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Genetic identity of two physonect siphonophores from Southern Ocean waters – the enigmatic taxon *Mica micula* and *Pyrostephos vanhoeffeni*

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Based on some coincident morphological characters and distribution, it was believed for a long time that Mica micula *was the post-larval stage of a species of* Bargmannia*, a genus having a very wide geographic distribution. Recent studies, however, have shown that it is much more likely to be the post-larval form of the physonect* Pyrostephos vanhoeffeni*, which is very common in both Antarctic and sub-Antarctic waters. Until now, molecular evidence to support this theory has been lacking. In the present study 34 nectophores of* P. vanhoeffeni *and four colonies of* M. micula *collected from three areas in the Southern Ocean were analysed for the 16S rRNA gene. Five haplotypes were identified, which formed two clearly distinct lineages. Three haplotypes were found exclusively in Admiralty Bay and were shared between individuals of both studied taxa, confirming that* M. micula *is indeed the post-larval stage of* P. vanhoeffeni*. Two additional haplotypes were found in one open ocean locality and in Admiralty Bay.*

Keywords: Siphonophora (*Pyrostephos vanhoeffeni*, *Mica micula*), Southern Ocean, genetic identity

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INTRODUCTION

Studies on gelatinous zooplankton, particularly cnidarian siphonophores, have increased in recent years. However, our knowledge of the biology, ecology and systematics of these animals, particularly in the Antarctic region, is still poor. Traditional net studies have often ignored Siphonophora in favour of more robust invertebrates, such as crustaceans. Siphonophores can be abundant and ecologically important oceanic hydrozoans (Totton & Bargmann, 1965; Kirkpatrick & Pugh, 1984; Mills, 2001; Boero *et al.*, 2008). There are approximately 180 described species; the group has the highest division of labour between zooids and the most precise organization of all colonial animals (Mapstone, 2014). Siphonophores are among the most abundant carnivores in the oceanic macroplankton (Pugh, 1984), and include the longest animals in the world, with colonies of some species exceeding 40 m in length (Dunn, 2009).

The identification of all parts of the fragile colonies which are usually separated during net sampling is the greatest challenge in the study of Siphonophora. Past descriptions of many species were based solely on damaged and often incomplete colonies. Species identity within the group is typically based on the morphology of at least one swimming bell. For other zooids of the colony, identification can be more problematic, however the sexual eudoxid stage is known in a number of calycophorans (Pugh, 1999b). But the evidence of a link between a eudoxid and an adult colony formerly treated as separate taxa using molecular markers has so far been shown in only one case. Using DNA barcoding techniques, Grossmann *et al.* (2013a) showed that *Eudoxia macra* (Totton, 1954), is the sexual stage of the small diphyomorph calycophoran *Lensia cossack* (Totton, 1941).

Pyrostephos vanhoeffeni Moser (1925) was first identified by Moser (1925) as a large colourful species with unusually modified palpons on the siphosome, later termed oleocysts (Totton & Bargmann, 1965). Totton provided the first accurate description and figures of the nectophores and tentilla of this species, fragments of which were first taken by the German Southpolar Expedition in 1902, just off the Antarctic Continent (in the Indian sector of the Southern Ocean) (Totton & Bargmann, 1965). *Pyrostephos vanhoeffeni*, although not abundant, is widely distributed throughout the Southern Ocean, as well as in sub-Antarctic waters and also further north (but only as far as 33°S to 40°S in the Pacific and Atlantic Oceans respectively) (Palma, 1986, 2006; Pagès & Kurbjeweit, 1994; Pagès *et al.*, 1994; Pagès & Schnack-Schiel, 1996; Panasiuk-Chodnicka & Żmijewska, 2010; Guerrero *et al.*, 2013; Lindsay *et al.*, 2014; Panasiuk-Chodnicka *et al.*, 2014; Palma *et al.*, 2016). It should be emphasized that *P. vanhoeffeni* occurs exclusively

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in the southern hemisphere, in contrast to all four *Bargmannia* species whose records come mostly from the North Atlantic (Pugh, 1999a).

