

## Phylogenetic relationships and taxonomy of the *Leptogium lichenoides* group (Collemataceae, Ascomycota) in Europe

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The taxonomy of the *Leptogium lichenoides* complex is revised here based on a morphological, ecological and molecular phylogenetic study. A phylogenetic analysis of phenotypic characters was compared to a phylogeny based on nrITS and  $\beta$ -tubulin data. Using these phylogenies, we concluded that what was commonly recognized as *Leptogium lichenoides* s.l. encompasses three distinct species. *Leptogium lichenoides* var. *pulvinatum* is now recognized as a separate species *L. pulvinatum* comb. nov. *Leptogium aragonii* sp. nov., a non-isidiate species with large thalli, is the second species part of this complex, and *L. lichenoides* s.str., as redefined here, is the only species of this group with isidia. We also found that *Leptogium lichenoides* s.l. is polyphyletic. *Leptogium pulvinatum* and *L. lichenoides* s.str. are more closely related to *L. gelatinosum* than to *L. aragonii*. The taxonomic status of *L. quercicola* is reduced to a variety of *L. pulvinatum*. Identification key, descriptions and distribution maps are presented for *Leptogium aragonii*, *L. gelatinosum*, *L. intermedium*, *L. lichenoides* s.str., *L. pulvinatum*, and *L. pulvinatum* var. *quercicola*.

**KEYWORDS:**  $\beta$ -tubulin, Collemataceae, *Leptogium lichenoides* group, lichens, nrITS, phylogeny, species complex

### INTRODUCTION

*Leptogium* (Ach.) Gray is a cosmopolitan genus of lichen-forming ascomycetes classified in the Collemataceae and represented by c. 250 species with about 40 infrageneric taxa found in Europe (Aragón & al., 2005). Several complexes have been studied within this morphologically and anatomically heterogeneous genus by different authors (Jørgensen, 1975, 1994, 1997; Jørgensen & James, 1983; Jørgensen & Tønsberg, 1999). However, different taxonomic questions relating to many species complexes and groups remain to be resolved. One of these is the *lichenoides* species complex, which includes four species in Europe: *L. intermedium* (Arnold) Arnold, *L. gelatinosum* (With.) J.R. Laundon, *L. lichenoides* (L.) Zahlbr. and *L. quercicola* Otálora & al. (Otálora & al., 2004). Although species of this group may display considerable morphological variability that hinders their identification, no taxonomic revision has been conducted for this species complex.

Nomenclaturally, the main difficulty resides in the numerous infraspecific taxa recognized within *L. lichenoides* (Zahlbruckner, 1924; Lamb, 1963; Jørgensen, 1994). These taxa show considerable morphological differences and could represent distinct species. However, a morphological and molecular systematic study is required to distinguish between phenotypic plasticity and genetically based morphological differences.

*Leptogium lichenoides* was originally described as *Tremella lichenoides* L. (1753). Many taxa have been introduced since then, some of which were correctly classified in the genus *Leptogium* when Gray (1821) recognized the genus *Leptogium* as a separate taxonomic entity from the genus *Collema*. Subsequently, Zahlbruckner (1924) included more taxa within the *lichenoides* species complex, and considered five different varieties (*L. lichenoides* var. *lichenoides*, var. *lophaeum*, var. *pulvinatum*, var. *sendtneri*, var. *subsiniatum*) as well as four different forms within var. *lichenoides* (f. *ateleum*, f. *fimbriatum*, f. *laevigatum*, f. *luxurians*).

Morphologically, the *lichenoides* complex includes species with thin, paper-like, bluish-grey to brown thalli, and a distinctly ridged upper surface. Thalli cross sections reveal a well-developed lower and upper cortex made up of a single layer of cells (Otálora & al., 2004).

*Leptogium gelatinosum* and *L. intermedium* are morphologically similar, although some differences in thallus anatomy and apothecial margin have been observed (Jørgensen, 1994; Aragón & Otálora, 2004). Both species are distinguishable from other taxa within the *lichenoides* complex, especially based on lobe margins, ridges on the lower surface of the thallus, and presence of apothecia, which are often numerous on thalli of *L. gelatinosum* (Coppins & Purvis, 1992; Jørgensen, 1994; Otálora & al., 2004).

*Leptogium lichenoides* is the most variable, abundant, and broadly distributed species of the complex in Europe (Wirth, 1980; Nimis, 1993; Llimona & Hladun, 2001). Several authors have pointed out the existence of marginal isidia in *Leptogium lichenoides*. However, Sierk (1964) who studied the genus *Leptogium* in North America, did not observe the presence of isidia, but observed that margins of the thallus can range from entire to finely lobulated or fimbriated. Sierk (op. cit.) concluded that in spite of the variability observed, and multiple infraspecific taxa proposed by other authors, it was more appropriate to not recognize distinct taxonomic units within *L. lichenoides*. Brodo & al. (2001) indicated that some marginal outgrowths of *L. lichenoides* can be interpreted as isidia, and on that basis they recognized two thallus types within *L. lichenoides* s.l.; i.e., with or without isidia. However, they specified that the degree to which marginal outgrowths resemble isidia varies, leading to problems in identification. Coppins & Purvis (1992) acknowledged that some specimens of *L. lichenoides* have small, simple or branched, cylindrical isidia-like extensions, which may be very densely produced, giving the appearance of fibrillose margins. Other authors (e.g., Nimis, 1993; McCune & Geiser, 1997) have also pointed out the high level of morphological polymorphism within *L. lichenoides*.

The diagnostic value of isidia (presence or not, shape and position) and lobes (colour and shape) were highlighted by the recognition of several new species, within *Collema* and *Leptogium* during the last 22 years: *L. magnussoni* Degel. & P.M. Jørg. and *L. subaridum* P.M. Jørg. & Goward (Jørgensen & Goward, 1994; Aragón & al., 2004), *L. furfuraceum* and *L. pseudofurfuraceum* (Jørgensen & James 1983; Jørgensen, 1997; Aragón & al., 2005), *Collema flaccidum* and *C. subflaccidum* (Coppins & Purvis, 1992). However, the value of these characters as being robust morphological markers that characterize monophyletic entities that can be recognized as species, has never been confronted with molecular evidence within the *L. lichenoides* complex.

The main objective of this study is to evaluate the accuracy of morphological traits in recognizing monophyletic entities within the *L. lichenoides* species complex using molecular markers. We chose the nuclear ribosomal internal transcribe spacer (nrITS) and  $\beta$ -tubulin as markers for this study, because they have been shown repeatedly to have the appropriate level of variation for phylogenetic studies at the species and population levels for lichen-forming fungi (Myllys & al., 2001; Printzen & Ekman, 2002; Martín & al., 2003; McDonald & al., 2003; Miadlikowska & al., 2003; Printzen & al., 2003; Grube & al., 2004; Molina & al., 2004; Divakar & al., 2005). On this basis, we provide a taxonomic revision of this species complex in Europe, including species descriptions and identification key.

## MATERIALS AND METHODS

**Specimens.** — This study is based on 2,000 specimens of the *Leptogium lichenoides* group from herbaria B, BM, BP, E, G, GB, GDA, H, KUOPIO, L, LD, MA, MACB, MAF, OULU, TU and S, as well as personal collections by the authors. The type material of *Leptogium lichenoides* (LINN), *Leptogium gelatinosum* (OXF), *L. intermedium* (M), and several varieties and forms of this group were also revised.

Eighteen specimens (collected from 2002 to 2004 in the Iberian Peninsula) were selected for DNA sequencing to represent the phenotypic variability within the *L. lichenoides* complex (Appendix 1). One specimen of *Leptogium saturninum* (Dickson) Nyl. and one specimen of *Collema undulatum* Laurer ex Flotow were selected as outgroup taxa based on results from previous Lecanorales and Peltigerales phylogenies (Wiklund & Wedin, 2003; Miadlikowska & Lutzoni, 2004). It was not possible to obtain DNA sequences for *L. intermedium*, so, this species was not included in the phylogenetic analyses.

**Morphological, anatomical and ecological studies.** — The morphological, anatomical and ecological characters studied were: presence/absence of isidia, thallus colour, thallus diameter, lobe width, lobe margin shape, apothecium shape, presence/absence of arachnoid white hairs or tomentum on lower surface, and habitat type. Based on substantial morphological variability detected during a preliminary survey of *L. lichenoides* s.l. and the lectotype examined, specimens were classified into three putative species: *L. lichenoides* s.str. = isidia marginal, *L. pulvinatum* = lobes erect and thallus margin lacinate to deeply divided or revolute and *L. aragonii* = lobes wider and planate with crenate margin (Fig. 1; Appendix 2).

