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# Southernmost records of *Escarpia spicata* and *Lamellibrachia barhami* (Annelida: Siboglinidae) confirmed with DNA obtained from dried tubes collected from undiscovered reducing environments in northern Chile

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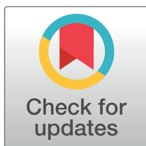

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## Abstract

Deep-sea fishing bycatch enables collection of samples of rare species that are not easily accessible, for research purposes. However, these specimens are often degraded, losing diagnostic morphological characteristics. Several tubes of vestimentiferans, conspicuous annelids endemic to chemosynthetic environments, were obtained from a single batch of deep-sea fishing bycatch at depths of around 1,500 m off Huasco, northern Chile, as part of an ongoing study examining bycatch species. DNA sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and an intron region within the hemoglobin subunit B2 (hbB2i) were successfully determined using vestimentiferans' dried-up tubes and their degraded inner tissue. Molecular phylogenetic analyses based on DNA sequence identified the samples as *Escarpia spicata* Jones, 1985, and *Lamellibrachia barhami* Webb, 1969. These are the southernmost records, vastly extending the geographical ranges of both species from Santa Catalina Island, California to northern Chile for *E*. *spicata* (over 8,000 km), and from Vancouver Island Margin to northern Chile for *L*. *barhami* (over 10,000 km). We also determined a 16S rRNA sequence of symbiotic bacteria of *L*. *barhami*. The sequence of the bacteria is the same as that of *E*. *laminata*, *Lamellibrachia* sp. 1, and *Lamellibrachia* sp.2 known from the Gulf of Mexico. The present study provides sound evidence forthe presence of reducing environments along the continental margin of northern Chile.

## Introduction

Deep-sea fishing bycatch provides a glimpse into the species co-occurring with commercial fishes and often comprises a way of recording rare species that are not easily accessible for research. However, bycatch is seldom reported, kept, or landed due to a lack of commercial

interest, administrative restrictions, and for other socio-economic reasons [1]. In addition to the inconsistent and fragmented nature of these records, examining such organisms is often hampered by their degradation before they reach researchers. Most often, the only available remains of deep-sea fishing bycatch are carapaces or shells, or, in the case of vestimentiferan tubeworms, their hardened chitin-protein tubes. Therefore, identification of vestimentiferans to species level is impeded by the absence of diagnostic soft parts.

Vestimentiferans are interesting members of the annelid family Siboglinidae as they lack a mouth and digestive organs, depending on endosymbiotic chemoautotrophic bacteria for nutrition in the adult phase [2]. To date, 20 vestimentiferan species within 10 genera have been recorded from hydrothermal vent fields [3, 4], cold-seep areas [5, 6], and organic falls [7–9]. In the Pacific Ocean, vestimentiferans are particularly diverse, with four genera identified in cold-seep areas: *Alaysia* Southward,1991; *Escarpia* Jones, 1985; *Lamellibrachia* Webb, 1969; and *Paraescarpia* Southward, Schulze & Tunnicliffe, 2002 [3, 10–12]. While the monotypic genus *Alaysia* is known from a hydrothermal vent field,several undescribed species of the genus have been collected from cold-seep areas around Japan [13]. *Escarpia* includes three described and a few undescribed species [14, 15]; *Lamellibrachia*, the most diverse group among vestimentiferans, consists of eight named and several species so far undescribed [16–18], while the genus *Paraescarpia* is monospecific.

In the cold-seep areas of the northeastern Pacific, two vestimentiferan species have been reported: *Escarpia spicata* Jones, 1985, known from off Santa Catalina Island, California to Middle American Trench (reviewed by Karaseva et al. [19]); and *Lamellibrachia barhami* Webb, 1969, known from British Columbia to Costa Rica (reviewed by Karaseva et al. [19]). However, to date, no vestimentiferan species have been identified to the species level in the southeastern Pacific. A record of "pogonophoran," which may represent a vestimentiferan species, was reported from a seep site in the Peruvian margin, off Paita ~5˚S [20], while an unidentified vestimentiferan species was recorded from the Concepción Methane Seep Area (CMSA) off Concepción ~36˚S [21]. This undescribed species is suggested to be most closely related to *Lamellibrachia luymesi* van der Land & Nørrevang, 1975 [22], described from the Gulf of Mexico, based on the partial sequence of the mitochondrial cytochrome *c* oxidase subunit I (COI) [21]. Furthermore, empty tubes of vestimentiferans have also been collected off Chile nearby the Taitao Peninsula, ~46˚S [23], and off El Quisco, ~33˚S [24].

