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A new riparian species of *Ramalina* (*Ramalinaceae*) from Brazil, with a key to neotropical saxicolous species

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Abstract: The new species *Ramalina fleigiae* from Brazil is described growing on rocks in riverbeds in high altitude grasslands of southern Brazil. It grows in areas with constant water flow, sometimes almost immersed, and always in exposed habitats. Through an integrative approach, the detailed description of *R. fleigiae* includes morphological, anatomical, ecological, chemical and molecular data. Ribosomal DNA-based phylogenies suggest that *R. fleigiae* is more closely related to a species that shares its habitat preference (*R. laevigata*) than to the morphologically and chemically similar *R. exiguella* and *R. gracilis*. *Ramalina fleigiae* and *R. laevigata* can be distinguished by thallus morphology (irregularly flat branches in *R. fleigiae* vs. flat to canaliculate in *R. laevigata*) and pattern of chondroid tissue, as genetic distances between them are compatible with the interspecific range. It is possible that many species of *Ramalina* still remain hidden within the morphological or chemical variation of currently accepted species. Combining ecological, anatomical and molecular data will improve our future understanding of this genus.

Key words: Ascomycota, fruticose thallus, lichenized fungi, rock, taxonomy

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Introduction

Ramalina Ach. is a cosmopolitan genus with *c*. 150–200 species (Kashiwadani & Nash 2004; Fletcher *et al*. 2009; Sérusiaux *et al*. 2010) which are known to be variable both morphologically and chemically (e.g. Culberson 1967; Sheard & James 1976; Rundel 1978; Krog & Østhagen 1980; Stevens 1987). However, recent studies have shown that different morphological and/or chemical characteristics, once thought to be caused by environmental factors, can also indicate distinct species (Kashiwadani *et al*. 2007; Ohmura *et al*. 2008; Hayward *et al*. 2014; Pérez-Vargas & Pérez-Ortega 2014).

Anatomical analyses can contribute to an improved understanding of *Ramalina* taxonomy; however, anatomical traits were not always mentioned in descriptions and consequently this information is frequently missing in the literature for several species. However, Kashiwadani and collaborators have ensured that the taxonomic significance of the cortex and chondroid tissue in the identification of *Ramalina* species has now been recognized (e.g. Kashiwadani 1986, 1987, 1992; Kashiwadani & Kalb 1993; Kashiwadani & Nash 2004; Kashiwadani *et al*. 2006, 2007).

In Brazil, *Ramalina* species occur in almost all environments, but especially in areas with high humidity, such as near the mountains and sea. Southern and south-eastern regions, characterized by a transitional subtropical/tropical climate, present the highest number of species and biomass (e.g. Kashiwadani & Kalb 1993; Spielmann 2006). Most species have a corticolous habit, occurring in forest borders and open areas. A few saxicolous

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species are found mainly along the coast on rocky shores, and on rocks that accompany the rivers in the plateau (Gumboski 2016). Kashiwadani & Kalb (1993) provided a good overview of the Brazilian species but, nonetheless, many regions and environments still lack adequate information about *Ramalina* species.

The aims of this work are to present a description of a new *Ramalina* species using morphological, anatomical, chemical and molecular data, and to provide a key to 32 neotropical saxicolous species based on a literature review and examination of herbarium material.

Material and Methods

Study region

The study region is located in the southern Brazilian highlands (>800 m a.s.l.), characterized by mosaics of grasslands (called 'Campos de Cima da Serra') and an Atlantic mixed forest (known as the Araucaria forest owing to the dominance of *Araucaria angustifolia*) (e.g. Rambo 1953; Boldrini 1997; Behling *et al.* 2004; IBGE 2004). Collection points were situated in waterfall areas: 'Cachoeirão dos Rodrigues' (Silveira River; 28°35'59·85"S, 49°59'19·89"W, *c.* 1150 m, municipality of São José dos Ausentes) and 'Cachoeira dos Venâncios' (Camisas River; 29°01'03·72"S, 50°15'31·55"W, *c.* 850 m, municipality of Cambará do Sul) (Fig. 1). According to Köppen (1936), the climate is categorized as Cfa (humid subtropical). The annual average temperature is 14·8 °C and the relative humidity *c.* 80% (Moreno 1961). In winter, the temperature falls to −8 °C, with frequent frosts and occasional snow formation. The annual rainfall is spread throughout the year and varies from 1500 to 2000 mm (Boldrini 1997).

