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# Molecular, morphological and chemical characterization of a poorly known lichen: the case of *Ramalina wirthii* (Ramalinaceae, Ascomycota)

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**Abstract.** The ascomycete genus *Ramalina* is one of the most remarkable examples of insular diversity and endemicity in lichen-forming fungi, as nearly half the species present in the Macaronesian region are endemic. Among the five archipelagos of the region, Azores is the one that hosts less endemic species: the fertile *R. azorica* and the sterile *R. wirthii*, both only known from the eastern islands of the archipelago. In a recent trip to the westernmost island of the Azores (Flores) we have discovered a population of *R. wirthii* with fertile thalli. In order to confirm the identification of these specimens, we conducted a molecular phylogenetic study based on the *ITS* region. In addition, we studied their morphology using 30 traits previously reported in the literature as relevant for the genus, and their secondary chemistry by thin layer chromatography. About half the morphological traits were not present in the original description of the species and are reported here for the first time. In addition, the specimens from Flores did not match with eight of the remaining characters. For this reason, they could be confused with other *Ramalina* species. Morphological and chemical differences with these taxa are discussed.
**Keywords.** Azores, biodiversity, endemism, Flores, Macaronesia, oceanic islands.

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## Introduction

Lichen-forming fungi have been traditionally regarded as organisms with wide distribution ranges, especially when compared to other sessile organisms such as vascular plants (Galloway, 2008). Endemic species were considered to be rare, but the use of molecular approaches has recently revealed this pattern to be much more common than previously thought (Sérusiaux *et al.,* 2011; Leavitt *et al.,* 2013; Moncada *et al.,* 2014, 2021; Lücking *et al.,* 2014; Dal Forno *et al.,* 2017; Simon *et al.,* 2018; Crespo *et al.,* 2020). One example can be found in the ascomycete genus *Ramalina* Ach., which includes c. 230 species (Lücking *et al.,* 2017) that form characteristic fruticose thalli of a yellowish-greenish color. It has a subcosmopolitan distribution, but there are five areas in which the genus shows great taxonomic diversity: The Andes (Marcano *et al.,* 2021), East Africa (Krog & Swinscow, 1976), Baja California (Nash *et al.,* 2002), Australasia (Stevens, 1987; Blanchon *et al.,* 1996) and Macaronesia, a region in which *Ramalina* reaches remarkable figures of endemicity (Krog & Østhagen, 1978, 1980a, b; Krog, 1990; Aptroot & Schumm, 2008; Pérez-Vargas & Pérez-Ortega, 2014; Spjut *et al.,* 2020).

Macaronesia is a biogeographical region formed by five volcanic archipelagos situated on the Atlantic Ocean (Azores, Madeira, Selvagens, Canary Islands and Cape Verde) and a swath of land in western Africa known as the continental Macaronesian enclave (Fernández-Palacios *et al.,* 2011). So far there are 59 species of *Ramalina* in Macaronesia, 28 of them endemic to the region (Hafellner, 1992; Arechavaleta Hernández *et al.,* 2005; Aptroot & Schumm, 2008; Hernández-Padrón & Pérez-Vargas, 2010; Pérez-Vargas & Pérez-Ortega, 2014; Sipman & Aptroot, 2020), but this figure is not constant across archipelagos. The most diverse are the Canary Islands (Hernández-Padrón & Pérez-Vargas, 2010; Pérez-Vargas & Pérez-Ortega, 2014), which host 43 species (ten

of them endemic to the archipelago), and the Madeira archipelago (Hafellner, 1992; Sipman & Aptroot, 2020), which harbors 34 species (six of them endemic). The least diverse are Cape Verde (Arechavaleta Hernández *et al.,* 2005), which contains 24 species (one of them endemic), and the Azores archipelago (Aptroot & Schumm, 2008), which hosts 17 species (two of them endemic).

The only available information on the two Azorean endemics, aside from their phylogenetic placement (see Sérusiaux *et al.,* 2010; Spjut *et al.,* 2020), is that presented in the original species descriptions (Aptroot & Schumm, 2008), as no further work has been carried out on them since. Both species, *Ramalina azorica* Aptroot & F. Schumm and *R. wirthii* Aptroot & F. Schumm, are saxicolous and are currently only known from the eastern islands of the archipelago: *R. azorica* from Pico, Terceira, Faial and São Miguel, and *R. wirthii* from Pico, Graciosa and São Miguel (Aptroot & Schumm, 2008), all appearing in the Nubia-Eurasia plate boundary (Vogt & Jung, 2018). *Ramalina azorica* is a fertile species that can be easily distinguished from other *Ramalina* by its characteristic cork-screwlike laciniae, while *R. wirthii* was described as a sterile species that could be differentiated from other *Ramalina* by its pycnidia and somewhat curled laciniae tips (Aptroot & Schumm, 2008).

