The phenotypical features used for distinguishing species within the *Cladonia furcata* complex are highly homoplasious

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Abstract: The *Cladonia furcata* complex here treated comprises *C. farinacea*, *C. furcata*, *C. multiformis*, *C. scabriuscula*, *C. stereoclada*, and *C. subrangiformis*. The well known taxonomic complexity of this group is caused by wide phenotypic variation within and high morphological similarity among the species. Here, we investigated the evolution of the phenotypic characters traditionally used to distinguish the species in this complex. A phylogenetic analysis of the *C. furcata* complex is presented here, based

on three loci (ITS rDNA, IGS rDNA and *rpb2*), representing specimens from a broad geographical range (Europe, North America and New Zealand). The phylogenetic reconstructions were performed using Maximum Likelihood and Bayesian analyses. In addition, 14 features traditionally used for species delimitation within this complex were mapped onto the Bayesian phylogeny. All the species currently accepted, with the exception of *C. stereoclada*, turned out to be polyphyletic. Most of the phenotypical characters studied are highly homoplasious with the exception of the podetium type. The solid podetia represent a diagnostic character of *C. stereoclada*.
Key words: *Cladonia, homoplasy, molecular phylogeny, phenotypic characters*

Introduction

It is of great importance in systematics to identify diagnostic characters that enable us to tell species apart. For a phenotypic trait to be useful as a diagnostic character it has to be constant within a taxon, undergoing only minor effects from environmental modifications (Davis & Heywood 1965; Winston 1999) and preferably easy to recognize (that is, the different states are unambiguous and easily observable). Features of this kind, however, are not easy to find: many of the characters used to circumscribe species in different groups of lichenized fungi are not diagnostic for different phylogenetic lineages (e.g. Lohtander *et al.* 1998; Ott *et al.* 2004; Buschbom & Mueller 2006; Nelsen & Gargas 2009; Velmala *et al.* 2009; Crespo & Lumbsch 2010; Leavitt *et al.* 2011*a*, *b*; Lumbsch & Leavitt 2011). One method of assessing whether the phenotypic characters are useful as diagnostic characters is to map them onto the phylogenetic tree of the group under study, and then verify whether the phylogenetically related OTUs share the same character states (Scotland *et al.* 2003).

Due to the fact that the genus *Cladonia* embraces a great number of species, many of which are morphologically extremely variable (Ahti 2000; Ahti & Stenroos 2013), the taxonomy of this genus can be considered one of the most intricate within macrolichens. Recent studies based on molecular characters have confirmed this complexity (Stenroos *et al.* 2002; Fontaine *et al*. 2010; Pino-Bodas *et al*. 2011; Steinová *et al*. 2013), indicating that many of the morphological characters used in the taxonomy of the genus are homoplasious and, consequently, that many species accepted on the basis of these characters are non-monophyletic.

The present study focus on the *Cladonia furcata* complex, here including *C. farinacea* (Vain.) A. Evans, *C. furcata* (Huds.) Schrad., *C. multiformis* G. Merr., *C. scabriuscula* (Delise) Nyl., *C. stereoclada* Abbayes, and *C. subrangiformis* Sandst. The taxa within the *Cladonia furcata* complex were formerly placed in Sect. *Ascyphiferae* Tornab. (type: *C. furcata*) of *Cladonia* (Ahti 2000). This section consisted of species characterized by an evanescent primary thallus, generally subulate podetia (with the exception of *C. multiformis*) with a dichotomous branching type, closed axils when young (later open and then often with lateral splits), with a corticate surface, a very strong central stereome and usually without soredia. Among these species, atranorin and fumarprotocetraric acid are the most frequent secondary metabolites (Huovinen *et al*. 1990; Ahti 2000). Using DNA sequences, Stenroos *et al.* (2002) showed that the section *Ascyphiferae* is polyphyletic, but *C. farinacea, C. furcata* and *C. scabriuscula* formed a monophyletic group. However, *C. farinacea* and *C. scabriuscula* were not monophyletic, hence the specimens from Chile were different from the North Hemisphere material (Stenroos *et al.* 2002).

