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Molecular studies reveal a new species of *Bryoria* in Chile

Carlos G. BOLUDA, Pradeep K. DIVAKAR, David L. HAWKSWORTH, Johana VILLAGRA and Víctor J. RICO

Abstract: *Bryoria araucana* sp. nov. is described from Chile on the basis of morphological, chemical and molecular data. It has a grey to dark greyish brown pendent thallus with the base usually black, branching angles mainly obtuse, terminal branches with few lateral branchlets acutely inserted, fumarprotocetraric acid, and often protocetraric and confumarprotocetraric acids. It is morphologically similar to the Northern Hemisphere *B. trichodes*, but lacks soralia and has inconspicuous concolorous or slightly darker pseudocyphellae. *Bryoria glabra* is also reported for the first time from the Southern Hemisphere. New phylogenetic data based on ITS, mtSSU and *MCM7* analyses suggest that *Bryoria* sect. *Bryoria* is polyphyletic and needs revision.

Key words: Conguillío National Park, lichen, *Parmeliaceae*, phylogeny, taxonomy

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Introduction

Bryoria Brodo & D. Hawksw. is the largest genus in the alectorioid clade of the family *Parmeliaceae* (Divakar *et al.* 2015), which inhabits temperate to alpine regions worldwide. It has been comprehensively studied in North America and northern Europe (Brodo & Hawksworth 1977; Myllys *et al.* 2011*a*); however, morphological simplicity and chemical variability make its taxonomy difficult, and recent molecular data have resulted in several changes (Velmala *et al.* 2009, 2014; Myllys *et al.* 2014). Recent studies have discovered additional new species in *Bryoria* from east-central Asia (Myllys *et al.* 2011*b*; Jørgensen *et al.* 2012), southern South America and the Antarctic (Olech & Bystrek 2004; Fryday & Øvstedal 2012). Temperate South America is a region with an unexpectedly low reported *Bryoria* diversity, suggesting that additional species may be awaiting discovery in the region. In the course of lichen research by one of us (JV) in the Conguillío National Park in Chile, samples of *Bryoria* growing on *Araucaria araucana* trees were collected. Molecular, morphological and chemical analyses of the specimens revealed the presence of two species: *Bryoria glabra* (Motyka) Brodo & D. Hawksw. and another that did not group with any known species in our ongoing morphological, chemical, and phylogenetic analyses. This second species is therefore described here as new.

C. G. Boluda, P. K. Divakar, D. L. Hawksworth, J. Villagra and V. J. Rico: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain. Email: carlos.g.boluda@gmail.com

D. L. Hawksworth is also at: Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; and Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Surrey TW9 3DS, UK.

Materials and Methods

The specimens collected were analyzed morphologically using standard methods (Smith *et al.* 2009) using a Nikon SMZ-1000 stereomicroscope and a Nikon Eclipse-80i microscope, and photographs were taken with a Nikon 105 mm f/2.8D AF Micro-Nikkor lens coupled to a Nikon D90 camera. Spot tests with C, K, KC, and Pd were carried out as explained in Brodo & Hawksworth (1977). For thin-layer chromatography (TLC), solvents A, B and C were used to run concentrated lichen extracts in 50 °C acetone spotted onto silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt), according to standard methods (Orange *et al.* 2010).

TABLE 1. *Specimen information and GenBank accession numbers for the taxa used in this study. Newly obtained sequences are in bold.*