According to Aragón & Otálora (2004) the main diagnostic anatomical characters for this group are lobe thickness, spore size and organization of thalline exciple. Thalli and apothecia were sectioned at a thickness of 14–16  $\mu$ m using a freezing microtome and stained with lactophenol cotton blue. Apothecial sections were mounted in distilled water. All light microscopy measurements were made in water mounts, with an oil-immersion lens. Measurements of thallus thickness were carried out on 210 specimens (10 measurements per specimen). Twenty measurements of randomly selected mature spores (width and length) and five measurements of the apothecial anatomy (thickness of thalline exciple, paraphyses, hymenium and epihymenium, and width and length of asci) were made for 44 fertile specimens.

An ANOVA was performed to test if differences among groups circumscribed in our preliminary survey (see above) were significant. Data were statistically analyzed using SPSS/PC+ version 3.0. A Tukey post hoc test was implemented to convert morphological continuous



Fig. 1. Thallus type and shape, corresponding to the morphological characters 1–9 (Appendix 2 in Taxon online issue). A, *Leptogium aragonii* (MTG) lobe morphology; B, *L. gelatinosum* thallus; C, *L. intermedium* thallus; D, *L. lichenoides* (MTA) thallus with isidiate margin (coralloid isidia); E, *L. pulvinatum* (MTV), lobe morphology showing lacinate margin; F, *L. pulvinatum* thallus; G, *L. pulvinatum* var. *quercicola* lobe morphology; H, *L. pulvinatum* var. *quercicola* thallus. All photos by M.A.G. Otálora.

characters into discrete characters (Fig. 2) following Lutzoni & Brodo (1995) and McDonald & al. (2003). A total of 17 morphological, anatomical and ecological characters (Appendix 2 in Taxon online issue) were included to build a phenotypic data matrix using MacClade 4.01 (Maddison & Maddison, 2001). Distribution maps show localities of specimens from the herbaria revised (see the Specimens section for list of herbaria) that were examined by the first author. Maps were drawn with the program Arcview GIS 3.2 and can be seen in Appendix 3 in Taxon online issue.

**DNA sequencing.** — Small samples prepared from fresh material were ground in liquid nitrogen. Total genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions with slight modifications described in Crespo & al. (2001). Dilutions (1 : 10 and 1 : 100) of genomic DNA were used for PCR amplifications of the nrITS region (including the 5.8S gene) and partial sequence of the protein coding  $\beta$ -tubulin gene. Fungal nrITS was amplified using the primer pair ITS1F (Gardes & Bruns, 1993) and ITS4 or ITS5 and ITS4 (White & al., 1990). We also replaced ITS1F and ITS5 by NSSU897R and NSSU1088R (Kauff & Lutzoni, 2002). The protein coding  $\beta$ -tubulin gene was amplified using the primers Bt3-LM and Bt10-LM (Myllys & al., 2001). Because only five samples could be amplified with this primer pair, we designed the specific primer Bt3-MO (5'-AAGTCAGCATCGGGAGTTTA-3') using FastPCR (Kalendar, 2005), which was used in combination with Bt10-LM to amplify the remaining samples. Amplifications were performed in 25  $\mu$ l volumes containing a reaction mixture of 2.5  $\mu$ l of 10X DNA polymerase buffer (Biotools; containing 2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100), 1.0  $\mu$ l of dinucleotide triphosphate (dNTPs) containing 10 mM of each base, 1.5  $\mu$ l of each primer (10  $\mu$ M), 0.75  $\mu$ l of DNA polymerase (1 U  $\times$   $\mu$ l<sup>-1</sup>), 13.75  $\mu$ l of dH<sub>2</sub>O and 4.0  $\mu$ l of dilution of genomic DNA. Amplifications were carried out in a Peltier thermal cycler (PTC-100) and performed using the following programs: initial denaturation at 95°C for 5 min, and 35 cycles at 94°C for 1 min, 54°C for 45 s (nrITS) or 58°C for 1 min ( $\beta$ -tubulin), 72°C for 1.5 min, followed by a final extension at 72°C for 5 min. PCR products were subsequently purified using the BioClean Columns kit (Biotools) according to the manufacturer's instructions. The purified PCR products were sequenced using the same amplification primers, as well as ITS2 and ITS1 (White & al., 1990) for the nrITS region. Both strands were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) with the following PCR 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 products were subjected to electrophoresis with a 3730 DNA analyser (Applied Biosystems).

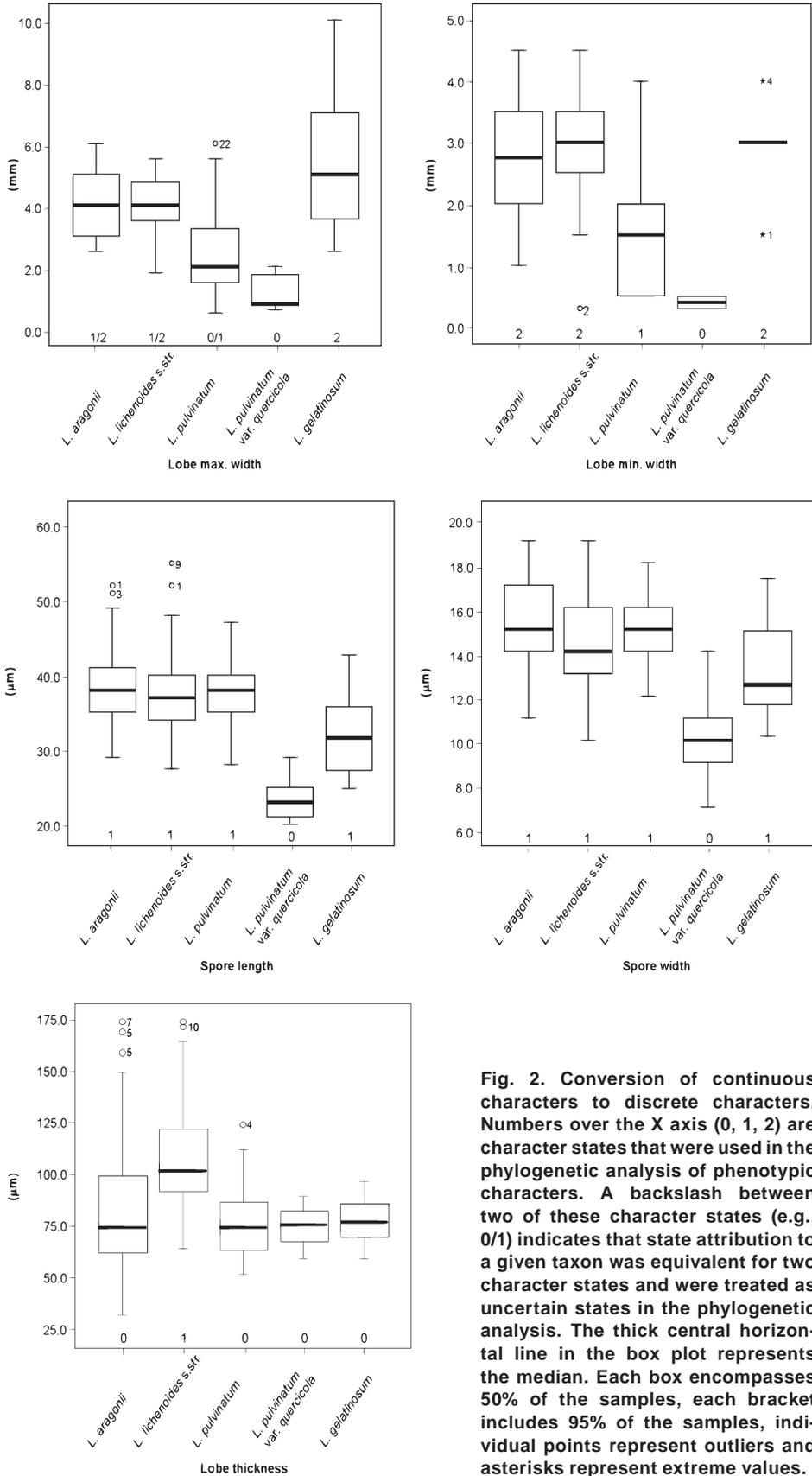
The nrITS and  $\beta$ -tubulin complementary strands were compared with the assistance of Windows SeqMan (DNA-Star) to check for reading errors. The nrITS and  $\beta$ -tubulin datasets were aligned using MacClade 4.01. Nucleotide sequences for  $\beta$ -tubulin were translated to amino acids to facilitate the alignment. Ambiguously aligned regions in the nrITS were excluded from the alignment. These regions were recoded using the program INAAASE (Lutzoni & al., 2000) and then used in phylogenetic analyses.

