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A phylogenetic study of the *Lecanora rupicola* group
(*Lecanoraceae, Ascomycota*)

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A molecular phylogeny of the *Lecanora rupicola* group is presented, based on ITS sequence analyses. The study includes saxicolous and corticolous members of the *Lecanora rupicola* group as well as other *Lecanora* species with pruinose apothecia. A phylogenetic hypothesis for species in *Lecanora s. lat.* and various other genera in *Lecanoraceae*, based on an alignment-free distance estimation technique, shows that the *Lecanora rupicola* group forms a monophyletic clade within *Lecanoraceae*. Affinities to the core group of *Lecanora* are not well supported, likewise the monophyly of *Lecanora s. str.* with other species groups in *Lecanora*, such as the lobate taxa (*Rhizoplaca*) is not supported. A more detailed analysis involving *Lecanora* species with pruinose apothecial discs was carried out with model-based Bayesian Markov chain Monte Carlo (B/MCMC) tree sampling. The results suggest the monophyly of the *Lecanora* species that are characterized by the presence of chromones. Corticolous as well as saxicolous species are included. *Lepraria flavescens* is closely related to the *Lecanora swartzii* subgroup, and the new name *Lecanora rouxii* nom. nov. is introduced for that species. Other *Lecanora* species with pruinose discs are not closely related to the *Lecanora rupicola* group.

INTRODUCTION

The classification of lichenized genera, as it evolved during the past two centuries, was guided primarily by morphological and chemical characters. This was straightforward since, in contrast to the mycelia of most non-lichenized fungi, lichen thalli provide a number of morphological characters that are easily observable. A higher number of thallus characters is usually found in lichens with a more complicated organization, i.e. with foliose or fruticose growth. This contributed to a present-day situation with numerous well-delimited foliose and fruticose genera, whereas some large crustose genera (e.g. *Arthonia, Buellia, Caloplaca, Lecanora*), are insufficiently circumscribed and understood using anachronistic concepts. Most lichenologists agree that such large crustose genera are heterogeneous, but due to the low number of synapomorphic characters only a few, sometimes monotypic, genera have been split from the large complexes.

*Lecanora*, a representing the largest order of lichenized fungi, *Lecanorales*, is a perfect example of a large and heterogeneous crustose genus. Due to the size of the genus (ca 300 spp.; Kirk et al. 2001) it is understandable that there exists no recent monograph of the whole genus, except for the posthumous publication of Motyka (1995, 1996a, b, c), which has been rejected as a nomenclatural work. Certain morphologically defined groups, have, however, been studied in detail, for example subgenus *Placodium* (Poelt 1958), the *Lecanora rupicola* group (Leuckert & Poelt 1989), the *Lecanora dispersa* group (Poelt, Leuckert & Roux 1995), corticolous species with pruinose discs (Lumbsch *et al.* 1997), species with a dark hypothecium (Lumbsch, Guderley & Elix 1996), or regional monographs of the *Lecanora subfusca* group (Brodo 1984, Miyawaki 1988, Lumbsch 1994, Jüriado 1998, Guderley 1999). The delimitation and relationships of these groups to others in the genus, could not be resolved by these studies, and requires molecular phylogenetic approaches.

Phylogenetic analyses in *Lecanora* were previously carried out by Arup & Grube (1998, 2000). These studies indicated that certain genera that were segregated from the huge core genus *Lecanora* due to their deviating growth forms, form monophyletic groups.
within \textit{Lecanora}. For example, \textit{Arctopeltis} and \textit{Rhizoplaca} were split from \textit{Lecanora} only due to their foliose growth form, yet the phylogenetic position of these genera within \textit{Lecanora} is supported by secondary compounds: \textit{Arctopeltis} containing chlorinated lichenanthones is placed within the \textit{Lecanora dispersa} group, which is usually rich in xanthones; and \textit{Rhizoplaca}, possessing usnic acid, is related to lobate \textit{Lecanora} species which share this compound. While these two genera are traditionally accepted as well-circumscribed segregates, other species groups are maintained in \textit{Lecanora}.

