

Article

DNA Barcoding of Moon Jellyfish (Cnidaria, Scyphozoa, Ulmaridae, *Aurelia*): Two Cryptic Species from the Azores (NE Atlantic, Macaronesia), and Evaluation of the Non-Indigenous Species (NIS)

Carlos J. Moura ^{*,†} , Bruno I. Magalhães  and João M. Gonçalves

Okeanos-Uac—Instituto de Investigação em Ciências do Mar, Universidade dos Açores, 9901-862 Horta, Portugal

* Correspondence: carlos.moura@mail.com

† Current address: Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

Abstract: Moon jellies are some of the most popular, widely distributed, and best-studied marine jellyfish. By the end of the past century only two or three *Aurelia* species were recognized, but with the rise of DNA barcoding studies, around thirty *Aurelia* species are presently accepted. Most of the species are morphologically indistinguishable and have restricted biogeography. We reveal, with COI, 16S, and ITS1-5.8S sequence data, two (pseudo-)cryptic species of *Aurelia*, potentially endemic to the Azores ecoregion, herein provisionally classified as *A. "cf. pseudosolida"* and *A. "misteriosa"*. These species are closely related to the Mediterranean lineages of *A. pseudosolida* and *A. persea*, respectively. In the Azores, the shape of the campanula and oral arms readily distinguishes the two species: the former with folded oral arms and globose campanula, and the latter with flattened campanula and thick and long oral arms. Previous reports of *A. solida* and *A. aurita* in the Azores should generally correspond to *A. "misteriosa"* and *A. cf. pseudosolida*, respectively. The phylogenetic (re-)examination of the available DNA barcodes of *Aurelia* only evidenced human-mediated dispersal for *A. coerulea*, *A. relicta*, and *A. aurita*. *Aurelia solida* cannot be yet considered NIS in the Mediterranean. More jellyfish DNA (meta)barcoding should reveal further cryptic diversity, biological invasions, and phylogeographic inferences.

Keywords: cryptic biodiversity; moon jellies; molecular taxonomy; biogeography; exotic species; biological invasions; MinION DNA sequencer; Macaronesia



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1. Introduction

The moon jellies (e.g., Figure 1), scyphozoans of the genus *Aurelia*, are among the best-studied, aquacultured, and globally distributed jellyfish. They are naturally present in open waters, including the deep sea, but are mostly found in neritic environments including estuaries and inlets [1–4]. Their metagenetic life cycle generally alternates seasonally between the asexual benthic morphs (planulocysts, podocysts, and polyps/scyphistoma) and the pelagic motile stages (ephyrae, sexual medusae, and planulae that may develop on the oral arms or manubrium of the females) (e.g., [4–8]). The local biotic and abiotic properties, such as temperature, salinity, solar irradiance, substrate, and food availability, may trigger these life-cycle transitions, correlating with their intensity and timing (e.g., [4,9–16]). The medusae, with a lifetime of ca. 4–8 months, but sometimes more [4], are considered the main dispersal phase of *Aurelia* spp. (e.g., [4,5]), for their active swimming capabilities (e.g., [17,18]), and their greatest ability to drift with oceanic currents (e.g., [19]). Nevertheless, the benthic stages of a few *Aurelia* species have been observed on artificial floating substrates (e.g., [20]), which adds the potential ability to raft with oceanic currents [20] and prosper in different biogeographic settings [21–24]. Transport through ballast waters may

also occur (e.g., [20]), in all life stages. It is also believed that some non-native populations of *Aurelia* could have been introduced by aquarists or aquacultures [25,26]. The known depth range of moon jellies extends between 0 and 3400 m depth [27].

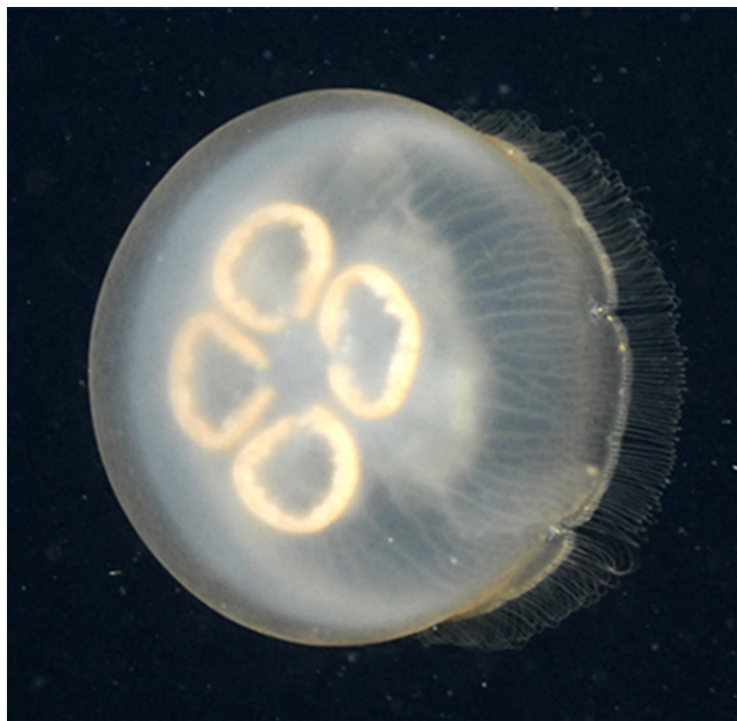


Figure 1. The most common moon jellyfish species in the Azores—*Aurelia* cf. *pseudosolida*. Photo credits: Nuno Vasco Rodrigues.

Presently, 26 nominal species of *Aurelia* are formally accepted [28], and seven putative species already identified by molecular methods still await recognition [29]. The taxonomy history of the *Aurelia* species is complex and has been quite disputed. Since the description of *A. aurita* by Linnaeus [30], 14 *Aurelia* species names were given at the beginning of the 20th century [28]. Mayer (1910) [1] then considered only “three reasonably well-defined types”. Later Kramp (1961) [31] recognized seven species, but by the end of the 20th century, only two species were generally accepted [5,32–34]. The increase in genetic studies in the 21st century started to unveil the cryptic diversity of *Aurelia* species (e.g., [21,22]), and species revalidations and redescriptions occurred [24,29,35,36]. Notably, Lawley et al. [29] recently demonstrated the extent of plasticity and inoperability of classic morphologic characters to differentiate molecularly defined species of *Aurelia* (something partially verified before by Chiaverano et al. [37], Scorrano et al. [24], and Chiaverano & Graham [38]), and consequently validated 14 *Aurelia* species diagnosed with nucleotide differences in COI and 16S “DNA barcodes”. That work is thus likely to induce other researchers to describe cryptic species (of *Aurelia*) based on nucleotide characters, incrementing formal species recognitions, and adding reliability to subsequent scientific studies involving those cryptic taxa (e.g., in biodiversity assessments and ecological, evolution, and biotechnology studies). Nevertheless, to facilitate visual species identification, it would still be preferable to provide morphological diagnostic characters to ease species differentiation [36], especially when they occur in sympatry or peripatry. Furthermore, more sampling and DNA barcoding may prove the uselessness of some DNA substitution mutations presently considered diagnostic to differentiate (putative) *Aurelia* species ([29]; this study) if these mutations are eventually found in closely related species, what may add taxonomic conflict at the end.

In the “Azores ecoregion” [39], roughly located in the middle of the North Atlantic, seldom reports of the nominal species *A. aurita* and *A. solida* exist ([40–44], and this study;

cf. Table S1). Mayer [1] reported an isolated report of *A. solida* close to Madeira Island, and to our knowledge, no other official reports of moon jellies reports exist in Macaronesia.

Regarding the recent recognition of many cryptic species within the genus *Aurelia* (e.g., [3,29]), we thus questioned which *Aurelia* species are present in the Azores and submitted to molecular analyses a reasonably high number of stranded medusa collected in the archipelago.

