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Population genetics, phylogeography and speciation of *Cystodytes* (Ascidiacea) in the western Mediterranean Sea

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Several morphotypes that so far have been attributed to the allegedly cosmopolitan ascidian *Cystodytes dellechiajei* occur in the Mediterranean Sea. Colour variation is the difference most frequently reported. In this study, we addressed the genetic structure of this ascidian in relation to geographical location and colour morph. Partial sequences of the gene cytochrome *c* oxidase subunit 1 (COI) were obtained from seven populations of the western Mediterranean, encompassing eight colour varieties. All population genetic analyses (exact test, pairwise F_{ST}, hierarchical analysis of molecular variance, multidimensional scaling, nested clade analysis) indicated clearly that differences between colour morphs are large enough to obscure any geographical differentiation when colours are combined within localities. When variance due to colour divergence was removed, however, a significant geographical variability between localities remained. The genetic divergence between the colour morphs analysed was significant in comparisons of the brown and purple forms with the others, but not among the green, blue, and white morphs. Phylogeographic analyses suggest that population fragmentation and range expansions have shaped the present-day distribution of the haplotypes. Taken together with existing chemotype information, our results indicate that several species are present in the area, and that a thorough revision of the genus is necessary.

ADDITIONAL KEYWORDS: ascidian – nested clade analysis – secondary metabolites.

INTRODUCTION

The existence of cosmopolitan distributions and intra- and interspecies variability in benthic invertebrates has been a long-standing source of taxonomic and biological controversy. For instance, species with a large distribution range may show morphological variants, related usually to their geographical or bathymetric distribution. However, the advent of molecular techniques has revealed a host of cryptic and sibling species in taxa formerly recognized as being distributed widely (Klautau *et al*., 1999; Knowlton, 2000; McGovern & Hellberg, 2003; Meroz-Fine *et al*., 2003). Therefore, morphology alone may be insufficient to assess species boundaries in marine invertebrates (Loukaci *et al*., 2004). Colour variation in particular has often been reported in marine environments, and shows complex patterns of relationship with genetic differentiation, thus precluding any general assertion about the validity of colour morphospecies (e.g. Wilson, Noack-Kunnmann & Meuer, 2000; McCartney *et al*., 2003; Le Gac *et al*., 2004; Mackenzie *et al*., 2004).

Although studies of population subdivision and speciation are crucial to our understanding and management of marine biodiversity and its biotechnological applications (Holland, 2000), the very concept of species is open to debate (Hull, 1997; Sites & Marshall, 2003). Templeton (2001) showed how formal phylogeographic analyses of genetic data can provide useful insights at the interface between intra- and interspecific evolution, within the framework of the cohesion species concept (Templeton, 1989). Although genetic variability is expected, true cosmopolitan species must maintain a certain degree of genetic cohesiveness, mediated by gene flow, throughout their distribution range. In marine environments, gene flow was thought

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to be secured by highly effective mechanisms of long-distance larval dispersal (Scheltema, 1986; Russo, Solé-Cava & Thorpe, 1994). Larvae of marine invertebrates, however, generally display limited dispersal capabilities (Jackson, 1986; Hunt, 1993; Hoskin, 1997) and gene flow between populations may become seriously restricted to short geographical scales.

Cosmopolitanism in colonial ascidians poses, therefore, a puzzling problem, as it seems to be at odds with their reproductive strategy. Larvae of colonial ascidians have short planktonic life spans that can vary from minutes to hours (Millar, 1971; Svane & Young, 1989). In addition, evidence to date suggests that successful fertilization in colonial ascidians generally occurs over distances of only a few tens of centimetres (Grosberg, 1991; Bishop, 1998; Yund, 1998). Therefore, the populations are sustained mainly by highly localized dispersal and recruitment, and are likely to display a marked genetic structure on a microgeographical scale (Yund & O'Neil, 2000). Not surprisingly, instances of sibling species have been revealed when ascidian species with variable morphology and wide distribution ranges have been studied using molecular markers (Aron & Solé-Cava, 1991; Dalby, 1997; Tarjuelo *et al*., 2001, 2004; Castilla *et al*., 2002). In addition, recent range expansions linked to human-mediated transport have added complexity to the issue of cosmopolitanism in ascidians (Lambert & Lambert, 1998; Castilla *et al*., 2002; Stoner *et al*., 2002).

Cystodytes dellechiajei (Della Valle, 1877) (Aplousobranchiata, Polycitoridae) is a colonial ascidian, distributed widely in both tropical and temperate waters. The general morphology of this species varies greatly. Although colour variation is the most apparent feature to have been reported, colony shape, texture, spicular composition, and zooid size may also vary, even within the same locality, without any clear distribution pattern (Turon, 1987; Méliane, 2002). In addition to colour variation, López-Legentil & Turon (2005) found spicular differences in Mediterranean specimens currently assigned to *C. dellechiajei*. However, when they mapped morphological characters onto a phylogenetic tree constructed using genetic data, little concordance was found. These authors concluded that the morphological traits studied were not sufficiently consistent to differentiate between *Cystodytes* species.

In this study, we analysed populations representative of the main colour morphs and geographical areas of the western Mediterranean, in an effort to explore the population subdivisions and phylogeographic relationships among them. We used mtDNA sequence data as a genetic marker that straddles the interface between population genetics and systematics (Avise *et al*., 1987; Avise, 2000). MtDNA has been used successfully to address speciation problems in ascidians (Tarjuelo *et al*., 2001, 2004; Turon *et al*., 2003).

We analysed the degree of differentiation between the locations studied, partitioned genetic variation according to geography and colour morphs, and performed a phylogeographic analysis (NCA, nested clade analysis; Templeton, 2004) of a haplotype network. In this way, we sought to clarify the relationship between the different colour morphs and genetic variation, and to determine whether *C. dellechiajei* should be reclassified as several species.

MATERIAL AND METHODS

Ascidian samples

Individuals of *Cystodytes* were collected from seven Mediterranean sampling areas: northern Catalonia (north-east Iberian peninsula), Mallorca–Menorca and Ibiza–Formentera (Balearic Islands), Cabo de Gata and Alborán Island (southern Iberian coast), Tunisia and Sicily (Fig. 1, Table 1). We considered each of these locations as populations because intrazone distances were small and so were assumed to be within the range of larval dispersion of this species. Preliminary analyses of genetic variation (within colour morphs) showed no significant intrazone structure. Sampling was undertaken by scuba diving in 2001 and 2002. In order to avoid problems with colony fission and clonality, colonies were collected at least 5 m apart from each other. The specimens were identified as being *C. dellechiajei* based on Turon (1987) and Kott (1990). The original colour of the colony was recorded prior to fixation in absolute ethanol (Table 1).

