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Outline of phylogeny and character evolution in *Rhizocarpon* (Rhizocarpaceae, lichenized Ascomycota) based on nuclear ITS and mitochondrial SSU ribosomal DNA sequences

PER G. IHLEN* and STEFAN EKMAN

Department of Botany, University of Bergen, Allégaten 41, N-5007 Bergen, Norway

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The phylogeny of the lichen genus *Rhizocarpon* (Rhizocarpaceae, lichenized Ascomycota) was investigated using nucleotide sequences from the ITS region of the nuclear ribosomal DNA and the SSU region of the mitochondrial ribosomal DNA from 13 species of *Rhizocarpon*, *Catolechia wahlenbergii* and *Poeltinula cerebrina*. Phylogenetic estimations were performed using maximum parsimony and Bayesian MCMC tree sampling. Twelve phylogenetic null hypotheses were tested using MCMC tree sampling. The evolution of five morphological characters was assessed by mapping them onto MCMC tree samples. The results indicate that *Rhizocarpon* in its current sense is polyphyletic and can only be made monophyletic if *R. hochstetteri* is excluded or *Poeltinula*, and possibly also *Catolechia*, are included. The root placement in the Rhizocarpaceae is ambiguous, either *Catolechia* or *Poeltinula* + *R. hochstetteri* being the sistergroup to the rest of the family. Previously suggested infrageneric arrangements based on presence or absence of the yellow substance rhizocarpic acid in the thallus or the septation of the ascospores are unnatural. Some species with grey or brown thallus may have evolved from a yellow ancestor. Spore septation and colour, amyloidity of the thalline medulla, and the presence of stictic acid complex and rhizocarpic acid are shown to have changed multiple times during the course of evolution. *Biological Journal of the Linnean Society*, 2002, **77**, 535–546.

ADDITIONAL KEYWORDS: Catolechia – lichen – MCMC – parsimony – Poeltinula.

INTRODUCTION

Rhizocarpon DC. is a large lichen genus containing approximately 200 species worldwide (Hawksworth *et al*., 1995). The most well-known representative of this genus is perhaps *R. geographicum* (L.) DC. (map lichen), which is widely used for dating rock surfaces. *Rhizocarpon* was, together with *Catolechia* Flot., *Epilichen* Clem. and *Poeltinula* Hafellner, placed in the Rhizocarpaceae by Hafellner (1984), a family which is mainly characterized by the lecideine exciple, asci with a distinct amyloid cap in the distal part of the tholus, and branched and anastomosed hamathecial filaments. Both *Rhizocarpon* and *Poeltinula* have a perispore, whereas this is absent in *Catolechia* and *Epilichen* (see, e.g. Hafellner, 1984). *Catolechia* has traditionally been referred to the Physciaceae (Zahlbruckner, 1931; as Buelliaceae; Henssen & Jahns, 1973).

The species of *Rhizocarpon* grow predominantly on siliceous rocks, whereas some grow on basic rocks and a few are parasites on other lichens (Purvis *et al*., 1992). They are mainly distributed in temperate, Arctic and Antarctic areas (Gelting, 1954; Runemark, 1956; Feuerer, 1991; Hawksworth *et al*., 1995; Fryday, 2000).

A variety of infrageneric arrangements in *Rhizocarpon* have been proposed. Fries (1874) assigned the species with one-septate ascospores to section *Catacarpon* and the species with four-celled to muriform ascospores to section *Eurhizocarpon*. A more recent and currently widely used classification was proposed by Thomson (1967), who included taxa containing the

*Corresponding author. E-mail: per.ihlen@bot.uib.no

Table 1. Species and specimens from which new nucleotide sequences were obtained. Herbarium acronyms follow Holmgren, Keuken & Schofield (1981)

Species	GenBank accession no.		Voucher information
	ITS	mtSSU	
Catolechia wahlenbergii (Ach.) Körb.	–	AF483175	1998, Engelstad (O)
Fuscidea intercincta (Nyl.) Poelt	AF483605	AF483172	Bjelland 59 (BG)
Poeltinula cerebrina (DC.) Hafellner	AF483606	AF483173	Mayrhofer *et al*. 12838 (GZU)
Rhizocarpon amphibium (Fr.) Th. Fr.	AF483611	AF483179	Muhr 11283 (BG)
Rhizocarpon copelandii (Körb.) Th. Fr.	AF483617	AF483185	Haugan H1530 (O)
Rhizocarpon distinctum Th. Fr.	AF483615	AF483183	Haugan H3703 (O)
Rhizocarpon geminatum Körb.	AF483614	AF483182	Haugan & Timdal 8055 (O)
Rhizocarpon geographicum (L) DC.	AF483619	AF483187	Ihlen 941 (BG)
Rhizocarpon hochstetteri (Körb.) Vain.	AF483607	AF483174	Haugan H1622 (O)
Rhizocarpon lavatum (Fr.) Hazsl.	AF483610	AF483178	Timdal 7586 (O)
Rhizocarpon norvegicum Räsänen	AF483618	AF483186	Timdal 9139 (O)
Rhizocarpon oederi (Weber) Körb.	AF483612	AF483180	Timdal 7540 (O)
Rhizocarpon petraeum (Wulfen) A. Massal.	AF483609	AF483177	Haugan H1387 (O)
Rhizocarpon polycarpum (Hepp) Th. Fr.	AF483616	AF483184	Haugan H1508 (O)
Rhizocarpon reductum (Ach.) A. Massal.	AF483608	AF483176	Ihlen 99 (BG)
Rhizocarpon suomiense Räsänen	AF483613	AF483181	Holtan-Hartwig & Timdal 4917 (O)

yellow substance rhizocarpic acid in subgenus *Rhizocarpon* and taxa lacking this substance in subgenus *Phaeothallus*. Within each subgenus, various taxonomic treatments have been presented (see review by Feuerer, 1991) and most of these emphasize secondary substances in the thallus, and coloration, septation and size of the ascospores as the most important characters to delimit species groups. Some groups of species are quite well understood, whereas others are in critical need of taxonomic revision. One such group is the non-yellow species with hyaline and muriform ascospores (Purvis *et al*., 1992; Fryday, 1996).

