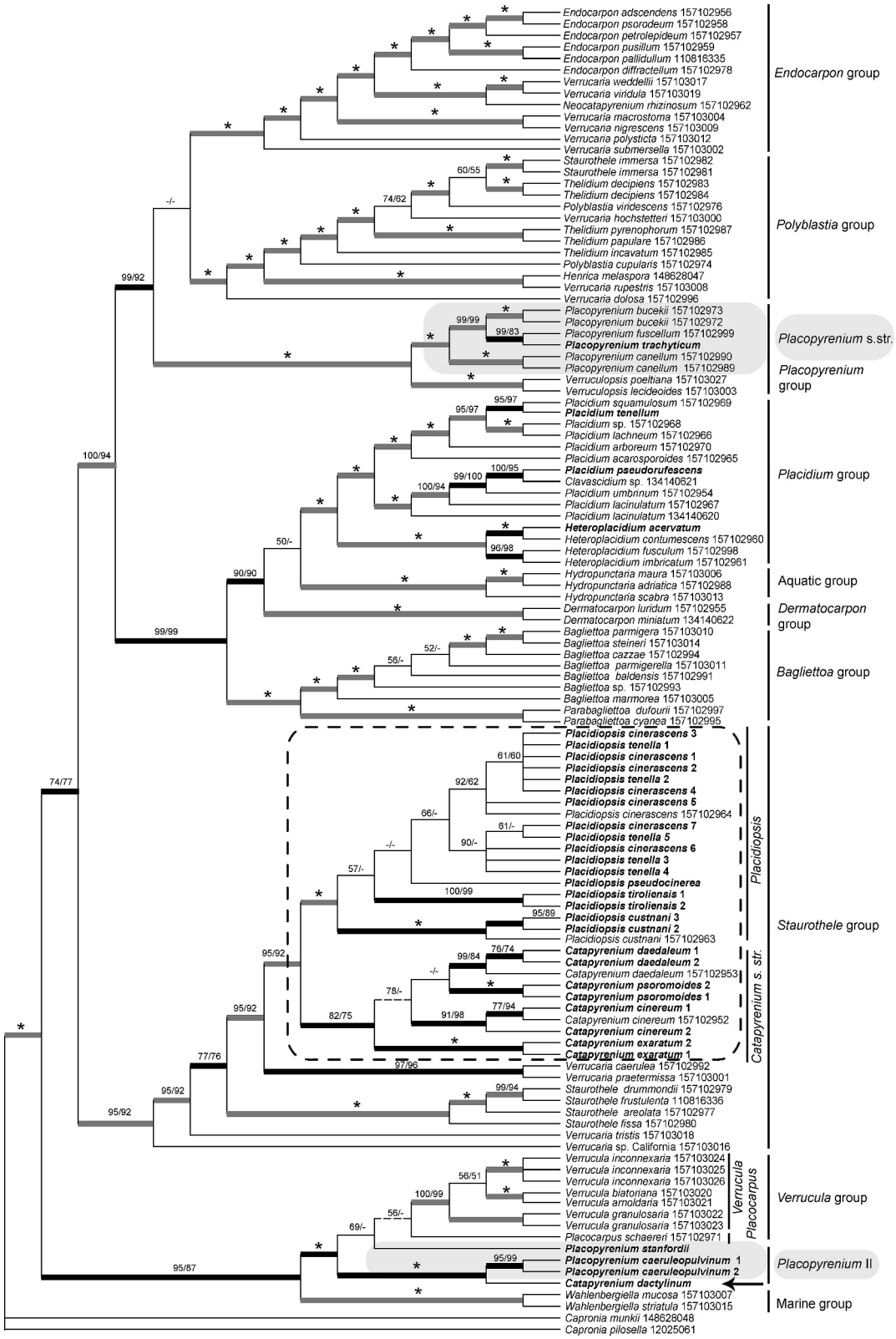

FIG. 1. Phylogenetic relationships within the Verrucariaceae based on nuLSU data for 114 specimens of Verrucariaceae and two specimens selected as outgroup from the Chaetothyriales. Strict consensus tree of three equally most parsimonious trees generated with wMP using a backbone constraint tree derived from Gueidan et al. (2007). Sequences downloaded from GenBank are followed by their GenBank identification (GI) numbers. Specimens sequenced for this study are in boldface and

at each of the unambiguously aligned and nonconstant sites was saved as a separate text file. This file was used as an input for program StMatrix 2.1. (Lutzoni and Zoller, Duke University, http://www.lutzonilab.net/downloads), which generates a step matrix (in Nexus format) by calculating frequencies of reciprocal changes from one state to another and converting them into costs of changes with the negative natural logarithm of the frequencies (Felsenstein 1981, Wheeler 1990).

