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

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Abstract: The *Cladonia furcata* complex treated here 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 and high morphological similarity among the species, for which reason we investigated the distribution in the phylogeny 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 phenotypic characters studied are highly homoplasious with the exception of the podetium type. The solid podetia represent a diagnostic character of *C. stereoclada*.

Key words: homoplasy, lichen, molecular phylogeny, phenotypic characters

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Introduction

It is of great importance in systematics to identify diagnostic characters that make it possible to distinguish species. For a phenotypic trait to be useful as a diagnostic character it has to be constant within a taxon, undergoing only minor changes from environmental modifications (Davis & Heywood 1963; Winston 1999), and preferably easy to recognize (that is, the different states are unambiguous and easily observable). Features of this kind, however, do not seem easy to find; many of the characters that had been 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 specimens 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),

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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 not monophyletic.

The present study focused 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 Northern 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 the considerable amount of literature on the subject (Asahina 1942; Evans 1954; Ullrich 1956; Schade 1964, 1966; Hennipman 1967; Pišút & 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* (Hariot 1887; Vainio 1887; Fink 1904; Abbayes 1937; Schade 1966; Hennipman 1967; Hawksworth 1969; Verseghy 1975; Hennipman & Sipman 1978; Clauzade & Roux 1985; Wirth 1995). For example, many authors have recognized *C. subrangiformis* as having infraspecific rank within *C. furcata* (Abbayes 1937; Schade 1966; Hennipman 1967; Hawksworth 1969; Verseghy 1975; Clauzade & Roux 1985; Wirth 1995; James 2009), while others consider both as species (Nimis 1993; Burgaz & Ahti 2009; Ahti & Stenroos 2013). The phenotypic characters that have been used in combination to distinguish the species of this complex include the secondary metabolites, the branching type, the branching angle, the presence of white medullary outgrowth, the presence, abundance and morphology of squamules, the presence of soredia, soredioid granules or phyllidia, the production of scyphi, and the podetium type (solid or hollow) (Sandstede 1922; Abbayes 1937; Huovinen *et al.* 1990; 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 using three molecular markers, and the phenotypic characters were mapped on the Bayesian tree, in order to address the following issues: 1) the monophyly of the currently accepted species, and 2) the degree of homoplasy of the phenotypic characters. We hypothesize that the phenotypic characters are highly homoplasious and most of the species polyphyletic.

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 a 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 represented in the species was successfully amplified. In the phylogenetic analyses, only the specimens with sequences of at least two loci were included. These specimens are listed in Table 1. *Cladonia pulvinella* S. Hammer and *C. corsicana* (Rondon & Vězda) Pino-Bodas *et al.* were used as outgroup taxa, based on

TABLE 1. *Specimen information and GenBank accession numbers for the species of the* Cladonia furcata *complex used in this study.*

