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Phylogenetic analyses support the recent placement of *Canoparmelia scrobicularis* into *Crespoa* (Parmeliaceae, lichenized Ascomycota)

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With 1 figure and 1 table

Abstract: A phylogenetic study based on the formal barcoding marker for fungi (ITS) was carried out within the *Parmotrema* clade to evaluate the recent combination of *Canoparmelia scrobicularis* into *Crespoa*, and to assess the monophyly of the genus *Crespoa*. Twenty-six sequences were newly generated, which were analyzed together with 14 retrieved from GenBank, representing a total 25 species of the genera *Austroparmelina* (5), *Canoparmelia* (4), *Crespoa* (5), and *Parmotrema* (11). The sequences were aligned and analyzed by MP, ML and BI methods. Our results have shown that *Crespoa scrobicularis* clearly constitutes a monophyletic group together with the other known *Crespoa* species, supporting its recent placement within *Crespoa* and the monophyly of the genus as currently defined.

Key words: *Austroparmelina*, *Canoparmelia*, *Crespoa*, *Parmotrema*, parmelioid lichens.

Introduction

Canoparmelia Elix & Hale, as traditionally circumscribed, includes species with relatively narrow, eciliate lobes, a pored epicortex, cell walls with isolichenan, and simple rhizines (Elix et al. 1986, Elix 1993). Phylogenetic studies based on molecular data have shown this parmelioid genus was actually highly polyphyletic, as it splits into four separate groups within the *Parmotrema* clade (Crespo et al. 2010a, Kirika et al. 2016). One of them, including four species of the *Canoparmelia crozalsiana* group, was later segregated in the genus *Crespoa* (D.Hawksw.) Lendemer & B.P.Hodk.

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(Hawksworth 2011, Lendemer & Hodkinson 2012). This genus was initially proposed by Hawksworth (2011) as the subgen. *Crespoa* D.Hawksw. within *Parmotrema* A.Massal. As its species differ markedly from *Parmotrema* in their thallus morphology, and considering the significant genetic distance between both taxa, Lendemer & Hodkinson (2012) elevated the group to the rank of genus. All *Crespoa* species have a rather distinctive thallus structure, with a strongly reticulately ridged and wrinkled upper surface, and medullary stictic and constictic acids, with the exception of *C. schelpei*, which has medullary protocetraric acid (Hawksworth 2011). More recently, Kirika et al. (2016) subsumed *Crespoa* at subgeneric level within *Parmotrema*, as initially proposed by Hawksworth (2011).

Recently, *Canoparmelia scrobicularis* (Kremp.) Elix & Hale has been transferred to *Crespoa* by Benatti & Lendemer (2014), based on its morphological and chemical characters, even though no molecular data was available to support this new combination. In this paper, we present a phylogenetic analysis within the *Parmotrema* clade based on the official fungal barcoding marker, the internal transcribed spacer region (ITS1, 5.8S, and ITS2) of the nrDNA, including species of the genera *Austroparmelina*, *Canoparmelia*, *Crespoa*, and *Parmotrema*, to evaluate the recent combination of *Canoparmelia scrobicularis* into *Crespoa* and to assess the monophyly of the genus as currently defined.

Materials and methods

Taxon sampling: Twenty-six sequences, belonging to 17 species, were newly generated and analysed together with 14 retrieved from GenBank (Benson et al. 2009, Sayers et al. 2009) representing a total of 25 species of the parmelioid genera *Austroparmelina* (5), *Canoparmelia* (4), *Crespoa* (5), and *Parmotrema* (11). The species *Usnea dasaea* Stirt. and *U. subdasaea* Truong & P. Clerc. were used as outgroup. Collection data and GenBank accession numbers of the specimens used in this study are detailed in Table 1.

Molecular methods: Portions of peripheral thalline lobes of fresh or recently collected samples were separated (20–30 mg) under dissecting microscope, ground with liquid nitrogen in porcelain mortars, and placed into 1.5 ml microcentrifuge tubes. Total DNA was isolated following a CTAB method for lichens (Crespo et al. 1999, Cubero et al. 1999, Cubero & Crespo 2002).

