

Mitochondrial and nuclear markers reveal a lack of genetic structure in the entocommensal nemertean *Malacobdella arrokeana* in the Patagonian gulfs

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Abstract *Malacobdella arrokeana* is an entocommensal nemertean exclusively found in the bivalve geoduck *Panopea abbreviata*, and it is the only representative of the genus in the southern hemisphere. To characterize its genetic diversity, population structure and recent demographic history, we conducted the first genetic survey on this species, using sequence data for the cytochrome oxidase I gene (COI), 16S rRNA (16S) and the internal transcribed spacer (ITS2). Only four different ITS2 genotypes were found in the whole sample, and the two main haplotypes identified in the mitochondrial dataset were present among all localities with a diversity ranging from 0.583 to 0.939. Nucleotide diversity was low ($\pi = 0.001\text{--}0.002$). No significant genetic structure was detected between populations, and mismatch distribution patterns and neutrality tests results are consistent with a population in expansion or under selection. Analysis of molecular variance (AMOVA) revealed that the largest level of variance observed was due to intrapopulation variation (100, 100 and 94.39 % for 16S, COI and ITS2, respectively). F_{st} values were also non-significant. The observed lack of population structure is likely due to high levels of genetic connectivity in combination with the lack or permeability of biogeographic barriers and episodes of habitat modification.

Keywords Nemertean · Entocommensal · Geoduck · Genetic variation · Patagonia

Introduction

Few studies in nemertean biology have examined the genetic structure of nemertean populations (Sundberg and Strand 2007; Thornhill et al. 2008; Andrade et al. 2011). Here, we present the first genetic characterization of the genetic diversity, population structure and recent demographic history of the entocommensal nemertean *Malacobdella arrokeana* Ivanov et al. 2002. Members of the genus *Malacobdella* belong to the order Hoplonemertea. Shared characteristics include direct development and non-feeding planuliform larvae (Maslakova and von Döhren 2009). *Malacobdella arrokeana* lives commensally within the mantle cavity of the bivalve geoduck *Panopea abbreviata* Valenciennes, 1839 and is the only species of the genus reported in the southern Atlantic Ocean (Ivanov et al. 2002). It attaches to host mantle tissues by means of its terminal sucker. In general, each bivalve hosts a single mature nemertean. Teso et al. (2006) and Vázquez et al. (2009) reported that from 99.4 to 100 % ($n = 762$ geoducks in total from the two studies) of *P. abbreviata* were inhabited by at least one specimen of *M. arrokeana* (with a maximum of 191 specimens in one clam). Populations of *M. arrokeana* and its host, *P. abbreviata* (also an endemic species of the South Atlantic Ocean), are abundant in the northern Patagonian gulfs; they live in aggregations, buried deeply (up to 40 cm in depth) in sand and mud substrata (Morsan et al. 2010 and references therein). The northern Patagonian San Matías, San José and Nuevo gulfs (Fig. 1A) occur at the limit of the Magellanic and

