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Cladonia verticillata (*Cladoniaceae*, Ascomycota), new record to Iberian Peninsula

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Abstract: Pino-Bodas, R.; Martín, M. P.; Stenroos, S. & Burgaz, A. R. 2013. *Cladonia verticillata* (Cladoniaceae, Ascomycota), new record to Iberian Peninsula. *Bot. Complut*. 37: 21-25.

The identity of a putative collection of *Cladonia verticillata* is assessed by means of ITS rDNA region. The phylogenetic analyses confirmed that this specimen belongs to *C. verticillata*, and this species is reported as new to Iberian Peninsula.

Key words: Ascomycota, *Cladonia cervicornis*, ITS rDNA, lichenized fungi

Resumen: Pino-Bodas, R.; Martín, M. P.; Stenroos, S. & Burgaz, A. R. 2013. *Cladonia verticillata* (*Cladoniaceae*, Ascomycota), nueva cita para la Península Ibérica. *Bot. Complut*. 37: 21-25.

La identidad de una supuesta muestra de *Cladonia verticillata* es evaluada por medio de la región ITS rDNA. Los análisis filogenéticos revelaron que esta muestra pertenece a *C. verticillata* y, por tanto, esta especie es una nueva cita para la Península Ibérica.

Palabras clave: Ascomycota, *Cladonia cervicornis*, ITS rDNA, hongos liquenizados.

INTRODUCTION

Cladonia verticillata (Hoffm.) Schaer. is a broadly distributed species, found in Europe, Asia, America and Australasia (Ahti 2007). It is characterized by its scyphose, corticate podetia that present proliferations growing from the scyphus center. Not long ago it was commonly considered as a subspecies of *C. cervicornis* (Ach.) Flot. However, van Herk & Aptroot (2003) deemed that the morphological differences between them were enough to raise *C. verticillata* to the species rank. This taxonomical change has been accepted by most of the authors (Ahti 2007, Burgaz & Ahti 2009).

Though *C. verticillata* had been previously reported from the Iberian Peninsula (Navás 1904, Tavares 1950, Seaward 1983, Valcárcel *et al*. 1993), the recent studies on Cladoniaceae in this area (Burgaz & Ahti 1998, 2009) excluded the species from the area, indicating that all the samples identified as *C. verticillata* were in fact specimens of *C. cervicornis*. During our studies of *Cladonia* in the Iberian Peninsula, we collected a sample morphologically resembling *C. verticillata*. The aim of this study was to clarify the identity of this sample of *Cladonia* by means of ITS rDNA region.

MATERIALS AND METHODS

Taxon sampling. Sequences of *C. verticillata* group (Ahti 2007) were downloaded from GenBank and other DNA sequences were generated in our previous studies (Stenroos *et al.* 2002; Pino-Bodas *et al.* 2010, 2011). In total 26 sequences from 15 taxa were downloaded (Table 1). *Cladonia cornuta* (L.) Hoffm., *C. foliacea* (Huds.) Willd. and *C. gracilis* (L.) Willd. were used as outgroup according with the results of Stenroos *et al.* (2002).

Molecular analyses. The DNeasy Plant Mini Kit (Quiagen) was used to extract DNA, according to the manufacturer's instructions. The primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) were used to amplify the ITS rDNA region. PCRs were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). The volume of reaction was 25 Ìl for each tube, with 0.4 mM final concentration of primers. The amplification program was 94 ºC for 5 min; 5 cycles of 94 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 1 min; and 33 cycles of 94 ºC for 30 s, 48 ºC for 30 s and 72 ºC for 1 min; with a final extension of 72 ºC for 10 min. PCR products were purified with

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Table 1
List of taxa included in this study with the respective sampling localities, collections numbers and GenBank accession numbers. In bold, the sequences generated in this study

