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rDNA ITS and β-tubulin gene sequence analyses reveal two monophyletic groups within the cosmopolitan lichen *Parmelia saxatilis*

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A considerable number of species of lichen-forming fungi have wide geographical distributions, but studies of their genetic variability are minimal. ITS rDNA sequences of 32 populations of *Parmelia saxatilis* from five continents revealed two monophyletic groups. β-tublin gene sequences from a subset of nine collections supported these conclusions. While the number of collections sequenced is limited, one monophyletic group (the Atlantic Population. AtP) was recognized as occurring in Arctic and Antarctic regions and also included collections from more atlantic sites. Samples from more mesic environments in the Mediterranean region belonged to a second monophyletic group (the Mediterranean Population, MeP). In addition, four subgroups were distinguishable within the Atlantic Population. Norstictic and protocetraric acids are reported from the species for the first time, the norstictic acid only being found in the Atlantic Population. Living thalli from the Atlantic Population were provenance-tested; specimens transported from the UK to central Spain where the Mediterranean Population occurs showed adverse symptoms after six months. These results demonstrate that there can be substantial large-scale genotypic variability within widespread lichen phenospecies, something which has implications for comparative ecological, physiological, and air pollution sensitivity studies as well as for lichen conservation.

INTRODUCTION

Particular lichen-forming fungi have long been recognized as having exceptionally wide global distributions, ranging from the Antarctic to the Arctic (Fries 1831, Hooker & Taylor 1844). While some of these have cosmopolitan patterns, others are restricted to high mountains (Walker 1985, Sato 1962, Hawksworth 1972), or are found only at high latitudes – and so are bipolar (Du Rietz 1940, Galloway & Aptroot 1995). Species concepts in these fungi are essentially based on morphology and consistency in secondary metabolites (Döring & Lumbsch 1998, Purvis 1997, Grube & Kroken 2000), but none has been extensively sampled and studied at the molecular level from throughout its global range. Molecular studies have already demonstrated the occurrence of cryptic phylogenetic species within phenotypically characterized lichen 'species' (Kroken & Taylor 2001) and infraspecific variation in some widespread lichens (Zoller, Lutzoni & Scheidegger 1999). Particular species have been found to exhibit molecular variation in material from disparate locations (Murtagh, Dyer & Crittenden 2000) or recolonizing under ameliorating ambient sulphur dioxide pollution conditions (Crespo *et al*. 1999), but no species occurring from pole to pole has previously been studied throughout its range.

One of the better known examples of a cosmopolitan lichen is *Parmelia saxatilis*. The morphological and chemical variation in this species has been regarded as minimal (Hale 1987, Hinds 1998), but some ecophysiological variation in relation to latitudinal gradients has been discovered (Schroeter 1991, Sancho *et al*. 2000) and a possible sulphur dioxide tolerant ecotype reported (Gilbert 1971). However, the extent of genotypic variation and the possibility of cryptic species within the phenospecies has not previously been addressed. A study at the molecular level is of particular interest as *Parmelia saxatilis* mainly reproduces asexually by isidia,

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Table 1. Specimens of *Parmelia* used in the study, with reference collection details and GenBank accession numbers.