Taxon	Locality and collection	Code	ITS rDNA	IGS rDNA	*RPB2*
C. farinacea	Canada, Nova Scotia, *D. H. S. Richardson* 200 (H)	1FARIN	KR818309	—	KR818487
C. farinacea	USA, Pennsylvania, *J.C. Lendemer* 12534 (H)	2FARIN	KR818321	KR818404	KR818496
C. farinacea	Chile, Region XII, *A. R. Burgaz* s.n. (MACB 92142)	3FARIN	KR818325	KR818409	—
C. farinacea	Chile, Region XII, *A. R. Burgaz* s.n. (MACB 92078)	4FARIN	KR818327	KR818411	KR818502
C. farinacea	Canada, Nova Scotia, *T. Ahti* 57238 (H)	LK19	AF455216	KR818472	KR818549
C. farinacea	Chile, Region XII, *A. R. Burgaz* s.n. (MACB 91976)	4SCABRI	KR818329	KR818413	KR818504
C. farinacea	USA, Hawaii, *Schumm & Frahm* 16957 (H)	CL179	KR818339	KR818423	KR818513
C. farinacea	Russia, Kamchatka, *A. P. Korablev* ex LECB 12-2010-143 (H)	CL362	KR818349	KR818434	—
C. farinacea	Russia, Dagestan, *G. Urbanavichus* 911168 (H)	CL363	KR818350	KR818435	—
C. farinacea	Russia, Kamchatka, *D.E. Himelbrant & I.S. Stepanchikova* ex LECB 12-2010-140 (H)	CL364	KR818351	KR818436	—
C. farinacea	USA, Hawaii, *Schumm & Frahm* 16957 (H)	CL395	KR818375	KR818463	KR818541
C. furcata	Spain, León, *A. R. Burgaz* s.n. (MACB 91055)	1FURC	KR818310	KR818394	KR818488
C. furcata	Spain, Segovia *A. R. Burgaz* s.n. (MACB 93519)	2FURC	KR818322	KR818405	KR818497
C. furcata	Spain, Lugo *A. R. Burgaz* s.n. (MACB 92559)	4FURC	KR818328	KR818412	KR818503
C. furcata	Portugal, Alto Alentejo, *A. R. Burgaz* s.n. (MACB 91087)	5FURC	KR818331	KR818416	KR818507
C. furcata	Spain, Mallorca, *A. R. Burgaz* s.n. (MACB 92764)	6FURC	KR818333	KR818418	—
C. furcata	USA, Virginia, *J. C. Lendemer* 9881 (FH 239444)	16FURC	KR818305	KR818391	KR818483
C. furcata	Denmark, Syddanmark, *E. S. Hansen*, Lich. Danici Exs. 382 (H)	22FURC	KR818314	KR818397	KR818489
C. furcata	Denmark, Capital Region, *E. S. Hansen*, Lich. Danici Exs. 251 (H)	23FURC	KR818315	KR818398	KR818490
C. furcata	Portugal, Madeira, *H. Väre* L1801 (H)	26FURC	KR818317	KR818400	KR818492
C. furcata	Finland, Etelä-Häme, *V. Haikonen* s.n. (H)	27FURC	KR818318	KR818401	KR818493
C. furcata	Finland, Etelä-Savo, *T. Rintanen* s.n. (H)	28FURC	KR818319	KR818402	KR818494
C. furcata	Finland, Åland, *M. Stjernberg* s.n. (H)	29FURC	KR818320	KR818403	KR818495
C. furcata	Italy, Sardinia, *H. Väre* L26119 (H)	30FURC	KR818324	KR818408	KR818499
C. furcata	USA, Georgia, *T. Ahti* 58283 (H)	AT638	AF455220	KP732369	KP732369
C. furcata	El Salvador, Santa Ana, *P. Clerc, C. Rojas & E. Morales* PC 2013/007 (H)	CL356	KR818344	KR818430	KR818518
C. furcata	New Zealand, South Island, *S. Stenroos* 5908 (H)	CL342	KR818342	KR818428	KR818516
C. furcata	Costa Rica, San José, *P. Clerc & C. Rojas* PC 2013/125 (H)	CL357	KR818345	KR818431	KR818519
C. furcata	Canada, New Brunswick, *J.C. Lendemer* 27921 (H)	CL358	KR818346	KR818432	KR818520
C. furcata	Denmark, Bornholm, *E.S. Hansen*, Lich. Danici Exs. 740 (H)	CL365	—	KR818437	KR818522
C. furcata	Denmark, Bornholm, *E.S. Hansen*, Lich. Danici Exs. 748 (H)	CL366	KR818352	KR818438	KR818523
C. furcata	USA, New York, *S. Stenroos* 5758 (H)	CL367	KR818353	KR818439	—
C. furcata	Russia, Dagestan, *G. Urbanavichus* 907167 (H)	CL368	KR818354	KR818440	KR818524
C. furcata	Russia, Dagestan, *G. Urbanavichus* 907156 (H)	CL370	KR818356	KR818442	KR818526
C. furcata	UK, Scotland, *V. Haikonen* 29237 (H)	CL371	KR818357	KR818443	KR818527
C. furcata	USA, Alabama, *E. Tripp* 1326 (H)	CL373	KR818359	KR818445	KR818529
C. furcata	Japan, Honshu, *Y. Ohmura* 6909 (H)	CL374	KR818360	KR818446	—
C. furcata	Spain, Canary Islands, *H. Väre* 20044 (H)	CL375	KR818361	KR818447	KR818530
C. furcata	USA, Alabama, *E. Tripp* 1325 (H)	CL376	KR818362	KR818448	KR818531
C. furcata	USA, Tennessee, *J. C. Lendemer* 29801 (H)	CL377	KR818363	KR818449	KR818532
C. furcata	USA, North Carolina, *J. C. Lendemer* 29724 (H)	CL378	KR818364	KR818450	KR818533

