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RESEARCH ARTICLE

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Sharpening the species boundaries in the *Cladonia mediterranea* complex (*Cladoniaceae*, Ascomycota)

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Key words

coalescence
Iberian Peninsula
integrative taxonomy
lichen forming fungi
Macaronesia
molecular systematic
species delimitation
taxonomy

Abstract The complex *Cladonia mediterranea* belongs to the section *Impexae* and is formed by *C. azorica*, *C. macaronesica* and *C. mediterranea.* These species are basically distributed in the Mediterranean and Macaronesian Regions. In the present work the limits between the species of this complex are re-examined. To this end, the morphological characters were studied along with the secondary metabolites and the DNA sequences from three loci (ITS rDNA, IGS rDNA and *rpb2*). The morphological data were studied by principal component analysis (PCA), while the DNA sequences were analyzed using several approaches available to delimit species: genealogical concordance phylogenetic species recognition, species tree (BEAST* and spedeSTEM) and cohesion species recognition. In addition, the genealogical sorting index was used in order to assess the monophyly of the species. The different procedures used in our study turned out to be highly congruent with respect to the limits they establish, but these limits are not the ones separating the prior species. Either the morphological analysis or the different approaches to species delimitation indicate that *C. mediterranea* is a different species from *C. macaronesica*, while *C. azorica* and *C. macaronesica*, which are reduced to synonyms of *C. portentosa*, constitute a separate lineage.

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INTRODUCTION

The development of molecular tools has brought about a more accurate species delimitation and a better understanding of the evolution of fungi. The definition of many species has changed. It is well-known that in many fungal groups a large number of morphological species hide cryptic species complexes (Bickford et al. 2007, Crespo & Lumbsch 2010). Despite the major methodological advances made in species delimitation, it still constitutes a challenge, since there does not exist a valid method that allows identification of independent evolutionary lineages in all the cases (Sites & Marshall 2003, Carstens et al. 2013). One of the most used criterion for species delimitation in fungi has been the Genealogical Concordance Phylogenetic Species Recognition (Taylor et al. 2000), that uses several unlinked loci and reciprocal monophyly to identify the species. In many cases this criterion has been very useful to distinguish divergent lineages (Kroken & Taylor 2001, Dettman et al. 2003, Ott et al. 2004, Fournier et al. 2005, Doyle et al. 2013, Morgado et al. 2013). Nevertheless, due to species divergence being a temporal process, this criterion can fail in cases of delimitation of closely related species that have diverged recently (Knowles & Carstens 2007). Some other facts such as hybridization, recombination and horizontal transfer can also cause the gene tree to be inconsistent with the species tree (Eckert & Carstens 2008). There are of course other procedures used for species delimitation in fungi (Wirtz et al. 2008, Gazis et al. 2011). One of them is Templeton's (1989) cohesion species recognition, that does not require species monophyly (Weisrock & Larson 2006, Wirtz et al. 2008, 2012). This method evaluates two hypotheses for the evolutionary lineages to be considered as species. The first of them (H1) is that there is only one evolutionary lineage in the studied group; the second (H2) is that the evolutionary lineages are genetically or ecologically exchangeable. The rejection of both hypotheses along with the congruence of H2 with the lineages found in H1 permits to delimit the cohesion species (Templeton et al. 2000). Numerous methods based on coalescence have recently been combined with the species delimitation procedures (O'Meara et al. 2006, Pons et al. 2006, Liu et al. 2009, Ence & Carstens 2011, Yang & Rannala 2010). They have the advantage of taking into account the incomplete lineage sorting and not requiring the reciprocal monophyly (Carstens & Knowles 2007, Fujita et al. 2012). These methods have been already applied in several works on species delimitation in lichenized fungi (Leavitt et al. 2011, 2012, 2013, Parnmen et al. 2012).

An emergent approach that is gathering increasing approval is the one that uses diverse data and analysis types to trace the most significant evidence, which permits the establishing of boundaries among species (Padial & de la Riva 2010, Gazis et al. 2011, Gebiola et al. 2012). This is the procedure that some authors call 'taxonomical integration' (Wiens & Penkrot 2002, Dayrat 2005, Will et al. 2005, Padial et al. 2009). According to Carstens et al. (2013), several analytical methods must be used in order to delimit species within a group of organisms, since each of the extant methods takes prior assumptions that not always fit the available data or the speciation scenery under screening.

Cladonia comprises most species within the family *Cladoniaceae*. According to Stenroos et al. (2002), *Cladonia* is a monophyletic genus that encompasses all the species of the former genus *Cladina* (Ahti 1961, 1984, 2000), represented by about 36 species from all over the world (Ahti 2000). This group, commonly known as reindeer lichens, is characterized by a crustose evanescent primary thallus, densely branched podetia, ecorticated, arachnoid surface, and by the absence of scyphi

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Table 1 Samples of *Cladonia mediterranea* complex used in this study with the GenBank accession numbers. The new sequences are in **bold**.