Through the application of a comprehensive multi-species DNA barcoding assessment of the marine gelatinous fauna occurring in the Azores, mainly sampled between 2019 and 2021, we found, and reveal here, genetic evidence for the existence of two cryptic species of *Aurelia* in the Azores. We present morphologic characteristics to differentiate the two *Aurelia* species occurring in the Azores and reclassified taxonomically (but provisionally) the moon jellies previously photographed or video recorded in the Azores. We putatively diagnose, with nucleotide combinations in DNA barcodes (COI and 16S markers), and morphologic characteristics, differences between the two species of *Aurelia* occurring in the Azores and against their evolutionary-close species. One *Aurelia* species in the Azores, herein named “*A. cf. pseudosolida*”, is highly related to, or the same species as, the recently described *A. pseudosolida* known from the Mediterranean. The other *Aurelia* species in the Azores, herein provisionally named “*A. misteriosa*”, is phylogenetically close to *A. persea*. We discuss the morphologic and genetic distinctiveness, and possible biogeographic isolation, of the moon jelly species occurring in the Azores. We also analyze all the available DNA barcode data to summarize which *Aurelia* species present distant and disjunctive biogeographic distributions due to human-mediated transport.

2. Materials and Methods

Stranded moon jellyfish (162 specimens) were collected manually conjunctly with other gelatinous fauna, on four beaches of two Azorean islands (Faial and São Miguel), by monthly monitoring for 6 consecutive days per month, at low tide, between February 2019 and January 2022. For each moon jelly collected, we recorded weight and bell diameter, and photographed the entire specimen. We then extracted a small tissue fragment with ethanol 96% for genetic analyses from 28 specimens, mostly found dead and lacerated. Only exemplars collected in 2021 were preserved in seawater with formalin (ca. 4%) and were deposited in the biological collection of the Campus of Horta—University of the Azores. Sampling data of the *Aurelia* specimens collected, as well as the previous records we found of *Aurelia* in the Azores, are presented in Table S1. Measurements and weights of the specimens collected are in Table S2.

The DNA barcoding laboratory methods applied to the 25 *Aurelia* samples collected in 2019 are described in Moura et al. [45], with the addition of the amplification and sequencing of ca. 420 bp of the nuclear region ITS1-5.8S using the primers ITSF (TAA-CAAGGTTTCCGTAGG) and ITSr (CTCAGACAGACATGCTCC) [22]. These samples were processed conjunctly with around 1000 samples of jellies and associated taxa (cf. methods in Moura et al. [45]), to sequence, in two runs with a MinION sequencer (©Oxford Nanopore Technologies), the mitochondrial markers 16S (ca. 600 bp) and COI (ca. 658 bp), and the nuclear marker ITS-1/5.8S. The correspondent PCR products were tracked using a pool of 13 bp primer tags used for demultiplexing (cf. Moura et al. [45]). The DNA barcodes generated were verified and eventually corrected, using Geneious Prime® 2021.1, as described by Moura et al. [45]. The three *Aurelia* samples collected in 2021 were processed posteriorly with similar methods, except for the PCR products that were purified separately and sequenced by an external company with a Sanger sequencer. The DNA sequences generated were deposited in GenBank under accession numbers ON419421 (COI), ON427537-ON427559 (16S), and ON427523-ON427536 (ITS-1/5.8S). Sequence data are presented in Table S3.

The DNA barcodes generated were incorporated in the nucleotide alignments provided by Lawley et al. [29], i.e., for the COI, 16S, ITS1-5.8S, and the “concatenated” dataset that also includes 28S sequence data. We further updated these alignments with the re-

cent nucleotide sequences of *Aurelia* provided by Brown et al. [35], Gawinski et al. [46], Rekstad et al. [47], Seo et al. [48], and Gari & Batistic [36]. The (re-) alignments were performed with MAFFT v7.450 in Geneious Prime[®] 2021.1 using the algorithm “E-INS-I” and the “Use legacy gap penalty” option, but not choosing to “preserve original sequence order”. The resultant alignments, intended to be similar to those of Lawley et al. [29], were visually inspected, but a few nucleotide positions were corrected manually. The 16S alignment presented by Lawley et al. [29] had several gap positions considered unnecessary, and, therefore, that alignment was realigned with the new sequences without the “Use legacy gap penalty” option, resulting in a 402 bp alignment (instead of 440 bp). As a result, note that the nucleotide differences used to diagnose *Aurelia* species according to Lawley et al. [29] are herein presented in different alignment positions in the 16S dataset. The resultant alignments are supplied in the Supplementary Materials (Alignments S1–S4).

The alignments were submitted to PHYML V3.0 [49] for 1000 bootstrap maximum likelihood phylogenetic tree building, choosing the “SMS: Smart Model Selection in PhyML” [50] and the remaining default settings. The resulting trees were finally edited using ITOL V5.0 [51] and INKSCAPE V1.0.2.

Uncorrected pairwise genetic distances (-distances) were determined, for the COI and 16S alignments, with MEGA V7.026 [52] to investigate intra- and inter-species sequence divergences.

The COI and 16S alignments were also analyzed with the DeSignate software [53], using its online platform (<https://designate.dbresearch.uni-salzburg.at>, accessed on 28 December 2022), to diagnose nucleotide differences of *A. cf. pseudosolida* and *A. “misteriosa”* in comparison to all publicly available sequences of *Aurelia* species and to the most closely related species.

3. Results

3.1. DNA Analyses

The COI marker was only sequenced in the sample of *A. “misteriosa”*. After several PCR attempts, with adjustments in the DNA volume and annealing temperature, we could not amplify and sequence the COI with the “Folmer primers” LCO1490 and HCO2198 [54] in any of the 27 samples of *A. cf. pseudosolida* submitted to molecular analyses. By contrast, the 16S marker was successfully amplified and sequenced, with Cunningham and Buss [55] primers, in 22 samples of *Aurelia* including the unique sample of *A. “misteriosa”*. The ITS-1/5.8S region was successfully sequenced, with Schroth et al. [22] primers, in 14 *Aurelia* samples including the sample of *A. “misteriosa”* (cf. Table S3).

3.1.1. Phylogenetic Position of *Aurelia* “Misteriosa”

Phylogenetic analyses on COI sequence data evidence (Figure S1), with high bootstrap support, that our unique sample of *Aurelia* “misteriosa” clusters with *A. persea* (Figure S1), which is solely known from the Eastern Mediterranean (Haifa Bay, Israel; [56]). The genetic distance between these two lineages is 5% (p-distance). *Aurelia* “misteriosa” and *A. persea* in turn group with *A. “sp. 18”*, which is only known in the Gulf of Mexico [57]. The COI sequence divergence (p-distance) is ca. 8.3% between *A. “misteriosa”* and *A. “sp. 18”*, and ca. 10.5% between *A. “sp. 18”* and *A. persea*, which is thus in the range of 6–10% genetic distance between most putative species of *Aurelia* [29]. These three species (i.e. *A. persea*, *A. “misteriosa”*, and *A. “sp. 18”*) in turn cluster with *A. relictata* (from the Mediterranean basin). The phylogenetic relationships between these four species present high bootstrap support uniquely with the COI sequence data.

The 16S sequence data also suggest, with high bootstrap support, that *Aurelia* “misteriosa” is closely related to *A. persea* (3% p-distance) (Figure S2). These two species group with *A. relictata* with high bootstrap support. These three species in turn cluster with *A. aurita* (bootstrap support of 97%) (Figure S2). No 16S sequence of *A. “sp. 18”* is yet available.

The available ITS1-5.8S sequence data suggest, with high bootstrap support, *Aurelia* “misteriosa” and *A. “sp. 18”* are sister species that in turn cluster with the clade of *A. relictata* (Figure S3). ITS1-5.8S sequences of *A. persea* are not available.

The concatenated phylogenetic analysis (Figure 2), similar to the COI dataset, indicates, with high bootstrap support, that *Aurelia* “misteriosa” and *A. persea* are sister species and that these two taxa in turn group with *A. sp. 18*. These three species then group with *A. relicta* with high bootstrap support. The phylogenetic position of these four taxa is not clear regarding the other known *Aurelia* species (Figure 2).



Figure 2. Maximum likelihood phylogenetic tree using the concatenated dataset (with the combined markers 16S, COI, ITS1-5.8S, and 28S) generated to analyze evolutionary relationships between 32 putative species of *Aurelia*. Bootstrap values are uniquely shown for branches between species if higher than 50%. Single marker phylogenies are shown in Figures S1–S3.

