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ITS1 variation among *Stichopathes* cf. *maldivensis* (Hexacorallia: Antipatharia) whip black corals unveils conspecificity and population connectivity at local and global scales across the Indo-Pacific

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Abstract

Due to the dearth of molecular markers variable enough to distinguish species of black corals, species delimitation in Antipatharia is still mainly based on morphological traits. One of the few markers available, the internal transcribed spacer (ITS) region located in ribosomal DNA, has already been used several times to infer phylogenetic relationships amongst antipatharians. However, this region was never considered in population-scale studies of shallow-water black corals till now. Here, we sampled two phenotypic variants of 41 specimens of the shallow-water antipatharian *Stichopathes* cf. *maldivensis* along the SW of Madagascar. The sampling area represents 200km of shore and encompasses the runoffs of

three rivers. We used direct sequencing to determine the main ITS1 types present in the genomes of the collected specimens, and retrieved sequences from GenBank for Indonesian specimens. Then, we constructed a haploweb to visualize possible species boundaries using the criterion of mutual allelic exclusivity. With this method, we confirm that the previously described phenotypic variants belong to a single species that apparently extends from Indonesia to Madagascar. Likewise, the three rivers that pour freshwater in southwestern Madagascar do not appear to act as a barrier for the larval dispersal of this species. This study highlights the usefulness of ITS markers within antipatharians at both species and population levels.

Introduction

Antipatharians (Cnidaria: Anthozoa), commonly called black corals, comprise approximately 250 species partitioned into seven families (Molodtsova and Opresko 2019). Most genera thrive at depths greater than 150 m (Molodtsova and Opresko 2017), but shallow-water species are found all around the world (*e.g.* Tazioli et al. 2007; Wagner 2015; Terrana et al. 2020). Despite this ubiquitous distribution, the systematics of black corals, especially shallow-water ones, has always been problematic because early descriptions lacked details of morphological features such as skeletal spines, colony shape and polyp morphology, on which the taxonomy of antipatharians is presently resting (Brugler et al. 2013). Improving these early descriptions is difficult as type specimens were often poorly preserved and some of them have gone missing. Moreover, many antipatharians display considerable intraspecific plasticity, potentially resulting in an overestimation of the total number of species (Lapian et al. 2007; Bo et al. 2012; Brugler et al. 2013). One issue with molecular studies of anthozoans in general, including antipatharians, is that the Folmer region of the mitochondrial cytochrome c oxidase subunit I (*cox1*) used as a general marker for DNA barcoding is unsuitable to distinguish among closely related species within this group due to slow evolution rates (France and Hoover 2002; Shearer et al. 2002; Hellberg 2006; McFadden et al. 2010). However, Brugler et al. (2013) showed that *cox1-cox3* with additional loci could be an adequate marker for species identification within Antipatharia, as well as for clarifying the phylogenetic relationships among them. These markers have also been used in recent species description (Opresko et al. 2020). To date, most genus-level studies of antipatharians have used the internal transcribed spacers 1 and 2 (ITS1 and ITS2, located in ribosomal DNA) to reconstruct phylogenetic trees (Lapian et al. 2007; Lapian 2009; Wagner et al. 2010; Bo et al.

2012; Bo et al. 2018). Both mitochondrial and nuclear markers revealed some genera to be polyphyletic whereas some species turned out to be more closely related than expected (Lapian et al. 2007; Lapian 2009; Wagner et al. 2010; Bo et al. 2012; Brugler et al. 2013; Bo et al. 2018).

One particularly revealing example is the whip black coral genus *Stichopathes*, which is widely spread and encompasses about 35 species. Twelve of the currently accepted species were described from the Indian Ocean (Terrana et al. 2020). One study distinguished four clades (called A, B, C and D) based on ITS sequences among Indonesian shallow-water specimens, the first three of which grouped together with branched species belonging to the genus *Antipathes* (Bo et al. 2012). A later publication split *Stichopathes* into two different families, Aphanipathidae and Antipathidae supported by both nuclear and mitochondrial markers as well as by morphological features (Brugler et al. 2013). The sequences analyzed in the latter study showed that specimens from relatively shallow waters (less than 100 m depth) grouped within Antipathidae whereas those clustering with Aphanipathidae were only found in the deep sea (between 490 and 1681 m depth). More recently, a phylogenetic tree reconstruction based on whole mitogenomes confirmed that *Stichopathes* clustered with members of the Aphanipathidae (Barrett et al. 2020). These results suggest that a whip-like morphology has appeared multiple times during antipatharian evolution, warranting an extensive taxonomic revision of the genus *Stichopathes*, and by extension of the families Antipathidae and Aphanipathidae.

