



## Characterising invasion processes with genetic data: an Atlantic clade of *Clavelina lepadiformis* (Ascidiacea) introduced into Mediterranean harbours

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### Abstract

We studied the genetic structure of populations of the Atlanto-Mediterranean ascidian *Clavelina lepadiformis* (Müller, 1776). A 369 bp segment of the COI mitochondrial gene was sequenced in Mediterranean and Atlantic populations from inside harbours, marinas and fjords (interior populations), and from the open rocky littoral (exterior populations). Previous work identified genetic differences between *C. lepadiformis* inhabiting Mediterranean harbours and the Mediterranean rocky littoral, however, the origin of these two clades remained speculative. Here we compared the Mediterranean populations with four Atlantic populations (two interior and two exterior). Gene differentiation and maximum likelihood analyses showed that the Atlantic forms were not divided into interior and exterior clades, and were closely related to the interior clade in the Mediterranean. The results support the hypothesis that both clades evolved allopatrically in the two seas, and that a recent colonisation of Mediterranean marinas from the Atlantic was caused by ship-hull transport. Colonisation of habitats by new genetic variants, morphologically indistinguishable from local populations, may be common among benthic invertebrates, and only genetic tools can uncover these cryptic invasions.

### Introduction

Colonisation of new geographical areas by marine species is often caused by transport on ships' hulls and in ballast waters, with harbours and marinas often being the entrance gates for new invasions (Carlton & Geller, 1993). This is especially true of invertebrate species with short-lived larvae, whose opportunities for long-distance dispersal may rely, in many cases, on rafting on human-made floating objects. Ascidians belong to this latter category due to their larval characteristics (Svane & Young, 1989), and the role of harbours and marinas in the introduction of non-indigenous ascidian species is increasingly recognised (Monniot et al., 1985, 1991; Monniot & Monniot, 1994; Lambert & Lambert, 1998). In the Mediterranean Sea, non-indigenous ascidian species have been reported

recently in harbours and marinas (Brunetti, 1979; Monniot, 1981; Zibrowius, 1991).

The contribution of maritime transport to benthic assemblages may go undetected if the coloniser is a genetic variant or a cryptic species that is morphologically indistinguishable from native populations/species. Such cryptic invasions may be common, with colonisers confined, at first, to marginal marine habitats such as harbours and lagoons, and eventually spreading to open habitats. While ecophenotypic variability may provide the first clue that a new variant has arrived in a region, only molecular tools can identify reliably these genetic variants and uncover cryptic colonisation of new areas. Indeed, there are instances of genetic discontinuities between supposedly conspecific populations inhabiting open littoral and marginal marine habitats (Dalby, 1997; Boisselier-Dubayle &

Gofas, 1999) that suggest the arrival of new genetic variants in the latter type of habitat.

*Clavelina lepadiformis* (Müller, 1776) is an Atlanto-Mediterranean species whose distribution ranges from Scandinavia (Millar, 1966) to the Aegean Sea in the eastern Mediterranean (Koukouras et al., 1995). Wirtz & Martins (1993) and Wirtz (1998) reported this species from the Azores and Madeira, and suggested that it may have been introduced by ships, spreading subsequently from island to island. In the Mediterranean, *C. lepadiformis* has been reported from harbours and shallow rocky littoral habitats (Harant, 1927; Turon, 1987; Ramos, 1988). In Atlantic waters, it is known from rocky bottoms down to 50 m, but is also present in sheltered environments including wharfs, harbours, fjords and shallow estuarine bays (Berrill, 1950; Millar, 1966; Vázquez, 1993). Here we use the term 'exterior populations' for those inhabiting open littoral rocky shores, and 'interior populations' for those inside harbours, marinas or fjords. A recent study of *C. lepadiformis* based on mitochondrial DNA sequence data (Tarjuelo et al., 2001) showed that Mediterranean populations inhabiting harbours (interior form) were genetically distinct from those thriving in the open habitats (exterior form). It was concluded that the two forms could be cryptic species. A biological study of both forms (De Caralt et al., 2002) revealed noticeable differences in parameters such as growth, biological cycles, chemical defence production and juvenile survival. Therefore, although the two clades were morphologically identical, genetic and ecophenotypic divergences were observed.