3.1.2. Phylogenetic Position of *Aurelia* “cf. *pseudosolida*”

The 16S sequence data indicates a well-supported cluster including *Aurelia* cf. *pseudosolida* specimens sampled in the Azores and the unique 16S sequence of *A. pseudosolida* available. The latter species, recently described from the Mediterranean, present the most divergent haplotype within that cluster (2–2.8% p-distance to haplotypes of *A. cf. pseudosolida*) which was not detected in the Azores (Figure S2). Recurrent NJ tree-building analyses recovered consistently *A. pseudosolida* as a sister clade to all the available 16S haplotypes of *A. cf. pseudosolida* (results not shown), but, conversely, several Bayesian tree reconstructions (results not shown) did not support it similarly to the ML analyses herein presented (Figure S2). Thirteen 16S haplotypes of *A. cf. pseudosolida* from the Azores were sequenced (with a maximum of 1.8% p-distance between them). The most common haplotype sequenced was found between April and June 2019 (on São Miguel Island and Faial Island) and in April 2021 on Faial Island. The phylogenetic relation of the cluster of *A. cf. pseudosolida* and *A. pseudosolida* with other *Aurelia* species remains unclear with the present 16S sequence dataset (Figure S2).

The ITS1-5.8S sequence data also suggest the close grouping of the Azores samples of *Aurelia* (excluding *A. “misteriosa”*) with *A. pseudosolida*, but the bootstrap support of that cluster is not high despite the small genetic distances between these haplotypes (Figure S3). Although fewer bp were used for phylogenetic analyses with the ITS1-5.8S dataset, a minor divergence of the haplotype of *A. pseudosolida* from the Mediterranean comparatively to the other Azorean haplotypes was noted. Nevertheless, similar to the available 16S sequence data, the only haplotype of *A. pseudosolida* known from the Mediterranean was not detected in the Azores (Figure S3).

The concatenated phylogenetic analysis (Figure 2) also suggests the clustering of *Aurelia* cf. *pseudosolida* conjunctly with *A. pseudosolida*. That clade in turn clusters with *A. solida* (without much bootstrap support) (Figure 2), which is in concordance with the morphologic similarities between these three taxa.

3.1.3. Review of the *Aurelia* Species with Widespread and Disjunct Biogeographic Distributions

COI sequence data evidence three species with wide and disjunct biogeographic distributions, namely: *Aurelia solida*, *A. coerulea*, and *A. aurita* (Figure S1). The clade of *A. solida* presents one haplotype from the Red Sea clustered with the Mediterranean haplotypes, but it is worth mentioning that the Red Sea haplotype is the most divergent (1.2–2.8% p-distance) and was not detected (yet) in the Mediterranean. *Aurelia coerulea* presents haplotypes in the Indo-Pacific, Mediterranean, and NE Atlantic. Two haplotypes of *A. coerulea* specimens were simultaneously found in the Mediterranean and Pacific. *Aurelia aurita* distributes in the North Atlantic, Black Sea, and SE and NW Pacific. Two haplotypes of *A. aurita* specimens were found simultaneously in the Black Sea and the North Sea (coast of the Netherlands), and one of these is also present in the Mediterranean (Figure S1).

The 16S sequence data highlights four species with widespread and disjunct biogeographic distributions: *Aurelia coerulea*, *A. solida*, *A. relictata*, and *A. aurita* (Figure S2). *Aurelia coerulea* is represented by 16S haplotypes in the Pacific, NE Atlantic, and the Mediterranean. One of these haplotypes was sampled simultaneously in the N Atlantic and N Pacific, another haplotype was collected in the Marmara Sea and N Pacific, and another haplotype was found in the Mediterranean Sea and N Pacific. *Aurelia solida* has haplotypes of specimens sampled in the Mediterranean and the Red Sea, but similar to the COI sequence data, the lineages seem segregated by marine area. *Aurelia relictata* was found in the NW Pacific (Japanese waters) and the Mediterranean and has specimens sampled with the same haplotype in these regions. *Aurelia aurita* is present in the N Atlantic and SE Pacific and has specimens with the same haplotype in these oceanic regions (Figure S2).

The ITS1-5.8S sequence data indicate disjunct distributions for the four species highlighted with 16S data, namely: *A. coerulea*, *A. solida*, *A. relictata*, and *A. aurita* (Figure S3). *Aurelia coerulea* occurs in the Pacific, NE Atlantic, and the Marmara Sea and has specimens

with the same haplotype in these three oceanic regions. *Aurelia solida* has different ITS1-5.8S sequences sampled from the Mediterranean and the Red Sea. *Aurelia relictata* is represented by ITS1-5.8S sequences from the SW Atlantic and the Mediterranean and has an identical haplotype between these regions. *Aurelia aurita* is represented by specimens with ITS1-5.8S haplotypes from the SE Pacific, N Atlantic, Black Sea, Sea of Marmara, and the Caspian Sea. One haplotype was found in *A. aurita* specimens sampled in the NE Atlantic and the Caspian Sea, while another haplotype was recovered in specimens from the Black Sea, White Sea, Argentina, and N Atlantic (Figure S3).

3.2. Systematic Account

Phylum: Cnidaria Verrill, 1865
 Class: Scyphozoa Goette, 1887
 Order: Semaestomeae Agassiz, 1862
 Family: Ulmaridae Haeckel, 1880
 Genus: *Aurelia* Lamarck, 1816
 Species: *Aurelia* cf. *pseudosolida*
 Figures 1, 3 and 4.

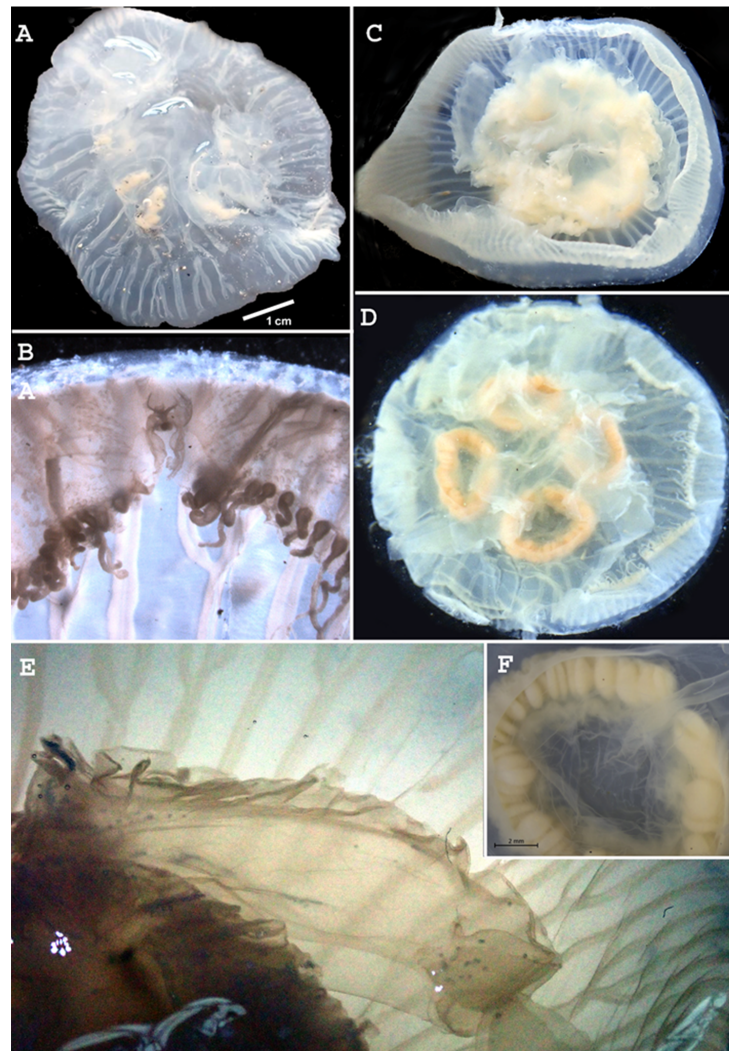


Figure 3. *Aurelia* cf. *pseudosolida*, collected in the Azores: field number Aurelia1PtoPim13Abr2021, 16S accession number ON427557 (A,B), field number ICTPLKT98—station 3.1, from 500 m (C) and 330 m depths (D–F). Photo credits: Carlos J. Moura (A–E), Bruno I. Magalhães (F).

