Syst. Biol. 57(1):141–156, 2008

ISSN: 1063-5157 print / 1076-836X online
DOI: 10.1080/10635150801910451

The Limitations of Ancestral State Reconstruction and the Evolution of the Ascus in the Lecanorales (Lichenized Ascomycota)

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Abstract.— Ancestral state reconstructions of morphological or ecological traits on molecular phylogenies are becoming increasingly frequent. They rely on constancy of character state change rates over trees, a correlation between neutral genetic change and phenotypic change, as well as on adequate likelihood models and (for Bayesian methods) prior distributions. This investigation explored the outcomes of a variety of methods for reconstructing discrete ancestral state in the ascus apex of the Lecanorales, a group containing the majority of lichen-forming ascomycetes. Evolution of this character complex has been highly controversial in lichen systematics for more than two decades. The phylogeny was estimated using Bayesian Markov chain Monte Carlo inference on DNA sequence alignments of three genes (small subunit of the mitochondrial rDNA, large subunit of the nuclear rDNA, and largest subunit of RNA polymerase II). We designed a novel method for assessing the suitable number of discrete gamma categories, which relies on the effect on phylogeny estimates rather than on likelihoods. Ancestral state reconstructions were performed using maximum parsimony and maximum likelihood on a posterior tree sample as well as two fully Bayesian methods. Resulting reconstructions were often strikingly different depending on the method used; different methods often assign high confidence to different states at a given node. The two fully Bayesian methods disagree about the most probable reconstruction in about half of the nodes, even when similar likelihood models and similar priors are used. We suggest that similar studies should use several methods, awaiting an improved understanding of the statistical properties of the methods. A *Lecanora*-type ascus may have been ancestral in the Lecanorales. State transformations counts, obtained using stochastic mapping, indicate that the number of state changes is 12 to 24, which is considerably greater than the minimum three changes needed to explain the four observed ascus apex types. Apparently, the ascus in the Lecanorales is far more apt to change than has been recognized. Phylogeny corresponds well with morphology, although it partly contradicts currently used delimitations of the Crocyniaceae, Haematommataceae, Lecanoraceae, Megalariaceae, Mycoblastaceae, Pilocarpaceae, Psoraceae, Ramalinaceae, Scoliciosporaceae, and Squamarinaceae. [Phylogeny; ancestral state reconstruction; discrete gamma categories; stochastic mapping; ascomycetes; lichens; Lecanorales; ascus]

Ascomycetes constitute the largest taxonomic group within Kingdom Fungi. Many fungi depend on an intimate parasitic, commensalistic, or mutualistic relationship with a second living organism. The largest mutualistic group among the fungi is formed by the lichens, close associations between a fungus, commonly an ascomycete, and a photobiont, nearly always a unicellular green alga or a cyanobacterium (or both). Approximately 40% of all known ascomycetes form lichens (Kirk et al., 2001; Taylor et al., 2004). Among the ascomycetes, the lichen symbiosis seems to have evolved one, two, or three times, followed by a series of independent losses of the symbiotic state (Lutzoni et al., 2001). Almost exclusively lichenized and the most species-rich among the ascomycetes, the class Lecanoromycetes in the sense of Eriksson (2006) and Miadlikowska et al. (2006) contains taxa with more or less disc- or cup-like fruiting bodies ("apothecia"). The class received high branch support for monophyly in molecular phylogenetic studies by Liu and Hall (2004) and Miadlikowska et al. (2006). Eriksson (2006) recognized three subclasses within the Lecanoromycetes: Acarosporomycetidae, Ostropomycetidae, and Lecanoromycetidae, the latter being by far the most species rich and containing a vast majority of all described lichens. Apart from being the most diverse in terms of species number, the Lecanoromycetidae displays amazing variation in morphological as well as chemical characters (Rambold and Hagedorn, 1998). They are of particular ecological interest, as this group contains a vast majority of the "macrolichens"; i.e., lichens with a foliose ("leafy") or fruticose ("shrubby") habit, which are responsible for much of the lichen biomass production in most of the many habitats where lichens occur.

Systematics in the Lecanoromycetes has been a matter of hot debate for more than two decades. Particular attention has been paid to the structure of the apical apparatus of the ascus; i.e., the apically thickened ascus wall layers above the maturing ascospore mass. These structures are commonly visualized by their bluish amyloid reaction, which can vary in intensity between layers. The variation in ascus apex characters was acknowledged early and pioneering work was conducted by French mycologists (for a summary see Chadefaud, 1973). Although variation was described and patterns were compared to existing taxonomy (e.g., Letrouit-Galinou, 1973; Honegger 1978, 1980), ascus apex characters were hardly in use for classification purposes in lichen fungi until they came into focus with the influential work of Hafellner (1984). Hafellner postulated that the ascus apex morphology would be invariable within taxa corresponding approximately to genera and families. The ascus type rapidly became a popular a priori criterion among lichen systematists for distinguishing genera and families and (in

numerous cases) for assigning newly described genera to family. An entire subordinal reclassification was presented that was based specifically on ascus apex morphology (Rambold and Triebel, 1992). Implicit in this view is that ascus characters are "conservative" and only rarely transform, and thus can be assumed to be useful tools to characterize natural groups. However, this assumption, which can be seen as typological, has been criticized (Ekman, 1996; Tibell 1998; Ekman and Wedin, 2000; Lumbsch et al., 2001). Yet, implicit or explicit assumptions on low rates of change in ascus characters still prevail in lichen systematics, particularly at genus and family level. It is noteworthy that variation in ascus apex characters has not acquired the same a priori weight in fungal classification outside the Lecanoromycetes. This is probably a reflection of the large amount of variation compared to other fungal groups. In other ascomycete groups with variable ascus apex characteristics, e.g., Helotiales (Verkley, 1992, 1993a, 1993b, 1994) and Xylariales (Læssøe and Spooner, 1994), a similar classification philosophy has not been adopted.

The core problem is that any assumption on the rate of change in ascus apex characters, whether small or large, remains untested. Ascus wall characteristics can be assumed to play a role in ascus dehiscence and ascospore discharge and dispersal (e.g., Henssen and Jahns, 1974; Honegger, 1982; Rambold, 1995). However, we know very little about the specific function of the various structures in the ascus apex and knowledge about ascus evolution is rudimentary. The purpose of this study was to provide an assessment of ascus evolution in light of phylogenetic data. We achieved that by extending the sampling of taxa and molecular markers in order to improve knowledge on the phylogeny of the order Lecanorales, the largest of the orders in the subclass Lecanoromycetidae. Lecanorales, taken here in the sense of Miadlikowska et al. (2006), is comprised of several thousands of species in 20 or so families (in the sense of Eriksson, 2006). It includes many familiar lichens like *Cladonia* (reindeer and cup lichens), *Parmelia* and related genera (shield lichens), *Alectoria* (witch's hair), *Usnea* (beard lichens), *Ramalina* (strap lichens), and *Sphaerophorus* (coral lichens). Furthermore, it contains more variation in ascus characters than any other order within the subclass, and it was the group of lichens that the original study by Hafellner (1984) was mostly focused on. This group of lichens tends to be poorly resolved or poorly or unevenly sampled in most molecular phylogenies published so far. With a phylogenetic estimate at hand, we inferred ancestral states and the amount of change in the evolution of the ascus within Lecanorales. In particular, we set out to test a variety of methods for ancestral state reconstruction in order to understand to what extent inferences were dependent on the particular method used.

Materials and Methods

Taxon and Gene Sampling

Representatives of 12 lecanoralean families and an additional 2 non-lecanoralean families in the' Lecanoromycetidae and a taxon of uncertain position in the Lecanoromycetes (*Helocarpon*), all taken in the sense of Eriksson (2006), were included in this study (Table 1). *Bellemerea diamarta*, *Lecidea fuscoatra*, and *L. silacea*, members of the Lecideaceae (Lecideales, Lecanoromycetidae), were chosen as outgroup because this group (together with the Peltigerales) constitutes one out of two potential sister groups to the Lecanorales, the other group being the Teloschistales (Miadlikowska et al., 2006). Anziaceae, Calycidiaceae, Cetradoniaceae, and Gypsoplacaceae in the sense of Eriksson (2006) were not included, Calycidiaceae because of unsuccessful PCR amplifications, the other three families because of lack of fresh material. Anziaceae should be included in the Parmeliaceae and Gypsoplacaceae is closely related to or should be included in that family (Thell et al., 2004; Arup et al., 2007). Calycidiaceae includes a single genus, *Calycidium*, with two species and is probably synonymous with or at least closely related to Sphaerophoraceae (Wedin, 2002). The Cetradoniaceae, which includes the single species *Cetradonia linearis* (Wei and Ahti, 2002), has been shown to be nested within the Cladoniaceae (Zhou et al., 2006; Miadlikowska et al., 2006). Altogether 58 species were included in this study. From each species, we included either previously published sequences or newly produced ones.

DNA Extraction, PCR Amplification, and DNA Sequencing

We attempted to obtain sequences from three different genes, the small subunit of the mitochondrial ribosomal RNA gene (mrSSU), the large subunit of the nuclear ribosomal RNA gene (nrLSU), and the largest subunit of RNA polymerase II (RPB1). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). PCR amplification of the mrSSU was performed as described by Andersen and Ekman (2005) or Wiklund and Wedin (2003). The nrLSU was amplified following the protocol of Wiklund and Wedin (2003), or using the primers ITS1F (Gardes and Bruns, 1993), nu-LSU-155-5', nu-LSU-401-3' (Döring et al., 2000), LR3, LR5, or LR6 (Vilgalys and Hester, 1990). The PCR mixture consisted of 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 800 μM total dNTPs (Promega), 0.7 μM of each primer, 1 U of the enzyme AmpliTaq Gold DNA polymerase (Applied Biosystems), and a variable amount of extracted DNA. The PCR cycling parameters included an initial hold at 95°C for 9 min, then denaturing at 95°C for 60 s, annealing at 62°C for 45 s, decreasing 1°C per cycle for the first 7 of the 42 cycles (touchdown), and polymerization at 72°C for 120 s for the first 7 cycles, then increasing polymerization by 3 s each cycle. RPB1 was amplified using the primers gRPB1-A and fRPB1-C (Matheny et al., 2002), and the PCR mixture consisted of 1× Herculase PCR buffer (Stratagene), 800 μM total dNTPs (Promega), 0.8 μM of each primer, 2.5 U of the enzyme Herculase (Stratagene), and a variable amount of extracted DNA. The PCR cycling parameters included an initial hold at 94°C for 2 min, then denaturing at 94°C for 1 min, annealing at 61°C for 90 s, decreasing 1°C per cycle

TABLE 1. Species included in this study with familial classification according to Eriksson (2006), GenBank accession numbers for each of the three genes, and voucher specimens (deposited in herbarium BG unless otherwise stated). *, newly obtained sequences. NA, genes for which data were not available.