Beside the xanthone-rich \textit{Lecanora dispersa} group, or various usnic acid containing groups, the \textit{L. rupicola} group is chemically distinct in \textit{Lecanora}. All species assigned to this group are characterized by apothecial discs covered by a conspicuous whitish to yellowish pruina, which is composed of sordidone (Huneck & Santesson 1969, Devlin et al. 1971). Members of this group comprise saxicolous crustose lichens, and an earlier monographic study accepted four species (Leuckert & Poelt 1989). Only two of these were included in the previous molecular studies of \textit{Lecanora} by Arup & Grube (1998, 2000).

Sordidone is a unique chromone, which usually occurs together with trace amounts of the dechlorinated accessory compound eugenitol. Chromones are only known from a few other lichens which are not closely related to \textit{Lecanora}, for example \textit{Siphula} and \textit{Haemanthoma} of the \textit{Lecanorales}, and the \textit{Roccellaceae} of the \textit{Arthoniales}. Certain chromosome derivatives are uncommon products also in few non-lichenized fungi (Turner & Aldridge 1983, Fujimoto et al. 2003, Lin et al. 2003), but so far, sordidone is only known from \textit{Lecanora} and one species assigned to \textit{Lepraria}. Apart from the \textit{Lecanora rupicola} group (sensu Leuckert & Poelt 1989), certain bark-inhabiting \textit{Lecanora} species also contain sordidone. The corticolous species with pruinose discs were recently revised by Lumbsch et al. (1997), who accepted four species which contain sordidone. One of these, \textit{L. carpinea}, was included by Arup & Grube (1998) who confirmed that this species is closely related to the saxicolous taxa of the \textit{L. rupicola} group.

In this contribution we present a phylogenetic analysis of \textit{Lecanora} species with pruinose discs to test whether they form monophyletic groups with other \textit{Lecanoraceae}, and whether any sordidone-lacking species are closely related to the \textit{Lecanora rupicola} group.

\section*{MATERIAL AND METHODS}

\subsection*{Specimens}
Lichen material for this study (Table 1) was borrowed from the herbarium GZU, from the private herbarium of Ulf Arup, and one specimen also of herbarium TSB.

\subsection*{DNA extraction, amplification, and sequencing}
Total DNA was extracted from individual thalli according to a modified CTAB method (Cubero et al. 1999) or using the DNeasy Plant Mini Kit (Qiagen, Vienna). DNA-extracts were used for PCR-amplification of the ITS regions including the 5.8S gene of the nuclear rDNA. Alternatively to DNA-extraction, we also used algal-free sections from the lichen medulla for direct amplification in some cases. Primers for amplification were ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). 50 \(\mu\)l PCR mix (10 mm Tris pH 8.3/50 mm KCl/1.5 mm MgCl\(_2\)/50 \(\mu\)g gelatine) contained 1.25 U polymerase (Taq DNA polymerase, Amerham), 0.2 mm of each of the four dNTPs, 0.5 \(\mu\)M of each primer and ca 10–50 ng genomic DNA. Products were cleaned using QIAquick PCR Purification Kit (Qiagen). Both complementary strands were sequenced using the BigDye Terminator Ready Reaction Kit (Applied Biosystems, Vienna) according to the manufacturers instructions. Sequences were run on an ABI 310 automated sequencer (Applied Biosystems) and assembled with AutoAssembler (Applied Biosystems).

Table 1 presents a list of specimens for which we sequenced the ITS rDNA. For the following species we retrieved sequences from GenBank: \textit{Biatora helvela} AJ247557, \textit{Biatora subduplex} AJ247540, \textit{Lecanora albescens} AF070033, \textit{L. allophana} AF159939, \textit{L. carpinea} AF070020, \textit{L. garovaglii} AF189718, \textit{L. muralis} AF070015, \textit{L. pruinosa} AF070018, \textit{Lepraria flavescens} AF517889, \textit{Protoparmelia badia} AF070023, \textit{Pyrrospora quernea} AF517930, \textit{Rhizoplaca chrysoleuca} AF159940, and \textit{R. peltata} AF159925.

