Cryptic species and genetic structure in *Didemnum granulatum* Tokioka, 1954 (Tunicata: Ascidiacea) from the southern Brazilian coast

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Abstract

Didemnum granulatum is a colonial fouling ascidian that lives in subtidal substrates, worldwide. It exhibits two morphotypes, orange and beige. In this study, we verified if the color morphotypes and/or the spatial distribution of specimens in different islands might be associated to patterns of genetic structure of a single species, or if they represent distinct cryptic species. Specimens were collected in four islands, along the coast of the Santa Catarina state. A segment of 490 bp from the mitochondrial gene cytochrome c oxidase subunit 1 (COI) was amplified from 45 samples. Twenty-one haplotypes were identified. The total haplotype diversity (0.912) and the total nucleotide diversity (0.044) were high. The global Fst of the populations analyzed was 0.97, with most of the variation occurring between orange and beige groups (82.19%). The variation found between populations within groups was 15.37%, and 2.45% within populations. Haplotype networks and the neighbor-joining tree showed clear genetic divergence between individuals of distinct colors, and between the islands. These evidences strongly support the presence of a complex of two cryptic species for *D. granulatum* occupying the studied area. Both species were also highly genetically structured between islands, suggesting that the conservation process of these populations is complex.

Keywords: ascidian, genetic diversity, population structure, islands, cryptic species.

Espécies crípticas e estrutura genética em *Didemnum granulatum* Tokioka 1954 (Tunicata: Ascidiacea) na costa sul do Brasil

Resumo

Didemnum granulatum é uma ascídia colonial incrustante de substratos consolidados infralitorâneos, e de ampla distribuição mundial. Variação na pigmentação é comum em invertebrados marinhos, e morfotipos de *D. granulatum* das cores laranja e bege foram detectados na região. Neste estudo, nós verificamos se os morfotipos de cor e/ou a distribuição espacial dos espécimens nas diferentes ilhas podem estar associados aos padrões de estruturação genética de uma única espécie, ou se eles representam espécies crípticas distintas. Os espécimens foram coletados em quatro ilhas ao longo da costa do estado de Santa Catarina. Um total de 45 amostras tiveram amplificados um segmento de 490pb do gene mitocondrial citocromo oxidase subunidade I (COI). Vinte e um haplótipos foram identificados. A diversidade haplotípica total (0.912) e a diversidade nucleotídica total (0.044) foram altas. O Fst global das populações analisadas foi 0.97, e a maior parte da variação ocorreu entre os grupos laranja e bege (82.19%). A variação encontrada entre as populações dentro dos grupos foi 15.37%, e 2.45% dentro das populações. A rede de haplótipos e a árvore de Neighbor-joining mostraram nítidas divergências genéticas entre os indivíduos de cores distintas, e entre as ilhas. Tais evidências sugerem a presença de um complexo de duas espécies crípticas de *D. granulatum* na região. Ambas as espécies foram fortemente estruturadas geneticamente entre as ilhas, o que reforça a necessidade de que tais atributos sejam considerados em medidas de conservação e proteção do ambiente marinho, mais especificamente nestas ilhas.

Palavras-chave: ascídia, diversidade genética, estrutura de população, ilhas, espécies crípticas.

1. Introduction

Understanding patterns of genetic diversity in populations of benthic marine invertebrates is necessary for assessing processes such as speciation and connectivity between populations, and also represents a powerful tool to evaluate marine protected areas (Shanks et al., 2003; Palumbi, 2004; Pérez-Portela and Turon, 2008). Studies considering connectivity between marine populations generate valuable insights for the species conservation and marine systems management. In biological terms, management strategies to assess marine protected areas play an important role on the local adaptation and speciation, on the gene flow between populations. Besides, it can diminish the risk of local extinction (Hedgecock et al., 2007). In general, nonplanktonic larvae (incubators with direct development) present short time dispersion in the oceans (Ayre et al., 1997). The gene flow of species with limited dispersion is thus restricted, generating reduction of intra-population genetic diversity through inbreeding and drift, and emphasizing genetic differences between populations (Crisp, 1978).