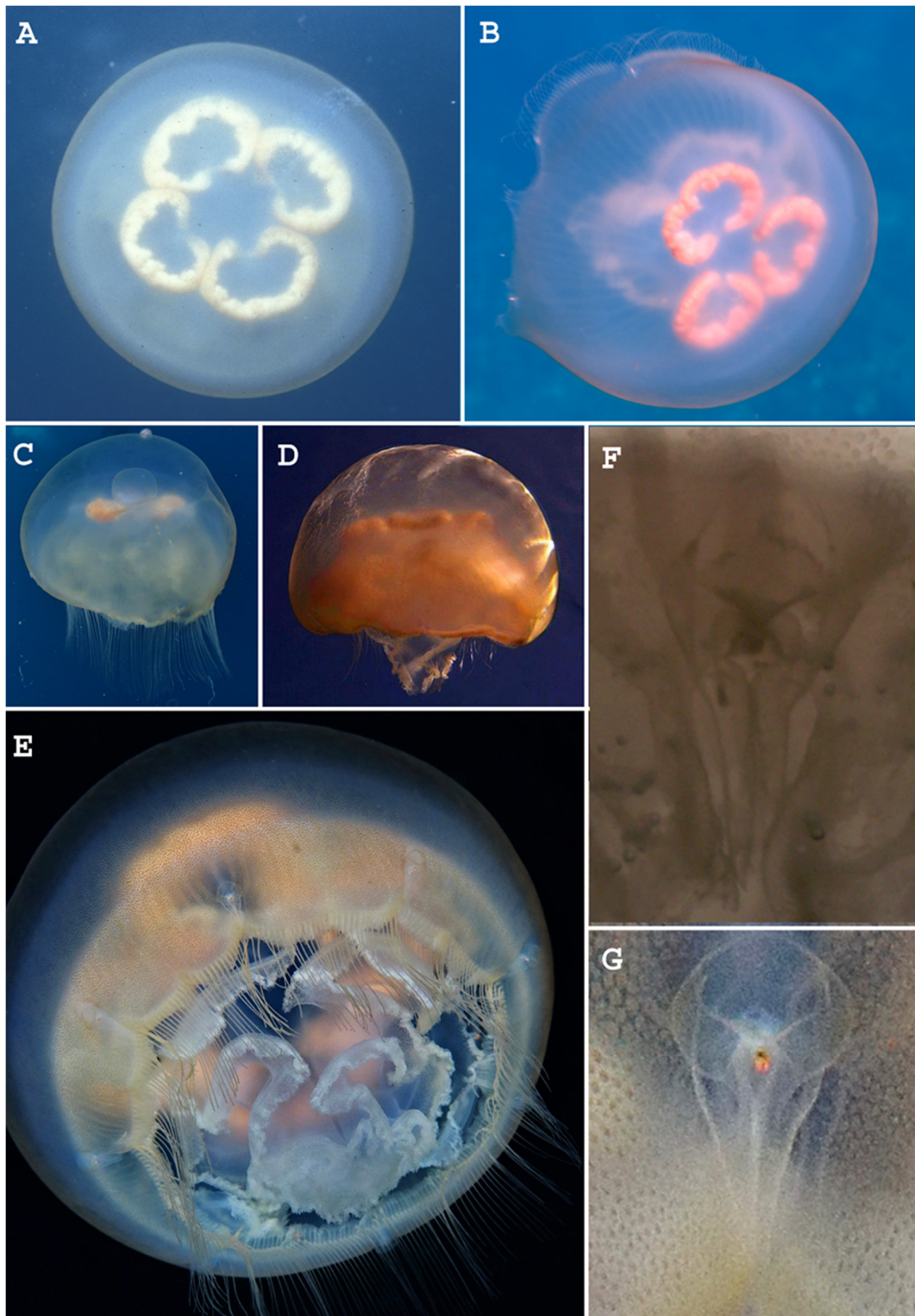


Figure 4. *Aurelia* cf. *pseudosolida* from the Azores: top view of medusae with four or three sets of gonads (A,B, respectively). Side views of medusae, highlighting the typical dome shape of the bell with thick mesoglea (C,D). Subumbrellar view, evidencing the typical folded oral arms and the eight rhopalia interspaced by marginal lappets with a middle indentation each (E). Rhopalia (F,G). The identification of these medusae was not checked with genetic analyses. Photo credits: Peter Wirtz/ImagDOP (A); Bruno I. Magalhães (B); João Bruges (C); Rolando Oliveira (D); Nelson Raposo (E,G); Carlos J. Moura (F).

Remarks: *Aurelia* cf. *pseudosolida*, from the Azores, are very similar, morphologically and genetically, to *A. pseudosolida* that was recently described and DNA barcoded from a single individual collected in the Adriatic Sea. It was much likely previously found in the Azores many times (Figures 1, 3 and 4; Video S1; Table S1), and recurrently named *A. solida*. We did not collect intact specimens to resolve the taxonomy of the Azorean specimens. Future findings and DNA barcoding of more specimens of *A. pseudosolida* from the Mediterranean should also help to determine its conspecificity or species differentiation from the Azorean specimens. Any of the seven molecular diagnostic characters indicated below might also be inoperable. The inobservance of the 16S and ITS1-5.8S haplotypes of *A. pseudosolida* in the Azores suggests, without much confidence, that the Azores jellies may correspond to a different species. Furthermore, the widely used DNA barcoding primers of Folmer et al. [54] could amplify the COI marker in *A. pseudosolida* but not the Azorean specimens of *A. cf. pseudosolida*. Morphologically, the structure of the rhopalia, namely the rhopalian canal and lappet, may eventually differentiate these two putative close species (cf. Figure 3B, Figure 4F,G and Figure 5E in Gari & Batistic [36]). See below for information on how to differentiate *A. cf. pseudosolida* from *A. "misteriosa"* morphologically, as both species may occur in sympatry.

