FACULTAD DE CIENCIAS VETERINARIAS Y BIOLÓGICAS

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"DNA BARCODING OF FIVE BENTHIC INVERTEBRATE TAXA FROM THE MACKELLAR COVE OF THE ANTARCTIC PENINSULA"

Trabajo de Investigación para optar el grado académico de:

Bachiller en Biología Marina

Presentado por:

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ACTA DE SUSTENTACIÓN

Lima, 20 de abril del 2020

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"DNA barcoding of five benthic invertebrate taxa from the Mackellar Cove of the Antarctic Peninsula"

Presentado por la estudiante:

- **Norma Mároli Merino Valencia**

Para optar el grado de Bachiller en **Biología Marina**

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- **Mg. Giovanna Sotil Caycho**

Luego de haber evaluado el trabajo de investigación y evaluado el desempeño de la estudiante en la sustentación, concluyen de manera unánime (X) / por mayoría simple ( ) calificar a:

Norma Maroli Merino Valencia			Nota (escrito): Dieciséis
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Los miembros del jurado firman en señal de conformidad.

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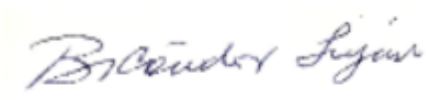

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DNA barcoding of five benthic invertebrate taxa from the Mackellar Cove of the Antarctic Peninsula

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Abstract

The Antarctic ecosystem presents benthic organisms with unique spatial and demographic distribution and exceptional adaptations to extreme conditions; however, the genetic diversity it hosts is little known. In order to characterize the molecular diversity of 25 benthic invertebrates collected in the Mackellar Cove during the Peruvian expedition ANTAR XXIV-2017, we analyzed nucleotide sequences obtained from the mitochondrial genes COI, ARNr 16S, and nuclear ARNr 18S and 28S (for molluscs). A morphological description of the 5 taxa between molluscs, bryozoans, polychaetes and crustaceans was made. The nucleotide sequences were recorded in open access databases (BOLD and GenBank) as *Margarella antarctica* (A1-A5), *Nacella concinna* (A11-A15), *Nematoflustra flagellata* (A16-A20), *Amphitrite kerguelensis* (A21-A25) and *Euphausia superba* (A26-A30).

Keywords: Benthos, COI, 16S, 18S, 28S, nucleotide diversity

INTRODUCTION

The Antarctic ecosystem, one of the most pristine ones in the world, contains high and endemic biodiversity, and a particular spatial and demographic distribution for benthic organisms (Ambroso, Salazar, Gili & Zapata, 2018). Since the first expeditions, species have been added to an inventory of invertebrates with a taxonomy based only on morphology. However, nowadays is easier to gain access to molecular techniques to evaluate this diversity at levels that were unattainable years ago (Stevens & Hogg 2006).

Lately, molecular markers have been used as great complementary tools to study intra and interspecific diversity in different taxonomic groups (Hebert, Cywinska, Ball & deWaard, 2003; Hebert & Gregory, 2005). The development of DNA barcoding has encouraged scientists to also apply molecular phylogenetic criteria, in both aquatic and terrestrial ecosystems, for ecological and systematic studies of biodiversity (Valentini *et al.* 2009). Therefore, this tool has been significantly useful to identify species which taxa are difficult to diagnose based only in morphology (Kerr *et al*. 2007).

Species identification studies based on nucleotide sequences of certain standardized regions of DNA are essential because they provide precise species discrimination. The mtDNA Cytochrome Oxidase I gene (COI), for example, usually shows levels of great interspecific divergence that leads to an accurate identification (Hebert *et al.* 2003). Until 2009, of all the Antarctic studies carried out, 74% of the genes used were rRNA 18S, 28S and 16S; 13% mtDNA COI; and the remaining 13%, rRNA 12S, ND1, CytB, ITS, Rhodopsin, H3 and rRNA 5.8S (Grant y Linse, 2009).

Researchers from all over the world have been studying the characteristics of the Antarctic ecosystem for more than 100 years (Echevarria & Paiva 2006); however, there's still an information gap in molecular studies, particularly for marine invertebrates (Grant y Linse, 2009). In fact, most of the Antarctic fauna is composed by these invertebrates (Clarke y Johnston, 2003). However, even now that molecular techniques were discovered as great tools to identify Antarctic marine invertebrates, the lack of available nucleotide sequence data in the public domain for comparison is very limiting (Webb et al., 2006).

DNA sequence records in public databases are still scarce for Antarctic fauna. Nevertheless, its development would provide better versatility applying this technology for specimens with difficult morphological identification (larval stages, cryptic species, phenotypic plasticity) or for community diversity studies based on environmental samples. In addition, knowing that this continent is the most isolated one with extreme living conditions, there's a growing interest in characterizing the genetic diversity of this ecosystem's organisms because of their exceptional adaptations to inhospitable and cold environments (Izaguirre & Mataloni, 2000). Consequently, creating a record of their genetic diversity would not only help complement taxonomic and ecological studies, but also contribute to measure the impact of climate change events and seek for future biotechnological applications.

The present study was made to molecularly characterize the five benthic invertebrate taxa collected in the Mackellar Cove, Antarctic Peninsula, during the Peruvian expedition ANTAR XXIV (2017), using sequences of the mitochondrial genes cytochrome oxidase subunit I (COI) and RNA 16S, and the nuclear rRNA minor subunit 18s. In addition, intraspecific nucleotide diversity was evaluated, comparing different markers. Finally, all sequences were recorded in the BOLD Systems database (http://www.boldsystems.org/) and GenBank (http://www.ncbi.nlm.nih.gov/).

MATERIALS AND METHODS

Sampling

Benthic specimens were collected during the Peruvian ANTAR XXIV expedition, in 2017, from Mackellar Cove (**Table 1**). Five taxa were preliminary morphologically identified and five individuals of each taxon were selected and fixed in ethanol (70%) for molecular analyses. A small piece of soft tissue from each individual was dissected and preserved in cryovials with ethanol 90%, and stored at 4°C. Material is available at the Colección Científica laboratory of the Universidad Científica del Sur.

Table 1. Details on the Mackellar Cove collection during the Peruvian expedition ANTAR XXIV – 2017.

Taxon	Station code	Latitude	Longitude	Depth (m)	Collection method
1 (A1-A5)	E11	-62,092622	-58,419861	15	Dredged (Dredge Van Veen 0.05m^2)
2 (A11-A15)	Intertidal	-62,103023	-58,460161	1	Manually
3 (A16-A20)	E11	-62,092622	-58,419861	15	Dredged (Dredge Van Veen 0.05m^2)
4 (A21-A25)	E02	-62,080806	-58,465083	30	Dredged (Dredge Van Veen 0.05m^2)
5 (A26-A30)	Stranded on beach	-62,091783	-58,468117	0	Manually

Morphological analysis

The species comparative remarks were examined for each taxon and each species' main diagnostic features were photographed using Leica Application Suite (LAS) stereomicroscopy. For the first taxon, the protoconch, teleoconch, umbilicus, operculum, and radula were examined and compared to previous descriptions from Lamy (1905) and Linse (2000). For the second taxon, shell outer and inner morphology was examined noting features as shell shape, coloration, ribs and marks. The mantle and cephalic tentacles were also photographed, as well as the radula. All the descriptive features were compared to González-Wevar and col. (2018) findings. For the third one, the colony and zooecium morphology, including operculum and vibraculum were observed, comparing them to the description from Waters (1904) and Hayward (1995). For the fourth one, the thoracic segments, cephalic lobes and tentacles, branchiae, and uncini were examined, comparing them to Hartman (1966) description. For the last taxon, the morpholical features observed were eyes shape and lappet, peduncle of the first antenna, rostrum, carapace shape, and mandibular palp were examined. All the observed features were compared to descriptions from Baker, Boden, and Brinton (1990), Bargmann (1937), John (1936), and Brinton, Ohman, Townsend, Knight, and Bridgeman (2000).

Molecular analysis (DNA extraction, PCR and sequencing)

DNA was extracted using CTAB buffer lysis, based on Sambrook et al. (1989). DNA was resuspended in nuclease-free water and quantified using a NanoDrop™ One spectrophotometer (ThermoFisher ScientificTM). Mitochondrial cytochrome c oxidase subunit I (COI), mitochondrial 16S rRNA, SSU 18S rRNA and 28S rRNA genes were amplified in a ProFlex PCR System (Applied Biosystems), using HotStartTaq Plus Master Mix (QIAGEN), with final concentrations of 1 - 2 ng/µl of genomic DNA, 0.1 - 0.2 µM of each primer and 1.5 - 2.25 mM of MgCl2. Primer sequences and PCR thermal cycling conditions are detailed in **Table 2**.

