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Using haplotype networks, estimation of gene flow and phenotypic characters to understand species delimitation in fungi of a predominantly Antarctic *Usnea* group (Ascomycota, Parmeliaceae)

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Abstract Species delimitations in the predominantly Antarctic and South American group of neuropogonoid species of the lichen-forming fungal genus *Usnea* are poorly understood. Morphological variability has been interpreted as a result of harsh ecological conditions, but preliminary molecular data have led to doubts about the current species delimitations in these lichenized fungi. We examined species boundaries using a phylogenetic approach and a cohesion species recognition method generating haplotype networks and looking at associations of phenotypic characters with clades found in the networks. In addition, we estimated gene flow among detected clades and currently circumscribed species. We identified several clades that were significantly associated with phenotypic characters, but did not necessarily agree with current species circumscriptions. In one case (*U. aurantiaco-atra/U. antarctica*), network analysis and the estimation of gene flow provided no evidence of distinct species. The distinctness of another species pair (*U. subantarctica/U. trachycarpa*) remains dubious, showing evidence for gene flow among currently accepted species.

Keywords Lichens · *Usnea* · Species delimitation · Cohesion species

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Introduction

Molecular data have revolutionized our understanding of recognition and delimitation of species. They have identified hidden "cryptic" diversity of species that are indistinguishable based on traditional morphological data. The systematic treatment of taxonomic groups, in which phenotypic traits are highly homoplasious or extremely variable, may also benefit greatly from the use of molecular data. However, the recognition of species using molecular markers remains challenging due to evolutionary processes, such as incomplete lineage sorting, hybridization, and peripatric speciation (Funk and Omland 2003; Kliman et al. 2000; Rieseberg and Brouillet 1994; Soltis et al. 2007; Soltis and Soltis 2009; Syring et al. 2007; for a discussion on species concepts and species delimitation, see de Queiroz 2007). While early molecular studies often applied a strict phylogenetic species concept, the introduction of the genealogical concordance phylogenetic species concept (GPSC) (Avise and Ball 1990; Dettman et al. 2003a, b; Fisher et al. 2000; Geiser et al. 1998; Kasuga et al. 2003; Kroken and Taylor 2001; Taylor et al. 2000) accommodated, at least partly, for incomplete lineage sorting by requiring

samples of several genes and evaluating concordance or discordance among gene trees. The GCPSR (genealogical concordance phylogenetic species recognition) combines aspects of phylogenetic, genealogical, and biological species recognition. Proponents of the method argue that, as long as the individuals in a study belong to a recombining population, phylogenies from different loci will show discordance. When there is no recombination between individuals, different gene phylogenies are expected to be concordant. Tree-length permutation tests, such as the incongruence length difference test (Farris et al. 1994), are used to distinguish between discordance and concordance. Discordance among gene partitions will introduce homoplasy into a multi-locus dataset with the consequence of an increased tree length. In the presence of significant differences of tree lengths (Farris et al. 1995), discordance is assumed. Strong support for nodes in multi-locus phylogenies is taken as evidence for the absence of recombination and for the presence of reproductively isolated species, while low support is taken as evidence of dataset incongruence as a result of recombination. Although GCPSR is popular and has been used successfully in various groups of filamentous fungi (Dettman et al. 2003b, 2006; Fisher et al. 2000; Geiser et al. 1998; Kasuga et al. 2003; Kroken and Taylor 2001; O'Donnell et al. 2004), there are several points of criticism that could be raised against it. These include the fact that homoplasy can be caused by numerous factors, including parallel evolution or reversals, and that simply a small number of genetic differences in a data set may lead to low support values. Further, GCPSR still requires monophyly of species. However, it is well known that peripatric speciation leads to paraphyly of widely distributed species with monophyletic species nested within paraphyletic ones. The monophyly of these "embedded" species is the result of genetic drift during founder events (Kliman et al. 2000). Moreover, gene genealogies of closely related species will often display non-monophyly, because of the stochastic nature of lineage sorting, the removal of ancestral polymorphisms from evolutionary lineages during speciation. The probability of observing reciprocal monophyly of emerging species on gene trees depends on the number and kind of genetic markers used (mitochondrial or nuclear) and the number of alleles sampled, but generally approaches 1 only on time scales≥5 N, i.e., five times the effective population size (Hudson and Coyne 2002). Therefore, in order to delimit closely related species based on genetic data, species recognition methods that do not require monophyly are needed. Several such methods, based on coalescence theory, have been proposed (Ané et al. 2007; Heled and Drummond 2009; Kubatko et al. 2009; Liu and Pearl 2007; Liu et al. 2009; Yang and Rannala 2010). Simulation studies indicate that these approaches perform reliably only with a large number of unlinked gene loci (e.g., Hird et al. 2010; Liu et al. 2009). Practically all coalescent-based methods that have been proposed so far require a priori assignment of gene sequences to species and are hence better suited to detect hidden "cryptic" diversity than to recognize species in taxonomically difficult groups, in which phenotypic data alone is insufficient to delimit taxa. This a priori species assignment has two major shortcomings: dubious or faulty species assignments may influence the species delimitation process, and morphological traits are usually not critically re-evaluated. The general mixed Yule coalescent method introduced by Pons et al. (2006) works independent of a priori species assignment by fitting a model to single locus datasets in which deep nodes are assumed to represent an interspecific birth process (speciation) and shallow nodes are the result of infraspecific coalescence. However, this method again assumes monophyly of species on the studied gene trees, and hence does not account for deep coalescent events. Furthermore, it has been shown that the number of inferred species can be inflated when the genetic diversity of populations is spatially structured (Lohse 2009). A method that goes without a priori assignments and monophyly of species is cohesion species recognition, introduced by Templeton (2001). Cohesion species recognition is based on the cohesion species concept (Templeton 1989), which defines species in terms of genetic and phenotypic cohesion with an emphasis on the mechanisms yielding cohesive populations. It is based on the assumption that cohesion mechanisms preserve phenotypic and genetic clusters (Coyne and Orr 2004). These mechanisms include (1) genetic exchangeability via gene flow and (2) demographic or ecological interchangeability. The cohesion species approach uses haplotype networks based on one or more gene loci (Templeton 2001) to delimit species by rejecting the null hypothesis of a random distribution of traits in clades of the network. When a significant association between clade and character is found, the likely explanation is that the genes responsible for the observed character are not spread randomly throughout the sample and thus recent recombination and gene flow are lacking, provided the genes are not linked to the genes under study. The method thus combines genetic with anatomical and morphological data, which allows one to simultaneously explore the taxonomic importance of "traditional" characters and infer possible species boundaries in a statistical framework.

In this paper, we use cohesion species recognition to delimit closely related species from the lichen genus *Usnea*—a genus in which species delimitation based on phenotypical characters is notoriously difficult. For our studies it was important to use a method that is able cope with a certain degree of incomplete lineage sorting not relying on strict monophyletic lineages. Incomplete lineage sorting is a common phenomenon in closely related, recently emerged species. In fact, the detection of slow genetic drift in a lichen (Printzen et al. 2003) makes it likely that ancestral polymorphisms might distort the outcome of phylogenetic analyses of closely related lichen species. And methods for delimiting closely related

lichens potentially have to struggle with this problem of incomplete lineage sorting between recently diverged species. Further, we wanted to combine morphological, chemical and genetic data in a statistical framework.

Lichens are symbiotic systems consisting of fungi and green algae or cyanobacteria (photobionts) that supply the symbiosis with carbohydrates through photosynthesis. Many of them are pioneer organisms colonizing new habitats including very cold and nutrient-poor areas, such as polar, high-mountain and desert regions (Lange 1992). Their poikilohydric and autotrophic life strategy allows them to develop on solid rocks and outlast adverse conditions in a desiccated state. Sexual propagation is restricted to characteristic fungal fruiting bodies (ascomata) producing meiospores. The photobionts reproduce only asexually. Most characters used to distinguish lichen species are related to their ascomata. However, species without apparent sexual reproduction are frequent and the number of useful morphological characters in these species is thus often very limited. Sterile individuals that are otherwise similar to fertile ones have often been described as separate species. The question of whether the sister taxa of these so-called "species-pairs" (Poelt 1970) merit species status has been answered differently by different authors (e.g., Articus et al. 2002; Kroken and Taylor 2001; Lohtander et al. 1998; Molina et al. 2002; Myllys et al. 1999, 2001, 2003). Hence, the delimitation of closely related or similar species is often controversial. To make things worse, many lichens display extreme morphological plasticity, making their identification difficult.

Several studies dealing with species delimitation in Parmeliaceae, the largest family of lichenized fungi, and based on molecular phylogenetic methods, showed that numerous "cryptic species" may be hidden under a single name resulting in a vast underestimation of species numbers (e.g., Crespo and Lumbsch 2010; Crespo and Perez-Ortega 2009; Lumbsch and Leavitt 2011; Molina et al. 2004). It has also been shown that many supposedly "cryptic" species are in fact distinguished by overlooked characters. For example, Argüello et al. (2007) showed that the worldwide distributed species *Parmelina quercina* splits into four "molecular" species, with subtle but unique morphological features assigned to each species. Divakar et al. (2005) described a new species in the *P. sulcata* complex using molecular as well as morphological data.

The fruticose (shrubby) lichen genus *Usnea* is an example of a notoriously "difficult" group within Parmeliaceae. The ca. 600 species of the genus can be found on every continent and in a wide range of habitats. Here we investigate a subgroup of the genus *Usnea*, the neuropogonoid species, which occur mainly on rocks in Antarctic and sub-Antarctic regions, in southern South America and in high mountain ranges of South America and New Zealand (Seymour et al. 2007; Walker 1985; Wirtz et al. 2006, 2008). Species of this group sometimes dominate the vegetation in Antarctic and alpine habitats of the Southern Hemisphere. Various authors have reviewed the group (Dodge 1973; Lamb 1939; Motyka 1936–1938; Walker 1985) and often distinguished species based on minor morphological or chemical differences. One group of neuropogonoid *Usnea* species—the *U. perpusilla* complex—has been studied previously using molecular markers (Wirtz et al. 2008). In this study, undetected genetic diversity was shown to correlate with hitherto unrecognized morphological characters, which led to the circumscription of partly undescribed species within this group. The present study extends these investigations to two additional species groups of neuropogonoid *Usnea* species, the *U. aurantiaco-atra* and the *U. sphacelata* group. Our study attempts to answer two specific question: (1) How many species (hierarchically nested clades) can we identify in these two lichen groups? And (2) which phenotypical characters are associated with the identified clades?