wMP analyses were performed with the heuristic search option with 1000 random addition sequences (RAS) replicates, TBR (tree bisection reconnection) branch-swapping and MULTREES option in effect. We performed a search in two steps for VD. In the first step we performed only 100 RAS (i.e. without branch swapping after the last sequence was added) while in the second step we searched for all equally parsimonious trees, saving all trees only when swapping a tree equal to or shorter than the shortest tree that we found in the first step.

All character states were treated as unordered, and gaps were considered as a fifth character. Nonparametric bootstrap (Felsenstein 1985) was used to assess statistical support of the clades with heuristic searches as described above on 1000 bootstrap datasets. Five RAS per bootstrap replicate were specified for VD and two for C-PD based on the efficiency of the searches based on the original datasets.

Analyses were performed three ways for VD bootstrap: saving no more than 10 trees per RAS in the first analysis, no more than 100 trees per RAS in the second and no more than 10 trees per RAS but with 10 000 bootstrap replicates in the third analysis. Bootstrap values were similar, consequently values from the first analysis are reported here (FIG. 1).

Preliminary analyses of VD failed to recover a well supported topology (results not shown). Based on multilocus phylogenies of the Verrucariaceae by Gueidan et al. (2007), from which our initial nuLSU alignment was derived and to which we added nuLSU sequences generated for our study, we constructed a topological constraint tree for a subset of the deep nodes and including only sequences from Gueidan et al. (2007) (i.e. backbone constraint). This backbone constraint tree was constructed by hand with McClade 4.06 (Maddison and Maddison 2003). Internodes were included in this tree when they were highly supported (i.e. $\geq$ 70% bootstrap values and $\geq$ 95% posterior probabilities) at least in two of the three analysis based on weighted maximum parsimony (wMP), maximum likelihood bootstrap (ML BS) and Bayesian analysis (MB) in the three-gene tree of Gueidan et al. (2007). Specimens sequenced for this study not included in the constraint (skeletal) tree were free to attach to any point on the trees during the search. Our approach did not assume any restrictions on the phylogenetic positioning of specimens sequenced for this study but enforced topological relationships between the remaining taxa only, as far as sufficiently supported in multigene phylogenies by Gueidan et al. (2007) that were based on more characters (i.e. nuLSU, nuSSU and *RPB*1).

Phylogenetic relationships and confidence for C-PD were inferred with weighted maximum parsimony, Bayesian analyses and bootstrap maximum likelihood on a combined nuITS-nuLSU dataset. The combinability of the datasets was assessed by separately comparing clades among trees based on the nuITS region and nuLSU (Mason-Gamer and Kellogg 1996). Because no conflict was detected it was assumed that the two datasets were congruent and could be combined.

The evolutionary models for Bayesian analyses were selected with the Akaike information criterion (AIC) as implemented in MrModeltest 2.2 (Nylander 2004). GTR+I+G was used for nuLSU, HKY+G for ITS 1 and ITS 2 and K80+I for ITS 5.8S in the C-PD. Datasets were analyzed with MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Two analyses of four chains were run 5 000 000 generations starting from an initial random tree. A tree was saved every 100th generation. A burn-in sample of 150, 200 and 100 trees for nuITS, nuLSU and the combined dataset respectively were discarded. The remaining trees were used to estimate branch lengths with the SUMT command of MrBayes, and posterior probabilities (PP) were calculated with the majority rule consensus tree command in PAUP*.