Taxon name	Specimen number	Locality	Chemistry	GenBank accession numbers		
				ITS	mtSSU	MCM7
Bryoria americana	1	Finland, Kainuu	Fum	HQ402677	HQ402636	KJ948017
B. americana	2	Canada, B. C.	Fum, Cfum, Pro	HQ402678	HQ402637	KJ948016
B. araucana	1	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975402**	**KP939085**	**KP975410**
B. araucana (holotype)	2	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975405**	**KP939082**	**KP975413**
B. araucana	3	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975404**	**KP939083**	**KP975412**
B. araucana	4	Chile, La Araucaría IX R.	Fum, Cfum,	**KP975403**	**KP939084**	**KP975411**
B. araucana	5	Chile, La Araucaría IX R.	Fum, Cfum	**KP975407**	**KP939081**	**KP975414**
B. araucana	6	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975406**	**KP939080**	**KP975415**
B. bicolor	1	Finland, Etelä-Häme	-	HQ402691	HQ402645	KJ948018
B. bicolor	2	Finland, Koillismaa	Bar, Pso, Fum	HQ402689	HQ402644	KJ948019
B. confusa		China, Yunnan	-	HQ402686	-	KJ948024
B. divergescens		China, Yunnan	Fum, Pro, Cfum, Qua	HQ402705	HQ402654	KJ948025
B. fastigiata		China, Yunnan	Fum, Pro, Cfum	HQ402706	HQ402655	-
B. fremontii	1	Canada, B. C.	No subs	FJ668503	FJ668436	KJ948028
B. fremontii	2	Finland, Koillismaa	Vul in soralia.	FJ668498	FJ668432	KJ948029
B. furcellata	1	Finland, Etelä-Savo	Fum, Pro, Cfum	HQ402722	HQ402667	KJ948031
B. furcellata	2	Canada, Manitoba	Fum, Pro, Cfum	HQ402721	HQ402666	KJ948030
B. fuscescens	1	Finland, Koillismaa	Fum, Pro, Cfum	GQ996291	GQ996332	KJ948035
B. fuscescens	2	Finland, Åland	Fum, Pro, Cfum	GQ996290	GQ996322	KJ948032
B. glabra	1	Canada, B. C.	Fum in soralia.	HQ402728	HQ402673	KJ948037
B. glabra	2	Finland, Koillismaa	Fum in soralia.	FJ668494	FJ668428	KJ948036
B. glabra	7	Chile, La Araucaría IX R.	Fum	**KP975408**	**KP939086**	**KP975417**
B. hengduanensis		China, Yunnan	Usn, Fum, Pro, Cfum	HQ402704	HQ402653	KJ948038
B. lactinea		China, Yunnan	Fum, Pro, Cfum	HQ402699	-	KJ948050
B. nadvornikiana	1	Finland, Kainuu	Bar, Ale, Fum, Cfum, Atr	HQ402718	HQ402663	KJ948053
B. nadvornikiana	2	Iran, East-Azarbaijan	Bar	HQ402720	HQ402665	KJ948052
B. nitidula	1	Sweden, Ångermanland	-	HQ402713	HQ402658	KJ948054
B. nitidula	2	Greenland	Fum, Pro, Cfum	HQ402711	HQ402656	KJ948055
B. poeltii		China, Yunnan	Fum	HQ402701	HQ402650	KJ948057
B. simplicior	1	Finland, Koillismaa	Fatty acids	HQ402714	HQ402659	KJ948063
B. simplicior	2	Russia, Sakha Republic	No subs	HQ402716	HQ402661	KJ948062
B. simplicior	3	Norway, Troms	No subs	**KP975409**	-	**KP975416**
B. smithii	1	Finland, Varsinais-Suomi	No subs	HQ402684	HQ402642	KJ948065
B. smithii	2	India, Uttaranchal	-	HQ402685	HQ402643	KJ948064
B. tenuis	1	Finland, Kainuu	Fum	HQ402694	HQ402648	KJ948074
B. tenuis	2	Sweden, Dalarna	Fum	HQ402695	HQ402649	KJ948073
B. trichodes	1	Canada, Newfoundland	Fum, Cfum, Pro, Atr	HQ402710	-	KJ948075
B. trichodes	2	Russia, Kamchatka	-	KJ947952	-	KJ948076
Pseudephebe pubescens		USA, Alaska	No subs	HQ402676	HQ402635	KJ948091

Chemistry as follows: Ale = alectorialic acid, Atr = atranorin, Bar = barbatolic acid, Cfum = confumarprotocetraric acid, Fum = fumarprotocetraric acid, Gyr = gyrophoric acid, Nor = norstictic acid, Pro = protocetraric acid, Pso = psoromic acid, Qua = quaesitic acid, Usn = usnic acid, Vul = vulpinic acid, No subs = no lichen substances detected.

For the best resolution in solvent C, the spotted plate was left to stand for 10 min before running in an acetic acid atmosphere.