Table 2. List of primers and PCR conditions. (*) primers used only for sequencing COI gene amplified. Gene, primer name, primer sequence (5'→3'), annealing temperature (ºC), primer reference.

Gene	Primer name	Primer sequence (5'→3')	PCR cycle conditions	~Amplicon length (bp)	Reference
COI	ZplankF1_t1 ZplankR1_t1	tgtaaaacgacggccagtTCTASWAATCATAARGATATTGG caggaaacagctatgacTTCAGGRTGRCCRAARAATCA	1X: 95°Cx5min, 38X: 95°Cx40s-45°Cx1min-72°Cx1min, 1X: 72°Cx7min	700	Prosser et al. (2013)
	*M13F *M13R	TGTAAAACGACGGCCAGT CAGGAAACAGCTATGAC			Messing (1983)
16S	16Sar 16Sbr	CGCCTGTTTATCAAAAACAT CCGGTCTGAACTCAGATCACGT	1X: 95°Cx5min, 38X: 95°Cx40s-50°Cx40s-72°Cx40s, 1X: 72°Cx7min	550	Palumbi et al. (1996)
18S	SSU-F SSU-R	AACCTGGTTGATCCTGCCAGT TGATCCTTCTGCAGGTTCACCTAC	1X: 95°Cx5min, 35X: 95°Cx30s-52°Cx1min-72°Cx2min, 1X: 72°Cx5min	1800	Medlin et al. (1988)
28S	LSU ECD2*R	TAGGTCGACCCGCTGAAYTTAAGCA CCTTGGTCCGTGTTTCAAGACGGG	1X: 95°Cx5min, 35X: 94°Cx1min-54°Cx1min-72°Cx1min, 1X: 72°Cx5min	900	Littlewood et al. (2001)

All PCR products were evaluated by electrophoresis in 1% agarose gels with TBE buffer. Products amplified with the expected size were purified using the AccuPrepPCR Purification kit (Bioneer) and sequenced in both directions on an ABI 3500 Genetic Analyzer. Sequences were edited and then aligned with MUSCLE (Edgar, 2004), with default parameters, and trimmed to the same length for further comparisons within dataset. Consensus sequences of each sample were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) from the NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The percentage considered for a valid match was more than 98%. All sequences were registered in BOLD under the Project Barcoding ANTAR Peruvian Expedition, and in GenBank database obtaining correspondent accession number **(Table 3).**

Genetic divergence

Genetic distances were calculated using Kimura-2-parameters (K2P) distance method in MEGA-X (Kumar et al. 2018). Inter-specific divergence was calculated considering the average of inter-specific distance (K2P distance) between species in each genus, with at least two species. Due to the very low nucleotide diversity observed in 16S and 18S in genus evaluated, COI and 28S genes were used for phylogenetic analysis. Maximum Likelihood method was evaluated in PhyML (Guindon et al., 2010) considering the best nucleotide model substitution/evolutionary model calculated in MEGA-X. Bootstrap was 1000 replicates.

RESULTS

MORPHOLOGICAL DESCRIPTION

Taxon 1: *Margarella antarctica* (Lamy, 1905)

Margarita antarctica (Lamy, 1905: 481, Fig.5); *Margarita antarctica* (Powell, 1960: 131; De Deambrosi 1969: 50)

The shell is small, wider than high, with a white surface without periostracum. It also shows a small white protoconch with 1.5 turns (Fig. 1A) and a teleoconch with spiral ribs in the first whorl and in half of the second one (Fig. 1E). The umbilicus is deep and open, and the aperture is large and oval; the corneous brownish operculum, with same size as the aperture, displays multispirals as well as a short growing edge (Fig. C). The radula is rhipidoglossate and bilaterally symmetric, with the formula ∞ : 5 : 1 : 5 : ∞, with transverse rows (Fig. 1G) and well-developed lateral, marginal and rachidian teeth. The lateral teeth are large with lateral wings in the outer margin. The rachidian tooth is deltoid, with triangulates lateral wings (Fig. 1I).

Our comparative remarks for *M. antarctica* seem to be the same as in Linse (2000), where the amount of protoconch turns, radula type and rachidian tooth are shown (Fig. 1B, 1H, 1J, respectively). In addition, the figures of the external morphology showing the open umbilicus, operculum type and shell characteristics are the same as seen in the original description's figures by Lamy (1905) (Fig. 1D, 1F).

The species name *Margarites antarctica* was proposed by Deambrosi based on the absence of the long basal projection on the internal part of the base; while *Margaritella*, Thiele's proposition, is a homonym of the genus of hexactinellid sponges *Margaritella* (Deambrosi, 1969; Thiele, 1891; Schmidt, 1880). Consequently, *Margarella* was proposed by Thiele (1893) as a replacement name (Zelaya, 2004). However, the presence of transverse rows with a small depression divided the group apart from *Margarites*, and placed under the name *Margarella antarctica* (Linse, 2000).

Taxon 2: *Nacella concinna* (Strebel, 1908)

Patella polaris (Hombron & Jacquinot, 1841: 191); *Nacella polaris* (Lamy, 1906: 10)

The thick shell is large, conical and raised dorsally, and non-translucent, laterally compressed in anterior part, and with an oval aperture. The apex is situated in this anterior part, approximately 30% of the shell's length. The surface shows weak primary and secondary radial ribs, as well as concentric growth lines (Fig. 2A). The shell's coloration varies between pale brown, dark brown, and grey. The shell's internal part is nacreous and dark bronzy (Fig. 2H). A series of one longer tentacle and three shorter ones alternate repetitively on the mantle tentacles (Fig. 2c). First lateral teeth located closely on the anterior edge of the basal plates are shown on the radula, in addition to a couple spoon-like cusps. While second lateral teeth are located wider spaced, with one spoon-like cusp and another bigger and broader one (Fig. 2E, 2G).

Nacella concinna seems to be a single genetic population in the maritime Antarctica but is visibly different from the one in South Georgia. The morphotypes were considered two different subspecies: *N. polaris polaris* and *N. polaris concinna* (Powell, 1951); however, genetic comparisons confirmed that the differences in shell height, shape and sculpture are due to phenotypic plasticity in one same species (González-Wevar et al., 2018). The figures shown in this study match the latest description for *N. concinna* in González-Wevar (2018) showing this species' shell type, tentacles and radula (Pag. 17, Fig. 8A-H).

Taxon 3: *Nematoflustra flagellata* (Waters, 1904)

Flustra flagellata (Waters, 1904: 27, pl. 2, fig. 1)

The colony is unilaminar (Fig. 3C, 3D) with dichotomously dividing fronds (Fig. 3A) with chitinized rhizoids. Autozooids show no spines, with an entirely membranous front. Avicularia vicarious, intercalated within autozooid rows. These autozooids are rectangular or linguiform. In addition, there's a vibracular chamber at the proximal end of each zoecium with very long vibraculae. The base of the vibraculum is unsymmetrical, and the central area shows several protuberances where muscles are attached, allowing movement in all directions. The amount of heterozooids, in a vibracularia shape, is almost the same as the autozooids. The operculum is thickly chitinized with an angular outline (Fig. 3E, 3G).

Waters (1904) placed the species under the name of *Flustra flagellata*, describing it as the only case of *Flustra* having vibracula. The comparative remarks for *N. flagellata* in our figures match the ones from the original description of *Flustra* in his finding (Fig. 1). Later, Moyano (1972) included *F. flagellata* in the new genus *Nematoflustra*, characterized by its unilaminar fronds and unique vibracularian heterozooids. Our photographed structures also show the same morphology drawn for *N. flagellata* in Hayward (1995: 72, fig. 63).

Taxon 4: *Amphitrite kerguelensis* (Mcintosh, 1876)

Terebella kerguelensis (Grube, 1877: 546)

The large body shows brown coloration, seventeen thoracic segments, and numerous abdominal setigerous segments (Fig. 4A). Four lobes are located on the cephalic region: two forward-facing and under the first branchiae, a ventral one, and a fan-shaped one that spreads ventrally from the base of the last branchiae (Fig. 4C). The long branchiae spring from three short trunks in each side (Fig. 4D). This species is characterized by the high dorsal collar on the fourth segment to which the posterior branchiae are attached.