Molecular data

Small samples (0·01–0·05 g) of *Ramalina* thalli were soaked in acetone overnight. The acetone was then removed with a pipette and the samples were reduced to powder with a ball mill and subsequently used for DNA extraction following the protocol described in Cubero *et al.* (1999). The markers selected were the ITS rDNA and IGS. PCR amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems) using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for the ITS rDNA region and IGSf and IGSr (Wirtz *et al.* 2008) for IGS. Each 25 µl reaction contained: 1 × Taq buffer (Promega), 0·2 mM of dNTP set, 0·2 µM of each primer, 25 mM of $MgCl_2$, 1U of DNA Polymerase (Promega) and *c.* 20–50 ng of DNA. The PCR conditions for ITS amplification were: 2 min at 95 °C for initial denaturation, followed by 30 cycles of 30 s at 95 °C, 1 min at 50 °C, 1 min at 72 °C, with a final elongation of 10 min at 72 °C. For IGS amplification the conditions used were: 2 min at 95 °C for initial

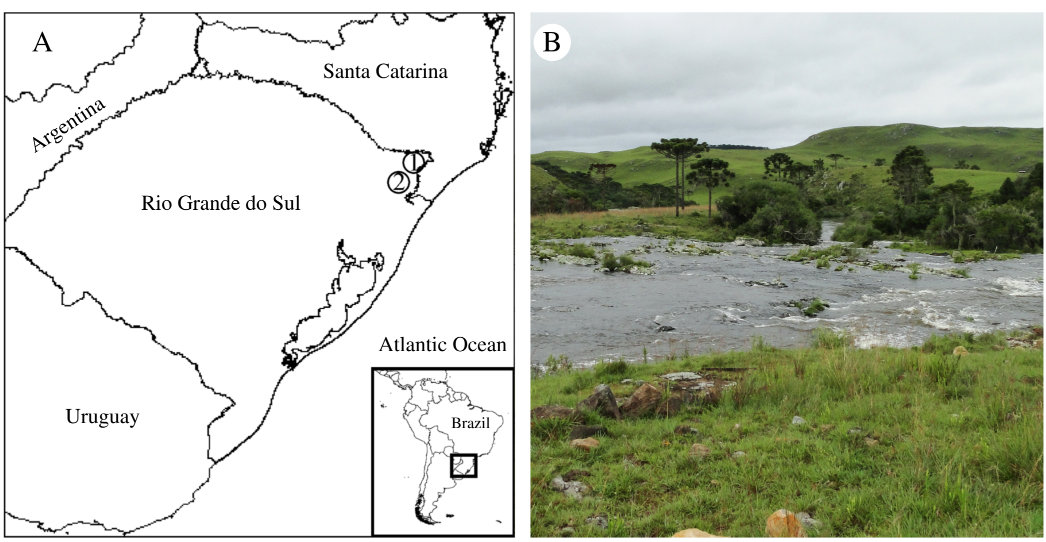

Fig. 1. A, map of the study region in Rio Grande do Sul State, southern Brazil, with collection area 'Cachoeirão dos Rodrigues' (1) and 'Cachoeira dos Venâncios' (2); B, Silveira River, Cachoeirão dos Rodrigues, habitat of the type specimen of *Ramalina fleigiae* sp. nov. In colour online.

denaturation, followed by 30 cycles of 30 s at 95 °C, 45 s at 46 °C, 1 min at 72 °C, with a final elongation of 10 min at 72 °C. PCR products were purified with ammonium acetate and ethanol, and sequenced in an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems).

Sequences from nine specimens of *Ramalina fleigiae* were obtained. We also analyzed sequences from material collected in southern Brazil: the saxicolous *R. laevigata* (three specimens) and the corticolous species *R. exiguella* (three specimens), *R. gracilis* (one) and *R. sprengelii* (two). In addition to their phylogenetic proximity, these species were selected as they share morphological, chemical (e.g. *R. exiguela*) and ecological (e.g. *R. laevigata*) features with *R. fleigiae*. Sequences from *R. celastri*, *R. glaucescens*, *R. inflexa*, *R. ovalis* and *R. siliquosa* used in the comparisons were obtained from GenBank. *Lecania erysibe* (Ach.) Mudd and *L. leprosa* Reese Næsb. & Vondrák were used as outgroup. Geographical origin, voucher details and additional information about the specimens are provided in Table 1.

Forward and reverse reads were assembled using Geneious 9.1.2 (http://www.geneious.com; Kearse *et al.* 2012). Sequences were aligned using the MAFFT algorithm (Katoh *et al.* 2002) in Geneious 9.1.2 (auto function for the best algorithm choice, gap penalty = 1·53, offset value = 0·123 and scoring matrix = 200 PAM/k = 2) and manually adjusted. The analyses were performed with two data sets: ITS only and ITS/IGS sequences combined. The best model for each matrix was obtained using the jModelTest2 program (Guindon & Gascuel 2003; Darriba *et al.* 2012). Maximum likelihood analyses were performed in PhyML (Guindon & Gascuel 2003) with 1000 bootstrap replicates for calculation of the nodal support, using the TrNef + G substitution model for the combined data set and TiM2 + G for the ITS data set. Bayesian analyses were estimated using the software BEAST 1.8.2 (Drummond *et al.* 2012) with the substitution model TN93 + G for both data sets. A Yule tree prior and a random starting tree were selected and the MCMC runs were performed using four chain lengths of 10 000 000, sampling trees every 1000th generation. In TreeAnnotator v1.8.2 (part of BEAST v1.8.2), the first 20% of trees was discarded as burn-in and the maximum clade credibility tree generated. The results were checked in Tracer v1.6 (Rambaut *et al.* 2014) for the MCMC convergence and the effective sample sizes (≥200). Conflicts between maximum likelihood and Bayesian analyses were assumed to be significant if two different relationships for the same taxa were supported with bootstrap values >75% and posterior probability >0·95 (Sérusiaux *et al.* 2010).

