The Lichenologist **43**(6): 553–559 (2011)
doi:10.1017/S0024282911000417

Phylogenetic relationships of the neuropogonoid core group in the genus *Usnea* (Ascomycota: *Parmeliaceae*)

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Abstract: Species of *Usnea* with black pigmentation in the cortex and dark apothecial discs are informally referred to as neuropogonoid lichens. Here we studied the phylogenetic relationships of the core group of neuropogonoid lichens using DNA sequence data of three loci: nuclear ITS and IGS rDNA and *RPB1*. Maximum likelihood and Bayesian analyses revealed monophyly of 11 neuropogonoid species, with *U. ciliata* and *U. subcapillaris* forming a separate lineage. The backbone of the phylogeny of the core group was not resolved with statistical confidence, but relationships of groups of two to three species received strong support (*U. acromelana* +*U. aurantiaco-atra; U. messutiae*+*U. pallidocarpa; U. sphacelata*+*U. subantarctica*+*U. trachycarpa; U. lambii*+*U. perpusilla*+*U. ushuaiensis*). The new combination *U. lambii* (Imshaug) Wirtz & Lumbsch comb. nov. is made and *U. messutiae* Wirtz & Lumbsch sp. nov. is described.

Key words: *Lecanorales*, lichens, *Neuropogon*, phylogeny

Introduction

Neuropogonoid lichens include species of the speciose genus *Usnea* that are morphologically characterized by a yellow thallus with a patchy or fasciated dark pigmented cortex and generally darkly pigmented apothecial discs. These lichens occur almost exclusively on siliceous rocks and are most common in polar regions (especially the Southern Hemisphere) and high altitudes of temperate and tropical regions. The group was formerly often treated as a separate taxonomic entity, at different levels. However, molecular studies employing ITS sequence data revealed that the group was polyphyletic with a core group nested within *Usnea* subgen. *Usnea* (Wirtz *et al*. 2006). Hence we use the informal name 'neuropogonoid' for these *Usnea* spp. that seem to have adapted to harsh environmental conditions by producing melanoid substances in the thalline cortex and apothecial discs. Subsequent studies on these lichens have focused on species circumscriptions using molecular data (Seymour *et al*. 2007; Wirtz *et al*. 2008; N. Wirtz, C. Printzen and H. T. Lumbsch, unpublished data) and on clarifying the extrolites present in the species (Elix *et al*. 2007). However, the phylogenetic relationships among species within the core group of neuropogonoid lichens, as circumscribed in Wirtz *et al*. (2006), have not been studied. Therefore, we assembled a complete data set of three genes (including two nuclear ribosomal and one protein-coding loci) of 45 samples representing 14 species (Wirtz *et al*. 2008; N. Wirtz, C. Printzen and H. T. Lumbsch, unpublished data) to address the phylogenetic relationships among these taxa.

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Material and Methods

Taxon sampling

Data matrices of 45 samples, including three samples of *U. acanthella* that were used as an outgroup following a recent molecular study (Wirtz *et al*. 2006), were assembled using sequences of nuclear IGS, ITS and nuclear, protein-coding *RPB1* sequences. Specimens and sequences used for the phylogenetic analyses are listed in Table 1. The data set mainly includes sequences used in studies previously addressing species delimitation (Wirtz *et al*. 2006, 2008; N. Wirtz,

C. Printzen and H. T. Lumbsch, unpublished data). Genbank accession numbers of newly obtained sequences are indicated in bold. Laboratory methods to obtain these sequences have been described in the above mentioned references.

Chemical analyses

The chemical constituents were identified using high performance thin-layer chromatography with solvent systems A and C (HPTLC) (Arup *et al.* 1993), and gradient-elution high performance liquid chromatography (HPLC) (Feige *et al.* 1993).