The colonial ascidians (Chordata, Tunicata) are marine invertebrates widely distributed in benthic systems. Although a free-swimming larval stage does occur, these species hatch their larvae and release them only at an advanced stage of development. Their natural dispersal ability is usually low and might reveal vicariance processes related to their geographical distribution (Ayre et al., 1997). Asexual reproduction and low ability of larval dispersion might be associated with maintenance of populations by highly localized dispersion (Jackson, 1986; Ayre et al., 1997; Pérez-Portela and Turon, 2008).

The sea squirt *Didemnum granulatum* Tokioka 1954 (Didemnidae) is a worldwide colonial fouling marine invertebrate. In southeastern and southern Brazil, it can be found along the seashores of the states of São Paulo (SP), Paraná (PR) and Santa Catarina (SC) (Rocha and Nasser, 1998) It grows mainly on rocky substrates, at depths > 4m (Seleghim et al., 2007). Specimens with variable body colors represent the species, with orange or brick red the predominant color of the larger, and white or beige of the smaller colonies (Rocha et al., 2005). A previous taxonomic study did not find patterns of different morphotypes based in color representing distinct species in ascidians of New Caledonia (Monniot et al., 1991).

However, genetic assessments have suggested that, despite morphologically similar, they might represent distinct species (Nobrega et al., 2004). Genetic differentiation between chromatic variations within a species complex has been recently described for ascidians, e.g., *Pyura stolonifera* (Heller, 1878) (Dalby, 1997), and *Cystodites dellechiajei* (Della Valle, 1877) However, it is worth emphasizing that intraspecific polymorphism also occurs within ascidians. For example, cytochrome c oxidase subunit 1 (COI) analysis suggested that two different morphotypes of the genus *Synoicon* represents variations within a single valid species (Wiernes et al., 2013). DNA sequences represent useful tools in understanding the connectivity of geographically distant populations, and also in revealing cryptic species complexes. In this context, mitochondrial (mt) genes seem appropriate to evaluate genetic diversity at the species level, because they usually present high mutational rates leading to intraspecific polymorphisms (Avise et al., 1987; Ballard and Whitlock, 2004).

We analyzed the genetic structure of populations of the sea squirt *D. granulatum* inhabiting coastal islands of the state of Santa Catarina, Brazil. Our aim was to verify if the color morphotypes and/or the spatial distribution of specimens in different islands might be associated to patterns of genetic structure of a single species, or if they represent distinct cryptic species. In this context, we aimed to contribute with future taxonomic studies on this ascidian, and also with management conservation programs for the studied areas. Our study was based on a segment of the *mt* encoding gene COI, a commonly used marker in molecular studies of ascidians (e.g., Pineda et al., 2011; Rocha et al., 2012; Bock et al., 2012; Wiernes et al., 2013).

2. Material and Methods

The specimens of *D. granulatum* were collected in four islands localized along the coast of SC as follows: 14 specimens in the Tamboretes Archipelago –TB (26°22 'S; 48°31'W), 13 in the central coast of the Arvoredo Island –AR (27°16'S; 48°24'W), 13 in Moleques do Sul Archipelago-MS (27°51'S; 48°26'W) and Five in Matadeiro Beach-MT (27°37'S; 48°28'W), in the Florianópolis island (Figure 1).

Small fragments of colonies were collected with a minimum of 2m of distance between them, at depths ranging between 6-15m, during the fall of 2008. The ascidians were anesthetized with menthol and kept on ice until fixation with 95% ethanol was performed. Then, they were stored at -20°C until processing. Approximately 40 zooids of each fragment, separated under stereomicroscope and washed in 50mL MilliQ water, were used for DNA extraction. Samples were digested in a solution of 5µL containing 20mg/ml proteinase-K and 500µL of lysis buffer (10mM Tris pH 7.4, 10mM NaCl, 200µL of 1M NaCl, 25 mM EDTA, 1% SDS), and then incubated at 56°C for approximately 1h.

