Polar Biol (2010) 33:1567–1576
DOI 10.1007/s00300-010-0848-2

ORIGINAL PAPER

Population genetic structure of the Antarctic ascidian *Aplidium falklandicum* from Scotia Arc and South Shetland Islands

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Received: 13 November 2009 / Revised: 5 June 2010 / Accepted: 7 June 2010 / Published online: 29 July 2010

Abstract In sessile marine organisms, gene flow between populations depends mainly on free-living reproductive stages (such as larvae and gametes), and usually the strength of genetic structure is related to the time spent in the plankton and physical factors as oceanographic conditions. In Antarctica, abyssal depths that surround the continent and the Polar Front are considered strong barriers for benthic marine fauna, keeping the continent isolated from other shelves. The only available shallow water habitats between South America and the Antarctic continent are those around the Scotia Arc Islands; there are no shallow water habitats between the other southern continents and Antarctica. In this work, ISSRs-PCR markers were used to study the genetic structure of populations of *Aplidium falklandicum*, a compound ascidian with short-lived lecitotrophic larvae. A highly significant genetic differentiation ($\Phi_{ST} = 0.405$; $P < 0.05$) and a pattern of isolation by distance were found. A genetic landscape approach identified a discontinuity in genetic diversity, coincident with the southernmost registered position of the Polar Front. For *A. falklandicum*, a species with presumably low capacity of long distance dispersal, the abyssal depths together with the large geographic distances create a barrier for gene flow.

Keywords *Aplidium falklandicum* · Population genetics · ISSR-PCR · Antarctic shallow shelf

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Introduction

The absence of genetic exchange between populations can lead to extreme genetic differentiation and ultimately to reproductive isolation and speciation. Consequently, it is important to estimate not only the dispersion potential of organisms but also their ability of leaving progeny in the recently colonized population, that is, of gene flow (Palumbi 1996). Gene flow not only counteracts divergence generated by genetic drift but also introduces variability into a population, making it less prone to extinction.

Antarctic shelf environments are isolated from those of the adjacent continents by the abyssal depths surrounding Antarctica (up to 5,000 m in some areas) and by a strong oceanic stream, the Antarctic Circumpolar Current (ACC) and its associated fronts (Clarke and Crame 1989). The formation of the ACC was led by the opening of the Drake Passage, in the Late Paleocene, which generated a new seaway between South America and Antarctica (Maldonado et al. 2003). The northern limit of the ACC is conformed by the Antarctic Polar Front (PF) (Dell 1972), a transition area

between Antarctic and sub-Antarctic waters extending to a depth of 1,000 m. This zone shows steep gradients of temperature, salinity and nutrients that act as a barrier to free north–south exchange of water, isolating Antarctic shelf communities geographically, climatically, thermally and oceanographically, therefore representing an important biogeographic boundary (Clarke et al. 2005; Barnes et al. 2006). Under these conditions of isolation, the Antarctic biota evolved in a cold but physically stable ecosystem with predictable seasonal pulses of biologic productivity, displaying nowadays a unique shelf community biodiversity and a high degree of endemisms (Dayton 1990; Clarke 1996; Clarke and Johnston 2003; Gili et al. 2006).

Many benthic species are present in the Magellanic Province (Subantarctic Region) and in the Antarctic Region (Clarke and Crame 1989; Arntz and Ríos 1999, Barnes and De Grave 2001; Ramos-Esplá et al. 2005). Therefore, several authors have inquired about the permeability of the PF and the different mechanisms to cross it displayed by different species; one possible mechanism would be through intrusions of eddies, the high abundance and biomass of euphausiids in the Subantarctic Region was related with a cold eddy from the Antarctic area (Bernard et al. 2007). On the other hand, two species of decapod crustaceans characteristic of the Subantarctic Region found in Antarctic waters may have crossed the PF through eddies (Thatje and Fuentes 2003). Other possible mechanisms to cross that barrier would include migration through abyssal depths in the case of euribathic species or migration through the shelves of the Scotia Arc Islands. These sets of islands offer a wide area of shallow platforms appropriate for benthic macrofauna settlement as it goes through the abyssal depths surrounding Antarctica, giving the chance to benthic organisms, specially with high dispersal potential to cross from South America to Antarctica and vice versa in a stepping stone process (Clarke and Crame 1989).