Specimen no. and Species	Country	Collector	Reference collection accession no.	GenBank accession no. nrDNA	GenBank accession no. β-tubulin
1. *P. saxatilis*	C Spain (Madrid)	A. Crespo	MAF 6884	AF350037	
2. *P. saxatilis*	C Spain (Madrid)	A. Crespo	MAF 6893	AF350038	
3. *P. saxatilis*	WC Spain (Castilla y León)	A. Crespo *et al.*	MAF 6896	AF350039	
4. *P. saxatilis**	C Spain (Madrid)	A. Crespo	MAF 6885	AF530040	AF391142
5. *P. saxatilis*	UK (New Forest, S England)	D. L. Hawksworth	MAF 6886	AF350041	
6. *P. saxatilis*	UK (Peak District, N England)	A. Crespo	MAF 7031	AF350042	
7. *P. saxatilis*	Spain (Canary Islands, Tenerife)	A. Crespo & J. J. Crespo de Las Casas	MAF 6889	AF350044	
8. *P. saxatilis*	Spain (Canary Islands, Tenerife)	A. Crespo & J. J. Crespo de Las Casas	MAF 6890	AF350045	
9. *P. saxatilis*	Switzerland	R. Honegger	MAF 6891	AF350043	
10. *P. saxatilis*	UK (N England)	A. Crespo	MAF 6888	AF350035	
11. *P. saxatilis*	NW Russia (Pen. Kola)	M. Schlensog	MAF 6880	AF350023	
12. *P. saxatilis*	CW Spain (Castilla y León)	A. Crespo *et al.*	MAF 6887	AF350032	
13. *P. saxatilis*	CW Spain (Castilla y León)	A. Crespo *et al.*	MAF 6883	AF350024	
14. *P. saxatilis*	NE USA (New Hampshire)	P. May	May Coll 5375	AF350033	
15. *P. saxatilis*	NE USA (New Hampshire)	P. May	May Coll 5374	AF350034	
16. *P. saxatilis*	Austria (Tirol)	J. Hafellner	GZUHafellner 52306	AF350031	
17. *P. saxatilis*	Austria (Tirol)	J. Hafellner	GZUHafellner l52044	AF350036	
18. *P. saxatilis*	Switzerland	R. Honegger	MAF 6892	AF350030	
19. *P. saxatilis*	N Sweden (Umea)	S. Ott	MAF 6804	AF350027	
20. *P. saxatilis*	N Sweden (Umea)	S. Ott	MAF 6882	AF350028	
21. *P. saxatilis*	Antarctica (South Shetlands)	L. G. Sancho	MAF 6897	AF350020	
22. *P. saxatilis*	Antarctica (South Shetlands)	L. G. Sancho	MAF 6898	AF350021	
23. *P. saxatilis**	Antarctica (Leoni Is.)	B. Schroeter	MAF 6803	AF350022	AF391138
24. *P. saxatilis*	S Chile (Patagonia)	L. G. Sancho	MAF 6894	AF350025	
25. *P. saxatilis**	S Chile (Patagonia)	L. G. Sancho	MAF 6895	AF350026	AF391138
26. *P. saxatilis*†	E Canada (British Columbia)			AF141370	
27. *P. saxatilis**	C China (Altay)	T. Ahti	MAF 7030	AF350029	AF391139
28. *P. saxatilis**	NW Russia (Pen. Kola)	M. Schlensog	MAF 7276	AY036989	AF301136
29. *P. saxatilis**	NW Russia (Pen. Kola)	M. Schlensog	MAF 7273	AY036990	AF391135
30. *P. saxatilis*	C Spain (Extremadura)	A. Crespo *et al.*	MAF 7286	AY036996	AF391140
31. *P. saxatilis*	C Spain (Extremadura)	A. Crespo *et al.*	MAF 7287	AY036997	AF391141
32. *P. saxatilis*	S Spain (Cádiz)	A. Crespo *et al.*	MAF 7668	AY114359	AY114357
33. *P. fertilis*	Hokkaido (Tokio) Japan	H. Hamada	MAF 7282	AY036982	AF391131
34. *P. sulcata*	UK (Sheffield, N England)	A. Crespo	MAF 6054	AY114358	

C, Central; CW, Central West; NW, North-west; NE, North-east; S, South.

* Collections used in the chemical analyses.

† Sequence obtained from GenBank.

structures including the fungal partner and symbiotic alga (*Trebouxia*), although in moist temperate or oceanic habitats thalli are occasionally fertile (Molina & Crespo 2000). In contrast, fertile specimens producing apothecia are unknown from polar regions and mountains above the tree-line.

We examined the rDNA ITS sequences in 32 populations of *Parmelia saxatilis* from a wide range of locations and discovered a substantial amount of specialization at the molecular level within the species and that there were two main monophyletic groups, including one that occurs in ecologically extreme cold habitats pole to pole. In order to further test this result, we also sequenced the β-tublin gene in nine representative collections, and the Type I intron in 20 collections that exhibited this. Selected collections were also studied microchemically and observations made on some transplanted thalli.