Materials and methods

Taxon sampling

The detailed cohesion species recognition analysis in this study includes samples assigned to six currently accepted species (Walker 1985) of neuropogonoid *Usnea* (Wirtz et al. 2006): *Usnea aurantiaco-atra*, *U. antarctica*, *U. acromelana*, *U. sphacelata*, *U. subantarctica* and *U. trachycarpa.* These specimens were collected from 19 sample localities, plus nine further collecting sites for the outgroup specimens (see Table 1). The phylogenetic analysis, which was meant to give an overview of the main species groups, also comprised a third species group, the *U. perpusilla* complex, which was studied in more detail in a previous study [see supplementary material Table 2, and Table 1 in Wirtz et al. (2008) for taxon sampling]. Additionally, we used an outgroup of two accepted species, *U. acanthella* (12 individuals) and *U. patagonica* (12 individuals), plus an undescribed new species from Peru, *Usnea* sp. (seven individuals), and two species from New Zealand, *U. ciliata* (two individuals) and *U. subcapillaris* (three individuals), as well as two additional sorediate specimens from New Zealand (see Table 1). Lichen material for the molecular studies was predominantly used in a fresh, dried state. Voucher material for each specimen is preserved at the herbarium of the Field Museum (F) in Chicago or as stated in Table 1.

Morphological and chemical examination

Morphological characters were chosen based on previous morphological studies (Clerc 1984, 1998; Ohmura 2001; Walker 1985; Wirtz et al. 2008) and include reproductive mode, surface ornamentation, holdfast, and thallus anatomy (Tables 2 and 3). Secondary metabolites play an important

Table 1 Investigated samples and species assignments using morphological data according to Walker (1985). Sampling locations, collectors, herbaria (F= The Field Museum, ASS=British Antarctic Survey, OTA=University of Otago Herbarium), number of sampled individuals per locality (*N*) and haplotypes (H) at sampling locations are given. Numbers in parentheses represent the number of specimens of each taxa sharing a haplotype and the number of haplotype individuals in total, if larger than one

Species	Sampling locality	Collector	Herbarium	*N*	Haplotypes
Usnea aurantiaco-atra group					
Usnea acromelana	Argentina, Santa Cruz, El Chalten 49º02'S, 72º55'W	N Wirtz & MI Messuti	F	3	H1[a] (3/4)
	Tierra del Fuego	N Wirtz & MI Messuti	F	8	H1[a] (1/4), H2, H3(5), H35
Usnea antarctica	Argentina, Santa Cruz, El Chalten 49º02'S, 72º55'W	N Wirtz & MI Messuti	F	4	H4, H37, H38, H39
	Argentina, Santa Cruz, Monte Aymond, 52º07'S, 69º31'W	N Wirtz & MI Messuti	F	8	H5(6), H21[a] (1/17), H22
	Tierra del Fuego	N Wirtz & MI Messuti	F	9	H8[a] (2/6), H18, H19, H21(3/17), H29, H36
	Antarctica, King George Island, Admiralty Bay	J Gloser	F	1	H7
	Antarctica, Livingston Island	HT Lumbsch	F	24	H6[a] (4/7), H8[a] (2/6), H9 (2), H10(2), H12(3), H13(4), H14[a] (2/3), H15(2), H16, H17, H23[a] (1/2),
	Antarctica, Deception Island	B Schroeter	F	2	H6[a] (2/7)
	Antarctica, Deception Island, Entrance Point	RIL Smith	F, AAS 11482	2	H27, H28
	Antarctic Peninsula, Leonie Island	Mairi	F	2	H31, H32
	Antarctic Peninsula, Adelaide Island, Rothera	RIL Smith	F, AAS 98–261, 11482	3	H14[a] (1/3), H30(2)
	Antarctic Peninsula, MB, Terra Firma	S Ott	F	1	H6[a] (1/7)
Usnea aurantiaco-atra	Tierra del Fuego	N Wirtz & MI Messuti	F	11	H20, H21[a] (4/17), H23[a] (1/2), H24, H42, H43, H44, H45
	Falkland Islands, Port Stanley	P Crittenden	F, AAS	1	H25
	Antarctica, King George Island, Admiralty Bay	K Láska	F, AAS	2	H33[a] (1/2), H34
	Antarctica, Livingston Island	HT Lumbsch	F	13	H8[a] (2/6), H11(2), H21[a] (5/17), H26[a] (1/2), H33[a] (1/2), H40, H41
	Antarctica, Deception Island	B Schroeter	F	4	H21[a] (4/17)
Usnea *cf* aurantiaco-atra	Antarctica, Signy Island, Poal Harbour	RIL Smith	AAS 10883	1	H26[a] (1/2)
				99	45
Usnea sphacelata group					
Usnea sphacelata	Svalbard, Spitsbergen	T Tønsberg; C Printzen	F	9	H5[a] (9/28)
	Canada, Melville Island	GW Scotter	F	4	H5[a] (4/28)
	Greenland, NW, Thule, Air Base 76º31'N, 68º24'W	V Alstrup	F	4	H5[a] (4/28)
	Greenland, Disko, Skarvefjeld 69º16'N, 53º35'W	AV Larsen	F	3	H17(3)
	Iceland, Central Highlands, Eyjafjarðarsyla, Skagafjarðarsyla	S Heiðmarsson; H Arinbjarnarson	F	9	H5[a] (9/28)
	Ecuador, Chimborazo, volcano	Z Palice & Soldán; Z Palice; Kulísek & Stancík	F	6	H5[a] (2/28), H8, H9(2), H10
	Argentina, Rio Negro, Cerro Catedral 41°16'S, 71°20'W	N Wirtz & MI Messuti	F	2	H3(2/3)
	Antarctic Peninsula, Alexander Isl. 71º52'S, 68º15'W	S Ott	F	2	H1[a] (1/7), H11
Usnea cf *sphacelata*	Antarctic Peninsula, Alexander Isl. 71º52'S, 68º15'W	S Ott	F	1	H1[a] (1/7)
Usnea subantarctica	Argentina, Rio Negro, Cerro Catedral 41°16'S, 71°20'W	N Wirtz & MI Messuti	F	5	H1[a] (1/7), H3(1/3), H4(3)
	Argentina, Santa Cruz, El Chalten 49º02'S, 72º55'W	N Wirtz & MI Messuti	F	12	H1[a] (1/7), H2, H6[a] (1/2), H7[a] (6/8), H14, H15, H27

Table 1 (continued)

Species	Sampling locality	Collector	Herbarium	*N*	Haplotypes
	Tierra del Fuego	N Wirtz & MI Messuti	F	3	H1[a] (1/7), H6[a] (1/2), H32
	Antarctic Peninsula, Lagoon Isl.	S Ott	F	2	H13, H18
	Antarctic Peninsula, Leonie Isl. 67º36'S, 68º21'W	S Ott	F	1	H12[a] (1/2)
	Antarctic Peninsula, Alexander Isl. 71º52'S, 68º15'W	S Ott	F	4	H1[a] (1/7), H7[a] (2/8), H16
Usnea trachycarpa	Argentina, Santa Cruz, Monte Aymond, 52º07'S, 69º31'W	N Wirtz & MI Messuti	F	8	H12[a] (1/2), H24, H25(2), H26, H28, H29, H30
	Tierra del Fuego	N Wirtz & MI Messuti	F	8	H1[a] (1/7), H19, H20, H21, H22(2), H23, H31
				83	32
Outgroup					
Usnea acanthella	Peru, Cusco, Challabamba, 13°11'S, 71°37'W	A Ramírez	F	2	
	Peru, Ancash, P.N. Huascaran, Quebrada Ulta, 09°08'S, 77°31'W	N Wirtz & HT Lumbsch & A Ramírez	F	2	
	Ecuador, Tungurahua, Llangamates, 01°15'S, 78°30'W	Z Palice	F	1	
	Ecuador, Carchi, Volcano Chiles, 00°48'N, 77°56'W	Z Palice	F	7	
Usnea patagonica	Argentina, Santa Cruz, El Calafate, 50°28'S, 72°47'W	N Wirtz & MI Messuti	F	4	
	Argentina, Santa Cruz, Monte Aymond, 52º07'S, 69º31'W	N Wirtz & MI Messuti	F	1	
	Peru, Ancash, P.N. Huascaran, Huarapasca, 09°53'S, 77°11'W	N Wirtz & HT Lumbsch & A Ramírez	F	4	
	Ecuador, Chimborazo, volcano	Z Palice	F	3	
Usnea sp.	Peru, Ancash, P.N. Huascaran, Quebrada Ulta, 09°08'S, 77°31'W	N Wirtz & HT Lumbsch & A Ramírez	F	7	
NZ-*Usnea*	New Zealand, Otago, Remarkables, 45°02'S, 168° 48'W	A Marky	OTA 49924	1	
	New Zealand, Otago, Pisa Range	A Knight	OTA 57994	2	
	New Zealand, Otago, Old Man Range	DJ Galloway	OTA 53656	1	
	New Zealand, Otago, Old Man Range	K Spencer	OTA 58829	3	

[a] Haplotypes found in more than one location

role in the recognition of lichen species. We examined chemical characters in a previous study (Elix et al. 2007) and used these data for statistical analyses.