Lichenologists have shown great interest in this complex of species, as well as in the morphological variability of the taxa, indicated by a considerable amount of

literature on the subject (Asahina 1942; Evans 1954; Ullrich 1956; Schade 1964, 1966; Hennipman 1967; Pišut & Wagner 1973; Paus 1997; Günzl 2004). The species are very similar and their circumscription has occasionally been questioned, which led several authors to consider some of these taxa as having infraspecific rank within *C. furcata* (Vainio 1887; Fink 1904; Hariot 1887; Abbayes 1937; Schade 1966; Hawksworth 1969; Hennipman 1967, Hennipman & Sipman 1978; Verseghy, 1975; Wirth 1995; Clauzade & Roux 1985). For example, many authors have recognized *C. subrangiformis* as having infraspecific rank within *C. furcata* (Wirth 1995; Abbayes 1937; Schade 1966; Hawksworth 1969; Hennipman 1967; Verseghy, 1975; Clauzade & Roux 1985; James 2009), while others consider both as species (Nimis 1993; Burgaz & Ahti 2009; Ahti & Stenroos 2013). The phenotypic characters that in combination have been used to distinguish the species of this complex include the secondary metabolites, the branching type, the branching angle, the presence of white medullary outgrowth, the production of squamules (presence, abundance and morphology), the presence of soredia or soredioid granules or phyllidia, the production of scyphi, and the podetium type (solid or hollow) (Sandstede 1922; Huovinen *et al.* 1990; Abbayes 1937; Wirth 1995; Ahti 2000; James 2009). However, intensively sampled molecular data have not been compiled to test these features in the circumscription of species in this complex

In this study, the phylogeny of the *Cladonia furcata* complex is inferred, and the phenotypic characters were mapped on the Bayesian tree, in order to address the following [MW1]issues: 1) the monophyly or polyphyly of the current species, 2) the homoplasy degree of the phenotypic characters. We hypothesize that the phenotypic characters are highly homoplasious and most of the species, as understood today, polyphyletic.phenotypic

Material and Methods

Taxon sampling

This study is based on 862 specimens (19 of *C. farinacea*, 583 of *C. furcata*, 18 of *C. multiformis*, 43 of *C. scabriuscula*, 19 of *C. stereoclada*, and 180 of *C. subrangiformis*) from the herbaria CANB, FH, H, MA, MACB, L, S and UPS. For molecular study 114 specimens were selected. The criteria for the selection were: 1) the entire morphological and chemical variability of each species was included, 2) the material originated from different geographical regions in order to represent as complete distribution of the species as possible, 3) the specimen had to be less than 10 years old to be suitable for DNA studies. Unfortunately, even the fresh specimens were not all successfully amplified for the three loci but most of the phenotypic variation was gathered in the specimens successfully amplified. In the phylogenetic analyses only the specimens with sequences of two loci at least were included. These specimens are listed in Table 1. *Cladonia pulvinella* S. Hammer and *C. corsicana* (Rondon & Vězda) Pino-Bodas, Burgaz & M. P. Martín were used as outgroup taxa based on their placement in our more extensive phylogenetic analyses (Pino-Bodas *et al*. 2012; Ahti *et al.* 2015; Stenroos *et al.* in prep).

Phenotypic characters

Fourteen phenotypic characters were selected based on the literature (Merrill 1909; Sandstede 1922; Abbayes 1937, 1946; Evans 1950; Ahti 2000). Table 2 lists the characters and their different states. The state of each phenotypic character was obtained by the morphological and chemical study of each specimen included in the phylogenetic study. The frequency of each character state per epecies based on a extensive number of specimens is provided in the supplementary material (Table S1 & S2). The morphological characters were studied in three podetia per specimen (the podetia were

randomly selected). The angles of all the branches in the three podetia were measured, then the average of all these values per specimen was used for the analysis. Only a few specimens showed different states for some of the characters (frequently for the characters axil type or squamules). In these cases the presence of squamules was taken as a character state for the specimen, since we considered that the podetia had the ability to develop squamules even though they did not show them. The axil type was coded as a third state (Table 2). The macroscopic characters were observed under the Olympus SZX9 stereomicroscope, while the anatomical character of stereome type was studied using an Olympus CX41 microscope at 400x. Secondary metabolites were studied by TLC according to the standardized procedures (White & James 1985), with solvent systems A and B.

