

Biodiversity Study of Black Coral (Order: Antipatharia) Collected from Manado, Indonesia based on rDNA Internal Transcribed Spacer (ITS) Sequences Analysis

HAPRY FRED NICO LAPIAN*

Faculty of Animal Science, Sam Ratulangi University, Manado 95115.

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ABSTRACT

Biodiversity of black coral (order Antipatharia) collected from the Bunaken Marine Park (Manado Sea, Indonesia) has been studied based on the sequence of the Internal Transcribed Spacers (ITS) region of rDNA gene. The results of the study showed that the 18 species of Antipatharia were considered to be separated in two family groups, family Myriopathidae and Antipathidae-Aphanipathida. A significant species-specific signal has been detected among the families of Antipathidae and Aphanipathida. However, more studies on different species were required to be clearly interpreted. The new species *Pseudocirrhopathes mapia*, the new genus *Reticulopathes*, and possibly a new taxon of the family Myriopathidae has been recognized based on ITS sequence data.

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Key words: biodiversity study, Manado, rDNA ITS, Antipatharia, black coral.

INTRODUCTION

Antipatharia Milne-Edwards and Haime, 1857 (Cnidaria, Anthozoa) is the order of Black Coral. Five families of Antipatharia have been established, Antipathidae, Cladopathidae, Leiopathidae, Myriopathidae, and Schizopathidae. About 200 species of black coral have been recorded. Black coral could be found in all oceans at depths ranging from those of shallow waters to two thousands meters (Pax et al., 1987)

Antipatharians have been traditionally classified according to morphological and anatomical characters. The primary characters that are usually used in this term of systematic are the general morphology of the corallum, the pattern of ramification, the spines arising on the skeleton surface, the morphology of the polyps and the number of their mesenteries. Milne-Edwards and Haime (1857) had been proposed one of the first systems of classification that is entirely based on the skeletal features. The polypar characteristics are considered important also in most recent taxonomic works where the number of mesenteries, the size and position of tentacles around the mouth and the

arrangement of zooids around the axis and the kind of ramification of the corallum are characters used in the genus separation (Cooper, 1909; Opresko, 1972).

In line with developing technology, more specific tools in biology such as electron microscope enhance placing of black coral in more specific taxon. The study of spines surface using scanning electron microscopy has recently been employed with success in taxonomic works, putting in evidence micro-differences in the deposition of skeletal material on the spines, which are often useful for species distinction.

At a specific level, although there is an increasing study of systematic of Antipatharia based on morphological and anatomical characters, the main problem in taxon definition by means of this approaches is the extreme plasticity of the colonies. The number of species obtained tends to overestimate because it may contains some ecotypes. For this reason, sequences data of DNA are an obvious source of additional evidences regarding black corals systematic relationships. Genetic consideration has been widely used to clarify one of the largest problems arising from identifying coral at the species level.

Genes of internal transcribed spacers (ITS) have been used for molecular markers at the species level of coral in order to study intra- and inter-specific diversity (Diekmann et al., 2001; van Oppen et al., 2002). Sequences of internal transcribed spacers of rDNA was also usefully used in resolving

♥ **Corresponding address:**
Kampus UNSRAT Kleak Bahu, Manado 95115
Tel.: +62-431-863786, Fax.: +62-431-822568
e-mail: hlapian@yahoo.it

phylogenetic relationship of closely related taxa in some coral (Odorico and Miller, 1997; Takabayashi et al., 1998; van Oppen et al., 2000; Lam and Morton, 2003; Chen et al., 2004). The internal transcribed spacer (ITS) region, consisting of the ITS1, 5.8S, and ITS2 sequenced from protists (Hunter et al., 1997), animals (Gonzalez et al., 1990), plants (Baldwin et al., 1995), fungi (Lee and Taylor 1992), and macrophyte algae (Coleman et al., 1994 and Goff et al., 1994), typically provide phylogenetic resolution at or below the species level in each of these groups.

In this paper, in order to investigate biodiversity of black coral lived in the sea of Manado, genetic relationships of 18 species of black coral belonged to nine genera and three families have been performed. The application of a molecular approach to the systematic of this group represents an interesting possibility to test, by an alternative method, the traditional morphological taxonomy.