As with their taxonomy, the population genetics of black corals remains poorly understood. The only study available to date for a shallow-water species used allozymes and detected significant genetic differentiation of populations of *Antipathes fjordensis* within fjords in New Zealand, suggesting a very limited dispersal of the larvae of this species (Miller 1997). Still, it remains unknown if this is the consequence of environmental barriers or of the limited ability of black coral larvae to disperse. Notably, the possibility that freshwater outflows from rivers act as natural barriers to larval dispersal of black corals has never been studied, although such impact of rivers on the dispersal of marine invertebrates has already been suggested for both sessile (see de Souza et al. 2017 for *Millepora* corals in Brazil) and benthic species (see Dong et al. 2012 for limpets or Du et al. 2016 for mantis shrimps in China).

The markers ITS1 and ITS2 have been used repeatedly for species delimitation and studies of population structures in cnidarians such as soft corals (Calderón et al. 2006), scleractinian corals (e.g. van Oppen et al. 2002; Pillay et al. 2006; Flot and Tillier 2006; Flot et al. 2010; Adjerooud et al. 2014; Terraneo et al. 2016; Huang et al. 2018; Kang et al. 2020) and in one

study dealing with black corals (Miller et al. 2010). These spacers are transcribed but non-coding, making them highly variable, but they are flanked by the highly conserved 18S, 5.8S and 28S genes and can therefore be readily PCR-amplified using generic primers. Moreover, the entire ribosomal DNA region is present in numerous copies that evolve in a concerted fashion (Dover 1982), resulting in a smaller effective population size compared to single-copy nuclear markers (Navajas and Boursot 2003).

To find out whether internal transcribed spacers can elucidate the species boundaries and connectivity of antipatharians, we chose to focus on Bo et al.'s (2012) clade D, tentatively identified as *Stichopathes* cf. *maldivensis* and widely distributed from Indonesia to Madagascar. This clade is abundant in the southwest of Madagascar where it is easily found over 200km of coast (Terrana et al. 2020). In the same way as in Sulawesi (Bo et al. 2012), two color morphs of *S.* cf. *maldivensis* are present in Madagascar (a brown morph and a white morph), and Bo et al. (2012) as well as Terrana et al. (2020) hypothesized that these morphs may be conspecific based on some shared morphological features. To test this hypothesis, we sequenced the ITS1 marker from a set of individuals of both morphotypes and conducted a haploweb analysis (Flot et al. 2010); a complementary species delimitation analysis which included sequences available in GenBank was performed using Automatic Barcode Gap Discovery (ABGD, Puillandre et al. 2012). We also used the same sequence data to investigate the connectivity of *S.* cf. *maldivensis* at two spatial scales: within Madagascar and across the Indo-Pacific. At the first scale, we hypothesized that the terrestrial runoffs of the three rivers in our study area act as natural barriers to the larval dispersal of these shallow-water black corals (which would result in distinct ITS1 haplotypes frequencies on either sides). At the second scale, we wanted to find out whether populations of *S.* cf. *maldivensis* separated by thousands of kilometers across the Indian-Ocean showed evidence of population differentiation.

Material and Methods

Sampling

Our sampling sites (Fig. 1) were located along the southwestern coast of Madagascar, which is divided by the runoffs of three main rivers. The Onilahy, the main one, covers about 32,000 km² with a mean flow estimated at 145m³s⁻¹ (Aldegheri 1972). The river flows all year round into the sea at the village of Saint Augustin, where there is also an oceanic trench of 1,000 m depth. With a watershed of about 7,500 km² and the average discharge is 35.4 m³s⁻¹

(Aldegheri 1972), the Fiherenana separates the Great Reef of Toliara from other sites located farther north. Finally, the mouth of the Manombo separates Ifaty from Soalary and Andavadaoka. Both the Fiherenana and the Manombo flow only during the wet season (from December to March).

Specimens were collected using SCUBA at depths comprised between 14m and 28m in November and December 2014, in May, November and December 2015 and in July and August 2016. In total, 41 colonies of *S. cf. maldivensis* were sampled, of which 7 came from Maromena, 3 from Soalara, 21 from the North Channel of Toliara, 1 from the South Channel of Ifaty, 5 from the North Channel of Ifaty and 4 from Andavadoaka (Fig. 1, Table 1, see Terrana et al. 2020 for GPS coordinates). Field identification of the specimens was performed following the descriptions in Terrana et al. (2020). *Stichopathes cf. maldivensis* has a unique phenotype in that area that allowed us to identify this species underwater. To avoid sampling repeatedly the same clones, colonies close to each other were avoided.