A cluster analysis was run in order to assess how the specimens gathered on the basis of the fourteen phenotypic characters. Then, the morphological groups were compared with the clades obtained in the phylogenetic analyses. The analysis was implemented in R version 3.1.2, using the cluster package (Maechler *et al.* 2014).

DNA extraction and sequencing

Prior to DNA isolation, secondary metabolites were extracted by soaking the samples in acetone for two hours; then the liquid was used for thin layer chromatography (TLC). The DNeasy Plant Mini Kit (Qiagen) or DNeasy Blood and Tissue Kit (Qiagen) was used to extract DNA, according to the manufacturer's instructions. No differences between these kits were found with respect the amount or quality of the genomic DNA obtained. The DNA was dissolved in 200 µl of buffer included in the kit. The following three nuclear loci were amplified: 1) ITS rDNA using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990); 2) *rpb2* was amplified using nested PCR with two pairs of primers, RPB2-5F/RPB2-7R (Liu *et al.*

1999) in the first PCR and RPB2dRaq/RPB2rRaq (Pino-Bodas *et al.* 2010) in the second PCR; and 3) IGS rDNA using IGSf/IGSr (Wirtz *et al.* 2008). PCRs were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). The volume of reaction was 25 μl for each tube, with a 0.4 mM final concentration of primers and 5 to 30 ng of extracted DNA. The same amount of DNA was used to amplify the three loci. The amplification programs were: 1) 94 ºC for 5 min; 5 cycles of 94 ºC for 30 s, 54 ºC for 30 s, and 72 ºC for 1 min; and 33 cycles of 94 ºC for 30 s, 48 ºC for 30 s, and 72 ºC for 1 min; with a final extension of 72 ºC for 10 min for ITS rDNA, 2) initial denaturation at 94 ºC for 5 min; 40 cycles of 95 ºC for 1 min, 52 ºC for 30 s, and 72 ºC for 2 min; with a final extension at 72 ºC for 10 min for *rpb2* and IGS rDNA. PCR products were purified using the QIAquick gel extraction Kit (QIAGEN, Hilden, Germany) or ExoSAP-IT (USB Corporation, OH, USA). The sequencing reactions were done at Macrogen (South Korea) service (www.macrogen.com), with the same primers used for the PCR.

Sequence alignment and phylogenetic analyses

The sequences were aligned manually with SE-AL v2.0a11 (Rambaut 2002) for each locus separately. The alignments did not have ambiguous regions and all the positions were included in the analyses. Each region was analysed by maximum likelihood (ML). The ML analyses were implemented using RAxML (Stamatakis 2006) assuming the GTRGAMMA model. The bootstrap searches were conducted with 500 pseudoreplicates using the rapid bootstrap algorithm. Congruence among the different topologies inferred from the loci was tested following Lutzoni *et al.* (2004). We consider nodes to be in conflict if different topologies are each supported with at least 70% bootstrap. The topology of ML analysis of IGS rDNA yielded 2 main unsupported clades and few small well-supported subclades. The topology of ITS rDNA yielded one

