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Phylogeography and speciation of colour morphs in the colonial ascidian *Pseudodistoma crucigaster*

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Abstract

Variation in pigmentation is common in marine invertebrates, although few studies have shown the existence of genetic differentiation of chromatic forms in these organisms. We studied the genetic structure of a colonial ascidian with populations of different colour morphs in the northwestern Mediterranean. A fragment of the *c* oxidase subunit 1 (COI) mitochondrial gene was sequenced in seven populations of *Pseudodistoma crucigaster* belonging to three different colour morphs (orange, yellow and grey). Maximum likelihood analyses showed two well-supported clades separating the orange morph from the yellow-grey morphotypes. Genetic divergence between these clades was 2.12%, and γ_{ST} values between populations of the two clades were high (average 0.936), pointing to genetic isolation. Nested clade and coalescence analyses suggest that a past fragmentation event may explain the phylogeographical origin of these two clades. Non-neutral mtDNA evolution is observed in our data when comparing the two clades, showing a significant excess of nonsynonymous polymorphism within the yellow–grey morphotype using the McDonald–Kreitman test, which is interpreted as further support of reproductive isolation. We conclude that the two clades might represent separate species. We compare the population genetic differentiation found with that estimated for other colonial and solitary ascidian species, and relate it to larval dispersal capabilities and other life-history traits.

Keywords: ascidians, coalescence analysis, maximum likelihood, nested analysis, phylogeography, population genetic structure

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Introduction

Speciation studies are at the heart of understanding mechanisms of evolutionary change. The first step in such studies is to identify populations that are subdivided, either ecologically, behaviourally or genetically. Different authors have posited various ideas about the relative importance of these different kinds of subdivision. Some species concepts (e.g. Mayr's Biological Species Concept) are entirely genetic with strictly geographical correlates associated with genetic structuring, whereas others (e.g. Templeton's Cohesion Concept) allow for exchange of genes while maintaining either phenotypic (including ecological and behavioural phenotypes) and/or genetic cohesion among species. We subscribe to the view that ecology can play a significant role in the speciation process (McKinnon *et al*. 2004) and are therefore interested in determining the associations of phenotypic traits with genetic subdivision. There is a variety of statistical frameworks within which one can rigorously test species boundaries (Sites & Marshall 2003). We have chosen the Templeton Cohesion Concept framework (Templeton 2001). The next step is that of testing the role of natural selection in the speciation process (McKinnon *et al*. 2004). In this study we utilize these approaches on a model group of marine invertebrates (ascidians) with morphological variation that appears to be partitioned at the microgeographical level. We also present molecular evidence suggesting that natural selection is playing a role in the speciation process, and couple this with suggestions for future work to explore further the role of natural selection.

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Diversity in colouration has largely been described in nature as being associated with polymorphism or with divergence. Whether it is one or the other is always an open debate that has to be solved before a biogeographical analysis is undertaken. Most of the time, the significance of such colour differences is unclear. It has been claimed that colour polymorphism may be maintained by disruptive selection through the effect of prey, predators and competitors in birds (Galeotti *et al*. 2003) or symbionts in corals (Mackenzie *et al*. 2004). Alternatively, strong genetic separation based on colouration has been found between cichlid species related to sexual selection playing an important role in speciation (Wilson *et al*. 2000). Colour morphospecies in hamlets (Serranidae) show complex genetic patterns; significant genetic differentiation was found in Puerto Rico and no differentiation in Panama, suggesting that interactions between mating behaviour and population history shape genetic differences in recent evolutionary radiations (McCartney *et al*. 2003).