DNA extraction was performed using a phenol-chloroform method (Sambrook et al., 1989). After extraction, the DNA templates were subsequently rehydrated in 50µl MilliQ water and kept on the bench for approximately 12h for elution at room temperature. The extraction products were stored at –20° C. COI was amplified by PCR, using the specific primers for ascidian: Tun-F (5'-GCT ACT AAT CAT AAA GAT ATT AG - 3 ') and Tun-R (5'-AAC TTG TAT TTA AAT GAT TAC C -3 ') (Stefaniak et al., 2009), at a concentration of 50 pmol. Each PCR mix was composed of 0.2mM of each dNTP (Invitrogen), 1.5mM of MgCl2, Buffer 1X, 100ng of DNA template and 0.1U/µl of Platinum Taq DNA polymerase (Invitrogen) in a final volume of 20 µl of reaction. The cycling conditions were performed according to Stefaniak et al. (2009). The purification of

PCR products was made using an initial precipitation with 70% isopropanol and centrifugation at 9,000g for 45min at 4°C, followed by washing with cold 70% ethanol and centrifugation at 9,000g for 20min at 4°C. Purified PCR products were rehydrated in 13µl MilliQ water.

The sequencing was carried out in a MegaBACE 1000 DNA Analysis System (GE / Amersham Biosciences Inc., Uppsala), using the sequencing kit DYEnamic ET Dye Terminator® (GE/Amersham Biosciences). We used a concentration of 500ng of the PCR products as template, 5.0 pmol of the same PCR primers and 4.2 ml DYEnamic® ET terminator reagent premix in reactions in a final volume of 10 µl. The cycling conditions were: initial denaturation of 95°C for 25s; 35 cycles of denaturation at 95°C for 15s, annealing at 55°C for 30s, and extension at 60°C for 120s.

The resulting products were purified with 70% isopropanol and centrifuged at 9,000g for 45min at room temperature. After discarding the supernatants, the precipitates were

