CLEMENTEANA 18, pp. 5-13
https://doi.org/10.62136/clementeana/18/art1

New Iberian records of *Caloplaca monacensis* (Leder.) Lettau

Nuevas citas ibéricas de *Caloplaca monacensis* (Leder.) Lettau

Olariaga I.[1,2]*
[1] Biology and Geology, Physics and Inorganic Chemistry department, Rey Juan Carlos University, C/ Tulipán s/n, Móstoles, 28933, Madrid, Spain.
[2] Aranzadi Society of Sciences, Mycology section, Zorroagagaina 11. PC 200014, Donostia-San Sebastián, Basque Country, Spain.
* ibai.olariaga@urjc.es

Abstract
Three new Iberian records of *C. monacensis* are reported and described. The material conforms to previous records of *C. monacensis*, except for the overall paler colour of the thalline exciple and thallus, differing from the *C. cerina* complex in darker apothecial discs, paler thalline exciple and thallus, and above all, in the granular thallus. In the analyses of the ITS region, sequences of *C. monacensis* do not form a supported clade, whereas in the Bayesian analysis two separated clades are retrieved, namely a main clade with nearly all the sequences, including a specimen from Soria sequenced here (ARAN-Fungi 19741), and another one that contains the only previous known Iberian collection from Cáceres (van den Boom 38821) that has a 218 b.p. insertion at the end of the 18S region and might belong to a related taxon. It is concluded here that further studies including other genetic markers are needed to understand the divergence of the ITS region within *C. monacensis*.

Key words: *Juniperus thurifera,* endangered species, species delimitation, *Caloplaca cerina*.

Resumen
Se citan y describen tres nuevas colecciones de *C. monacensis*. El material coincide con descripciones previas de la especie, salvo en el color más pálido del excípulo talino y el talo, y difiere a su vez de las especies del complejo *C. cerina* en el color más oscuro de los apotecios, el talo más pálido, y sobre todo, en el talo granuloso. En nuestros análisis de la región ITS *C. monacensis* no forma un clado apoyado filogenéticamente, e incluso, forma dos clados en el análisis bayesiano; uno formado por casi todas las secuencias de *C. monacensis*, incluyendo un espécimen de Soria (ARAN-Fungi 19741), y otro que incluye el único espécimen conocido previamente para la Península Ibérica (van den Boom 28821). Además, este último espécimen posee una inserción de 218 p.b. al final de la región 18S y podría pertenecer a otro taxon. Se discute la necesidad de realizar estudios más profundos utilizando otros marcadores genéticos y que ayuden a interpretar la divergencia de la región ITS en *C. monacensis*.

Palabras clave: *Juniperus thurifera,* especies amenazadas, delimitación de especies, *Caloplaca cerina*.

Sociedad Española de Liquenología

Introduction

The large heterogenous genus *Caloplaca* Th. Fr. s.l. has been progressively segregated into multiple genera (Arup et al. 2013), though generic delimitation in the *Teloschistaceae* is still unsettled (Llewellyn et al. 2023). The species around *Caloplaca cerina*, type of *Caloplaca* (Dodge & Baker 1938), form a small monophyletic group characterized by a white to dark grey crustose thallus lacking anthraquinones, but with presence of Sedifolia-grey pigment (K+ violet), and lecanorine —considered zeorine by Šoun et al. 2011)— apothecia with yellow to orange discs (Arup et al. 2013).

Due to the large morphological variation many names have been described in the group. Šoun et al. (2011) revisited the *C. cerina* group using morphological and molecular data of the ITS region and recognized 20 phylospecies in the Northern Hemisphere, most of which could also be separated through morphological characters, and of which at least 4 are present in the Iberian Peninsula, namely *C. cerina* s.l. (clades A and D3), *C. chlorina* (Flot.) H. Olivier (see also van den Boom & Rico 2006), *C. monacensis* (Leder.) Lettau and *C. stillicidiorum* (clades 1 and 5). *Caloplaca squamuloisidiata* van den Boom & V.J. Rico, despite resembling morphologically the species of the *C. cerina* group, does not belong to it (Vondrák et al. 2008).