When the senior author started a taxonomical study of these species in the Nordic countries, based on anatomy, morphology and chemistry, it soon became apparent that an outline of the phylogeny of the genus and of the evolution of characters claimed to be important for the grouping of species was highly desirable. As these aims are difficult to reach with morphological, anatomical and chemical data alone, we believe a better understanding can be attained by the use of DNA sequence data. Owing to the large number of species in *Rhizocarpon*, we clearly want to point out that this contribution is meant as a pilot project.

MATERIAL AND METHODS

Sampling of taxa and specimens

Thirteen species of *Rhizocarpon*, representing major groups of species and the morphological diversity within the genus, were sampled for total DNA extraction. Furthermore, *Catolechia wahlenbergii* (Ach.) Körber and *Poeltinula cerebrina* (DC.) Hafellner were included, at first as potential outgroups. However, later doubts about the position of the root in the Rhizocarpaceae (and hence the suitability of *Catolechia* and *Poeltinula* as outgroups) led us to choose a taxonomically somewhat more distant outgroup, *Fuscidea intercincta* (Nyl.) Poelt. Voucher information is provided in Table 1. The selected specimens were collected between 1986 and 1999.

Selected nucleotide sequences

An initial study of the phylogeny, using only sequences from the ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA, indicated that many relationships remained ambiguous due to poor resolving power of the data. By adding part of the SSU region of the mitochondrial ribosomal DNA, sufficient resolution was obtained. Mitochondrial ribosomal DNA sequences have been widely used in phylogenetic studies on non-lichenized fungi, but only a few have so far been published on lichens (e.g. Crespo, Blanco, & Hawksworth, 2001; Schmitt *et al*., 2001).

DNA extraction, PCR amplification and sequencing

Three to five apothecia from each specimen were removed and roughly cleaned with a razor blade. Total DNA was extracted using the DNeasy Plant Mini Kit

(Qiagen). PCR amplification of the ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA was performed using the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al*., 1990), whereas the SSU region of mitochondrial ribosomal DNA was amplified using the primer pairs mrSSU1–mrSSU2R (Zoller, Scheidegger & Sperisen, 1999) and mrSSU2–MSU7 (Zoller *et al*., 1999; Zhou & Stanosz, 2001). In addition to the extracted DNA, the PCR mixture contained 2.5 mM $MgCl_2$, 200 μM of each of the four dNTPs, 0.7 μM of each primer, 1.5 U of a DNA polymerase, either AmpliTaq Gold (Applied Biosystems) or Herculase (Stratagene), and an Mg^{2+} free buffer in the concentration recommended by the manufacturer. The same PCR programme was used for the amplification of both genes: a 9-min (AmpliTaq Gold) or a 4-min hold (Herculase) at 94°C, six cycles including denaturation at 94°C for 60 s, annealing at 62°C (decreasing by 1°C for each cycle) for 60 s, and extension at 72°C for 105 s, followed by 34 cycles with denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 105 s plus an addition of 3 s per cycle, and finally a hold at 72°C for 10 min before the reaction was cooled to a constant 4°C. PCR products were electrophoresed in a 1% agarose gel, visualized using ethidium bromide and subsequently cleaned using the QiaQuick Spin kit or the QiaQuick Gel Extraction kit (Qiagen). For both genes, sequencing was performed with the amplification primers, using the Big Dye Terminator kit (Applied Biosystems) according to the manufacturer's protocol except that reactions were half-size. The final extension products were cleaned using an NaAc precipitation protocol and subjected to automatic sequencing on an ABI 377 with the XL upgrade or an ABI 3700 robot (Applied Biosystems). Sequences were assembled and edited using the software Sequencher 3.1.1 (Gene Codes Corp.). Pieces of nuclear SSU and LSU at either end of the ITS region were removed.

Sequence alignment

A preliminary alignment of the newly obtained sequences and two ITS sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov), *Rhizocarpon geographicum* (AF250805) and *Catolechia wahlenbergii* (AF250792), were constructed using the on-line version of the software DCA 1.0, Divide-and-Conquer Multiple Sequence Alignment (Stoye, 1998) (available on-line at http://bibiserv.techfak.uni-bielefeld.de/dca/). This alignment was manually optimized. When aligning the outgroup, *Fuscidea intercincta*, to the ingroup, a few regions in the outgroup proved to deviate extensively from the corresponding regions in the ingroup, despite alignment in these regions of the ingroup being unambiguous. To avoid potential problems with rooting against an effectively random sequence, and at the same time avoid excluding unambiguous alignment in the ingroup, the sequence of the outgroup in these regions was recoded as missing data. A similar recoding as missing data was performed in one very short region in *R. lavatum* (Fr.) Hazl. and one in *R. oederi* (Weber) Körber, where these taxa were difficult to align to the rest of the data set, the remaining alignment being unambiguous. Primer sequences at either end of the mitochondrial SSU were excluded from the phylogenetic analysis.