In a recent trip to Flores Island, the westernmost island of the Azores archipelago, already in the American plate, we found fertile thalli of *R. wirthii*. Here we study these specimens by molecular, morphological and chemical means, point out differences between these specimens and the original description, and discuss the relevance of some of the characters in the species identification and possible confusion with other *Ramalina*.

## Materials and methods

### Studied material

The ten specimens characterized in this study were collected in a small transect (c. 400 m in length) in Lajes das Flores, near Fajã Grande, on the western shore of Flores Island (39°27'04"N, 31°15'26"W), at an altitude of 150 m asl. The specimens were found growing on rock in exposed situations and on stone walls separating cultivated fields, oriented towards the prevailing winds. All thalli have been deposited in the TFC-Lich herbarium (Universidad de La Laguna), with numbers between 17101-17110.

### Molecular characterization

Six specimens encompassing the morphological variability observed in the samples were selected for a molecular study. We separated a small thallus fragment (c. 1 $mm^2$) of the apical zone of each specimen, as this was the area in which the presence of epiphytic microalgae and fungi was not apparent. Sample preparation was carried out under a Nikon SMZ800 stereomicroscope. Samples were stored at -80°C and, after one hour of freezing, were pulverized using a TissueLyser II (Qiagen) with two crystal beads. Genomic DNA was extracted using E.Z.N.A.® Forensic DNA Kit (Omega Bio-Tek). We amplified the nuclear internal transcribed spacer (*ITS*), which has been regarded as a universal barcode region for fungi (Schoch *et al.,* 2012), using primers ITS1F-KYO2 and ITS4-KYO2 (Toju *et al.,* 2012). PCR reactions were carried out in a total volume of 15 µL, containing 2 µL of template DNA, 0.5 µL of each primer (10 µM), 6.5 µL of MyTaq™ Red Mix (Bioline) and 5.5 µL of distilled water. The amplification program consisted of an initial denaturation at 95°C for 2 min; 8 cycles of 94°C for 1 min, 58°C for 1 min (decreasing 0.5°C each cycle) and 72°C for 1 min 30 s; 28 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min 30 s; with a final extension at 72°C for 7 min. PCR products were run in 1% agarose gels stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific). DNA was sequenced in Macrogen Spain (Madrid, Spain).

We downloaded from GenBank twelve additional sequences of the species that were found to be phylogenetically close to *Ramalina wirthii* (Spjut *et al.,* 2020): *R. cribrosa* De Not., *R. nodosa* Krog & Østh., *R. portuensis* Samp., *R. subgeniculata* Nyl. and *R. subpusilla* (Nyl.) Pit. & Harm. We also downloaded the only *R. wirthii* sequence available in GenBank, obtained from a thallus collected on the island of Pico (Sérusiaux *et al.,* 2010). These, together with the newly generated sequences, were aligned using MAFFT v.7.308 (Katoh *et al.,* 2002) as implemented in Geneious Prime v.2023.0.4. We selected the FFT-NS-I x1000 algorithm, the 200PAM / k = 2 scoring matrix, an offset value of 0.123 and a gap open penalty of 1.53. We trimmed sequence ends prior to DNA alignment. No manual adjustments were performed. We divided the *ITS* into three subregions, namely *ITS1*, *5.8S*, and *ITS2*, and used the resulting matrix for phylogenetic inference. We used Maximum Likelihood (ML) and Bayesian approaches for inferring phylogenetic relationships. The ML tree was calculated using RAxML (Stamatakis, 2014). We performed 1000 rapid bootstrap pseudoreplicates to evaluate nodal support. The Bayesian tree was calculated using MrBayes 3.2.7 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Starting with a random tree, two simultaneous, parallel four-chain runs were executed over $1x10^7$ generations, and sampled after every 1000th step. The first 20% of data was removed as burn-in. Convergence of chains was cheeked using Tracer 1.7.1 (Rambaut *et al.,* 2018). The 50% majority-rule consensus tree was calculated from the remaining trees. Nodes with bootstrap values equal or higher than 70% and/or with posterior probabilities equal or higher than 0.95 were considered to be significantly supported. RAxML and MrBayes were run in the CIPRES Science Gateway (Miller *et al.,* 2011). Additionally, we generated a haplotype network with the *R. wirthii* sequences using the TCS method (Templeton *et al.,* 1992). GenBank accession numbers are available in Table 1.