Many marine invertebrates show high variability in terms of size, shape and colour. This presents the problem of species definition, as some variants may in fact correspond to different species that have not been recognized by traditional methods. Indeed, with the advent of molecular techniques, a bewildering array of cryptic species has been uncovered in many groups of marine organisms (reviewed in Knowlton 2000). Much variation (morphological, chemical or otherwise) attributed to intraspecies adaptation to local environmental conditions corresponded with different species (e.g. Klautau *et al*. 1999; Miller *et al*. 2001; McGovern & Hellberg 2003; Meroz-Fine *et al*. 2003). In ascidians variation in pigmentation is common, and chromatic varieties have not, in general, been given any valid taxonomic status (Monniot *et al*. 1991). In *Botryllys schlosseri*, polychromatism is regulated by a few Mendelian loci and is clearly an intraspecific feature (Sabbadin 1982; Yund & O'Neil 2000). López-Legentil & Turon (2004) concluded that colour variation in *Cystodytes dellechiajei* correlate with genetic differences but may not be enough to differentiate species. On the other hand, Dalby (2000) found genetic differentiation between colour varieties of *Pyura stolonifera*. Similarly, studies of morphological or ecological varieties of other ascidian species have usually revealed strong genetic barriers, suggesting that formerly recognized species should be split (Aron & Solé-Cava 1991; Degnan & Lavin 1995; Tarjuelo *et al*. 2001; Turon *et al*. 2003).

Populations of colonial ascidians are likely to be highly genetically structured over short distances (tens of Km), as their larvae undergo a prolonged embryogenesis, hatch with juvenile rudimentary structures already formed and can settle within minutes (Svane & Young 1989; Young *et al*. 2002), thus providing a good model for the study of microgeographical genetic patterns. When larval dispersal of colonial ascidians has been monitored in the field, short-distance dispersal has been substantiated (Grosberg 1987; Olson & Macpherson 1987; Davis & Butler 1989; Bingham & Young 1991). Genetic evidence also shows that dispersal in colonial ascidians is more restricted than that of broadcasting solitary forms (Ayre *et al*. 1997).

The colonial ascidian *Pseudodistoma crucigaster*, described by Gaill (1972), is endemic to the Mediterranean Sea where it occupies rocky littoral surfaces (Turon 1987). It has been found along the western Mediterranean arch between France and the Gibraltar Straits, including the Balearic Islands (Gaill 1972; Lafargue *et al*. 1986; Turon 1990, 1993; Ramos *et al*. 1991, 1992). It is a perennial form and broods up to three big larvae per zooid during its reproductive season (Tarjuelo & Turon 2004). Larvae of this species settle down in the laboratory within a few hours (personal observation) and are defended against predation, although the basis of their unpalatability, either chemical or due to tunic toughness, has not been ascertained (Tarjuelo *et al*. 2002). Three chromatic varieties, lacking any other morphological feature to allow a formal taxonomic distinction, have been found: yellow, grey and orange (Turon 1987, 1993). The different colour morphs of *P. crucigaster* inhabit the same type of ecological habitat, and their populations, although not in sympatry, are interspersed over relatively short distances (tens of kilometres). They are therefore a suitable model to test the combined effects of short-distance dispersal and genetic differentiation and relate them to chromatic variation and species boundaries.

In the present work, we quantify mtDNA variability in seven populations of *P. crucigaster* in the western Mediterranean covering the two main areas of the known distribution of the species (Iberian littoral and Balearic Islands) and all colour morphs described so far. Using the *c* oxidase subunit 1 (COI) gene we hope to clarify whether these morphs are related to genetic differentiation. Also, we contrast these results with those from previous studies on colonial and solitary ascidians (Ayre *et al*. 1997; Tarjuelo *et al*. 2001) to investigate whether there is any relationship between larval dispersal capabilities and degree of genetic structure in populations of ascidians.