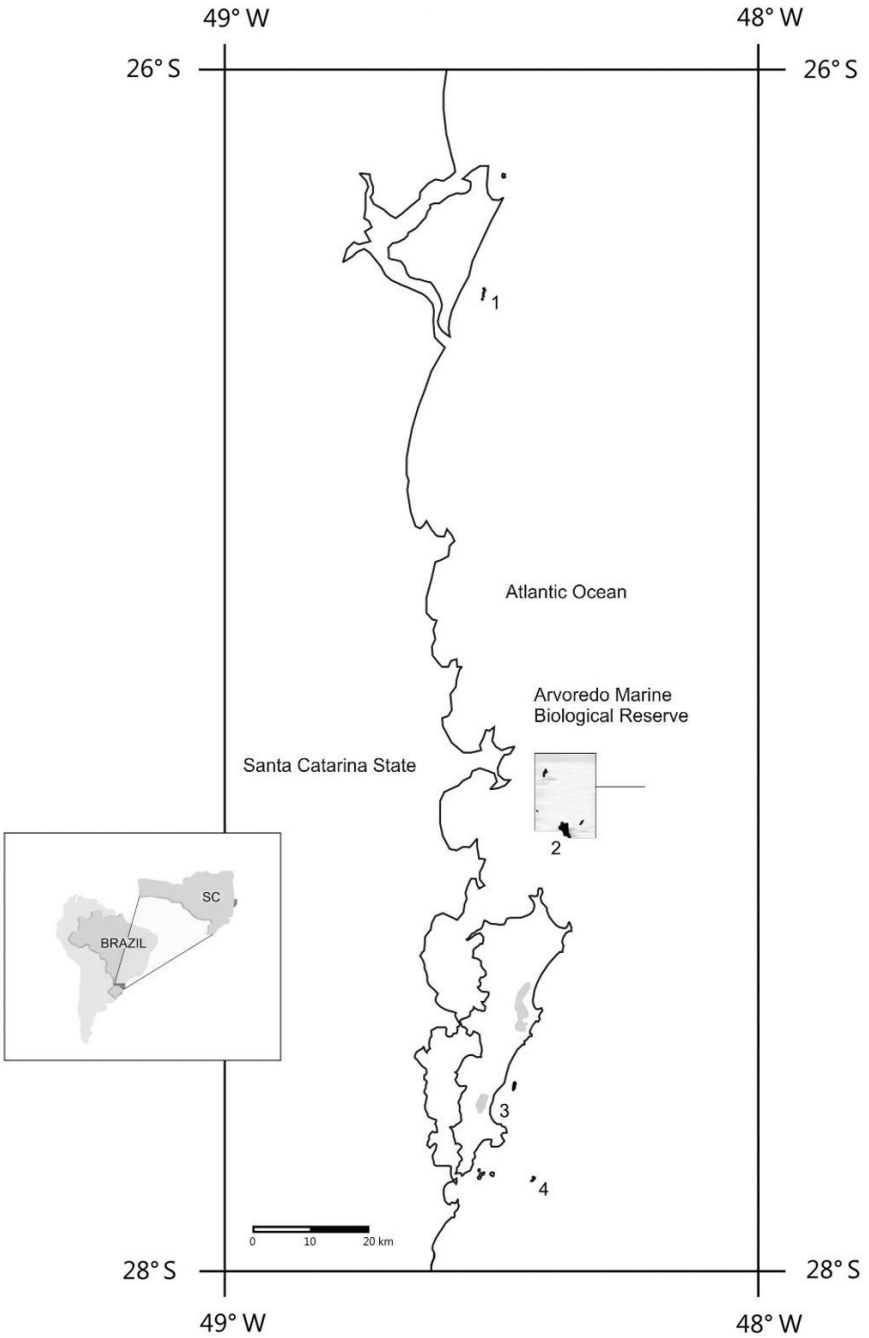

Figure 1. Sampling sites of the *D. granulatum* on the Santa Catarina coast. 1, Tamboretes Archipelago (TB); 2, Arvoredo Island (AR); 3, Matadeiro's beach (MT); 4, Moleques do Sul Archipelago (MS).

washed with 150µl of cold 70% ethanol and followed by another centrifugation at 9,000g for 15min at 4°C. Subsequently, the supernatants were discarded using a spin down, to remove excess of ethanol. After incubation of the plates at 37°C for 15min for evaporation of ethanol residues, samples were diluted in 8µl of application buffer, containing 70% formamide and 1mM EDTA.

Consensus sequences (GenBank accession numbers JQ780668 to JQ780688) were edited in Phred v. 0.20425 (Ewing et al., 1998), Phrap v. 0.990319 (http://www.phrap.org), and Consed 14.0 (Gordon et al., 1998). Sequence alignments and analyses of genetic distances were performed with ClustalW, using BioEdit version 7.0.9.0. (Hall, 1999) as an interface. MEGA 3.1 was also used to construct trees by neighbor joining (NJ) based on the K2p model and maximum parsimony (MP), both with branch topology tested by 10,000 bootstrap replicates. *Didemnum vexillum* Kott, 2002 (GenBank accession number EU742677) was used as outgroup terminal. We also used the median joining (MJ) network analysis (Bandelt et al., 1999) to depict the relationships between haplotypes, as implemented in the Network 4 software (http://www.fluxus-engineering.com). The identification of the substitution model was made on HIV sequence database (http://www.hiv.lanl.gov/cgi-bin/findmodel). Population pairwise Fst's and /st's, analyses of molecular variance (AMOVA) (Excoffier et al., 1992) between populations, exact tests of population differentiation, Tajima's D and Fu's Fs neutrality tests, and haplotype (h) and nucleotide (pi) diversities were calculated with Arlequin version 3.1 (Excoffier et al., 2005).

3. Results

3.1. Characterization of morphotypes

We encountered two morphotypes of *D. granulatum* in two collection points: MS (nine orange individuals and four beige) and TB (eight orange individuals and six beige). In the other two points, we found only the orange morphotypes: 15 in AR and five in MT. Actually, the beige morphotype showed different degree of colours, varying from white to beige (Figure 2).

3.2. Characterization of haplotypes

We obtained 45 COI sequences 490 bp long, for which 21 haplotypes were defined by 73 polymorphic sites (15%). Sixty-four polymorphic sites were parsimony informative, with 23 non synonymous mutations (31.5%). The overall h was 0.912 ± 0.028 (mean ± standard deviation), ranging from 0.250 ± 0.180 for the TB population, to 0.916 ±0.072 for the MS population. The overall pi was 0.0443 (± 0.00581), ranging from 0.00051 ±0.00072 for TB, to 0.00694 ± 0.00476 for TBbg.