TABLE 1. *Continued*

Taxon	Locality and collection	Code	ITS rDNA	IGS rDNA	*RPB2*
C. furcata	USA, Vermont, *M. Sundue & O. Peter* 2719 (H)	CL379	KR818365	KR818451	—
C. furcata	Finland, Etelä-Häme, *V. Haikonen* 27704 (H)	CL383	—	KR818454	KR818535
C. furcata	Finland, Etelä-Häme, *V. Haikonen* 29424 (H)	CL384	KR818368	KR818455	KR818536
C. furcata	Finland, Etelä-Häme, *V. Haikonen* 29446 (H)	CL385	KR818369	KR818456	KR818537
C. furcata	Finland, Uusimaa, *R. Pino-Bodas* s.n. (H)	CL386	KR818370	KR818457	KR818538
C. furcata	USA, Oregon, *B. McCune* 3045 (H)	CL393	—	KR818461	KR818539
C. furcata	Germany, Saxony, *J. Steinová* 599 (PRC 851)	CL396	KR818376	KR818464	KR818542
C. furcata	Austria, Styria, *J. Steinová* 179, *M. Cardinalle & M. Grube* (PRC 8513)	CL397	KR818377	KR818465	KR818543
C. furcata	Czech Republic, Central Bohemia, *Z. Palice* 13549 (PRC)	CL398	KR818378	KR818466	KR818544
C. furcata	Greece, Thasos, *H. Sipman & T. Raus* 58677 (H)	CL406	KR818379	KR818467	—
C. furcata	Sweden, Uppland, *M. Wedin* 8377 (S)	MWE47	KR818387	KR818481	KR818558
C. furcata	Sweden, Uppland, *M. Wedin* 8378 (S)	MWE48	KR818388	KR818482	KR818559
C. furcata	Sweden, Västergötland, *M. Wedin* 8382 (S)	MWE179	KR818382	KR818476	KR818553
C. furcata	Sweden, Västergötland, *M. Wedin* 8395 (S)	MWE180	KR818383	KR818477	KR818554
C. furcata	Sweden, Uppland, *M. Wedin* 8403 (S)	MWE181	KR818384	KR818478	KR818555
C. furcata	Croatia, Dubrovnik-Neretva, *A. R. Burgaz* s.n. (MACB 101098)	18SUBR	KR818307	KR818392	KR818485
C. multiformis	USA, Nova Scotia, *T. Ahti* 57065 (H)	LK70	AF455213	KR818474	KP732370
C. multiformis	Canada, Yukon, *J. C. Lendemer* 29155 (H)	CL372	KR818358	KR818444	KR818528
C. multiformis	USA, Alaska, *T. Ahti* 69715 (H)	CL387	KR818371	KR818458	—
C. multiformis	Canada, Manitoba, *T. Ahti* 62709 (H)	CL389	KR818372	KR818459	—
C. multiformis	Canada, Manitoba, *T. Ahti* 62784, *Piercey-Normore & Booth* (H)	CL390	KR818373	KR818460	—
C. multiformis	Mexico, *J. Steinová* 442 (H)	CL416	—	KR818468	KR818545
C. scabriuscula	USA, Alaska, *S. Walker* s.n. (H)	1SCABRI	KR818311	KR818395	—
C. scabriuscula	Canada, Newfoundland, *T. Ahti* 56969 (H)	LK11	AF455217	KR818471	KP732371
C. scabriuscula	China, Hunan, *T. Koponen et al.* 54509 (H)	LK58	AF455218	KR818473	KR818550
C. scabriuscula	Chile, Magallanes, *T. Feuerer* 60212 (TUR)	LK88	AF455219	KR818475	KR818552
C. scabriuscula	New Zealand, Otago, *S. Stenroos* 5918 (H)	CL1	KR818337	KR818422	KR818512
C. scabriuscula	New Zealand, Otago, *S. Stenroos* 5920 (H)	CL7	KR818380	KR818469	KR818546
C. scabriuscula	New Zealand,West Coast, *S. Stenroos* 5946 (H)	CL12	KR818338	KR818427	—
C. scabriuscula	New Zealand, Southland, *S. Stenroos* 5779 (H)	CL36	KR818347	KR818426	—
C. scabriuscula	UK, Scotland, *S. Stenroos* 6076 (H)	CL98	KR818381	KR818470	KR818547
C. scabriuscula	New Zealand, Southland, *S. Stenroos* 5895 (H)	CL341	KR818341	KR818425	KR818515
C. scabriuscula	New Zealand, Southland, *S. Stenroos* 5773 (H)	CL343	KR818343	KR818429	KR818517
C. scabriuscula	USA, Alaska, *S. Talbot & J. Myers* UNI059-55 (H)	CL381	KR818366	KR818452	KR818534
C. scabriuscula	USA, Alaska, *S. Talbot & J. Myers* UNI066-13 (H)	CL382	KR818367	KR818453	—
C. scabriuscula	USA, Alaska, *S. Talbot & Sa. Talbot* ADA706 (H)	CL394	KR818374	KR818462	KR818540
C. stereoclada	Azores, São Miguel, *F. Berger* 18357 (H)	1STEREO	KR818312	KR818396	—
C. stereoclada	Azores, Terceira, *A. F. Rodriguez* s.n. (H)	2STEREO	KR818313	KR818406	—
C. stereoclada	Spain, Canary Islands, *A. R. Burgaz* s.n. (MACB 97913)	3STEREO	KR019556	KR019558	KR818500
C. stereoclada	Spain, Canary Islands, *A. R. Burgaz* s.n. (MACB 97911)	4STEREO	KR818330	KR818414	KR818505
C. stereoclada	Spain, Canary Islands, *P. Alanko* 128470a (H)	CL138	KR019557	KR019559	—