Molecular methods and phylogenetic analyses

Sample preparation, DNA isolation, PCR and direct sequencing were performed as described previously (Wirtz et al. 2008). We generated DNA sequences of three gene loci: (1) nuclear ITS rDNA, (2) a fragment at the 3'-end of the nuclear IGS rDNA, and (3) a fragment of the protein-coding RPB1 gene. Sequences were assembled using SeqMan 4.03 (DNASTar, Madison, WI) and edited manually. Sequence data of each marker were aligned separately in BioEdit (Hall 1999) using ClustalW (Thompson et al. 1994), adjusted manually, trimmed at both alignment ends to exclude missing data and concatenated. A Bayesian analysis (MrBayes 3.1; Huelsenbeck and Ronquist 2001) with the complete dataset (306 taxa), including all specimens of previous *Usnea* studies (Lumbsch and Wirtz 2011; Wirtz et al. 2008) was performed. The Bayesian analysis was performed assuming the general time reversible model of nucleotide substitution (Rodriguez et al. 1990) including estimation of invariant sites, assuming a discrete gamma distribution with six rate categories, and allowing site specific rates (GTR+I+G+SS) as well as using the covarion (Tuffley and Steel 1998) option of MrBayes. We have used this model since GTR+I+G was identified by Modeltest (Posada and Crandall 1998) as the most appropriate substitution model and the covarion model (which is not tested for in programs such as Modeltest) allows for varying distribution of rates across sites. Posterior probabilities (PP) were approximated by sampling trees using a MCMC method. A run with 4,000,000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every hundredth tree was saved into a file. We used AWTY (Nylander et al. 2008) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. The first 4,000 trees were deleted as the "burn in" and a 95%

Table 2 Results of contingency table tests for the *U. aurantiaco-atra* group. Clade level, character and character states and the probability *P*, assuming the null hypothesis of a random distribution of character states, are shown. 4-step clades are 4–1 and 4–2, 3-step clades are 3–1, 3–2, 3–3 and 3–4. The Bonferroni-corrected significance threshold varies according to the number of tests on the same dataset (4-step clades (14): 0.004; 3-step clades (14): 0.004)

Clade level	Character	Character state	Probability *P*
4-step clades	Reproductive mode[a]	Apothecia – soralia – both	0.103^{NS}
3-step-clades	Reproductive mode[a]	Apothecia – soralia – both	<0.001*
4-step clades	Frequency of soralia	Moderate – merged	0.954^{NS}
3-step clades	Frequency of soralia[a]	Moderate – merged	0.186^{NS}
4-step clades	Position of soralia	Side branches – throughout	0.004 *
3-step clades	Position of soralia	Side branches – throughout	0.032^{NS}
4-step clades	Isidiomorphs	Lacking – present	0.018^{NS}
3-step clades	Isidiomorphs	Lacking – present	0.088^{NS}
4-step clades	Papillae	Lacking/rare – frequent	<0.001*
3-step clades	Papillae[a]	Lacking/rare – frequent	<0.001*
4-step clades	Fibrils	Lacking – present	0.717^{NS}
3-step clades	Fibrils	Lacking – present	0.005^{NS}
4-step clades	Annulations	Lacking – main branches – throughout	<0.001*
3-step clades	Annulations[a]	Lacking – main branches – throughout	<0.001*
4-step clades	Base of secondary branches[a]	Cylindrical – narrowed	<0.001*
3-step clades	Base of secondary branches[a]	Cylindrical – narrowed	<0.001*
4-step clades	ß-orcinol depsidone	Lacking – present	0.001*
3-step clades	ß-orcinol depsidone	Lacking – present	<0.001*
4-step clades	Norstictic acid	Lacking – present	<0.001*
3-step clades	Norstictic acid[a]	Lacking – present	<0.001*
4-step clades	Salazinic acid	Lacking – present	<0.001*
3-step clades	Salazinic acid	Lacking – present	<0.001*
4-step clades	Quaesitinic acid	Lacking – present	0.392^{NS}
3-step clades	Quaesitinic acid	Lacking – present	0.048^{NS}
4-step clades	(Fumar-)protocetraric acid	Lacking – present	0.195^{NS}
3-step clades	(Fumar-)protocetraric acid	Lacking – present	<0.001*
4-step clades	Hypostrepsilic acid chemosyndrome	Lacking – present	0.021^{NS}
3-step clades	Hypostrepsilic acid chemosyndrome[a]	Lacking – present	0.018^{NS}

[a]Calculated with exact $r \times c$ two-sided contingency table test due to small cell values

consensus tree was calculated of the remaining 36,001 trees, considering all strongly supported branches with PPs≥0.95.

Congruence of data

To test for potential conflict among data sets, a phylogenetic approach was chosen: MP bootstrap analyses were performed on each individual data set, and 75% bootstrap consensus trees were examined for conflict (Lutzoni et al. 2004). Since no conflicts (i.e., well supported differences in the topology) were found, multi-gene data sets were used in the network analyses.

Network analyses

Haplotype networks were calculated separately for the *U. aurantiaco-atra* and *U. sphacelata* groups using TCS 1.21 (Clement et al. 2000). Gaps were coded as fifth character state. TCS calculates statistical networks under a 95% parsimony probability criterion (Templeton et al. 1992) for connections among haplotypes. Following standardized nesting rules (Crandall 1996; Templeton et al. 1987; Templeton and Sing 1993) we inferred the evolutionary hierarchy manually. Loops, which represent localized uncertainty caused by ambiguous linkages, were dealt with following Pfenninger and Posada (2002) and Posada and Crandall (2001).

Tests of contingency tables and analyses of variance

We tested whether we can reject the null hypothesis of random distribution of traits in clades of the network. Under the cohesion species concept (Templeton 2001), significant departure from random distribution of traits

Table 3 Results of contingency table tests for the *U. sphacelata* group. Clade level, character and character states and the probability *P*, assuming the null hypothesis of a random distribution of character states, are shown. The Bonferroni-corrected significance threshold varies according to the number of tests on the same dataset: 4-step clades (17): 0.003; 3-step-clades (17): 0.003

Clade level	Character	Character state	Probability *P*
4-step clades	Reproductive mode	Apothecia – soralia	0.171
3-step-clades	Reproductive mode[a]	Apothecia – soralia	<0.001*
4-step clades	Frequency of soralia	Moderate – merged	0.002 *
3-step clades	Frequency of soralia[b]	Moderate – merged	0.007^{NS}
4-step clades	Position of soralia	Side branches – throughout	<0.001*
3-step clades	Position of soralia[b]	Side branches – throughout	<0.001*
4-step clades	Isidiomorphs	Lacking – present	0.722^{NS}
3-step clades	Isidiomorphs[a]	Lacking – present	0.312^{NS}
4-step clades	Papillae	Lacking/rare – pigmented – unpigmented - both	0.021^{NS}
3-step clades	Papillae[a]	Lacking/rare – pigmented – unpigmented - both	0.012^{NS}
4-step clades	Form of papillae	Gross – fine	0.253^{NS}
3-step clades	Form of papillae[a]	Gross – fine	<0.001*
4-step clades	Fibrils	Lacking – present – frequent	0.006^{NS}
3-step clades	Fibrils[a]	Lacking – present – frequent	<0.001*
4-step clades	Position of fibrils	Throughout – main branches	0.016^{NS}
3-step clades	Position of fibrils[a]	Throughout – main branches	0.126^{NS}
4-step clades	Annulations	Lacking – present	0.061^{NS}
3-step clades	Annulations[a]	Lacking – present	0.297^{NS}
4-step clades	Base of secondary branches	Cylindrical – broadened	0.007^{NS}
3-step clades	Base of secondary branches[a]	Cylindrical – broadened	0.002*
4-step clades	Colour of holdfast	Pale – black – brown	<0.001*
3-step clades	Colour of holdfast[a]	Pale – black – brown	<0.001*
4-step clades	Cortex cross section	Shiny – very shiny – matt	<0.001*
3-step clades	Cortex cross section [a]	Shiny – very shiny – matt	<0.001*
4-step clades	ß-orcinol depsidone	Lacking – present	<0.001*
3-step clades	ß-orcinol depsidone[a]	Lacking – present	<0.001*
4-step clades	Norstictic acid	Lacking – present	<0.001*
3-step clades	Norstictic acid[a]	Lacking – present	<0.001*
4-step clades	Salazinic acid[a]	Lacking – present	0.637^{NS}
3-step clades	Salazinic acid[a]	Lacking – present	0.002*
4-step clades	Quaesitinic acid[a]	Lacking – present	0.578^{NS}
3-step clades	Quaesitinic acid[a]	Lacking – present	0.003*
4-step clades	Hypostrepsilic acid chemosyndrome	Lacking – present	0.601^{NS}
3-step clades	Hypostrepsilic acid chemosyndrome[a]	Lacking – present	0.195^{NS}

[a] Calculated with exact $r \times c$ two-sided contingency table tests due to small cell values

[b] Omitting clade 3–3 and 3–4, because of the lack of soralia

is interpreted as indicating independent evolution of these traits in the different clades and thus a lack of gene flow among them. We used contingency tables for discrete traits and analyses of variance (ANOVAs) (among clades) for continuous traits to test for associations between phenotypic character states and the phylogenetic structure of the nested haplotype network. These tests were employed for each single character on the 3- and 4-step levels in the *U. aurantiaco-atra* and the *U. sphacelata* group of the nested networks (Tables 2 and 3) using the online tool at: http://www.physics.csbsju.edu/stats. ANOVAs of the 3-step level clades were also calculated using the aforementioned online tool.

Nucleotide polymorphism and estimation of gene flow

In order to see whether the cohesion species inferred by the network analyses were genetically more coherent than the species as traditionally circumscribed, we calculated nucleotide polymorphism statistics for both groups of individuals, including Θ (Watterson 1975) and average pairwise distance Π (Nei 1987, eq. 10.5.) using SITES (Hey and Wakeley 1997). We used the same program to assess gene flow among phylogenetically (based on molecular data) and morphologically circumscribed species including a calculation of the numbers of shared and fixed alleles and pairwise fixation indices (F_{ST}) (Hudson et al. 1992). Calculations were

performed for all pairwise comparisons of species within each of the two groups studied.

Results

Alignments, phylogenetic analyses and haplotype network analyses

The phylogenetic analysis was performed using ITS, IGS and *RPB1* fragments of 306 *Usnea* specimens in total, with 101 specimens belonging to the *U. aurantiaco-atra* group, 86 to the *U. sphacelata* group and 80 to the *U. perpusilla* group. A total of 38 specimens were outgroup specimens; 170 ITS sequences, 197 IGS sequences and 185 *RPB1* sequences were newly generated for this study and are available under Genbank accession numbers JQ314680–JQ314849 (ITS), JQ314850–JQ315046 (IGS) and JQ314495–JQ314679 (*RPB1*). Detailed Genbank accession numbers for all samples are given in the supplementary material (Tables 1 and 2). The combined dataset for the phylogenetic analyses included 1,574 nucleotides (383 bp: IGS, 511 bp: ITS, 680 bp: *RPB1*).

