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Cryptic species of *Clavelina* (Ascidiacea) in two different habitats: harbours and rocky littoral zones in the northwestern Mediterranean

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Abstract Marinas and harbours provide ideal sites for the study of population genetics of marine invertebrates with restricted dispersal capabilities. They combine a confinement effect, particular ecological conditions (pollution, turbidity), and the possibility of high gene flow through ship-borne propagules, which greatly increases the natural dispersal capability of sexual and asexual propagules in many species with short-lived larvae. We studied the genetic structure of populations of the ascidian *Clavelina lepadiformis* living inside and outside harbours in the north-western Mediterranean. A 500-bp segment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene was sequenced in three populations from inside harbours (interior form) and in three populations from the rocky littoral (exterior form). Two congeneric Mediterranean species, *Clavelina* sp. and *C. dellavallei*, were used for comparison. We found that the interior and exterior forms of *C. lepadiformis* belong to two distinct clades, with a genetic divergence of 5%. Gene-flow values among these forms were insignificant. The lack of gene flow and the genetic divergence suggest that the interior and exterior forms of *C. lepadiformis* are in fact cryptic species rather than differentiated populations of the same species. Levels of gene flow were higher among interior habitats than among exterior habitats, a pattern likely maintained by

genetic exchange through ships. We discuss the possible origins of the present-day distribution of these cryptic species. We contend that the study of species living both inside and outside these particular habitats will reveal more instances of genetic discontinuities allowing local adaptations.

Introduction

Ascidians are solitary and colonial filter-feeding organisms that can be found in all benthic marine environments and constitute one of the major components of the benthic macrofauna. High growth rates and an array of toxic metabolites enable ascidians to successfully compete for available substrates and play an important role as fouling organisms and epibionts on submerged structures (Monniot et al. 1991). They are also good indicators of water quality, due to their capability of concentrating a certain amount of toxic elements in their tissues, such as heavy metals and hydrocarbons (Monniot et al. 1990, 1993, 1994). *Clavelina* is a genus of colonial ascidians belonging to the family Polycitoridae, which in the Mediterranean comprises five different species (Brunetti 1987): *C. lepadiformis* (Müller 1776); *C. dellavallei* (Zirpolo 1925); *C. nana* Lahille, 1890; *C. phlegrea* Salfi, 1929; and *C. sabbadini* Brunetti, 1987. *Clavelina* is often used as a model for the morphology and physiology of the whole group of ascidians (Brien 1948). The zooids are hermaphroditic, and larvae are brooded in the branchial sac of each zooid until hatching. Larvae of colonial ascidians have short planktonic life-spans that can vary from minutes to hours (Berrill 1935; Millar 1971; Svane and Young 1989). After attachment and metamorphosis, the larva gives rise to an oozoid which, by means of asexual reproduction, originates a colony of zooids, basally interconnected by stolons. Colonies of *Clavelina* are formed by a few to several hundred zooids linked by stolons, each encased in a thin, transparent tunic but retaining functional independence.

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Our study is focused on *Clavelina lepadiformis*, a species that was first described in the eighteenth century in Atlantic waters. *C. lepadiformis* is an Atlanto-Mediterranean species whose known distribution ranges from Scandinavia (Millar 1966) to the Aegean Sea in the eastern Mediterranean (Koukouras et al. 1995). This species is found in two different habitats in the study area (NE Spain, Western Mediterranean; Fig. 1); it colonizes rocky littoral surfaces (hereafter, exterior habitat), and marinas and harbours (hereafter, interior habitat).