Some vestimentiferan tubes lacking diagnostic soft parts were also collected as bycatch of deep-sea fishing off Huasco, northern Chile, in September 2017. As part of an ongoing project investigating the bycatch of deep-sea fishing in northern Chile [25–31],the present study reports two species of vestimentiferans, identified to the species level through molecular phylogenetic analyses based on DNA sequences determined using dried-up tubes and tissue.

## Materials and methods

### Sampling

Ten anterior parts and some fragments of vestimentiferan tubes lacking posterior parts were collected as bycatch of longline fishing by the fisheries vessel (FV) Rocio III during fishing of *Dissostichus eleginoides* Smitt, 1898 (Patagonian toothfish or Chilean sea bass) fishing, at a depth of about 1,500 m off Huasco (28˚S, 71˚W; accurate coordinates are not available), Región de Atacama, northern Chile, in September 2017. As this material was serendipitously collected in the fish bycatch (discarded material), no permit was necessary for the current research. Siboglinids are not endangered nor protected by local law. The substrata of tubes were not collected. Four tube samples (two anterior parts and two fragments) containing degraded tissues (may be trophosome of the worms) were used for morphological and

molecular analyses. The samples were stored at room temperature in Chile (around 18˚C) from September 2017 to March 2018, after which they were used for DNA extraction. Following DNA extraction, four specimens were preserved at –20˚C.

## Polymerase chain reaction (PCR) and sequencing

Total DNA was extracted both from the tissue left inside each of four tubes (probably consisting of trophosomes) and from the tubes themselves, using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), following a normal protocol of manufacturer's recommendations. Tube pieces were carefully cut from parts of the tube where obvious tissue were absent, but see the "DNA extraction from dried-up vestimentiferan tubes" section in Discussion. Fragments of the mitochondrial COI gene (658 bp) were amplified by PCR using a primer set LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAG GGTGACCAAAAAATCA-3′) [32]. Fragments of an intron region within the hemoglobin subunit B2 (hbB2i; ~660 bp) were amplified with the following primer sets: hbB2i_F (5′-TCC ATCGCCCCAGGCTGTCTTC-3′); and hbB2i_R (5′-GCCTTGAATTCGTTGCTGTT-3′) [33]. A mitochondrial gene (16S rRNA; 1409 bp) of symbiotic bacteria was amplified with primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACT T-3′) [34].

The PCR mixtures for vestimentiferans contained 16 μl DDW, 0.13 μl TaKaRa Ex Taq Hot Start Version (TaKaRa Bio Inc., Kusatsu, Japan), 2.5 μl 10× Ex Taq Buffer, 2.0 μl dNTP mixture (2.5 μM each), 0.3 μl forward and reverse primers (20 μM each), and 4.0 μl template DNA. For bacteria, the PCR mixtures contained 7.3 μl DDW, 0.1 μl TaKaRa Ex Taq Hot Start Version, 1.3 μl 10× Ex Taq Buffer, 1.0 μl dNTP mixture (2.5 μM each), 0.65 μl forward and reverse primers (10 μM each), and 2.0 μl template DNA. PCR amplifications were performed as follows: initial denaturation at 94˚C for 120 s; followed by 35 cycles consisting of denaturation at 94˚C for 30 s, annealing at 42˚C (COI) or 53˚C (hbB2i) for 40 s, extension at 72˚C for 20 s; and a final extension at 72˚C for 300 s. Exceptions included annealing at 52˚C for 20 s and 105 s of extension for bacterial 16S rRNA. Obtained PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) and then sequenced using the same primer sets asforPCR. Sequencing reactions were prepared using a BigDye Terminator Cycle Sequence Kit v3.1 (Applied Biosystems [ABI], Foster City, CA). Nucleotide sequences were determined using an ABI 3130xl automated DNA sequencer after being purified with a BigDye XTerminator Purification Kit (ABI).