Sequence alignments and phylogenetic analysis

Alignments were made using Clustal W (Thompson *et al.* 1994). Ambiguously aligned regions were removed manually. The alignments were analyzed by maximum likelihood (ML) and a Bayesian approach (B/MCMC). Maximum likelihood analysis was performed using the program GARLI (Zwickl 2006), employing the general time reversible model of nucleotide substitution (Rodriguez *et al.* 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories. Bootstrapping (Felsenstein 1985) was performed based on 2000 replicates. The B/MCMC analysis was conducted using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), using the same substitution model as in the ML analysis. A run with 20 M generations starting with a random tree and employing four simultaneous chains was executed. Every 100th tree was saved into a file. The first 500 000 generations (i.e. the first 5000 trees) were deleted as the 'burn in' of the chain. We used AWTY (Nylander *et al.* 2007) to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. Of the remaining 390 000 trees (1925 000 from each of the parallel runs), a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades that received bootstrap support equal or above 70% under ML and posterior probabilities $\geq$ 0·95 were considered as strongly supported. Phylogenetic trees were visualized using Treeview (Page 1996).

Results and Discussion

A matrix of 1650 unambiguously aligned nucleotide position characters was produced; 1403 characters in the alignment were constant. ML analysis yielded a maximum likelihood tree that did not contradict the Bayesian tree topology. In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following mean (variance): LnL = −4584·318 (0·86), while the likelihood of the ML tree was –4582·382.

Since the topologies of the ML and B/MCMC analyses did not show any strongly supported conflicts, only the tree of the ML analysis is shown (Fig. 1). All species in the circumscriptions based on recent network-based studies (Wirtz *et al.* 2008) form strongly supported monophyletic groups, with the exception of *U. trachycarpa*, which is paraphyletic with a monophyletic *U. subantarctica* nested within it. The latter is consistent with N. Wirtz, C. Printzen and H. T. Lumbsch (unpublished data) who discussed that the distinction of these two species requires additional studies. The two species, *Usnea ciliata* and *U. subcapillaris*, form a strongly supported lineage separate from the remaining species of the neuropogonoid core group. The remaining eleven species form a strongly supported monophyletic group. The backbone of the topology within this group lacks support. However, relationships of groups of two to three species each receive statistical support. *Usnea aurantiaco-atra* (incl. *U. antarctica*) is sister to *U. acromelana* with strong support, as shown previously (Wirtz *et al.* 2006). *Usnea pallidocarpa* and the new species *U. messutiae*, which is formally described below, form a strongly supported monophyletic group. The latter was referred to previously as *Usnea* sp. 2 and *U. pallidocarpa* as *U.* sp. 1 (Wirtz *et al.* 2008). *Usnea pallidocarpa* was recently described (Lumbsch *et al.* 2011). The bipolar *Usnea sphacelata* is sister to *U. subantarctica* and *U. trachycarpa*. Another bipolar species is *U. lambii*, which has a strongly supported sister-group relationship to *U. perpusilla*, and these two taxa form a sister clade to *U. ushuaiensis*. *Usnea patagonica* is the earliest deviating lineage in the group, agreeing with previous results (Wirtz *et al.* 2006) but in both studies this phylogenetic placement lacks support.

This study and previous analyses (Wirtz *et al.* 2006, 2008; Seymour *et al.* 2007) show that the diversity of neuropogonoid lichens has been underestimated by a morphology-based species concept and this is in agreement with studies in other groups of

TABLE 1. *Species and specimens used in the present study, with GenBank accession numbers of IGS, ITS and* RPB1 *sequences used in this study. New sequences in bold.*

