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Molecular studies on *Punctelia* species of the Iberian Peninsula, with an emphasis on specimens newly colonizing Madrid

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Abstract: The first molecular phylogenetic study on a range of *Punctelia* species is reported, focussing on specimens growing in the Iberian Peninsula. Material of seven species was included in the analysis. Forty sequences were generated from nuITS and mtSSU rDNA in 20 specimens, and the resultant majority rule consensus tree from the combined analyses shows four major clades. *Punctelia ulophylla* is confirmed as a distinct species, *P. reddenda* is basal to *P. borreri*, and *P. perreticulata* groups with *P. subrudecta*. Samples identified as *P. rudecta* from the Canary Islands and China occupy different basal positions; the complex merits further study. *Punctelia borreri* and *P. subrudecta* are mainly coastal in the Iberian Peninsula, but are now reported from the central plateau for the first time; newly colonizing thalli have been found in a park in Madrid which is regularly spray-watered and where sulphur dioxide levels have fallen over the last two decades.

Key words: air pollution, Ascomycota, *Flavopunctelia*, invasive species, lichens, *Parmeliaceae*, phylogeny

Introduction

Punctelia s. str., is a relatively small genus in the *Parmeliaceae*, comprising about 30 species, which was originally introduced by Krog (1982). It is characterized by a grey, punctiform pseudocyphellatae upper surface, and unciform to filiform conidia, and is presumed to have its centre of speciation in South America and Africa (Elix 1993). Seven species of the genus have been reported from Europe to date: *P. borreri* (the type species of the genus), *P. perreticulata, P. reddenda, P. rudecta, P. stictica, P. subrudecta*, and *P. ulophylla* (Clauzade & Roux 1985; van Herk & Aptroot 2000; Llimona & Hladun 2001).

Punctelia as circumscribed by Krog (1982) was heterogeneous. She placed in *Punctelia* subgen. *Flavopunctelia* species of the *Parmelia flaventior* group (Hale 1980), which differed from those of subgen. *Punctelia* in having bifusiform conidia and being yellow-green due to the presence of usnic acid in the cortex. In addition, Common (1981) reported that the hyphal walls of representatives of this group had a different polysaccharide chemistry from other parmelioid lichens. *Flavopunctelia* was subsequently raised to generic rank by Hale (1984), and molecular studies show that *Flavopunctelia* is the sister group of *Punctelia* (Blanco *et al.* 2004*a*).

The most abundant species of *Punctelia* s. str. in the western Mediterranean are *P. borreri* and *P. subrudecta*. Both are commonly sympatric in all coastal regions of the Iberian Peninsula (Llimona & Hladun 2001; Pugh-Jones 2002), although *P. subrudecta* is apparently more continental (Nimis 1993) occurring much further inland. *Punctelia perreticulata* (Adler & Ahti 1996) occurs in oceanic sites in the Mediterranean region (Nimis 1993; Logán *et al.* 2000; van Herk & Aptroot 2000) but is not common. *Punctelia reddenda* has been reported from a few localities in southern Europe but is also

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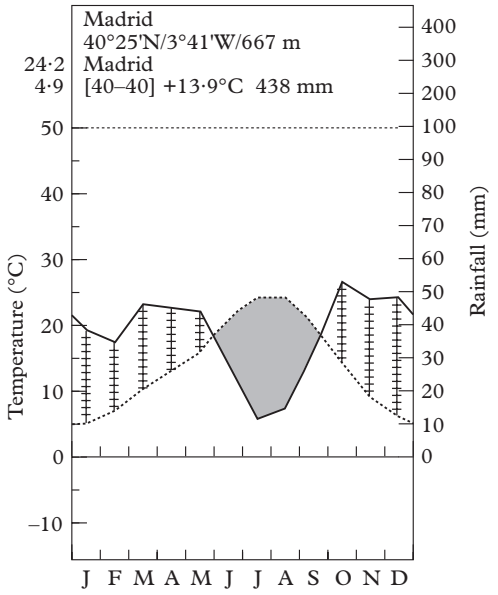

FIG. 1. Climatic diagram for Madrid (from *www.globalbioclimatis.org*). · · · temperature; —— rainfall.

rather scarce in the region (Atienza 1990; Llimona & Hladun 2001; Pugh-Jones 2002). *Punctelia ulophylla*, which was not accepted as distinct from *P. subrudecta* by Santesson *et al.* (2004), has not so far been reported from Spain (Llimona & Hladun 2001), Italy (Nimis 1993), or Portugal (Tavares 1945; Pugh-Jones 2002).