## Phylogenetic analysis

A total of 71 COI sequences of vestimentiferans and two sequences of other siboglinid species were used for phylogenetic analysis. Accession numbers obtained from GenBank are shown after the taxonomic names in the resultant tree. There were no indels resulting in an unambiguous alignment for the COI. Phylogenetic trees were reconstructed using Bayesian inference and maximum likelihood (ML) methods, based on the COI dataset. Bayesian analysis was performed using MrBayes v3.1.2. [35], with the setting "branch lengths unlinked." Partitioning scheme and best-fit substitution models were estimated using PartitionFinder v2.1.1. [36] with "model selection" set to "AICc," "branchlengths" set to "unlinked," and using the "-raxml" option [37]: TRN + Γ+ I for the first + second codon positions of COI; GTR + Γ + I for the third codon position of COI. Since the TRN model was not implemented in MrBayes,it was replaced by the GTR model. Two parallel runs were made for 5,000,000 generations (with a sampling frequency of 1,000), using the default value of four Markov chains. The initial 25% of samples were discarded, and the subsequent 75% were used to confirm that the four chains

reached stationary distributions, referring to the average standard deviation of split frequencies [35]. The ML analysis was performed using RAxML v7.2.6 [37]. The rapid bootstrap analysis was used to identify the best-scoring ML tree in a single program run, and to identify 500 bootstrap replicates under the GTR + Γ + I substitution model for all partitions.

DNA sequences of hbB2i of 42 *Escarpia* species and of five *Seepiophila jonesi* (outgroup) were aligned using MAFFT v7.294b with the default option [38]. Only three bp of a deletion was found in *S. jonesi*, resulting in non-ambiguous alignment. A phylogenetic analysis was conducted to identify the *Escarpia* specimens to the species level, based on the hbB2i sequences. Bayesian inference and ML methods were employed with the same options as the analysis for the COI genes. The GTR + G model was estimated as the best-fit substitution model with PartitionFinder v2.1.1. for the Bayesian analysis. All trees were edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

## Results

### Tube morphology and associated species

The tubes of GK608 and GK621 included anterior regions but lacked posterior ones, whereas those of GK605 and GK607 lacked both anterior and posterior regions. For GK621, the outer width of the top funnel opening was 14.9 mm,whereas its base was 11.7 mm (Fig 1). Unlike the other tubes, GK608 did not form conspicuous funnels, presenting a smooth surface (Fig 1) with its top opening measured at 14.0 mm. The only organisms attached to the tubes were unidentified species of limpets.

### PCR amplification of the DNA extracted from vestimentiferan tissue and tubes

DNA sequences were successfully obtained from the dried-up three vestimentiferan tissue (GK605, GK607, and GK608) and two tubes (GK605 and GK621). The partial sequences of the mitochondrial COI (658 bp) of GK605 and GK621 were identical, making it impossible to determine whether the specimens were derived from different individuals. Although PCR was successful for the other tissue and tube samples, the sequences were not determined by direct sequencing.

