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DNA Barcode-Assisted Inventory of the Marine Macroalgae from the Azores, Including New Records

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Abstract: Up to the present study, only 8.5% of the 522 macroalgal species reported at the Azores have sequences deposited in GenBank and BOLD public repositories. The sequences of four genetic markers (*cox*1, *rbc*L, UPA, *tuf*A) were obtained for recently collected samples from two Azorean islands. DNA barcode-assisted identification was conducted on newly generated and unpublished sequences from public repositories. A literature review of recently published studies, including the molecular identifications of Azorean macroalgae, was also performed. The results confirm the occurrence of 51 species (including subspecific ranks) and provide four new records, namely, three cryptogenic species (*Olokunia boudouresquei*, *Padina gymnospora*, and *Ulva lacinulata*) and one introduced species (*Ulva australis*). This study contributes 23 DNA barcodes generated for the first time to the Azores, which now has 10.5% of its marine flora represented in public repositories. Additionally, UPA sequences were generated for the first time for the five taxa.

Keywords: *cox*1; cryptogenic species; DNA barcoding; introduced species; oceanic islands; public repositories; *rbc*L; *tuf*A; UPA

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1. Introduction

The Azores Archipelago, located in the mid-Atlantic ridge, is composed of nine islands separated into three main groups (eastern, central, and western), spread over 620 km from SE to NW, 1465 km from mainland Portugal and 1925 km from Newfoundland (Canada; Figure 1A). The marine flora of the Azores has been extensively studied since 1843 [1]. Currently, it comprises 522 macroalgal taxa [2], of which 97 belong to the Ochrophyta, 349 to the Rhodophyta, and 76 to the Chlorophyta, with the number of species known to occur on each island varying from 43 in Corvo to 328 in São Miguel [3,4]. Regarding the origin of the taxa, 42 are currently reported as introduced species [2], from which 9 are classified as invasive [5], 43 as cryptogenic or of unknown origin [3,6,7], and the remaining 437 being possibly native, from which 8 are Azorean endemic [7].

Macroalgae is a term used to refer to macroscopic, aquatic, photosynthetic organisms composed of filaments or simple tissues belonging to three different Phyla: brown algae (Ochrophyta), red algae (Rhodophyta), and green algae (Chlorophyta; [8]). The identification of macroalgal species is usually challenging due to the simple morphology and anatomy, the high rates of convergence, high phenotypic plasticity, and the limited information on life histories [9]. Comparative DNA sequences analyses have been used to assist the morphology-based studies of macroalgae since the 1990s [10–12], and the support of molecular markers became the standard approach to assess the algal species-level diversity [13]. Several studies involving the molecular-assisted identification have contributed to revealing the molecular diversity of the macroalgae occurring in the Azores (as listed in Section 3.3).

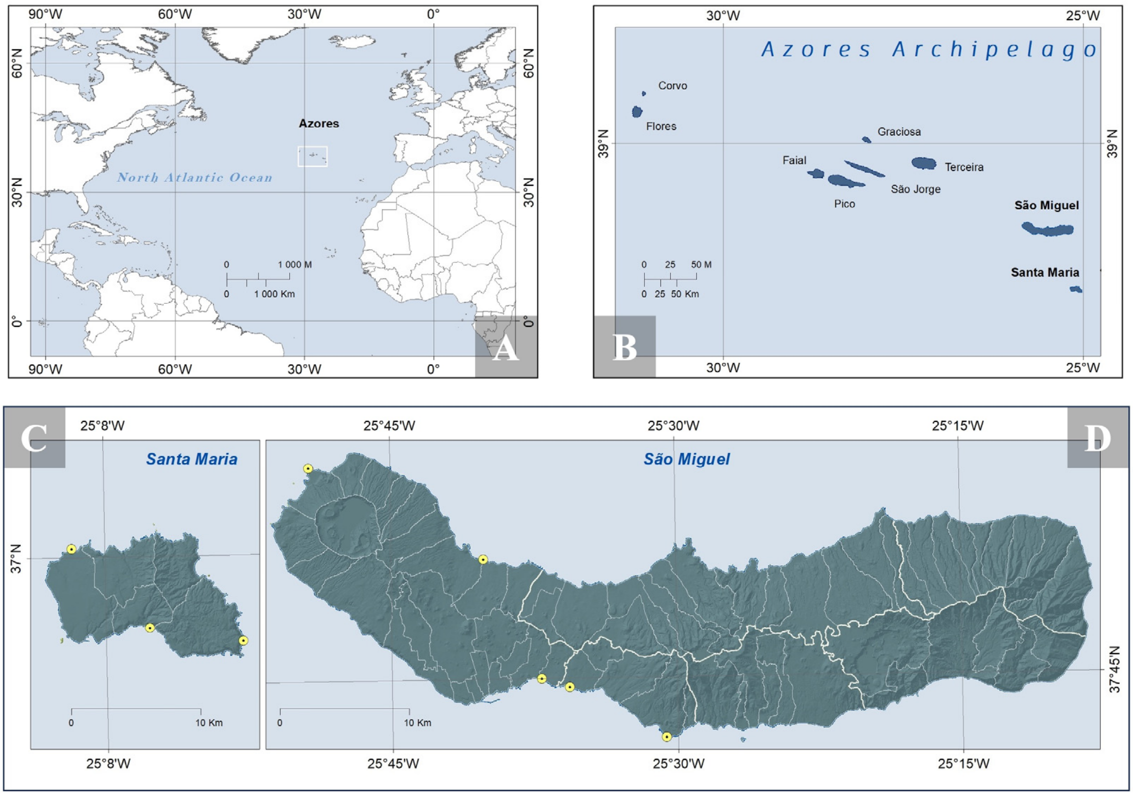

Figure 1. Map of the study area. (**A**) Azores and its position in the North Atlantic; (**B**) The Azores Archipelago; (**C**) Santa Maria Island; and (**D**) São Miguel Island. Collection sites are marked with yellow circles (see Table 1 for further details).

Table 1. Collection sites in the Azores.

