Laurencia mediterranea sp. nov. (Ceramiales, Rhodophyta) from the central Mediterranean Sea

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Abstract

The identification of macroalgal species within the *Laurencia* complex is challenging, due to the presence of inconspicuous morphological characters, extensive variation in these traits and a diverse biogeography. Moreover, for a number of these species, no DNA sequence data are available in online databases. For this study, five algal specimens, tentatively assigned to *Laurencia*, were collected from Malta in the central Mediterranean Sea and studied using an integrative systematics approach. An analysis of the data resulted in the description of a new species, *Laurencia mediterranea* using combined morphological and molecular criteria, including COI-5P and *rbc*L. Morphologically *L. mediterranea* sp. nov. was distinct from other *Laurencia* spp. in the type of holdfast, the colour and shape of the thallus, the presence of secondary pit connections, lenticular thickenings and *'corps en cerise'*. Moreover, a new genetic species cluster indicated a separate taxon at the species level. Our study demonstrates that genetic sequences having clear links to sample metadata and digital herbaria are indispensable for macroalgal biodiversity research. This approach could offer a solution for algal taxonomy in cases where biogeography is important and limited sequence data exist in online DNA libraries.

Keywords: algal biodiversity; DNA barcoding; herbarium; *Laurencia*; Malta.

Introduction

Inaccuracies in the morphological identification of marine algae are a common occurrence worldwide. This happens due to the existence of morphologically cryptic taxa that possess inconspicuous morphological features or exhibit great morphological plasticity. Such challenges have previously been highlighted for the Florideophyceae and Ulvophyceae in the central Mediterranean, but they also exist in other algae (Bartolo et al. 2020~~,; Bartolo et al.~~ 2022a, b, c). A case in point is the classification of *Laurencia* spp. within the *Laurencia* complex, in which the morphological identification of species can prove to be challenging (Rousseau et al. 2017).

Such species misidentifications have also occurred in the Mediterranean Sea. In fact, until 1981, *Laurencia* specimens growing in the Mediterranean and possessing "*corps en cerise*" (globular, hyaline bodies) were identified as *Laurencia obtusa* (Hudson) J.V.Lamouroux. However, this characteristic was found not to be exclusive to *Laurencia* spp., according to Verlaque (1981). Since then, a more thorough morphological and molecular analysis has revolutionised the identification of *Laurencia* spp., leading to the recognition of species having distinctive morphological features (Rousseau et al. 2017). Examples include the elevation of taxa such as *Yuzurua* (K.W. Nam) Martin-Lescanne from a subgeneric to generic rank (Martin-Lescanne et al. 2010), the description of new genera, such as *Ohelopapa* F.Rousseau, Martin-Lescanne, Payri *et* L.Le Gall (Rousseau et al. 2017) and the transfer of species between genera of the *Laurencia* complex, for instance from *Chondrophycus* (J.Tokida *et* Y.Saito) Garbary ~~&~~*et*

J.T.Harper to *Palisada* (Yamada) K.W.Nam (Cassano et al. 2012a). Studies have demonstrated how various species have been misidentified between different regions, for instance taxa previously cited as *Laurencia filiformis* (C. Agardh) Montagne, *L. majuscula* (Harvey) A.H.S Lucas, *L. obtusa, L. arbuscula* Sonder, *L. composita* Yamada and *L. microcladia* Kützing in Brazil (Cassano et al. 2012a) and *L. majuscula* in the Canary Islands (Machín-Sánchez et al. 2014), in fact all are representative the same taxonomic entity, *L. dendroidea* J. Agardh, whose type locality is Brazil. Such studies underline the value of molecular sequences and the importance of phylogenetic studies in the description of algal diversity (Cassano et al. 2012a; Machín-Sánchez et al. 2014; Martin-Lescanne et al. 2010; Rousseau et al. 2017).

In fact, the combination of molecular and morphological studies of marine algae growing around the coastline of the Maltese islands, has recently revealed new microalgal (Schembri and Zammit 2022; Zammit et al. 2021) and macroalgal diversity (Bartolo et al. 2021, 2022a, b, c). Moreover, new microalgal strains and species have been described from diverse habitats in Malta on the basis of morphological, molecular and biochemical criteria (Zammit 2018, 2019; Zammit et al. 2010, 2012; Zammit and Agius 2022).

Herbarium specimens have become essential in ensuring the traceability of DNA barcodes and in this regard, the use of herbaria as an obligatory voucher specimen for macroalgal DNA barcodes has even been suggested (Rimet et al. 2021). In fact, linking sample metadata in digital herbaria to DNA barcodes, provided these data are available, might indeed offer the ideal solution to the current data quality limitations of DNA reference libraries (Rimet et al. 2021).