Bayesian analysis (Fig. 1) resulted in three distinct species complexes—the *U. aurantiaco-atra*, *U. perpusilla* and *U. sphacelata* group—as well as four significantly delimited outgroup taxa. As in previous studies, *U. patagonica* is placed at the base of the three species complexes and appears to be an early deviating neuropogonoid lineage (Lumbsch and Wirtz 2011; Wirtz et al. 2006). The New Zealand *Usnea* samples form a well separated and highly supported group as well, which forms a sister-group to all other neuropogonoid core group species (see also Lumbsch and Wirtz 2011). Species boundaries within this early diverging group are still equivocal and need to be addressed in a separate study. The two outgroup taxa, the Peruvian *Usnea* sp. and *U. acanthella* each form strongly supported clades. Both species have been shown previously to deviate from neuropogonoid *Usnea* and to cluster within the subgenus *Usnea* (Wirtz et al. 2006).

The *U. aurantiaco-atra* group formed a strongly supported monophyletic group, splitting into two well-supported sister clades *U. acromelana* and *U. aurantiaco-atra/U. antarctica*, with fertile and sorediate specimens intermixed and closely related (Figs. 1 and 2). Some subgroups within this clade have high support values and comprise individuals either only with soralia or soralia combined with apothecia, but there are also mixed groups with apothecia or soralia. The sorediate *U. acromelana* includes specimens from Patagonia and Tierra del Fuego (Fig. 2). The *U. perpusilla* complex was a paraphyletic group and has already been discussed in detail by Wirtz et al. (2008). The *U. sphacelata* group formed a monophyletic but unsupported group (Figs. 1 and 3). The traditionally sexually reproducing *U. trachycarpa* is a polyphyletic group split into a small, supported group of five individuals with apothecia named as *U. trachycarpa* 1 and a larger group, which includes specimens with apothecia or soralia, *U. trachycarpa* 2. One specimen identified as *U. trachycarpa* based on morphology (196–2), is placed basal to the well supported *U. sphacelata* clade. Within the basal *U. trachycarpa* individuals a strongly supported clade of *U. subantarctica* specimens is found. Two of them have soralia and yellowish apothecia (A!S!). The strictly sorediate *U. sphacelata* clade is well supported.

For the haplotype network analyses two specimens of the *U. aurantiaco-atra* group (175–3 and 175–4) and three specimens of the *U. sphacelata* group (189–3, 119–1 and 78) were excluded from the alignment due to length or quality issues. Furthermore, five ambiguous alignment positions of the ITS and 56 positions of the *RPB1* were excluded at the beginning or the end of the sequence alignments, resulting in 1,513 unambiguously aligned nucleotides (383 bp: IGS, 506 bp: ITS, 624 bp: *RPB1*), which were used for the haplotype network analyses in the *U. aurantiaco-atra* and *U. sphacelata* group. A spliceosomal intron in the *RPB1* fragment was not excluded, since it was present in all sequences. 57 sites were variable in the *U. aurantiaco-atra* data set without any gaps or missing data, resulting in a total of 45 haplotypes with a haplotype diversity h=0.946. The *U. sphacelata* dataset included 51 variable sites without any missing data, gaps at three positions and a total of 32 haplotypes with a diversity of h=0.841.

The statistical parsimony analysis generated a haplotype network for the *Usnea aurantiaco-atra* group connecting all 45 haplotypes into a single network (Fig. 4a). The inferred nesting design resulted in groups up to the 4-step hierarchical nesting level. Haplotypes represent the 0-step level in the network, which are nested into 25 1-step clades, ten 2-step, four 3-step, and two 4-step clades (Fig. 4b). Two loops, depicted in Fig. 1 as dashed lines, were resolved according to Posada and Crandall (2001) and Pfenninger and Posada (2002). The haplotypes H7 and H32 as well as H20 and H22 were assigned to their adjacent central haplotypes with more than one individual. Clade 3–4 represents all specimens morphologically identified as *U. acromelana*. Specimens identified as *U. aurantiaco-atra* and *U. antarctica* and one individual tentatively assigned to *U. aurantiaco-atra* were distributed across clades 3–1, 3–2 and 3–3. All individuals in clade 3–1 were from the Antarctic and reproduced mainly asexually via soredia or showed both reproductive modes. They were a priori identified as *U. antarctica*. Only the two specimens combined into haplotype H11 were identified as *U. aurantiaco-atra* because they lacked soralia. Clade 3–2 with the central major haplotype H21 as well as clade 3–3 comprised specimens from southern South America and the

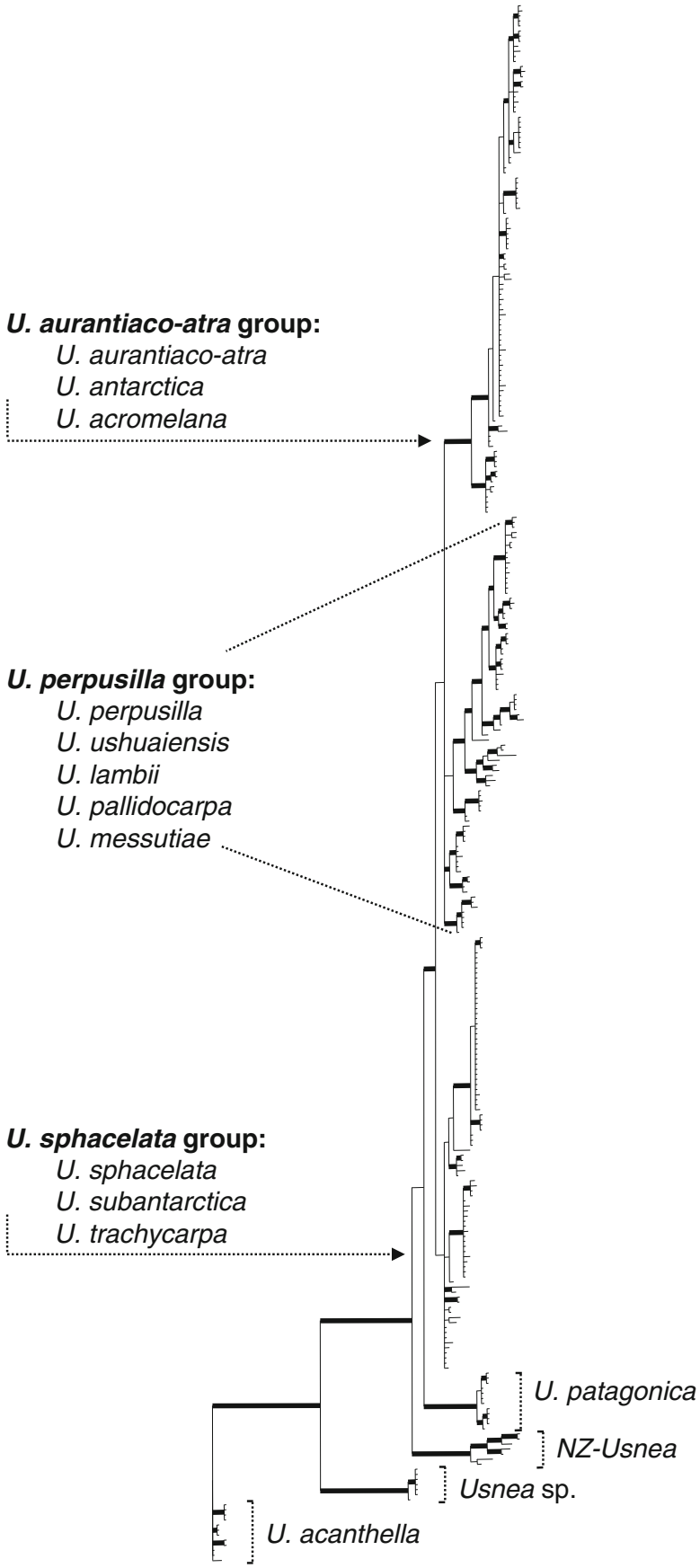


Fig. 1 50%-majority-rule consensus tree resulted from a Bayesian inference of a combined data set of ITS, IGS and *RPB1* sequences of 306 taxa. Posterior probabilities≥ 0.95 are depicted by bold branches

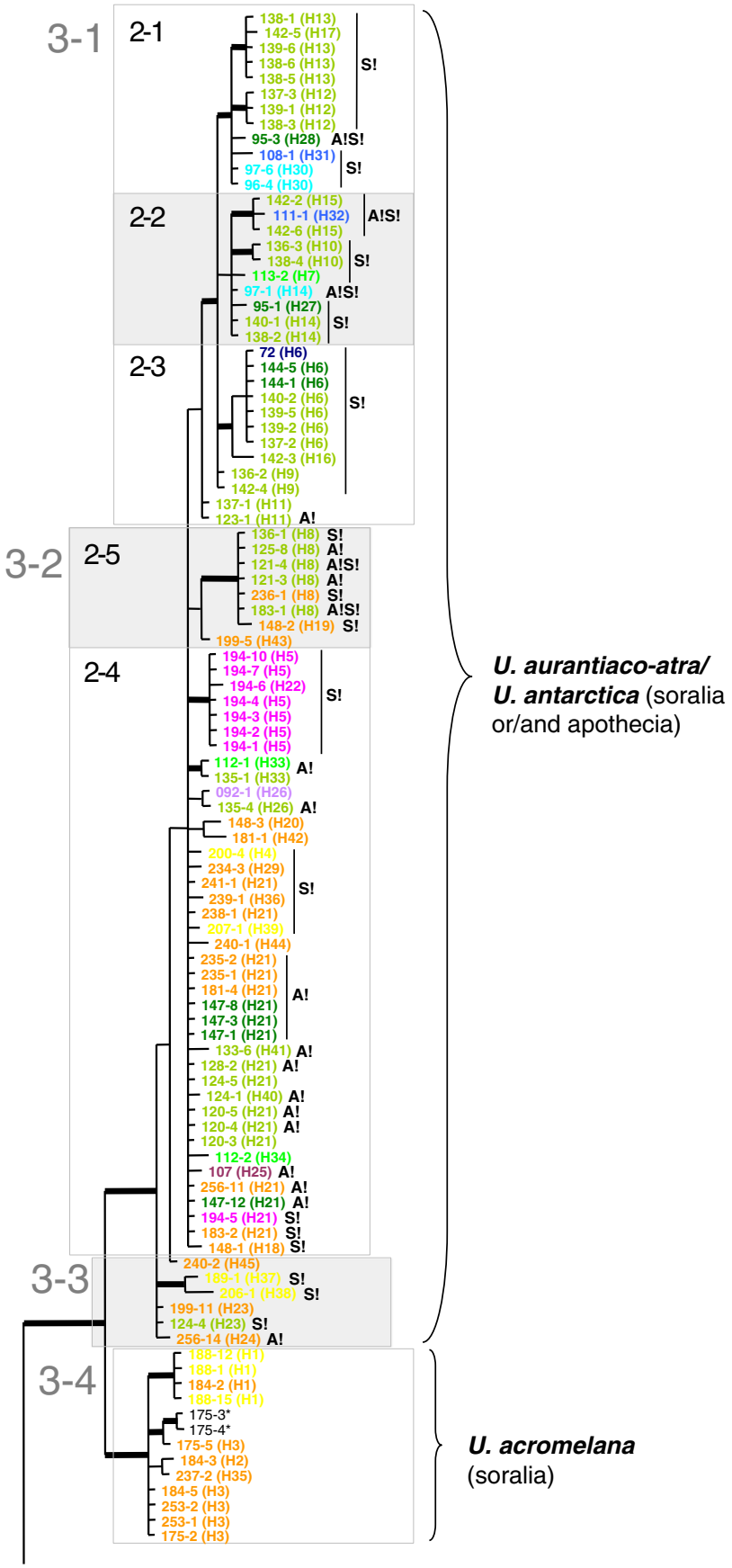


Fig. 2 Enlargement of the *Usnea aurantiaco-atra* group of the Bayesian inference (Fig. 1) depicting 101 taxa. Posterior probabilities≥0.95 are visualized by bold branches. Colors match the sampling site colours in Fig. 4a. *A!* fertile specimen with apothecia, *S!* vegetative reproduction via soralia. Most important clades of the nested clade analysis (Fig. 4a,b) are plotted on the phylogenetic tree