Other descriptive remark is the presence of four well-developed flaps on the anterior segments, and the last one is the largest one, extending up to the side reaching the base of the third gill (Fig. 4C). The neuropodial chaetae are avicular, with a large fang surmounted by smaller teeth (Fig. 4E).

Both comparative remarks photographed are also described by McIntosh (1855) in the original description, showing, the same entire animal enlarged but partly encased in tube (pl. 49, fig. 1). The four cephalic lobes and the three pairs of branchiae trunks seen here are also described in Hartman (1966).

Taxon 5: *Euphausia superba* (Dana, 1850)

The eye is round and small to medium size. The first segment in the peduncle of the first antenna is wide and with a distal lappet that extends to part of the second segment; it's almost as wide as the second segment's base (Fig. 5A). A long lobe is also projected horizontally from the upper surface of the second segment onto the third one. The third segment shows a keel (Fig. 5C). The rostrum is short and rounded at the tip, extending until approximately the middle of the eye (Fig. 5G).

The carapace has cervical grooves in the antero-lateral part of the head, in both sides, behind each eye (Fig. 5E). The distal segment of the mandibular palp is very long, at least seven times longer than other Antarctic species (Fig. 5H). The thoracic legs are much longer than in other species of the same genus. The merus of the first five legs extend forward until the end of the first segment of the first antenna's peduncle. They are feathered with setae forming a filtering basket (Fig. 5I).

The comparative remarks found for *E. superba* match Baker, Boden, and Brinton (1990) description, where they presented figures of some descriptive structures such as the species round eyes, the presence of the distal wide lappet and the very long thoracic legs. This last characteristic was also pointed for *E. superba* by Bargmann (1937). The short rostrum was also described by John (1936) in figures, also mentioning that it may be a shorter in males than in females. The rest of comparative remarks mentioned in the present description were also presented by Brinton, Ohman, Townsend, Knight, and Bridgeman (2000).

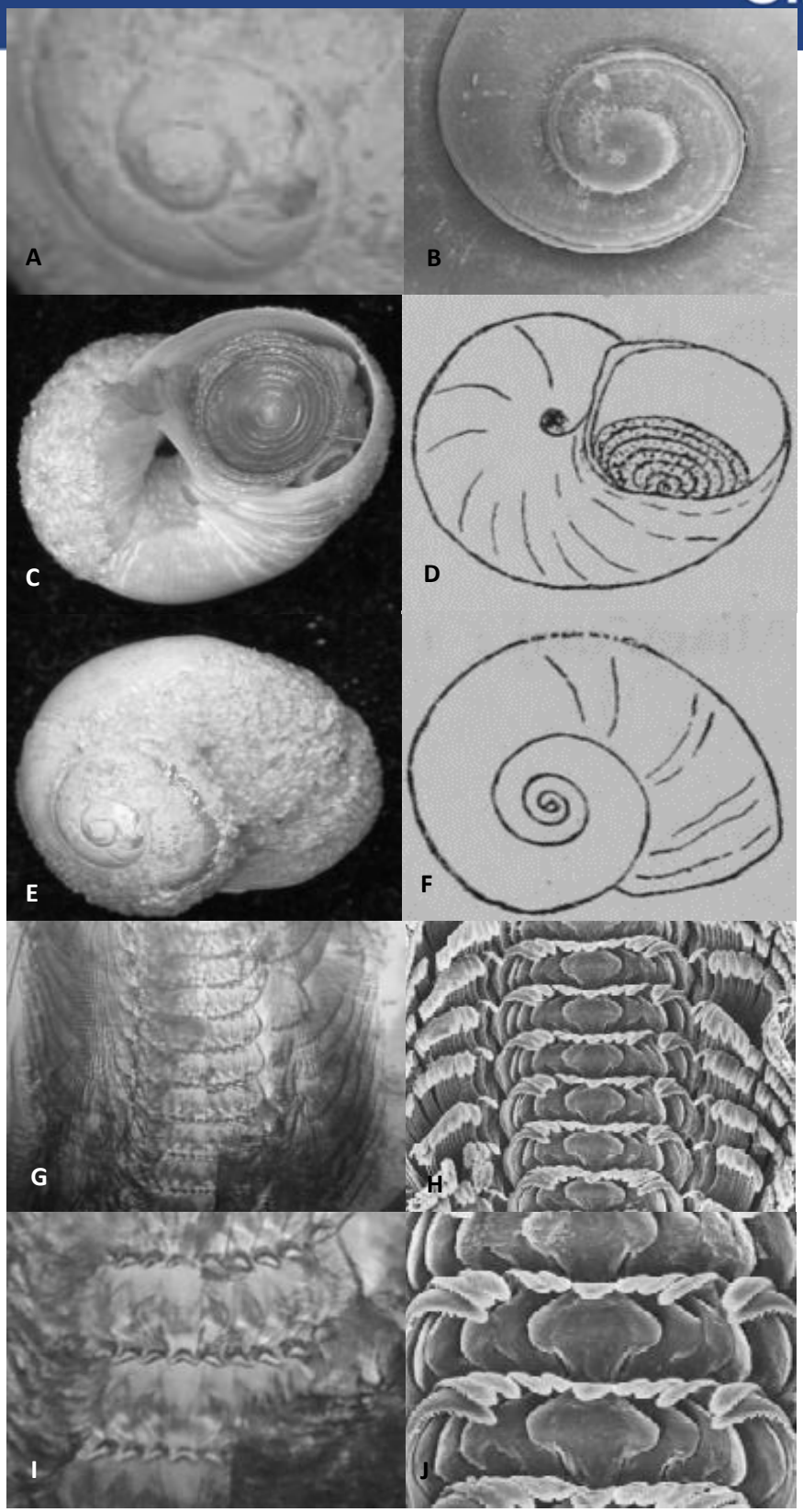

Figure 1. Morphological descriptive characters in *Margarella antarctica* A: protoconch, C: umbilicus and operculum, E: teleoconch whorls, G: radula, I: central complex and inner marginal teeth. D and F taken from Lamy (1905); B, H, and J, from Linse (2000).