High values of h were found for MS and MT, MS beige and TB (Table 1). Low values of h were found for AR and TB (Table 1). The number of haplotypes (H) per population ranged from 2 for TB, to 6 for MS (Table 2). The haplotype VII was the only one shared by two populations (MS and MT). The exact tests for population differentiation revealed significant heterogeneity on haplotypes distribution ($p<0.05$).

Figure 2. Chromatic variation of the sea squirt *Didemnum granulatum* in the coastal region of Santa Catarina.

Table 1. Sample size (N), haplotype types (H), haplotype diversities (h), nucleotide diversities (pi) (mean ± standard deviation). AR: Arvoredo; MT: Matadeiro; MS: Moleques do Sul; TB: Tamboretes.

		N	H	h	pi
Orange	AR	13	3	0.295 (±0.156)	0.00063 (±0.00077)
	MT	5	4	0.900 (±0.161)	0.00327 (±0.00202)
	MS	9	6	0.916 (±0.072)	0.00385 (±0.00274)
	TB	8	2	0.250 (±0.180)	0.00051 (±0.00072)
Beige	MS	4	3	0.833 (±0.222)	0.00204 (±0.00202)
	TB	6	4	0.866 (±0.129)	0.00694 (±0.00476)
	Total	45	21	0.912 (±0.028)	0.04438 (± 0.00581)

Table 2. Absolute haplotypes frequencies of *D. granulatum* in Santa Catarina islands. (AR: Arvoredo; MT: Matadeiro; MS: Moleques do Sul; TB: Tamboretes).

Haplotypes	Populations						
	White				Beige		
	AR	MT	MS	TB	MS	TB	Overall
I	11						11
II	1						1
III	1						1
IV		1					1
V		1					1
VI		1					1
VII		2	2				4
VIII			1				1
IX			1				1
X			1				1
XI			2				2
XII			2				2
XIII					2		2
XIV					1		1
XV					1		1
XVI				7			7
XVII				1			1
XVIII						2	2
XIX						2	2
XX						1	1
XXI						1	1
TOTAL	15	5	9	8	4	6	45

3.3. Phylogenetic and Population Genetic Analysis

The substitution model estimated to construct the NJ tree was Transition Model plus gama (TIM) with Alfa distribution of Gamma = 0.77730. Three monophyletic groups were observed on the NJ tree (Figure 3), with high genetic divergence between the two groups MS06-TB49 (beige coloration group) and TB09-TB79 (orange coloration group).

When compared with other gene bank sequences of *Didemnum*, both groups were separately monophyletic. However, they probably don´t form a monophyletic clade when grouped: *Didemnum incanum* and *Didemnum* sp (both species represented by individuals from Australia – GENBANK-Accession JQ692626 to JQ692628 and JQ731736 to JQ731748) were phylogenetic close of the orange clade (Figure 3).

The global Fst for the six populations was 0.97, with 82.19% of the variations occurring between the clades orange and beige, 15.37% found between populations, and 2.45% within populations.

The Table of populations pairwise Fst's (Table 3) showed no significant values ($p>0.08$) between orange populations of MT and MS (Table 4) showing that they are not genetically divergent. All other Fst values were high and significant ($p<0.05$), ranging from 0.216 between the beige populations of MS and MT, to 0.990 between populations of AR and MS. Our results show that all the remaining pairs of populations were genetically distinct.

The pairwise genetic distances between the two color morphotypes were high (0.106), and the genetic distance within the orange (0.016) and the beige (0.006) morphotypes were low (Table 5). These results are in agreement with those of high genetic divergence between the two color morphotypes depicted in the NJ tree (Figure 4) and the MJ network (Figure 5).

The MJ networks (Figure 5) for the species haplotypes indicated the existence of two clusters: one composed of 14 haplotypes (H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H16, H17) exclusive to the orange, and the other of seven haplotypes (H13, H14, H15, H18, H19, H20, 21) exclusive to the beige specimens. The clusters were separated by 43 mutation steps, and fourteen mutations steps from the other three populations split the TB population. The H7 was the only haplotype shared by two different populations (MS and MT).