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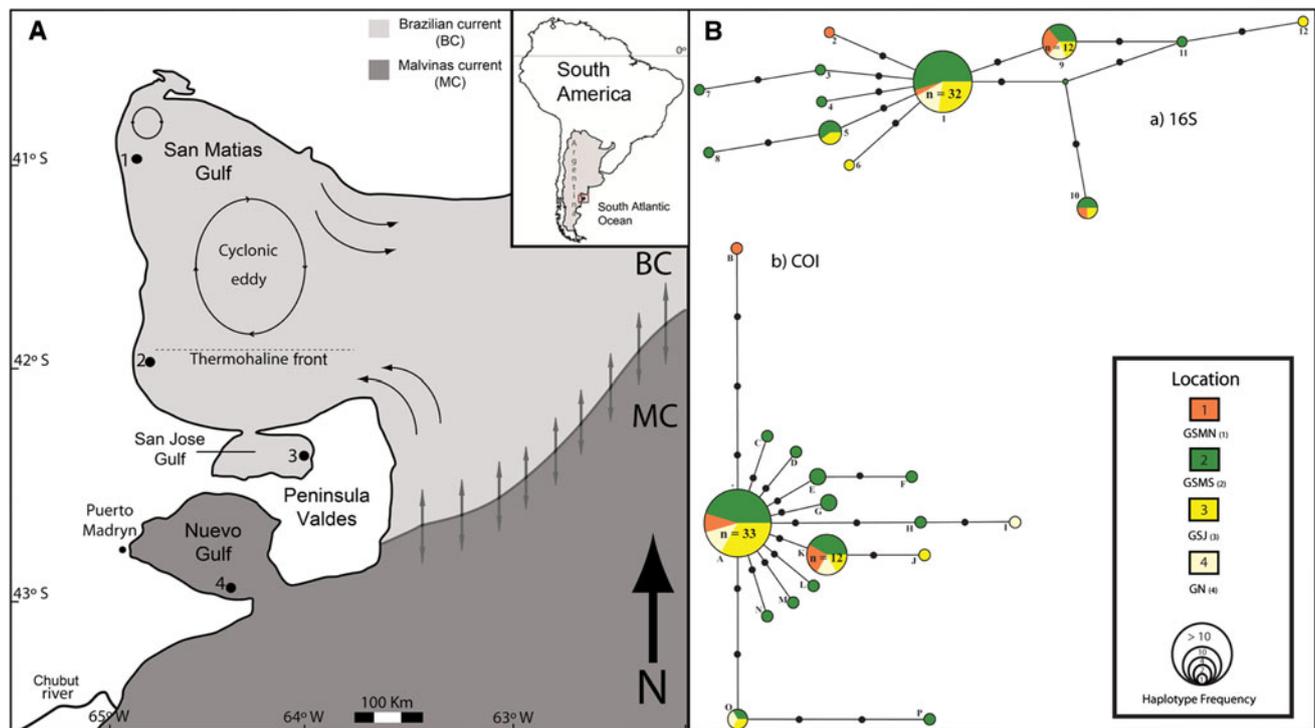


Fig. 1 **A** Southern Atlantic Ocean populations of the geoduck *Panopea abbreviata* were collected from: 1 El Sótano (northwest coast of San Matías gulf), 2 Puerto Lobos (southwest coast of San Matías gulf), 3 Punta Conos (San José gulf) and 4 Bahía Craker (Nuevo gulf). In all areas, samples were collected at depths between 5 and 25 m. **B** Networks of North Patagonian gulf populations of *Malacobdella arrokeana* analyzed, using 95 % connectivity level. **a** Network based on 16S mtDNA. **b** Network based on COI mtDNA. Alphanumeric names (designated by letters corresponding to COI

sequences and numbers corresponding to 16S sequences) are provided for each haplotype. Sampled haplotypes are indicated by colored circles, according to the geographical region from which the sample was collected as in **A**; missing or unsampled haplotypes are indicated by black dots. Each branch indicates a single mutational difference. Circle size is proportional to observed haplotype frequency; observed number of ancestral haplotypes is indicated by “*n*=” (the number of times each haplotype was obtained). *GSMN* northern San Matías gulf, *GSMS* southern San Matías gulf, *GSJ* San José gulf, *GN* Nuevo gulf

Argentinian biogeographic provinces. Each of these provinces has different oceanographic conditions (Scarabino 1977, Boschi 2000). The latitudinal thermal gradient in this area is controlled by the shallow and cold Malvinas current that flows northwards and by the warm Brazilian current that flows southwards (Martínez and del Río 2002). Peninsula Valdes is considered the confluence zone and a natural barrier. However, some taxa are represented in both provinces (Boschi 2000); this is the case for *M. arrokeana* and its host.

Previous studies on other marine invertebrates showed differences among Patagonian gulf populations. For example, Real et al. (2004) reported genetic variation between northern and southern San Matías populations of the scallop *Aequipecten tehuelchus*, estimating that genetic distances were higher between San Matías populations than between these populations and San José gulf population. *Panopea abbreviata* specimens from different populations of the San Matías gulf indicate different rates of individual growth between populations located on either side of the thermohaline front (Morsan et al. 2010). Considering the

supposedly limited larval dispersal capacity of *M. arrokeana*, its strict association with a sessile bivalve and the particularities of its habitats (in gulfs isolated due to different causes), a certain level of genetic structure among *M. arrokeana* populations is expected.