MATERIALS AND METHODS

Sample collection

Eighteen species of black coral belonged to three families and 9 genera of the order have been used in this study. All the specimens were collected by SCUBA diving on the shallow-water reefs of the Bunaken Marine Park (North Sulawesi, Indonesia) within a depth range of 10-50 m.

DNA Analysis

Antipatharian ITS rDNA sequences were obtained after isolation, amplification, and sequencing process. All procedures of this molecular analysis were conducted using facilities of the Molecular Genetic Laboratory of *Istituto di Biologia e Genetica, Dipartimento di Scienze del Mare, Facolta di Scienze, Universita Politecnica delle Marche, Italy*.

Isolation

Using protocol of Qiamp tissue kit (QIAGEN) the genomic DNA was isolated using primer RA2 5'-GTCCCTGCCCTTTGTACACA-3' and primer ITS 2.2 5'-CCTGGTTAGTTTCTTTCTCCTCCGC-3' (Wörheide, 1998) for the internal transcribed spacer regions (ITS1 and ITS2).

Amplification

Thirty cycles of PCR were conducted using a *Perkin Elmer GeneAmp PCR System 2400* under following profiles: 94°C for 30 s; 52°C for 30 s; and 72°C for 60 s using HotStarTaq Master Mix Kit (Qiagen). The PCR product was then purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's recommended protocol.

Sequencing

Cycle sequencing reactions were done using DNA Sequencing Kit (Applied Biosystem) according to the

protocol provided by the manufacturer with the same primers used in PCR. To obtain the sequences in both directions the forward primer 5'-CAACGGGCGGGCTGGTGCACA-3' and the reverse primer 5'-TGCGCACCAGCCCGCCCGTTG-3' designed on the 5.8S rDNA have been used. The sequencing reaction product was purified using Qiagen DyeEx Spin Kit and then was sequenced on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems). The sequences are about 850 nt. long except for *Cirrhopathes spiralis* (partial sequences of 642 nt). The nucleotide sequences have been deposited in GenBank (accession numbers AM404315-AM404329).

Phylogenetic analysis for biodiversity observation

The trees were produced by maximum parsimony (MP), maximum likelihood (ML), and neighbour-joining (NJ) methods, using the PAUP 4.8 beta version (Swofford, 1998) program. For the heuristic ML analysis, the optimum substitution model was determined using the Model test 3.06 program (Posada and Crandall, 1998). Once the appropriate model was determined (TrNef + G) (Tamure and Nei, 1993), ML analysis was performed with all the parameter values of the model (Gamma distribution shape parameter = 0.2411; substitution model: A-C 1.000; A-G 2.3660; A-T 1.000; C-G 1.000; C-T 3.4329; G-T 1.000; equal base frequencies). *Porites lutea* was used as outgroup.

RESULTS AND DISCUSSION

Results

Sequencing of the ITS region (spanning a partial sequence of the 5' end of the 18S gene, complete ITS1-5.8S-ITS2 and partial sequence of the 3' end of the 28S gene) results sequences ranged between 804 (*Pseudocirrhopathes mapia*) and 912 (*Cirrhopathes* sp.) nucleotides for the species of the family Antipathidae, and between 812 (*Myriopathes myriophylla*) and 836 (*Antipatella subpinnata*) nucleotides for the species of the family Myriopathidae, while the sequence of *Rhipidipathes reticulata* (Aphanipathidae) was 832 nucleotides long.

Figure 1 show that the 18 species of Antipatharia considered are separated in two groups, one containing the genera belonging to the family Myriopathidae and the other belonging to the families Antipathidae-Aphanipathidae. The distance matrix shows that in the first group genetic distances among the Indonesian Myriopathidae species *Cupressopathes abies*, *Myriopathes myriophylla* and *Myriopathes* sp.1, *Myriopathes* sp.2 and the Mediterranean species *Antipatella subpinnata* were very small (0.25% to 2.6%). The second group is divided into three well-differentiated clades: the first group, in basal position, is composed of the new species *Pseudocirrhopathes mapia*, the second is

made up of the genera *Cirripathes*, *Rhipidipathes*, and *Reticulopathes*, the last clade is made up of the genera *Stichopathes* and *Antipathes* and the species *Cirripathes* cf. *anguina* 2. The analysis showed that the species belonging to *Cirripathes* genus are not monophyletic. In fact although *Cirripathes spiralis* and *Cirripathes* sp. show a genetic distance of 0.99%, the two specimens of *Cirripathes anguina* display, respect to those mentioned above, a genetic distance not smaller than 9% and between them of 11.20%. Similarly, *Stichopathes* sp.2 is not clustered with *Stichopathes* sp.1 although shows a genetic distance of 3.53%. In the *Cirripathes*-*Rhipidipathes*-*Reticulopathes* group, the new genus *Reticulopathes* differs from the other species belonging to this group by genetic distances ranging from 11.06% (*Cirripathes* cf. *anguina* 1) to 14.80% (*Cirripathes* sp.).