Locality	Collectors	Date	Coordinates
São Miguel Island			
Caloura, Cerco	D. Gabriel, A. F. Santos	21, 24-July-2020	37.708154 N 25.509782 W
Atalhada, Cruzeiro	D. Gabriel	9-June-2020	37.744306 N 25.594341 W
Livramento, Praia do Pópulo	D. Gabriel	8-June-2020	37.750304 N 25.618740 W
São Vicente de Ferreira, harbour	D. Gabriel	27-September-2022	37.834432 N 25.668619 W
Mosteiros, tide pools	D. Gabriel	28-September-2022	37.899311 N 25.821360 W
Santa Maria Island			
Maia	D. Gabriel, A. I. Ferreira	27-August-2022	36.939619 N 25.014179 W
Praia Formosa	D. Gabriel, A. I. Ferreira	25-August-2022	36.949958 N 25.094974 W
Anjos, Poça do Carro	D. Gabriel, A. I. Ferreira, S. Cabral	25-August-2022	37.005955 N 25.161788 W

DNA barcoding is an identification system based on the comparison between the sequence of a genetic marker from a given specimen against a database of sequences from an *a priori* defined species [13]. The variety of markers used in DNA-assisted morphological studies presents a challenge in comparing newly sequenced specimens with the sequences available in public databases [9]. A few standard markers are currently used as barcodes for macroalgae, mainly *rbcL* for the three Phyla, combined with *cox*1 for Ochrophyta and Rhodophyta, and *tuf*A for Chlorophyta [14]. Sequencing from different species and worldwide populations, as well as the provision of the links between sequences and proper species names [13], are needed to build an extensive DNA barcode library to facilitate the easy identification of algae for ecological, legislative, and commercial purposes [14].

The present study aims to provide an inventory of the marine macroalgae with species identity supported by molecular data reported from the Azores. The inventory is based on sequences deposited in GenBank and BOLD public repositories, as well as newly sequenced vouchers. The native statuses regarding the species' origins were evaluated for the Azores, and the year of the first reports to these islands were provided. This study also intends to increase the sequences available for DNA barcoding from the Azores Archipelago in public databases.

2. Materials and Methods

2.1. Sampling

Samples were collected by hand at low tide from five sites in São Miguel and three sites in Santa Maria Islands (Western Group; Figure 1 and Figure S1; Table 1 and Table S1) between June 2020 and September 2022. Specimens were photographed in situ (underwater) and/or in vivo (in a container with seawater or wet on herbarium paper) using an Olympus Tough TG-5 waterproof camera (OM Digital Solutions Corporation, Tokyo, Japan; Figure 2 and Figure S2). In each case, a voucher was pressed onto herbarium paper, with subsamples kept in silica gel for subsequent molecular work. Vouchers are stored with Daniela Gabriel (address above), who performed a preliminary identification based on general morphology and will eventually be deposited in the Ruy Telles Palhinha Herbarium at the University of the Azores, Ponta Delgada (Code AZB).

2.2. DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted using the methods described by [15]. PCR amplification was performed in 20 µL reaction mixtures with MyTaq Polymerase and Mix (Bioline) following the manufacturer's instructions: 4 µL of MyTaq Mix (enhanced buffer containing dNTPs and $MgCl_2$), 1 µL of MyTaq Polymerase, 1 µL of each primer (10 µM), 2 µL of DNA, and 11 µL of H_2O. Published primers were used to amplify the mitochondrion-encoded gene Cytochrome c oxidase subunit 1 DNA barcode region (*cox*1, ~664 bp; [9]) and the chloroplast-encoded genes ribulose-1,5-bisphosphate carboxylase large subunit (*rbc*L, ~1350 bp; [16–19]), elongation factor tu (*tuf*A, ~800 bp; [20,21]), and LSU (23S) domain V (UPA, ~370 bp; [22]—Table S2). PCR conditions were optimized and are as follows: (1) *rbc*L: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 47 °C for 45 s, and 72 °C for 90 s, with a final extension of 72 °C for 10 min; (2) *tuf*A: initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 20 s, 52 °C for 15 s, and 72 °C for 55 s, with a final extension of 72 °C for 5 min; (3) UPA: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 10 min; and (4) *cox*1: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. Sanger DNA sequencing was performed with StabVida (Lisbon, Portugal) using the produced PCR fragments with the respective sets of primers.

Forward and reverse sequence reads were edited and concatenated using Geneious Prime 2023.2 (Biomatters Inc., Boston, MA, USA) and later deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank; accessed on 21 December 2023), with the accession numbers OR944632-OR944652 and OR961484-OR961498. Newly generated sequences were compared to those on the GenBank sequence database through Basic Local Alignment Search Tool—NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 21 December 2023). Sequences with more than 99% identity match were considered to belong to the same species [23]. Sequence names from GenBank were confirmed with the most recent publications containing these sequences and the most updated taxonomy available on Algaebase (https://www.algaebase.org; accessed on 21 December 2023).

Figure 2. In situ pictures of some marine macroalgae from the Azores analyzed in the present study. Ochrophyta: (**A**) *Fucus spiralis*; (**B**) *Padina gymnospora*; and (**C**) *Scytosiphon lomentaria*. Rhodophyta: (**D**) *Amphiroa beauvoisii*; (**E**) *Jania verrucosa*; and (**F**) *Lomentaria articulata*. Chlorophyta: (**G**) *Ulva australis*; (**H**) *Ulva compressa*; and (**I**) *Ulva lacinulata*.

2.3. Compilation of Publicly Available Sequences for the Azores

The information of DNA sequences currently available on GenBank and Barcode Of Life Data System—BOLD (https://www.boldsystems.org; accessed on 21 December 2023), hereafter public repositories, with "Azores" included in the sampling locality, were retrieved. Records retrieved from BOLD linked to GenBank suppressed sequences were not considered. Unpublished sequences, i.e., sequences deposited in public repositories but not included in the peer-reviewed literature, were submitted to BLAST for identification. The sequences of vouchers identified at the species level were used to produce an inventory of the Azorean marine flora supported by molecular identification. Sequence names were updated as described in Section 2.2 and classified according to their native distributional range as described in Section 2.4.

2.4. Evaluation of Native Status in the Azores

Taxa confirmed to be present in the Azores were classified as native, i.e., occurring within the species' native distributional ranges; endemic, i.e., occurring only in the Azores; introduced, i.e., occurring outside the species native distributional range; invasive, i.e., with documented impacts on the Azorean biodiversity; or cryptogenic, i.e., not clearly classified as native or introduced in the archipelago. The species known distributional ranges were retrieved from Algaebase, and their eventual report as being invasive or posing a threat of invasion elsewhere was checked in the databases Information system on aquatic non-indigenous and cryptogenic species—AquaNIS (http://www.corpi.ku.lt/databases/index.php/aquanis; accessed on 21 December 2023), SeaLifeBase (www.sealifebase.org), Centre for Agriculture and Bioscience International Invasive Species Compendium—CABI: (www.cabi.org/ISC; accessed on 21 December 2023), Global Invasive Species Database—GISD (www.iucngisd.org; accessed on 21 December 2023), European Alien Species Information Network—EASIN (https://easin.jrc.ec.europa.eu/easin; accessed on 21 December 2023), and Invasive and Exotic Species of North America list—IESNA (www.invasive.org; accessed on 21 December 2023). The date of the first record of each species in the Azores is also provided.