Polymerase chain reaction (PCR) amplifications of the entire internal transcribed spacer region – ITS1, 5.8S, and ITS2 (ITS) of the nrDNA were performed using primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). The final volume of 25 µl contained: 1 × buffer of PCR (200 mM Tris HCL-pH 8.4 and 500 mM KCl), 1.25 U/µl of Taq polymerase, 2 mM of $MgCl_2$, 0.5 µM of each primer, 0.2 mM of dNTPs, 1 µl of DNA (50 ng/µl) and sterilized deionized water. PCR reactions were set up under the following conditions: one initial denaturalization phase at 94°C for 5 min, followed by 25 cycles of 1 min at 94°C, 1 min at 62°C and 1.5 min at 72°C, ending with a final extension of 5 min at 72°C. PCR products were confirmed by electrophoresis in 1.4% agarose gels stained with ethidium bromide, and photographed under UV. Forward and reverse strands were sequenced by Macrogen © (Seoul, South Korea) and assembled. The obtained sequences were read and manually edited using Chromas version 2.0 (McCarthy 1996).

Sequence alignment: The alignment was initially performed with the multiple-sequence alignment program Muscle (Edgar 2004) through MEGA 5.0 (Tamura et al. 2011), using default parameters, and then manually checked. Ambiguously aligned regions were removed from the alignment using Gblocks version 0.91b (Castresana 2002), using options for a relaxed selection of blocks as recommended by the software for short alignments.

PHYLOGENETIC ANALYSES: The ITS alignment was analysed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. MP analysis was performed with MEGA 5.0 (Tamura et al. 2011), with heuristic search using the subtree-pruning-regrafting (SPR) branch swapping algorithm, with search level 1 (Nei & Kumar 2000), in which the initial trees were obtained by the random addition of sequences (10 replicates). Ten thousand bootstrap replicates were performed to assess confidence values for trees. To assess homoplasy levels, the consistency (CI), retention (RI), and composite indexes were calculated. A consensus tree was visualized and edited with TreeGraph 2.4.0-456 beta (Stöver & Müller 2010).

ML analysis was carried out with RaxML version 7.0.3 (Stamatakis 2006), on the T-REX (Tree and reticulogram REConstruction) web server (Boc et al. 2012; http://www.trex.uqam.ca/index.php?action=raxml&project=trex), with the rapid hill-climbing algorithm and 1000 non-parametric bootstrap inferences, with the model of evolution set to GTRGAMMA, searching for the best-scoring maximum likelihood tree in a single run. The best ML tree was visualized and edited with FigTree version 1.3.1 (Rambaut 2009).

Nucleotide substitution model for BI analysis was selected with jModeltest 2.1.4 (Darriba et al. 2012) for the entire ITS region with the corrected Akaike Information Criterion (AICc), as recommended by Posada & Buckley (2004), and the Bayesian Information Criterion (BIC). BI was performed with MrBayes version 3.2 (Ronquist et al. 2012) assuming the symmetrical model of nucleotide substitution (Zharkikh 1994) including a proportion of invariable sites and a discrete gamma distribution with six rate categories (SYM+I+G), which was the model with lowest –lnL value with AICc, and the second with BIC. Two simultaneous runs starting with an UPGMA starting tree with 4 Markov Monte Carlo chains were run for 2.000.000 generations, and saving every 100th sampled tree into a file. Convergence of chains of each replicate was checked using Tracer version 1.6.0 (Rambaut et al. 2014), to ensure an effective sampling size (ESS) over 200. The first 25% of sampled trees were discarded as burn-in. The remaining samples of each run were combined and a 50% majority consensus tree was calculated.