The brown morphotype (Fig. 2a) was found at all the stations (n=35) whereas the white morphotype (Fig. 2b) was only present in Maromena, the North Channel of Toliara and Andavadoaka (n=6). During each dive, pieces of 2 to 3 cm² of tissues were sampled with a sharp blade at the apex of the colony and directly placed into labeled vials, then transferred in absolute ethanol once back to shore.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the Invisorb Spin Tissue Minikit (Invitek) according to the manufacturer's protocol and stored at -20°C. The internal transcribed spacer 1 (ITS1) was first amplified using the primers F18S1 (5'-CGATYGAAYGGTTTAGTGAGGC-3') and ITScl (5'-CATTTGCGTTCAAAGATTTCG-3') of Flot et al. (2011). As ITS1 was not amplified in all samples using these primers, we designed the new primers T18S (5'-GGATGCCGAGAAGTTGTTCA-3') and T58S (5'-TTCACACTACTTATCGCACCTG-3') using Primer3 (Untergasser et al. 2012) in the conserved regions 18S and 5.8S based on sequences retrieved from GenBank. Both sets of primers were expected to amplify full-length ITS1 along with flanking portions of the 18S and 5.8S genes. T18S is annealing around 37 bp downstream F18S1 at the 3' end of the 18S gene, while T58S is annealing around 21 bp upstream of ITScl at the 5' end of the 5.8S gene. For both primer pairs, amplifications were carried out in 50µl reaction mixes using the Red'y'StarMix (Eurogentec) with the following conditions: initial denaturation and polymerase activation at 95°C for 10 min; 50 cycles of

denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and elongation at 72°C for 1 min; and 10°C for 5 min. Amplicons were Sanger-sequenced in both directions (Genewiz, UK) using the same primers as for amplification.

Haplotype determination and haploweb analysis

Chromatograms were assembled and cleaned using Sequencher 4 (Gene Codes, see “Sequences_Madagascar.SPF” in Supplementary material). The chromatograms were analyzed following the procedure for nuclear sequence markers described in Fontaneto et al. (2015). Phasing was trivial for homozygous colonies (that had no double peaks) and for colonies that had only one double peak (i.e., comprising two ITS1 sequence types that differ at a single position). Colonies in which numerous double peaks were observed, as expected from the superposition of two ITS1 sequence types of unequal lengths (Flot et al. 2006), were phased using the online program Champuru (Flot 2007; <http://jfflot.mnhn.fr/champuru/>) whenever both the forward and reverse chromatograms were available, or (for one specimen for which sequencing had failed in one direction) using the software Indelligent v.1.2 (Dmitriev and Rakitov 2008; <http://dmitriev.speciesfile.org/indel.asp>). Finally, colonies that had only a few double peaks, as expected from the superposition of two ITS1 sequence types of equal lengths differing at several positions, were phased using the programs SeqPHASE (Flot 2010; <https://eeg-ebe.github.io/SeqPHASE/>) and PHASE (Stephens et al. 2001); in eight cases where the resulting phasing was uncertain, i.e. where the posterior probability of the most likely haplotype pair was less than 0.90, we resorted to scrutinizing peak heights in order to disentangle the two haplotypes.

The phased sequences of all individuals were then aligned in MEGA7 using MUSCLE (Tamura et al. 2011). Each SNP (single-nucleotide polymorphism) observed in the alignment was verified in the chromatograms. The resulting phased sequences were deposited in GenBank under accession numbers MT901478-MT901551 (Table 1).

A haploweb (Flot et al. 2010) was built using the median-joining algorithm (Bandelt et al. 1999) implemented in the online software HaplowebMaker (Spöri and Flot, 2020; available online at <https://eeg-ebe.github.io/HaplowebMaker/>), with curves connecting the haplotypes found co-occurring in heterozygous individuals (see “Haploweb_cladeD.json” and “Haploweb_colortype.json” in Supplementary Material). Indels were treated as missing data, i.e. columns containing indels were masked during analysis.

The fixation index F_{ST} between Indonesian and Malagasy populations as well as between Malagasy populations was calculated using the program DnaSP version 6 (Rozas et al. 2017).

Phylogenetic analyses and automatic barcode gap delimitation

ITS1 sequences of black corals from different parts of the world (Table 1) were retrieved from Lapien et al. (2007), Bo et al. (2009), Wagner et al. (2010) and Bo et al. (2012) and aligned with the sequences from this study using MAFFT's E-INS-i mode (Katoh et al. 2005) with default gap penalties (see "Alignement_phylo_tree.fas" in Supplementary Material)..

An automatic barcode gap delimitation (ABGD; Puillandre et al. 2012) analysis of the resulting alignment was performed using the ABGD webserver (<https://bioinfo.mnhn.fr/abi/public/abgd/>) with default parameters.

A maximum-likelihood analysis was performed using MEGA7 (Kumar et al. 2016) with 1000 bootstrap replicates, using the general time-reversible (GTR) substitution model with a gamma distribution with invariant sites (G+I) as determined by Modeltest (Posada 2006). Indels and missing data were treated using the 'pairwise deletion' mode.