Small colonies comprising a single nectophore (2 mm in length) and stem were collected by Margulis (1982) from Antarctic waters and introduced as *Mica micula*. Further specimens were described later by Pagès & Gili (1989). *Mica micula* colonies showed some characteristics associated with the family Pyrostephidae, such as the presence of stenoteles and a smaller spherical kind of nematocyst on the tentilla of the tentacles (Pugh, 1999a; Mapstone, 2009), but stenoteles also occur in a range of other siphonophore tentilla (Mapstone, 2014). Other morphological characters were imprecise. The colonies collected so far indicate that this species is limited to Antarctic waters. The ill-defined nature of the pneumatophore (suggesting it is still developing), the simple structure and singularity of the nectophore and the presence of a single gastrozooid without any other distinguishable siphosomal structures suggest that this taxon is the post-larval or siphonula stage of a physonect (Pagès & Gili, 1989). These authors suggested that *M. micula* might be a post-larvae of *Bargmannia elongata*, another representative of Pyrostephidae in Antarctic waters, although recent studies undermined this hypothesis (Grossmann *et al.*, 2013b).

Recently, Grossman *et al.* (2013b) published a redescription of *Mica micula* with notes on its distribution and identity. These samples were obtained during the 2008 Collaborative East-Antarctic MARine Census (CEAMARC), and all 18 specimens were collected in the area of Mertz Glacier, within the limits of the Antarctic Convergence. However, no *Bargmannia* nectophores or bracts were found amongst these samples, thus it was concluded that it is much more likely to be the post-larval form of the physonect *Pyrostephos vanhoeffeni*, which is very common in both Antarctic and sub-Antarctic waters.

In recent years the importance of molecular studies application to resolve taxonomical challenges has grown significantly. The idea of DNA barcoding, first proposed by Hebert *et al.* (2003), is now widely used across many animal phyla (e.g. Heimeier *et al.*, 2010; Jinbo *et al.*, 2011 and references therein). The mitochondrial cytochrome c oxidase subunit 1 (COI), for which several protocols as well as universal and also specific primers already exist, is the most commonly used gene (e.g. Folmer *et al.*, 1994; Hoareau & Boissau, 2010; Geller *et al.*, 2013 and references therein). Several investigations of Hydrozoa using the COI gene have aided in species identifications and indicated cryptic diversity in some taxa (e.g. Bucklin *et al.*, 2010; Ortman *et al.*, 2010; Laakmann & Holst, 2014). However, other authors suggested that the mutation rate of this gene is too slow for hydrozoans (Shearer *et al.*, 2002). Moreover, Lindsay *et al.* (2015b) pointed out that two COI GenBank siphonophore sequences published by Ortman *et al.* (2010) actually represent ostracod or protist contaminants so they are misleading. As a result, another mitochondrial gene 16S rRNA is more frequently used, works well for most pelagic hydrozoans, and many more sequences are available for this gene for hydrozoans on GenBank (Zheng *et al.*, 2014; Lindsay *et al.*, 2015b). Dunn *et al.* (2005) used the 16S rRNA gene to study phylogenetics within the order Siphonophora, and this mitochondrial gene also allowed for positive identification of *Eudoxia macra* as the eudoxid stage of *Lensia cossack* (Grossmann *et al.*, 2013a).

Grossmann *et al.* (2013b) studied the morphology of *Mica micula* colonies and assumed that this siphonophore is most probably the post-larval stage of *Pyrostephos vanhoeffeni*, in contrast to the suppositions of some other authors (Margulis, 1982; Pugh, 1999a; Mapstone, 2009). However, Grossmann *et al.* (2013b) also stated that further research applying genetics to the problem is needed and could give the final answer to this question. The aim of the present study therefore is to use the molecular methods to check the genetic affinity of *M. micula* with *P. vanhoeffeni*.