Additional tools, such as maximum likelihood (ML) distance (number of substitutions per site, s/s) calculations in PAUP* v4.0b10 (Swofford 2003) and the Automatic Barcode Gap Discovery (http://wwwabi.snv.jussieu.fr/public/abgd/; Puillandre *et al.* 2012), were used to delimit *R. fleigiae*. The default settings (Pmin = 0·001, Pmax = 0·1, and 20 numbers of distribution) and four gap width values were tested (0·5, 0·7, 1·0 and 1·2) using Jukes-Cantor distances.

Morphological, anatomical and chemical analyses

Specimens were removed with a knife and dried at room temperature. Previously collected specimens from herbaria ICN and UFP were also studied, allowing us to identify the collection site and obtain fresh specimens.

Specimens were examined using standard techniques with stereoscopic (×20–40) and light microscopes (×400–1000). Freehand sections of the thalli and apothecia were mounted in water. Spot tests were conducted according to Huneck & Yoshimura (1996) and Orange *et al.* (2001), including observation under UV light and thin-layer chromatography (TLC) using solvent C.

The key to the 32 saxicolous species of *Ramalina* from the Neotropical region was based partially on literature (e.g. Nylander 1870; Malme 1934; Grassi 1950; Kashiwadani 1987; Kashiwadani & Kalb 1993; Marcano & Morales Méndez 1994; Aptroot & Bungartz 2007; Kashiwadani *et al.* 2007) and partially on herbarium specimens. The material examined was sourced from the following regions and herbaria: Argentina (CTES, MIN, MPUC, MSC), Bahamas (MSC), Brazil (BM, BHCB, CGMS, CTES, F, G, H, HAS, ICN, MPUC, SP, UFP, UPCB, TUR, W), Chile (BM, CTES, G, H, MIN, MSC, O, WRSL), Colombia (H), Cuba (G, H, MICH, MSC), Dominican Republic (MSC), Ecuador (MSC), Falkland Islands (MSC), Guadalupe (MICH, MIN), Guatemala (F, MSC), Guyana (BM), Haiti (MSC), Honduras (BM), Jamaica (H, MIN, MSC), Mexico (WIS, WRSL), Panama (F), Paraguay (CTES, H), Peru (DUKE, H, M, W), Puerto Rico (F, MICH, MSC), Uruguay (DUKE, ICN, MPUC) and Venezuela (MIN). In addition to the species described as exclusively saxicolous, we also included species rarely recorded as corticolous. *Ramalina siliquosa* (Huds.) A. L. Sm, a doubtful record for South America (e.g. Argentina; Calvelo & Liberatore 2002), was integrated into the key.

Results and Discussion

Molecular data

The ITS and combined sequence data sets contained 549 and 958 unambiguously aligned sites, respectively. The specimens *Ramalina fleigiae* 1 to 8 presented identical sequences (in both markers) and thus only one sequence was used in the analyses. Phylogenetic reconstructions generated by maximum likelihood and Bayesian analyses, for both data sets, did not show incongruences and so only the Bayesian phylogenetic inferences are shown in Fig. 2. The species clustered in the two main clades have distinct morphological and anatomical features, as well as different geographical origins and habitats. Therefore, there are

Table 1. *Specimen information together with the GenBank Accession numbers for the collections used in Fig. 2 Newly obtained sequences are in bold.*

			GenBank Acc. no.	
Specimen	Voucher information	Reference	ITS	IGS
Lecania leprosa	Slovakia	Næsborg 2008	AM292698	AM600969
L. erysibe	Denmark	Næsborg 2008	AM504061	AM504080
Ramalina celastri 1	Rwanda	Sérusiaux *et al.* 2010	GU827295	-
R. celastri 2	New Zealand	Hayward *et al.* 2014	KF583540	KF594450
R. celastri 3	New Zealand	Hayward *et al.* 2014	KF583542	KF594452
R. exiguella 1	Brazil. Rio Grande Do Sul State, *E. Gumboski* 4068 (ICN)	Present study	**KY171853**	**-**
R. exiguella 2	Brazil. Santa Catarina State, *A. Gerlach* 875b (ICN)	Present study	**KY171852**	**-**
R. exiguella 3	Brazil. Santa Catarina State, *A. Gerlach* 873 (ICN)	Present study	**KY171851**	**-**
R. fleigiae 1	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5049 (ICN)	Present study	**KY171854**	**KY006649**
R. fleigiae 2 (type)	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5050 (ICN)	Present study	**KY171855**	**KY006656**
R. fleigiae 3	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5051 (ICN)	Present study	**KY171856**	**KY006655**
R. fleigiae 4	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5052 (ICN)	Present study	**KY171857**	**KY006654**
R. fleigiae 5	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5053 (ICN)	Present study	**KY171858**	**KY006648**
R. fleigiae 6	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5554 (ICN)	Present study	**KY171859**	**KY006653**
R. fleigiae 7	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5055 (ICN)	Present study	**KY171860**	**KY006652**
R. fleigiae 8	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5056 (ICN)	Present study	**KY171861**	**KY006651**
R. fleigiae 9	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5057 (ICN)	Present study	**KY171866**	**KY006650**
R. glaucescens	New Zealand	Hayward *et al.* 2014	KF583545	KF594455
R. gracilis	Brazil. Paraná State, *A. Gerlach* 780b (ICN)	Present study	**KY171862**	**KY006647**
R. inflexa	New Zealand	Hayward *et al.* 2014	KF583546	KF594456
R. laevigata 1	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5033 (ICN)	Present study	**KY171863**	**KY006644**
R. laevigata 2	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5046 (ICN)	Present study	**KY171864**	**KY006645**
R. laevigata 3	Brazil. Rio Grande Do Sul State, *E. Gumboski* 5047 (ICN)	Present study	**KY171865**	**KY006646**
R. ovalis 1	New Zealand	Hayward *et al.* 2014	KF583554	KF594464
R. ovalis 2	New Zealand	Hayward *et al.* 2014	KF583556	KF594466
R. ovalis 3	New Zealand	Hayward *et al.* 2014	KF583557	KF594467
R. siliquosa	United Kingdom	Groner & LaGreca 1997	U84587	-
R. sprengelii 1	Brazil. Santa Catarina State, *F. Beilke* S. N. (ICN)	Present study	**KY171867**	**-**
R. sprengelii 2	Brazil. Santa Catarina State, *A. Gerlach* 539a (ICN)	Present study	**KY171868**	**-**