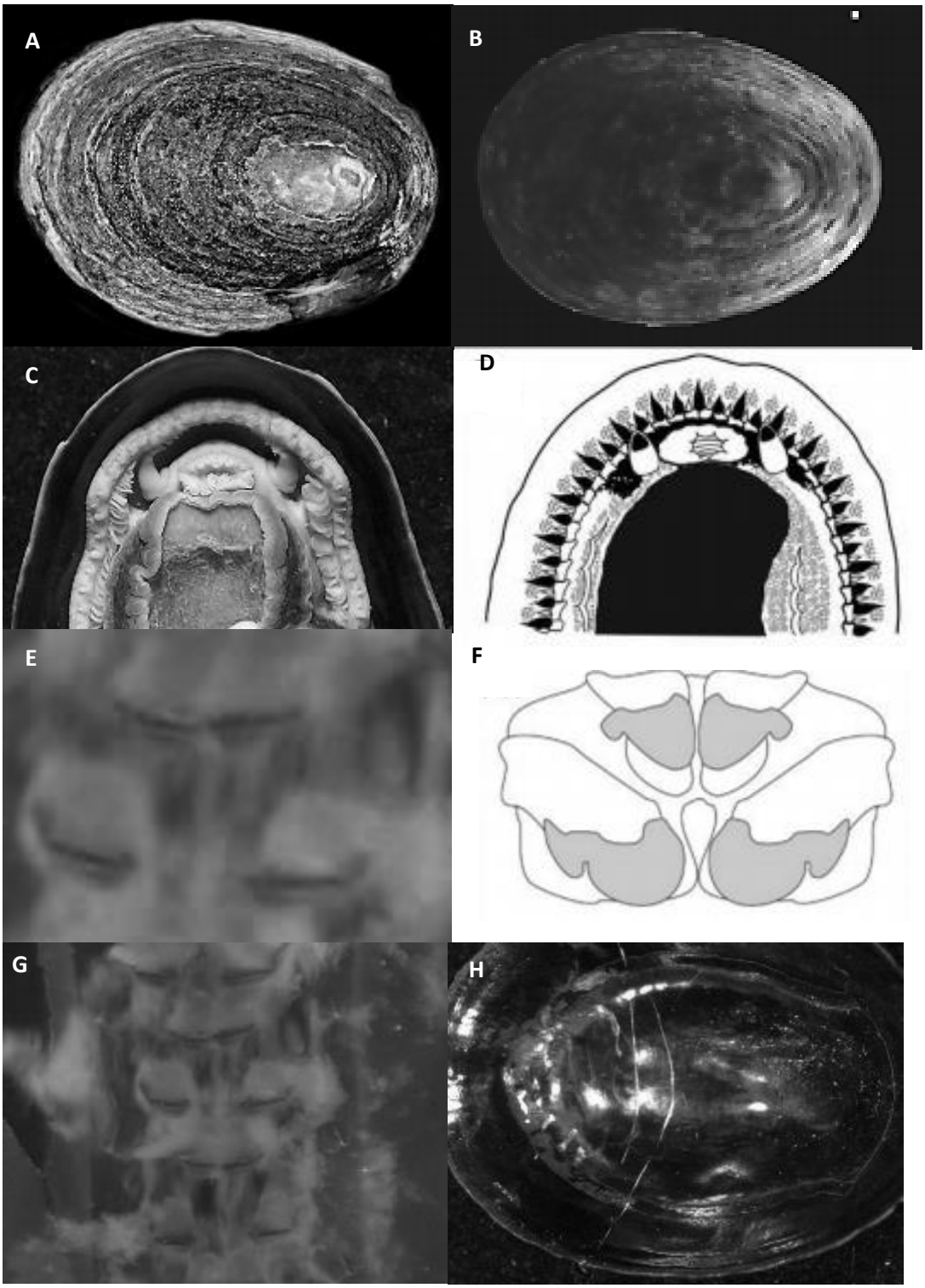

Figure 2. Morphological descriptive characters in *Nacella concinna* A: shell morphology, C: mantle and cephalic tentacles, E and G: radula, H: inner part of the shell. B, D and F taken from González-Wevar (2018).

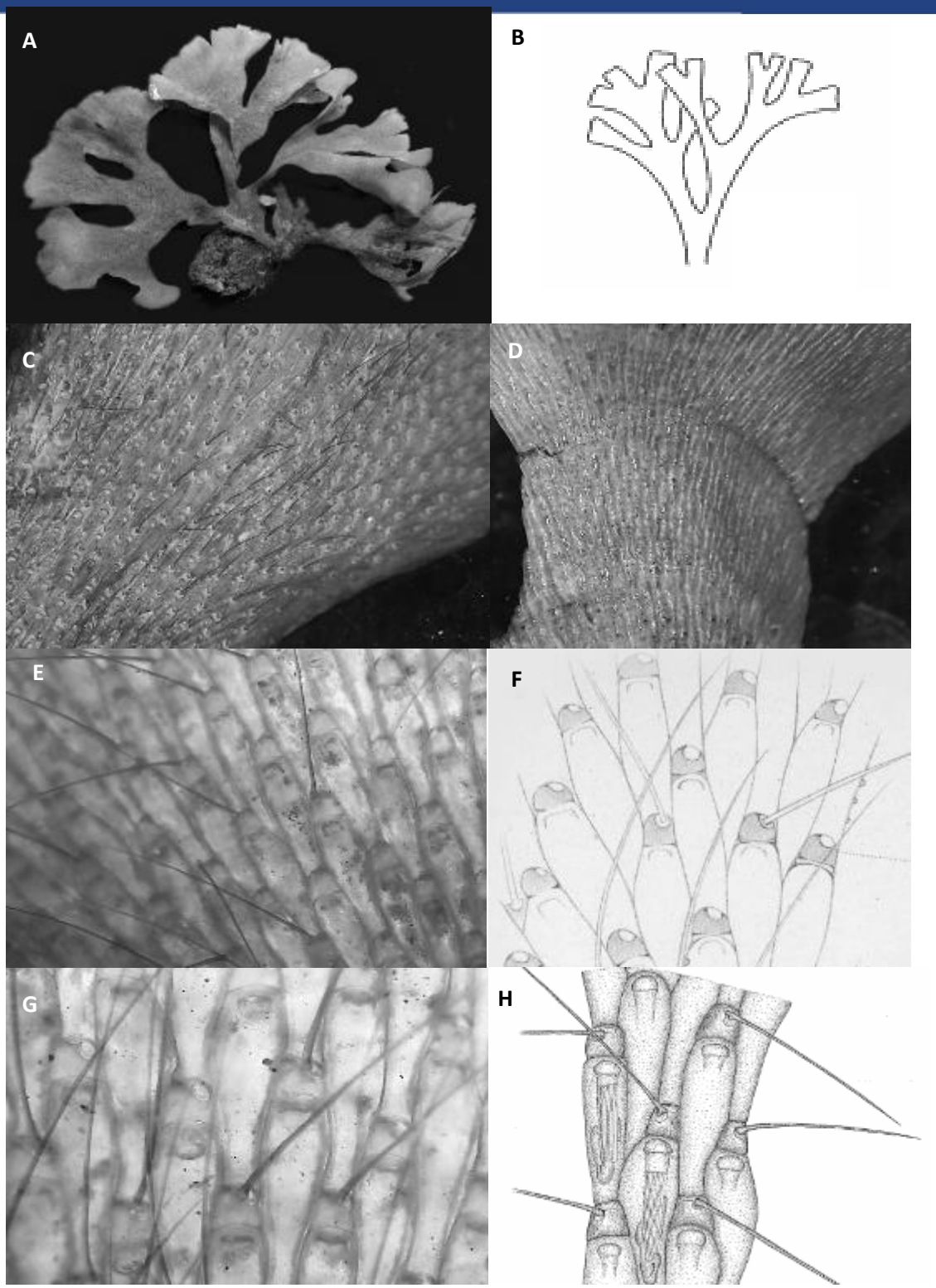

Figure 3. Morphological descriptive characters in *Nematoflustra flagellata* A: colony morphology, C: frond's front (with zooecium), D: frond's back (without zooecium). E and G: operculum and vibraculum. F taken from Waters (1904), B and H taken from Hayward (1995).