Materials and methods

Samples

We collected samples of *P. crucigaster* (Aplousobranchiata) from seven different localities in the western Mediterranean during 1998 and 1999 (Fig. 1). Samples were obtained by self-contained underwater breathing apparatus (scuba) diving in the open rocky seashore. See Table 1 for details of localities and colour morphs. Colonies were preserved in 95% EtOH and stored at –20 °C until processed, when several zooids were extracted from each colony with forceps. The sequence of another aplousobranch species, *Aplidium*

Table 1 Haplotype frequencies, nucleotide diversity (π) and number of polymorphic sites, within populations and for the total data set, in the populations of *Pseudodistoma crucigaster* studied. Colour morphs are indicated by the codes: O (orange), Y (yellow) and G (grey)

P. crucigaster Population	Colour Morph	Haplotypes											Total	π	No. of polymorph sites
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI			
Tossa Mar	O	15	—	—	—	—	—	—	—	—	—	—	15	0.00000	0
Cala Ratjada	O	2	3	2	—	—	—	—	—	—	—	—	7	0.00248	3
Cap Catalunya	O	—	7	—	—	—	—	—	—	—	—	—	7	0.00000	0
Blanes	Y	—	—	—	—	7	2	1	1	1	—	—	12	0.00139	4
Cadaqués	Y	—	—	—	4	—	—	—	—	—	—	—	4	0.00000	0
Illa Imperial	Y	—	—	—	6	—	—	—	—	—	—	1	7	0.00050	1
Cap Llebeig	G	—	—	—	4	—	—	—	—	—	1	—	5	0.00139	2
Total		17	10	2	14	7	2	1	1	1	1	1	57	0.01160	

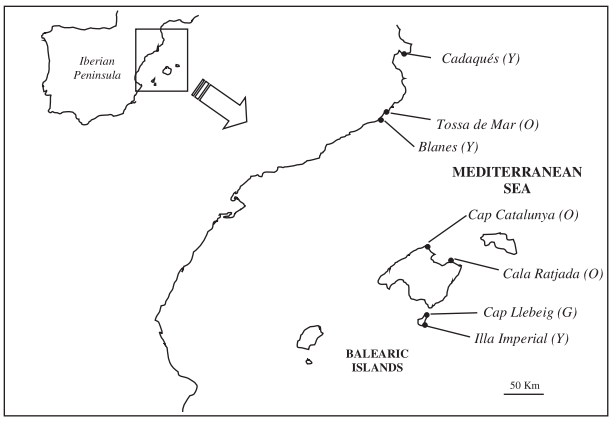

Fig. 1 Map of Spain showing the different sampling localities of this study. Colour morphs in parentheses (G, grey; O, orange; Y, yellow).

elegans (Giard, 1872), was obtained from Turon & López-Legentil (2004) and used as an outgroup (Accession no. AY600971).

We extracted mitochondrial DNA using the protocol for *Drosophila* sp. described in Latorre *et al*. (1986). We used the universal primers LCO1490 and HCO2198 described in Folmer *et al*. (1994) to obtain a fragment of the cytochrome COI gene. Amplification was performed in a 20 μL reaction volume (2 μL 10× buffer containing 15 mM $MgCl_2$, 3 μL dNTPs (1 mM), 1 U *Taq* polymerase, 0.4 μL of each primer (25 μM) and 1 μL DNA) with an initial denaturing of 96 °C for 3 min, 35 cycles (95° for 55 s, 42° for 55 s and 72° for 1 min 30 s) and a final extension at 72° for 5 min, on a Perkin Elmer 9600 polymerase chain reaction (PCR) machine. The same primers were used for the sequencing reaction with the ABI-Big-Dye Ready-Reaction kit of Perkin Elmer. The PCR products were sequenced on an ABI Prism 377XL automated sequencer at the Scientific and Technical Services of the University of Barcelona. Sequences were aligned with CLUSTALX (Thompson *et al*. 1997) and confirmed by eye.

Phylogenetic analysis

The best-fit model of nucleotide substitution for our data was selected by statistical comparisons of the likelihood scores for 56 different models of evolution with the Akaike information criterion (AIC), as implemented in MODELTEST 3.0 (Posada & Crandall 1998). A haplotype tree was estimated in PAUP* (Swofford 1998) [heuristic search; 1000 replicates of random stepwise addition and tree-bisection–reconnection (TBR)] under the maximum likelihood criterion (Felsenstein 1981) with the parameter estimates obtained under the best-fitting model of substitution. Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein 1985).