Materials and methods

Sampling

Malacobdella arrokeana specimens were sampled in an area that covers the entire Argentinian *P. abbreviata* distribution sensu Scarabino (1977), from Mar del Plata (800 km North of GSM, coast of Argentina 38°S) to Puerto Deseado (550 km south of the Nuevo gulf) at depths ranging from 15 to 20 m. Specimens were only found in three northern Patagonian gulfs: San Matías-north, El Sótano (*GSMN*, *n* = 7), and San Matías-south, Puerto Lobos (*GSMS*, *n* = 35); San José (*GSJ*, *n* = 17) and Nuevo (*GN*, *n* = 8) (Fig. 1A). The clams were collected by SCUBA

diving, using a hydro-jet to dislodge them from the substratum.

Clams were transported isolated in plastic bags and opened in the laboratory by severing both anterior and posterior adductor muscles. All of the *M. arrokeana* inside each clam were removed alive using a scalpel, and immediately fixed in absolute ethanol. The low numbers of samples from the GSMN and GN populations were due to low *P. abbreviata* population densities at greater depths (25–30 m).

DNA extraction, PCR amplification, and sequencing

Nemertean (and voucher geoducks) specimens were stored in absolute ethanol. DNA was extracted from preserved tissue using the DNeasy extraction Kit (Qiagen, Inc.) according to the manufacturer's protocol. Partial nemertean COI, 16S and ITS2 sequences were amplified by PCR using the following primers: 16sar-L and 16sbr-H (Palumbi et al. 1991) for 16S; LCO1490 (Folmer et al. 1994) and COI-H (Machordom et al. 2003) for COI and ITS2-3d (White et al. 1990) and ITS2-4r (5'-AGT TTY TTT TCC TCC GCT TA-3') (modified from White et al. 1990) for ITS2. Amplifications were carried out in a 50 μ l of final volume reaction containing 5 μ l of 10 \times buffer (containing 10 \times 2 mM MgCl₂), 1 μ l dNTPs mix (10 mM), 0.8 μ l of each primer (10 μ M), 0.4 μ l of *Taq* DNA polymerase (5 U/ μ l) (Biotools) and 1–3 μ l of genomic DNA. Thermocycling for the COI fragment included an initial 4 min denaturation step at 94 $^{\circ}$ C, followed by 40 cycles of 45 s at 94 $^{\circ}$ C, 1 min at 46.5 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C. The cycle ended with 10 min of sequence extension at 72 $^{\circ}$ C. For 16S and ITS2, we used the same cycle parameters, but annealing temperatures were 42 and 47 $^{\circ}$ C, respectively. Products were visualized under blue light in 0.8 % agarose gels stained with SYBR Safe (Invitrogen), with co-migrating 100 bp or 1 Kb ladder molecular weight markers. The amplification products (approximately 700 bp for each gene) were purified by ethanol precipitation. Sequencing of selected fragments was performed for both strands in an automatic ABI 3730 sequencer (Applied Biosystems Inc.) using BigDye Terminator kits.

Approximately 1900 base pairs were sequenced for *M. arrokeana* (658 bp for the mitochondrial COI, 533 bp for 16S and approximately 700 bp for the nuclear ITS2) (GenBank accession numbers JX220535–JX220725).

Data analysis

Sequences of each sample analyzed were refined by strand comparison, and primers sequences cut using the Sequencher program (Gene Code Corporation) and aligned using Se-AL 2.0a11 (Rambaut 2002). Haplotype networks analyses were

performed using TCS 1.18 (Clement et al. 2000) and Network 4.5 (www.fluxus-engineering.com), with default parameters, 95 % limit connection and gaps considered as either a fifth character state or as missing data. Only the 16S data set presented a reticulation.

For each fragment, and when appropriate for a concatenated matrix of COI, 16S and ITS2 data, genetic parameters of differentiation (e.g., number of haplotypes, haplotype and nucleotide diversities, F-statistics, AMOVA [considering each locality separately and also grouped by gulf], mismatch distribution, Tajima's *D*, Fu's *F_s* tests of neutrality) were obtained using the following software: Arlequin 3.5 (Excoffier and Lischer 2010) and DnaSP 5.0 (Librado and Rozas 2009).