This study deals with *C. monacensis*, a species of the *C. cerina* group only known from a single locality in Cáceres (Šoun et al. 2011: 127) and that we have collected twice recently. Given that *C. monacensis* is a forgotten species recognized by few authors, never cited in the Iberian literature and possibly endangered in Poland (Kubiak & Wilk 2016), encourage us to publish those new records with the hope that *C. monacensis* is found in the future in further locations.

Materials and methods

Morphological study

This study is centered around three specimens of *C. monacensis* recorded in Spain and deposited in the ARAN herbarium. Specimens were studied with a light microscope. Anatomical characters were observed from hand-cut sections observed in water. Statistics are based on measurements of 20 spores from each collection: L_m = mean length, W_m = mean width and $Q_m = L_m / W_m$. Extreme values are given in parentheses. Only spores with well-developed septa were measured

DNA extraction, PCR amplification, sequencing and alignment

DNA was extracted from dried material, using the DNeasy Plant Mini Kit (Qiagen). Primer combination used was ITS5-ITS4 (White et al. 1990). The same primers were used for sequencing. PCRs were conducted in Applied Biosystems GeneAmp® PCR System 9700 and 2720 Thermal Cyclers. Amplifications were performed through the following program: initial denaturation at 95 °C for 5 min, followed by 35–45 cycles of 95 °C for 45–60 s, 50–55 °C for 50 s, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were purified using the enzymatic method Exo-sap-IT (USB Corporation, Santa Clara, California, USA). PCR products were visualized in a 1 % agarose gel, stained with GelRedTM (Biotium Inc.) and a UV trans-illuminator. Purified PCR products were sequenced at Macrogen Spain service.

Sequences were edited and assembled using Sequencher v. 4.1.4 (Gene Codes Corp., Ann Arbor, MI) and deposited in GenBank. Additional sequences used by Šoun et al. (2011) were downloaded from GenBank, keeping two or three sequences from each clade, except for the clade of *C. monacensis* of which all available sequences were included. The

dataset was automatically aligned in Aliview (Larsson 2014), and manually adjusted. Ends were trimmed and a 218 b.p. insertion in the beginning of the ITS region was deleted from sequences HM538493 and HM538494 identified as *C. monacensis*. The ITS data matrix was subjected to Maximum Likelihood (ML) and Bayesian analyses. The ML analysis was conducted in IQ-TREE (Nguyen et al. 2015) performing 1000 ultrafast bootstrap replicates. The partitioning scheme and models were estimated through the Bayesian information criterion of ModelFinder (Kalyaanamoorthy et al. 2017) integrated into IQ-TREE. The Bayesian analysis was carried out in MrBayes v. 3.2.7 (Ronquist et al. 2012), using two parallel runs of eight Metropolis-coupled Markov chain Monte Carlo (MCMCMC) chains for 10 M generations, starting from a random tree, and sampling one tree every 100th generation from the posterior distribution. Substitution models were sampled across the GTR space during the MCMC simulation (Ronquist et al. 2012). Stationarity was assumed when average standard deviation of split frequencies fell below 0.01. A burn-in sample of 50 000 trees was discarded. To assess branch confidence, a 50 % majority rule consensus tree was computed with the remaining 150002 trees using the SUMT command of MrBayes. Maximum Likelihood Boostrap values and Bayesian posterior probability (PP) values ≥ 95 and ≥ 0.95 were considered to be significant, respectively.

Results

The ITS alignment comprised 48 sequences and 549 characters. The ML analysis of our dataset resulted in a single best ML tree of -lnL = 2141.819. Bayesian analyses reached an average standard deviation of split frequencies below 0.01 after 1 900 000 generations. The most likely tree of the Maximum Likelihood analysis is provided in FIG. 1 alongside Maximum Likelihood and Bayesian posterior probabilities. Basal nodes are not supported whereas most shallow clades are supported in both analyses. All sequences of *C. monacensis* included in the analyses form a clade that is not supported in the ML analysis (ML-BP 84), whereas in the MB analysis sequences of *C. monacensis* fall in two clades, namely the main clade comprising most sequences (BPP 0.82), including specimen ARAN-Fungi 19741 and a clade containing specimens v.d. Boom 38821 (HM538493) and JV3236 (HM538494), from Spain and Bulgaria, respectively. The main *C. monacensis* clade encompasses further three more supported clades, as well as sequences without a clear affinity.

