Research Article

Marta V. Freitas, Marco Simões, Christian Valdez, Clélia Afonso, Beatriz G. Trindade, Ana Correia, Teresa Baptista and Teresa Mouga*

Identification of Gracilariaceae (Rhodophyta) of central Portugal by histological and genetic methods

https://doi.org/10.1515/bot-2021-0017
Received February 24, 2021; accepted September 17, 2021; published online December 8, 2021

Abstract: This study aims to identify different populations of Gracilariaceae collected from the central coast of Portugal through light microscopy, anatomical observations and genetic tools, essential approaches to correctly assign species identity. Samples were obtained from Ria de Aveiro (AV), Figueira da Foz (FFBC, FFMD), and Lagoa de Óbidos (LOBR, LOEV, LOBS). Although histological observations offered a visual representation of the characteristic pseudoparenchymatous organization, they did not allow a clear distinction among the species. The amplification of a ~700 bp fragment of the mitochondrial cytochrome oxidase I gene, and its sequencing enabled us to assign the populations FFBC and LOBS to *Gracilaria gracilis*, and the populations AV, FFMD, LOBR, and LOEV to *Agarophyton vermiculophyllum*. This contribution will help phycologists to correctly identify the Portuguese populations of *Gracilaria sensu lato* at the species level, which will be crucial in ensuring that future studies and industrial exploration accurately target the correct species.

Keywords: genetic analysis; *Gracilaria* sp.; gracilarioids; seaweed histology; species identification.

1 Introduction

Gracilaria is a red seaweed genus extensively cultivated in East Asia (FAO 2020), both for its high market value as a source of agar, and as a food ingredient (Santelices 2014). *Gracilaria gracilis* is not only regarded as a valuable agarophyte (Gioele et al. 2017; Mensi 2019), but it is also the subject of extensive research worldwide regarding the optimization of culture methods and its biotechnological potential (Ben Said et al. 2018; Francavilla et al. 2013).

Within *Gracilaria sensu lato*, developmental and anatomical features, morphological homoplasies present in vegetative and reproductive phases, and the presence of cryptic species complexes, have occasionally been subject to new observations and nomenclatural changes, rendering any morphology-based taxonomic activities challenging (Gurgel et al. 2018; Iyer et al. 2004; Steentoft et al. 1995).

The high growth rates and biochemical potential of the gracilariods promoted their potential value among commercial seaweed growers. In addition to *G. gracilis*, authors have reported other gracilarioid species present on Portuguese shores, such as *Gracilaria bursa-pastoris* (Matos et al. 2006; Valente et al. 2006), *Gracilaria cornea* (Valente et al. 2006), *Gracilaria multipartita* (Pereira 2014; Polifrone et al. 2005), *Gracilariopsis longissima* (formerly reported as *Gracilaria verrucosa*; Ardré 1970) and *Agarophyton vermiculophyllum* (formerly known as *Gracilaria vermiculophylla*; Abreu et al. 2011; Mendes et al. 2013; Rueness 2005; Silva and Abreu 2014). However, it remains difficult to identify

Marta V. Freitas and Marco Simões contributed equally to this work.

***Corresponding author: Teresa Mouga**, MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal, E-mail: mougat@ipleiria.pt. https://orcid.org/0000-0003-4849-8840
Marta V. Freitas, MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal; and Department of Life Sciences, MARE – Marine and Environmental Sciences Centre, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal, E-mail: marta.freitas@ipleiria.pt. https://orcid.org/0000-0002-1164-224X
Marco Simões, Christian Valdez, Clélia Afonso, Beatriz G. Trindade, Ana Correia and Teresa Baptista, MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal, E-mail: marco.a.simoes@ipleiria.pt (M. Simões), christiandres1@hotmail.com (C. Valdez), clelia@ipleiria.pt (C. Afonso), biatrindade_93@hotmail.com (B.G. Trindade), anacorreia.2817@gmail.com (A. Correia), teresa.baptista@ipleiria.pt (T. Baptista). https://orcid.org/0000-0002-4167-5237 (C. Afonso). https://orcid.org/0000-0003-1168-7461 (T. Baptista)

these seaweeds to the species level based on morphology alone. This is especially pertinent for *A. vermiculophyllum*, which is currently reported as an invasive species in Europe (Krueger-Hadfield et al. 2017, 2018; Rueness 2005), including Portugal (Abreu et al. 2010, 2011; Presidência do Conselho de Ministros 2019; Villanueva et al. 2010). For this reason, work has been done on the identification and phenotyping of *A. vermiculophyllum* as well as on its reproductive system (Krueger-Hadfield et al. 2016, 2017; Sotka et al. 2018). Having proper additional tools and methodologies to accurately identify species is paramount, especially in the context of commercial seaweed cultivation, and in Portugal where the cultivation of alien species is strictly overseen by law (Presidência do Conselho de Ministros 2019).