Two simultaneous and independent runs of Bayesian analyses were performed using MrBayes v3.0b4 (Ronquist and Huelsenbeck 2003), using the model GTR+I+G. Four Markov chains were run simultaneously for 10^6 generations, and trees were sampled every 100 cycles, resulting in a total of 10^4 trees. The first $2.5 \cdot 10^3$ trees with pre-asymptotic likelihood scores were discarded as 'burnin'. The remaining trees were used to compute Bayesian posterior probabilities. Trees were rooted using myriopathids as an outgroup.

Results

Black corals were found to be present in variable abundances in the shallow waters of the southwestern coast of Madagascar: a black coral bed was observed in the North Channel of Toliara, whereas black corals were rarer in other locations. Nevertheless, the morphospecies *S. cf. maldivensis* was found in all the stations.

The 41 specimens collected in south-western Madagascar yielded ITS1 sequences of 360 bp after manual trimming. Among them, 8 individuals were homozygotes, 9 were length-variant heterozygotes and 24 were heterozygotes without length variation (five colonies with a single double peak, six with two double peaks, nine with three double peaks, three with four double peaks and one with five double peaks). In total, 11 SNPs were found among the ca. 360 bases of the phased alignment (representing 3% of the sites), all of which were bi-allelic. The

maximum intra-individual variation recorded was 1.39%, whereas the overall mean distance among the individuals we collected was <0.01%.

The phased alignment of *S. cf. maldivensis* from south-western Madagascar included a total 74 sequences from 41 individuals, representing 10 distinct haplotypes. The most common haplotype was shared between 24 specimens (Fig. 3), whereas 5 haplotypes were singletons (*i.e.*, each of them was observed in a single specimen). The two color types white and brown shared 5 haplotypes co-occurring between individuals, with 5 haplotypes were private to the brown morphotype (Fig. 3). All the haplotypes found in white morphotype specimens were frequent haplotypes also found in brown morphotype specimens. All the specimens of both types shared a single pool of ITS1 sequences regardless of their location (Fig. 3), *i.e.* they belonged to the same field for recombination (FFR) *sensu* Doyle (1995).

The phased alignment was combined with sequences of 9 *Stichopathes* Clade D specimens from SE Asia, for a total of 50 specimens. The alignment comprised 83 sequences in total and was 360 nucleotides long after manual trimming, among which 9 positions were variable. All the specimens sampled along the southwestern coast of Madagascar shared haplotypes (Fig. 4). The North Channel of Toliara was the location with the highest haplotype diversity: nine out of ten Malagasy haplotypes were represented there. Five out of these ten haplotypes were also observed in at least one other location in SW Madagascar, and three Malagasy haplotypes were also observed in specimens from Indonesia except for Mentawai.

The fixation index between Indonesian population and Malagasy population was $F_{ST}=0.093$. It was also calculated between populations of the southwest of Madagascar with the hypothesis that the three rivers would separate black corals into four distinct populations: in all cases, F_{ST} was null.

The consensus tree obtained using Bayesian analysis is shown in Figure 5 with its posterior probabilities. In the bootstrap consensus tree obtained using maximum likelihood, all *Stichopathes* species grouped in the same clades as for Bayesian inference. The only notable difference is that the clade encompassing species of the genus *Rhipidipathes* was the sister group of *Antipathes* + *Stichopathes* + *Cirrhopathes* in the Bayesian analysis (PP=1) whereas it was the sister group of *Cirrhopathes* + *Antipathes curvata* in the ML analysis (with a rather low bootstrap value of 55%). In the ML analysis, the *Rhipidipathes* + *Cirrhopathes* + *Antipathes curvata* clade was the sister group of the clade comprising all the other *Antipathes* species as well as all *Stichopathes* species (BS=89%).

Clades were congruent with those of Bo et al. (2012), and the specimens from Madagascar grouped with *Stichopathes* specimens from Clade D (Bo et al. 2012) originating from Ambon,

Bali, Bunaken, Raja Ampat and Mentawai. All *Stichopathes* species belonged to Antipathidae. Other *Stichopathes* species grouped with other genera but all belonged to the Antipathidae. *Stichopathes* cf. *occidentalis* from the NW Atlantic grouped with *Antipathes caribbeana* and *Stichopathes* sp. AMBA5 from the Clade A grouped with *Antipathes grandis*. Specimens from Clade C all grouped together. Specimens from Clade B grouped in a clade together with various *Antipathes* species. ABGD species delimitation (see bar on the right side of Figure 5) confirmed that all Clade D sequences were conspecific, and considered Bo's Clade B and Clade C as two distinct species as well. Bo's Clade A, on the other hand, was split into two distinct species using this approach.