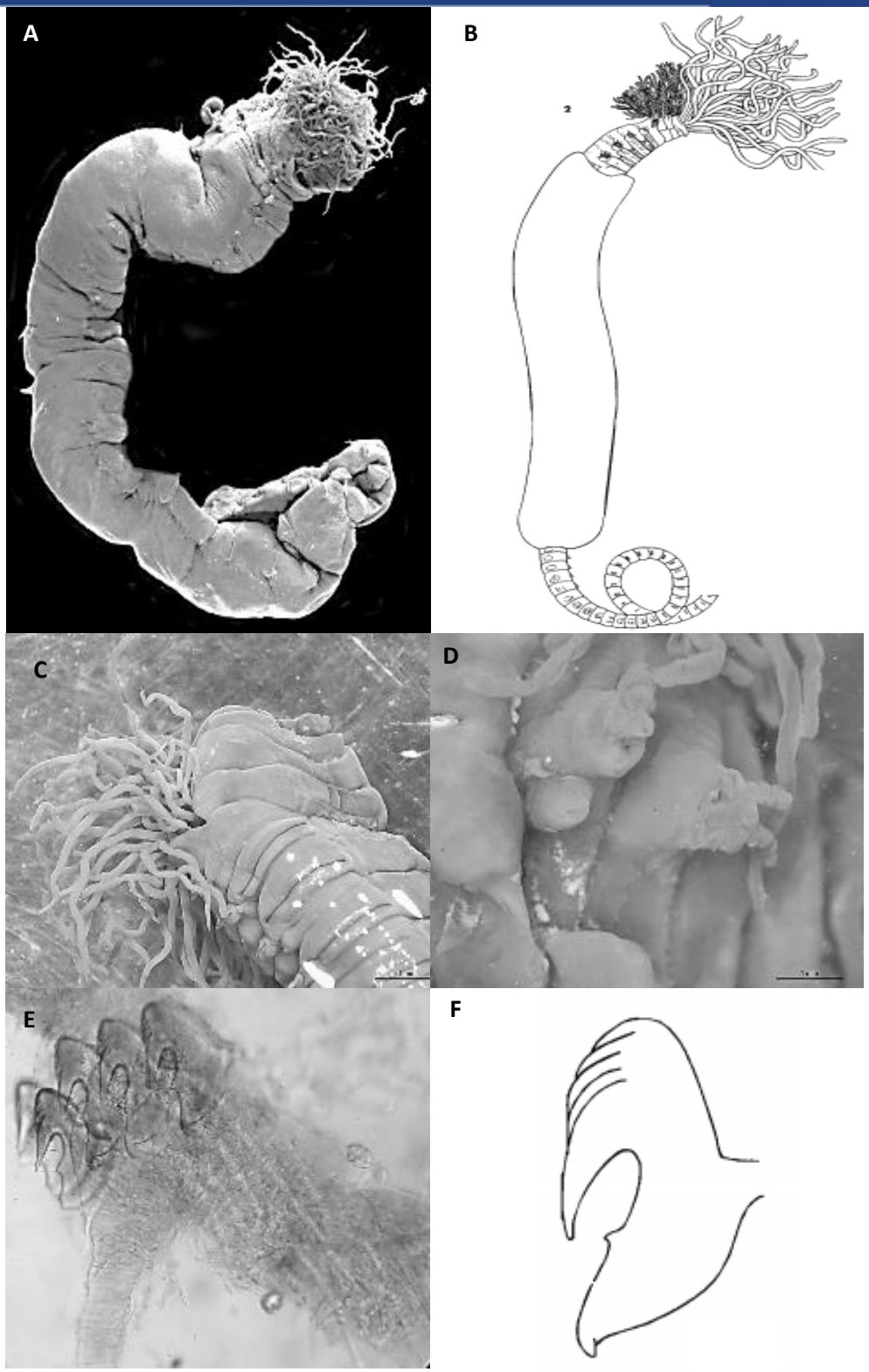

Figure 4. Morphological descriptive characters in Amphitrite kerguelensis. A: entire enlarged animal, C: cephalic lobes and tentacles, D: branchiae trunks, E: avicular uncini and long fang. B taken from MacIntosh (1885), and F, from Hartman (1966).

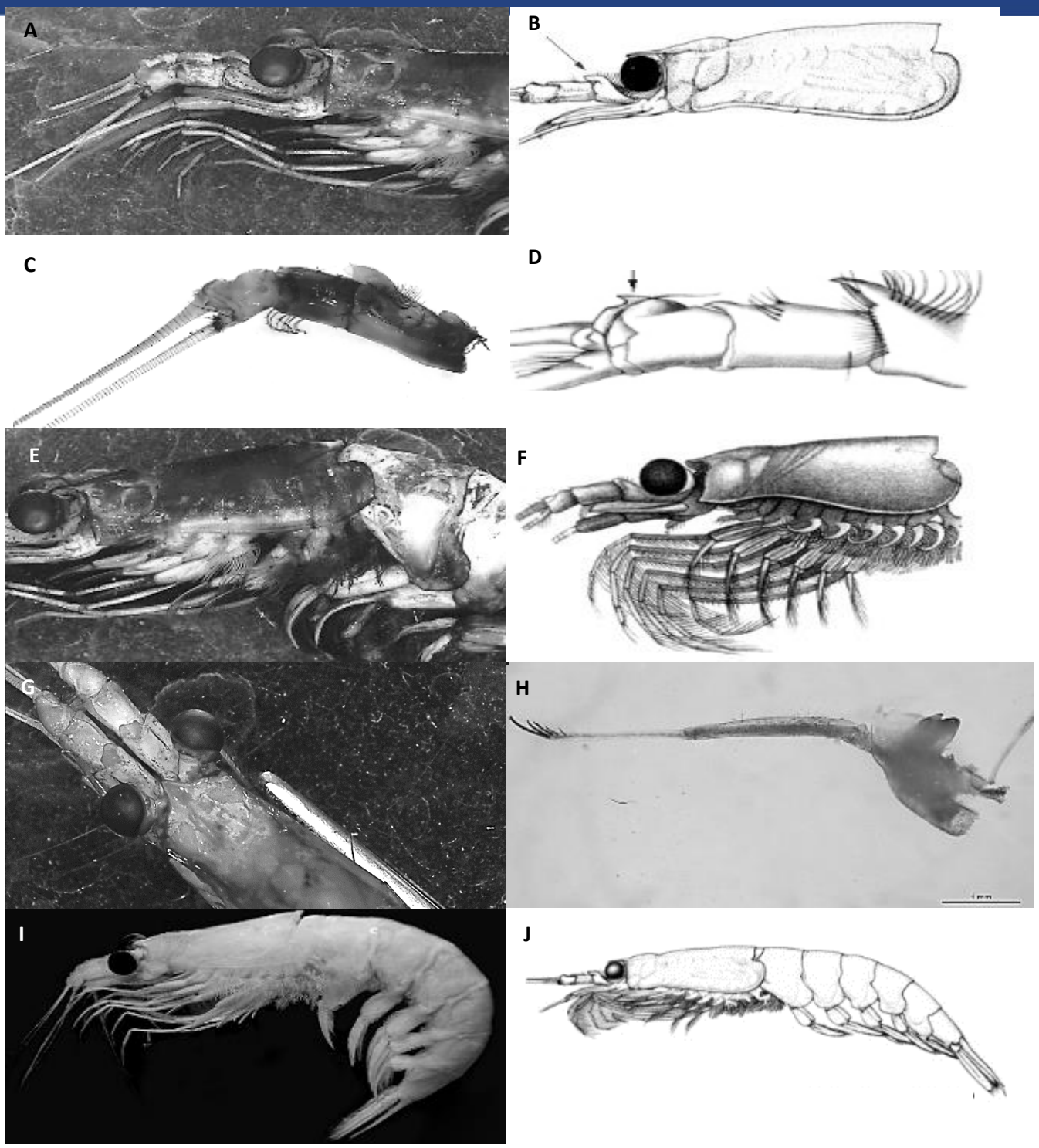

Figure 5. Morphological descriptive characters in *Euphausia superba.* A: eyes and lappet. C: keel on peduncle of first antenna. E: cervical groove. G: rostrum (dorsal view). H: mandibular palp. I: entire animal. B and J taken from Baker, Boden, and Brinton (1990), D from Hansen (1912), and F, from Bargmann (1937).

MOLECULAR ANALYSES

Molecular identification

The comparison of the obtained sequences using the COI gene with the ones from GenBank and BOLD-Systems databases allowed to reach the species level in three taxa. The second taxon was identified as *Nacella concinna* (>99%). The fourth taxon was identified as *Amphitrite kerguelensis* (>99%), and the last taxon was identified as *Euphausia superba* (>99%) (**Table 4**).

The rRNA 16S and 18S genes also identified the last taxon as *Euphausia superba* (100%); however, failed to successfully identify the other taxa to a species level. The homology percentage for the other sequences obtained through rRNA 16S were below the 98% due to the lack of previous nucleotide sequences for those species (**Table 4**). Even though the homology percentage was high (>98%) using the rRNA 18S gene, several sequences corresponding to different species had the same elevated percentages for one taxon. The region was identical for remote taxa, meaning that the gene was too conserved interspecifically for those remaining taxa.

The rRNA 28S gene, which was used complementarily for the identification of the gastropods' samples, identified the first taxon as *Margarella antarctica* (> 99%) and confirmed that the second one was *Nacella concinna* (>99%) (**Table 4**).

Intraspecific diversity

Low levels of intraspecific diversity were observed for the COI gene, the sequences obtained for the first taxon being 100% identical; 5 variable sites (4 transitions, 1 transversion) for *Nacella concinna* with a maximum intraspecific distance of 0.005; and 3 variable sites of the transition type in *Amphitrite kerguelensis* (distance 0 to 0.005). The greatest diversity value was found for *Euphausia superba*, with 16 variable sites (13 transitions and 4 transversions) and an intraspecific distance of 0.003 to 0.023 **(Table 5).**

Gene rRNA 16S showed no intraspecific diversity between the identified groups, obtaining 5 sequences 100% identical for each taxon analyzed. Low levels of intraspecific diversity were also observed for the rRNA 18S. Sequences obtained for *Amphitrite kerguelensis* as well as for *Euphausia superba* were 100% identical, and for both the first and fourth taxon, the intraspecific distance was 0 to 0.00058 (1 transversion and 1 transition, respectively) **(Table 6).**