DNA extraction and sequencing

In order to maximize the yield of DNA extractions, we separated the zooids from the tunic and spicular cap-

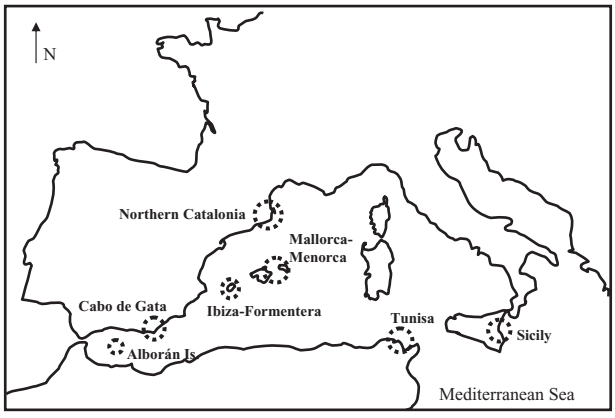

Figure 1. Map showing the Mediterranean zones sampled: northern Catalonia, Cabo de Gata, Alborán Island, Ibiza–Formentera, Mallorca–Menorca, Tunisia, and Sicily. Is, island.

Table 1. *Cystodytes dellechiajei* populations sampled in the western Mediterranean and their geographical location, along with the original colour of the colonies, the sample size (*N*) and the haplotype distribution within locations and colours

Geographic locations	Colour	*N*	Haplotypes
Northern Catalonia	Purple	10	H2, H10, H20, H21, H22
	Blue	4	H6
	White	4	H3, H7, H24
	Green	1	H16
Mallorca–Menorca	Brown	2	H18, H19
	Green	10	H4, H14, H16, H23, H25, H27, H34
	Blue	3	H14, H25, H26
Ibiza–Formentera	Brown	2	H1
	Green	4	H30, H31
	Blue	4	H5, H28, H29
Cabo de Gata	Green	6	H8, H32, H33
Sicily	Blue	1	H14
	Pink	1	H8
	Yellow	1	H15
	White	2	H14
Tunis	Orange	1	H10
	Green	3	H9, H11
	White	2	H12, H13
Alboran Island	Green	2	H6
	Unknown	2	H10, H36
	White	2	H6

sules under a binocular microscope. The zooids were then kept in absolute ethanol at –25 °C until used. MtDNA was extracted from the zooids using the 'Quiamp Mini Kit' (Quiagen). Sequences were obtained for a segment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene. Universal primers HCO2198 and LCO1490 (Folmer *et al*., 1994) were used to amplify the purple, brown, white, and orange forms, whilst primers AvA and AvB (López-Legentil & Turon, 2005) were more successful for amplifying the blue, green, pink, and yellow colour morphs. Amplification was performed in a 20 μL reaction volume with 0.4 μL each primer (25 μM), 0.5 μL dNTPs (10 mM), 2 μL 10× reaction buffer containing 15 mM $MgCl_2$ (Promega), 1U Taq polymerase (Promega), and 1 μL template DNA. When using the universal primers UniA and UniB described in Folmer *et al*. (1994), an initial denaturation step of 94 °C for 2 min was followed by 35 amplification cycles (denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1.5 min), and a final extension step at 72 °C for 7 min, in a Perkin Elmer 850 DNA amplifier. The AvA and AvB primers were used with an initial denaturation step of 94 °C for 2 min and 35 cycles of 94 °C for 35 s, 45 °C for 45 s and 72 °C for 1 min, with a final extension step at 72° for 5 min, in a Perkin Elmer 9700 DNA amplifier.

The sequencing reaction was carried out with an ABI Big-Dye Ready-Reaction kit (Perkin Elmer) using the same primers as in the amplification step. Sequences were obtained using an ABI Prism 377XL automated sequencer. The nucleotide sequences obtained in this study were added to the extant genetic database (López-Legentil & Turon, 2005) and have been deposited in GenBank (accession numbers AY523042–AY523057, AY523060–AY523076 and AY821784.

Population genetic analysis

Sequences were aligned using SeqPup version 0.6 and alignments were confirmed by eye. Tajima's D-statistics were estimated with DnaSP version 3.53 (Rozas & Rozas, 1999) to test whether selective pressures were acting on the substitutions.

Further analyses were performed using Arlequin vers. 2000 (Schneider, Roessli & Excoffier, 2000). Nucleotide diversity and haplotype diversity (Nei, 1987) were calculated for each population. The exact test of population differentiation (Raymond & Rousset, 1995) was used to test the null hypothesis of a random distribution of the different haplotypes among geographical locations and colours, and its significance was estimated from 10 000 random permutations of the original data matrix. We calculated pairwise F_{ST} values and their significance through permutation tests (1000 replicates) for both the geographical location and the main colour morphs (purple, blue, white, green and brown). To test the isolation by distance between populations (Rousset, 1997), we performed a Mantel test with 10 000 permutations. Analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was performed to test hierarchical models of genetic variability using pairwise differences as a measure of divergence. We tabulated our data according to two grouping categories: geographical location and colour morphotype. These data were then used to test two models. In the first model, genetic variance was partitioned into the following levels: among geographical location, between morphotypes within geographical location, and within morphotypes at each location. In the second model, we estimated the relative variance contribution of the following components: between colour morphs, between locations within each colour morphotype, and within locations for each colour morph. Comparison of the results provided an estimate of the relative contribution of geographical distance and morphotype to the overall genetic variability.