Species numbers	Source	Family	Genbank accesion		
			mrSSU	nrLSU	RPB1
Adelolecia pilati	Austria, *Ekman 3373*	Ramalinaceae	AY567713	AY300826	AY756379*
Bacidia rosella	Sweden, *Ekman 3117*	Ramalinaceae	AY300877	AY300829	AY756412*
Bellemerea diamarta	Sweden, *Wedin 6822* (UPS)	Porpidiaceae	AY756398*	AY756336*	AY756406*
Bilimbia sabuletorum	Norway, *Ekman 3091*	Ramalinaceae	AY567721	AY756346*	AY756413*
Byssoloma leucoblepharum	Portugal, *Ekman 3502*	Pilocarpaceae	AY567778	AY756317*	AY756380*
Calopadia foliicola	Costa Rica, *Lücking 16011*	Pilocarpaceae	AY567782	AY756318*	AY756381*
Catinaria atropurpurea	Norway, *Holien 9178* (TRH)	Ramalinaceae	NA	AY756347*	NA
Cladonia digitata	Sweden, *Ekman 3424*	Cladoniaceae	AY756366*	AY756319*	AY756414*
Cladonia peziziformis	Norway, *Ekman 3295*	Cladoniaceae	AY567716	AY756320*	AY756415*
Crocynia gossypina	Costa Rica, *Lücking 16052*	Crocyniaceae	AY567766	NA	NA
Fellhanera bouteillei	Sweden, *Ekman 3417*	Pilocarpaceae	AY567787	AY756348*	AY756382*
Fellhanera subtilis	Germany, *Tønsberg 28199*	Pilocarpaceae	AY567786	AY756321*	NA
Frutidella caesioatra	Norway, *Andersen 91*	Ramalinaceae	AY567765	AY756349*	AY756383*
Glyphopeltis ligustica	South Africa, *Brusse 4947* (UPS)	Psoraceae	AY756399*	AY756337*	NA
Haematomma ochroleucum	Norway, *Ekman 3184*	Haematommataceae	AY756367*	AY756350*	NA
Halecania alpivaga	Sweden, *Nordin 5504* (UPS)	Catillariaceae	AY756368*	NA	NA
Helocarpon crassipes	Sweden, *Kanz & Printzen 5459* (hb Printzen)	?	AY567728	AY756322*	AY756384*
Herteliana taylorii	Ireland, *Hertel 39599* (UPS)	Ramalinaceae	AY756369*	AY756351*	AY756385*
Hypogymnia physodes	Sweden, *Mattsson 4005* (UPS) Sweden, *Wedin 6623* (UPS)—RPB1 only	Parmeliaceae	AY756400*	AY756338*	AY756407*
Lecania atrynoides	United Kingdom, *Coppins 15594 & O'Dare* (E)	Ramalinaceae	AY756370*	AY756352*	AY756416*
Lecanora aff.*allophana*	Sweden, *Ekman 3434*	Lecanoraceae	AY567710	AY756353*	NA
Lecanora intumescens	Norway, *Ekman 3162*	Lecanoraceae	AY567715	AY300841	AY756386*
Lecidea atrosanguinea	Norway, *Ekman 3438*	Lecideaceae	AY762094*	AY756354*	NA
Lecidea fuscoatra	Sweden, *Wedin 6860* (UPS)	Lecideaceae	AY756401*	AY756339*	AY756408*
Lecidea silacea	Sweden, *Wedin 6865* (UPS)	Lecideaceae	AY756402*	AY756340*	AY756409*
Lecidea turgidula	Sweden, *Ekman 3416*	Lecideaceae	AY567788	AY756323*	AY756387*
Lecidella meiococca	Sweden, *Ekman 3101*	Lecanoraceae	AY567714	AY300842	NA
Lepraria bergensis	Norway, *Tønsberg 28875*	Stereocaulaceae	AY756371*	AY756324*	AY756417*
Lepraria lobificans	Norway, *Tønsberg 28224*	Stereocaulaceae	AY756372*	AY756325*	AY756418*
Lopadium disciforme	Norway, *Ekman s.n.*	Pilocarpaceae?	AY756373*	AY756355*	NA
Megalaria grossa	Norway, *Tønsberg 26038*	Megalariaceae	AY762095*	AY756356*	AY756419*
Micarea adnata	Norway, *Andersen 48*	Pilocarpaceae	AY567751	AY756326*	AY756388*
Micarea alabastrites	Norway, *Andersen 17*	Pilocarpaceae	AY567764	AY756327*	AY756389*
Micarea erratica	Sweden, *Arup 99192* (hb Arup)	Pilocarpaceae	AY567737	AY756328*	AY756390*
Micarea micrococca	Norway, *Andersen 34*	Pilocarpaceae	AY567749	AY756330*	NA
Micarea sylvicola	Sweden, *Ekman 3629*	Pilocarpaceae	AY567768	AY756331*	AY756392*
Miriquidica garovaglii	Norway, *Ekman s.n.*	Lecanoraceae	AY567711	AY756357*	AY756420*
Mycoblastus sanguinarius	Sweden, *Wedin 6932* (UPS) Norway, *Ekman s.n.*—RPB1 only	Mycoblastaceae	AY756403*	AY756341*	AY756393*
Platismatia glauca	Sweden, *Mattsson 4007* (UPS) Sweden, *Granberg s.n.* (UPS)—RPB1 only	Parmeliaceae	AY756404*	AY756342*	AY756410*
Protoblastenia rupestris	Norway, *Johnsen 19.02.2002*	Psoraceae	NA	AY756358*	AY756421*
Protomicarea limosa	Norway, *Andersen 92*	Psoraceae?	AY567733	AY756332*	NA
Psilolechia leprosa	Norway, *Tønsberg & Botnen 27362*	Pilocarpaceae	AY567730	AY756333*	AY756395*
Psilolechia lucida	Norway, *Andersen 8*	Pilocarpaceae	AY567729	AY756334*	NA
Psora decipiens	Norway, *Ekman 3327* Sweden, *Wedin 6452* (UPS)—nrLSU only	Psoraceae	AY567772	AY756343*	AY756396*
Psora rubiformis	Norway, *Ekman 3343*	Psoraceae	AY756374*	AY756359*	NA
Psorula rufonigra	USA, *Nordin 5265* (UPS)	Psoraceae	AY756405*	AY756344*	AY756411*
Pyrrhospora quernea	Sweden, *Ekman 3019*	Lecanoraceae	AY567712	AY300858	NA
Ramalina fastigiata	Norway, *Ekman 3616*	Ramalinaceae	AY756375*	AY756360*	AY756422*
Rolfidium coccocarpioides	Mauritius, *Krog & Timdal MAU61/08* (O)	Ramalinaceae	AY762096*	AY756361*	AY756423*
Schadonia fecunda	Austria, *Hafellner 43170* (GZU)	Ramalinaceae	AY756376*	AY756362*	NA
Scoliciosporum intrusum	Norway, *Ekman s.n.*	Lecanoraceae	AY567767	AY756329*	AY756391*
Scoliciosporum umbrinum	Norway, *Ekman 3005*	Lecanoraceae	AY567719	AY300861	NA
Sphaerophorus globosus	Island, *Högnabba 101* (UPS)—mrSSU U.K., *Wolseley 18/2/98* (BM)—nrLSU Norway, *Tønsberg 30589*—RPB1	Sphaerophoraceae	AY256751	AY756345*	AY756424*
Squamarina lentigera	Norway, *Haugan & Timdal 4801* (O)	Ramalinaceae?	AY756377*	AY756363*	AY756425*
Stereocaulon pileatum	Norway, *Tønsberg 27339*	Stereocaulaceae	AY567718	AY756335*	AY756426*
Stereocaulon tomentosum	Norway, *Tønsberg 27335* Sweden, *Wedin 5089* (UPS)—nrLSU only	Stereocaulaceae	AY756378*	AY340569	AY756427*
Tephromela atra	Sweden, *Ekman 3105*	Ramalinaceae	AY762097*	AY756364*	AY756428*
Toninia cinereovirens	Norway, *Haugan & Timdal 7953* (O)	Ramalinaceae	AY567724	AY756365*	AY756429*

for the first 7 of the 40 cycles (touchdown), and polymerization at 72°C for 120 s for the first 7 cycles, then increasing polymerization by 3 s each cycle. Direct sequencing of PCR products in both directions was performed using the PCR primers. Cycle sequencing was carried out using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an ABI Prism 3700 DNA analyzer (Applied Biosystems). Sequences were assembled using SeqMan II, version 4.05 (DNASTAR).

Sequence Alignment

Sequences were aligned using SAM (Sequence Alignment and Modeling software system) version 3.4 (Hughey et al., 2003; Hughey and Krogh, 1996; available on-line at http://bioweb.pasteur.fr/seqanal/motif/sam-uk.html), followed by manual adjustment. Ambiguous regions of the alignments were excluded from further analyses. Introns in RPB1 were excluded; only open reading frames were used in subsequent analyses.