**Phylogenetic analysis.** — Individual weighted maximum parsimony analyses MP1 and MP3 were first performed on nrITS and  $\beta$ -tubulin, respectively, with only unambiguous aligned sites, using PAUP\* version 4.0b10 (Swofford, 2002). Symmetric step matrices were assembled using STMatrix 2.1 (Stefan Zoller & François Lutzoni; <http://www.lutzonilab.net/downloads>) following the methodology of Gaya & al. (2003). For the nrITS region, two step-matrices were implemented, one for ITS1 and ITS2 and another for the 5.8S. Each codon position of  $\beta$ -tubulin was subjected to a specific step matrix as well as one spliceosomal intron, for a total of six step matrices being implemented simultaneously when the phylogenetic search was performed on the combined dataset. A second parsimony analysis restricted to nrITS (MP2) was performed using both unambiguous and coded ambiguous aligned regions. Nine regions of the nrITS were recoded with INAAASE. These recoded regions were each subjected to a specific step matrix (Lutzoni & al., 2000).

The combinability of the datasets was assessed by comparing highly supported clades (bootstrap values  $\geq$  70%) among trees based on the ITS region or  $\beta$ -tubulin, exclusively (Mason-Gamer & Kellogg, 1996). Because no conflict was detected, it was assumed that the two datasets were congruent and could be combined. Thus, two additional MP analyses were conducted on this combined dataset: MP4 (nrITS unambiguous regions +  $\beta$ -tubulin) and MP5 (nrITS + INAAASE coded characters +  $\beta$ -tubulin). The last parsimony analysis was made using the morphological matrix (MP6).

For each MP analysis, a heuristic search of 1,000 random addition sequences (RAS) was conducted, with TBR branch-swapping, the multrees option was in effect and gaps were treated as a fifth character state for unambiguously aligned sites. Constant characters were excluded. Bootstrap analyses (Felsenstein, 1985) were used to estimate phylogenetic uncertainty with heuristic searches as described above on 1,000 bootstrap datasets. Two RAS per bootstrap replicate were specified based on the high resolving power of the original data when 1,000 RAS were implemented.

Using the Akaike Information Criterion (AIC) as implemented in MrModeltest 2.2 (Posada & Crandall, 1998; Nylander, 2004) we selected the optimal models of nucleotide substitution for each data partition of the nrITS



**Fig. 2.** Conversion of continuous characters to discrete characters. Numbers over the X axis (0, 1, 2) are character states that were used in the phylogenetic analysis of phenotypic characters. A backslash between two of these character states (e.g., 0/1) indicates that state attribution to a given taxon was equivalent for two character states and were treated as uncertain states in the phylogenetic analysis. The thick central horizontal line in the box plot represents the median. Each box encompasses 50% of the samples, each bracket includes 95% of the samples, individual points represent outliers and asterisks represent extreme values.

and  $\beta$ -tubulin (Table 1). Both individual (MB1/nrITS and MB2/ $\beta$ -tubulin) and combined (MB3/nrITS +  $\beta$ -tubulin) datasets were also analyzed using MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). The same six data partitions as in the MP analyses were considered for the Bayesian analysis of the combined dataset. A run of 5,000,000 generations starting from an initial random tree and employing four simultaneous chains was executed. A tree was saved every 100th generation. We plotted the log-likelihood scores of sample points against generation time, which revealed that a stable equilibrium was reached quickly for these datasets (Huelsenbeck & Ronquist, 2001). The first 4,800, 6,000 and 5,000 saved trees for MB1, MB2 and MB3, respectively, were discarded as “burn-in”. Using PAUP\* 4.0b10 (Swofford, 2002), majority rule consensus trees were assembled using the remaining sampled trees and posterior probabilities were calculated for each node. The phylogenetic trees were drawn using TreeViewPPC 1.5.3 (Page, 1998).

## RESULTS

**Morphology.** — The examination of specimens revealed clear differences between the putative species within *Leptogium lichenoides* s.l. and the other species of the group. The ANOVA analysis of continuous characters also detected significant differences between them. These differences were most striking in the minimum values of lobe width. No putative species was identical to another in all characters (Figs. 1, 2; Table 2).

**Phylogenetic analyses.** — The nrITS sequences were highly variable in length (455–580 bp). The first 100 bp and seven ambiguously aligned regions (31 sites) were excluded. The final length of the nrITS alignment was 523 sites, of which 214 were variable, and 80 were parsimony informative (15.29%). The number of parsimony informative characters increased to 87 when INAASE coded characters were added to the nrITS data matrix. In contrast, virtually no length differences were detected

**Table 1. Maximum likelihood best-fit evolutionary models and parameters selected by the Akaike Information Criterion (AIC) for each data partition.**

	ITS1+ITS2	5.8S	$\beta$ -tubulin 1st codon position	$\beta$ -tubulin 2nd codon position	$\beta$ -tubulin 3rd codon position	$\beta$ -tubulin intron
Model selected <sup>a</sup>	TVM+G	TrNef	TrN	TVM	TVM+I	JC+I
–Lln	856.79	245.42	384.3198	363.9562	938.5582	265.1414
AIC	1,729.5941	494.8535	778.6395	741.9124	1,893.1165	532.2828
Proportion of invariable sites (I)	0	0	0	0	0.3196	0
Rates heterogeneity among sites (G)	1.2453	Equal <sup>b</sup>	Equal <sup>b</sup>	Equal <sup>b</sup>	Equal <sup>b</sup>	Equal <sup>b</sup>

<sup>a</sup>See Posada & Crandall (1998) for the definition of these abbreviations. <sup>b</sup>Equal rates for all sites.

**Table 2. Standard deviations and ranges (in parentheses) for diagnostic morphological characters of the *Leptogium lichenoides* species complex.**

Characters	<i>L. aragonii</i>	<i>L. gelatinosum</i>	<i>L. intermedium</i>
Thallus diam. (cm)	3.0–7.0	2.5–5.0	0.5–2.0
Lobe width			
Min. (mm)	(1.4) 1.7–3.5 (4.5)	(1.5) 2.1–3.7 (4.0)	(0.2) 0.2–0.8 (1.0)
Max. (mm)	(2.5) 3.3–6.0 (7.0)	(2.5) 3.0–6.6 (7.0)	(0.8) 0.8–0.9 (1.2)
Thallus thickness ( $\mu$ m)	(32.5) 53.7–114.7 (175.0)	(60.0) 68.9–87.3 (97.5)	(50.0) 55.7–84.2 (95.0)
Spore			
Width ( $\mu$ m)	(11.0) 13.5–17.1 (19.0)	(10.2) 10.9–14.8 (17.3)	(8.1) 9.2–12.3 (13.6)
Length ( $\mu$ m)	(29.0) 33.2–43.6 (52.0)	(24.8) 26.1–36.7 (42.7)	(21.2) 23.3–31.6 (36.3)
	<i>L. lichenoides</i> s.str.	<i>L. pulvinatum</i>	<i>L. pulvinatum</i> var. <i>quercicola</i>
Thallus diam. (cm)	2.5–6.0	1.0–4.0	0.5–2.0
Lobe width			
Min. (mm)	(0.3) 1.9–3.8 (4.5)	(0.5) 0.6–2.4 (4.0)	(0.3) 0.3–0.5 (0.5)
Max. (mm)	(1.8) 2.8–4.9 (5.5)	(0.5) 1.1–3.8 (6.0)	(0.6) 0.6–1.8 (2.0)
Thallus thickness ( $\mu$ m)	(65.0) 83.2–131.4 (175.0)	(52.0) 61.5–94.6 (125.0)	(60.0) 66.2–84.8 (90.0)
Spore			
Width ( $\mu$ m)	(10.0) 12.4–16.3 (19.0)	(12.0) 14.2–16.6 (18.0)	(7.0) 8.6–11.3 (14.0)
Length ( $\mu$ m)	(27.5) 32.4–42.5 (55.0)	(28.0) 33.1–41.5 (47.0)	(20.0) 20.5–25.9 (29.0)