MATERIAL AND METHODS

Lichen material

In all, 32 populations of *Parmelia saxatilis* from extreme habitats from the arctic to the antarctic, and also from high and lower altitudes in more temperate regions at intermediate latitudes, were sequenced (Table 1). The rDNA ITS region was studied in all (Figs 1–2), the β-tubulin gene also in nine (Figs 3–4), and the intron in 20 that possessed one (Fig. 5). Voucher specimens are preserved in the reference collections of the Departamento de Biologia Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid (MAF).

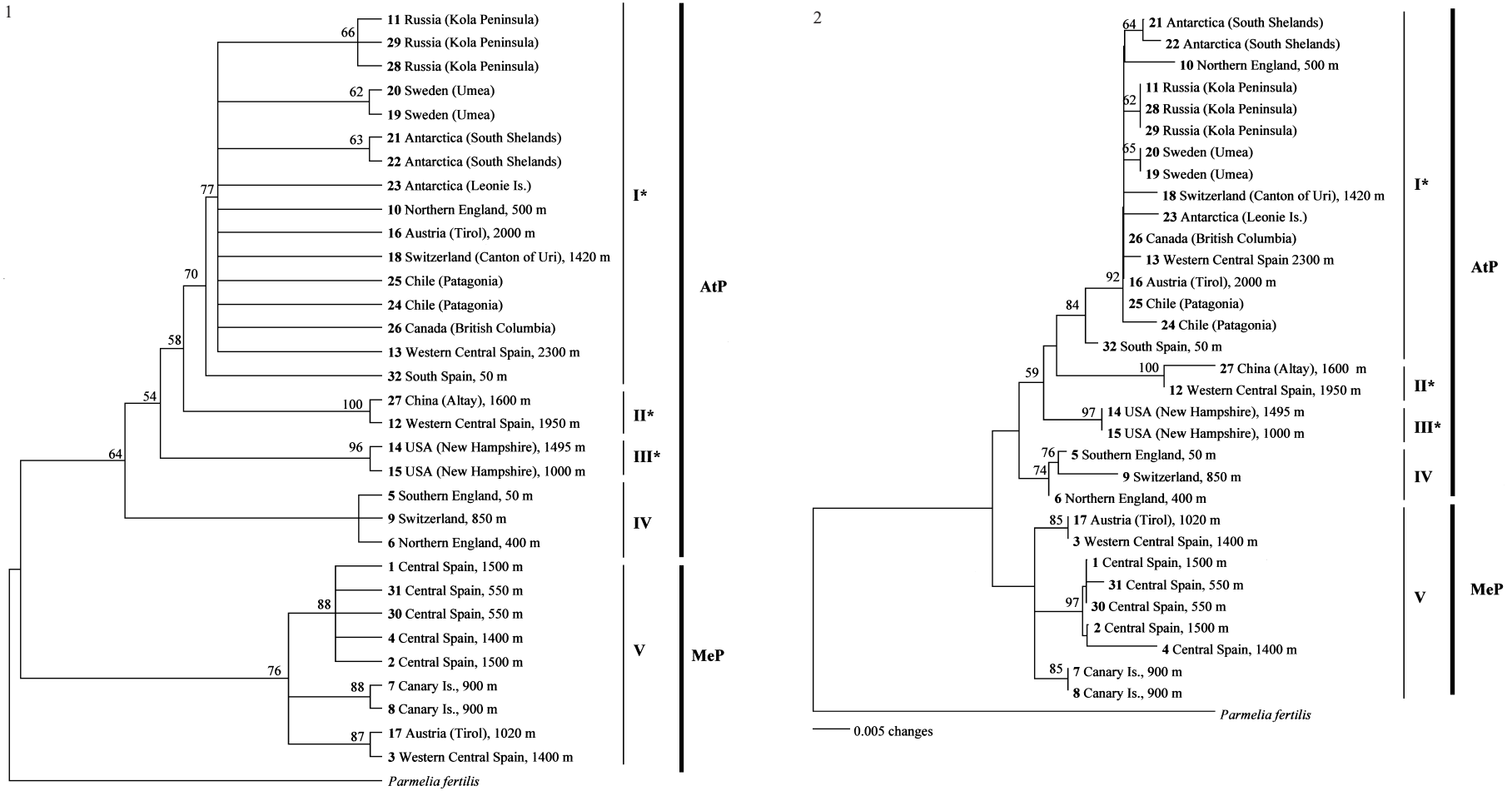
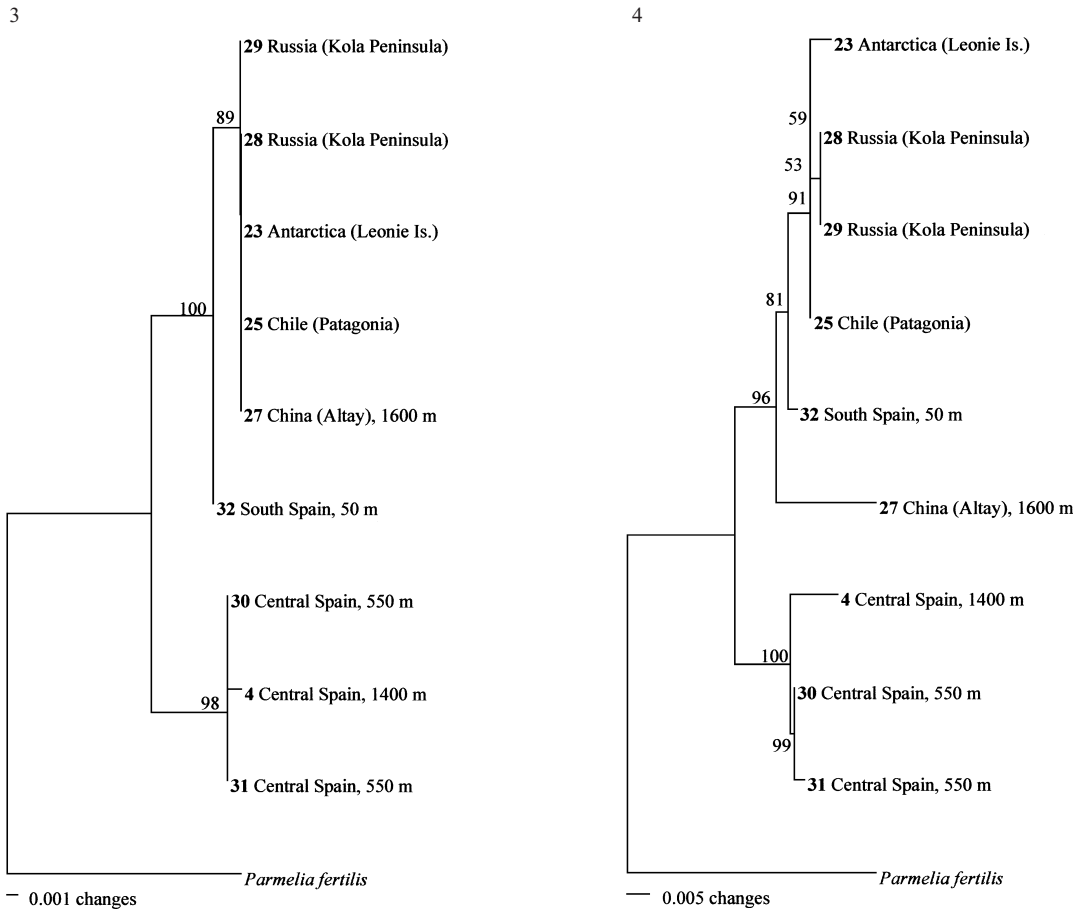