Molecular methods have been used to solve taxonomic and identification problems of seaweeds, including red algae (Hassan et al. 2019). Gene markers that have been used to identify *Gracilaria* are the 5′ region of cytochrome oxidase subunit I (COI-5P) (Ferrer et al. 2019; Saunders 2005), the plastid-encoded large subunit of ribulose-1,5-biphosphate carboxylase (*rbc*L) (Gurgel et al. 2018), the intergenic spacer between the cytochrome oxidase subunits 2 and 3 (cox2–3 spacer) (Yow et al. 2013), the internal transcribed spacers (ITS) (Destombe et al. 2010) and the COI-5P which presents high interspecific variability, helping to identify red seaweeds at species level (Ferrer et al. 2019).

The current study aims to identify gracilarioid species collected along the Central coast of Portugal through histological observation and genetic methods. Specifically, it aims to precisely identify specimens of *Gracilaria gracilis*, *Agarophyton vermiculophyllum*, or *Gracilariopsis longissima*, all of which have previously been reported at our sampling sites. This is essential to assign correct species identity to sampled units, and to identify the local populations, helping to ensure that future studies and commercial seaweed exploration are focused on the correct target species.

2 Materials and methods

2.1 Gracilariod sampling

Gracilarioid samples were collected from one site in Ria de Aveiro (AV, 40° 38′59″N, 8°43′3″W), two sites in Figueira da Foz: Buarcos beach (FFBC, 40°9′57″N, 8°53′5″W) and Mondego Estuary (FFMD, 40°7′55″N, 8°50′37″ W), and three sites in Lagoa de Óbidos: Braço da Barrosa (LOBR, 39°24′19″ N, 9°11′12″W), Escola de Vela (LOEV, 39°24′36″N, 9°12′8″W), and Braço do Bom Sucesso (LOBS, 39°24′1″N, 9°13′11″W) (Figure 1). Individual sections of thalli destined for histological procedures were immediately fixed in buffered formalin (4%) for 24 h, and then in 70% ethanol, before processing, whereas portions of thalli destined for genetic analysis were stored at −20 °C upon arrival at the laboratory.

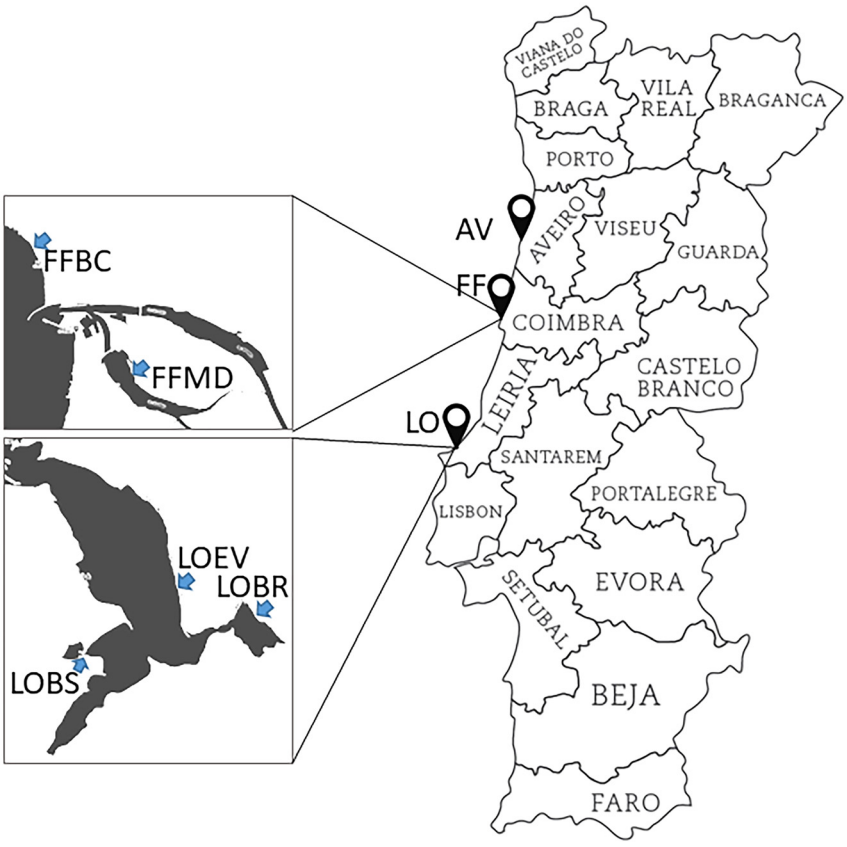

Figure 1: Map of Portugal showing *Gracilaria* sp. sampling sites Ria de Aveiro (AV), Figueira da Foz (FF), Lagoa de Óbidos (LO). Inset are FF and LO locations: FFBC: Figueira da Foz: Buarcos Beach, FFMD: Figueira da Foz: Mondego Estuary, LOBS: Lagoa de Óbidos: Bom Sucesso Branch, LOEV: Lagoa de Óbidos: Escola de Vela, and LOBR: Lagoa de Óbidos: Barrosa Branch.