3. Results

3.1. DNA-Barcoding of Newly Generated Sequences

A total of 36 sequences from four different markers were produced for the 24 collected samples, namely, ten sequences of *cox*1, four of *tuf*A, seven of *rbc*L, and 15 of UPA (Table S1). The newly generated sequences comprise 24 barcodes for 14 species present in the Azores, with 22 originally published barcodes (Appendix A). UPA barcodes belonging to five species are published here for the first time (*Laurencia pyramidalis*, *Jania pedunculata* var. *adhaerens*, *Jania verrucosa*, *Gelidium microdon*, and *Lomentaria articulata*).

3.2. Analyses of Publicly Available Sequences

A total of 373 DNA sequences from Azorean vouchers were retrieved from public repositories, namely, 147 sequences of *cox*1, ten of *cox*2, 74 of *cox*3, 14 of LSU, two of *nad*1, four of *psa*A, 17 of *psb*A, 89 of *rbc*L, eight of *rbc*L-*rbc*S spacer, one of *rpl*16, and seven of *tuf*A (Table S3). Unpublished sequences were subject to molecular analyses to assess their correct identities (Appendix B). Of the 58 taxa retrieved, 13 were identified only at the generic level. The assembled publicly available sequences comprise 66 barcodes for 45 species present in the Azores.

3.3. DNA-Based Species Inventory

The molecular analyses of the newly generated sequences (Section 3.1) and the sequences retrieved from public repositories (Section 3.2) uncovered 55 species (including subspecific ranks), with 20 species belonging to Ochrophyta, 28 to Rhodophyta, and 7 to Chlorophyta (Table 2). The uncovered taxa represent 51 known species of Azorean marine flora and 4 new species: 3 cryptogenic species (*Olokunia boudouresquei*, *Padina gymnospora*, and *Ulva lacinulata*) and 1 introduced species (*Ulva australis*).

Table 2. Inventory of the Azorean marine flora supported by molecular identification, with targeted genetic markers deposited in public repositories, first time reported, and the origin status in the Azores. Sequences deposited in public repositories but not included in peer-reviewed literature are referred to as "unpublished". Taxa marked with "*" represent updates for the Azorean marine flora.

Taxa	Genetic Markers	First Report to the Azores	Status in the Azores	Comments
Phylum **Ochrophyta** Class **Phaeophyceae** Order **Dictyotales** Family **Dictyotaceae**				
Dictyota cyanoloma Tronholm, De Clerck, A.Gómez-Garreta & Rull Lluch	*psb*A [24,25]	2006 [24]	Native [7]	
Dictyota dichotoma (Hudson) J.V.Lamouroux	*psb*A; *cox*1 [24,25]	1928 [26]	Native [7]	
Dictyota implexa (Desfontaines) J.V.Lamouroux	*psb*A; *cox*1 [24,25]	1870 [27]	Native [7]	
Dictyota pleiacantha Tronholm *	*psb*A [25]	2005 [25]	Native (This study)	Phylogenetic reconstruction of *Dictyota pleiacantha* based on multiple genes (*psa*A, *psb*A, *rbc*L, *cox*1; [28]) indicates that the species comprises two clades, one occurring in the Canary Islands (type locality), Madeira and the Azores, therefore a Macaronesian endemic, and another occurring on the Western Atlantic coasts.
Lobophora delicata Camacho & Fredericq	*cox*3 [28]	2018 [28]	Native (This study)	According to Vieira et al. [25,29], the Azores are within the species' native distributional range.
Padina gymnospora (Kützing) Sonder *	UPA (This study)	First report (This study)	Cryptogenic (This study)	The species currently has a worldwide distribution and is not reported as introduced or invasive elsewhere.
Rugulopteryx okamurae (E.Y.Dawson) I.K.Hwang, W.J.Lee & H.S.Kim	*psb*A, *rbc*L [4]	2019 [4]	Introduced [4]	This species exhibits a highly invasive behavior in the Azores [5,30].
Order **Ectocarpales** Family **Scytosiphonaceae**				
Colpomenia sinuosa (Mertens ex Roth) Derbès & Solier	*cox*1, *rbc*L, UPA (Unpublished; this study)	1894 [29]	Native [7]	
Hydroclathrus tilesii (Endlicher) Santiañez & M.J.Wynne	*psa*A, *cox*3 ([31]; as *Hydroclathrus stephanosorus*)	2016 [31]	Introduced [2]	
Petalonia binghamiae (J.Agardh) K.L.Vinogradova	*cox*1 (Unpublished)	1989 ([32]; as *Endarachne binghamiae*)	Introduced [2]	
Scytosiphon lomentaria (Lyngbye) Link, nom. cons.	*cox*1, UPA (Unpublished; this study)	1911 ([33]; as *Scytosiphon lomentarius*)	Native [7]	Although the binomial *Scytosiphon simplicissimus* has priority over *Scytosiphon lomentaria*, the latter was maintained due to its widespread and consistent use for the species [34].
Zonaria tournefortii (J.V.Lamouroux) Montagne	*cox*1 (Unpublished; [25])	1928 [26]	Native [7]	
Order **Fucales** Family **Fucaceae**				
Fucus spiralis Linnaeus	*cox*1, UPA (Unpublished; this study)	1911 ([33]; as *Fucus platycarpus* var. *spiralis*)	Cryptogenic [7]	