MATERIALS AND METHODS

Samples for this study were collected from three areas in the Southern Ocean: on a transect from Cape Town (South Africa) to the Weddell Sea (1 station – T1), on a transect from the Antarctic Peninsula to South America through Drake Passage (2 stations – D1, D2) and in Admiralty Bay, King George Island, South Shetland Islands (3 stations – AB1–AB3) (Table 1, Figure 1). Samples from the transects Cape Town (South Africa) – Weddell Sea and Drake Passage were collected between December 2009 and January 2010, during the cruise on RV 'Akademik Ioffe', while those from Admiralty Bay were collected during the 33rd Polish Antarctic Expedition (Austral summer 2008/2009). Sampling was performed with a WP2 plankton net (200 μm mesh size) and a Neuston net (500 μm). Thirty-four nectophores of *Pyrostephos vanhoeffeni*, and four colonies of *Mica micula* after identification to species level were preserved in 99.5% ethanol (Table 1, Table S1). DNA extraction from all specimens was performed according to a standard phenol-chloroform method after Hillis *et al.* (1996). The initial digestion with proteinase K was performed for one hour. Air-dried DNA pellets were eluted in 100 μl of TE buffer, pH 8.00, stored at 4°C until amplification, and subsequently at −20°C for

Table 1. Characteristics of the samples used for the present work.

Species	Station code	No. of nectophores/colonies used	Depth (m)	Date	Sampling location	
					Lat.	Long.
P. vanhoeffeni	T1	1	300–0	10.12.2009	43°16′S	8°16′E
P. vanhoeffeni	D1	14	200–0	03.01.2010	62°19.769′S	63°48.37′W
P. vanhoeffeni	D2	4	100–0	05.01.2010	60°20.87′S	64°30.59′W
P. vanhoeffeni	AB1	15	470–0	20.12.2008	62°08.90′S	58°29′40′W
Mica micula	AB2	3	1–0	28.11.2008	62°08.90′S	58°29′40′W
M. micula	AB3	1	1–0	31.12.2008	62°08.90′S	58°29′40′W

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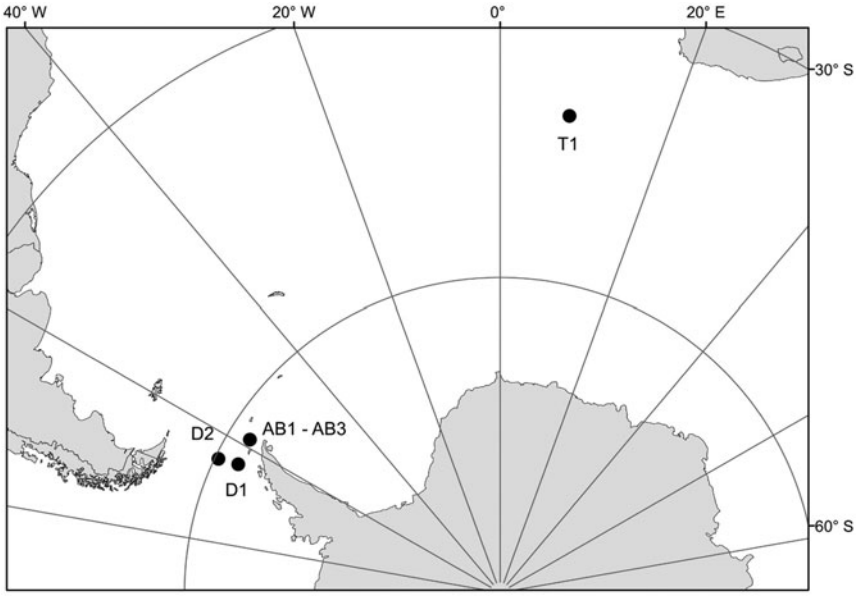

Fig. 1. Sampling points. T1, D1, D2, AB1–AB3 – station codes; see Table 1 for details.

long-term storage. A fragment of 16S ribosomal RNA (16S rRNA; ~580 bp fragment) was amplified using 'primer 1' and 'primer 2' from Cunningham & Buss (1993) with DreamTaq Green PCR Mastermix (Thermo Scientific). The protocol for the PCR reaction was 94°C for 5 min, 35 cycles (94°C for 60 s, 51°C for 60 s, 72°C for 90 s); finally fragments were elongated at 72°C for 5 min. Sequences were obtained using the BigDye sequencing protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. The sequences were aligned with MAFFT v7.308 algorithm (Katoh *et al.*, 2002) in Geneious 10.1.2, leading to 38 sequences of 561 bp each.