Fig. 1–2. Fig. 1. Parsimony tree based on ITS 1–5.8S-2 rDNA sequences. Bootstrap values over 50% are indicated.* Sequences with Type I intron in the position 1516 of the SSU rDNA. **Fig. 2.** Neighbour-joining distance tree based on ITS 1–5.8-2 rDNA sequences. Bootstrap values over 50% are indicated, and the bar indicates 0.005 Kimura-2 distance units.* Sequences with Type I intron in the position 1516 of the SSU rDNA.



Figs 3–4. Fig. 3. Comparison of ITS 1–5.8-2 rDNA and β-tubulin gene sequence data for a subset of the collections. **Fig. 3.** Neighbour-joining distance tree based on ITS1–5.8-2 rDNA sequences. **Fig. 4.** Neighbour-joining distance tree based on β-tubulin gene for the subset of collections in Fig. 3. In Figs 3–4, bootstrap values over 50% are indicated, and the bar indicates 0.001 and 0.005 Kimura-2 distance units respectively.

DNA extraction and PCR amplification

Total DNA was extracted using the Dneasy Plant Mini Kit (Qiagen) with minor modifications as described elsewhere (Crespo, Blanco & Hawksworth 2001). Amplification of the ITS region of the rDNA gene cluster was undertaken with the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). ITS1F is specific for fungi and located at the 3′ end of the SSU gene of the rDNA, whereas ITS4 has been described as a 'universal' primer and hybridizes at the 5′ end of the LSU gene (Gardes & Bruns 1993). Amplification of the partial β-tubulin sequence was carried out using Bt3-LM and Bt10-LM primers (Myllys, Lohtander & Tehler 2001).

Dilutions (1:10) of the total DNA were used for amplification. Amplification was carried out in 50 μl aliquots consisting of 5 μl 10× buffer (Biotools), 5 μl 25 mM $MgCl_2$, 1 μl dinucleotide triphosphate (dNTP) containing 10 mM of each base, 2.5 μl of each primer 10 μM, 1.25 μl of DNA polymerase (Biotools) and 8 μl of a 50-fold dilution of stock DNA. The PCR conditions were: 5 min at 94 °C; denaturation 1 min at 94 °, annealing 1 min at 54 ° (ITS region) or 1 min at 55 ° (partial β-tubulin gene), extension 2 min at 72 ° (35 cycles), and 5 min at 72 °. The PCR products were purified (Biotools' PCR purification kit) and sequenced by the PRISM dRhodamine Terminator Cyndle sequencing Ready Reaction kit (Applied Biosystems) with detection on a 373A stretch automated sequencing apparatus (Applied Biosystems). Complementary strands were compared with Windows SeqMan (DNAStar) to check for reading errors and alignment was optimised manually.

All data were analysed with PAUP* version 4.0 (Swofford 1999). For the ITS region, the total fragment was sequenced comprising the 3′ end of the small subunit gene, ITS 1, the 5.8S gene, ITS 2 and the 5′ terminus of the large subunit gene. The flanking regions of the SSU, LSU rDNA and the end of ITS 2 presented several ambiguities and were excluded from the analyses. A sequence matrix of 466 nucleotide positions was analyzed, and 38 parsimony- informative characters were detected. The Type I introns at the position 1516 SSU rDNA found in 20 populations investigated were also sequenced. A matrix of 163 characters was obtained.