Phylogeographic analysis

We estimated a haplotype network using the TCS vers. 1.18 program (Clement, Posada & Crandall, 2000), which implements the statistical parsimony method described by Templeton, Crandall & Sing (1992). This method estimates an unrooted tree and provides a 95% plausible set for the relationships between the haplotypes. We solved ambiguities (loops) in the resulting network by applying the criteria summarized by Pfenninger & Posada (2002). We then nested the network by hand, using the procedures described by Templeton, Boerwinkle & Sing (1987) and expanded by Crandall (1996). Symmetrically stranded intermediate clades were nested according to the method described by Templeton & Sing (1993). Once the nesting design was complete, we performed a NCA (Templeton *et al*., 1987) on the clades with geographical and genetic information using the Geodis vers. 2.0 program (Posada, Crandall & Templeton, 2000). A matrix of geographical distances (in km) between the localities was input into the program, and distance data were automatically coded as clade distances (Dc, a measure of geographical dispersion of individuals bearing haplotypes of a given clade) and nested clade distances (Dn, a measure of how far individuals with haplotypes belonging to a given clade are from individuals bearing haplotypes of the immediate higher-step clade). NCA aims to distinguish recurrent gene flow from historical events as determinants of the present-day distribution of haplotypes (Templeton, 1998). The program looks for significant associations between genetic and geographical distances Dc and Dn, using randomization procedures. Significant associations can then be interpreted in terms of biological processes using the inference key described by Templeton (2004).

A muldimensional scaling analysis (MDS) was also performed on a matrix of genetic distances estimated as the number of pairwise differences with the Mega vers. 2.1 program (Kumar *et al*., 2001). The MDS was run in two dimensions with the Kruskal linear algorithm using the statistical software Systat vers. 9.

RESULTS

A total of 67 sequences of the ascidian *C. dellechiajei* were obtained, and eight colour morphs were recorded. Purple, green, blue, white and brown were the most abundant colour morphs, whilst orange, yellow, and pink colonies were found only occasionally (Table 1, see Fig. 2 for some examples). After alignment and trimming, we obtained sequences for the COI mitochondrial gene with a final length of 617 bp. Thirty-four different haplotypes were identified with a total of 154 variable sites (Table 1). Nucleotide variation was scattered across the entire sequenced region but was restricted mainly to the third base position of the codon. Thus, 153 (99.35%) of the nucleotide substitutions yielded synonymous changes, and only one was a transition (A–G) resulting in a nonsynonymous substitution. Tajima's D-statistic was not significant ($D = 0.48880$, $P > 0.10$) for the entire dataset, showing no evidence of selection acting on this locus, and indicating that a neutral model cannot be rejected.

Population genetics

The main parameters describing variability within populations, nucleotide diversity (π), haplotype frequencies within populations (H), and haplotype diversity (h) as a measure of within-population variation (Nei, 1987), are summarized in Table 2.

The results of the exact test for population differentiation based on haplotype frequencies between the different locations and between colour morphs in both cases revealed significant heterogeneity in the distribution of haplotypes ($P < 0.05$). Pairwise tests for genetic differentiation among geographical locations based on F_{ST} values showed that all pairwise comparisons involving the Tunisian population displayed significant genetic divergence. Likewise, almost all comparisons of the northern Catalonian population with the other locations showed significant divergence (Table 3). The F_{ST} analysis in which the samples were grouped according to the main colour morphs (purple, blue, green, white, and brown), disregarding their geographical origin, showed a strong genetic differentiation of the purple and brown morphs from all the others. In contrast, differences between blue, green, and white morphs were not significant (Table 4). The mean ± SE pairwise F_{ST} value between geographical locations was significantly lower than that between colour morphs (0.21 ± 0.03 vs. 0.55 ± 0.11, respectively; *t*-test, $P < 0.01$).

There was no evidence of isolation by distance between the geographical locations (Mantel test, $P = 0.66$). In fact, whilst the most distant populations (Sicily and Alborán Island, separated by a distance of 1618.28 km) were not significantly different, the reverse was true of the closest ones, Cabo de Gata and Alborán Island (144.64 km apart).

The hierarchical AMOVA analysis (Table 5) based on geographical origin revealed a negligible and nonsignificant (0.59%) variance component associated with between-group (i.e. geographical location) differentiation. Thus intergroup differences did not explain any significant variance other than that accounted for by comparisons between colours within geographical location (*c*. 76%) and within colours within location (23%), both of which were significant. In contrast, if we partitioned our model to consider location nested within colour morphs (Table 5), then most of the vari-