Results

The ITS matrix included sequences from 40 taxa and 457 unambiguously aligned nucleotide position characters in the final dataset, including 26 newly generated sequences (Table 1). A total of 56 bp were excluded from the analyses. MP analysis yielded three equally parsimonious trees with a length of 437. The value for CI was 0.511002, and the RI was 0.797776. Since the topologies of the consensus tree, the best ML tree, and the Bayesian phylogenetic tree (BI) did not show any supported conflict, only the best ML tree with all support values is shown (Fig. 1).

Four major clades were recovered, corresponding to each genus included in the analyses – *Parmotrema*, *Crespoa*, *Austroparmelina*, and *Canoparmelia*. All genera were recovered monophyletic with a strong support value, except for the clade including *Parmotrema* species. *Crespoa* was shown as phylogenetically more closely related to *Parmotrema*, although this relationship was unsupported. Two highly supported clades were distinguished within *Crespoa*, one of them with the species *C. inhaminensis*, *C. schelpei*, *C. carneopruinata*, and *C. crozalsiana* (100/100/1.0) and the other including only *C. scrobicularis* (99/99/0.99). The species of *Canoparmelia* are grouped into three well supported clades, one with *C. austroamericana*, and *C. caroliniana* (89/91/1.0), the second with *C. texana* (92/-/0.95), and the third with *C. cryptochlorophaea* (100/100/1.0).

Table 1. Collection data and GenBank accession numbers of the specimens used in this study. Newly generated sequences are indicated in bold.

Species	Locality	Collector (s) and collection number	GenBank accession no.	Reference
Austroparmelina conlabrosa	Australia, n/d	Elix 38801	GU183183	Crespo et al. (2010b)
Austroparmelina elixia	Australia, Rutherfords Creek	Elix n/d	DQ273859	Argüello et al. (2007)
Austroparmelina endoleuca	Australia, n/d	Elix 38805	GU183184	Crespo et al. (2010b)
Austroparmelina endoleuca	Australia, n/d	Elix 38802	GU183185	Crespo et al. (2010b)
Austroparmelina labrosa	South Africa, n/d	Crespo et al. n/d	GU183186	Crespo et al. (2010b)
Austroparmelina pruinata	Australia, Turanning Natural Reserve	McCrum s/n	EF042905	Crespo et al. (2007)
***Canoparmelia austroamericana* 1**	**Argentina, Chaco**	**Michlig & Niveiro 2309**	**KY929407**	**This study**
***Canoparmelia austroamericana* 2**	**Argentina, Chaco**	**Michlig & Niveiro 2301**	**KY929408**	**This study**
***Canoparmelia caroliniana* 1**	**Argentina, Misiones**	**Michlig et al. 2770**	**KY929409**	**This study**
Canoparmelia caroliniana 2	USA, North Carolina, Swain Co.	Tripp 1478	KP659627	Lendemer & Ruiz (2015)
***Canoparmelia cryptochlorophaea* 1**	**Argentina, Misiones**	**Michlig et al. 2839**	**KY929410**	**This study**
***Canoparmelia cryptochlorophaea* 2**	**Argentina, Misiones**	**Michlig et al. 2827**	**KY929411**	**This study**
Canoparmelia cryptochlorophaea 3	USA, South Carolina, Berkeley Co.	Lendemer 41059	KP659638	Lendemer & Ruiz (2015)
***Canoparmelia texana* 1**	**Argentina, Misiones**	**Michlig et al. 2713**	**KY929412**	**This study**
***Canoparmelia texana* 2**	**Argentina, Misiones**	**Michlig et al. 2747**	**KY929413**	**This study**
Canoparmelia texana 3	USA, Tennessee, Blount Co.	Lendemer 29616	KP659642	Lendemer & Ruiz (2015)
Crespoa carneopruinata	Costa Rica, Sarchi	Lücking 15171a	EF042904	Crespo et al. (2007)
Crespoa crozalsiana	**Argentina, Misiones**	**Michlig et al. 2801**	**KY929414**	**This study**
Crespoa inhaminensis	n/d	n/d	GU994544	Crespo et al. (2010a)
Crespoa schelpei	n/d	n/d	GU994546	Crespo et al. (2010a)
***Crespoa scrobicularis* 1**	**Argentina, Misiones**	**Michlig et al. 2722**	**KY929415**	**This study**
***Crespoa scrobicularis* 2**	**Argentina, Misiones**	**Michlig et al. 2783**	**KY929416**	**This study**

