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## Short Communication

## Lack of COI variation for *Clavelina oblonga* (Tunicata, Ascidiacea) in Brazil: Evidence for its human-mediated transportation?

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### Abstract

Recent studies indicate that ascidians are efficiently dispersed by human transport. We have chosen the mitochondrial gene cytochrome c oxidase subunit I (COI) to address whether *Clavelina oblonga* is an introduced species in the Brazilian coast. Colonies of *C. oblonga* were sampled in different localities along Atlantic coasts of USA, Panama, and Brazil. The sequencing of 92 colonies resulted in three haplotypes for the species, two unique to Florida and the other shared by exemplars collected in Brazil and Panama; the latter haplotype is identical to the published sequence of Azores. Our evidence, including the absence of *C. oblonga* in the country's northern tropical waters, its association with artificial habitats and lack of COI variation suggest that the species has been introduced in the southeastern and southern Brazilian coasts. Previous records (85 years old) suggest that it could be a relatively long-term introduction.

**Key words:** cytochrome c oxidase subunit I; sea squirt; southwestern Atlantic; species introduction

### Introduction

Ascidians have been shown to be efficiently dispersed between different oceans and hemispheres by human transport (Rius et al. 2008; Stefaniak et al. 2009; Bock et al. 2012). *Clavelina oblonga* Herdman, 1880 (Aplousobranchia, Clavelinidae) is an Atlantic colonial species well known in American tropical waters. It was first described from Bermuda and its known distribution includes the United States (South Carolina, Florida), Caribbean Sea (Rocha et al. 2005) and Brazil (Rocha and Kremer 2005). When recorded at the eastern Atlantic: Azores Islands (Monniot and Monniot 1994), Cape Vert (Hartmeyer 1912) and Senegal (Pérès 1951) the species was considered as introduced for these areas.

The distribution of *Clavelina oblonga* along the Brazilian coast attracted our attention, since it is restricted to southeastern and southern cooler waters; but is without records in northern warmer waters, as would be expected,

considering its presence in the Caribbean Sea. Disjunctive distribution of any species (especially along a continuous coast) contributes to evidence of introduction (Chapman and Carlton 1991). Other criteria proposed by these authors in order to define introductions are (1) lack of previous records in the region; (2) extension of range after the first encounter; (3) access to human mechanism(s) of dispersal; (4) association with known introductions; (5) prevalence in, or restriction to, artificial or altered environments; (6) global distribution; (7) insufficient active dispersion capabilities. *Clavelina oblonga* only meets these criteria partially. The oldest report of this species from the Brazilian coast (Van Name 1945) seems to correspond to specimens collected in 1925 by Luederwaldt. This date also represents the beginning of ascidian studies in Brazil. Following studies are dated at least 30 years later (Rodrigues 1962). Although *C. oblonga* was found both on artificial and natural rocky substrates, it was never reported from boat hulls, but these have not been

extensively surveyed in southern Brazilian coast. The species is not particularly associated with any introduced species.

The COI is widely used as a genetic marker in evolutionary studies, particularly to define different populations and closely related taxa of metazoans (Hebert et al. 2003). The first half at the 5' end of the COI (Folmer region) has been suggested to reveal patterns of cryptic speciation and genetic structure within lineages of *Clavelina lepadiformis* (Müller, 1776) (Tarjuelo et al. 2001) and to detect introduction of that species in the Mediterranean Sea (Turon et al. 2003) and western Atlantic (Reinhardt et al. 2010). It has also successfully elucidated introduction events both for ascidians (e.g., Castilla et al. 2002; Turon et al. 2003; Rius et al. 2008), and other marine invertebrate groups (e.g. Mackie et al. 2006; Darling et al. 2008). Within members of Clavelinidae, the COI variability has also been used to refine species boundaries of *Clavelina* and *Pycnoclavella* (Pérez-Portela and Turon 2008). The objective of this study was to address whether *C. oblonga* is an introduced species in the Southwestern Atlantic (Brazilian coast), based on comparisons of COI diversity among colonies collected at that area and other ones from the eastern and western Atlantic (that study and cited literature).

