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Phylogenetic and morphological analysis of Antarctic lichen-forming *Usnea* species in the group *Neuropogon*

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Abstract: *Usnea* species of the *Neuropogon* group are amongst the most widespread and abundant macrolichens in Antarctic regions. Four principal species, *U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata* and *U. subantarctica*, have been described on morphological grounds. However, identification to species level is often difficult and atypical morphologies frequently arise. Over 400 specimens were collected on the Antarctic Peninsula and Falkland Islands. Both morphological and molecular characters (ITS and *RPB1*) were used to compare samples to clarify taxonomic relationships. Morphological characteristics used included presence of apothecia, apothecial rays, soredia, papillae, fibrils, pigmentation and the diameter of the central axis as a proportion of branch diameter. Results revealed a very close relationship between *U. antarctica* and *U. aurantiaco-atra*, suggesting that they might constitute a species pair or be conspecific. *Usnea sphacelata* was comprised of at least two genetically distinct groups with no clear differences in morphology. One group included the first reported fertile specimen of this species. *Usnea subantarctica* was phylogenetically distinct from the other main Antarctic *Usnea* species, but clustered with *U. trachycarpa.* Genetic variation was evident within all species although there was no clear correlation between geographic origin and genetic relatedness. Phylogenetic analyses indicated that species circumscription in the *Neuropogon* group needs revision, with the principal species being non-monophyletic. None of the morphological characters, or groups of characters, used in this study proved to be completely unambiguous markers for a single species. However, axis thickness was supported as being informative for the identification of monophyletic lineages within the group.

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Key words: Ascomycota, bipolar, lichens, Parmeliaceae, species circumscription, taxonomy

Introduction

Lichens constitute the principal components of the terrestrial biota on seasonal ice free terrain in the Antarctic (Longton 1988, Øvstedal & Smith 2001). Amongst the most widespread and abundant macrolichens are species of *Usnea* of the *Neuropogon* group (Parmeliaceae, lichenized Ascomycetes). Members of this grouping are characterized by several unique features that, in combination, distinguish them from other *Usnea* species - such as the presence of black pigment in the upper thallus, a dark brown apothecial disc and a mainly saxicolous habitat (Walker 1985, Øvstedal & Smith 2001, Ohmura & Kanda 2004). There has been debate over the exact taxonomic status of the *Neuropogon* group based on molecular and morphological data (Ohmura 2002, Articus 2004, Ohmura & Kanda 2004, Wirtz *et al*. 2006). It has generally been recognized as a subgenus within *Usnea* (e.g. Lamb 1964, Walker 1985) but Ohmura & Kanda (2004) proposed that the *Neuropogon* group instead be recognised as a distinct section within the subgenus *Usnea*. Articus (2004) on the other hand suggested accepting the *Neuropogon* group as a separate genus, which was rejected by Wirtz *et al.* (2006). The distribution of the group is restricted principally to the high Andes and southernmost South America, the Falkland Islands, Australasia and Antarctica, with the greatest abundance and species diversity occurring in ice free areas of the Antarctic Peninsula, where *Usnea* species may develop stands covering a few to several hundred hectares (Walker 1985, Øvstedal & Smith 2001, Ott 2004).

Walker (1985) has produced the most recent comprehensive account of *Neuropogon* in which she summarizes preceding systematic research. She recognises 15 morphological species of which four are locally co-dominant macrolichens in continental Antarctica and the Antarctic Peninsula (*U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata* and *U. subantarctica*) (Walker 1985, Øvstedal & Smith 2004) (Fig. 1). *Usnea antarctica* is reported to have the widest distribution of any Antarctic macrolichen (Øvstedal & Smith 2001); it is circumpolar with its main centre of abundance on the Antarctic Peninsula, and extends into the sub-Antarctic islands, southernmost South America, the Falkland Islands and the