northern maritime Antarctic. Individuals reproduced either via apothecia or soredia or showed both reproductive mechanisms and were a priori identified as *U. aurantiaco-atra*, *U. antarctica* and *U.* cf. *aurantiaco-atra*, where the identification was not clear. All specimens in clade 3–4 and all but one individual in clade 3–3 were from southern South America.

The haplotype network for the *Usnea sphacelata* group connected all 32 haplotypes of the involved species in a single network (Fig. 5a). The inferred nesting design resulted in groups up to the 4-step hierarchical nesting level. Again, haplotypes represented the 0-step level in the network, which were nested into 22 1-step clades, 11 2-step clades, five 3-step clades, and two 4-step clades (Fig. 5b). The largest clade 3–5 comprised all specimens from the northern hemisphere, e.g., Spitsbergen, Canada, Greenland and Iceland, as well as most individuals from a tropical mountain range in Ecuador and six individuals from Patagonia. All northern hemispherical and tropical specimens were a priori identified as *U. sphacelata* and most of them belonged to the same haplotype H5, which was separated by only one mutational step from six Patagonian specimens (H3 and H4). Four of the Patagonian individuals were referred to *U. subantarctica* and two (within H4) were referred to *U. sphacelata* using Walker's (1985) identification key. All individuals mentioned reproduced asexually.

The fertile individuals in the network were genetically quite variable. Clade 3–4 was composed of southern South American specimens from Tierra del Fuego and Monte Aymond, Santa Cruz in Argentina. These were a priori identified as *U. trachycarpa* with brown or yellowish apothecia except for a specimen in H28 (clade 2–9), which was sterile and isolated genetically by several mutational steps from clades 2–8 and 2–3. Clade 3–3 comprised two *U. trachycarpa* individuals with brown apothecia from Monte Aymond, which were separated from individuals in clade 3–2 by four and nine mutational steps, respectively. Clade 3–2 was composed of Patagonian, but also one Ecuadorian (H10) and five Antarctic individuals (H1, H11, H12) and included morphologically characteristic *U. trachycarpa* specimens with brown apothecia (e.g., H1, H12, H25), some individuals with yellowish apothecia (H19, H21) as well as non-fertile individuals with soralia, which were a priori identified as *U. sphacelata* (H1, H11), *U. subantarctica* (H1, H12) or tentatively as *U.* cf. *sphacelata* (H1). Most specimens in clade 3–1 with the central haplotype H7 were collected in Patagonia and Tierra del Fuego. Four individuals in H7, H13, H16, H18 were from the southern maritime Antarctic. In clade 3–1, all individuals with soralia were identified as *U. subantarctica*, including two specimens with yellowish apothecia and soralia (H6, H13). Two individuals without soralia or apothecia were a priori identified as *U. trachycarpa* (H23, H31).

Association of phenotypic characters with clades

We examined the morphological characters listed in Tables 2 and 3. The secondary metabolites of the studied groups were discussed in detail elsewhere (Elix et al. 2007); relevant substances, substance classes and chemosyndromes are listed in Tables 2 and 3. Morphological characters studied include the reproductive mode, presence and location of vegetative diaspores, the structure of the thallus surface, including presence of papillae, fibrils and annulations, and the thickness of the central cord as defined by Clerc (1998).

The results of the contingency table tests and ANOVA are given in Tables 2, 3 and 4. In some labeled cases Fisher's exact test was employed, since tables were sparsely populated. We interpreted significant association of a priori defined characters with clades on the haplotype network as evidence for the presence of distinct evolutionary lineages. In the *U. aurantiaco-atra* group (Table 2), clades at both nesting levels differed significantly in the presence of papillae, annulations and the form of the base of secondary branches, which tended to be narrowed in clade 3–4. Presence of the β-orcinol depsidones norstictic and salazinic acids also differed significantly at both nesting levels. Four-step level clades were significantly different in the position of soralia, which are restricted to secondary branches in clade 4–2. Clades at the 3-step nesting level differed significantly in their reproductive mode and the presence of the fumarprotocetraric acid chemosyndrome. An inspection of the contingency tables revealed that some of the significant results were due to differences between clade 3–4 and the other clades. When the contingency table test was repeated without clade 3–4, the reproductive mode also differed significantly between the remaining clades. Beside the reproductive mode, there was also a statistical significance between clades 3–1, 3–2 and 3–3 in the frequency of fibrils and the production of salazinic and fumarprotocetraric acids. Only half of the individuals from clade 3–1, but 85% from clade 3–2 and 100% from clade 3–3 had fibrils. Salazinic and fumarprotocetraric acid were detected in about a third of the samples from clade 3–1, c. 75% in clade 3–2 and all of the individuals in clade 3–3.

Because these morphological and chemical trends correlate with a latitudinal geographic gradient, we were unable to decide whether they represent adaptations to environmental differences or indicated the presence of different species. We therefore treated the three clades as a single evolutionary lineage in the following analyses. Other characters, such as position of soralia, papillae or annulations, base of secondary branches and the presence of norstictic acid were not significantly different between clades 3–1, 3–2 and 3–3.

In the *U. sphacelata* group (Table 3), clades at the 3- and 4-step nesting level differed significantly in the position of soralia, the color of the holdfast, and the cortex cross

section. Chemical characters that differed significantly between clades at both nesting levels include the presence of β-orcinol depsidones and norstictic acid. Most specimens in clade 3–1 contained only norstictic acid, while individuals from clade 3–5 had no medullary substances, and clades 3–2, 3-3- and 3–4 were characterized by the presence of salazinic and quaesitinic acids in addition to norstictic acid. The distribution of these two substances was significant only at the 3-step level. Other characters that differed only at the 3-step level were the reproductive mode, the form of papillae (fine in clade 3–1, rather coarse in clades 3–2, 3–3 and 3–4), the frequency of fibrils (higher in clades 3–2, 3–3 and 3–5), and the form of the base of secondary branches (broadened in less than half of the individuals in clade 3–1). Four-step level clades also differed in the frequency of soralia (more frequent and restricted to the main branches in clade 4-2/3-5). Other characters did not differ significantly among clades. The ANOVA results (Table 4) revealed a significant difference of the thickness of the central cord between 3-step clades in both *Usnea* groups studied. In the *U. aurantiaco-atra* group the central cord in clade 3–4 was significantly thinner than in the other clades. In the *U. sphacelata* group the mean thickness was considerably higher in clade 3–5.

Polymorphism statistics and estimation of gene flow

For the calculation of polymorphism statistics and gene flow estimation in the *Usnea aurantiaco-atra* group, we assumed that all specimens in clades 3–1, 3–2 and 3–3 belonged to one polymorphic species *U. aurantiaco-atra* with different reproductive modes (see above). Clade 3–4 corresponded to *U. acromelana*. The *Usnea sphacelata* group was split into the asexually reproducing *U. sphacelata* (clade 3–5), the mostly sexually reproducing and very polymorphic *U. trachycarpa* (clades 3–4, 3–3, 3–2) and the mostly asexually reproducing *U. subantarctica* (clade 3–1). Overall, the number of polymorphic sites was highest in the IGS data set, while *RPB1* only included a few polymorphic sites (Table 5). The Θ-values varied between 0.0009 (*U. acromelana*) and 0.151 (the genetically circumscribed *U. aurantiaco-atra*) in the IGS data set, and between 0.0011 and 0.0075 (morphologically and genetically circumscribed *U. acromelana*, respectively) for ITS. The *RPB1* data set showed less variability with Θ-values varying between 0.0004 (*U. sphacelata*) and 0.0024 (morphologically delimited *U. trachycarpa*). Values for Π varied between 0.0002 (RPB1 in *U. sphacelata* and morphologically circumscribed *U. subantarctica*) and 0.0078 (ITS in morphologically delimited *U. subantarctica*) (Table 5). In most cases Θ and Π for the genetically delimited lineages were similar or lower than those for the species as circumscribed by morphological and chemical characters.