big clade and other unsupported small clades. Inside the main clade several supported subclades resulted. The topology of *rpb2* yielded 3 main clades, one of them with the same specimens that the main clade found in the ITS rDNA analysis. Few small were well-supported subclades were found in the *rpb2* analysis. No incongruity was detected. MrModeltest 2.3 (Nylander 2004) was used for selecting the most appropriate nucleotide substitution model for each locus using the AIC criterion. Two combined datasets were constructed, one of them containing the specimens for which at least two loci were amplified and the other one containing the specimens for which all three loci were amplified. The combined datasets were treated as five partitions: ITS rDNA, IGS rDNA and each of the three codon positions of *rpb2*, respectively, and analysed by ML (on the same conditions for each locus separately) and a Bayesian approach. The Bayesian analysis was carried out using MrBayes 3.2 (Ronquist *et al.* 2012).. The model SYM+I+G was applied to each partition of *rpb2,* while the SYM+G model was used for ITS rDNA and GTR+G was applied to IGS rDNA. The posterior probabilities were approximated by sampling trees using Markov Chain Monte Carlo (MCMC). The posterior probabilities of each branch were calculated by counting the frequency of trees visited during MCMC analysis. Two simultaneous runs with 20,000,000 generations each, starting with a random tree and employing 4 simultaneous chains, were executed. Every 1000th tree was saved into a file. The convergence was assessed with the average standard deviation of split frequencies (< 0.01) and in Tracer v. 1.5 (Rambaut & Drummond 2009) plotting the likelihood versus generation number. In addition, the split probabilities were checked in AWTY (Nylander *et al.* 2008). The first 1,000,000 generations (i.e. the first 1000 trees) were deleted as the "burn in" of the chain. A 50% majority-rule consensus tree was calculated using the "sumt" command of MrBayes.

Mapping and homoplasy of phenotypic characters

The fourteen phenotypic characters previously described (Table 2) were mapped on the 50% majority-rule consensus tree from the Bayesian analysis based on the dataset containing the specimens for which all three loci were amplified.

The homoplasy for the fourteen characters was estimated by the consistence index (CI) and retention index (RI), using MESQUITE 2.75 (Maddison & Maddison 2011). Both parameters were calculated for each character on the 50% majority-rule consensus tree from the Bayesian analysis of the three loci dataset.

Results

Phylogenetic analyses

In this study 261 new sequences were generated: 89 of ITS rDNA, 97 of IGS rDNA and 75 of *rpb2* (Table 1). The concatenated dataset with sequences of all three loci contained 73 taxa and 1876 characters, 1586 were constant and 145 were parsimony informative. The average of the number of different nucleotides among the sequences of the ingroup was 8.5 in ITS rDNA, 6.7 in IGS and 6.7 in *rpb2*. The number of different nucleotides including the outgroup was 9.5 in ITS rDNA, 7.2 in IGS rDNA and 7.0 in *rpb2*. The ML analysis yielded a tree with -LnL = 5947.213, while the Bayesian analysis resulted in arithmetic mean of -LnL = 6164.412. The trees from ML and Bayesian analyses had the same topology, so only the Bayesian tree is shown (Fig. 1). The *C. furcata* complex was resolved into three major clades. All the species except *C. stereoclada* turned out to be polyphyletic. Clade A was well-supported and contained specimens of *C. furcata*, *C. farinacea*, *C. scabriuscula*, *C. subrangiformis* and *C. stereoclada*. Several well-supported subclades (six) were recovered (Fig. 1). One subclade included four specimens of *C. scabriuscula;* another subclade included all the

specimens of *C. stereoclada*; another had two specimens of *C. farinacea*; another included two specimens of *C. furcata* and the remaining two subclades comprised specimens of *C. furcata* and *C. subrangiformis.* Clade B included three specimens, one of *C. furcata* and two of *C. subrangiformis*. Clade C was supported only in the Bayesian analysis (57% bootstrap in the ML analysis). This clade included specimens of *C. furcata*, *C. scabriuscula*, *C. farinacea*, *C. multiformis* and *C. subrangiformis* (Fig. 1). Five well-supported subclades were recovered, one of them with two specimens of *C. farinacea* and one specimen of *C. scabriuscula* from Chile; another with two specimens of *C. farinacea* from North America; two subclades contained two specimens of *C. furcata* each; and other subclade with one specimen of *C. subrangiformis* and two specimens of *C. furcata*.

