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Genetic diversity of the *Pennaria disticha* Goldfuss, 1820 (Cnidaria, Hydrozoa) complex: new insights from Brazil

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Abstract

Analyzing the mitochondrial 16S gene from specimens collected along the Brazilian coast, we expanded the current genetic diversity and phylogeny of the Christmas tree hydroid, *Pennaria disticha* Goldfuss, 1820 complex. A total of seven clades were retrieved, of which three were recovered among the newly added specimens—two were already known from different locations in previous studies and a new one was formed by a specimen collected near an isolated coastal island in the São Paulo state—demonstrating that *P. disticha* also represents a complex of cryptic species in the southwestern Atlantic. Species delimitation analyses recovered three to five clades as separate species within *P. disticha*; moreover, the haplotype network confirmed the already suggested scenario of multiple invasive trajectories and helped untangle the genetic relationships between clades. The data presented herein, besides broadening *P. disticha* genetic diversity, reinforce the importance of barcoding analyses of local populations in cryptic species studies.

Keywords Phylogenetic · Barcoding · 16S · Southwestern Atlantic · Cryptic species

Introduction

Species are considered 'cryptic' if their morphological boundaries are unclear (Bickford et al. 2007; Pfenninger and Schwenk 2007) resulting in more than one species being assigned under one species name. In the last decades, molecular approaches, such as phylogenetics and DNA barcoding, have shed light on cryptic lineages across different habitats

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and taxa (Hebert et al. 2004; Bekker et al. 2016; Maggioni et al. 2016; Bringloe and Saunders 2019). As different species might respond differently to the environmental variables (Heads 2015), the identification of cryptic species has serious implications for nature conservation (Bickford et al. 2007). The identification of cryptic species has also become of crucial importance when it involves the detection of exotic species (Geoffroy et al. 2012; Stouthamer et al. 2017; Bezeng and van der Bank 2019; Hernández-Triana et al. 2019). During the last decades, biological invasions have worldwide become one of the major causes of the deterioration of natural communities and loss of biodiversity (Bax et al. 2003; Simberloff et al. 2013; Chan and Briski 2017). In particular, shipping in combination with ballast water and biofouling, on both regional and global scales, is the most significant vector of non-native species introduction in the marine realm (Rocha et al. 2013; Bailey 2015; Verna et al. 2016; Peters et al. 2019).

The Christmas tree hydroid, *Pennaria disticha* Goldfuss, 1820, is a conspicuous species that forms large pinnate colonies on hard substrates. Considered to have a wide geographical distribution in tropical and temperate waters (Schuchert 2006; Calder 2019), it has a complex taxonomical history (synonymy in Calder 1988). Until recently, *P. disticha* has been interpreted as showing a high intraspecific variability, which led to the description of a series of nominal species in

the nineteenth and first half of the twentieth century as its synonyms (Schuchert 2020). Moreover, this hydroid has been reported as invasive in various locations in the Indian and Pacific oceans, such as the Palmyra Atoll (Knapp et al. 2011), the Galápagos Archipelago (Collins et al. 2020), South Africa (Mead et al. 2011), and Hawaii (Boone 1938; Godwin et al. 2006; Miglietta et al. 2015). In one of these locations, it has also been observed to be an aggressive competitor for space in natural and artificial substrates (Knapp et al. 2011). Due to its frequent presence on artificial substrates and algae, it has been hypothesized that *P. disticha* could disperse by rafting on floating material, such as algae and debris (Cornelius 1992), and through ship fouling and ballast water (Godwin et al. 2006; Carlton and Eldredge 2009). Although some studies suggested that the Christmas tree hydroid is native to the Atlantic (e.g. Cohen and Carlton 1995; Carlton and Eldredge 2009), it has in general been regarded as cryptogenic at various localities (e.g. Mead et al. 2011; Rocha et al. 2013; Calder et al. 2019).