The final matrix was submitted to TreeBASE (http://www.treebase.org/) and filed under matrix accession number M3579. It consists of 58 species and 2421 unambiguously aligned positions, 1186 of which were variable (99 out of 232 in RPB1 first codon positions, 83 out of 232 in RPB1 second codon positions, 225 out of 231 in RPB1 third codon positions, 434 out of 794 in mrSSU, and 345 out of 932 in nrLSU). Most species in our matrix are represented by all three genes, but 15 are represented by only two genes and three (*Catinaria atropurpurea*, *Crocynia gossypina*, and *Halecania alpivaga*) by only one gene (Table 1). Seventeen out of 18 taxa with missing data from at least one gene lack RPB1, the technically most difficult gene to amplify with PCR.

Phylogenetic Analyses

Phylogenetic analyses were carried out using Bayesian Markov chain Monte Carlo (MCMC) inference. Likelihood model selection was carried out in two steps. First, the best among 56 reversible models was selected using decision theory (Minin et al., 2003) as implemented in the Perl script DT-ModSel in combination with PAUP* 4.0 beta 10 (Swofford, 2003) for (1) the entire data set including all three genes (unpartitioned), (2) each gene separately (three partitions), and (3) mrSSU and nrLSU separately as well as each of first, second, and third codon positions in the RPB1 separately (five partitions). Subsequently, the degree of model partitioning was selected using Bayes factor (Kass and Raftery, 1995), the computation of which involved the harmonic mean estimator of model likelihood (Newton and Raftery, 1994). The MPI version of the software MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) was used to compare an unpartitioned model with a model with three partitions and then compare a model with three and a model with five partitions. For partitioned data, the likelihood model included allowing interpartition rate heterogeneity (Yang, 1996) but assuming branch lengths to be proportional across partitions. Rate matrix parameters, state frequencies, the gamma shape parameter, and proportion of invariable sites were unlinked across partitions. The number of discrete gamma categories, as in the decision theory test, was kept at default four in all cases in order not to obscure the effect of partitioning. Bayesian prior distributions included treating all tree topologies as equally likely, a uniform (0.1, 50) distribution for the gamma shape parameter, a uniform (0, 1) distribution for the proportion of invariable sites, a flat (1, 1, 1, 1, 1, 1) Dirichlet for the rate matrix, a flat (1, 1, 1, 1) Dirichlet for the state frequencies (except when the model dictated state frequencies to be equal), and exponentially distributed branch lengths with inverse scale parameter 10. For each model, three parallel runs were performed, each with eight chains, seven of which were incrementally heated with a temperature of 0.15. The appropriate degree of heating (temp = 0.15) was determined by observing swap rates between chains in preliminary runs. Analyses were diagnosed for convergence every 10^5 generations in the last 50% of the tree sample and automatically halted when convergence was reached. Convergence was defined as a standard deviation of splits (of frequency ≥ 0.1) between runs below 0.01. Every 250th tree was sampled. Tracer 1.3 (Rambaut and Drummond, 2005) was used to check for problems with arbitrarily truncated posterior distributions (concerns only the gamma curve shape parameter) and for adequate effective sample sizes (ESS) for all model parameters. Both tests using Bayes factor for model adequacy identified "very strong" support for the more complex model (Kass and Raftery, 1995). The harmonic mean estimator has been reported to be unstable and indeed sometimes an unreliable estimator of model likelihood (Kass and Raftery, 1995; Bos, 2002; Lartillot and Philippe, 2006), particularly when MCMC sampling is limited and when the dimensionality of the models being compared is very different. In our case, however, Bayes factors were very large and harmonic mean likelihoods very similar across identical runs.

Once appropriate models had been selected, a choice had to be made concerning the number of categories to utilize in the discretized distribution of the gamma rate heterogeneity model for each partition. Common approaches in the literature involve accepting four categories, which is often the default, or adding one category incrementally until the likelihood of a given tree stops improves more than a prespecified amount. However, no criteria have been described that can help define what this "prespecified amount" of likelihood should be. We chose a different approach to the problem, which involves assessing the effect the number of categories has on phylogeny estimates rather than on likelihoods. We started by generating a sample of 100 "reasonably good trees" by performing a neighbor-joining bootstrap using LogDet distances in PAUP*. The likelihood of each tree in the sample was then evaluated using a varying number of gamma categories from 2 to 25 under the appropriate model. Each tree could then be assigned a likelihood rank from 1 (the best) to 100 (the worst) for each number of gamma categories. For each addition of

a category, going from $i-1$ to i categories, we calculated the sum of squares of differences in rank across the $j = 100$ trees in the sample, $d_i = \sum_{j=1}^{100} (r_{i-1} - r_i)^2$, where r is the rank. For identical rankings $d_i = 0$. The smallest possible difference between two unidentical rankings is $d_i = 2$; i.e., when two trees consecutive in ranks under $i-1$ categories swap ranks when going to i categories. Using SPSS 13.0 (SPSS Inc., Chicago, IL), we performed a regression of d_i against i. The best curve fit was found for an S model (all $P < 0.01$), which has the shape $d_i = \exp(a + b/i)$, where a and b are shape parameters to be estimated. In most cases (except for RPB1 third positions and the complete RPB1 data set), d_i did not approach 0 or even 2 for values of i up to 25. Therefore, we picked the highest number of i on the horizontal axis that resulted in d_i decreasing by at least 2 (the smallest meaningful improvement) on the vertical axis, going from $i-1$ to i.

As a consequence of the above relative model adequacy tests, partitions were assigned the following submodels: SYM+I+dΓ9 (RPB1 all positions), TIM+I+dΓ12 (RPB1 first positions), TVMef+I+dΓ12 (RPB1 second positions), TVM+I+dΓ9 (RPB1 third positions), TVM+I+dΓ13 (mrSSU), and TrN+I+dΓ11 (nrLSU). For the full data set, five partitions were used (the three positions in RPB1 as well as mrSSU and nrLSU). For model denotations, we refer to the documentation to the software ModelTest 3.7 (Posada and Crandall, 1998). Because of restrictions in model selection imposed by MrBayes, we had to approximate the best models with either simpler two-rate models or more complex six-rate models. In order not to overestimate confidence in a limited set of trees (Huelsenbeck and Rannala, 2005), we decided to apply independent GTR+I+dΓ models to all partitions (Rodríguez et al., 1990; Yang, 1993, 1994a, 1994b, 1996; Gu et al., 1995), except the complete RPB1 data set and the second-codon position RPB1 partition, for which SYM+I+dΓ models were used. This model differs from the GTR model only in assuming nucleotide frequencies to be equal.

Congruence between the three genes was investigated a priori by performing Bayesian MCMC analyses on each gene separately. Analyses were identical to the ones described above, except that 20 chains were used in the case of nrLSU. Majority-rule consensus trees were constructed from post–burn-in tree samples. We defined incongruence between genes to mean the presence of conflicting nodes with high support (Mason-Gamer and Kellogg, 1996), which is taken here to mean 95% or higher posterior probability. The final posterior tree sample used for producing a consensus tree and performing analyses of ascus evolution was obtained by running MrBayes with the combined data, allowing for interpartition rate heterogeneity but assuming branch lengths to be proportional across partitions. In each run, 20 parallel chains were used.

Analyses of Character Evolution

The amyloid reaction of the apical apparatus of the ascus was classified into discrete states, closely following consensus in lichen systematics. The coding of character states in individual taxa was based on literature (Hafellner, 1984; Hertel and Rambold, 1988; Haugan and Timdal, 1992; Purvis et al., 1992; Thell et al., 1995; Ekman, 1996; Ekman and Wedin, 2000) and, whenever necessary our own observations (for the staining procedure, see Ekman, 1996). Four different ascus types were found to be present among the taxa included in this study (Fig. 1): (1) *Lecanora*-type, with a pale axial body penetrating through all of the dark-amyloid layer; (2) *Micarea*-type, with a thin and dark tube-structure with more or less parallel sides, often less distinctly amyloid at the top, and with a pale axial body; (3) *Psora*-type, with a wide, dark tube-structure that diverges and often becomes wider towards the top, without a pale axial body; and (4) *Bacidia*-type, with a conical or bell-shaped, pale axial body. We did not recognize the *Biatora*-type ascus as distinct from the *Bacidia*-type ascus, because we find the presence or absence of a dark layer around the axial body to be variable among closely related taxa or even within species. All ascus types known to occur among the lecanoraleans were present in our taxon sample, except the *Cetraria*-type ascus, which appears to be restricted to the genus *Cetraria* and related genera inside the Parmeliaceae (Thell et al., 1995). The *Cetraria*-type ascus has been considered to be very similar to and probably derived from the *Lecanora*-type ascus (Mattsson and Wedin, 1999; Wedin et al., 2000). In two cases, *Bilimbia sabuletorum* and *Crocynia gossypina*, ascus classification was ambiguous. Our primary choice was to classify the ascus of these taxa as *Bacidia*-type, being well aware that this can be questioned. The *Bacidia*-type ascus is fairly clearly visible in young asci, whereas its appearance seems to transform into *Micarea*-type with age, although structures become very unclear and difficult to interpret. However, for the purpose of evaluating the sensitivity of our results to this coding, we also performed all analyses of character evolution with these taxa coded as *Micarea*-type.

We defined here the order Lecanorales as the monophyletic group of taxa united by the deepest node receiving 99.99% posterior probability. The remaining 0.01% posterior probability for nonmonophyly of the Lecanorales comes from the long-branch taxon *Psorula* appearing nested among non-lecanoralean taxa in three trees of the posterior sample, never that nonlecanoraleans appeared inside the lecanoraleans. We decided to condition analyses of character evolution on lecanoralean monophyly and to exclude non-lecanoralean taxa from further analysis. Including nonlecanoraleans would have meant basing inferences on a scarce sample of distantly related nonlecanoraleans. Taxon sampling density and branch lengths between ingroup and outgroup have been shown to affect ancestral state reconstructions (Schultz and Churchill, 1999; Salisbury and Kim, 2001). Our condition also meant ignoring the 0.01% chance that *Psorula* is not a lecanoralean. This is anyway much less than the stochastic error in the MCMC approach described below.