The analyses based on the concatenated data set including specimens with sequences for 2 or 3 loci generated a ML tree with -LnL = 6972.790 and in the Bayesian analysis was -LnL = 6817.049. Both trees had the same topology. This topology was an unresolved phylogeny (supplementary material) for the *C. furcata* complex. Thus we used the three loci tree for mapping the phenotypic characters.

Phenotypic groups and homplasy

Fig. 2 shows the dendrogram yielded in the cluster analysis based on the phenotypic charactes studied. It identifies three main groups, one with the two specimens of *C. stereoclada*; one with most of the specimens of *C. subrangiformis* and three specimens of *C. furcata*; and the third including the specimens of *C. farinacea*, *C. multiformis*, *C. scabriuscula* and most specimens of *C. furcata*. Inside the last group, the specimens trended to gather in species[MW2]. However, there was a low correlation between the phenotypic affitinies and the phylogenetic results.

No phenotypic characters supported any of the main clades (A-C) obtained in the molecular phylogeny (Fig. 3). The solid podetia were exclusive for the subclade constituted by *C. stereoclada*. The other phenotypic characters did not show a clear phylogenetic tendency. For example, psoromic acid, which is an exclusive feature of *C. subrangiformis*, was present in five subclades; scyphi, which is a diagnostic character of *C. multiformis*, were present in two separate places in the clade C.

Table 3 summarizes the CI and RI values for the different traits examined. Most of the characters were highly homoplasious, with CI and RI values close to 0.0. Only the solid podetium was not homoplasious (CI = 1.0, RI = 1.0).

Discussion

The phylogeny of the *C. furcata* complex is inconsistent with the species based on phenotypic characters. [MW3]All but one of the studied phenotypic characters that were previously used for species circumscription in the *C. furcata* complex have here been shown to be highly homoplasious (Table 2). This is consistent with previous studies that had also found high homoplasy levels for phenotypic characters within Cladoniaceae (Pino-Bodas *et al.* 2011; Parnmen *et al.* 2012). For example, the presence of scyphi (Pino-Bodas *et al.* 2011), or the presence of soredia (Stenroos *et al.* 2002) were shown to be highly homoplasious in other groups of Cladoniaceae. The podetium type is the only non-homoplasious character and solid podetia represent an autapomorphy for *C. stereoclada*.

The lack of incongruence between [MW4]the studied phenotypic characters and the molecular phylogeny can be due to the influence of the environmental conditions on those characters (Osyczka *et al*. 2014). It is well known that a large number of *Cladonia*

species are morphologically extremely variable (Abbayes 1937; Clauzade & Roux 1985; Ahti & Stenroos 2013). *Cladonia furcata* is especially variable in morphology, which has led several authors to distinguish numerous infraspecific taxa (Vainio 1887; Fink 1904; Thomson 1968; Ozenda & Clauzade 1970; Egan 1972). Nevertheless, most authors currently consider much of the morphological variation of *C. furcata* to be an effect of phenotypic plasticity (Ahti 1977), or to represent development stages of this species (Jahns & Beltman 1973; Jahns *et al.* 1978). In addition, many intermediate forms have been described among *C. furcata*, *C. subrangiformis*, *C. scabriuscula* and *C. farinacea* (Abbayes 1937; Brodo & Ahti 1996; Stenroos *et al.* 2002).

Up to now few ecological studies have addressed the influence of environmental factors on the morphology of *Cladonia* species (Schade 1966; Vagts *et al*. 1994; Paus 1997; Günzl 2004; Osyczka & Rola 2013). For example, in the *C. pocillum*/*C. pyxidata* complex Kotelko & Piercey-Normore (2010) showed that the morphology of the primary thallus (the main character used to separate *C. pyxidata* from *C. pocillum*) is an adaptation to differences in soil pH and that diverse phylogenetic lineages are tolerant to different soil pH values.