Discussion

Using ITS1 to delineate black coral species

Allele sharing-based approaches to species delimitation, such as haplowebs, rely on heterozygous individuals to delineate species (Fontaneto et al. 2015). Even though polymorphism is generally high for marine species (Leffler et al. 2012), intra-individual ITS diversity in black corals (be it single nucleotide polymorphisms and/or length polymorphism) had only been documented previously for two deep-sea species within the genus *Stichopathes* (Miller et al. 2010). The latter study found a total variability of 2.7% in ITS1 of 16 specimens of *S. variabilis* (including substitutions and indels), corresponding to 8 haplotypes, and 1.6% variation in ITS1+ITS2 of 17 specimens of *S. filiformis*. The intra-specific variation of ITS1 is similar between *S. variabilis* and *S. cf. maldivensis* (3% and 10 haplotypes), although these two species are taxonomically and ecologically distinct. In other black coral species, ITS variability was only been reported in the genus *Antipathes* (Wagner et al. 2010; Brugler et al. 2013). On the other hand, double peaks in ITS chromatograms were not mentioned in the phylogeny of Mediterranean species (Bo et al. 2018); the phylogeny of tropical shallow-water species (Lapian et al. 2007; Lapian 2009); the revision of the family Schizopathidae (MacIsaac et al. 2013); nor in studies on the genera *Leiopathes* (Ruiz-Ramos et al. 2015), *Pseudocirrhipathes* (Bo et al. 2009) and *Stichopathes* (Bo et al. 2012). This almost certainly points to a general oversight: in the case of *Leiopathes*, for instance, examination of the chromatograms communicated by the authors revealed the presence of numerous double peaks that had not been considered. Reanalyzing their data paying specific attention to double peaks yielded a haploweb in which all haplotypes were connected via heterozygous individuals, confirming the authors' original conclusion that all *Leiopathes glaberrima*

individuals sequenced in their study were conspecific, regardless of their color morph (Flot 2020).

In this study, we carefully searched for double peaks in our chromatograms and found that most of the individuals sequenced harbored two distinct sequence types. As these sequence types were found in various combinations and occurred alone in some individuals, we interpreted them as haplotypes and therefore called individuals harboring no double peak “homozygotes” and individuals harboring double peaks “heterozygous” for this marker. The fact that the assortment of ITS1 haplotypes we reconstructed matched closely the expectation of random mating in a panmictic population validated our interpretation and confirmed that our sample size was adequate: examination of the haploweb shown in Figure 3 shows that it has reached saturation in terms of number of individuals. The most frequent haplotypes (represented with large circles on the haplowebs) were often found co-occurring in heterozygous individuals (represented with curves connecting two haplotypes), whereas rare haplotypes (represented with small circles on the haplowebs) were usually found co-occurring with frequent haplotypes and not with other rare haplotypes. This suggests that, as observed in previous articles (see *e.g.* discussion in Flot et al. 2011), ITS behaves as a “regular” diploid marker when sequenced directly, which contrasts with the often extreme diversity of ITS paralogs revealed by cloning (Flot et al. 2006) and next-generation sequencing (Brabec et al. 2016). Saturation was not reached concerning the Indonesian individuals included in the haploweb shown in Figure 4, as evidenced by the three disconnected haplotypes in the network, but as these samples were from other studies we were unable to address this limitation.

By phasing ITS and identifying heterozygous individuals, we were able to apply two complementary approaches to species delimitation: haplowebs, an allele sharing-based approach, and ABGD, a distance-based approach (Flot 2015). These two methods were identified in a previous simulation study as the most effective single-locus approaches to species delimitation, in contrast to tree-based approaches such as Generalized Mixed Yule Coalescent method (GMYC) and Poisson Tree Processes (PTP) that often overestimate the actual number of species (Dellicour and Flot 2018). Moreover, the same study advocated that, since ABGD and haplowebs exhibit very distinct patterns of bias, when both approaches yield congruent results one can be confident in the resulting delimitation. Here, both haploweb and ABGD concurred in suggesting that Bo et al.’s Clade D corresponds to a single species, which one may call *Stichopathes* cf. *maldivensis* pending taxonomic revision (Terrana et al. 2020). For the other clades in Bo et al. (2012), however, we were only able to use one of the two

methods, ABGD, as haplowebs require heterozygous individuals but all the individuals analyzed in previous studies were reported as homozygous.

Using ITS1 to analyze black coral connectivity patterns

Based on the Miller (1997) study that had found significant genetic differentiation between sampling sites within fjords for *Antipathes fjordensis*, it was expected that black corals had a very limited larval dispersal, over distances of tens of kilometers only. By contrast, the ITS1 sequences and F_{ST} values we obtained do not suggest any barrier to dispersal along the 200 km of shore we sampled, with includes the discharges of three rivers thought to represent potential natural barriers to the dispersal of black corals larvae.