The origin of the two clades (interior and exterior), however, remained speculative. Two hypotheses were advanced by Tarjuelo et al. (2001): firstly, parapatric speciation occurred in the Atlantic between populations in open shores and those in sheltered, marginal marine environments. The form evolving in these marginal habitats would have been preadapted to colonise man-made harbours and constructions. The two clades could have colonised the corresponding Mediterranean habitats, possibly after the Messinian salinity crisis of the Mediterranean (late Miocene, between 5 and 6 MY, Maldonado, 1985). The second hypothesis was that the two clades diverged allopatrically from an original population split in the Mediterranean and Atlantic basins since the Miocene, and that the interior form represents the Atlantic clade that has colonised recently Mediterranean harbours through ship traffic (Tarjuelo et al., 2001).

Here, we examine the origin and relationships between the two Mediterranean clades by adding to the existing genetic database representatives of interior and exterior populations of the Atlantic. Specifically, we address the following questions: is there a genetic divergence between interior and exterior populations in the Atlantic, as is the case in the Mediterranean? What are the relationships between the Atlantic populations and the two Mediterranean clades?

## Materials and methods

We studied 73 individuals of *Clavelina lepadiformis* belonging to two exterior and two interior populations from Atlantic shores, and three exterior and three interior populations from the Spanish Mediterranean (Fig. 1). The Mediterranean populations studied were: Cadaqués and Tossa de Mar (exterior populations), Estarrit and Port Ginesta (interior populations), and Blanes (which had both exterior and interior populations, Fig. 1). The Atlantic populations sampled were: Smatiolmarma, at the Gullmarsfjorden (Sweden), Vigo (NW Spain), Sesimbra (Portugal), and Sao Miguel Island (Azores). The Portuguese mainland and Azorean populations sampled were from exterior habitats, while the Vigo sampling point was inside the Baiona Port; the Swedish locality was inside a fjord. The Mediterranean locations were the same as in Tarjuelo et al. (2001).

We also sequenced two specimens of *C. dellavallei* (Zirpolo, 1925) from Cabo de Gata (SE of Spain) and one specimen of *C. oblonga* Herdman, 1880 from Faial Island (Azores) as outgroups.

Mitochondrial DNA was extracted from ethanol-preserved samples using the protocol for *Drosophila* sp. described in Latorre et al. (1986). We used specific primers designed for a region of cytochrome oxidase I in this species (Tarjuelo et al., 2001). The amplification yielded a fragment of ca. 500 bp that, after alignment and trimming, reduced to 369 positions for the analyses. No gaps were needed in the alignment.

Amplification was performed in a 20  $\mu$ l total reaction volume with 0.4  $\mu$ l of each primer (25  $\mu$ M), 3  $\mu$ l dNTP's (1 mM), 2  $\mu$ l 10X buffer containing 15 mM MgCl<sub>2</sub> (Promega), 1U Taq polymerase (Promega) and 1  $\mu$ l DNA. A single soak at 96 °C for 3 min was followed by 35 cycles (denaturation at 95 °C for 55 s, annealing at 47 °C for 55 s, and extension at 72 °C for 1.5 min), and a final extension at 72 °C for 5 min, on a Perkin-Elmer 9600 machine. The same primers were