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instructions (Crespo *et al.* 2001; Divakar *et al.* 2012). Three loci were amplified: 1) nrITS, with ITS1FKYO2 (5'-TAG AGG AAG TAA AAG TCG TAA-3') and ITS4KYO2 (5'-RBT TTC TTT TCC TCC GCT-3'; Toju *et al.* 2012) primers; 2) mSSU rDNA, with mtSSU1 (5'-AGC AGT GAG GAA TAT TGG TC-3') and mtSSU3R (5'-ATG TGG CAC GTC TAT AGC CC-3'; Zoller *et al.* 1999) primers; and 3) the low copy protein coding gene *MCM7*, with MCM71348rev (5'-GAY TTD GCI ACI CCI GGR TCW CCC AT-3') and MCM7-709f (5'-ACI MGI GTI TCV GAY GTH AAR CC-3'; Schmitt *et al.* 2009) primers. For amplification, a reaction mixture of

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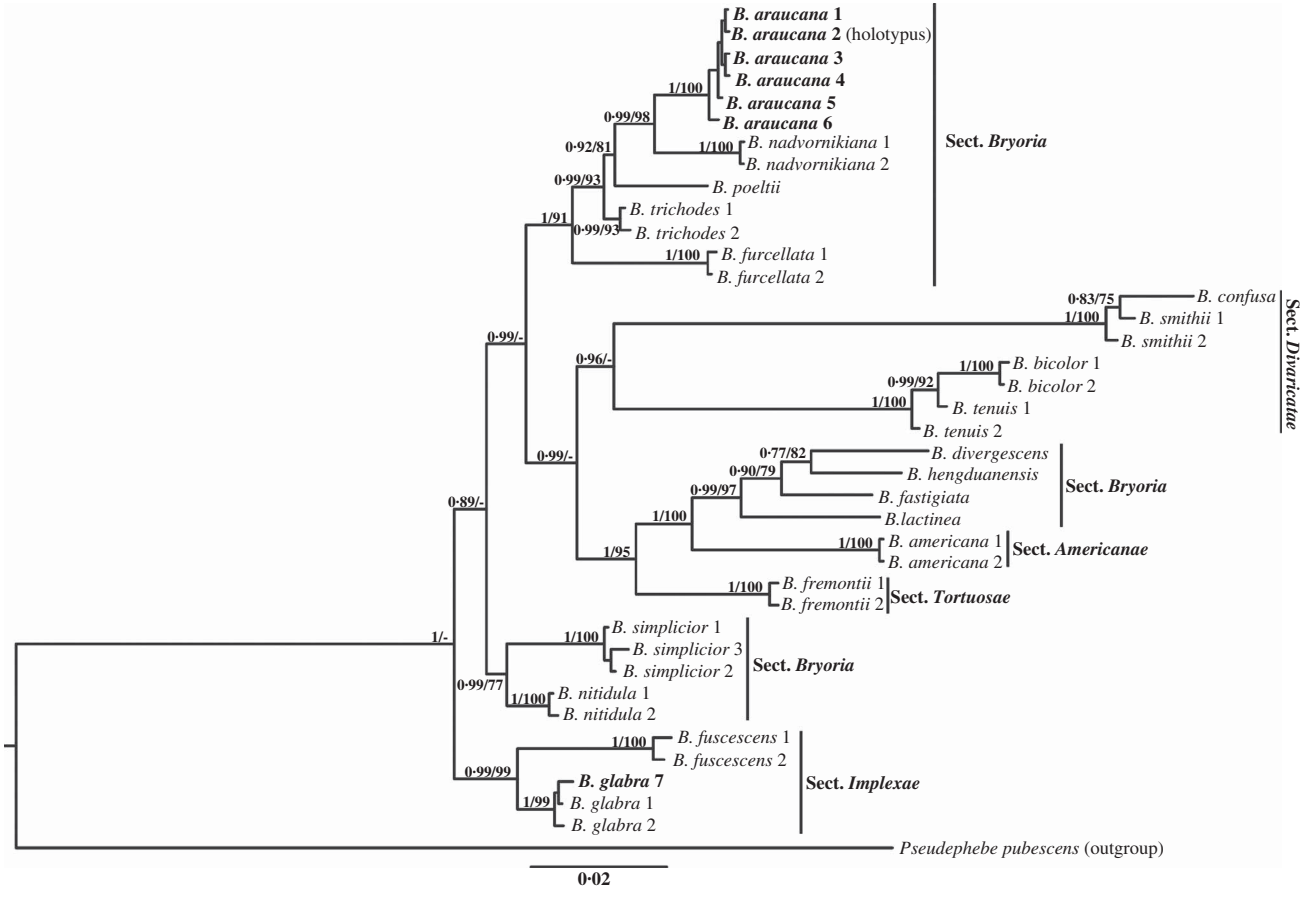

FIG. 1. Phylogenetic relationships of *Bryoria* species used in this study, 38 samples representing 19 species, based on ITS, mtSSU, and *MCM7* markers analyzed in a concatenated data matrix. Tree topology depicts the results of the Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Posterior probabilities and bootstrap values, when coincident with the Bayesian tree, are given on the node branches. Sections according to Myllys *et al.* (2011*b*). *B. glabra* (bold) = Chilean specimen. *B. araucana* (bold) = new species. *Peudephebe pubescens* used as outgroup.