Taxa	Voucher specimen	Chemistry[1]	Code	GenBank numbers		
				IGS rDNA	ITS rDNA	*rpb2*
C. azorica	Azores Islands, São Miguel, Lago do Foco, *Haikonen* 26865 (H)	FUM, PERL, USN	1AZO	**KP941478**	**KP941520**	–
	Azores Islands, São Miguel, Serra de Aqua de Pau, *Väre* L1791 (H)	FUM, PERL, USN	2AZO	–	**KP941535**	–
	Madeira, Queimados, *Pérez-Vargas* (TFC)	FUM, PERL, USN	1894	**KP941461**	**KP941516**	**KP941544**
	Canary Islands, La Palma, Los Sauces, *Pérez-Vargas* (TFC)	FUM, PERL, USN	1866	–	–	**KP941547**
	Madeira, Pico Ruivo, *Pérez-Vargas* (TFC)	FUM, PERL, USN	1898	**KP941463**	**KP941525**	**KP941545**
	Azores Islands, Terceira, Sierra de Santa Bárbara, *Pérez de Paz* (TFC)	FUM, PERL, USN	1856	**KP941474**	**KP941511**	–
	Azores Islands, Pico, *Pérez de Paz* (TFC)	FUM, PERL, USN	1855	–	–	**KP941537**
	Madeira, Queimados, *Pérez-Vargas* (TFC)	FUM, PERL, USN	1900	**KP941476**	**KP941510**	–
	Madeira, Folhadal, *Pérez-Vargas* (TFC)	FUM, PERL	1897	**KP941475**	**KP941509**	**KP941548**
C. boryi	USA, Massachusett, Plymouth County, *Burgaz* (MACB)	USN, ZEO	1904	**KP941495**	**KP941532**	**KP941563**
C. cenotea	Denmark, *Vondrák* 6965 (MACB)	SQUA	1CENO	**KP941497**	FN868596	HM243221
C. confusa	Brazil, Minas Gerais, *Stenroos* 5091 (TUR)	–	LK46	**KP941492**	AF458296	**KP941559**
	Bolivia, Santa Cruz, *Flakus & Plata* 22689 (H)	–	CL271	**KP941491**	**KP941536**	**KP941560**
C. deformis	USA, New Hampshire, Grafton County, *Burgaz* (MACB)	RHO, USN	1905	**KP941496**	**KP941533**	**KP941570**
C. macaronesica	Canary Islands, La Gomera, Laguna Grande, *Pérez-Vargas* (TFC)	PERL, USN	1849	**KP941470**	**KP941503**	**KP941538**
	Canary Islands, La Gomera, Montaña de la Zarza, *Pérez-Vargas* (TFC)	PERL, USN	1848	**KP941462**	**KP941502**	–
	Canary Islands, Tenerife, Pico del Ingles, *Pérez-Vargas* (TFC)	PERL, USN	1847	**KP941464**	**KP941501**	**KP941546**
	Canary Islands, Tenerife, El Pijaral, *Pérez-Vargas* (TFC)	PERL, USN	1846	**KP941459**	**KP941500**	–
	Canary Islands, Tenerife, El Bailadero, *Pérez-Vargas* (TFC)	PERL, USN	1845	**KP941458**	**KP941499**	**KP941556**
	Canary Islands, Tenerife, El Bailadero, *Pérez-Vargas* (TFC)	PERL, USN	1852	**KP941472**	**KP941534**	**KP941539**
	Canary Islands, La Gomera, Roque de la Zorcita, *Hernandez-Padrón & Pérez de Paz* (MACB)	PERL, USN	1863	**KP941460**	**KP941512**	–
	Canary Islands, La Gomera, Cumbres de Tajaqué, *Pérez-Vargas* (TFC)	PERL	1853	–	**KP941505**	**KP941543**
	Canary Islands, La Gomera, Laguna Grande, *Pérez-Vargas* (TFC)	PERL, USN	1854	**KP941473**	–	**KP941540**
	Canary Islands, Tenerife, El Bailadero, *Pérez-Vargas* (TFC)	PERL, USN	1850	**KP941471**	**KP941504**	**KP941541**
C. mediterranea	Balearic Islands, Ibiza, Sant Joseph de Sa Talaia, *Pino-Bodas* (MACB)	PERL, USN	1MED	**KP941489**	**KP941524**	**KP941569**
	Portugal, Algarve, Maria Vinagre, *Burgaz* (MACB)	PERL, USN	1861	**KP941483**	**KP941522**	**KP941551**
	Portugal, Beira Litoral, Figueira da Foz, *Burgaz* (MACB)	PERL, USN	1862	**KP941484**	–	**KP941552**
	Portugal, Beira Litoral, Vagos, *Pino-Bodas* (MACB)	PERL, USN	1871	–	**KP941523**	**KP941553**
	Portugal, Beira Litoral, Areao, *Pino-Bodas* (MACB)	PERL, USN	1876	**KP941485**	**KP941521**	–
	Portugal, Beira Litoral, Mira, *Pino-Bodas* (MACB)	PERL, USN	1880	**KP941486**	**KP941513**	**KP941554**
	Portugal, Beira Litoral, Mira, *Pino-Bodas* (MACB)	PERL, USN	1883	**KP941487**	**KP941514**	**KP941550**
	Canary Islands, Gran Canaria, El Palmital, *Pérez-Vargas* (TFC)	PERL, USN	1895	**KP941488**	**KP941517**	**KP941555**
	Canary Islands, Gran Canaria, El Palmital, *Pérez-Vargas* (TFC)	PERL, USN	1896	**KP941490**	**KP941518**	**KP941568**
C. portentosa subsp. *pacifica*	USA, Alaska, Aleutian Islands, *Talbot & Myers* UNI 064-24 (H)	PERL, USN	CL308	**KP941479**	**KP941528**	**KP941557**
	USA, Alaska, Aleutian Islands, *Talbot* WOS 025-24 (H)	PERL, USN	CL340	**KP941480**	**KP941529**	**KP941558**
C. portentosa subsp. *portentosa*	Madeira, Folhadal, *Pérez-Vargas* (TFC)	PERL, USN	1902	**KP941477**	**KP941519**	**KP941549**
	Madeira, Queimados, *Pérez-Vargas* (TFC)	PERL	1893	–	**KP941515**	**KP941542**
	Portugal, Beira Litoral, Areao, *Pino-Bodas* (MACB)	PERL, USN	1875	**KP941466**	**KP941506**	–
	Portugal, Beira Litoral, Areao, *Pino-Bodas* (MACB)	PERL	1878	–	**KP941507**	**KP941564**
	Portugal, Beira Litoral, Canicrira, *Pino-Bodas* (MACB)	PERL, USN	1884	**KP941469**	**KP941508**	–
	United Kingdom, Scotland, *Stenroos* 6074 (H)	PERL, USN	CL92	**KP941482**	**KP941530**	**KP941565**
	United Kingdom, Scotland, *Stenroos* 6094 (H)	PERL, USN	CL97	**KP941481**	**KP941531**	**KP941567**
	United Kingdom, Scotland, *Sanderson* (MACB)	PERL, USN	1/13	**KP941465**	**KP941498**	**KP941566**
	Spain, Valencia, Utiel, *Burgaz* (MACB)	PERL, USN	8/13	**KP941467**	**KP941526**	–
	Spain, Burgos, Pineda de la Sierra, *Burgaz* (MACB)	PERL, USN	9/13	**KP941468**	**KP941527**	–
C. pycnoclada	Chile, Osorno, *Feuerer* 60257 (TUR)	–	AT509	**KP941494**	AF458297	**KP941561**
	Chile, Osorno, *Feuerer* 60275 (TUR)	–	AT510	**KP941493**	AF458298	**KP941562**

[1] FUM = fumarprotocetraric acid; PERL = perlatolic acid; RHO = rhodocladonic acid; SQUA = squamatic acid; USN = usnic acid; ZEO = zeorin.

and soredia (Ahti 1961, 1984). Furthermore, Stenroos et al. (2002) showed that *Cladina* is a monophyletic group (Group *Cladinae*) divided into two clades, one corresponding to the old section *Impexae* and the other to the sections *Cladina* and *Tenues*. However, some studies indicated that the Group *Cladinae* is not monophyletic (DePriest et al. 1999, 2000, Guo & Kashiwadani 2004) but the old section *Impexae* is monophyletic. The section *Impexae* is represented in Europe by *C. azorica*, *C. macaronesica*, *C. mediterranea*, *C. portentosa* and *C. stellaris*. The problematic *C. mediterranea* complex includes three of these species, viz. *C. azorica*, *C. macaronesica* and *C. mediterranea*. *Cladonia azorica* is reported to be widespread in Madeira, Azores, Ireland, England and Iceland (Ahti 1961, Ruoss 1989, James 2009, Ahti & Stenroos 2013), while *C. macaronesica* is known from the Canary Islands, Madeira and Azores (Ahti 1961). *Cladonia mediterranea* has the broadest distribution, from Portugal to Turkey, southwestern Britain and the Canary Islands (des Abbayes & Duvigneaud 1947, Ruoss 1989, James 2009, Hernández-Padrón & Pérez-Vargas 2009).

It is still unclear whether *C. azorica*, *C. macaronesica* and *C. mediterranea* represent independent species or not. Ahti (1977) synonymized *C. azorica* with *C. macaronesica*; but later on, he again recognized *C. azorica* (Ahti 1984), whereas Ruoss (1989) concluded that *C. mediterranea* and *C. macaronesica* were conspecific. However, in many floristic works *C. macaronesica* and *C. mediterranea* are still treated as separate species (Hafellner 1995, Flores Rodrígues & Aptroot 2005, Carvalho et al. 2008, Hernández-Padrón & Pérez-Vargas 2009, Gabriel 2012). Sicilia et al. (2009) refer to *C. mediterranea* group because of the high morphological variation they found, while they pointed out the necessity of molecular studies for clarifying the taxonomy of this complex. To date, analyses using DNA sequence data have not been carried out.

The aim of the present work is to study the species delimitation in the *Cladonia mediterranea* complex using different approaches and several data sources: DNA sequences from three loci (ITS rDNA, IGS rDNA and *rpb2*), morphological data and chemical data.

MATERIAL AND METHODS

Sampling

The specimens were collected during 2011 from the Canary Islands, Madeira, Azores and the coast of Portugal. To complete the sampling, specimens deposited at MACB and H were included. In all, 40 specimens of *C. azorica*, *C. macaronesica*, *C. mediterranea* and *C. portentosa* were selected (Table 1). We included *C. portentosa*, which is a common species in the Iberian Peninsula and Macaronesia, because of its morphological resemblance to the other three species (Ahti 1961, Ruoss 1989, Orange 1993, Burgaz & Ahti 2009, Sicilia et al. 2009, Ahti & Stenroos 2013). Two specimens of *C. pycnoclada* and two of *C. confusa* were also included, both South American members of the section *Impexae*; they have been considered by some authors to be close to *C. azorica* (des Abbayes 1946, Tavares 1952). *Cladonia deformis*, *C. boryi* and *C. cenotea* were chosen as outgroup species, according to the results of Stenroos et al. (2002).