### Morphological characterization

The morphological study was based on 30 traits previously reported in the literature as relevant for the genus (Krog & Østhagen, 1980a; Stevens, 1987; Krog, 1990; Kashiwadani & Kalb, 1993; Pérez-Vargas & Pérez-Ortega, 2014): (1) laciniae length, (2) laciniae width, (3) surface brightness

Table 1. GenBank accession numbers and geographic origin of the specimens used for phylogenetic inference.

| Species | Locality | Voucher | GenBank | Source |
|---|---|---|---|---|
| *Ramalina cribrosa* | Corsica | LG 1526 | MN811267 | Spjut *et al.* (2020) |
| *R. cribrosa* | Corsica | LG 2113 | MN811278 | Spjut *et al.* (2020) |
| *R. nodosa* | Tenerife | BR 10767 | GU827301 | Sérusiaux *et al.* (2010) |
| *R. nodosa* | | HBG 17057 | FJ871093 | Direct submission |
| *R. nodosa* | | HBG 17058 | FJ871094 | Direct submission |
| *R. portuensis* | Scotland | LG R175 | GU827298 | Sérusiaux *et al.* (2010) |
| *R. portuensis* | Newfoundland | DUKE 402772 | MW242873 | Direct submission |
| *R. subgeniculata* | Madeira | BR 10489 | GU827300 | Sérusiaux *et al.* (2010) |
| *R. subpusilla* | Pico | LG R249 | GU827302 | Sérusiaux *et al.* (2010) |
| *R. subpusilla* | Porto Santo | ALV 8538 | MN587017 | Sipman & Aptroot (2020) |
| *R. subpusilla* | Porto Santo | ALV 8539 | MN587018 | Sipman & Aptroot (2020) |
| *R. subpusilla* | Porto Santo | ALV 8540 | MN587019 | Sipman & Aptroot (2020) |
| *R. wirthii* | Pico | LG R306 | GU827304 | Sérusiaux *et al.* (2010) |
| *R. wirthii* | Flores | TFC 17101 | OR947438 | This study |
| *R. wirthii* | Flores | TFC 17106 | OR947439 | This study |
| *R. wirthii* | Flores | TFC 17107 | OR947440 | This study |
| *R. wirthii* | Flores | TFC 17109 | OR947442 | This study |
| *R. wirthii* | Flores | TFC 17110 | OR947443 | This study |
| *R. wirthii* | Flores | TFC 17108 | OR947441 | This study |

(matt-shiny), (4) contorted laciniae (presence-absence), (5) fenestrations (presence-absence), (6) pseudocyphellae (presence-absence), (7) pseudocyphellae position, (8) pseudocyphellae length, (9) anatomical type (see Krog & Østhagen 1980a; Kashiwadani & Kalb 1993), (10) cortex thickness, (11) apothecia (presence-absence), (12) apothecia position, (13) apothecia color, (14) apothecia shape, (15) apothecia diameter, (16) presence of pruina in the apothecia, (17) thalline margin, (18) branching pattern of the paraphyses, (19) paraphyses width, (20) asci length, (21) asci width, (22) ascospore length, (23) ascospore width, (24) ascospore septation, (25) pycnidia (presence-absence), (26) pycnidia color (black-pale), (27) pycnidia position, (28) pycnidia tuberculated (yes-no), (29) conidia length, and (30) conidia width. These traits were measured under a Nikon SMZ800 stereomicroscope and a Zeiss Primo Star compound microscope. Quantitative traits were independently measured ten times in each thallus. Ascospores and conidia were measured from multiple apothecia and pycnidia, respectively. Quantitative traits are reported in the results section as (smallest absolute measurement-) 10th percentile - 90th percentile (-largest absolute measurement).