4. Discussion

Our study is the first to present data from genetic diversity and population genetic structure for *D. granulatum*. Our results suggest a clear genetic divergence pattern for populations of *D. granulatum* inhabiting Santa Catarina, both between populations from different islands, as well

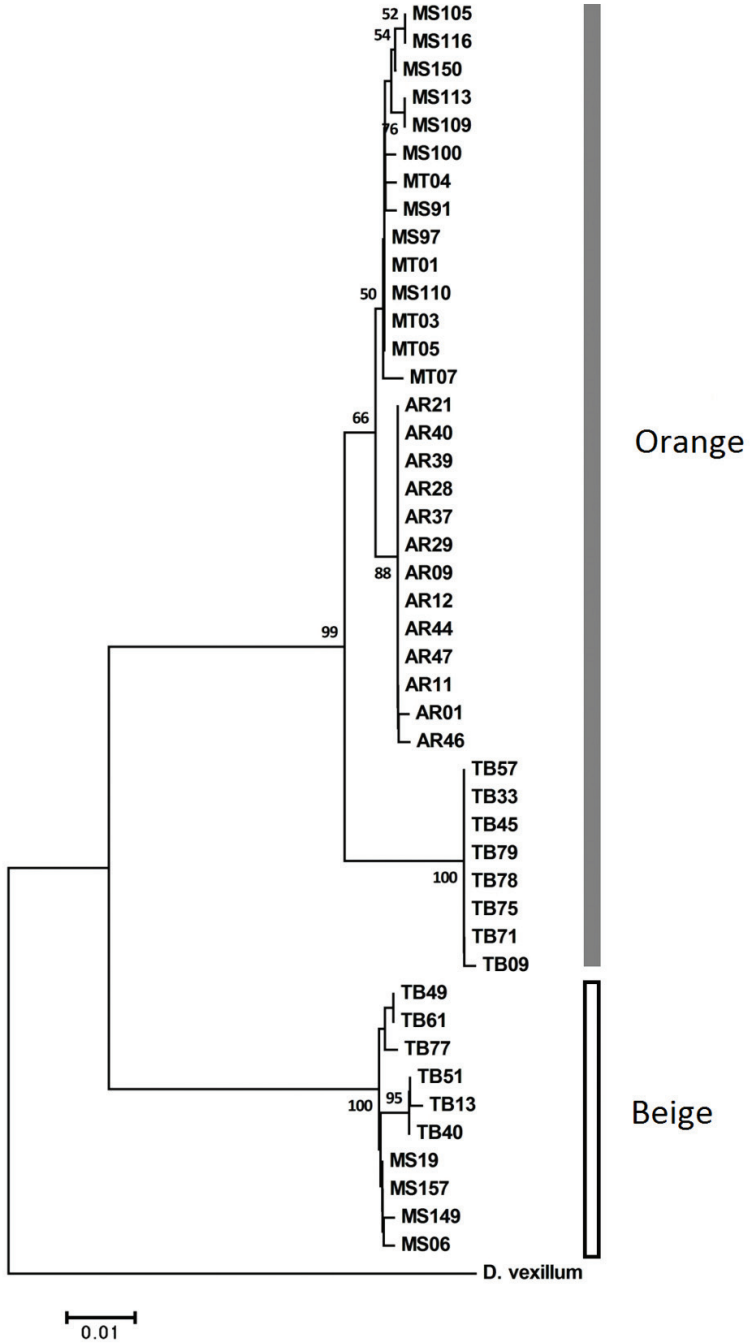

Figure 3. Neighbor-joining tree of *D. granulatum*, using TIM evolution model. Bootstrap support values above 50% are shown on the branches. *D. vexillum* sequence was used as outgroup.

as between populations of distinct color morphotypes. This genetic divergence is shown in the haplotype network (Figure 4), which displays 43 mutational steps between the beige and the orange lineages (approximately 15% of divergence, a value that lies in divergence mean at COI for congeneric species of Chordata- Hebert et al. (2003)), suggesting that they are probably distinct species.; thus, our study is in line with other studies that show the existence of complexes of cryptic species in the seas not recognized by morphological characteristics only (e.g. Palumbi 1994; Knowlton 2000; Pérez-Portela and Turón 2008).