Taxonomy

Caloplaca monacensis (Leder.) Lettau, *Hedwigia* 52(3-4): 240. 1912.

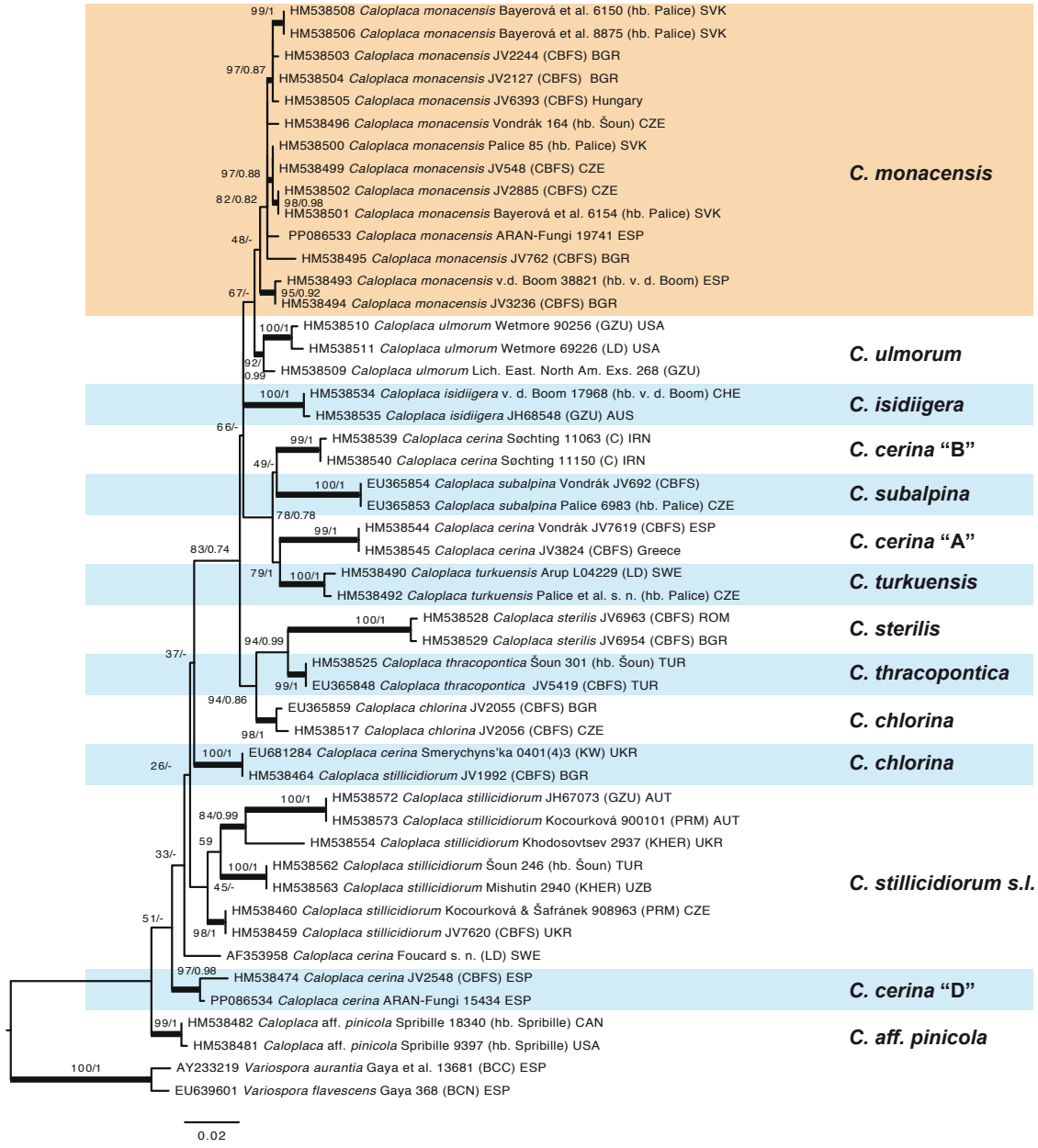
≡ *Pyrenodesmia monacensis* Leder., *Ber. Bayer. Bot. Ges.* 4: 26. 1896.