Aplidium falklandicum Millar 1960 (Polyclinidae) is a colonial benthic sessile ascidian whose distribution includes SW Atlantic (Patagonia), Malvinas/Falkland Islands, South Georgia Island, South Orkney Islands, Antarctic Peninsula and Kerguelen Islands (Millar 1960). Zooids that constitute a colony are completely independent clonemates. In Polyclinidae, sexual reproduction occurs when sperm released by a hermaphroditic zooid enters through the oral siphon of a zooid from the same or another colony and fertilizes eggs in the atrium, self-fertilization being a possible trait in this species. The embryonic stage consists of a pelagic tadpole larva with a locomotory tail that allows some movements; despite this, it can be easily carried out by ocean currents. All ascidian larvae are lecithotrophic and their life span in colonial ascidians ranges from a few minutes to a couple of hours and is extremely short when compared with other taxa (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 by genetically identical zooids (Stoner and Weissman 1996). The larval stage is responsible for the dispersion of the species and it is also the most vulnerable one. Considering its short lived larvae, *A. falklandicum* would be useful as model for testing the dispersal potential of sessile benthic species across the Scotia Arc Islands.

In this study, the genetic variability of five populations of *A. falklandicum* from the South Shetland, South Orkney and South Georgia Islands was quantified using Inter Simple Sequence Repeats-PCR (ISSR-PCR) as genetic markers. The genetic structure was analyzed, and the possible role of geographic distance as a barrier to gene flow in this species with presumable low dispersive capabilities was evaluated.

Materials and methods

Study material

A total of 53 colonies of *A. falklandicum* were collected from the Scotia Arc and Shetland Islands during LAMPOS ANT XIX-5 expedition of the "RV Polarstern" in April 2002, and during the BENTART-06 Antarctic Project of the "BIO Hespérides" in January and February 2006. Two stations were sampled in the Scotia Arc: South Georgia Island (SGI; 54° 27′ S, 35° 41′ W; 257 m depth; $n = 12$) and South Orkney Islands (SOI; 60° 58′ S, 43° 26′ W; 400 m depth; $n = 12$). Three stations were sampled in the Shetland Islands: one at Livingston Island (Liv; 63° 01′ S, 60° 32′ W; 215 m; $n = 10$) and two at Low Island, Low1 (63° 43′ S, 62° 21′ W; 86 m depth; $n = 10$) and Low2 (63° 43′ S, 62° 24′ W; 97 m depth; $n = 9$) (Fig. 1). All samples were collected with an Agassiz trawl and kept in 100% ethanol until processing.

Genomic DNA extraction and amplification conditions

Genomic DNA was purified using the salt purification method described in protocol 1 of Bruford et al. (1992). Zooids were processed by removing the tunic, gut and larvae if any to avoid amplifications from possible contaminants like commensal organisms and gut content.

Genetic variability was studied employing Inter Simple Sequence Repeats (ISSR-PCR) as molecular markers (Zietkiewicz et al. 1994; Bornet and Branchard 2001). The ISSR-PCR technique is based on the amplification of nuclear DNA sequences delimited by two inverted microsatellites, with primers composed of a specific microsatellite sequence. Each primer usually amplifies several loci; polymorphism is revealed as presence or absence of a band