Table 2. *Cont.*

Taxa	Genetic Markers	First Report to the Azores	Status in the Azores	Comments
Family **Sargassaceae**				
Cystoseira pustulata (Ercegovic) Neiva & Serrão *	*cox*1, *cox*3, *nad*1, *psa*A (Unpublished; [35,36])	1986 ([37]; as *Cystoseira compressa and C. humilis*)	Native (This study)	According to Neiva et al. [36], *Cystoseira compressa* and *C. humilis* are apparently absent in the Azores and should be referred to as *C. pustulatus*. According to Neiva et al. [36], the Azores are within the native distributional range of *Cystoseira pustulata*. Sequences not found in the search described in Section 2.3 for missing the locality in GenBank were added to the list by the authors who knew the article where the sequences were published [35].
Ericaria selaginoides (Linnaeus) Molinari & Guiry	*cox*1 [36]	1986 ([37]; as *Cystoseira tamariscifolia*)	Native ([38]; as *Cystoseira tamariscifolia*)	
Gongolaria abies-marina (S.G.Gmelin) Kuntze	*cox*1, cox3, *nad*1, *psa*A (Unpublished; [36])	1938 ([39]; as *Cystoseira abies-marina*)	Native [7]	
Sargassum cymosum C.Agardh	*cox*1 (Unpublished)	1986 [37]	Native [7]	
Order **Ralfsiales**				
Family **Pseudoralfsiaceae**				
Pseudoralfsia azorica Parente, Fletcher & G.W.Saunders *	*cox*1, *rbc*L [40]	2009 [40]	Endemic [40]	
Order **Sphacelariales**				
Family **Stypocaulaceae**				
Halopteris filicina (Grateloup) Kützing	*cox*1 (Unpublished)	1938 ([39]; as *Sphacelaria filicina*)	Native [7]	
Halopteris scoparia (Linnaeus) Sauvageau	LSU (Unpublished)	1911 [33]	Native [7]	
Phylum **Rhodophyta**				
Class **Florideophyceae**				
Order **Bonnemaisoniales**				
Family **Bonnemaisoniaceae**				
Asparagopsis armata Harvey	*cox*1 (Unpublished)	1988 [41]	Introduced [2]	Previous reports *Asparagopsis armata* (see list from [42]) are based on *A. delilei*, which is currently a synonym of *A. taxiformis*.
Asparagopsis taxiformis (Delile) Trevisan	*cox*1, *cox*2, LSU, *rbc*L-*rbc*S spacer (Unpublished; [43])	1928 ([44]; as *Asparagopsis delilei*)	Introduced [2]	
Order **Ceramiales**				
Family **Rhodomelaceae**				
Herposiphonia tenella (C.Agardh) Ambronn	*rbc*L [3]	2018 [3]	Native [3]	
Laurencia pyramidalis Bory ex Kützing	*cox*1, *rbc*L, UPA ([45]; This study)	2011 [45]	Native [7]	
Laurencia viridis Gil-Rodríguez & Haroun	*cox*1, *rbc*L [45]	1981 [46]	Native [38]	
Laurenciella marilzae (Gil-Rodríguez, Sentíes, Díaz-Larrea, Cassano & M.T.Fujii) Gil-Rodríguez, Sentíes, Díaz-Larrea, Cassano & M.T.Fujii	*cox*1, *rbc*L [45]	2011 [45]	Native [7]	
Lophocladia trichoclados (C.Agardh) F.Schmitz	*rbc*L [3]	2018 [3]	Native [3]	
Lophosiphonia simplicissima Díaz-Tapia	*rbc*L [3]	2018 [3]	Native [3]	
Melanothamnus macaronesicus Rodríguez-Buján & Díaz-Tapia	*rbc*L [47]	2018 [47]	Native (This study)	According to Rodriguez-Bujan et al. [47], *Melanothamnus macaronesicus* was only found in the Canary Islands and the Azores; therefore, it is a Macaronesian endemic.

Table 2. *Cont.*

Taxa	Genetic Markers	First Report to the Azores	Status in the Azores	Comments
Melanothamnus pseudoforcipatus Díaz-Tapia	*rbc*L [3]	2018 [3]	Cryptogenic [3]	
Osmundea oederi (Gunnerus) G.Furnari	*cox*1, *rbc*L [48]	2010 [45,48]	Native [7]	
Osmundea pinnatifida (Hudson) Stackhouse	*cox*1, *rbc*L [45]	1938 ([39]; as *Chondria pinnatifida*)	Native [7]	
Polysiphonia villum J.Agardh	*rbc*L [3]	2018 [3]	Native [3]	
Vertebrata barbarae Muñoz-Luque & Díaz-Tapia	*rbc*L [49]	2018 [49]	Endemic [49]	Sequences not found in the search described in Section 2.3 for missing the locality in GenBank were added to the list by the authors who knew the article where the sequences were published [49].
Vertebrata cymatophila (Børgesen) Muñoz-Luque	*rbc*L [49]	2018 [49]	Native (This study)	According to Díaz-Tapia et al. [49], *Vertebrata cymatophila* was only found in the Canary Islands and the Azores; therefore, it is a Macaronesian endemic. Sequences not found in the search described in Section 2.3 for missing the locality in GenBank were added to the list by the authors who knew the article where the sequences were published [49].
Order **Corallinales**				
Family **Corallinaceae**				
Corallina berteroi Montagne ex Kützing	*cox*1, *rbc*L (Unpublished; [50]; as *Coralllina caespitosa*)	2013 [51]	Native [7]	Sequences not found in the search described in Section 2.3 for missing the locality in GenBank were added to the list by the authors who knew the article where the sequences were published [50].
Jania pedunculata var. *adhaerens* (J.V.Lamouroux) A.S.Harvey, Woelkerling & Reviers	*cox*1, UPA (This study)	1986 ([37]; as *Jania adhaerens*)	Native [7]	Studies based on lectotype material have supported the recognition of *Jania pedunculata* var. *adhaerens* as a variety rather than a distinct species [52]. However, the synonym *Jania adhaerens* is still widely used.
Jania verrucosa J.V.Lamouroux, nom. rejic.	*cox*1, *rbc*L, UPA (This study)	1993 [53]	Native [7]	Although studies based on lectotype material have indicated that *Jania verrucosa* is a name of uncertain nomenclatural application [54], it is still widely used.
Family **Lithophyllaceae**				
Amphiroa beauvoisii J.V.Lamouroux	*cox*1, UPA (This study)	1886 ([55]; as *Amphiroa exilis*)	Native [7]	Gain [33] is the first to report *Amphiroa beauvoisii* to the Azores based on Piccone [55]. Nevertheless, Piccone reported *A. exilis* (currently a valid species) with various synonyms, including *A. pustulata*. (currently a synonym of *A. beauvoisii*). Although it is unknown how or when the species identity was clarified, the date of the original record was maintained.
Order **Gelidiales**				
Family **Gelidiaceae**				
Gelidium microdon Kützing	*rbc*L, UPA (This study)	1938 ([39]; as *Sphaerococcus corneus* var. *spinulosus*)	Native [7]	
Order **Gelidiales**				
Family **Pterocladiaceae**				
Pterocladiella capillacea (S.G.Gmelin) Santelices & Hommersand	*cox*1 (Unpublished)	1886 ([55]; as *Pterocladia capillacea*	Native [7]	
Order **Gigartinales**				
Family **Cystocloniaceae**				
Hypnea musciformis (Wulfen) J.V.Lamouroux	*cox*1, *rbc*L, UPA (This study)	1911 [33]	Cryptogenic [7]	