2.2 Histological methods

The samples were dehydrated in an automatic tissue processor (Leica Biosystems TP1020) in the following ethanol series: 80% (1 h), 96% (three successive 2 h periods), 99% (three successive 1 h periods), followed by 1 h immersion in xylene and ethanol 99% (1:1), and 2 h clearing in xylene. Cleared samples were then immersed in a solution of xylene and paraffin for at least 15 min, until paraffin embedding and blocking. Transverse sections of 5 µm thickness were cut with a rotary microtome (Sakura Accut-Cut SRM 200), set in permanent slides, and stained with toluidine blue (FFMD, LOEV, and LOBS) or hematoxylin and eosin (AV, FFBC, and LOBR) prior to image viewing. Slides were observed under an optical microscope (Leica DM2000 LED) coupled with a digital camera (Leica MC 170 HD) for image capture.

2.3 Molecular analysis

For amplification by the polymerase chain reaction (PCR) of the mitochondrial marker cytochrome *c* oxidase subunit 1 gene (COI-5P), the forward (M13LF3) and reverse (M13Rx) primers (Saunders and Moore 2013) were used. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. PCR was performed using 1× OneTaq Standard Reaction Buffer, 200 µM dNTPs, 0.2 µM of each forward and reverse primer, 1.25 units of OneTaq Polymerase enzyme (New England Biolabs), and ~0.75 ng DNA from each population in a total volume of 50 µL. PCR program consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 68 °C, with a final extension step at 68 °C for 5 min.

Amplifications were checked by electrophoresis in 1.0% agarose gel stained with Orange DNA loading dye (Thermo Scientific). The purification of the amplified fragment was performed with the GeneJET PCR Purification Kit (Thermo Fisher), according to manufacturer instructions, and sequenced by the Sanger method using the same forward and reverse primers at the Stab Vida company (www.stabvida.com). The obtained sequences were visualized in Chromas software (version 2.4), assembled in DNASTAR Lasergene MegAlign software (version 7.0) and edited by hand in DNASTAR SeqBuilder software (version 7.0), according to the quality sequence. Species identification was done using the Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information (NCBI) database (Zhang et al. 2000). After the bioinformatic analysis the sequences were submitted to GenBank with the following accession numbers: FFBC (MW465693), LOBR (MW465694), FFMD (MW465695), LOEV (MW465696), AV (MW465697) and LOBS (MW465698).

3 Results and discussion

3.1 Morphology and anatomy

Initial morphological studies identified LOBR as *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M.Irvine *et* Farnham, FFMD and AV as *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N. Norris *et* Fredericq, and LOEV, FFBC and LOBS as *Gracilaria gracilis* (Stackhouse) Steentoft, L.M.Irvine *et* Farnham. Besides macroalgal identification guides, AlgaeBase (algaebase.org) and Portuguese Seaweeds Website (http://macoi.ci.uc.pt/) databases were used, to confirm the presence of those species in the sampling sites. Anatomical observations assigned the samples LOBR and LOEV to the species *A. vermiculophyllum* (Figure 2A and C). The sections (Figure 2B and D provide representative examples) show two layers of radially elongated cortical cells, and two layers of subcortical cells. There is an abrupt transition between subcortex and medulla, with 6–8 medullary cell layers 400–550 µm in diameter, as reported by Muangmai et al. (2012) and Gurgel et al. (2018). Reproductive structures were not observed for these populations, although seasonal observations were performed between April 2017 and November 2018. Figure 2B also assigns the LOEV sample to *A. vermiculophyllum*, although morphology initially identified it as *G. gracilis*. Thus, anatomical studies were a beneficial tool to complement species identification approaches.

Through morphological observations we believe that all the populations observed in LOBR, LOEV, FFMD and AV are tetrasporophytes of *A. vermiculophyllum*, morphologically similar to male gametophytes, which are much more branched and thinner than cystocarp-bearing female gametophytes. Whereas samples from LOBR, FFMD and AV were often found loose on muddy substrata under *Ul*va sp. in the intertidal zone, LOEV samples were found attached to rocks or pebbles on sandy bottoms, in accordance with the observations of Muangmai et al. (2012). *Agarophyton vermiculophyllum* may grow up to 80 cm, but usually reaches up to 30 cm. The reddish-brown cylindrical branches are alternately, irregularly or unilaterally branched 3–4 times (Muangmai et al. 2012). There is large variation in growth form, ranging from bushy to long and straggly (Bunker et al. 2017). A discoid holdfast is often absent due to fragmentation of the thalli.