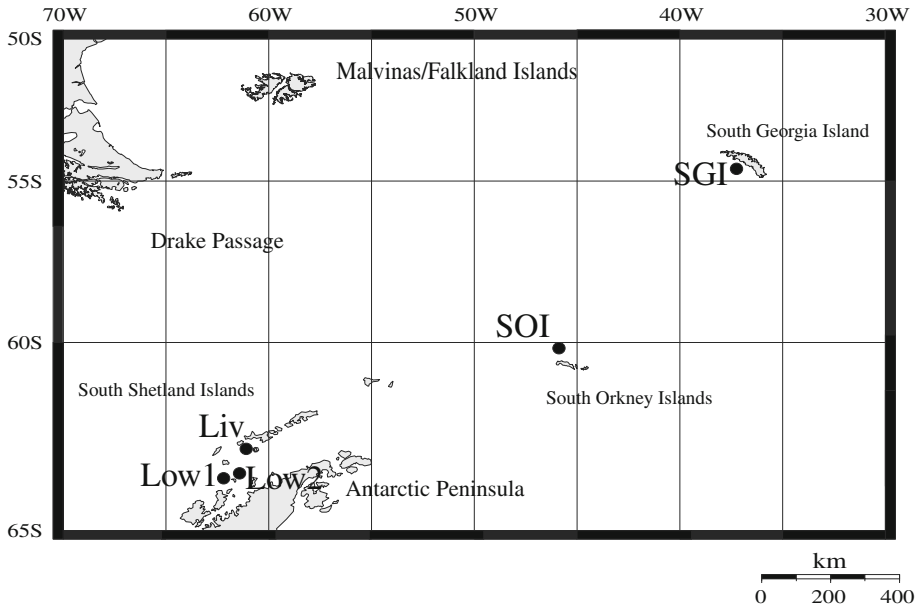

Fig. 1 Location of the five studied populations of *Aplidium falklandicum* in the west side of the Antarctic Peninsula and in the Scotia Ridge

of a given molecular size and therefore, these markers segregate as dominant. Despite its dominant mode of inheritance, this technique has several advantages. First, it does not require prior sequence knowledge. Second, it reveals high levels of polymorphism. Third, as amplified sequences lie between microsatellite repeats, they are usually located in non-coding regions of the nuclear genome and, therefore, selective neutrality is highly probable. Finally, ISSR primers are longer (typically 18–22 bp) and have higher annealing temperatures (around 50°C) than RAPD primers; therefore, the band patterns obtained with this technique are highly repeatable (Segman et al. 2006).

Polymerase chain reaction (PCR) amplifications were performed in each 10 µl reaction, consisting of 6 pmol primer, 200 µM dNTP, 1 × Buffer (SIGMA®), 2.5 mM $MgCl_2$, 0.2 U RED*Taq* DNA Polymerase (SIGMA®), 5% DMSO, and 10 ng of genomic DNA. Amplifications were carried out in an UNO II Biometra thermocycler using the following cycling profile: an initial denaturation step of 94°C for 4 min followed by 27 cycles of 1 min at 94°C, 1 min at the annealing temperature (50–54°C depending on the primer), and 4 min extension at 72°C, followed by a final 7-min extension at 72°C. Eight primers were screened: $(AC)_{10}AA$, $(TG)_{10}GG$, $(GT)_{10}TG$, $(TG)_{10}GC$, $(AC)_{10}AG$, $(TG)_{10}$, $(TCC)_{10}$ and $(GACA)_5$. PCR products were electrophoresed through 1.2% agarose gels for 4 h in 0.5 × TBE buffer at 120 V, along with a known molecular size standard. Gels were stained with ethidium bromide, visualized in an UV transilluminator and photographed.

Primers used for further analyses were selected on the basis of number, spacing and intensity of bands amplified and polymorphisms revealed: $(AC)_{10}AA$, $(AC)_{10}AG$, $(TCC)_{10}$ and $(GACA)_5$.

In order to verify if colonies were formed by cloned zooids exclusively, five randomly chosen zooids from three colonies from the stations SGI and SOI were extracted and amplified independently, prior to the processing of all samples. PCR products were electrophoresed in a single gel to corroborate that band patterns among zooids from the same colony are identical.

Genetic data analysis

Two bands were assumed to be homologous if they had the same molecular weight. A presence/absence matrix for all scorable bands for all individuals was generated. The used approached did not required the assumption of Hardy–Weinberg equilibrium, as this information is not available for *A. falklandicum* and it cannot be obtained from dominant markers. Genetic diversity within populations was estimated using the marker diversity index (M), defined analogously to Nei's heterozygosity (Nei 1978; Kapralov et al. 2006). The proportion of unmatched ISSR bands between every pair of individuals within a population was calculated. These values were averaged to obtain M within a population. One-way analysis of variance (ANOVA) was used to compare M among populations. In order to compare the differences in mean M among populations, a Student–Newman–Keuls (SNK) multiple comparisons procedure was used with the program Statistica 6 (StatSoft, Inc.) (StatSoft, Inc., STATISTICA 2001). Homogeneity of variances was tested using the Cochran C′ Test. A correlation between M diversity index and depth was performed with Statistica 6 (StatSoft, Inc.) (StatSoft Inc., STATISTICA 2001), in order to identify if the genetic variability was related to depth.