Ambiguous alignment, appearing in gap-rich regions of both the ITS and the mitochondrial SSU, was recoded according to the method described by Lutzoni *et al*. (2000), as implemented in the software INAASE 2.2b. The calculations were checked manually and corrected where necessary. This strategy preserves some of the information present in ambiguous alignment without violating positional homology. Complex, hypervariable regions, e.g. at the end of ITS1, were not recoded but simply excluded from further analysis. Furthermore, the outgroup was not part of the recoding process but recoded as missing data, again to avoid potential problems with random rooting. Matrices recoded with the INAASE procedure can only be analysed under parsimony. Consequently, in the Bayesian analysis, ambiguous alignment was excluded instead of recoded. Finally, the alignment was submitted to TreeBASE (http://www.herbaria.harvard.edu./treebase) where it is filed under matrix accession number M1142. The first 539 alignment positions in this matrix are ITS, the remainder mitochondrial SSU.

Phylogenetic analyses

Tree inference was carried out under the maximum parsimony optimality criterion and a likelihood-based Bayesian tree sampling procedure. The parsimony analysis was performed as implemented in the software PAUP* 4.0b8 (Swofford, 2001). Character state changes were weighted equally, except the INAASE recoded characters, which were weighted according to their step matrices. Gaps were treated as a fifth character state. A heuristic search with 1000 random-addition sequence replicates was performed using tree bisection-reconnection (TBR) branch-swapping, and with the MulTrees option on and the steepest descent and collapse zero-length branches options off. Branch lengths were assigned using ACCTRAN character state optimization. Branch support of the individual clades was estimated using a bootstrap analysis with 1000 replicates and using the previous search parameters, except that only 10 random-addition sequence replicates were performed in each bootstrap replicate.

To avoid inconsistencies due to a distant outgroup, the outgroup was excluded from the bootstrap analysis.

A likelihood ratio test as implemented in the software Modeltest 3.04 (Posada & Crandall, 1998) was performed to estimate the best-fitting likelihood model. The test favoured the TrN model of Tamura & Nei (1993), including substitution rate heterogeneity among nucleotide sites according to a gamma model (Yang, 1993) and a proportion of invariable sites (Gu, Fu & Li, 1995). Gamma distributed rates were modelled as discrete categories, each represented by its mean (Yang, 1994). It was found that the likelihood increased asymptotically with increasing number of gamma categories. Hence, the optimal number of gamma categories was set by (starting with two) increasing the number of categories in increments of one and treating each increase as a likelihood ratio test with one degree of freedom. In this way, the optimal number of gamma categories turned out to be five.

In order to estimate the posterior probability of phylogenetic trees, a Bayesian tree-sampling technique was used (reviewed by Lewis, 2001), as implemented in the software MrBayes 2.0 (Huelsenbeck & Ronquist, 2001). This technique employs a Markov chain Monte Carlo (MCMC) procedure for the sampling of trees in proportion to their probability of occurrence under the likelihood model in use. Likelihood settings included estimating a reversible rate matrix with six substitution types, rate heterogeneity across sites modelled according to a discrete gamma model with five categories and the shape parameter estimated, and base frequencies estimated. Bayesian priors were set to uniform distributions for branch lengths, the rate matrix, and the gamma shape parameter, and to a dirichlet for the base frequencies. The MCMC was allowed to run for 1 380 000 generations starting from a random tree, sampling every tenth tree. The first 30 000 generations were later discarded as the burn-in period. Four parallel chains, incrementally heated by a temperature of 0.2°C, were run. A majority-rule consensus tree with average branch lengths and probability of branches was computed from a sample of 50 000 trees.

Hypothesis testing and evolution of character states

Twelve phylogenetic null hypotheses were tested using part of the MCMC tree sample described above. The probability of the null hypothesis being correct equals the frequency of trees in an MCMC tree sample that are in agreement with the hypothesis under consideration (Huelsenbeck & Bollback, 2001; Lewis, 2001). Here, 5000 trees per null hypothesis were sampled. The frequency of the trees agreeing with the null hypothesis was calculated by applying constraint-based tree filters in PAUP*.

The evolution of five supposedly important morphological characters for grouping species in the Rhizocarpaceae, i.e. spore pigmentation, spore septation, amyloid reaction of the thalline medulla, presence of substances in the stictic acid complex and presence of rhizocarpic acid (Table 2), was reconstructed by mapping these characters onto trees in an MCMC sample. In this way, uncertainty in the phylogeny estimation will be taken into account in the character evolution inference (Huelsenbeck, Rannala & Masly, 2000). The optimization of character state change onto trees was performed under the maximum parsimony criterion, as implemented in the software MacClade 3.08 (Maddison & Maddison, 1992), although algorithms for performing this under the maximum likelihood criterion have been described (e.g. Pagel, 1994, 1999). Maximum parsimony, rather than maximum likelihood, was chosen here as the optimality criterion because it is computationally simpler, because software is readily available, and because the maximum likelihood and maximum parsimony estimates of amount of change on a tree are always identical given a single character (Penny & Hendy, 2001). An identical approach, using parsimony-based reconstruction of character evolution on MCMC tree samples, was utilized by Huelsenbeck *et al.* (2000). However, under parsimony the actual placement of a character state change on a branch (and hence its direction, i.e. gain or loss) is often ambiguous, whereas the total number of changes is not. For each morphological character, the total number of changes (irrespective of direction) was counted on 5000 trees from an MCMC sample. Subsequently, the direction of the changes was taken into consideration by counting the number of gains and losses in the 5000 trees under the two extremes of character-state optimization on a tree, accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN). ACCTRAN will favour few gains deep in the tree followed by later losses (reversals). DELTRAN, on the other hand, will favour independent gains (parallelisms) high up in the tree, thereby minimizing the number of losses. The probability of a certain number of state changes or a certain number of gains and losses corresponds to the frequency in the tree sample of trees with that number of reconstructed state changes/gains/losses.