25 µl was used containing 18 µl sterile water, 2·5 µl ×10 buffer with 2 mM $MgCl_2$, 0·5 µl dNTPs (10 mM of each base), 1·25 µl of each primer at 10 µM, 0·625 µl of DNA polymerase (1U $µl^{-1}$), and 1–2 µl DNA template. In failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2·5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers: 10 mM Tris-HCl ph 9·0, 50 mM KCl, 1·5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK) adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM, and 1·5 µl of DNA template.

Amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou) using the following parameters for ITS rDNA and mtSSU rDNA: initial denaturation 5 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1·5 min at 72 °C, and a final extension of 10 min at 72 °C. For *MCM7* we used a touchdown cycling process: initial denaturation 10 min at 94 ºC, then followed by 4 cycles of 45 s at 94 ºC, 50 s at 56 ºC, 1 min at 72 ºC; 4 cycles of 45 s at 94 ºC, 50 s at 54 ºC, 1 min at 72 ºC; 36 cycles of 45 s at 94 ºC, 50 s at 52 ºC, 1 min at 72 ºC and a final extension of 8 min at 72 ºC. PCR products were cleaned using illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer´s instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid).

DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison) and MEGA5 (Tamura *et al.* 2011). Some GenBank sequences from Myllys *et al.* (2011*b*; Table 1) were added to the file and aligned using MAFFT version 7 (Katoh & Standley 2013), with the G-INS-I alignment algorithm, a scoring matrix of 1 PAM/k = 2, and 0·1 as offset value. Gblocks version 0.91b (Castresana 2000) was used to delete non-conserved GAPs, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The alignments of each region and the concatenated one were analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches, with *Pseudephebe pubescens* as outgroup to root the tree (Divakar *et al.* 2015). For maximum likelihood (ML) tree reconstruction, the program RAxML v7.2.8 (Stamatakis 2006) implemented on the Cipres Science Gateway (Miller *et al.* 2010) was used. We selected the GTRGAMMA model, which includes a parameter (Γ) for rate heterogeneity among sites and chose not to include a parameter to estimate the proportion of invariable sites (Stamatakis 2006; Stamatakis *et al.* 2008). Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For Bayesian reconstruction, MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003) was used, assuming the general time reversible model (Rodriguez *et al.* 1990) and a discrete gamma distribution with six rate categories (GTR + G). The nucleotide-substitution model and parameters were selected using the Akaike Information Criterion as implemented in jModelTest (Posada 2008). A run with four million generations, starting with a random tree and employing eight simultaneous chains, was executed. Every 400th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using TRACER v1.5 (http://beast.bio.ed.ac.uk/Tracer) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium value (Huelsenbeck & Ronquist 2001), discarding the trees obtained before stationarity was reached. Posterior probabilities (PPs) were obtained from the 50% majority-rule consensus of sampled trees after excluding the initial 25% as burn-in. The phylogenetic trees were drawn using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Results and Discussion

The tree obtained from the concatenated ITS, mtSSU and *MCM7* dataset (Fig. 1) is mainly based on sequences published by Myllys *et al.* (2011*b*), who performed a parsimony analysis obtaining five infrageneric sections. Here we subjected those sequences to maximum likelihood and Bayesian analyses, resulting in a different and better supported tree topology. This discrepancy may not be due to the phylogenetic reconstruction method, but to different sampling and the loci used. Sections *Americanae*, *Divaricatae*, *Implexae* and *Tortuosae* are resolved as monophyletic, but in different tree locations than those of Myllys *et al.* (2011*b*). Section *Implexae* appears as basal rather than derived, and sections *Americanae* and *Tortuosae* are no longer basal. Section *Divaricatae* seems justified, but section *Bryoria* was recovered as polyphyletic and split into three monophyletic groups. In view of this, sections *Americanae*, *Tortuosae* (with one sequenced species each) and *Bryoria* (polyphyletic) will evidently need revision after more detailed analysis has been undertaken. At the species level, *Bryoria tenuis* appears paraphyletic with *B. bicolor*, and *B. smithii* paraphyletic with *B. confusa*, but due to the small number of specimens included in this study it would be premature to propose any change here.