The alignment for the B/MCMC analysis was produced using a linear Hidden Markov Model (HMM) implemented in the software SAM (Hughes & Krogh 1996; http://www.cse.ucsc.edu/research/compbio/sam.html).

\subsection*{Phylogenetic analyses}
To assess the relationship of the \textit{Lecanora rupicola} group in a larger phylogenetic context, we constructed an alignment that included representatives of other genera within \textit{Lecanoraceae}. Ambiguous alignment positions needed to be discarded from further analyses. Data exclusion can be done empirically or using described algorithms (Castresan 2000, Löytynoja & Milinkovitch 2001), which also reduces the information content of the data set. One alternative to data exclusion is a recoding of ambiguous alignment portions (Wheeler 1999, Lutzoni \textit{et al.} 2000) for use in parsimony analyses. However, a maximum likelihood distance method that retains the full information of sequences is also available. The program Statalign (Thorne, Kishino & Felsenstein 1991, Thorne & Kishino 1992, Thorne & Churchill 1995) does not require a distance alignment, it calculates pairwise maximum likelihood distances among all possible pairs of
sequences. The resulting distance matrix, containing the distances and their standard deviations, is subjected to tree inference using the program Modfitch35, which is a modified version of the Fitch program (Felsenstein 1989), included in the Statalign package. Branch supports can also be assessed by randomization with Statalign, which is called a pseudobootstrap approach by Thorne & Kishino (1992). The support values obtained by this approach are rather conservative when compared to traditional bootstrap techniques.

The phylogenetic hypothesis for *Lecanora*-species with pruinose discs was constructed using a Bayesian approach as implemented in the program MrBayes (Huelsenbeck & Ronquist 2001). The general time reversible substitution model (Rodriguez *et al.* 1990) with estimation of invariant sites and assuming a discrete gamma distribution with four rate categories (GTR + I + Γ) was used for likelihood calculations. The nucleotide substitution model was selected using a likelihood ratio test (Huelsenbeck & Crandall 1997) with the program MrModeltest (Nylander 2002), a simplified version of Modeltest v3.06 (Posada & Crandall 1998). For other parameters default settings were used. The Markov Chain Monte Carlo (MCMC) analysis was run for 2 000 000 generations, with 12 chains starting from a random tree, and using the default temperature of 0.2. Every hundredth tree was sampled, while the first 100 000 generations were discarded as burn-in. A consensus phylogram showing the distances and their standard deviations, is subjected to tree inference using the program Treeview (Page 1996).

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**RESULTS**

The tree for *Lecanoraceae* based on the alignment-free maximum likelihood distance approach is represented in Fig. 1 as a circular tree. Not all genera are supported
by pseudobootstrap support in Statalign, however, it should be noted that the support values are rather conservative in comparison with 'normal' bootstrap techniques (Thorne & Kishino 1992). Protoparmelia, Biatora and the Lecanora rupicola group are well-supported by pseudobootstrap values. Lecanora s. str. is supported only by 58%. Lobate species of Lecanora group together. The Lecanora muralis group with L. muralis and L. garovaglii are supported by a pseudobootstrap of 70%. Rhizoplaca peltata, Lecanora pruinosa and R. chrysoleuca are basal to this assemblage. However, all these lobate species are clearly distinct from Lecanora s. str. and from the Lecanora rupicola group. The two species of Lecidella form one clade in the tree but receive a pseudobootstrap support of less than 50%; the same is true for Tephromela. Pyrrhospora, Carbonea, Scoliciosporum and Bryonora which have no clear affiliation to other genera.