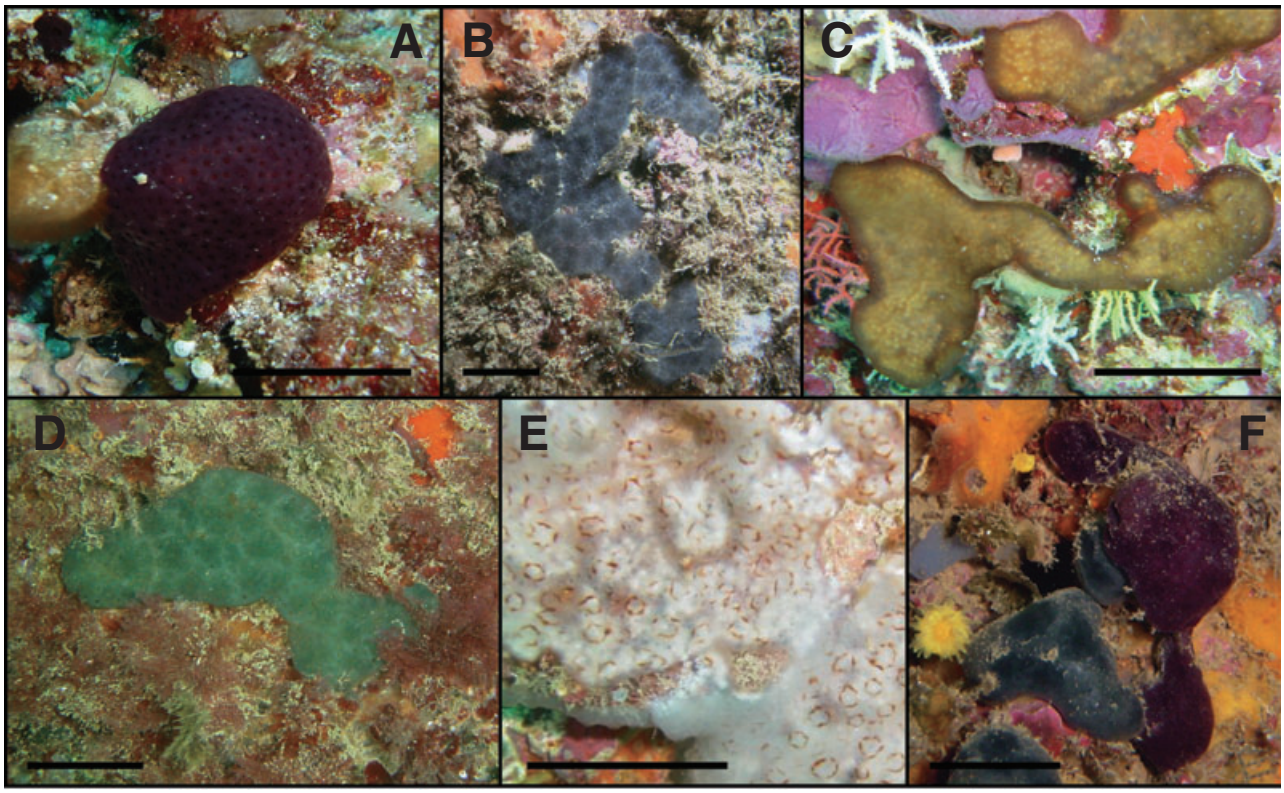

Figure 2. Some representative colour morphs of *Cystodytes* spp: A, purple morph; B, blue morph; C, brown morph; D, green morph; E, white morph with brown circles; F, an example of interaction between blue and purple morphs. Scale bars = 2 cm.

ance (*c*. 59%) was concentrated between colour morphs, with a much smaller variance (27%) explained by comparisons between locations (within colours), and a still lower residual variance within locations and colour morphs (14%). All components, however, were significant in a permutation test ($P < 0.001$).

PHYLOGEOGRAPHY

The parsimony haplotype network is presented in Figure 3. The maximum number of steps for a 95% parsimonious connection between haplotypes (i.e. with a 95% confidence level that no multiple substitution had occurred at some site) was ten mutations. We obtained eight groups of haplotypes that were separated by more than ten changes. Group A was the only one in which we found loops (two of them) connecting haplotypes. These ambiguities were easily solved using the frequency criterion (Pfenninger & Posada, 2002) that states that low-frequency haplotypes are probably connected to haplotypes with high frequency. The different groups of haplotypes appeared well separated in the two-dimensional space (stress of the configuration = 0.17) of the MDS ordination based on a matrix of genetic distances (Fig. 4). In particular, the largest group (A, with 19 haplotypes) formed a cluster at the positive end of dimension 1, well separated from the remaining groups. Graphical representation of the colour morphs that predominated in the eight groups (Fig. 3) showed that whilst Group A was formed mainly by the blue, green, and white morphs, only one or two colours were found in the remaining groups.

The eight groups of haplotypes found in the parsimony network were treated subsequently as independent cladograms (Fig. 3). No attempt was made to group these terminal units because the number of changes in the shortest connections between them was generally well above the parsimony limit of ten. Only Group A had a four-step nesting design; the other groups featured one- or two-level clades. The results of the NCA analysis showed that most associations were not significant, indicating a lack of geographical structure in the data, but a few clades showed significant associations that permitted phylogeographic inferences (Table 6). For Clade 2–5 the outcome was inconclusive, while for Clade 3–3 (which included haplotypes found

Table 2. Parameters of the populations studied: nucleotide diversity (π,) haplotype diversity (h) and its standard error (SE), with haplotype frequencies

	Mallorca–Menorca	Sicily	Ibiza–Formentera	Northern Catalonia	Tunisia	Cabo de Gata	Alborán Island
π	0.038	0.049	0.049	0.081	0.072	0.006	0.072
h	0.924	0.9	0.889	0.912	0.933	0.600	0.6
SE	0.053	0.161	0.075	0.04	0.122	0.215	0.215
H1	0	0	0.2	0	0	0	0
H2	0	0	0	0.105	0	0	0
H3	0	0	0	0.0526	0	0	0
H4	0.133	0	0	0	0	0	0
H5	0	0	0.2	0	0	0	0
H6	0	0	0	0.211	0	0	0.667
H7	0	0	0	0.0526	0	0	0
H8	0	0.2	0	0	0	0.667	0
H9	0	0	0	0	0.333	0	0
H10	0	0	0	0.211	0.167	0	0.167
H11	0	0	0	0	0.167	0	0
H12	0	0	0	0	0.167	0	0
H13	0	0.2	0	0	0.167	0	0
H14	0.267	0.4	0	0	0	0	0
H15	0	0.2	0	0	0	0	0
H16	0.0667	0	0	0.0526	0	0	0
H18	0.0667	0	0	0	0	0	0
H19	0.0667	0	0	0	0	0	0
H20	0	0	0	0.0526	0	0	0
H21	0	0	0	0.105	0	0	0
H22	0	0	0	0.0526	0	0	0
H23	0.0667	0	0	0	0	0	0
H24	0	0	0	0.105	0	0	0
H25	0.133	0	0	0	0	0	0
H26	0.0667	0	0	0	0	0	0
H27	0.0667	0	0	0	0	0	0
H28	0	0	0.1	0	0	0	0
H29	0	0	0.1	0	0	0	0
H30	0	0	0.3	0	0	0	0
H31	0	0	0.1	0	0	0	0
H32	0	0	0	0	0	0.167	0
H33	0	0	0	0	0	0.167	0
H34	0.0667	0	0	0	0	0	0
H36	0	0	0	0	0	0	0.167

Table 3. Pairwise F_{ST} values among locations

	Mallorca–Menorca	Sicily	Ibiza–Formentera	Northern Catalunya	Tunisia	Cabo de Gata	Alboran Island
Mallorca–Menorca	0						
Sicily	0	0					
Ibiza–Formentera	0.071	0.088	0				
Northern Catalunya	0.299*	0.219*	0.232*	0			
Tunisia	0.491*	0.314*	0.340*	0.246*	0		
Cabo de Gata	0.249	0.153	0.251*	0.330*	0.569*	0	
Alboran Island	0.061	0.008	0.004	0.051	0.274*	0.18519*	0

*$P < 0.05$.

in northern Catalonia, Balearic Islands, Sicily, and Tunisia) a contiguous range expansion was substantiated. For Clade 4–1 (which linked the whole of Group A), a past fragmentation event explained the geographical distribution of the haplotypes. In agreement with Templeton (2004), a past fragmentation event was favoured over a long-distance colonization because of the number of steps involved (see missing intermediates in Fig. 3) separating the haplotypes found in Ibiza–Formentera (Clade 3–1) from the other ones. Finally, at the level of the whole cladogram, another fragmentation event was inferred, this time being caused mainly by the separation of the haplotypes found in Tunisia (Clade 1–18) from the remainder.

