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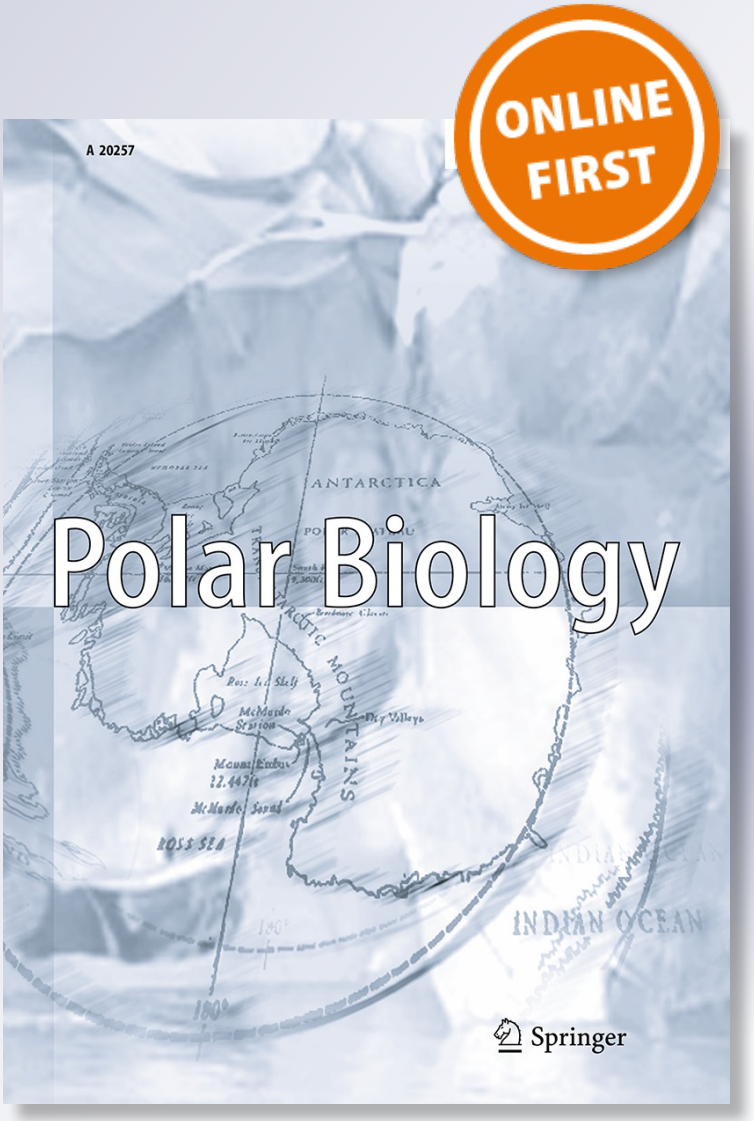

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ORIGINAL PAPER

Genetic variability and differentiation among polymorphic populations of the genus *Synoicum* (Tunicata, Ascidiacea) from the South Shetland Islands

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Abstract In Antarctica, ascidians are among the most conspicuous and abundant organisms in benthic ecosystems and many species present wide distribution patterns. Two similar forms of ascidians of the colonial genus *Synoicum* were sampled along the South Shetland Islands, one greenish-yellow with elongated colony stalks and the other yellow-orange with shorter stalks and more rounded colony bodies. The taxonomic analyses indicated that the greenish-yellow form corresponded to the description of the species *S. ostentor* and the yellow-orange form to that of *S. adareanum.* However, molecular analyses using the cytochrome oxidase I gene did not detect clear differences between both forms. The frequency and geographic distribution of haplotypes indicate that some degree of gene flow may be occurring, strongly suggesting that both morphotypes did not achieve a complete reproductive isolation yet, that they can still interbreed and should therefore be considered as a single species. Surprisingly, these results are not in line with recent studies of Antarctic fauna using a similar approach, which revealed several cases of morphologically indistinguishable but genetically distinct species.

Keywords *Synoicum* · COI · Morphotypes · Genetic differentiation

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Introduction

The Antarctic Ocean has unique characteristics, mostly because unlike the other major oceans, it became isolated about 30 million years ago in the Eocene, with the establishment of the Antarctic Circumpolar Current (ACC) and its associated fronts (Lawver et al. 2011). The ACC acts as a barrier to a free north–south exchange of water, isolating Antarctic shelf communities geographically, climatically, thermally and oceanographically, therefore forming an important biogeographic boundary (Clarke and Crame 1989; Clarke et al. 2005; Barnes et al. 2006). It now displays a unique shelf biota, with ascidians among the most important taxa in the benthic communities (Dayton 1990; Clarke 1996; Clarke and Johnston 2003; Gili et al. 2006).

Synoicum adareanum (Herdman 1902; family Polyclinidae) is a colonial ascidian commonly reported on hard substrates in shallow Antarctic waters. Its wide distribution extends from some subantarctic islands, throughout the Scotia Arc into the Antarctic Peninsula and all along the Continental Antarctic coastline. The clearly visible zooids are clonemates arranged in the tunic forming a circle around a common cloaca that is shared by 6 to 10 zooids (Kott 1969). A similar species, *Synoicum ostentor* Monniot and Monniot 1983, shares some characteristics with the

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former such as similar colony shape and arrangement of the zooids. *S. ostentor* has been found in areas quite distant from each other: it has been reported at the South Orkney Islands, Balleny Island and Wilkes Land, and its presence is not common (Monniot and Monniot 1983; Monniot et al. 2011). According to Monniot and Monniot (1983), the diagnostic features of both species are as follows: the number of stigmata rows, the number of stigmata per half-row and the position of the ovary. *S. ostentor* may have been misidentified and confused with *S. adarenaum* in many ecological and biochemical studies, because the differences between both species are noticeable only after inspection of the zooids at microscopic level. The in vivo coloration in both species is not mentioned, probably because determinations have been made on formaldehyde fixed material (Kott 1969; Monniot and Monniot 1983; Monniot et al. 2011). Kott (1969), however, mentions one collector who observed that living colonies of *S. adareanum* were orange. Recently, Koplovitz et al. (2011) found that *Synoicum adareanum* from the area of the US Palmer Station (Anvers Island, Western Antarctic Peninsula) differing in the form of the colonies, showed marked differences in the anti-diatom activity of secondary metabolites. No reference of the color of the colonies was made. At the South Shetland Islands, two similar forms of *Synoicum* are common: one greenish-yellow with elongated colony stalks and other yellow-orange with shorter colony stalks and more rounded heads (Tatián, personal observation). It is probable that previous taxonomists considered both forms as *S. adareanum* since most of the descriptions (Herdman 1902; Van Name 1945; Millar 1960; Kott 1969; and others) included under *S. adareanum* the whole range of the 3 differential features of the zooids used by Monniot and Monniot (1983) to separate the species. Since these color differences have not been reported in the previous descriptions for either species, the question arises if these two forms correspond to intra-specific polymorphisms within a single species, or to different species.