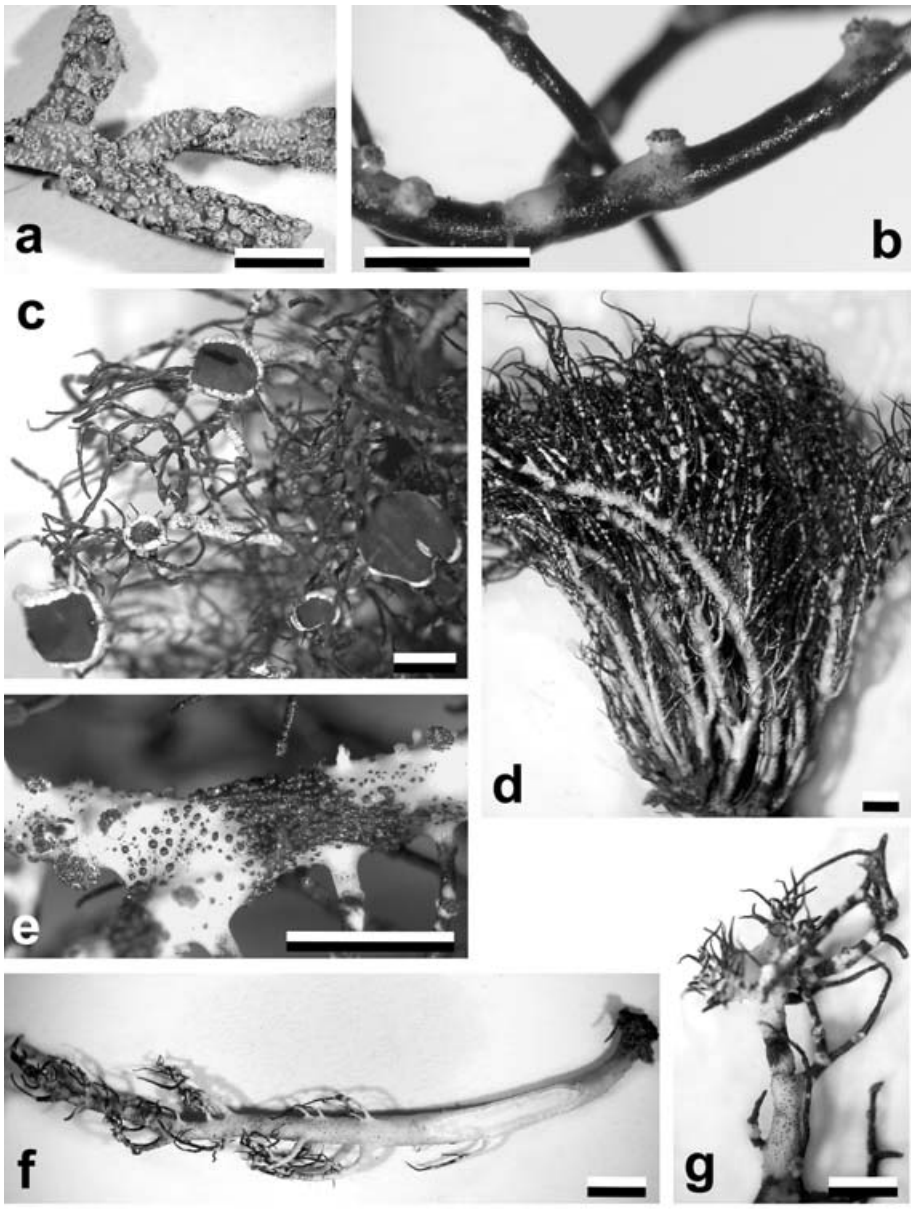

Fig. 1. Characteristic morphological attributes of *Usnea* (*Neuropogon* group) species studied. *U. antarctica* thallus with papillae and **a.** plain soralia, and **b.** 'stalked' soralia, **c.** *U. aurantiaco-atra* thallus with black apothecia, **d.** *U. sphacelata* growth habit and **e.** thallus detail with black papillae and emerging soralia, **f.** *U. subantarctica*, growth habit of sorediate specimen and **g.** detail of fertile specimen with small black papillae and an apothecium with marginal rays. Scale bars = 5 mm.

South Island of New Zealand. The species may exhibit early developmental features favouring success in extreme environments (Ott 2004). *Usnea aurantiaco-atra* has a less ubiquitous distribution than *U. antarctica* being absent from continental Antarctica and New Zealand, but present in the Falkland Islands, sub-Antarctic islands, southernmost South America and the western coasts of the Antarctic Peninsula (Lamb 1964, Walker 1985, Øvstedal & Smith 2001). *Usnea sphacelata* is the only known bipolar species in the group. It has a circumpolar distribution in both the Antarctic and the Arctic, the former extending into the Antarctic Peninsula and southern South America, and is also found in the northern Andean regions of South America and the South Island of New Zealand (Thompson 1984, Goward 1999, Walker 1985, Øvstedal & Smith 2001). *Usnea subantarctica* is found in southernmost South America and on the Antarctic Peninsula. Two further species are found in southern South America and the sub-Antarctic islands, but have more restricted distributions on the Antarctic Peninsula: *Usnea acromelana* (also found in Australasia) occurs infrequently on the west coast and northernmost tip, and *U. trachycarpa* is only known from the south-west region (Walker 1985, Øvstedal & Smith 2001). Remaining members of the subgenus are not found in Antarctic regions, but instead have distributions in South America and Australasia (Walker 1985).

Despite the visual prominence and numerical importance of *Neuropogon* in Antarctica, identification to species level is often difficult owing to pronounced variability in certain species. Morphological variability is typical of the genus as a whole, which is universally regarded to be difficult and poorly understood (e.g. James *et al*. 1992, Clerc 1998,

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Table I. Typical morphologies of the principal Antarctic species of *Usnea* studied in the present work (*U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata*, *U. subantarctica* and *U. trachycarpa*) as described by Walker (1985) and Øvstedal & Smith (2001).

	U. antarctica	*U. aurantiaco-atra*	*U. sphacelata*	*U. subantarctica*	*U. trachycarpa*
Axis[1]	Thick axis (>50%)	Thick axis (>50%)	Thin axis (20–40%)	Thin axis (30–50%)	Thin axis (30–50%)
Soralia	Numerous, over the whole thallus	Absent	Numerous, usually confined to ultimate branches	Numerous, usually confined to ultimate branches	Absent
Papillation	Primary branches grossly papillate (unpigmented)	Verrucose to grossly papillate above	Minute, often pigmented papillae	Small pigmented papillae	Foveolate to richly papillate above
Fibrils	Rare	Rare	Usually absent	More or less extensive.	Numerous on all branches
Pigmentation[2]	± variegated above, ± continuously pigmented towards apices	± variegated above, ± continuously pigmented towards apices	Conspicuously variegated above or continuously pigmented towards apices	Variegated or continuously black towards apices	± Continuously pigmented towards apices
Apothecia rays colour	Rare, subterminal Not present Black/brown	Frequent, subterminal Rare Black to pale yellow	Not seen Not applicable Not applicable	Rare Present Pink or yellow to dark brown	Present Present Pink to brown
Geographical range	Circumpolar Antarctic; main centre of distribution around the Antarctic Peninsula. Also New Zealand (South Island), southernmost South America, Falkland Is.	Widespread and abundant in most habitats though less ubiquitous than *U. antarctica*. Found in southernmost South America, Falkland Is. Sub-Antarctica: South Georgia, Bouvetøya. In Antarctica: South Orkney Is, South Shetland Is, Antarctic Peninsula.	Only known bipolar species of the subgenus. Arctic distribution is almost circumpolar. Known from Greenland, Iceland, Svalbard, Franz Joseph Land, Novaya Zemlya, Jan Mayen, and a few islands of Arctic Canada. Also: South America, New Zealand (South Is). In Antarctica: Antarctic Peninsula (southwards from *c*. 65ºS on west coast, but extending to northern tip at 63ºS on east coast. Continental Antarctica.	Southernmost South America. In Antarctica: Antarctic Peninsula (mainly northern Trinity Peninsula and adjacent east coast islands, northern Marguerite Bay). The species is absent from continental Antarctica and the islands of the Scotia Arc, but may eventually be found elsewhere in the sub-Antarctic regions.	Southern South America, Falkland Is. Sub-Antarctica: Heard Is, Îles Kerguelen, In Antarctica: South Orkney Is, South Shetland Is (King George Is, Admiralty Bay, Ullman Point), south-western Antarctic Peninsula (especially islands in northern Marguerite Bay, Charcot Is).

[1]See text for formula. [2]Black to violaceous black pigment.

Articus *et al.* 2002) and in which most of the described species seem to be connected by a continuous array of transitional forms (Clerc 1998). *Neuropogon* species have been distinguished mainly by general habit of the thallus, mode of branching, pigmentation, surface ornamentation, branch anatomy, morphology and frequency of soralia, papillae, fibrils and apothecia, and chemistry (Walker 1985, Øvstedal & Smith 2001). Walker (1985) notes that published descriptions of *Usnea* (subg. *Neuropogon*) spp. have sometimes proved misleading, especially when used to identify specimens collected near the limits of a species range where atypical morphologies frequently ariseBoth Walker (1985) and Øvstedal & Smith (2001) report immature specimens of *U. sphacelata* and *U. subantarctica* to be morphologically indistinguishable, especially on the Antarctic Peninsula. The close morphological similarities amongst Antarctic *Neuropogon* are further illustrated by Walker's (1985) suggestion that *U. aurantiaco-atra* and *U. antarctica*, *U. perpusilla* (a South American species) and *U. sphacelata*, and *U. trachycarpa* and *U. subantarctica* might each constitute a 'species pair' of a fertile primary species and a derived sterile, sorediate secondary species (Poelt 1970). Putative species pairs have been identified elsewhere in several lichen genera (e.g. Tehler 1982, Mattson & Lumbsch 1989, Poelt 1994). Indeed, there is continuing debate about how lichen species in general are defined, what characters should be used and their relative diagnostic values in taxonomic analysis. The dual nature of lichens, the difficulty of obtaining axenic cultures and the current impossibility of making experimental crosses makes discussing lichen species concepts difficult in the context of modern species definitions (e.g. see Clerc 1998, Bridge & Hawksworth 1998, Grube & Kroken 2000, Taylor *et al.* 2000). Most lichen taxonomy still relies on morphological, chemical and geographical information. Only recently have molecular biological techniques been applied to the taxonomy of lichens and in particular to the genus *Usnea* (e.g. Ohmura 2002, Articus 2004, Ohmura & Kanda 2004, Wirtz *et al.* 2006). To date, there have been no detailed studies using molecular data to address species relationships within the *Neuropogon* group.