The fact that we did not detect any difference in ITS1 haplotype composition among our field sites suggests that *Stichopathes* cf. *maldivensis* presents a much higher dispersal ability compared to the only other black coral species examined to date, *Antipathes fjordensis*. A newly discovered shallow oceanic current (the Southwest Madagascar Coastal Current, SMACC) was recently characterized as describing a gyre along the SW of the country (Ramanantsoa et al. 2018). Its limits exactly correspond to the area of this study, and it is estimated that it carries water masses from Andavadoaka to Maromena in 11 days, with a flow acceleration around the bay of Toliara (Ramanantsoa et al. 2018). We hypothesize that the larval dispersal of black corals might be influenced by this current that forces larvae to settle within the bay, explaining the high occurrence of black corals in this place compared to other locations in the SW. This hypothesis is also supported by the fact that all the specimens from the southwestern part of Madagascar share ITS1 haplotypes.

The hypothesis that *Stichopathes* cf. *maldivensis* is able to disperse widely is corroborated by the unexpected observation that this species extends from Madagascar to Indonesia. This finding of identical ITS haplotypes across large geographical distance is reminiscent of a similar finding in *Lophelia* corals, for which identical haplotypes were discovered in the Ionian and Barents seas (Flot et al. 2013). In the case of *Lophelia*, it was hypothesized that deep-water currents such as the Mediterranean outflow carry larvae very efficiently over large distances.

To date, there is no information regarding the biology and ecology of black coral larvae, except from unpublished data cited in Miller (1998) that reported a limited movement of the crawling sexual planulae of *A. fjordensis*. However, no larvae from any other black coral species have been observed since then and therefore no conclusions can be drawn. Present hypotheses regarding larval dispersal in black corals are mainly based on models of oceanic

currents (Cardona et al. 2016), on genetic data (Miller 1998; Ruiz-Ramos et al. 2015) and/or on morphological features of the gametes (Terrana and Eeckhaut 2019). For instance, very large oocytes as seen in the deep-sea species *Dendrobathypathes grandis* (Lauretta and Penchaszadeh 2017) or spermatocysts released in buoyant spheres as seen in the shallow-water species *Cupressopathes pumila* (Gaino and Scoccia 2009) suggest enhanced dispersal abilities. The sexual reproductive cycle of *S. cf. maldivensis* remains unknown; however, tropical shallow-water *Stichopathes* species are reported to have small oocytes similar in size to other shallow-water species such as *Cirrhopathes anguina* (Wagner et al. 2010; Terrana and Eeckhaut 2019), which would typically presumed to lead to short-lived larvae. More information on the biology of black coral larvae will be required to make sense of the very high apparent dispersion potential of *Stichopathes cf. maldivensis* and to understand whether such broadly distributed species is an exception or the rule among antipatharians.

Limitations of single marker usage and future studies

Despite relying on a relatively short, single nuclear marker, the methods employed in this study is specifically aimed at discriminating potential cryptic species. No species boundary was detected in our haplowebs as all individuals sequenced belong to the same field for recombination, a finding confirmed using the independent approach ABGD. When using haplowebs to delineate species, the length of the marker used is not as important as proper phasing of the haplotypes. Dealing with short sequences allows to perform accurate phasing of the haplotypes of heterozygous individuals with a reliable call of double peaks. Indeed, the usefulness of a single short marker such as ITS1 or ITS2 for species delimitation has already been shown in large-scale population genetic structures of scleractinian corals (see for instance Flot et al. 2011; Adjerdoud et al. 2014; Terraneo et al. 2016; van Der Ven et al. 2020). However, future studies focusing on *Stichopathes cf. maldivensis* across the Indian Ocean may uncover cryptic species, as larval dispersal is closely depending on environmental conditions and oceanic currents.

Prior to the phylogenetic analyses in this study, we conducted a thorough morphological comparison between Malagasy and Indonesian specimens (Terrana et al. 2020) that allowed us to directly hypothesized that our specimens belonged to one of the clade formed by tropical shallow-water *Stichopathes* formerly described by Bo et al. (2012). However, future studies involving new species descriptions or data from large black coral surveys should consider new high-throughput sequencing techniques to infer phylogenetic relationships, such as Ultra-

Conserved Elements (Quattrini et al. 2018). Over the last few years, UCEs have demonstrated their ability to solving long-standing taxonomic uncertainties among anthozoans, including a recent first study on antipatharians (Horowitz et al. 2020).

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Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure 1. Sampling areas in the southwest of Madagascar. The total number of specimens collected at each location is shown, as well as the relative abundance of the two color morphs.

Figure 2. Color morphs of *Stichopathes cf. maldivensis* found on the southwestern coast of Madagascar. a: brown type, b: white type. Scales: a, b: 1 cm.

Figure 3. Haplotype network of ITS1 sequences of the 41 *Stichopathes* cf. *maldivensis* collected along the southwestern coast of Madagascar. The alignment comprised 74 sequences corresponding to 10 distinct haplotypes, each of them represented by a pie chart with an area proportional to the number of individuals harboring this haplotype. Straight lines connecting haplotypes represent evolutionary paths, with mutations shown as bars. Curved lines connect haplotypes found co-occurring in heterozygous individuals, with a thickness proportional to the number of heterozygous individuals connecting two haplotypes.