The presence of atranorin is one of the main characters used to distinguish *C. subrangiformis* from *C. furcata* (Sandstede 1922; Evans 1954). Atranorin is generally lacking in *C. furcata*, although in certain areas *C. furcata* specimens containing atranorin have been found (Huovinen *et al.* 1990; Etayo & Burgaz 1997; Ahti 2000; Huneck *et al.* 2004), especially in eastern North America, where Evans (1954) included them in *C. subrangiformis* (see Hale & Culberson 1960; Ahti 1962). Furthermore, this substance is not constantly present in *C. subrangiformis* as chemotypes lacking atranorin were found (Burgaz & Ahti 1992, 2009; Ahti & Stenroos 2013). In the other *Cladonia* species of this complex atranorin is not constant either. For instance, material

of *C. farinacea* from South America often contains atranorin, while samples from North America and Asia lack this substance (Huovinen *et al.* 1990; Stenroos *et al.* 1992). Also, *C. scabriuscula* can rarely contain atranorin. The presence of psoromic acid is apparently restricted to some specimens of *C. subrangiformis.* The chemotypes with psoromic acid are particularly abundant in the Iberian Peninsula but are also known in Sweden (Burgaz & Ahti 2009; Ahti & Stenroos 2013). Psoromic acid is known to be variable and often rare in many *Cladonia* species, for example in *C. arbuscula* (Ruoss & Huovinen 1989), *C. rappii* A. Evans (Ahti 2000), *C. foliacea* (Huds.) Willd. (Burgaz & Ahti 2009, under *C. convoluta*), *C. symphycarpa* (Osyczka & Skubala 2011), *C. acuminata* (Ahti & Stenroos 2013), and *C. fruticulosa* (Stenroos 1988).

Most of the species in the *C. furcata* complex constantly contain fumarprotocetraric acid (Huovinen *et al.* 1990), which is absent only in some specimens of *C. subrangiformis* (Burgaz & Ahti 2009), so being no use as diagnostic character[MW5].

. Bourgeanic acid is present in some specimens of *C. subrangiformis* (four of them were included in the phylogenetic analyses). These specimens do not appear phylogenetically closely related to each other in our analyses, showing that this character has a weak phylogenetic signal. For other *Cladonia* species it has been shown that the presence of fatty acids may have little taxonomical significance, as is the case of *C. subturgida* (Pino-Bodas *et al.* 2012*a*), while the presence of bourgeanic acid is diagnostic to disthinguish *C. humilis* from *C. conista* (Pino-Bodas *et al.* 2012*b*, 2013). This is the first time that bourgeanic acid is found in *C. stereoclada*.

Ahti (2000) indicated that the internal stereome surface in *C. furcata* is striate in general. The stereome surface in *Cladonia* can be smooth, papillate, striate, reticulate or tomentose (Ahti 2000). In our study only two types of stereomes were found: smooth

and striate. The specimens which share the same stereome type are not closely related (Fig. 3B). Additional studies on the morphological variation and the taxonomic value of stereome types are necessary to evaluate their taxonomic significance. However, our data suggest that its taxonomic significance is low in the *C. furcata* complex.

The podetial surface is one of the main characters used for separating species in the genus *Cladonia* (Ahti 2000). In the *C. furcata* complex all species have corticate podetia with the exception of *C. farinacea*, which is partially sorediate. However, some authors found differences in the cortex surface between *C. furcata* and *C. subrangiformis* (Wirth 1995). In the latter the surface is usually wrinkled and glossy, while it is smooth and less shiny in *C. furcata*. Our analyses show that specimens with different cortex types are not phylogenetically separated.

The presence of white medullary outgrowths (often tuberculose) was a diagnostic character used by Sandstede (1922; protologue) to distinguish *C. subrangiformis* from *C. furcata* but later he considered that the presence of atranorin is more important, since white medullary outgrowths could be a response to unfavorable conditions (Sandstede 1931). Other authors have considered that these outgrowths appear in response to the accumulation of calcium oxalate in calcareous habitats (James 2009). Several studies have shown that lichens in calcareous soils often produce calcium oxalate to remove the excess of calcium (Ascaso *et al.* 1982; Wadsten & Moberg 1985; Edwards *et al.* 1991). Therefore, this character is probably influenced by the type of substrate. Other *Cladonia* species with these structures include *C. macroceras*, *C. ecmocyna*, or *C. rangiformis* (Schade 1957; Ahti 1980; Burgaz & Ahti 1992).