In an attempt to improve standards for biodiversity studies in morphologically cryptic species from the central Mediterranean Sea, we have adopted these principles to facilitate identification of *Laurencia* spp. specimens. To this end, samples were obtained from mature algal specimens growing around the Maltese coastline. These were preserved in herbaria, observed microscopically and processed for DNA analysis. Sequencing of the mitochondrial cytochrome c oxidase subunit 1 (COI-5P) gene and the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) ensued. The phylogenetic relationships of the Mediterranean specimens with other *Laurencia* spp. having a diverse biogeography were analysed.

Materials and Methods

Four coastal sites off Malta, in the Mediterranean Sea, were selected on the basis of varying degrees of anthropogenic pressures on the marine habitat. These are shown Figure 1 and Figure S1 and include an aquaculture facility at St. Paul's Islands, a desalination outfall site at Ċirkewwa and the popular tourist areas at Golden Bay and St. Paul's Bay. Thalli of mature red algae were sampled at an approximate depth of 2 m. The provenance of samples, including spatial data, was recorded by means of a hand-held Garmin 78s Marine Global Positioning System (GPS) device (Kansas, United States). Underwater photographs were taken with an Olympus TG-4 camera (Tokyo, Japan).

Five specimens were collected in all from these locations and these were labelled C182, C183, C197, C200 and C201 (Table 1; Figures 1, S1). All specimens were epilithic in the infralittoral zone (Figure S2). Algal specimen C182 was collected from the waters close to St. Paul's Island, C183 from a pier in

Golden Bay, C197 from Ċirkewwa, while both C200 and 201 were collected from the waters beneath the Wignacourt Tower in St. Paul's Bay.

Fragments of the thalli of macroscopic algae were stored in cetyltrimethylammonium bromide (CTAB) for subsequent DNA extraction. The remaining parts were dried on Bristol paper for preservation in a herbarium. The herbarium sheets were digitally photographed, and stored at the Malta Algal Culture Collection (MACC) (Zammit 2016) and the Herbarium of the University of Malta.

Sections were prepared from rehydrated herbarium material. These were stained with 1% aqueous aniline blue acidified with dilute HCl or acetocarmine, and were mounted in 50% dilute corn syrup (Rousseau et al. 2017). The samples were studied using a Nikon Eclipse Ti-S inverted microscope (Tokyo, Japan) connected to a Nikon Digital DS-Fi 1 camera (Tokyo, Japan) and a Zeiss Axio Imager.D2™ (Oberkochen, Germany) compound microscope. Taxonomic keys (Furnari et al. 2001, Rodríguez-Prieto et al. 2013) were utilised to morphologically identify the species. AlgaeBase (Guiry and Guiry 2023) was consulted for current taxonomy and nomenclature.

DNA was extracted from the samples stored in cetyltrimethylammonium bromide (CTAB), using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts USA). Partial COI-5P and *rbc*L sequences were amplified using the primers listed in Table 2. PCR amplifications were performed in a total volume of 50 µl, containing

approximately 100 ng of DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), supplemented to give a final concentration of 1.8 mM $MgCl_2$, 0.625 U of OneTaq Quick Load 2x Master Mix with Standard Buffer (New England Biolabs Inc., Massachusetts, United States) and 0.5 pmol of each primer.

Amplifications were carried out in a GeneAmp thermocycler PCR system 2700 (Applied Biosystems, Foster City, CA, USA) or T3000 thermocycler (Biometra, Jena, Germany) according to the PCR programmes listed in Table 3. PCR products were verified on 1% (w/v) agarose gel. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced via a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, California, USA).

The sequences were manually checked and compared to published sequences using the Basic Local Alignment Search Tool (BLAST) of the United States National Centre of Biotechnology Information (NCBI) (Zhang et al. 2000). The 5′ and 3′ ends were trimmed due to variable sequence lengths, which resulted in a final length of 664 bp for the COI-5P alignment and 1,287 bp for the *rbc*L alignment. A total of 49 COI-5P sequences and 64 *rbc*L sequences were included and further sequence data are reported in Supplementary Table S1.

The two datasets were analysed for the genus *Laurencia*. Multiple alignments of the *rbc*L and COI-5P biomarkers were performed using the MAFFT algorithm L-INS-I (Katoh and Standley 2013) on the NGPhylogeny portal (Lemoine et al. 2019). MEGA-X (Kumar et al. 2018) was used for calculation of

inter- and intraspecific COI-5P and *rbc*L distances using the pairwise distance model (p-distance).