Besides morphological analyses, in recent years, molecular markers have made it possible to distinguish intraspecific polymorphisms from phenotypic traits that characterized different species in ascidians. For example, color differences in populations of *Botryllus schlosseri* have been found to be determined by several Mendelian loci, and therefore, differences in coloration are an intraspecific polymorphism (Sabbadin 1982; Yund and O'Neil 2000). On the other hand, a correlation between external shape and coloration with the presence of different species has been reported for the colonial ascidians *Pseudodistoma crucigaster* (Tarjuelo et al. 2004), species of *Cystodytes* (López-Legentil et al. 2005) and of *Pycnoclavella* (Pérez-Portela et al. 2007) from the Mediterranean Sea, and *Didemnum molle* from the Ryukyu Islands in Japan (Hirose et al. 2010).

In Polyclinidae, sexual reproduction occurs when sperm released by a hermaphroditic zooid enters through the oral siphon of another zooid from the same or another colony and fertilizes oocytes in the atrium, self-fertilization being a possible trait in this family. The embryonic stage consists of a pelagic tadpole larva with a tail that allows some movements, and it can be easily driven by currents. Given that the larval stage is responsible for dispersion in sessile organisms, species with short-lived larvae or direct development are, in general, expected to have low dispersal potential and therefore to show a strong genetic structure (reviewed in Thatje 2012 for Antarctic invertebrates). All ascidian larvae are lecithotrophic, that is, they do not feed while in the water column and depend on the yolk reserves. Therefore, their life spans are short when compared with other taxa; in colonial ascidians, the free swimming time of larvae ranges from a few minutes to several hours (Svane and Young 1989). When the larva disperses from a mother colony, it rapidly settles down and metamorphoses into a founder individual (oozooid) that, like other clonal invertebrates, grows through asexual reproduction into a multi-individual colony composed of genetically identical zooids (Stoner and Weissman 1996). Demarchi et al. (2010) found a highly significant genetic differentiation among populations of the polyclinid *Aplidium falklandicum* from Low and Livingston Islands. Therefore, it can be expected that *Synoicum* will also present highly differentiated populations at the same geographic scale.

Mitochondrial DNA, and specifically the COI gene, has been used successfully to address speciation and species boundaries problems in several ascidian species and in phylogenetic and phylogeographic studies of this group (Tarjuelo et al. 2001, 2004; Turon et al. 2003; López-Legentil and Turon 2006; López-Legentil et al. 2006; Pérez-Portela and Turon 2008; Hirose et al. 2009; Goldstien et al. 2010; Haydar et al. 2011; Pineda et al. 2011). In the present study, we used morphological analyses and molecular markers (cytochrome oxidase subunit I gene, COI) to evaluate the genetic structuring of four *Synoicum* populations from the Bransfield Strait (Western Antarctic Peninsula) and assess whether the different morphotypes observed in *Synoicum* ascidians along the South Shetland Islands correspond to different or to a single species.

Materials and methods

Sample collection

A total of 53 colonies of *Synoicum* were collected from the South Shetland Islands during the BENTART-06 Antarctic Project of the "BIO Hespérides" in January and February 2006, and during the summer campaign of the Argentine

Antarctic Institute at Potter Cove, in January 2009. One sampling station was located at Deception Island (63°01′S, 60°32′W; 215 m depth; $n = 11$), at which all individuals belonged to the yellow-orange morphotype (YO from now on). Another two stations were sampled at Low Island: Low 45 (63°43′S, 62°21′W; 86 m depth; $n = 14$), where 10 individuals belonged to the greenish-yellow (YG) morphotype and 4 individuals to the YO morphotype and Low 46 (63°43′S, 62°24′W; 97 m depth; $n = 20$), where 10 individuals were YG and the other 10, YO. The last station was sampled at King George (25 de Mayo) Island (Potter Cove; 62°13′S, 58°42′W; 20 m depth; $n = 8$), where all individuals were YO. Samples from Deception, Low 45 and Low 46 were collected with an Agassiz trawl, while samples from Potter Cove were obtained by SCUBA diving. Colonies from Deception, Low 45 and Low 46 sampling stations were photographed immediately after collection. Sampling by SCUBA diving at Potter Cove allowed in situ observations of the colonies and underwater pictures. Sampling locations are shown in Fig. 1 and photographs of the colonies showing the in vivo color are presented in Online Resource 1. Once they were collected, colonies were relaxed by submerging them for several hours in sea water with menthol crystals. Then, a small portion of each colony was dissected, fixed in ethanol 95 % and stored at −20 °C until genetic analysis. Zooids of each ethanol-preserved colony were separated using histological instruments under a binocular microscope and processed by removing the tunic, gut and larvae, if any, to avoid amplification from possible contaminants like commensal organisms and gut contents. The bulk of the colonies were fixed in 2.5 % formaldehyde in sea water, to allow the examination of zooids and morphological analysis.

Morphological observations

Morphological characters that differentiate *S. adareanum* from *S. ostentor* according to Monniot and Monniot (1983): number of stigmata rows, number of stigmata per half-row and position of the ovary were analyzed under a stereoscope from material fixed in formaldehyde. Three colonies and 10 zooids per colony of each morphotype were examined per sampling location.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the post-abdomen of an individual zooid per colony using the salt purification method described in protocol 1 of Bruford et al. (1992), followed by ethanol precipitation. Once extracted, DNA was stored in double-distilled water at 4 °C until PCR amplification. Double-stranded products for the mitochondrial cytochrome oxidase I gene (COI) were amplified via the polymerase chain reaction (PCR) using the universal primers of Folmer et al. (1994), and purified and sequenced in both directions at Macrogen (USA). However, only four of the fifteen haplotypes found among the 53 zooids sequenced, showed high homology with COI sequences of other species of the family Polyclinidae, after a BLAST search in GenBank. The remaining haplotypes showed high homology with bacterial COI genes and may belong to endosymbionts (Riesenfeld et al. 2008). Therefore, specific primers were designed starting from the consensus COI sequence for species of the family Polyclinidae obtained from GenBank and those sequences of *Synoicum* obtained with the universal primers that showed high homology with them. Designed primer sequences and names were as follows: PolCOI-F (5′-TTGATCTGCTCCTHCTTAGA-3′) and PolCOI-R (5′-CCACTAGARTGTGCTAARCC-3′). The expected size of the amplicon is 351 bp.