Species name	Sample code	Voucher	IGS	ITS	*RPB1*
Usnea acanthella	169–2	Ecuador, Carchi. 6 Mar 2003. *Z. Palice* s.n. (F)	JF283533	DQ235483	JF283498
U. acanthella	289–1	Peru, Parque Nacional Huascaran, *N. Wirtz, H. T. Lumbsch* 19320c & *A. Ramirez* (F)	JF283534	DQ235482	JF283499
U. acanthella	268–1	Peru, Cusco. *A. Ramirez* 1770 (USM)	JF283535	DQ235480	JF283500
U. acromelana	188–1		JF283518	DQ235516	JF283484
U. acromelana	184–3		JF283519	DQ235514	JF283485
U. acromelana	175–2		JF283520	DQ235515	JF283486
U. aurantiaco-atra	123–1		JF283515	JF283507	JF283481
U. aurantiaco-atra	147–1		JF283516	JF283508	JF283482
U. aurantiaco-atra	256–14		JF283517	DQ235519	JF283483
U. aurantiaco-atra (sorediate)	138–6		JF283512	DQ235517	JF283478
U. aurantiaco-atra (sorediate)	72		JF283513	DQ235523	JF283479
U. aurantiaco-atra (sorediate)	236–1		JF283514	DQ235524	JF283480
U. ciliata	276–1	New Zealand, Otago. *A. Marky* 49924 (OTA)	JF283539	DQ235475	JF283504
U. ciliata	287–1	New Zealand, Otago. *K. Spencer* 58829 (OTA)	JF283540	DQ235476	JF283505
U. ciliata	N5	New Zealand, Canterbury, *Printzen* 9531 (FR)	**JF283541**	**JF283511**	**JF283506**
U. lambii	251–2		EF528390	EF492205	EF528469
U. lambii	167–1		EF528376	EF492191	EF528455
U. lambii	212–1		EF528362	EF492179	EF528442
U. messutiae	209–1		EF528393	EF492208	EF528472
U. messutiae	66		EF528372	EF492187	EF528451
U. messutiae	204–4		EF528359	EF492176	EF528439
U. pallidocarpa	193–2		EF528333	DQ235512	EF528413
U. pallidocarpa	193–5		EF528334	EF492154	EF528414
U. pallidocarpa	210–2		EF528338	EF492157	EF528418
U. patagonica	63	Ecuador, Chomborazo. *Z. Palice* 2614 (F)	JF283530	DQ235487	JF283495
U. patagonica	266–2	Peru, Parque Nacional Huascaran. *N. Wirtz, H. T. Lumbsch* 19364i & *A. Ramirez* (F)	JF283531	DQ235486	JF283496
U. patagonica	191–3	Argentina, Santa Cruz. *N. Wirtz* PA-2 & *M. I. Messuti* (F)	JF283532	DQ235485	JF283497
U. perpusilla	227–4		EF528348	DQ235491	EF528428
U. perpusilla	208–19		EF528327	DQ235490	EF528407
U. perpusilla	221–2		EF528343	EF492161	EF528423
U. sphacelata	243–2		JF283521	DQ235494	JF283487
U. sphacelata	117–1		JF283522	DQ235492	JF283488
U. sphacelata	225–1		JF283523	DQ235495	JF283489
U. subantarctica	205–1		JF283524	DQ235505	JF283490
U. subantarctica	265–1		JF283525	JF283509	JF283491
U. subantarctica	165–2		JF283526	DQ235508	JF283492

TABLE 1. *Continued*

Species name	Sample code	Voucher	IGS	ITS	*RPB1*
U. subcapillaris	283–1	New Zealand, Otago. *D. J. Galloway* 53636 (OTA)	JF283536	DQ235477	JF283501
U. subcapillaris	286–1	New Zealand, Otago. *K. Spencer* 58829 (OTA)	JF283537	DQ235478	JF283502
U. subcapillaris	N13	New Zealand, Canterbury, *Printzen* 9531 (FR)	**JF283538**	**JF283510**	**JF283503**
U. trachycarpa	182–3		JF283527	DQ235497	JF283493
U. trachycarpa	197–3		JF283528	DQ235498	JF283494
U. trachycarpa	173–1		JF283529	DQ235496	EF179793
U. ushuaiensis	179–1		EF528395	EF492210	EF528474
U. ushuaiensis	179–4		EF528397	EF492212	EF528476
U. ushuaiensis	258–1		EF528350	EF492167	EF528430