### Chemical characterization

We studied the secondary chemistry of the specimens using thin layer chromatography (TLC), following Culberson (1972) and Orange *et al*. (2001). Thallus fragments were immersed on acetone by approximately one hour to extract lichen substances. These acetone extracts were applied to TLC Silica gel 60F254 glass plates (Merck KGaA, Germany) and run in solvent system C (Orange *et al.,* 2001). We used glass plates to check for fatty acids. The plates were left to dry during one hour and then we applied 10% sulfuric acid and placed then on a heating plate at 120 °C. Once they were fully developed, we examined them in a CN-6 darkroom cabinet (Vilber Lourmat Sté, France) under a wavelength of 365 nm.

## Results

### Molecular characterization

In this study we generated six new sequences belonging to *Ramalina wirthii*. The DNA alignment had a length of 511 bp, of which 80 were variable positions and 21 singleton sites. The Bayesian analysis converged, with average standard deviation of split frequencies at termination below 0.01. Most species were recovered as strongly supported clades in both the Bayesian and ML analyses, with the only exception of *R. portuensis* and *R. subpusilla*. There was, however, some level of topological incongruence between the two analyses. The Bayesian analysis recovered *R. cribrosa* as sister to the clade formed by *R. subpusilla* and *R. portuensis*, while the ML analysis recovered this species as sister to *R. nodosa*. Statistical support was lacking in both cases. In both analyses the specimens collected in Flores Island clustered with the *R. wirthii* sequence from Pico, forming a strongly supported clade sister to *R. subgeniculata* (Figure 1). There were 15 variable positions between the two species. The TCS analysis revealed that each *R. wirthii* specimen carried a different *ITS* haplotype (there were ten variable positions among them), with the specimens from Flores being more closely related among themselves than with the specimen from Pico (Figure 2).