Morphology and secondary metabolites

The morphological characters studied were selected on the basis of the original descriptions of the species (des Abbayes & Duvigneaud 1947, Ahti 1961, 1978) and other studies (Ruoss 1989, Burgaz & Ahti 2009). Fourteen quantitative morphological characters were measured (length of podetia, width of podetia, number of branches, dichotomous branches percentage, trichotomous branches percentage, tetrachotomous branches percentage, branching angles, length of internodes, length of last branch, thickness of podetia, thickness of medulla, thickness of stereome, number of open axils, number of closed axils). For each specimen, the measures were taken in one to three podetia, according to the available material. All the macroscopic characters were measured by means of a digital slide gauge (0.01 mm precision) under a binocular stereomicroscope (Olympus SZX9). Transverse sections of the podetia were made free-hand, and the microscopical measures were taken at 400× magnification using an Olympus CX41 microscope, in distilled water. The matrix containing the fourteen characters previously mentioned was analyzed using principal component analysis (PCA). This analysis was performed with the Canoco 4.5 program (ter Braak & Šmilauer 2002). The variables were centered and standardized before the PCA. The values for the first two components were plotted (Fig. 1). In Fig. 1a the data were coded according to the morphological identification. Using the same matrix the discriminatory descriptors were inferred from the lenght of the vector and its correlation with the respective axes, so Fig. 1b represents the correlation of the different morphological variables with the components.

The secondary metabolites were studied in all the specimens using the solvents A (Toluene: dioxane: acetic acid) and B (Hexane: diethylether: formic acid) (White & James 1985).

DNA extraction, PCR and sequencing

Genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The DNA was eluted in the final step in 200 ml of elution buffer provided by the manufacturer. The following three nuclear loci were sequenced: ITS rDNA, *rpb2* and IGS rDNA. The primers and PCR programs are described in Pino-Bodas et al. (2013). The amplifications were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). PCR products were purified with ExoSAP-IT (USB Corporation, OH, USA). The sequencing was performed at Macrogen Europe service (www.macrogen.com), with the same primers used for the PCR.

Phylogenetic analyses

The consensus sequences from forward and reverse templates were assembled and edited in Sequencher™ 4.1.4 program (Gene Codes Corporation, Inc, Ann Arbor, Michigan, USA). The sequences of each locus were manually aligned in BIOEDIT 7.0 (Hall 1999). No ambiguous positions were found and all the positions of the alignments were included in the analyses. Each region was analyzed separately by Maximum Likelihood (ML) using RAxML 7.0.3 (Stamatakis et al. 2005), under the model GTRGAMMA. Fast bootstrap was run with 500 pseudoreplicates. The congruence among the different topologies inferred from the loci was tested following Hillis & Bull (1993): each clade with more than 75 % bootstrap support was scanned for conflict among loci. We considered the existence of a conflict whenever a clade was supported with a bootstrap (more than 75 %) in one locus, while it was not supported in another locus, and the individual sequences of this clade were part of another clade with bootstrap support 75 %. In the combined datasets, only the specimens with sequences at least for 2 genes were included. The combined dataset was analyzed by Maximum Parsimony (MP), ML and Bayesian analyses. MP analyses were performed in PAUP* v. 4.0.b.10 (Swofford 2003) using the heuristic searches with 1 000 random taxon-addition replicates, with TBR branch swapping and MulTrees option in effect, equally weighted characters. Gaps were treated as missing data. For the confidence analysis, the bootstrap (Felsentein 1985) was applied, with 1 000 replicates and heuristic searchs.

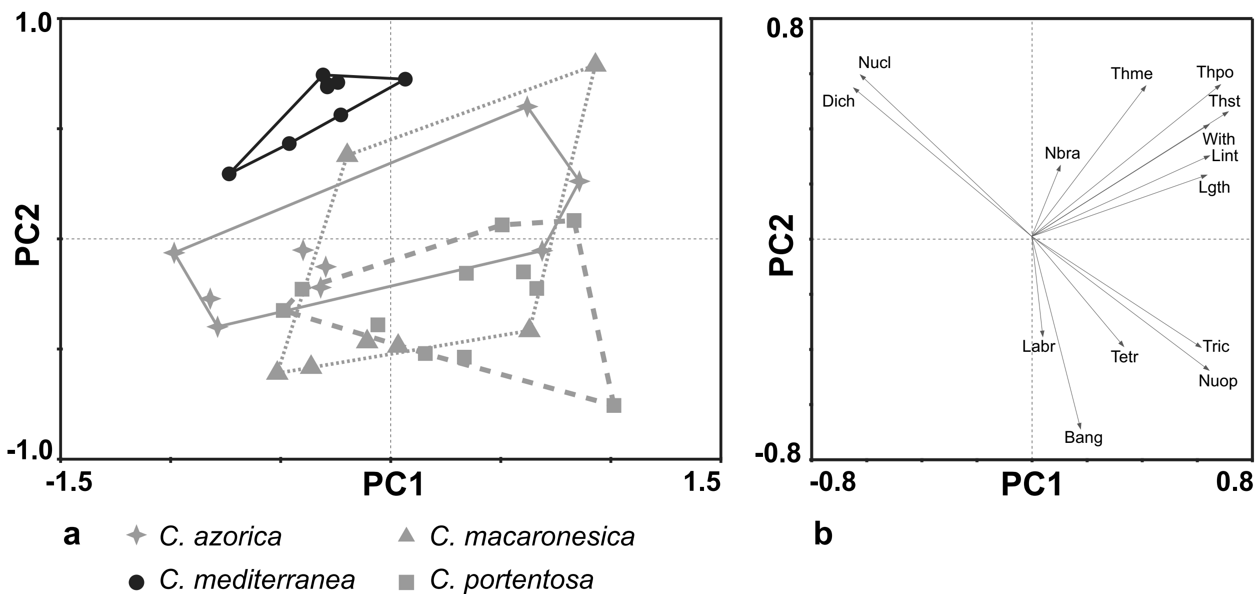

Fig. 1 Results of PCA analysis. a. PCA scatterplot with all the standardized variables and species studied; b. vector plot on PC1 and PC2. The length of vectors show the importance of each character. Bang: branching angles; Dich: dichotomous branches percentage; Labr: length of last branch; Lgth: length of podetia; Lint: length of internodes; Nbra: number of branches; Nucl: number of closed axils; Nuop: number of open axils; Tetr: tetrachotomous branches percentage; Thme: thickness of medulla; Thpo: thickness of podetia; Thst: thickness of stereome; Tric: trichotomous branches percentage; With: width of podetia.

The ML analysis was performed in the same conditions as the single gene datasets but considering 5 partitions: ITS rDNA, IGS rDNA and each codon position of *rpb2*. The best fit substitution model for each region was calculated using MrModeltest 2.3 (Nylander 2004), with Akaike information criterion. The models selected and used in the Bayesian analysis were: GTR+G for IGS rDNA, SYM+G for ITS rDNA and SYM+I for *rpb2*. The Bayesian analysis was carried out using MrBayes 3.2 (Ronquist et al. 2012). 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 the MCMC analysis. Two simultaneous runs with 10 000 000 generations, each starting with a random tree and employing 4 simultaneous chains, were executed. Every 1 000th tree was saved into a file. The convergence was assessed checking that the average standard deviation of split frequencies was < 0.01. In addition, the compare and slide commands in AWTY (Nylander et al. 2008) were used. Afterwards, the 50 % majority-rule consensus tree was calculated after removing the first 2 500 000 generations (i.e. the first 2 500 trees) using the 'burn in' command.

Tests of monophyly and genealogical sorting index

In order to assess the hypotheses that *C. azorica*, *C. macaronesica* and *C. portentosa* were monophyletic, constraint trees were constructed. These alternatives topologies were supplied to RAxML to search the 'best' ML tree. The GTRGAMMA model was used. Shimodaira-Hasegawa test (SH; Shimodaira & Hasegawa 1999) and expected likelihood weight test (ELW; Strimmer & Rambaut 2002) were performed using the program TREE-PUZZLE 5.2 (Schmidt et al. 2002) with 1 000 replicates resampled using the RELL method.