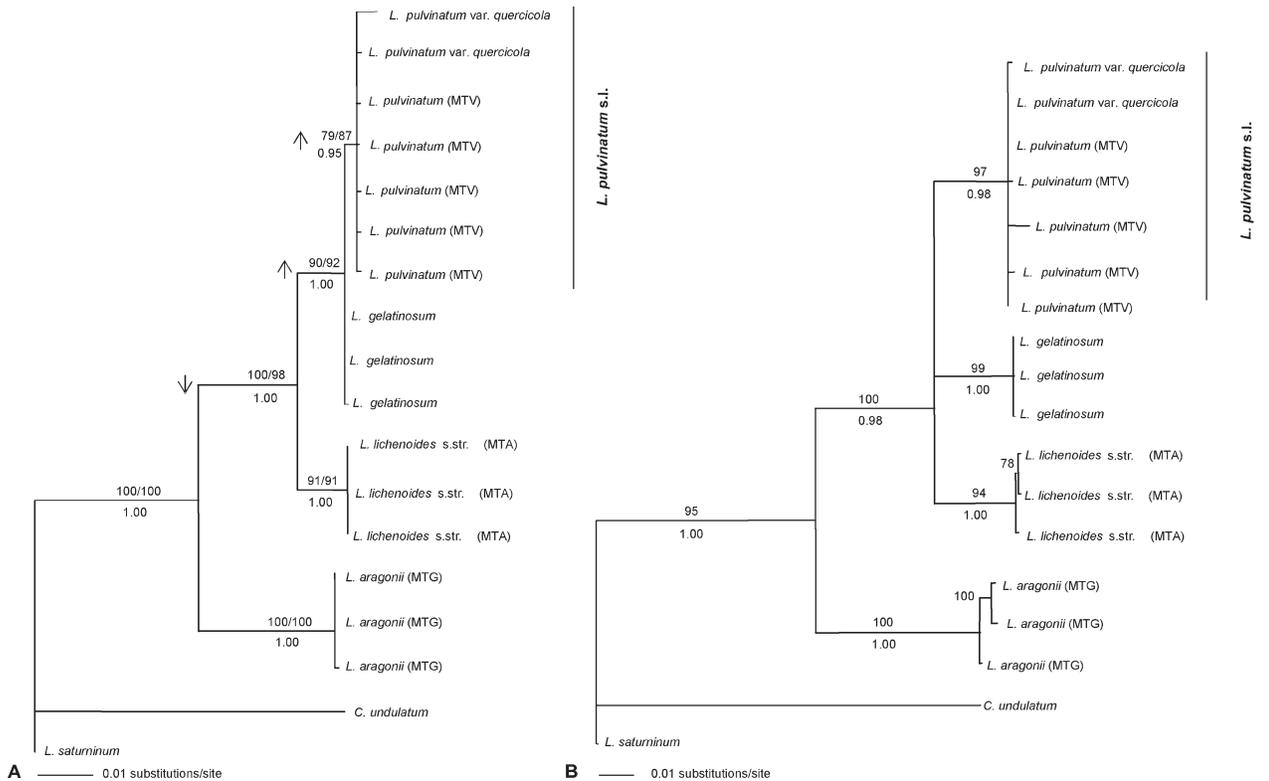
for  $\beta$ -tubulin nucleotide sequences (762–764 bp). The final  $\beta$ -tubulin alignment consisted of 767 sites, of which 223 were variable. A total of 91 sites were parsimony informative (11.8%). Starting at position 460, an intron of 56 bp was present in all  $\beta$ -tubulin sequences.

Eight equally most parsimonious trees of 447.9 steps (one single island, hit 996 times) were obtained from MP1 (nrITS dataset). The analysis using INAASE characters in addition to the unambiguously aligned nrITS sites (MP2) revealed four equally most parsimonious trees (tree length = 487.90 steps, one island hit 996 times). Both majority rule consensus topologies were identical, but bootstrap support values were different for three branches. The only noteworthy difference is an increase in one bootstrap value from 79% to 87% resulting from the addition of INAASE characters (Fig. 3A).

The Bayesian analysis was performed using only unambiguously aligned sites (MB1). The majority rule consensus tree of 45,200 sampled trees was similar to the parsimony topologies and no significant conflict was detected among these three topologies (MP1, MP2, MB1 trees). Only the Bayesian phylogram with posterior

probabilities and bootstrap support from MP1 and MP2 is shown here (Fig. 3A). The nrITS phylogeny revealed three well-supported monophyletic groups: *Leptogium aragonii*, *L. lichenoides* s.str., and *L. pulvinatum* (including var. *quercicola*). The three individuals of *Leptogium gelatinosum* were not resolved using this molecular marker, forming a polytomy with *L. pulvinatum* s.l.

Parsimony analysis of  $\beta$ -tubulin dataset (MP3) revealed two equally most parsimonious trees (tree length = 615.65 steps), and the strict consensus topology was identical to the MB2 tree (shown in Fig. 3B). As for the tree based on nrITS data, this topology revealed two main sister clades, one formed by *Leptogium pulvinatum* s.l., *L. gelatinosum* and *L. lichenoides* s.str., and the second clade only constituted by *L. aragonii*. The resolving characteristics of nrITS and  $\beta$ -tubulin are complementary.  $\beta$ -tubulin resolved relationships among *L. gelatinosum* individuals and *L. pulvinatum* s.l., whereas nrITS resolved relationships among *L. gelatinosum*, *L. lichenoides* s.str. and *L. pulvinatum* s.l., where *L. gelatinosum* is more closely related to *L. pulvinatum* s.l. than to *L. lichenoides* s.str. (Fig. 3).



**Fig. 3.** Single locus phylogenies of the *L. lichenoides* species complex using Bayesian analyses. *Leptogium saturninum* and *Collema undulatum* were used as outgroup taxa. Internal branches with pp < 0.8 and bootstrap < 70 were collapsed using TreeviewPPC 1.5.3. A, nrITS-based phylogeny summarizing 45,200 post burn-in trees. Numbers above each branch indicate bootstrap support of MP1/MP2 analyses (MP1 = nrITS sites only, MP2 = nrITS sites + INAASE characters). Posterior probabilities are shown below branches. Arrows show branches where INAASE encoding enhanced or diminished bootstrap support. B,  $\beta$ -tubulin phylogeny summarizing 44,000 post burn-in trees. Numbers above branches represent MP bootstrap values (MP3 analyses). Posterior probabilities are found below branches.

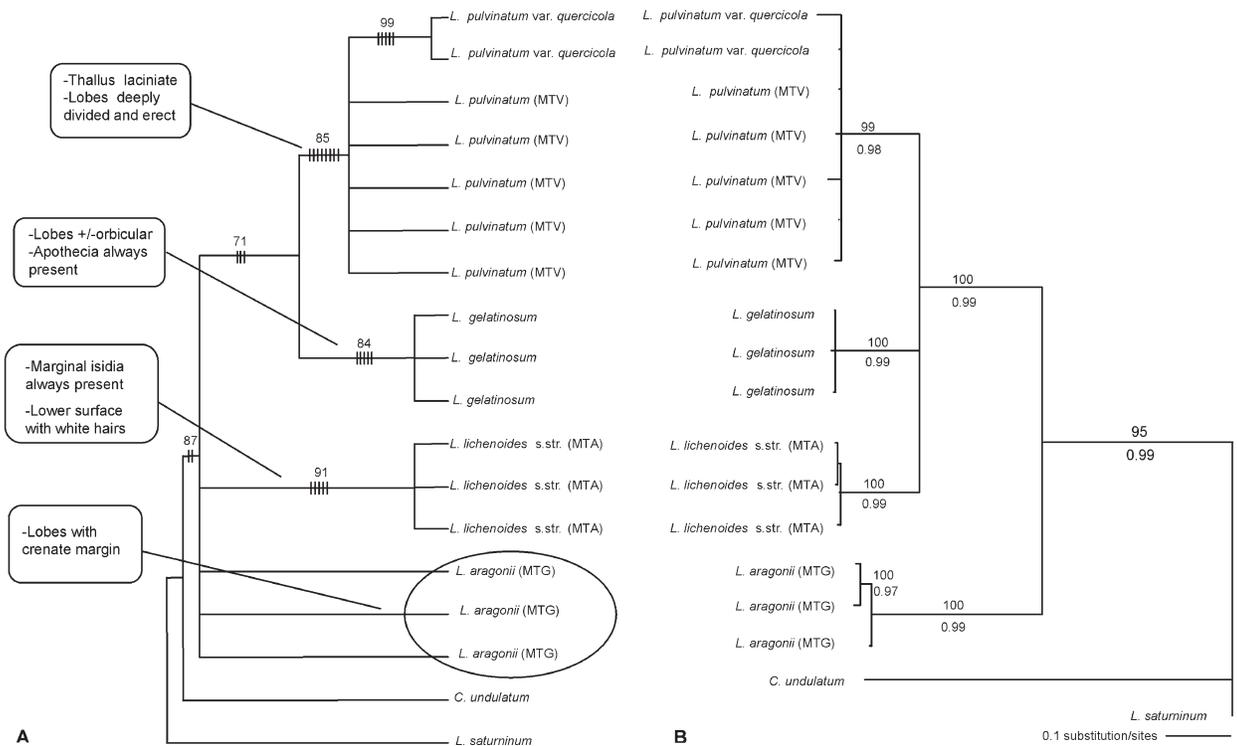
The nrITS matrix with INAASE characters (MP2 dataset) was combined with the  $\beta$ -tubulin dataset for the MP5 analysis, because monophyletic groups were better supported in MP2 than MP1 analyses due to the additional signal provided by the INAASE characters. The MP5 analysis produced two equally most parsimonious trees (score = 1,102.66 steps, hit = 195 times). The topology was identical to the MB3 majority rule consensus tree (Fig. 4B). This final topology revealed four well-supported monophyletic entities, recognized here as distinct *Leptogium* species. Three of these species (*L. aragonii*, *L. lichenoides* s.str., *L. pulvinatum*) belong to what was previously recognized as *L. lichenoides* s.l., and the fourth monophyletic group corresponds to *L. gelatinosum*.