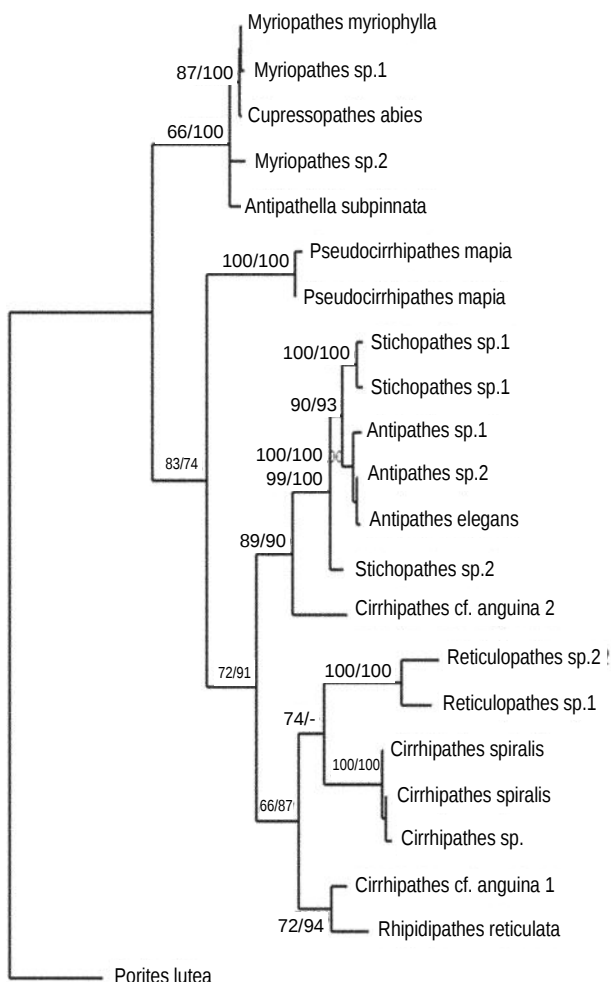


Figure 1. Phylogenetic tree obtained by Maximum Likelihood and Maximum Parsimony of the antipatharian species based on internal transcribed spacers rDNA. On the left ML value, on the right MP value.

The phylogenetic tree obtained by Neighbor-Joining method (Figure 2) presents the same tipology

of the described tree with the exception of the species *Reticulopathes* which are separated from the *Stichopathes*-*Antipathes* group and the *Cirripathes*-*Rhipidipathes* group which is supported by a low bootstrap value (54%).

