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Mitochondrial and nuclear ribosomal DNA data do not support the separation of the Antarctic lichens *Umbilicaria kappenii* and *Umbilicaria antarctica* as distinct species

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Abstract: The Antarctic endemics *Umbilicaria kappenii* and *U. antarctica* are morphologically close, but mainly distinguished by their reproductive strategies. *Umbilicaria antarctica* propagates by means of thalloconidia. *Umbilicaria kappenii* lacks thalloconidia, but exhibits a variety of asexual propagules: soredia, adventive lobes and thallyles. We have now employed molecular data from three gene regions to examine the phylogenetic relationships of these two morphotypes. The phylogeny of ten samples and four outgroup taxa (*Umbilicaria decussata*, *U. krascheninnikovii*, *U. nylanderiana*, *U. umbilicarioides*) was reconstructed using Bayesian and maximum parsimony analyses of a combined data set of nuclear ITS, nuclear LSU rDNA and mitochondrial LSU rDNA sequences. Forty two new partial sequences of 14 specimens were generated. Our results indicate that all samples morphologically referred to *U. antarctica* and *U. kappenii* form a monophyletic group. A topology separating the two morphotypes as phylogenetic species is significantly rejected with the data set. It is proposed to place *U. kappenii* into synonymy with *U. antarctica*.

Key words: Antarctica, Bayesian statistics, combined analysis, lichens, species concept, *Umbilicaria*.

Introduction

Our knowledge of the taxonomy and phylogeny of the lichen-forming fungal genus *Umbilicaria* (*Umbilicariaceae*, Ascomycota) has increased recently as a result of morphological, chemical and molecular studies, including those of Codogno *et al.* (1989), Hestmark (1990, 1997), Ivanova *et al.* (1999), Krog & Swinscow (1986), Narui *et al.* (1996), Poelt & Nash (1993), Posner *et al.* (1992), Sancho *et al.* (1992), Sipman & Topham (1992), Smith (2001), and Wei & Jiang (1993). However, several problems remain and the genus includes poorly understood and morphologically and chemically variable species. Numerous species are recognized by only a few characters, such as secondary metabolites or presence and absence of vegetative propagules, such as soredia or isidia. Molecular data provide additional characters to rigourously test the validity of such species circumscriptions (Grube & Kroken 2000).

Currently, eleven *Umbilicaria* species are recorded from the Antarctic region (Øvstedal & Smith 2001), including three endemic species: *U. cristata* C.W. Dodge & G.E. Baker, *U. antarctica* Frey & I.M. Lamb and *U. kappenii* Sancho, B. Schroet. & Vallad. Among these, the two latter are morphologically close and indeed most specimens currently classified in *U. kappenii* were formerly identified as *U. antarctica* (Sancho *et al.* 1998). However, although they show a similar external morphology and sometimes share the same ecological niches,

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differences between these species in morphology of the lower surface and the propagation were demonstrated by Sancho, Schroeter and Valladares (1998). *Umbilicaria antarctica* has a black lower surface and propagates mainly by means of thalloconidia but also frequently develops adventive lobes on the upper as well as on the lower surface. These adventive lobes provide an additional mode of vegetative reproduction but also might enlarge the photosynthetically active surface (S. Ott & J. Zimmer, unpublished). In contrast, *U. kappenii* has a basically ash-grey or purple-brown lower surface that is blackened close to the umbilicus only. Furthermore, it lacks thalloconidia, but exhibits a variety of asexual propagules, such as soredia, adventive lobes and thallyles. The adventive lobes of *U. kappenii* show a high degree of plasticity in morphology and are frequently developed from soredia that are formed on the upper surface (S. Ott & J. Zimmer, unpublished). Such asexual propagules are commonly used characters to distinguish morphologically similar species in *Umbilicaria* taxonomy. Well known species with asexual propagules include the parasoredial *U. grisea* Hoffm. and *U. hirsuta* (Sw. ex Westr.) Hoffm. in the northern Hemisphere or the southern hemispherical sorediate *U. soralifera* (Frey) Krog & Swinscow (Krog & Swinscow 1986; Codogno *et al.* 1989). In other groups of lichen-forming fungi, such as the genus *Physcia*, *Usnea* or several arthonialean genera (Articus *et al.* 2002; Lohtander *et al.* 1998*a*, 1998*b*; Myllys *et al.* 1999, 2001), species formerly distinguished mainly on the basis of different reproductive strategies, have been found to form single monophyletic groups. Therefore we decided to test this character in a member of the genus *Umbilicaria*. We have chosen the two species *U. antarctica* and *U. kappenii* as models to examine the taxonomic use of this character set in *Umbilicaria*, since preliminary molecular studies showed only a slight difference in ITS sequences of the two species (Romeike *et al.* 2002).