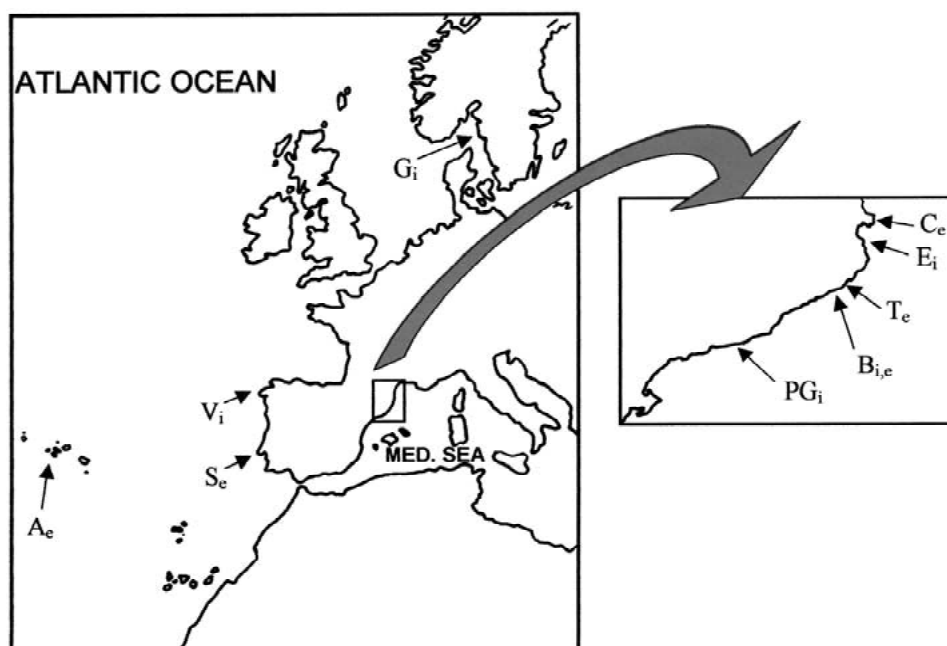


Figure 1. Map showing the position of the populations sampled. Codes of localities: (A) Azores Islands, (B) Blanes, (C) Cadaqués, (E) Estartit, (G) Gullmarsfjorden, (PG) Port Ginesta, (S) Sesimbra, (T) Tossa, (V) Vigo. Subscripts e and i mean exterior and interior types of habitat, respectively.

used for the sequencing reaction, and the sequences were obtained using the ABI Big-Dye Ready-Reaction kit of Perkin Elmer on an ABI Prism 9700 automated sequencer belonging to the Scientific and Technical Services of the University of Barcelona.

Nucleotide diversity within and between populations ( $\pi$ , Nei, 1987, equation 10.5; Lynch & Crease, 1990), divergence between populations ( $D_{xy}$ , Nei, 1987, equation 10.20), and haplotype diversity (Nei, 1987, equation 8.4) were measured. Genetic divergence was estimated by measuring  $\Gamma_{ST}$  ( $\gamma_{ST}$ ) (Nei, 1982). The significance of estimates of genetic differentiation was tested with a permutation (randomisation) test. Gene flow was calculated using the standard relationship  $\gamma_{ST} = 1/(1+2N_{ei}m)$  to obtain  $N_{ei}m$ , the effective number of migrants, where  $N_{ei}$  is the inbreeding effective population size and  $m$  is the migration rate per generation. These estimates are contingent on the assumptions of the island model of population structure (Wright, 1951). The population genetic parameters were estimated and analysed using DnaSP v.3.97 (Rozas & Rozas, 1999) and Arlequin v. 2000 (Schneider et al., 2000).

Phylogeographic information in the data was studied by maximum likelihood analysis of the sequences. To select the best-fit model of nucleotide substitution

for the COI data set, the hierarchical likelihood ratio tests approach implemented in Modeltest 3.0 (Posada & Crandall, 1998) was applied to a NJ cladogram obtained from the data. A haplotype tree was then estimated under the maximum likelihood criterion (ML; Felsenstein, 1981), with the parameter estimates obtained under the best-fit model. A heuristic search with 1000 replicates of random stepwise addition and tree bisection-reconnection (TBR) branch-swapping was implemented. As we used mid-point rooting, there was no need to force the ingroup to be monophyletic. Confidence in the resulting nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985). Phylogenetic analyses were implemented in PAUP\* (Swofford, 1998).