Harbour environments differ from open-sea environments in several respects. The former contain nutritionally rich waters that sustain an intense proliferation of bacteria, constituting an important source of food for filter-feeding invertebrates like ascidians. In addition, the movement of ships mixes the water both horizontally and vertically, which keeps the food particles in suspension (Monniot et al. 1985; Papadopoulou et al. 1998; Dhainaut et al. 2000). Moreover, harbours are sheltered places, where the survival and attachment of ascidian larvae is more successful (Monniot et al. 1985, 1991). On the other hand, negative effects on the epifauna can result from higher pollution levels in these habitats. Contents of heavy metals and total hydrocarbons in the sediments and water column can be significantly higher in harbours than in the open sea (Fichet et al. 1998; Commendatore et al. 2000). In one of the marinas studied, Blanes harbour, concentrations of copper, cadmium and hydrocarbons in the sediments are significantly higher than in the exterior habitat, as well as the levels of copper in the water and of particulate organic nitrogen (Pinedo 1998; Cebrian 2001). *C. lepadiformis* inside the harbour accumulate about six times more copper and eight times more lead than the form living outside the harbour (De Caralt 2001). These environmental factors might influence the growth and survival of filter-feeders and pose different selective pressures on their populations in the two environments.

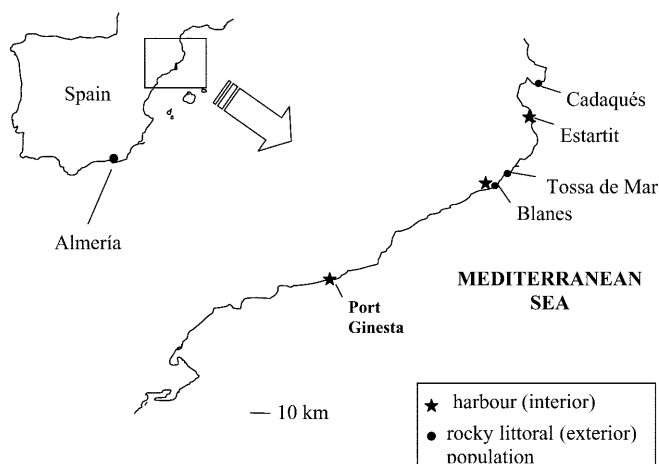


Fig. 1 Map of Spain showing the location of the different sampling sites

No morphological differences were observed in *C. lepadiformis* related to the different habitats, although the average size and zooid density of the interior form are higher than in the exterior habitat (De Caralt 2001). Likewise, some different biological features have been observed in the two forms: the reproductive period in the interior form starts some weeks earlier than in the exterior form and the former lacks the aestivation period of several months found in exterior populations (Turon 1988; De Caralt 2001). Short planktonic dispersal and water confinement within harbours may substantially hinder genetic exchange between interior and exterior habitats.

The objectives of this study were (1) to test the hypothesis that *Clavelina* populations in the marinas and in the exterior habitat show a high degree of genetic isolation and that this fact, combined with contrasting environmental conditions between the two habitats, may have resulted in the establishment and maintenance of two different genetic clades; and (2) to compare the genetic variation of *Clavelina* in each of the two habitats and to relate them to the dispersal mechanisms acting in each habitat.

Materials and methods

Ascidian samples

Ascidian colonies were collected from six different localities along the Catalan coast of the Mediterranean (NE Spain) by SCUBA diving in 1998 and 1999 (Fig. 1). Colonies morphologically attributable to *Clavelina lepadiformis* were found in two different habitats: in the marinas (interior form); and the open rocky seashore (exterior form). Several zooids of each colony were collected, preserved in EtOH, and stored at -20°C until processed. After removing the tunic, gut contents and branchial parasites (if any), two or three zooids of each colony were used for the mtDNA extraction. For the rocky littoral habitat, 3 specimens were collected from Cadaqués (C; $42^{\circ}16.54'N$, $3^{\circ}17.48'E$), 15 from Tossa de Mar (T; $41^{\circ}43.12'N$, $2^{\circ}56.24'E$), and 10 from Blanes (Be; $41^{\circ}40.24'N$, $2^{\circ}48.12'E$). For the interior habitat, 11 specimens were collected in the marina of Estartit (E; $42^{\circ}3.6'N$, $3^{\circ}12.36'E$), 15 in the marina of Blanes (Bi; separated only by tens of metres from the exterior population), and 9 in Port Ginesta (G; $41^{\circ}15.30'N$, $1^{\circ}55.18'E$). It should be noted that the harbours studied were built in this century, Port Ginesta being only 10 years old. Six colonies of *Clavelina dellavallei* from Almería (Playazo de Rodalquilar; $36^{\circ}51.36'N$, $02^{\circ}00.12'W$) and one of *Clavelina* sp. from Tossa de Mar were collected from the rocky seashore and used as an outgroup. This *Clavelina* sp. is a new species that will be formally described elsewhere (X. Turon, current research).