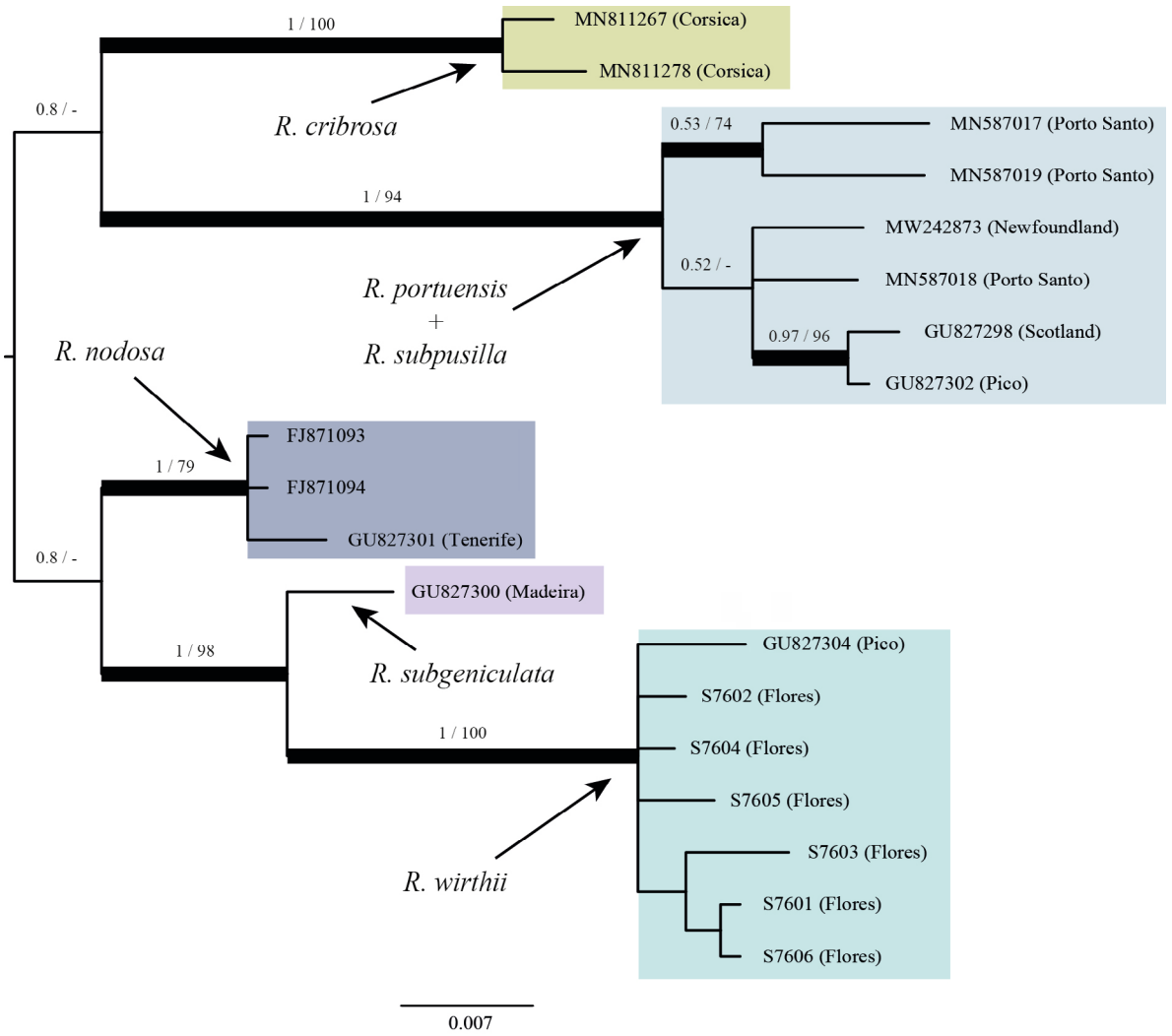


Figure 1. Phylogenetic tree inferred by Bayesian and Maximum Likelihood analyses of the *ITS* region including *Ramalina wirthii* and closely related species. Posterior probabilities and bootstrap values are shown over the branches. Supported branches are highlighted in bold. The tree was rooted following the topology obtained by Spjut *et al*. (2020).

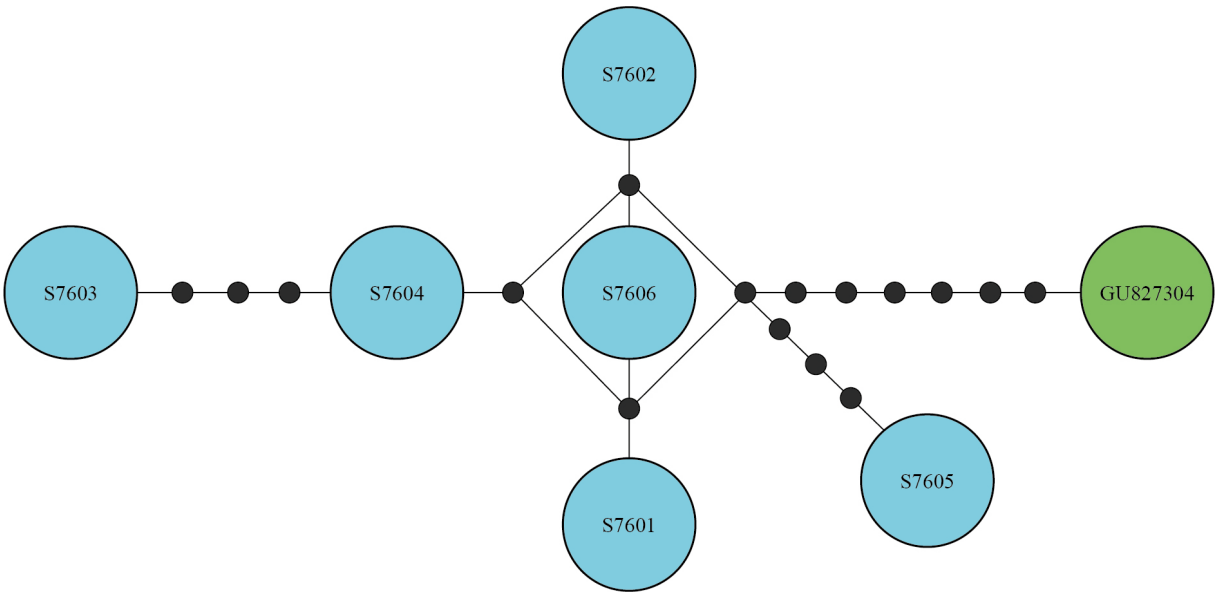


Figure 2. TCS network of the *Ramalina wirthii ITS* haplotypes. Color of the circles represents geographic origin: specimens from Flores are depicted in blue and from Pico in green. Black-filled small circles indicate missing haplotypes.

## Morphological and chemical characterization

Sixteen of the thirty morphological characters measured in this study were not present in the original description of *R. wirthii* and are here reported for the first time (Table 2). In addition, the morphology of the Flores specimens did not fully match with many of the remaining characters provided in the description. These specimens had slightly larger and wider laciniae, but also had long marginal pseudocyphellae, presented abundant marginal apothecia with mature ascospores, and their pycnidia were mainly marginal and mostly non tuberculated. Figure 3 illustrates these characters (Figure 3A-F), ascospore septation (Figure 3G) and thallus anatomy (Figure 3H). Regarding the chemical characterization, most specimens contained both salazinic and protocetraric acid, while a few thalli contained salazinic acid only.