TABLE 1. *Continued*

Taxon	Locality and collection	Code	ITS rDNA	IGS rDNA	*RPB2*
C. subrangiformis	Spain, Soria, *A. R. Burgaz* s.n. (MACB 91155)	2SUBR	KR818323	KR818407	KR818498
C. subrangiformis	Spain, Soria, *A. R. Burgaz* s.n. (MACB 102468)	3SUBR	KR818326	KR818410	KR818501
C. subrangiformis	Spain, Guadalajara, *A. R. Burgaz* s.n. (MACB 102802)	4SUBR	—	KR818415	KR818506
C. subrangiformis	Spain, Asturias, *A. R. Burgaz* s.n. (MACB 102466)	5SUBR	KR818332	KR818417	KR818508
C. subrangiformis	Portugal, Beira Alta, *A. R. Burgaz* s.n. (MACB 102469)	7SUBR	KR818334	KR818419	KR818509
C. subrangiformis	Spain, Madrid, *A. R. Burgaz* s.n. (MACB 102467)	8SUBR	KR818335	KR818420	KR818510
C. subrangiformis	Spain, Mallorca, *A. R. Burgaz* s.n. (MACB 96253)	9SUBR	KR818336	KR818421	KR818511
C. subrangiformis	China, Xinjiang, *A. Abbas* 200265 (FH 302266)	11SUBR	KR818303	KR818389	—
C. subrangiformis	The Netherlands, Limburg, *A. Aptroot* 57907 (H)	14SUBR	KR818304	KR818390	—
C. subrangiformis	Turkey, Zonguldak, *K. Yazici* 12 (H)	17SUBR	KR818306	—	KR818484
C. subrangiformis	Bosnia & Herzegovina, Sarajevo, *A. R. Burgaz* s.n. (MACB 101105)	19SUBR	KR818308	KR818393	KR818486
C. subrangiformis	Sweden, Uppland, *M. Wedin* 8376 (S)	MWE46	KR818386	KR818480	KR818557
C. subrangiformis	Sweden, Öland, *M. Wedin* 9491 (S)	MWE182	KR818385	KR818479	KR818556
C. subrangiformis	Spain, Mallorca, *Schumm, Frahm & Lüth* 16302 (H)	CL197	KR818340	KR818424	KR818514
C. subrangiformis	Russia, Dagestan, *G. Urbanavichus* 920180 (H)	CL360	KR818348	KR818433	KR818521
C. subrangiformis	Russia, Dagestan, *G. Urbanavichus* 907162 (H)	CL369	KR818355	KR818441	KR818525
C. subrangiformis	Turkey, Zonguldak, *K. Kinalioglu* s.n. (H)	24FURC	KR818316	KR818399	KR818491

their placement in our more extensive phylogenetic analyses (Pino-Bodas *et al.* 2012*a*; Ahti *et al.* 2015; S. Stenroos, R. Pino-Bodas, H. T. Lumbsch, S. Parnmen, A. Thell, P. Clerc, A. R. Burgaz, M. P. Martín, F. Högnabba, T. Ahti, unpublished data).

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 species, based on an extensive number of specimens, is provided in the supplementary material (Tables S1 & S2, supplementary material available online). The morphological characters were studied in three randomly selected podetia per specimen. The angles of all 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 an Olympus SZX9 stereomicroscope, while the anatomical character of stereome type was studied using an Olympus CX41 microscope at ×400. Secondary metabolites were studied by TLC according to 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 14 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 (R Core Team 2013), 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 2 h; then the liquid was used for 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 the kits were found with respect to 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

TABLE 2. *List of phenotypic characters studied and their states in the* Cladonia furcata *complex.*

Character	States of character
Atranorin	0 = absence, 1 = presence
Psoromic acid	0 = absence, 1 = presence
Bourgeanic acid	0 = absence, 1 = presence
Fumarprotocetraric acid	0 = absence, 1 = presence
Stereome type	0 = smooth, 1 = striate
Surface of the podetia	0 = smooth, 1 = wrinkled
White medullary outgrowths	0 = absence, 1 = presence
Squamules	0 = absence, 1 = presence
Axils	0 = open, 1 = closed, 2 = both
Soredia	0 = absence, 1 = presence
Podetium type	0 = hollow, 1 = solid
Scyphi	0 = absence, 1 = presence
Branching type (thorny branches)	0 = absence, 1 = presence
Branching angle	0 = < 70°, 1 = > 70°