species with very distinct characteristics in the same clade, such as very long and somewhat cylindrical branches to mainly short and clearly flattened branches, as well as distinct patterns of chondroid tissue. The phylogenetic relationships in these *Ramalina* groups lack clear phenotypic and/or biogeographical affinities.

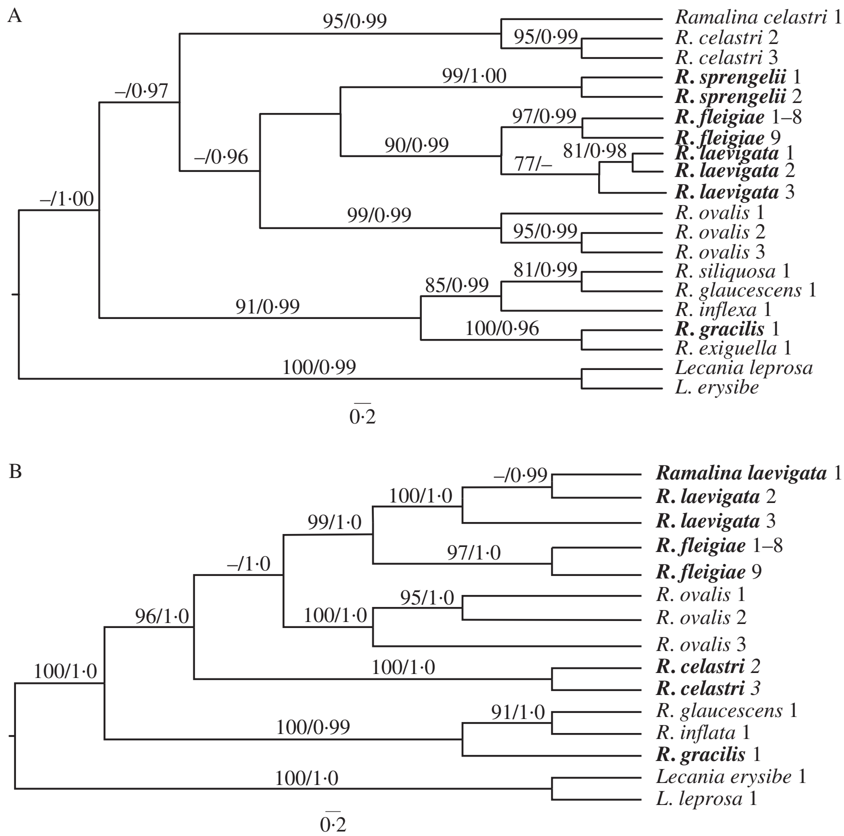

FIG. 2. Bayesian maximum clade credibility tree based on (A) ITS sequences and (B) the concatenated data set (ITS and IGS sequences) showing the phylogenetic relationships among *Ramalina* species, including *R. fleigiae* sp. nov. ML boostrap ≥70 (before the slash) and posterior probability ≥0·95 (after the slash) are shown above branches. Specimen details are given in Table 1. Specimens for which new sequences were obtained in this study are shown in bold.