Clustering analyses

Clustering for the first taxon (A1-A5) using the mitochondrial cytochrome oxidase I gene (COI) though Maximum Likelihood (Tamura-Nei with Gamma distribution and Invariable sites) showed the sequences in a single clade, with more than 99% robustness, differentiated from the rest. The clustering also shows organisms from the genus *Calliostoma*, genus from the same family Calliostomatidae. *Photinula coerulescens*, the species with the higher homology percentage for COI (**Table 4**), wasn't included in the clustering. Many of the nucleotide sequences present in BoldSystems are not accessible because the data has not been released yet, and this was the case for *P. coerluscens*, where the sequences were only available for Blast but not for downloads for further analyses (**Fig. 6)**. A single clade for *Margarella antarctica* was also seen with the rRNA 28S

sequences. This clade not only included the obtained sequences but also another *Margarella antarctica* recorded previously in the database, confirming the identity of the specimens. The sequences are also grouped with two other species from the same genus (**Fig. 7).**

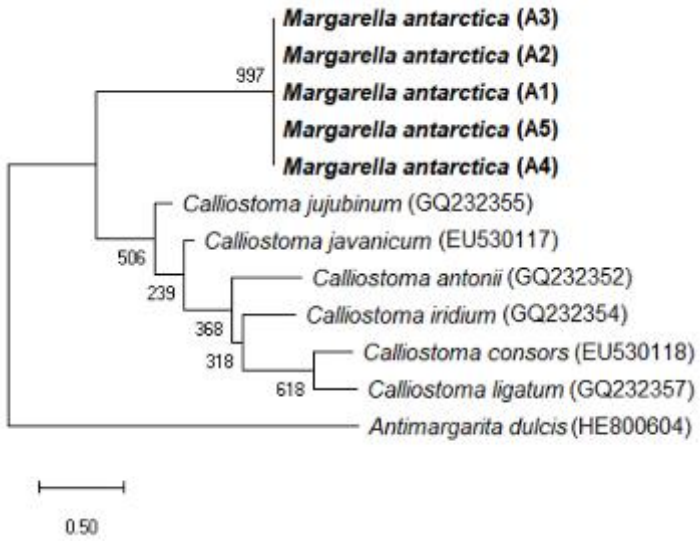

Figure 6. Maximum likelihood tree (T93+G+I) for COI in *Margarella antarctica*.

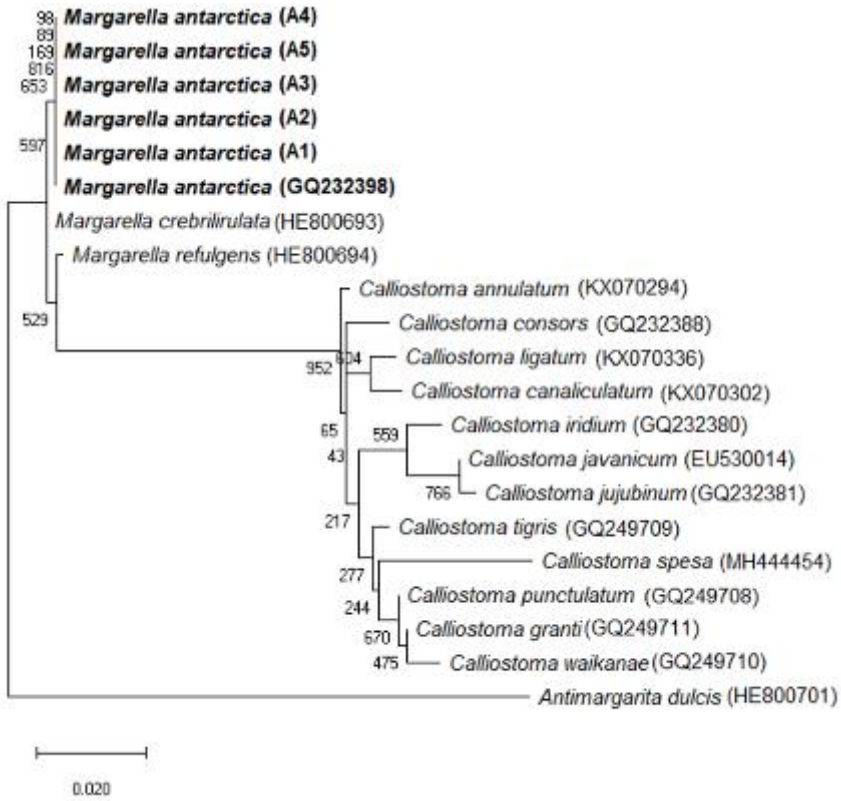

Figure 7. Maximum likelihood tree (T93+G+I) for 28S in *Margarella antarctica*.

The second taxon, through the mtDNA COI reflects a single clade along with other *Nacella concinna* sequences recorded in the GenBank and a subgroup of *Nacella delesserti* within the same clade with a high percentage of bootstrap. The percentage of identity for this group turned out to be high for *Nacella concinna*, but also for *Nacella delesserti* (<98%) (**Fig. 8**). However, clustering with rRNA 28S reflects differentiated subgroups, where second and third taxon sequences are found in a distinct clade from other sequences of *Nacella concinna* as well as of *Nacella delesserti* **(Fig. 9)**.

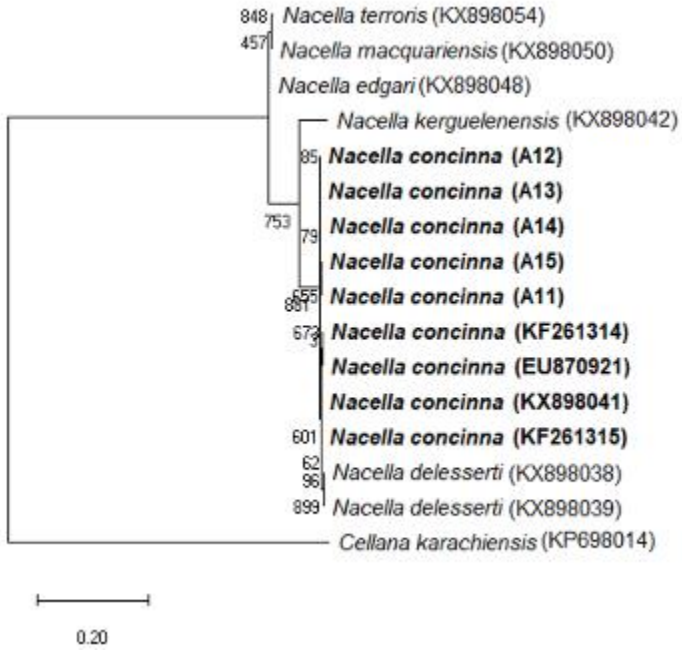

Figure 8. Maximum likelihood tree (HYK+G) for COI in *Nacella concinna.*

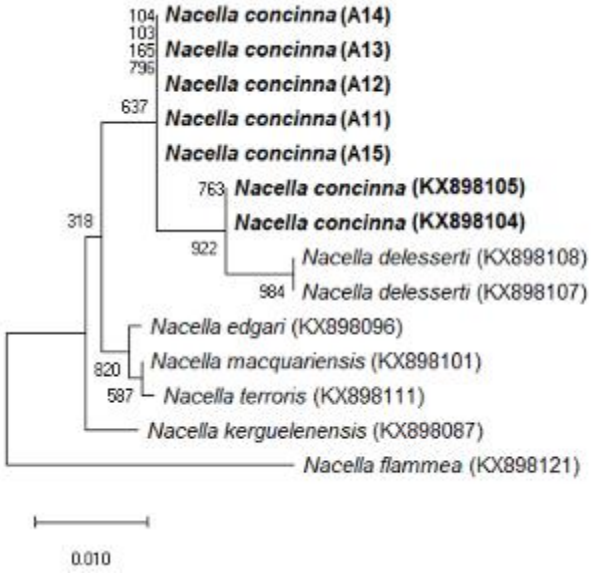

Figure 9. Maximum likelihood tree (HYK+G) for 28S in *Nacella concinna.*

Nematoflustra flagellata sequences (fourth taxon) formed a single clade with high percentage of robustness (>99%). However, due to the lack of sequences records for this species, the obtained sequences failed to group with any other related species (**Fig. 10**).