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 5–30 ng of extracted DNA. The same amount of DNA was used to amplify the three loci. The amplification programs were: 1) 94°C for 5 min; 5 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and 33 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min; with a final extension of 72°C for 10 min for ITS rDNA; 2) initial denaturation at 94°C for 5 min; 40 cycles of 95°C for 1 min, 52°C for 30 s, and 72°C for 2 min; with a final extension at 72°C 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, Ohio, USA). The sequencing reactions were performed 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 analyzed 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 considered nodes to be in conflict if different topologies were each supported with at least 70% bootstrap. The topologies of the single locus ML trees were all poorly resolved. In the IGS rDNA tree, two main unsupported clades and a few small well-supported subclades were found. The topology of ITS rDNA yielded one large clade and other unsupported small clades. Inside the main clade, several supported subclades resulted. The topology of *RPB2* yielded three main clades, one of them with the same specimens that the main clade found in the ITS rDNA analysis. Few small well-supported subclades were found in the IGS rDNA 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 containing the specimens for which at least two loci were amplified and the other 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 analyzed 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 14 phenotypic characters described previously (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 14 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 of which were constant and 145 were parsimony-informative. The average 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 an 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 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 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 another subclade had one specimen of *C. subrangiformis* and two specimens of *C. furcata*.

The analyses based on the concatenated dataset including specimens with sequences for 2 or 3 loci generated a ML tree with –lnL = 6972·790, and –lnL = 6817·049 in the Bayesian analysis. Both trees had the same topology. This topology was an unresolved phylogeny (see Supplementary Material, Figure S1 available on-line) for the *C. furcata* complex. As a result, the three loci were used to map the phenotypic characters.

Phenotypic groups and homplasy

Figure 2 shows the dendrogram yielded in the cluster analysis based on the phenotypic characters studied. It shows three main groups, one with the two specimens of *C. stereoclada*; a second with most of the specimens of *C. subrangiformis* and three specimens of *C. furcata*; and a third one including the specimens of *C. farinacea*, *C. multiformis*, *C. scabriuscula* and most specimens of *C. furcata*. Inside the latter group, the specimens tended to gather in species. However, there was a low correlation between the phenotypic affinities and the phylogenetic results.

No phenotypic characters supported any of the main clades (A–C) obtained in the molecular phylogeny (Fig. 3). Solid podetia were characteristic for the subclade constituted by *C. stereoclada*. The other phenotypic characters did not show a clear phylogenetic structure. For example, the presence of psoromic acid, which is a descriptive trait for *C. subrangiformis*, occurred in five subclades; the presence of scyphi, which is diagnostic for *C. multiformis*, occurred in two positions in clade C.