Population structure was analyzed employing the analysis of molecular variance (AMOVA) in order to test the null hypothesis of panmixia among populations. A matrix of Nei and Li (1985) genetic distances between pairs of individuals was calculated from the presence/absence matrix with RAPDplot (Black 1995). The AMOVA analysis was run using the program WINAMOVA 1.55 (Excoffier 1995), which calculates the components of the genetic variance and the Φ_{ST} indices for the whole population and between pairs of populations. The Φ_{ST} value, which is analogous to F_{ST} (Wright 1931), is an estimator of the level of genetic differentiation. To test for significant deviation of between groups variation from within-group variation, 1,000 permutations were conducted to obtain a *P* value.

Different approaches to investigate genetic landscape features were used. A Mantel test was performed in order to test for correlation between geographic (straight-line) and genetic (Φ_{ST} values) distances with the program TFPGA 1.3 (Miller 1998). A plot called "Genetic Landscape Shapes" was obtained with the program AIS (Miller 2005), which is a graphical representation of diversity patterns across the landscape. This analysis starts by constructing a connectivity network of the sampling locations based on a Delaunay triangulation. Then, it assigns a genetic distance to the landscape coordinate at the midpoint of each network edge connecting two populations, and uses an interpolation procedure to infer genetic distances between points located in a uniform grid superimposed on the sampled landscape. This procedure allows the identification of putative barriers to gene flow and has been used widely in vagile and sessile species and can resolve population substructure across different geographic scales (Manel et al. 2003; Holderegger and Wagner 2006). A neighbor-joining tree of Nei and Li (1985) genetic distances between pairs of individuals was constructed with the program Phylip ver 3.5 (Felsenstein 1993) in order to visualize the genetic relationships between individuals.

Results

The clonal nature of colonies was confirmed running side by side the amplification products from zooids of the same colony. This allowed to pool the DNA from zooids of the same colony and deal with large amounts of DNA (Fig. 2).

The four primers chosen revealed a total of 79 bands (between 16 and 22 for each primer), ranging from 300 to 1,900 bp (Table 1), and only 2 bands were monomorphic. A total of 25 bands were unique for the South Shetland Islands populations, not shared by SGI and SOI, while these two populations had 14 unique bands, not shared with the South Shetland populations. This means that 49.6% of the amplified bands were not shared between South Shetland populations and SGI-SOI. SGI had two unique bands. Almost 50% of the monomorphic bands in any of the three South Shetland populations were present neither in SGI nor in SOI.

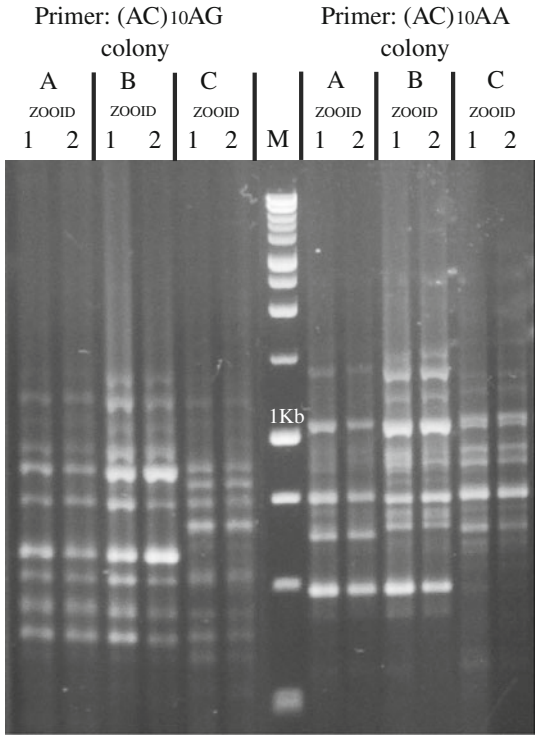

Fig. 2 ISSRs band patterns of two zooids of each of three colonies of *Aplidium falklandicum* obtained with two ISSR primers

Table 1 ISSRs primers used and number of amplified bands in the PCR reaction

Sequence	Polymorphic bands	Monomorphic bands
$(AC)_{10}AG$	16	–
$(AC)_{10}AA$	19	–
$(AC)_7C$	20	2
$(GACA)_4$	22	–
Total	79	

Marker diversity (M) ranged from 0.233 to 0.364 (Table 2). SNK multiple comparison procedure showed significant differences between two groups: (A) SGI, Low1 and Low2; and (B) SOI and Liv. M diversity index and depth were positively correlated ($r = 0.7$; $P < 0.05$).