Table 2. Comparison between the *Ramalina wirthii* specimens collected in Flores and the original description of the species (Aptroot & Schumm, 2008).

| Character | *R. wirthii* from Flores | Original description |
|---|---|---|
| Laciniae length | (1.6-) 2.5-6.7 (-7.4) cm | Up to 6 cm |
| Laciniae width | (0.4-) 0.9-2.4 (-3.9) mm | Up to c. 2 mm |
| Surface brightness | Matt | - |
| Contorted laciniae | Generally non-contorted | Somewhat curled towards the tips |
| Fenestrations | Rare | - |
| Pseudocyphellae | Present | Present |
| Pseudocyphellae position | Marginal and laminal | - |
| Pseudocyphellae length | (0.5-) 1-4 (-15) mm. Marginal pseudocyphellae much longer than the laminal ones. | Dots or small lines |
| Anatomical type | Anatomy *decipiens*-type | - |
| Cortex thickness | 17-27 (-30) µm | - |
| Apothecia | Present | Rare, not well developed |
| Apothecia position | Marginal | Laminal |
| Apothecia color | Disc pale orange colored | - |
| Apothecia shape | At first concave or flat, becoming deeply convex at maturity. Often geniculated. | - |
| Apothecia diameter | (1.2-) 1.4-3.3 (-3.6) mm | - |
| Presence of pruina in the apothecia | Present | - |
| Thalline margin | At first present, becoming extremely reduced or even absent at maturity. | - |
| Paraphyses | Simple, not enlarged apically | - |
| Paraphyses width | 1 µm thick | - |
| Asci length | (38-) 39-43 (-44) µm | - |
| Asci width | (9-) 10-12 µm | - |
| Ascospore length | 10-12 (-13) µm | Ascospores not seen |
| Ascospore width | 4-6 µm | Ascospores not seen |
| Ascospore septation | Generally 1-septate, more rarely 3-septate. | Ascospores not seen |
| Pycnidia | Present | Present |
| Pycnidia color | Pale | Pale |
| Pycnidia position | Mainly marginal, in some specimens also laminal. | Laminal |
| Pycnidia tuberculated | Mostly non tuberculated | Surrounded by thallus warts |
| Conidia length | (3) 4-5 µm | - |
| Conidia width | 1 µm | - |
| Secondary chemistry | Salazinic ± protocetraric acid | Salazinic ± protocetraric acid |