## Material and methods

Ninety-two colonies of *Clavelina oblonga* were collected on artificial and natural substrates in different localities along Atlantic coasts of USA (n=3), Panama (n=4) and Brazil (n=85) (Table 1). Additionally, three colonies of *Clavelina picta* Verrill, 1900 were collected in Cuba for comparison purposes (Table 1). Most colonies were deposited as vouchers (hologenophores, *sensu* Pleijel et al. 2008) at the Ascidiacea collection in the Zoology Department, Universidade Federal do Paraná, Brazil (DZUP) (Table 1).

Total genomic DNA was isolated from tissue of individual zooids (without tunic), and extracted with the DNeasy Blood and Tissue Kit (QIAGEN). The primer set LCO1490 + HCO2198 (Folmer et al. 1994) was used for PCR and sequencing of COI. PCRs were carried out in 25µl volumes, using illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare). Cycling conditions for the amplification were: 94°C, 5' – 35x: 94°C, 30"; 50-55°C, 30"; 72°C, 45" – 72°C, 3'. PCR products were cleaned with

illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing was performed on an ABI PRISM® 3100 Genetic Analyzer, using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Complementary strands were combined and edited with CodonCode Aligner (CodonCode Corporation) and compiled using Geneious Pro v5.3.6 (Drummond et al. 2010). The resulting sequences were deposited in GenBank (Table 1).

An alignment of all sequences obtained in this study, plus one of *C. oblonga* from Azores, Portugal (Table 1), was constructed in Geneious using default settings. The number of haplotypes, synonymous and nonsynonymous substitutions, the nucleotide diversity ( $\pi$ ), and the percentage values of variable sites were estimated by pairwise analyses using DnaSP v5 (Librado and Rozas 2009).

The tree search considered 13 ingroup terminals; including three of *C. oblonga* and the others of congenics sequenced for the present study or downloaded from GenBank (Table 1). The clavelinids *Pycnoclavella flava* Monniot, 1988 and *Pycnoclavella* aff. *tabella* Kott, 1990 were used as outgroup terminals (Table 1). All sequences were aligned and verified in Geneious prior to tree reconstruction. Phylogenetic analysis was undertaken using Maximum Likelihood (ML) with PhyML 3.0 (Guindon et al. 2010). GTR+I+G was selected as the best-fit model of evolution for the dataset using jMODELTEST v. 0.1.1 (Posada 2008), through corrected Akaike Information Criterion. An initial tree was estimated using neighbor joining followed by a heuristic search performed implementing the estimated model parameters ( $p\text{-inv} = 0.3360$ ;  $\gamma\text{ shape} = 0.3100$ ) and using nearest-neighbor-interchange (NNI) branch swapping. Nodal support was estimated by ML bootstrapping (n=100), with values of  $\leq 75\%$  considered as not significant on the tree.

## Results

The sequencing of a fragment of COI from 92 colonies of *Clavelina oblonga* resulted in three haplotypes: haplotypes 1 (672bp) and 2 (598bp) unique to Florida, and haplotype 3 (672bp) shared by exemplars collected in Brazil and Panama (Table 1) ( $\pi = 0.05797 \pm 0.02528$ ; number of singleton variable sites = 52; 91.2% of identical sites). Additionally, the comparison of 617 bp of haplotype 3 and the complete

**Table 1.** Sequences of *Clavelina* and *Pycnoclavella* included in the phylogenetic analysis, with localities and GenBank accession numbers. For the sequences obtained in this study, substrate types, geographic coordinates, number of colonies analyzed (N), haplotype designation (Hap), and DZUP numbers are also provided.