The Fst values were higher between populations of distinct colors than between populations of distinct islands in all comparisons (Table 3). The genetic distance between

the beige and orange clades (10.6%) was higher than the values found by other studies addressing ascidians with different color morphotypes. The mean genetic distance found between two color variants from the Mediterranean colonial ascidian *Pycnoclavella communis* (Pérez-Portela, Duran and Turon, 2007) was 8.55%, suggesting that they would represent two different species (Pérez-Portela and Turon, 2008). Another study also regarding a colonial ascidian, *Pseudodistoma crucigaster* Gaill 1972, suggests speciation associated to the different color morphotypes that present genetic divergence of only 2.12% (Tarjuelo et al., 2004).

Comparing AMOVA values, *P. crucigaster* showed 90.9% of the genetic variation between orange and yellow-grey clades (Tarjuelo et al., 2004). For *D. granulatum*, the

Table 3. Population pairwise Fst using Kimura 2p distance method. AR: Arvoredo; MT: Matadeiro; MS: Moleques do Sul; TB: Tamboretes.

	AR	MT	MS	MS (Beige)	TB	TB (Beige)
AR						
MT	0.80974					
MS	0.77859	0.12831				
MS (Beige)	0.99041	0.97101	0.96486			
TB	0.98241	0.95188	0.93142	0.99078		
TB (Beige)	0.97427	0.94530	0.94828	0.21569	0.97029	

Table 4. P-values.

	AR	MT	MS	MS (Beige)	TB	TB(Beige)
AR						
MT	0.0000±0.000					
MS	0.0000±0.000	0.0849±0.009				
MS (Beige)	0.0000±0.000	0.0107±0.003	0.0009±0.001			
TB	0.0000±0.000	0.0009±0.001	0.0000±0.000	0.0009±0.001		
TB (Beige)	0.0000±0.000	0.0029±0.002	0.0000±0.000	0.0478±0.006	0.0009±0.001	

Table 5. Pairwise genetic distances (K2p) between (underlined number) and within beige and orange populations of *D. granulatum.*

	Beige	Orange
Beige	0.006	
Orange	0.106	0.016

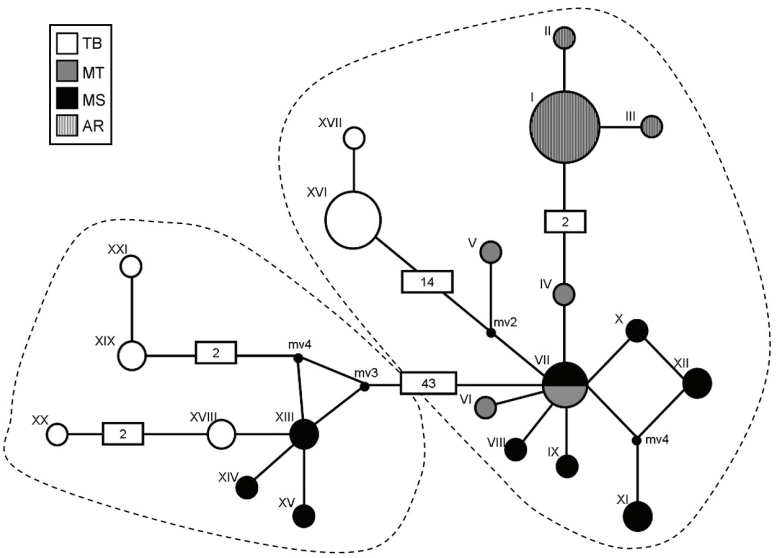

Figure 4. Median-joining (MJ) network describing the relationship among the 21 *D. granulatum* COI haplotypes. Simple line: 1 evolutionary step between the haplotypes. The numbers inside the box means the mutational points number if greater than one. The circle area is proportional to the frequency of each haplotype and the colours indicate the four different populations.