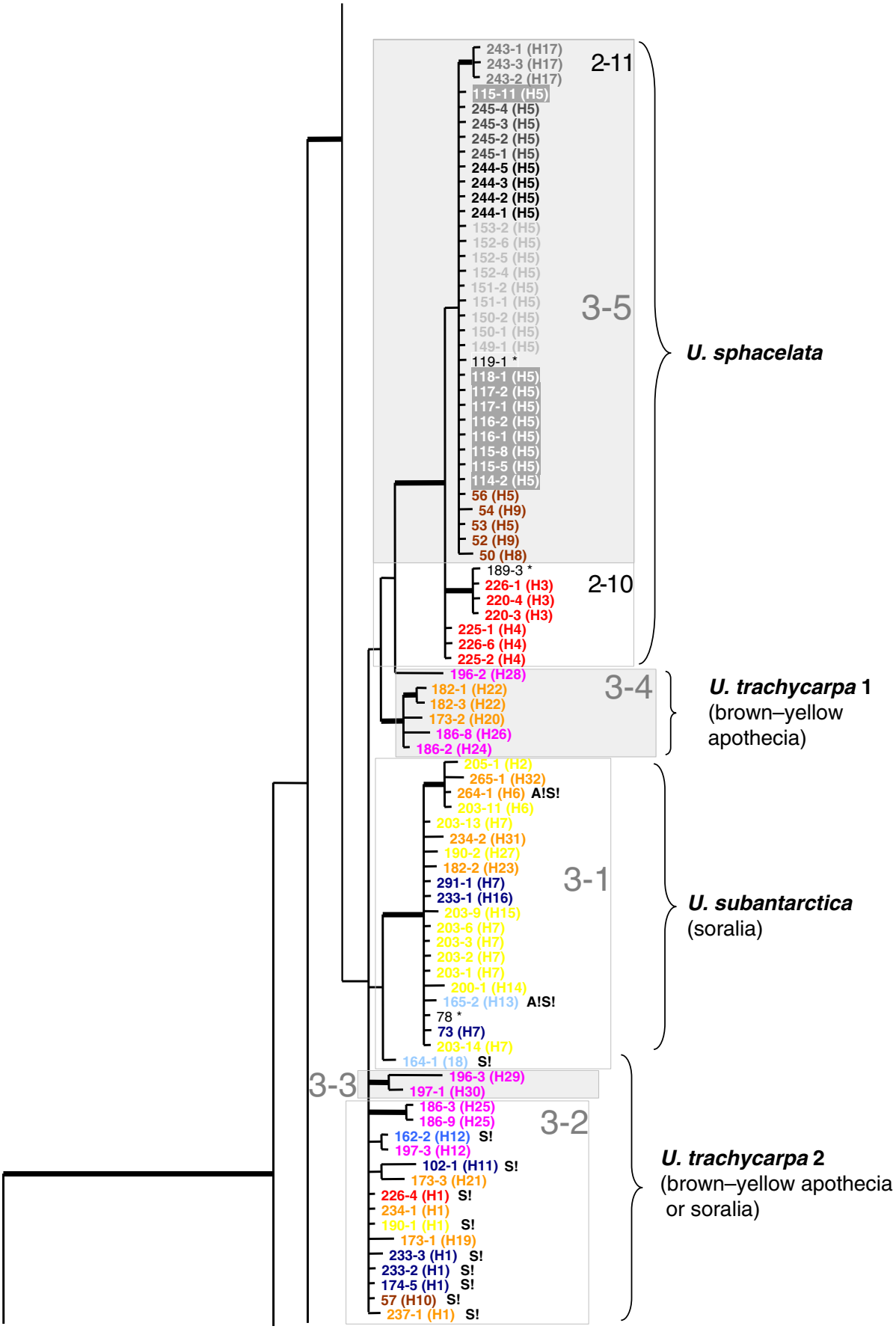
Fig. 3 Enlargement of the *U. sphacelata* group of the Bayesian inference (Fig. 1) depicting 86 taxa. Posterior probabilities≥0.95 are visualized by bold branches. Colors match the sampling site colours in Fig. 5a. *A!* fertile specimen with apothecia; *S!* vegetative reproduction via soralia. Most important clades of the nested clade analysis (Fig. 5a, b) are plotted on the phylogenetic tree

Most pairwise comparisons between the species in each group (Table 6) revealed fixed allele differences in at least two examined loci. Notable exceptions were the morphologically circumscribed *U. antarctica* and *U. aurantiaco-atra*, which showed no fixed differences but between two and four shared alleles at each locus. All morphologically delimited species of the *U. sphacelata* group shared between five and ten alleles at the ITS locus. This number dropped considerably when lineages were delimited based on genetic data. Correspondingly, overall levels of genetic differentiation (as expressed by F_{ST}-values) were higher in genetically circumscribed species. Although polymorphism values did not differ dramatically between the *U. aurantiaco-atra* and the *U. sphacelata* groups, the F_{ST}-values for the *RPB1* locus were exceptionally low in the *U. sphacelata* group.

Discussion

We have used molecular, morphological and chemical data to address the species circumscription in two neuropogonoid groups of the genus *Usnea.* This genus is morphologically and chemically extremely variable and species recognition based on phenotypic characters has been difficult, because it has often been unclear whether variation represents morphological plasticity of taxa or characterizes different species (Clerc 1998; Øvstedal and Lewis Smith 2001). Hence it is not surprising that molecular data indicate that the current phenotype-based species circumscription in the two examined species groups of Antarctic neuropogonoid *Usnea* species is in need of revision. This is in agreement with previous studies on neuropogonoid *Usnea* spp. (Seymour et al. 2007; Wirtz et al. 2008) that demonstrated non-monophyly of some currently accepted species. Our study goes further in that it investigates the relationships between genetical, morphological and chemical differences between putative species without assuming strict monophyly of species. By testing alternative morphological species distinctions based on the molecular data, this approach enables not only rejection of traditional species delimitations but suggests alternative classifications.

In the case of the South American *U. acromelana*, our analysis supports the currently accepted species concept. Several morphological characters provide significant evidence for its separation from *U. aurantiaco-atra*/*U. antarctica*, such as the absence of papillae and frequent occurrence of cortex annulations in clade 3–4 as well as the form of the


243-1 (H17)
243-3 (H17)
243-2 (H17)
2-11
115-11 (H5)
245-4 (H5)
245-3 (H5)
245-2 (H5)
245-1 (H5)
244-5 (H5)
244-3 (H5)
244-2 (H5)
244-1 (H5)
153-2 (H5)
152-6 (H5)
152-5 (H5)
152-4 (H5)
151-2 (H5)
151-1 (H5)
150-2 (H5)
150-1 (H5)
149-1 (H5)
3-5
119-1 *
118-1 (H5)
117-2 (H5)
117-1 (H5)
116-2 (H5)
116-1 (H5)
115-8 (H5)
115-5 (H5)
114-2 (H5)
56 (H5)
54 (H9)
53 (H5)
52 (H9)
50 (H8)
189-3 *
226-1 (H3)
220-4 (H3)
220-3 (H3)
2-10
225-1 (H4)
226-6 (H4)
225-2 (H4)
U. sphacelata
196-2 (H28)
182-1 (H22)
182-3 (H22)
173-2 (H20)
186-8 (H26)
186-2 (H24)
3-4
U. trachycarpa 1
(brown–yellow
apothecia)
205-1 (H2)
265-1 (H32)
264-1 (H6) A!S!
203-11 (H6)
203-13 (H7)
234-2 (H31)
190-2 (H27)
182-2 (H23)
291-1 (H7)
233-1 (H16)
203-9 (H15)
203-6 (H7)
203-3 (H7)
203-2 (H7)
203-1 (H7)
200-1 (H14)
165-2 (H13) A!S!
78 *
73 (H7)
203-14 (H7)
3-1
U. subantarctica
(soralia)
164-1 (18) S!
196-3 (H29)
197-1 (H30)
3-3
186-3 (H25)
186-9 (H25)
162-2 (H12) S!
197-3 (H12)
102-1 (H11) S!
173-3 (H21)
226-4 (H1) S!
234-1 (H1)
190-1 (H1) S!
173-1 (H19)
233-3 (H1) S!
233-2 (H1) S!
174-5 (H1) S!
57 (H10) S!
237-1 (H1) S!
3-2
U. trachycarpa 2
(brown–yellow apothecia
or soralia)

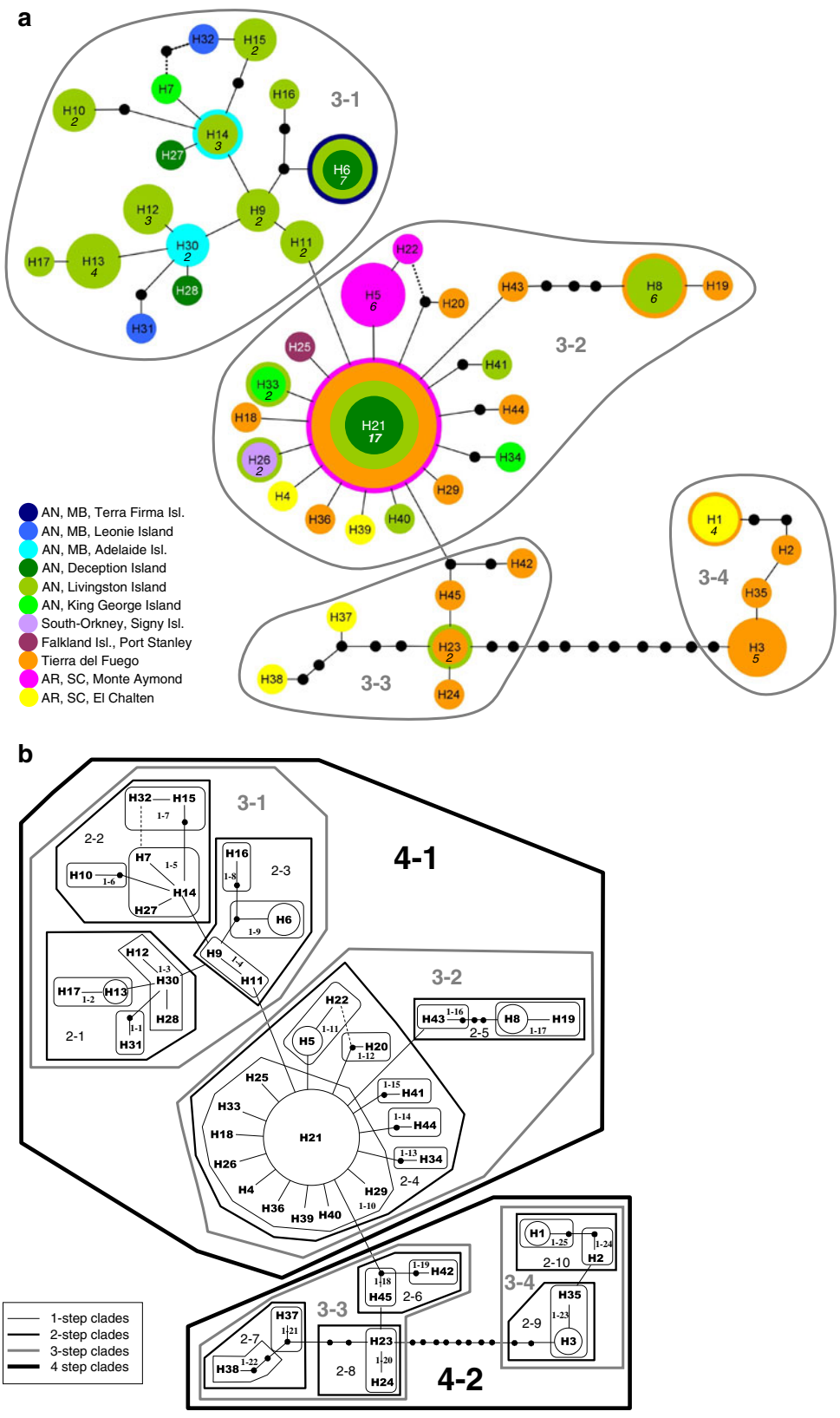


Fig. 4 95% probability haplotype network for the *U. aurantiaco-atra* group based on a combined dataset of ribosomal IGS and ITS and protein-coding *RPB1* sequences. **a** Haplotypes are represented by coloured circles according to the sample localities. The circle size is proportional to the number of sequences sharing a haplotype, except for haplotype H21 which was reduced in size because it comprised too many individuals. Numbers of individuals for each haplotype exceeding one are given in italics. Black dots represent unsampled haplotypes and connective lines one mutational step. **b** Same network as in A with hierarchical nesting design (Crandall 1996) superimposed. 1-step clades represent the basic clade level. The clade levels 1 to 4 are indicated by different line types and label fonts

base of secondary branches, which is commonly narrowed in *U. acromelana*. Chemically, the presence of the β-orcinol depsidones norstictic and salazinic acids differed significantly at both nesting levels in the *U. aurantiaco-atra* group, since