Through DNA barcoding of a fragment of the mitochondrial large ribosomal subunit gene (16S rRNA) (widely considered an excellent marker for barcoding and diversity determination within the class Hydrozoa [Moura et al. 2012a, 2018; Zheng et al. 2014]), *P. disticha* was shown to consist of a complex of at least six different clades belonging to up to three phylogenetically separate species (yet unnamed), rather than a species with a cosmopolitan distribution (Miglietta et al. 2015, 2019). Moreover, Miglietta et al. (2019) suggested that the clades with broad multi-ocean distributions were probably the product of multiple and independent invasive trajectories. Also, the same study puts forward that *P. disticha* needs to be re-evaluated using molecular approaches to improve the understanding of which specific clades local populations belong to. However, both studies are lacking an extensive sampling of *P. disticha* from the southwestern Atlantic coast.

The Brazilian coast, where *P. disticha* has a wide and well-studied distribution (Migotto et al. 2002), is recognized as the most important biogeographic province in the southwestern Atlantic (Floeter et al. 2008). Here, using samples collected along the Brazilian coast, we expanded the current genetic diversity and phylogeny of the *P. disticha* complex and demonstrate that it represents a complex of cryptic species in the southwestern Atlantic.

Materials and methods

Sample collection

Colonies of *Pennaria disticha* were collected between 2007 and 2018 by free diving or scuba diving from shallow-water benthic localities along the Brazilian coast where they were immediately fixed in ethanol 99.5% for molecular studies. The other previously sampled colonies (preserved in ethanol 99.5% or formalin 10%) were sourced from the Museum of Zoology of the University of São Paulo (MZUSP) collections. A list of all specimens used in this study is provided in Fig. 1 (details in Online Resources 1).

DNA extraction, PCR amplification, and sequencing

Samples preserved in formalin 10% were washed repeatedly in phosphate-buffered saline (PBS 1X, pH 7.2) to remove the fixative from the tissue. Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's animal tissue protocol. After extraction, a fragment of the mitochondrial large ribosomal subunit gene (16S rRNA) was amplified using SHA and SHB primers (Cunningham and Buss 1993) under the following PCR conditions: 1 min at 94 °C, then 35 cycles of 94 °C for 15 sec, 50 °C for 1:30 min, and 72 °C for 2:30 min with a final extension at 72 °C for 5 min. Amplified products were visualized in 1% agarose gel electrophoresis and two different protocols were utilized for sequencing: (i) PCRs were purified with illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced into both directions on an ABI PRISM 3100 using Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and (ii) PCRs were purified using ExoSAP and sequenced in both directions using the platform Applied Biosystems® Sanger Sequencing 3500 Series Genetic Analyzers. The obtained sequences were assembled and edited using the software Geneious R9.0.5 (Kearse et al. 2012) or with CodonCode Aligner v4 (CodonCode Corporation, Dedham, MA, USA). New sequences determined in the present study were deposited in GenBank (accession numbers: MT408048-MT408071).

Phylogenetic analyses

Newly obtained sequences of 16S rRNA were aligned with *P. disticha* sequences from Miglietta et al. (2015, 2019) and additional sequences available in GenBank (see Online Resources 2). *Hydrocoryne iemanja* Morandini, Stampar, Migotto & Marques, 2009 was used as outgroup since *Hydrocoryne* was previously shown to be a sister taxon of the genus *Pennaria* (Nawrocki et al. 2010; Montano et al. 2015). Sequences were aligned using MAFFT v.7 (Katoh et al. 2019), using the Auto mode option, and inspected by the naked eye and manually edited using MEGA X (Kumar et al. 2018). GTR + G was retrieved as the best-fit model for the alignment using JModelTest 2.1.10 (Darriba et al. 2012) under the Akaike information criterion and Bayesian information criterion. Bayesian inference (BI) and Maximum Likelihood (ML) analyses were performed using MrBayes 3.2.6