Taxa	Locality and collection	GenBank Nº
C. andesita	Kenya, *Chuah-Petiot* 947, TUR	AF453844
C. apodocarpa	USA, North Carolina, *Ahti* 60198, H	AF455237
C. calyciformis	Australia, *Wall* s.n., TUR	AF455176
C. cervicornis 1	Spain, Cádiz, *A. R. Burgaz*, MACB 91631	FM211897
C. cervicornis 2	Spain, Guadalajara, *A. R. Burgaz*, MACB 90738	FM205904
C. cervicornis 3	Spain, Madrid, *A. R. Burgaz*, MACB 90840	FM205905
C. cervicornis 4	Spain, Cuenca, *A. R. Burgaz*, MACB 90718	FM205906
C. cervicornis 5	Spain, Huelva, *A. R. Burgaz*, MACB 91610	FM205916
C. cervicornis subsp. *mawsonii*	Kerguelen, *Poulsen* RSP-1044, TUR	AF455178
C. clathrata	Brazil, Minas Gerais, *Stenroos* 5085a, TUR	AF455185
C. cornuta subsp. *cornuta*	Finland, Uusimaa, *R. Pino-Bodas*, MACB 101646	JN811385
C. crinita	Brazil, Minas Gerais, *Stenroos* 4963, TUR	AF455186
C. firma	Spain, Guadalajara, *A. R. Burgaz*, MACB 91619	FM205907
C. foliacea	Portugal, Trás-os-Montes, *A. R. Burgaz*, MACB 90503	FM205898
C. gracilis subsp. *gracilis*	Spain, Palencia, *A. R. Burgaz*, MACB 94216	JN811386
C. pulvinata 1	Spain, Orense, *A. R. Burgaz*, MACB 91646	FM205911
C. pulvinata 2	Portugal, Trás-os-Montes, *A. R. Burgaz*, MACB 94339	FM205917
C. pulvinata 3	Spain, Segovia, *A. R. Burgaz*, MACB 95598	FM205913
C. rappii 1	Australia, New South Wales, *Wall* s.n., TUR	AF455177
C. rappii 2	Thailand, Phu Hin Rong Kla National Park, *S. Parnmen* SP269, RAMK	EU113293
C. rappii 3	Bhutan, *Søchting* 8205, H	AF453843
C. staufferi	Australia, New South Wales, *Hammer* 7051, H	AF455179
C. verticillata 1	Canada, Manitoba, *Normore* 1624	DQ530193
C. verticillata 2	Canada, Manitoba, *Normore* 2401	DQ530206
C. verticillata 3	Canada, Manitoba, *Normore* 2370	DQ530207
C. verticillata 4	Canada, Newfoundland, *Ahti* 56951, H	AF453845
***C. verticillata* 5**	**Canada, Ontario, *A. R. Burgaz*, MACB 103716**	KC776933
***C. verticillata* 6**	**USA, New Hampshire, *A. R. Burgaz*, MACB 103718**	KC776934
***C. verticillata* 7**	**The Netherlands, Gelderland, Garderen, *T. Ahti* 72002 & *A. Aptroot*, H**	KC776935
***C. verticillata* 8**	**Spain, Burgos, Villasur de Herreros, *A. R. Burgaz*, MACB 103717**	KC776932

ExoSAP-IT (USB Corporation, OH, USA) and sequencing was carried out in Macrogen (South Korea) service (www.macrogen.com). The sequences were edited using Sequencher™ (Gene Codes, Ann Arbor, MI, USA). The alignment was done manually, using SE-AL v2.0 (Rambaut 2002) program. Maximun Parsimony (MP) analysis was done in PAUP 4.0b10 (Swofford 2003), using heuristic searches with 100 random taxon-addition replicates with TBR branch swapping and MulTrees option in effect, equally weighted characters and gaps treated as missing data. Bootstrap analysis was used with 1000 replicates and the heuristic option. The ML analyses were implemented in RAxML 7.04 (Stamatakis 2006), assuming the GTRGAMMA model. The nucleotide substitution model was choosen using MrModeltest (Nylander 2004). SYM+G was the best-fitting evolutionary model according to AIC criterion. The Bayesian analysis was carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). The posterior probabilities were approximated by sampling trees using Markov Chain Monte Carlo (MCMC). 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. Tracer v. 1.0 (http://evolve.zoo.ox.ac.uk/