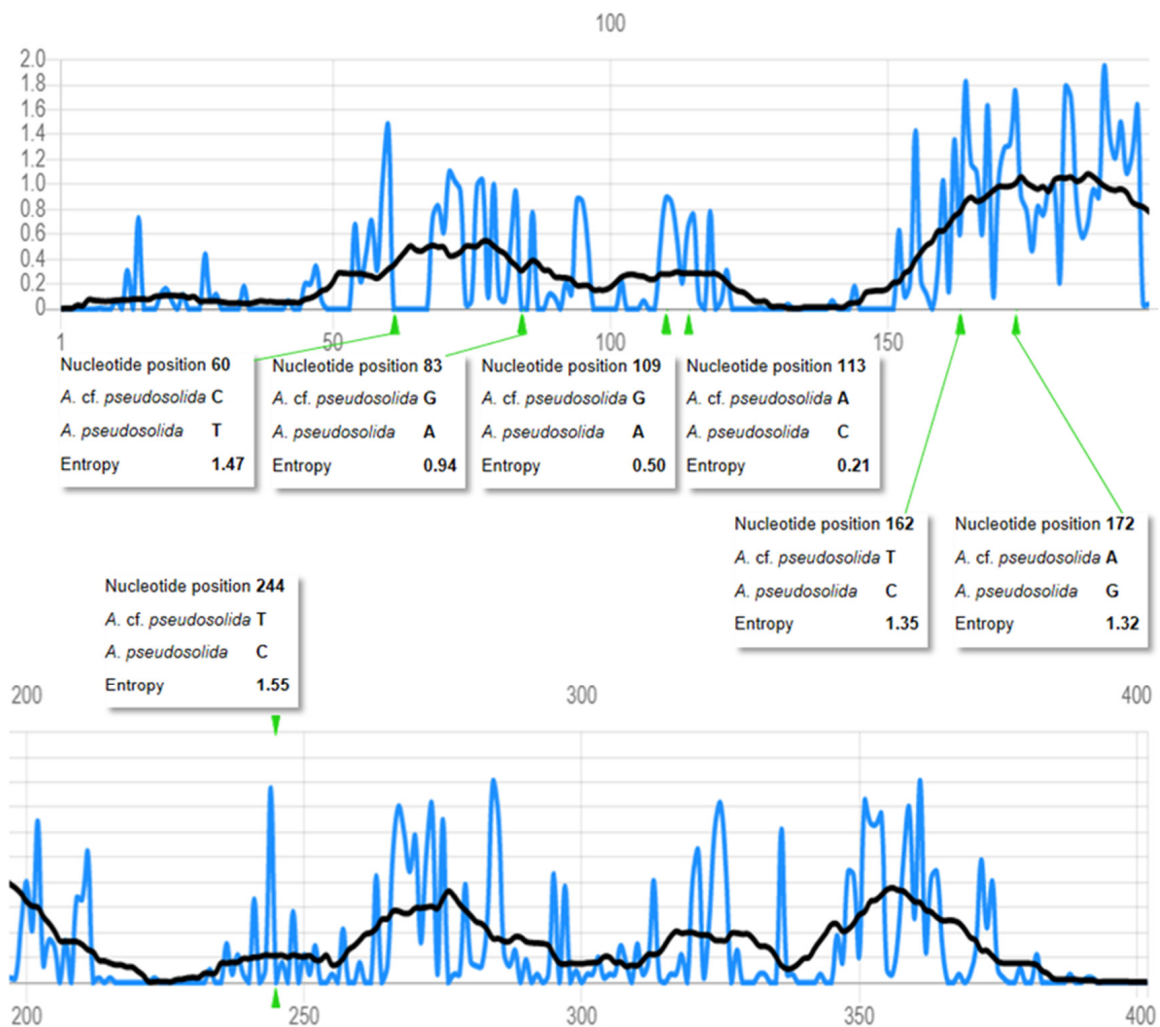


Figure 5. Entropy plot (blue), indicating (green arrows) “binary” character states between *Aurelia* cf. *pseudosolida* and *A. pseudosolida*, of nucleotide positions in the 16S dataset alignment. The black line represents the entropy moving average.

Distribution: The Azores archipelago (Table S1). If synonymous with *A. pseudosolida*, the distribution extends to the Mediterranean. This taxon may eventually expand occasionally its range distribution until the Madeira archipelago, considering the unconfirmed report of *A. "solida"* in Madeira by Mayer [1].

Diagnosis: In the 16S alignment, there were seven “binary” diagnostic characters (cf. terminology in Hütter et al. [53]) regarding the available 16S sequence of *Aurelia pseudosolida* (Figure 5; DeSignate analyses S1). If also compared to *A. solida*, which may cluster with these two putative species, of these seven putative diagnostic characters, the positions 83, 162, and 172 may be uniquely exclusive to *A. cf. pseudosolida*.

Species: *Aurelia "misteriosa"*

Figures 6 and 7.

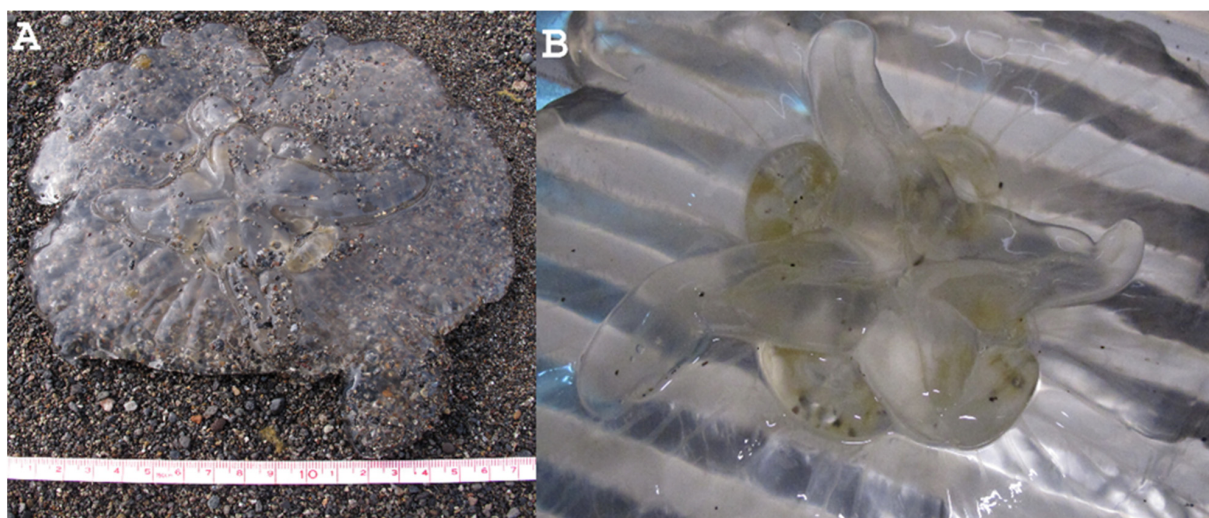


Figure 6. *Aurelia "misteriosa"*: (A) Entire specimen and (B) details of the oral arms, gonads, and radial canals. Photo credits: Bruno I. Magalhães.

Remarks: Only one specimen, collected in poor condition and later lost was submitted to molecular analyses. This taxon probably refers to a distinct undescribed species, likely already reported from the Azores (Figure 7; Table S1) mostly under the name *A. aurita*. It is closely related to *A. persea*, which is only known from the Eastern Mediterranean (Israel). *Aurelia "sp. 18"* is currently known from the Gulf of Mexico and clusters with the previous pair of species (Figure 2). Presently, it is not possible to compare or diagnose morphologic characteristics with these two related species, which were poorly described phenotypically.

In the Azores, *A. "misteriosa"* is easily distinguished from *A. cf. pseudosolida* by the wide and flattened shape of the medusae bell. The campanula diameter and the medusa size are much higher in *A. "misteriosa"* (Figure 8). The oral arms of *A. "misteriosa"* are also comparatively longer, thicker, and much less folded (contrast Figures 3 and 4 with Figures 6 and 7).

Distribution: Only known from the Azores archipelago (Table S1).

Diagnosis: In the COI alignment, there were five “combined asymmetric” diagnostic characters (cf. terminology in Hütter et al. [53]) not shared with all the other COI sequences of *Aurelia* represented (DeSignate analyses S2). In comparison with the available COI sequences of *A. persea*, *A. relictata*, and *A. "sp. 18"*, this cryptic species is herein diagnosed by seven “binary”, one “asymmetric”, and ten “combined asymmetric” differences in nucleotide positions. Note that position 511 has much less entropy, being maybe the most relevant diagnostic character with the COI marker (Figure 9; DeSignate analyses S3).