Haplotype network and nested clade analysis (NCA)

A haplotype network was estimated using the program TCS (Clement *et al*. 2000), which implements the statistical parsimony (SP) algorithm described by Templeton *et al*. (1992). The procedure described in Crandall (1996) was applied to nest the haplotype network. An NCA was performed in order to differentiate between population history events and population structure (Templeton *et al*. 1995; Templeton 1998). The NCA was carried out using the program GEODIS 2.0 (Posada *et al*. 2000) and an inference key (Templeton 2004). Recent critiques of this approach have been raised recently (Knowles & Maddison 2002), although based on limited simulation studies (only 10 replications). Templeton (2004) has shown that these critiques were largely unfounded. Furthermore, using empirical data he demonstrated that the inference key used did contain some problems which he corrected in the current key used in the present study. Finally, as suggested by multiple authors, we have used a variety of statistical

approaches to test for population subdivision (see below).

Population genetic and demographic analyses

We estimated nucleotide diversity within and between populations (Nei 1987; Lynch & Crease 1990), gamma$_{ST}$ (γ_{ST}) values (Nei 1982) between pairs of populations, and genetic differentiation using the *Snn* statistic (Hudson 2000). We additionally attempted to obtain migration rates using the software MIGRATE 1.7.3 (P. Beerli, available at http://evolution.genetics.washington.edu/lamarc/migrate.html), that implements coalescent estimates of migration rates (Beerli & Felsenstein 2001). However, although we implemented several stratagies to assure convergence (long chains, chain heating, multiple replicates) the estimates obtained appeared to be problematic (large overflow in several cases), due presumably to the low divergences resulting in a relatively flat likelihood surface. Therefore, these results are not presented. Similar difficulties were found in simulation studies of MIGRATE (Abdo *et al*. 2004). Tajima's *D* (Tajima 1989) and the McDonald & Kreitman (1991) test were used to contrast whether patterns of variation among the different colour morphs were consistent with predictions of the neutral model. For detecting population growth, we computed the raggedness index based on the mismatch distribution (Harpending 1994), Fu's F_S test (1997) and the R_2 test (Ramos-Onsins & Rozas 2002). All these analyses were performed with the program DNASP 4.0 (Rozas *et al*. 2003).

An analysis of molecular variance (AMOVA) was performed to partition total variance components into those between and within clades predicted by the phylogenetic analysis using the ARLEQUIN program (Schneider *et al*. 2000).

Coalescence analysis

The population parameter $\theta = N_e\mu$ (where μ is the mutation rate per sequence per generation and N_e the effective population size) was estimated using the program FLUCTUATE (Kuhner *et al*. 1998). To obtain estimates of the ages of the mutation events defining the clades within *P. crucigaster*, we used the coalescence simulation approach (Griffiths & Tavaré 1994) implemented in the program GENETREE version 8.3 (R. C. Griffiths). The effect of subdivision among populations was considered in the age estimates by using the estimates of *Nm* derived from γ_{ST} values assuming an island model of gene flow. One of the assumptions of the coalescence theory is the existence of a molecular clock, and we tested its occurrence with a likelihood ratio test Felsenstein (1988). We used the mutational rate $\mu = 2.86$/MY/locus from ectotherm mtDNA (Morita 1999).