RESULTS

ITS sequences were obtained from all 15 species for which PCR amplification had been attempted. Similarly, the first part of the mitochondrial SSU (between primers mrSSU1 and mrSSU2R) was successfully amplified and sequenced in 16 species (the same spe-

Table 2. Summary of morphological and chemical characters of *Catolechia wahlenbergii*, *Poeltinula cerebrina* and *Rhizocarpon* species mapped onto sequence-based MCMC tree samples

	Character				
Species	Ascospore colour	Ascospore septation	Amyloid medulla	Stictic acid complex	Rhizocarpic acid
Catolechia wahlenbergii	Brown	1-septate	No	Absent	Present
Poeltinula cerebrina	Hyaline	1-septate	No	Absent	Absent
Rhizocarpon amphibium	Hyaline	Eumuriform	No	Absent	Absent
Rhizocarpon copelandii	Brown	1-septate	No	Present	Absent
Rhizocarpon distinctum	Hyaline	Submuriform	Yes	Present	Absent
Rhizocarpon geminatum	Brown	Eumuriform	No	Present	Absent
Rhizocarpon geographicum	Brown	Eumuriform	Yes	Absent	Present
Rhizocarpon hochstetteri	Hyaline	1-septate	No	Present	Absent
Rhizocarpon lavatum	Hyaline	Eumuriform	No	Absent	Absent
Rhizocarpon norvegicum	Brown	1-septate	Yes	Present	Present
Rhizocarpon oederi	Hyaline	3-septate	Yes	Absent	Absent
Rhizocarpon petraeum	Hyaline	Eumuriform	No	Present	Absent
Rhizocarpon polycarpum	Hyaline	1-septate	Yes	Present	Absent
Rhizocarpon reductum	Hyaline	Eumuriform	No	Present	Absent
Rhizocarpon suomiense	Hyaline	Eumuriform	No	Present	Absent

cies in which ITS had been sequenced, plus *Catolechia wahlenbergii*). However, the second part of the mitochondrial SSU, between primers mrSSU2 and MSU7, was more difficult to amplify, and sequences were obtained from only 10 species. Although incomplete, this information was used in the phylogenetic analyses.

The final combined matrix of the 17 sequences contained 1267 unambiguously aligned sites and 11 characters recoded with INAASE (the latter excluded in the Bayesian tree inference). In total, 357 characters were variable and 192 parsimony-informative.

The tree-search under parsimony, based on the combined ITS–mtSSU matrix, yielded a single most parsimonious tree 944 steps in length, and with consistency index (CI) = 0.57, retention index (RI) = 0.43, and rescaled consistency index (RC) = 0.24 (indices calculated with uninformative characters excluded). This tree, with bootstrap branch support, is shown in Figure 1(A). Tree-searches were also performed on the ITS and mtSSU matrices separately. Taken separately, however, the matrices have poor resolving power and the degree of conflict is difficult to assess (results not shown).

A majority-rule consensus tree (with all compatible groups), with average branch lengths and probabilities of branches, resulting from the Bayesian MCMC tree sampling procedure, is shown in Figure 1(B). The likelihood parameters had the following average values (± one standard deviation) in the tree sample: base frequencies $\pi_A = 0.303 \pm 0.012$, $\pi_C = 0.201 \pm 0.010$, $\pi_G = 0.222 \pm 0.011$, $\pi_T = 0.273 \pm 0.011$, rate matrix $r_{AC} = 1.978 \pm 0.680$, $r_{AG} = 5.300 \pm 1.580$, $r_{AT} = 1.978 \pm 0.647$, $r_{CG} = 3.670 \pm 1.267$, $r_{CT} = 12.256 \pm 3.851$, $r_{GT} = 1.0 \pm 0$, gamma shape parameter $\alpha = 0.448 \pm 0.114$, and the proportion of invariable sites $p_{invar} = 0.372 \pm 0.084$.

Probabilities of 12 phylogenetic null hypotheses being correct are presented in Table 3. The probability distribution of the total number of total state changes and the number of gains and losses (under ACCTRAN and DELTRAN, respectively) for the five selected morphological characters (spore colour, spore septation, amyloid reaction, presence of stictic acid complex and presence of rhizocarpic acid), based on the mapping of these characters on MCMC samples of 5000 trees per character, are shown in Tables 4 and 5, respectively.