The AMOVA analysis showed that 40% of the total variation found was attributable to differences among populations. The Φ-statistic revealed significant genetic differentiation ($\Phi_{ST} = 0.405$, $P < 0.001$) (Table 3), indicating that genetic heterogeneity was present a this spatial scale. Pairwise estimations of Φ_{ST} were significant in all cases but one (Table 4). The highest value was obtained between SGI and Low1 ($\Phi_{ST} = 0.587$, $P < 0.001$) the most distant locations,

Table 2 Marker diversity index (M) for populations of *Aplidium falklandicum*

Pops	M	Groups
SGI	0.241	A
SOI	0.364	B
LIV	0.345	B
Low1	0.233	A
Low2	0.258	A

Different letters indicate significant differences between groups obtained with Student–Newman–Keuls (SNK) multiple comparison procedure

Table 4 Φ_{ST} values between pairs of populations of *Aplidium falklandicum* (below the diagonal); above the diagonal statistical significance

Pops	SGI	SOI	LIV	Low1	Low2
SGI	–	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
SOI	0.399	–	$P < 0.001$	$P < 0.001$	$P < 0.001$
LIV	0.514	0.313	–	$P < 0.001$	$P < 0.001$
Low1	0.587	0.477	0.088	–	$P = 0.043$
Low2	0.553	0.445	0.105	0.085	–

while between the closest ones (Low1 and Low2), the differentiation was the lowest ($\Phi_{ST} = 0.085$, $P < 0.05$) (Table 4). The Mantel test showed a significant correlation between geographic and genetic distances ($r = 0.95$; $P < 0.05$), indicating an isolation by distance pattern where genetic exchange decreases with geographic distance.

The interpolation analysis (Fig. 3) found the greatest peak of genetic discontinuity between SOI and SGI and a smaller one between Low2 and Liv, differentiating tree main groups: (a) Low1, Low2 and Liv, (b) SOI, and (c) SGI.

The cluster analysis grouped the individuals in three clusters, one corresponding to SGI individuals, other to SOI and the third comprising individuals from Liv, Low1 and Low2 (Fig. 4).

Discussion

This study presents the first analysis of population genetic structure in an Antarctic ascidian species. ISSRs markers demonstrated its power to assess genetic variability information in *A. falklandicum*. This technique has recently become more used in population genetic studies of marine organisms, such as bivalves, fishes, mussels, red coral, leopard sharks, limpets, and arctic ascidians (Hou et al. 2006; Maltagliati et al. 2006; Lewallen et al. 2007; Varela et al. 2007; de Aranzamendi et al. 2008; Demarchi et al. 2008).

A positive correlation between M diversity index and depth was observed. Iceberg scouring could explain the increase in the genetic diversity in deeper waters. Ice is one of the major features in structuring benthic communities in Antarctic shallow waters (Gutt and Starmans 2001). It acts in geological scales as a wiper for sessile communities in glacial maxima, and in an ecological scale as a regional disturbing factor (Gutt et al. 1996; Gili 2001; Clarke et al. 2004; Gili et al. 2006). Ice can affect community structure and population genetic diversity by removing a considerable amount of biomass with the consequent loss of individuals and alleles. There is previous evidence that ice scouring reduces species diversity and abundance; in areas with high ice-scouring frequency, there is considerable low species diversity, and the recovery rate of communities is very slow (Brown et al. 2004). Gutt and Piepenburg (2003) found in analysis of seabed videos at a local scale, that iceberg undisturbed megabenthic assemblages were significantly more diverse than disturbed habitats. Smale (2008) found that the relative abundance of the low mobility species was significantly greater at low ice disturbance levels. Along the Antarctic Peninsula, icebergs can impact the bottom up to 150–200 m depth (Dowdeswell and Bamber 2007). Low1 and Low2 stations were sampled at depths less than 100 m, and therefore they would be affected by scouring that could cause fluctuations in the population effective size and the observed loss of genetic variability. SOI and Liv stations, which were sampled in shelves deeper than 200 m, housed the highest diversity. Iceberg scouring would have a slighter effect on these populations. SGI population was sampled at 257 m, but showed the lowest M value, suggesting that the low genetic diversity would not be a result of ice scouring. A possible explanation could