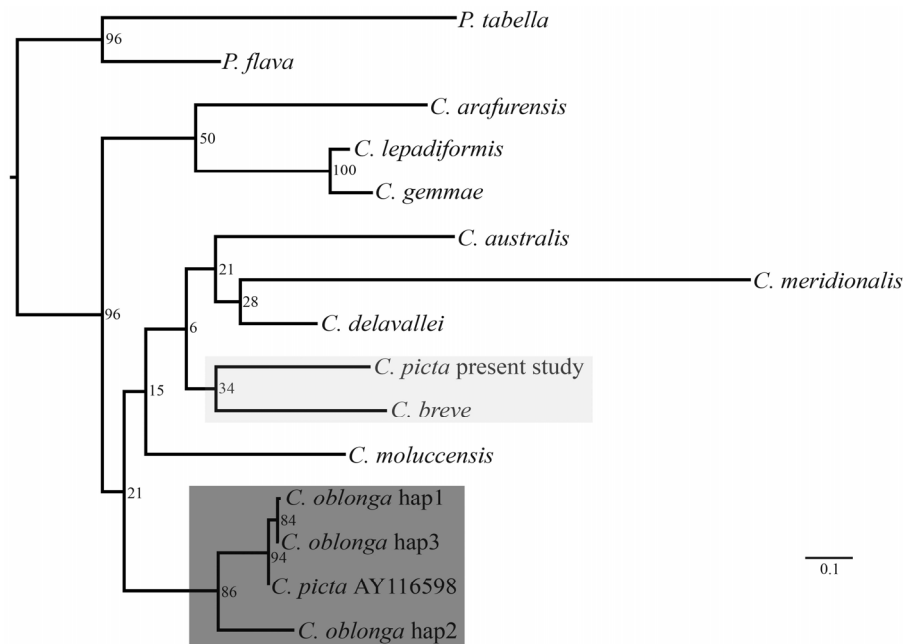
Species	Locality	Substrate	GPS Coordinates	N	Hap	GenBank	DZUP
<i>C. oblonga</i>	Cape Canaveral, Florida, USA	Pier, hanging brick	28°24'56"N, 80°37'24"W	01	1	JN703738	—
	Fort Pierce, Florida, USA	Pier column	27°27'21"N, 80°18'34"W	02	1 2	JN703738 JN859182	CLA-46
	Bocas del Toro, Panama	Piercolumn	09°20'08"N, 82°14'50"W	04	3	JN703739	CLA-20
	Pontal da Cruz, Brazil	Pier column	23°46'53"S, 45°23'46"W	04	3	JN703739	—
	Praia do Cabelo Gordo, Brazil	Natural rocks	23°49'42"S, 45°25'22"W	03	3	JN703739	CLA-013
	Yacht Club IlhaBela, Brazil	Pier column	23°46'27"S, 45°21'20"W	11	3	JN703739	—
	Saco do Poço, IlhaBela, Brazil	Natural rocks	23°45'30"S, 45°15'12"W	04	3	JN703739	—
	Ilha do Mel, Brazil	Pier column	25°34'12"S, 48°18'58"W	04	3	JN703739	CLA-09
	Ilha da Galheta, Brazil	Natural rock, subtidal	25°35'06"S, 48°19'26"W	01	3	JN703739	—
	Tenenge, Brazil	Pier column	25°33'08"S, 48°21'52"W	04	3	JN703739	CLA-25
	São Francisco do Sul, Brazil	Mussel socks	26°12'27"S, 48°33'57"W	02	3	JN703739	—
	Arquipélago das Graças, Brazil	Natural rock, subtidal	26°10'51"S, 48°20'06"W	03	3	JN703739	—
	Ilha Deserta, Brazil	Natural rock, subtidal	27°16'31"S, 48°19'57"W	08	3	JN703739	—
	Ilha Arvoredo, Brazil	Natural rock, subtidal	27°16'59"S, 48°22'26"W	07	3	JN703739	CLA-03
	Penha, Brazil	Mussel socks	26°46'48"S, 48°36'44"W	09	3	JN703739	CLA-05
	Ribeirão da Ilha, Brazil	Oyster lanternets	27°44'08"S, 48°33'52"W	24	3	JN703739	CLA-22
	Saco da Ponta Grossa, Brazil	Natural rocks	23°46'56"S, 45°14'01"W	01	3	JN703739	CLA-42
<i>C. picta</i>	Azores, Portugal			-	3	AY603106*	
	Havana, Cuba	Coral reef	23°05'20"N, 82°30'23"W	-	-	JN703740	CLA-44
<i>C. arafurensis</i>	Tanzania			-	-	AM706463*	
<i>C. australis</i>	Jervis Bay, Australia			-	-	AM706464*	
<i>C. breve</i>	Wasini, Kenya			-	-	AM706465*	
<i>C. meridionalis</i>	Australia			-	-	AM706468*	
<i>C. moluccensis</i>	Guam			-	-	AM706472*	
<i>C. gemmae</i>	Spain			-	-	AJ884573*	
<i>C. lepadiformis</i>	Spain			-	-	AY603104*	
<i>C. delavallei</i>	Spain			-	-	AY603105*	
" <i>C. picta</i> "	Florida, USA			-	4	AY116598*	
<i>Pycnoclavella aff. tabella</i>	Mafia, Tanzania			-	-	AM706488*	
<i>P. flava</i>	Kenya			-	-	AM706476*	