A maximum likelihood (ML) search for the most likely tree was completed on 1000 replicates with RAxML-VI-HPC (Stamatakis et al. 2005) with a GTRMIX model of molecular evolution. An additional constrained ML tree search (with the same backbone constraint tree mentioned above) was conducted on VD, and 1000 BS replicates were run for both datasets.

RESULTS

Verrucariaceae dataset.—The data matrix included 1390 characters after exclusion of ambiguous regions and introns. Fourteen of the ambiguous regions were recovered by INAASE characters, and three of them by arc characters. Nine hundred four characters were excluded from the wMP analysis because they were

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lack this number. Internodes with BS support from wMP and ML $\geq$ 70% are highlighted with thicker lines. Internodes with black lines were not part of the skeletal backbone constraint tree. Internodes with gray lines were part of the backbone constraint tree. Internodes with dashed lines were part of the backbone tree but not recovered with high support. The support values are ordered as wMP/ML BS. An asterisk over a bold branch indicates that this internode has a bootstrap support value of 100% for both wMP and ML. A dash indicates bootstrap support values $<$ 50%. Members of *Placopyrenium* are in gray boxes, showing the polyphyly of the genus. Dashed line box delimits *Catapyrenium s. str.* and *Placidiopsis* clades, which is the ingroup of the phylogenetic tree (FIG. 2). An arrow shows the position of *Catapyrenium dactylinum* outside *Catapyrenium s. str.*