Ancestral state reconstructions of discrete morphological characters on phylogenies can be done in a series

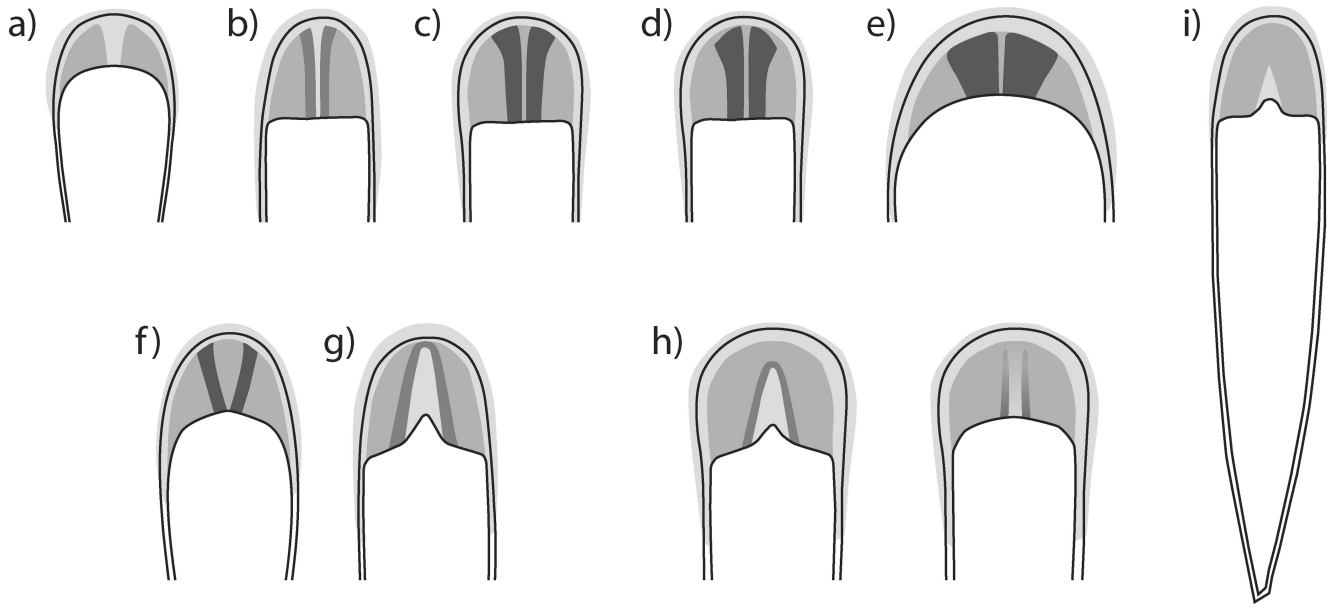

FIGURE 1. Ascus types occurring in the Lecanorales. Illustrations show the blue amyloid reaction depicted as shades of grey. Apical part of ascus in a to h, entire ascus in i. (a) *Lecanora*-type in *Micarea intrusa*. (b) *Micarea*-type in *Micarea micrococca*. (c) *Psora*-type in *Micarea sylvicola*. (d) *Psora*-type in *Micarea erratica*. (e) *Psora*-type in *Schadonia fecunda*. (f) *Psora*-type in *Protomicarea limosa*. (g) *Bacidia*-type in *Bacidia heterochroa*. (h) *Bacidia*-type (left) in young ascus of *Crocynia gossypina*; borderline *Micarea*-type in older ascus (right). (i) *Bacidia*-type in *Bacidia arceutina*.

of ways. Reconstructions can be performed on a single tree (e.g., a maximum parsimony or a maximum likelihood tree) or on a Bayesian posterior tree sample. Unlike single-tree reconstructions, the latter approach has the advantage of taking uncertainty in the tree topology and branch lengths, thereby effectively integrating out these parameters from the question in focus. However, ancestral state reconstruction itself on each of the trees in a posterior tree sample can be performed under either the maximum parsimony optimality criterion, the maximum likelihood optimality criterion (Lutzoni et al., 2001, following Pagel, 1997, 1999), or using a Bayesian approach. Only fully Bayesian approaches take phylogenetic uncertainty as well as uncertainty in the state reconstruction into account. There are two slightly different Bayesian methods available, the strategy described by Huelsenbeck and Bollback (2001) extended to morphological characters and the strategy of Pagel et al. (2004), with the additions by Pagel and Meade (2006). We performed ancestral state reconstruction for deep nodes with high support in the Lecanorales using the maximum parsimony and maximum likelihood optimality criteria on our Bayesian posterior sample of trees as well as both of the fully Bayesian methods. We defined "deep nodes" to mean nodes that unite two or more families according to the results of the phylogenetic analysis and "high support" as 95% or better posterior probability. Eight such nodes exist. All analyses were performed using the complete posterior tree sample from the Bayesian phylogenetic analysis of the combined data set. Before proceeding with the reconstructions, we checked trees for the node-density artefact (Fitch and Bruschi, 1987; Fitch and Beintema, 1990) using the method described by Webster et al. (2003) and Venditti et al. (2006) and implemented with a Web interface at http://www.evolution.reading.ac.uk/pe/index.html.

Maximum parsimony and maximum likelihood ancestral state reconstructions were performed using Mesquite 1.12 (Maddison and Maddison, 2006), counting only unequivocal states. Because Mesquite cannot handle unknown states, taxa with such states were pruned from the trees prior to analysis (but preserving branch lengths). ML reconstructions were performed under a single-rate Mk likelihood model for discrete morphological characters described by Lewis (2001). This is the only morphological model implemented in this version of Mesquite. The likelihood decision threshold, the minimum difference in likelihood between the best and the next best state needed for assigning a state to a node, is potentially crucial. However, the effect of varying this parameter is rarely investigated. We used thresholds of 2.0 (a commonly used value that was originally proposed by Pagel, 1999) as well as 0.0 (or, actually, 10^{-8}, the lowest possible value, because a threshold of precisely zero is not allowed).

Ancestral state reconstruction using the Huelsenbeck and Bollback (2001) procedure was performed using the software SIMMAP 1.0 build 12092006-1.0-B2.3 (Bollback, 2006). SIMMAP implements only a single-rate model of transformation for multistate characters. Two different priors were used on the overall transformation rate, rate being defined here as tree length: (1) A "minimum change prior"; i.e., a fixed rate of 3. This prior is consistent with the idea that ascus apex characters completely reflect relationships. If so, there is no homoplasy in the

ascus character and three transformations are enough to explain the four observed states (one of which is plesiomorphic in the entire group). (2) A "parsimony prior," which is consistent with the idea that we should assume a priori no more change than indicated by the results of the phylogenetic analysis; i.e., the amount of change needed to explain the observed states in the terminals of the trees in the posterior sample. This distribution was obtained by counting the number of transformations under maximum parsimony using MacClade 4.08 (Maddison and Maddison, 2005). Character states were treated as unordered. SIMMAP assumes prior distributions of tree lengths to be gamma distributed. Therefore, we set the shape parameters of the gamma distribution (α, β) to make it imitate as closely as possible the maximum parsimony distribution of tree lengths. The gamma distribution was discretized using 50 categories. The number of realizations from the prior distribution was set to one per tree when the rate was fixed (because further draws from the prior would not affect the calculations) and 50 per tree when the rate was gamma distributed. Total tree lengths were rescaled to one before placing the morphology prior.

Ancestral state reconstructions using the strategy of Pagel et al. (2004) and Pagel and Meade (2006) were performed using the software BayesTraits 1.0. Unlike SIMMAP, BayesTraits allows the user to explore a variety of models or even to integrate over models with the use of reversible-jump Markov chain Monte Carlo (the latter recommended in the software documentation). In a first analysis, we used reversible-jump MCMC on an unrestricted model with twelve transformation rates. In the second analysis, we used reversible-jump MCMC on a single-rate model by constraining all transformation rates to be equal. In the third analysis, we used a fixed gamma-distributed prior on transformation rates (instead of reversible-jump) and a single-rate model. The second and third analyses were performed in order to make the model and priors comparable to the model and priors used by SIMMAP. In the two first analyses, we used a uniform prior distribution on the likelihood models and uniform (0, 10) hyperpriors on the shape and scale parameters to seed a gamma-distributed prior distribution on the rate coefficients. The fixed gamma distribution in the third analysis was set to mimic the "parsimony prior" in SIMMAP as closely as possible by dividing the mean and standard deviation of the fitted gamma distribution on parsimony tree length by the average (molecular) likelihood tree length in our posterior tree sample and by the number of transformation rate parameters (12). All analyses were run for 10^9 MCMC generations, the first 10^8 of which were later removed as burn-in. A sample from the posterior was taken every 5000 generations. The proposal mechanism was adjusted to target an acceptance rate of ca. 50%. Each analysis was conducted three times in order to check for convergence problems. Similar harmonic mean likelihoods across identical runs indicated that convergence had been reached and samples were consequently pooled. BayesTraits does not provide a summary of the MCMC results. Consequently, software needed to extract the marginal posterior probabilities of each state at each node (integrated over priors, tree topologies, and branch lengths) from the BayesTraits output was written in REALBasic (REAL Software Inc., Austin, TX). This kind of marginalization was performed in order to make results directly comparable to results reported by SIMMAP.

Finally, we obtained posterior distributions of the number of character state transformations of the ascus across the Lecanorales using (1) maximum parsimony as implemented in the software MacClade 4.08 (Maddison and Maddison, 2005) and (2) the Bayesian stochastic mapping procedure outlined by Huelsenbeck et al. (2003) and Ronquist (2004), as implemented in SIMMAP (Bollback, 2006). This method, which is conceptually different from and should not be confused with the ancestral state reconstruction algorithm implemented in the same software, is based on the "Bayesian mutational mapping" method described by Nielsen (2002). We used the same Bayesian tree sample and the same rate priors as in the ancestral state reconstructions using SIMMAP. The number of realizations from the prior distribution was set to 100 per tree. Software needed to extract (1) the posterior probability distribution of the number of state changes across trees and (2) the posterior probability distribution of the frequency of transformations between character states from the SIMMAP output was written in REALBasic. We made three replicates of each analysis, final results being obtained by summing across analyses.