Bayesian Inference (BI) was performed using MrBayes v. 3.2.7 (Ronquist et al. 2012) on the NGPhylogeny portal (Lemoine et al. 2019) and Maximum Likelihood (ML) analyses were carried out using MEGA X (Kumar et al. 2018). The ML analyses were conducted using the general time reversible + gamma distribution + invariable sites (GTR + G + I) model (Nei and Kumar 2000). This was determined from the Maximum Likelihood scores implemented in jModelTest 2.1 software (Darriba et al. 2012), with 1000 bootstrap replicates. BI analyses were run with the GTR + G + I model parameters estimated independently for each partition, with four Monte Carlo Marko Chains for 2 million generations. Nodal support was assessed by calculating the posterior probability (PP) values for each node of the resulting consensus tree after a burn-in value of 25% of the trees. Both ML and BI analyses produced trees with a similar topology. Viewing and editing of the phylogenetic trees were carried out in FigTree v. 1.4.4 (Rambaut 2012).

Results

Morphological observations

The five specimens, from different locations in Malta, were morphologically similar (Figures 2-6), and a detailed taxonomic description is given in the section 'taxonomic treatment'. To assist in distinguishing between *L. mediterranea* sp. nov and other *Laurencia* species occurring in the Mediterranean, a comparison of morphological characteristics is given in Table 4.

Table 4 includes *L. caduciramulosa* Masuda ~~&~~*et* S. Kawaguchi, *L. chondrioides* Børgesen, *L. epiphylla* Boisset *et* Lino, *L. intricata* J.V. Lamouroux, *L. dendroidea*, *L. 'microcladia'* Kützing, *L. minuta* Vandermeulen, Garbary ~~&~~*et* Guiry subsp. *scammaccae* G. Furnari ~~&~~*et* Cormaci, *L. obtusa* (Hudson) J.V. Lamouroux and *L. pyramidalis* Bory ex Kützing. This table is updated from the study by Serio et al. (2004) to include the following: the new species, *L. mediterranea* sp. nov. and the facts that *L. glandulifera* (Kützing) Kützing is presently regarded as a synonym of *Chondrophycus glandulifer* (Kützing) Lipkin *et* P.C. Silva and *L. majuscula* is a synonym of *L. dendroidea* (Guiry and Guiry 2023).

The closely related taxa *Laurencia chondrioides, L. epiphylla* and *L. minuta* subsp. *scammaccae* were distinguished from *L. mediterranea* sp. nov. by certain morphological features (Furnari et al. 2001; Serio et al. 2004; Verlaque 1981): *L. epiphylla* and *L. minuta* subsp. *scammaccae* due to their minute size, having a height of 2 cm and 3 mm, respectively. Moreover, these two species have a discoid, rather than a stoloniferous holdfast. *Laurencia chondrioides* is distinct from the new species due to sparse, irregularly alternate to rarely subopposite or subverticillate branching, with branches and branchlets often inserted at a right angle. Branching reaches up to 3 orders in *L. mediterranea* sp. nov., with branches and branchlets spaced and opposite to verticillately arranged. *Laurencia mediterranea* sp. nov. differed from the morphologically similar species *L. chondrioides.* In fact, among the morphological characteristics of *L. chondrioides* are the following: sparse branching, which is irregularly alternate, rarely subopposite or subverticillate with up to 3 orders and branches and

branchlets that are often inserted at a right angle (Furnari, 2001; Hofmann, 2014). On the other hand, *L. mediterranea* sp. nov. has branching up to 3 orders, with branches and branchlets spaced and opposite to verticillately arranged. The colour of the thallus also differs, with *L chondrioides* possessing a light pink to rosy-red thallus (Hofmann 2014) while *L. mediterranea* sp. nov. is yellow-orange with a rose-carmen holdfast. Additionally, no lenticular thickening is present in medullary cell walls of *L. chondrioides*, which, on the other hand, was occasionally present in *L. mediterranea* sp. nov.

Morphologically, *L. mediterranea* sp. nov. was also distinct ~~to~~ from *L. viridis* which was described by Gil-Rodriguez and Haroun (1992) as having dark green thalli arising from a stoloniferous holdfast, one single *'corp en cerise'*, no cell projections and no lenticular thickenings.

Molecular data

Nine DNA sequences of COI-5P and *rbc*L barcodes were obtained during this study. These were submitted to GenBank and were assigned the following accession numbers: OR570859 to OR570863 for COI-5P and OR567415 to OR567418 for *rbc*L.

The COI-5P sequences obtained for C182, C183, C197, C200 and C201 gave a 97% identity in all cases to *Laurencia obtusa* having accession number KX258828 (Rousseau et al. 2017). The *rbc*L sequences for C183, C197, C200 and C201 were most closely related to *L. obtusa* (98%) with GenBank accession number KX146185 (Rousseau et al. 2017) and topotype sequence AF281881 (McIvor et al. 2000), as well as *L. viridis* Gil-Rodríguez ~~&~~*et* Haroun (98%) with

accession number EF685999 (Gil-Rodríguez et al. 2009). The COI-5P and *rbc*L sequences of *L. microcladia* specimens, only share a 94-95 % identity with the Maltese specimens.