Fig. 1 Map showing the localities where specimens of *Pennaria disticha* Goldfuss, 1820 of this study were sampled, showing their voucher number and clade distributions
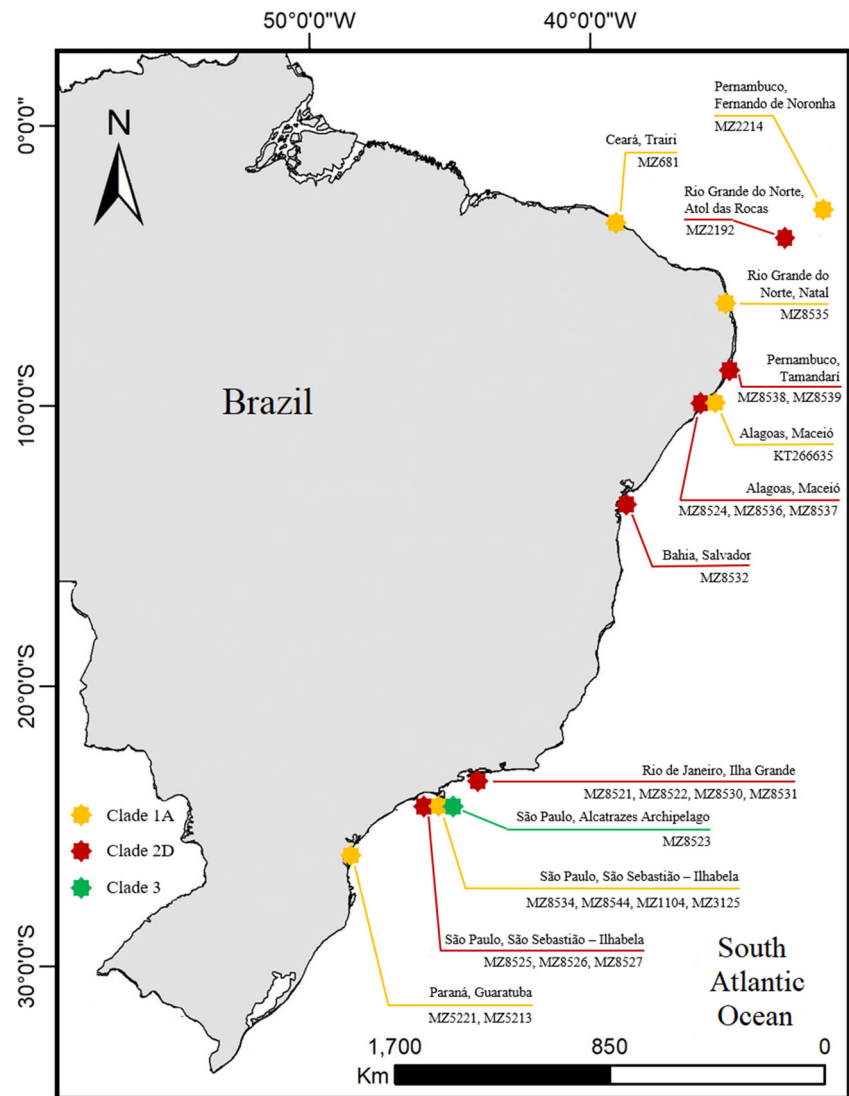
(Ronquist et al. 2012) and PhyML as a plugin of Geneious (Guindon et al. 2010), respectively. For the BI, four parallel Markov chain Monte Carlo runs were carried out for 2.5 million generations with trees sampled every 100 generations and burn-in was set to the first 25% of the generations. The average standard deviation of the split frequency value was < 0.01 for the Bayesian analysis. ML reconstruction was performed with 1000 bootstrap replicates. Within and between clades genetic distances (Kimura 2-parameter, 500 bootstraps) were calculated in MEGA X, clades arranged as in Fig. 2.

Species delimitation analyses and haplotype network

Species delimitation was performed using two different analyses: the (i) automatic barcoding gap discovery (ABGD) method and the (ii) Poisson tree processes (PTP) method. ABGD (Puillandre et al. 2012) was performed via the website http://wwwabi.snv.jussieu.fr/public/abgd/ and the most

conservative result was selected. PTP (Zhang et al. 2013) was performed via the website https://species.h-its.org/ptp/ for 5×10^5 MCMC generation with burn-in set to 25%, using the resulting Bayesian tree as an input. Moreover, the 16S rRNA alignment was collapsed into haplotypes using the online tool DnaCollapser (Villesen 2007) and a haplotype network was constructed with PopART 1.7 (Leigh and Bryant 2015) using the median-joining algorithm (Bandelt et al. 1999) and default settings.