The Bayesian analysis included additional Lecanora species with pruinose apothecial discs, as well as Pyrrhospora quernea and Lecidella elaeochroma as members of other genera, while Protoparmelia badia was used as the outgroup taxon. The likelihood parameters in the sample had the following average values (± one standard deviation): rate matrix $r(GT) = 1.000$ ($± 0$), $r(CT) = 7.980$ ($± 2.811$), $r(CG) = 1.515$ ($± 0.151$), $r(AT) = 2.733$ ($± 0.452$), $r(AG) = 4.266$ ($± 0.952$), $r(AC) = 2.113$ ($± 0.317$), base frequencies $\pi(A) = 0.204$,$\pi(C) = 0.297$,$\pi(G) = 0.157$,$\pi(T) = 0.342$.
Phylogeny of the *Lecanora rupicola* group

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\[ \pi(C) = 0.295 \pm 0.033, \pi(G) = 0.265 \pm 0.033, \pi(T) = 0.236 \pm 0.033,\]

the proportion of invariant sites \( p(\text{invar}) = 0.202 \pm 0.005 \). The majority-rule consensus tree of 19,001 sampled trees is shown in Fig. 2.

*Lecanora* species containing sordidone form a monophyletic group with 100% posterior probability (*Lecanora rupicola* group clade, Fig. 2). Within this clade, the *L. swartzii* subgroup sensu Leuckert & Poelt (1989) is well supported. The *Lecanora swartzii* clade consists of *L. swartzii*, including the two subspecies *nylanderi* and *caulescens*, the sorediate *L. lojkaeana* and the sterile *Lepraria flavescens*, which has a basal position to the other species. A sister group to this assemblage are corticolous and saxicolous species (posterior probability 77%). At the basis are two clades, *Lecanora albelliformis* and *L. subcarnea* - group.
specimens of Lecanora carpinea, while the other corticolous taxa form a sister assemblage to the <i>L. rupicola/ L. bicincta</i> complex. Specimens of the corticolous <i>L. subcarpinea</i>, <i>L. carpinea</i> and <i>L. leptyrodes</i> form a highly supported group (100% pp). The <i>L. rupicola/L. bicincta</i> complex receives a posterior probability of 100%, but the relationships within this complex remain unresolved. Hence, the traditional morphological concept, which distinguishes <i>L. rupicola</i> from <i>L. bicincta</i>, is not supported by ITS data.

Other corticolous and saxicolous species with pruinose discs do not form a monophyletic group with species of the <i>Lecanora rupicola</i> group. Especially the species of the former <i>Lecanora pallida</i> group (<i>Lecanora caesiorubella</i>, <i>L. albella</i>, and <i>L. subcarnea</i>) that have been discussed to be close to <i>L. rupicola</i> and <i>L. carpinea</i> (Choisy 1929, Eigler 1969) form a separate clade together with the pantropical <i>L. farinacea</i>. The position of two representatives of related genera of <i>Lecanora, Pyrrhospora quernae</i> and Lecidella elaeochroma, is poorly supported by posterior probabilities and morphology.

In the distance analysis and the Bayesian analysis, the included <i>Lecanora</i> species are not supported as a monophyletic group. While <i>Lecanora s. str</i> (here with <i>L. epibryon, L. allophana</i> and <i>L. horiza</i>) and the <i>Lecanora rupicola</i> group are supported as groups and seem closer to each other, lobate Lecanoras appear more distant, and group together with <i>Rhizoplaca</i> species. The position of <i>Rhizoplaca</i> species within <i>Lecanora</i> has also been shown by Arup & Grube (2000) by parsimony analyses.