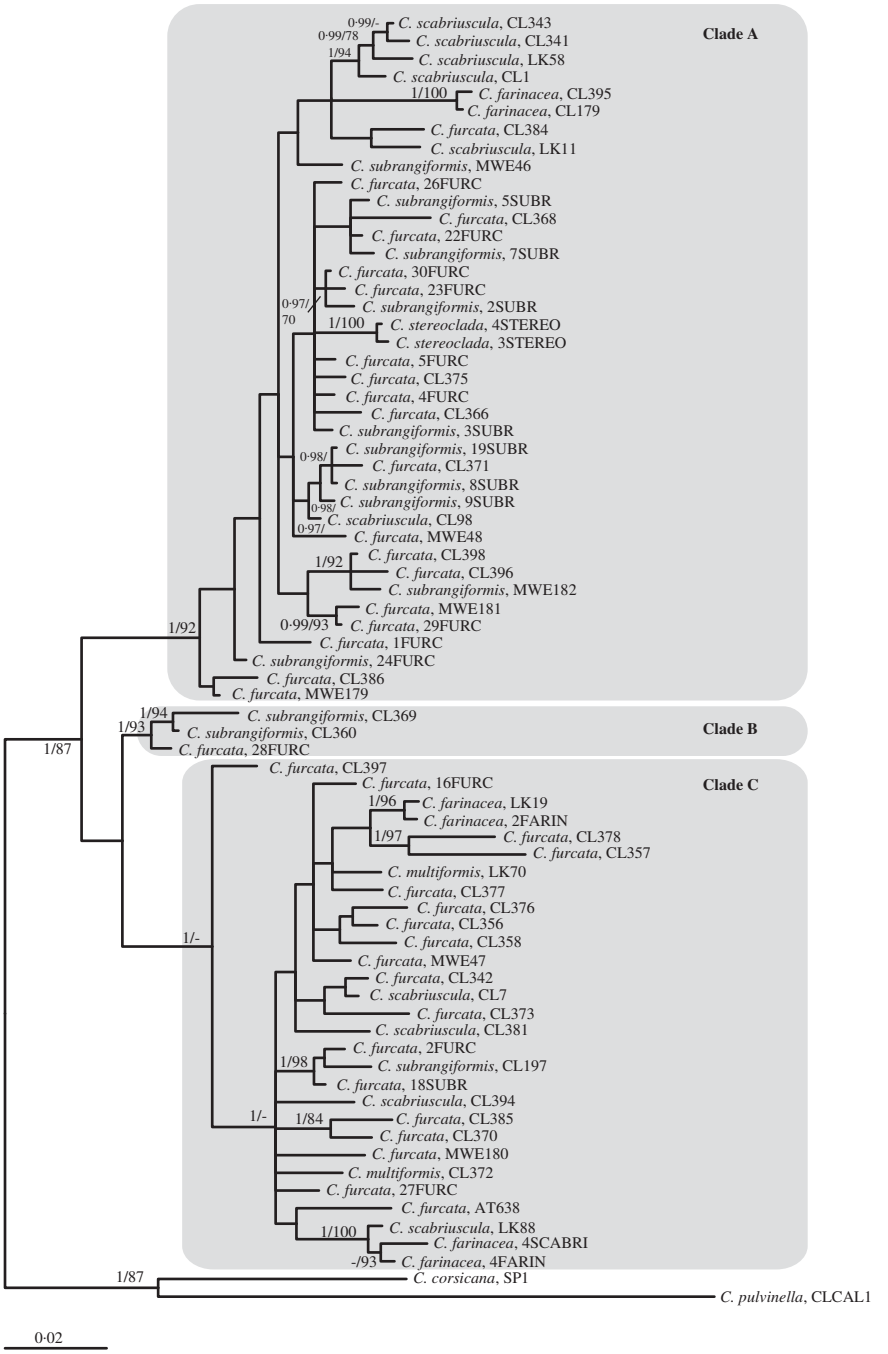

FIG. 1. Molecular phylogeny of the *Cladonia furcata* complex. This is a 50% majority-rule consensus tree from a Bayesian analysis based on the concatenated dataset of ITS rDNA, IGS rDNA and *RPB2*. Posterior probability (first figure) ≥0·95 and bootstrap (second figure) ≥70% are indicated at the branches.