The uncorrected p-distance and the Kimura 2-parameter (K2P) model (Kimura, 1980) were used to determine sequence divergence in MEGA V7.0.18 (Kumar *et al.*, 2016). A Neighbour-joining (NJ) tree was built based on the p-distance with both transition and transversion substitutions included and pairwise deletion chosen. Node support was inferred with a bootstrap analysis (1000 replicates). The sequences of *Bargmannia amoena* and *B. elongata*, the only representatives of the family Pyrostephidae with available 16S data, were also used in the analysis (GenBank accession numbers AY935292 and AY935321, respectively). The sequence of *Apolemia rubriversa*, another representative of Physonectae, was used to root the tree (GenBank accession number KF214713). All sequences were deposited in GenBank with the accession numbers KY370929–KY370966 (Table S1). Relevant voucher information, taxonomic classifications, and sequences are accessible through the public data set 'DS-PVSO' on the Barcode of Life Data Systems (BOLD; http://www.boldsystems.org) (Ratnasingham & Hebert, 2007).

RESULTS

Among the 38 sequences obtained, five haplotypes were distinguished. One of them, represented by a single sequence, differed from the others by only one insertion and in the NJ tree was not treated as a separate entity. Due to the fact that some nectophores from the same samples shared haplotypes, it was assumed that they belonged to the same colony fragmented during collection. In further analyses they were not treated as separate units. This resulted in final examination of six colonies of *P. vanhoeffeni* and four colonies of *M. micula*. The values of overall uncorrected p-distance and the K2P distance between haplotypes were very similar (0.041 and 0.043, respectively). The haplotype divergence ranged from 0 to 0.063 in case of p-distance and from 0 to 0.066 for K2P distance (Table 2). The NJ tree showed that all sequences from the present study constituted a single branch further divided into two distinct clades with high support (bootstrap 100%) (lineages A, B) (Figure 2). The lineage A consisted of three haplotypes of both *Mica micula* and *Pyrostephos vanhoeffeni*, present exclusively in Admiralty Bay. The individuals belonging to the second Molecular Operational Taxonomic Unit

Table 2. Comparison of the genetic distance between haplotypes found calculated using uncorrected p-distance (above diagonal – grey tint) and Kimura 2-parameter (K2P) (below diagonal).

	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5
Haplotype 1		0.002	0.004	0.059	0.059
Haplotype 2	0.002		0.002	0.061	0.061
Haplotype 3	0.004	0.002		0.063	0.063
Haplotype 4	0.062	0.064	0.066		0
Haplotype 5	0.062	0.064	0.066	0	

The distance between haplotype 4 and 5 is zero due to one insertion in the latter that is not recognized as a mutation in both measures.

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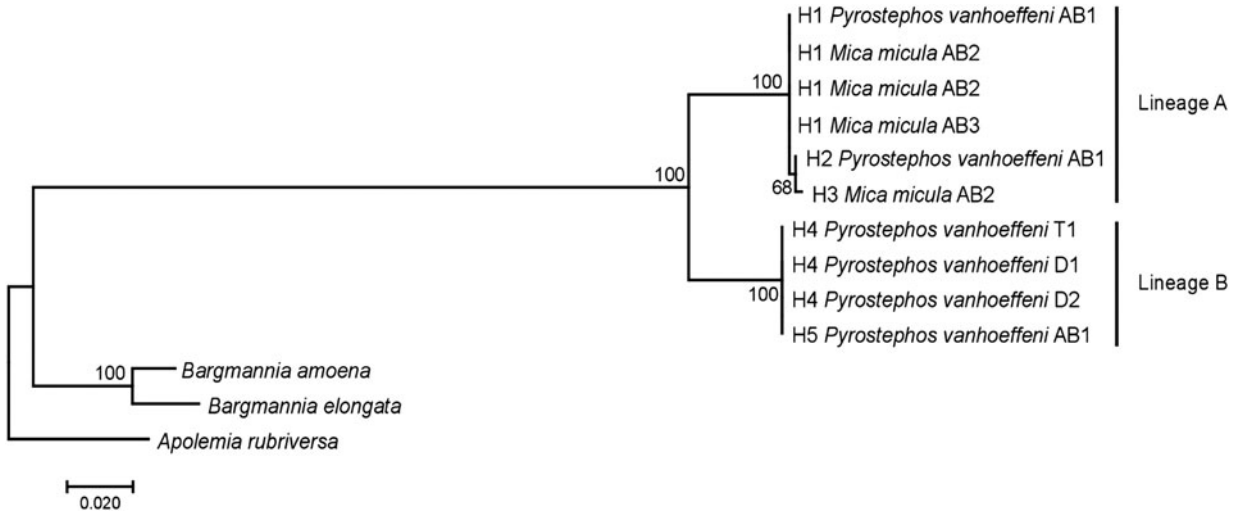