DISCUSSION

The trees inferred under maximum parsimony and the Bayesian tree-sampling procedure differ, to some extent, in overall topology and branch support (Fig. 1A,B). Under parsimony, the outgroup branch is fairly short and the root of the Rhizocarpaceae placed along the branch leading to *Poeltinula cerebrina* and *Rhizocarpon hochstetteri* (Körb.) Vain., whereas in the Bayesian inference, the outgroup branch is long and the ingroup rooted along the branch leading to *Catolechia wahlenbergii*. Despite attempts to keep the outgroup branch short, e.g. by only rooting the ingroup against the conserved parts of the *Fuscidea* outgroup

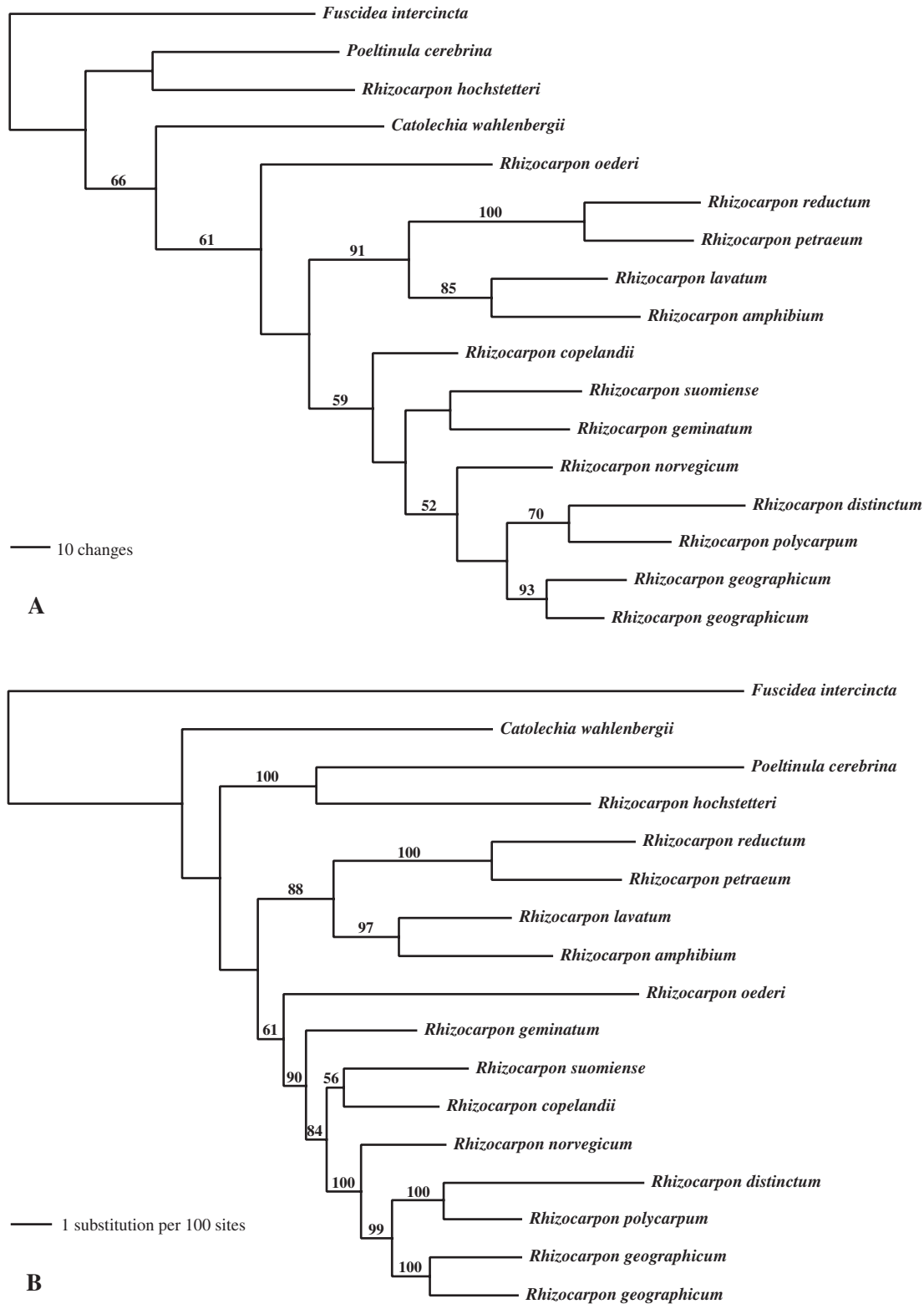

Figure 1. (A) Single optimal tree obtained from a combined ITS and mtSSU matrix under the maximum parsimony criterion. Tree length = 944. Branch support refers to bootstrap values in per cent. (B) Majority-rule consensus tree with average branch lengths based on 50 000 trees from a Bayesian MCMC tree sampling procedure. Branch support refers to the probability (%) of occurrence.

Table 3. Probabilities of 12 phylogenetic null hypotheses being correct. Each test is based on an MCMC tree sample of 5000 trees. Probabilites significant at ≤ 0.1% are denoted '***' and at ≤ 1% '**'; 'ns' refers to values that are not signicant at $P \leq 5\%$

Null hypothesis	Probability (%)
Rhizocarpon is monophyletic	0.04***
Rhizocarpon species containing rhizocarpic acid form a monophyletic group[1]	0.34**
Rhizocarpon species not containing rhizocarpic acid form a monophyletic group[2]	0.00***
Rhizocarpon species containing 1-septate ascospores form a monophyletic group[3]	0.00***
Rhizocarpon species containing multicelled ascospores form a monophyletic group[4]	0.00***
Rhizocarpon species containing hyaline ascospores form a monophyletic group	0.00***
Rhizocarpon species containing coloured ascospores form a monophyletic group	0.00***
Rhizocarpon with sub- and eumuriform ascospores form a monophyletic group	0.00***
Rhizocarpon species with an amyloid medulla form a monophyletic group	0.48**
Rhizocarpon with stictic acid complex form a monophyletic group[5]	0.00***
Rhizocarpon reductum and *R. lavatum* are sister taxa[6]	0.00***
The two-spored asci has originated once from the fusion of spores in an eight-spored ascus	5.14 ns