Table 4. Pairwise F_{ST} values among main colours

	Purple	Blue	White	Green	Brown
Purple	0				
Blue	0.866*	0			
White	0.644*	0.127	0		
Green	0.759*	0.041	0.045	0	
Brown	0.809*	0.906*	0.579*	0.763*	0

*$P < 0.05$.

DISCUSSION

Our results revealed a noticeable variability between colour morphs that was large enough to obscure any geographical differentiation when colours were com-

Table 5. Two-level AMOVAs considering both geographical location and colour

AMOVA	d. f.	Sum of squares	Variance component	% of variation	*P*
Among locations	6	406.51	0.117	0.59	0.595
Among colours within locations	10	520.48	15	76.02	< 0.001
Within colours within locations	45	207.8	4.62	23.39	< 0.001
Among colours	4	636.53	12.28	56.11	< 0.001
Among locations within colours	11	254.53	6.44	29.42	< 0.001
Within locations within colours	42	133.05	3.17	14.47	< 0.001

Table 6. Results of the nested clade analysis (NCA)

Clade	Clade members	Dc	Dn	Inference
2-5	1-7 (interior)	165.96	462.4	1-2-11-17
	1-8 (tip)	702.15	642.57 (L.)	Inconclusive outcome
	I-T	–536.19	–180.16	
3-3	2-6 (interior)	82.98 (S)	289.72 (S)	1-2-11-12
	2-5 (tip)	621.09	503.52	Continuous range expansion
	I-T	–538.11 (S)	–213.78 (S)	
4-1	3-3 (interior)	483.75	559.48	1-2-3-5-15
	3-1 (tip)	0 (S)	402.97 (S)	Past fragmentation
	3-2 (tip)	591.43	619.90 (L.)	
	I-T	98.02	15.03	
Whole cladogram	1-17 (tip)	0	1071.60 (L.)	1-2-3-5-15
	1-18 (tip)	0 (S)	776.42 (L.)	Past fragmentation
	1-19 (tip)	0	361.44	
	1-20 (tip)	0	419.03	
	2-7 (tip)	400.90	506.54	
	2-8 (tip)	0	397.67	
	2-9 (tip)	441.72	502.43	
	4-1 (tip)	557.43	549.37	

Only clades with significant geographical associations as detected by the permutation test are included in the table. For each clade we list the members (either tips or interiors), the corresponding clade (Dc, in km) and nested clade (Dn) distances, and the interior-tip contrasts (I-T). We indicate significantly small (S) or large (L.) distances. The 'Inference' column describes the steps followed in the inference key of Templeton (2004) applied to that clade and the biological process inferred.

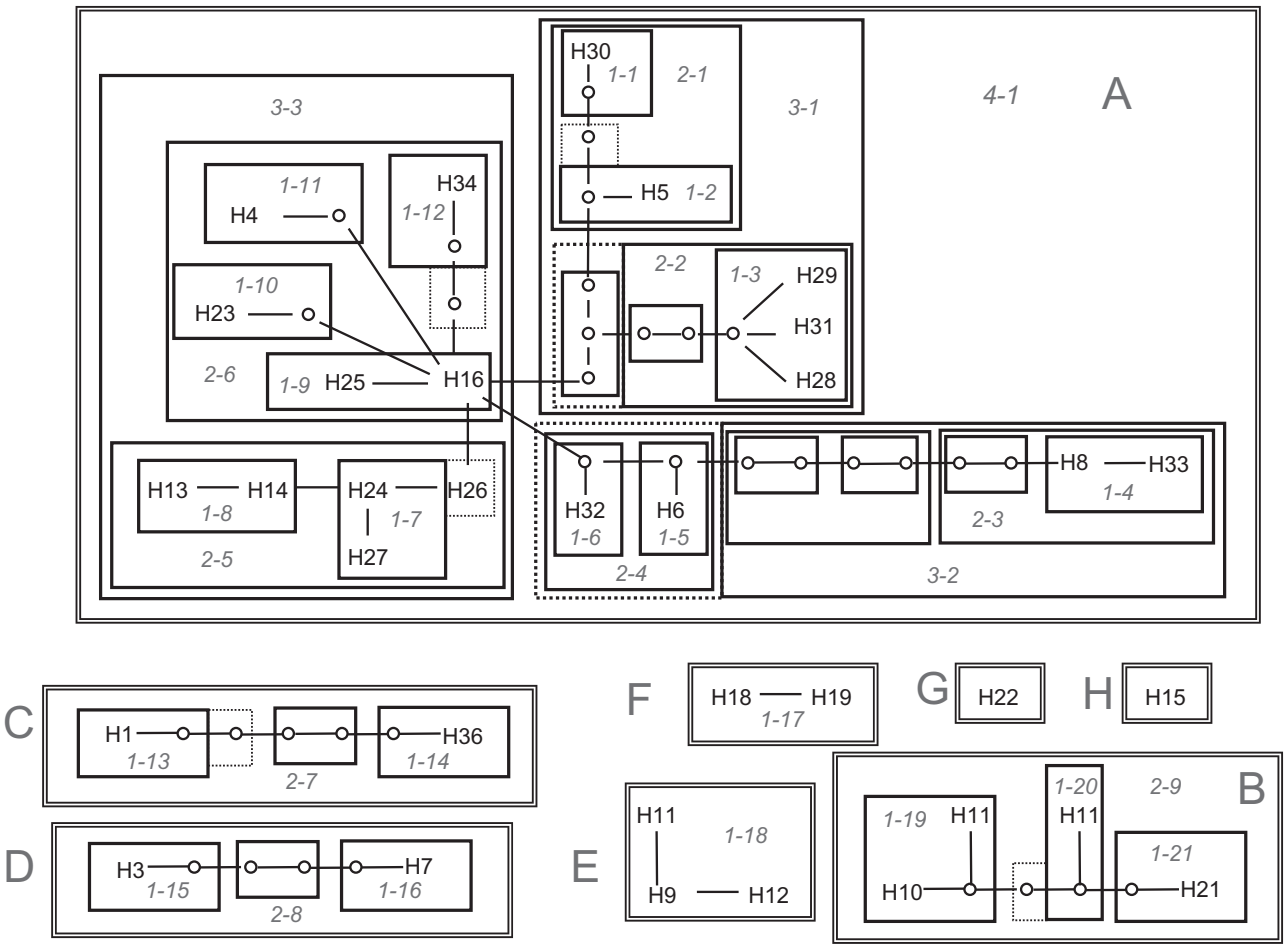

Figure 3. Statistical parsimony cladogram obtained from the TCS program. Each line represents a mutational step. Missing intermediates are indicated by 'o'. The eight groups that could not be connected unambiguously are designated A–H. The nesting design adopted is shown with boxes, and boxes with dotted margins indicate the assignment of symmetrically stranded clades.