Fig. 2. Neighbour-joining (NJ) tree of 16S rRNA sequences representing each studied colony based on uncorrected p-distance; the numbers in front of the nodes indicate bootstrap support (1000 replicates, only the values higher than 50% are presented); T1, D1, D2, AB1–AB3 – station codes – see Table 1 for details. Sequences of *Bargmannia amoena*, *B. elongata* and *Apolemia rubriversa* retrieved from GenBank.

(MOTU) identified (lineage B), represent two haplotypes of *P. vanhoeffeni* and were found in localities in the open sea in the Southern Ocean and in Admiralty Bay. The haplotype from Admiralty Bay differs from that in the open ocean by a single insertion. The average distance of the sequences forming lineage A was 0.002 (both p-distance and K2P), while that between the lineages A and B was 0.060 of p-distance and 0.063 of K2P (Table 3). The distance between both *Bargmannia* species and the two discovered clades ranged from 0.272 to 0.280 for p-distance. In the case of K2P the values were from 0.345 to 0.357. Similar values of sequence divergence were observed between the two discovered lineages and *Apolemia rubriversa*. Nucleotide differences between sequences from clades A and B were 6.5–6.8%.

DISCUSSION

In our study molecular techniques were used to investigate two species of Southern Ocean siphonophores, namely the enigmatic taxon *Mica micula* (Figure 3a), and a taxon which is quite common and abundant in these waters – *Pyrostephos vanhoeffeni* (Figure 3b).

Table 3. Genetic distance calculated using p-distance and Kimura 2-parameter (K2P) within and between distinguished lineages and outgroups.

	p-distance		K2P	
	Mean	**SD**	**Mean**	**SD**
Lineage A	0.002	0.002	0.002	0.002
Lineage B	0	0	0	0
Lineage A *vs* Lineage B	0.060	0.002	0.063	0.002
Lineage A *vs B. elongata*	0.280	0.001	0.356	0.003
Lineage B *vs B. elongata*	0.280	0.002	0.357	0
Lineage A *vs B. amoena*	0.272	0.002	0.345	0.002
Lineage B *vs B. amoena*	0.275	0	0.349	0
Lineage A *vs A. rubriversa*	0.283	0.002	0.361	0.003
Lineage B *vs A. rubriversa*	0.275	0	0.347	0
B. elongata vs B. amoena	0.033	–	0.034	–
B. elongata vs A. rubriversa	0.103	–	0.111	–
B. amoena vs A. rubriversa	0.090	–	0.096	–

Mean - mean value, S.D. - standard deviation.