The centre of the Iberian Peninsula (Meseta Castellana) is a continental plateau region averaging *c.* 700 m in altitude with a meso-Mediterranean climate characterized by dry or extremely dry summers (Rivas-Martínez *et al.* 2002; www.globalbioclimatis.org, Fig. 1). In spite of the abundance of *P. borreri* and *P. subrudecta* in all coastal and suboceanic regions of the Iberian Peninsula, and also inner lowland areas (Aragón & Rico 1997), no *Punctelia* species have previously been reported from the central plateau (Crespo 1975; Crespo & Barreno 1975; Crespo *et al.* 1977; Crespo & Bueno 1982; Llimona & Hladun 2001; Fig. 4).

The city of Madrid is located in the centre of the continental plateau area of the Meseta Castellana at around 650 m within the upper mesomediterranean thermotype belt which has a low dry ombrotype character (Rivas-Martínez *et al.* 2002). As in many other European cities, sulphur dioxide and particulate air pollution have been decreasing over the last two decades, falling from *c.* 80 $\mu g\, m^{-3}$ in 1980 to *c.* 18 $\mu g\, m^{-3}$ in 2000 (Fig. 3), and lichens are recolonizing trees in urban gardens and parks. Among the *Parmeliaceae*, *Flavoparmelia soredians*, *Flavopunctelia flaventior, Melanelixia glabra*, and *Parmelina tiliacea* are the only species so far reported from the less polluted areas in the periphery of the city (Crespo & Bueno 1982), but are continuing to expand. In the course of our ongoing studies of the responses of lichens to changes in air pollution levels in the city, we have recently detected several thalli of *Punctelia* species. In almost all of the cases the lichen samples were growing in humid microhabitats on moderately or very old trees; the thalli were all small (less than 1·5 cm diam.), and the colour of the lower surface and other critical characters were not properly developed making identification difficult.

We therefore used molecular techniques to identify the juvenile colonizing specimens, and also to examine the phylogeographic and morphological relationship of our recent collections of *Punctelia* species, which proved to be almost all those known in Europe. ITS rDNA sequences were selected for comparison as these are the most valuable molecular markers at the population and species levels, whereas mitochondrial SSU sequences are more conserved and better suited for studies of phylogenetic relationships at the generic level (Crespo *et al.* 2001; Blanco *et al.* 2004*a*,*b*).

Material and Methods

Taxon sampling

Most of the parks in Madrid were surveyed, and *Punctelia* specimens from those and other parts of Europe were sampled. Sequence data of the nuITS rDNA, and mtSSU rDNA were obtained from six *Punctelia* and one *Flavopunctelia* species respectively. A total of 40 new sequences were generated from 20 specimens (Table 1). *Flavopunctelia flaventior* was used

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TABLE 1. Punctelia *and* Flavopunctelia *specimens included in the study, with details of location, collectors, herbarium code and GenBank accession numbers.*