bined within localities. Thus, F_{ST} values were low when comparing samples grouped according to geographical origin, although some comparisons were significant (e.g. most comparisons involving northern Catalonia and Tunisia). In contrast, the divergence between the main colour morphotypes was significant in comparisons of the brown and purple morphs with the others. No significant genetic distance was detected between the green, blue, and white morphs. Overall, *F*st values were significantly higher between colour morphs than they were between geographical locations. The AMOVA clearly illustrated this same pattern. The contrasting outcomes according to the partitioning of the model indicate the differences between colour morphs to be sufficiently large to blur any geographical differentiation when colours were combined. When variance due to colour divergences was taken out, however, there remained a significant variability between the geographical areas.

The haplotype network also showed wide genetic variability, and up to eight separate groups of haplotypes appeared that could not be connected parsimoniously. The MDS ordination matched the results of the haplotype network, and an important role of colour morphs in the groupings seems evident. The largest group (A) showed a mixture of blue, green, and white colours, again indicating the lack of differentiation of these particular morphs, whilst other groups appeared more clearly linked to either a single or a few morphotypes (purple, brown).

When we performed the NCA, only a few of the clades showed significant associations between geography and genetic variability. This was surprising given the long branches that separated the diverse groups, indicating deep divergence. The pattern may be explained by secondary expansions of the diverse groups in the areas studied after an initial, possibly allopatric, divergence. These range expansions would

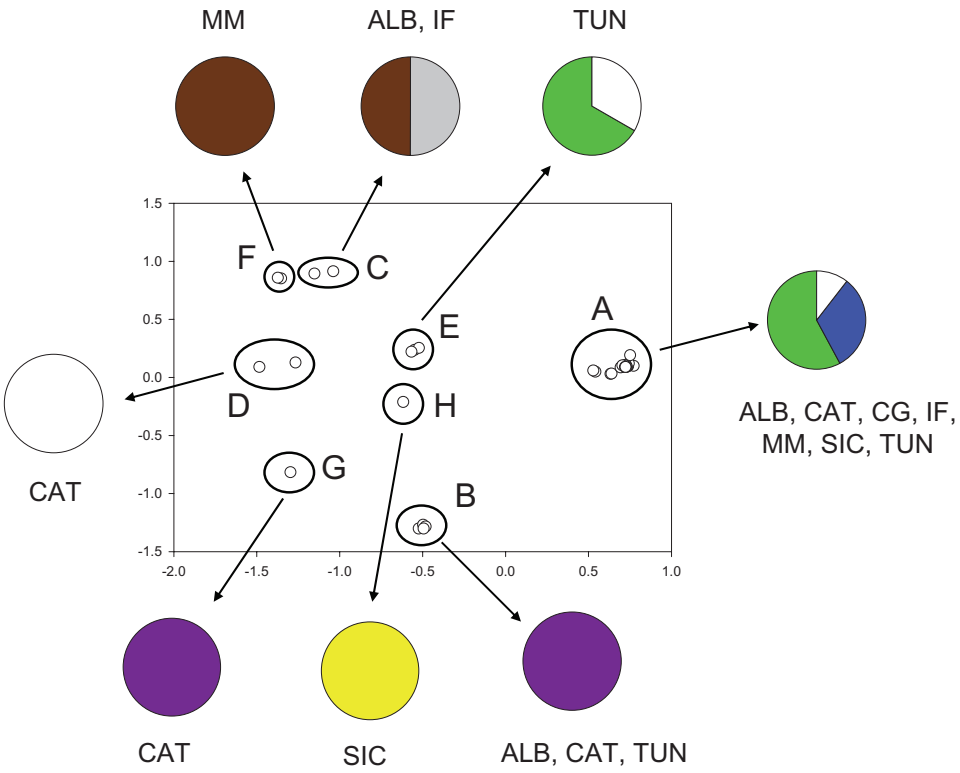

Figure 4. Results of the muldimensional scaling analysis, with the groups (A–H) obtained in the haplotype cladogram overlaid. For each group the pie chart indicates the proportion of haplotypes featuring the main colours considered. When one haplotype contained representatives of more than one colour morph, we assigned the colour that was recorded more often in terms of number of individuals. The grey shading in the pie corresponding to Group C indicates haplotypes whose colour was not recorded. The abbreviations beside the pie charts indicate the geographical locations at which the haplotypes of that group have been found. ALB, Alborán Island; CAT, northern Catalonia; CG, Cabo de Gata; IF, Ibiza–Formentera; MM, Mallorca–Menorca; SIC, Sicily; TUN, Tunisia.

have erased most of the geographical signal. In this context, the maintenance of genetic differences strongly points to reproductive isolation of at least some of these forms. Past fragmentation is a relevant biological inference when trying to infer species boundaries (Templeton, 2001), as it falsifies the hypothesis that the samples came from a single evolutionary lineage. Our NCA indicates that a past fragmentation event occurred that caused the divergence of some of the main groups, as well as divergence within some clades (e.g. within Group A). These results, coupled with some evidence of range expansion in other clades (e.g. Clade 3–3), support the idea of a secondary expansion of already diversified lineages in the western Mediterranean.