Figure 4. Haplotype network of ITS1 sequences of the 41 colonies of *Stichopathes* cf. *maldivensis* of Madagascar together with sequences of *Stichopathes* clade D from Indonesia. The alignment comprised 83 sequences corresponding to 13 distinct haplotypes. Each color represents a location, the curves represent haplotypes found co-occurring in heterozygous individuals, the thickness of the lines represents the frequencies of co-occurrences between haplotypes, and the pie charts represent each haplotype with their size depending on their frequency.

Figure 5. Consensus phylogenetic tree obtained by Bayesian inference using ITS1 sequences of *Stichopathes* specimens from SW Madagascar, NW Atlantic and SE Asia, along with other antipatharian species, and rooted using myriopathids as an outgroup. A single sequence was included for each of the 10 Malagasy haplotypes. Numbers above the nodes of the tree represent posterior probabilities, as well as bootstrap values when branch topology was similar between both analyses, respectively. Groupings based on morphology (*Stichopathes* clades according to Bo et al. 2012) and molecular data (haplowebs and ABGD) are shown as colored bars on the right side. Branch tips with squares highlight species belonging to Antipathidae, with triangles to Aphanipathidae and with circles to Myriopathidae.

Table 1. Species, localities, accession numbers and references of the sequences used in this study.

Family and species	Locality	Accession #	References
Antipathidae			
<i>Allopathes desbonni</i>	Louisiana, USA	FM882171	Bo et al. 2009
<i>Antipathes atlantica</i>	Hawai'i, USA	HM060621	Wagner et al. 2010
<i>Antipathes caribbeana</i>	Netherlands Antilles	GU296498	Wagner et al. 2010
<i>Antipathes curvata</i>	South China Sea	GU296497	Wagner et al. 2010
<i>Antipathes elegans</i>	Bunaken, Indonesia	AM404317	Lapian et al. 2007
<i>Antipathes furcata</i>	Hawai'i, USA	HM060620	Wagner et al. 2010
<i>Antipathes grandis</i>	Hawai'i, USA	GU296493	Wagner et al. 2010
<i>Antipathes griggi</i>	Hawai'i, USA	GU296496	Wagner et al. 2010
<i>Antipathes</i> sp.	Bunaken, Indonesia	AM404315	Lapian et al. 2007
<i>Antipathes</i> sp. 2	Bunaken, Indonesia	AM404321	Lapian et al. 2007
<i>Antipathes</i> sp. 3	Bunaken, Indonesia	HE600716	Bo et al. 2012
<i>Cirrhipathes spiralis</i>	Bunaken, Indonesia	AM404320	Lapian et al. 2007
<i>Cirrhipathes</i> sp.	Bunaken, Indonesia	AM404319	Lapian et al. 2007
<i>Pseudocirrhipathes mapia</i>	Bunaken, Indonesia	FM882167	Bo et al. 2009
<i>Pseudocirrhipathes mapia</i> 2	Bunaken, Indonesia	FM882168	Bo et al. 2009
<i>Stichopathes</i> cf. <i>occidentalis</i>	NW Atlantic Ocean	HM060618	Wagner et al. 2010
<i>Stichopathes</i> BALA48 clade A	Bali, Indonesia	HE600722	Bo et al. 2012
<i>Stichopathes</i> AMBA5 clade A	Ambon, Indonesia	HE600721	Bo et al. 2012
<i>Stichopathes</i> BUK11 clade B	Bunaken, Indonesia	HE600719	Bo et al. 2012
<i>Stichopathes</i> INDO19 clade B	Bunaken, Indonesia	HE600720	Bo et al. 2012
<i>Stichopathes</i> AMBA6 clade C	Ambon, Indonesia	HE600717	Bo et al. 2012
<i>Stichopathes</i> MENT52 clade C	Mentawai, Indonesia	HE600718	Bo et al. 2012
<i>Stichopathes</i> AMBA45 clade D	Ambon, Indonesia	HE600710	Bo et al. 2012
<i>Stichopathes</i> BALA46 clade D	Bali, Indonesia	HE600711	Bo et al. 2012
<i>Stichopathes</i> BUK14b clade D	Bunaken, Indonesia	HE600713	Bo et al. 2012
<i>Stichopathes</i> BUNA14 clade D	Bunaken, Indonesia	AM404318	Bo et al. 2012
<i>Stichopathes</i> MENT8b clade D	Mentawai, Indonesia	HE600715	Bo et al. 2012
<i>Stichopathes</i> RJ15 clade D	Raja Ampat, Indonesia	HE600712	Bo et al. 2012
<i>Stichopathes</i> RJ16 clade D	Raja Ampat, Indonesia	HE600708	Bo et al. 2012
<i>Stichopathes</i> RJ22 clade D	Raja Ampat, Indonesia	HE600709	Bo et al. 2012
<i>Stichopathes</i> RJ35 clade D	Raja Ampat, Indonesia	HE600714	Bo et al. 2012
<i>Stichopathes</i> cf. <i>maldivensis</i> PN4	North Toliara, Madagascar	MT901503, MT901504	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN015	North Toliara, Madagascar	MT901512, MT901513	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN016	North Toliara, Madagascar	MT901511	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN028	North Toliara, Madagascar	MT901509, MT901510	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN030	North Toliara, Madagascar	MT901507, MT901508	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN033	North Toliara, Madagascar	MT901505, MT901506	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN058	North Toliara, Madagascar	MT901501, MT901502	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN061	North Toliara, Madagascar	MT901499, MT901500	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN066	North Toliara, Madagascar	MT901497, MT901498	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN068	North Toliara, Madagascar	MT901495, MT901496	This study