We have used a multilocus approach to address the issue of species distinction, since single locus approaches may be biased by the locus having not completed the process of lineage sorting (Taylor *et al.* 2000; Kroken & Taylor 2001). Nuclear ITS and LSU rDNA were selected as nuclear phylogenetic markers that have been used previously to examine relationships between closely related species of lichen-forming fungi (e.g. Articus *et al.* 2002). A mitochondrial gene was also targeted to provide a third independent set of data; one derived from a gene guaranteed to have evolved independently of the nuclear rDNA. The mitochondrial LSU rDNA was chosen, since fungal specific primers are available and the gene was shown to be useful at infraspecific level in the lichen-forming fungal genus *Biatora* (Printzen 2002).

A Bayesian approach was used that allows efficient analysis of data sets while employing complex nucleotide substitution models in a parametric statistical framework (Larget & Simon 1999; Huelsenbeck *et al.* 2001). Bayesian phylogenetics also allows simultaneous estimation of uncertainty in the phylogenetic topology, as well as hypothesis testing of alternative topologies, since posterior probabilities of alternative trees can be calculated (Huelsenbeck *et al.* 2000). In addition a maximum parsimony analysis was performed.

Materials and Methods

Taxon sampling

Sequence data of the nuITS rDNA and nuLSU rDNA and mtLSU rDNA were collected from a total of six *Umbilicaria* species. Forty two new sequences were obtained from the 14 specimens listed in Table 1. The following Antarctic *Umbilicaria* species were used as outgroups: *U. decussata*, *U. krascheninnikovii*, *U. nylanderiana* and *U. umbilicarioides*.

Molecular methods

Small samples prepared from herbarium specimens were ground with sterile pestles in 1·5 ml reaction tubes precooled with liquid nitrogen. Additional grinding with quartz sand and 200 µl lysis buffer at room temperature was followed by extraction of total genomic DNA using the DNEasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Dilutions of the total DNA were used for PCR amplifications of the genes coding for the nuclear ITS and LSU rDNA,

TABLE 1. *Species and specimens of* Umbilicariaceae *from Antarctica used in the current study*

Species	Locality	Collector (s)	Herbarium acc. No.	GenBank acc. no.		
				nuLSU	nuITS	mtLSU
U. antarctica Frey & I.M. Lamb—1	Lagoon Isl.	S. Ott	hb. Ott 2001	AY 603107	AY 603123	AY 603135
U. antarctica—2	Lagoon Isl.	S. Ott	hb. Ott 2002	AY 603108	AY 603124	AY 603136
U. antarctica—3	Lagoon Isl.	S. Ott	hb. Ott 2003	AY 603109	AY 603125	AY 603137
U. antarctica—4	Lagoon Isl.	S. Ott	hb. Ott 2004	AY 603110	AY 603126	AY 603138
U. antarctica—5	Lagoon Isl.	S. Ott	hb. Ott 2005	AY 603111	AY 603127	AY 603139
U. antarctica—6	Lagoon Isl.	S. Ott	hb. Ott 2006	AY 603112	AY 603128	AY 603140
U. decussata Zahlbr.	Lagoon Isl.	S. Ott	hb. Ott 2007	AY 603113	AY 603122	AY 603141
U. kappenii Sancho, B. Schroet. & Vallad.—1	Leonie Isl.	S. Ott	hb. Ott 2008	AY 603114	AY 603129	AY 603142
U. kappenii—2	Leonie Isl.	S. Ott	hb. Ott 2009	AY 603115	AY 603130	AY 603143
U. kappenii—3	Lagoon Isl.	S. Ott	hb. Ott 2010	AY 603116	AY 603131	AY 603144
U. kappenii—4	Livingston Isl.	H.T. Lumbsch 19047a & L. Sancho	F	AY 603117	AY 603132	AY 603145
U. krascheninnikovii Zahlbr.	Livingston Isl.	H.T. Lumbsch 19046a & L. Sancho	F	AY 603118	AY 603134	AY 603146
U. nylanderiana Zahlbr.	Livingston Isl.	H.T. Lumbsch 19046b & L. Sancho	F	AY 603119	AY 603133	AY 603147
U. umbilicarioides Krog & Swinscow	Lagoon Isl.	S. Ott	hb. Ott 2011	AY 603120	AY 603121	AY 603148