Results

The final 16S rRNA alignment of 136 sequences was 565 bp in length (1 outgroup and 135 *P. disticha* sequences). Among them, 24 were sequences from specimens studied herein, while the remaining were obtained from GenBank (see Online Resource 2). All the 24 newly generated sequences
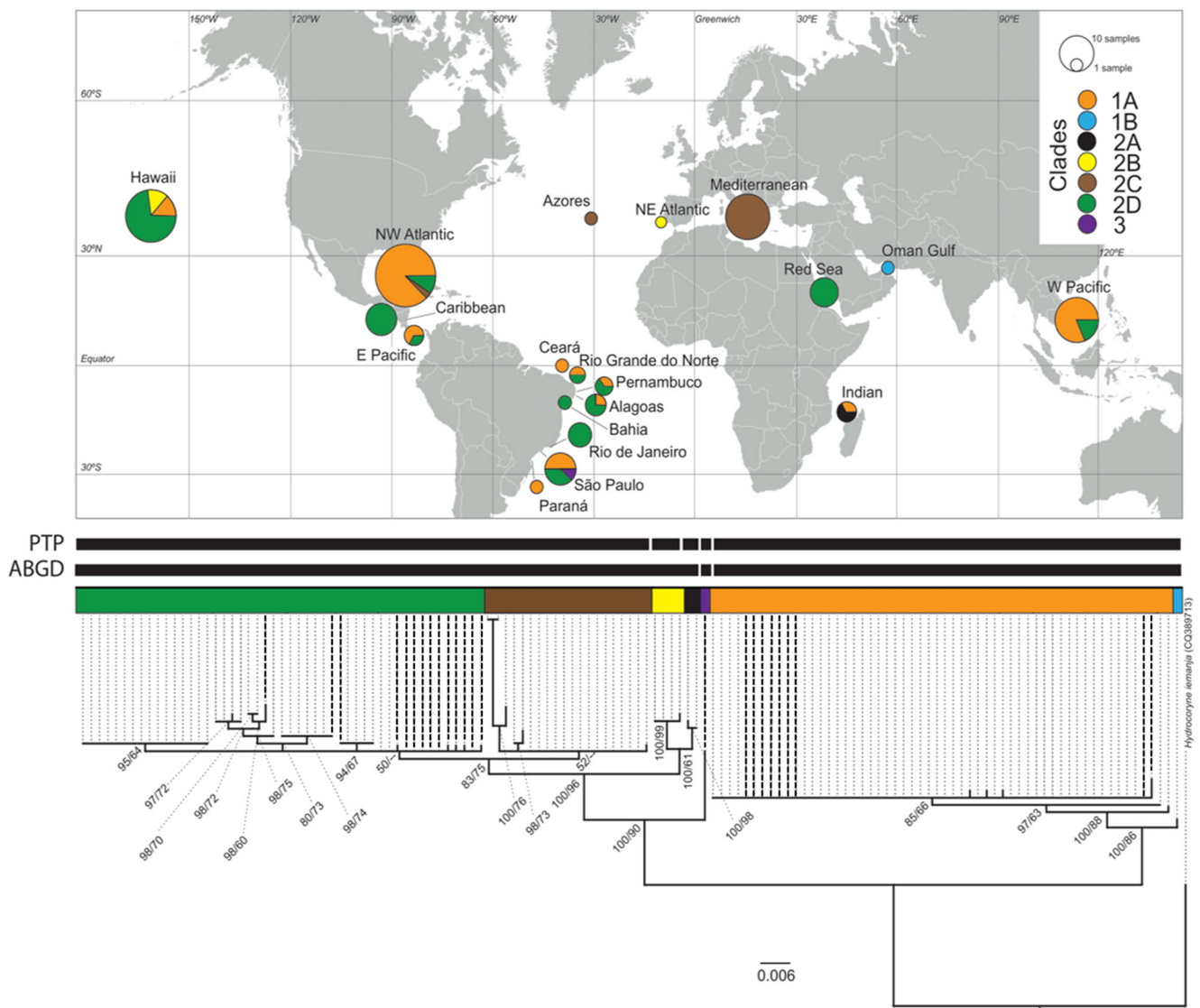
Fig. 2 Global map with *Pennaria disticha* Goldfuss, 1820 distribution and relative abundance of clades, and phylogenetic tree recovered by Bayesian inference—clades support values are posterior probabilities from Bayesian inference and bootstrap values from maximum likelihood analyses, respectively (sequences from this study are represented by bold lines). In the middle, representation of the species delimitation analyses from the ABGD and PTP test

had good quality (forward and reverse chromatograms with no ambiguous peaks) and were retrieved from specimens preserved in ethanol (22) and also from specimens preserved in formalin (2). Phylogenetic trees obtained from BI and ML analyses were identical; therefore, only the Bayesian topology with branch support indicated by Bayesian posterior probability (PP) scores and ML bootstrapping supports (BS) are shown in Fig. 2. Overall, the resulting phylogenies increased the complexity of two previously described clades (see Miglietta et al. 2019) and proposed a new one. Below we describe each clade focusing on those that include sequences from the Brazilian coast specimens retrieved herein.