Type sequences are available from GenBank (Mediterranean haplotypes, accession # AF368352 and AF368353; Atlantic haplotypes, accession # AY211529, AY211530 and AY211531).

## Results

Thirteen different haplotypes were found in the samples (Table 1). Overall, there were 32 variable sites (8.67%), of which only 5 yielded non-synonymous changes. 14 fixed nucleotide positions

Table 1. *Clavelina lepadiformis*. Haplotype frequencies, nucleotide diversity within populations ( $\pi$ ) and number of polymorphic sites (PS) in the populations studied. The codes used in Figure 1 to designate the sampling points are indicated in parentheses, e and i denote exterior and interior populations, respectively

<i>C.lepadiformis</i> Population	Haplotypes													Total	$\pi$	PS
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII			
Cadaques (C), e	2	1	-	-	-	-	-	-	-	-	-	-	-	3	0.0022	1
Tossa (T), e	7	-	1	7	-	-	-	-	-	-	-	-	-	15	0.0024	3
Blanes (B), e	4	-	-	-	2	1	3	-	-	-	-	-	-	10	0.0027	3
Estartit (E), i	-	-	-	-	-	-	-	3	7	1	-	-	-	11	0.0017	2
Blanes (B), i	-	-	-	-	-	-	-	4	11	-	-	-	-	15	0.0012	1
Port Ginesta (PG), i	-	-	-	-	-	-	-	6	2	-	1	-	-	9	0.0022	3
Vigo (V), i	-	-	-	-	-	-	-	-	5	-	-	-	-	5	0	0
Sesimbra (S), e	-	-	-	-	-	-	-	-	-	-	-	1	-	1	0	0
Gullmarsfjorden (G), i	-	-	-	-	-	-	-	-	2	-	-	-	-	2	0	0
Açores (A), e	-	-	-	-	-	-	-	-	1	-	-	-	1	2	0.0162	6
Totals	13	1	1	7	2	1	3	13	28	1	1	1	1	73		

(3.79%, of which 2 represented non-synonymous changes) discriminated the Mediterranean exterior populations from the group formed by the Mediterranean interior and Atlantic populations. There were no coincident haplotypes between exterior and interior Mediterranean populations, or between exterior Mediterranean and Atlantic populations, while haplotype IX was shared and was the most abundant both in the interior Mediterranean and the Atlantic samples (Table 1). The nucleotide diversity ( $\pi$ ) pooling Mediterranean exterior populations was 0.0037, in Mediterranean interior populations it was 0.0018 and in Atlantic populations it was 0.0063. The values of haplotype diversity were 0.728, 0.550, and 0.378, respectively. When Mediterranean exterior populations were compared with Mediterranean interior+Atlantic samples, the average number of nucleotide differences between these two groups was 20.048 (5.42%), and the average number of nucleotide substitutions per site ( $D_{xy}$ ) was 0.054.

Table 2 shows pairwise values of  $\gamma_{ST}$  between the studied populations and the corresponding estimates of gene flow ( $N_{ei}m$ , subject to the island model of population structure). Again, two groups formed by Mediterranean exterior and Mediterranean interior+Atlantic samples were evident, as reflected by low genetic divergence and high gene flow within groups, and high genetic divergence and low gene flow between groups. Within Mediterranean exterior populations, average  $\gamma_{ST}$  was  $0.143 \pm 0.035$  (mean  $\pm$  SE), and within Mediterranean interior+Atlantic popula-

tions it was  $0.192 \pm 0.035$ . In contrast, average  $\gamma_{ST}$  value between the two groups was  $0.891 \pm 0.015$ . The values of genetic differentiation between the two groups of populations were significant (permutation test at  $p=0.05$ ) in all pairwise comparisons except for the comparisons of Cadaqués with Gullmarsfjorden and Azores (possibly as a result of low sample size in the latter two populations). Conversely, none of the pairwise measures within groups was significant except the comparison between Port Ginesta and Vigo. The estimates of gene flow were lower in between-group comparisons ( $N_{ei}m = 0.063 \pm 0.010$ ) than in within-group pairs ( $N_{ei}m = 3.23 \pm 0.77$  and  $5.31 \pm 1.97$  for exterior Mediterranean and interior Mediterranean+Atlantic groups, respectively).