In both phylogenetic trees resulting for *rbc*L (Figure 7) and COI-5P (Figure 8), the Maltese specimens clustered together in a clade that was separate from other closely-related congeners and this was highly supported in both BI and ML analysis (*rbc*L: 1.00 and 96% and COI-5P: 1.00 and 100%).

The *L. mediterrranea* sp. nov. clade in the *rbc*L phylogenetic tree comprised four sequences. Intraspecific p distances within this clade were 0.00%. This was within the <2.00% species limit generally accepted for *rbc*L marker (Cassano et al. 2009; Díaz-Larrea et al. 2007; Metti 2022; Metti et al. 2015; Nam et al. 2000). The closest-related sequences to the *L. mediterranea* sp. nov. clade belonged to *L. obtusa* and *L. viridis*, that were both well-supported clades. The pairwise distance between the topotype *L. obtusa* sequence AF281881 and the *L. mediterranea* sp. nov. sequences was 2.6%. The pairwise distance between the *L. mediterranea* sp. nov. sequence and *L. viridis* was 2.2%. This was higher than the accepted species limits and much higher than the intraspecific divergence between the species in question.

The COI-5P results were congruent with the *rbc*L results. The *L mediterranea* sp. nov. clade comprising of 5 sequences was well-supported. The clade grouped within the *Laurencia* genus. The intraspecific p distances within the *L mediterranea* sp. nov. were 0.00%. The closest neighbouring taxa were *L. obtusa* and *L. viridis,* with a pairwise distance of 3.6% and 4.3% respectively.

Supplementary Table S2 provides the metadata for the macroalgal samples and DNA barcodes (including GenBank accession numbers) of the digitized herbarium specimens produced in the current study. These results, taking morphological, biogeographical and molecular data, as well as phylogenetic analysis into consideration, suggest that the Maltese specimens belong to a novel *Laurencia* species, which is being formally described below.

Taxonomic treatment

***Laurencia mediterranea* A.G. Bartolo ~~&~~*et* G. Zammit sp. nov. (Figures 2-6)**

Description. Submerged cartilaginous thalli to 15 cm long, arising from a holdfast that is attached to the substratum by prostrate creeping stolons. The colour of the tangled axes of the base is carmen pink, while the rest of the thallus is yellow to orange in colour. Thalli with three orders of branching; branches and branchlets arranged oppositely to verticillately. The diameter of the main axes is 800-1200 µm and that of the ultimate axes 200-300 µm. The ultimate branches are short and clavate. In transverse section, the epidermal cells are polygonal to elongate, not palisade-like and connected to each other by secondary pit connections. The epidermal cells occasionally project over the surface of the cortex near the apices. In transverse section, lenticular thickenings may be present in the medullary cells. Four pericentral cells per axial segment could be observed in fresh material, and often one (occasionally two to three) inclusion or '*corp en cerise*' form in each epidermal cell.

The tetrasporangia are produced from the third and fourth pericentral cells in a parallel arrangement to the branching axis and are located in distal regions of branches. In the female gametophyte, cystocarps have a diameter of 700-750 µm and a length of 600-750 µm and are located on branches and branchlets. Mature carpospores have a diameter of 80-100 µm and a length of 200-250 µm. In the male gametophyte, spermatangia are formed in apical depressions at the end of the branches, which broaden to diameters of 400-500 µm and depths from 150-170 µm.

Diagnosis. The specimen had thalli up to 15 cm long that were attached to the substratum by a stoloniferous holdfast. They were cartilaginous in texture, branching up to 3 orders, with branches and branchlets spaced and opposite to verticillately arranged (Figures 2- 4). The ultimate branches were short and clavate (Figure 4). In fresh material, often one '*corp en cerise*' per epidermal cell was detected.

In transverse section, the epidermal cells appeared polygonal to elongate (Figure 5), included secondary pit connections and occasionally projected over the surface near the apices. Each axial segment had four pericentral cells (Figure 5B). In transverse section, the epidermal cells were not palisade-like and lenticular thickenings were present on medullary cells (Figure 6).