Mitochondrial DNA was extracted using the protocol for *Drosophila* spp. described in Latorre et al. (1986). Sequences were obtained for a segment of 500 bp of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene. COI is a protein-coding gene with highly variable sections, ideal for comparisons between closely related groups of organisms (Van Syoc 1995). We designed specific primers for this gene, based on a sequence obtained for one of the populations with the universal primers UniA and UniB described in Folmer et al. (1994). The sequences of the primers that we specifically designed for this group of ascidians are 5'GTACTGAGCTTTCACAAACTGGGCAAT3' (forward) and 5'TGAAA-AAGAAATAGGATCTCTCCTTCC3' (reverse).

Amplification was performed in a 20- μl total-reaction volume with 0.4 μl of each primer (25 μM), 3 μl dNTPs (1 mM), 2 μl 10 \times buffer containing 15 mM MgCl_2 (Promega), 1 U *Taq* polymerase

Table 1 *Clavelina lepadiformis*. Haplotype frequencies, nucleotide diversity within populations (π), %AT and number of polymorphic sites in six populations

<i>C. lepadiformis</i> Population	Haplotypes											Total	π	%AT	No. of poly- morphic sites
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI				
Cadaqués (C)	2	1	–	–	–	–	–	–	–	–	–	3	0.0022	70.65	1
Tossa (T)	7	–	1	7	–	–	–	–	–	–	–	15	0.0024	70.46	3
Blanes exterior (Be)	4	–	–	–	2	1	3	–	–	–	–	10	0.0027	70.70	3
Estartit (E)	–	–	–	–	–	–	–	3	7	1	–	11	0.0017	68.98	2
Blanes interior (Bi)	–	–	–	–	–	–	–	4	11	–	–	15	0.0012	69.03	1
Ginesta (G)	–	–	–	–	–	–	–	6	2	–	1	9	0.0022	68.86	3
Totals	13	1	1	7	2	1	3	13	20	1	1	63			

Phylogenetic analyses

The comparisons between the different likelihood scores for each model of evolution showed that the HKY model (Hasegawa et al. 1985) was the best-fit model among those evaluated for our data. This model incorporates unequal base frequencies [$\pi_{(A)}=0.2741$, $\pi_{(T)}=0.4080$, $\pi_{(C)}=0.1314$, $\pi_{(G)}=0.1864$], and a transition/transversion ratio (ti/tv = 9.8309). An ML haplotype tree revealed four different clades (Fig. 3), with interior and exterior forms of *C. lepadiformis* recovered as well supported monophyletic groups (with bootstrap support of 74% and 80%, respectively). The sister group of the exterior form was found to be *Clavelina* sp. However, we also tested the hypothesis of a monophyletic *C. lepadiformis* (interior + exterior) by estimating an ML tree with the constraint that all *C. lepadiformis* haplotypes were included in the same clade. This constrained tree was not significantly different from our best tree (Shimodaira–

Hasegawa test, $P=0.47$), so the monophyly of *C. lepadiformis* could not be rejected with certainty.