Table 2. *Cont.*

Taxa	Genetic Markers	First Report to the Azores	Status in the Azores	Comments
Order **Nemastomatales** Family **Nemastomataceae**				
Predaea feldmannii subsp. *azorica* Gabriel	*rbc*L [56]	1990 ([57]; as *Predaea feldmannii*)	Endemic [56]	
Family **Schizymeniaceae** *Platoma cyclocolpum* (Montagne) F.Schmitz	*rbc*L [58,59]	1986 [37]	Native [7]	
Schizymenia apoda (J.Agardh) J.Agardh	LSU, *rbc*L [59–61]	1894 ([29]; as *Schizymenia obovata* and *S. undulata*)	Native [7]	
Order **Peyssonneliales** Family **Peyssonneliaceae**				
Olokunia boudouresquei (Yoneshigue) Pestana, Lyra, Cassano & J.M.C.Nunes *	*rbc*L (Unpublished; as *Cruoriopsis crucialis*)	First report (This study)	Cryptogenic (This study)	*Olokunia boudouresquei* is mostly an Atlantic species, with few reports to Australia and New Zealand. Due to the taxonomic confusion within Peyssonneliaceae [62,63], further studies are necessary to assess the species status in the Azores.
Peyssonnelia coriacea Feldmann	LSU, *rbc*L ([60,64]; as *Peyssonnelia squamaria*)	1990 [65]	Native ([7]; as *Peyssonnelia squamaria*)	Reappraisal of sequences of *Peyssonnelia* species indicates that the species reported from the Azores as *P. squamata* (KR732900) may be *P. coriacea* [62,63]. The sequencing of type material is necessary to confirm the new identity.
Order **Rhodymeniales** Family **Lomentariaceae** *Lomentaria articulata* (Hudson) Lyngbye	*cox*1, UPA (This study)	1911 [33]	Native [7]	
Phylum **Chlorophyta** Class **Ulvophyceae** Order **Bryopsidales** Family **Caulerpaceae** *Caulerpa prolifera* (Forsskål) J.V.Lamouroux	*tuf*A [66]	2013 [67]	Introduced [2]	
Caulerpa webbiana f. *disticha* Vickers	*tuf*A (Unpublished)	2002 ([68]; as *Caulerpa webbiana*)	Introduced ([68]; as *Caulerpa webbiana*)	This species has an invasive behavior in the Azores [68,69].
Family **Codiaceae** *Codium adhaerens* C.Agardh	*rpl*16, *rbc*L [70]	1912 [33]	Native [7]	
Order **Ulvales** Family **Ulvaceae**				
Ulva australis Areschoug *	*tuf*A (This study)	First report (This study)	Introduced (This study)	Molecular analyses point to a greater genetic diversity of *Ulva australis* in the Northwest Pacific, with populations in Australia, New Zealand, California, Chile, and Europe deriving from multiple introductions [71–73]. In European waters, *Ulva australis* was discovered at several scattered locations in the 1990s and early 2000s, including the Delta region of the Netherlands, the Dutch Wadden Sea [74], off Brittany [75], the Iberian Peninsula, from the Bay of Biscay to Portugal [76,77] and the Mediterranean Sea (e.g., [78–80]). Possible introduction vectors include ballast water, hull fouling, and oyster transplants.
Ulva compressa Linnaeus	*tuf*A (This study)	1938 [39]	Native [7]	
Ulva lactuca	*rbc*L ([81]; as *Ulva fasciata*)	1938 [39]	Cryptogenic [7]	Loughane et al. [81] provided an *rbc*L sequence (EU484418) of a voucher identified as *Ulva fasciata*. Later studies including type material [82] corrected the voucher identity to *Ulva lactuca*.

Table 2. *Cont.*

Taxa	Genetic Markers	First Report to the Azores	Status in the Azores	Comments
Ulva lacinulata (Kützing) Wittrock *	*tuf*A (This study)	First report (This study)	Cryptogenic (This study)	According to Hughey et al. [83], there is a common taxonomic confusion with this species, which has been mistakenly reported as *U. laetevirens* (JQ048945), *U. scandinavica* (MW570777), *U. armoricana* (AB097632), and *U. rigida* (EU484395). Genetic investigations of *Ulva* spp. in the Baltic Sea and the Atlantic proximities showed that *U. rigida* was absent from the studied areas while *U. lacinulata* was relatively frequent [84]. The worldwide distribution of *Ulva* species needs to be reappraised based on sequences from type material due to the general misuse of species names [85]. Additionally, further studies are necessary to assess the real identity of *U. rigida, which is* currently reported to be native to the Azores [7].

3.4. Updates to the Azorean Marine Flora

The most recent update to the number of marine macroalgae in the Azores was published by Gabriel et al. [2], who reported the occurrence of 522 taxa (97 Ochrophyta, 349 Rhodophyta, and 76 Chlorophyta). The revision of the recent peer-reviewed literature revealed that three species should be added (*Pseudoralfsia azorica*, *Dictyota pleiacantha*, and *Cystoseira pustulata*) and two species should be removed (*Cystoseira compressa* and *C. humilis*) from that account based on new species descriptions, new reports, or taxonomic clarification of those taxa [25,36,40]. Together with the new reports of the present study (*Olokunia boudouresquei*, *Padina gymnospora*, *Ulva australis*, and *Ulva lacinulata*), the updated marine flora comprises 527 species (99 belonging to Ochrophyta, 350 to Rhodophyta, and 78 to Chlorophyta).

4. Discussion

The present study increases our knowledge of the Azorean marine flora and increases the number of taxa with sequences deposited in public repositories from 45 to 55. The temporal trend of sequences published in GenBank indicates a substantial increase since 2021 (Figure 3). Because the submission date of sequences deposited in BOLD is not specified, we were unable to detect such a trend in this public repository. The number of marine macroalgae known to the Azorean was also raised from 522 to 527. The number of introduced species reported to the Azores has also increased from 42 to 43, while the number of cryptogenic species has increased from 43 to 46.

Only 10.5% of the Azorean marine flora is currently represented in public repositories, which poses a limitation for barcoding studies, including environmental barcoding (e-barcoding). This percentage may be underestimated since some authors fail to inform the voucher locality when submitting sequences to public repositories. The examples of sequences from the Azores that were not retrieved in our search (as described in Section 2.3) are those from *Corallina berteroi*, *Cystoseira pustulata*, *Vertebrata barbarae*, and *V. cymatophila*, and species were added to the inventory only because the authors knew the publications where they were published [35,49,50]. The identification of taxa deposited in public repositories is to be considered with caution since accuracy cannot always be verified in the articles where the sequences were published, and the sequences of type material are seldom available [85,86].

Most sequences from Azorean vouchers retrieved from public repositories were published in taxonomic studies and produced in the context of phylogenetic reappraisals (e.g., [25,28,36,45,47–49,56,59,61,83]. Besides the fact that morphological observations supported the identifications, these studies sometimes provided the sequences of type material (e.g., *Dictyota pleiacantha* and *Laurencia pyramidalis*). For the newly generated sequences, the standard barcodes *cox*1, *rbc*L, and *tufA* were enough to discriminate the species [14], and the sequences of type material were used whenever available (e.g., *Hypnea musciformis* and *Ulva lacinulata*).