**DISCUSSION**

**Relationships in Lecanora s. lat.**

The trees presented here show the heterogeneity of <i>Lecanora s. lat</i>. Some morphologically distinct segregates of the large genus, such as Tephromela and others, are now widely accepted and supported (Fig. 1). Other groups which are still maintained in <i>Lecanora</i>, however, appear to be more distinct from the core group of <i>Lecanora</i> than morphological data alone would suggest. This is the case for groups with lobate <i>Lecanora</i> species, which were formerly classified as subgenus Placodium (Poelt 1958). The name Protoparmeliosis was introduced for <i>Lecanora muralis</i> (a member of Placodium) by Choisy (1929), but never accepted by other authors. Later, Hafellner (1984) suggested taking up this name if the <i>Lecanora muralis</i> group were to be ranked at genus level. If Protoparmeliosis were accepted in a broader sense, it might also include other usnic acid-containing <i>Lecanora</i> species, e.g. the <i>Lecanora polytropa</i> group, which contains both lobate and crustose members (e.g. <i>L. concolor, L. disperso-areolata, L. intricata, L. polytropa</i>). On the other hand, it might be considered better to accept the older name <i>Rhizoplaca</i> as a genus name also for certain groups of lobate <i>Lecanora</i> species, but a solution of this question should also consider data from another genetic locus.

While usnic acid-containing monophyletic groups in <i>Lecanora</i> are diverse in morphology, some primarily crustose lineages are distinct from each other in their secondary compound patterns. The rank of the <i>Lecanora dispersa</i> group, where chlorinated xanthones are found as the major constituents, is a matter for future debate. It forms a moderately supported group with the clades of lobate and usnic acid containing <i>Lecanora</i> species (Arup & Grube 1998). Here, we focus on a group characterized by the presence of a chemical compound, which is not found in other lichens.

The sordidone-containing <i>Lecanora rupicola</i> group is clearly supported as a group in our trees with 100% posterior probability and 94% pseudobootstrap support. This unites all <i>Lecanora</i> species with sordidone in a monophyletic group. The genus name Glaucomaria was already introduced by Choisy (1929) for <i>Lecanora rupicola</i>, and later also used in Hafellner (1984). However, we hesitate to accept this name at a particular taxonomic rank before further studies clarify its relationships to other Lecanoraceae. The <i>Lecanora rupicola</i> group includes corticolous and saxicolous species, which mostly have a crustose growth habit, with continuous to areolate thalli. Only <i>L. swartzii</i> subsp. <i>caulescens</i> has a more or less fruticose growth.

Besides sordidone and the accessory compound eugenitol, all species also contain atranorin. Most saxicolous taxa produce roccellic acid, and in some of them different xanthones of the thiophanic acid series and arthothelin series are present.

**Relationships within the Lecanora rupicola group**

The Bayesian analysis revealed the relationships within the <i>Lecanora rupicola</i> group and supports the concept of Leuckert & Poelt (1989), who divided the <i>Lecanora rupicola</i> group in two subgroups: the <i>swartzii</i>- and the <i>rupicola</i>-subgroup. The <i>swartzii</i>-subgroup is characterized by ascomatal margins, which possess an algal-free, strongly conglutinate eucortex and a more or less loose medullar plectenchyma in inner parts.

Substantial variation of growth form is present in the <i>Lecanora swartzii</i> subgroup. <i>L. swartzii s. str</i> is a strictly crustose species, whereas ssp. <i>caulescens</i> is characterized by an almost shrubby habit. This is connected with the development of a true cortex (eucortex) of the thalli, which extends from the cortex of the apothecial margin. Leuckert & Poelt (1989) thought that chemical characters indicate that the sub-species <i>caulescens</i> emerged from ssp. <i>swartzii</i>. We have no support for this hypothesis and at present, it is also unclear at what rank <i>L. swartzii</i> ssp. <i>caulescens</i> should be classified. The single specimen of the sterile <i>L. lojkaeana</i> is clearly positioned within the <i>L. swartzii</i> subgroup, which agrees with data from secondary chemistry (Leuckert & Poelt 1989).
The position of the sordidone-containing ‘Lep- rraria’ flavescens in this subgroup is not surprising. Ekman & Tønsberg (2002) already showed the relationship of this species with Lecanora, but have not focused on a more detailed placement within the genus. A new name has to be selected: the epithet ‘flavescens’ is not available in Lecanora, as there is already a Lecanora flavescens (Bagl.) Bagl. 1879 (a synonym of Lecanora rupicola ssp. sulphurata (Ach.) Leuckert & Poelt 1989 (Nimis 1993). For this reason the name Lecanora rouxii is introduced here.