The sequences generated using *R. fleigiae* specimens clustered in well-supported clades with *R. laevigata*. Both of these species occur in riparian and saxicolous habitats, produce usnic acid only, and also present clearly distinct morphological and anatomical features. *Ramalina fleigiae* is densely branched in the upper half, irregularly flat to subterete, up to 1·5 mm wide, while *R. laevigata* is moderately branched and has distinct dorsiventral branches up to 2·0(–4·0) mm wide (Fries 1825; Malme 1934). In contrast, *R. fleigiae* is not closely related to *R. exiguella* and *R. gracilis*, two morphologically and chemically similar species. All have shrubby thalli, irregularly flat to subterete, branches both symbiotic propagules and a lack of medullary acids. While *R. fleigiae* occurs only on rocks above 1100 m, *R. exiguella* and *R. gracilis* occur mainly on trunks, twigs or rocks, and are strictly coastal species. Furthermore, *R. exiguella* and *R. gracilis* have linear to long linear pseudocyphellae while *R. fleigiae* is characterized by mainly irregularly ellipsoid to short linear pseudocyphellae.

Analyses of the ITS region revealed that *R. fleigiae* specimens presented ML distances between 0 (identical) and 0·001 s/s. The distances among the other species analyzed varied between 0·023 s/s (*R. fleigiae* × *R. laevigata*) and 0·210 s/s (*R. fleigiae* × *R. inflexa*; *R. fleigiae* × *R. gracilis*). For

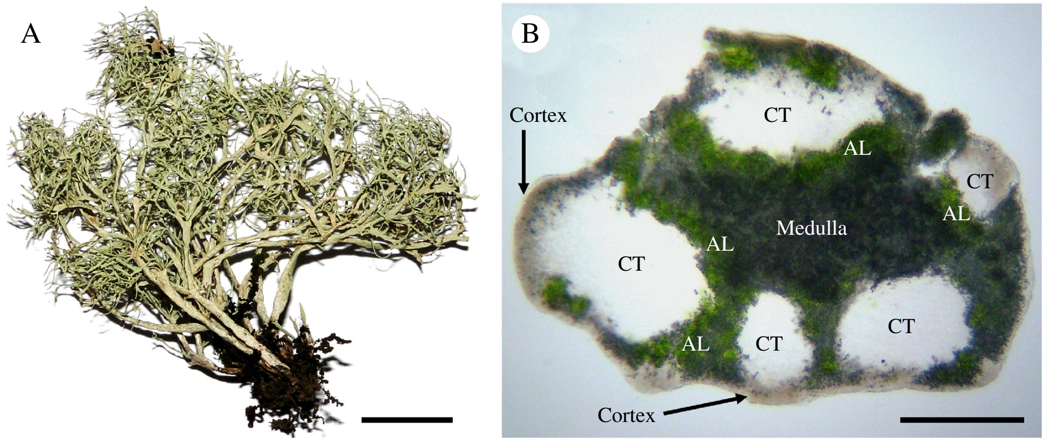

FIG. 3. *Ramalina fleigiae*. A, thallus (holotype) with solid, irregularly flat to subterete branches; B, TS main branch (holotype) showing the distinct cortex, the discontinuous, uncracked chondroid tissue (CT), algal layer (AL) below the cortex and around the chondroid tissue, and the medulla in the grey central portion. Scales: A = 1 cm; B = 200 µm. In colour online.

FIG. 4. *Ramalina fleigiae* (arrow) growing on mid-river rock, very near the water indicating they may be prone to temporary submergence (Silveira River, Cachoeirão dos Rodrigues, southern Brazil). In colour online.

comparison, the mean distance between *R. ovalis* and *R. celastri* (recognized as distinct species by Hayward *et al.* 2014) was 0·107 s/s, much higher than the distances between *R. fleigiae* and *R. laevigata*. All the settings tested in the ABGD analysis resulted in 10 distinct groups, corresponding to the species identification and corroborating the delimitation of *R. fleigiae* as a new species.

Morphological, anatomical and chemical analyses

Ramalina fleigiae Gumboski, Eliasaro & R. M. Silveira sp. nov.

MycoBank No.: MB 824641

Differing from *Ramalina exiguella* Stirt. by the thallus with branches originating from a single holdfast, most densely branched in the upper half of the thallus, and further distinguished by numerous irregular ellipsoid to short linear pseudocyphellae on the surface and margin of its branches.

Type: Brazil, Rio Grande do Sul State, municipality of São José dos Ausentes, locality of 'Cachoeirão dos Rodrigues', on a rock in the middle of Silveira River, *c.* 1150 m alt., 28°35'59·85"S, 49°59'19·89"W, 20 January 2015, *E. Gumboski* 5050 (ICN—holotype; UPCB, SP, H, F—isotypes). GenBank Accession no.: KY171855

(Fig. 3)

Thallus saxicolous, shrubby, up to 5·0 cm tall and 6·0 cm wide, whitish green in the field, becoming stramineous in the herbarium; densely branched in the upper half in most well-developed thalli; branches originating from a single holdfast, consisting of dense groups of 2–7 branches rooting in a common, necrotic base up to 0·7 cm high; branching dichotomously anisotomic; *main branches* ramify mostly from the upper half; branches solid, rigid, irregularly flat to subterete, somewhat inflated, but not hollow, distinctly flattened mainly at their tips; main branches 0·4–1·5 × 0·2–1·1 mm broad; *secondary branches* 0·15–0·60 × 0·1–0·5 mm wide; apex somewhat truncated to slightly acute, sometimes curved appearing hook-shaped; surface opaque, irregular, ± striate owing to the presence of abundant pseudocyphellae resembling maculae; symbiotic propagules absent; *pseudocyphellae* numerous on lamina and margins, present across both the main and secondary branches, depressed on level surface, irregular ellipsoid to shortly linear, 0·15–1·0 × 0·05–0·15 mm, rarely orbicular, 0·1–0·2 mm diam.; *cortex* distinct, 10–35 µm thick; chondroid tissue not cracked, discontinuous, in irregular bundles intermixed with medulla (but sometimes the major portion of the branches); main branches 80–620(–700) µm thick; secondary branches usually with one ±circular to irregular bundle, 30–210 µm thick; *medulla* compact; algal layer below the cortex and around the chondroid tissue, 15–70 µm thick.