Polymerase chain reactions were carried out in 50 µl volumes. In addition to the DNA template from each specimen (1 µl from the undiluted DNA), PCR included 5 µl 10× reaction buffer [750 mM Tris–HCl pH 8.8, 200 mM $(NH_4)_2$ SO_4, 0.1 % Tween-20], 5 µl $MgCl_2$ 25 mM, 0.75 µl of a 20 mM stock of each dNTP (dATP, dCTP, dGTP, dTTP), 1.5 µl of a 10 mM stock of each PolCOI-F and PolCOI-R primers, 0.3 µl of recombinant Taq DNA Polymerase (5 U/µl stock; Thermo Scientific EP0402, Brazil) and ddH_2O to final volume. PCR amplification was performed in a Px2 Thermal Cycler, Thermo ELECTRON CORPORATION, for 3 min at 94 °C, 35 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min and extension at 72 °C for 1 min. A post-treatment of 7 min at 72 °C and a final cooling at 4 °C were performed. Negative controls for each primer were included in the amplifications in order to detect bands caused by contamination. PCR products were visualized on 1 % agarose gels stained with ethidium bromide, visualized under UV and photographed. The double-stranded PCR products were purified and sequenced by Macrogen Inc. (USA) using the same primers as for the PCR. Sequences were analyzed using the program CHROMAS version 2.23 (McCarthy 1998) and manually edited. In order to rule out the amplification of non-ascidian sequences, both strands were sequenced and a BLAST search on each haplotype was performed. Sequences were aligned with CLUSTAL X (Thompson et al. 1997) and confirmed by eye. No gap was needed in the alignment, and all sequences were able to be translated into amino acid sequences without stop codons. The final sequence length after alignment and trimming was 304 pb. Once aligned, sequences for all individuals were collapsed into haplotypes.

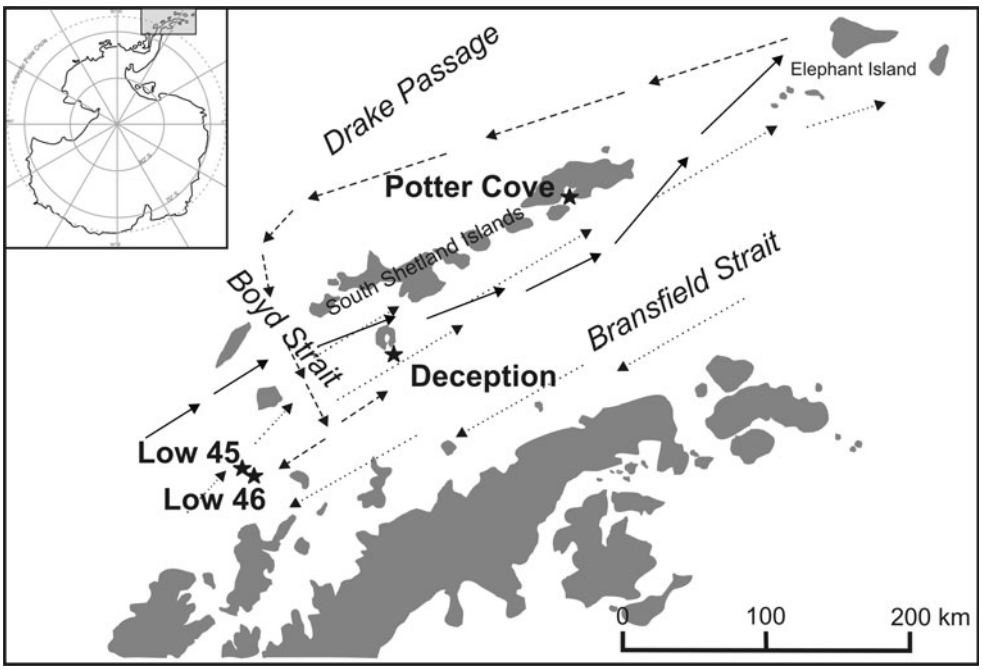

Fig. 1 Map of the study area showing the location of the sampling sites (*stars*). The *arrows* indicate the direction of the major water currents, drew according to the description in Savidge and Amft (2009). *Straight line*: South Shetland Islands Jet, *dashed line*: Shallow Polar Slope Current, *dotted line*: cyclonic circulation of Bransfield Strait

Population genetic analyses

Using the DNAsp 5.10 program (Librado and Rozas 2009), several indices of genetic diversity within sampling stations were estimated: nucleotide diversity (π), the number of polymorphic sites (S; Nei 1987; Lynch and Crease 1990), haplotype diversity (Hd; Nei 1987) and the average number of nucleotide differences.

Pairwise genetic distances were calculated between haplotypes under the Tamura-Nei (TrN) model of nucleotide substitution with a gamma parameter value of 0.032, using the ARLEQUIN 3.11 program (Excoffier et al. 2005). The TrN distance was selected according to the best-fit model of nucleotide substitution for our data (GTR+G) by statistical comparisons of the likelihood scores for 56 different models of evolution with the Akaike information criterion, as implemented in Modeltest 3.0 (Posada and Crandall 1998). This distance matrix was used to perform an analysis of molecular variance (AMOVA) and to calculate differentiation between pairs of samples with the Φ_{ST} index. Φ_{ST} is analogous to Wright's (1969) measure of variance in allelic frequencies among populations (F_{ST}), but also takes into account the mutational differences among haplotypes (Excoffier et al. 1992). AMOVA and Φ_{ST} were calculated considering geographic location, and geographic location and morphotype. Significance of all indices was tested through 10,000 permutations. The Arlequin program was used for these calculations. The correlation of pairwise Φ_{ST} values and geographic distance was assessed by means of a Mantel test, performed with Arlequin.

The relationships among haplotypes were estimated using different approaches. First, phylogenetic trees were obtained using maximum parsimony (MP) and neighbor-joining (NJ) with PAUP 4.0b10 (Swofford 2001), and Bayesian analysis (BA) with MrBayes 2.1.3 (Ronquist and Huelsenbeck 2003). The MP tree was estimated by performing a heuristic search with 1,000 replicates of random stepwise additions of taxa and a tree bisection reconnection branch swapping. The NJ tree was constructed on the basis of TrN distances among haplotypes. In both cases, 1,000 bootstrap replications were performed to construct a 50 % majority rule consensus tree, to evaluate the stability of the nodes. The BA was performed by partitioning the data set by codon position. Models of nucleotide evolution selected by MrModeltest 2.3 (Nylander 2004), according to the Akaike criterion were: GTR for the first, F81+I for the second and HKY for the third codon positions. Four chains of the Markov chain Monte Carlo algorithm were run simultaneously for 5 million generations, on two independent runs. Trees were sampled every 1,000 generations and the first 25 % of them were discarded as burn in. For this analysis, we also included a COI sequence of *S. adareanum* from Terre Adelie (BoLD Database ASCAN011-10) and used *Ciona intestinalis, Clavelina lepadimorfis* and *Herdmania momus* as outgroups (GenBank accession numbers NC_004447.2, NC_012887.1 and NC_013561.1, respectively). Second, a haplotype network was obtained using the NETWORK program (Bandelt et al. 1999), which implements the Median Joining Network algorithm to estimate relationships among haplotypes without assuming bifurcating relationships.