A maximum likelihood analysis was performed on the 13 haplotypes found. Two haplotypes found in the congeneric *C. dellavallei* from the Mediterranean and one sequence of *C. oblonga* from the Azores Islands were used as outgroups. The hierarchical comparison between likelihood scores of each model of evolution showed that Kimura's three-substitution-type model (Kimura, 1981) with unequal base frequencies was the best-fitting model.

The results of the ML haplotype tree show (Fig. 2) that Mediterranean interior and Atlantic haplotypes cluster together in a monophyletic clade with 88% bootstrap support, while the Mediterranean exterior haplotypes formed another group, with the same support.

Table 2. *Clavelina lepadiformis*.  $\Gamma_{ST}$  ( $\gamma_{ST}$ ) values (above the diagonal) and gene flow estimates ( $Nm$ , below the diagonal) for the different populations studied (nps: no polymorphic sites). The Sesimbra datum was not included as it consisted of a single sequence. Comparisons between the two recognized clades (exterior Mediterranean and interior Mediterranean+Atlantic) are marked by double-line boxes. (e) and (i) denote exterior and interior populations, respectively

	Cad. (e)	Tossa (e)	Blanes (e)	Blanes (i)	P. Gin. (i)	Estartit (i)	Vigo (i)	Gullm. (i)	Açores (e)
Cadaques (e)		0.13	0.09	0.92	0.91	0.91	0.98	0.97	0.85
Tossa (e)	3.29		0.21	0.92	0.90	0.90	0.89	0.79	0.73
Blanes (e)	4.54	1.87		0.93	0.91	0.92	0.93	0.87	0.80
Blanes (i)	0.04	0.04	0.03		0.13	0.02	0.08	0.04	0.32
P. Ginesta (i)	0.05	0.05	0.05	3.23		0.08	0.29	0.17	0.32
Estartit (i)	0.05	0.05	0.04	28.49	5.85		0.12	0.06	0.30
Vigo (i)	0.01	0.06	0.04	5.50	1.25	3.58		Nps	0.42
Gullmarsfj. (i)	0.02	0.13	0.08	11.69	2.46	7.26	nps		0.33
Azores (e)	0.09	0.18	0.13	1.07	1.05	1.16	0.70	1.00	