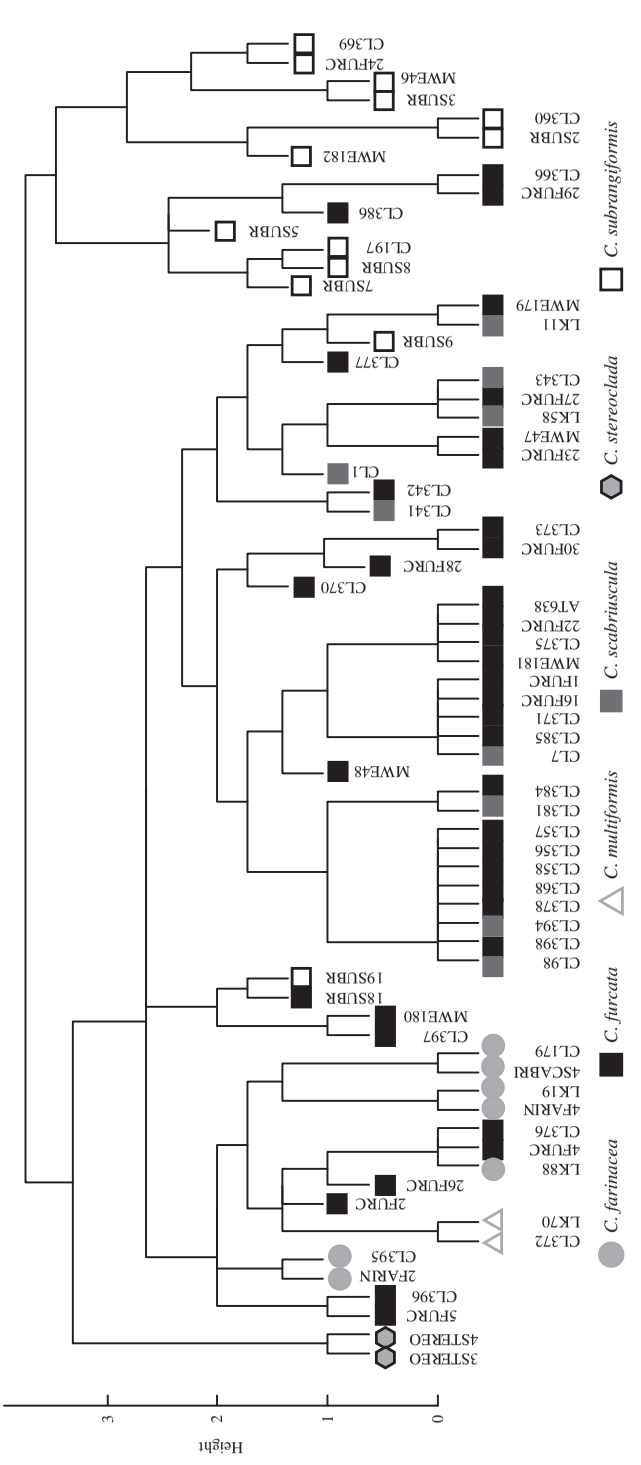

FIG. 2. Dendrogram of a cluster analysis calculated from 14 phenotypic characters of the *Cladonia furcata* complex (see Table 2). The different symbols represent species, labels correspond with the code used in the molecular phylogeny (Fig. 1, Table 1).

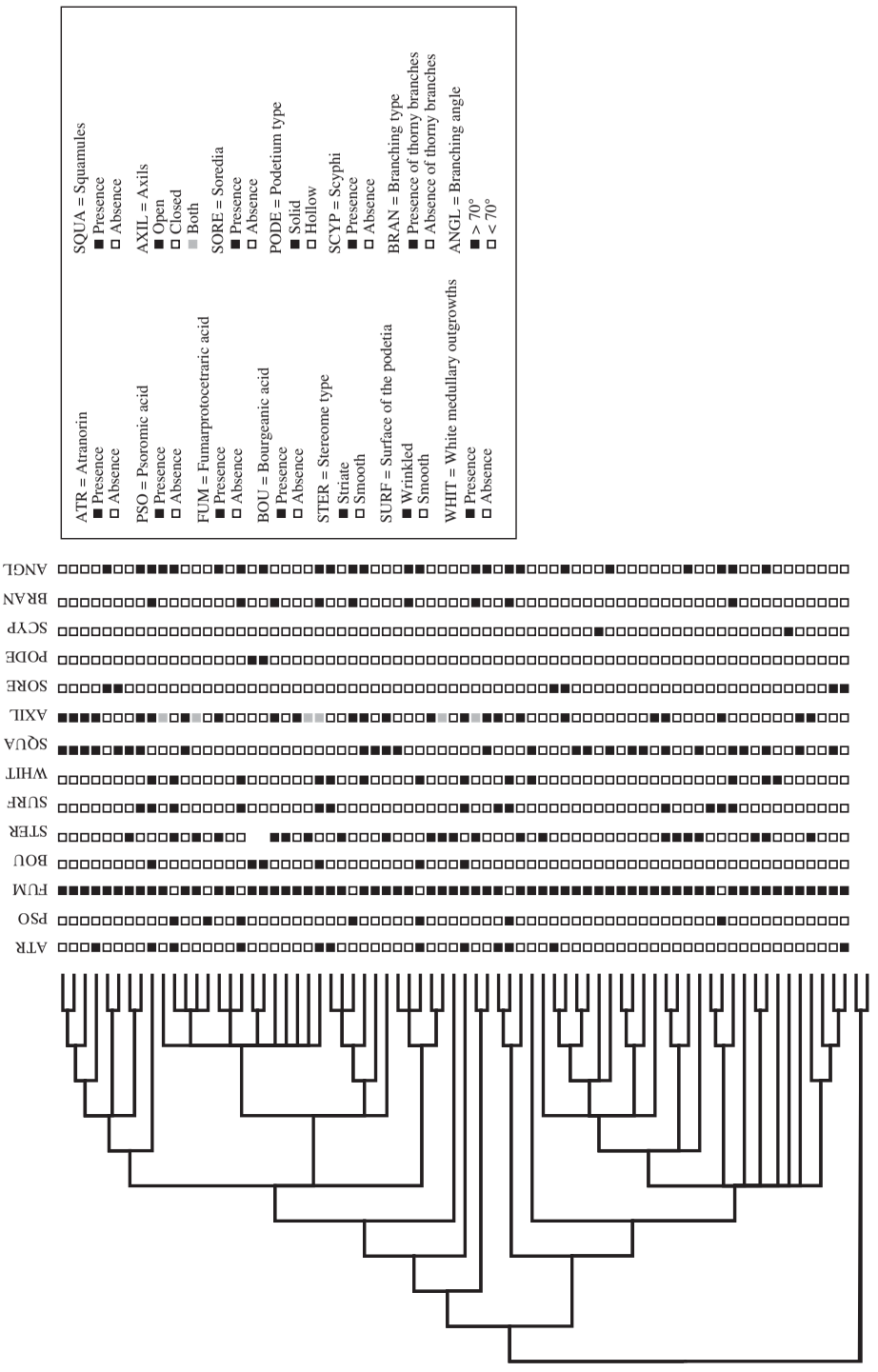

FIG. 3. Phenotypic character states of the *Cladonia furcata* complex mapped on the Bayesian tree based on the concatenated dataset including only the specimens with sequences for all three loci.