Squamulose podetia can be present in most of the studied taxa. In *Cladonia scabriuscula* squamules are always present and are more abundant than in the other

species and peltate. The name "var. *pinnata* (Flörke) Vain." is often used to denote the squamulose specimens of *C. furcata*. Numerous species of *Cladonia* are able to develop squamules but not always present them. Vainio (1887) pointed out that populations living in humid areas tend to develop more squamules than those that grew in drier habitats. This observation agrees with the fact that *C. scabriuscula* is a species common in oceanic areas (Krog 1968; Brodo & Ahti 1996; Ahti & Stenroos 2013). Günzl (2004) found that specimens of *C. furcata* living in shady places more often had squamules than those growing in sunny habitats. Vagts *et al.* (1994) transplanted *C. furcata* thalli to soils with different chemical composition, finding that eventually many thalli developed squamules. All these observations indicate that the presence and abundance of squamules, at least in the *C. furcata* complex, are dependent on environmental factors, such as humidity and light.

Cladonia furcata, C. farinacea, C. scabriuscula, and *C. multiformis* usually have open axils, whereas in *C. subrangiformis* are closed, although either state can be present in most taxa (Ahti & Stenroos 2013), as it has been observed in the specimens under study (Fig. 4C). Podetia with longitudinal fissures and perforated axils in *Cladonia* have been observed in habitats with high humidity and shade (Sembdner 1958), but Günzl's observations (2004) did not corroborate these observations for *C. furcata*. However, he observed that older podetia, often bearing apothecia, have the perforated axils showing longitudinal fissures, as did other authors (Burgaz & Ahti 2009; Ahti & Stenroos 2013). This suggests that the different states represent different developmental stages, which would explain the lack of phylogenetic signal of the character.

In other *Cladonia* groups it has been found that specimens with sorediate podetia can be present in several lineages, as is the case of *Cladonia coccifera* (Steinová *et al.* 2013). In general, it has been shown that in some lichen genera the presence of soredia

is a poor diagnostic character (Tehler *et al.* 2004, 2009; Ferencova *et al.* 2010; Lumbsch & Leavitt 2011).

This podetium type was not homoplastic in the *C. furcata* complex and solid podetia represent an synapomorphy for *C. stereoclada*. This is a rare character in *Cladonia*; only two species in the genus have solid podetia, *C. solida* Vainio and *C. stereoclada* (Ahti 2000). *Cladonia solida* has not been included in any phylogenetic study. However, it is morphologically very different from *C. stereoclada* and we do not think that these species are phylogenetically closely related. Therefore, this state could have appeared several times independently in the genus *Cladonia*. Some other genera of Cladoniaceae also have solid podetia (*Cetradonia* or *Gymnoderma*), but in these the podetia are lacking algae (Wei & Ahti 2002).

The ability to produce perforated scyphi is used to distinguish *C. multiformis* from *C. furcata*. However, not all the podetia of *C. multiformis* have scyphi at every ontogenetic stage and when scyphi are lacking the species can be very difficult to distinguish from *C. furcata* (Merrill 1909; Hammer 1995). Fontaine *et al*. (2010) found that the formation of scyphi is a character affected by convergent evolution, and hypothesized that scyphi fulfil the function of retaining water near the developing apothecia. This hypothesis could explain the inconstancy of scyphi in many species of *Cladonia*; their presence would be linked to the advanced development stages in which apothecia grow.