Although the COI gene is well suited for discriminating between closely related species (Hebert, Ratnasingham & de Waard, 2003), some caution in interpreting our results is necessary as we considered only one mitochondrial gene, and the study of others, especially those of biparentally inherited nuclear locus, may yield different results. Nevertheless, the results found here agree with previous chemical analyses that revealed important qualitative differences in the alkaloid composition of some of the morphotypes studied here (López-Legentil *et al*., 2005a). The blue and green forms, corresponding to Group A of this study, feature C9-unsubstituted alkaloid pyridoacridines, whilst purple forms, corresponding here to Group B, have sulphur-containing pyridoacridines. Secondary chemistry, therefore, matches the groupings obtained in this study. In addition, López-Legentil *et al*. (2005b) found differences in the reproductive cycles of the blue and purple morphs occurring in the same locality (Groups A and B). All this, along with the existence and maintenance of diverse morphotypes in the same locality (up to four colour variants were found at the same place), argues strongly against the notion of a single cohesion species for the morphs studied. The shortest genetic divergence between haplotypes of Groups A and B was 9.88%, which, assuming a mutational rate for ectoderm

mtDNA of 2.86/Myr/locus (Morita, 1999) gives us a scenario of at least *c*. 3.5 Myr of separation and probably more given that multiple substitutions must surely have occurred. Although this datum is only indicative because there is no calibrated molecular clock for this group, this time of divergence is consistent with the idea that these groups diverged after the Messinian salinity crisis (5–6 Mya, Maldonado, 1985) in the Mediterranean Sea, coinciding with a period of ample scope for colonization of new habitats and speciation.

In conclusion, we found a complex distribution pattern of genetic variance, explained mainly by clear differentiation between some (but not all) colour morphs. Divergence among geographical localities was also evident when the different morphotypes were considered separately. Population fragmentation and range expansions seem to have shaped the present-day distribution of the haplotypes. Taken together with chemotype and life-cycle information, our results indicate that several species are present in the area, as has already been suggested by some authors (Turon, 1987; Brunetti, 1994; Méliane, 2002; López-Legentil *et al*., 2005a; López-Legentil *et al*., 2005b). Clearly, previous reports of *C. dellechiajei* in the Mediterranean Sea should be considered with caution. How many different species are present, and which one (if any) should keep the name *C. dellechiajei* will only be clarified after analysis of more material from the Mediterranean and other seas.

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REFERENCES

Aron S, Solé-Cava AM. 1991. Genetic evaluation of the taxonomic status of two varieties of the cosmopolitan ascidian *Botryllus niger* (Ascidiaceae: Botryllidae). *Biochemical Systematics and Ecology* **19:** 271–276.

Avise JC. 2000. *Phylogeography. The history and formation of species*. Cambridge: Harvard University Press.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel T. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* **18:** 489–522.

Bishop JDD. 1998. Fertilization in the sea: are the hazards of broadcast spawning avoided when free-spawned sperm fertilize retained eggs? *Proceedings of the Royal Society* **265:** 725–731.

Brunetti R. 1994. Ascidians of the northern Adriatic Sea. Aplousobranchia I. *Bollettino Di Zoologia* **61:** 89–96.

Castilla JC, Collins AG, Meyer CP, Guíñez R, Lindgberg DR. 2002. Recent introduction of the dominant tunicate, *Pyura praeputialis* (Urochordata, Pyuridae) to Antofagasta, Chile. *Molecular Ecology* **11:** 1579–1584.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1660.

Crandall KA. 1996. Multiple interspecies transmissions of human and simian T-cell Leukemia/Lymphoma virus type I sequences. *Molecular Biology and Ecology* **13:** 115–131.

Dalby JE Jr. 1997. Dimorphism in the ascidian *Pyura stolonifera* near Melbourne, Australia, and its evaluation through field transplant experiments. *Marine Ecology* **18:** 253–271.

Excoffier L, Smouse P, Quattro J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479–491.

Folmer O, Hoeh W, Black M, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Grosberg RK. 1991. Sperm-mediated gene flow and the genetic structure of a population of the colonial ascidian *Botryllus schlosseri*. *Evolution* **45:** 130–142.

Hebert PDN, Ratnasingham S, de Waard JR. 2003. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London* **270:** S96–S99.

Holland BS. 2000. Genetics of marine invasions. *Hydrobiology* **420:** 63–71.

Hoskin MG. 1997. Effects of contrasting modes of larval development on the genetic structures of populations of three species of prosobranch gastropods. *Marine Biology* **127:** 647–656.

Hull DL. 1997. The ideal species concept – and why we can't get it. In: Claridge MF, Dawa HA, Wilson MR, eds. *Species: the units of biodiversity*. London: Chapman & Hall, 357–380.

Hunt A. 1993. Effects of contrasting patterns of larval dispersal on the genetic connectedness of populations of two intertidal starfish, *Patiriella calcar* and *P. exigua*. *Marine Ecology Progress Series* **92:** 179–186.

Jackson JBC. 1986. Modes of dispersal of clonal benthic invertebrates: Consequences for species distributions and genetic structure of local populations. *Bulletin of Marine Science* **39:** 588–606.

Klautau M, Russo CAM, Lazoski C, Boury-Esnault N, Thorpe JP, Solé-Cava AM. 1999. Does cosmopolitanism result from overconservative systematics? A case study using

the marine sponge *Chondrilla nucula*. *Evolution* **53:** 1414–1422.

Knowlton N. 2000. Molecular genetic analyses of species boundaries in the sea. *Hydrobiology* **420:** 73–90.

Kott P. 1990. The Australian ascidiacea. Part 2. Aplousobranchia (1). *Memoirs of the Queensland Museum* **29:** 1–266.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2 – molecular evolutionary genetics analysis, vers. 2.1. [Computer Software and Manual]. Tempe, Arizona: Arizona State University.

Lambert CC, Lambert G. 1998. Non-indigenous ascidians in southern California harbors and marinas. *Marine Biology* **130:** 675–688.

Le Gac M, Féral JP, Poulin E, Veyret M, Chenuil A. 2004. Identification of allopatric clades in the cosmopolitan ophiuroid species complex *Amphipholis squamata* (Echinodermata). The end of a paradox? *Marine Ecology Progress Series* **278:** 171–178.

López-Legentil S, Dieckmann R, Bontemps-Subielos N, Turon X, Banaigs B. 2005a. Qualitative variation of alkaloids in colour morphs of *Cystodytes* (Ascidiacea). *Biochemical Systematics and Ecology* **33:** 1107–1119.