and the mitochondrial LSU rDNA. Primers for amplification were: (a) for the nuclear ITS rDNA: My1700f (Helms *et al.* 2003), ITS4 (White *et al.* 1990), (b) for the nuclear LSU rDNA: nu-LSU-155-5′ (Döring *et al.* 2000), LR6 (Vilgalys website), and (c) for the mitochondrial LSU rDNA: ML4A and ML3C (Printzen 2002). Amplifications were performed in reaction mixtures of 0·5–10 µl diluted DNA, 12·5 µl Hot Star Taq Master Mix (Qiagen) containing DNA-polymerase, $MgCl_2$ and dNTPs. 0·5 µl of each primer (10 µM) and dH_2O were added to 25 µl.

The amplifications were carried out in an automatic thermocycler Biometra TGradient and performed using the following programs. ITS rDNA: initial activation of the Taq-polymerase and denaturation of the DNA-template at 95°C for 15 min, and 33 cycles of: 94°C for 1 min, 53°C for 1 min, 72°C for 1·5 min, and a final extension at 72°C for 10 min. The PCR amplifications for mitochondrial and nuclear LSU rDNA were performed using the following touch-down programs: initial activation of the Taq-polymerase and denaturation of the DNA-template at 95°C for 15 min and 10 cycles of: 94°C for 1 min, 52°C for 1 min (with a decrease of −1°C in each cycle), and 72°C for 1·5 min, 28–40 cycles of: 94°C for 1 min, 45°C for 1 min, and 72°C for 1·5 min, and a final extension at 72°C for 10 min.

Fragments were cleaned using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions, and sequenced using the ABI Prism™ Dye Terminator Cycle Sequencing Ready reaction kit (PE Biosystems). The following cycle sequencing profile was used: denaturation for 3 min at 94°C and 25 cycles at: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems). Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually adjusted.

Sequence alignments

The nuITS, nuLSU and mtLSU data sets were aligned using Clustal X (Thompson *et al.* 1994), separately for the three genes. Regions that could not be aligned unambiguously were excluded from the phylogenetic analysis.

Phylogenetic analysis

The alignment was analysed using the programs PAUP* 4.0b10 (Swofford 2003) and MrBAYES 3.0 (Huelsenbeck & Ronquist 2001). The data were analysed using a Bayesian (Larget & Simon 1999; Huelsenbeck *et al.* 2000) and a maximum parsimony approach. Posterior probabilities were approximated by sampling trees using a Markov Chain Monte Carlo (MCMC) method. The posterior probabilities of each branch were calculated by counting the frequency of trees that were visited during the course of the MCMC analysis.

The program MrBAYES was employed to sample the trees. The analysis was performed assuming the general time reversible model (Rodriguez *et al.* 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G) for the single-gene and the combined analyses. No molecular clock was assumed. A run with 2 000 000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file.

We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) and determined that stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). The initial 1000 trees were discarded as burn-in before stationarity was reached. Using PAUP*, majority-rule consensus trees were calculated from 19 000 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes. In addition maximum parsimony (MP) trees were inferred using the heuristic search option with 200 random sequence additions. Gaps were treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap support (Felsenstein 1985) for each clade was tested based on 2000 replications, using the heuristic bootstrap option of PAUP* 4.0. Phylogenetic trees were drawn using TREEVIEW (Page 1996).