lichenized fungi (Crespo & Pérez-Ortega 2009; Crespo & Lumbsch 2010). Our three-gene data set did not allow us to resolve the backbone of the phylogeny within the main group of neuropogonoid lichens. This is probably due to the low amount of variable characters in our data set (*c.* 85% of the characters in the alignment are constant). Currently, a major challenge of DNA sequence-based approaches to species delimitation and phylogeny of groups of closely related species is the lack of fungal specific primers for fast-evolving loci. Recently, primers for two protein-coding genes have been published (Schmitt *et al.* 2009) with great potential to resolve phylogenetic relationships. It has been demonstrated that they outperformed other commonly used loci at higher phylogenetic levels (Aguileta *et al.* 2008), but there are no data at hand to determine if these genes allow more robust phylogenetic estimates for closely related taxa.

Although phylogenetic analyses strongly suggest that dark pigmentation of cortex and/or apothecial discs has evolved several times independently in the genus *Usnea*, there has not been any study to test whether this character state can be reversed, and there is also no clear picture on how often the pigmentation has evolved. These issues need to be addressed using a much larger taxon sampling, including more species groups of non-neuropogonoid species of *Usnea*.

The results of our phylogenetic analyses require two nomenclatural changes; the combination of *Neuropogon lambii* into *Usnea* (the reference to "*Usnea lambii* Imshaug" in Elix *et al.* 2007 was erroneous) and the description of *U. messutiae* as a new species (Fig. 2), both of which are proposed below.

Taxonomy

Usnea lambii (Imshaug) Wirtz & Lumbsch comb. nov.

MycoBank no.: MB 561368

Basionym: *Neuropogon lambii* Imshaug, *Rhodora* **61:** 154 (1954).

Usnea messutiae Wirtz & Lumbsch sp. nov.

MycoBank no.: MB 561369

A *Usnea pallidocarpa* soralis punctiformis continentes et apotheciis destitis differt.

Typus: Argentina, Santa Cruz, *c.* 30km N of El Chalten, N of National Park 'Los Glaciares', Cerro Creston, above Laguna del Desierto, below Glaciar Huemul, *c.* 800 m alt, 9 December 2003, *N. Wirtz & M. I. Messuti* PA-1 (F—holotypus).

(Figs 2 & 3)

Thallus approx. 3–4 cm tall, arising from a proliferating, mostly unpigmented holdfast with main branches tapered towards the holdfast; erect, shrubby and richly branched with terete branches and usually one stronger