### Phylogenetic analyses of vestimentiferans

Since Bayesian and ML analyses of the COI dataset generated similar tree topologies and support values, only the Bayesian tree is shown with posterior probabilities (PP) and ML bootstrap values (BS) (Fig 2). As shown in Fig 2, all sequences of vestimentiferans collected from off the coast of northern Chile were included in highly supported clusters. Three of these vestimentiferans (GK605, GK607, and GK621) were clustered with *Lamellibrachia barhami* (PP = 1.00, BS = 95%), while the other vestimentiferan (GK608) was included in a cluster comprising *Escarpia laminata* Jones, 1985; *Escarpia southwardae* Andersen, Hourdez, Jolivet, Lallier and Sibuet, 2004; and *Escarpia spicata* (PP = 1.00, BS = 98%). The phylogenetic relationships of the *Escarpia* species are not clear from the COI tree, as *E. laminata* and *E. spicata* were not monophyletic. The Bayesian tree based on the hbB2i sequences of the *Escarpia* species showed that a single cluster was recovered for *E. laminata* (PP = 0.99, BS = 76%) and a sub-cluster, which includes four sequences, was recognized for *E. spicata* with weak support values (PP = 0.86, BS = 55%), whereas clusters were not recovered for *E. southwardae* nor the rest of *E. spicata* (Fig 3). GK608 was included in the *E. spicata* clade.