Analysis of the biogeographic distribution of *Pyrostephos vanhoeffeni*, *Mica micula* and *Bargmannia elongata* showed that the distribution of the two first is limited to the southern hemisphere (Figure 4). In contrast, the distribution of *B. elongata* is much broader, with individuals occurring in Canadian Pacific waters (Mapstone, 2009), off California and San Diego and in the NE Atlantic (Pugh, 1999a; Dunn *et al.*, 2005), in the Gulf of Mexico (Pugh & Gasca, 2009) as well as in Japanese waters (Lindsay & Hunt, 2005; Lindsay, 2006) and in the Indo-Pacific (Lindsay *et al.*, 2015a) (Figure 5). Both *P. vanhoeffeni* and *B. elongata* have been observed in the east and west Antarctic regions (Margulis, 1982; Pugh *et al.*, 1997; Toda *et al.*, 2010; Grossmann *et al.*, 2013b), but overall there are many more records for *P. vanhoeffeni* in this area than for *B. elongata*. *Mica micula* has been recorded in the East Antarctic region (Grossmann *et al.*, 2013b), Admiralty Bay (King George Island, South Shetlands Archipelago) and in the Atlantic sector of the Southern Ocean (Pagès & Gili, 1989) (Figures 4 and 5). The last authors collected one nectophore of *B. elongata* and two colonies of *M. micula*, but no associated specimens or nectophores of *P. vanhoeffeni*. Summarizing, the records for *B. elongata* in the Southern Ocean show very little correlation with the areas of distribution of *M. micula*, whereas the distribution of *Pyrostephos vanhoeffeni* overlaps well with that of *M. micula*.

A study of 16S rRNA sequences clearly show that *Mica micula* is the post-larval stage of *Pyrostephos vanhoeffeni* (Figure 2). Similar studies resulted in the recognition of another enigmatic siphonophore taxon – *Eudoxia macra* – as the eudoxid stage of the small diphyid *Lensia cossack* (Grossmann *et al.*, 2013a). Some authors have suggested that *M. micula* might be a post-larva of *Bargmannia elongata*, the only other representative of family Pyrostephidae identified in Antarctic waters (Pagès & Gili, 1989; Pugh, 1999b), but this has been questioned due to the non-coincidence of distribution records of these two pyrostephid taxa (Grossmann *et al.*, 2013b). Our study also shows that the genetic distance

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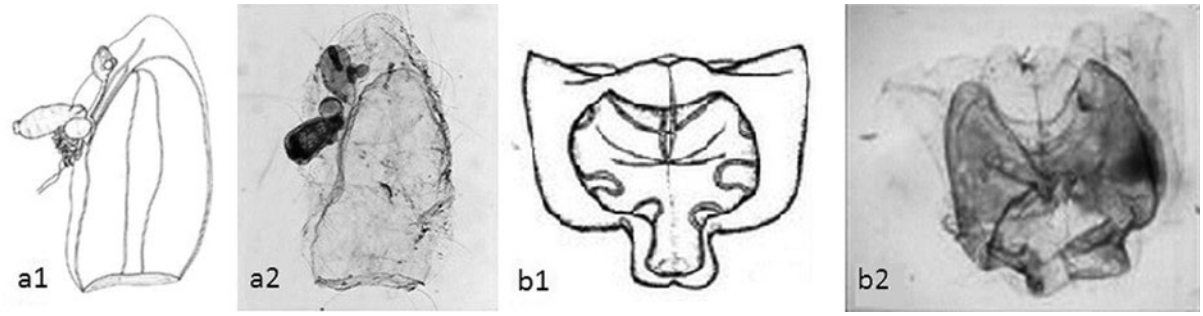

Fig. 3. *Mica micula* – young colony: a1 – from Pagès & Gili (1989), a2 – A. Panasiuk, *Pyrostephos vanhoeffeni* – nectophore: b1 – from Alvarino *et al.* (1990), b2 – A. Panasiuk.

between sequences of *M. micula* and *B. elongata* is greater than the inter-family distances within some other hydrozoans (Zheng *et al.*, 2014).