Here we attempt to clarify taxonomic relationships between the four principal Antarctic species of *Usnea* (subgenus *Neuropogon*): *U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata* and *U. subantarctica*. Both morphological and molecular characters were used to compare a large collection of specimens sampled from a wide geographic range from the Antarctic Peninsula and the Falkland

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Table II. Details of collection sites for *Usnea* subgenus *Neuropogon* species and numbers of specimens collected at each. Species identifications were based on morphological characters according to Walker (1985) and Øvstedal & Smith (2001).

Location	*U. acromelana*	*U. antarctica*	*U. aurantiaco-atra*	*U. sphacelata*	*U. subantarctica*	*U. trachycarpa*	Total no. of specimens collected per site
Mars Oasis, Alexander Island, Antarctica, 71°52'S, 68°15'W	0	0	0	37	66	0	103
Rothera Research Station and local islands, Ryder Bay, Antarctica 67°33'–36'S, 68°04'–20'W (Anchorage Island, Lagoon Island, Léonie Island , Killingbeck Island, Reptile Ridge, Rothera Point)	1	195	3	27	63	2	291
Jubany Research Station, King George Is., Antarctica, 62°14'S, 58°38'W	0	3	4	0	0	0	7
Livingston Island, Antarctica, 62°37'-40'S, 61°05'–32'W	0	1	2	0	0	0	3
Tierra del Fuego, Lapataia Bay, Argentina, 54°50'S, 68°26'W	0	1	0	0	0	1	2
Stanley, Falkland Islands, South Atlantic, 51°45'S, 57°56'W	0	0	10	0	0	3	13
Chimborazo, Ecuador, 51°20'S, 75°05'W	0	0	0	2	0	0	2
Svalbard, Spitzbergen, Norway, 79°17'–18'N, 16°04'E	0	0	0	2	0	0	2
Total number of specimens collected for each species	1	200	19	68	129	6	423

Islands, and to a lesser extent from southern South America. Typical morphological characteristics and distributions of the species under consideration in this paper are given in Table I. Morphological characters selected for analysis were those used by Walker (1985) and Øvstedal and Smith (2001) to distinguish the four species. Molecular analysis is based on DNA sequences from the RNA polymerase II largest subunit (*RPB1*) gene and internal transcribed spacer (ITS) region of the ribosomal RNA-encoding gene unit, as used elsewhere in phylogenetic studies (e.g. Matheny *et al.* 2002, DePriest 2004) and in assembling the Fungal Tree of Life (Spatafora *et al.* 2006).

Materials and methods

Collection, identification and morphological analysis

Specimens of *Usnea* subgenus *Neuropogon* were collected on the Falkland Islands and at four locations on the Antarctic Peninsula between November 2001 and March 2002. Additional specimens were subsequently collected from Tierra del Fuego, Ecuador and Svalbard during 2003. Details for each location are shown in Table II. Thalli were removed from rocks by cutting through the holdfast with a scalpel. Samples were air dried and a preliminary identification made using the key to *Usnea* subgenus *Neuropogon* provided by Øvstedal & Smith (2001). An

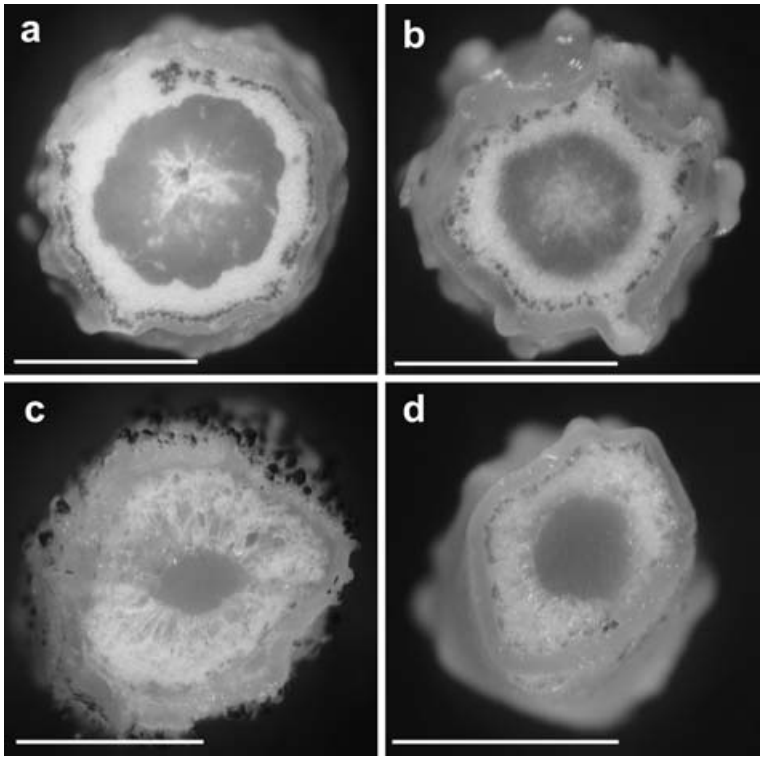

Fig. 2. Transverse sections of main branches of *Usnea* (*Neuropogon* group) species studied. **a.** *U. aurantiaco-atra*, **b.** *U. antarctica*, **c.** *U. subantarctica*, **d.** *U. sphacelata*. The proportion of the branch diameter occupied by the central axis is 61, 56, 27 and 39%, respectively. Scale bars = 1 mm.

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Table III. Details of *Usnea* species selected for DNA analysis.