Remarks: In the past, *Laurencia mediterranea* sp. nov. has often been misidentified in the waters surrounding the Maltese islands as *L. microcladia* Kützing. However, there are both morphological and genetic differences between the new species *Laurencia mediterranea* sp. nov. from the Mediterranean Sea and

the *L. microcladia* from the US Virgin Islands with the range of type locality, the West Indies (Popolizio et al. 2022). The branching of the algal thallus in the two species is markedly different. In *L. microcladia*, branches are clustered and create a whorled appearance, with densely clustered ultimate branches resulting in a verrucose appearance (Popolizio et al. 2022). On the other hand, *L. mediterranea* sp. nov. specimens exhibit three orders of branching, with branches and branchlets arranged oppositely to verticillately. The holdfasts of both species differ, being discoidal in *L. microcladia* and stoloniferous in *L. mediterranea* sp. nov.. The morphological differences between these two distinct species and with other species within this genus are consistent with genetic studies. In fact, the *rbc*L and COI-5P sequences corroborate the notion that *L. mediterranea* sp. nov. is a new species.

Holotype. (here designated): Strain C200, St. Paul's Bay, Malta, Mediterranean Sea (35.94960000° N 014.40093333° E), 2 m depth. Deposited at the Herbarium of the University of Malta. GenBank sequences with accession number: *rbc*L (OR567418) and COI-5P (OR570861).

Isotype. Deposited as C201, sampled from St. Paul's Bay (Malta). All relevant information is provided in Table S2.

Paratypes. Collected as macroscopic thalli and dried as herbarium material. Deposited as C182, C183, C197 and C201, corresponding to specimens sampled from St. Paul's Islands (Malta), Golden Bay (Malta), Ċirkewwa (Malta) and St. Paul's Bay (Malta) respectively. Further information provided in Table S2.

Etymology. The epithet '*mediterranea*' describes the location where the type material was collected in the Mediterranean Sea. It also collectively describes other locations where this morphospecies was previously recorded under other binomials.

Habitat. Epilithic thalli growing submerged in sea water at a temperature of 25 °C in the upper infralittoral zone at a depth of 2m. The sampling locations are described in Table 1.

Discussion

The five marine algal specimens being studied here, C182, C183, C197, C200 and C201, were identified as *Laurencia mediterranea* sp. nov. All other *Laurencia* spp. that were previously recorded in the Mediterranean Sea and genetically sequenced were found to be distinct via the analysis of both morphological and genetic traits.

Since the work of Yamada (1931), various morphological characters have been considered valuable for delimiting species and varieties within the *Laurencia* complex. These include the presence or absence of lenticular thickening and projection of the cortical cell walls over the surface. However, more recent studies by Cassano et al. (2012a), have shown that these characters could be highly variable within individuals of the same population. For instance, *L. dendroidea* J. Agardh has often been misidentified due to morphological variation regarding the presence or absence of lenticular thickening, the projection of the cortical cell walls, its basal system, as well as differences in habitat (Cassano et al. 2012a). Such morphological variation may be related to environmental parameters; for

instance, *Laurencia* specimens growing in moderately exposed intertidal and subtidal zones or areas with high-energy waves, not only had smaller thalli but also rare lenticular thickening that was absent altogether in some cases (Cassano et al. 2012a). In contrast, specimens growing up to three metres in depth in calm waters of the subtidal zone, tended to have longer thalli and variable lenticular thickening (abundant to rare or even absent). In the lower intertidal zone characterised by vigorous water movement, specimens always possessed lenticular thickening (Cassano et al., 2012a).

The comparison and subsequent segregation of *Laurencia mediterranea* specimens from *L. caduciramulosa, L. intricata, L. dendroidea, L. obtusa* and *L. pyramidalis* (Table 4), proceeded via a consideration of morphological criteria (Serio et al. 2004), as well as molecular analyses of the *rbc*L and COI-5P genes (Figures 7, 8). As regards the publicly available DNA data, 181 COI-5P and 280 *rbc*L sequences in GenBank represent the 138 species currently included in the genus *Laurencia* (Guiry and Guiry 2023). The five *Laurencia mediterranea* COI-5P sequences obtained in this study registered a low genetic identity (97%) to *L. obtusa* LBC0053 from Banyuls-sur-Mer, France, and are thus considered to belong to a different species. The *rbc*L sequences produced in this study did not provide a close identity to any other DNA sequences in GenBank.

Since *rbc*L sequences were available for *Laurencia caduciramulosa, L. intricata, L. dendroidea, L. obtusa* and *L. pyramidalis* in GenBank, these could be included in the phylogenetic analysis, the result of which is shown in Figure 7. Our *rbc*L phylogenetic tree demonstrates that *Laurencia mediterranea* clustered in a clade separate from *L. caduciramulosa, L. intricata, L. dendroidea, L. obtusa*

and *L. pyramidalis* and this was highly supported by both BI and ML analyses (1.00 and 97%). On the other hand, COI-5P sequences were available for *L. dendroidea, L. obtusa* and *L. pyramidalis* in genetic databases, however none were available for *L. caduciramulosa* and *L. intricata.* The COI consensus tree in Figure 8 shows that *L. mediterranea* clustered separately from *L. dendroidea, L. obtusa* and *L. pyramidalis* and this was well supported by both BI and ML analyses (1.00 and 100%).