Cladogram estimation and nested analysis

The number of steps for parsimonious connections among haplotypes with a 95% confidence was estimated to be eight. The cladogram estimation procedure resulted in four distinct networks (Fig. 4), all of which were separated by more than eight mutational steps. The interior and exterior forms of *C. lepadiformis* fell into two independent networks separated by 17 steps. *Clavelina* sp. was differentiated from the interior and exterior networks of *C. lepadiformis* by 21 and 22 steps, respectively, while *C. dellavallei* was separated from the two *C. lepadiformis* networks by 54 (interior form) and 55 (exterior form) steps. In turn, *Clavelina* sp. and *C. dellavallei* networks were separated by 58 steps. The geographical association test showed a significant association between haplotypes and localities (Blanes exterior, Blanes interior, Tossa de Mar, Cadaqués, Port Ginesta, and Estartit) (Monte Carlo significance value <0.0001), and between haplotypes and habitats (interior and exterior; Monte Carlo significance value <0.0001). Evidence of a contiguous range-expansion event was found with the nested geographical analysis of genetic variation for both the interior and the exterior forms of *C. lepadiformis*. The results of the nested geographical analysis are shown in Fig. 5.

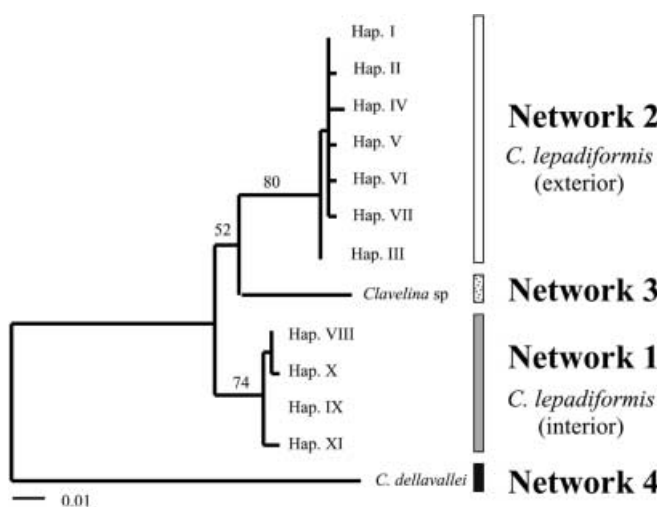


Fig. 3 Maximum-likelihood tree for the haplotypes, estimated using the model of evolution HKY. Bootstrap values with maximum-likelihood criterion are shown for the branches with more than 50% support. Each taxon corresponds to a haplotype (Hap.), designated by a roman number. Coloured bars group all the haplotypes nested in the same network in Fig. 4

Population genetic parameters

Mean genetic divergence between pairs of interior and exterior populations was 0.052. Nucleotide diversity was lower for the interior form (0.0018) than for the exterior form (0.0032). The mean value of gene flow between interior and exterior habitats was insignificant ($Nm=0.044$), whereas the mean γ_{ST} value between these two habitats was high ($\gamma_{ST}=0.91$). Measures of gene flow among the interior populations (Blanes, Estartit and Port Ginesta) were higher than those obtained