The cell organization displayed by the samples corresponding to *A. vermiculophyllum* is also observed in *Gp. longissima*. According to Steentoft et al. (1995), the sections show a deep cortex of two cell layers and a subcortex that is distinct from the medulla, with the major difference that the medulla of *Gp. longissima* is only 3–5 medullary cell layers in diameter. *Gracilariopsis longissima* occurs in sheltered intertidal to subtidal zones, either attached to bedrock and stones or drifting freely. The cystocarps are subspherical, wider than they are high, with a slight basal constriction and an apical central pore (Steentoft et al. 1995). Although morphological identification of the sample LOBR indicated *Gp. longissima*, due to its strong bushy appearance and irregular branching as described by Steentoft et al. (1995), histological analysis revealed it was

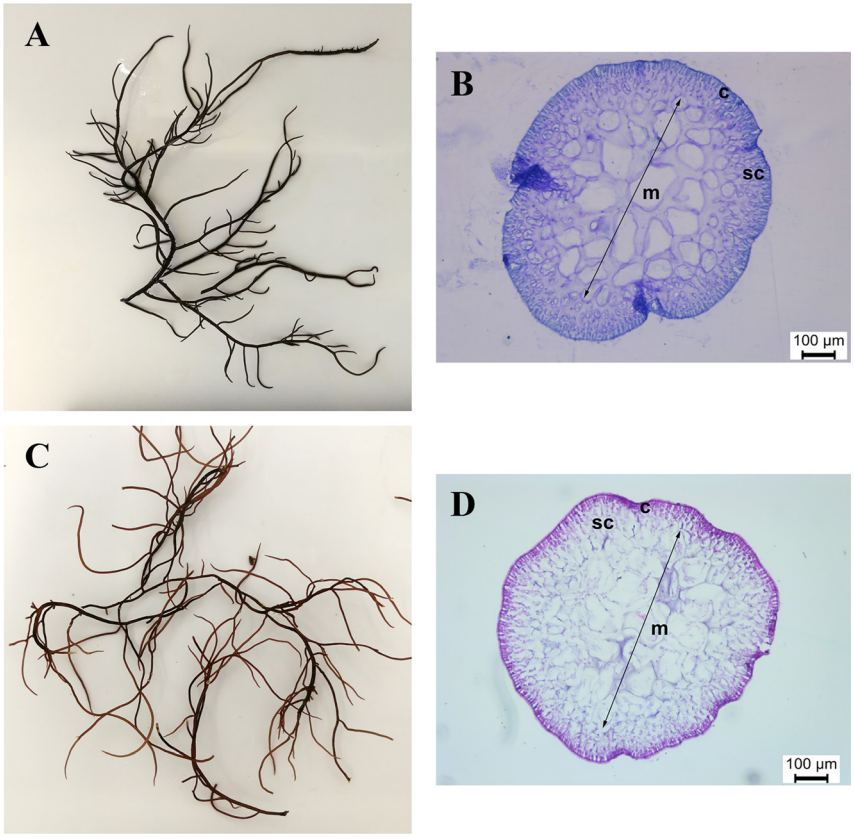

Figure 2: Macroscopic image of (A) gracilarioid from LOEV, genetically identified as *Agarophyton vermiculophyllum*, with (B) corresponding transverse section (10×) stained with toluidine blue, showing cortex (c), subcortex (sc), and medulla (m). Macroscopic image of (C) gracilarioid from LOBR, genetically identified as *Agarophyton vermiculophyllum*, with (D) corresponding transverse section (10×) stained with hematoxylin and eosin, showing cortex (c), subcortex (sc), and medulla (m).

6–7 medullary cell layers in diameter, thus potentially identifying the species also as *A. vermiculophyllum*. Such uncertainty increases due to the absence of reproductive structures which would provide further details to enable identification of these species.

Histological observations support the assignment of the samples FFBC and LOBS to *Gracilaria gracilis* (Figure 3A). The sections (Figure 3B being a representative example) show an easily visible cortex of two cell layers of small, densely pigmented, isodiametric cells (5–8 mm). A smooth transition between cortex and medulla is noticeable, with the medulla displaying 8–9 cells in diameter and a large central cell. The subcortex is formed by smaller cells than the medullary ones, but with no clear distinction between the two, and with the medullary cells growing in size towards the center of the thallus, as also described by Steentoft et al. (1995). In contrast to Bunker et al. (2017), however, the central medullary cells are larger than other medullary cells.