Thorny branches are characteristic of *C. furcata* "var. *palamea*" and *C. subrangiformis,* which are difficult to distinguish (Burgaz & Ahti 2009). In the present study, five specimens of *C. furcata* and four of *C. subrangiformis* with thorny branches have been included but they are not [MW6]closely related. Some authors' field studies indicate that the specimens of *C. furcata* with thorny branches develop in sunny habitats

(Hillmann & Grumman 1957; Vagts *et al.* 1994), which would indicate that the presence of such a branching type is due to environmental factors.

The branching angle is one of the characters used to separate *C. furcata* from *C. subrangiformis* (Wirth 1995; Burgaz & Ahti 2009; Ahti & Stenroos 2013). In *C. subrangiformis* the angles tend to be wider than in *C. furcata*, often ranging from right to obtuse, sometimes beyond 120º.

The results of our phylogenetic analysis are congruent with the results of several previous studies and are inconsistent with the traditional species circumscriptions. Günzl's phylogenetic analysis (2004) based on ITS rDNA could not separate German specimens of *C. furcata* and *C. subrangiformis*. Stenroos *et al.* (2002) showed that *C. farinacea* and *C. scabriuscula* are not monophyletic. Fontaine *et al.* (2010) reported that *C. multiformis* was monophyletic, but in our analysis this taxon turned out to be polyphyletic (Fig 1, Fig 1S). We think that this disagreement is influenced by those authors´ inclusion of *C. multiformis* in an analysis of *C. gracilis* group, which is not phylogenetically close to the *C. furcata* group (Stenroos *et al.* 2002).The sequences produced by Fontaine *et al.* (2010) were included in our analysis of ITS rDNA, and we found that these sequences do not form one monophyletic clade (data not shown). In fact, Fontaine *et al.* (2010) mention the large genetic variation of *C. multiformis*.

According to our results, a study focused on species delimitation should be carried out in order to clarify the taxonomy of the *C. furcata* complex. Additional loci, including more variable loci than the ones used here, should be studied in the future to clarify the delimitation of the species within this group. In addition, other phenotypic characters should be investigated. In the family Parmeliaceae, the anatomical characters, such as ultrastruture of the cortex and ascomata have supported the clades (Argüello et al. 2007; Divakar et al. 2010).

In conclusion, only one out of the six species included in our analyses, *C. stereoclada*, was monophyletic and the presence of solid podetia is a diagnostic character to distinguish it. This is the species with the most restricted distribution, limited to Macaronesia and some areas of Ireland and Scotland (James 2009). Otherwise[MW7], the present study indicates that the phenotypic characters used to distinguish species within the *C. furcata* complex are highly homoplasious and, consequently, the species circumscribed on the base of these characters are polyphyletic.

This study was supported by the Spanish Ministry of Economy & Competitiveness (CGL2013-41839-P). MW acknowledges support from the Swedish Research Council (VR 621-2009-5372, VR 621-2012-3990). R. P-B was funded by Marie Curie IEF-program (PIEF-GA-2013-625653).

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FIG.1 Molecular phylogeny of the *Cladonia furcata* complex. This is a 50% majority rule consensus tree from a Bayesian analysis based on the concatenated dataset of ITS rDNA, IGS rDNA and *rpb2*. Posterior probability ≥ 0.95 and Bootstrap ≥ 70% are indicated at the branches.

FIG. 2. Dendrogram from the cluster analysis calculated from fourteen phenotypic characters (see table 2). The different symbols represent species, labels correspond with the code used in the molecular phylogeny (Fig. 1, Table 1).

FIG. 3. Phenotypical character states mapped on the Bayesian tree based on the concatenated dataset including only the specimens with sequence for the three loci.

SUPPLEMENTARY MATERIAL

FIG. 1S. Molecular phylogeny of *Cladonia furcata* complex including specimens with at least two loci. 50% majority rule consensus tree from the Bayesian analysis based on the concatenate dataset of ITS rDNA, IGS rDNA and *rpb2*. Posterior probability ≥ 0.95 and Bootstrap ≥ 70% are indicated on the branches.

Table S1 & S2. Frequency of the different character states in each species of the *Cladonia furcata* complex.