Results

Sequence variation

A total of 57 sequences for *P. crucigaster* were obtained for the COI mitochondrial gene. The final length after alignment and trimming was of 578 base pairs (bp) and free of gaps. All unique haplotypes have been deposited in EMBL (Accession nos AJ748708–AJ748718). Within *P. crucigaster*, 11 different haplotypes were identified (Table 1) with 22 polymorphic sites, six of them with nonsynonymous changes (Fig. 2). Of the nucleotide substitutions, 63.6% occurred at third codon positions. Transitions accounted for 68% of the nucleotide substitutions and A–T proportion was high (68%). The orange morphotype had three haplotypes and the yellow morphotype seven haplotypes; no haplotype was shared between the two morphs. We found two haplotypes in the grey colour morph, one of them shared with the yellow form (haplotype IV, Table 1). Three polymorphic sites were found within the orange form, all resulting in synonymous changes (Fig. 2). Six polymorphic sites were detected within the yellow morphotype, all yielding nonsynonymous substitutions. The grey morphotype featured two polymorphic sites, both of them being synonymous changes. Eleven fixed nucleotide positions, all of them producing synonymous changes, differentiated the orange from the other two morphotypes (Fig. 2). The ratio of nonsynonymous to synonymous substitutions in the total data set was 0.0274.

Phylogenetic analysis

The model of evolution selected for our data was a special case of the general time reversible (GTR) model (Tavaré 1986) with all sites evolving at the same rate and unequal base frequencies ($\pi_A = 0.24$, $\pi_C = 0.13$, $\pi_T = 0.45$, $\pi_G = 0.18$)

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                                      **   *
                          *11111122222222455
                        ** 3433577801244589302
                        3576958412019935451512
Consensus               TTGTATTGTTACATTTTAAGGC
Hap I          (17)     .....C.ACCGT.C...GTA.T
Hap II         (10)     ..A..C.ACCGT.C...GTA.T
Hap III         (2)     ..AA.C.ACCGTGC...GTA.T
Hap IV         (14)     ......................
Hap V           (7)     ....G.................
Hap VI          (2)     .A..G.................
Hap VII         (1)     ....G...........G.....
Hap VIII        (1)     G...G.................
Hap IX          (1)     ....G..........A......
Hap X           (1)     ......C.......C.......
Hap XI          (1)     ...................T.
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Fig. 2 Sequence variation among 11 mtDNA haplotypes for 578 bp of COI gene in *Pseudodistoma crucigaster*. Numbers at the top indicate site positions and the asterisks show those sites yielding nonsynonymous changes. Number of individuals of each haplotype is shown in parentheses next to the haplotype name.