Clade 1 was recovered with high statistical support (100 PP and 86 BS). Clade 1B (*sensu* Miglietta et al. 2019) remained

unvaried with only one sequence from the Oman Gulf (Indian Ocean). Sequences from five different Brazilian states (Ceará, Rio Grande do Norte, Pernambuco, São Paulo, Paraná) were grouped within the clade 1A. In clade 2, all subclades previously indicated in Miglietta et al. (2015, 2019) were recovered. Nevertheless, while clades 2A and 2B were recovered with high statistical support (100 and 98, 100 and 99 for PP and BS respectively), clades 2C and 2D displayed low support values, which corroborate their paraphyletic status suggested in Miglietta et al. (2019). Overall, clades 2A, 2B, and 2C remained unvaried, while 2D grouped the specimens from six Brazilian states (from north to south): Rio Grande do Norte, Pernambuco, Alagoas, Bahia, Rio de Janeiro, and São Paulo. Finally, a new clade formed by a specimen from the

Alcatrazes Archipelago (São Paulo state), here named clade 3, was recovered with 100 PP and 90 BS support. Within-clade genetic distances were extremely low ranging from 0.1 ± 0.1 to 0.5 ± 0.1 (except for clade 2C [2.0 ± 0.4], probably due to the presence of the three highly divergent sequences from Italy and Azores [KT984714-KT984716]), while interclade genetic distances were higher ranging from 1.1 ± 0.5 to 9.8 ± 1.3 (Table 1).

Species delimitation analyses indicated three to five species: while ABGD recovered clade 1, clade 2, and clade 3 as distinct species, PTP analyses recovered clade 1, clade 2A, clade 2B, clade 2C/D, and clade 3 as representing different species (Fig. 2).

A total of 29 haplotypes were obtained from the 16S rRNA *P. disticha* sequences. Among them, 19 (65.5%) were single-tons, and the remaining (10 haplotypes) were shared among geographically close or distant populations. The two most widespread haplotypes were Hap_1—shared by individuals from West Pacific, Brazil, Northwest Atlantic, and Hawaii; and Hap_23—observed from West and East Pacific, Brazil, Northwest Atlantic, and Hawaii. The haplotype clusters separated by multiple mutation steps (Fig. 3) corroborate the phylogenetic reconstruction and the species delimitation recovered by the PTP analyses.

Discussion

Although the broad-based phylogenetic tree obtained with the 16S rRNA gene (Fig. 2) is consistent with the results from previous studies Miglietta et al. (2015, 2019), it essentially depicts that the nominal *P. disticha* is formed of at least three monophyletic groups (i.e. clade 1, clade 2, and clade 3) of which two have broad geographic distributions. These three groups are each recognized as species hypotheses by species delimitation analyses, which seem to be confirmed by the genetic distances between clades. Although a widely accepted genetic distance threshold to differentiate hydrozoan species using 16S rRNA sequences has not been established to date, the genetic differentiation of this locus between multiple clades of *P. disticha* (ranging from 1.1 ± 0.5 to 9.8 ± 1.3, Table 1) is consistent with multiple studies that revealed the existence of cryptic species within, for instance, *Cordylophora* (3.3–6%) (Folino-Rorem et al. 2009), *Nemertesia* (up to 4.8%) (Moura et al. 2012b), *Millepora* (up to 5.8%) (Arrigoni et al. 2018), scleractinian-associated *Zanclea* (2.1–9.5%) (Montano et al. 2015), and Alcyonacea-associated *Pteroclava krempfi* (Billard, 1919) (up to 7.5%) (Maggioni et al. 2016).