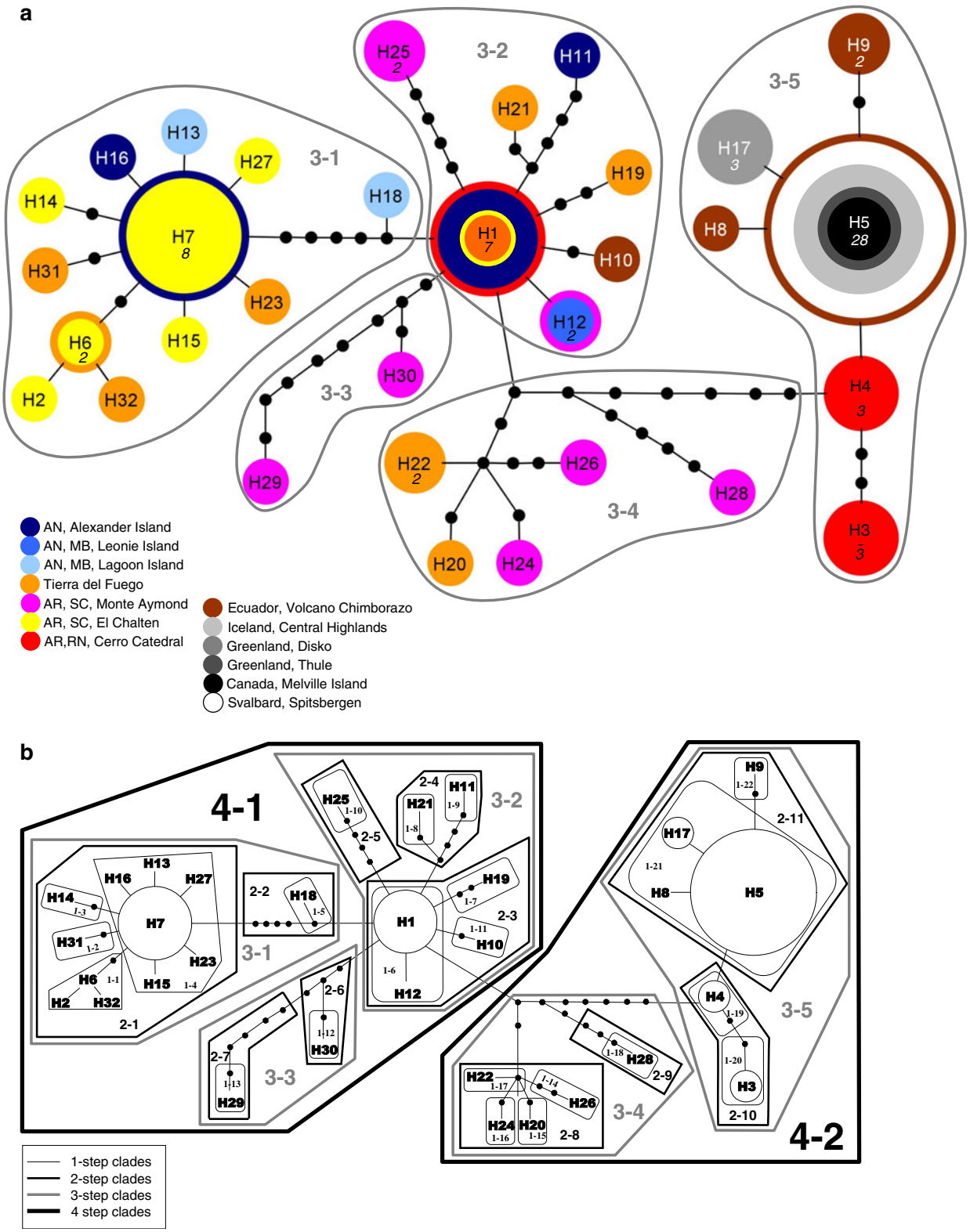


Fig. 5 **a** 95% probability haplotype network for the *U. sphacelata* group based on a combined dataset of ribosomal IGS and ITS and protein-coding *RPB1* sequences. The 3-step nesting level is shown. Haplotypes are represented by colored circles according to the sample localities. The circle size is proportional to the number of sequences sharing a haplotype, except for haplotype H5 comprising too many individuals to be depicted. Numbers of individuals for each haplotype exceeding one are given in italics. Black dots represent unsampled haplotypes and connective lines one mutational step **b** Same network as in A with hierarchical nesting design (Crandall 1996) superimposed. 1-step clades represent the basic clade level. The clade levels 1 to 4 are indicated by different line types and label fonts

Table 4 ANOVA results

Clade level	Character	*F*-statistic	Probability *P*
U. aurantiaco-atra			
3-step clades	Thickness of the central cord in % of the thallus' diam.	7.790	<0.001*
U. sphacelata			
3-step clades	Thickness of the central cord in % of the thallus' diam.	14.25	<0.001*

these are both constant features in *U. acromelana*, occurring only sporadically or in combination with other depsidones in the other clades of the *U. aurantiaco-atra* group. However, the type material of *U. acromelana* is from New Zealand, and Walker (1985) mentioned that the species is more common in Australasia than in Patagonia and the Antarctic Peninsula. Unpublished genetic data indicate that most *U. acromelana* specimens from New Zealand are not related closely to Patagonian individuals, but rather to the fertile Australasian species *U. ciliata*. Further studies to clarify these relationships and are underway.

A question that has been investigated repeatedly by lichenologists is the distinction of so-called lichen species pairs. Traditionally, these species were distinguished based on their reproductive modes ("primary" ─ fertile vs. "secondary" ─ sorediate species) (Mattsson and Lumbsch 1989; Poelt 1970, 1972), but molecular data have repeatedly provided evidence that samples that differ only in the presence or absence of soredia often belong to a single lineage (Articus et al. 2002; Buschbom and Mueller 2006; Cubero et al. 2004; Lohtander et al. 1998; Myllys et al. 2001; Ott et al. 2004). A specific example in our study concerns the delimitation of *U. aurantiaco-atra* and *U. antarctica*. The two species differ in their dispersal strategies (fruiting bodies in *U. aurantiaco-atra*, predominantly vegetative soredia in *U. antarctica*) but were also shown to form a single clade in previous phylogenetic analyses based on a smaller taxon sampling (Lumbsch and Wirtz 2011; Seymour et al. 2007). Our extended dataset shows that reproductive mode is indeed not strictly correlated with genetic differences; e.g., in clade 3–2 individuals with soralia and individuals with apothecia commonly share one and the same haplotype (H21, H8).

Table 5 Polymorphism statistics for morphologically ("old") and genetically ("new") circumscribed *Usnea* species. *N*=number of samples, N_{poly}=number of polymorphic sites, H=number of unique haplotypes; Θ=estimate of 4Nμ per base pair using the number of polymorphic sites; Π=estimate of 4 Nμ per base pair using the average pairwise differences. Differences in sample numbers between "old" and "new" species are due to the fact that we were unable to assign some samples to species using only morphological characters

Species	*N*		N_{poly}		H		Θ		Π	
	Old	New	Old	New	Old	New	Old	New	Old	New
			IGS							
U. acromelana	11	11	1	1	2	2	0.0009	0.0009	0.0013	0.0013
U. antarctica	56	88	20	29	16	23	0.0125	0.0151	0.0074	0.0065
U. aurantiaco-atra	31		11		10		0.0072		0.0031	
U. sphacelata	39	40	9	3	5	3	0.0056	0.0019	0.0025	0.0009
U. subantarctica	27	20	11	5	7	3	0.0075	0.0037	0.0061	0.0026
U. trachycarpa	16	23	22	14	11	12	0.0165	0.0142	0.0012	0.0086
			ITS							
U. acromelana	11	11	2	2	3	3	0.0014	0.0013	0.0017	0.0017
U. antarctica	56	88	12	16	14	19	0.0052	0.0066	0.0024	0.0022
U. aurantiaco-atra	31		8		9		0.0039		0.0018	
U. sphacelata	39	40	10	3	6	4	0.0047	0.0014	0.0024	0.0010
U. subantarctica	27	20	16	11	9	8	0.0082	0.0061	0.0078	0.0022
U. trachycarpa	16	23	18	16	8	9	0.0011	0.0075	0.0059	0.0030
			RPB1							
U. acromelana	11	11	1	2	2	2	0.0006	0.0006	0.0009	0.0009
U. antarctica	56	88	2	2	3	3	0.0007	0.0006	0.0009	0.0009
U. aurantiaco-atra	31		2		3		0.0008		0.0004	
U. sphacelata	39	40	1	1	2	2	0.0004	0.0004	0.0002	0.0002
U. subantarctica	27	20	2	3	3	4	0.0008	0.0014	0.0002	0.0005
U. trachycarpa	16	23	5	4	6	5	0.0024	0.0017	0.0012	0.0007

Table 6 Numbers of fixed differences and shared polymorphisms and fixation indices (F_{ST}) for comparisons of morphologically ("old") and genetically ("new") circumscribed *Usnea* species