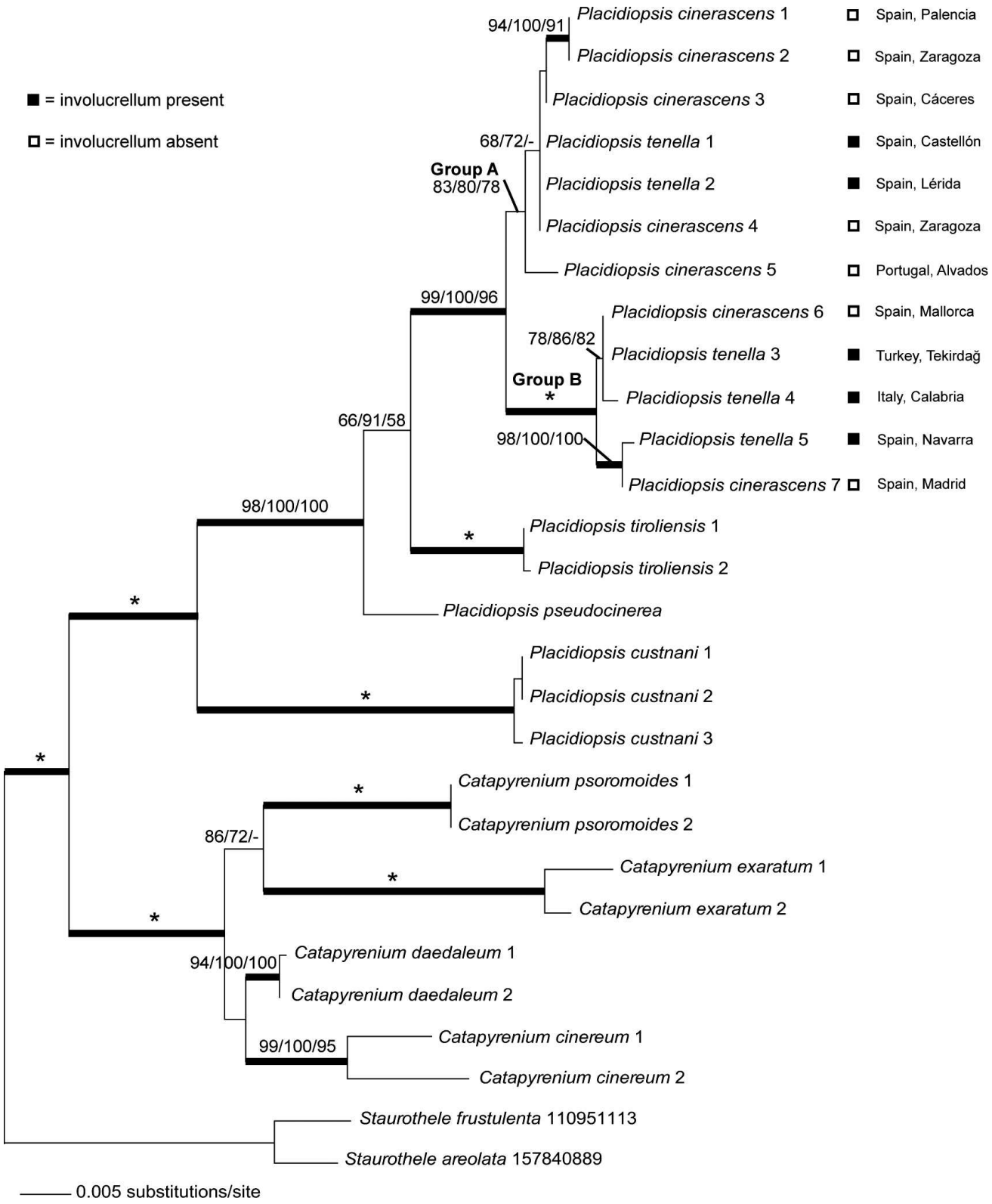

FIG. 2. Phylogenetic relationships among 28 members of *Catapyrenium s. str.* and *Placidiopsis*, based on ML analysis of concatenated nuITS and nuLSU datasets. Two species of *Staurothele* were chosen as outgroup and are followed by their GenBank identification (GI) numbers. ML BS, PP and wMP BS are represented with the first, second and third numbers associated with branches. Branches in boldface indicate a support of PP ≥ 95%, and both ML and wMP BS ≥ 70%. An asterisk on a bold branch indicates that this internode has a value of 100% for all support estimates. A dash indicates bootstrap support

constant. Of the remaining sites, 338 were parsimony informative. Constant sites were included for maximum likelihood analyses.

The weighted maximum parsimony analysis with the backbone constraint tree resulted in three equal, most parsimonious trees, 3782.47 steps long, which were part of one island hit 663 times out of 1000 RAS. Resolution and support at deeper nodes largely reflect the backbone constraint in our analyses, but the presence of unconstrained taxa in their expected clades indicates that phylogenetic signal was not guided by our backbone constraint alone. This is the case for *Heteroplacidium acervatum*, *Placidium pseudorufescens* and *Placidium tenellum* that appear in the *Placidium* group sensu Gueidan et al. (2007), together with other members of *Heteroplacidium* and *Placidium* genera with high support values (FIG. 1).

All specimens of *Placidiopsis* and *Catapyrenium s. str.* (except *C. dactylinum*) sequenced by us, and which were free to attach anywhere on the tree, connected to the skeletal monophyletic quartet formed by *Placidiopsis cinerascens*, *P. custnani*, *Catapyrenium cinereum* and *C. daedaleum* from Gueidan et al. (2007). All new specimens we included connected to their respective genera thereby recovering the sister relation between these two genera (FIG. 1). In the clade containing specimens of *Catapyrenium s. str.* the internode part of the constrained backbone tree was recovered with high support value only with wMP (dashed line in FIG. 1). The core of *Catapyrenium s. str.* species are significantly supported by both analyses (82% wMP BS, 75% ML BS). This supported node includes all species of *Catapyrenium s. str.* except *C. dactylinum*. All *Placidiopsis* specimens included in this study formed a highly supported monophyletic group (100% wMP BS and ML BS). However interspecific relationships within this genus were not fully resolved. In the *Staurothele* group (sensu Gueidan et al. 2007) relationships not previously supported were highly supported in our analyses, such as the sister relationship between *Verrucaria caerulea* and *V. praetermissa* (wMP BS = 97%, ML BS = 96%) and the monophyletic group formed by these two species with *Catapyrenium s. str.*, *Placidiopsis* and *Staurothele* species (wMP BS = 77%, ML BS = 76%).