Lectotypus: Germany, an alten Strassenpappeln nicht weit vom Warthof bei Giesing, München, March 1896, leg. Lederer, M-0023624 (Arnold, *Lich. Monacenses Exs. 1896* no. 422, sub *Pyrenodesmia monacensis*).

FIGS. 2-5

Thallus granular, composed of coarse, irregularly globose granules, sometimes elongated and shortly finger-like, isidioid, 100–270 µm broad, pale greenish grey, often forming small, up to 300 µm wide, convex white squamules around apothecia, sometimes with minute erect hairs, 10–50 x 2–3 µm. Prothallus not observed.

Apothecia lecanorine, usually abundant, solitary to crowded, 0.5–1.4 mm diam., urn-shaped when immature, then expanded and disciform, sessile. *Disc* initially concave, then plane, slightly convex at the end, dark orange, sometimes with a faint white pruina. *Thalline exciple* white to pale grey, often rough, sometimes furrowed, raised over the disc, reduced



▲ FIG. 1. Best tree of the Maximum Likelihood analysis of selected ITS sequences of the *Caloplaca cerina* group. Maximum Likelihood ultrafast bootstrap values (ML-BP) / Bayesian Posterior probabilities (BPP) are shown on branches, ordered as ML-BP/BPP. Thickened branches received support at least in one analysis (ML-BP ≥95 % and/or BPP ≥0.95). GenBank accession numbers, original identification, voucher data, and ISO country code are provided in this order for each taxon.

◀ FIG. 2. *Caloplaca monacensis* (ARAN-Fungi 19741).

FIG. 3. A. *Caloplaca monacensis* (ARAN-Fungi 19554). B. *C. monacensis* (ARAN-Fungi 19741). C. *C. monacensis* (ARAN-Fungi 19741), showing a granular thallus. D. *C. monacensis* (ARAN-Fungi 19741), showing areolate thallus near young apothecia and pycnidia. E. *Caloplaca cerina*, clade D (ARAN-Fungi 15434), general aspect. F. *Caloplaca cerina*, clade D (ARAN-Fungi 15434), close-up, showing a more grey smooth thallus.

in old apothecia, 40–70 µm thick, sometimes pruinose, especially when young, due to hyaline crystals insoluble in K, with scattered erect, cylindrical to narrowly conical hairs, obtuse, slightly thick-walled, 8–11 × 2–2.5 µm. *True exciple* indistinct. *Epihymenium* orange, covered with granules. *Hymenium* 65–90 µm thick, hyaline. *Hypothecium* 40–60 µm thick, hyaline. *Algal layer* 150–300 µm thick. *Photobiont* green algae, not trebouxioid, 8–14 µm diam. *Asci* 43–53 × 9–15 µm, 8-spored. *Paraphyses* simple to branched, upper cells 2.5–3 µm broad. *Ascospores* polarilocular, ellipsoid to narrowly ellipsoid, (10–)11–15(–16) × (3.5–)