Our results also revealed two clearly separated genetic lineages of *Pyrostephos vanhoeffeni* in the Antarctic (Figure 2). Lineage (A) represented by three haplotypes was restricted solely to Admiralty Bay, whereas specimens assigned to the other lineage (B) came from several regions including Drake Passage, Admiralty Bay, and also the South African region of the Atlantic Ocean. Apart from the sequence from Admiralty Bay (differing by one insertion), this widespread lineage (B) is represented by a single haplotype, which suggests constant gene flow. What is more, the colony from the South African region was collected north of the Antarctic Convergence. That indicates that the differences in water temperature observed north and south of it do not prevent mixing of the populations. This is in contrast to the findings by Grossmann *et al.* (2013a) who recorded the existence of two genetically distinct populations of another siphonophore, *Lensia achilles* associated with different water masses. It is also worth noting that genetic diversity observed in Admiralty Bay is noticeable as four out of five haplotypes recorded in this study were present only in this small embayment. The diversity expressed by K2P within both lineages was very low, whereas between them it amounted to 0.063 (Table 3). This value falls well within the intra-species distances observed in different *Lensia* species (Lindsay *et al.*, 2015b). Grossmann *et al.* (2013a, 2015) have observed also the cryptic diversity within this genus. In this case, the genetic distances between populations of several morphospecies were distinctly higher (up to 0.25), compared with usually recorded intraspecific values of 0.01 to 0.16 (Lindsay *et al.*, 2015b). However, one must take into account that in the case for *Lensia*, the nominal species with large intra-species genetic distances were sampled in different geographic locations that are not expected to exhibit gene flow in modern times, namely Japan and Antarctica, so the geographic distance between sampling localities is much greater than in

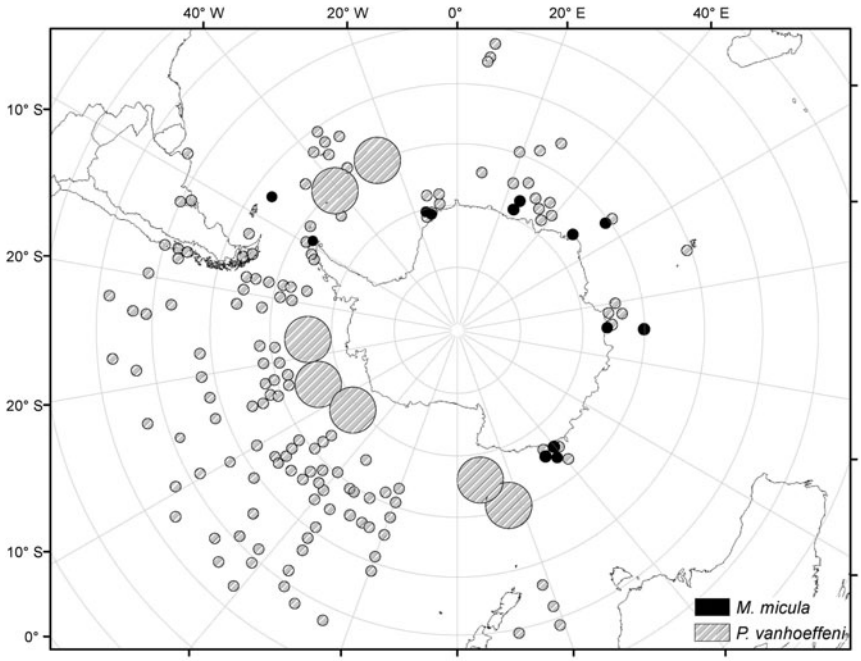

Fig. 4. Distribution/records of *Pyrostephos vanhoeffeni* and *Mica micula* based on available data (from Hardy & Gunther, 1935; Alvarino, 1971; Pagès & Gili, 1989; Alvarino *et al.*, 1990; Margulis, 1992; Pagès & Kurbjeweit, 1994; Pagès *et al.*, 1994; Pakhomov *et al.*, 1994; Pagès & Schnack-Schiel, 1996; Palma & Rosales, 1997; Pugh *et al.*, 1997; Pagès & Orejas, 1999; Palma & Aravena, 2001; Fuentes *et al.*, 2008; Panasiuk-Chodnicka & Żmijewska, 2010; Toda *et al.*, 2010, 2014; Grossmann *et al.*, 2013b; Guerrero *et al.*, 2013; Lindsay *et al.*, 2014; Panasiuk-Chodnicka *et al.*, 2014); size of the circle indicates the frequency of records.