\* sequences accessed from GenBank

sequence from the Azores did not reveal any differences between them. The three aligned haplotypes (final alignment of 597bp) were translated with the ascidian mitochondrial genetic code available in DnaSP, and no stop or ambiguous codons were observed. Of the 199

codons analyzed, 48 synonymous substitutions and four replacement changes were detected.

The final alignment constructed for phylogenetic analysis resulted in 566 sites after the exclusion of leading and trailing gaps; the ML tree is shown in Figure 1, with values of nodal



**Figure 1.** Maximum likelihood cytochrome c oxidase subunit I tree for the species of *Clavelina* included in this study. The numbers on nodes indicate percentage of bootstrap values ( $\geq 75\%$  considered as significant). Scale bar indicates the number of substitutions per site. The clade of *Clavelina oblonga* + contaminated '*C. picta*' from Genbank is highlighted in dark gray, while the group in which *C. picta* sequenced for the present study nests is highlighted in light gray.

support  $\geq 75\%$  depicted below branches. The three haplotypes of *C. oblonga* formed a clade, in which *C. picta* from Florida nested with significant nodal support (ML bootstrap = 86%). Haplotypes 1 and 3 of *C. oblonga* were highly similar to *C. picta* from Florida (98.4% of identical sites). Interestingly, the previously published sequence of *C. picta* from Florida and the sequence of the same species from Cuba (present study) were highly divergent (~16%).

## Discussion

Contrasting with other species of stolidobranch ascidians (Rius et al. 2008; Pineda et al. 2011), the results of the present study indicate a low number of haplotypes for the sampled specimens of *C. oblonga*. Surprisingly, while we found two haplotypes (1, 2) after analyzing only three individuals from Florida, the haplotype 3 was fixed for the seven individuals of Panama and the 85 individuals sequenced from Brazil, suggesting the introduction of *C. oblonga* in the latter country, even though our results are not conclusive under a genetic perspective. On the other hand, haplotypes 1 and 3 of *C. oblonga*

were highly similar to *C. picta* from Florida (98.4% of identical sites). The COI similarity and the phylogenetic analysis suggest that Stach and Turbeville (2002) misidentified *C. picta*, and that its sequence (Genbank accession number AY116598) represents in fact a fourth haplotype of *C. oblonga*.

Like *C. oblonga*, the Atlantic-Mediterranean *C. lepadiformis* occurs on rocky littoral zones and marinas (Tarjuelo et al. 2001). That species is a complex of two cryptic lineages, one restricted to the rocks at the Mediterranean, and the other by specimens occurring in the Atlantic and the artificial Mediterranean substrates (Turon et al. 2003). The latter lineage is composed by six haplotypes, of which one is shared between Mediterranean and Atlantic; these results were interpreted by Turon et al. (2003) as evidence of an introduction from the eastern Atlantic to the Mediterranean by ship-hull transportation.