Catapyrenium s. str. was not recovered as monophyletic because *C. dactylinum* was found to be sister of *Placopyrenium caeruleopulvinum*. These two species, together with *Placopyrenium stanfordii*, belong to a well supported clade that first split from the remaining members of the Verrucariaceae, including *Placocarpus schaereri*, as well as the monophyletic genera *Verrucula* and *Wahlenbergiella*. These *Placopyrenium* species (*Placopyrenium* II) together with *Catapyrenium dactylinum*, *Placocarpus schaereri* and the *Verrucula* group formed a clade that was highly supported by both analyses (100% BS support). The previously supported (Gueidan et al. 2007) and constrained relationship between *Placocarpus schaereri* and the *Verrucula* clade was recovered but without significant support values (dashed line in FIG. 1).

The clade containing both species of *Placopyrenium* (*P. caeruleopulvinum* and *P. stanfordii*) and *Catapyrenium dactylinum* is only distantly related to the *Placopyrenium* group sensu Gueidan et al. (2007). This latter group comprises the type species of *Placopyrenium* (*P. bucekii*) and two *Verrucaria* species recently combined as *Placopyrenium* by Navarro-Rosinés et al. (2007) (i.e. *P. canellum* and *P. fuscellum*). Another species of *Placopyrenium*, *P. trachyticum*, not previously analyzed by Gueidan et al. (2007) was found to be closely related to *P. fuscellum* (FIG. 1). Despite results from Gueidan et al. (2007, 2009), which reported genus *Placopyrenium* as monophyletic, the addition of *Placopyrenium caeruleopulvinum* and *P. stanfordii* to their dataset resulted in *Placopyrenium s.l.* being polyphyletic. We refer to the clade containing the type species of *Placopyrenium*, as *Placopyrenium s. str.*, whereas we labeled the paraphyletic group containing the remaining species of *Placopyrenium* we analyzed as *Placopyrenium* II (FIG. 1).

Furthermore we obtained high bootstrap support for the *Placopyrenium* group (*Placopyrenium s. str.*) forming a monophyletic group with *Endocarpon* and *Polyblastia* groups sensu Gueidan et al. (2007) and for the clade composed of the *Placidium*, *Aquatic* and *Dermatocarpon* groups. The latter clade was found to be well supported as sister of *Baggliettoa*. We must regard these relationships as tentative although well supported because only one ribosomal gene was used here, necessitating the use of a skeletal backbone constraint tree to resolve the phylogenetic placement of 16 additional species (representing five genera) within the Verrucariaceae tree of Gueidan et al. (2007).

Catapyrenium-Placidiopsis *dataset.*—The combined data matrix included 495 nuITS and 969 nuLSU

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values < 50%. In the *Placidiopsis cinerascens-tenella* clade the presence/absence of involucrellum and geographical location of where the samples were obtained are shown after the name of each specimen.

TABLE III. Mean, standard deviation and range (in parentheses) of measurements (in micrometers) for *Placidiopsis cinerascens* and *P. tenella* specimens belonging to groups A and B, (FIG. 2)

Characters (μm)	Group A	Group B	significance
Thallus thickness	(150) 236.4 ± 47.1 (320)	(110) 213.9 ± 48.9 (300)	n.s.
Epinecral layer thickness	(3) 13.2 ± 8.1 (32.5)	(5) 18.8 ± 10.9 (50)	n.s.
Upper cortex thickness	(5) 20.8 ± 8.6 (37.5)	(5) 17.2 ± 7.1 (35)	n.s.
Upper cortex cells diameter	(4) 7 ± 1.3 (10)	(5) 7.3 ± 1.6 (11)	n.s.
Algal layer thickness	(62.5) 109.8 ± 25.3 (150)	(50) 101.6 ± 34.42 (175)	n.s.
Algal cells diameter	(3) 6.6 ± 1.7 (12)	(4) 6.5 ± 1.8 (11)	n.s.
Medullary cells diameter	(4) 7.7 ± 1.7 (11)	(5) 7.3 ± 1.3 (10)	n.s.
Rhizohyphae thickness	(3) 3.2 ± 0.4 (4)	(2.5) 3.2 ± 0.5 (4)	n.s.
Perithecium diameter	(140) 212 ± 40.5 (300)	(140) 191.7 ± 30.9 (250)	n.s.
Exciple thickness	(10) 17.2 ± 4.9 (32)	(10) 16 ± 3.6 (23)	n.s.
Ascospore length	(12) 16.6 ± 1.6 (21)	(12) 15.5 ± 1.7 (21)	n.s.
Ascospore width	(5.5) 6.1 ± 0.38 (7)	(5) 6.2 ± 0.4 (7)	n.s.

n.s.= no significant differences ($P > 0.05$).

characters after exclusion of ambiguous regions and introns. From the 1464 unambiguously aligned sites, 1167 were excluded from the wMP analysis because they were constant. Of the remaining sites 227 were parsimony informative. Constant sites were included for the maximum likelihood and Bayesian analyses.