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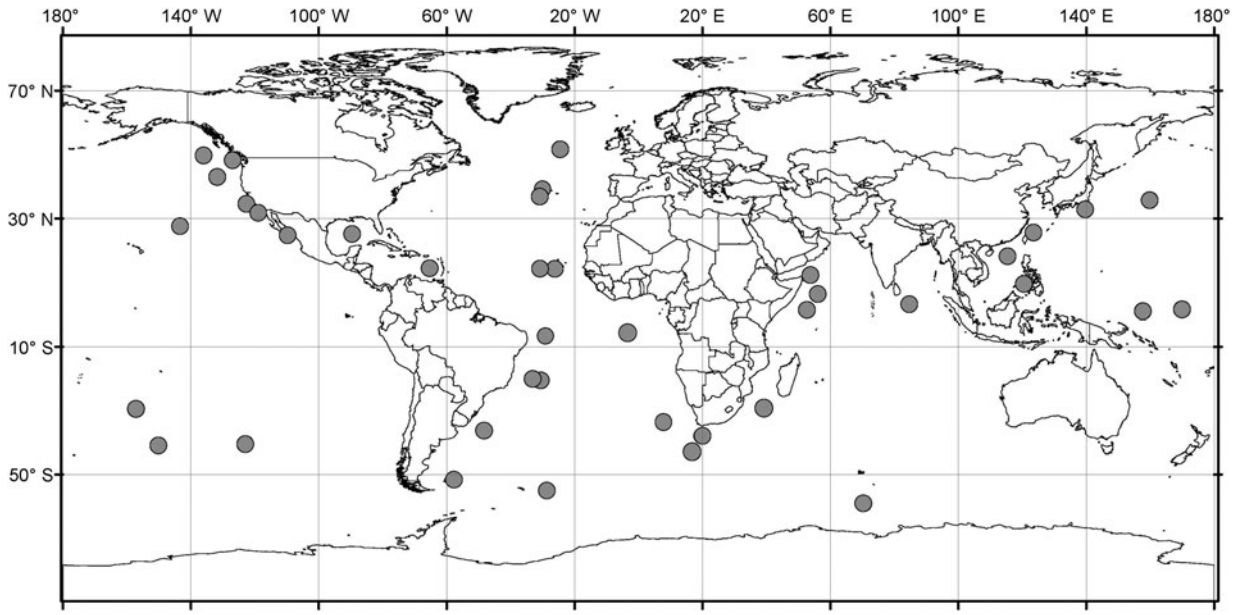

Fig. 5. Distribution/records of *Bargmannia elongata* based on available data (from Alvarino, 1963, 1971; Margulis, 1980, 1992; Alvarino *et al.*, 1990; Pugh, 1999a; Dunn, 2005; Lindsay, 2006; Hosia *et al.*, 2008; Mapstone, 2009; Pugh & Gasca, 2009; Grossmann *et al.*, 2015); with data from GBIF (Global Biodiversity Information Facility) and OBIS (Ocean Biogeographic Information system).

the present study (Grossmann *et al.*, 2013a). Higher genetic diversity (0.12) was also recorded for *L. achilles* specimens from two different water masses – one sub-arctic and one sub-tropical (Grossmann *et al.*, 2013a). On the other hand, it is worth noting that the genetic distance between sequences of two species of *Bargmannia* used in the present study is considerably lower than the one between the two lineages of *P. vanhoeffeni* (Table 3). Also Zheng *et al.* (2014) who studied pelagic Hydrozoa from the order Leptothecata found that the intra-specific variation of the 16S rRNA gene was considerably lower in these cnidarians than in the siphonophores studied here (with a maximum value of K2P reaching 0.016). At the same time the interspecies distances of this parameter observed by these authors varied from 0.062 to 0.642.

The phylogeography of the recognized lineages remains an open issue. Lineage B has a wide geographic range and may represent the population of circum-Antarctic distribution, extending also north of the Antarctic Convergence. On the other hand lineage A may indicate a population of limited distribution. The study of further material from additional Antarctic localities, including both detailed morphological investigations and additional molecular analyses, could address this question.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0025315418000218

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