The phylogenetic search based on phenotypic data using maximum parsimony as the optimization criterion included 17 anatomical, ecological and morphological characters. Fifteen characters were parsimony informative. Seven equally most parsimonious trees were recovered by this analysis, which are summarized using a strict consensus tree (Fig. 4A). As for the nrITS and  $\beta$ -tubulin datasets, the combined molecular dataset provided resolution complementary to the morphological dataset. *Leptogium*

*pulvinatum* var. *quercicola* forms a very well supported monophyletic group nested within the *L. pulvinatum* clade using morphological data, while it is not resolved with nrITS and  $\beta$ -tubulin combined. Reversely, *L. aragonii* is not resolved nor rejected as a monophyletic group with phenotypic data, but is well-supported as a monophyletic group with nrITS and  $\beta$ -tubulin combined. The morphological and molecular data independently support three well-supported monophyletic groups, and the molecular data supports one extra monophyletic group (Fig. 4). These four monophyletic entities are recognized as distinct species here.

### TAXONOMY OF THE *LEPTOGIUM LICHENOIDES* GROUP

The results of this work suggest that *Leptogium lichenoides* s.l. comprises three well-supported monophyletic species. One of them corresponds to *L. lichenoides* s.str. with marginal isidia, in accordance with the lectotype (*Tremella lichenoides* LINN1279.9). The second corresponds to *Leptogium aragonii*, which is a new species described



**Fig. 4.** Phylogenetic relationships within the *Leptogium lichenoides* species complex based on phenotypic and molecular data. *Leptogium saturninum* and *Collema undulatum* were used as outgroup taxa. **A**, Strict consensus of seven equally most parsimonious trees based on the phenotypic data set. Numbers above branches represent bootstrap support values. Diagnostic character states are shown for each morphospecies. Unequivocal changes associated with each branches are stippled. **B**, Phylogram derived from a Bayesian analysis of a combined data set including nrITS and  $\beta$ -tubulin nucleotide sequences. Numbers above branches indicate bootstrap support from the MP5 analyses. Bayesian posterior probabilities from the MB3 analyses are shown below each branch.

here. The third corresponds to what was previously described as variety *pulvinatum*, which we recognize here as *L. pulvinatum* comb. nov. In contrast, the previously recognized species *Leptogium quercicola* did not form a monophyletic group based on molecular data. In spite of this, we consider that there are sufficient distinctive morphological character states (Fig. 4A) to give it a variety status. Therefore, it is considered here as a new combination descending in taxonomic rank from species to variety.

We present here a key of the *L. lichenoides* group using the synapomorphies of each group.

1. Thallus minutely foliose or minutely shrubby, up to 2 cm in diam.; lobes < 1.5 mm wide . . . . . **2**
1. Thallus distinctly foliose, > 2 cm in diam.; lobes > 1.5 mm wide . . . . . **3**
2. Lobes mainly erect to semi-erect, forming dense fruticulous cushions. Individual lobes often deeply divided, elongated, lacinated to subcylindrical-complanated, apothecia rarely present, spores 7–14 × 20–29 µm. . . . . ***L. pulvinatum* var. *quercicola***
2. Lobes flat, horizontally spreading. Individual lobes entire or finely divided. Apothecia always present, spores 8–13 × 22–36 µm. . . . . ***L. intermedium***
3. Lobes ± orbicular with margin entire or finely divided; abundant apothecia always present . . . . . ***L. gelatinosum***
3. Lobes elongate, with margins crenate, denticulate, lacerate or fimbriate; apothecia rarely present . . . . **4**
4. Margin lobes isidiate; isidia elongate, cylindrical to coralloid . . . . . ***L. lichenoides* s.str.**
4. Isidia absent . . . . . **5**
5. Lobes flat, horizontally spreading, with margin crenate, over pleurocarpous mosses . . . . . ***L. aragonii***
5. Lobes erect to semi-erect, fimbriate, deeply divided, with margin orbiculate-complanate, forming a dense pulvinulum, over mosses . . . . . ***L. pulvinatum***

***Leptogium aragonii* Otálora, sp. nov.** – Holotype: Spain, Cantabria, Fuente Dé, pista al puerto Pan de Ruedas, 43°08'29.94" N, 5°10'20.64" W, 1,142 m, haiedo, *Otálora 153* (MA-Lichen 16029).

Thallus foliaceus, *L. lichenoidis* similis sed differt isidiis nullis, lobis orbiculatis, complanatis et marginibus integris, sordide cinerea aut viridis.

**Morphology.** – Thallus 3–8 cm broad, formed by flattened and oblong to orbicular lobes, 2–7 mm wide, margins entire to poorly lacerate, slightly undulate over the substrate; upper surface with a few ridges to wrinkled, grey-greenish to brown, always mate; lower surface with few and thin simple hairs towards the lobes base. *Apothecia* sessile, usually rare, but they could be numerous in some thalli, 0.25–0.80 mm diam.; thalline exciple well developed, concolorous with the thallus, sometimes lobulate; disc concave,

brown to red brown. *Conidiomata* rarely observed, marginal, immersed, with dark ostiole (Fig. 1A).

**Anatomy.** – Thallus heteromerous, 54–115 µm thick, cortex paraplectenchymatous, brown coloured, consisting of a single row of elongated cells (5–10 µm length × 3–8 µm wide); photobiont *Nostoc* 4–6 µm diam., spherical to ellipsoidal cells. Hairs 3–4 µm in diameter developed from the lower cortex. *Apothecia* with thalline margin, 25–40 µm wide; proper margin paraplectenchymatous, laterally 10–12 µm, widening at the base to 20–55 µm; hymenium hyaline, 180–220 µm high; epihymenium orange-brown, 2–4 µm; paraphyses simple, 1.0–1.5 µm thick; subhymenium yellowish to orange, 30–55 µm thick; asci cylindrical-clavate, (20–)22.1–36.1(–41) × (110–)120–158(–162) µm, 8-spored. Spores ellipsoid, submuriform to muriform, (11–)13.5–17.1(–19) × (29–)33.2–43.6(–52) µm. Conidia not observed.

**Etymology.** – The epithet *aragonii* refers to the Spanish lichenologist Gregorio Aragón in recognition of his contribution and interest for the genus *Leptogium*, especially in the Iberian Peninsula.

**Habitat and distribution.** – This species is widely distributed throughout Europe, occurring in preserved forests from 200 m in northern regions to 1,800 m in the southern part of Europe. It grows on pleurocarpous mosses close to the base of many tree species such as *Acer* spp., *Fraxinus angustifolia*, *Quercus rotundifolia*, *Q. faginea*, *Q. pyrenaica*, *Pinus pinaster*, *Olea europea*, and shrubs such as *Ilex aquifolium* and *Phillyrea angustifolia*. It also occurs over mossy walls or calcareous rocks within forests. *Leptogium aragonii* frequently appears together with *Collema furfuraceum* Du Rietz, *C. nigrescens* (Huds.) DC., *Lobaria pulmonaria* (L.) Hoffm., *Pannaria conoplea* (Pers.) Bory, and *Nephroma parile* (Ach.) Ach. Specimens were also seen from Japan and North America.

**Remarks.** – *Leptogium aragonii* shows an intermediate morphology between *L. lichenoides* s.str. and *L. pulvinatum*. *Leptogium lichenoides* s.str. is similar to *L. aragonii* in its overall thallus shape, because both present flat and wider lobes than other species of the group, but the lobes of *L. lichenoides* are divided and possess isidiate margins, while *L. aragonii* lobes are divided with lacerate and non-isidiate margins. In relation to *L. pulvinatum*, this species shows clear differences in the lobes and habitat. For example, *L. aragonii* has white hairs on the lower surface of the central part of the thallus, while *L. pulvinatum* does not. Another similar taxon, *Leptogium gelatinosum*, has entire margins and semi-erect lobes, as well as abundant apothecia, while *L. aragonii* has only isolated lobes with few apothecia.

***Leptogium gelatinosum* (With.) J.R. Laundon** in Lichenologist 16: 219. 1984 ≡ *Lichen gelatinosus* With., Bot. Arr. Veg. Gr. Brit.: 710. 1776 – Lectotype (designated

by: J.R. Laundon in Lichenologist 16: 219. 1994): [icon in] Dillenius, Hist. Musc.: 145, tab. 19, fig. 43!. 1742.

**Morphology.** – Thallus foliose, muscicolous, 2.5–4.5 cm broad, greenish to dark brown, formed by rounded to orbicular and semi-erect lobes, from 1.5 to 7.0 mm wide, upper surface strongly wrinkled, lobes with margin entire to irregularly cut, usually shiny; lower surface similar, but paler. *Apothecia* sessile, very numerous, 1–2 mm in diam., disc concave to planate, red brown; thalline margin smooth and paler than lobes. *Conidiomata* not found (Fig. 1B).