The alignment of the partial β-tubulin gene sequences

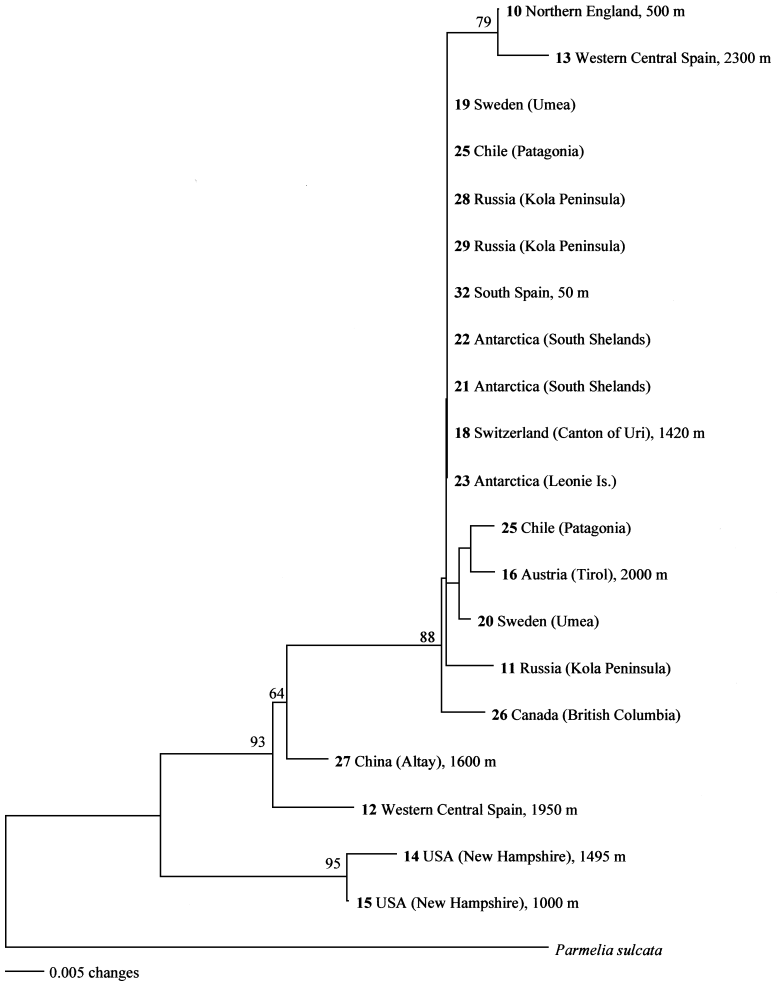


Fig. 5. Neighbour-joining distance tree based on Type I intron in position 1516 sequences in those collections with this intron. Bootstrap values over 50% are indicated, and the bar indicates 0.005 Kimura-2 distance units.

did not result in any gaps or ambiguous regions, and in this case a matrix of 780 nucleotide positions was analyzed, of which 37 positions were variable.

A maximum parsimony analysis was carried out. Because of the high number of trees generated, the option of saving 10000 trees was selected. Gaps were treated as missing values. The heuristic search option was used, employing random addition sequences and 100 replicates generated by the tree bisection reconnection (TBR) method of branch swapping and MulTrees option in effect. 1000 bootstrap replicates (Felsenstein 1985) were used for the identification of well supported monophyletic groups.

Four neighbour-joining trees based on Kimura 2 parameter distances were constructed, again with bootstrap percentages determined for 1000 resamplings of the data sets.

Parmelia fertilis was used as out-group for the ITS (Figs 1–3) and β-tubulin (Fig. 4) sequences, and *P. sulcata* for the intron analysis as that has a Type I intron in the same position, 1516 in the SSU (Fig. 5).

Transplant experiment

Two thalli of *Parmelia saxatilis* forming rosettes of 8 and 9.5 cm diam respectively growing on a single Millstone Grit (Carboniferous) boulder collected at an altitude of 85 m near Belper, Derbyshire (national grid reference SK(43)/ 347458) in the UK Peak District, were transported dry by road over four days from the UK to Mataelpino (Comunidad de Madrid) at 1080 m in the Sierra Guadarama of Central Spain, arriving on 9 August 2001. The boulder was placed in a position with modest shade comparable to that where it had been growing in the UK and where other parmelioid lichens were present on granite, and observed each month. During this period the temperature ranged from $-17\,^{\circ}$ to $+46\,^{\circ}$.

Phenolic composition

Secondary chemical compounds present in six samples (Table 1) were identified by thin layer chromatography according to the method described by Elix & Ernst-Russell (1993).

RESULTS

Molecular studies

The parsimony and neighbour-joining trees constructed

from the ITS sequences revealed a similar topology. Two main monophyletic groups within the phenospecies *Parmelia saxatilis* (Figs 1–2) were evident. The first (AtP) contained several subgroups (I–IV). Subgroup I included all the samples from the coldest regions: Arctic, Antarctica, Patagonia, and boreal and mountainous areas of temperate Europe and North America – but also one from a more mesic area (Table 1, collection 32). Subgroup II, the sister group of subgroup I, is a clade grouping one sample from the Altay region of China (1600 m, collection 27) and another from the west of the mountains of the Sistema Central in Spain (1950 m, collection 12). The two last terminal subgroups clustered samples from both sides of the Atlantic Ocean, one from the mountains in temperate eastern North America (subgroup III, collections 14–15), and a sister group of three from oceanic areas in western Europe (subgroup IV, collections 5–6, and 9).

The sequences in the AtP subgroups I, II and III all had a Type I intron (Gargas, DePriest & Taylor 1995, Thell 1999) at position 1516 in the SSU. The genetic distance among the 15 samples in subgroup I from arctic and antarctic regions was minimal (Fig. 2), and these collections were the sister group of a single sample (collection 32) in this subgroup that came from a warm but atlantic locality.