Table 3 Results of analysis of molecular variance in five populations of *Aplidium falklandicum*

Source of variation	Sum of squares	Degrees of freedom	Variance components	Percentage of variation	Φ_{ST}	*P*
Among pops	3.630	4	0.075	40.46	0.405	<0.05
Within pops	5.325	48	0.111	59.54		
Total	8.955	52	0.186	100		

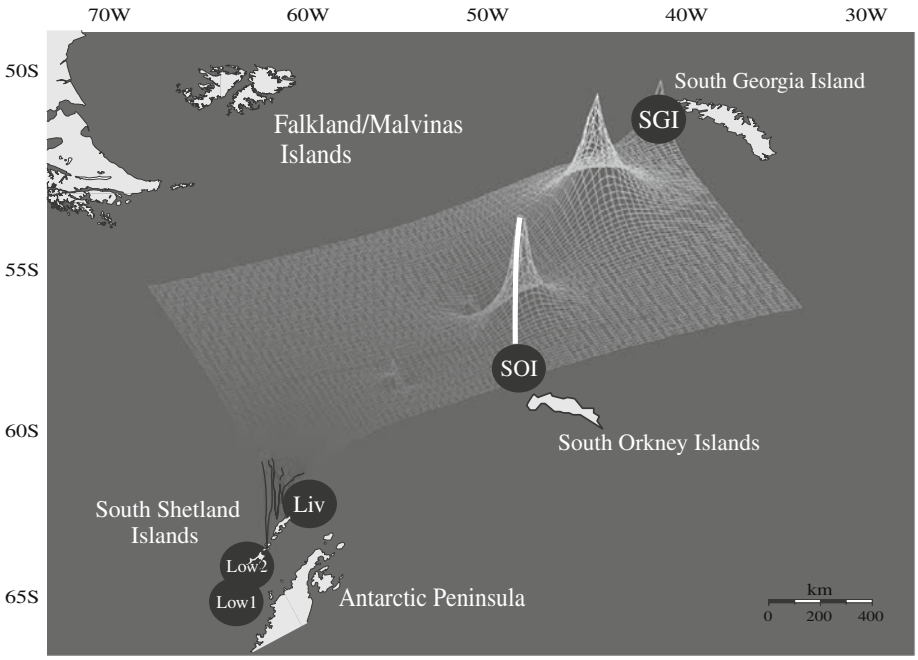

Fig. 3 Genetic landscape shape interpolation plot of ISSR data, where heights reflect genetic discontinuities and valleys genetic similarities

be the instability of the system due to the natural north–south shifts of the PF position. In early literature, the PF position was described to be 50°S (Hedgpeth 1969), but recent cruises, the ANDEEP and LAMPOS of the *RV* Polarstern, have collected data indicating that PF could occasionally be south of the South Georgia Island (Grabbert et al. 2003; Arntz 2005; Barnes 2005). Moreover, there is other important front in the ACC, the southern ACC front (SACCF) that has been registered sometimes north of South Georgia and sometimes wrapping anticyclonically around the island and retroflecting north (Thorpe et al. 2002). Changes in the PF position expose South Georgia Island to a continuous environmental instability and might affect establishment of recruits and migrant larvae, resulting in a low effective population size, which will translate into a loss of genetic variability. Low migration rates would also decrease the effective size of SGI and consequently its genetic variability. However, it might be useful to sample south of the PF, in a homogeneous area before accepting the iceberg scouring hypothesis.