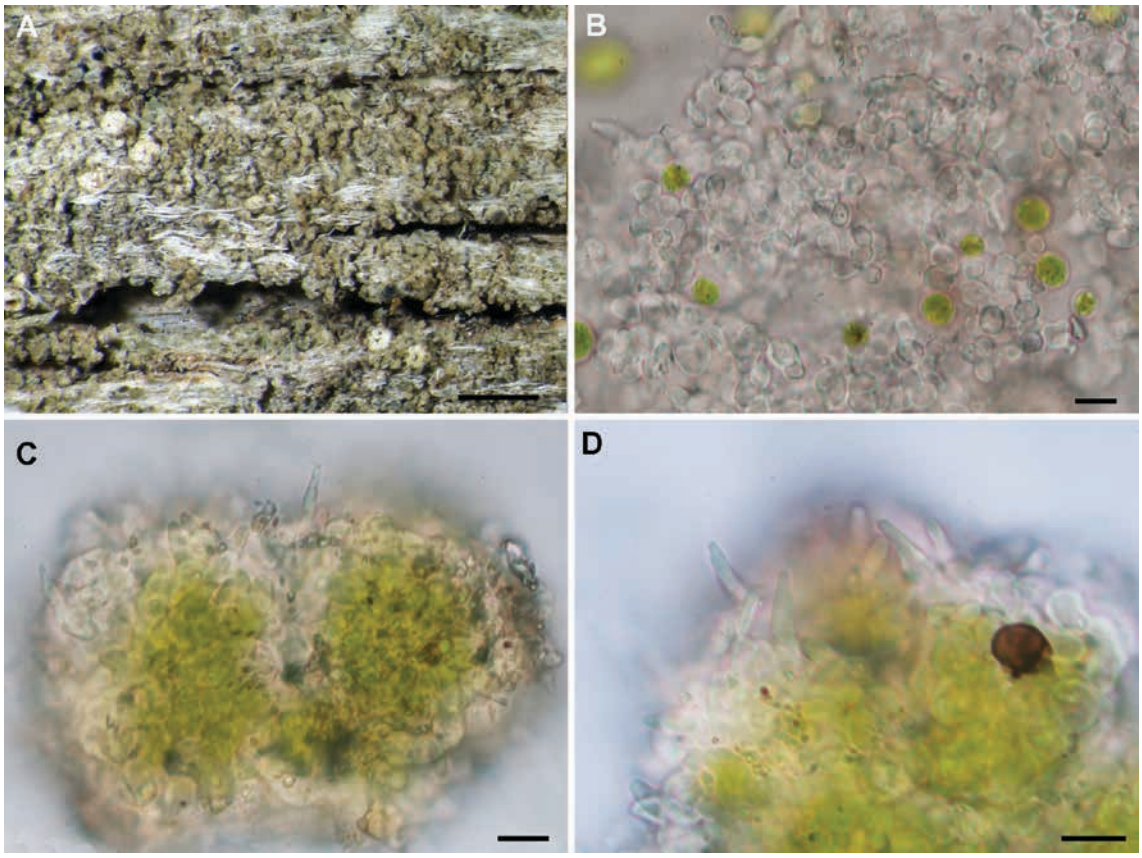


FIG. 4. *Caloplaca monacensis* (ARAN-Fungi 19741). A. Granular thallus. B. Cells of the surface of the thallus. C. Granule with protruding hairs. D. Detail of thallus hairs. Scale bars: A:1 mm B-D:10 µm.

5–6 µm, septum (3.0–) 3.5–5 (–5.5) µm wide (L_m=12.4–14.4, W_m=5.2–6, Q_m=2.1–2.8, ratio septum width/spore length 0.30–0.34. Pycnidia sometimes present, immersed, with a slightly raised ostiole, grey. Conidia bacilliform, some slightly curved, 3–4.5 × 0.8–1 µm.

Material examined: ESPAÑA. **Cuenca**: Las Majadas, Pajar de Zenón, 40.2686379 -1.8908692 (±10 m), euthrophic bark on isolated *Juniperus thurifera*, 16-Oct-2022, leg. M. Prieto & I. Olariaga, ARAN-Fungi 19554. **Soria**: Borobia, El Frontón, 41.64645 -1.91389 (±10 m), on bark of *Juniperus thurifera* in open forest, 17-Oct-2021, leg. I. Olariaga, ARAN-Fungi 19741. Ciria, Cañada de los Pozos, 41.6343711 -1.92507179 (±10 m), on bark of *Juniperus thurifera* in open forest, 17-Oct-2021, leg. I. Olariaga, ARAN-Fungi 19767.

Additional material examined: *Caloplaca cerina*. ESPAÑA. **Huesca**: Benasque, Cerler, 42.594261 0.542438 (±5 m), on bark of *Fraxinus excelsior*, 8-Aug-2020, leg. M. Prieto & I. Olariaga, ARAN-Fungi 15434.

Comments

Caloplaca monacensis is easily distinguished in the field by its lecanorine apothecia with a dark orange disc, pale grey, thalline exciple often rough, white to very pale grey and an entirely granular thallus with a few areoles near apothecia. Thalli of Iberian material possess erect cylindrical hairs as cited by Šoun et al. (2011: 126). We have observed also similar —but shorter— hairs on the thalline exciple.