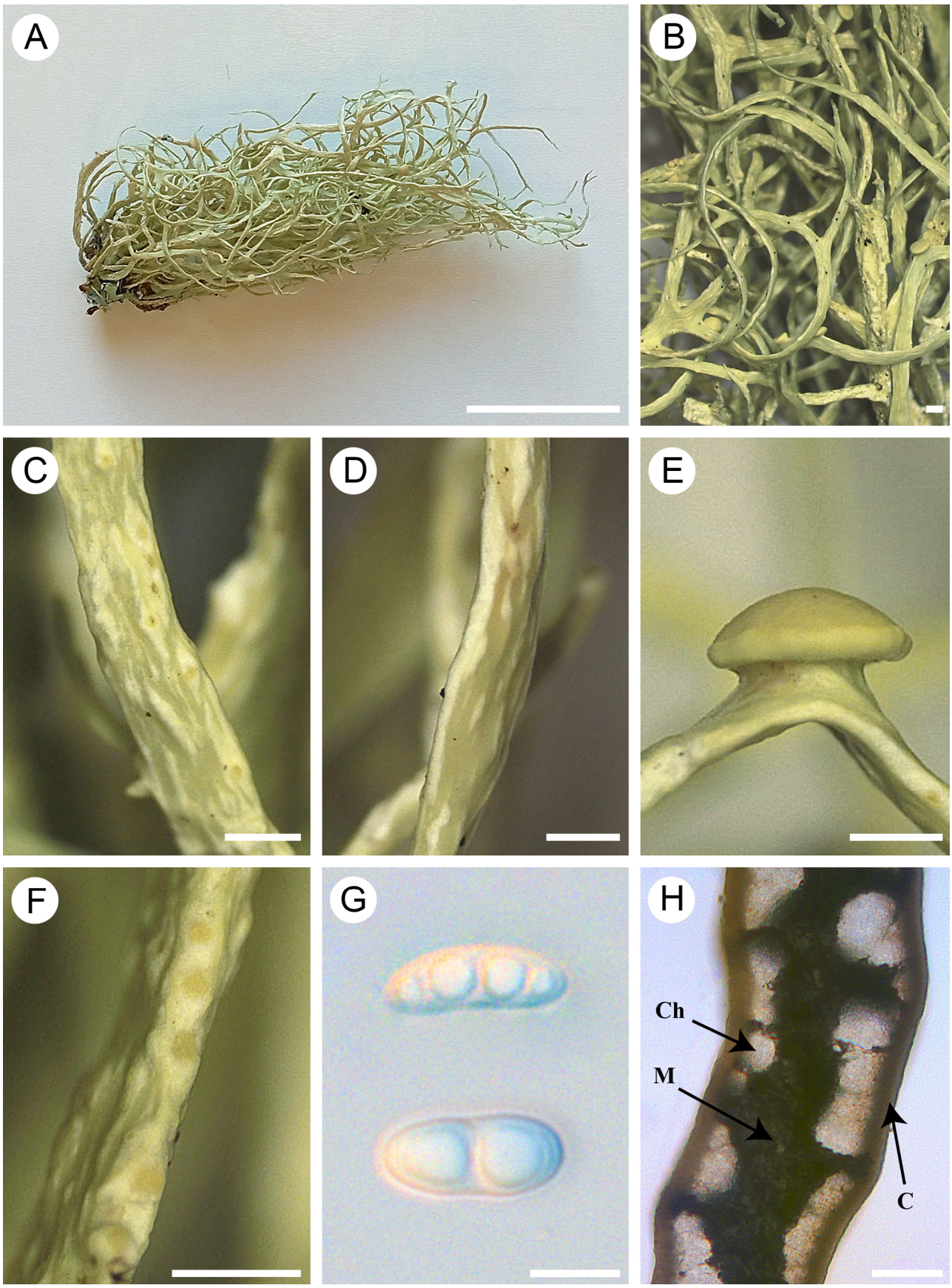


Figure 3. *Ramalina wirthii* collected in Flores Island (TFC-Lich 17106). Macroscopic and microscopic characters. A, Habit; B, Laciniae detail; C, Laminar pseudocyphellae; D, Marginal pseudocyphellae; E, Apothecium; F, Pycnidia; G, Ascospores; H, Thallus anatomy. Thalline layers are indicated as C (cortex), Ch (chondroid tissue) and M (medulla). Scale bars: A: 2 cm; B-F: 1 mm; G: 5 µm; H: 100 µm.