The significant genetic differentiation obtained for the five populations (overall $\Phi_{ST} = 0.405$) was that expected for isolated populations with restricted gene flow. For benthic organisms, patterns reflecting genetic structure and the extent of gene flow may be the result of the interplay of larval development, dispersal potential and local recruitment, coupled with oceanographic features such as oceanographic circulation (gyres and fronts) and physicochemical parameters (temperature, salinity) (Palumbi 1994; Grosberg and Cunningham 2001; Wares et al. 2001). *A. falklandicum*, as the majority of ascidians, has exclusively lecitotrophic larvae, and time as a free living tadpole larva lasts from minutes to hours. Thus, the limited dispersal potential leads to a reduction in gene flow between distant populations. *A. falklandicum* showed a decrease in genetic similarity among populations with the increase of geographic distance: SGI and Low1, the more distant populations were the most differentiated genetically ($\Phi_{ST} = 0.587$; $P < 0.05$) and Low1 and Low2 (the closest populations) showed the lowest differentiation ($\Phi_{ST} = 0.085$; $P < 0.05$) suggesting more gene flow between them than between the more distant ones. The cluster analysis also highlighted the potential gene flow among the South Shetland Islands populations, as individuals from Low1, Low2 and Liv were clustered together. SOI and SGI were grouped as separate branches, with SOI being closer to South Shetland Island populations than to SGI. A strong genetic structuring was also found in other species of colonial ascidians such as *Pseudodistoma crucigaster* from southwestern Mediterranean (Tarjuelo et al. 2004), *Botryllus schlosseri* from New Zealand (Ben-Shlomo et al. 2001), and from Maine, USA, in a microgeographic scale (Yund and O'Neil 2000). Additionally, there is evidence of restricted gene flow in other species of Antarctic marine organisms. In an allozyme survey of the Antarctic limpet *Nacella concinna*, differences in allele frequency were detected between populations located in South Georgia and in South Orkney Island (Beaumont and Wei 1991). Wilson et al. (2007) reported restricted gene flow in the crinoid *Promachocrinus kerguelensis* of the Antarctic Peninsula, South Sandwich Is., South Georgia and Bouvetøya Islands, a surprising find because of the presence of the ACC which assist in directing water from one population to the other. Brierley et al. (1993) found absence of panmixia in populations of the squid *Martialia hyadesi*