Analyses of the Chilean specimens (Table 1; Fig. 1) revealed the presence of *Bryoria glabra*, the first record from the Southern Hemisphere, and a set of different specimens that did not group with any known species. These were phylogenetically close to the Northern Hemisphere *Bryoria nadvornikiana* (Gyeln.) Brodo & D. Hawksw. but they contained fumarprotocetraric rather than barbatolic acid as the

FIG. 2. *Bryoria araucana*, holotype. A, habitat; B, habit; C, detail of branching pattern; D & E, detail of pseudocyphellae. Scales: B = 1 cm; C = 1 mm; D = 0·15 mm; E = 0·25 mm.

main substance. The Chilean specimens were quite different from *B. nadvornikiana* morphologically in that they lacked extensive blackened bases and short, perpendicular, lateral, spinule-like branches. Additionally, they were morphologically and chemically similar to the Northern Hemisphere *Bryoria trichodes* (Michx.) Brodo & D. Hawksw. but lacked soralia, although soralia are not found in every specimen of *B. trichodes*.

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The material is therefore described here as a new species.

The Species

Bryoria araucana Boluda, D. Hawksw. & V. J. Rico sp. nov.

MycoBank No.: MB811960

Resembles the Northern Hemisphere circumboreal *Bryoria trichodes*, but is distinct molecularly, without soralia, and with less conspicuous pseudocyphellae.

Type: Chile, IX Región de La Araucanía, Provincia de Cautín, Comuna de Melipeuco, Conguillío National Park, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71° 37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 31 August 2014, *J. Villagra* 2 (MAF-Lich. 19718—holotype). GenBank accession numbers: KP975405 (ITS), KP939082 (mtSSU), and KP975413 (MCM7).

(Fig. 2)

Thallus pendent to subpendent, 6–12 cm long; isotomic to anisotomic dichotomously branched, angles between dichotomies mainly obtuse, rarely acute; branches terete, even, main branches at base 0·2–0·4 mm diam., tips to 0·1 mm diam.; terminal portions with few lateral branchlets acutely inserted. *Surface* dark grey to dark greyish brown, shiny, base ordinarily black; cortex prosoplectenchymatous. *Soralia* and *isidia* lacking. *Pseudocyphellae* inconspicuous, depressed, fusiform, concolorous to slightly darker than the thallus, sometimes faintly pruinose, straight or twisted, up to 1·5 mm long. *Photobiont* trebouxioid.

Apothecia and *conidiomata* unknown.

Chemistry. Inner cortex and medulla C−, K−, KC−, PD+ yellow turning red, sometimes faint. TLC: fumarprotocetraric acid as the main substance, with protocetraric and confumarprotocetraric acids in trace amounts.

Etymology. Named after the IX Región de la Araucanía in Chile, which is the only known area for the species, as was the case in the name *Araucaria araucana*.

Distribution and ecology. Known only from the type locality and immediate surroundings in the Parque Nacional Conguillío, IX Región de La Araucanía (Chile), occurring on trunks of *Araucaria araucana* in mature open forests (Fig. 2A). Those forests are characteristic of the upper supratemperate bioclimatic belt with the ultraperhumid rainfall regime of the South American Temperate Region (Amigo & Ramírez 1998). Furthermore, the mean annual precipitation in the area, which falls mainly as snow, is *c.* 2000 mmy^{-1}, and the mean annual temperature is 8·6 °C, with dry and hot short summers (Di Castri & Hajek 1976). *Bryoria araucana* is more frequent on the north-facing trunks exposed to humid winds, growing with *Coelopogon epiphorellus*, *Protousnea dusenii*, *P. magellanica*, *P. poeppigii*, and *Platismatia glauca*. On the south-facing sides of the trunks it is less frequent, growing with *Nephroma antarcticum*, *Pseudocyphellaria coriifolia*, *P. flavicans*, and *P. granulata*. It may be anticipated that *B. araucana* will be found to have a wider distribution in the temperate forests of the Southern Hemisphere that are almost unexplored for alectorioid lichens.