Similar to *C. lepadiformis*, studies of the widespread distribution of the ascidian *Botryllus schlosseri* Pallas 1766 (Bock et al. 2012), the bryozoan *Bugula neritina* (Linnaeus, 1758) (Mackie et al. 2006), and the green crab

*Carcinus maenas* (Linnaeus, 1758) (Darling et al. 2008) suggest that while the majority of the COI haplotypes found for their populations are restricted to specific localities, only one was widely introduced. Although there are only four haplotypes for *C. oblonga* (this study; Stach and Turbeville 2002) sampled in part of its known distribution, such pattern also might be plausible to explain the wide distribution of the haplotype 3.

A second hypothesis to explain the lack of COI variation among individuals widely distributed (Brazil, Panama, Azores) is the unusual conservation of the gene. The only other known instance of widely distributed ascidians lacking COI variation is the case of *Ecteinascidia turbinata* Herdman, 1880 (Phlebobranchia; Perophoridae) (López-Legentil and Turon 2007). In that study, the sequencing of 163 colonies from the western and eastern Atlantic, the Caribbean and the Mediterranean, revealed a single haplotype fixed for all populations. On the other hand, contrasting patterns of other characters were found for *E. turbinata*, including colour variation of colonies and high intraspecific diversity of the nuclear gene *hsp70*. We did not find any remarkable differences in morphology among the studied colonies of *C. oblonga* that could be indicative of population structure. Future tests of the hypothesis of highly conservation of COI for populations of this species will need to rely on the sequencing of nuclear genes.

Consistent with the lack of genetic variation detected among samples from Brazil, Caribbean and Azores, the ability of the species to colonize a wide range of natural and artificial substrates, and the observation that *C. oblonga* thrives well in calm and polluted waters (Monniot 1972) such as those encountered in ports and marinas, favour the hypothesis of its introduction in Brazil. Recently, the eastern Atlantic lineage of *C. lepadiformis* has also been detected in the northwestern Atlantic (Reinhardt et al. 2010). The existence of a congeneric invasive lineage with a similar life cycle, introduced into non-native areas via modern navigation, allows us to infer that boat hulls might also transport colonies of *C. oblonga*.

Unfortunately, we cannot infer the specific source area of the invasive haplotype 3, due to lack of phylogeographic information. Due to the highest genetic diversity among Florida samples (three haplotypes), we might speculate that the

native range of the species is somewhere in the tropical northwest Atlantic. The presence of *C. oblonga* in Jamaica and Cuba was reported by personal observations (I. Goodbody and A. Zanuy, respectively). Recently, our efforts to collect *C. oblonga* in Jamaica (2003-2004) and in Cuba (2010), including natural and artificial substrates, were not successful, due its apparent disappearance in these areas. Local extinctions of some introduced populations of marine organisms recently established are known, representing evidence that those populations might also be non-native in those areas. In the coast of Brazil, the ascidian *Eudistoma carolinense* Van Name, 1945 was common on intertidal rocky shores in the state of Santa Catarina in the 1990s (Rocha and Moreno 2000) but has not been found in the last 10 years (personal observation, RMR); the bivalve *Isognomon bicolor* (C.B. Adams, 1845) also established on rocky shores in southeastern and south Brazil in the 1980s, but populations in Rio de Janeiro suffered a great mortality in 2006 (López 2008).

In conclusion, our results suggest that *C. oblonga* might have been introduced in the southeastern and southern Brazilian coasts; these include a disjunctive distribution (absence in the country's northern tropical waters), its association with artificial habitats, and lack of COI diversity among the studied populations. The first collections of ascidians made by Luederwaldt in 1925 in São Sebastião, São Paulo (animals identified by Van Name, 1945) had already included *C. oblonga*, suggesting a relatively long-term introduction (at least 85 years old).

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