To test the hypothesis of correlation between genetic and geographic distance, we performed 1000 randomizations of a Mantel test using the program IBDWS (Jensen et al. 2005) and the *F_{st}* and linearized *F_{st}* (i.e., $F_{st}/(1 - F_{st})$) against the logarithm of geographic distance. Demographic population history was inferred by comparing mismatch distributions of pairwise nucleotide differences among haplotypes of each gene, both separately and combined, using Arlequin 3.5.

Results

Specimens of *P. abbreviata* were only found from the San Matías gulf to the Nuevo gulf, and no evidence of living or empty shells was reported south of the Nuevo gulf. All but 3 of the geoducks (2 from GSMS and 1 from GSJ) sampled contained a single adult specimen of *M. arrokeana*. The two mentioned clams from GSMS hosted 3 and 6, and the clam from GSJ 7 immature nemerteans, respectively, but no mature ones.

Sixteen exclusive haplotypes were found for COI, twelve for 16S and four for ITS2. The two most frequent haplotypes observed for COI and 16S (found in 33 and 12 specimens for COI and 32 and 12 for 16S) were shared among all localities. Haplotype diversity (*H_d*) was relatively high, ranging from 0.583 to 0.939 (Table 1). The different networks for each gene or for the matrix that included the 3 gene regions (Fig. 1B, ITS2 not shown) revealed a high number of rare haplotypes, differing primarily by a single mutational step with the most frequent haplotype/genotype. Therefore, low nucleotide diversity (π) of 0.001–0.002 was observed (Table 1). The majority of the rare haplotypes belonged to the immature specimens, which did not have the most frequent haplotypes (these were only found in mature specimens).

There was almost no genetic differentiation among populations: AMOVA, either examining each population separately or grouped by gulfs, revealed that most of the

Table 1 Genetic population parameters recorded for *M. arrokeana* in the four different populations

	16S					COI				
	<i>n</i>	Hd	π	Fu's	Ta'D	<i>n</i>	Hd	π	Fu's	Ta'D
GSMN	5	0.700	0.002	-0.475	0.243	7	0.714	0.001	-0.237	0.206
GSMS	32	0.671	0.002	-4.163*	-1.362	32	0.778	0.002	-9.171***	-2.058*
GSJ	16	0.683	0.002	-3.011	-0.793	15	0.447	0.001	-0.064	-1.009
GN	8	0.607	0.001	-0.478	-0.448	8	0.750	0.002	-0.197	-1.280
TOTAL	61	0.673	0.002	-6.973***	-1.436	63	0.687	0.002	-10.960***	-2.034*

	ITS-2					3 MARKERS				
	<i>n</i>	Hd	π	Fu's	Ta'D	<i>n</i>	Hd	π	Fu's	Ta'D
GSMN	7	0.809	0.001	-1.390	-0.654	5	1.000	0.001	-2.371	-0.190
GSMS	35	0.569	0.001	-1.576	-0.793	30	0.954	0.001	-11.974	-1.867
GSJ	17	0.323	0.000	-1.038	-1.069	13	0.871	0.001	-5.391	-0.945
GN	8	0.750	0.001	-1.096	-0.304	8	0.964	0.001	-2.870	-0.958
TOTAL	67	0.583	0.001	-0.893	-0.387	56	0.939	0.001	-26.19	-1.727

GSMN and GSMS northern and southern populations of the San Matías gulf, GSJ San José gulf, GN Nuevo gulf, *n* number of samples, Hd haplotype diversity, π nucleotide diversity, Fu's and Ta'D represent the values obtained in Fu's *F_s* and Tajima's *D* tests, respectively. * $p < 0.05$; *** $p < 0.001$

observed variance was due to intrapopulation variation (100, 100, and 94.39 % for 16S, COI, and ITS2, respectively). F_{st} values for the four populations were negative for both of the mitochondrial genes and not statistically significant. Since the range for F_{st} values is 0–1, but due to software limitations, negative values are sometimes provided for very small F_{st} values, such figures should be considered zero (Long 1986), indicating no differentiation among populations. For ITS2, F_{st} was 0.056, which was also not significant ($p = 0.067$). Mantel tests showed no relationships between the different F_{st} values ($Z = 0.0059$, $r = -0.1583$, $p = 0.62$) or the linearized F_{st} values ($Z = 0.0132$, $r = -0.1611$, $p = 0.64$), with respect to geographic distances.