Ascomata and *conidiomata* not found.

Chemistry. Cortex and medulla: K−, C−, KC−, PD−, UV−. TLC: usnic acid.

Etymology. This new species is dedicated to Dr Mariana Fleig, who devoted much of her life to the study of lichens and contributed enormously to the knowledge of Brazilian lichenology. Fleig also collected the oldest known herbarium specimens (in ICN) of this new species.

Distribution and habitat. The species described here is known only from two localities in the north-eastern plateau of Rio Grande do Sul State (Fig. 1). *Ramalina fleigiae* colonizes only rocks in the riverbed and waterfalls that are exposed to direct sunlight; some thalli were almost submerged in the water (Fig. 4) at *c.* 1150 m elevation. All thalli are probably temporarily submerged during occasional floods. No thallus was found on rocks that are further from the river. The species probably occurs in nearby rivers but due to very small population sizes these might easily have been overlooked.

The species was not found in similar environments visited by the first author in the adjacent states of Santa Catarina and Paraná.

Discussion. *Ramalina fleigiae* is a distinct species, known mainly for its saxicolous habit and branching pattern (originating from the same base, densely branched upper half). Branches are solid, irregularly flat to subterete (Fig. 3A), up to 1·5 mm wide, with numerous irregular ellipsoid to short linear pseudocyphellae on the lamina and margins. Anatomically, a distinct cortex with a discontinuous and not cracked chondroid tissue characterizes the species (Fig. 3B). There are no secondary metabolites in the medulla.

Similar species are *R. gracilis* (Pers.) Nyl. and *R. exiguella* Stirt. but both have thalli growing from a delimited holdfast, angular branches in cross-section, long linear pseudocyphellae and no distinct cortex; these two species are also strictly coastal (Fleig 1988; Kashiwadani & Kalb 1993). *Ramalina osorioi* Kashiw. *et al.* is a similar saxicolous species and has a shrubby thallus with solid branches. However, it is moderately branched, has distinctly dorsiventral branches and produces psoromic acid (Kashiwadani *et al.* 2007). *Ramalina continentalis* Malme is a saxicolous species that inhabits rocks along rivers in Brazil, Paraguay and Uruguay (e.g. Malme 1934; Osorio 1972). However, it has distinctly flattened and has much larger branches of 5–10(–20) mm width (Malme 1934).

Ramalina furcellangulida Aptroot, endemic to the Galapagos Islands, has no distinct holdfast. Branches are irregularly angular in section, typically not flattened, often with rounded, irregular, laminal warts and linear pseudocyphellae, rarely with few punctiform tuberculate pseudocyphellae. This species produces divaricatic or salazinic and/or sekikaic acid (Aptroot & Bungartz 2007).

Ramalina siliquosa (Huds.) A. L. Sm. is shrubby to pendulous and its main branches are more or less flattened, rarely entirely terete; however, its secondary branches are mostly furcate, rarely terete, and the surface is more or less matt, rugose, or frequently even foveolate, occasionally with well-developed furrows (Krog & James 1977). In this species the chondroid tissue is clearly to moderately cracked (Kashiwadani 1992). *Ramalina cuspidata* (Ach.) Nyl. has simple or sparingly furcate branches which rarely become densely branched towards the apices. Branches are mostly terete towards the base and remain so above or gradually become flattened (Krog & James 1977). The holotype (H!) has a distinct 8–10 µm thick cortex and the chondroid tissue is clearly cracked, continuous, and 50–220 µm thick.

Ramalina geniculatella Aptroot, endemic to the Galapagos Islands, is initially shrubby, becoming pendant with age, branches are mostly flattened and partly terete, and most thallus parts have conspicuous whitish linear pseudocyphellae (Aptroot 2008). An examination of the holotype (B!) showed that the cortex is absent, and the chondroid tissue is not cracked and forms almost regular bundles mixed with medulla.

Ramalina elegantula P. M. Jørg. has a caespitous thallus with linear thin branches, up to 0·5 mm wide. The pseudocyphellae are linear and marginal (Jørgensen 1977). The cortex is distinct, 10–20 µm thick, and the chondroid tissue is continuous and uncracked, 60–105 µm thick (O—holotype!). *Ramalina leptocarpha* Tuck. has a shrubby to subpendulous thallus. The branches are lanceolate, plane to somewhat canaliculated, and the pseudocyphellae are linear to ellipsoid, laminal, or rarely marginal. *Ramalina bajacalifornica* Bowler & Rundel has a shrubby to subpendulous thallus and flat or weakly canaliculated branches; pseudocyphellae are laminal or rarely marginal, short linear, somewhat elevated. This species produces salazinic acid in the medulla (Kashiwadani & Nash 2004).