Parmotrema argentinum	**Argentina, Corrientes**	**Michlig & Niveiro 2590**	**KY929417**	**This study**
Parmotrema austrosinense	**Argentina, Corrientes**	**Michlig & Niveiro 2671**	**KY929418**	**This study**
***Parmotrema cetratum* 1**	**Argentina, Corrientes**	**Michlig & Niveiro 2586**	**KY929419**	**This study**
***Parmotrema cetratum* 2**	**Argentina, Corrientes**	**Michlig et al. 2682**	**KY929420**	**This study**
***Parmotrema flavomedullosum* 1**	**Argentina, Misiones**	**Michlig et al. 2732**	**KY929421**	**This study**
***Parmotrema flavomedullosum* 2**	**Argentina, Misiones**	**Michlig et al. 2816**	**KY929422**	**This study**
***Parmotrema masonii* 1**	**Argentina, Corrientes**	**Michlig & Niveiro 2573**	**KY929423**	**This study**
***Parmotrema masonii* 2**	**Argentina, Corrientes**	**Michlig & Niveiro 2679**	**KY929424**	**This study**
Parmotrema melanochaetum	**Argentina, Misiones**	**Michlig et al. 2695**	**KY929425**	**This study**
Parmotrema muelleri	**Argentina, Misiones**	**Michlig et al. 2512**	**KY929426**	**This study**
Parmotrema praesorediosum	**Argentina, Corrientes**	**Michlig & Niveiro 2679**	**KY929427**	**This study**
Parmotrema recipiendum	**Argentina, Corrientes**	**Michlig & Niveiro 2674**	**KY929428**	**This study**
***Parmotrema reticulatum* 1**	**Argentina, Chaco**	**Michlig et al. 2632**	**KY929430**	**This study**
***Parmotrema reticulatum* 2**	**Argentina, Chaco**	**Michlig et al. 2609**	**KY929429**	**This study**
***Parmotrema tinctorum* 1**	**Argentina, Chaco**	**Michlig et al. 2613**	**KY929431**	**This study**
***Parmotrema tinctorum* 2**	**Argentina, Misiones**	**Michlig et al. 2746**	**KY929432**	**This study**
Usnea dasaea	Peru, n/d	n/d	JQ837305	Troung et al. (2013)
Usnea subdasaea	Galapagos, n/d	n/d	JQ837329	Troung et al. (2013)