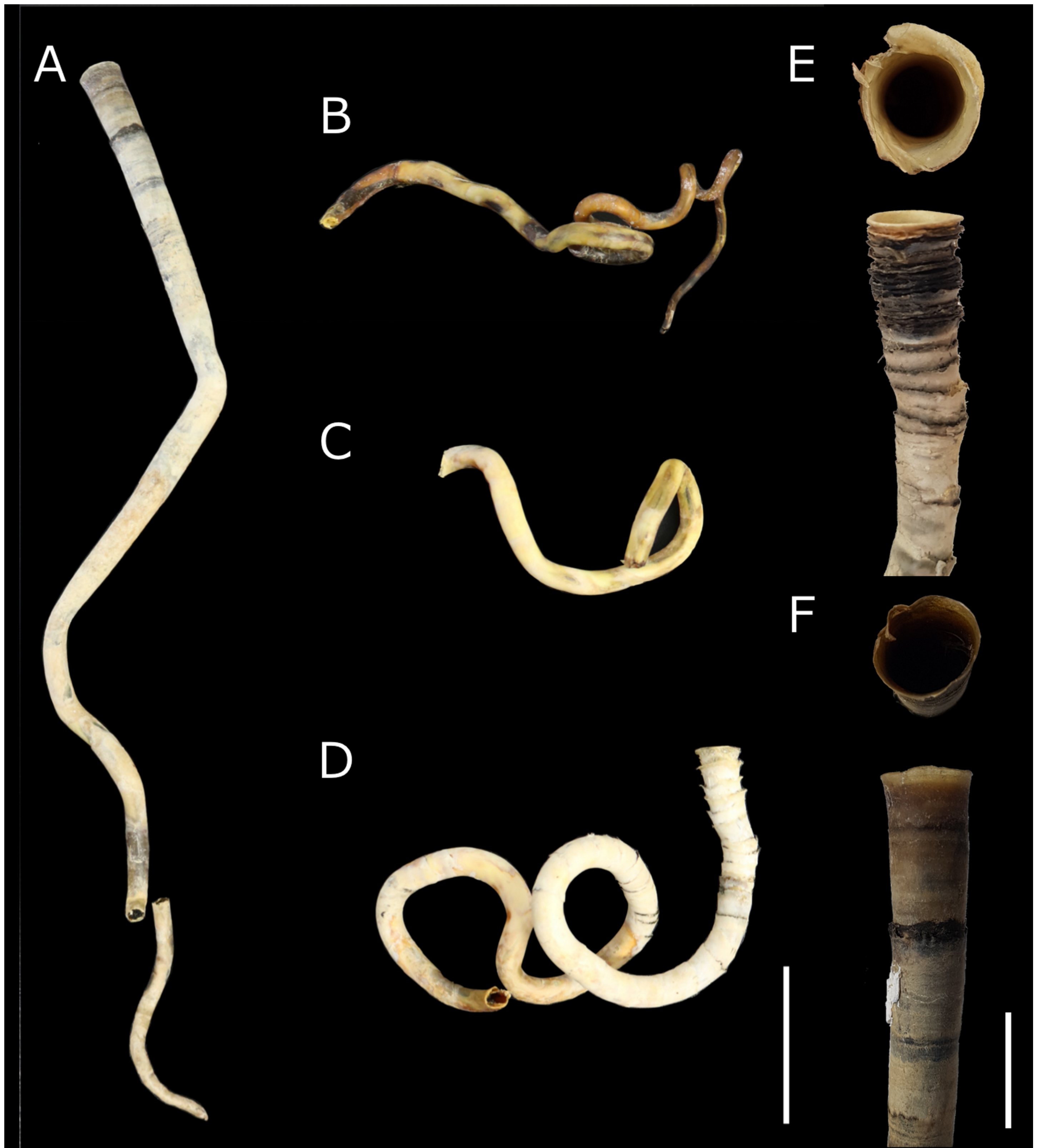


**Fig 1. Vestimentiferan tubes.** *Escarpia spicata* (A, GK608); *Lamellibrachia barhami* (B, GK607; C, GK605; D, GK621); other vestimentiferan tubes which were not used for molecular analyses (E, F). Scale bar = 5 cm for A–D, 2 cm for E and F.

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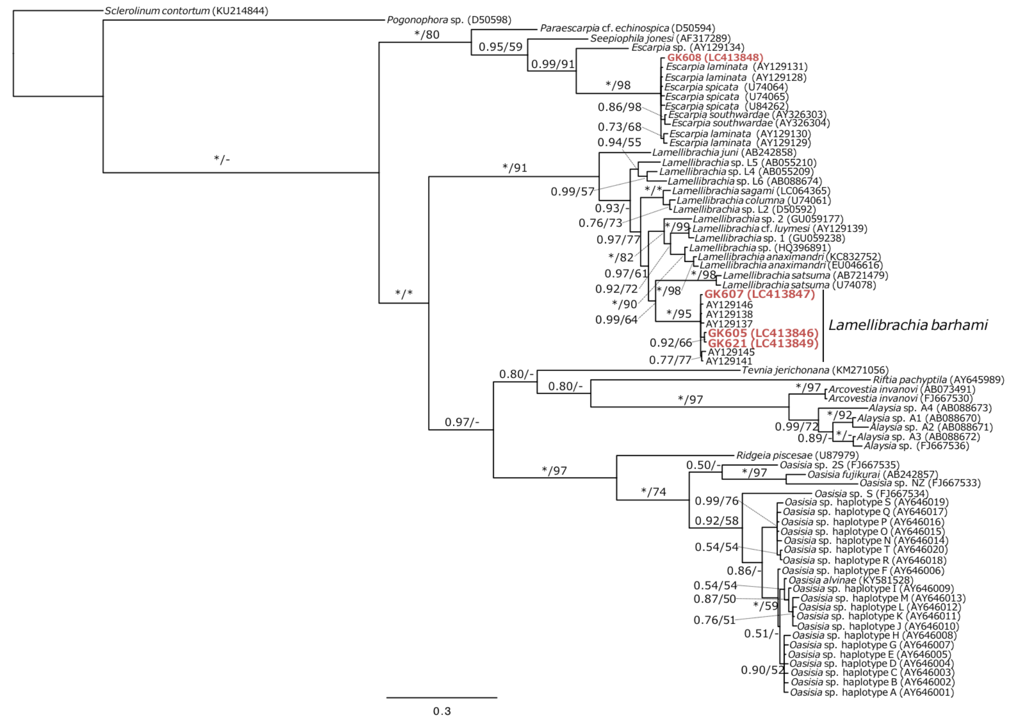


**Fig 2. Bayesian phylogeny of vestimentiferans based on the COI gene sequences (up to 1270 bp).** The numbers above the branches indicate the posterior probability (PP), followed by the percentage of maximum likelihood bootstrap probabilities (BS) above 50%. Asterisks indicate values of 1.00 (PP) or 100% (BS) and hyphens do value below 50% (BS).