**Anatomy.** – Thallus 50–120 µm thick; cortex with isodiametric cells, 6–8 µm diam., often brown only on upper cortex; homiomorous medulla of loosely interwoven hyphae; photobiont *Nostoc* cells of 4–8 µm diam., spherical to ellipsoid, arranged in short chains of 4–9 cells throughout thallus, sometimes more abundant near upper cortex. *Apothecia* with thalline margin 50–80 µm wide; proper margin euparaplectenchymatous, 20–30 µm thick; subhymenium yellowish, 20–35 µm thick; hymenium 175–220 µm high with brown to red epithecium; paraphyses simple, 1.0 µm thick, apices 3–4 µm wide; asci cylindrical-clavate, with 8 spores, (10.0–)10.5–14.5(–15.0) × (100.0–)112.1–137.9(–140.0) µm. Spores ellipsoid, submuriform to muriform, (10.2–)10.9–14.8(–17.3) × (24.8–)26.1–36.7(–42.7) µm.

**Habitat and distribution.** – *Leptogium gelatinosum* grows among mosses on rocks, soils and rarely on bark of *Quercus*, *Juniperus*, *Pinus* and *Populus* species. It usually prefers basic habitats, although it can be found over mosses on granitic rocks. It prefers well-preserved forests. *Leptogium gelatinosum* is broadly distributed in Europe, from sea level in Nordic countries to 2,000 m in the Alps and the Eurosiberian region. In the Mediterranean region, it usually grows in slopes with humid conditions. This is a common species in Europe but less frequent than *Leptogium pulvinatum*. It appears with species such as *Collema furfuraceum*, *Leptochidium albociliatum* (Desm.) M. Choisy, *Leptogium corniculatum* (Hoffm.) Minks, *L. tenuissimum* (Hoffm.) Körb. and *Polychidium muscicola* (Sw.) Gray.

**Remarks.** – *Leptogium gelatinosum* resembles some big specimens of *L. pulvinatum* with weakly divided lobes, but the latter normally does not have apothecia over all thalli, and has revolute margins. Some fertile specimens of *Leptogium aragonii* could be confused with this species, but *L. gelatinosum* is more strongly wrinkled, shiny and dark. Other similar species are *Leptogium polycarpum* P.M. Jørg. & Goward, which is an American species with four spores per ascus, and *L. gelatinosum*, which has 8-spored asci. The most similar species is *L. intermedium*, which has smaller thalli.

***Leptogium intermedium*** (Arnold) Arnold in Flora 68: 212. 1885 ≡ *Leptogium minutissimum* var. *intermedium* Arnold in Flora 50: 122. 1867 – Lectotype

(designated by: P.M. Jørgensen in Lichenologist 26: 12. 1994): Switzerland, near Zurich, leg. Hepp [Flechten Europas no. 212] (M-024289!).

**Morphology.** – Thallus minutely foliose, 0.5–2.0 cm in diameter, lobes with margin entire and ascending, 0.2–1.0 mm broad, blue green to grey, usually mat, with ridges or slightly wrinkled; lower surface similar, but paler. *Apothecia* common and usually numerous, sessile and laminal, 0.5–1.5 mm diam., disc concave and brown; thalline margin smooth and paler than lobes. *Conidiomata* not found (Fig. 1C).

**Anatomy.** – Thallus 50–95 µm thick; upper and lower cortex with a single layer of isodiametric cells, 5–8 µm diam.; medulla of densely interwoven hyphae; photobiont *Nostoc* in short chains of 4–8 cells, cells 4–6 µm diam. *Apothecia* with thalline margin, 35–60 µm wide; proper margin subparaplectenchymatous, 5–10 µm thick; subhymenium brownish, 25–50 µm thick; hymenium 95–180 µm high, with brownish epithecium; paraphyses simple, 1 µm thick, slightly thickened at the apex; asci cylindrical-clavate, (12.0–)14.4–20.0(–21.2) × 85.4(–90.0)–124.5(–150.0) µm, 8-spored. Spores ellipsoid, submuriform to muriform, (8.1–)9.2–12.3(–13.6) × (21.2–)23.3–31.6(–36.3) µm.

**Habitat and distribution.** – This species grows on calciferous soils, mossy rocks and mossy trunks of old trees. It is widespread in Europe but is not common. It prefers shady locations with high humidity, from 400 m to subalpine regions. *Leptogium intermedium* shares its habitat with *Collema subnigrescens* Degel., *Leptogium aragonii*, *L. subtile* (Schrad.) Torss., *Nephroma laevigatum* Ach., *Fuscopannaria ignobilis* (Anzi) P.M. Jørg. and *Peltigera collina* (Ach.) Röhl.

**Remarks.** – As mentioned above, *Leptogium intermedium* looks like a diminutive form of *L. gelatinosum*. However, there are morphological differences such as in the shape of the thallus and upper surface ridges, as well as differences in spore size (Table 2). The medulla of *L. intermedium* has densely interwoven hyphae, while *L. gelatinosum* has loosely interwoven hyphae. Differences are also observed in the thalline exciple, which is smooth and thin in *L. intermedium* and rough and thick in *L. gelatinosum*. *Leptogium intermedium* can also be confused with *L. imbricatum* P.M. Jørg. (1994) and *L. subtile*, but both species have paraplectenchymatous thalli, and the lobes of the latter are arranged in rosettes.

***Leptogium lichenoides*** (L.) Zahlbr., Cat. Lich. Univ. 3: 136. 1924 ≡ *Tremella lichenoides* L., Sp. Pl. 2: 1157. 1753 – Lectotype (designated by: P.M. Jørgensen & al. in Bot. J. Linn. Soc. 115: 371, f. 67. 1994): (LINN 1276.9! lower specimen)  
= *Leptogium lichenoides* f. *fimbriatum* (Ach.) Zahlbr. Cat. Lich. Univ. 3: 140. 1924 ≡ *Collema fimbriatum* Hoffm., Deutschl. Fl.: 104. 1796 ≡ *Parmelia lacera*

var. *fimbriata* (Hoffm.) Ach., Methodus: 226. 1803  
 ≡ *Collema lacerum* var. *fimbriatum* (Hoffm.) Ach.,  
 Lichenogr. Universalis: 658. 1810.

**Morphology.** – Thallus muscicolous, foliose, 2.5–6.0 cm broad. Lobes flat to semi-erect, deeply divided, papyraceous, blue-grey to greenish colours, wrinkled to distinctly ridged with isidiate margins; lower surface with fine hairs in the middle of the thallus. *Isidia* cylindrical to coralloid, 0.1–0.7 mm long. *Apothecia* sessile, 0.25–0.7 mm diam., disc concave, red to red brown, thalline margin well developed concolorous with thallus, frequently isidiate, with cylindrical to coralloid *isidia*. *Conidiomata* not found (Fig. 1D).

**Anatomy.** – Thallus heteromerous, 65–175 µm thick; upper and lower cortex formed by a layer of isodiametric to elongated cells, 6–12 µm in diam., hyaline to faintly coloured; medulla of loosely interwoven hyphae among *Nostoc* chains; *Nostoc* in chains of 10–15 subglobose cells, individual cell 5–8 µm diam. *Apothecia* with thalline margin, 20–70 µm wide; proper margin euparaplectenchymatous, 20–40 µm thick; subhymenium yellowish, 25–50 µm thick; hymenium 65–135 µm high with brown to red epithecium; paraphyses simple, 1 µm thick and thickened at the apex, up to 2–3 µm thick; asci cylindrical-clavate, 8-spored, (10.0–)11.4–17.0(–18.0) × (90.0–)94.5–115.4(–120.0) µm. Spores ellipsoid, submuriform to muriform, (10.0–)12.4–16.3(–19.0) × (27.5–)32.4–42.5(–55.0) µm.

**Nomenclatural remarks.** – When Linnaeus (1753) described *Tremella lichenoides*, he assigned this name to a specimen from Sweden (LINN 1276.9, a portion of which was selected as lectotype for *Leptogium lichenoides* by Jørgensen & al., 1994: 371). In the protologue, Linnaeus did not mention the presence of *isidia* and did not describe the lower surface of the thallus. When we examined the lectotype and other specimens from the same collection, marginal *isidia* were found in planate lobes and white hairs were observed on the lower surface of thalli.

**Habitat and distribution.** – *Leptogium lichenoides* occurs among mosses, on the base of *Fraxinus*, *Acer*, *Populus*, *Quercus*, *Abies* and *Fagus* trees, although it is also possible to find it directly on soils, rocks and walls within well-preserved forests, generally over calcareous soils. However, a few specimens have been found on acidic substrates. This species is widespread in Europe, from Mediterranean to Nordic countries, from 400 to 1,500 m in altitude. It is usually accompanied by other cyano-lichens such as *Lobaria amplissima* (Scop.) Forssell, *L. pulmonaria* (L.) Hoffm., *Nephroma laevigatum*, *N. resupinatum* (L.) Ach., *Pannaria conoplea* (Pers.) Bory, *Pameliella tryptophylla* (Ach.) Müll. Arg. and *Peltigera horizontalis* (Huds.) Baumg.