Another species included in our phylogenetic analysis was *Laurencia microcladia* that was recently described in a study by Popolizio et al. (2022) from the Virgin Islands within its type locality in the West Indies. These authors provided the full morphological description for *L. microcladia*, as well as the first COI-5P and *rbc*L markers for this species. However, these authors completely dismissed any prior morphological descriptions of *L. 'microcladia'* from the Mediterranean Sea (Popolizio et al. 2022). In fact, previous records exist of *L. 'microcladia'* that were identified and described morphologically in a number of studies (Verlaque 1981; Furnari et al. 2001; Tsiamis et al. 2014; Tsiamis and Panagiotidis 2016). The Mediterranean *Laurencia 'microcladia'* was first reported by Verlaque (1981) from the Lavezzi Islands, off the coast of Corsica (France) and from Baie de Port-Cros in the Var, in the south of France. It was subsequently recorded from various localities in the Mediterranean Sea, including Spain, the Balearic Islands, Sardinia, western Italy, Sicily and the adjacent islands, the Adriatic Sea, Greece, Egypt, Algeria and Morocco (Gómez Garreta et al. 2001).

Our findings demonstrate that *L. mediterranea* differs biogeographically, morphologically, as well as genetically from *L. microcladia*. In fact, *L. mediterranea* specimens possess a stoloniferous holdfast, with spaced branches and branchlets that are opposite to verticillately arranged (Figures 2-4) (Furnari et al. 2001; Serio et al. 2004).

Moreover, molecular data and phylogenetic analysis based on the *rbc*L and COI-5P gene sequences (Figures 7, 8) further distinguish the two species and demonstrate that the *L. mediterranea* strains C182, C183, C197, C200 and C201 form a cluster separate to *L. microcladia* from Bermuda and the US Virgin Islands.

Other *Laurencia* spp. having unique biogeographies were initially misidentified due to their cryptic morphology. For example, the recently described species *Laurencia longiramea* Cassano, G.N. Santos, J.M.C. Nunes, M.C. Oliveira ~~&~~*et* M.T. Fujii from Brazil was previously identified as *L. clavata* Sonder (now regarded as a synonym to *Corynecladia clavata* (Sonder) J. Agardh) with the type locality in Australia (Cassano et al. 2019). In the Mediterranean, *Laurenciella marilzae* (Gil-Rodríguez, Sentíes, Díaz-Larrea, Cassano *et*~~&~~ M.T.Fujii) Gil-Rodríguez, Sentíes, Díaz-Larrea, Cassano *et*~~&~~ M.T.Fujii, was often misidentified as *Laurencia dendroidea* (as *L. majuscula*), with its type locality in Brazil and this only came to light when *L. marilzae* was described from the Canary Islands (Cassano et al. 2012b). In fact, it has recently been demonstrated that the diversity within *Laurencia* has been grossly underestimated (Cassano et al. 2012b; 2019).

Finally, we also considered the historical *Laurencia obtusa* var. *gracilis* (C. Agardh) Zanardini that was originally described as *Chondria obtusa* var. *gracilis* C. Agardh (Agardh 1822; Popolizio et al. 2022) and subsequently transferred to *Laurencia* by Kutzing, with the type locality in the Adriatic Sea (Kützing 1865). The syntype localities for this species are considered to be as follows: the Mediterranean Sea, Cádiz in Spain, Brazil, the West Indies, the Ratak Chain, Marshall Islands (Guiry and Guiry 2023). Subsequently, *L. obtusa* var. *gracilis* was also reported from the Adriatic Sea, the Black and Azov Seas, France, Tunisia, Libya (Gómez Garreta et al. 2001), Turkey (Taşkın and Atakan 2013), the Balearic Islands, Greece and Spain (Guiry and Guiry 2023).

Laurencia obtusa var. *gracilis* was considered *taxon inquirendum*, with the possibility of being a synonym to *L. 'microcladia'* by Furnari et al. (2001), since it is morphologically similar to it. This variety is also considered to be a synonym to *L. 'microcladia'* in subsequent literature (Tsiamis et al. 2014; Tsiamis and Panagiotidis 2016). Nevertheless, *L. obtusa* var. *gracilis* is presently considered an accepted taxon on AlgaeBase, with a comment indicating *taxon inquirendum* status (Guiry and Guiry 2023). In the present study, both the *rbc*L (Figure 7) and COI-5P (Figure 8) phylogenetic analyses demonstrate clearly that the Maltese specimens do not belong to a variety of *L. obtusa*, since they cluster separately to *L. obtusa*, which is well-supported by both BI and ML analyses (*rbc*L 1.00/97 and COI 1.00/100). Thus, while different species of *Laurencia* have previously been identified morphologically from Maltese waters, including *L. 'microcladia', L. minuta* subsp. *scammaccae* and *L. obtusa* (Cormaci et al. 1997), this study describes a new species *L. mediterranea*, based on an integrative

systematics approach and provides the first nine (4 *rbc*L and 5 COI-5P) genetic sequences for this species. For the first time, this study provides linked data for *L. mediterranea* as a distinct species from *L. microcladia.* It demonstrates the importance of including sampling location linked to digital herbaria, because this helps resolve misidentifications due to cryptic morphology, but distinct biogeography.