According to Steentoft et al. (1995), *G. gracilis* occurs attached by a small discoid holdfast to bedrock, pebbles or rocks, in sandy substratum in lower intertidal zones and rock pools, under running water at low tide, but in protected sites. These features characterize the sampling sites of FFBC and LOBS, with the former a region with a shoreline rich in protected rock pools, and the latter a region sheltered within a semi-enclosed lagoon system connected to the Atlantic Ocean. The holdfast of *G. gracilis* measures 10 mm in diameter and produces 2–4 orders of lateral, irregularly branched dark-red axes, up to 120 cm in length, and 2.5 mm in diameter. Reproductive cystocarpic thalli were often observed throughout the year (Figure 4A). Cystocarps are subspherical, as wide as they are high (0.5–1 mm), with an apical off-centre pore when the cystocarp is immature and mamillate with a marked basal constriction, and a central pore upon maturation.

Gracilaria gracilis tetrasporangia are not visible to the naked eye (Bunker et al. 2017; Steentoft et al. 1995) (Figure 4B). This species could be observed in a "short-growth" stage in the Buarcos sample site (FFBC) and in a "long-growth" stage in the Bom Sucesso sample site (LOBS). Thus, the only distinguishing anatomical characteristics between the three species are (1) the abrupt transition between the cortex and the medulla, a feature that does not

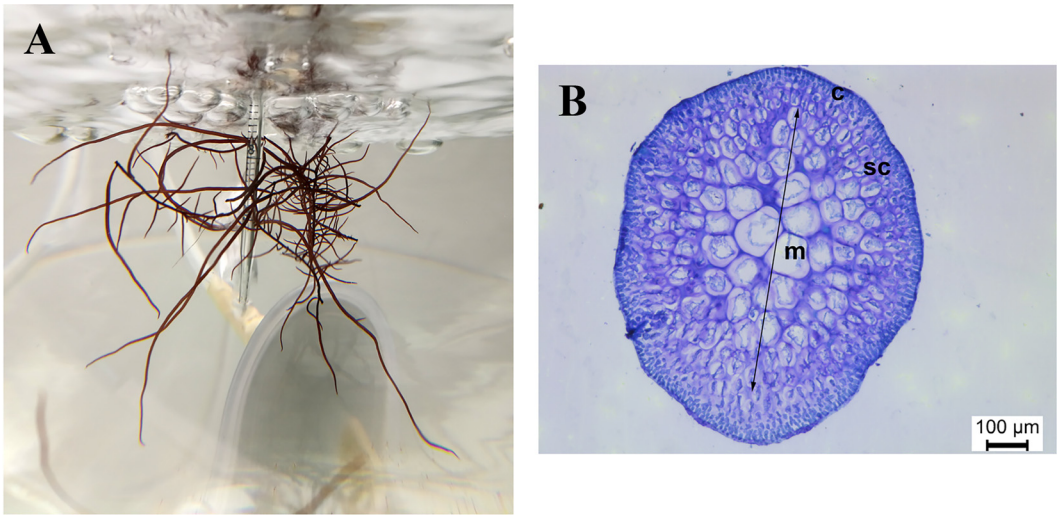

Figure 3: Thallus of (A) gracilarioid from LOBS, genetically identified as *Gracilaria gracilis*, with (B) corresponding transverse section (10×) stained with toluidine blue, showing cortex (c), subcortex (swc), and medulla (m).

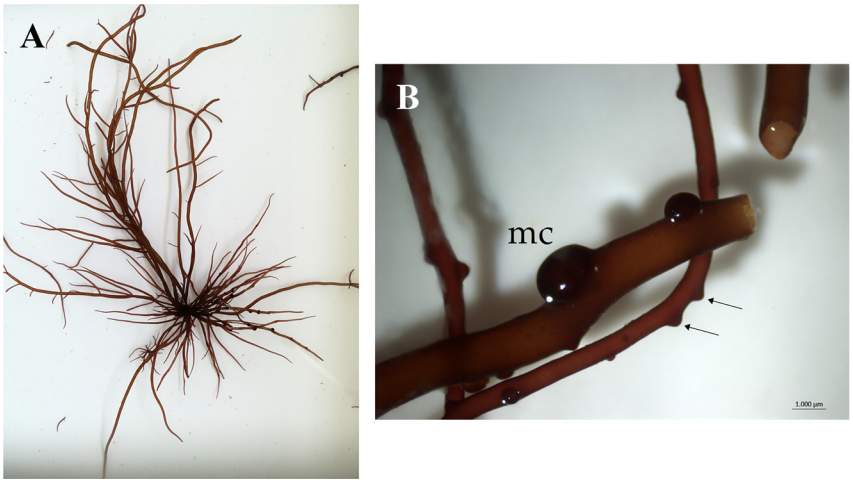

Figure 4: Macroscopic image (A) of gracilarioid, genetically identified as *Gracilaria gracilis*, bearing cystocarps, and (B) close-up of immature (small) and mature (large) cystocarps observed under a stereomicroscope (Zeiss Stemi 2000-C). Mamillate mature cystocarp (mc) and immature cystocarps (arrows) are visible.