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## DNA sequence of symbiotic bacteria

Direct sequencing allowed a 16S rRNA sequence of symbiotic bacteria to be obtained from the tissue of vestimentiferan GK607. No variable sites were found between the sequence of symbiotic bacteria of GK607 and that of *Escarpia laminata* (Accession No. HE983329, 1335 bp), *Lamellibrachia* sp. 1 (HE983327; 1471 bp), and *Lamellibrachia* sp. 2 (HE983328, 1372 bp; HE983337, 1279 bp), all of which were collected from the Gulf of Mexico at depths of 2,335–2,604 m [39]. The 16S rRNA sequence of symbiotic bacteria obtained fromGK607 is almost same as the sequence of symbiotic bacteria obtained from *Lamellibrachia barhami* (AY129103, 1361 bp) collected from the Vancouver Island Margin at a depth of 1,300 m, with only a single nucleotide substitution [40].

# Discussion

## Tube morphology

Tube morphology was insufficient to identify vestimentiferan specimens to the species level. All tubes presented a hard, wood-like texture, similar to those of vestimentiferans inhabiting cold-seep areas, including species of the genera *Escarpia*, *Lamellibrachia*, *Paraescarpia*, and *Seepiophila*. The samples GK605 and G607 lacked both anterior and posterior parts, making species identification from tube morphology impossible. A coiled tube with funnels (GK621) resembled that of *Lamellibrachia barhami*, shown in fig 25 by Webb [41]. Such entangled tubes are unknown in any other vestimentiferans, especially in terms of anterior region of tubes. Although a smooth, straight tube, lacking evident funnels (GK608) resembles those of *Escarpia* species [3, 15]; some *Lamellibrachia* species also lack conspicuous funnels in the

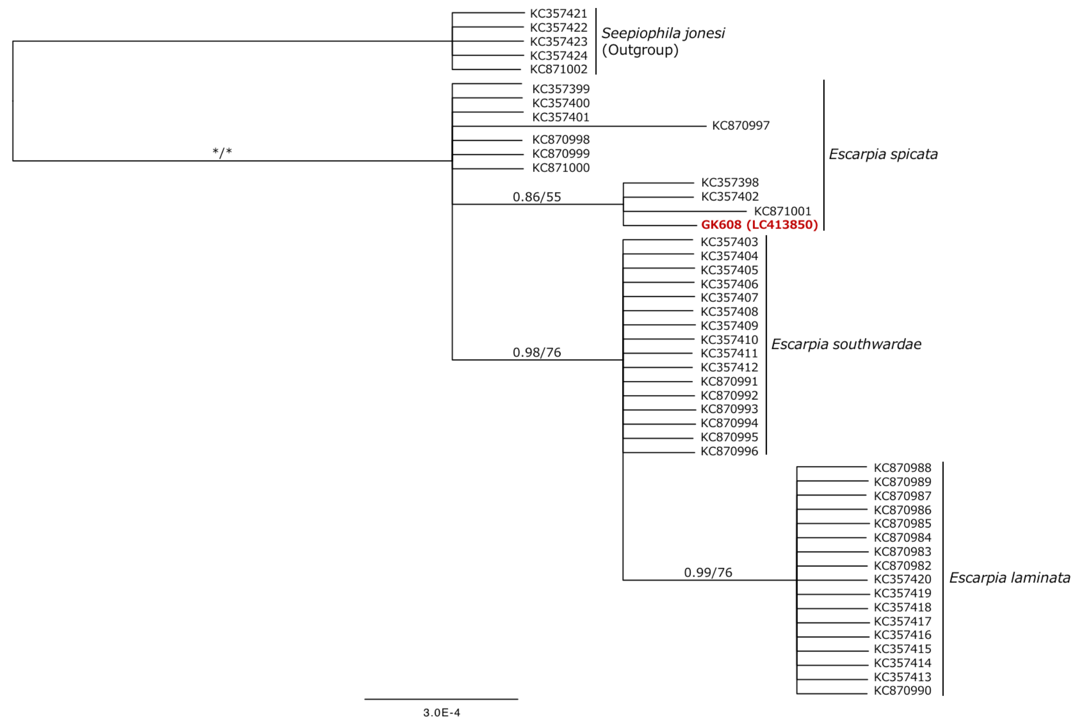


**Fig 3. Bayesian phylogeny of *Escarpia* species based on the hbB2 gene sequences (up to 660 bp).** *Seepiophila jonesi* was included as an outgroup. The numbers above the branches indicate the posterior probability (PP), followed by the percentage of maximum likelihood bootstrap probabilities (BS) above 50%. Asterisks indicate values of 1.00 (PP) or 100% (BS).