Of the three different clades found in sympatry along the Brazilian coast, clades 1A and 2D co-occurred among almost all sampled localities (i.e. Brazilian states), while the newly recovered clade 3 was detected only in the Alcatrazes Archipelago, one of the most isolated coastal islands off the Southeastern Brazilian mainland. The Archipelago, currently considered to harbor one of the most well-conserved rocky reef faunas in Brazil, displays a high marine biodiversity (ICMBio 2017), including endemic species (Lanna et al. 2007). Unfortunately, sample conditions were not adequate for obtaining enough material to enable a detailed morphological analysis, but future fresh or better-preserved specimens might disclose differences between the Alcatrazes specimens and those of other *P. disticha* clades.

Several other hydroids previously considered to have a cosmopolitan distribution were found to comprise cryptic species complexes. Schuchert (2014) revealed different lineages in *Plumularia setacea* (Linneus, 1758), highlighting that additional evidence for cryptic species is provided when genetic distant lineages occur in sympatry (the case of some haplotypes in the *P. disticha* complex), therefore proving that these lineages are reproductively isolated.

Previously, it has also been described that different clades belonging to hydrozoan species complexes, with high interclade genetic distances, were found associated with different substrates. Montano et al. (2015) and Maggioni et al. (2017, 2018, 2020) revealed a high diversity in hydroids belonging to the genus *Zanclea* found in association with distinct scleractinian and bryozoan genera. Similarly, Montano et al. (2017) showed that cryptic species in the *Pteroclava krempfi* species complex were associated with different octocoral families. Likewise, calcifying hydrozoans (*Millepora*) that overgrow live scleractinian corals constituted a highly divergent clade in comparison to those from abiotic substrates (Takama et al. 2018). Furthermore, mitochondrial

Table 1 Genetic distances within and between *Pennaria disticha* clades. Standard deviations are in parentheses. n/c = not calculable

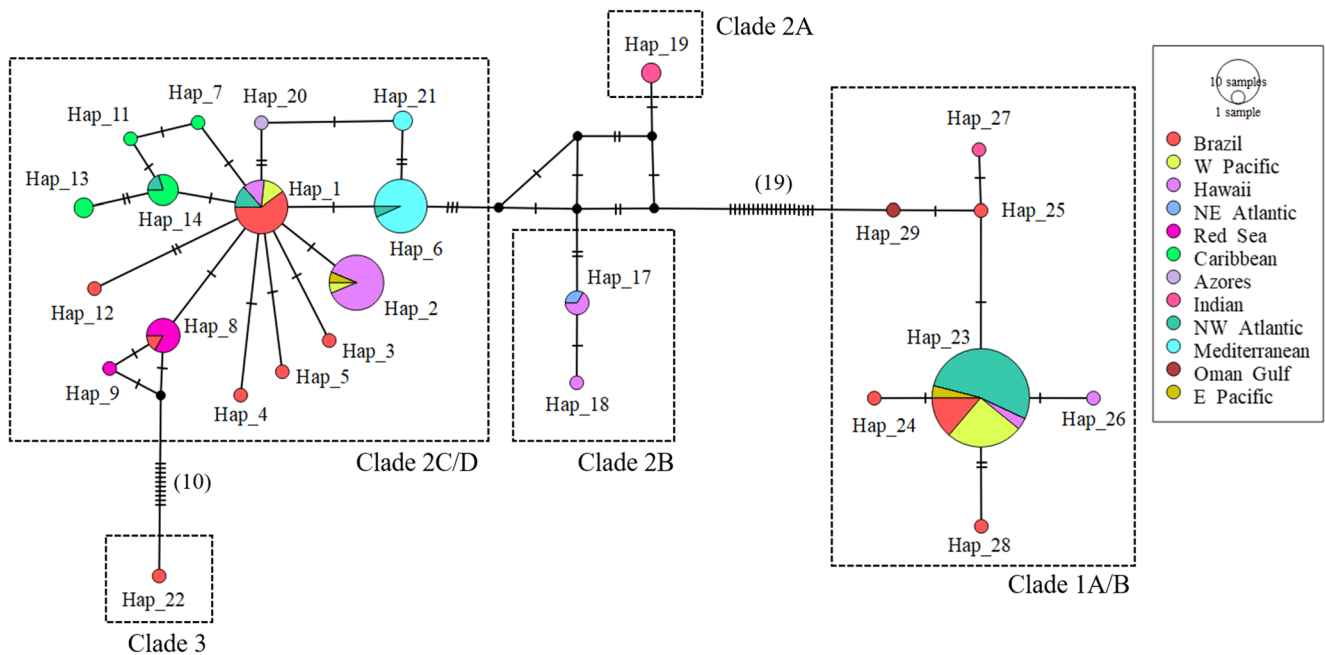| Clades	1A	1B	2A	2B	2C	2D	3
1A	0.1 (0.1)						
1B	1.1 (0.5)	n/c					
2A	8.9 (1.3)	8.0 (1.2)	0.2 (0.2)				
2B	9.1 (1.3)	8.2 (1.2)	2.0 (0.6)	0.1 (0.1)			
2C	9.8 (1.3)	9.0 (1.2)	3.4 (0.7)	3.4 (0.7)	2.0 (0.4)		
2D	8.8 (1.3)	7.9 (1.2)	2.6 (0.6)	2.6 (0.6)	1.5 (0.3)	0.5 (0.1)	
3	8.8 (1.3)	7.9 (1.2)	5.9 (1.1)	6.4 (1.1)	6.6 (1.1)	5.4 (1.0)	n/c