The genealogical sorting index (GSI) was used to assess the level of genealogical exclusivity (Cummings et al. 2008) for *C. azorica* and *C. macaronesica*. The GSI value was not calculated for *C. portentosa* because we have more specimens of this species than of *C. azorica* and *C. macaronesica*, and the GSI has bias when unequal sampling size among the groups. The GSI was calculated for the ML tree of each locus and GSI_T was calculated for the set of ML trees of the three loci. The significance was calculated using 10 000 permutations on the online platform at http://www.genealogicalsorting.org/. The GSI supports monophyly when this value is > 0.90 and the P-value < 0.001 according with Cummings et al. (2008) and Gazis et al. (2011).

Species tree

Two methods were used to calculate the species tree. First, the species tree under multispecies coalescent model was estimated using *BEAST implemented in BEAST (Drummond & Rambaut 2007), including only the specimens with sequence for the three loci. The specimens were assigned to species based on their morphology (*C. azorica*, *C. macaronesica*, *C. mediterranea* and *C. portentosa*). The model GTR+G was assigned to ITS and IGS partitions, and GTR+I to *rpb2* partition, selecting birth-death speciation process with an uncorrelated relaxed lognormal clock (Drummond et al. 2006) and a constant size population. For the remaining priors the defect values were kept. The analysis was run for 50 000 000 generations, sampling every 1 000. The convergence was calculated with TRACER 1.5 (Rambaut & Drummond 2007). After discarding the first 10 000 000 generations the effective sample size for all the parameters of the evolutionary model reached values > 200. The tree was summarized with TREEANNOTATOR 1.7.5 (Rambaut & Drummond 2013) using maximum clade credibility tree option for the target tree type.

In the second method SpedeSTEM (Ence & Carstens 2011) was used to calculate the species tree. This method is based on coalescence that applies several loci gene trees to calculate the maximum likelihood species tree (Kubatko et al. 2009). This program allows not only to validate the species generated by other procedures, but also to delimit species with no a priori assignment of individuals. In this study only discovering analyses were made according to Satler et al. (2013). The ML gene trees were generated in PAUP (using the models estimated in MrModeltest), including *C. mediterranea*, *C. macaronesica*, *C. portentosa*, *C. azorica* and the outgroup. SpedeSTEM requires the specimens to be present in all the gene trees, and so only those can be studied for which it was possible to generate sequences for the three loci. SpedeSTEM needs a θ value for scaling the

branch lengths in the species trees it produces. The θ value for each locus was calculated in DnaSP v. 5 (Librado & Rozas 2009), being $\theta = 0.04437$ for IGS rDNA, $\theta = 0.04073$ for ITS and $\theta = 0.02804$ for *rpb2*. The analysis was repeated for several θ values: the average value of the three loci ($\theta = 0.03771$), $\theta = 0.02$, $\theta = 0.03$ and $\theta = 0.04$, to avoid the issues the program can have when calculating the likelihood for low θ values (Giarla et al. 2014).

Species delimitation by cohesion species recognition

Haplotypes networks under statistical parsimony with a confidential interval of 95 % were generated with TCS 1.21 (Clement et al. 2000) for each locus (ITS rDNA, IGS rDNA and *rpb2*). For the ITS rDNA analysis all the sequences of *C. portentosa* from GenBank (FR799166, FR799167, JQ695921, JQ695922, JQ695323) were included. Gaps were coded as missing data. The haplotypes were gathered manually in clades according to the rules of Templeton et al. (1987). This algorithm identifies clades united by mutational steps. The x-step clades are successively grouped in x+1-step clades and the final level of nested clades includes the complete network. The loops were resolved following the three criteria (frequency, topology and geographical) proposed by Pfenninger & Posada (2002). Table of contingence and Kruskal-Wallis analyses were done to test the null hypothesis (H2) of no significant association between haplotype variation and phenotypical variation. The quantitative variables with more contribution to separate the groups in the PCA analysis were analyzed by Kruskal-Wallis. The statistical analyses were performed in STATGRAPHICS 5.1 program. The clades 2-4 of ITS rDNA and 2-4 of *rpb2* could not be included in the statistical analyses because they contained only one specimen. The pairwise fixation index F_{ST} (Weir & Cockerham 1984) was calculated with the program DnaSP. This value was used to assess whether gene flow exists among the cohesive species delimited.

RESULTS

Morphological analysis and secondary metabolites

Fig. 1 shows the results of PCA. The first two principal components PC1 and PC2 summarize 51.44 % of the total variance (29.93 % and 21.51 %, respectively). The analysis distinguished two groups (Fig. 1a). The first group contains all the specimens of *Cladonia mediterranea* (on the upper left area of the scatterplot) and the other group is formed by the rest of the species analyzed, *C. azorica*, *C. macaronesica* and *C. portentosa* (on the center of the scatterplot). The analysis shows a continuous morphological variation in the second group with a high degree of overlapping between the three species. The characters that most contribute to separate *C. mediterranea* from the other group were the dichotomous branching percentage and the number of closed axils (Fig. 1b).

The secondary metabolites found in each specimen are listed in Table 1. All the specimens of *C. mediterranea* and *C. macaronesica* contained perlatolic and usnic acids. One specimen of *C. azorica* lacked usnic acid, containing only fumarprotocetraric and perlatolic acids. The other specimens contained fumarprotocetraric, perlatolic and usnic acids. Three specimens of *C. portentosa* lacked usnic acid (*C. portentosa* subsp. *portentosa* f. *subimpexae*). The other specimens contained perlatolic and usnic acids.

Datasets and phylogenetic analyses

In this study 113 new sequences (39 from ITS rDNA, 40 from IGS rDNA and 34 from *rpb2*) have been generated, the GenBank accession numbers are listed in Table 1. The concatenated dataset contained 1 781 characters, 136 of which were parsimony-informative. The locus which contained more parsimony-informative positions was ITS rDNA with 50 positions, followed by IGS rDNA with 47 positions and *rpb2* with 39 positions.

The separated analyses of ITS rDNA and IGS rDNA yielded trees with the same topology (not shown). Three well supported clades appeared. One clade contained all the samples of *C. mediterranea*, the second clade contained the samples of *C. confusa* and *C. pycnoclada* and the third included all the samples of *C. azorica*, *C. macaronesica* and *C. portentosa*. The ML analysis of *rpb2* yielded a tree with 4 clades, one with all the samples of *C. mediterranea*, two clades containing samples of *C. azorica*, *C. macaronesica* and *C. portentosa*, and the fourth with the samples of *C. confusa* and *C. pycnoclada*. Only the *C. confusa* and *C. pycnoclada* clade was supported. No conflict among the loci was found and the datasets were combined. The MP analysis based on the concatenated dataset yielded trees of 357 steps long, CI = 0.8711 and RC = 0.9170. The ML analysis yielded a tree with a likelihood value of −LnL = 4431.65, while the mean likelihood of the Bayesian tree sampling was −LnL = 4620.16. The tree topology was the same in all the analyses, so only the 50 % consensus majority tree from Bayesian analysis is shown (Fig. 2). Three clades appeared in all the analyses. One of them contained all the specimens of *C. mediterranea*, the second the specimens of *C. pycnoclada* and *C. confusa*, and the third the specimens of *C. azorica*, *C. macaronesica* and *C. portentosa*. The *C. mediterranea* clade was basal to the group. This clade was well supported in MP and ML analyses but not in the Bayesian analysis (0.74 posterior probability). The clade formed by *C. pycnoclada* and *C. confusa* was supported in all the analyses, and the third clade had high support in all the analyses. In the ML and Bayesian analyses two unsupported subclades can be distinguished in