Fu's *Fs* (Fu 1997) was used to test for selective neutrality of the mutations using ARLEQUIN 3.11 (Excoffier et al. 2005). To evaluate a possible historical population expansion event, the distribution of the frequencies of pairwise differences between individuals was compared with the expected distribution of a model of population expansion as implemented in ARLEQUIN (Mismatch distribution, Rogers and Harpending 1992; Excoffier and Schneider 1999). The validity of the estimated demographic model was tested through the statistical distribution of SSD (sum of squared differences) with parametric bootstrap (Excoffier and Schneider 1999). The raggedness index (Harpending 1994) was estimated to test the deviation from unimodal mismatch distribution. Several *Fs* values and mismatch distributions were calculated considering: the whole data set, haplotypes found only in YO individuals, haplotypes found only in YO individuals plus shared haplotypes between the two morphotypes, and finally considering YG haplotypes and shared haplotypes between both morphotypes.

Results

Morphological observations

The zooids exhibited constant differences in the two morphotypes.

YO morphotype: orange-yellow colonies with short stalks and rounded heads. The thoraces were long, with 14–16 stigmata rows in the branchial sac. Stigmata per half-row varied from 24 to 26. The ovary was situated in the upper part of the post-abdomen, close below the intestine (abdomen).

YG morphotype: greenish-yellow colonies with elongated stalks and no rounded heads. The thoraces showed 21–22 stigmata rows in the branchial sac. Stigmata per half-row were from 31 to 34. The ovary was situated at a half way through the post-abdomen, far below the intestine (abdomen).

Population genetic analyses

A total of 53 sequences were obtained for the COI mitochondrial gene. The final length after alignment and trimming was of 304 base pairs (bp) and free of gaps. Seventeen different haplotypes were identified (Table 1) with 11 polymorphic sites. All unique haplotypes have been deposited in Genbank (accession numbers JX121258 to JX121274). The detail of the haplotypes found in each single colony is presented in Online Resource 2.

The number of haplotypes found in each sampling station, the number of polymorphic sites (*S*) and values of nucleotide diversity are shown in Table 1. The haplotype frequencies per sampling station are also presented in Online Resource 3. Nucleotide diversity over all samples is 0.009. Haplotype diversity is 0.763 ± 0.058 (mean ± SD), and the average number of nucleotide differences is 2.938. Potter Cove and Deception Island present the highest values of nucleotide diversity as well as of haplotype diversity. Only two haplotypes are shared between the two morphotypes (Hap VII and Hap VIII). The YO morphotype presents thirteen exclusive haplotypes, and the YG, two (Hap X and XI). From the fifteen haplotypes present in the YO form (thirteen exclusive, and two shared with YG), eleven polymorphic sites were found. The YG morphotype features two polymorphic sites that coincided with two of the eleven polymorphic sites from YO. Of the seventeen haplotypes found, only four are shared among sampling sites (Hap I, Hap II, Hap VII, Hap VIII), and the percentage of singletons (haplotypes represented by a single member in the data set) is 52.94 %. The most frequent haplotype (Hap VIII, observed in 47.17 % of the individuals and shared between YO and YG forms in Low 46) is broadly distributed, occurring at most localities within the study area (Low 46, Low 45 and Deception). The second most frequent haplotype (Hap VII) presents similar characteristics as Haplotype VIII. Deception presents three exclusive haplotypes (Hap VI, Hap IX and Hap XVII), as do Low 45 (Hap IV, Hap VI and Hap XI) and Low 46 (Hap III, Hap X and Hap XVI), while Potter Cove has four exclusive haplotypes (Hap XII, Hap XIII, Hap XIV and Hap XV).

Results of the AMOVA show that a significant part of the total variability can be assigned to the "among sampling sites" component, no matter the structure tested (Table 2). However, despite 16.5 % of the total variation corresponding to differences between groups of samples of different morphotypes, this value is not statistically significant (Table 2). Pairwise Φ_{ST} values are presented in Table 3; a significant correlation was found between genetic differentiation and genetic distance when morphotype was not considered ($r = 0.813$; $p = 0.041$; Online Resource 4a). Potter Cove was the most differentiated sample. Deception showed a significant genetic differentiation with Low 45 but not with Low 46, while there was no differentiation between sampling sites in Low Island (Table 3a). If morphotypes are taken into account, samples of the YG morphotype showed no genetic differentiation and the YO morphotype of Low 46 was less differentiated from YG samples than from the YO sample of Low 45 station (Table 3b). Indeed, separating sampling stations by morphotype yielded a non-significant correlation between Φ_{ST} and geographic distance ($r = 0.193$; $p = 0.245$; Online Resource 4b), which became significant when YO samples from Low 45 were eliminated from the analysis ($r = 0.906$; $p = 0.041$; Online Resource 4c). This is

Table 1 Number of haplotypes, nucleotide diversity (π), haplotype diversity (*Hd*) and number of polymorphic sites (*S*) found in *Synoicum* from four geographic locations at the South Shetland Islands. Haplotypes shared by YO and YG morphotypes are underlined

Sampling site	Haplotypes																	Total	π	*Hd*	*S*
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII				
Deception	–	1	–	–	–	2	2	4	1	–	–	–	–	–	–	–	1	11	0.012	0.855	10
Low 46	–	–	1	–	–	–	4	13	–	1	–	–	–	–	–	1	–	20	0.004	0.558	5
Low 45	1	–	–	1	2	–	1	8	–	–	1	–	–	–	–	–	–	14	0.008	0.681	7
Potter Cove	1	1	–	–	–	–	–	–	–	–	–	2	1	1	2	–	–	8	0.011	0.929	9
Total																		53	0.009	0.763	

Table 2 Analysis of molecular variance (AMOVA) for *Synoicum* samples, testing alternative spatial structures

Structure tested	Source of variation	Percentage of variation	*F* statistics	Probability
Sampling site (not considering morphotype)	Among sampling site	28.87	$F_{ST} = 0.289$	0
	Within sampling site	71.13		
Sampling site and morphotype	Among populations	43.18	$F_{ST} = 0.432$	0
	Within populations	56.82		
Two groups: YO sites versus YG sites	Among groups	16.52	$F_{CT} = 0.165$	0.133
	Among sampling sites within groups	30.68	$F_{SC} = 0.367$	0
	Within sampling sites	52.80	$F_{ST} = 0.472$	0