Fig. 3 Most parsimonious median-joining networks of *Pennaria disticha* Goldfuss, 1820 complex inferred from the gene 16S rRNA. The size of the circles is proportional to the frequencies of specimens sharing the same haplotype. The colors of the circles correspond to the localities (see legend)

genotypes of the pinnate hydroid *Aglaophenia latecarinata* Allman, 1877 were strongly correlated with seaweed substrate (*Sargassum*), indicating that the species-specific substrate pattern could be the result of substrate selection by planula larvae or substrate availability (Govindarajan et al. 2019). Therefore, the different *P. disticha* clades may have different substrate preferences, a characteristic that has so far been overlooked.

The comparison of genetic distances and haplotype network in *P. disticha* (Fig. 3) suggests that clade 3 is separated from the other ones by multiple mutations with no apparent gene flow with clades 1A/B, 2A, and 2B, instead being closely related only to clade 2C/D. Moreover, the network analysis suggests that clades 2C and 2D might represent one single clade, as well as clades 1A and 1B. Thus, our analyses brought the number of *P. disticha* lineages in the Atlantic Ocean to five (or four, if considering the clade 2C/D as one). It is important to highlight that several of the sampled localities in the Atlantic Ocean are found near harbors with intense ship traffic (Kaluza et al. 2010), this factor probably leading to an easy global dispersion of the *P. disticha* lineages through biofouling or ballast waters.

Interestingly, as some of the haplotypes (e.g. Hap_6 and Hap_8) are shared between samples from very distant regions, the idea that the cosmopolitan distribution of clade 2C/D may be the outcome of multiple separate introduction events seems to be confirmed (Miglietta et al. 2015). Moreover, the network shows the presence of gene flow and strict relationship between Hap_1 (with wide geographic distribution) and the Mediterranean (Hap_6 and Hap_21) and Azores (Hap_20) haplotypes, suggesting a scenario of multiple introductions

that made recent interbreeding possible between populations from distant locations.

The present study shows that a denser sampling of *P. disticha* specimens in a so far not well-studied area can offer more comprehensive results about which specific clades local populations belong to and uncover unexpected genetic complexity. The future use of nuclear markers, which have a slow evolutionary rate in hydrozoans, in addition to mitochondrial sequences might shed light on the dispersal history of this cryptic species complex.

Compliance with ethical standards

Conflict of Interest   The authors declare that they have no conflict of interest.

Ethical approval   All applicable international, national, and/or institutional guidelines for animal testing, animal care and use of animals were followed by the authors.

Sampling and field studies   All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities.

Data availability   New sequences determined in the present study were deposited in the GenBank repository (accession numbers: from MT408048 to MT408071). Online Resource 1 lists all studied specimens and their respective voucher catalog number (respective sampling and preservative information), and GenBank accession number for each sequence.

Author contribution   AEM conceived the research. CFV, AEM and KBN designed the study. CFV and KBN conducted experiments. CFV and MVK analyzed data. All authors wrote, read, and approved the manuscript.

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