**Remarks.** – *Leptogium lichenoides* is easily distinguishable from other species of the group, because it is the only species with marginal cylindrical *isidia*. Also, the thallus of *L. lichenoides* is bigger and has thicker lobes than *L.*

*pulvinatum* and *L. gelatinosum* (Fig. 2). There are significant differences in thallus thickness, with *L. lichenoides* having among the highest values for this group (Table 2). Another similar taxon is *Leptogium juressianum* Tav., with marginal cylindrical *isidia*, which, however, lacks a wrinkled surface and has arachnoid hairs on the upper surface. The molecular analysis suggests that it is a monophyletic species clearly separated from *Leptogium pulvinatum* and *L. aragonii*.

***Leptogium pulvinatum* (Hoffm.) Otálora, comb. et stat.**

**nov.** ≡ *Collema pulvinatum* Hoffm., Deutschl. Fl.: 104. 1796 ≡ *Parmelia lacera* var. *pulvinatum* (Hoffm.) Ach., Methodus: 226. 1803 ≡ *Collema lacerum* var. *pulvinatum* (Hoffm.) Ach., Lichenogr. Universalis: 658. 1810 ≡ *Leptogium lacerum* subsp. *pulvinatum* (Hoffm.) Cromb., Monogr. Lich. Britain 1: 70. 1894 ≡ *Leptogium lichenoides* var. *pulvinatum* (Hoffm.) Zahlbr., Cat. Lich. Univ. 3: 142. 1924 – Lectotype (designated by: P.M. Jørgensen in Lichenologist 26: 10. 1994): [icon in] Dillenius, Hist. Musc.: tab. 19, fig. 43!. 1742.

**Morphology.** – Thallus foliose, pulvinate, 1–4 cm, formed by erect lobes, compactly united. Individual lobes often deeply divided, elongated, lacinated and revolute; both surfaces wrinkled, shiny, greyish-brown to dark brown in colour; erect lobes 2–6 mm wide. *Apothecia* sessile, rare, 0.2–0.8 mm in diam.; thalline margin well developed, concolorous with thallus; disc concave, brown to red brown. *Conidiomata* not found (Figs. 1E, F).

**Anatomy.** – Thallus heteromerous, 50–120 µm thick; upper and lower cortex brown-coloured, with a single layer of irregularly isodiametric cells, 6–9 µm diam.; medulla of smooth interwoven hyphae; photobiont *Nostoc* in short chains, individual cells 5–7 µm diam. *Apothecia* with thalline margin, 20–70 µm wide; proper margin euparaplectenchymatous, 10–40 µm thick; subhymenium yellowish, 25–50 µm thick; hymenium 65–135 µm high with brown to red epithecium; paraphyses simple, 1.5 µm thick and thickened at the apices up to 5 µm; asci cylindrical-clavate, (12.0–)14.4–21.2(–22.0) × (80.0–)85.4–124.5(–125.0) µm, 8-spored. Spores ellipsoid, submuriform to muriform, (12.0–)14.2–16.6(–18.0) × (28.0–)33.1–41.5(–47.0) µm.

**Nomenclatural remarks.** – The lectotype of *Collema pulvinatum* Hoffm. (1796), was designated by Jørgensen (1994) and corresponds to a Dillenian illustration cited by Hoffmann (1796), which represents a cushion-form like *Leptogium pulvinatum*. Although accepted in the *Index Fungorum*, following Zahlbruckner (1924) and Lamb (1963), the combination “*Leptogium pulvinatum* (Hoffm.) Cromb.” was not validly published by Crombie (1894) who considered this taxon as a subspecies within *Leptogium lacerum* Gray (*L. lacerum* was regarded as a synonym of *L. lichenoides* by Zahlbruckner, 1924) and mentioned that it could almost be regarded as a distinct species, but he did not treat it at that level in his publication.

**Habitat and distribution.** – This species is the most common and abundant of the *L. lichenoides* complex species in Europe. *Leptogium pulvinatum* occurs among mosses at the base of trees or occasionally directly over bark of different trees such as *Abies pinsapo*, *Quercus rotundifolia*, *Q. faginea*, *Q. pyrenaica*, *Q. suber*, *Pinus pinaster*, *Olea europaea* and *Fraxinus angustifolia*. It is also often found on walls, rocks or soil in open zones over acrocarpous mosses, from the coast to the mountains. When growing on soils or rocks it shares habitats with *Fulgensia schistidii* (Anzi) Poelt and *Candelariella aurella* (Hoffm.) Zahlbr.

**Remarks.** – This species is easily distinguishable from the rest of the group, because the lobes are deeply divided and the margin is lacinate. Neither *Leptogium aragonii*, *L. gelatinosum* nor *L. intermedium* have divided lobes. Some specimens of *L. pulvinatum* with slightly erect lobes and long lacinia can be confused with *L. lichenoides* s.str., although the margin of *L. lichenoides* has cylindrical isidia. *Leptogium pulvinatum* has shiny lobes while *L. aragonii* and *L. lichenoides* are characterized by mat lobes. However, there are no clear differences in anatomical characters between this taxon and the rest of the group. There are differences in lobe width (Fig. 2). According to our molecular phylogenetic analyses, this species represents a lineage distinct from *Leptogium lichenoides* s.str.

***Leptogium pulvinatum* var. *quercicola*** (Otálora, Aragón, I. Martínez & M.C. Molina) Otálora **comb. et stat. nov.** ≡ *Leptogium quercicola* Otálora, Aragón, I. Martínez & M.C. Molina in *Lichenologist* 36: 199. 2004 – Type: Spain (MA-lichen 15217! holotype; MA-lichen 15206! isotype).

**Morphology.** – Thallus 0.5–2.0 cm in diam., with a minutely foliose base, which develops many crowded, narrow, and erect to semi-erect lobes, forming dense fruticulose cushions; individual lobes deeply divided, elongated, lacinated to subcylindrical-complanated; upper surface wrinkled, shiny, greenish-brown to dark brown colour; erect lobes 60–100 µm wide and up to 1 mm long, external lobes wider, 200–240 µm. *Apothecia* sessile, very rare, 0.2–0.8 mm in diam.; thalline margin well developed, concolorous with thallus; disc concave, brown to red brown. *Conidiomata* not found (Fig. 1G–H).

**Anatomy.** – Thallus heteromerous, 60–90 µm thick; upper and lower cortex brown-coloured, with a single layer of isodiametric cells, 8–9 µm diam.; medulla of densely interwoven hyphae; photobiont *Nostoc* in short chains, individual cells 5–7 µm in diam. *Apothecia* with thalline margin 25–40 µm wide; proper margin paraplectenchymatous, laterally 10–15 µm, basally 25–45 µm. Hymenium 100–125 µm high; epihymenium brown, 3–5 µm thick; paraphyses simple 1.0–1.5 µm thick, 2.5–3.0 µm apically; hypothecium yellowish, 25–35 µm thick;

asci cylindrical-clavate, (12.0–)13.0–19.1(–20.0) × (80.0–)82.1–102.9(–105.0) µm, 8-spored. Spores ellipsoid, submuriform to muriform, with rounded apices, (7.0–)8.7–11.3(–14.0) × (20.0–)20.5–25.9(–29.0) µm.

**Habitat and distribution.** – *Leptogium pulvinatum* var. *quercicola* grows on bark of *Abies pinsapo*, *Quercus rotundifolia* and *Q. faginea*. It is found only in central and southern regions of the Iberian Peninsula. It grows mainly in humid valleys and ravines, inside well-preserved forests, from the meso- to supra-Mediterranean belts (750–1,280 m). This species frequently occurs together with *Collema fasciculare*, *Fuscopannaria ignobilis* (Anzi) P.M. Jørg., *F. mediterranea* (Tav.) P.M. Jørg., *Koerberia biformis* A. Massal., *Leptogium furfuraceum* (Harm.) Sierk, *L. subaridum*, *L. teretiusculum*, *Lobaria pulmonaria* and *Waynea adscendens* V.J. Rico.

**Remarks.** – This variety usually develops smaller and dense fruticulose thalli, with narrower lobes than *L. pulvinatum* s.str. (Table 2). There is a clear difference in spore size, *L. pulvinatum* var. *quercicola* having smaller spores (Table 2). This taxon was only found growing directly over bark of *Quercus* trees, while *L. pulvinatum* var. *pulvinatum* grows on bark, rocks and soil among mosses. With regard to the distribution of *L. pulvinatum* var. *quercicola*, it should be emphasized that we did not find specimens from any of the herbaria we visited or requested material that could be classified as part of this new variety. *Leptogium pulvinatum* var. *quercicola* is morphologically similar to *L. teretiusculum* and some corticolous individuals of *L. tenuissimum*, both having smooth narrow lobes with segmented branches, and are cellular throughout their thalli, whilst *L. pulvinatum* var. *quercicola* is heteromerous.