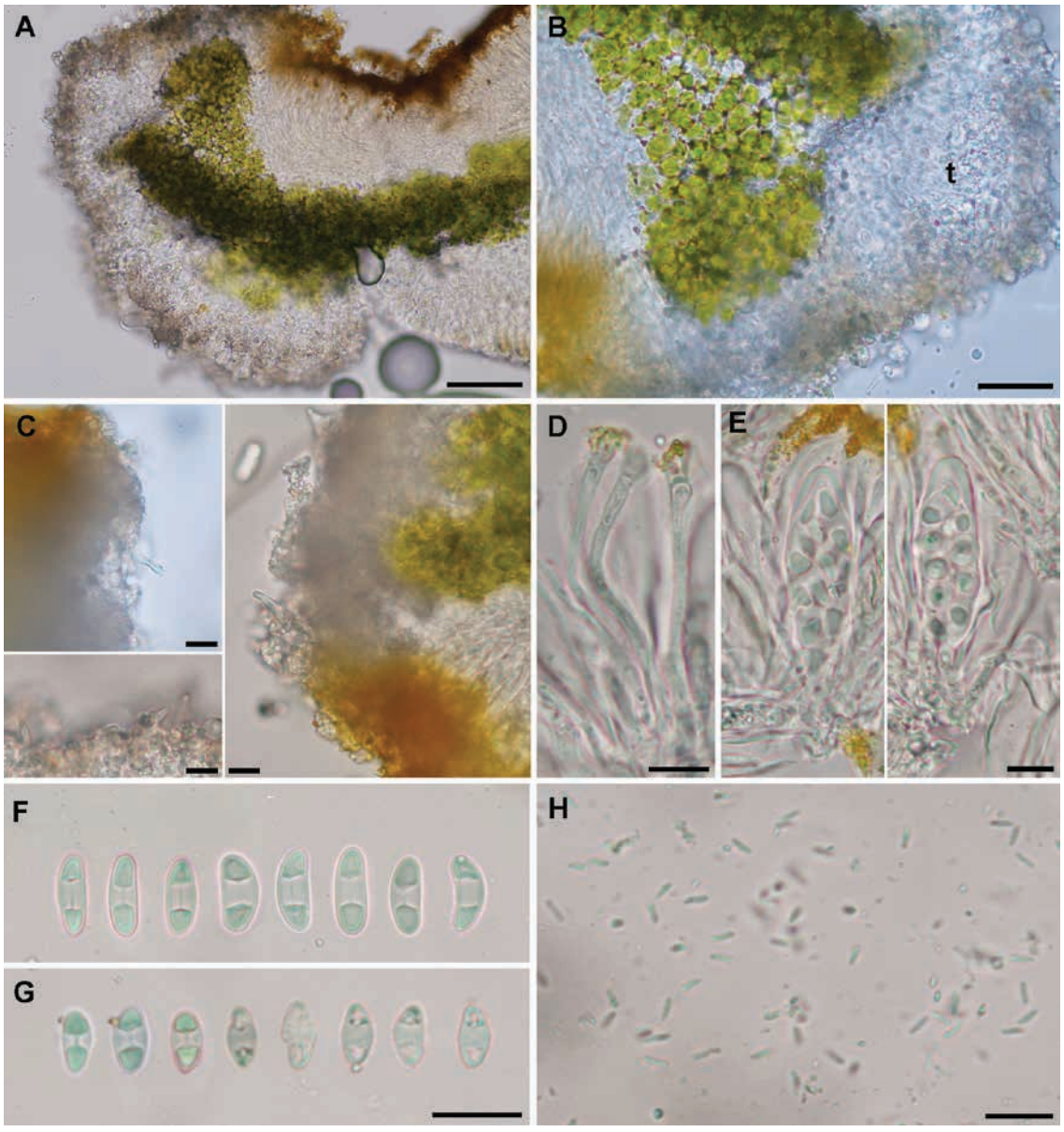


FIG. 5. Microscopic characters of *Caloplaca monacensis*. A. Apothecial section (ARAN-Fungi 19741). B. Close-up of apothecial margin, showing the thalline exciple (marked with a t) (ARAN-Fungi 19741). C. Hairs from the surface of the thalline exciple (ARAN-Fungi 19741). D. Paraphyses (ARAN-Fungi 19554). E. Asci (ARAN-Fungi 19554). F. Spores (ARAN-Fungi 19554). G. Spores (ARAN-Fungi 19741). H. Conidia. Scale bars: A: 50 µm; B-G: 10 µm. Mounting medium water.