TABLE 3. *Homoplasy values for each character in the* Cladonia furcata *complex.*

Character	CI	RI
Atranorin	0·083	0·083
Psoromic acid	0·142	0·000
Fumarprotocetraric acid	0·142	0·000
Bourgeanic acid	0·166	0·166
Stereome type	0·058	0·2
Surface	0·1	0·25
White medullary outgrowths	0·076	0·00
Squamules	0·05	0·24
Axils	0·076	0·111
Soredia	0·2	0·333
Solid podetium	1·0	1·0
Scyphi	0·333	0·00
Branches	0·1	0·0
Angle type	0·045	0·086

CI = consistence index, RI = retention index.

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. All but one of the studied phenotypic characters that were previously used for species circumscription in the *C. furcata* complex have been shown here to be highly homoplasious (Table 2). This is consistent with previous studies that 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 congruence between the phenotypic characters studied and the molecular phylogeny may 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 developmental 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* rarely contains 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 always contain fumarprotocetraric acid (Huovinen *et al.* 1990), which is absent only in some specimens of *C. subrangiformis* (Burgaz & Ahti 2009), hence being non-diagnostic.

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 taxonomic significance, as is the case for *C. subturgida* (Pino-Bodas *et al.* 2012*a*), while the presence of bourgeanic acid is diagnostic in distinguishing *C. humilis* from *C. conista* (Pino-Bodas *et al.* 2012*b*, 2013). This is the first time that bourgeanic acid has been 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 taxonomic value of stereome types are necessary to evaluate their taxonomic significance. However, our data suggest that their 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 tuberculous) 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 unfavourable 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 substratum. Other *Cladonia* species with these structures include *C. macroceras*, *C. ecmocyna*, and *C. rangiformis* (Schade 1957; Ahti 1980; Burgaz & Ahti 1992).

Squamulose podetia can be present in most of the taxa studied. 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 produce squamules, but do not always develop them. Vainio (1887) pointed out that populations living in humid areas tend to develop more squamules than those growing in drier habitats. This observation is

supported by 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 had squamules more often than those growing in sunny habitats. Vagts *et al.* (1994) transplanted *C. furcata* thalli to soils with different chemical compositions, 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* they are closed, although either state can be present in most taxa (Ahti & Stenroos 2013), as was 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 in the case of *Cladonia coccifera* (Steinová *et al.* 2013). In general, it has been shown that the presence of soredia is a poor diagnostic character in some lichen genera (Tehler *et al.* 2004, 2009; Ferencova *et al.* 2010; Lumbsch & Leavitt 2011).

The podetium type did not show homoplasy in the *C. furcata* complex, and solid podetia represent an autapomorphy of *C. stereoclada*. This is a rare character in *Cladonia*; only two species in the genus have solid podetia, namely *C. solida* Vainio and *C. stereoclada* (Ahti 2000). *Cladonia solida* has not been included in any phylogenetic study. Some other genera in *Cladoniaceae* also have species with solid podetia (*Cetradonia* and *Gymnoderma*), but in these genera the algae are lacking from the podetia (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 lack of consistency in the production of scyphi in many species of *Cladonia*; their presence would be linked to the advanced developmental 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 were included but they are not phylogenetically closely related. Some authors' field studies indicate that the specimens of *C. furcata* with thorny branches develop in sunny habitats (Hillmann & Grummann 1957; Vagts *et al.* 1994), which would suggest 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 120°.

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 and Supplementary Figure S1). We think that this disagreement is caused by the inclusion of *C. multiformis* in an analysis of the *C. gracilis* group, which is only distantly related 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*, anatomical characters such as ultrastructure of the cortex and ascomata have supported clades found in phylogenetic analyses (Argüello *et al.* 2007; Divakar *et al.* 2010).

In conclusion, only one out of the six species included in our analyses, namely *C. stereoclada*, was monophyletic. This is also a species with the most restricted geographical range, limited to Macaronesia and some areas of Ireland and Scotland (James 2009).

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Supplementary Material

For supplementary material accompanying this paper visit http://10.1017/S0024282915000225

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