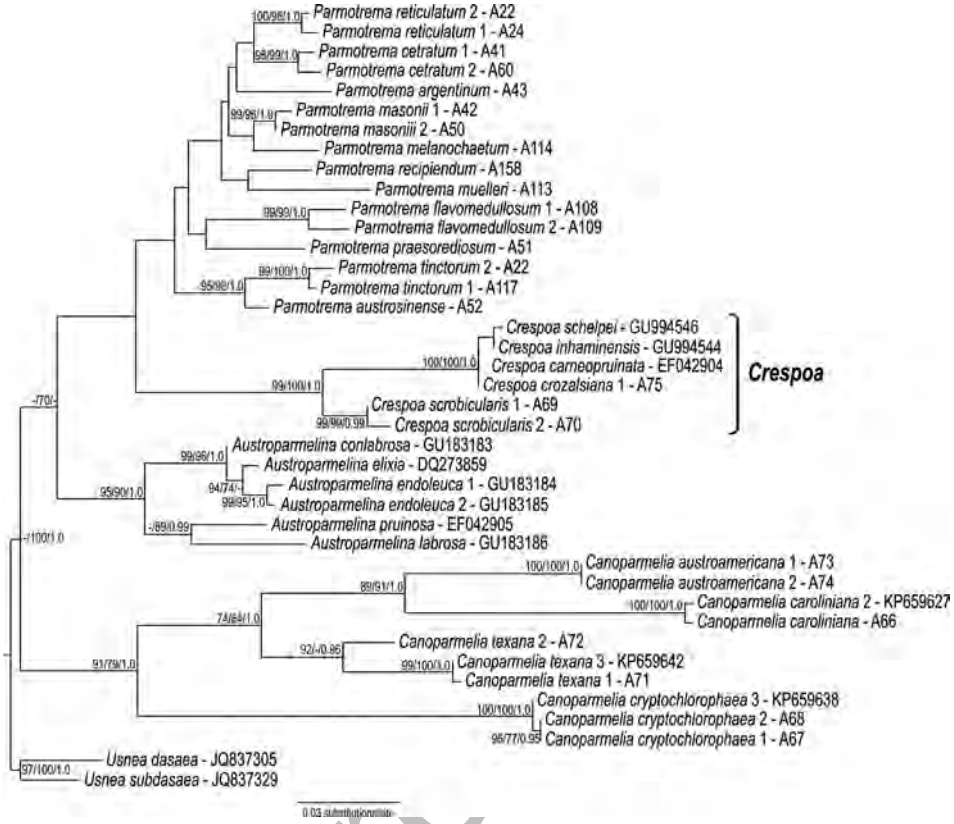

Fig. 1. Phylogenetic placement of *C. scrobicularis* within the *Parmotrema* clade inferred from the ITS region of the nrDNA. The tree is rooted using *Usnea* as outgroup. The numbers on internal branches indicate bootstrap (≥70%) and posterior probabilities (≥0.95) support values in the following format: MP-BP/ML-BP/B-PP.

Discussion

The phylogenetic analyses based on molecular data from the ITS support both the recent placement of *Canoparmelia scrobicularis* into *Crespoa*, and the monophyly of the genus *Crespoa* as currently circumscribed. The monophyly of *Austroparmelina*, *Canoparmelia* s. str., and *Parmotrema* in the ITS topology is consistent with previous studies (Blanco et al. 2005, 2006, Crespo et al. 2010a–b, Divakar et al. 2013, 2015, Kirika et al. 2016).

Crespoa was created to accommodate species of the *Canoparmelia crozalsiana* group (Hawksworth 2011, Lendemer & Hodkinson 2012), as molecular data have shown they constitute a well-supported monophyletic group separate from other species of *Canoparmelia* (Crespo et al. 2010a–b). Apart from molecular data, there are few

characters in parmelioid lichens that could be consider truly synapomorphic (Crespo et al. 2011). Although more recently Kirika et al. (2016) and Divakar et al. (2017) subsumed this group as a subgenus within *Parmotrema*, we agree with Lendemer & Hodkinson (2012) and consider this as a separate genus from *Parmotrema*.

Crespoa scrobicularis (Kremp.) Benatti & Lendemer is characterized by its distinctly scrobiculate thallus, and medullary stictic and constictics acid, which clearly place it within *Crespoa*, and the absence of vegetative propagules (Benatti & Lendemer 2014). As thalline anatomical studies in Parmeliaceae have shown that the cortical cell arrangement is quite distinct among parmelioid genera (Barbosa & Marcelli 2010), comparative anatomical studies on selected species of *Canoparmelia* and *Crespoa* were recently made by Zanetti et al. (2015) to find additional characters to support the distinction between both genera. These studies have revealed significant differences in cortical and algal layers between *Canoparmelia* and *Crespoa*. In addition, they found that the structure of upper and lower cortices of *C. scrobicularis* is highly similar to that in *Crespoa* species, supporting the new combination of the species. Our phylogenetic analyses based on molecular data from the ITS, recovered *Crespoa* as a strongly-supported monophyletic group with *C. scrobicularis* nested within, clearly supporting its recent placement within that genus.

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