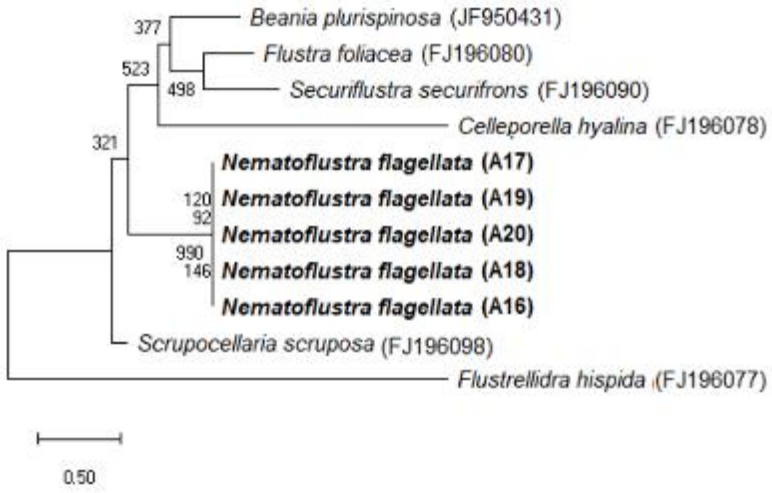

Figure 10. Maximum likelihood tree (HYK+G) for COI in *Nematoflustra flagellata*.

The clustering for *Amphitrite kerguelensis* showed a single clade, with high percentage of robustness (>99%), differentiated from those of the same genus. Our obtained sequences had a significant homology percentage with another previously recorded sequence belonging to *A. kerguelensis* (**Table 4**). However, these previous record in Bold Systems database is not yet released for public access. Therefore, it wasn't available for cluster analyses of any other further study (**Fig. 11**).

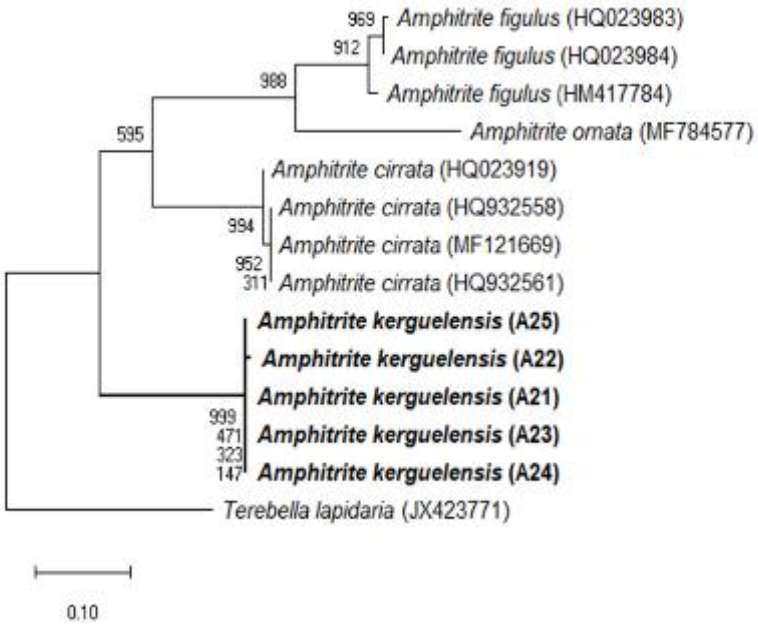

Figure 11. Maximum likelihood tree (T93+I) for COI in *Amphitrite kerguelensis*.

Table 3. Accession codes of nucleotide sequence records for COI (both GenBank and BoldSystems), 16S, 18S and 28S.

Code	Species	Molecular markers				
		COI		16S	18S	28S
		GenBank	Bold Systems			
A1	*Margarella antarctica*	MN941957	ANPE001-19	MN941904	MN941922	MN941932
A2	*Margarella antarctica*	MN941961	ANPE002-19	MN941908	MN941926	MN941936
A3	*Margarella antarctica*	MN941960	ANPE003-19	MN941907	MN941925	MN941935
A4	*Margarella antarctica*	MN941959	ANPE004-19	MN941906	MN941924	MN941934
A5	*Margarella antarctica*	MN941958	ANPE005-19	MN941905	MN941923	MN941933
A11	*Nacella concinna*	MN941966	ANPE011-19	-	-	MN941941
A12	*Nacella concinna*	MN941965	ANPE012-19	-	-	MN941940
A13	*Nacella concinna*	MN941964	ANPE013-19	-	-	MN941939
A14	*Nacella concinna*	MN941963	ANPE014-19	-	-	MN941938
A15	*Nacella concinna*	MN941962	ANPE015-19	-	-	MN941937
A16	*Nematoflustra flagellata*	MN941975	ANPE016-19	MN941913	MN941931	-
A17	*Nematoflustra flagellata*	MN941974	ANPE017-19	MN941912	MN941930	-
A18	*Nematoflustra flagellata*	MN941973	ANPE018-19	MN941911	MN941929	-
A19	*Nematoflustra flagellata*	MN941972	ANPE019-19	MN941910	MN941928	-
A20	*Nematoflustra flagellata*	MN941971	ANPE020-19	MN941909	MN941927	-
A21	*Amphitrite kerguelensis*	MN941947	ANPE021-19	-	-	-
A22	*Amphitrite kerguelensis*	MN941951	ANPE022-19	-	MN941916	-
A23	*Amphitrite kerguelensis*	MN941950	ANPE023-19	-	MN941915	-
A24	*Amphitrite kerguelensis*	MN941949	ANPE024-19	-	-	-
A25	*Amphitrite kerguelensis*	MN941948	ANPE025-19	-	MN941914	-
A26	*Euphausia superba*	MN941956	ANPE026-19	MN941903	MN941921	-
A27	*Euphausia superba*	MN941955	ANPE027-19	MN941902	MN941920	-
A28	*Euphausia superba*	MN941954	ANPE028-19	MN941901	MN941919	-
A29	*Euphausia superba*	MN941953	ANPE029-19	MN941900	MN941918	-
A30	*Euphausia superba*	MN941952	ANPE030-19	MN941899	MN941917	-

DISCUSSION

The lack of nucleotide sequence records in open access databases, such as NCBI and Bold Systems, difficults species identification reflected in the low homology percentage ("Identity"). Levels below 98% are insufficient to allocate the scientific name of the species to the analyzed sample. Here, the percentage of species identified with the COI gene is higher than 16S and 18S genes, showing the

progress and increase of nucleotide sequence information with that marker recently for Antarctica. This greater number of sequences recorded with the Cytochrome oxidase I may be due to current large-scale projects, such as the Barcode initiative of Life (http://www.barcodinglife.org/) of Bold Systems. This marker provides important information about Antarctic marine genetic diversity. For example, it has facilitated the discovery of numerous new and complex species of cryptic species that are commonly found as part of the Antarctic marine fauna (Grant, James & Steinke, 2010).

Since rRNA 18S sequences can be obtained from poorly preserved specimens, they could be very useful for higher taxonomic levels (Heimeier, Lavery and Sewell; 2010). However, in this case it didn't provide us the level of desired discrimination for species identification. In several taxa, the percentages of identity were high and similar among the match options, but they represented organisms from taxonomically remote groups. Therefore, the fact that this gene is very conserved did not allow identification at the species level for the analyzed samples.

Therefore, 18S and 28S nuclear genes could be used to elucidate the phylogenetics of higher taxa (Dreyer and Wägele, 2002; Taylor et al., 2007; Vonnemann et al., 2005). While mitochondrial genes COI and 16S could be used for studies within the same genus or between different genera (Lörz and Held, 2004; Balzer et al., 2000). However, it is necessary to complement the information with genetic data from markers other than COI since it doesn't work for all groups of organisms. For example, foraminifera can't be identified using cytochrome oxidase I, but regions within the 18S rRNA can be used to identify up to the species level (Pawlowski et al., 2003).

Additionally, analyzing samples from very different taxa, as in this case, require different sets of primers. As seen in the results, the samples were not sequenced with all molecular markers, but the products were sequenced depending on what set amplified successfully. Webb et al. (2006) used several sets of pairs of primers, including those for 16S and COI genes, to sequence and obtain matches for Antarctic larvae.

The first taxon wasn't identified at a species level using the COI, 16S, or 18S obtained sequences compared to public databases due to the lack of sequences recorded for this group. Most recent Antarctic molecular studies are based on phylogeny of mollusks together with malacrostrac crustaceans (Brasier et al., 2016). However, even in these groups, only few families are completely studied, or have recorded and released sequences. Although there are several projects interested in these groups from years ago, they mainly focus on one or two species.