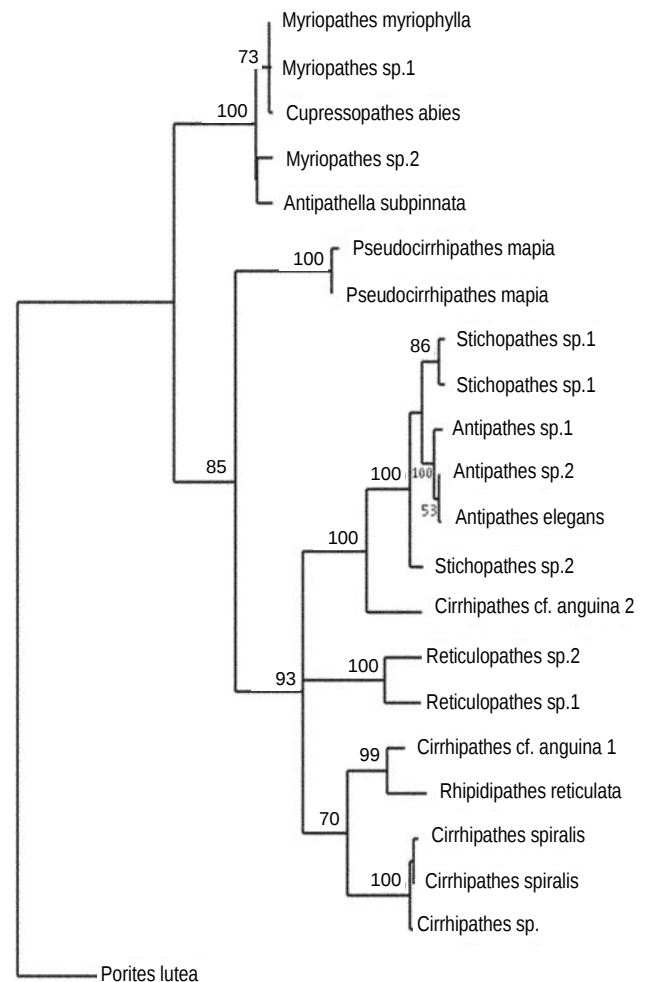


Figure 2. Phylogenetic tree obtained by Neighbor-Joining method.

Discussion

The results demonstrated that ITS genes are able to give information regarding biodiversity of black coral lived in around Manado. These results are interesting because they represent the opportunity to set, with the help of an alternative method, the value of the traditional taxonomy based on morphological characters. The data obtained from this analysis support the difference among the families Antipathidae, Aphanipathidae and Myriopathidae with the first two more closely related. The family of Myriopathidae and Antipathidae are genetically divergent from one another and obviously are not considered as sister taxa. The degree of genetic variation differs inside the families Antipathidae and Myriopathidae. In the Myriopathidae, the analysis by means of DNA sequences is not able to separate the

species of *Myriopathes* and *Cupressopathes* and a clear difference is detected only with the Mediterranean *Antipathella subpinnata*. Particularly the similarity among the species of *Cupressopathes* and *Myriopathes* could suggest the possibility of a reticulate evolution due to hybridization among these species (Diekmann et al., 2001; van Oppen et al., 2002). Hybridization among these species affects the similarity as shown both in genetic and morphological characters. The degree of hybridization of these genera might be very high since they share many ecological and reproductive traits. Morphologically similar species frequently live in close proximity to each another and have overlapping reproductive periods, thus opportunities for interspecific encounters among gametes. This hypothesis could clarify the high range of morphological variability of the colonies (size, height, thickness, branching) of the specimens "*Cupressopathes*-like".

Although the genetic distance among the species of Myriopathidae is not able to differentiate among them, it could be identified in the tree a group of new species or at least a new genus of *Myriopathes*-like antipatharians. When it is compared to the morphological data, the description confirms the existence of either new species or new genus of Myriopathidae.

ITS sequences data subdivided the Antipathidae cluster into different clades. The first clade comprises *Antipathes* sp.1, *Antipathes* sp.2, and *Stichopathes*. The second clade comprises the genus *Cirripathes*, while the last clade includes *Reticulopathes*. This present study has also shown that *Cirripathes* cf. *anguina* and *Cirripathes spiralis* are not clustered in a monophyletic group which indicates that placement of these two species need to be re-examined.

The separation of *Rhipidipathes reticulata* from the *Cirripathes* and *Antipathes* groups supports, although with low bootstrap values, the recent establishment (Opresko, 2004), based on polyp morphology and on the size and shape of spines, of the family Aphanipathidae. The agreement between the genetic variability and spine morphology, suggests that this morphological character is largely independent from the environmental cues and therefore particularly suitable for a diagnostic verification. To some extents, the ITS region of rDNA provides a great systematic resolution for Antipatharians and it's useful to distinguish among high taxa of the order. Further analysis of the data set supports the establishment of new genera and species. At the tip of the tree, *Pseudocirripathes mapia*, as a new undescribed species, is significantly different from the other groups.

CONCLUSION

The study showed that gene of ITS are able to distinguished a variety among three families of black

coral (Antipatharia) collected from the Bunaken Marine Park (Manado Sea, Indonesia). A Significant species-specific signal has been detected among the families Antipathidae and Aphanipathidae, even studies on different species of the last were needs to be clearly interpreted. The new species *Pseudocirripathes mapia*, the new genus *Reticulopathes*, and possibly a new taxon of the family Myriopathidae have been recognized based on ITS sequence data.

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