RESULTS

Phylogenetic Analyses

Bayesian analyses of phylogeny halted automatically after 2.3×10^6 (RPB1), 8.9×10^6 (mrSSU), and 14.3×10^6 (nrLSU) generations, the last half of each posterior sample being stationary. The analysis of topological incongruence revealed no cases of discordance between the three genes. Consequently, we performed an analysis of the combined data. Treating the combined analyses as unpartitioned resulted in an analysis that halted automatically after 10×10^6 generations, whereas dividing the data into three partitions, corresponding to the three genes, resulted in an analysis that halted after 6.9×10^6 generations. However, the final analysis of the combined data upon which all downstream analyses were based consisted of five partitions, as support was strong for this. This analysis halted automatically after 6.8×10^6 generations, the last half of the posterior sample being stationary. Effective sample sizes ranged from several hundreds to tens of thousands for all parameters in all analyses, and arbitrary truncations of posterior distributions were not detected. A majority-rule consensus tree with all compatible groups, average branch lengths, and posterior probabilities of branches from the Bayesian posterior tree sample (48,000 trees) is provided in Figure 2. In this figure, we also indicate a revised but tentative family classification that incorporates the newly obtained phylogenetic information by imposing the smallest possible change

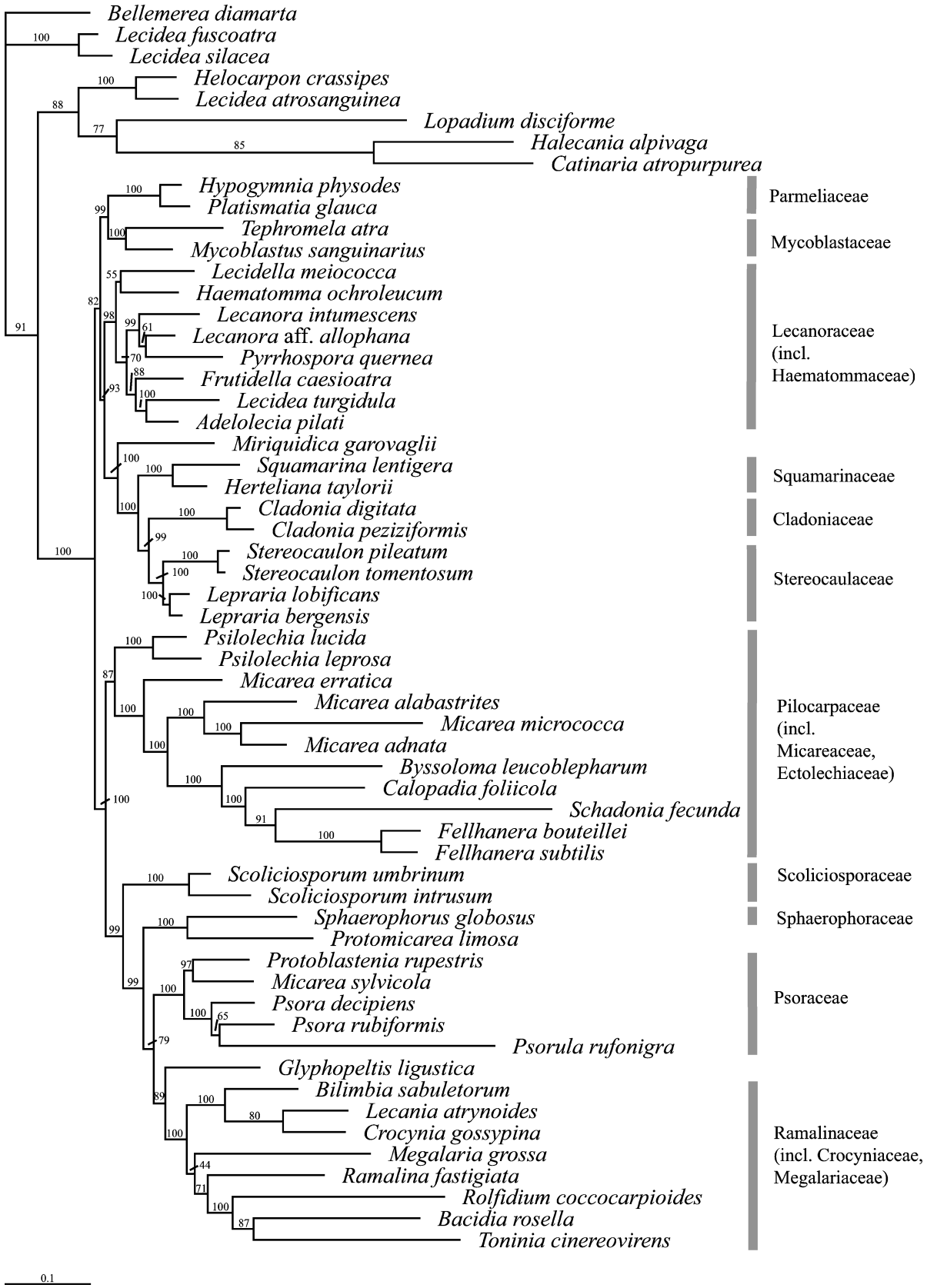

FIGURE 2. Majority-rule consensus tree with all compatible groups and with average branch lengths, based on a Bayesian posterior tree sample comprising 48,000 trees. A 4×(GTR+I+Γ)+(SYM+I+Γ) likelihood model with interpartition rate heterogeneity was used. Bayesian posterior probabilities are displayed at nodes (in %). The familial classification predicted by this tree is indicated in the right margin.

on the existing classification. Members of two genera, *Lecidea* and *Micarea*, stand out as being represented by taxa on widely spaced branches. The genus name *Lecidea* has been used on a variety of unrelated taxa. The type species is *L. fuscoatra*, included in our study. The type species of *Micarea* is *M. prasina*, which is closely related to *M. micrococca*, also included in our study. Consequently, *Lecidea turgidula*, *L. atrosanguinea*, and *Micarea sylvicola* should be referred to other genera, which are most likely undescribed.

Analyses of Character Evolution

Observed character states and eight well-supported deep nodes for which ancestral state reconstructions were performed are indicated in Fig. 3. Ancestral state reconstructions using maximum parsimony and maximum likelihood on a Bayesian posterior tree sample (Mesquite) as well as two fully Bayesian methods (SIMMAP, BayesTraits) are summarized in Table 2. The total number of state transformation counts in the ascus in the Lecanorales, using stochastic mapping (SIMMAP) and parsimony (MacClade), are provided in Table 3. Table 4 shows the stochastic mapping transformation counts broken down into frequencies of transformations between the four states. No significant node-density artefact was detected. Reversible-jump MCMC in BayesTraits visited 124,532 different models under the preferred coding of states and 170,655 under the alternative coding. In both cases, the median number of model parameters was two, the 95% equal-tail credible interval ranging from one to three. Twice the difference in harmonic mean likelihood between reversible-jump MCMC and the single-rate model is approximately 5.

DISCUSSION

Ancestral State Reconstruction

Ancestral state reconstructions using maximum parsimony, maximum likelihood, or Bayesian approaches to map morphological or ecological traits on molecular phylogenies are becoming increasingly popular and frequent in the literature. They all rest on two assumptions, the first of which is rate constancy; i.e., that the expected rate of character change in the character being reconstructed is constant throughout the tree. Rate constancy is probably violated in most real cases (Schluter et al., 1997; Cunningham et al., 1998). Morphological evolution in the fossil record often displays prolonged stasis interrupted by bursts of change (see Eldredge et al., 2005, for a review as well as an attempt to clarify underlying mechanism, and Gavrilets, 2004:185–191, for models of parapatric speciation in support of these observations). However, rate constancy may still be a reasonable approximation (Schluter et al., 1997), although this remains to be tested. The second assumption, which is related to rate constancy, is that branch lengths carry information on the probability of phenotypic change; i.e., that states at each end of a long branch are less likely to be identical than states at each end of a short branch. For branch length information obtained from genetic data to be relevant to ancestral state reconstructions of morphological or ecological traits, one has to assume that a correlation exists between the rate of genetic change and the rate of morphological/ecological change. Such a correlation was reported by Omland (1997), but Cunningham (1999) cautioned that this may not always be the case; e.g., during adaptive radiations. Bromham et al. (2002) and Davies and Savolainen (2006) went further, arguing that the rate of phenotypic change (assumed to be under directional selection, at least periodically, is either independent or only partially explained by the rate of neutral genetic change. However, these investigations are associated with major methodological issues such as small sample size, influence of priors on branch lengths, branch-length estimations from single genes, use of average branch lengths as a measure of genetic change (rather than the full uncertainty in the MCMC sample), use of oversimplified likelihood models, and inadequate convergence monitoring. These sources of error may erode any chance of recovering a correlation between genetic and morphological/ecological change even if it exists. As pointed out by Bromham et al. (2002), a correlation between morphological/ecological and molecular rates of change is only expected if a process has affected the entire genome; e.g., generally increased rates of mutation or effects of population size dynamics. However, genome-wide mutation rates have indeed been suggested to be affected by small population size (Kliman et al., 2000; Woolfit and Bromham, 2003), environmental energy (Davies et al., 2004), generation time (Martin and Palumbi, 1993; Mooers and Harvey, 1994), DNA replication accuracy (Hebert et al., 2002; Xu et al., 2006), "metabolic rate" (Martin and Palumbi, 1993), and indirect effects of lifestyle transitions (Lutzoni and Pagel, 1997). The apparent lack of a universal molecular clock (e.g., Thomas et al., 2006), even between closely related taxa, is also an indication that genome-wide processes affecting mutation rate may be ubiquitous. Consequently, branch lengths may well contribute important information to ancestral state reconstruction, although the quality and quantity of bias in ancestral state reconstructions of morphological characters on trees obtained from molecular data remains to be established. We therefore conducted additional ancestral state reconstruction using parsimony, which does not make use of branch lengths.