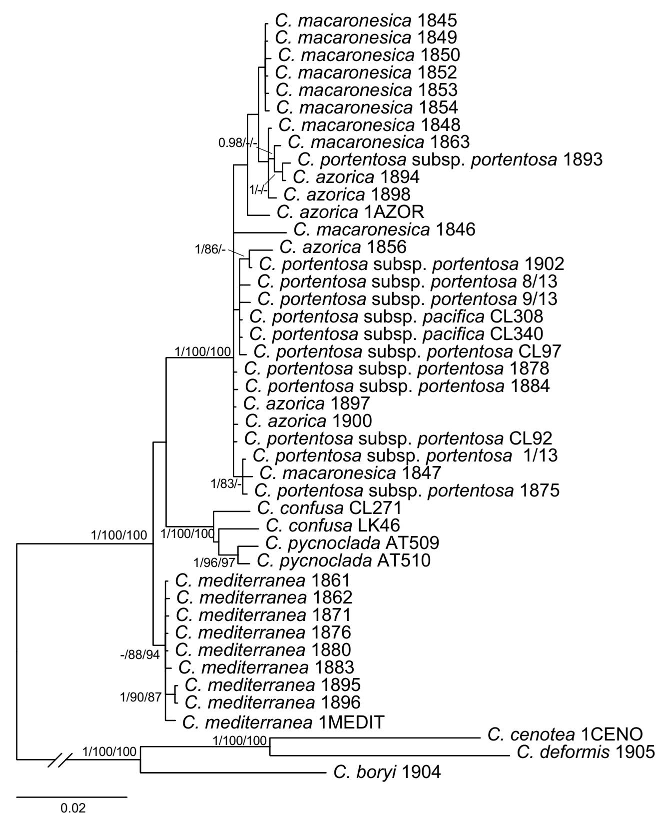

Fig. 2 Phylogeny of *Cladonia mediterranea* complex. The 50 % consensus majority tree of the Bayesian analysis based on concatenated dataset. The values on the branches are the posterior probability from Bayesian analysis (≥ 0.95), bootstrap values from ML analysis (≥ 75 %) and bootstrap values from MP analysis (≥ 75 %).

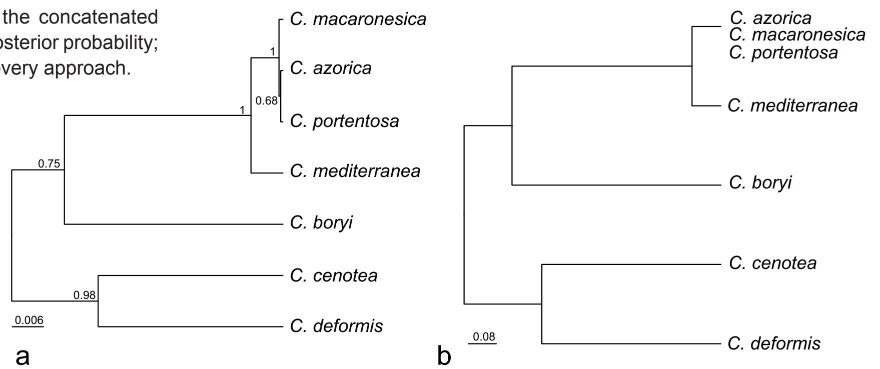

Fig. 3 a. Species tree inferred in *BEAST based on the concatenated dataset. The values on the branches correspond to the posterior probability; b. species tree estimated in spedeSTEM based on discovery approach.

the third clade. One of them was constituted by some samples of *C. macaronesica* and *C. azorica* and the other was formed by all the specimens of *C. portentosa*, some specimens of *C. macaronesica* and some specimens of *C. azorica*.

Species trees

The results of the *BEAST analysis are shown in Fig. 3a. *Cladonia mediterranea* was well supported, *C. azorica* and *C. portentosa* form a clade but the node was not significantly supported with posterior probability. The clade clustering *C. azorica*, *C. macaronesica* and *C. portentosa* was significantly supported. The results from SpedeSTEM analyses were similar for different θ values. In all the cases the model that obtained most support was the one that considers 2 lineages in the ingroup (Fig. 3b). One of them was composed only by *C. mediterranea* and the other by *C. macaronesica*, *C. portentosa* and *C. azorica.* The probability of this model was wi = 0.99 for θ = 0.03771, wi = 1.0 for θ = 0.02, wi = 0.99 for θ = 0.03 and wi = 0.99 for θ = 0.04. The probability for alternative models was wi = 0.0.

Hypotheses and GSI

The SH and ELW significantly rejected the three hypotheses:

a. the monophyly of *C. azorica* (SH, P-values = 0.0090 and ELW P-value = 0.0009);
b. the monophyly of *C. macaronesica* (SH, P-values = 0.0290 and ELW P-value = 0.0423);
c. the monophyly of *C. portentosa* (SH, P-value = 0.0270 and ELW P-value = 0.0142).

The GSI test results are shown in the Table 2. The GSI values for *C. azorica* were similar among the different loci and the P-values rejected the monophyly in all the loci. The GSI values for *C. macaronesica* were low in ITS rDNA and *rpb2*, and not significant. However, the GSI value of IGS rDNA was 0.5806 and significant. The GSI_T rejected the exclusive ancestry for both species.

Networks and nested clade analyses

A total of fifteen haplotypes of ITS rDNA were identified, connected in a single network (Fig 4a). Two haplotypes were unique for *C. mediterranea*, two were unique for *C. macaronesica*, two were unique for *C. azorica* and five were unique for *C. portentosa*. The other four haplotypes were shared by samples of different species (C. *macaronesica*, *C. portentosa* and *C. azorica*). The IGS rDNA network analysis yielded a total of six haplotypes connected into a single network (Fig. 4b). All the samples of *C. mediterranea* were represented in one unique haplotype, one haplotype was unique for *C. azorica*, and one was unique for *C. portentosa*. The other three haplotypes were shared by samples of *C. macaronesica* and *C. azorica*; *C. macaronesica* and *C. portentosa*; or *C. macaronesica*, *C. portentosa* and *C. azorica*. The *rpb2* network analysis yielded nine haplotypes connected into a single network (Fig. 4c), four of them were unique for *C. mediterranea*, one for *C. macaronesica* and one was unique for *C. azorica*. The other three haplotypes were shared by samples of different species.

The nested clade analyses generated five 1-step clades, four 2-step clades and two 3-step clades for ITS rDNA; for IGS rDNA, three 1-step clades and two 2-step clades were generated; and for *rpb2* six 1-step clades, four 2-step clades and two 3-step clades were generated. All the specimens of 2-2 clade from IGS rDNA and 3-1 clade from ITS were identified as *C. mediterranea*, while the 3-2 clade of *rpb2* contained all the specimens of *C. mediterranea* and one of *C. macaronesica*. The specimens of *C. macaronesica*, *C. portentosa* and *C. azorica* appeared together in the 3-2 clade of ITS rDNA, 2-1 clade of IGS rDNA and 3-1 clade of *rpb2*. The specimens grouped together in the 2-1 clade of IGS rDNA, 3-2 clade of ITS rDNA and 3-1 clade of *rpb2* are from Macaronesia, North America and Europe while the specimens of the clades 2-2 of IGS rDNA and 3-2 of *rpb2* are from the Canary Islands and the Iberian Peninsula.

Table 3 and 4 summarize the results of the contingency table analyses. In the analyses of the 3 networks (ITS rDNA, IGS rDNA and *rpb2*) significant differences in characters were observed (Table 3). These characters include medulla (loose/compact), branching pattern (isotomic/anisotomic/subisotomic) and algal layer (continuous/discontinuous). These differences were observed between the 3-step clades of ITS rDNA and *rpb2* and 2-step clades in IGS rDNA. No significant differences in the presence/absence of fumarprotocetraric acid among these clades were found. The results of the contingency table analyses at 2-step level are presented in Table 4. In ITS rDNA, most of the significant differences were detected among the 2-1 clade and the other clades; in *rpb2* significant differences

Table 2 Genealogical sorting index and probability values under the null hypothesis that the samples labeled as putative species are monophyletic.

Species	ITS rDNA		IGS rDNA		*rpb2*		GSI_T	
	GSI	P-value	GSI	P-value	GSI	P-value	GSIt	P-value
C. azorica	0.1429	0.1798	0.2114	0.0659	0.2614	0.0463	0.1429	0.1782
C. macaronesica	0.1556	0.1376	0.5806	1e-04*	0.1795	0.3308	0.1556	0.1343

* denotes significant result.