## Discussion

In this study we discovered a population of *Ramalina wirthii* in the island of Flores and characterized the new specimens by molecular, morphological and chemical means. We have found important differences with the original description of the species, which was based on specimens collected in the eastern islands of the archipelago (Aptroot & Schumm, 2008). Many of these differences, but not all, relate to the apothecial characters, reported here for the first time. Because of their morphological differences with the type material, we were not able to correctly identify the Flores specimens using the key provided by Aptroot & Schumm (2008). The main taxa with which the species could be confused are some of the species of the *R. decipiens* group, *R. decipiens* Mont., *R. hamulosa* Krog & Østh., *R. maderensis* Motyka, *R. nematodes* (Nyl.) Krog & Østh. and *R. pluviariae* Krog & Østh. (Krog & Østhagen, 1980a; Sérusiaux *et al.,* 2010; Spjut *et al.,* 2020; Blázquez *et al.,* 2024); some of the species of the *R. bourgaeana* group, *R. bourgaeana* Mont. ex Nyl. and *R. crispatula* Despr. ex Nyl. (Krog & Østhagen, 1980a; Sérusiaux *et al.,* 2010; Spjut *et al.,* 2020); two endemic species from Saint Helena, *R. geniculatella* Aptroot and *R. sanctae-helenae* Aptroot (Aptroot, 2008); an endemic species from Porto Santo, *R. jamesii* Krog (Krog, 1990); and a European maritime species, *R. cuspidata* (Ach.) Nyl. (LaGreca *et al.,* 2020). Of these species six have been recorded in the Azores archipelago: *R. bourgaeana* (Aptroot & Schumm, 2008), *R. crispatula* (Berger & Priemetzhofer, 2008), *R. cuspidata* (Tavares, 1952), *R. decipiens* (Tavares, 1952), *R. maderensis* (Blázquez *et al.,* 2024) and *R. nematodes* (Aptroot, 1989). *R. wirthii* can be easily distinguished from *R. bourgaeana* by thallus anatomy, as the former presents *decipiens*-type anatomy and the later *bourgaeana*-type (Krog & Østhagen, 1980a). *Ramalina wirthii* can also be separated in this way from *R. crispatula*, which also presents *bourgaeana*-type anatomy (Krog & Østhagen, 1980a). The confusion with *R. maderensis* may stem from the longitudinally arranged pseudocyphellae, often spurred apothecia and *decipiens*-type anatomy shared by the two species. However, both species can be easily separated by apothecial characters, as *R. maderensis* has concave or flat apothecia with a persistent thalline margin (Krog & Østhagen, 1980a) while *R. wirthii* does not. Among the remaining taxa in the *R. decipiens* group, *R. wirthii* can be separated straightaway from *R. nematodes* and *R. pluviariae*, as both species are characterized by the lack of a cortex (Krog & Østhagen, 1980a, b). The differentiation with the remaining two species of the group, *R. decipiens* and *R. hamulosa*, can be achieved by the aforementioned apothecial characters, as *R. hamulosa* is a sterile species (Krog & Østhagen, 1980a) and apothecia in *R. decipiens* are concave or flat and always present a thalline margin, often with a black ring surrounding the hamathecium (Krog & Østhagen, 1980a; Blázquez *et al.,* 2024) which we have not observed in the *R. wirthii* specimens. *R. wirthii* can be easily separated from the two St. Helena endemics, as both *R. geniculatella* and *R. sanctae-helenae* are large, pendant species that lack holdfasts (Aptroot, 2008). The species can be distinguished from the Porto Santo endemic *R. jamesii*, as this species is much smaller (up to 3 cm, Krog, 1990) and presents a cross section almost entirely filled up with chondroid tissue (Krog, 1990), while in *R. wirthii* this tissue appears as separated strands adjoining the cortex and, to a lesser extent, embedded in the medulla. The remaining species, *R. cuspidata*, is a European taxon (LaGreca *et al.,* 2020) whose separation from *R. wirthii* is rather straightforward, as the former species grows on wind-exposed seashore rocks, is markedly blackened at the base, and presents pycnidia with black ostioles (Cannon *et al.,* 2021), none of which is true for *R. wirthii.*

In addition to the previously discussed morphological differences, secondary chemistry is of great help to differentiate *R. wirthii* from these species. *R. wirthii* often contains salazinic and protocetraric acid at the same time (Aptroot & Schumm 2008), a combination that does not occur in the remaining taxa. In fact, most of the species here discussed contain secondary metabolites that are not present in *R. wirthii*, namely boninic, bourgeanic, divaricatic, lecanoric, norstictic, stictic and 4-*O*-demethylbarbatic acids (Krog & Østhagen, 1980a; Krog, 1990; Aptroot, 2008; Cannon *et al.,* 2021). The few specimens that contained only salazinic acid could be confused with *R. decipiens*, but the two species can be easily separated by the apothecial characters discussed in the previous paragraph. The presence of triseptate ascospores also has some diagnostic value. These spores are rather rare in *R. wirthii,* but when found they may be diagnostic. Most *R.* species produce 1-septate ascospores only (Aptroot & Schumm, 2008; Cannon *et al.,* 2021). There are some species in Australia (Stevens, 1987), Brazil (Kashiwadani & Kalb, 1993) and New Zealand (Blanchon *et al.,* 1996) that occasionally produce 2-septate, 3-septate and even 4-septate spores but, to our knowledge, *R. wirthii* is the only Macaronesian *Ramalina* to present ascospores with this septation.

## Conclusions

The *Ramalina wirthii* population discovered in Flores Island shows remarkable morphological differentiation with the original description of the species. Apothecial characters have been described for the first time.

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## Authorship contribution

MB: Conceptualization, Data curation, Formal analysis, Research, Methodology, Visualization, Writing - first draft, Writing - review and editing; ABP: Research, Writing - review and editing; IPV: Conceptualization, Fundraising, Research, Methodology, Management of the project, Writing - review and editing.

## Conflict of interest

None.

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