Species	Locality	Altitude (m)	Collector(s)	Herbarium acc. no.	GenBank acc. no.	
					nuITS	mtSSU
P.subrudecta	Spain, Madrid, Capital, Parque del Oeste	600	*Crespo & Gasca*	MAF-10245	AY613396	AY613416
P. subrudecta	China, Yunnan, Lu Nana County, near Shihin and Stone Forest, *Pinus yunnanensis* forest	1909	*Crespo, Blanco & Argüello*	MAF-10244	AY613395	AY613415
P. subrudecta A	Germany, Northrhine-Westfalia, Leichlingen, Valley of the Wupper	426	*Zimmermann*	MAF-10243	AY613393	AY613413
P. subrudecta	Canary Island, Tenerife, Las Mercedes	900	*Crespo*	MAF-10242	AY613397	AY613417
P. subrudecta	Spain, Galicia, Gondomar, Parque Natural de Monte Alhoya	620	*Divakar*	MAF-10241	AY613392	AY613412
P. subrudecta B	Germany, Düsseldorf	40	*Crespo*	MAF-10248	AY613398	AY613418
P. subrudecta C	Germany, Düsseldorf	40	*Crespo*	MAF-10250	AY613394	AY613414
P. borreri	Spain, Madrid, Parque del Oeste	600	*Crespo & Argüello*	MAF-10240	AY613409	AY613429
P. borreri	Spain, Cádiz, Arcos de la Frontera, Molino del Pilón	180	*Crespo, Gasca, Blanco & Argüello*	MAF-10255	AY613405	AY613425
P. borreri	Spain, Galicia, Gondomar, Parque Natural de Monte Alhoya	620	*Divakar*	MAF-10254	AY613404	AY613424
P. borreri	Spain, Castellón, Puerto de Eslida	650	*Crespo, Barreno & Divakar*	MAF-10238	AY613399	AY613419
P. borreri	Canary Island, Tenerife, Las Mercedes	1200	*Crespo*	MAF-10246	AY613400	AY613420
P. borreri	Portugal, Coimbra, plaza de la universidad	45	*Crespo*	MAF-10237	AY613401	AY613421
P. aff. *rudecta*	China, Yunnan, Wuching County, Shizishan, Lion Mountain, secondary forest, predominantly conifers	2260	*Hawksworth*	MAF-10253	AY613402	AY613422
P. rudecta	Canary Island, Tenerife, Las Mercedes	1200	*Crespo*	MAF-10256	AY613403	AY613423
P. ulophylla A	Germany, Düsseldorf	40	*Crespo*	MAF-10251	AY613406	AY613426
P. ulophylla B	Germany, Düsseldorf	40	*Crespo*	MAF-10249	AY613407	AY613427
P. pereticulata	Spain, Castellón, Puerto de Eslida	450	*Crespo, Barreno & Divakar*	MAF-10239	AY613391	AY613411
P. reddenda	Chile, Valdivia	20	*Sancho*	MAF-10247	AY613410	AY613430
Flavopunctelia flaventior	Spain, Córdoba, Las Ermitas	150	*Argüello*	MAF-10252	AY613408	AY613428

as outgroup since it has been shown to be the sister group to *Punctelia* in previous molecular studies (Blanco *et al.* 2004*a*).

Molecular methods

Small samples prepared from freshly collected and frozen herbarium specimens were ground with sterile plastic pestles. Total genomic DNA was extracted using the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions with slight modifications as described in Crespo *et al.* (2001). Dilutions of the total DNA were used for PCR amplifications of the genes coding for the nuclear ITS (nITS) and mitochondrial SSU (mtSSU) rRNA. Fungal nITS rDNA was amplified using the primers ITS1F (Gardes & Bruns 1993), ITS4 (White *et al.* 1990), ITS1-LM (Myllys *et al.* 1999) and ITS2-KL (Lohtander *et al.* 1998); and mitochondrial SSU rDNA was amplified using the primers mrSSU1 and mrSSU3R (Zoller *et al.* 1999), MSU1 and MSU7 (Zhou & Stanosz 2001). Amplifications were performed in 50 µl volumes containing a reaction mixture of 27·5 µl of dH_2O, 5 µl of 10 × DNA polymerase buffer (Biotools) (containing $MgCl_2$ 2 mM, 10 mM Tris–HCl, pH 8·0, 50 mM KCl, 1 mM EDTA, 0·1% Triton X-100), 1 µl of deoxynucleotide triphosphate (dNTPs), containing 10 mM of each base, 2·5 µl of each primer (10 µM), 1·25 µl of DNA polymerase (1 U/µl). Finally, 40 µl of this mixture was added to 10 µL of DNA of each sample.

The amplifications for ITS rDNA and SSU mitochondrial rDNA were carried out in an automatic thermocycler (Techne Progene) and performed using the following programs: (1) ITS rDNA: 94°C for 5 min, and 30 cycles of: 94°C for 1 min, 54–60°C for 1 min, 72°C for 1·5 min, and a final extension at 72°C for 5 min; and (2) SSUmt rDNA: 94°C for 5 min and 30 cycles of: 94°C for 1 min, 56–58°C for 1 min, and 72°C for 1·5 min, and a final extension at 72°C for 5 min.