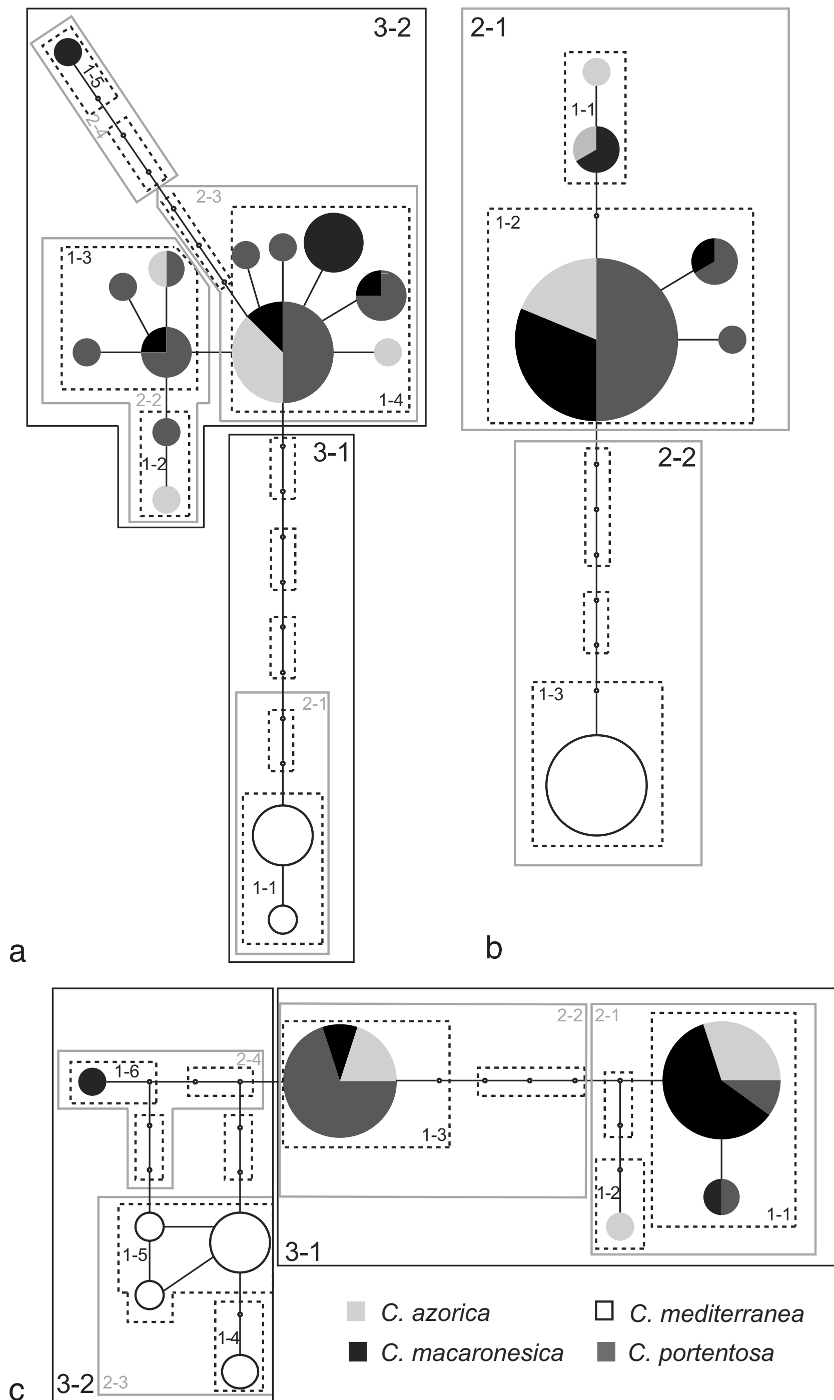

Fig. 4 Haplotype networks at 95 % of probability of *C. mediterranea* complex based on a. ITS rDNA; b. IGS rDNA; c. *rpb2*. The circles represent the haplotypes and the size is proportional to haplotype frequency. The small circles represent missing haplotypes. The discontinuous lines outline the 1-step nested clades, the grey lines outline the 2-step nested clades and the black lines outline the 3-step nested clades.

were detected among the 2-3 clade and the other clades; and in IGS rDNA significant differences were detected among the 1-3 clade and the other clades.

Table 5 shows the Kruskal-Wallis results. Significant differences were obtained for all of the characters among the 3-step clades in ITS rDNA and *rpb2* and the 2-step clades in IGS rDNA. However, there were not significant differences among all the 2-step clades (see Tukey test, Table 6). No significant differences were found among the 2-step clades in *rpb2* for dichotomous branching rate and trichotomous branching rate.

The F_{ST} values between the 3-step clades of ITS rDNA and *rpb2* and the 2-step clades of IGS rDNA are shown in Table 7. In all the comparations the values were high. The lowest value was between the clades appearing in *rpb2*.

DISCUSSION

This work addresses the species delimitation in the *C. mediterranea* complex using two data sources: phenotypical data (morphological and chemical) and DNA sequences from three nuclear genes. The DNA data were analyzed by different methods often used for species delimitation (gene trees, species trees, haplotype networks) and they were highly congruent. When the analyses performed with different type of data show congruent results (as in this case), the concordant inferred boundaries likely correspond to existing biological entities. According to our results, the most probable scenario is the one that comprises two species.

The analyses of the morphological data reveal that *C. mediterranea* is different from the remaining species. The genealogical

Table 3 Contingency test results for association of haplotype clades and phenotypical characters at highest step clade level for each locus.

Comparation level	Character	P-value
ITS rDNA		
3-step	Presence / lack of fumarprotocetraric acid	0.193
3-step	Isotomic / anisotomic or subisotomic pattern	0.001*
3-step	Compact / loose medulla	0.000*
3-step	Continuous / discontinuous algal layer	0.000*
IGS rDNA		
2-step	Presence / lack of fumarprotocetraric acid	0.211
2-step	Isotomic / anisotomic or subisotomic pattern	0.007*
2-step	Compact / loose medulla	0.000*
2-step	Continuous / discontinuous algal layer	0.000*
rpb2		
3-step	Presence / lack of fumarprotocetraric acid	0.111
3-step	Isotomic / anisotomic or subisotomic pattern	0.004*
3-step	Compact / loose medulla	0.000*
3-step	Continuous / discontinuous algal layer	0.000*

* denotes significant result.

Table 4 Contingency test results for association of haplotype clades and phenotypical characters.

Comparation level	Character	P-value
ITS rDNA		
2-1 to 2-2	Presence / lack of fumarprotocetraric acid	0.429
2-1 to 2-3	Presence / lack of fumarprotocetraric acid	0.171
2-2 to 2-3	Presence / lack of fumarprotocetraric acid	0.412
2-1 to 2-2	Compact / loose medulla	0.001*
2-1 to 2-3	Compact / loose medulla	0.000*
2-2 to 2-3	Compact / loose medulla	0.704
2-1 to 2-2	Continuous / discontinuous algal layer	0.007*
2-1 to 2-3	Continuous / discontinuous algal layer	0.000*
2-2 to 2-3	Continuous / discontinuous algal layer	0.296
2-1 to 2-2	Isotomic / anisotomic or subisotomic pattern	0.001*
2-1 to 2-3	Isotomic / anisotomic or subisotomic pattern	0.002*
2-2 to 2-3	Isotomic / anisotomic or subisotomic pattern	0.406
IGS rDNA		
1-1 to 1-2	Presence / lack of fumarprotocetraric acid	0.179
1-1 to 1-3	Presence / lack of fumarprotocetraric acid	0.091
1-2 to 1-3	Presence / lack of fumarprotocetraric acid	0.348
1-1 to 1-2	Compact / loose medulla	0.662
1-1 to 1-3	Compact / loose medulla	0.002*
1-2 to 1-3	Compact / loose medulla	0.487
1-1 to 1-2	Continuous / discontinuous algal layer	0.450
1-1 to 1-3	Continuous / discontinuous algal layer	0.018*
1-2 to 1-3	Continuous / discontinuous algal layer	0.000*
1-1 to 1-2	Isotomic / anisotomic or subisotomic pattern	0.134
1-1 to 1-3	Isotomic / anisotomic or subisotomic pattern	0.160
1-2 to 1-3	Isotomic / anisotomic or subisotomic pattern	0.001*
rpb2		
2-1 to 2-2	Presence / lack of fumarprotocetraric acid	0.166
2-1 to 2-3	Presence / lack of fumarprotocetraric acid	0.054
2-2 to 2-3	Presence / lack of fumarprotocetraric acid	1.000
2-1 to 2-2	Compact / loose medulla	0.045*
2-1 to 2-3	Compact / loose medulla	0.000*
2-2 to 2-3	Compact / loose medulla	0.022*
2-1 to 2-2	Continuous / discontinuous algal layer	0.191
2-1 to 2-3	Continuous / discontinuous algal layer	0.000*
2-2 to 2-3	Continuous / discontinuous algal layer	0.006*
2-1 to 2-2	Isotomic / anisotomic or subisotomic pattern	0.208
2-1 to 2-3	Isotomic / anisotomic or subisotomic pattern	0.015*
2-2 to 2-3	Isotomic / anisotomic or subisotomic pattern	0.003*

* denotes significant result.