López-Legentil S, Ruchty M, Domenech A, Turon X. 2005b. Life cycles and growth rates of two morphotypes of *Cystodytes* (Ascidiacea) in the western Mediterranean. *Marine Ecology Progress Series* **296:** 219–228.

López-Legentil S, Turon X. 2005. How do morphotypes and chemotypes relate to genotypes? The colonial ascidian *Cystodytes* (Ascidiacea, Polycitoridae). *Zoologica Scripta* **34:** 3–14.

Loukaci A, Muricy G, Brouard J-P, Guyot M, Vacelet J, Boury-Esnault N. 2004. Chemical divergence between two sibling species of *Oscarella* (Porifera) from the Mediterranean Sea. *Biochemical Systematics and Ecology* **32:** 893–899.

Mackenzie JB, Munday PL, Willis BL, Miller DJ, Van Oppen MJH. 2004. Unexpected patterns of genetic structuring among locations but not colour morphs in *Acropora nasuta* (Cnidaria; Scleractinia). *Molecular Ecology* **13:** 9–20.

Maldonado A. 1985. Evolution of the Mediterranean basins and a detailed reconstruction of the Cenozoic paleoceanography. In: Margalef R, ed. *Western Mediterranean*. Oxford: Pergamon Press, 1760.

McCartney MA, Acevedo J, Heredia Rico C, Quenoville B, Bermingham E, Mcmillan WO. 2003. Genetic mosaic in a marine species flock. *Molecular Ecology* **12:** 2963–2973.

McGovern TM, Hellberg ME. 2003. Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoan *Bugula neritina*. *Molecular Ecology* **12:** 1207–1215.

Méliane I. 2002. Contribution to the knowledge of the ascidian fauna in the South East of Tunisia. Masters Thesis, University of Alicante.

Meroz-Fine E, Brickner I, Loya Y, Ilan M. 2003. The hydrozoan coral *Millepora dichotoma*: speciation of phenotypic plasticity? *Marine Biology* **143:** 1175–1183.

Millar RH. 1971. The biology of ascidians. *Advances in Marine Biology* **9:** 1–100.

Morita T. 1999. Molecular phylogenetic relationships of the deep-sea fish genus *Coryphaenoides* (Gadiformes: Macrouridae) based on mitochondrial DNA. *Molecular Phylogenetics and Evolution* **13:** 447–454.

Nei M. 1987. *Molecular evolutionary genetics*. New York: Columbia University Press.

Pfenninger M, Posada D. 2002. Phylogeographic history of the snail *Candidula unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migration and secondary contact. *Evolution* **56:** 1776–1788.

Posada D, Crandall KA, Templeton AR. 2000. Geodis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9:** 487–488.

Raymond M, Rousset F. 1995. An exact test for population differentiation. *Evolution* **49:** 1280–1283.

Rousset F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145:** 1219–1228.

Rozas J, Rozas R. 1999. DnaSP, vers. 3.0: an integrated program for molecular population genetics and molecular evolution analyses. *Bioinformatics* **15:** 174–175.

Russo CAM, Solé-Cava AM, Thorpe JP. 1994. Population structure and genetic variation in two tropical sea anemones (Cnidaria, Actinidae) with different reproductive strategies. *Marine Biology* **119:** 267–276.

Scheltema RS. 1986. Long-distance dispersal by planktonic larvae of shallow-water benthic invertebrates among central Pacific islands. *Bulletin of Marine Science* **39:** 241–256.

Schneider S, Roessli D, Excoffier L. 2000. *Arlequin*, vers. 2000. *Software for population genetics data analysis.* http://anthro.unige.ch/arlequin.

Sites JJ, Marshall J. 2003. Delimiting species: a renaissance issue in systematic biology. *Trends in Ecology and Evolution* **18:** 462–470.

Stoner DS, Ben-Shlomo R, Rinkevich B, Weissman IL. 2002. Genetic variability of *Botryllys schlosseri* invasions to the east and west coasts of the USA. *Marine Ecology Progress Series* **243:** 93–100.

Svane I, Young CM. 1989. The ecology and behavior of ascidian larvae. *Oceanographic Marine Biology Annual Review* **27:** 45–90.

Tarjuelo I, Posada D, Crandall KA, Pascual M, Turon X. 2001. Cryptic species of *Clavelina* (Ascidiacea) in two different habitats: harbours and rocky littoral zones in the northwestern Mediterranean. *Marine Biology* **139:** 455–462.

Tarjuelo I, Posada D, Crandall KA, Pascual M, Turon X. 2004. Phylogeography and speciation of colour morphs in the colonial ascidian *Pseudodistoma crucigaster*. *Molecular Ecology* **13:** 3125–3136.

Templeton AR. 1989. The meaning of species and speciation: a genetic perspective. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland, Massachusetts: Sinauer Associates, 3–27.

Templeton AR. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7:** 381–397.

Templeton AR. 2001. Using phylogenetic analyses of gene

trees to test species status and processes. *Molecular Ecology* **10:** 779–791.

Templeton AR. 2004. Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology* **13:** 789–810.

Templeton AR, Boerwinkle E, Sing CF. 1987. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics* **117:** 343–351.

Templeton AR, Crandall KA, Sing CF. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132:** 619–633.

Templeton AR, Sing CF. 1993. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics* **134:** 659–669.

Turon X. 1987. Estudio de las ascidias de las costas de Cataluña e Islas Baleares. PhD Thesis, University of Barcelona.

Turon X, Tarjuelo I, Duran S, Pascual M. 2003. Characterizing invasion processes with genetic data: an Atlantic clade of *Clavelina lepadiformis* (Ascidiacea) introduced into Mediterranean harbours. *Hydrobiologia* **503:** 29–35.

Wilson AB, Noack-Kunnmann K, Meuer A. 2000. Incipient speciation in sympatric Nicaraguan crater lake cichlid fishes: sexual selection versus ecological diversification. *Proceedings of the Royal Society of London (B)* **267:** 2133–2141.

Yund PO. 1998. The effect of sperm competition on male gain curves in a colonial marine invertebrate. *Ecology* **79:** 328–339.

Yund PO, O'Neil PG. 2000. Microgeographic genetic differentiation in a colonial ascidian (*Botryllus schlosseri*) population. *Marine Biology* **137:** 583–588.