Compared species pair		IGS			ITS			RPB1		
		Shared	Fixed	F_{ST}	Shared	Fixed	F_{ST}	Shared	Fixed	F_{ST}
U. acromelana vs. *U. antarctica*		0	4	0.750	0	4	0.833	0	1	0.729
U. acromelana vs. U. aurantiaco-atra	old	0	5	0.875	0	4	0.848	0	1	0.749
U. antarctica vs. *U. aurantiaco-atra*		4	0	0.178	3	0	0.024	2	0	0.352
U. acromelana vs. *U. aurantiaco-atra* (incl. *U. antarctica*)	new	0	4	0.779	0	4	0.838	0	1	0.708
U. sphacelata vs. *U. subantarctica*	old	0	6	0.629	5	0	0.664	0	0	0.027
	new	0	4	0.884	0	5	0.921	0	0	0.017
U. sphacelata vs. *U. trachycarpa*	old	0	4	0.518	5	0	0.647	0	0	0.027
	new	0	2	0.663	2	1	0.814	0	0	0.027
U. subantarctica vs. *U. trachycarpa*	old	0	4	0.139	10	0	0.263	0	0	0.019
	new	1	0	0.347	0	1	0.778	0	0	0.011

The reasons underlying the variability in vegetative reproduction in lichenized fungi are poorly understood but it is usually argued that vegetative reproduction enables species to circumvent the disadvantages of relichenization associated with sexual reproduction in lichens (Buschbom and Barker 2006; Buschbom and Mueller 2006). It is interesting to note that the frequency of apothecia in our sample declines from north to south. It is therefore also possible that the harsh environmental conditions in the southern maritime Antarctic are unfavorable for sexual reproduction. One reason for this might be a lack of compatible mating partners. This would, however, only affect obligateley outbreeding (heterothallic) species. There are currently no data on the mating system of the *Usnea* species studied here. Within other groups of lichenized ascomycetes, inbreeding (homothallic) species have been found to be common (Honegger et al. 2004; Honegger and Zippler 2007; Murtagh et al. 2000), on the other hand, a heterothallic breeding system has been shown to be present in the Antarctic lichen *Cladonia galindezii* and other species of this genus (Seymour et al. 2005). Judging by the frequency with which apothecia are formed, mating conditions in the northern maritime Antarctic, in particular on the South Shetland Islands, must be ideal for *U. aurantiaco-atra*. This interpretation is also supported by reports of gigantism of *U. aurantiaco-atra* and *U. antarctica* in the maritime Antarctic (Øvstedal and Lewis Smith 2001; our own observations). In Patagonia the species are less frequent and specimens are not as gigantic, but mating conditions seem to be similar.

The distribution of chemical characters within the *U. aurantiaco-atra* group is also significant, but again the pattern reflects a north–south gradient. Just one-third of individuals in the exclusively Antarctic clade 3–1 (Fig. 4a) produce depsidones, while in clade 3–2 three-fourths and in clade 3–3 and 3–4 all individuals produce β-orcinol-depsidones. Further, fertile individuals from maritime Antarctica, which occur mainly in clade 3–2 tend to produce depsidones more frequently than sorediate ones, which are found mainly in clade 3–1, whereas sorediate as well as fertile individuals from South America very regularly produce a variety of depsidone metabolites. There is obviously a north–south gradient of secondary metabolite production with increased production in warmer, northerly habitats to near absence in cold, southerly habitats. Many depsidones have antimicrobial activity or function as repellents against grazing invertebrates. Therefore this geographic trend might just reflect an increased need of being protected against herbivores or competitors in more northerly habitats. The fact that fibrils in clade 3–1 are less common than in clades 3–2 and 3–3 might be explained by the consistent occurrence of soralia in clade 3–1. Fibrils tend to break off the thallus easily and then function as vegetative propagules, which are not necessary in individuals/taxa with a high output of vegetative soredia fulfilling the same task. Based on these results we propose to include the sorediate *U. antarctica* in the circumscription of *U. aurantiaco-atra*.

Another potential species pair in the studied group is *U. subantarctica* and *U. trachycarpa*, which were a priori distinguished based mainly on the presence or absence of soralia and vegetative characters such as fibrils and pigmentation (Walker 1985). The distinction of these two taxa remains somewhat dubious based on the paucity of fixed alleles and the low F_{ST} values. However, it is striking that F_{ST} values at the IGS and ITS locus between *U. sphacelata, U. trachycarpa* and the newly circumscribed *U. subantarctica* are considerably higher than those for the same species in their traditional circumscription (Table 6). In its new circumscription *U. subantarctica* is confined to one clade of the network and shows much lower polymorphism than *U. trachycarpa*, which occupies the three clades 3–2, 3–3 and 3–4 (Table 5, Fig. 5). *U. trachycarpa*, which previously had been described as a solely sexual reproducing species with brownish apothecia (Walker 1985), comprises also sorediate specimens, sharing

haplotypes with fertile specimens (H1, H12). There is no striking character delimiting sorediate *U. trachycarpa* and *U. subantarctica* specimens, but rather a combination of variable but significant characters, which include the persistent occurrence of norstictic acid in *U. subantarctica* (3–1) and an irregular occurrence of norstictic, salazinic and quaesitinic acids in clades 3–2, 3–3 and 3–4. Papillae in individuals of clade 3–1 were commonly dark pigmented and fine, whereas the individuals of clades 3–2, 3–3 and 3–4 had rather coarse papillae. The base of secondary branches was frequently broadend in clade 3–1 and agreeing with Walker (1985), the frequency of fibrils was higher in clades 3–2, 3–3 and 3–4 than in clades 3–1 and also 3–5. One hypothesis regarding the origin of *U. subantarctica* could be a split from *U. trachycarpa* or its ancestor by peripatric speciation. This hypothesis is concordant with a certain degree of incomplete lineage sorting, which we did observe. Additional sampling will be necessary to elucidate whether *U. subantarctica* and *U. trachycarpa* represent distinct lineages or not. It is also necessary to further investigate the genetically isolated haplotypes combined in clades 3–3 and 3–4. Whether these few samples belong to further, perhaps undescribed cryptic species was impossible to assess, so that we prefer to keep them under *U. trachycarpa* at present.

Samples in clade 2–10 (H3, H4) (Figs. 2 and 5a) that were a priori identified as *U. subantarctica* appear to belong to Patagonian *U. sphacelata*. In the northern hemisphere, *U. sphacelata* tends to be morphologically more homogeneous than in the southern hemisphere (clade 2–11 versus 2–10) and a morphological distinction of Patagonian *U. subantarctica* and *U. sphacelata* specimens can be difficult. The only reliable and significant characters for a differentiation of both species are the constant occurrence of β-orcinol depsidones (norstictic acid) in *U. subantarctica* specimens from South America combined with soralia restricted to secondary branches and a usually about 20% thinner central cord in *U. subantarctica* (ANOVA results, Table 4). *U. sphacelata* lacks secondary metabolites apart from dibenzofuranes and has soralia that are commonly found on the main branches. In addition *U. subantarctica* specimens from South America tend to have a very shiny cortex cross section and either a rather black or a light holdfast as well as papillae that are always finely dark pigmented, whereas *U. sphacelata* cross sections tend to be rather matt and the holdfast is commonly brownish. Genetically the bipolar *U. sphacelata* (clade 3–5) is well supported as a distinct lineage, further clarifying the confused delimitation of this species. In a previous study, Wirtz et al. (2008) already demonstrated that a similar species *U. lambii* (Elix et al. 2007; Wirtz et al. 2008), which was included in *U. sphacelata* by Walker (Walker 1985), is related more closely to *U. perpusilla*. Both species have an antitropical distribution. However, as far as our data show, *U. sphacelata* in contrast to *U. lambii* does not occur in Antarctica but predominantly in the northern hemisphere, the high Andes and Patagonia. Populations from the northern hemisphere are genetically almost uniform (clade 2–11). The fact that the most common northern haplotype was also found in Ecuador indicates the possibility of ongoing gene flow between the Andean and northern hemispheric populations. It is also possible that historical long-distance dispersal, possibly during the last ice ages, when *U. sphacelata* from South America shifted its range northwards, enabled the species to disperse across the equator into the northern hemisphere. However, the low overall variability, the sampling gaps along the Andean Cordillera and the small sample sizes from Andean collection sites do not allow us to conclusively explain the origin of this distribution pattern at present. A denser sampling and the investigation of other genetic markers, e.g., microsatellites, will be necessary to further understand the distribution and gene flow among populations in this group of lichens.

The comparison of pairwise F_{ST} values was used as a method to assess whether the cohesion species delimited in our study were genetically more uniform and better segregated from each other than the traditionally separated species. In the *U. aurantiaco-atra* group, this approach proved to be impossible because we found evidence that two of the three species previously distinguished within the group likely form a single evolutionary lineage. The pairwise F_{ST} values for the remaining two species *U. acromelana* and *U. aurantiaco-atra* were similar to those obtained by comparing *U. acromelana* to the traditionally circumscribed species. This was to be expected, because *U. acromelana* was the only species in this study whose delimitation remained unchanged. Within the *U. sphacelata* group, the "new" cohesion species appear genetically much better delimited against each other (Table 6). For the IGS and ITS loci pairwise F_{ST} values between the cohesion species are up to three times higher than those for the traditional species. This pattern was not found for the *RPB1* locus, which is probably due to the fact that the number of polymorphic sites and the genetic diversity of *RPB1* was much lower than that of IGS and ITS.

Our approach of using a non-tree-based method to circumscribe species coupled with estimation of gene flow has been successful in identifying distinct lineages among the examined groups of *Usnea* species. As in previous molecular studies on the genus *Usnea* (Articus et al. 2002; Seymour et al. 2007; Wirtz et al. 2008), no single morphological and chemical character represents an unambiguous marker for a single species. The distinct lineages identified in this study can be characterized phenotypically only by combinations of different characters. This is, however, a usual phenomenon in lichen taxonomy. In the case of *Usnea*, the possibility of evaluating the taxonomic importance and usefulness of single characters in the light of genetic data makes cohesion

species recognition a powerful tool to investigate species delimitations. Some nomenclatural consequences of our results can be drawn only when the remaining species groups of neuropogonoid *Usnea*, particularly the Australasian taxa, have been thoroughly studied. We therefore have to postpone the presentation of a revised classification and identification key until our ongoing studies have been completed.

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