Table 3 Φ_{ST} values between (a) sampling stations of *Synoicum,* and (b) sampling stations considering morphotype. YO: yellow-orange morphotype, YG: yellow-green morphotype

(a)	Potter Cove	Deception	Low 45	Low 46
Potter Cove	–			
Deception	0.358**	–		
Low 45	0.659***	0.119*		
Low 46	0.409***	−0.042	0.046	–

(b)	Potter Cove (YO)	Deception (YO)	Low 45 (YO)	Low 46 (YO)	Low 45 (YG)	Low 46 (YG)
Potter Cove (YO)	–					
Deception (YO)	0.334***	–				
Low 45 (YO)	0.226	0.281*	–			
Low 46 (YO)	0.513***	0.023	0.550**	–		
Low 45 (YG)	0.673***	0.146	0.779**	0.008	–	
Low 46 (YG)	0.673***	0.146	0.779***	0.008	−0.111	–

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

because haplotypes VII and VIII (shared between morphotypes) were found in YO and YG individuals in Low 46, while in Low 45, they were found only in YG individuals, probably due to the low number of YO individuals that could be successfully sequenced in that station (only four).

In the two sampling sites where both morphotypes were found in sympatry, Tamura-Nei pairwise genetic distances between haplotypes (Table 4) showed that the highest genetic distances were those between YO haplotypes and haplotypes VII and VIII (between 3 and 6 % of sequence divergence). Genetic distances between exclusive YO

haplotypes, and those between YO haplotypes and YG exclusive haplotypes (Hap X and Hap XI), presented intermediate values (between 2 and 4 % of sequence divergence). The lowest distances were found between Haplotypes VII and VIII and between these and the YG haplotypes (1–2 % of sequence divergence).

Bayesian analyses placed haplotypes VII and VIII in the basal position (posterior probability 0.87). However, this was not supported by MP and neighbor-joining analyses (Fig. 2). These haplotypes were widely distributed in most sampling stations (Low 45, Low 46 and Deception), and in both morphotypes. Most of the remaining nodes showed low support in the three analyses. The haplotype network (Fig. 3) showed that Haplotypes VII and VIII, shared between morphotypes and widely distributed, are closely related, as they are separated by only one synonymous substitution from each other. The other two haplotypes shared among sampling sites were present in two YO samples (Hap I present in Low 45 and Potter Cove, and Hap II in Deception and Potter Cove) and were more distantly related between each other and with haplotypes VII and VIII. Relationships among exclusive haplotypes from Low 45, Low 46 and Deception showed no relationship with geographic location, while Potter Cove's exclusive haplotypes were grouped together and located at the edge of the network.

According to Fu (1997), *Fs* should be regarded as significant if $p < 0.02$. Therefore, significant and negative estimates for Fu's *Fs* were obtained for the whole data set ($Fs = -6.029$, $p = 0.012$) and for YO haplotypes ($Fs = -5.587$, $p = 0.004$), while *Fs* yielded marginally non-significant values when calculated for YO plus shared haplotypes ($Fs = -4.423$, $p = 0.035$), or YG plus shared haplotypes ($Fs = -1.450$, $p = 0.050$). A significant negative value of *Fs* is evidence for an excess number of alleles, as would be expected from recent population expansion or from genetic hitchhiking. The mismatch distribution analysis obtained for all analyses showed non-significant values of raggedness index and SSD (Fig. 4), supporting the hypothesis of demographic expansion. However, it is worth noting that the observed distribution of pairwise differences for all haplotypes, although not

Table 4 Tamura-Nei genetic distances between haplotypes (Hap) from Low 45 and Low 46. Bold letters indicate haplotypes found exclusively in YO individuals and italics indicate those found in the YG morphotype. Underlined haplotypes were shared between both populations and were found in individuals from both morphotypes in Low 46, and only in YG individuals, in Low 45

Low 45	**Hap I**	**Hap IV**	**Hap V**	Hap VII	Hap VIII	*Hap XI*
Hap I	–					
Hap IV	4.079	–				
Hap V	3.039	3.041	–			
Hap VII	3.076	5.105	4.065	–		
Hap VIII	4.084	6.161	5.105	1.008	–	
Hap XI	2.034	4.079	3.039	1.008	2.016	–

Low 46	Hap VIII	Hap VII	*Hap X*	**Hap XVI**	**Hap III**
Hap VIII	–				
Hap VII	1.008	–			
Hap X	1.008	2.016	–		
Hap XVI	3.039	4.079	2.031	–	
Hap III	5.135	4.079	4.127	2.031	–

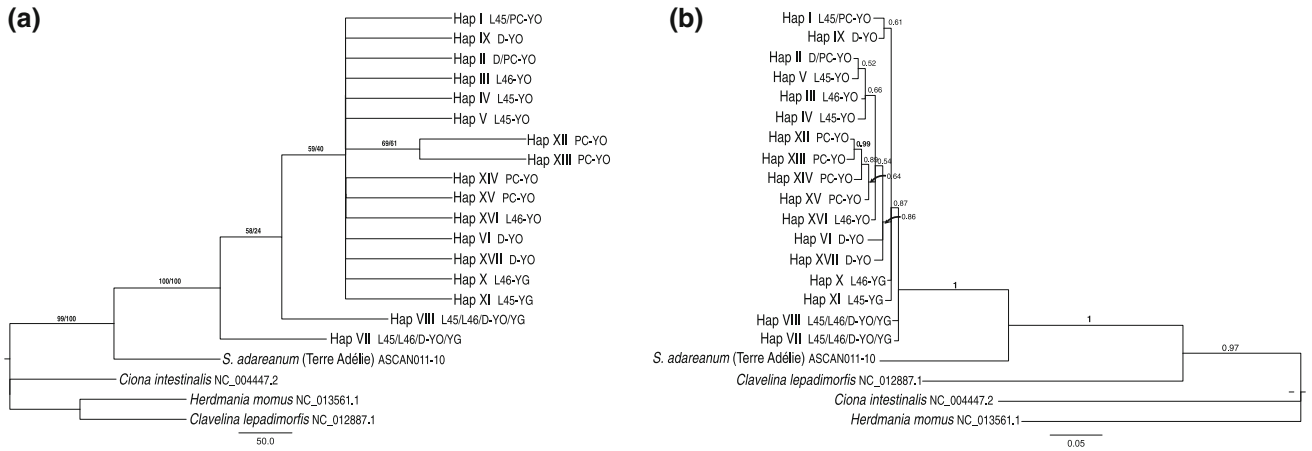

Fig. 2 Phylogenetic relationships among *Synoicum* haplotypes. (**a**) Neighbor-joining tree from Tamura-Nei genetic distances. The topology of a maximum parsimony tree was identical. Bootstrap values after 1,000 replications for both methods are shown *above* the *branches*: before the slash are values for the neighbor-joining tree and after the slash those for the maximum parsimony tree. (**b**) Bayesian tree, the posterior probabilities of each *branch* are shown *above* the *branches* are. *Clavelina lepadimorfis*, *Herdmania momus* and *Ciona intestinalis* were used as outgroups. YO: yellow-orange morphotype, YG: yellow-green morphotype, D: Deception, PC: Potter Cove, L45: Low 45, L46: Low 46