The PCR products were then cleaned using the Bioclean Columns kit (Biotools) according to the manufacturer's instructions. The cleaned PCR products were sequenced using the same primers as used in the amplifications. The ABI Prism™ Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) was used with the following settings: denaturation for 3 min at 94°C, 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems). Partial SSU rDNA sequences, sometimes including an intron at the end of 3′ (SSU), were removed before alignment. Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually adjusted.

Sequence alignments

An alignment procedure employing a linear Hidden Markov Model (HMM) as implemented in the SAM software (Hughey & Krogh 1996) was used. Sequences of 20 specimens (Table 1) were aligned separately for the two genes. Regions that could not be aligned with statistical confidence were excluded from the phylogenetic analysis.

Phylogenetic analysis

The alignment was analyzed using the program MrBAYES 3.0 (Ronquist & Huelsenbeck 2003). The polarity of characters was assessed by outgroup comparison, using *Flavopunctelia flaventior* as the outgroup (see above). The data were analyzed using a Bayesian approach (Huelsenbeck *et al.* 2000; Larget & Simon 1999). Posterior probabilities were approximated by sampling trees using a Markov Chain Monte Carlo (MCMC) method. The posterior probabilities of each branch were calculated by counting the frequency of trees that were visited during the course of the MCMC analysis.

The program MrBAYES was employed to sample the trees, and the analysis was performed assuming the general time reversible model (Rodriguez *et al.* 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G) for the single-gene and the combined analyses. No molecular clock was assumed. A run with 1 000 000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file.

We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) and determined that stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). The initial 600 trees were discarded as burn-in before stationarity was reached. Using PAUP* (Swofford 2003), a 95% majority-rule consensus tree was calculated from 9400 trees sampled after reaching likelihood convergence, to calculate the posterior probabilities of the tree nodes using MrBAYES. Unlike non-parametric bootstrap values (Felsenstein 1985), these are estimated probabilities of the clades under the assumed model (Rannala & Yang 1996), and hence posterior probabilities equal to and above 95% are considered significant supports.

We used a Bayesian approach to examine the heterogeneity in phylogenetic signal between the two data sets (Buckley *et al.* 2002). For the two genes and the concatenated analyses, the set of topologies reaching 0·95 posterior probability was estimated. The combined analysis topology was then compared for conflict with the 0·95 posterior intervals of the single gene analyses. If no conflict was evident, it was assumed that the two data sets were congruent and could be combined (Bull *et al.* 1993).

Results

A total of 20 new mitochondrial SSU rDNA, and 20 new nuclear ITS rDNA sequences were generated. We produced a matrix of

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812 unambiguous nucleotide position characters in the mtSSU and 489 in the nuITS. The final alignment of the 20 collections studied (Table 1) was 1301 positions in length and 156 characters were variable.

The likelihood parameters in the sample had the following average values (± one standard deviation): likelihood (LnL) = −3215·37 (± 0·243), base frequencies π(A) = 0·282 (± 0·002), π(C) = 0·199 (± 0·002), π(G) = 0·215 (± 0·002), π(T) = 0·304 (± 0·002), rate matrix r(AC) = 1·912 (± 0·04), r(AG) = 8·778 (± 0·224), r(AT) = 5·367 (± 0·12), r(CG) = 1·964 (± 0·585), r(CT) = 15·229 (± 0·324), r(GT) = 1·0 (± 0), the gamma shape parameter alpha = 0·517 (± 0·001), and the pinvar = 0·714 (± 0·004).

The 95% majority-rule combined consensus tree (nr ITS and mtSSU) of 9400 sampled trees is shown in Fig. 2. Four different clades can be recognized (Clades I, II, III and IV):

Clade I joins the two samples of *Punctelia ulophylla* from Düsseldorf (Germany).

Clade II includes one sample of *P. rudecta* from Tenerife (Canary Islands).

Clade III includes seven samples; *P. reddenda* from the urban area of Valdivia (Chile) as basal sister group (1·00 pp) to six *P. borreri* samples. The collection from Tenerife is basal (1·00) in relation to all the samples of *P. borreri* from the Iberian Peninsula. The specimen from the urban park in Madrid is more closely related (1·00 pp) to one from Castellón (close to the Mediterranean coast) than to the other samples.