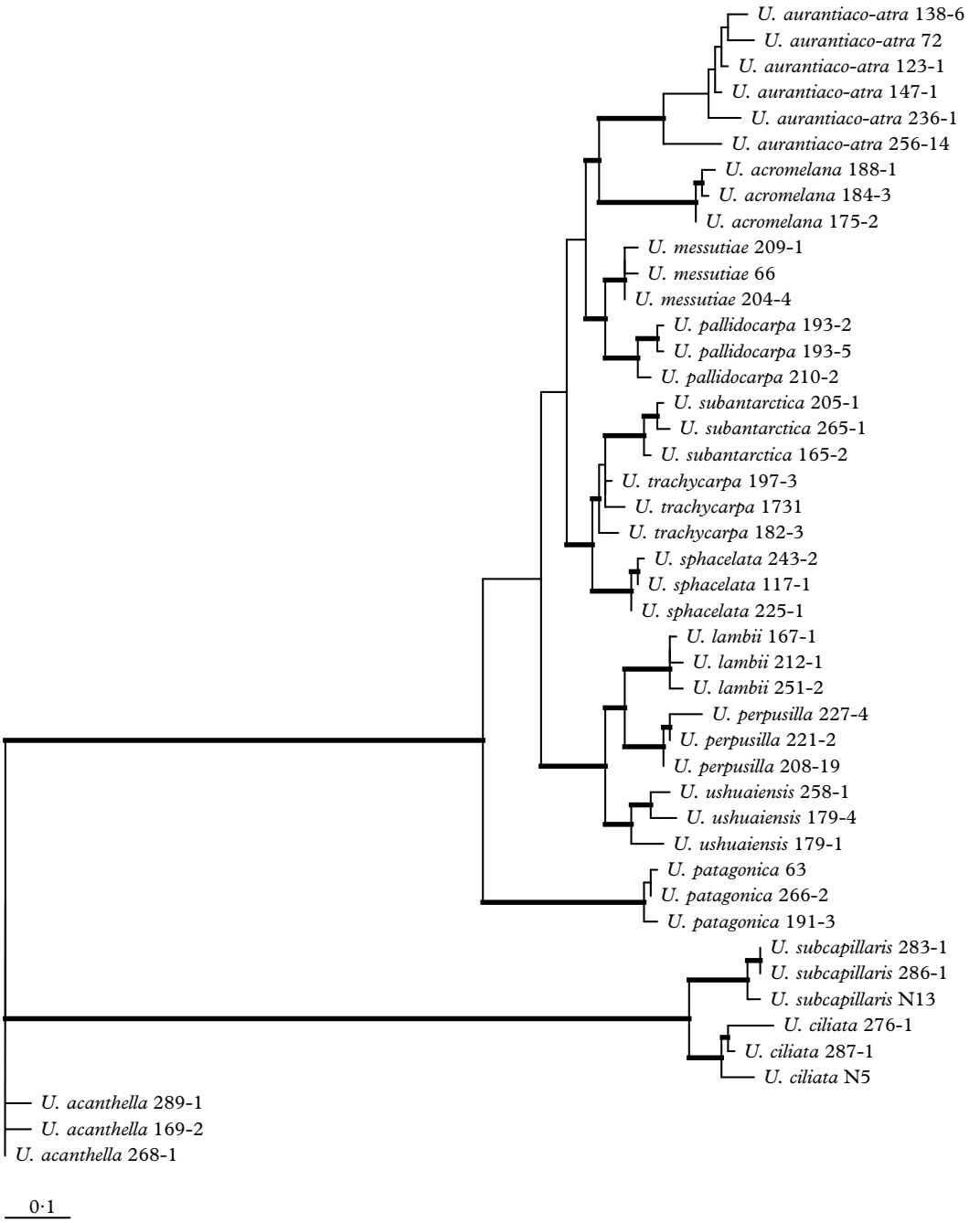

FIG. 1. Phylogenetic relationships of the neuropogonoid core group in *Usnea* as inferred from a concatenated alignment of nuclear ribosomal IGS and ITS DNA, and *RPB1* sequences. This is the optimal tree under maximum likelihood. Branches in bold received likelihood bootstrap support values above 70% and posterior probabilities equal to or above 0·95.

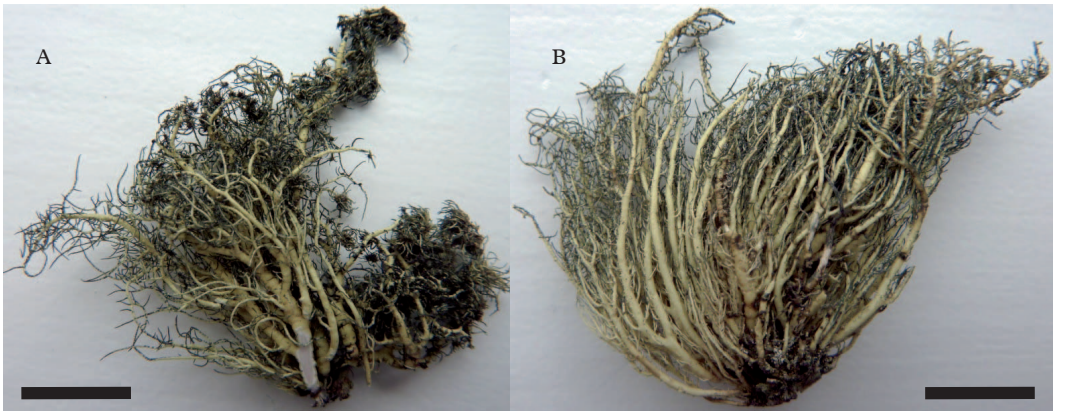

FIG. 2. *Usnea messutiae*, habit (holotype, F). A & B, thalli. Scales: A & B = 1 cm. In colour online.