Conservation status. Although the new species seems not to be frequent, it occurs in a protected area (Parque Nacional Conguillío, Chile). No special actions to conserve the species are currently required.

Additional specimens examined. Bryoria araucana **Chile:** *IX Región de La Araucanía, Provincia de Cautín*: Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'14·83"S, 71°37'01·06"W, 1211 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2013, *J. Villagra* 5 & 6 (MAF-Lich. 19723, 19724); *ibid.*, 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 1, 3 & 4 (MAF-Lich. 19719, 19720, 19721).

Bryoria glabra **Chile:** *IX Región de La Araucanía, Provincia de Cautín*: Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 7 (MAF-Lich. 19722).

Bryoria araucana and the Northern Hemisphere species *B. trichodes* form divergent independent clades which are well supported

TABLE 2. *Comparison of the chemistry, main morphological characters and distribution of five phylogenetically related species of* Bryoria *including* B. araucana *based on our observations and bibliographic references (Brodo & Hawksworth 1977; Bystrek 1969; Myllys* et al. *2011a; Wang & Chen 1994).*

	B. araucana	*B. nadvornikiana*	*B. poeltii*	*B. trichodes*	*B. furcellata*
Main chemistry	Fum	Bar, Ale, ± Atr, Fum in soralia	Fum	Fum, Chlor	Fum
Thallus	Pendent	Caespitose (base) to pendent	Caespitose (base) to pendent	Pendent	Caespitose
Pseudocyphellae	Inconspicuous, dark grey-brown	Inconspicuous, white	Conspicuous, dark brown-black	Conspicuous, white to brownish	Absent
Soralia	Absent	Tuberculate to fissural, white	Tuberculate to fissural, dark, spinulose	Rare, fissural, white	Fissural, white, spinulose (tufts)
Spinules or spinulose branches	On terminal portions, sparse	Lateral, sparse to frequent	Sparse, also on soralia	Lateral, sparse	Sparse to frequent
Colour	Dark grey-brown, base usually darker	Pale to dark brown-violet, base generally black	Dark brown to black	Pale to dark brown	Pale to dark brown, base often darker
Distribution	Chile, South America	Europe, Africa, Asia, Hawaii, North America	Himalayas	Asia, North America	Europe, Macaronesia, Asia, Oceania, North and Central America

Ale = Alectorialic acid, Atr = Atranorin, Bar = Barbatolic acid, Chlor = Chloratranorin, Fum = Fumarprotocetraric acid.

(Fig. 1). The two species are very similar in morphology and chemistry (cf. Brodo & Hawksworth 1977), but *B. araucana* develops less conspicuous pseudocyphellae, lacks atranorin, and apothecia and soralia are unknown. *Bryoria nadvornikiana*, *B. poeltii* (Bystrek) Brodo & D. Hawksw. and *B. furcellata* (Fr.) Brodo & D. Hawksw. are phylogenetically related species, but they can be distinguished by the characters shown in Table 2. Based on the molecular results, restricted distribution, development of inconspicuous pseudocyphellae, and absence of soralia, the new species is well supported.

The *Bryoria glabra* specimen appears to be the first record from the Southern Hemisphere (Fig. 1; Table 1). It is characterized by repeatedly oval, whitish soralia and regular branching, with rounded and obtuse angles between the branches, and contains fumarprotocetraric acid (Brodo & Hawksworth 1977; Myllys *et al.* 2011*b*).

Five additional *Bryoria* species are reported in the literature from southern South America (Argentina and Chile): *Bryoria bicolor* (Ehrh.) Brodo & D. Hawksw. (Calvelo & Liberatore 2002), *B. chalybeiformis* (L.) Brodo & D. Hawksw., *B. mariensis* Øvstedal *et al.* (Fryday & Øvstedal 2012), *B. implexa* (Hoffm.) Brodo & D. Hawksw., and *B. austromontana* P. M. Jørg. & D. J. Galloway (Øvstedal & Lewis Smith 2004). *Bryoria araucana* is distinguished from all these species by the corticolous pendent habit, lack of soralia, branches 0·2–0·4 mm diam., and the sometimes dark basal parts. However, we consider some of these literature records dubious, and in need of verification through molecular analyses.

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