Two endemic saxicolous species of the Azores are morphologically similar to *R. fleigiae*. They differ by their branching and the pattern of pseudocyphellae, and by the production of secondary metabolites. *Ramalina azorica* Aptroot & Schumm has few antler-like branches or dense branches that become terete towards the tips. The pseudocyphellae are linear. It produces divaricatic acid together with an unknown pigment. *Ramalina wirthii* Aptroot & Schumm has branches that are mostly flattened, or in parts irregularly rounded, with several of the branches usually becoming terete toward the tips. It produces salazinic acid, usually together with protocetraric acid (Aptroot & Schumm 2008).

Ramalina jamesii Krog, *R. portosantana* Krog and *R. timdaliana* Krog, all endemic to Porto Santo Island near Madeira, are saxicolous and have shrubby thalli. However, *R. jamesii* has subterete and uneven branches, and fragmentation areas are common. The pseudocyphellae are shortly linear to ellipsoid. It produces divaricatic acid, salazinic acid and triterpenoids. *Ramalina portosantana* has subterete or more or less flat branches, and the surface is rugulose with irregularly reticulate ridges which can develop pseudocyphellae. The cortex is very thick, up to 80 µm. The chondroid tissue occasionally adjoins the cortex but mostly forms numerous strands intermixed with the medulla. This species produces salazinic acid. *Ramalina timdaliana* is richly branched and the branches are subterete to somewhat flat, often with shortly linear pseudocyphellae. It produces divaricatic acid and triterpenoids (Krog 1990).

Ramalina pluviariae Krog & Østh. is saxicolous and has subterete branches, and the pseudocyphellae are mainly linear, more or less anastomosing (Krog & Østhagen 1980). It has no cortex and the chondroid tissue is discontinuous and clearly cracked, 70–350 µm thick (O—holotype!). *Ramalina hamulosa* Krog & Østh. has subterete, angular to flattened branches, with numerous short branchlets terminating in nodules or hook-shaped structures. The pseudocyphellae are linear, restricted mostly to the margin of flattened branches (Krog & Østhagen 1980). The cortex is distinct, 30–40 µm thick, and the chondroid tissue is discontinuous, cracked, and 10–275 µm thick (BM—isotype!).

Ramalina australiensis Nyl., a rarely saxicolous species, has thalli that are 3–10(–20) cm long, with sparse branching into the basal region of the thallus which becomes densely branched at the tips. The primary branches are usually subterete and thin, rarely thick, compressed and coarse (Stevens 1987). The chondroid tissue is clearly cracked, discontinuous, and 140–300 µm thick (H-NYL—holotype!). *Ramalina tropica* G. N. Stevens has branches arising from the base with common lateral branchlets. The pseudocyphellae are linear, within which tuberculate extensions are sometimes present. The presence of salazinic and protocetraric acids is characteristic for this species (Stevens 1987).

Additional specimens examined. **Brazil:** *Rio Grande do Sul State*: municipality of Cambará do Sul, Cachoeira dos Venâncios, on rock, 2000, *W. B. Sanders* 00108.3B (UFP 29592); municipality of Jaquirana, locality of Cachoeira dos Venâncios, on rock, *c.* 850 m, 29°01'03·72"S, 50°15'31·55"W, 2015, *E. Gumboski* 5054, 5055 (ICN), 5056, 5057 (SP); municipality of São José dos Ausentes, source of Rio do Marco, riverbed, on rocks, 1994, *M. Fleig* 6643, 6660 (ICN 99838); locality of 'Cachoeirão dos Rodrigues', on rock in the middle of the river (Silveira River), *c.* 1150 m, 28°35'59·85"S, 49°59'19·89"W, 2015, *E. Gumboski* 5049, 5051, 5052 (ICN), 5053 (JOI).

Key to saxicolous species of *Ramalina* from the Neotropical region (including the Galapagos but excluding the South Atlantic islands)

1 Branches hollow 2
 Branches solid (even with a lax medulla) 3

2(1) Branches 0·1–1·0(–2·0) mm wide, sorediod granules produced at the apices of the branches .... **R. roesleri** (Hochst. ex Schaer.) Nyl.
 Branches up to 4·0 mm wide, without soredioid granules on branches .... **R. turgida** Kremp.

3(1) Soredia present 4
 Soredia absent 20

4(3) Branches mainly terete to subterete, or irregular in thickness but not distinctly dorsiventral 5
 Branches flat, canaliculate, lanceolate or palmate 9

5(4) Branches mainly terete 6
 Branches mainly irregular in thickness 8

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6(5) Medulla K− or K+ pinkish .... **R. fragilis** Aptroot & Bungartz
Medulla K+ yellow to red.. 7

7(6) Cortex distinct, chondroid tissue cracked.................................... **R. rigida** Ach.
Cortex indistinct, chondroid tissue not cracked.... **R. sorediosa** (B. de Lesd.) Landrón

8(5) Branched from a spreading holdfast; soredia subgranular to granular...................
.... **R. subfarinacea** (Nyl. ex Cromb.) Nyl.
Branched from a narrow holdfast; soredia farinose .... **R. farinacea** (L.) Ach.