A Bayesian approach was used to examine the heterogeneity in phylogenetic signal among the three data partitions (Buckley *et al.* 2002). For the three gene portions and the concanated analyses, the set of topologies reaching 0·95 posterior probability was estimated. The combined analysis topology was then compared for conflict with the 0·95 posterior intervals of the single gene analyses. If no conflict was evident, it was assumed that the two data sets were congruent and could be combined. If conflict was evident, the two data sets were interpreted as incongruent and thus the combined analysis might be potentially misleading (Bull *et al.* 1993).

Hypothesis testing

The null hypothesis of the two described *Umbilicaria* spp. (*U. antarctica*, *U. kappenii*) as distinct phylogenetic species was tested using a MCMC tree sampling procedure as described above. For the hypothesis testing a run as described above was performed with the same settings as in the estimation of the phylogeny using the combined data set. 10 000 trees at the equilibrium state were used from this analysis. The probability of the null hypothesis being correct is calculated by counting the presence of this topology in the MCMC sample (Lewis 2001; Lumbsch *et al.* 2004). The frequency of trees in the MCMC sample agreeing with the null hypothesis was calculated using the filter command in PAUP* with the constraint describing the null hypothesis.

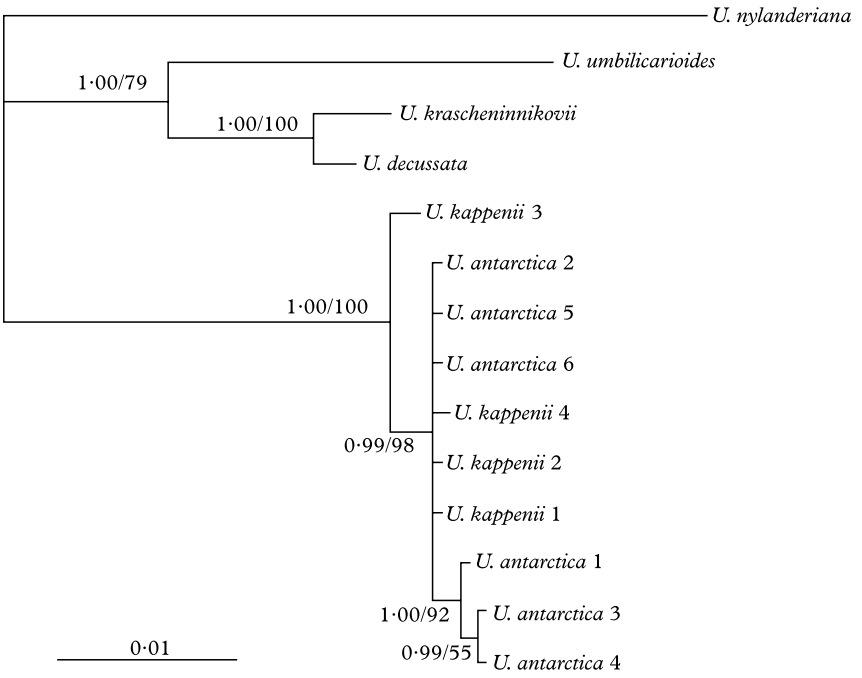

FIG. 1. Majority-rule consensus tree, based on 19 000 trees from a B/MCMC tree sampling procedure from a combined data set of nuLSU, nuITS and mtLSU rDNA. Posterior probabilities $\geq 0{\cdot}95$ are indicated at branches, followed by bootstrap values obtained from a MP analysis.

Results and Discussion

A total of 14 new sequences each of mitochondrial LSU rDNA, nuclear ITS rDNA, and nuclear LSU rDNA was generated for this study (Table 1). The sequences were aligned to produce a matrix of 2088 unambiguously aligned nucleotide position characters, 131 of them being variable. The Bayesian approach for testing data sets for incongruence indicated that the topology of the majority-rule consensus tree from the combined analysis lies within the 0·95 posterior intervals for the three separate data sets (data not shown) and hence a combined analysis was performed.