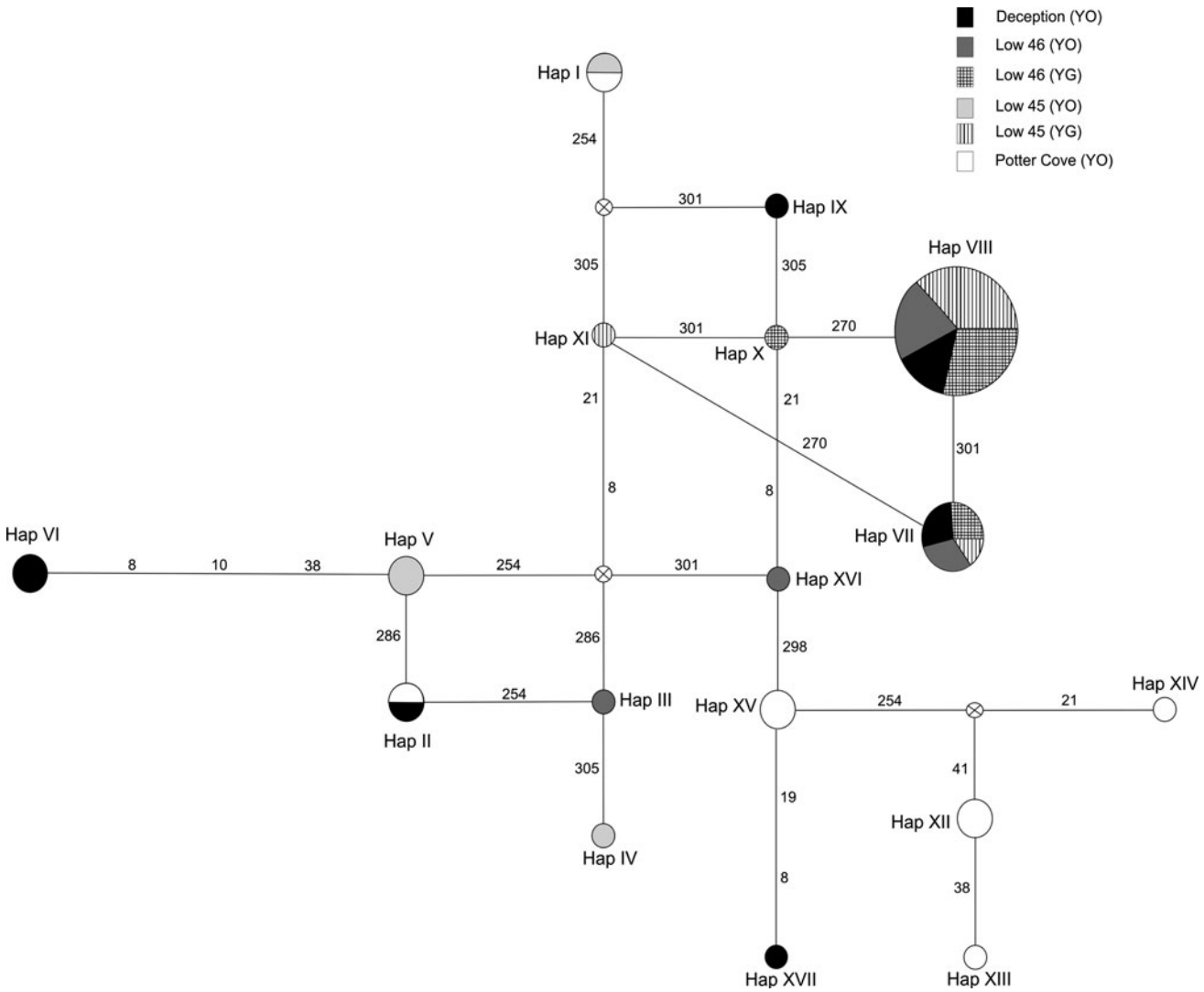

Fig. 3 Haplotype network of COI mitochondrial DNA sequence data. *Circles* represent haplotypes and *circle* diameter is proportional to haplotype frequency. *Each haplotype* is represented by the abbreviation Hap and a roman number. *Numbers above white circles with a cross in the center* show intermediate haplotypes not found in the study. *Numbers above the branches* are the positions of the mutated sites

significantly different from that expected under a population expansion, showed a bimodal shape, indicating the presence of two (or more) groups of divergent lineages. The parameter τ showed different values, being 5.824 for all haplotypes, 5.103 for YO plus shared haplotypes, 4.281 for YO haplotypes and 0.492 for YG plus shared haplotypes. These results indicate that time since the last demographic expansion event was longer for YO haplotypes than for YG ones, and the population expansion of YO morphotype would be more ancient.

Discussion

This study presents the first analysis of population genetic structure using a mitochondrial DNA marker in an ascidian species with an Antarctic and Sub-Antarctic distribution. Besides that, combined with morphological observations, it tries to clarify if the two morphotypes of *Synoicum* found at the South Shetland Islands correspond to different species or to intra-specific polymorphisms.

The morphological analysis showed that each morphotype may correspond to a different species according to Monniot and Monniot (1983). The taxonomic features that characterize YO zooids were consistent with those that define *Synoicum adareanum*, while those that characterized YG zooids were consistent with the features that define *Synoicum ostentor*. However, there were no significant differences in the COI gene between both forms living in sympatry (Table 3), and therefore, in the next paragraphs, the colonies will continue to be named as YG and YO morphotypes.