Species	Specimen number	% Axis[1]*	Sorediate	Papillate	Fibrilate	Apothecia present	Apothecia rays	Apothecia colour	Morphology[2] Description	Group	Location[3]
U. antarctica	39, 66, 67, 68, 72, 76, 130, 137-2, 148-1, 154	(63)–*70*–(78)	+	+	--	--	--		Typical	A	RP, RP, RP, RP, RP, RP, Lé, Li, TF, LH
U. antarctica	115, 125	68, 72	+?	+	--	+	--	Black	*U. aurantiaco-atra*-like apothecia	B	Lé, Lé
U. antarctica	78, 79	65, 67	+	+	+?	--	--		Fibrilate?	C	RP, RP
U. aurantiaco-atra	107-2, 205, 208, 210, 211, 120-5, 124-1	(62)–*71*–(81)	--	+	+?	+	--Brown/black		Typical	D	FI, FI, JB, JB, JB, Li, Li
U. aurantiaco-atra (*U. sphacelata*)[4]	212	40	+?	+	+	+	--	Black	Sorediate? Fertile *U. antarctica*?	E	RP
U. aurantiaco-atra	214	66	+?	+	--	+	--	Black	Sorediate? Fertile *U. antarctica*?	E	RP
U. sphacelata	62, 115-8, 222, 263	(28)–*32*–(34)	+	+	--	--	--		Typical	F	Ec, Sv, RR, MO
U. sphacelata (*U. acromelana*)[4]	233	51	+	--	--?	--	--		Wrinkled. Papillate? Difficult morphology	G	RP
U. sphacelata (*U. subantarctica*)[4]	277	21	+	--?	--?	--	--		Wrinkled. Papillate? Difficult morphology	G	MO
U. sphacelata	275, 276, 280	(22)–*25*–(27)	+	--?	--?	--	--		Wrinkled. Papillate? Difficult morphology	G	MO, MO, MO,
U. sphacelata	42, 118-1	49–50	+	+	--	--	--		Axis *c.* 50%	H	Ec, Sv
U. sphacelata	269, 274	23–26	+	--?	--?	--	--		*U. acromelana* type annulations.	I	MO, MO
U. subantarctica	293, 305, 309, 310	(18)–*34*–(46)	+	+	+	--	--		Typical	J	RR, RP, RP, RP
U. subantarctica	165-1	34	+?	+?	+?	+	+	Pink	*U. trachycarpa*-like apothecia	K	La
U. subantarctica	284	27	+	+	+	+	+Brown/orange		*U. trachycarpa*-like apothecia	K	RR
U. subantarctica	286, 287, 288	(16)–*27*–(33)	+	+	+	+	+	Pink	*U. trachycarpa*-like apothecia	K	RR, RR, RR
U. subantarctica	73	33	+	+	--	--	--		Possibly *U. sphacelata*? Weathered thalli.	L	MO
U. subantarctica	290	27	+	+	+	--	--		Possibly *U. sphacelata*? Weathered thalli.	L	RR
U. trachycarpa	173-1	46	--	--	+	+	+	Pink	Typical	M	TF
U. trachycarpa	411	68	--	+	+	+	+	Pink	Abnormal axis	M	FI
U. trachycarpa	164-1	34	--	--	--	ND	ND		Probably *U. sphacelata*. Weathered thalli	N	La

[1]Minimum, maximum and mean (italicized) value are given for groups of three or more samples (*see below).
[2]Based on descriptions in Walker (1985) and Øvstedal & Smith (2001).*% axis values for all specimens collected in this investigation are as follows (minimum and maximum in parentheses, mean ± 95% confidence interval in italics): *Usnea antarctica* (47) *71 ± 1* (87), *n* = 198, number of specimens with values < 50% = 2; *U. aurantiaco-atra* (44) *63 ± 6* (81), *n* = 15, number of specimens with values < 50% = 3; *U. sphacelata* (16) *32 ± 2* (60), *n* = 66, number of specimens with values > 50% = 2; *U. subantarctica* (14) *31 ± 1* (58), *n* = 128, number of specimens with values > 50% = 2.
[3]Ec = Ecuador, FI = Falkland Islands, JB = Jubany Base, La = Lagoon Island, Lé = Léonie Island, Li = Livingston Island, MO = Mars Oasis, RP = Rothera Point, RR = Reptile Ridge, Sv = Svalbard, TF = Tierra del Fuego; locations are listed sequentially with respect to specimen numbers (see Table II for details of collection sites).
[4]These specimens were originally determined as *U. aurantiaco-atra* 212 (redetermined as *U. sphacelata* 212), *U. sphacelata* 233 (redetermined as *U. acromelana* 233) and *U. sphacelata* 277 (redetermined as *U. subantarctica* 277). New identifications were based on molecular data and a subsequent and more thorough morphological and chemical analysis. Characters followed by '?' were difficult to determine. 'ND' denotes not determined (due to loss in transport).

attempt was made to collect specimens with a diverse range of thallus morphologies including fertile and infertile specimens. Lichens were stored at -20°C prior to, during and following transport back to the UK until required for further morphological and molecular analysis.

Species determinations were later confirmed or otherwise changed based on the detailed descriptions of Walker (1985). In addition to collections of the four main Antarctic *Neuropogon* species, one specimen of *U. acromelana* and six specimens of *U. trachycarpa* were also among the collections and included in the analysis. Using a stereo dissecting microscope (Nikon SMZ1000), the presence or absence of fibrils, papillae, soralia and pigmentation were recorded for each thallus (Fig. 1). Where apothecia were present, the colour of the apothecial disk and the presence or absence of apothecial rays were recorded. Cross-sections of the thallus branches were examined and the diameters of both the central axis (a) and the whole branch (b) were measured twice along radii at 90 degrees to each other and the axis value expressed as a percentage of the whole (i.e.

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a/b x 100) (see Walker 1985, Hancock & Seppelt 1988) (Fig. 2). Where possible, measurements were further replicated using 2–3 different branches per thallus. Specimens of each species were sorted into groups according to whether they had a 'typical', or an 'atypical' thallus morphology according to descriptions in Walker (1985). Representative thalli of *U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata* and *U. subantarctica* from each morphological sub-group as well as the *U. acromelana* and *U. trachycarpa* samples were selected for further DNA analysis (Table III).

Voucher specimens for each morphological species according to Walker (1985) and Øvstedal & Smith (2001) have been deposited in BM with the following specimen numbers: F. Seymour 79 & 125 for *U. antarctica*; F. Seymour 211 & 214 for *U. aurantiaco-atra*; F. Seymour 269 & 274 for *U. sphacelata*; F. Seymour 284, 288, 290 & 293 for *U. subantarctica*.

Chemical analysis

Lichen compounds were determined in selected specimens by thin layer chromatography (TLC) using the same protocols as Øvstedal & Smith (2001).

DNA extraction

Samples for DNA analysis were thoroughly rinsed in sterile water to remove grit or organic debris then freeze dried and stored at -80°C. DNA was extracted directly from whole thalli using a DNeasy® Plant Maxi Kit (Qiagen) according to the manufacturer's instructions. The integrity and yield of DNA was checked by electrophoresis with lambda DNA standards followed by staining with ethidium bromide (Andrews 1991).