Table 5 Kruskal-Wallis results for association of haplotype clades and phenotypical characters.

Comparation level	Character	Estatistic	P-value
ITS rDNA			
3-step	Dichotomous branching rate (%)	5.53597	0.0186*
3-step	Trichotomous branching rate (%)	3.90411	0.0481*
3-step	Closed axils rate (%)	12.4813	0.0004*
2-step	Dichotomous branching rate (%)	8.26747	0.0407*
2-step	Trichotomous branching rate (%)	9.39649	0.0244*
2-step	Closed axils rate (%)	12.5187	0.0058*
IGS rDNA			
2-step	Dichotomous branching rate (%)	10.6712	0.0010*
2-step	Trichotomous branching rate (%)	7.5518	0.0059*
2-step	Closed axils rate (%)	11.8636	0.0057*
1-step	Dichotomous branching rate (%)	10.9286	0.0042*
1-step	Trichotomous branching rate (%)	7.64197	0.0219*
1-step	Closed axils rate (%)	13.5391	0.0011*
rpb2			
3-step	Dichotomous branching rate (%)	5.16176	0.02308*
3-step	Trichotomous branching rate (%)	4.64772	0.03109*
3-step	Closed axils rate (%)	11.268	0.00078*
2-step	Dichotomous branching rate (%)	5.63538	0.1307
2-step	Trichotomous branching rate (%)	6.65459	0.0837
2-step	Closed axils rate (%)	14.1365	0.00272*

* denotes significant result.

Table 6 Tukey's multiple comparison test for significant results of the Kruskal-Wallis analyses.

	Dichotomic	Trichotomic	Closed axil
ITS rDNA			
2-1 to 2-2	*	ns	*
2-1 to 2-3	ns	ns	*
2-1 to 2-4	ns	ns	ns
2-2 to 2-3	ns	ns	ns
2-2 to 2-4	ns	ns	ns
2-3 to 2-4	ns	ns	ns
IGS rDNA			
1-1 to 1-2	ns	ns	ns
1-1 to 1-3	*	*	*
1-2 to 1-3	ns	ns	ns
rpb2			
2-1 to 2-2	–	–	ns
2-1 to 2-3	–	–	ns
2-1 to 2-4	–	–	*
2-2 to 2-3	–	–	*
2-2 to 2-4	–	–	ns
2-3 to 2-4	–	–	ns

ns = not significant; * = significant with 95 % of probability.

Table 7 Pairwise F_{ST} for each clade defined in the networks.

Locus	Comparisons	F_{ST}
ITS rDNA	3-1 to 3-2 step clade	0.87659
IGS rDNA	2-1 to 2-2 step clade	0.93114
rpb2	3-1 to 3-2 step clade	0.69796

phylogenetic species recognition (GPSR) was congruent with the results of the analysis of the morphological data (Fig. 2). *Cladonia mediterranea* formed a monophyletic clade well supported in MP and ML analyses, but not in the Bayesian analysis. The hypotheses tests (SH and EWL) significantly rejected the alternative topologies, in which *C. azorica*, *C. macaronesica* and *C. portentosa* were divided into independent monophyletic groups. Since the incomplete lineage sorting could be responsible for the lack of monophyly of *C. azorica*, *C. macaronesica* and *C. portentosa*, we applied the GSI test to evaluate the degree of genealogic divergence. The monophyly of *C. azorica* and *C. macaronesica* was not supported by this test. The species trees generated by means of *BEAST and SpedeSTEM gave rise to two well-supported species (Fig. 3). These analyses are congruent with the gene trees and the morphological analysis, leading to consider *C. azorica*, *C. macaronesica* and *C. portentosa* as a unique species, and *C. mediterranea* as a different one. The cohesion species recognition requires, in addition to rejecting the two null hypothesis, that the groups delimited during the evaluation of H1 be congruent with H2 hypothesis (Templeton et al. 2000). This congruence happens at 3-step clade level in ITS rDNA and *rpb2* and at 2-step clade level in

IGS, since at an inferior level (2-step clade level in ITS and *rpb2* and 1-step clade level in IGS) significant results were obtained, but not among all the clades. The morphological differences occur between the clades that contain samples of *C. mediterranea* and the remaining clades, while there are no significant differences between the clades that contain the samples of *C. azorica*, *C. macaronesica* and *C. portentosa* (2-2, 2-3 and 2-4 in ITS rDNA, 2-1, 2-2, 2-4 in *rpb2* and 1-1 and 1-2 in IGS rDNA). Strong evidence for the fact that *C. mediterranea* is a different species from *C. macaronesica*, is that all the samples of *C. mediterranea* are confined to a unique clade in all the haplotype networks. In addition, *C. mediterranea* shows high levels of genetic differentiations, according with the F_{ST} values.

The analyses of the morphological data and also numerous analyses based on the DNA sequences are consistent, indicating that *C. mediterranea* is an independent evolutionary lineage and *C. azorica*, *C. macaronesica* and *C. portentosa* are conspecific. Thus our results reject the taxonomical proposal that *C. mediterranea* and *C. macaronesica* are conspecific (Ruoss 1989). This author studied the branching pattern and the characteristics of the algal layer and concluded that *C. macaronesica* and *C. mediterranea* were the same species. The diagnostic characters used to distinguish these species were the following: length of the internodes are longer in *C. mediterranea* than in *C. macaronesica*; the algal layer is continuous in *C. mediterranea* and discontinuous in *C. macaronesica*; a compact medulla present in *C. mediterranea* and a lax medulla in *C. macaronesica*; the axils are frequently closed in *C. mediterranea* and generally open in *C. macaronesica* (Ahti 1961). The PCA analyses carried out in this work show that the most relevant variables to distinguish *C. mediterranea* from the remaining species are the percentage of dichotomous branching and the number of closed axils. According to Ruoss (1989) *C. mediterranea* had more closed axils than *C. macaronesica*. However, we think that the internodal length does not contribute to the separation of *C. mediterranea* from the remaining samples, since *C. portentosa* has internodes of similar length or even with greater variation (Burgaz & Ahti 2009). Burgaz & Martínez (2008) found that the podetial wall is thicker in *C. mediterranea* than in *C. portentosa*; however, in our analysis this character had a scant contribution to distinguish *C. mediterranea* from the other species. The morphological characters that distinguish *C. mediterranea* are: the presence of a continuous algal layer, the presence of a compact medulla, the prevalence of isotomy, with dichotomous branching and closed axils. These characters are the originally used ones to describe the species (des Abbayes & Duvigneaud 1947).