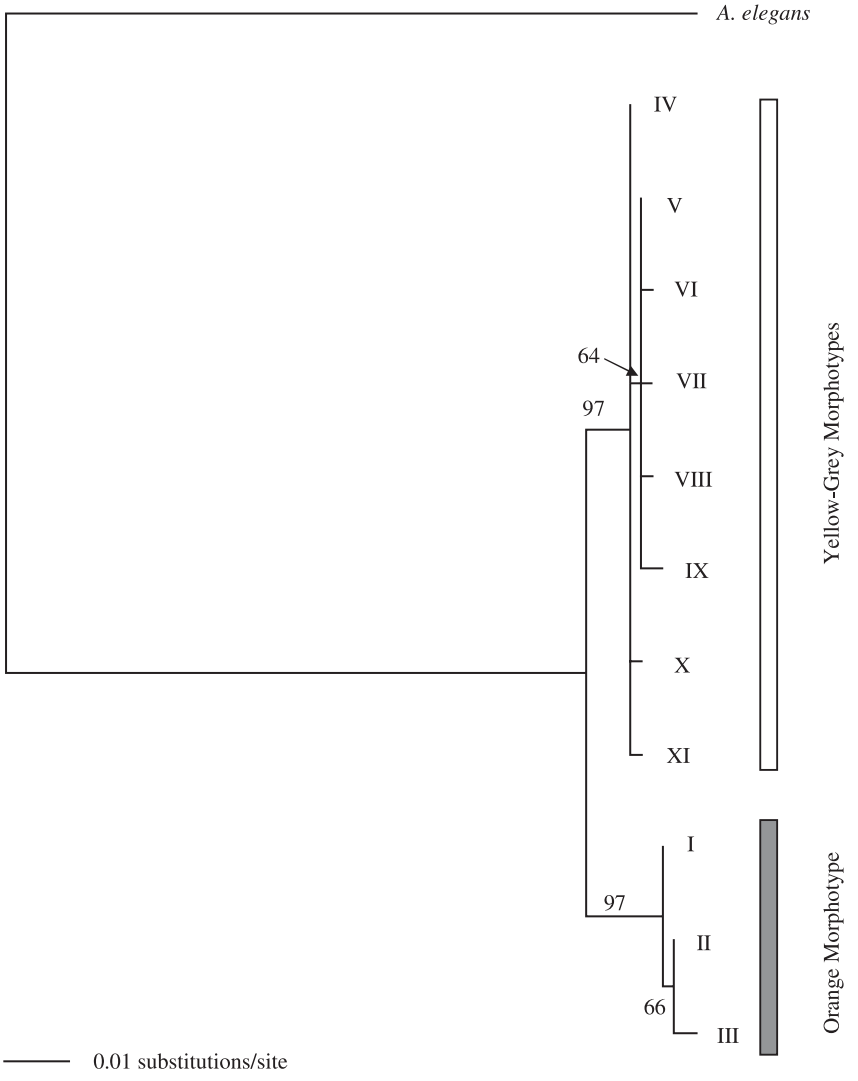

Fig. 3 Maximum likelihood tree for *Pseudodistoma crucigaster* haplotypes. Bootstrap values higher than 50% are shown.

and unequal relative substitution rates (R_{AC} = 1.00, R_{AG} = 8.18, R_{AT} = 3.44, R_{CG} = 3.44, R_{CT} = 4.25, R_{GT} = 1.00). An ML haplotype tree was obtained under this model (Fig. 3), where two monophyletic clades were inferred, one corresponding to the orange morphotype (haplotypes I, II and III) and another clade corresponding to the yellow and grey morphotypes together. Support for this grouping was high (97% bootstrap).

Haplotype network and NCA

We found two subnetworks separated by 11 substitutions, corresponding to the yellow–grey clade (Network 2-1) and the orange clade (Network 2-2) (Fig. 4). These clades could not be connected unambiguously in one single network because the maximum number of substitutions to establish a parsimonious connection between two haplotypes, at the 95% confidence level, was 10 for this alignment.

The NCA suggested that the oldest event was a historical fragmentation between the orange and the yellow–grey morphotypes (Fig. 5), an inference that is reinforced by the long branch that separates these two clades. For the yellow–grey clade, all the haplotypes from one of the localities (Blanes) grouped on clade 1-3, separated from the rest of the haplotypes (Fig. 4). In clade 2-1 the NCA also suggested a past fragmentation event as being responsible for the separation within the yellow–grey clade, which is mainly the result of the isolation of the one-step clade (1-3) of Blanes (Fig. 5). As for the orange morphotype, the NCA suggested that its overall distribution might be explained by range expansion. Within clade 1-4 of the orange form the NCA could not resolve fully between range expansion or restricted gene flow (Fig. 5).

Population genetic and demographic analyses

The number of polymorphic sites and values of nucleotide diversity within each population are shown in Table 1.