PCR amplification and sequencing of ITS region and RPB1

Amplification of the ITS1-5.8S-ITS2 region was performed using the primer pair ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). ITS1-F is specific to higher fungi, hybridizing to a sequence at the 3' end of the small subunit (SSU) rRNA gene (Gardes & Bruns 1993) whilst ITS4 is a universal primer that hybridizes to the 5' end of the large subunit (LSU) rRNA gene (White *et al.* 1990). Amplification of part of the *RPB1* gene was performed using the degenerate primer pair gRPB1-Af (5'-GADTGTCCDGGDCATTTTGG-3') and fRPB1-Cr (5'-CNGCDATNTCRTTRTCCATRTA-3'), which were slightly modified by B.D. Hall and N. Wirtz (personal communication 2004) from those described by Matheny *et al*. (2002).

PCR was performed using cycle parameters modified from White *et al.* (1990) and Matheny *et al.* (2002). Amplification reactions contained 200 µmol each of dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies), 2.5 µl of a 10 fold buffer containing $MgCl_2$, 1 unit of FastStart HiF Taq polymerase (Roche), 50 pmol of each primer, 1–10 ng genomic DNA and ultra pure water (Sigma) to a final volume of 25 µl. An initial denaturation step of 95ºC for 5 min was followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min (ITS region) or 56ºC for 1 min (*RPB1*) and 72ºC for 1 min before an elongation stage of 72ºC for 5 min. PCR products were gel purified by electrophoresis in 1.6% agarose gels, then slices containing the PCR fragment were excised and DNA separated from the agarose using the QIAEX II Gel Extraction Kit (Qiagen) according to the manufacturers instructions. Sequencing reactions were performed by MWG Biotech (Edersberg, Germany) using the same primer sets utilized in PCR experiments.

Phylogenetic analysis

Two approaches were used to infer relationships between taxa. Multiple sequence alignments of the combined ITS and *RPB1* DNA sequence data were generated using the CLUSTALW program (Thompson *et al.* 1994). Gaps were excluded and the arising alignments were analysed by maximum likelihood (ML) and Bayesian inference approaches. ML analysis was performed using the program PAUP*4.0s (Swofford 2000). A heuristic search of 200 random taxon addition replicates was conducted with TBR branch-swapping and the MulTrees option in effect. A 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 was used (GTR+I+G). Separate analyses of the two datasets were performed. Since no hard conflict (supported by at least 70% bootstrap support) was evident, it was assumed that datasets were congruent and hence a combined analysis was performed. Nonparametric bootstrap (Felsenstein 1985) was used to assess robustness of clades, running 200 pseudoreplicates with the same settings as in the heuristic search. Only clades that received bootstrap support equal to or above 70% were considered as strongly supported.

The program MrBAYES 3.1.2 (Huelsenbeck & Ronquist 2001) was employed to sample trees using a Bayesian inference Markov Chain Monte Carlo (B/MCMC) method. The analyses were performed assuming the general time reversible model of nucleotide substitution (Rodríguez *et al.* 1990) including estimation of invariant sites, 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. Parallel runs with 2 000 000 generations starting with a random tree and employing eight simultaneous chains each were executed. Every 100th tree was saved into a file. The first 200 000 generations (i.e. 2000 trees) were deleted as the "burn in" of

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the chains. 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) to ensure that stationarity was achieved after the first 200 000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). Of the remaining 18 000 trees from both runs (i.e. 36 000 trees) a majority rule consensus tree with average branch lengths was calculated using the "sumt" option of MrBayes. Posterior probabilities were obtained for each clade. Posterior probabilities equal to and above 95% were considered strong supports.

Any specimen that appeared to have been wrongly identified on morphological grounds, given the results of the phylogenetic analyses, was subsequently re-examined and tested chemically using thin-layer chromatography as described by Walker (1985).

Results

Distribution

A total of 423 specimens of *Usnea* from the *Neuropogon* grouping were collected primarily from four main locations on the Antarctic Peninsula (Mars Oasis, Rothera Research Station, Jubany Base, Livingston Island) and from the vicinity of Stanley, East Falkland Island. Additional specimens were collected from South America and Svålbard in the Arctic for comparative purposes (Table I). Details of collection sites, species distributions and numbers of specimens collected are given in Table II. The number of samples obtained at any one site reflects the time available for collecting at that location. *Usnea antarctica*, *U. subantarctica* and *U. sphacelata* were the most frequently sampled species (94%) (Table II). Despite the disparity between sites in the number of samples collected, there is some evidence that the species have different distributions. For example, at the most southerly location (Mars Oasis) only *U. sphacelata* and *U. subantarctica* were collected suggesting that the other species are either absent or occur less frequently. At Rothera, all six species were present; *U. sphacelata* and *U. subantarctica* were still well represented (31%), but *U. antarctica* was the most frequently collected species (67%). In addition, a specimen (233) originally thought to be *U. sphacelata* was later confirmed as *U. acromelana* (Table III). On moving northward to Jubany and Livingston Island, the most southerly occurring species *U. sphacelata* and *U. subantarctica* were no longer encountered. Finally, *U. aurantiaco-atra* and *U. trachycarpa* were the only species collected from the Falkland Islands.

Morphological analysis

A subset of 50 representative specimens was selected for further morphological analysis (Table III). Morphological groups A, D, F, J and M are 'typical' for *U. antarctica*, *U. aurantiaco-atra*, *U. sphacelata*, *U. subantarctica* and *U. trachycarpa*, respectively. *Usnea antarctica* groups B and C contain specimens that were considered best placed in *U. antarctica* but have intermediate characteristics. Group B contains specimens that have *U. aurantiaco-atra*-like apothecia and ± poorly developed soralia. Although these specimens were placed in a separate morphological group, Walker (1985) notes that *U. antarctica* is known to produce apothecia, if only rarely, in locations experiencing optimum conditions; these apothecia are similar in appearance to those of *U. aurantiaco-atra* in terms of colour and morphology. Specimens in Group C had fibril-like branches developing from some papillae while the wide axis (≥ 65%) and lack of apothecia were inconsistent with *U. subantarctica* or *U. trachycarpa*, and the abundant soralia were inconsistent with *U. aurantiaco-atra*.

Usnea aurantiaco-atra group E includes sorediate specimens that on morphological grounds might alternatively be fertile specimens of *U. antarctica*. The presence of soredia in *U. aurantiaco-atra* has not previously been recorded. Within this group, *U. aurantiaco-atra* specimen 212 was later shown to be *U. sphacelata* following molecular analysis (see below) and TLC (usnic acid only); again, this is the first record of a fertile specimen of this species. The intermediate *U. sphacelata* specimens in groups G, H and I, all have atypical and highly variable morphologies. The specimens in group G had highly wrinkled and cracked thalli on which the presence or absence of papillae was difficult to determine. *Usnea sphacelata* 233 (usnic, salazinic and norstictic acids) and 277 (usnic acid) were later shown to be *U. acromelana* and *U. subantarctica*, respectively, as a result of a separate molecular analysis and TLC. Fertile specimens of *U. subantarctica* were designated a distinct group (Group K). In agreement with Walker (1985), such fertile specimens were encountered infrequently and were observed to have *U. trachycarpa*-like apothecia with respect to colour and morphology. The absence of soredia in *U. trachycarpa* (group M) distinguishes it from fertile *U. subantarctica*.