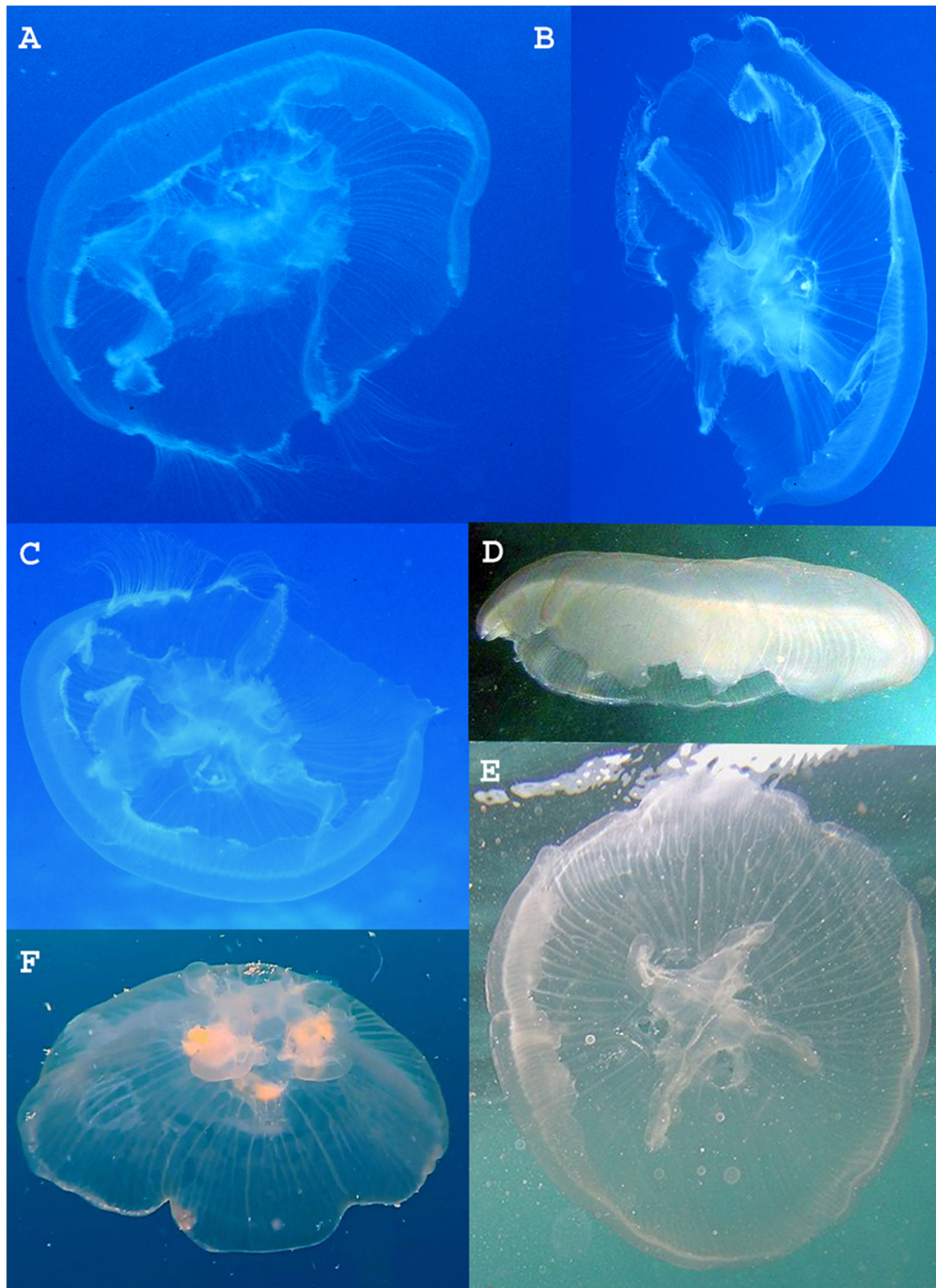


Figure 7. *Aurelia "misteriosa"*: details of medusa, evidencing the typical thick and long oral arms and the wide and flattened campanulae (A–D). *Aurelia "misteriosa"* observed while diving at Santa Maria Island in May 1999 (A–C), and on São Miguel Island in May 2015 (D–F). The identification of these medusae was not checked with genetic analyses. Photo credits: Jorge Fontes/ImagDOP (A–C); Hélio Filipe (D,E); Gui Costa (F).

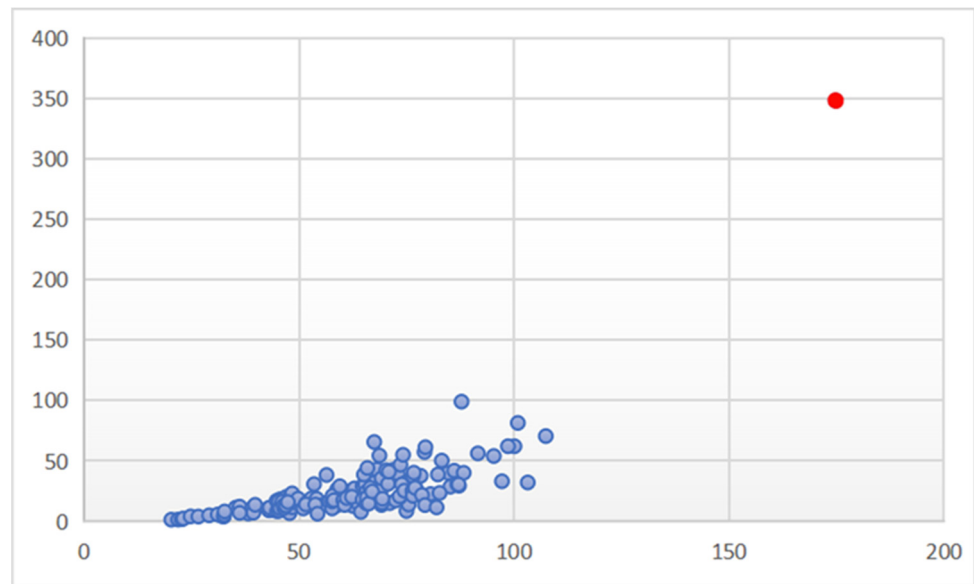


Figure 8. Maximum diameter in mm (x-axis) and corresponding weight in g (y-axis) of moon jellies collected in the Azores. For *Aurelia*. cf. *pseudosolidata* blue dots, and *A.* “misteriosa” the red dot.

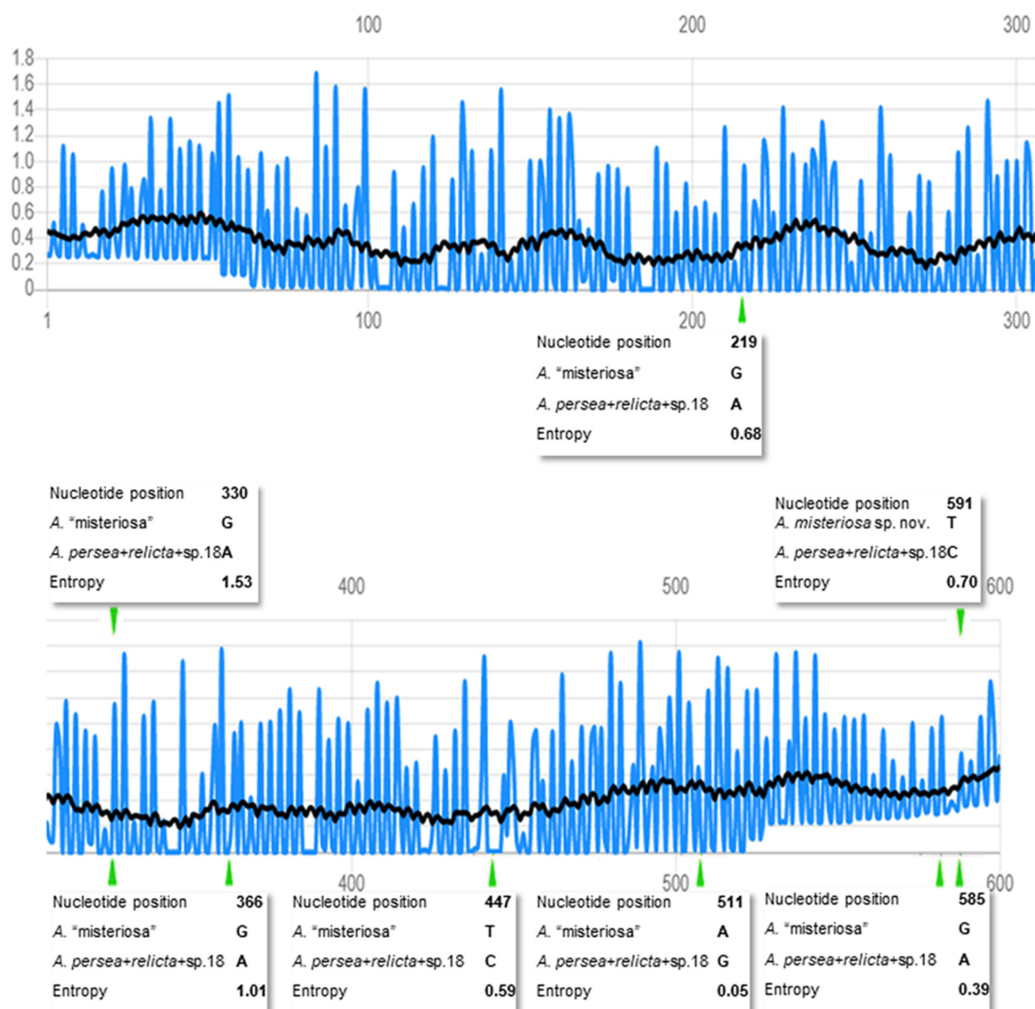


Figure 9. Entropy plot (blue), indicating (green arrows) “binary” character states between *Aurelia* “misteriosa” and *A. persea*, *A. relicta* and *A.* “sp. 18”, of nucleotide positions in the COI dataset alignment. The black line represents the entropy moving average.