González-Wevar et al. (2018) identified *Nacella concinna* and *Nacella delesserti* as two different species through morphological and molecular methods (COI + 28S), where *N. concinna* (Strebell, 1908) is distributed by the Maritime Antarctic, including rocky ecosystems without ice of the Antarctic Peninsula, as well as the South Shetland Islands, South Georgia, South Orkney, Bouvet, Elephant Island, Seymour Island, Paulet Island, Wander Island, Anvers Island and Petermann Island. While *N. delesserti* is distributed by the Marion Islands, Prince Edward and Crozet, far away from the study area. Additionally, they reported that *N. concinna* has phenotypic plasticity in the shape and height of its shell, which greatly difficults its correct identification. It is known to be distributed in the Maritime Antarctic as a single genetic population, but it differs morphologically from those distributed in South Georgia (González-Wevar et al., 2013). Also, phylogenetic reconstructions show that both Antarctic limpets are sister species whose separation occurred approximately 0.35 million years ago (González-Wevar et al., 2017).

Therefore, all the evidence from the COI and 28S marker, together with the clustering analysis, percentage of homology, morphological description, and distribution indicate that this species is *Nacella concinna*. However, there are other markers that could be used as cytochrome b, repeated intersimple sequences (De Aranzamendi, Gardenal, Martin & Bastida, 2009), 12s and 16s (Goldstein, Gemmel & Schiel, 2006), or rRNA 28s (González-Wevar et al., 2018). These genes would be effective for a higher level of taxonomic resolution, as has been used in other molecular studies of characterization of the genus *Nacella*.

There's a great number of Antarctic cryptic species, especially for the marine invertebrate group. For example, organisms such as bryozoans, which are quite cryptic, have a high degree of difficulty for morphological identification. Not being able to detect cryptic species not only reduces the degree of real biodiversity in the area, but also makes it harder to study ecological relationships with other organisms and speciation patterns (Dodson et al., 2003; Knowlton, 2000). To know precisely the species richness and the taxonomy in Antarctic environments, molecular techniques such as phylogenetics and bar codes can be used. Gómez and col. (2007) were able to accurately identify species from the cryptic complex of the bryozoan *Celleporella hyalina*, using the DNA barcode. Also, Porter and Hayward (2004) managed to characterize species of the genus *Alcyonidium* (Antarctic bryozoan), using morphological, reproductive and molecular methods. Although interest in these organisms is rising due to their antimicrobial activity and ecological importance in the marine benthos, the records of Antarctic sequences for this group are still minimal.

Polychaetes are also included within the documented cryptic species for Antarctica along with some echinoderms and nemerteans (Janosik & Halanych, 2010). Polychaetes represent one of the most dominant taxa of the Antarctic benthic communities conforming about 70% of the macrofauna (Gambi, Castelli & Guizzardi, 1997) with high levels of abundance. The first project that used the DNA barcode to identify polychaetes was made by Carr and col. (2011) where 1876 specimens from Alaska surroundings and the Arctic were sequenced. Also, Brasier and col. (2016) managed to record the sequences of 15 species of Antarctic polychaetes using the COI gene and 16S regions. These data suggest that the Antarctic benthos have a species richness much greater than previously thought thanks to the identification of cryptic species that were previously overlooked. However, as seen in the present study, the sequences recorded for Antarctic species are still scarce for the great diversity that is now estimated.

Once a complete database for Antarctic species is available, accurate species detection techniques will be used and costs, currently considered as relatively expensive, will decrease significantly. During the last ten years, a molecular uprising in taxonomy occurred added to a stronger conventional taxonomic framework; however, there are still many species that can only be identified by few specialists (Webb, Barnes, Clark & Bowden, 2006). A molecular method needs to be strongly discriminatory to reach success as an identification tool; former DNA sequencing is essential to build reference collections with free access (Deagle et al., 2003, Hare et al., 2000).

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ANNEXES

Table 4. Molecular species identification obtained through mitochondrial markers (COI, 16S) and nuclear markers (18S, 28S) and compared to GenBank and BOLD Systems databases. bp=base pairs, ID=homology percentage.

Code	COI			16S			18S			28S		
	Species	bp	ID (%)	Species	bp	ID (%)	Species	bp	ID (%)	Species	bp	ID (%)
Taxa 1												
A1	*Photinula coerulescens*	658	81.19	*Margarella refulgens*	549	95.81	Trochidae	1763	99.43	*Margarella antarctica*	734	99.05
A2	*Photinula coerulescens*	658	81.19	*Margarella refulgens*	549	95.81	Trochidae	1763	99.20	*Margarella antarctica*	734	99.05
A3	*Photinula coerulescens*	658	81.19	*Margarella refulgens*	549	95.81	Trochidae	1763	99.49	*Margarella antarctica*	734	99.05
A4	*Photinula coerulescens*	659	81.13	*Margarella refulgens*	549	95.81	Trochidae	1763	99.38	*Margarella antarctica*	734	99.05
A5	*Photinula coerulescens*	658	81.44	*Margarella refulgens*	549	95.81	Trochidae	1763	99.49	*Margarella antarctica*	734	99.05
Taxa 2												
A11	*Nacella concinna*	658	99.85	-	-	-	-	-	-	*Nacella concinna*	860	99.04
A12	*Nacella concinna*	658	99.85	-	-	-	-	-	-	*Nacella concinna*	860	99.04
A13	*Nacella concinna*	658	99.85	-	-	-	-	-	-	*Nacella concinna*	860	99.04
A14	*Nacella concinna*	658	99.85	-	-	-	-	-	-	*Nacella concinna*	860	99.04
A15	*Nacella concinna*	658	99.85	-	-	-	-	-	-	*Nacella concinna*	860	99.04
Taxa 3												
A16	*Scrupocellaria scruposa*	658	81.40	*Beania plurispinosa*	514	83.91	*Callopora dumerilii*	1723	99.08	-	-	-
A17	*Scrupocellaria scruposa*	658	81.40	*Beania plurispinosa*	514	83.91	*Callopora dumerilii*	1723	99.08	-	-	-
A18	*Scrupocellaria scruposa*	658	81.40	*Beania plurispinosa*	514	83.91	*Callopora dumerilii*	1723	99.13	-	-	-
A19	*Scrupocellaria scruposa*	658	81.40	*Beania plurispinosa*	514	83.91	*Callopora dumerilii*	1723	99.14	-	-	-
A20	*Scrupocellaria scruposa*	658	81.40	*Beania plurispinosa*	514	83.91	*Callopora dumerilii*	1723	99.13	-	-	-
Taxa 4												
A21	*Amphitrite kerguelensis*	658	99.06	-	-	-	-	-	-	-	-	-
A22	*Amphitrite kerguelensis*	658	99.25	-	-	-	*Terebella lapidaria*	1720	99.02	-	-	-
A23	*Amphitrite kerguelensis*	658	99.06	-	-	-	*Terebella lapidaria*	1720	99.01	-	-	-

A24	*Amphitrite kerguelensis*	658	99.06	-	-	-	-	-	-	-	-	-
A25	*Amphitrite kerguelensis*	658	99.06	-	-	-	*Terebella lapidaria*	1720	99.01	-	-	-
Taxa 5												
A26	*Euphausia superba*	658	99.33	*Euphausia superba*	514	100	*Euphausia superba*	1735	100	-	-	-
A27	*Euphausia superba*	658	99.50	*Euphausia superba*	514	100	*Euphausia superba*	1735	100	-	-	-
A28	*Euphausia superba*	658	99.84	*Euphausia superba*	514	100	*Euphausia superba*	1735	100	-	-	-
A29	*Euphausia superba*	658	100	*Euphausia superba*	514	100	*Euphausia superba*	1735	100	-	-	-
A30	*Euphausia superba*	658	99.83	*Euphausia superba*	514	100	*Euphausia superba*	1735	99.94	-	-	-

Table 5. Intraspecific diversity of the analyzed samples with COI gene.

COI				
Code	Intraspecific distance	Variable sites	Substitution type	
			Transitions	Transversions
01 al 05	0	0	0	0
06 al 15	0 - 0.005	5	4	1
16 al 20	0	0	0	0
21 al 25	0 - 0.005	3	3	0
26 al 30	0.003 - 0.023	16	13	4

Table 6. Intraspecific diversity of the analyzed samples with 18S gene.

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18S				
Code	Intraspecific distance	Variable sites	Substitution type	
			Transitions	Transversions
01 al 05	0 - 0.00058	1	0	1
06 al 15	-	-	-	-
16 al 20	0 - 0.00058	1	1	0
21 al 25	0	0	0	0
26 al 30	0	0	0	0