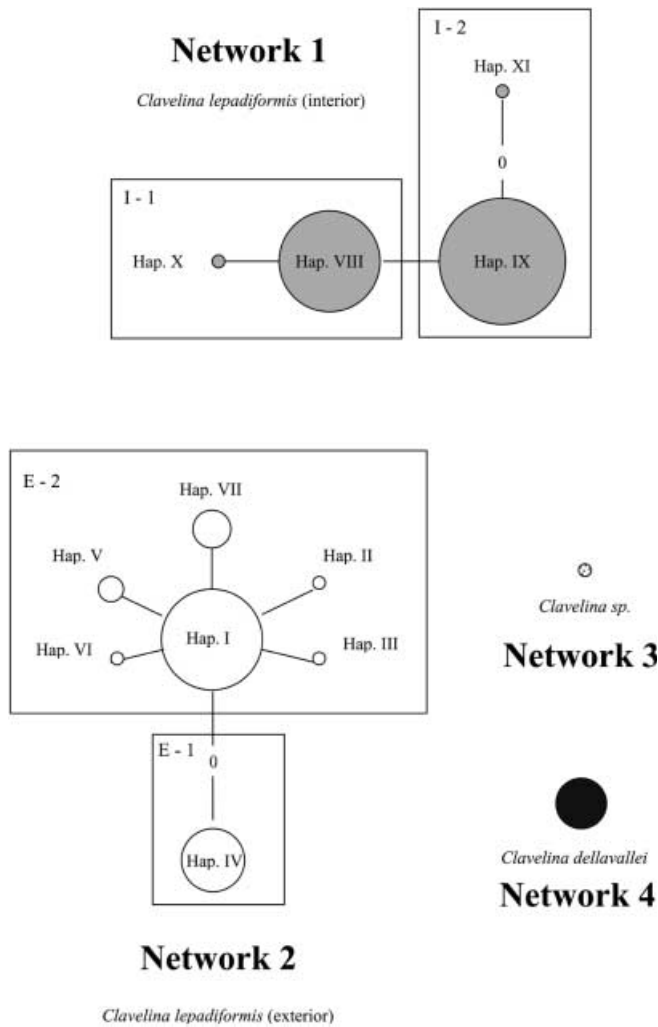


Fig. 4 TCS networks. Lines represent one mutational step between haplotypes, and the 0 indicates a missing intermediate. Boxes show the haplotypes nested together into one-step clades. Each network was subjected to an independent analysis and the codes (I-1, I-2, E-1 and E-2) correspond to the notation used in the nested analyses. *Clavelina lepadiformis* haplotypes (Hap.) are indicated by roman numbers, and haplotype frequencies (number of individuals within each haplotype) are represented by the area of the circles

among exterior populations: Nm of 12.5 ± 8.02 and 3.23 ± 0.77 , respectively (mean \pm SE; see Table 2). A significant genetic differentiation between localities (based on a χ^2 test; Hudson et al. 1992) was found among exterior populations ($\chi^2 = 26.42$, $P = 0.009$), whereas no significant genetic differentiation was detected among interior populations ($\chi^2 = 10.60$, $P = 0.101$). Pooling gene-flow values within interior and exterior habitats, we obtained a significantly larger mean value (Mann–Whitney U test, $P < 0.01$) than that observed between pairs of interior and exterior populations: Nm of 7.8783 ± 4.159 and 0.044 ± 0.002 , respectively (mean \pm SE, see Table 2). The AMOVA showed significant genetic differences between the two habitats (interior and exterior), among populations

within these habitats, and within populations ($P < 0.05$). Most of the genetic variation was found within populations (57.9%), followed by the variation among habitats (33.4%), and that among populations within habitats (8.72%).

Divergence times

The asymptotic identity was estimated from the data to be 0.302. With this value, and assuming a molecular clock, the calibration of Lynch and Jarrell (1993) indicated that the interior and exterior forms of *C. lepadiformis* diverged 8.9 MY ago (95% confidence interval 5–36 MY ago).

Discussion

The interior and exterior forms of *Clavelina lepadiformis* are identified as two very divergent and distinct genetic clades. There seems to be no evidence for gene flow between these two forms, and the apparent Nm (mean of 0.044) is, most probably, a consequence of their shared ancestral population. The lack of gene flow among the two forms, and their genetic divergence estimated by the phylogenetic and statistical parsimony analyses, suggest that *C. lepadiformis* interior and exterior are cryptic species rather than two forms of the same species. The relationships among the four main genetic clades found on the phylogenetic tree, *C. lepadiformis* interior, *C. lepadiformis* exterior, *Clavelina* sp., and *C. dellavallei*, could not be fully resolved, owing to the absence of substitutions occurring in the interior branch that separates them. Most of the differences are autapomorphies in the branches that lead to the four clades. This generated a lack of phylogenetic signal at this level, and the *C. lepadiformis* monophyly could not be rejected, although in our best tree (the tree with the highest log-likelihood score) *Clavelina* sp. appears as the sister taxon to the exterior form.