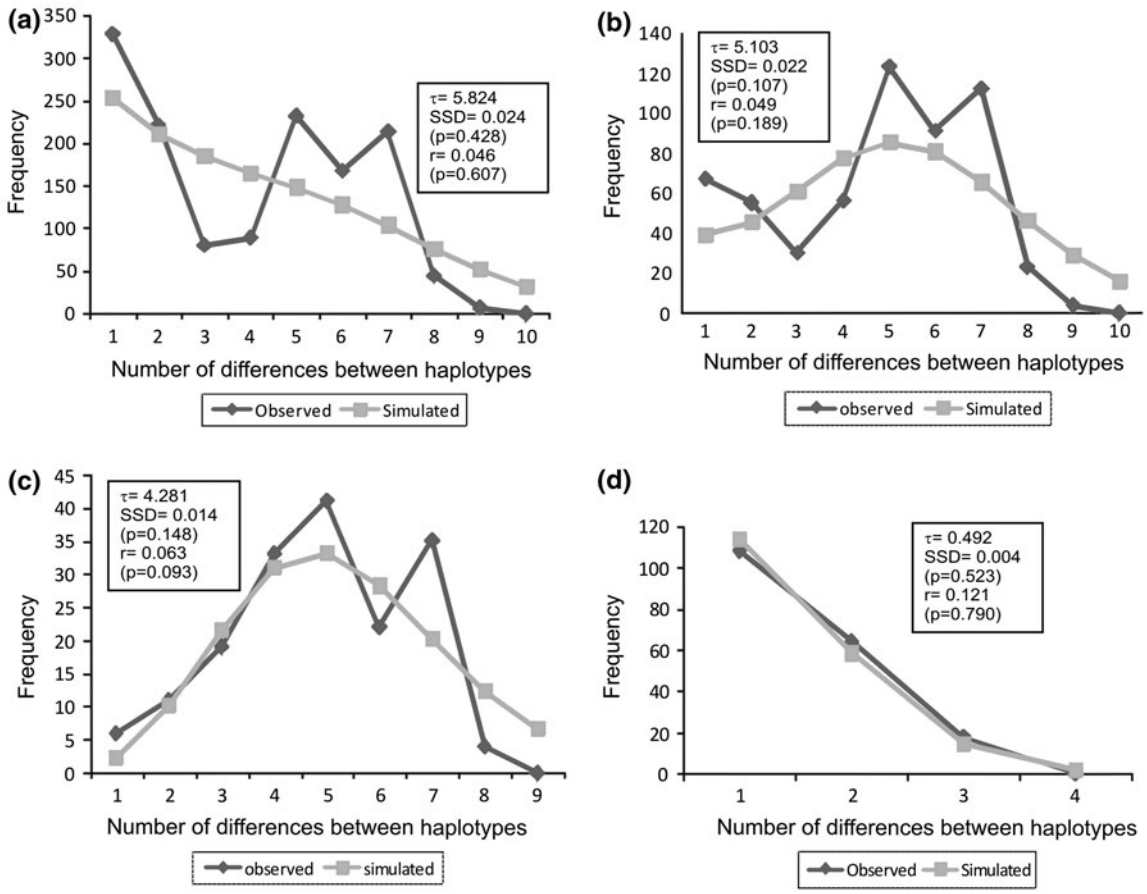

Fig. 4 Predicted patterns of mismatch distribution for (**a**) all haplotypes, (**b**) YO haplotypes and shared haplotypes between the two morphotypes, (**c**) YO haplotypes and (**d**) YG haplotypes and shared haplotypes between morphotypes. τ: time in generations since the last demographic expansion; SSD: sum of squared differences; r: raggedness index. *P*-values of SSD and of *r* are indicated in *brackets*

Seventeen haplotypes were found out of 53 individuals sequenced. Levels of haplotype diversity in the COI gene of *Synoicum* samples ($Hd = 0.558$–0.929) were in the range found in other colonial ascidian species, for example, *Botryllus schlosseri* ($Hd = 0.153$–0.700; López-Legentil et al. 2006), *Cystodytes dellechiajei* ($Hd = 0.600$–0.933; López-Legentil and Turon 2006) and *Pycnoclavella communis* ($Hd = 0.248$–0.903; Pérez-Portela and Turon 2008). These studies were all performed on species from the Mediterranean Sea; population genetic studies about levels of variability from populations of colonial species from other oceans, are unfortunately scarce. Nucleotide diversity was generally smaller in *Synoicum* samples ($\pi = 0.004$–0.012) compared to other colonial species (*B. schlosseri*: $\pi = 0.008$–0.079, op. cit.; *C. dellechiajei*: $\pi = 0.006$–0.081, op. cit.; *P. communis*: $\pi = 0.0004$–0.0445, op. cit.; *Pseudodistoma crucigaster*: $\pi = 0.000$–0.011, Tarjuelo et al. 2004). Goodall-Copestake et al. (2012) identified two sources of bias when comparing variability measures using COI: low sample sizes and not using strictly homologous positions because, usually, fragments of different lengths and of different parts of the COI gene are used. Regarding the first issue, the authors demonstrated by using subsampling analyses that samples of $N > 5$ were enough to discriminate extremes of high and low COI diversity, while samples bigger than 25 were required for greater accuracy. Therefore, given that sample sizes in our work range from 8 to 20 (Table 1), we should be able to discriminate whether *Synoicum* populations show higher or lower variability levels than other ascidians. Regarding the second issue, Goodall-Copestake et al. (2012) recommend re-assembling data in the literature, in order to compare strictly homologous sites. In our work, levels of variability were calculated on an unusually short fragment of COI gene. Therefore, we selected those papers that published the complete data set (or information to re-assemble it), aligned sequences with ours and recalculated *Hd* and π. We found only two such ascidian publications, those of

Tarjuelo et al. (2004) on *Pseudodistoma crucigaster*, and of Pérez-Portela and Turon (2008) on *Pycnoclavella communis*. Results of the re-analysis are shown in Online Resource 5. *Hd* remained in the range of that of *Synoicum*, while π was smaller in *P. crucigaster*, and similar in *P. communis*. Therefore, we can reasonably assume that variability levels in *Synoicum* populations are in the range of that found in colonial ascidian species of temperate waters.

Rogers (2007) and Allcock and Strugnell (2012) reviewed studies on genetic structure in organisms from the Southern Ocean and pointed out that much of Southern Ocean fauna would have survived the Quaternary Glaciations in situ. They identified two general patterns: organisms which might have survived glaciations in refuge on the continental shelf, or by retreating to sub-Antarctic islands, are likely to show low genetic variability due to bottlenecks and founder effects, while those eurybathic species that took refuge on the continental slope are likely to show higher levels of variability, because they would have been able to maintain higher population sizes. The levels of variability found in *Synoicum* samples point to persistence on the continental slope for this species.