The weighted parsimony analysis of the combined dataset resulted in one equal, most parsimonious tree of 956.99 steps, which was part of one island hit 996 times out of 1000 RAS. The majority rule consensus tree of 99 800 sampled trees from Bayesian analysis was similar to the parsimony and maximum likelihood topologies, and no conflict was detected. The most likely tree from the ML analysis is presented (FIG. 2) with branch lengths and support values.

All analyses supported two sister monophyletic genera (100% PP, ML BS and wMP BS), consisting of *Placidiopsis* and *Catapyrenium s. str.* (excluding *C. dactylinum*). Species of *Placidiopsis* lacking rhizines, including *P. cinerascens*, *P. tenella*, *P. pseudocinerea* and *P. tiroliensis*, formed a well supported monophyletic group sister of *P. custnani*, a species of *Placidiopsis* with a rhizine-like central holdfast.

A clade consisting of both *Placidiopsis cinerascens* and *P. tenella* was highly supported (99% ML BS, 100% PP and 96% wMP BS). These species were distinguished based on the presence or absence of an involucrellum (Breuss 1996b, Nimis and Martellos 2004) (FIG. 2). However the first author's observations led her to conclude that the presence of an involucrellum is not a reliable trait because it is not always associated with all ascomata in the same specimen or even in the same squamule of *P. tenella*. Therefore *Placidiopsis tenella* cannot be distinguished from *P. cinerascens* with this character.

Although the clade consisting of both *Placidiopsis cinerascens* and *P. tenella* seems to have phylogenetic structure, showing a subclade with 100% support in all analyses, (group B in FIG. 2), and another subclade supported by ML and wMP BS (group A in FIG. 2), the pattern of presence/absence of an involucrellum for the specimens selected for this study does not correlate with the association of these specimens with groups A or B. A more detailed morphological and anatomical study of these two species confirms this result. No significant differences were found between groups A and B in the measured variables (TABLE III), and therefore no molecular or phenotypic traits support the maintenance of *Placidiopsis cinerascens* and *P. tenella* as distinct species. To find some differences between these two subclades we considered whether the specimens of each subclade showed some ecological or geographical trend, but members of both subclades live in the same ecological conditions (i.e. calciferous soils in dry, open habitats) and no geographical pattern seems to explain these two monophyletic groups (FIG. 2). Based on these results, *Placidiopsis tenella* is here reduced to synonymy with *Placidiopsis cinerascens*.

TAXONOMY

Placidiopsis cinerascens (Nyl.) Breuss, Plant Syst. Evol. 148:315. 1985 – Holotypus: Gallia merid., Beaucaire, W. Nylander (H-NYL 4021!)

= *Placidiopsis tenella* (Nyl.) Zahlbr., Catal. Lich. Univ. I: 240. 1921 – Lectotypus designated by Cl. Roux in herbarium; Oran, Balansa (H-NYL 3944!)

MycoBank MB 515120.

Note: We do not know the exact date of publication of *Endocarpon tenellum* in Annal Sci Nat (Nylander 1853b), however searching through some indirect

references we think that it could not have been published before the end of 1853. The publication of *Endocarpon cinerascens* in Bot Not (Nylander 1853a) is dated Oct–Nov 1853. With this information we decided to consider *Endocarpon cinerascens* as having priority over *E. tenellum*.

Placidiopsis cinerascens includes specimens with the upper surface greenish gray to brownish, with a variable epinecral layer and perithecia with or without apical involucrellum. The species is distributed in central Asia, Mediterranean Europe and Morocco, Mexico and SW North America in Mediterranean and arid climates.