[1]This corresponds to subgenus *Rhizocarpon sensu* Thomson (1967). [2]This corresponds to subgenus *Phaeothallus sensu* Thomson (1967). [3]This corresponds to section *Catacarpon sensu* Fries (1871). [4]This corresponds to section *Eurhizocarpon sensu* Fries (1874), who defined three-septate, sub- and eumuriform ascospores as multicelled. [5]In Rhizocarpaceae, the dominant members of the stictic acid complex are stictic and norstictic acids. [6]*Rhizocarpon reductum* (former *R. obscuratum*) has frequently been confused with *R. lavatum* and accordingly they have been regarded as each other's closest relatives (e.g. Timdal & Holtan-Hartwig, 1988).

sequence, the likelihood-based Bayesian inference method reveals the outgroup as distant, possibly due to an excess of unusual substitution types. It is not possible to conclude which rooting (if any) is correct, but if parsimony has underestimated the length of the outgroup branch a consequence of this could be artificial rooting. Clearly, *Fuscidea* is suboptimal as an outgroup. However, it was the best we were able to find during the process of this study. The taxonomical position of the Rhizocarpaceae is uncertain, although most authors place it in the Lecanorales (e.g. Eriksson & Hawksworth, 1998). The two inference methods also differ in the position of *R. oederi* and in the position of *R. geminatum* Körb., *R. suomiense* Räsänen and *R. copelandii* (Körb.) Th. Fr. relative to each other. However, branch support is fairly poor in both trees for relationships involving these taxa.

Among identical branches between the two inference methods, highly supported parsimony branches with a weaker Bayesian counterpart are absent, whereas strongly supported branches under Bayesian interference, which are weakly supported under parsimony, are found in three instances. These branches include (1) *P. cerebrina* and *R. hochstetteri*, (2) *R. norvegicum* Räsänen, *R. distinctum* Th. Fr., *R. polycarpum* (Hepp.) Th. Fr., and *R. geographicum*, and (3) *R. geographicum*, *R. distinctum* and *R. polycarpum*. In all, the disagreement between the two inference methods must be said to be medium.

The trees obtained under both maximum parsimony and Bayesian inference indicate that *Rhizocarpon* is polyphyletic, and the null hypothesis concerning the monophyly of the genus is rejected (Table 3). This is obviously due to the fact that *R. hochstetteri* appears on the same clade as *Poeltinula*. Potential remedies to this situation, if correct, are either to exclude *R. hochstetteri* from *Rhizocarpon* or to include in *Rhizocarpon* at least *Poeltinula*, and possibly also *Catolechia* if the parsimony tree is correct. *Poeltinula* and *Catolechia* have been regarded as distinct genera in the Rhizocarpaceae (Hafellner, 1984). A preliminary evaluation of the morphological, anatomical and chemical characters of *Catolechia* (Hafellner, 1978), *Poeltinula* (Hafellner, 1984; Gilbert & Coppins, 1992) and *R. hochstetteri* (Fryday, 1996) by the senior author indicates that the variation pattern in these characters cannot easily be explained in the light of the molecular data. *Catolechia* appears to be distinct from *Rhizocarpon* by, for example, its lack of perispore, presence of torus, and a squamulose, thick and ridged thallus. *Poeltinula*, on the other hand, has a distinct perispore, and is separated from *Rhizocarpon* also by having ascospores reacting red with nitric acid and shortly lirelliform to angular apothecia with a thick black exciple. In addition, to include more *Rhizocarpon* species in future phylogenetic analyses, a detailed comparative investigation of the morphology and ontogeny of *R. hochstetteri* and the presently accepted species of *Poeltinula* should be carried out in order to evaluate their taxonomical position. Characters which

Table 4. Probability distribution (%) of the total number of state changes in five morphological characters (Table 2). Each character was mapped onto an MCMC tree sample including 5000 trees. Characters: A = ascospore colour (hyaline or coloured), B = ascospore septation (one-septate or eumuriform; three-septate and submuriform states not counted since they were autapomorphic in the data set), C = amyloid reaction of the thalline medulla (non-amyloid or amyloid), and D = presence of substances in the stictic acid complex in the thallus (absent or present). E = presence of rhizocarpic acid in thallus (absent or present). All characters had two states, and hence a minimum of one state change in any tree

No. of steps	Probability (%)
A	
4	7.4
5	89.2
6	3.4
B	
5	8.5
6	85.3
7	6.2
C	
2	100
D	
4	87.8
5	12.2
E	
2	0.4
3	99.6

may deserve closer study in this respect are the development and morphology of the hamathecial filaments (Ihlen, 2002). Furthermore, more molecular data are needed to investigate the possibility of the position of *R. hochstetteri* being artefactual due to a paralogous sequence. Paralogy has been known to distort phylogenetic reconstruction (see, for example, Mayol & Rosselló, 2001), but this phenomenon has never been demonstrated with certainty in lichenized ascomycetes.