Lecanora rouxii (Fig. 3) grows in similar rain-protected habitats as do L. swartzii and L. lojkaeana, but in contrast to the latter it prefers limestone. This is quite unusual since no other member of this group occurs on limestone. Only *L. bicincta* has been collected from marble once, but on superficially decalcified spots (Willfling 1998).

The *rupicola*-subgroup in the sense of Leuckert & Poelt (1989) has amphiethecia without large intercellular spaces in inner parts and the algal cells are also found in outer parts. Two species are distinguished in this subgroup: *L. bicincta* is characterized by a dark ring lining the outer edge of the hymenium (Fig. 4) and formed by the dark-pigmented apical cells of the para-thecium. In *L. rupicola* these cells are hyaline and a dark ring is not present (Fig. 5). In our analysis the clade with members of the morphospecies *Lecanora rupicola* and *L. bicincta* is not resolved, hence their diagnostic morphological characters do not correlate with the ITS phylogeny. We assume that the development of a dark ring, typical for *L. bicincta*, is to some extent influenced by environmental conditions. The distribution of compounds in the lichen thalli has been used by Leuckert & Poelt (1989) for the characterization of subspecies, but molecular support for the evolutionary significance of such compounds needs further studies. Interestingly, three Australian samples of this species complex group together, but additional data are needed to resolve whether Australian material is genetically distinct.

The corticolous members do not form a monophyletic entity, and two specimens of *L. carpinea* are separate from other samples of the taxon. Morphologically these two corticolous *L. carpinea* samples are distinct from other specimens placed in this species, and we assume that these could possibly represent a separate species, yet to be described. *L. carpinea* (Fig. 6) is rich in diverse morphotypes, and may consist of several species which still need to be delimited. Samples of *L. subcarpinea* (Fig. 7) do form a monophyletic group; this species is also well characterized by ascomata, which become quite large (up to 2 mm diam), and by the contents of psoromic acid. *L. leptyrodes* (Fig. 8) groups together with one sample of *L. carpinea*. Also in this case the position of *L. carpinea* could be due to the variation in this species and because only one sample of *L. leptyrodes* was included. Morphologically, *L. leptyrodes* is easily distinguished by the characteristic, thickish apothecial margins, which have a loose, hydrophobic pseudocortex.

Other sordidone-containing *Lecanora* species, (e.g. *L. subpallens*) have not yet been analysed because we had no appropriate material for molecular analyses but it is likely that these also belong to the *Lecanora rupicola* group. The same is certainly true for other described subspecific taxa in the group, i.e. *L. rupicola* subsp. *efflorescens*, subsp. *subplanata*, subsp. *arctoa*, and *L. swartzii* subsp. *nuorensis*.

**Lecanora species with pruinose discs**

Lecanora species with pruinose discs that lack sordidone are clearly excluded from the *Lecanora rupicola* group in our analyses. We did not attempt to include a larger number of taxa in case their placement in other species groups was rather clear from previous publications. For example epihymenial pruinans are rather common in the *Lecanora dispersa* group (Poelt & Leuckert 1995, Arup & Grube 1998). Some species were considered to be more closely related to the *Lecanora rupicola* group, such as the ‘*Lecanora albella* group’ (the *L. pallida* group; circumscribed as species with whitish pruinose discs, lacking large crystals in the margins, and K+ yellow thallus) and species of the closely related ‘*L. subcarnea* group’; they clearly form a separate group. The same is true for the clade with *L. intumescens* and *L. cateleia*. While we could show that these species groups are distinct from the *Lecanora rupicola* group the clarification of taxonomic ranks in the *Lecanoraceae* requires a marker which is less variable than ITS.

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