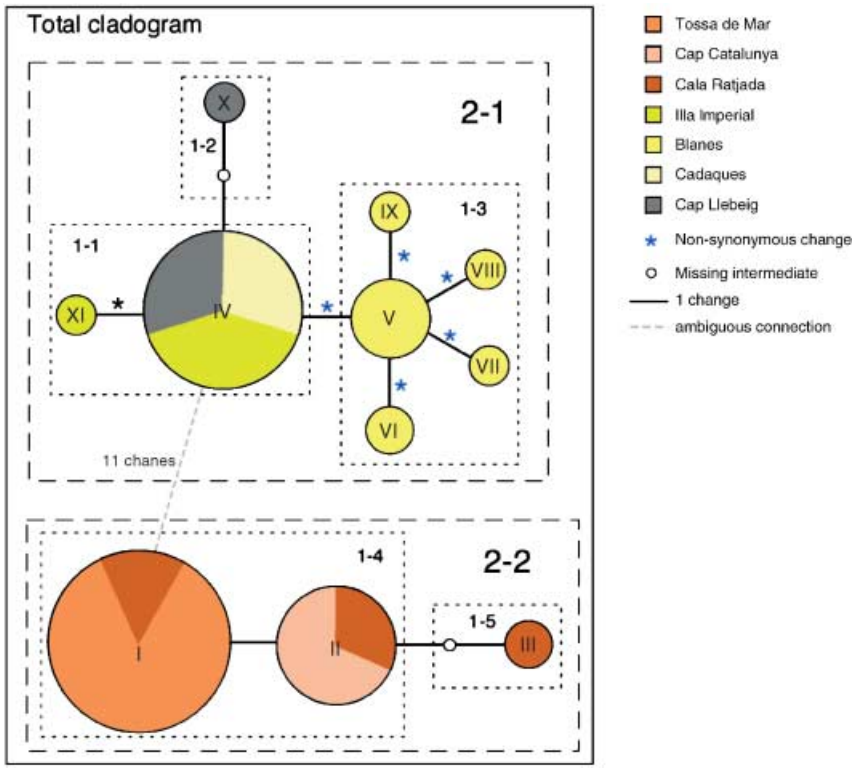

Fig. 4 TCS networks for *Pseudodistoma crucigaster*. Each haplotype is designated by a roman numeral and haplotype frequencies are represented by the area of the circle. Boxes group the haplotypes nested together into one-step and two-step clades.

Haplotype			1-step clade			2-step clade		
Name	*Dc*	*Dn*	*Name*	*Dc*	*Dn*	*Name*	*Dc*	*Dn*
IV	183.15	176.46						
XI	0	102.31	**NS**			**(Yellow-Grey Morphotypes)**		
I-T	183.15	74.14	1-1	176.57	201.04^{L}			
X	0	0	1-2	0	181.51			
V	0	0						
VI	0	0						
VII	0	0						
VIII	0	0						
IX	0	0	1-3	0^{S}	176.44	**1-2-3-4-9-No: Past. Frag.**		
			I-T	176.57^{L}	24.21	2-1	191.75^{L}	172.26^{L}
I	80.24^{S}	135.56^{L}						
II	22.90^{S}	142.97^{L}	**1-2-3-5-6-'too few clades' RE/RGF**			**(Orange Morphotype)**		
I-T	57.33^{L}	–7.408^{S}	1-4	140.08^{S}	139.44			
III	0	0	1-5	0	130.25	**1-2-11-12-No: CRE**		
			I-T	140.08	9.19^{S}	2-2	140.78^{S}	149.47^{S}

Total cladogram	**1-2-3-4-9-No: Past Frag**

Fig. 5 Results of the nested geographical analysis for *Pseudodistoma crucigaster*. Column *Name* is the name of the clade, *Dc* is the clade distance and *Dn* the nested clade distance at each one of the levels of the analysis (haplotype, one-step and two-step levels). The row *I-T* indicates the average difference between interior and tip clades. Superscript S means that the statistic was significantly small and superscript L that the statistic was significantly large (both at the 5% level). The lines in bold describe the steps followed in the inference key and the conclusion reached by this method: NS (not significant), Past Frag (past fragmentation), RE (range expansion), RGF (restricted gene flow) and CRE (contiguous range expansion).

Table 2 Gamma ST (γ_{ST}) values among the populations of *Pseudodistoma crucigaster* and significance of the *Snn* statistic for detecting genetic differentiation obtained by a permutation test

	Tossa Mar	Blanes	Illa Imperial	Cap Llebeig	Cap Catalunya	Cala Ratjada
Cadaqués	1*	0.416*	0.057 NS	0.1 NS	1*	0.874*
Tossa Mar		0.948*	0.984*	0.963*	1*	0.429*
Blanes			0.473*	0.398*	0.929*	0.866*
Illa Imperial				0.106 NS	0.980*	0.888*
Cap Llebeig					0.957*	0.853*
Cap Catalunya						0.167*

*$P < 0.001$; NS, not significant.