We performed ancestral state reconstruction (Fig. 3, Table 2) of the eight nodes with 95% or better support that unite two or more families, as circumscribed in Fig. 2. In general, maximum parsimony as well as maximum likelihood reconstructions with a zero likelihood decision threshold, followed by the SIMMAP approach, tend to provide rather "certain" answers in the sense that much of the posterior probability is focused on a single state. Maximum likelihood reconstructions with the decision threshold set to 2 ln likelihood units are much less certain, indicating that in many cases there are several almost equally good state assignments. BayesTraits, on the other hand, tends to disperse much of the posterior

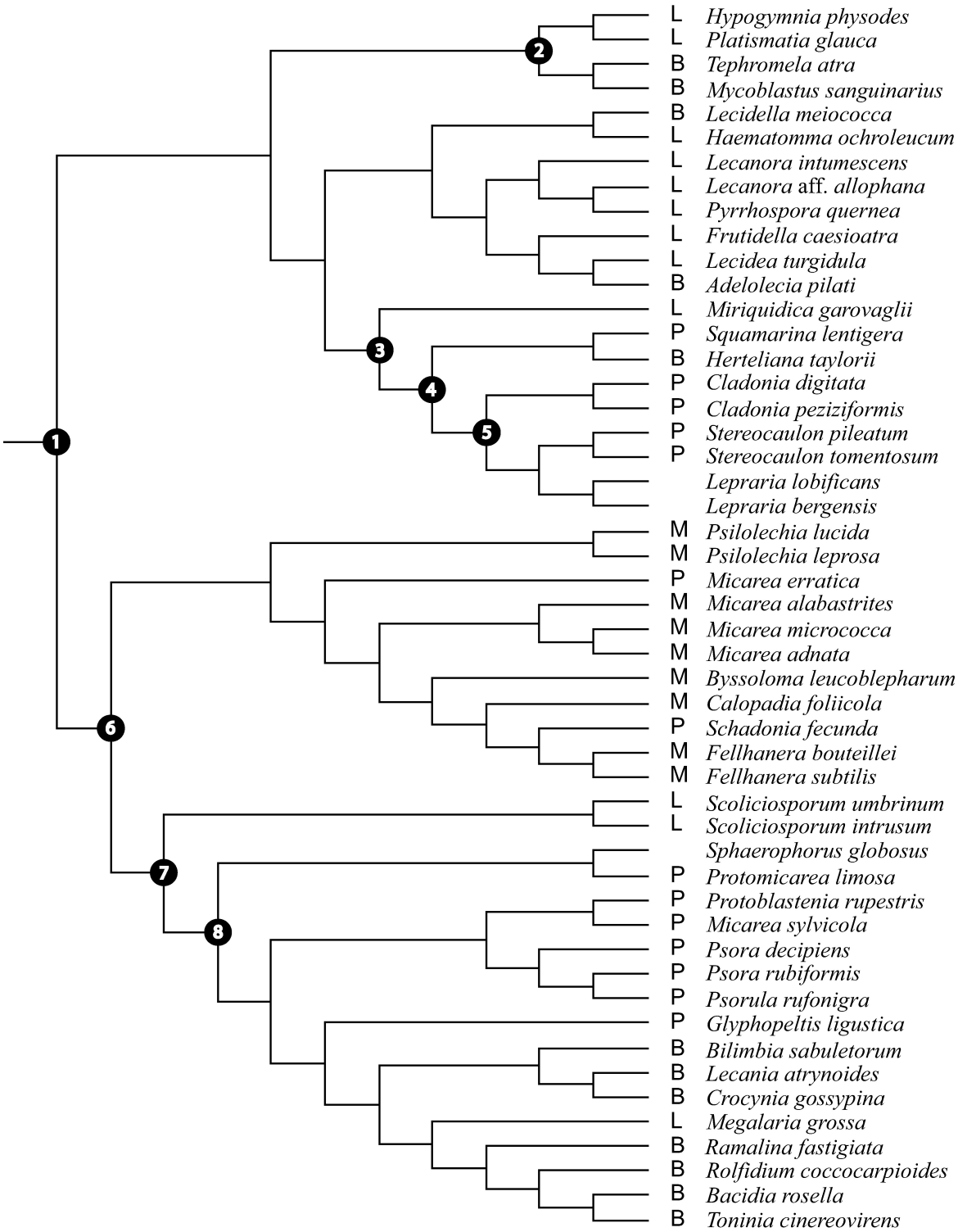

FIGURE 3. Nodes in the Lecanorales for which ancestral states were reconstructed. Eight nodes were reconstructed, viz. the ones uniting two or more families and having a posterior probability of at least 95%. Character states: L = *Lecanora*-type ascus; M = *Micarea*-type ascus; P = *Psora*-type ascus; B = *Bacidia*-type ascus. A few character states are unknown, because fruiting bodies (*Lepraria*) or mature asci (*Sphaerophorus*) are not produced. Alternatively, the character states of *Bilimbia* and *Crocynia* can be coded as M.

TABLE 2. Posterior probabilities of ancestral states in the ascus of Lecanorales, reconstructed using maximum parsimony (MP), maximum likelihood (ML) on a Bayesian posterior tree sample, as well as two fully Bayesian methods: SIMMAP (Bollback, 2006) and BayesTraits (BT; Pagel et al., 2004; Pagel and Meade, 2006). For reconstructed nodes, see Fig. 3. ML reconstructions were performed under a single-rate model with the likelihood decision threshold (LDT) set to 0 and 2.0, respectively. ML probabilites with LDT = 2 as well as MP probabilities do not necessarily add to 1 because only unequivocal reconstructions were counted. SIMMAP analyses were performed under a single-rate model using two different priors on tree length, one fixed at tree length = 3 and one gamma distributed that approximates the distribution obtained when counting change using parsimony on the MCMC tree sample. BayesTraits analyses were performed under a single-rate model (1-rate) as well as under reversible-jump Markov chain Monte Carlo (rj), where models are visited in proportion to their posterior probabilities. Gamma-distributed priors were used, either seeded by shape parameter hyperpriors (hp) or with fixed shape parameters chosen to mimic the gamma prior in SIMMAP as closely as possible (fixed gamma). Marginal probabilities are reported, obtained by integrating over trees and priors. All probabilities are conditional on the node existing. The corrected probability of the node adopting a state and the node existing (Pagel et al., 2004) can be approximated by multiplying with the posterior probability of the node (given behind the node number).

			ML		SIMMAP		BT		
Node (PP)	State	MP	LDT = 0	LDT = 2	Fixed	Gamma	rj hp	1-Rate hp	1-Rate fixed gamma
(A) *Bilimbia* and *Crocynia* coded as *Bacidia*-type ascus (preferred coding)									
1 (1.000)	*Lecanora*	0.887	1.000	0.846	0.954	0.906	0.905	0.885	0.839
	Micarea	0.000	0.000	0.000	0.029	0.049	0.047	0.052	0.049
	Psora	0.000	0.000	0.000	0.016	0.038	0.032	0.048	0.044
	Bacidia	0.000	0.000	0.000	0.001	0.006	0.016	0.015	0.013
2 (0.992)	*Lecanora*	0.999	1.000	0.994	0.958	0.906	0.516	0.304	0.301
	Micarea	0.000	0.000	0.000	0.000	0.001	0.005	0.021	0.018
	Psora	0.000	0.000	0.000	0.000	0.001	0.021	0.021	0.018
	Bacidia	0.000	0.000	0.000	0.041	0.092	0.457	0.654	0.662
3 (0.998)	*Lecanora*	1.000	1.000	0.853	0.931	0.811	0.483	0.188	0.187
	Micarea	0.000	0.000	0.000	0.000	0.001	0.020	0.024	0.022
	Psora	0.000	0.000	0.000	0.069	0.186	0.419	0.736	0.745
	Bacidia	0.000	0.000	0.000	0.000	0.002	0.079	0.052	0.046
4 (1.000)	*Lecanora*	0.000	0.000	0.000	0.000	0.000	0.073	0.005	0.004
	Micarea	0.000	0.000	0.000	0.000	0.000	0.009	0.005	0.004
	Psora	1.000	1.000	0.580	1.000	0.998	0.836	0.947	0.955
	Bacidia	0.000	0.000	0.000	0.000	0.001	0.082	0.042	0.037
5 (0.990)	*Lecanora*	0.000	0.000	0.000	0.000	0.000	0.027	0.010	0.008
	Micarea	0.000	0.000	0.000	0.000	0.000	0.020	0.010	0.008
	Psora	1.000	1.000	1.000	1.000	1.000	0.939	0.969	0.975
	Bacidia	0.000	0.000	0.000	0.000	0.000	0.014	0.010	0.008
6 (0.996)	*Lecanora*	0.875	0.968	0.241	0.775	0.657	0.367	0.063	0.062
	Micarea	0.000	0.031	0.000	0.153	0.223	0.507	0.615	0.618
	Psora	0.000	0.001	0.000	0.072	0.117	0.109	0.306	0.307
	Bacidia	0.000	0.000	0.000	0.000	0.003	0.017	0.016	0.014
7 (0.991)	*Lecanora*	0.876	0.967	0.107	0.049	0.064	0.565	0.254	0.253
	Micarea	0.000	0.000	0.000	0.008	0.030	0.016	0.022	0.020
	Psora	0.001	0.033	0.000	0.943	0.905	0.350	0.678	0.687
	Bacidia	0.000	0.000	0.000	0.000	0.002	0.068	0.046	0.041
8 (0.993)	*Lecanora*	0.000	0.000	0.000	0.000	0.001	0.106	0.006	0.005
	Micarea	0.000	0.000	0.000	0.000	0.000	0.010	0.006	0.004
	Psora	0.994	1.000	0.672	1.000	0.999	0.811	0.947	0.956
	Bacidia	0.000	0.000	0.000	0.000	0.001	0.073	0.041	0.035
(B) *Bilimbia* and *Crocynia* coded as *Micarea*-type ascus (alternative coding)									
1 (1.000)	*Lecanora*	0.887	1.000	0.787	0.954	0.896	0.860	0.853	0.864
	Micarea	0.000	0.000	0.000	0.030	0.054	0.033	0.063	0.060
	Psora	0.000	0.000	0.000	0.016	0.042	0.044	0.060	0.056
	Bacidia	0.000	0.000	0.000	0.001	0.008	0.063	0.024	0.020
2 (0.992)	*Lecanora*	0.999	1.000	0.979	0.958	0.897	0.584	0.315	0.312
	Micarea	0.000	0.000	0.000	0.000	0.001	0.014	0.030	0.027
	Psora	0.000	0.000	0.000	0.000	0.002	0.024	0.030	0.027
	Bacidia	0.000	0.000	0.000	0.041	0.100	0.378	0.624	0.635
3 (0.998)	*Lecanora*	1.000	1.000	0.670	0.931	0.792	0.486	0.193	0.191
	Micarea	0.000	0.000	0.000	0.000	0.001	0.026	0.035	0.031
	Psora	0.000	0.000	0.000	0.069	0.204	0.363	0.697	0.712
	Bacidia	0.000	0.000	0.000	0.000	0.003	0.124	0.074	0.066
4 (1.000)	*Lecanora*	0.000	0.000	0.000	0.000	0.001	0.077	0.011	0.009
	Micarea	0.000	0.000	0.000	0.000	0.000	0.020	0.011	0.009
	Psora	1.000	1.000	0.224	1.000	0.998	0.747	0.912	0.926
	Bacidia	0.000	0.000	0.000	0.000	0.002	0.157	0.065	0.056
5 (0.990)	*Lecanora*	0.000	0.000	0.000	0.000	0.000	0.033	0.020	0.016
	Micarea	0.000	0.000	0.000	0.000	0.000	0.024	0.020	0.016
	Psora	1.000	1.000	0.963	1.000	1.000	0.914	0.941	0.952
	Bacidia	0.000	0.000	0.000	0.000	0.000	0.029	0.020	0.016
6 (0.996)	*Lecanora*	0.875	0.967	0.123	0.775	0.638	0.357	0.069	0.066
	Micarea	0.000	0.032	0.000	0.154	0.237	0.418	0.614	0.620
	Psora	0.000	0.001	0.000	0.071	0.121	0.146	0.296	0.296
	Bacidia	0.000	0.000	0.000	0.000	0.004	0.079	0.020	0.017
7 (0.991)	*Lecanora*	0.876	0.966	0.038	0.049	0.068	0.571	0.262	0.260
	Micarea	0.000	0.001	0.000	0.008	0.038	0.030	0.037	0.032
	Psora	0.001	0.033	0.000	0.943	0.891	0.310	0.650	0.662
	Bacidia	0.000	0.000	0.000	0.000	0.002	0.089	0.051	0.046
8 (0.993)	*Lecanora*	0.000	0.000	0.000	0.000	0.001	0.170	0.013	0.010
	Micarea	0.000	0.000	0.000	0.000	0.000	0.034	0.020	0.016
	Psora	0.962	1.000	0.390	1.000	0.998	0.709	0.926	0.938
	Bacidia	0.000	0.000	0.000	0.000	0.001	0.087	0.040	0.036