Clade IV includes nine samples. All seven *P. subrudecta* collections studied group together, along with *P. perreticulata* from Castellón (Spain), and one sample close to *P. rudecta* from Yunnan (China). However, while falling into the same group, the relationships of these last two specimens was not resolved.

Discussion

The few scattered specimens of *Punctelia* detected in the urban park of Madrid (Parque del Oeste) grow in deep bark crevices at the base of one old *Pinus*, and also more rarely on some other trees, especially of *Populus*. All the samples were young and small, up to 1·5 cm diam., and as a consequence, morphological features were unable to provide a reliable identification. The two samples included in the analysis clearly correspond to two different taxa (Fig. 4); one from *Populus* nested with all *P. borreri* samples, and the other from *Pinus* with those of *P. subrudecta*. The sulphur dioxide and particulate air pollution in the park, as in most areas of the city of Madrid, have been decreasing for the last two decades (Fig. 3) and the peripheral position and the size (2 km^2) of the park favour the air quality inside. Nimis (1993) pointed out that both species belong to *Xanthorion* communities (Barkmann 1958), i.e. nutrient-enriched substrata. Although extensive collections have been made in Madrid by earlier workers (Crespo 1975; Crespo & Barreno 1975; Crespo *et al.* 1977; Crespo & Bueno 1982; Llimona & Hladun 2001), no *Punctelia* species have ever been reported from the area, or from adjacent regions in the centre of the Iberian Peninsula, neither from urban nor rural sites (Fig. 4).

These two *Punctelia* species are common, and partially sympatric, in more oceanic (less continental) regions of southwestern Europe (Llimona & Hladun 2001; Pugh-Jones 2002). In Europe generally, especially in Belgium, France, Germany and The Netherlands, *P. borreri* is more coastal and less common than *P. subrudecta* (Hale 1965; Targé & Lambinon 1965), but appears to be expanding its range (Spier & van Herk 1997). *Punctelia borreri* has a much more strongly pronounced southern distribution in the British Isles (Hawksworth 1972; Seaward 1995) and does not extend northwards as far as Fennoscandia (Santesson *et al.* 2004). In North America, *P. borreri* is only known from Ohio and West Virginia while *P. subrudecta* is much more widespread (Brodo *et al.* 2001).

We suggest that the most likely explanation for the occurrence of both species in the Parque del Oeste is the special watering