occur in *G. gracilis*, (2) the smaller size of the central medullary cell, a feature that we could not confirm in *G. gracilis*, and (3) the number of medullary cells, being fewer in *Gp. longissima*. There are no clearly distinct morphological variations between the three species, although *G. gracilis* seems usually longer and less branched, displaying a strong dark-red color in its "long-growth" stage.

Cystocarps were not observed in the *A. vermiculophyllum* observed in this study, regardless of the sampling site, therefore hindering any attempt to distinguish between the three species through cystocarp observation. This observation is consistent with the findings of Krueger-Hadfield et al. (2016), who studied *A. vermiculophyllum* populations along their introduced range in European and North American coastlines, and report the widespread dominance of vegetative fragmentation, attesting to genetic signals. According to the authors, *A. vermiculophyllum* was favoured by vegetative fragmentation when invading European and North American coastlines, while the Pacific Ocean's native populations kept reproducing sexually. This happened due to the ecological shift from the Pacific's hard substratum (this substratum is a requirement for spore recruitment and settlement), to the European and North American soft mudflats, in which vegetative fragmentation was advantageous (Krueger-Hadfield et al. 2016). Krueger-Hadfield et al. (2016) also name sites where the presence of reproductive female thalli, bearing cystocarps, were occasionally reported, but none of these were in Portugal. However, Abreu et al. (2011)

observed unattached populations of *A. vermiculophyllum* in Ria de Aveiro and reported the abundant presence of reproductive female *A. vermiculophyllum* thalli bearing cystocarps, as well as tetrasporophytes, but no mature male thalli in three sampling sites within Ria de Aveiro.

The gracilarioids are one of the most difficult seaweed families to identify at the species level (Bunker et al. 2017) based on morphology alone, so that identification is easily subject to error. The transverse sections of the analyzed specimens are anatomically alike, especially in the "short-growth" stage, leaving lingering doubts on the anatomy of the gracilarioid thalli. While it was possible to identify both *G. gracilis* and *A. vermiculophyllum* without doubt, questions remained about whether sample LOBR was *A. vermiculophyllum* or *Gp. longissima*, due to the similarities mentioned above. A photographic registry showing details on cell organization is provided, which may be useful for future identification (Figures 2B, D and 3D).

3.2 Species identification through the COI-5P gene

In order to confirm the taxonomic identification, a fragment of ~700 bp was successfully amplified and sequenced for all samples. The obtained sequences were cross-checked with the NCBI database, showing that all queried nucleotide sequences had significant matches with known sequences, with FFBC (MW465693) and LOBS (MW465698) samples matching 100% with *Gracilaria gracilis* COI sequences, and AV (MW465697), FFMD (MW465695), LOEV (MW465696), and LOBR (MW465694) samples matching 100% with *Agarophyton vermiculophyllum* COI sequences. All six sequences matched *G. gracilis* or *A. vermiculophyllum* with an expected value (E value) of zero, determining the significance level between the obtained DNA sequences and the COI DNA sequences of algae from the GenBank database, implying a high similarity match (Karlin and Altschul 1990). This means that the identification through the taxonomic and the molecular methods agree, confirming and corroborating the classic method used. In addition, the results demonstrate that the molecular tools and the COI-5P gene region enabled identification and differentiation of morphologically similar algae, again proving that the high interspecific variability of the COI-5P gene allows us to distinguish red algae at the genus and species level (Kim et al. 2012; Kucera and Saunders 2012; Lyra et al. 2015; Ng et al. 2017), specifically gracilariods.

The semi-enclosed lagoon system of Lagoa de Óbidos harbors both *G. gracilis* and *A. vermiculophyllum*, yet current observations indicate that these species do not share the same habitat type. *Agarophyton vermiculophyllum* from sampling sites at LOBR, FFMD, and AV was abundantly scattered along a muddy substratum, along with the green seaweed *Ulva* sp. This is consistent with the observations of Krueger-Hadfield et al. (2016) on the habitat colonization of this invasive macroalgae. According to the authors, the presence of holdfasts in *A. vermiculophyllum* is only observed in the native populations in the northwest Pacific Ocean, whereas their invasive counterparts underwent an ecological shift from attached to unattached thalli to successfully invade the soft-sediment habitats in Europe and North America. On the other hand, *G. gracilis* in LOBS, and FFBC were collected from clearer running waters, with a holdfast attached to pebbles and other debris, or partially buried under a sandy substratum. The sampling site of LOEV corresponds to a transitional area between these two substrata with the samples here identified as *A. vermicullophylum*.