Group L contains specimens that were difficult to classify due to extensive weathering to the thallus and they exhibited morphologies with elements of both *U. subantarctica* and *U. sphacelata* e.g. absence of, or poorly developed fibrils, and narrow axis. The specimen assigned to group N was not fertile at the time of analysis as the apothecia had been destroyed during transport.

Molecular analysis

DNA yields for the 50 specimens analysed (Table III) typically ranged between 20–200 ng μl^{-1} (0.4–4 µg total) genomic DNA per 100 mg dry weight thallus tissue (data not shown). Single amplicons of approximately 550 bp and

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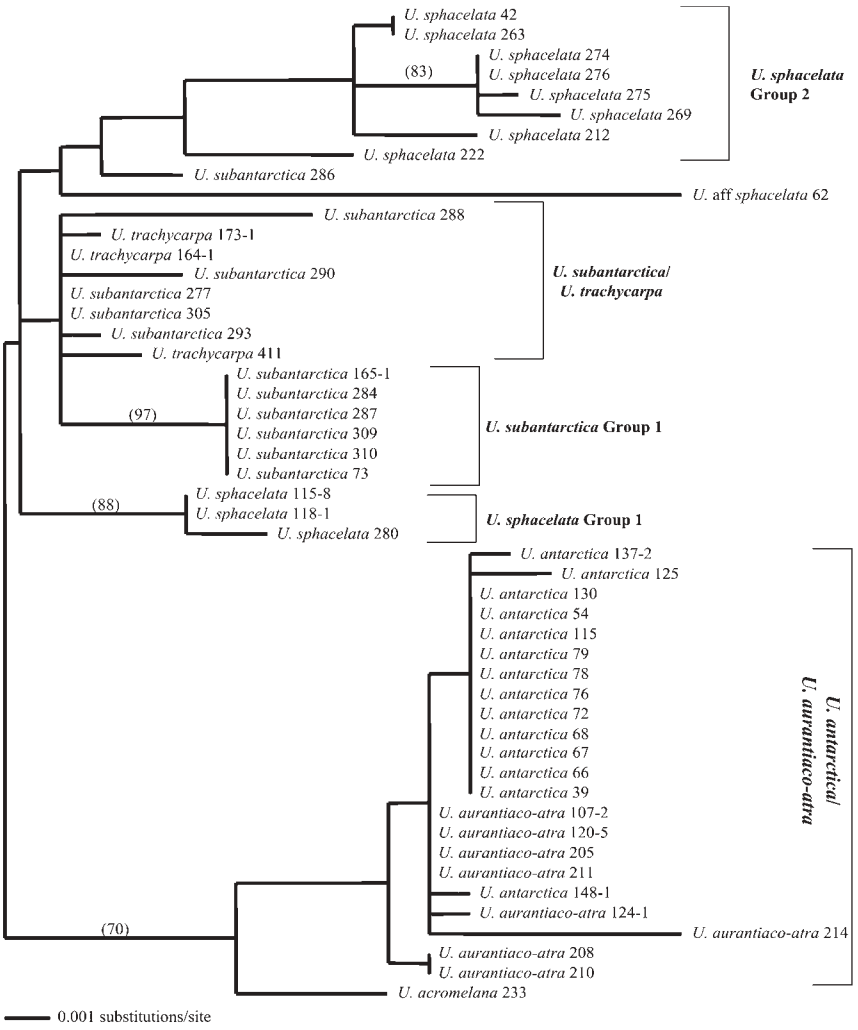

Fig. 3. Phylogenetic tree showing relationships between *Usnea* species based on molecular data, with tree derived from maximum likelihood analysis of combined ITS region and *RPB1* gene sequence data. Numbers at each node indicate bootstrap support (200 pseudoreplicates), only values ≥ 70% are shown.

800 bp were successfully amplified for the ITS1-5.8S-ITS2 rDNA region and the *RPB1* gene, respectively, for all 50 specimens. After alignment and exclusion of gaps the ITS region yielded 452 characters of which 53 were variable (11.7%), whilst the *RPB1* fragment yielded 671 characters of which 19 were variable (2.8%). The resulting sequences were combined and aligned to produce a matrix of 1123 unambiguously aligned nucleotide position characters with a total of 72 variable positions.

Phylogenetic trees derived from the ML and Bayesian analyses shared some characteristics, although differences in clustering were apparent for a subset of samples. Five main groups could be recognised in both analyses (Figs 3 & 4). The *U. antarctica*/*U. aurantiaco-atra* group contained all of the *U. antarctica* and *U. aurantiaco-atra* specimens in a well-supported monophyletic clade (ML and Bayesian analyses). Within this clade almost all *U. antarctica* formed a distinct sub group, although bootstrap analysis did not provide support for the distinction between *U. antarctica* and *U. aurantiaco-atra*. The majority of the *U. antarctica* specimens (mainly from the vicinity of Rothera Point) had identical sequences. Greater variation was seen among the *U. aurantiaco-atra* specimens. *Usnea antarctica* 148-1 from Tierra del Fuego bore soredia but was located among the *U. aurantiaco-atra* specimens. Thallus 233 had originally been identified as *U. sphacelata* but was found to be most closely related to the *U. antarctica*/*U. aurantiaco-atra* group. Subsequent morphological analysis and further molecular analysis with a larger population of *Neuropogon* species revealed this specimen to be *U. acromelana* (N. Wirtz *et al*. unpublished results). The thickness of the axis (cf. Table III) turns out to be a reliable character for determining this species.

Both methods of phylogenetic analysis showed that *Usnea sphacelata* was polyphyletic, dividing into at least two distinct groups (Figs 3 & 4). Group 1 was strongly supported by bootstrap analysis and posterior probabilities and contained three *U. sphacelata* specimens. Interestingly, two of these specimens were collected from the Arctic (115-8 and 118-1) and the other from Mars Oasis in the Antarctic (280). A second group contained a strongly supported sub-group of four specimens from Mars Oasis (269, 274, 275 and 276), the most southerly collection site, together with three further samples (212, 263 and 42). Two of the latter