According to Goodall-Copestake et al. (2012), another source of bias in the calculation of *Hd* and π is the inclusion of cryptic species in single species samples, which would increase the estimates. In our data set, this could occur in estimates of Low 45 and Low 46 samples, which included both morphotypes (Table 1). However, *Hd* and π were the lowest in these samples. In shallow Antarctic waters, ice acts as a regional disturbing factor of high impact; along the Antarctic Peninsula and the South Shetland Islands, icebergs can impact the bottom up to 150–200 m depth and are an important factor in shaping the benthic system on the Antarctic shelf (Gutt 2000; Dowdeswell and Bamber 2007). In the Antarctic colonial ascidian *Aplidium falklandicum*, Demarchi et al. (2010) found that populations sampled at more than 200 m depth showed significantly more variability than those sampled at less than 200 m, which coincided with the limit of disturbance by ice scouring. The authors proposed that ice affected genetic diversity by removing a considerable amount of biomass with the consequent loss of individuals and alleles. The four geographic samples analyzed for *Synoicum* were obtained at depths of 200 m or less, and therefore, they may be affected by ice action that could cause fluctuations in the population effective size. But additionally, Low 45 and Low 46 stations are much more exposed to the ACC than the other stations (Fig. 1). This can have a double effect on benthic populations: on the one hand, the ACC would sweep away larvae from parent populations more efficiently in Low stations, and on the other hand, it would drive icebergs and increase its frequency compared to the other sampled stations that are more sheltered. Then, these disturbance factors could contribute to fluctuations in the population's effective size and the decreased levels of genetic variability found in the Low stations. On the other hand, Potter Cove is located in a more protected area, where the effects of these disturbances are probably lower, which would explain the higher genetic variability found at this station.

Patterns of genetic structure and the extent of gene flow in benthic organisms could be the result of the interaction between the larval development, dispersal potential and local recruitment, coupled with oceanographic features such as the circulation of the water masses (Palumbi 1994; Grosberg and Cunningham 2001; Wares et al. 2001). Taking into account that the larval stage is responsible for organism dispersal and, in colonial ascidians, larvae are short-lived (from few minutes to hours; Svane and Young 1989), a strong genetic structure due to very low levels of gene flow is expected in these species (Thatje 2012). In this work, the levels of genetic differentiation among geographic samples and morphotypes and their correlation with geographic distance indicate that gene flow among populations has been sufficient for a long enough time to establish isolation by distance pattern. Despite the low dispersal potential of ascidian larvae, gene flow exists, probably facilitated by different marine currents over the West Antarctic Peninsula (Fig. 1), which would explain the presence of shared haplotypes as well as some degree of connectivity between separate locations within the study area. Passive transport by ocean currents has been proposed as a mechanism to explain gene flow in Antarctic species with low dispersal potential as the brooding brittle star *Astrotoma agassizii* (Hunter and Halanych 2008), the benthic notothenioid fish *Gobionotothen gibberifrons* (Matschiner et al. 2009) and the amphipod *Eusirus perdentatus* (Baird et al. 2011).

The haplotype network showed that the shared haplotypes (Hap VII and Hap VIII) were also the most frequent and widely distributed and that most haplotypes were present in a single sampling site. This type of haplotype network has been named "parochial" by Allcock and Strugnell (2012). According to these authors, it is found in Antarctic organisms that during the Pleistocene glaciations persisted in large refugial areas and therefore maintained their genetic diversity because they did not suffer major bottlenecks. If post-glacial expansion occurred from multiple refugia before the acquirement of reproductive isolation, secondary contact would result. Hints of a recent population expansion in *Synoicum* were revealed by *Fs* and the Mismatch distribution test. The negative and significant values of the Fu's *Fs* test, along with non-significant values of raggedness index and SSD and the shape of the haplotype network, support the idea of a demographic expansion. The observed distribution of pairwise differences

obtained from the Mismatch distribution showed a bimodal distribution, which is usually observed when two (or more) groups of divergent haplotypes are included in the analysis, either because there is a strong genetic structure, a secondary contact between divergent forms has occurred, or when a spatial expansion followed by low levels of gene flow occurred (Ray et al. 2003; Excoffier et al. 2009). Although the number of samples analyzed in this work does not allow us to discriminate among these options, it should be noted that τ values (time in generations since the last demographic expansion) obtained from the Mismatch distribution analysis support the idea that the expansion of YO morphotype may be more ancient than that of the YG morphotype ($\tau = 4.281$ and $\tau = 0.492$, respectively). Also, Tamura-Nei genetic distances showed that sequence divergence between YO haplotypes and between them and shared or YG haplotypes was higher than among YG haplotypes, indicating that YO haplotypes are more ancient than YG ones, since they required more time to accumulate differences. Therefore, evidence so far points to a secondary contact among forms differentiated in allopatry, but still pertaining to the same biological species. Even though the phylogenetic trees failed to resolve most of the relationships among haplotypes, the haplotype of *S. adareanum* from Terre Adelie was in a basal position, followed by Hap VII and VIII, all with high support (Fig. 2, BA). These results suggest that shared haplotypes VII and VIII are those closer to the common ancestor.

In conclusion, morphological and genetic analyses were not consistent in separating the two forms at the species level. The morphological approach indicated that both morphotypes of *Synoicum* found along the South Shetland Islands corresponded to a different species of the same genus: the YO morphotype to *S. adareanum* and the YG morphotype to *S. ostentor*, according to the previous descriptions (Monniot and Monniot 1983; Monniot et al. 2011). However, the COI gene showed no differences at interspecific level. Considering the biological definition of species, each morphotype would not correspond to a full species, because the analyses presented in this work indicate that both morphotypes did not achieve a complete reproductive isolation yet, can still interbreed and that some degree of gene flow is still occurring. This was certainly unexpected given the numerous reported cases worldwide and especially in Antarctica, where molecular approaches were able to resolve several cryptic species, that morphology-based taxonomy could not (Bernardi and Goswami 1997; Held 2003; Raupach and Wägele 2006; Bickford et al. 2007; Allcock et al. 2011). These results are relevant not only due to the ecological importance of *Synoicum* in Antarctic benthic communities, but also for its biochemical potential. As reported by Koplovitz et al. (2011), *S. adareanum* from the Western Antarctic Peninsula differing in the form of the colonies showed marked differences in the anti-diatom activity of secondary metabolites. Besides, a secondary metabolite, Palmerolide A, which has an important cytotoxic activity against certain types of melanoma, was obtained from *S. adareanum* (Diyabalange et al. 2006; Jiang et al. 2007; Nicolaou et al. 2007; Riesenfeld et al. 2008). However, these authors did not mention any color differences in the sampled colonies, and therefore, it is possible that both or only one form was studied. Considering that there is some genetic difference between the forms, it is also possible that these forms produce different secondary metabolites. As chemical variation related to color and genotype has been previously reported in other colonial ascidians (López-Legentil and Turon 2005), future work should consider proper identification of the ascidian species using molecular, chemical and morphological descriptions.

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