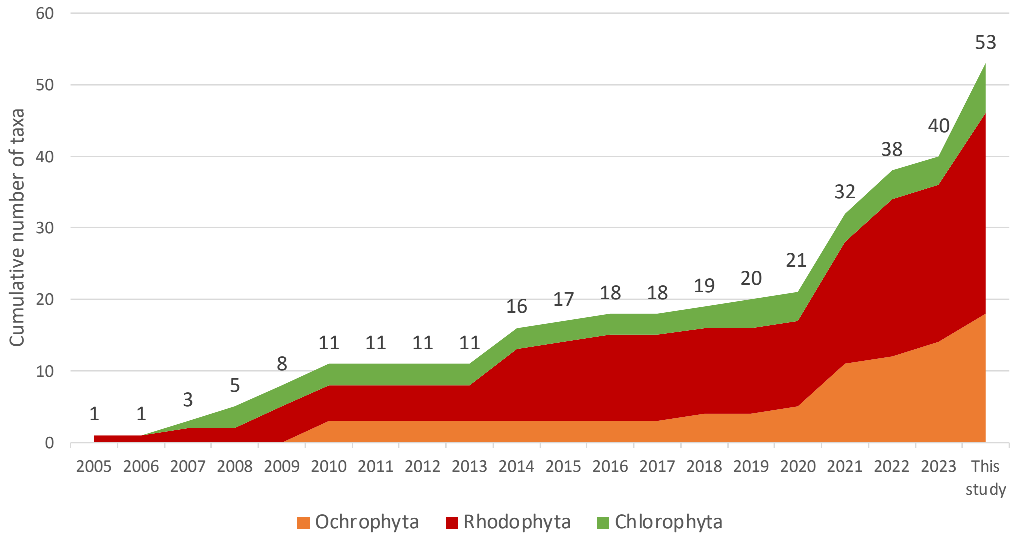

Figure 3. Cumulative number of taxa from Azorean vouchers with sequences deposited in GenBank. The number above line represents the sum of Ochrophyta, Rhodophyta, and Chlorophyta.

UPA, a universal marker for photosynthetic organisms, was also used in this study despite the low efficacy of this genetic marker to discriminate certain closely related species [14,87]. Easily amplified with a single pair of primers, UPA can identify a general group of algae and indicate the choices of primers to sequence other informative markers [88]. For that reason, UPA sequences are provided for most newly generated sequences, with five species receiving their first sequence of this marker. We were unable to produce DNA sequences other than UPA for only two vouchers, namely, *Padina gymnospora* and *Sytosiphon lomentaria*. Therefore, a further sequencing of other genetic markers is necessary to confirm the species' identities.

The groups of macroalgae have different percentages of taxa represented in public repositories when compared to the known marine flora of the Azores. Ochrophyta has 20% of its taxa with known sequences, having the highest followed by Chlorophyta with 9% and Rhodophyta with 8% (Figure 4). This variation reflects the fact that most studies that generated the molecular data (Table 2) focused on specific taxa. This is the first study to provide DNA barcodes for Azorean macroalgae from vouchers that were not specifically targeted but were collected for being conspicuous in the sampling sites.

Sampling was performed in the initial stages of the *Rugulopteryx okamurae* invasion on the southern coast (2020) of São Miguel Island [4,30]. As the invasion progressed, sampling was performed in the initial stages of the *Rugulopteryx okamurae* invasion on the northern and western coasts (2022; [4,30]). No specimen of *Rugulopteryx okamurae* was observed during the samplings performed on Santa Maria Island, though the collectors were not focused on searching for this species. The sampled taxa are not easily observed now due to the mentioned invasion.

Even though this was a small DNA-barcoding study, i.e., based on the collection of conspicuous specimens from a small number of sites, only in the intertidal zone of two islands, this study contributed to increasing the percentage of taxa from the Azorean marine flora represented in public repositories from 8.5% to 10.5%. Besides contributing to DNA barcodes from the Azores, the study also led to the uncovering of four new records for the archipelago, showing that the Azorean marine flora is still poorly known.

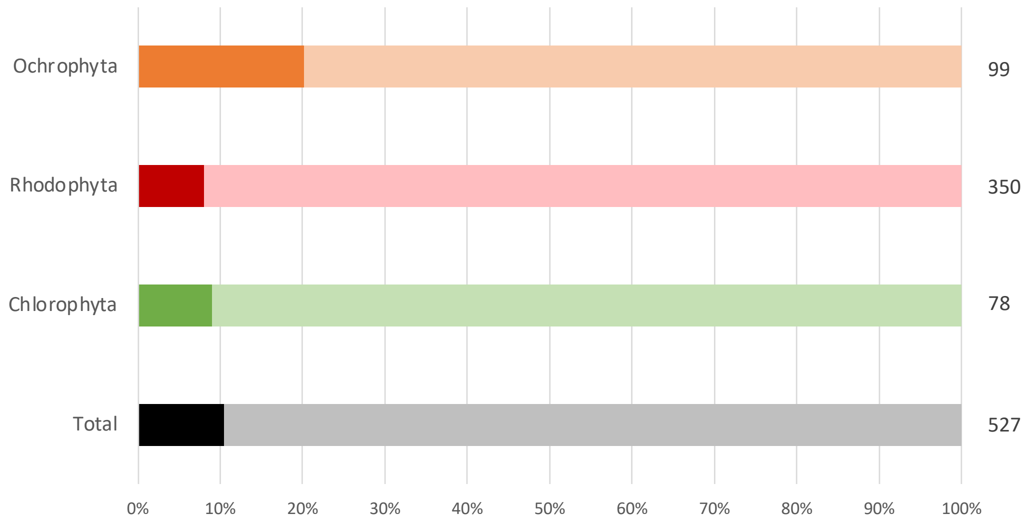

Figure 4. Percentage of taxa known to the Azorean marine flora with sequences deposited in public repositories. Dark colors indicate taxa with sequences, and light colors indicate taxa without sequences.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/phycology4010004/s1, Figure S1: Collection sites. São Miguel: (A) Caloura; (B) Atalhada; (C) Praia do Pópulo; (D) São Vicente de Ferreira; and (E) Mosteiros. Santa Maria: (F) Maia; (G) Praia Formosa; and H) Anjos; Figure S2: In vivo pictures of some marine macroalgae from the Azores analyzed in the present study. Ochrophyta: (A) *Colpomenia sinuosa*; (B) *Fucus spiralis*; (C) *Padina gymnospora*; and (D) *Scytosiphon lomentaria*. Rhodophyta: (E) *Amphiroa beauvoisii*; (F) *Gelidium microdon*; (G) *Hypnea musciformis*; (H) *Laurencia pyramidalis*; and (I) *Lomentaria articulata*. Chlorophyta: (J) *Ulva australis*; (K) *Ulva compressa*; and (L) *Ulva lacinulata*. Scale bar = 1 cm; Table S1: Collection information and GenBank accession numbers for the newly collected samples used; Table S2: Primers and PCR conditions for the different genetic markers used [9,16–22]; Table S3: Sequences from Azorean vouchers retrieved from GenBank and BOLD, with the respective references [3,4,24,25,28,31,36,40,43,45,47,48,50,56,58–61,64,66,70,81,89,90].