The origin of the present-day parapatric distribution of the two clades of what has been so far called *Clavelina lepadiformis* remains speculative at present. The species was originally described in Scandinavian waters and has an Atlanto-Mediterranean distribution (Pérès 1958). In the Mediterranean the species has been cited in harbours and in shallow rocky littoral habitats (Harant 1927; Turon 1987), corresponding to our interior and exterior clades. In Atlantic waters it is known from rocky bottoms down to 50 m, but is also present in sheltered environments like wharfs, harbours, fjords and shallow estuarine bays (Berrill 1950; Millar 1966; Vázquez 1993). One possible hypothesis for the origin of the two clades is that a parapatric speciation occurred in the Atlantic between populations on open shores and those in sheltered, marginal-marine environments, where gene flow is restricted and where they faced different conditions (Boisselier-Dubayle and Gofas 1999). The form evolving in

Fig. 5 *Clavelina lepadiformis*. Results of the nested geographical analysis. The first three columns correspond to the haplotype level, while the second three columns correspond to the second level of the analysis. Columns: *Name* is the name of the clade, *Dc* is the clade distance and *Dn* is the nested clade distance. The row *I-T* indicates the average difference between interior and tip clades. *Superscript S* means that the measure was significantly small and *L* that this distance was significantly large (both at the 5% level). The rows 1–2–11–12(No): CRE under the shaded boxes represent the steps followed in the inference key (Templeton 1998) and the conclusion reached by this method: CRE contiguous range expansion. For a graphical representation of these results, see Fig. 4

Haplotypes			1-step clades		
Name	<i>Dc</i>	<i>Dn</i>	Name	<i>Dc</i>	<i>Dn</i>
VIII	84.49	84.8	Network 1 (Interior)		
X	0	88.09			
<i>I-T</i>	84.49	-3.27	I-1	86.73 ^L	73.28 ^L
IX	57.83	60.54	I-2	63.31 ^S	64.64 ^S
XI	0	101.63	1-2-11-12(No): CRE		
<i>I-T</i>	57.83	-41.08			
IV	0	0	E-1	0 ^S	14.70 ^S
I	32.87	29.61	E-2	31.65 ^L	27.2 ^L
II	0	73.44	1-2-11-12(No): CRE		
III	0	17.64			
V	0	18.76	Network 2 (Exterior)		
VI	0	18.29			
VII	0	19.25			
<i>I-T</i>	32.87	4.03			

Table 2 *Clavelina lepadiformis*. Γ_{ST} (γ_{ST}) values (above the diagonal) and gene-flow estimates (Nm , below the diagonal) for the different populations studied

	Cadaqués (exterior)	Tossa (exterior)	Blanes (exterior)	Blanes (interior)	Ginesta (interior)	Estartit (interior)
Cadaqués (exterior)	–	0.13	0.09	0.92	0.91	0.91
Tossa (exterior)	3.29	–	0.21	0.92	0.90	0.90
Blanes (exterior)	4.54	1.87	–	0.93	0.91	0.92
Blanes (interior)	0.04	0.04	0.03	–	0.13	0.017
Ginesta (interior)	0.05	0.05	0.05	3.23	–	0.078
Estartit (interior)	0.05	0.05	0.04	28.49	5.85	–

these marginal habitats would have been preadapted to colonize the present-day man-made harbours and constructions. This split could be more than 8 MY old, according to the molecular clock. After the Messinian salinity crisis of the Mediterranean (in the late Miocene, between 5 and 6 MY ago), when the Gibraltar Strait opened (Maldonado 1985), the two clades could colonize the corresponding Mediterranean habitats, where they can be found at present. This would agree with the process of contiguous range expansion found in our analysis.