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anterior region [10, 18]. Moreover, vestimentiferans show considerable plasticity in terms of tube morphology [6, 42, 43], making identification to the species level from tube morphology difficult.

## DNA extraction from dried-up vestimentiferan tubes

DNA sequences were successfully obtained from vestimentiferans dried-up tissue and tubes. Although a previous study extracted DNA from the degraded tissue of *Lamellibrachia* species [44], to our knowledge the present study is the first time that DNA is successfully amplified from vestimentiferan tubes, which are constituted by chitin and protein secreted by vestimentiferans [45, 46]. Discarded tissues that are included in secretions, such as mucus, can be a source of DNA [47]. By experimentally immersing *Riftia* tubes into a hydrothermal vent field for 180 days, *Riftia* tubes were estimated to degrade within 2.5 years of the death of the organism [48]. DNA extraction from tubes may, therefore, allow identification of the vestimentiferan species, by using vacant tubes without the soft parts, which usually prevents identification to the species level. Although the tubes which were used in the present study were carefully cut to exclude dried tissue from DNA extraction, in future studies DNA extraction using degraded tubes that is completely free from remains of vestimentiferan tissue would be needed, to eliminate false positives for DNA present in the tubes.

## Identification based on molecular phylogeny of vestimentiferans from Chile

The COI phylogenetic tree shows that three vestimentiferan sequences (GK605, GK607, and GK621) were clustered with *Lamellibrachia barhami* with high support values (Fig 2).

Although one vestimentiferan sequence (GK608) was clustered with three *Escarpia* species, the COI analysis did not allow for identification to the species level (Fig 2). The specimen was clustered with *Escarpia spicata* in the hhBb2i phylogenetic tree (Fig 3). The hhBb2i sequence is known as a useful indicator to identify the three *Escarpia* species, which are not discriminated by COI sequences [33]. Thus, the present results allow the identification of the vestimentiferan tubes collected from off Chile confidently to be *L. barhami* and *E. spicata*.

## Implications for vestimentiferan biogeography and phylogeography

The present study generated new records of two vestimentiferan species from the Chilean waters, including the first record of the genus *Escarpia*. *Lamellibrachia barhami* was previously identified along the continental margin of the northeastern Pacific from the Vancouver Island Margin to Costa Rica, at depths of 1,000–2,400 m (reviewed by Karaseva et al. [19]; they regarded the "Vigo worm" collected from off Spain as *L. barhami*, although the 28S rRNA sequence of the Vigo worm considerably differs from that of *L. barhami* [44], thus we did not include the record from off Spain here), at both hydrothermal vent fields and cold-seep areas. *Escarpia spicata* was previously identified in chemosynthetic environments from off Santa Catalina Island, California, to the Middle American Trench at depths of 1,240–2,756 m (reviewed by Karaseva et al. [19]). The present record of both species considerably extends their southernmost limit: the geographic range of *L. barhami* is over 10,000 km for straight-line distance and that of *E. spicata* is over 8,000 km. Vestimentiferans inhabiting hydrothermal vent fields (i.e., *Riftia pachyptila* Jones, 1981; and *Tevnia jerichonana* Jones, 1985) present a wide geographical distribution across the eastern Pacific [19]; the present study provides the first record of cold-seep vestimentiferans with a broad distribution across the eastern Pacific.