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Data Availability Statement: The data presented in this study are available in Supplementary Tables S1 and S3. DNA sequences can be retrieved from GenBank and BOLD using the accession numbers in the mentioned tables.

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Appendix A

DNA barcode-assisted analysis of samples collected and sequenced in this study as described in Section 3.1 (Table A1).

Table A1. DNA barcode-assisted analysis of samples collected and sequenced in this study. Sequences marked with * represent the first DNA sequence of the marker for the species based on Azorean vouchers, and sequences marked with + represent the first DNA sequence of the marker for the species.

Taxa	Genetic Marker	GenBank Accession Numbers	Top Matches	Comments
Ochrophyta				
Colpomenia sinuosa	*rbc*L *	OR944643 OR944644	*Colpomenia sinuosa*, 99.39% similarity, Hawaiian Islands (AB578988) and Korea (AY398468)	
	UPA *	OR961485	*Colpomenia sinuosa*, 99.74% similarity, China (OR094252) and Hawaiian Islands (EF426596)	
Fucus spiralis	*cox*1	OR944632	*Fucus spiralis*, 100% similarity, the Atlantic USA coast (EU646722) *Fucus vesiculosus*, 100% similarity, Norway (MN184303)	Phylogeny reconstructions based on complete mitochondrion and chloroplast genomes support the recognition of *Fucus spiralis* var. *vesiculosus* [91], although this taxon is not currently recognized in Algaebase. Until further taxonomic decisions, and since the presence of *F. vesiculosus* in the Azores is not confirmed by morphological studies [7], *F. spiralis* is maintained as the identity of the Azorean voucher.
	UPA *	OR961486 OR961487 OR961488	*Fucus spiralis*, 99.75% similarity, the Pacific USA coast (MG922855) *Fucus vesiculosus*, 99.75% similarity, Portugal (FM957154)	
Padina gymnospora	UPA *	OR961489	*Padina gymnospora*, 99.20% similarity, Hawaiian Islands (EF426588)	UPA sequences from *Padina pavonica*, the only *Padina* species currently reported to the Azores, are not available for comparison.
Scytosiphon lomentaria	UPA *	OR961490	*Scytosiphon promiscuous*, 100% similarity, Japan (MK107984) *Hapterophycus canaliculatus*, 100% similarity, Korea (MF591718) *Petalonia binghamiae* (as *Endarachne binghamiae*), 100% similarity, Korea (MF460360) *Scytosiphon lomentaria*, 100% similarity, China (MK798154) *Petalonia fascia*, 100% similarity, the Atlantic USA coast (KF367780) and China (KC782886)	Phylogeny reconstructions based on a complete chloroplast genome show that *Scytosiphon promiscuus* and *Petalonia binghamiae* are different taxa [92], though UPA sequences retrieved from their complete chloroplast genome are 100% similar.

Table A1. *Cont.*

Taxa	Genetic Marker	GenBank Accession Numbers	Top Matches	Comments
Rhodophyta				
Amphiroa beauvoisii	*cox*1 *	OR944633 OR944634 OR944635 OR944636	*Amphiroa beauvoisii*, 100% similarity, Brazil (MG521348)	Phylogenetic reconstructions based on four genetic markers indicate that *Amphiroa beauvoisii* is a species complex [87], with the Azorean vouchers nested in "clade 2".
	UPA *	OR961491 OR961492	*Amphiroa beauvoisii*, 100% similarity, Brazil (MG521307) and the Atlantic USA coast (KF367784)	
Gelidium microdon	*rbc*L *	OR944645	*Gelidium microdon*, 100% similarity, the Atlantic Spanish coast (KT920271)	
	UPA *+	OR961493	This is the first UPA sequence for *Gelidium microdon*	
Hypnea musciformis	*cox*1 *	OR944637 OR944638	*Hypnea musciformis*, 99.85% similarity, Tunisia (MG030775) and Italy (MF597663)	A sequence from the complete chloroplast genome generated from a topotype of *Hypnea musciformis* was used for comparison (OL964154; [93]).
	*rbc*L *	OR944646	*Hypnea musciformis*, 99.93% similarity, Italy (OL964154), France (EU346011), and the Mediterranean Spanish coast (KT428787)	
	UPA *	OR961494	*Hypnea musciformis*, 100% similarity, Italy (OL964154) and the Atlantic USA coast (KJ202096)	
Jania pedunculata var. *adhaerens*	*cox*1 *	OR944639	*Jania* sp., 100% similarity, the Indian South African coast (LC071780) *Jania adhaerens*, 99.85% similarity, China (OP168510)	Based on the lectotype, *Jania pedunculata* var. *adhaerens* should be referred to as a variety rather than a distinct species [52].
	UPA *+	OR961495	This is the first UPA sequence for *Jania pedunculata* var. *adhaerens*	
Jania verrucosa	*cox*1 *	OR944640	*Jania verrucosa*, 98.95% similarity, the Indian South African coast (LC071781)	Although the similarity between *cox*1 sequences was slightly beyond 99%, this match was used to discriminate the species due to the higher similarity observed between the *rbc*L sequences.
	*rbc*L *	OR944647	*Jania verrucosa*, 99.89% similarity, New Zealand (KM369140)	
	UPA *+	OR961496	This is the first UPA sequence for *Jania verrucosa*	

Table A1. *Cont.*

Taxa	Genetic Marker	GenBank Accession Numbers	Top Matches	Comments
Laurencia pyramidalis	*rbc*L	OR944648	*Laurencia pyramidalis*, 100% similarity, the Atlantic France (FJ785316), the Canary Islands (KF492791), the Azores (KF492787), and the Mediterranean Turkish coast (MZ153331)	
	UPA *+	OR961497	This is the first UPA sequence for *Laurencia pyramidalis*	
Lomentaria articulata	*cox*1 *	OR944641	*Lomentaria articulata*, 100% similarity, the Atlantic French coast (KJ960811)	
	UPA *+	OR961498	This is the first UPA sequence for *Lomentaria articulata*	
Chlorophyta				
Ulva australis	*tuf*A *	OR944649	*Ulva australis*, 100% similarity, the Atlantic French coast (MT160640), Italy (LR732070), the Netherlands (MZ272366), and Australia (MK125443) *Ulva pertusa*, 100% similarity, China (MN853875)	
Ulva compressa	*tuf*A *	OR944650	*Ulva compressa*, 99.85% similarity, Australia (JN029285)	
Ulva lacinulata	*tuf*A *	OR944651 OR944652	*Ulva lacinulata*, 100% similarity, Croatia (MW543061) and Portugal (MT160657) *U. laetevirens*, 100% similarity, Italy (MF544109), Tunisia (MF614791), and Korea (MT625105)	A sequence from the complete chloroplast genome generated from the lectotype of *Ulva lacinulata* was used for comparison (MW543061; [83]).