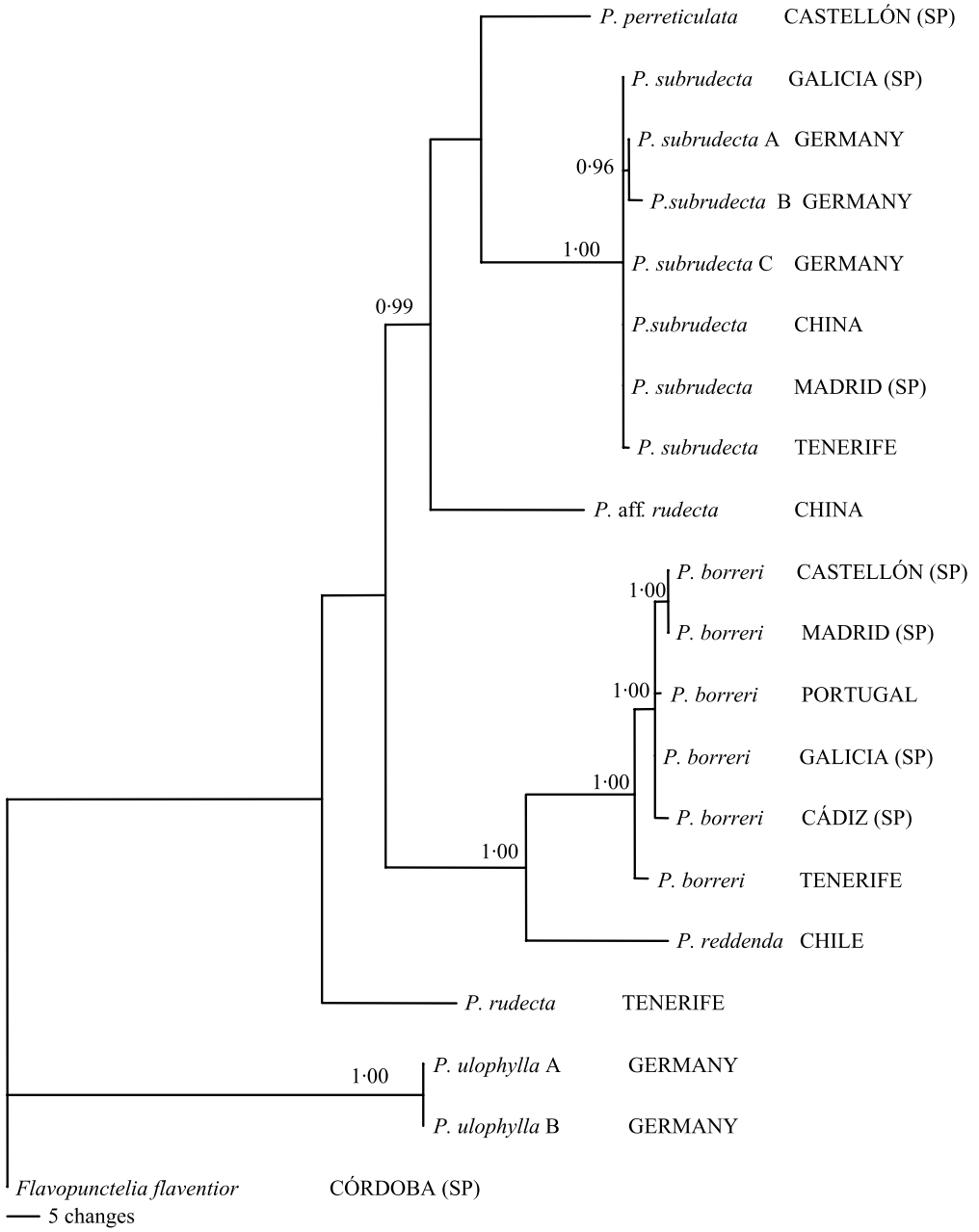

FIG. 2. 95% majority rule consensus tree of 9400 trees visited during a B/MCMC sampling procedure using nrITS and mtSSU gene sequences. Numbers at the nodes are posterior probability values above or equal to 95%.

process that is being currently used. From 1999, the irrigation system in this park has consisted of an extremely fine spray provided by a turbine (PGC, Hunter®) activated following a computer-controlled automated programme designed to reach a constant relative humidity level in the air. This favourable circumstance is further

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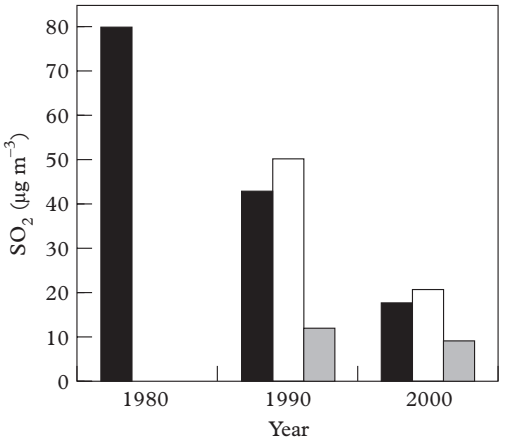

FIG. 3. Annual averages of sulphur dioxide air pollution in Madrid. Based on information supplied by the Servicio de Gestión de Residuos y Calidad Ambiental del Ayuntamiento de Madrid. ■, average all Madrid stations; ▒, station 1 km west of the Parc del Oeste; □, 3 km west of the Parc.

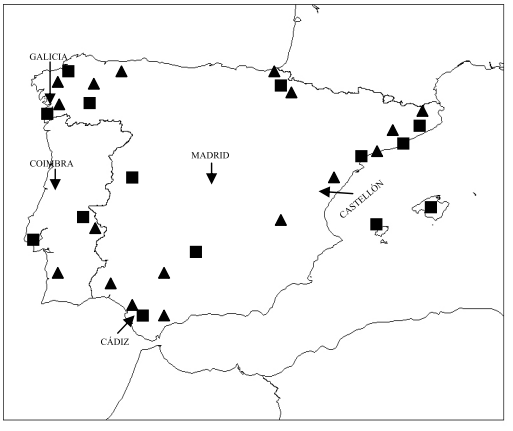

FIG. 4. Distribution of *Punctelia borreri* (■) and *P. subrudecta* (▲) in the Iberian Peninsula and Balearic Islands. Based on literature reports and herbarium specimens. Arrows indicate sites of specimens sequenced (see Table 1).