Despite an 8,000 km distance, *L. barhami* from Monterey Canyon (e.g., AY129137, AY129138) and from Chile (GK607, LC413847) are identical in terms of shared sites (633 bp) of the COI gene, indicating a close intra-specific relationship, similar to that of other eastern Pacific vestimentiferans (e.g., *R. pachyptila* and *T. jerichonana)* for which shared COI haplotypes were reported between specimens from the northeastern and southeastern Pacific, although different haplotypes dominate at north and southern localities [49, 50]. This little genetic divergence in the COI gene may be attributed to the slow evolutionary rate in the gene [40] or to a recent radiation of vestimentiferan species. In general, deep-sea benthic invertebrates show a wide geographical distribution with little genetic divergence [50–54], and the present study provides another example of such a pattern. Further analyses including more specimens are needed to further discuss the phylogeography of *L. barhami*. Unfortunately, there are still no appropriate DNA markers available for intra-specific phylogeography of *E. spicata*.

Vestimentiferan species play an important role in structuring the benthic community by providing microhabitats for other organisms [55, 56]. The chitinous tubes of vestimentiferans increase the spatial heterogeneity in soft bottoms and are used as substrata for colonization of various epibenthos, in terms of their taxon and body size [57–64]. Although only unidentified limpets were found in the surfaces of examined tube specimens, hidden communities of these vestimentiferans would harbor epibenthos and extend their southern limits.

## Symbiotic bacteria of *Lamellibrachia barhami* from off Chile

The 16S rRNA sequence of symbiotic bacteria was determined through direct sequencing of the total DNA extracted from the degraded tissue of *L. barhami* (GK607). Although vestimentiferans host multiple symbiont lineages [65], Gammaproteobacteria are dominantly present in the trophosome, thus their sequences may be determined by direct sequencing. The present

sequence was identical to Gammaproteobacteria-affiliated 16S rRNA sequences obtained from *E. laminata*, *L.* sp. 1, and *L.* sp. 2 from the Gulf of Mexico [66]. A similarity in the sequences of symbiotic bacteria of GK607 and those of vestimentiferans inhabiting the Gulf of Mexico support previous reports that close relationships have been shown for symbiotic bacteria of vestimentiferans separated by great distances [40, 66].

## Conclusions

The present study represents an additional case study that the bycatch of deep-sea commercial fishing provides valuable information about rare species (see Introduction).

We successfully extracted total DNA from dried-up tissue and tubes of vestimentiferans, and showed that dried tubes, in addition to degraded tissue [44], are usable to obtain DNA. The Molecular phylogenetic analysis based on the COI gene successfully identified *Lamellibrachia* specimens, which are difficult to identify from the morphological characters of tubes. In addition to the COI gene, the hbB2i sequences were useful to identify the *Escarpia* species, as was reported by Cowart et al. [33]. Although the duration of DNA in the vestimentiferan tubes remains unknown, extracting DNA from the tubes is thus useful to identify tube-building species.

Our records of *E. spicata* and *L. barhami* from Chile considerably extend the previously-known geographic distribution of these two species; *E. spicata* was previously known to exist north of Mexico, whereas *L. barhami* was known to exist north of Costa Rica. A patchy distribution of reducing environments may account for the sparse records of vestimentiferans in the southeastern Pacific. A broad geographic species distribution is, however, not uncommon among deep-sea organisms, sometimes through a whole stretch of a submarine ridge or a continental margin [49, 50, 52].

The presence of these vestimentiferans provides a sound evidence for the occurrence of reducing environments along the continental margin in the northern Chile. Heterogeneous environments may partly explain the high biodiversity existing in the fishing grounds of *Dissostichus eleginoides*, whose habitat is related to such reducing environments [67].

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**Funding acquisition:** Genki Kobayashi.

**Methodology:** Genki Kobayashi.

**Project administration:** Genki Kobayashi, Juan Francisco Araya.

**Validation:** Genki Kobayashi, Juan Francisco Araya.

**Visualization:** Genki Kobayashi, Juan Francisco Araya.

**Writing – original draft:** Genki Kobayashi, Juan Francisco Araya.

**Writing – review & editing:** Genki Kobayashi, Juan Francisco Araya.

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