The second monophyletic group, MeP (Figs 1, 2, subgroup V) included samples from southern Europe and North Africa (the Canary Islands) growing in essentially Mediterranean climates. None of the collections in subgroup V contained an intron in the SSU region.

In the parsimony analysis, all five subgroups had bootstrap values over 50%, and the two main groups AtP and MeP held with values of 64% and 76% respectively.

The resulting topology from the ITS analysis was tested by additional sequences. Partial β-tubulin sequences of nine samples gave identical results to those from ITS trees of the same collections (Figs 3–4). A genetic distance tree based on the intron sequences of 20 collections in subgroups I, II and III also gave the same result (Fig. 5), with collections from the most extreme environments having a bootstrap value of 88% with scarcely any genetic difference between those from the coldest areas.

Transplant studies

The material remained a typical healthy cover until November 2001 and then started to deteriorate, central parts of the thalli becoming discoloured pinkish and breaking away from the rock. By February 2002, most of the central parts of the thalli had fallen away, and by April some of the marginal areas had also been lost, although fragmented grey marginal crescents 6–15 mm wide remained. The material was left to follow any further changes or recovery that might occur.

Chemical studies

All six collections contained atranorin, chloroatranorin, protocetraric, and salazinic acids, but differed in the other accessory compounds detected. Norsticic acid occurred in three collections from Antarctica, China and Chile (Table 1, collections 23, 25 and 27), and lobaric in one of those from Chile (Table 1, collection 25).

DISCUSSION

The topology of the main trees (Figs 1–2) distinguished two principle monophyletic groups, refered to as the Atlantic Population (AtP) and Mediterranean Population (MeP). Complementary trees based on β-tubulin gene sequences (Figs 3–4) and on intron sequences in collections which had an SSU intron (Fig. 5), provided additional evidence as to the robustness of the main trees and confirmed the topology. The AtP includes all collections from both harsh cold environments as well as more oceanic or atlantic regions. We suggest that AtP and MeP could have originated in Eurasiatic mesic mountain conditions, the region including China, where *Parmelia s. str.* has previously been suggested to have its centre of diversity (*cfr* Hale 1987). Indeed about half of the accepted species of *Parmelia s. str.* (*ca* 40) are known from Asia, while Europe has only seven and North America ten.

In several cases (Figs 1–2), samples from distant regions are placed together in the trees (e.g. Russia and Antarctica, in subgroup I), while some other samples from neighbouring localities are split. In such cases, the majority of localities have similar bioclimatic conditions. In the MeP, collections 1, 2, 3, 4, 30, and 31 from central Spain are all from sites with a continental climate with a wide range of temperatures between summer maximum and winter minimum, but collections 12 and 13 from the same general area but more westerly oceanic sites are in quite different clades, subgroups I and II in the AtP. Samples from central Asia (Altay Region, collection 27) and from mountains in western central Spain (Sierra de Gredos in the western Sistema Central, collection 12) are grouped in the same terminal clade (subgroup II) and also share an oceanic climate. Collection 13 from a nearby Spanish mountain occurred at a much higher altitude (2300 m) with an extremely cold climate in winter belonged to the same clade as all the polar and boreal samples (subgroup I). Similarly, in other clades of the tree the topology also brought together samples from different geographical localities but which had a similar ecology (Frey & Lösch 1998). In the case of group MeP, the strong bootstrap support for collections from distinctly Mediterranean environments is striking.

It may be pertinent that the two transplanted thalli from the AtP region reacted adversely with some parts having died after eight months in an environment where the MeP population thrives. This provenance

test (Walser & Scheidegger 2002) should be repeated with more samples from different parts of the AtP range to test whether the correlation is valid, but is not inconsistent with the hypothesis as to the climatic preferences of the two monophyletic populations, the demonstration of ecophysiological variability (Sancho *et al*. 2000), and suggestions of ecotypic variation (Gilbert 1971) within the species. The type of damage shown is typical of that the species exhibits when stressed by sulphur dioxide air pollution (Gilbert 1971).