9(4) Branches flat, dorsiventral, 3·0–6·0(–12·0) mm wide...
....**R. terebrata** Hook. f. & Taylor
Branches variably flat, 0·2–3·0 mm wide.. 10

10(9) Medulla K+ yellow → red 11
Medulla K−, K+ yellow or K+ pinkish 13

11(10) Branches distinctly canaliculate .... **R. subpollinaria** Nyl.
Branches not canaliculate... 12

12(11) Branches with short conspicuous laminal striae along their entire length; soralia lateral, subapical to apical, capitate or labriform (Venezuela)...........................
.... **R. microphylla** V. Marcano & A. Morales
Branches irregularly striate from cartilaginous cortical strands; soralia mostly apical, rather discrete but occasionally confluent, capitate or irregular (Galapagos) ......
.... **R. polyforma** Aptroot

13(10) Medulla K+ pinkish (homosekikaic acid and sekikaic acid)............................. 14
Medulla K− or K+ yellow.. 15

14(13) Surface of branches shiny, smooth.............. **R. intermedia** (Delise ex Nyl.) Nyl.
Surface of branches wrinkled, irregularly striate (Galapagos).......... **R. polyforma**

15(13) Medulla PD + golden yellow (psoromic acid) .... **R. dissecta** Kashiw.
Medulla PD − 16

16(15) Soredia marginal and/or terminal 17
Soredia laminal, sometimes also marginal and/or terminal 19

17(16) Soredia mostly terminal (Galapagos) .... **R. polyforma**
Soredia marginal only 18

18 (17) Secondary compounds absent from the medulla..................... **R. incana** Kashiw.
Divaricatic acid present..**R. stoffersii** Sipman

19(16) Branches flattened to partly terete usually towards the apices, sometimes palmate; medulla with evernic acid agg. (UV+ bluish white).. ..**R. pollinaria** (Westr.) Ach.
Branches usually palmately to irregularly branched; medulla with bourgeanic acid (UV−)... **R. lacera** (With.) J. R. Laundon

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20(3) Branches terete to subterete, angular in cross-section or irregularly flat............ 21
Branches clearly flat.. 26

21(20) Branches mainly irregularly flat, usually angular in cross-section 22
Branches terete to subterete 24

22(21) Branches irregularly angular in cross-section, surface often with laminal warts; pseudocyphellae linear .... **R. furcellangulida**
Branches terete to flat, surface smooth, rugose or furrowed but without warts; pseudocyphellae orbicular to ellipsoid 23

23(22) Main branches irregularly flat to subterete, somewhat inflated, at their tips usually distinctly flattened; chondroid tissue not cracked........................... **R. fleigiae**
Main branches somewhat flattened and secondary branches subterete to terete; chondroid tissue cracked...**R. siliquosa**

24(21) Medulla K− (protocetraric acid) .... **R. gallowayi** Kashiw *et al.*
Medulla K+ yellow → red (salazinic acid) 25

25(24) Branches with many pale, warty patches at the apical portion........................
.... **R. dasypoga** Tuck.
Branches without warty patches..........**R. santanensis** V. Marcano & A. Morales

26(20) Branches mainly to distinctly canaliculate 27
Branches flat, usually dorsiventral but not distinctly canaliculate 28

27(26) Pseudocyphellae marginal, linear; ascospores 18–30 × 3–6 µm.............................
....**R. puiggarii** Müll. Arg.
Pseudocyphellae marginal, puntiform to sublinear; ascospores 10–12 × 4–5 µm ....
.... **R. darwiniana** Aptroot & Bungartz

28(26) Medulla K+ yellow → red (salazinic acid or norstictic acid)............................ 29
Medulla K−, K+ yellow or K+ pinkish 31

29(28) Branches lanceolate; norstictic acid present.......................... **R. fasciata** Kremp.
Branches flat but not lanceolate; salazinic acid present.................................... 30

30(29) Surface of branches smooth to rugose; pseudocyphellae tuberculate to punctiform
....**R. complanata** (Sw.) Ach.
Surface of branches conspicuously striate; pseudocyphellae linear ....
....**R. sideriza** Zahlbr.

31(28) Medulla K+ pinkish (sekikaic acid) .... **R. fasciata** Kremp.
Medulla K− or K+ yellow.. 32

32(31) Medulla PD+ yellow (psoromic acid).. **R. osorioi**
Medulla PD− 33

33(32) Thallus with a narrow holdfast; branches up to 15 mm wide....... **R. continentalis**
Thallus with a common holdfast; branches up to 4 mm wide............................ 34

34(33) Branches 2·0–4·0 mm wide; ascospores 13–16 × 3–4 µm ....**R. laevigata**
Branches 0·5–2·0 mm wide; ascospores 11–14(–17) × 4–5 µm ....
....**R. pilulifera** Taylor

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