In the 16S alignment, there was one “binary” diagnostic character regarding all the other *Aurelia* sequences available at alignment position 414. Six “combined asymmetric” alignment positions might serve also to diagnose *A. “misteriosa”* (DeSignate analyses S4). In comparison to the available 16S sequences of *A. persea* and *A. relictata*, there were six “binary” diagnostic characters and three “combined asymmetric” nucleotidic combinations (Figure 10; DeSignate analyses S5).

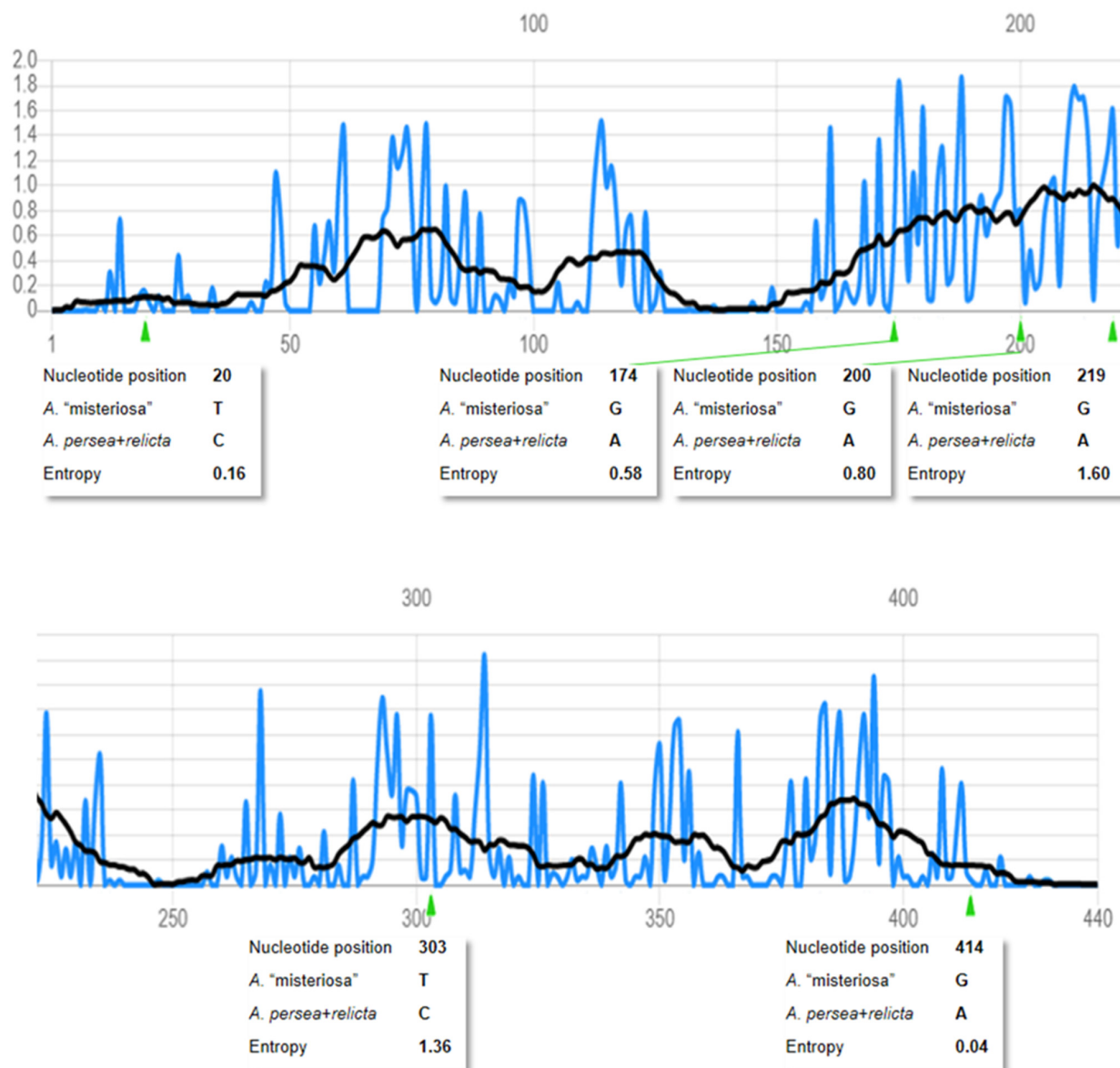


Figure 10. Entropy plot (blue), indicating (green arrows) “binary” character states between *Aurelia* “*misteriosa*” and *A. persea* and *A. relictata*, of nucleotide positions in the 16S dataset alignment. The black line represents the entropy moving average.

3.3. Moon Jellies Reports in the Azores

In our beach monitoring, we detected stranded medusae of moon jellies only in the spring of 2019 and 2021. In 2019, we observed and collected a relatively high abundance of *Aurelia* between March and June, both on Faial Island and São Miguel Island. In 2021, *Aurelia* specimens were only found on Faial Island between April and June. We reported more moon jellies in April 2021. In 2019 we reported more *Aurelia* strandings in June.

We found 33 additional distinct reports of *Aurelia* in the Azores between the 19th century (Haeckel, 1880) and the year 2021 (see Table S1). This additional data reveals

historical occurrences of moon jellies in the Azores between February and September but mostly in May and June. Most reports of *Aurelia* medusa thus coincide with the spring season in the Azores.

Aurelia cf. *pseudosolida* is the most common species of moon jelly in the Azores, at least in shallow waters. The flattened-bell forms likely corresponding to *A. "misteriosa"* seem comparatively much less common in the Azores and have been reported at least since 1999 (Table S1). Nevertheless, that species might have been reported under the name *A. aurita* but without morphologic description, illustration, or photography.

To our knowledge, no records of *Aurelia* polyps exist in the Azores.

4. Discussion

Our study reveals, with DNA barcoding data, two cryptic (eventually new) species of *Aurelia* species co-occurring in the marine waters of the Azores, herein temporarily classified as *A. "misteriosa"* and *A. "cf. pseudosolida"*. These taxa should have been previously identified in the Azores, roughly, either as *A. aurita* or *A. solida*, respectively, considering the morphology of these nominal species, the characteristics of the specimens collected, and the available information extracted from the literature and jellyfish photographs. The most common *Aurelia* species in the coastal waters of the Azores is *A. cf. pseudosolida*, which has a thick and dome-shaped campanulae and is indeed morphologically (and genetically) similar to *A. solida* (cf. Figures 3 and 4). *Aurelia* cf. *pseudosolida* is, however, more closely related genetically and morphologically to *A. pseudosolida* that was recently described from a single specimen collected in the Adriatic Sea. Further DNA barcoding of *A. pseudosolida* in the Mediterranean, as well as the collection of moon jellies in the Azores in better morphologic condition, will determine the taxonomic identity of the Azores specimens. We refrained to consider these as *A. pseudosolida* because: (1) we did not find the 16S or ITS1-5.8S haplotypes of *A. pseudosolida* in the Azores (namely in the 22 and 13 specimens with 16S and ITS1-5.8S sequences generated, respectively) (cf. Figures S2 and S3); and (2) the "Folmer primers" successfully amplified and sequenced the COI marker in *A. pseudosolida* but did not amplify the marker in any of the 23 specimens of *A. cf. pseudosolida* from which we obtained 16S and/or ITS1-5.8S sequences. Additionally, *A. pseudosolida* may have rare occurrence in the Mediterranean considering that only one specimen has been recorded so far, and the inobservance of *Aurelia* species in the Lusitania province or other Macaronesia islands (except a unique report of *A. (cf.) solida* in Madeira) raises the question whether the Azores specimens are genetically isolated from *A. pseudosolida*. Morphologically, we note small putative differences in the structure of the rhopalia when contrasting Figure 5E in Gari et al. [36] with Figures 3B and 4F,G. More specimens of both species have to be collected in good condition, DNA barcoded, and morphologically inspected to check if the Azores specimens refer to a distinct new species or to *A. pseudosolida*. The unique report of *A. (cf.) solida* from Madeira, provided by Mayer [1], may refer to a synonymous or closely related species.