The lichen always contains atranorin, chloroatranorin and salazinic acid, but differs in the accessory compounds present, lobaric acid, protolichesterinic acid, and unidentified fatty acids having been reported (Hale 1987). Hinds (1998) also mentions the occurrence of consalazinic acid; that, atranorin and salazinic acid were present in all the samples we examined. However, our discoveries of accessory norstictic and protocetraric acids appear to be the first reports of these compounds from the species. That norstictic acid was found as an accessory only in the AtP monophyletic group, but in material from sites as disparate as Chile, China, and Antarctica, suggests that occurrences of this compound in low concentrations may be a special feature of the AtP. This hypothesis, however, needs further testing by more sensitive chemical analytical methods on a wider range of samples.

This pattern of distribution is surprising within what is recognized as a single species, where genetic exchange would be expected to be open and consequently result in geographic correlations (Lohtander, Källersjö & Tehler 1998). In the case of *P. saxatilis*, however, some geographically close sites have different genotypes as discussed above.

Asexual propagules composed of both fungal and algal partners ('isidia') are always produced by *P. saxatilis* and are its main method of propagation and dispersal. Indeed, where the air is polluted by sulphur dioxide and the lichen is under stress no apothecia are formed and isidia are the only kind of diaspores produced (Hawksworth, Rose & Coppins 1973). Collections in subgroup I are dispersed geographically through an essentially atlantic pathway which crosses the whole meridian semicircle from pole to pole. These collections are from geographically distant regions (Arctic, Antarctica, boreal Europe and North America, Patagonia and several high mediterranean and temperate mountains) are essentially from areas where the species does not form apothecia, and where it must be dispersed asexually by isidia. Since the genetic distance among the three sets of sequences tested was in all cases minimal (Figs 2, 3–4 and 5) a common origin of this asexual clade is supported. The issue then is how such a wide distribution could have been achieved. Studies on lichen dispersal and establishment on a global scale are minimal, but the arrival in Antarctica of propagules of lichens and bryophytes from several thousand km away has been demonstrated (Kappen & Straka 1988, Marshall 1996), and asexual propagules of lichens can be detected on birds (Bailey & James 1979). The dispersalist view supported by Aptroot (*in* Galloway & Aptroot 1995) considers bipolar disjunctions of a species to result from a relatively early origin and then subsequent dispersal; this concept fits well with our results. In the context of a single monophyletic group, this statement is particulary pertinent as the genetic distance between the samples is extremely low. Consequently relatively recent dispersion of asexual propagules may have been occurred both by birds or by the same general wind circulation that drives to the dispersion of pollutants from temperate regions to both polar regions (Nazaroff 1996). However, while ascospores and soredia may be taken up in wind currents, and there is experimental evidence for wind dispersal of soredia (Armstrong 1988), it is difficult to visualize larger propagules such as the isidia of *P. saxatilis* which have to be detached mechanically rather than by wind currents becoming air-borne in any numbers. Indeed, we have not found any references to isidia being found in atmospheric air samples, and for this reason consider dispersal by birds, themselves affected by global wind patterns, as the most probable hypothesis.

Despite the distinctness of the two monophyletic groups AtP and MeP, and also the genetic isolation of subgroup 1, no morphological or clear chemical differences were found in the samples, although more comprehensive morphometric and chemical studies would have been desirable. The distributions do, however, suggest that there are ecophysiological differences between AtP and MeP in particular as discussed above. In accordance with the pragmatic morphospecies concept maintained in fungi generally (Vinuesa *et al*. 2001), even where incompatibility tests and molecular evidence show biological species are present (Petersen & Hughes 1999), it is not appropriate to formally recognize even the two main groups revealed, AtP and MeP, taxonomically here. More extensive data sets are needed to explore further the factors contributing to the observed patterns. It is, however, becoming clear that considerable genetic variation occurring within widespread lichen phenospecies may be the rule rather than the exception (*cfr* Crespo *et al*. 1999, Grube & Kroken 2000). This has wide implications for both conservation strategies and comparative ecological studies, including ecophysiological investigations and responses to air pollutants. Authors working with the same morphospecies may be dealing with different genotypes which thus should not be directly compared, and where conservation strategies are being considered transplant and other actions may not be maintaining the full genotypic range of a species.

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