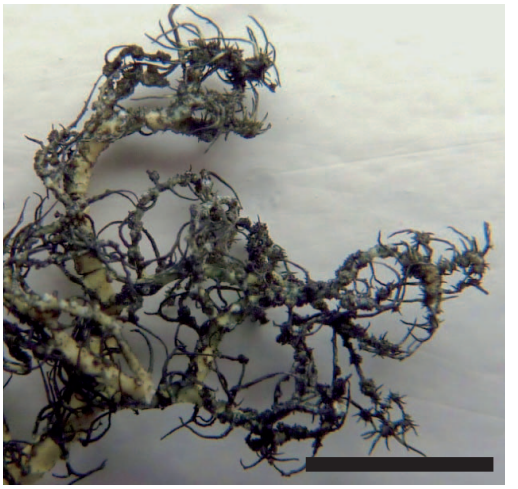

FIG. 3 *Usnea messutiae*, soralia and isidiomorphs (holotype, F). Scale 0·5 cm. In colour online.

ramifying main branch, sometimes eventually bifurcating at the tip; thallus surface yellow-green, foveolate, rarely smooth; main branches unpigmented, side branches unpigmented or sparsely variegated with bands of black pigment or entirely black towards the tips. *Cortex* annulations common, sometimes pigmented. Papillae rare; unpigmented and black pigmented fibrils common. Medulla dense. *Axis* thick, (35)–47–(60)% of branch diam. *Soralia* punctiform, hemispherical, convex, fusing, mostly on tips of side branches and developing from fibril scars. *Isidiomorphs* frequent, mainly blackish, sometimes growing into fibril-like structures, developing within soralia.

Apothecia not seen.

Pycnidia not seen.

Chemistry. ±Hypostrepsilic acid chemosyndrome (Elix *et al*. 2007), ±unidentified substance, and usnic acid.

Etymology. The epithet honours our friend and colleague, the Argentinean lichenologist Maria Ines Messuti (Bariloche).

Distribution and habitat. Known from a few localities in the Andean Cordillera in southern South America (Argentina) and Ecuador. It is an alpine species found on rocks at higher altitudes about 800 m in southern South America in communities with *U. aurantiaco-atra*, *U. acromelana*, *U. subantarctica*, *U. trachycarpa* and *U. sphacelata* and at high altitudes of about 5000 m in Ecuador in a community with *U. sphacelata*.

Usnea messutiae (referred to as *U.* sp. 2 in Wirtz *et al*. 2008) is characterized by a mostly unpigmented dense thallus with pigmented tips, fibrils, soralia and isidiomorphs. Its thallus surface is rough. Soredia are mostly convex and frequently growing into isidiomorphs and longer fibril-like structures. The new species is similar to *U. subantarctica*, but is distinguished by a more dense growth

form, almost no papillae, a dense medulla and thick axis. It is also similar to *U. acromelana* because of pigmented thallus annulations, dark soralia and a thick axis, but distinguished by a rough, ornamented thallus surface with faveoles and an unpigmented, proliferating holdfast.

Additional specimens examined. **Argentina:** *Rio Negro*: Cerro Catedral, 1850 m alt., *N. Wirtz & M .I. Messuti* PA-24 (F).—**Ecuador:** Chimborazo, volcano, alt. 4900 m, *Z. Palice & Soldán* 4291 (F); Chimborazo, alt. 4600 m, *Z. Palice* 2614 (F).

This study was financially supported by a grant from the German Science Foundation (DFG) to HTL and a DAAD scholarship to NW. We are grateful to Maria Ines Messuti (Bariloche), who organized a field trip through Patagonia to Tierra del Fuego, and to Christian Printzen (Frankfurt) for collecting lichen samples in New Zealand for this project.

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Accepted for publication 08 *May* 2011