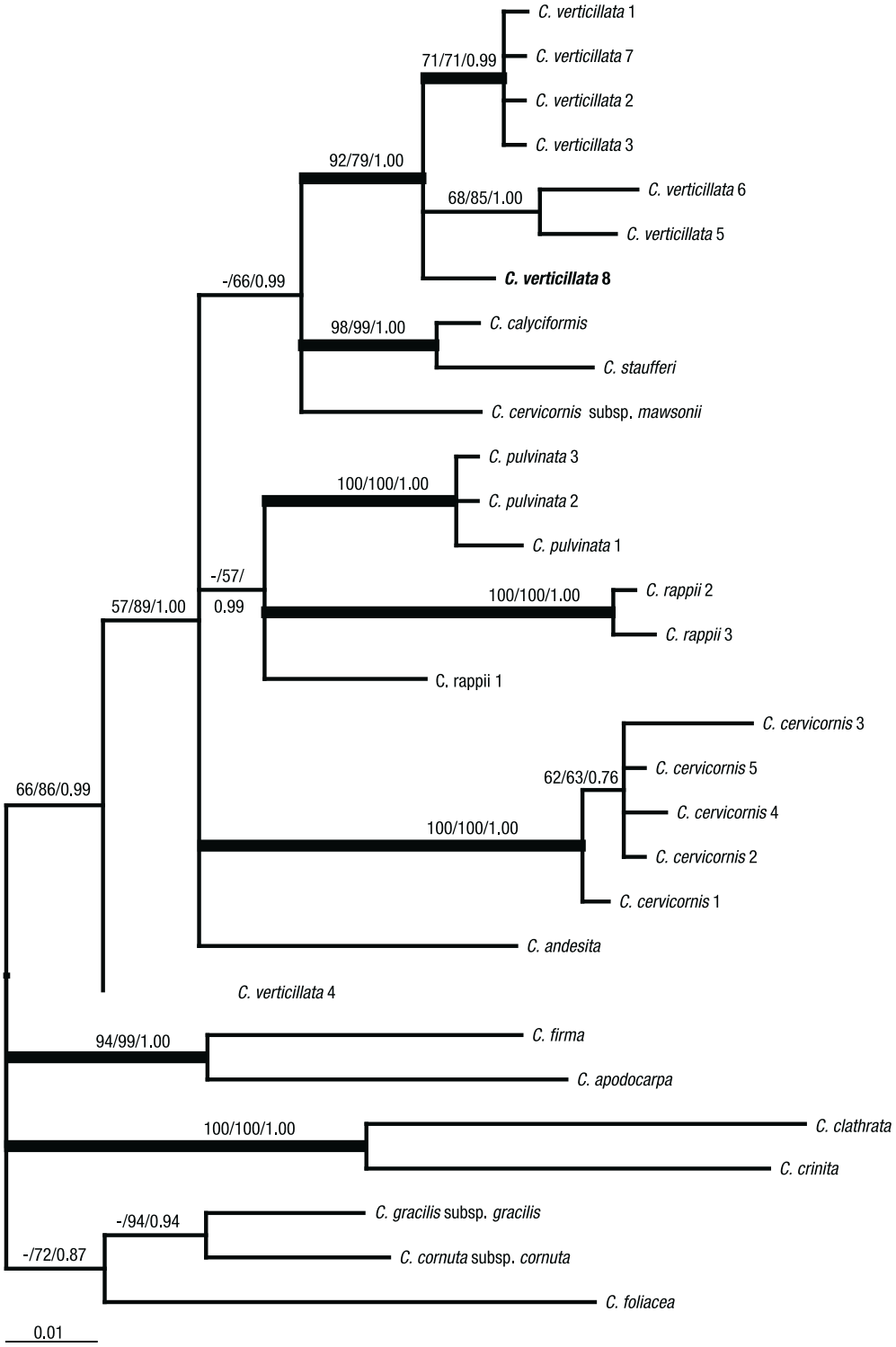

Fig. 1– Phylogeny of the *Cladonia verticillata* group. 50% Majority Rule Bayesian tree based on ITS rDNA region. Branches supported with posterior probability ≥ 0.95 and bootstrap > 70% are indicated in bold. Bootstrap for MP/Bootstrap for ML/posterior probability for Bayesian analysis at branches.

software.html?id=tracer) was used to determine when the chains reached the stationary stage and to assess the number of generations that should be discarded as burn-in. The 50% majority-rule consensus tree was calculated using MrBayes "sumt" command, deleting the first 2.000.000 generations.