There have been a number of previous efforts to identify gracilarioids in Portugal, through genetic methods. The presence of *G. gracilis* was reported in Ria de Aveiro (Abreu et al. 2011) and the Tejo estuary (Destombe et al. 2010). The presence of *A. vermiculophyllum* has been reported in Ria de Aveiro (Abreu et al. 2011; Krueger-Hadfield et al. 2016, 2017; Saunders 2009), and Faro (Krueger-Hadfield et al. 2016, 2017; Rueness 2005). The presence of *Gp. longissima* was also reported in Ria de Aveiro (Abreu et al. 2011; Saunders 2009) and Faro (Rueness 2005). To the best of our knowledge, ours are the first records of *A. vermiculophyllum* and *G. gracilis* for Figueira da Foz, Buarcos, and Lagoa de Óbidos, which are supported by genetic evidence. Therefore, the occurrence of these gracilarioids in the aforementioned locations, supported by genetic methods, adds extra evidence to the existing data from other Portuguese locations, compiled by authors such as Rueness (2005), Destombe et al. (2010), Abreu et al. (2011), and Krueger-Hadfield et al. (2016, 2017).

To date, the NCBI database holds only 19 entries for the COI gene of *G. gracilis*, when compared to 214 entries corresponding to *A. vermiculophyllum* (and 311 entries corresponding to *G. vermiculophylla*). While the objective of the present work focused on assigning the sampled populations to genetic identities, further studies may be done to assess evolutionary lineages between these populations, to confirm the recent findings of previous authors (Gurgel et al. 2018; Lyra et al. 2015). An assessment of reproductive modes and population structure through genetic tools (Krueger-Hadfield et al. 2016, 2017), and a fine-tuned resolution of the present distribution of both *G. gracilis* and *A. vermiculophyllum* along the Portuguese coast, following Krueger-Hadfield et al. (2018), are also worth considering.

Similarly, several studies have used molecular tools to unequivocally resolve species complexes and taxonomic

identities in the class Florideophyceae (Saunders and Lehmkuhl 2005), including the order Gracilariales (Destombe et al. 2010; Gargiulo et al. 2006; Goff et al. 1994; Gulbransen et al. 2012; Gurgel et al. 2003, 2018, 2020; Gurgel and Fredericq 2004; Lyra et al. 2015). Taxonomic resolution has also been determined within the orders Gigartinales (Le Gall and Saunders 2010; Payo et al. 2013; Saunders 2005), Bangiales (Robba et al. 2006), Rhodymeniales (Saunders 2005), and Corallinales (Hind and Saunders 2013; Torrano-Silva et al. 2018). Likewise, studies have helped to identify species in regional seaweed communities (Alshehri et al. 2019), with occasional confirmation of exotic species unveiled by molecular tools (Montes et al. 2017). Macroalgal-related works supplementing morphological observations with molecular identifications offer reliability and are common practice nowadays. According to Saunders and Moore (2013), there is value in the assignment of biological entities to genetic species, with simple PCR reactions and sequence reads, to unravel species identities with greater certainty for routine identifications (Andreakis and Schaffelke 2012). Even to the most seasoned phycologists, the accurate identification of macroalgae is occasionally elusive, due to a range of particularities such as simple morphology and anatomy. This may lead to convergence, outstanding phenotypic plasticity in response to the environment, and poorly understood life histories (Saunders 2005). Therefore, the use of additional tools in taxonomic resolution of macroalgal systematics should be welcomed and increasingly adopted.

4 Conclusions

The present work assigned the correct species identity to the sampled taxa, a task which cannot be performed by visual observations alone due to the high morphological similarity between species. We are able to report the presence of both the native *G. gracilis* and the invasive *A. vermiculophyllum* on Portuguese shores. This was confirmed by anatomical and genetic methods, with their distribution likely to depend on the geographic location and substratum type. Considering the potential negative consequences that invasive species such as *A. vermiculophyllum* may have on native ecosystems and native species, including *G. gracilis*, as well as the legal restrictions currently in place regarding their commercial exploration, it is imperative that species identification is done accurately. In this context, the present report shows that species identity assessment using tools beyond morphological observations alone, is fundamental to properly identifying specimens. This has implications for both academic studies and commercial culture involving these species.