A second possible explanation is that, if our molecular clock overestimates the timing of divergence, both clades could be the result of the original Atlantic species colonizing the Mediterranean after the Miocene, and a new species evolved allopatrically in the Mediterranean, where conditions are different from those in the Atlantic. This divergence could have been favoured by the existence of effective barriers against gene flow between Atlantic and Mediterranean marine populations (Tintoré et al. 1988). In fact, instances of genetic discontinuities between north-east Atlantic and Mediterranean marine populations can be found in the literature, either concerning conspecific populations or congeneric species-pairs of several groups (e.g. soft corals, McFadden 1999; cirripedes, Pannacciulli et al. 1997; bivalves, Sa-

avedra et al. 1993, Quesada et al. 1995; limpets, Côte-Real et al. 1996; pelagic crustacea, Zane et al. 2000; fish, Borsa et al. 1997). The interior form could be the result of the Atlantic clade having invaded the Mediterranean recently by way of ships, and thriving in an environment, the inside of harbours, that is more similar to the turbid and richer Atlantic waters than the more clean and oligotrophic waters of the exterior habitats. A similar hypothesis of ancient and recent invasions of the Mediterranean from Atlantic populations has been suggested to explain the origin of two distinct genetic lineages in Mediterranean populations of the genus *Ophiothrix* (Baric and Sturmbauer 1999). Whether the two *Clavelina* species originated parapatrically or allopatrically, and which of them should retain the original name *C. lepadiformis*, can only be ascertained by future studies covering an arch from Scandinavian waters to the eastern Mediterranean, including exterior rocky shores and natural and man-made marginal-marine habitats.

Gene flow among the exterior populations is lower than among interior populations. These low values of gene flow agree with the short free-living larval period of this species which hinders larval dispersal at long distances. However, the higher gene-flow levels found

among interior populations cannot be explained by larval dispersal only. A likely explanation would be the dispersal of adult colonies through the marinas by means of maritime traffic. Larvae could settle on ships' hulls, the new individuals could then be carried to other marinas and mate with the colonies present there. Observations of this phenomenon in ascidians have been made in the past by other authors (Monniot et al. 1985).

The highest genetic variation found in the exterior form (both at the nucleotide and the haplotype level) is consistent with the fact that the exterior habitat is much more heterogeneous and rich in microhabitats. However, the lower genetic variability in harbours could also be explained if these populations have gone through a bottleneck at the time the Mediterranean was colonized. This would imply that the hypothesis of the interior form being introduced recently from the Atlantic is correct. Intense ship traffic among harbours would have maintained the genetic homogeneity of those populations.

Marinas and similar environments provide ideal field sites for the study of population genetics of invertebrates. They combine a confinement effect, particular ecological conditions, and the possibility of high gene flow facilitated by ships, which overcomes the (usually restricted) dispersal capability of sexual and asexual propagules. Compared to open-sea populations, those in harbours are expected to feature a more homogeneous genetic structure and higher gene flow among them, as found in the present study. Likewise, selection for characteristics that allow a better adaptation to this particular kind of environment can result in a quick genetic divergence between interior and exterior populations in species that can thrive in both habitats. Some instances of genetic isolation of invertebrate populations in closed habitats, such as lagoons and harbours, have been reported (e.g. solitary ascidians, Dalby 1997; gastropoda, Boisselier-Dubayle and Gofas 1999). We anticipate that more instances will be documented as more species are investigated from a genetic point of view, and the occurrence of cryptic species in these marginal-marine environments may turn out to be more common than is presently recognized. Studies on the population dynamics and genetic structure of species adapted to harbours or those able to live in both habitats can have profound ecological, evolutionary and biotechnological (e.g. adaptations to pollution) implications.

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