<i>Stichopathes</i> cf. <i>maldivensis</i> PN071	North Toliara, Madagascar	MT901493, MT901494	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN073	North Toliara, Madagascar	MT901492	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN080	North Toliara, Madagascar	MT901490, MT901491	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN086	North Toliara, Madagascar	MT901489	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN088	North Toliara, Madagascar	MT901487, MT901488	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN090	North Toliara, Madagascar	MT901485, MT901486	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN096	North Toliara, Madagascar	MT901483, MT901484	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN102	North Toliara, Madagascar	MT901520, MT901521	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN132	North Toliara, Madagascar	MT901518, MT901519	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN136	North Toliara, Madagascar	MT901516, MT901517	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> PN137	North Toliara, Madagascar	MT901514, MT901515	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IFPS07	South Ifaty, Madagascar	MT901536, MT901537	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IF196	North Ifaty, Madagascar	MT901544	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IF197	North Ifaty, Madagascar	MT901542, MT901543	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IF198	North Ifaty, Madagascar	MT901541	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IF199	North Ifaty, Madagascar	MT901539, MT901540	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> IF200	North Ifaty, Madagascar	MT901538	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> SOA232	North Ifaty, Madagascar	MT901482	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> SOA236	North Ifaty, Madagascar	MT901480, MT901481	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> SOA237	Soalara, Madagascar	MT901478, MT901479	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR259	Soalara, Madagascar	MT901534, MT901535	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR264	Soalara, Madagascar	MT901532, MT901533	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR265	Maromena, Madagascar	MT901530, MT901531	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR267	Maromena, Madagascar	MT901528, MT901529	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR270	Maromena, Madagascar	MT901526, MT901527	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR273	Maromena, Madagascar	MT901524, MT901525	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> MAR275	Maromena, Madagascar	MT901522, MT901523	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> AND284	Maromena, Madagascar	MT901550, MT901551	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> AND286	Maromena, Madagascar	MT901549	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> AND289	Andavadoaka, Madagascar	MT901547, MT901548	This study
<i>Stichopathes</i> cf. <i>maldivensis</i> AND290	Andavadoaka, Madagascar	MT901545, MT901546	This study
Aphanipathidae			
<i>Aphanipathes</i> cf. <i>sarothamnoides</i>	Palau, North Pacific	FM882166	Bo et al. 2009
<i>Aphanipathes</i> <i>pedata</i>	Florida, USA	FM882170	Bo et al. 2009
<i>Rhipidipathes</i> <i>reticulata</i>	Bunaken, Indonesia	AM404322	Lapian et al. 2007
<i>Rhipidipathes</i> <i>reticulata</i>	Bunaken, Indonesia	HE600723	Bo et al. 2009
<i>Rhipidipathes</i> <i>reticulata</i>	Bunaken, Indonesia	HE600724	Bo et al. 2009
<i>Phanopathes</i> <i>rigida</i>	Louisiana, USA	FM882169	Bo et al. 2009
Myriopathidae			
<i>Antipathella</i> <i>subpinnata</i>	Messina Strait, Italy	AM404329	Bo et al. 2009
<i>Cupressopathes</i> <i>abies</i>	Bunaken, Indonesia	AM404324	Lapian et al. 2007
<i>Cupressopathes</i> <i>pumila</i>	Bunaken, Indonesia	AM404326	Lapian et al. 2007
<i>Cupressopathes</i> sp. 1	Bunaken, Indonesia	AM404325	Lapian et al. 2007
<i>Cupressopathes</i> sp. 2	Bunaken, Indonesia	AM404323	Lapian et al. 2007
<i>Myriopathes</i> <i>myriophylla</i>	Bunaken, Indonesia	AM404328	Lapian et al. 2007
<i>Myriopathes</i> sp.	Bunaken, Indonesia	AM404327	Lapian et al. 2007