Mismatch distributions for each gene and population, and for combined matrices showed the same expansion/selection model profile, except for COI sequences from Nuevo gulf specimens, which resulted in a bimodal curve. In addition, Fu's *F_s* and Tajima's *D* values were mainly negative (Table 1) and significant when the number of specimens analyzed was sufficiently large for both mitochondrial markers. Considering all of the populations together, Fu's *F_s* and Tajima's *D* values were both significant only for COI, and Fu's *F_s* was only significant for 16S. Although both parameters were also negative for ITS2, no significance was detected.

Discussion

No *P. abbreviata* populations were found outside of the Patagonian gulfs, and *M. arrokeana* was never found

outside of its host or in other bivalves. The difficulty in host sampling (as animals are deeply buried in the sediment) makes extraction by trawling impossible, and the low visibility in waters outside the northern Patagonian gulfs makes manual extraction difficult. Furthermore, north of the Patagonian gulfs, *P. abbreviata* lives in deeper waters (at depths of 70–200 m) (F. Scarabino, personal communication). The low density observed in Nuevo gulf might denote the distribution limit of *P. abbreviata*.

In our study, a lack of genetic differentiation was observed among populations living in the different gulfs. This result is consistent with previous studies in nemertean genetic populations. Rogers et al. (1997) found evidence of low genetic differentiation in populations of two different free-living nemertean species, which are separated by large geographic distances (thousands of km). Thornhill et al. (2008) and Sundberg and Strand (2007), using partial COI and 16S gene sequences, also detected a homogeneous genetic structure in populations of the heteronemertean *Parborlasia corrugatus* from South America and of *Riseriallus oculatus* from Spain and Wales, respectively. Thornhill et al. (2008) only observed genetic structure on either side of Drake Passage, a strong barrier to gene flow.

Several aspects of nearshore oceanographic conditions in the Patagonian gulfs are consistent with the geographical patterns in gene flow observed in this study. Along the Patagonian coast, the tidal range is among the highest in the world, generating strong currents and significant variations in sea level (Palma et al. 2004). The shelf water of the Malvinas current enters from south of the San Matías gulf, determining a water residence time in the San Matías

gulf of approximately 300 days (Rivas and Beier 1990). Low levels of genetic differentiation related to larval retention and oceanographic conditions were previously shown in many invertebrate species populations along the Pacific coast (Sotka et al. 2004; Kelly and Palumbi 2010). Besides, the Argentinean coasts suffered several glacial periods (following the Pleistocene, as well as in the Late Miocene and Holocene) that resulted in changes in sea level, salinity and temperature (Martínez and del Río 2002). Due to such changes, new available niches might have led to the rapid expansion of cold-water species, such as *P. abbreviata* and therefore to its closely associated entocommensal, *M. arrokeana*. Population expansion from restricted areas into newly available habitats from marine refugia could explain the loss of genetic structure of nearshore fishes and invertebrates (Sotka et al. 2004 and references therein).

Interestingly, only one mature *M. arrokeana* is usually found inside a single geoduck. When more than one *M. arrokeana* were found inside a single *P. abbreviata*, those nemerteans were immature (Ivanov et al. 2002; Teso et al. 2006; Vázquez et al. 2009; this study). This finding indicates an exclusion process during maturation of the nemertean, likely due to intraspecific competition for space (see Bush and Lotz 2000), chemical inhibition of maturation between individuals (Teso et al. 2006), or both. Our results showed that the three cases in which more than one nemertean was found per geoduck, the corresponding specimens presented a proportionately larger number of rare haplotypes (mature-common, immature-rare). Although based on the low numbers of immature specimens examined here, we wonder whether, in addition to the factors mentioned above, a selection of common haplotypes could be acting on these populations. Future research efforts, such as analyzing more cases of multiple specimens in a single clam in combination with the analysis of highly variable markers (e.g., microsatellites), will help shed light on the potential selection pressures affecting populations of *M. arrokeana*.

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