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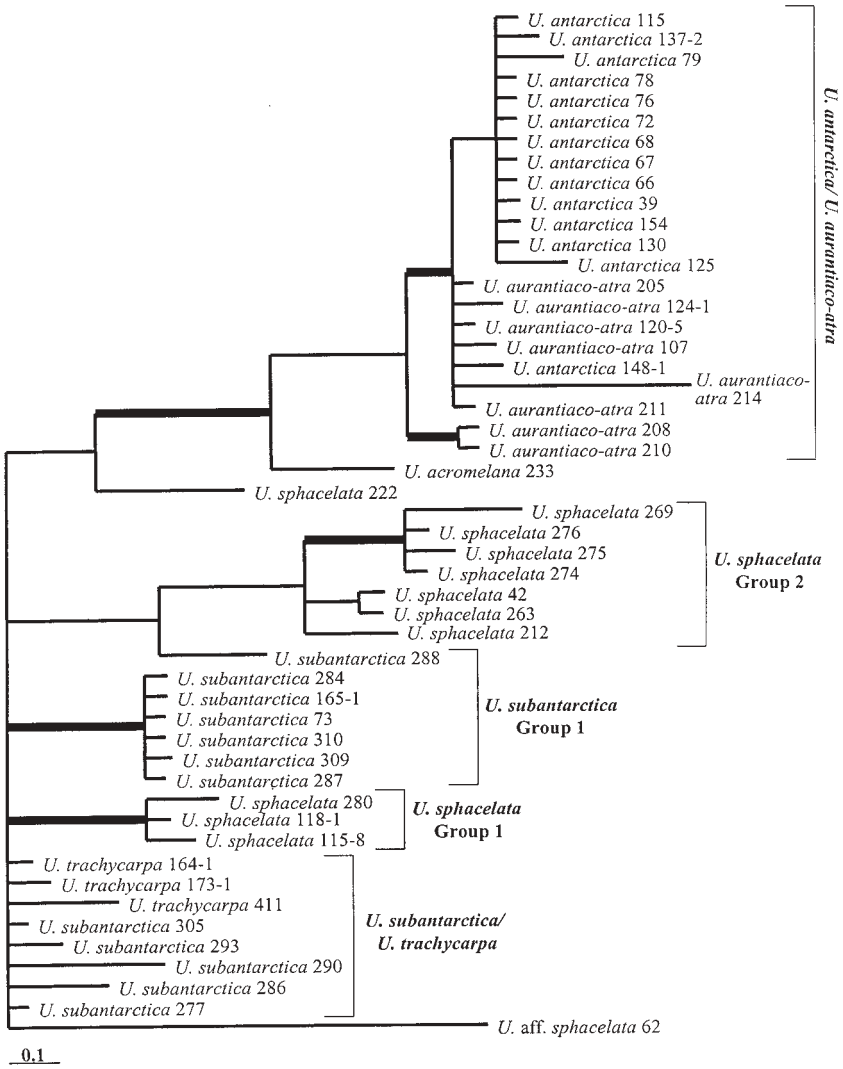

Fig. 4. 50%-majority-rule consensus tree showing relationships between *Usnea* species, based on 36 000 trees from a B/MCMC tree sampling procedure from combined ITS region and *RPB1* gene sequence data. Posterior probabilities ≥ 0.95 are indicated as bold branches.

specimens, one from Mars Oasis (263) and one from Ecuador (42) had identical sequences. Specimen 212 had originally been identified as a fertile *U. aurantiaco-atra* specimen but was found to cluster within the *U. sphacelata* group 2.

The fourth group consisted of a strongly supported subset of six *U. subantarctica* specimens from different geographic origins on the Antarctic peninsula (73, 165-1, 284, 287, 309, 310). These specimens had identical ITS nrDNA and RPB1 sequences. Finally, a loose grouping was evident containing the remaining *U. subantarctica* specimens together with three samples of *U. trachycarpa*. Interestingly, two *U. subantarctica* specimens (277 and 305) shared identical ITS and *RPB1* sequences with a specimen of *U. trachycarpa* (164-1). A single specimen (62) from Ecuador, identified on morphological grounds as *U. sphacelata*, appeared as an outgroup in both analyses and was named *U.* aff. (affiliated to) *sphacelata*. This specimen is likely to be assigned to *Usnea patagonica*, a South American species. Sequence data from representative isolates of the five main groups have been deposited at Genbank under the accession numbers: DQ235496, DQ767952–DQ767965 and EF179795–EF179806 for the ITS region; DQ658417, EF179782–EF179794 and EF193046–EF193058 for RPB1 .

Although the overall tree topologies resulting from the maximum likelihood and Bayesian analyses were similar, there were some notable exceptions. *Usnea sphacelata* sample 222 was placed near to the *U. sphacelata* group 2 according to maximum likelihood, but was allied to the *U. antarctica*/*U. aurantiaco-atra* group in the Bayesian analysis. Similarly, *U. subantarctica* specimen 288 was placed within the broad *U. subantarctica*/*U. trachycarpa* grouping according to maximum likelihood, but was allied to *U. sphacelata* group 2 in the Bayesian analysis.

Discussion

Phylogenetic analysis based on DNA sequence data has been used to great effect to resolve issues of lichen taxonomy over recent years. This has included investigation of support for class and order relationships, assessment of

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whether genera are monophyletic or paraphyletic, and evaluation of the taxonomic status of closely related taxa (e.g. Lohtander *et al*. 1998; Martin *et al*. 2000, Tehler & Källersjö 2001, Articus *et al.* 2002, DePriest 2004, Miadlikowska & Lutzoni 2004, Ott *et al.* 2004, Reeb *et al*. 2004, Schmitt & Lumbsch, 2004, Divakar *et al.* 2005). In the present study we used a phylogenetic approach, combined with thorough morphological studies, to clarify taxonomic relationships between the four principal Antarctic species of *Usnea*. Analysis of DNA sequences is especially valuable because it overcomes problems encountered with morphological characters that are subject to environmental influence and are thus less reliable, as seen particularly in morphologically variable *Usnea* species (Clerc 1998, Øvstedal & Smith 2001). We used both ITS region and *RPB1* gene sequence data, noting that the ITS data showed a relatively higher number of variable positions. The phylogenetic analyses provided various novel insights into the taxonomy of *Neuropogon* species in Antarctica as described by Walker (1985).

Firstly, both the maximum likelihood and Bayesian analyses demonstrated a very close relationship between *U. antarctica* and *U. aurantiaco-atra*. The species formed a single well-supported grouping. Within this most *U. antarctica* samples formed a distinct cluster, including four morphologically problematic 'intermediate' forms. However, one *U. antarctica* sample from Tierra del Fuego was placed among the *U. aurantiaco-atra* samples. These results add support to the suggestion that *U. antarctica* and *U. aurantiaco-atra* might constitute a species pair, with *U. aurantiaco-atra* representing the fertile non-sorediate form and *U. antarctica* the sterile sorediate form (Walker 1985), or indeed they might constitute a single species i.e. be conspecific. Molecular data have elsewhere shown that the putative species pair *U. florida* and *U. subfloridana* (subgenus *Usnea*) formed one monophyletic group of intermixed specimens suggesting that they were morphological variants of a single species (Articus *et al.* 2002). A similar situation has been described for the lichens *Umbilicaria kappenii* and *Umbilicaria antarctica*, and it was suggested that these be treated as the single species *Umbilicaria antarctica* (Ott *et al.* 2004). However, sequencing of further loci is needed in order to resolve the detailed relationship between *U. antarctica* and *U. aurantiaco-atra,* before confirmation of synonymy might allow these species to be formally united.