It appears that this character may be of taxonomic significance as such hairs appear to be absent in other species of the group.

The three specimens studied here are macroscopically identical, but specimen ARAN-Fungi 19554 has longer spores (FIG. 5F) than ARAN-Fungi 19741 (FIG. 5G), probably due to the fact that they were collapsed, old or immature in the latter. The Iberian material examined here conforms otherwise to the description by Šoun et al. (2011), except for the overall colour of the thallus and thalline exciple that is paler in our material. *Caloplaca cerina* s.l., apparently a species complex,

usually differs by having a paler orange-yellow disc, darker grey thalline exciple, and a usually smooth thalline exciple and thallus. According to Šoun et al. (2011: 122), however, the thallus of specimens of clade D of *C. cerina* may be warted. *Caloplaca squamuloisidiata*, despite not belonging to the *C. cerina* group (Vondrák et al. 2008) resembles *C. monacensis*, from which it differs in having well-developed isidia (up to 500 µm), lacking hairs on thallus and thalline exciple surface and being saxicolous on acid rocks (van den Boom & Rico 2006).

At molecular level (FIG. 1), the ITS sequence of specimen ARAN-Fungi 19741 nests in the *C. monacensis* clade, not supported in any analysis as in Šoun et al. (2011). This is here interpreted as a lack or resolution of the ITS region to discriminate species within the *C. cerina* group, probably due to incomplete lineage sorting (Ulf Arup, pers. comm.). Interestingly, sequences attributed to *C. monacensis* show some sequence divergence. The ITS sequence of specimens van den Boom 38821 (HM538493, collected in Cáceres) and JV3236 (HM538494), both nesting outside the main *C. monacensis* clade in the Bayesian analysis, have a 218 b.p. long insertion at the end of the 18S that is missing in the sequence of ARAN -Fungi 19741 (PP086533) and the rest of sequences in the main clade of *C. monacensis.* The presence of this insertion, of possible evolutionary significance, would require further investigation. Despite the lack of support in the ITS analyses, we think that the Iberian material studied here should be referred to *C. monacensis*. The use of other markers for the study of the *C. cerina* group will allow a further assessment of current species hypotheses, as well as for understanding existing ITS-sequence divergence within *C. monacensis*.

The three Iberian specimens of *C. monacensis* studied here were collected in open woodlands with ancient *Juniperus thurifera* trees. Accompanying species were *Alyxoria varia* (Pers.) Ertz & Tehler, *Athallia pyracea* (Ach.) Arup, Frödén & Søchting, *Diplotomma pharcidium* (Ach.) M. Choisy, *Myriolecis hagenii* Ach.) Śliwa, Zhao Xin & Lumbsch, *Rinodina mayrhoferi* A. Crespo and *Xanthoria parietina* (L.) Th. Fr. Nevertheless, *C. monacensis* has been cited from several phorophytes, such as *Acer, Fagus, Fraxinus, Juglans, Ostrya, Pistacia, Populus, Pyrus, Quercus, Salix, Tilia* and *Ulmus* (Šoun et al. 2011; Kubiak & Wilk 2016). Vondrák et al. (2009) cited a collection on shaded calcareous rocks as well. Our search for further records of *C. monacensis* in the Iberian Peninsula yielded only one more record from Cáceres (van den Boom 38821; Šoun 2011; Šoun et al. 2011), which requires further study. It is very possible that *C. monacensis* has been mistaken for *C. cerina* s.l. or other species in the past (Šoun et al. 2011; Kubiak & Wilk 2016). We hope this contribution helps to find further material of *C. monacensis* in the Iberian Peninsula, and help to include more markers of new specimens in phylogenies to be able to better understand the divergence of the ITS region.

Acknowledgements

I feel indebted to María Prieto for her support, guidance and nice companionship during field excursions, as well as to the colleagues of the Aranzadi Science Society for efficiently processing the specimens deposited in ARAN-Fungi.

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