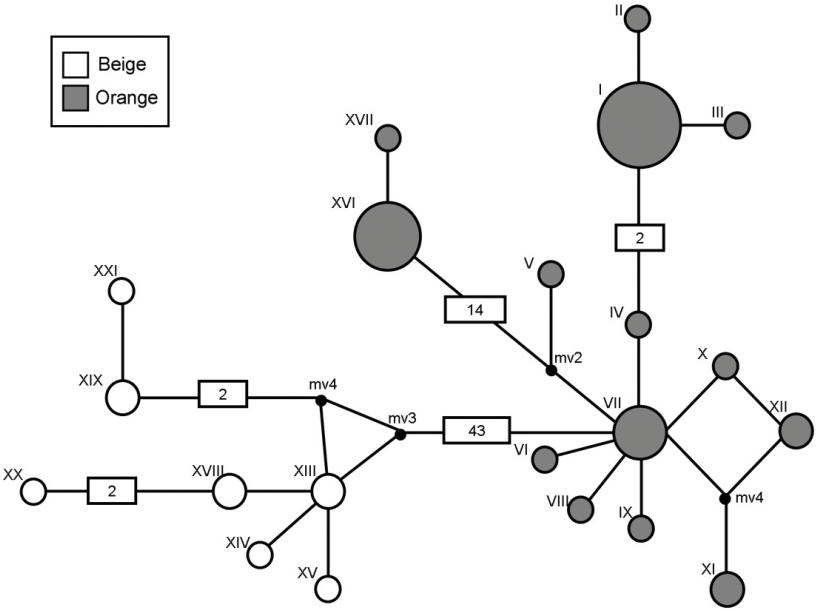

Figure 5. Median-joining (MJ) network describing the relationship among the 21 mtDNA haplotypes and the cromatic variation. Simple line: 1 evolutionary step between the haplotypes. The number inside the Box means the mutational points number IF greater than one. The circle area is proportional to the frequency of each haplotype and the colours indicate the samples colours.

variation between the two color morphotypes (orange and beige) was 82.19%. *Cistodytes dellechiajei* (Della Valle, 1877), another colonial Mediterranean ascidian, also presented high genetic divergence between populations with distinct color morphotypes (76.02%) and inhabit different localities (29.42%) (López-Legentil and Turon, 2006).

Additional morphological and other biological studies, such as cross-fertilization might be helpful in revealing complexes of cryptic species (Pérez-Portela and Turon, 2008). López-Legentil and Turon (2006) consider that the distribution of haplotypes is related to population fragmentation and geographic expansion. Despite the high pairwise Fst values for beige and orange populations from MS and TB (100 km far from each other), the haplotype and nucleotide diversities were high, suggesting some gene flow between close or distant populations. The frequency of private haplotypes for the studied populations of *D. granulatum* (95%) suggests restricted gene flow between them as found for *P. communis* (Pérez-Portela and Turón op cit). Our results reinforces the idea that localized dispersion and local self-recruitment is the dominant pattern for populations of colonial ascidians, contrasting with other marine groups that have high dispersion rates (Ayre et al., 1997).

When the genetic diversity of two morphotypes were separately analyzed, we were not able to recognize a *stepping stones* pattern. In stepping stone model (Kimura, 1953), colonization always occurs from the most geographic near population, what makes the genetic diversity changes gradually in the same way the geographical distance increases. The observed patterns demonstrated that the diversity of orange populations of TB and AR and the beige populations have not an origin in the adjacent islands.

4.1. Implications for conservation

There is no evidence that human activity have caused these patterns. Human activities are often associated with genetic homogenization (mixture of lineages and species in same locations) and loss of genetic diversity (including extinction). Indeed, the high genetic diversity and the presence of private haplotypes (or even lineages) found for each sampled island are not sign of recent colonization or recent bottle neck. On the other hand, a recent decrease in population size could result in loss of intermediate haplotypes similarly to the loss of intermediate alleles in microssatellites described by Garza and Williamson (2001). The patterns found in Santa Catarina suggest that the source of genetic diversity is mutation rather than gene flow. Considering that populations have been stable for a long time, the main obstacle for their conservation may be the exogamic depression that may occur in the future caused by human activity.

The fact that such population divergences were found in this restricted area creates a problem to conservation of this (these) species. Since there are subdivisions and cryptic species in this restricted area, the global diversity pattern of *D. granulatum* must be highly complex. Tests for exogamic depression may reveal the consequences of genetic homogenization before this becomes a reality. Finally, we suggest the need of further systematic studies, including the investigation of new morphological and molecular characters.

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