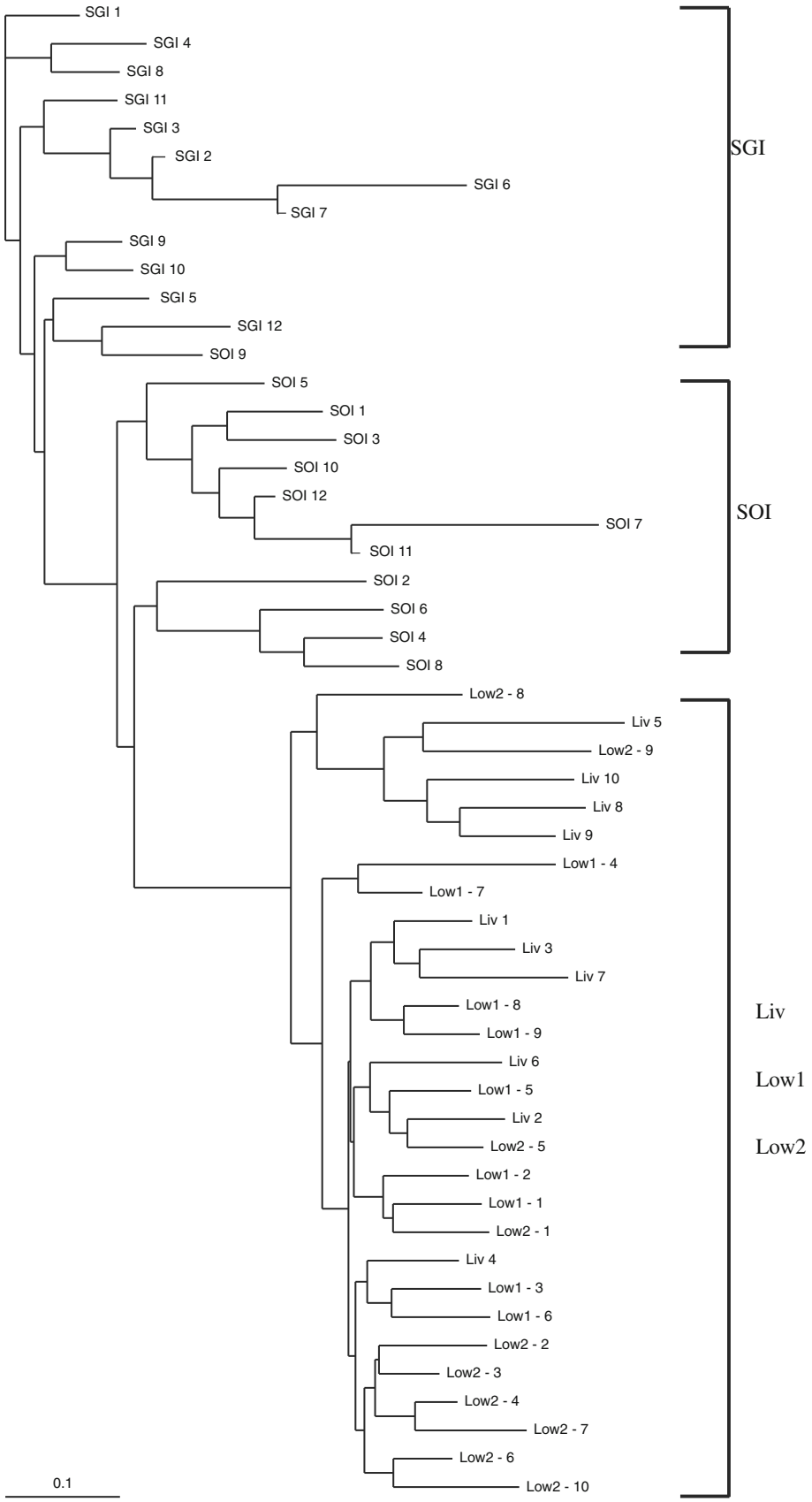

Fig. 4 Neighbor-joining tree of Nei and Li's (1985) genetic distances between pairs of individuals of *A. falklandicum*

from the Patagonian shelf and the Antarctic PF Zone, suggesting that the PF exerts a barrier effect for dispersal. The Antarctic sea slug (*Doris kerguelenensis*) showed deep mitochondrial divergences within populations on both sides of the Drake Passage, this nudibranch is a direct developer and has long generation times and despite its reduced dispersal capabilities has a very broad circumantarctic and Magellanic (southern South America) distribution (Wilson et al. 2009). Linse et al. (2007) have identified strong genetic structure in the brooding bivalve *Lissarca notorcadensis* between Antarctic and sub-Antarctic groups, and within the sub-Antarctic specimens sampled throughout the Scotia arc, providing evidence for reproductive isolation.

The population genetic pattern known as "isolation by distance" (IBD) describes genetic variations between populations originated by spatially restricted gene flow. *A. falklandicum* shows a decrease in genetic similarity among populations in accordance of increasing geographic distance, more distant populations are the most differentiated genetically, and Low1 and Low2 (the nearest populations) showed less differentiation. However, the genetic differences are higher between the pair SGI-SOI ($\Phi_{ST} = 0.399$) than between SOI-Liv populations ($\Phi_{ST} = 0.313$) which are almost at the same geographic distance. Consistently, in the landscape genetic plot a peak representing genetic discontinuity is situated between SGI and SOI stations and not between SOI and Liv stations, suggesting that not only the geographic distance is responsible for the lack of gene flow. This genetic discontinuity would indicate a barrier to genetic exchange, which could be explained by temporal changes in the position of the PF, sometimes south of SGI and sometimes north of the islands. When the PF passes north of the islands, some migration would be allowed, but when it passes south of the islands gene flow would be prevented. The fact that for some ascidians species, SGI is the southernmost limit for exclusive Magellan species and the northernmost limit for exclusive Antarctic species, and thus displaying affinities with these two biogeographic regions (Hedgpeth 1969, Tatián et al. 2005), would support this conclusion. The inclusion of *A. falklandicum* populations north of SGI, would help to further understand the role of Scotia Arc Islands as biogeographic bridge for benthic macrofauna, between Antarctica and the Magellanic region.

Conclusions

In summary, in *A. falklandicum,* a species with low dispersive capabilities, geographic distance between populations is the main factor that restricts genetic exchange among populations. But in the case of South Georgia islands, there could be a combined effect of the large geographic distances and the temporal shifts in the PF position, which could be the main factor in determining the genetic discontinuity. The Scotia Ridge shallow shelves south of South Georgia islands would be acting as a bridge that favors gene flow between populations of *A. falklandicum* allowing exchange with SGI only when the PF passes north of the island.

Acknowledgments We thank the crew and participants of the LAMPOS Expedition ("R/V Polarstern") and BENTART-06 (BIO Hespérides) for their help and assistance. We are gratefully acknowledged to Noemi Gardenal, who gave her help for the laboratory techniques and also to Wolf Arntz and Ana Ramos for their constant support. Financial and logistic supports were provided by Alfred Wegener Institute, CONICET, Spanish Council of Scientific and Technical Research (CICYT), Spanish Antarctic Programmes and Instituto Antártico Argentino (IAA) and FONCyT.

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