The boundaries among *C. azorica*, *C. macaronesica* and *C. portentosa* were not supported by any of the analyses carried out in this work. *Cladonia azorica* was distinguised from *C. macaronesica* mainly by the presence of fumarprotocetraric acid and by having a greater number of trichotomous branching, although the dichotomous pattern is also prevailing in this species (Ahti 1961). But our analyses did not show a correlation between the presence of fumarprotocetraric acid and a greater number of trichotomous branching. In previous works based on the study of the morphological variation, the species status of *C. azorica* and *C. macaronesica* had already been questioned (Ahti 1977). Although no previous study has suggested that *C. portentosa* is conspecific with the latter, Orange (1993) pointed out that in Britain it was impossible to distinguish *C. azorica* from *C. portentosa* only by means of morphological characters. The morphological similarities of *C. macaronesica* and *C. azorica* with *C. portentosa* are clear, even with *C. mediterranea* (Fig. 5), and the identification keys and floristic works usually point out the possible confusion of *C. portentosa* with these other species (James 2009, Sicilia et al. 2009). But *C. portentosa* is generally distinguished by the prevailing trichotomous branching and an anisotomous pattern, where a main axis is clear. Nonetheless, *C. portentosa* is a very variable species, either morphologically or chemically (des Abbayes 1939, Ahti 1961, 1978, Burgaz & Martínez 2008). Within this taxon several forms and subspecies have been described. *Cladonia portentosa* subsp. *pacifica*, growing in western North America, is more slender and deflexed than *C. portentosa* subsp. *portentosa* and shows a greater number of dichotomous branches (Ahti 1978, Brodo & Ahti 1996). *Cladonia portentosa* subsp. *pacifica* f. *decolorans* is a chemotype that lacks usnic acid, turning to a greyish shade (Brodo & Ahti 1996). *Cladonia portentosa* subsp. *portentosa* f. *subimpexa* also lacks usnic acid (Ahti 1978). The ITS rDNA sequences of *C. portentosa* subsp. *pacifica* and *C. portentosa* subsp. *portentosa* were recently compared and it was found that there was no genetical difference between them (Smith et al. 2012). Our analyses confirm these results. The two specimens of *C. portentosa* subsp. *pacifica* here included share a haplotype with some of *C. portentosa* subsp. *portentosa* samples in each of the 3 loci.

In other species of the Group *Cladinae* (Stenroos et al. 2002) similar results have been found. This is the case of *C. arbuscula*, for which several subspecies were defined on the basis of the morphological and chemical variation. However, much of this variation is not correlated with the genetic variation (Piercey-Normore et al. 2010). The authors attribute the high variation within this species to environmental agents such as lighting, humidity, nutrients and thallus age. The warm temperatures throughout the year in Macaronesia, which causes a continuous development of the podetia, could be the environmental agent that determines *C. portentosa* to develop a prevailing dichotomous branching, instead of trichotomous. Ahti (1961) had already pointed out that in southern Europe (Portugal) *C. portentosa* tended to produce dichotomous branching, being easily mistaken for *C. mediterranea*, with which it often coexists (Burgaz & Ahti 2009).

Our results indicate that *C. confusa* and *C. pycnoclada* are related, while in the phylogeny submitted by Stenroos et al. (2002) *C. confusa* appeared closely related to *C. terra-novae* and *C. portentosa*. However, in our analyses *C. confusa* is not monophyletic, which could reveal a lack of genetic homogeneity of this species. Further studies, based on a wide range of sampling, should be made to confirm this observation.

TAXONOMY

In this section we present formally the taxonomical changes.

Cladonia portentosa (Dufour) Coem., Bull. Acad, Roy. Sci. Belgique, ser. 2, 19: 49. 1865.

Basionym. *Cenomyce portentosa* Dufour, Ann. Gen. Sci. Phys. 8: 69. 1821.
Type. France, Landes, Saint-Sever-sur-Adour, 1818, *J.-M. Dufour* (PC-Herb. Desmazières lectotype, Ahti, Ann. Bot. Fenn. 15: 8. 1978).

= *Cladonia azorica* Ahti, Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo 32, 1: 36. 1961.
Type. Portugal, Azores, Pico, 25 Sept. 1961, *A.G. da Cunha & L. Sobrinho* (LISU holotype).

= *Cladonia macaronesica* Ahti, Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo 32, 1: 37. 1961.
Type. Portugal, Madeira, Entre as Queimadas e o Caldeirão Verde, 1951, *C.N. Tavares* 4583 (LISU holotype).

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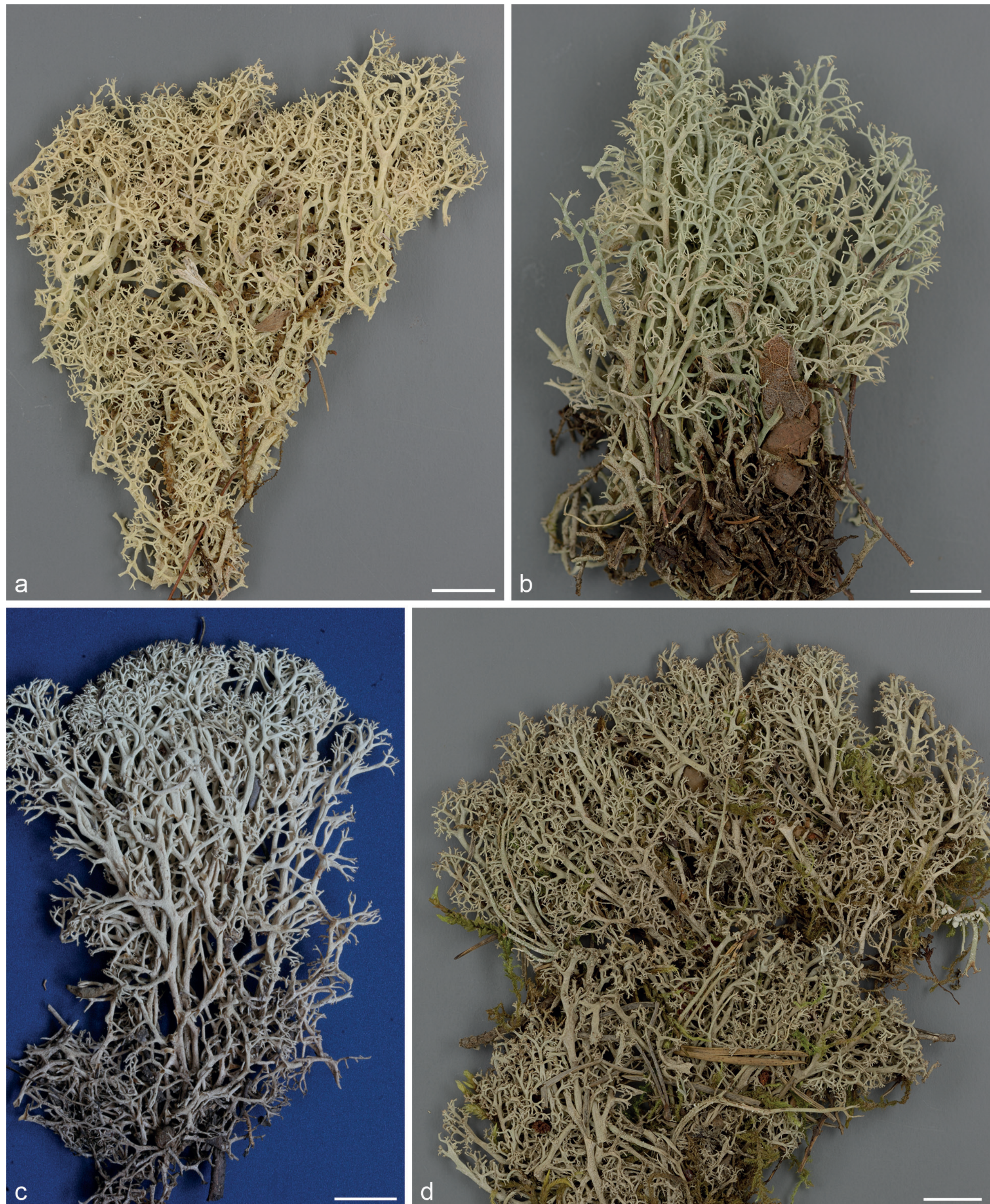

Fig. 5 Photographs of the four species studied, showing the general configurations of podetia a. *Cladonia azorica* (*Haikonen* 26865, H); b. *C. macaronesica* (*Pérez-Vargas* s.n., TFC 10602); c. *C. mediterranea* (*Burgaz* s.n., MACB 61559); d. *C. portentosa* (*Stenroos* 6074, H).

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