The branch including *R. reductum* (Ach.) A. Massal. and *R. petraeum* (Wulfen) A. Massal. as well as the branch including *R. lavatum* and *R. amphibium* (Fr.) Th. Fr. are strongly supported under both inference methods. All of these species have hyaline and eumuriform ascospores, but the species of the former clade produce stictic acid, while the species of the latter clade lack lichen substances. An alternative scenario, that *R. reductum* (formerly '*R. obscuratum*') and *R. lavatum* are sister taxa (Timdal & Holtan-Hartwig, 1988), was tested as a null hypothesis and rejected (Table 3). A recent taxonomical study of the non-yellow species with hyaline and muriform ascospores in the Nordic countries by the first author confirms that *R. reductum* is closest to *R. petraeum*. It is also noteworthy that both *R. amphibium* and *R. lavatum*, which constitute one clade, are usually found in moist habitats (Fryday, 2000; Gilbert & Giavarani, 2000).

The position of *R. copelandii*, *R. suomiense* and *R. geminatum* relative to each other differs between the two inference methods, although the Bayesian analysis provides slightly better support for the branches involving these taxa. In the parsimony tree, the only two species in this investigation with two-spored asci, *R. suomiense* and *R. geminatum*, are sister species. In the Bayesian tree, on the other hand, *R. suomiense* is sister to *R. copelandii*, a pattern which is not correlated with any apparent morphological character. However, a test of the null hypothesis that the two-spored ascus has originated once from the fusion of spores in an eight-spored ascus (which translates to *R. suomiense* and *R. geminatum* forming a monophyletic group) cannot be rejected using Bayesian tree sampling (Table 3). Poelt (1987) considered the fusion of eight-spored asci to two-spored asci to be an example of reduction, and regarded it to be of minor value in evaluating relationships. Fewer and larger ascospores have been proposed to be an adaptation to spore-survival for long periods of time, since the spores contain more nutritional resources (Hawksworth, 1987).

A pattern found in both phylogenetic trees is that the branch including *R. distinctum* and *R. polycarpum* is the sistergroup to *R. geographicum*, and that *R. norvegicum* is sister to all of these taxa. This pattern is very well supported in the Bayesian tree but not in the parsimony tree. The yellow pigment rhizocarpic acid, causing the yellowish colour of the thallus, is present in *R. geographicum* and *R. norvegicum* but absent from *R. distinctum* and *R. polycarpum*. This indicates that that the widely used classification of *Rhizocarpon* in yellow and non-yellow species, i.e. species with and without rhizocarpic acid (subgenus *Rhizocarpon* and subgenus *Phaeothallus*, respectively), proposed by Thomson (1967), is artificial. This is also supported by the rejection of the two null hypotheses stating that the two subgenera that are based on presence or absence of rhizocarpic acid are monophyletic (Table 3). The brown species *R. distinctum* and *R. polycarpum* are found inside a grade of yellow species. It should be noted that all species on this branch possess an amyloid medulla. The null hypothesis stating that species with an amyloid medulla form a monophyletic group was rejected, surely because an amyloid medulla is present also in *R. oederi*, as observed by Feuerer (1978) and Foucard (2001). However, some individuals of this species are known to

Table 5. Probability distribution (%) of the number of gains and losses in five morphological characters (Table 2), each optimized onto an MCMC tree sample including 5000 trees under ACCTRAN, accelerated transformation (bold) and DELTRAN, delayed transformation (normal), respectively. For an explanation of the characters A to E, see Table 4

	Number of losses						
No. of gains	0	1	2	3	4	5	6
A							
0	–	–	–	–	–	**5.0**	**3.4**
1	–	–	–	**5.2**/4.9	**40.0**/1.8	–	–
2	–	–	**2.0**/2.1	**8.3**/1.2	–	–	–
3	–	**0.1**/0.3	**35.3**/74.9	–	–	–	–
4	–	**0.7**/9.9	–	–	–	–	–
5	1.5	–	–	–	–	–	–
6	3.4	–	–	–	–	–	–
B							
0	–	–	–	**5.1**	**0.02**	**3.0**	–
1	–	**0.7**/0.2	**0.3**	**54.2**	**0.3**	–	–
2	**0.04**	**5.7**/5.6	**2.7**/0.3	**0.04**	–	–	–
3	**2.5**/2.7	**2.7**/0.1	**2.5**	–	–	–	–
4	**20.2**/84.9	2.0	–	–	–	–	–
5	4.2	–	–	–	–	–	–
C							
0	–	–	**0.1**	–	–	–	–
1	–	**5.3**	–	–	–	–	–
2	**94.6**/100	–	–	–	–	–	–
D							
0	–	–	–	–	–	**0.7**	–
1	–	–	–	**4.1**/0.2	**7.9**	–	–
2	–	–	**6.5**/0.2	**2.5**	–	–	–
3	–	**77.3**/87.3	**0.9**/2.6	–	–	–	–
4	–	**0.1**/9.6	–	–	–	–	–
5	0.1	–	–	–	–	–	–
E							
0	–	–	–	–	–	–	–
1	–	–	**0.2**	–	–	–	–
2	**0.4**/0.4	**99.2**/0.1	–	–	–	–	–
3	**0.2**/99.5	–	–	–	–	–	–

have a non-amyloid medulla (Purvis *et al*., 1992; Foucard, 2001).