The hypothesis that the sample identified as *C. verticillata* forms a monophyletic group with *C. cervicornis* samples was tested with Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa 1999) and the expected likelihood weight (ELW) tests (Strimmer & Rambaut 2002), implemented in Tree-PUZZLE 5.2 (Schmidt *et al.* 2002).

RESULTS AND DISCUSSION

In this study, four new sequences of ITS rDNA region have been generated (Table 1). The ITS alignment contained 565 unambiguous characters, 84 of which were parsimony-informative and 413 were constant. The MP analysis generated 81 equally parsimonious trees, 240 steps long, with CI = 0.7167 and RC = 0.5515. ML analysis generated a tree with likelihook value of LnL = –2126.72, while Bayesian analysis likelihook was LnL = –2158.97. The phylogenetic trees yielded in the three analyses had the same topology and only the 50% majority-rule consensus tree of the Bayesian analysis is shown (Fig. 1).

The specimen identified as *C. verticillata* from Spain gathers together with other samples of *C. verticillata* from Europe and North America, in a well supported clade. All the samples of *C. verticillata* except one from Canada joined in this clade. The hypothesis tests rejected that the putative specimen of *C. verticillata* from Spain belongs to *C. cervicornis* (SH, *P-value* = 0.01; ELW, *P-value* = 0.0013). The phylogenetic analyses give evidence that *C. verticillata* is present in the Iberian Peninsula. This species is morphologically closely related to *C. cervicornis*. In fact, it is difficult to tell them apart (Ahti 1980), particularly in Europe. Even their secondary metabolites are the same, viz. the fumarprotocetraric acid complex. Traditionally, the main characters used to differentiate them were that *C. verticillata* has slenderer podetia and more proliferatiing tiers than *C. cervicornis* (Poelt & Vûzda 1977, Wirth 1995, Clauzade & Roux 1985). However, the study by van Herk & Aptroot (2003) revealed that the podetia of both taxa are often indistinguishable in the European populations, though they can be separated by the primary thallus characters. *Cladonia verticillata* has small squamules with white lower surface, while *C. cervicornis* has big and deeply incised squamules with grey to pink lower surface. The specimen assessed here has squamules 3.5-6 mm long, with white lower surface (Fig. 2), and podetia 16-40 mm tall, with up to three successive proliferations. All these characters are consistent with those given by van Herk & Aptroot (2003).

Fig. 2– Habit of *Cladonia verticillata* (Spain, Burgos, Villasur de Herreros, *A. R. Burgaz*, MACB 103717).

In the Mediterranean region, *C. verticillata* s. str. was probably first reported in Italy (Nimis 1993), where it is more common in the North, in the Alps, but it was found in some localities in the south of Italy (Puglia, Basilicata and Sicilia) as well.

Other well supported clades appeared on the phylogenetic tree, such as those of *C. cervicornis*, *C. pulvinata* (Sandst.) Herk & Aptroot, and two specimens of *C. rappii* A. Evans (not monophyletic). These results are similar to those given by Stenroos *et al*. (2002) and Pino-Bodas *et al*. (2010). Ahti (2007) indicated that the status of several species in the *C. verticillata* group is uncertain and a deep worldwide study is necessary to clarify the limits among the species. The core species of this group, *C. verticillata,* probably hides more than one species. In fact, in our analyses one sample of this species did not form a group with the other samples. Further genetic studies will be necessary to verify the genetic homogeneity, or lack of it, in the populations of *C. verticillata*.

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