It is possible that other records of *L. microcladia* from the Mediterranean Sea are misidentifications, but this can only be resolved with molecular data and we cannot state at this time that *L. microcladia* does not occur in the Mediterranean marine flora.

The limitations of publicly available genetic data, as well as recommendations for future DNA studies, have recently been highlighted by a number of authors (Leigh et al. 2021; Mathur et al. 2021; Rimet et al. 2021). The importance of a link between algal DNA barcodes, sample metadata and digital herbaria has become evident in studies of marine algal biodiversity, biogeography and global change, especially due to the projected marine habitat degradation as a consequence of climate change in the near future (IPCC 2021).

Acknowledgements

This research was funded by an award to GZ (grant REP-2022-001), for the Project 'GreASE', financed by the Malta Council for Science & Technology, for and on behalf of the Foundation for Science and Technology, through the FUSION: R&I Research Excellence Programme'. AGB was supported by the ENDEAVOUR Scholarship Scheme (Malta)- Group B – National Funds. FCK

would like to acknowledge the TOTAL Foundation (Paris) and the MASTS pooling initiative (The Marine Alliance for Science and Technology for Scotland), which is funded by the Scottish Funding Council (grant reference HR09011).

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Figure 1. Map of the study area, with blue dots indicating the sampling sites. The four sites are Golden Bay = G, = Ċirkewwa = Ċ, St. Paul's Islands = SI, St. Paul's Bay = ST. Source for BaseMap: Esri, HERE, Garmin, FAO, NOAA, USGS, © OpenStreetMap contributors, and the GIS User Community.

Figure 2. Herbarium images of *Laurencia mediterranea* sp. nov. **A** C183, **B** C200 (holotype), with the stoloniferous holdfast clearly visible. Scale bar= 1cm.

Figure 3. Herbarium image of *Laurencia mediterranea* sp. nov. C201 (isotype). Scale bar= 1cm.

Figure 4. Photographic detail of *Laurencia mediterranea* sp. nov. C201; **A** the thallus is yellow-orange with a holdfast that is a rose carmen in colour. Branches and branchlets are spaced, with up to three branching orders and oppositely to verticillately arranged. **B** Ultimate branchlets are short and clavate. Scale bars (A) 0.5 and (B) 1 cm.

Figure 5. Light micrographs of *Laurencia mediterranea* sp. nov. C201 stained with aniline blue; **A** Transverse section of a branch showing the medullary cells **B** Transverse section showing axial cell, labelled 'a' and four pericentral cells, labelled 'p'. Scale bars (A) 100 μm and (B) 50 μm.

Figure 6. Light micrographs of *Laurencia mediterranea* sp. nov. C200 (holotype) stained with acetocarmine; **A, B** Transverse sections of a branch showing thickening in cell walls (black arrows) of the medullary cells. Scale bars (A, B) 50 μm.

Figure 7. Phylogenetic tree of *Laurencia* species inferred from the plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) sequences. Both Bayesian Inference (BI) and Maximum likelihood (ML) analyses were carried out. The numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (> 0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents the number of nucleotide substitutions per site.

Figure 8. Phylogenetic tree of *Laurencia* species inferred from the mitochondrial cytochrome c oxidase subunit 1 (COI) sequences. Both Bayesian Inference (BI) and Maximum likelihood (ML) analyses were carried out. Numbers on branches are Bayesian posterior probabilities (BPP) and bootstrap (BS) values (>

0.7 and 70% respectively). An asterisk (*) indicates full support (= 1.00 and 100%). The scale bar represents the number of nucleotide substitutions per site.

Supplementary information

The following supplementary material is available:

Supplementary Figure S1. The collection sites in Malta (A) St. Paul's Islands = SI, (B) Ċirkewwa = Ċ, (C) Golden Bay = G (D) St. Paul's Bay = ST.

Supplementary Figure S2. Underwater photographs showing typical algal communities growing at the collection sites (A-B) Golden Bay, (C-D) St. Paul's Bay.

Supplementary Table S1. Sequence information data used in this study.