Fries (1874) divided *Rhizocarpon* into taxa possessing one-septate ascospores, section *Catocarpon*, and taxa possessing multicelled ascospores, section *Eurhizocarpon* (this includes three-septate, sub- and eumuriform spores). The monophyly of the two sections was rejected, however (Table 3). In this study, species of *Rhizocarpon* with one-septate ascospores include *R. copelandii*, *R. hochstetteri*, *R. norvegicum* and *R. polycarpum*, while the rest have three-septate, sub- or eumuriform ascospores. In addition, the null hypothesis stating that taxa possessing sub- and eumuriform ascospores are monophyletic was rejected. This group is similar to Fries' section *Eurhizocarpon* except that *R. oederi*, with three-septate ascospores, is not included.

The colour of the ascospores has been considered an important character for separating the species in *Rhizocarpon* (e.g. Timdal & Holtan-Hartwig, 1988; Fryday, 1996). Among the species treated here, dark ascospores are found in *R. copelandii*, *R. geographicum*, *R. geminatum* and *R. norvegicum*, whereas other taxa have colourless spores. The monophyly of taxa possessing coloured spores as well as the monophyly of taxa with colourless spores were rejected (Table 3). Although ascospore characters in *Rhizocarpon* have proved to be of significant value to delimit taxa at the species level (Fryday, 2000; Ihlen, 2002), they are apparently of minor value for elucidating relationships within the genus.

In addition to the yellow rhizocarpic acid, colourless substances in the stictic acid complex are found in several species of *Rhizocarpon*. Other secondary sub-

stances than rhizocarpic acid and the stictic acid complex are unusual, gyrophoric and psoromic acids possibly being the least uncommon. The null hypothesis stating that species containing the stictic acid complex form a monophyletic group was rejected, however, and this character appears to be of limited value in predicting relationships within the genus.

The evolution of five morphological characters claimed to be important to the prediction of relationships between species of the Rhizocarpaceae, i.e. spore pigmentation, spore septation, amyloid reaction of the thalline medulla, presence of substances in the stictic acid complex and presence of rhizocarpic acid, was reconstructed by mapping each of these characters onto 5000 trees in an MCMC sample (Table 4). Although having been considered conserved characters, most of them appear to have changed more frequently than would have been expected. However, given the limited number of taxa included in this analysis, the results should be interpreted as a minimum estimate of the amount of change. Spore colour is likely to have changed five times, spore septation six times, amyloidity of the thalline medulla twice, presence of stictic acid complex four times and presence of rhizocarpic acid three times, although other possibilities cannot be ruled out. When dividing the total number of state changes into gains and losses (Table 5), only one character, the amyloidity of the medulla, stands out as relatively uncomplicated. ACCTRAN as well as DELTRAN indicate that amyloidity has been gained twice and never lost. Apparently, amyloidity has been gained independently in *R. oederi* and in the 'yellow' group, also including *R. distinctum* and *R. polycarpum*. It should be noted that some individuals of *R. oederi* are in fact non-amyloid (Purvis *et al*., 1992). The presence of rhizocarpic acid in the thallus is slightly more complicated. ACCTRAN indicates that it has been gained twice and lost once, whereas DELTRAN indicates that it has been gained three times and never lost. Consequently, rhizocarpic acid was either gained independently in *Catolechia*, *R. norvegicum* and *R. geographicum*, or it has was gained in *Catolechia*, in the group including *R. norvegicum*, *R. geographicum*, *R. distinctum* and *R. polycarpum,* and lost in the clade including the latter two species. The latter scenario suggests that the non-yellow species *R. distinctum* and *R. polycarpum* have evolved from a yellow ancestor, a possibility which should be further investigated. For the other three characters, the evolution in terms of gains and losses is less clear. The clearest picture is displayed by the presence of substances in the stictic acid complex. In the interval limited by ACCTRAN and DELTRAN, three gains and one loss is by far the most likely scenario, although other possibilities, ranging from almost every combination from one gain and four losses to four gains and one loss, cannot be ruled out. In the most probable scenario, stictic acid complex has been gained independently in *R. hochstetteri*, the branch containing *R. petraeum* and *R. reductum*, and in the branch containing *R. copelandii*, *R. distinctum*, *R. geographicum*, *R. geminatum*, *R. norvegicum*, *R. polycarpum* and *R. suomiense*, but then lost again in *R. geographicum*. When it comes to spore colour and septation, no clear picture emerges. In short, changes in spore colour are probable to be in the range from no gains and five losses to four gains and one loss, changes in spore septation from one gain and three losses to four gains and no losses. Clearly, this case demonstrates the danger of relying on one or a few 'optimal' trees for reconstructing the evolution of characters (Huelsenbeck, Rannala & Masly, 2000). A weakness in the analysis of character evolution was that the character states 'three-septate' and 'submuriform' ascospores were autapomorphic and hence, by definition, had a single origin in our phylogenetic trees (for clarity, gains of these states were not counted). Therefore, to obtain a better understanding of how spore septation evolved, more species of *Rhizocarpon* with three-septate or submuriform spores should be included in future analyses.

This study is, to our knowledge, the first attemt to address phylogeny and character evolution in *Rhizocarpon*. Conclusions should not be regarded as definitive, but as working hypotheses that can direct future work. Accordingly, we do not carry out formal nomenclatural changes. To reach more sound conclusions, future studies of the phylogeny of the genus should include a better outgroup, more species (*c*. 200 are known) and more individuals of each species, especially when, for example, different chemotypes or morphotypes are known within a species, or when taxa are distributed over wide geographical ranges.

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