Appendix B

DNA barcode-assisted analysis of sequences deposited in public repositories but not included in the peer-reviewed literature as described in Section 3.2 (Table A2).

Table A2. DNA barcode-assisted analysis of sequences deposited in public repositories but not included in the peer-reviewed literature (unpublished sequences). Sequence marked with * represents the first DNA sequence of the marker for the species based on Azorean vouchers.

Taxa	Genetic Marker	GenBank/ BOLD Accession Numbers	Top Matches	Comments
Ochrophyta				
Colpomenia sinuosa	*cox*1	PHAZO004-10 PHAZO117-10 PHAZO118-10 PHAZO119-10 PHAZO203-10 PHAZO204-10	*Colpomenia sinuosa*, 99.6–100% similarity, Australia (MZ054832)	
Cystoseira pustulata	*cox*1	OL434655 (as *Cystoseira pustulata*) PHAZO043-10 (as *Cystoseira compressa*)	*Cystoseira pustulata*, 100% similarity, the Canary Islands (OK480304), the Azores (MF768044), and the Mediterranean Spanish coast (MF768042)	
Fucus spiralis	*cox*1	PHAZO060-10 PHAZO062-10 PHAZO063-10	*Fucus spiralis*, 100% similarity, the Atlantic USA coast (EU646722) *Fucus vesiculosus*, 100% similarity, Norway (MN184303)	Phylogeny reconstructions based on complete mitochondrion and chloroplast genomes support the recognition of *Fucus spiralis* var. *vesiculosus* [91], although this taxon is not currently recognized in Algaebase. Until further taxonomic decisions, and since the presence of *F. vesiculosus* in the Azores is not confirmed by morphological studies [7], *F. spiralis* is maintained as the identity of the Azorean voucher.
Gongolaria abies-marina	*cox*1	PHAZO156-10 (as *Cystoseira abies-marina*)	*Gongolaria abies-marina*, 100% similarity, Azores (OK480429)	
Halopteris filicina	*cox*1	PHAZO003-10 PHAZO005-10 PHAZO008-10 PHAZO041-10 PHAZO042-10 PHAZO153-10	*Halopteris filicina*, 99.8% similarity, Mediterranean French coast (EU579868)	
Halopteris scoparia	LSU	OL434652	*Halopteris scoparia*, 99.2% similarity, Tunisia (OK646025)	
Petalonia binghamiae	*cox*1	PHAZO002-10 (as *Petalonia* cf. *fascia*)	*Petalonia binghamiae*, 100% similarity, China (MF374731; as *Endarachne binghamiae*).	

Table A2. *Cont.*

Taxa	Genetic Marker	GenBank/ BOLD Accession Numbers	Top Matches	Comments
Sargassum cymosum	*cox*1	OL434657 PHAZO044-10 PHAZO045-10 PHAZO116-10 (as *Sargassum* sp.)	*Sargassum natans*, 100% similarity, Curaçao (OM460646), from the Northwestern Atlantic Ocean and the Caribbean Sea (KY084907—KY084908, KY084912 *Sargassum fluitans*, 100% similarity, from the Gulf of Mexico (MT470904)	Phylogenetic reconstructions based on *cox*1, ITS-2, and *rbc*L [94] indicate that both species should be regarded as synonyms of *S. cymosum*. This name is used despite the lack of a formal amendment to *S. cymosum* since the species is reported to the Azores [7], unlike *S. natans* and *S. fluitans*.
Scytosiphon lomentaria	*cox*1	PHAZO060-10 PHAZO062-10 PHAZO063-10	*Scytosiphon lomentaria*, 99.8–100% similarity, Argentina (ON568311)	
Zonaria tournefortii	*cox*1	OL434658	*Zonaria tournefortii*, 100% similarity, the Canary Island (MW224392) and the Atlantic USA coast (KF367749)	
Rhodophyta				
Asparagopsis armata	*cox*1	OL434653	*Asparagopsis armata*, 100% similarity, the Atlantic French coast (KJ960344) and the UK (AB774223)	
Asparagopsis taxiformis	*cox*1	OL434654	*Asparagopsis taxiformis*, 100% similarity, Italy (KJ398158) and the Mediterranean Spanish coast (AB774217)	
Corallina berteroi	*cox*1 *	HQ919507	*Corallina caespitosa*, 100% similarity, the Atlantic Spanish coast (KF460944)	Reappraisal of *Corallina* species based on type material [95] suggested that the cosmopolitan species *C. caespitosa* should be referred to as *C. berteroi*.
		HQ919508	*Corallina caespitosa*, 100% similarity, the Atlantic Spanish coast (KF460946) and the Atlantic French coast (KJ960596)	
			HQ919502 HQ919503 HQ919504 HQ919505 HQ919506 HQ919509 HQ919510 HQ919511	*Corallina caespitosa*, 99.2–99.6% similarity, the Azores (KP834357)
Olokunia boudouresquei	*rbc*L	EU349143 (as *Cruoriopsis crucialis*)	*Olokunia boudouresquei*, 100% similarity, New Zealand (KC998949; as *Peyssonnelia boudouresquei*)	This sequence is reported as included in Krayesky et al. [60], but no reference to the sequence is found in this article.

Table A2. *Cont.*

Taxa	Genetic Marker	GenBank/ BOLD Accession Numbers	Top Matches	Comments
Osmundea pinnatifida	*rbc*L	KU647694/ REDEU882-13	*Osmundea pinnatifida*, 100% similarity, Ireland (AF281875)	
Pterocladiella capillacea	*cox*1	OL434656	*Pterocladiella capillacea*, 100% similarity, Tunisia (MG030796) and the Mediterranean French coast (OL809719)	
Chlorophyta				
Caulerpa webbiana f. *disticha*	*tuf*A	FM956073	*Caulerpa webbiana*, 100% similarity, Red Sea (AJ417958), Australia (KF314152), and the Hawaiian Islands (KJ957133)	Further studies of the different formae within the species are needed to confirm the subspecific rank of the Azorean voucher.

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