## DISCUSSION AND CONCLUSION

This study revealed that *Leptogium lichenoides* s.l. comprises three species: *L. aragonii*, *L. lichenoides*, and *L. pulvinatum*. *Leptogium aragonii* is described here as a new species, whereas *L. pulvinatum* var. *pulvinatum* is a new combination. The division of *L. lichenoides* s.l. into three different species is in agreement with our initial preliminary recognition of three morphospecies. Furthermore, our study revealed that the presence of isidia is a synapomorphy and must be considered as an important taxonomical trait, despite the fact that in the past this character was not thought to be a key diagnostic feature within this complex (Sierk, 1964; Coppins & Purvis, 1992).

Although molecular data have resolved relationships within this species complex, there are some differences between the analyses. The nrITS data did not detect the monophyly of *Leptogium gelatinosum* that was revealed independently by the phylogenetic analysis of β-tubulin and morphological data (Figs. 3, 4). However, the nrITS

tree did show that *L. gelatinosum* shares a most recent common ancestor with *L. pulvinatum* s.l., whereas the relationship between these two species and *L. lichenoides* s.str. is unresolved when phylogenetic analyses are restricted to  $\beta$ -tubulin, forming a trichotomy. We were unable to sequence the first portion of nrITS (c. 100 bp adjacent to the small subunit RNA gene) for most of our samples, due to the existence of multiple mononucleotide repeats. The exclusion of this portion of nrITS-1 implies an important loss of phylogenetic information (Myllys & al., 1999; Martín & al., 2000; Miadlikowska & al., 2003). Because these two markers are complementary in resolving relationships within this complex, we recommend that, at least, both markers be used in future studies of this complex. The suitability of  $\beta$ -tubulin as a molecular marker to reveal phylogenetic relationships among taxonomic entities has been broadly discussed, especially gene duplications and their implications for phylogenetic studies (Begerow & al., 2004). Despite the complications associated with paralogy, this marker has been successfully used in phylogenetic studies of basidiomycetes and ascomycetes, including lichen-forming fungi (Begerow & al., 2004; Myllys & al., 2001, 2005; Molina & al., 2004; Articus, 2004; Divakar & al., 2005).

This phylogenetic study suggests that the recently described *Leptogium quercicola* (Otálora & al., 2004) should be considered as a variety of *L. pulvinatum*. This species was described based on clear morphological, anatomical and ecological differences. The low amount of genetic variation found in *L. pulvinatum* s.l. for the nrITS and  $\beta$ -tubulin markers combined suggests that these morphological differences observed within this species s.l. relate more to phenotypic plasticity than genetics. It is possible that this taxon is in the process of speciating, or is the result of a recent speciation, and the molecular markers used here are not sufficiently sensitive to detect this recent divergence. Based on these results, it is preferable at this time to recognize *L. quercicola* as a variety of *L. pulvinatum*, until this question is resolved using faster evolving markers.

Finally, we conclude that in Europe the *Leptogium lichenoides* complex comprises five species, *L. aragonii*, *L. gelatinosum*, *L. intermedium*, *L. lichenoides* and *L. pulvinatum*, which are all broadly distributed in this part of the world. *Leptogium intermedium* and *L. pulvinatum* var. *quercicola* are the least frequently collected taxa of this complex. *Leptogium pulvinatum* var. *pulvinatum* is the most commonly found species of this group in Europe. It was sampled from very different types of habitats, including very dry environmental conditions where only acrocarpous bryophytes can be found. The new non-isidiate species *L. aragonii* was revealed as a monophyletic unit by molecular and morphological characters independently. This species grows on pleurocarpous mosses at the base of many types of trees and over mossy walls and calcareous rocks within forests.

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**Appendix 1. Voucher, locality and habitat, as well as GenBank accession numbers for specimens used in the molecular phylogenetic analyses of this study.**

Taxon name <sup>a</sup>	Locality and substrate	Voucher	GenBank acc. number	
			nrITS	β-tubulin
<i>Collema undulatum</i>	Spain, Málaga, saxicolous	MA Lich 16036	DQ466044	DQ466062
<i>Leptogium aragonii</i>	Spain, Jaén, terricolous (mosses)	MA Lich 16026	DQ466040	DQ466058
<i>L. aragonii</i>	Spain, Toledo, terricolous (mosses)	MA Lich 16028	DQ466041	DQ466059
<i>L. aragonii</i>	Spain, Cantabria, terricolous (mosses)	MA Lich 16029	DQ466042	DQ466060
<i>L. gelatinosum</i>	Spain, Toledo, terricolous (mosses)	MA Lich 16020	DQ466037	DQ466055
<i>L. gelatinosum</i>	Spain, Madrid, terricolous (mosses)	MA Lich 16022	DQ466038	DQ466056
<i>L. gelatinosum</i>	Spain, Granada, terricolous (mosses)	MA Lich 16021	DQ466039	DQ466057
<i>L. lichenoides</i> s.str.	Spain, Asturias, terricolous (mosses)	MA Lich 16023	DQ466045	DQ466063
<i>L. lichenoides</i> s.str.	Spain, Cantabria, terricolous (mosses)	MA Lich 16025	DQ466047	DQ466065
<i>L. lichenoides</i> s.str.	Spain, Jaén, terricolous (mosses)	MA Lich 16027	DQ466046	DQ466064
<i>L. pulvinatum</i>	Spain, Madrid, epiphytic (mosses)	MA Lich 16030	DQ466030	DQ466048
<i>L. pulvinatum</i>	Spain, Asturias, epiphytic (mosses)	MA Lich 16032	DQ466036	DQ466054
<i>L. pulvinatum</i>	Spain, Cuenca, epiphytic (mosses)	MA Lich 16031	DQ466032	DQ466050
<i>L. pulvinatum</i>	Spain Málaga, terricolous (mosses)	MA Lich 16083	DQ466031	DQ466049
<i>L. pulvinatum</i>	Spain, Guadalajara, terricolous (mosses)	MA Lich 16033	DQ466033	DQ466051
<i>L. pulvinatum</i> var. <i>quercicola</i>	Spain Toledo, corticolous (bark)	MA Lich 16034	DQ466034	DQ466052
<i>L. pulvinatum</i> var. <i>quercicola</i>	Spain, Jaén, corticolous (bark)	MA Lich 16035	DQ466035	DQ466053
<i>L. saturninum</i>	France, Midi-Pyrénées, corticolous (bark)	MA Lich 16024	DQ466043	DQ466061

<sup>a</sup>Identifications and names are based on conclusions of this study.

**Appendix 2. Characters and character states included in the morphological data matrix.**

Character	Character state
1. Thallus size	0 = <2 cm, 1 = 2–5 cm, 2 = >5 cm
2. Thallus color	0 = green, 1 = bluish-green, 2 = dark brown
3. Thallus upper surface luster	0 = shiny, 1 = mat
4. Lobe orientation	0 = erect, 1 = planate
5. Lobe margin	0 = crenate ( <i>L. aragonii</i> ), 1 = lacinate ( <i>L. pulvinatum</i> ), 2 = isidiate ( <i>L. lichenoides</i> s.str.), 3 = entire <sup>a</sup>
6. Lobe max. width	0 = <2.5 mm, 1 = 2.5–4.0 mm, 2 = >4.0 mm
7. Lobe min. width	0 = <0.5 mm, 1 = 0.5–1.5 mm, 2 = >1.5–4.0 mm
8. Lobe upper surface	0 = wrinkled, 1 = smooth
9. Isidia position	0 = absent, 1 = upper surface, 2 = marginal
10. Lower cortex	0 = smooth, 1 = arachnoid white hairs, 2 = tomentum ( <i>L. saturninum</i> )
11. Upper and lower cortex	0 = absent, 1 = present
12. Lobe thickness	0 = <100 µm, 1 = 100–140 µm, 2 = >140 µm
13. Spore shape	0 = ellipsoidal, 1 = linear oblong (fusiform)
14. Spore width	0 = <10 µm, 1 = >10 µm
15. Spore length	0 = 20–30 µm, 1 = >30 µm
16. Apothecia frequency	0 = >50% of thallus, 1 = <50%
17. Substrate	0 = corticolous (directly on bark), 1 = muscicolous on corticolous mosses, 2 = muscicolous on terricolous mosses

<sup>a</sup>See Fig. 1.

Appendix 3. European distribution of: A, *Leptogium aragonii*; B, *L. gelatinosum*; C, *L. intermedium*; D, *L. lichenoides*; E, *L. pulvinatum* (● = *L. pulvinatum* var. *quercicola*). The maps only include the localities of the specimens revised from the European herbaria.