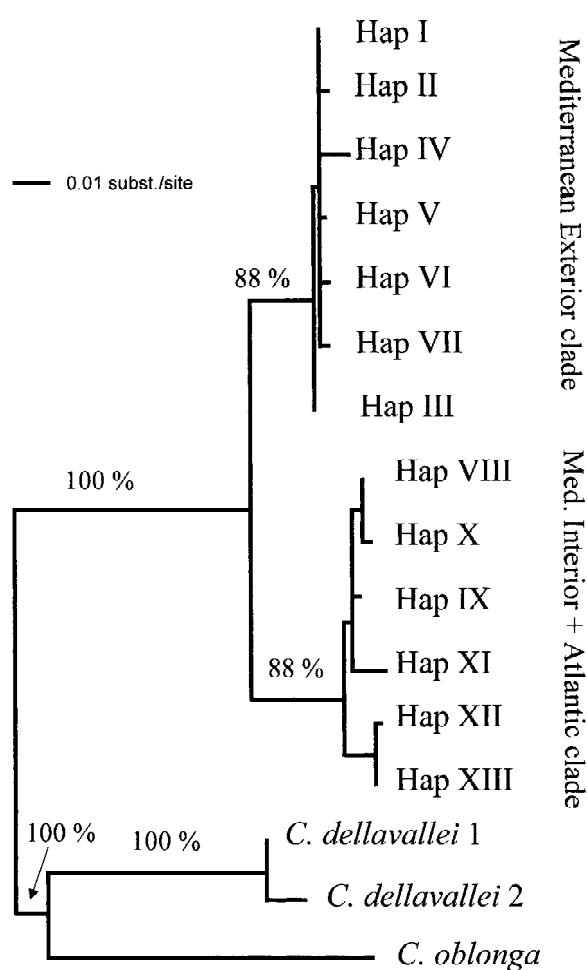


Figure 2. Maximum likelihood cladogram of the haplotypes found in *Clavelina lepadiformis* and the outgroups. Codes of haplotypes as in Table 1. Bootstrap support for the clades is indicated. See text for details.

## Discussion

Present results provide strong evidence that the interior Mediterranean form of *Clavelina lepadiformis* originated from the Atlantic clade. The high genetic similarity between the Atlantic interior populations (Vigo and Sweden) and the exterior ones (Azores, Portugal) indicates that there is only one clade in the Atlantic. This Atlantic clade thrived subsequently in an environment (inside of Mediterranean harbours) that was more similar to the turbid and richer Atlantic waters than the clearer and more oligotrophic open waters of the Mediterranean. Its presence in harbours suggests a colonisation related to ship traffic between ports. Low genetic divergence, and high gene flow estimates between Atlantic and interior Mediterranean populations, suggests strongly that ship traffic favours genetic exchange overwhelming the dispersal ability of the short-lived (hours to days, Tarjuelo, 2001) larvae of this ascidian.

The favoured hypothesis, that the two clades evolved allopatrically in the Mediterranean and Atlantic basins, is in agreement with other instances of genetic discontinuities in conspecific populations or congeneric species-pairs of benthic invertebrates between the north-east Atlantic and Mediterranean (Saavedra et al. 1993; Quesada et al., 1995; Côté-Real et al., 1996; Panacciulli et al., 1997; McFadden, 1999). The presence of two clades of the brittle star *Ophiothrix* in the Mediterranean could, similarly, be the result of allopatric divergence in the Atlantic and Mediterranean, followed by a second invasion of the Mediterranean by the Atlantic clade, as suggested by Baric & Sturmbauer (1999).

The level of nucleotide diversity, as well as the number of haplotypes found, is similar in the two main clades recognized, indicating an ancient separation that has allowed the two forms to undergo genetic differentiation. As the Mediterranean basin suffered a strong drying out period during the Messinian (between 5 and 6 million years ago), it seems likely that the species first originated in the Atlantic and colonised the Mediterranean after the Messinian crisis. This time scale would provide ample time for genetic differentiation of the two clades, followed by a recent invasion of Mediterranean marinas by the Atlantic clade. There are insufficient data to establish a precise time frame for this invasion, but Harant (1927) reported the presence of *C. lepadiformis* in harbours of the western Mediterranean. Although we are not making formal taxonomic distinctions, both genetic and biological data (De Caralt et al., 2002) indicate that the two clades might be given species status. As the original species description was based on Scandinavian specimens, the original name would belong to the Atlantic form and a new name could be given to the exterior Mediterranean form.

The extent of cryptic introductions of invertebrate species may be important and deserves further attention. Increased recreational sailing in the Mediterranean, and the proliferation of marinas in the past decades, provide additional points for step-by-step colonisation of alien forms, even with low natural dispersal abilities. In terms of conservation, not only is the arrival of non-indigenous species important, but so too are the invasions of new genetic variants. We believe that more studies of populations inhabiting marginal marine environments, supposedly conspecific with local species, may reveal more instances of genetic exchange through harbour traffic. Genetic tools are a useful method to investigate these cryptic invasions, as they allow the identification of sources, patterns of dispersal and degree of gene flow with local forms.

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