The presently suggested algal identities coupled with the corresponding morphological and molecular data will be particularly useful whenever future studies target wild gracilariods populations and require their assignment to a specific species identity.

Acknowledgements: The authors would like to thank Andreia Raposo for assisting in histological preparations, João Duarte for the image data photography. Credits are also due to Professor Paula Cardoso and Luke Devonald for reviewing the English language. Macroscopic and microscopic images are reproduced with the permission of Marta V. Freitas and João Duarte, respectively.

Author contributions: Conceptualization, Teresa Mouga. and Marta V. Freitas; methodology: Marta V. Freitas, Marco Simões, Christian Valdez, Beatriz G. Trindade, Ana Correia; validation, Marta V. Freitas, Marco Simões, Clélia Afonso, Teresa Baptista, Teresa Mouga; formal analysis, Teresa Mouga. Marco Simões, and Marta V. Freitas; resources, Teresa Baptista; writing –original draft preparation, Marta V. Freitas, Marco Simões; writing –review and editing, all authors; supervision, Clélia Afonso, Teresa Baptista, Teresa Mouga.; funding acquisition, Teresa Baptista. All authors have read and agreed to the published version of the manuscript.

Research funding: This research was funded by Fundação para a Ciência e Tecnologia (FCT), through the strategic project UIDB/04292/2020 granted to MARE, and the project Operational Programme MAR2020 through the project 16-02-01-FMP-84 – SeaWeedFeeds. Fundação para a Ciência e Tecnologia (FCT) also supported this research through the individual doctoral grant UI/BD/150957/2021 attributed to Marta V. Freitas.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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Bionotes

Marta V. Freitas
MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal
Department of Life Sciences, MARE – Marine and Environmental Sciences Centre, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal
marta.freitas@ipleiria.pt
https://orcid.org/0000-0002-1164-224X

Marta V. Freitas is a PhD student of University of Coimbra, and develops her work at MARE – Marine and Environmental Sciences Center in both Polytechnic of Leiria and University of Coimbra. She holds a degree in Marine Biology and Fisheries, a MSc degree in Conservation Biology and a MSc degree in Marine Resources Biotechnology. Her research and interests focus on seaweed biotechnology and seaweed culture. She published five scientific papers and participated in several international scientific conferences.

Marco Simões
MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal
marco.a.simoes@ipleiria.pt

Marco Simões is a PhD student of Porto University and MARE-Polytechnic of Leiria. He has a MSc degree in Biotechnology of Marine Resources and a BSc in Marine Biology and Biotechnology. He has developed work on diverse molecular areas, genetic and metabolic pathways of plants, pathogen detection in fish samples, microorganism and algae species identification, metagenomic

analysis and microsatellite markers. He has also helped to direct some students and participated on the publication of seven posters and five scientific papers.

Clélia Afonso
MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal
clelia@ipleiria.pt
https://orcid.org/0000-0002-4167-5237
Clélia Afonso has a PhD in Agronomic Engineering (Plant Biotechnology), and is a post doc in Cellular and Microbial Physiology. She is a member of MARE – Center for Marine and Environmental Sciences and Adjunct Professor of the School of Tourism and Technology of the Sea from the Polytechnic of Leiria. She has participated in several research projects a published several papers. Research interests: Applied Biotechnology, namely by the use of seaweeds, microalgae and plants and detection of their bioactivities and nutritional properties.

Teresa Baptista
MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal
teresa.baptista@ipleiria.pt
https://orcid.org/0000-0003-1168-7461

Teresa Baptista is a professor in the School of Tourism and Maritime Technology in Aquaculture. She has a Master's degree from Bangor University School of Ocean Sciences and is a specialist in aquaculture. She is responsible for the Aquaculture Lab of the MARE Leiria Polytechnic and has worked on the study of the biology of species with a focus on bivalve molluscs. She has also developed research work in pathology in aquaculture with the development of pathology diagnosis techniques as well as the study of host-pathogen interaction, with time course assays. She was the coordinator of Seaweedfeeds, the project that supported this paper.

Teresa Mouga
MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, Edifício CETEMARES, Av. Porto de Pesca, 2520-641 Peniche, Portugal
mougat@ipleiria.pt
https://orcid.org/0000-0003-4849-8840

Teresa Mouga is a senior professor in the School of Tourism and Maritime Technology (ESTM), Polytechnic of Leiria, since 2000, chair of the Scientific Committee of ESTM, since November 2019 and a researcher at MARE- Marine and Environmental Sciences Center. Her main domain of specialization are the growth and cultivation of seaweeds, seaweed bioactivities and surveys of littoral seaweeds to study the ecology of invasive and native species. She has published 17 scientific papers, three books, two book chapters, besides other written scientific contributions, and participated/managed more than 20 research projects.