TABLE 3. State transformation counts using stochastic mapping on a Bayesian posterior tree sample. Values provided are medians with the 95% equal-tail credible intervals within brackets. Analyses were performed using SIMMAP with a single-rate model and two different priors on rate (tree length), one fixed at tree length = 3 and one gamma-distributed that approximates the distribution obtained when counting change using parsimony on the posterior tree sample. Calculations were performed under the preferred coding of character states (*Bilimbia* and *Crocynia* coded as *Bacidia*-type ascus) as well as under an alternative coding (*Bilimbia* and *Crocynia* coded as *Micarea*-type ascus). For comparison, transformation counts under parsimony are provided.

	SIMMAP		
	Fixed	Gamma	Parsimony
Preferred coding	14 (12–16)	16 (13–21)	11 (11–12)
Alternative coding	16 (14–18)	19 (15–24)	13 (12–14)

probability over more than one state, particularly when reversible-jump MCMC is used. Although reversible-jump MCMC identified models with just two parameters as the most probable ones, differences between ancestral state posterior probabilities under reversible-jump MCMC and a constrained single-parameter model are sometimes striking, as in the case of nodes 2, 3, and 7. This implies that choice of model can have serious impact on the reconstruction of ancestral states. Indeed, a Bayes factor of 5 corresponds to "positive" support for a more complex model (Kass and Raftery, 1995) and indicates that more than one rate is on average better than a single rate at describing the observed character states, given the uncertainty in tree topology and branch lengths. Mooers and Schluter (1999), on the other hand, discouraged the use of more than a single transformation rate in binary characters across trees with less than ca. 100 terminal taxa. However, Leschen and Buckley (2007) observed one seemingly unrealistic result using stochastic mapping on a 53-taxon phylogeny that they ascribed to poor fit of the underlying single-rate model. Ancestral state reconstructions by SIMMAP seem rather insensitive to priors, although (as expected) uncertainty is slightly greater for the possibly more realistic gamma-distributed prior compared to a rather extreme fixed prior. In BayesTraits, seeding a gamma-distributed rate prior with hyperpriors on the shape parameters versus using a fixed shape on the gamma distribution has, in our case, very limited effect on the state reconstructions under a single-rate model. However, the most disquieting observation in the entire investigation is that methods sometimes ascribe high confidence to different ancestral character states (Table 2). Maximum parsimony and maximum likelihood generally agree very well. SIMMAP puts the highest confidence in the same character state as maximum parsimony and maximum likelihood in seven out of eight nodes. BayesTraits results seem to depend more on the model and the priors. Under reversible-jump MCMC, the highest confidence is ascribed to the same states as maximum parsimony and maximum likelihood in seven out of eight nodes, whereas single-rate reconstructions only agree with maximum parsimony and maximum likelihood in four out of eight nodes. The most conspicuous result is that the two fully Bayesian methods disagree partially when they use similar single-rate models and similar gamma-distributed priors on rate. In fact, SIMMAP and reversible-jump MCMC by BayesTraits disagree about the most probable character state in two out of eight nodes (6, 7), and distribute state probabilities very differently in another two nodes (2, 3). SIMMAP and the single-rate model in BayesTraits disagree about the most probable character state in three out of eight nodes (2, 3, 6), whereas the degree of confidence in the most probable state is clearly different in

TABLE 4. Posterior probabilities of transformation frequencies between four character states in the ascus of the Lecanorales. Calculations were made using stochastic mapping on a Bayesian posterior tree sample. Reported values are medians and 95% equal-tail credible intervals. For each transition between states, four medians and credible intervals are provided, obtained using (A) the preferred coding of character states and a fixed prior on tree length (three steps); (B) the preferred coding of characer states and a gamma-distributed prior on tree length that approximates the distribution obtained when counting change using parsimony on the MCMC tree sample; (C) the alternative coding of character states and a fixed prior on tree length; and (D) the alternative coding of character states and a gamma-distributed prior on tree length.

		To:			
From:		*Lecanora*	*Micarea*	*Psora*	*Bacidia*
Lecanora	A	—	0.07 (0.00, 0.13)	0.08 (0.06, 0.18)	0.21 (0.13, 0.29)
	B	—	0.06 (0.00, 0.13)	0.11 (0.05, 0.20)	0.19 (0.11, 0.27)
	C	—	0.06 (0.00, 0.13)	0.07 (0.06, 0.16)	0.19 (0.13, 0.25)
	D	—	0.05 (0.00, 0.12)	0.10 (0.04, 0.18)	0.17 (0.09, 0.24)
Micarea	A	0.00 (0.00, 0.08)	—	0.15 (0.07, 0.23)	0.00 (0.00, 0.07)
	B	0.00 (0.00, 0.13)	—	0.14 (0.05, 0.24)	0.00 (0.00, 0.11)
	C	0.00 (0.00, 0.13)	—	0.13 (0.06, 0.21)	0.06 (0.00, 0.13)
	D	0.05 (0.00, 0.14)	—	0.13 (0.05, 0.22)	0.06 (0.00, 0.17)
Psora	A	0.08 (0.00, 0.21)	0.07 (0.00, 0.20)	—	0.15 (0.13, 0.25)
	B	0.11 (0.00, 0.22)	0.06 (0.00, 0.19)	—	0.15 (0.07, 0.25)
	C	0.07 (0.00, 0.19)	0.06 (0.00, 0.19)	—	0.13 (0.06, 0.21)
	D	0.10 (0.00, 0.20)	0.06 (0.00, 0.18)	—	0.13 (0.05, 0.22)
Bacidia	A	0.07 (0.00, 0.14)	0.00 (0.00, 0.07)	0.00 (0.00, 0.08)	—
	B	0.07 (0.00, 0.19)	0.00 (0.00, 0.10)	0.06 (0.00, 0.16)	—
	C	0.06 (0.00, 0.13)	0.06 (0.00, 0.13)	0.00 (0.00, 0.07)	—
	D	0.06 (0.00, 0.17)	0.06 (0.00, 0.15)	0.05 (0.00, 0.14)	—

a fourth node (7). Obviously, ancestral state reconstructions need to be conducted with more than one method while awaiting clarification of the statistical properties of each method. We do not know exactly why methods provide such diverging answers, but the reason does not seem to be found in model use or specification of priors. There are some minor differences between the methods: BayesTraits estimates the joint posterior distribution of rate parameters across trees under a variety of models that can be defined by the user. "Rate," in this context, multiplied by expected tree length equals the expected number of state changes across the tree. Probabilities of states at each node are sampled from the posterior using MCMC. SIMMAP, on the other hand, places a prior on tree length and analytically calculates the marginal distribution of states at each node, this distribution equaling what one would obtain by recording the states at nodes using an infinite number of stochastic mappings on a tree sample.