Supplementary Table S2. The metadata and DNA barcodes of herbarium specimens belonging to *Laurencia mediterranea* sp. nov.

Tables

Table 1. Provenance of samples including spatial data. Algae were submerged in sea water at a depth of 2 m.

Strain	Location	Latitude	Longitude	Site description	Collection date
C182	Saint Paul's Bay	35.96546667° N	014.40515000° E	St. Paul's Island	10 June 2017
C183	Golden Bay	35.93458333° N	014.33835000° E	Sandy beach, near pier	15 June 2017
C197	Ċirkewwa	35.98603333° N	014.33841667° E	Near desalination plant	13 June 2017
C200	Saint Paul's Bay	35.94960000° N	014.40093333° E	Beneath Wignacourt Tower	12 June 2017
C201	Saint Paul's Bay	35.94960000° N	014.40093333° E	Beneath Wignacourt Tower	12 June 2017

Table 2. List of primers used in this study including the sequence and reference for each.

Gene	Primer Name	Primer No.	Sequence	Reference
COI	GazF2	1	CCAACCAYAAAGATATWGGTAC	Lane et al. 2007
	DumR1	2	AAAAAYCARAATAAATGTTGA	Saunders 2005
*rbc*L	F7	3	AACTCTGTAGAACGNACAAG	Gavio and Fredericq 2002
	R753	4	GCTCTTTCATACATATCTTCC	Freshwater and Rueness 1994
*rbc*L	F645	5	ATGCGTTGGAAAGAAAGATTCT	Lin et al. 2001
	*rbc*Start	6	GTTCCTTGTGTTAATCTCAC	Freshwater and Rueness 1994

Primer pair	Initial denaturation	Amplification			Final extension	Reference
		Denaturation	Annealing	Elongation		
1 and 2	2min at 95 °C	30s at 94 °C	40s at 50 °C	40s at 72 °C	5min at 72 °C	Peña et al. 2015
3 and 4	4min at 96 °C	60s at 94 °C	60s at 49 °C	90s at 72 °C	8min at 72 °C	Lin et al. 2001, modified
5 and 6	4min at 96 °C	60s at 94 °C	60s at 49 °C	90s at 72 °C	8min at 72 °C	Lin et al. 2001, modified

Table 3. PCR programme conditions used for each primer combination.

Table 4. Comparison of *Laurencia* spp. from the Mediterranean Sea (updated from Serio et al. 2004).

ND = Not detected.

Laurencia spp. in the Mediterranean	Attachment	*Corps en cerise*	Cell projections	Lenticular thickening
L. caduciramulosa Masuda *et* Kawaguchi	Stoloniferous	ND	Present	Present
L. chondrioides Børgesen	Discoid holdfast and stolons	ND	Present	Absent
L. epiphylla Boisset *et* Lino	Discoid	ND	Absent	Present
L. intricata J. V. Lamouroux	Stoloniferous	2 to 4	Present	Absent
L. dendroidea J.Agardh	Stoloniferous	1 to 2	Present	Absent
***L. mediterranea* sp. nov.**	Stoloniferous	1	Absent or present	Present
L. minuta subsp. *scammaccae* Furnari *et* Cormaci	Discoid	ND	Present	Present
L. obtusa (Hudson) J. V. Lamouroux	Stoloniferous	1	Absent	Absent
L. pyramidalis Bory ex Kützing	Stoloniferous	1	Absent or present	Absent

Graphical Abstract Text

A morphological and molecular description of *Laurencia mediterranea* sp. nov. (Ceramiales, Rhodophyta) from Malta in the central Mediterranean Sea.

Author Biographies

Angela G. Bartolo holds a PhD in Marine Biology (University of Aberdeen). Her research addresses DNA-methods in Malta. She holds a dual Master's degree MSc/MS from the University of Malta and James Madison University (Virginia) where she focused her research on sea water quality through the use of ecological indicators. Angela also works on the UN Barcelona Convention, EU Marine Strategy Framework Directive and Water Framework Directive.

Gabrielle Zammit is an Associate Professor at the University of Malta. Her research is mainly concerned with the molecular biology and biochemistry of cyanobacteria and algae from a variety of habitats, both terrestrial and marine. Over the past twenty years, she has also lectured and supervised undergrad and postgraduate students enrolled at different institutions internationally.

Frithjof C. Küpper has held the Chair in Marine Biodiversity at the University of Aberdeen since 2011, after 8 years at the Scottish Association for Marine Science, studying the biodiversity and biochemistry of marine plants/algae. He found that iodide serves as an inorganic antioxidant in kelp, the first described from a living system, impacting atmospheric and marine chemistry. A certified scientific diver, Frithjof has worked in the Mediterranean, South Atlantic (Ascension, Falklands), Antarctica, the Arctic and the Gulf.