Notes on the valid name of *Placidiopsis cartilaginea* (Nyl.) Vainio. Breuss (1996b) used the name *P. cartilaginea* in his revision of genus *Placidiopsis*, explaining the use of this name by Gueidan et al. in 2007. Even though *Verrucaria cinerea* var. *cartilaginea* was described in 1853 (i.e. before *Placidium custnani* A. Massal. [1856]), based on Article 11.2 of the ICBN this name has no priority due to the rank in which it was published. Therefore the accepted name is *Placidiopsis custnani* (A. Massal) Körber, Parerga Lich. 1963:305, which justifies the use of this name by Gueidan et al. in 2009.

DISCUSSION

The use of the nuLSU locus alone is clearly insufficient for resolving with high confidence relationships among a relatively large number of taxa representing species diversity across the Verrucariaceae. However it was sufficient to infer phylogenetic relationships of 16 species with regard to a core set of species for which relationships are fairly well established based on multilocus datasets. The efficiency and accuracy of tree searches are conditional on the quality of the backbone (skeletal) constraint trees used. In this study the backbone implemented was based on the most comprehensive phylogeny on Verrucariaceae conducted by Gueidan et al. (2007) with three loci (*RPB*1, nuLSU and nuSSU). We used this backbone tree, capturing known relationships within the Verrucariaceae, to first determine which taxa sequenced for this study were part of the *Staurothele* group, our main focus (FIG. 1). Based on this result we defined our ingroup, centered on *Catapyrenium s. str.* and *Placidiopsis*, for a more in-depth phylogenetic analysis based on nuITS and nuLSU, without the use of a backbone constraint tree.

The implementation of a backbone constraint tree let us take into consideration what is known based on exhaustive multilocus phylogenies when conducting phylogenetic searches to infer relationships for additional taxa but on the basis of only one locus. Contrary to constraint trees that include all OTU of the dataset analyzed, internodes of backbone constraint trees will not necessarily be recovered in bootstrap analyses. For example the internode supporting the sister relationship of *Placocarpus schaereri* and *Verrucula* received a bootstrap support value barely above 50% with wMP and below 50% with ML or the constrained relation between *Catapyrenium cinereum* and *C. daedaleum* was recovered only by wMP (FIG. 1).

This study demonstrates that *Catapyrenium s. str.* is not monophyletic because *C. dactylinum* is closely related to *Placopyrenium caeruleopulvinum*, *Placopyrenium stanfordii* and to a clade consisting of parasitic *Verrucula* species and *Placocarpus schaereri*. This unexpected result could be explained by the presence of pycnidia on the specimen of *C. dactylinum*, being the only member of *Catapyrenium s. str.* with pycnidia. Other species of *Catapyrenium s. str.* lack pycnidia as do species of *Placidiopsis*. However members of *Verrucula* and *Placocarpus schaereri*, along with *Catapyrenium dactylinum*, share *Dermatocarpon*-type pycnidia. Various authors have suggested that pycnidium type could be diagnostic for delimiting genera within the Verrucariaceae (Breuss 1990, Harada 1993, Gueidan et al. 2007). In addition *C. dactylinum* lacks a rhizohyphal weft (a constant feature in *Catapyrenium s. str.*), attaching to the substrate by its elongated basal ends. *Placocarpus schaereri* (Breuss 1985), *Placopyrenium caeruleopulvinum* and *P. stanfordii* (Breuss 2002c) attach to the substrate by a central stipe. Both basal ends and stipes are rhizine-like structures originating from medullar tissue. Moreover all four taxa share a constant feature, the bluish-gray thallus produced by granular pruina. While looking for more shared characters among these species we realized that although *Catapyrenium dactylinum* is squamulose the squamules are aggregated, giving an areolate aspect to the thallus, similar to *Placopyrenium caeruleopulvinum*, which develops a crustose areolated thallus with convex to bullate areoles.