enhanced by protection of the microclimate afforded by the, in parts, almost closed-canopy trees. The resultant environment is consequently more similar to the climate in coastal regions of the Iberian Peninsula where the two species naturally co-exist. How the propagules of the two lichens, presumably soredia rather than ascospores, reached the park is unclear, but it does have large numbers of visitors (especially students) from different parts of Spain and other European regions and is rich in bird-life. The only two other parmelioid species occurring in the park (*Parmelina tiliacea* and *Flavoparmelia soredians*) are common in the region and must be seen as normal recolonists.

Interestingly, *P. subrudecta* in particular appears to be increasingly invading urban and suburban areas in central and western Europe (Hawksworth & McManus 1989; Nimis 1993; Spier & van Herk 1997) as an active colonist where ambient sulphur dioxide and particulate air pollution levels have fallen sufficiently to permit its invasion. In these cases, the species was already known as a normal component of the lichen biota of surrounding and adjacent rural regions and does not appear to have established itself in different climatic regimes as has been the case in Madrid, from which no member of the genus has previously been reported.

In the phylogenetic tree (Fig. 2) the two samples examined from the Parque del Oeste are representatives of the clades of *P. borreri* and *P. subrudecta*. One of them joined with *P. subrudecta* from Europe (two urban areas of Germany, and one from north-west Spain), Asia (China, Yunnan Province) and one from north-west Africa (Canary Islands). Since *P. ulophylla* has been reported as frequent in more northern parts of central and western Europe (Belgium, France, Germany, Luxembourg, The Netherlands, Poland, Slovakia and Switzerland; van Herk & Aptroot 2000) occurring with *P. subrudecta*, we included some samples collected from an urban park of Düsseldorf where both species were present for comparison. *Punctelia ulophylla* had long been considered as a variety or synonym of the species now known as *P. subrudecta*, until van Herk & Aptroot (2000) resurrected it as a species. Indeed, although the species is counted as included in a group with *P. subrudecta* and *P. perreticulata* based on chemical (lecanoric acid present) and morphological features (pale lower surface) the species is placed in the phylogenetic tree

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in a monophyletic clade with unresolved relationship but isolated from the *P. subrudecta* group. *Punctelia ulophylla* should consequently not be synonymized with *P. subrudecta* as proposed by Santesson *et al.* (2004). On the other hand, the southern pantropical species *P. perreticulata* emerged as phylogenetically related to the *P. subrudecta* group. In addition, an isidiate species from China, morphologically close to *P. rudecta*, occupied a basal position in the same group with strong support (0·99 pp).

The second sample of *Punctelia* collected in the Madrid park joined with the *P. borreri* samples from different oceanic localities in the Iberian Peninsula (Fig. 2). All these samples clustered as a monophyletic clade (1·00 pp) that is the sister group of one sample from the laurisilva forest in the macaronesian region (Tenerife, Canary Islands). Interestingly, this monophyletic clade (1·00 pp) of *P. borreri* is strongly supported as a sister group to a collection of *P. reddenda* from the Chilean city of Valdivia, which has similar climatic conditions to those of the macaronesian region. The sample named as *P. rudecta*, also from the Canary Islands, seems to be basal to both main clades but lacked statistical support.

With respect to the generic circumscription of *Punctelia*, Figure 2 implies that the genus is not monophyletic. This result is contrary to the molecular phylogeny implied in the analysis of Blanco *et al.* (2004*a*) where the genus appears as the sister group to *Flavopunctelia*. However, it was not the aim of this study to address generic concepts, and data from more species and from more genes are needed to resolve this issue. Additional information will be presented and the matter addressed in detail elsewhere.

The question of the identity of the two isidiate collections referred to *P. rudecta* here needs to be investigated further, including additional specimens from North America, from which the species was first described, and also other parts of Asia. Interestingly, Culberson (1962) noted that specimens named as *Parmelia ruderata* from Japan had somewhat more flattened isidia than those of *P. rudecta*, with which he synonymized the species; morphological as well as molecular studies are evidently required.

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