Given the disagreement between methods discussed above, our ancestral state reconstructions are associated with considerable uncertainty. However, it appears likely that the ancestral ascus in the Lecanorales was of *Lecanora*-type (Table 2, node 1). Indeed, there is no strong evidence to contradict the supposition that all observations of the *Lecanora*-type ascus, except in *Megalaria*, are plesiomorphic. However, uncertainties at nodes 2, 3, 6, and 7 (Table 2) indicate that one cannot entirely ignore the possibility that the *Lecanora*-type ascus was gained independently on several occasions. Early during the evolution of the order, the *Lecanora*-type ascus seems to have transformed, directly or via other states, to *Psora*-type on at least two independent instances (Table 2, nodes 3 to 5 and 6 to 8). The *Bacidia*-type ascus seems in at least most cases to be a rather recent invention that was gained more than once.

State Transformation Counts

Estimates of the total number of transformations (Table 3) show that, as expected, stochastic mapping puts more probability to higher numbers of transformations (Huelsenbeck et al., 2003) than parsimony. Where parsimony puts its confidence in the interval 11 to 14, stochastic mapping suggests that the number of state changes is likely to be 12 to 24. Stochastic mapping on our tree sample appears to be somewhat sensitive to the prior distribution of tree lengths. If one assumes a "minimum change prior," i.e., no more change than is necessary to explain the number of character states observed (and therefore uses a fixed prior with three state changes), there is a 95% probability that the number of state changes is 12 to 16 or 14 to 18, depending on the character state coding. This prior is strong and probably unrealistic. If we instead let our prior beliefs in the stochastic mapping be guided by the distribution of parsimony tree lengths in the posterior tree sample (using the gamma distribution prescribed by SIMMAP), then there is a 95% probability that the number of state changes is 13 to 21 or 15 to 24, depending on the character state coding. When priors are generated from the data to be analyzed, as was the case for the "parsimony prior," the analysis is often referred to as "empirical Bayes" (Carlin and Louis, 2000; Robert, 2001).

In addition to counting the total number of character state transformations, we also mapped the frequency of the 12 individual transformations (Table 4). We used here the frequency rather than the absolute number of transformations in order to make estimates independent of total tree length. First of all, frequency estimates are largely concordant and do not seem to depend much on the particular coding scheme or prior distributions of tree lengths. Furthermore, only in four types of transformations do the lower bounds of the credible interval not include zero. In other words, these are the only four transformations that are likely to have occurred with a reasonable degree of certainty, given that the model and priors are adequate. By far most frequent is the transformation from the *Lecanora*-type to the *Bacidia*-type of ascus, implying that closure of the upper part of the axial body is a relatively small evolutionary step. Transformations from the *Psora*-type to the *Bacidia*-type and from the *Micarea*-type to the *Psora*-type are also relatively frequent, followed by the less common transformation from the *Lecanora*-type to the *Psora*-type of ascus.

Transformation counts using the "parsimony prior" are likely to be significantly more realistic than a fixed "minimum change prior." Irrespective of the specific prior or the character state coding, posterior probability distributions indicate that the number of state transformations is considerably greater than the minimum three needed to explain the observed number of states (one plesiomorphic state and three apomorphic). Apparently, the ascus in the Lecanorales is, from an evolutionary point of view, far more flexible and apt to change than has been previously thought. Yet, we may have underestimated the true number of transformations. Our phylogeny incorporates only a fraction of the species referred to the Lecanorales. A denser sampling would probably have revealed further state changes in the terminal parts of the phylogeny. Clearly, a critical reevaluation of how ascus characters are being used in lichen systematics is badly needed.

Phylogeny and Classification

Our phylogenetic estimates are largely concordant with morphology, although at odds with current classification in some cases (compare Table 1 and the classification suggested in Fig. 2). We chose here an outgroup consisting of three members of Lecideaceae, belonging to the Lecideales in the Lecanoromycetidae (Miadlikowska et al., 2006). The status of the branch uniting *Helocarpon*, *Lecidea atrosanguinea*, *Lopadium*, *Halecania*, and *Catinaria* is unclear. These taxa are most likely distantly related and may or may not have affiliations with the Lecanorales. We view the position of these taxa in the tree with suspicion, as they constitute two out of three cases in our study for which sequence data was only obtained from a single gene. A reasonable working delimitation of the Lecanorales is the group of taxa united by the most basal ingroup branch receiving 100% posterior

probability (Fig. 2). A revised taxonomic classification of this order into families being supported (or at least not contradicted) by our phylogenetic estimate is indicated in Fig. 2. All deviations between our suggested classification and the currently most widely used (Eriksson, 2006) represent cases where taxa have been assigned to their current place in the system according to their ascus type. Some families are homogeneous, some heterogeneous with regard to ascus type. Above the level of family, ascus type appears to have very limited predictive power on relationships. This is not unexpected, however frustrating it may be to lichen systematists who seek morphological and chemical characters that can be used to circumscribe higher taxonomical entities. Historically, character sets used by lichen taxonomists for delimiting families and higher taxa in Lecanorales, e.g., growth habit, ascospore septation, and the formation of the apothecial margin, have later proven to have limited predictive value, either in light of molecular phylogenies or additional morphological data. Apparently, translating molecular phylogenies into higher-level classifications that can be explained by morphology and chemistry is an increasingly difficult task. One path that may turn out to be fruitful is to direct future morphological studies towards ascomatal ontogeny, the study of which is still in its infancy (Döring and Lumbsch, 1998; Döring and Wedin, 2000; Lumbsch et al., 2001). Most importantly, however, is not to a priori attribute too much weight to one set of morphological characters in classification but to assess the predictive value of character states a posteriori in the light of phylogenetic analyses as well as multivariate variation patterns incorporating a variety of morphological, anatomical, and chemical characters.

ACKNOWLEDGMENTS

We thank Maria Ladstein and Carin Olofsson for laboratory assistance and Beate Helle Ingvartsen for the drawings in Fig. 1. Ove E. Eriksson provided valuable feedback and discussions on ascus characters and their use in classification. Jonathan Bollback kindly provided information on the statistical properties of SIMMAP and the interpretation of results. The Research Council of Norway (project no. 128388/420) and the Swedish Research Council (project nos. NFR B5101-20005187, VR 629-2001-5756, VR 621-2002-349, and VR 621-2003-303) are gratefully acknowledged for financial support that made this investigation possible. We would like to thank Thomas Buckley and Timothy James for constructive criticism that helped to improve this manuscript.

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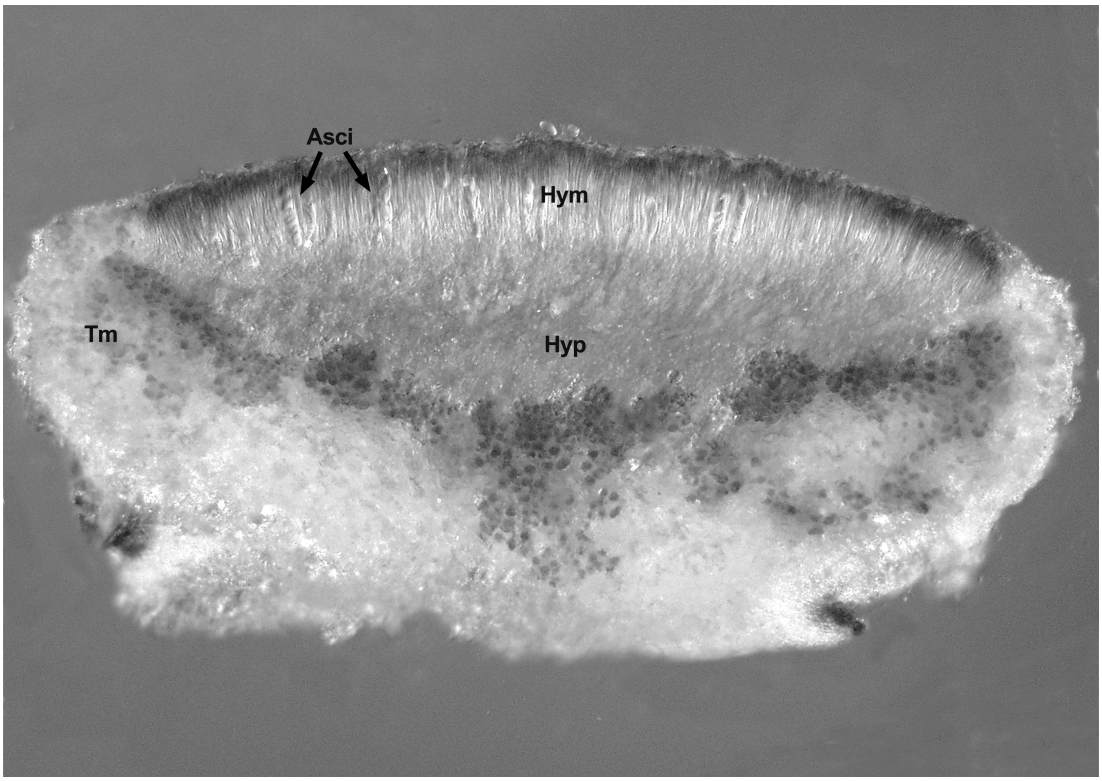
First submitted 28 June 2007; reviews returned 10 September 2007; final acceptance 30 November 2007
Associate Editor: Thomas Buckley



Section through a fruiting-body (diameter c. 1 mm) of a species of *Lecanora*, the genus of lichen fungi that lent its name to the family Lecanoraceae and the order Lecanorales. The hymenium ("Hym") is pigmented above and consists of asci ("Asci") and supporting filaments. Each ascus contains eight one-celled ascospores and an apex structure that has been used extensively in Lecanorales taxonomy. Below the hymenium is the hypothecium ("Hyp"). The hymenium and hypothecium are surrounded by a thalline margin ("Tm") that contains cells of the green alga *Trebouxia* (the dark, ellipsoid structures). Photo by Ulf Arup, Lund University.