It is not surprising that *Placopyrenium caeruleopulvinum* and *P. stanfordii* occur in the same clade together with *Placocarpus schaereri*. Hasse (1913) pointed out that *Placopyrenium stanfordii* (as *Dermatocarpon zalhbruckneri* Hasse) was morphologically similar to *Placocarpus schaereri* (as *Dermatocarpon monstrosum* [Schaer.] Vain.) but differ in its uniform thallus and size of the ascospores. *P. caeruleopulvinum* and *P. stanfordii* are very similar, according to

Thomson (1987). Both species have a pale excipulum with only the mouth darkened, and the ascospores are larger in *Placopyrenium stanfordii*.

James et al. (2006) and Lawrey et al. (2007) have noted that genera within a given clade may have different life strategies. *Verrucula* species are parasitic, while *Placocarpus schaereri* has been reported to be parasitic only in the juvenile state (Zehetleitner 1978, Gueidan et al. 2009). However *P. caeruleopulvinum*, *P. stanfordii* and *Catapyrenium dactylinum* are not parasitic (Breuss 2000, 2002c, 2009). In our analysis the nonparasitic species formed a basal grade. The same evolutionary trend has been suggested to have taken place within the *Verruculopsis* group, according to Navarro-Rosinés et al. (2007).

Contrary to *Placopyrenium caeruleopulvinum* and *P. stanfordii*, *P. trachyticum* was found to belong to the *Placopyrenium* clade sensu Gueidan et al. (2007), here called *Placopyrenium s. str.* together with *P. buceki* and the recently combined *P. canellum* and *P. fuscellum* (Navarro-Rosinés et al. 2007). These members of *Placopyrenium* are characterized by having black-rimmed areoles or dark brown margins (Orange 2004, Breuss 2009). To date, *Placopyrenium* has been recognized as a monophyletic genus (Gueidan et al. 2007, 2009; Savić et al. 2008), but our results show the polyphyly of *Placopyrenium s.l.* A detailed study of this genus will be needed before proposing any taxonomical changes for species *P. caeruleopulvinum*, *P. stanfordii* and *C. dactylinum*.

Although the generic classification in family Verrucariaceae was based traditionally on ascospore septation (apart from structure of the thallus, presence or absence of hymenial alga, structure of the upper cortex and pycnidia; Zahlbruckner 1921–1922, Zschacke 1933–1934, Harada 1993, Breuss 1996a), recent molecular studies have shown that the ascospore type is often homoplasious and groups based on this character alone can result in polyphyly (Gueidan et al. 2007, 2009; Savić et al. 2008; Savić and Tibell 2009). Gueidan et al. (2007, 2009) questioned the monophyly of *Catapyrenium s. str.*, and *Placidiopsis* and suggested the possibility of combining these two genera into one genus. Results from our phylogenetic study support the recognition of *Catapyrenium s. str.* (excluding *C. dactylinum*) and *Placidiopsis* as distinct genera according to the traditional taxonomy of both genera (Breuss 1996a, b), with ascospore septation being a synapomorphic character for defining *Placidiopsis*.

In summary *Catapyrenium s. str.* is not monophyletic unless *C. dactylinum* is excluded from this genus and the same is true for *Placopyrenium* unless *P. stanfordii* and *P. caeruleopulvinum* are excluded, which we recommend for both genera but cannot implement formally until the affiliation of these excluded species is determined through a detailed systematic study focusing on the *Verrucula-Placopyrenium-Wahlenbergiella* clade. Species delimitation has been problematic between *Placidiopsis cinerascens* and *P. tenella*. We have observed that the diagnostic involucrellum trait is present in specimens of both clades (FIG. 2), even though this character is not always present in all perithecia in the same thallus. The development of the involucrellum may vary and depends on the development of the thallus, which also depends on ecological conditions, such as exposure and humidity, according to Savić et al. (2008) and Thüs (2002). Because this character is not a constant feature we think it should not be employed for delimiting these two species. Both species were described by Nylander (1853a, b) who reported different ascospore lengths for these two species. In our study anatomical and morphological analyses have not shown differences between specimens of *Placidiosis cinerascens* and *P. tenella* other than the expected within species level of variation. *Placidiopsis cinerascens* and *P. tenella* cannot be distinguished genotypically or phenotypically. Thus, *P. tenella* is reduced to synonymy with *P. cinerascens*.

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