Aurelia "misteriosa" is the other *Aurelia* species currently occurring in the Azores. It is readily distinguished from *A. cf. pseudosolida* by a flattened, less thick (and wider) campanula and longer oral arms. *Aurelia "misteriosa"* also seems, so far, to be endemic from the Azores and is much less frequent than *A. cf. pseudosolida* at least in coastal waters and in recent times. *Aurelia "misteriosa"* was recovered as the sister species of *A. persea* (Figure 2), which is only known from the eastern Mediterranean. These two taxa in turn group with *A. "sp. 18"* from the Gulf of Mexico, forming subsequently a clade with *A. relictata* from the Mediterranean (Figure 2). *Aurelia "misteriosa"* is probably a distinct new species considering its relatively high genetic and geographic distances between *A. persea* and *A. "sp.18"*, for example, if comparing that between *A. smithsoniana* and *A. cebimarensis*. However, because we only collected a single degraded specimen that was lost, we leave a formal description of *A. "misteriosa"* for future sampling and DNA barcoding of intact specimens of the species (and *A. persea*). Nevertheless, is worth mentioning that practically no morphologic information is currently available for the medusa of *A. persea* and *A. "sp. 18"*

(cf. Table S8 of Lawley et al. [29]), complicating efforts to diagnose morphologic similarities or differences between these three close taxa. Considering the morphologic information available for the medusa of *A. relict*a (cf. Table S8 of Lawley et al. [29]), we note that *A. "misteriosa"* also presents campanulae with 8 lobes, but the bell diameter is considerably higher in the Azores specimen collected (although, of course, the medusa bell grows along with the life cycle development). *Aurelia "misteriosa"* is whitish and translucent (Figures 6 and 7), similar to *A. relict*a and *A. perse*a, but presents yellow gonads (Figure 6) in contrast to the supposedly white gonads of *A. perse*a (cf. Table S8 of Lawley et al. [29]).

Making use of an updated compilation of all DNA barcodes available for the *Aurelia* genus, we further analyzed biological invasions from haplotype distributions. We verified that 28 out of the 32 *Aurelia* species currently recognized with genetic analyses (considering the 2 putative species from the Azores as distinct) seem to present restricted geographical distributions (Figure 2). On the other hand, there is clear scientific evidence that *A. solida*, *A. coerulea*, *A. relict*a, and *A. aurita* (*sensu stricto*) are moon jellyfish species effectively widely distributed. We note, however, that *A. solida*, which has been considered a Lessepsian migrant (e.g., [24]), i.e., a species thought to enter into the Mediterranean through the Suez Canal, actually presents genetic lineages from the Mediterranean that were never detected (yet) in the Red Sea. It is thus premature to assume a human-mediated migration for *A. solida*. *Aurelia coerulea* was detected in the Indo-Pacific, NE Atlantic, Mediterranean, and Marmara Sea. *Aurelia relict*a was detected in Japan, the SW Atlantic, and the Mediterranean Sea. *Aurelia aurita* is confirmed present in the N Atlantic, SE and NW Pacific, Caspian Sea, Marmara Sea, and the Black Sea. The wider distributions of *A. coerulea*, *A. relict*a, and *A. aurita* are probably a consequence of the global trade of ornamental marine species, which results in (often inadvertent) subsequent introductions into natural marine habitats not necessarily through the medusa phase. Boat traffic, and namely the accidental transport of *Aurelia* polyps on hulls, and pelagic life stages in ballast waters may also partially explain the wide distribution of these three *Aurelia* species. The adaptability of a few *Aurelia* species to prosper simultaneously in cold and warm waters is remarkable and surely explains the thriving of these three taxa in distant locations with notable abiotic differences.

Moon jellies may sting bathers (affecting tourism), and in high aggregations, may impact local power and desalination plants, net fisheries, fish farms [58], and ultimately trophic-web dynamics (e.g., [59,60]) with potential consequences on fisheries and natural habitat structuring. Therefore, given the pervasion of cryptic species diversity within the genus *Aurelia* (e.g., as noted in this study) and other jellyfish (e.g., [45]), it is advisable to monitor jellyfish blooms conjunctly with DNA barcoding to ascertain the native or exotic status of species and (try) to mitigate potential economic and/or ecological negative effects, particularly in the presence of NIS. The 16S marker is of special utility to DNA barcode jellyfish and determines phylogenetic associations, being much more easily amplified and sequenced than the COI and ITS1-5.8S markers. The ITS1-5.8S marker may sometimes not be enough to differentiate some (moon) jellyfish species (cf. Figure S3) and presents many ambiguous positions which may condition the reliability and rapidity of molecular analyses. The COI marker, with a good phylogenetic signal to differentiate jellyfish species, cannot be yet amplified with a universal set of primers. Therefore, the 16S and COI markers should be both used, in a complementary way, to DNA (meta)barcode jellyfish species.

5. Conclusions

More new species of *Aurelia* still await discovery and formal taxonomic recognition. It is necessary to DNA barcode, simultaneously with the 16S and COI mitochondrial markers, more *Aurelia* (and other jellyfish taxa) across their entire geographical range of distribution to better comprehend the extent of cryptic species diversity and respective biogeographic distributions. Accurate DNA-based species identifications will further help to detect eventual NIS occurrences. We further recommend recording and presenting morphologic characteristics of the different taxa DNA barcoded to facilitate visual species differentiation and to clarify the (still) much controversial taxonomy of the genus.

Future morphologic and DNA barcoding analyses on intact moon jellies from the Azores and closely related lineages will disclose the correct taxonomic identity of the two cryptic moon jelly species herein presented. Non-indigenous species (NIS) of *Aurelia* (and other taxa) may be better tracked with DNA (meta)barcoding methods. Ideally, to prevent the further spread of NIS of jellyfish outside their natural biogeographic range: (1) the traffic/market of live jellyfish has to be prohibited, (2) ship-hull cleaning practices should be more frequent, and (3) ballast-water treatments need to be enforced and be more effective prior discharges. NIS should start to be detected earlier using regular DNA (meta) barcoding monitoring in ports (and other important marine areas) worldwide.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d15030323/s1>, Alignment S1: COI alignment, Alignment S2: 16S alignment, Alignment S3: ITS1-5.8S alignment, Alignment S4: concatenated analysis alignment, Figure S1: Maximum likelihood phylogenetic tree generated with all the available COI sequences of the *Aurelia* genus (and two sequences of *Drymonema dalmatinum* as the outgroup), Figure S2: Maximum likelihood phylogenetic tree generated with all the available 16S sequences of the *Aurelia* genus (and two sequences of *Drymonema dalmatinum* as the outgroup), Figure S3: Maximum likelihood phylogenetic tree generated with all the available ITS1-5.8S sequences of the *Aurelia* genus (and two sequences of *Drymonema dalmatinum* as the outgroup), DeSignate analyses S1: DeSignate analyses between *Aurelia* cf. *pseudosolida* and *A. pseudosolida* using the 16S alignment, DeSignate analyses S2: DeSignate analyses between the COI sequence of *Aurelia* "misteriosa" and all the other COI sequences of *Aurelia*, DeSignate analyses S3: DeSignate analyses between the COI sequence of *Aurelia* "misteriosa" and the available COI sequences of *A. persea*, *A. relictata*, and *A. "sp.18"*, DeSignate analyses S4: DeSignate analyses between the 16S sequence of *Aurelia* "misteriosa" and all the other 16S sequences of *Aurelia*, DeSignate analyses S5: DeSignate analyses between the 16S sequence of *Aurelia* "misteriosa" and the available 16S sequences of *A. persea* and *A. relictata*, Table S1: *Aurelia* observations in the Azores [61–65], Table S2: Maximum diameter and weight of *Aurelia* species from the Azores, Table S3: Sequence metadata, Video S1: *Aurelia* cf. *pseudosolida* swimming (credits: Carlos J. Moura).

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