Secondly, *Usnea sphacelata* was shown to comprise at least two genetically distinct subsets (groups 1 and 2 in Figs 3 & 4). Since both groups contain a mix of specimens considered to have typical and intermediate morphologies for this species, it appears that distinction on morphological grounds alone will be difficult, if not impossible. Group 1 is bipolar suggesting either that evolution in *U. sphacelata* has been slow following pole-ward migration of Group 1 populations from common refugia during de-glaciation, or that long-distance dispersal might have occurred relatively recently with insufficient time for significant differentiation, or that transport of propagules between Antarctica and the Arctic occurs relatively frequently. Thomson (1984), in his discussion of the bipolar distribution of *U. sphacelata*, points out that south polar skuas occasionally migrate into the Arctic (see Salomonsen 1976); Arctic terns and Wilson's storm petrels are other potential vectors (e.g. Alerstam 1990, Montalti & Soave 2002). Significantly, Group 2 of *U. sphacelata* included a specimen (212) that had originally been identified as *U. aurantiaco-atra* based partly on presence of apothecia, but was reassigned to *U. sphacelata* on molecular grounds. This placement was later confirmed by chemotyping. If this specimen is indeed *U. sphacelata*, it is the first ever reported fertile specimen of this species. However, the identity of *Usnea sphacelata* s.str. requires further study and one of us (NW) is currently working on this problem.

The fourth main species of study, *U. subantarctica*, was shown to be phylogenetically distinct from the other principal Antarctic *Usnea* species, including *U. sphacelata* to which it bears a very close resemblance in the field (Øvstedal & Smith 2001). Both sterile specimens and specimens bearing apothecia were found in one strongly supported sub-clade from the Antarctic Peninsula, confirming that the species may rarely undergo sexual reproduction (Walker 1985). Three specimens of *U. trachycarpa* were also included in the phylogenetic analysis. Intriguingly, these were found to cluster within a broader *U. subantarctica* grouping. This lends support to the suggestion by Walker (1985) that *U. trachycarpa* and *U. subantarctica* might also represent a species pair. However, comparison with further isolates of *U. trachycarpa* will be necessary before such conclusions may be drawn.

Finally, there were also other general observations from the data obtained. Genetic variation, as judged by limited sequence divergence, was evident within all of the species although there was no clear correlation between geographic origin and extent of genetic relatedness. The presence of genetic variation in coding regions might be of importance to allow response to environmental change in the Antarctic region (Seymour *et al.* 2005b, Wasley *et al.* 2006). Limited genetic variation was also detected within populations of the Antarctic lichens *Buellia frigida* and *Umbilicaria decussata* (Dyer & Murtagh 2001, Romeike *et al.* 2002). One factor contributing to genetic variation might be sexual reproduction involving outcrossing. For the species *U. antarctica* and *U. subantarctica* both fertile (i.e. bearing apothecia) and sterile specimens were found at the same localities. These species have previously been described as rarely fertile (Walker 1985). The precise reasons for lack of sexuality in some specimens are unclear, but it is possible that conditions are unfavourable for sex in certain environments, and/or these species might exhibit

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heterothallic (obligate outbreeding) breeding systems requiring the presence of a compatible mating partner for sex to occur (Seymour *et al.* 2005b). A heterothallic breeding system has recently been demonstrated in the Antarctic lichen *Cladonia galindezii* and related temperate species of *Cladonia* (Seymour *et al*. 2005a), although homothallic (self fertile) breeding systems are evident elsewhere in lichens (Murtagh *et al.* 2000, Honegger *et al.* 2004).

The phylogenetic analysis suggests that none of the morphological characters, or groups of characters, used in this study are completely unambiguous markers for a single species. The most reliable character was the diameter of the central axis relative to the branch diameter. With few exceptions, thalli in which the relative axis diameter was ≤ 50% belong to the putative species pair *U. aurantiaco-atra* /*U. antarctica* (Table III). These species are then best separated by a combination of the presence of abundant soralia (*U. antarctica*; cf *U. aurantiaco-atra* in which soralia are absent or poorly developed) or frequent to abundant apothecia (*U. aurantiaco-atra*; cf *U. antarctica* in which apothecia are absent or infrequent). Thalli with a relative axis diameter ≥ 50% belong to either *U. sphacelata* or the putative species pair *U. trachycarpa*/*U. subantarctica*. *Usnea trachycarpa* is then separated on the basis of lack of soralia and presence of apothecia (typically pink and with rays) while *U. subantarctica* is best distinguished from *U. sphacelata* by the presence of fibrils. Note that while axis diameter was found to be a sound distinguishing character in the present study, Hancock & Seppelt (1988) found significant overlap in this character in the 45–55% range between *Usnea sphacelata* and *U. antarctica* in the Windmill Islands.

In summary, the phylogenetic analyses indicate that species circumscription in the *Neuropogon* group needs revision, as also discussed by Wirtz *et al.* (2006). *Usnea aurantiaco-atra, U. antarctica, U. sphacelata, U. subantarctica* and *U. trachycarpa* were non-monophyletic, with *U. aurantiaco-atra* being paraphyletic and basal to most specimens of *U. antarctica*, whilst *U. sphacelata* was found to be polyphyletic. However, the phylogenetic analyses also supported some morphological characters as described by Walker (1985), such as axis thickness, as informative for the identification of monophyletic lineages within the group. The phylogenetic groupings based on the molecular data were consistent for most samples with those suggested by the morphological data, including samples with both typical and intermediate characters. Thus, an apparently sorediate form of *U. aurantiaco-atra* (214), epapillate and annulated variants of *U. sphacelata* (269, 274), and fertile specimens of *U. subantarctica* with apothecial rays (165-1, 284, 286, 287, 288) all had been named correctly according to the DNA analysis. However, according to the molecular analyses 6% of isolates had been incorrectly assigned based on morphological data. All of these samples (212, 233 and 277) had certain morphological features that were difficult to determine. This emphasises the utility of molecular data as a means to resolve taxonomic uncertainties and confirm species identifications made in field studies. Despite this there was difficulty in assigning some specimens based on molecular data and it is conceivable that occasional interspecies hybridization might occur in the field. While morphology appears to be powerful in identification and separation of some species, unrelated monophyletic groups may share a similar morphology (e.g. *U. sphacelata* groups 1 and 2). On the other hand some morphologically distinguishable taxa are not clearly separated in the molecular analyses (e.g. *U. antarctica* vs *U. aurantiaco-atra*). Further work is now needed to clarify the exact status of species pairs within the *Neuropogon* group and to extend analyses to include specimens from a much wider geographic region of sampling.

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