The likelihood parameters in the sample had the following average values ($\pm$ one standard deviation): base frequencies $\pi(A) = 0{\cdot}268$ ($\pm 0{\cdot}007$), $\pi(C) = 0{\cdot}236$ ($\pm 0{\cdot}007$), $\pi(G) = 0{\cdot}235$ ($\pm 0{\cdot}007$), $\pi(T) = 0{\cdot}261$ ($\pm 0{\cdot}006$), rate matrix $r(AC) = 1{\cdot}543$ ($\pm 0{\cdot}193$), $r(AG) = 3{\cdot}290$ ($\pm 0{\cdot}188$), $r(AT) = 1{\cdot}096$ ($\pm 0{\cdot}214$), $r(CG) = 0{\cdot}453$ ($\pm 0{\cdot}187$), $r(CT) = 5{\cdot}139$ ($\pm 0{\cdot}194$), $r(GT) = 1{\cdot}0$ ($\pm 0{\cdot}0$), and the gamma shape parameter alpha $= 0{\cdot}079$ ($\pm 0{\cdot}001$).

In the majority-rule consensus tree of 19 000 sampled trees (Fig. 1), the ten samples of *U. antarctica* and *U. kappenii* form a strongly supported monophyletic group [posterior probability (pp) 1·0]. Within this group there is only very slight genetic diversity: three of the samples examined of each of *U. antarctica* and *U. kappenii* have identical sequences. One sample of *U. kappenii* differs and the other samples form a well supported group (pp 0·99). Two samples of *U. antarctica* have a sister-group relationship (pp 0·99) and form another strongly supported group (pp 1·0) with a third deviating specimen. *Umbilicaria decussata* and *U. krascheninnikovii* appear as well supported sister-groups (pp 1·0) and again form a sister-group with *U. umbilicarioides*. The additional MP analysis revealed the same topology as the

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Bayesian analysis. One most parsimonious tree 165 steps long was found with consistency index 0·81 and retention index 0·87.

Since the placement of samples of *U. antarctica* and *U. kappenii* in one monophyletic group in the 50% majority rule consensus tree sampled during the Markov Chain Monte Carlo run contradicts the current classification of these morphotypes into two species, we ascertained whether our data set has sufficient phylogenetic signal to significantly reject alternative topologies that may be present in suboptimal trees not represented in the consensus tree. For this, Bayesian hypothesis tests were performed. An alternative topology separating two phylogenetic species based on the presence of soredia is rejected at $P \leq 0{\cdot}0001$.

The present results indicate that the morphotypes with thalloconidia and those with soredia currently distinguished at species level form one phylogenetic species that exhibits a high plasticity of developmental morphology and of reproductive strategy (Zimmer 1999). These results agree with those of studies in other groups of lichen-forming fungi (Articus *et al.* 2002; Lohtander *et al.* 1998*a*, 1998*b*; Myllys *et al.* 1999, 2001), in which soredia were found to be poor indicators of species delimitations. Soredia appear to occur sporadically in populations. Whether other modes of amphigenous diaspores in lichen fungi, such as isidia, are more reliable taxonomic characters, needs to be studied. Preliminary studies (A. Crespo, pers. comm.) suggest that isidiate species at least in *Parmeliaceae* and *Physciaceae* represent phylogenetic species. Furthermore, the presence and absence of fragile branches functioning as isidia-equivalents in the *Sphaerophorus globosus* complex were shown to distinguish two phylogenetic species (Högnabba & Wedin 2003).

Our results suggest that *U. kappenii* should be reduced to synonymy with *U. antarctica*, a phylogenetic species with a remarkable variety of modes of reproduction and developmental biology (Zimmer 1999). It remains to be determined whether high plasticity of propagation is a common feature inherent in *Umbilicaria* species or is caused by the harsh environmental conditions in Antarctica. *Usnea* species of the subgenus *Neuropogon* colonizing terrestrial Antarctic sites reflect a tendency amongst Antarctic macrolichens to exhibit a high plasticity of reproductive strategy (Ott & Romeike, 2004).

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