Nucleotide diversity over all sequences was 0.0116. Haplotype diversity was 0.815 ± 0.027 (mean ± SD) and the average number of nucleotide differences 6.69. The average number of nucleotide substitutions per site among all populations was 0.0129 ± 0.0022, while between the orange and the yellow–grey clades it was 0.0212 ± 0.0004.

Gamma ST values (Table 2) were considerably higher when comparing populations between the two main clades (0.936 ± 0.05, mean ± SD) than within clades (yellow–grey: 0.258 ± 0.19; orange: 0.532 ± 0.43). A significant overall genetic differentiation was observed among all populations as well as between population pairs with the exception of those within the yellow–grey clade other than Blanes (Table 2). The AMOVA used to determine the partitioning of variation between the two clades and among populations within clades found most variation between the orange and yellow–grey clades (90.9%), while variation among populations within clades explained 5.54% of the total variance and variation within populations was only 3.57%; none the less, all variance components were significant ($P < 0.05$), again indicating a high genetic structure at several levels.

Levels of haplotype and nucleotide diversity were higher in the yellow-grey clade (Table 3). The Tajima's *D*-test was performed independently for synonymous and nonsynonymous substitutions. The statistic acquires positive values when there is an excess of alleles at intermediate frequencies, and negative values when there is an excess of low frequency variants. Overall, the test was negative and not significant for nonsynonymous mutations ($D = -1.437$ $P > 0.10$), while it was positive and significant for synonymous mutations ($D = 2.336$ $P < 0.05$). This indicates that synonymous polymorphisms are found at intermediate frequencies, due probably to an admixture of different genetic variants. Additionally, each clade was analysed separately and Tajima's *D* had positive values for the orange and negative values for the yellow–grey, although they were not significant (Table 3). Population expansion was detected for the yellow–grey clade with Fu's F_S and R_2 but not by the raggedness test (Table 3), confirming previous reports that mismatch distribution tests are very conservative (Ramos-Onsins & Rozas 2002).

Table 3 Results of the demographic parameters for the two clades with their standard deviation or significance where applicable

	Orange	Yellow–grey
Haplotype diversity	0.552 ± 0.062	0.701 ± 0.075
Nucleotide diversity	0.00133 ± 0.00132	0.00186 ± 0.00033
D	0.014 NS	−1.474 NS
Raggedness	0.1456 NS	0.0771 NS
F_S	0.945 NS	−3.813*
R_2	0.1281 NS	0.0733*

*$P < 0.05$; NS, not significant.

The McDonald–Kreitman (MK) test of neutrality was performed comparing the orange and the yellow–grey clades to the outgroup species *Aplidium elegans*. Significant departure from neutrality was detected within the yellow–grey clade ($P = 0.014$, PR = 6, PS = 2, FR = 35 and FS = 84). When the grey variety was not included in the analyses the significance increased ($P = 0.001$) as the excess of amino acid variation in our sample was exclusive of the yellow morph. However, no deviation was observed within the orange clade ($P = 0.557$, PR = 0, PS = 3, FR = 35 and FS = 84). Therefore, there was an excess of amino acid variation within the yellow–grey but not within the orange clade. The MK test was also significant when the two clades were compared ($P = 0.012$, PR = 6, PS = 5, FR = 0 and FS = 11), and also excluding the grey morph ($P = 0.002$).

Coalescence analysis

Our data are compatible with the hypothesis of the existence of a molecular clock ($P = 0.52$); therefore, the use of coalescence theory to infer the age of mutations seems justified. The estimated value for the population mutational parameter θ was 2.2 per locus.

The estimated age for the fragmentation event between the orange and the yellow–grey morphs; that is, the time to their most recent common ancestor (TMRCA), was 5 ± 1.3 MYA (mean ± SD) while the estimated age for the divergence within the yellow–grey clade (marked by mutation no. 5) was 0.45 ± 0.3 MYA (Fig. 6).

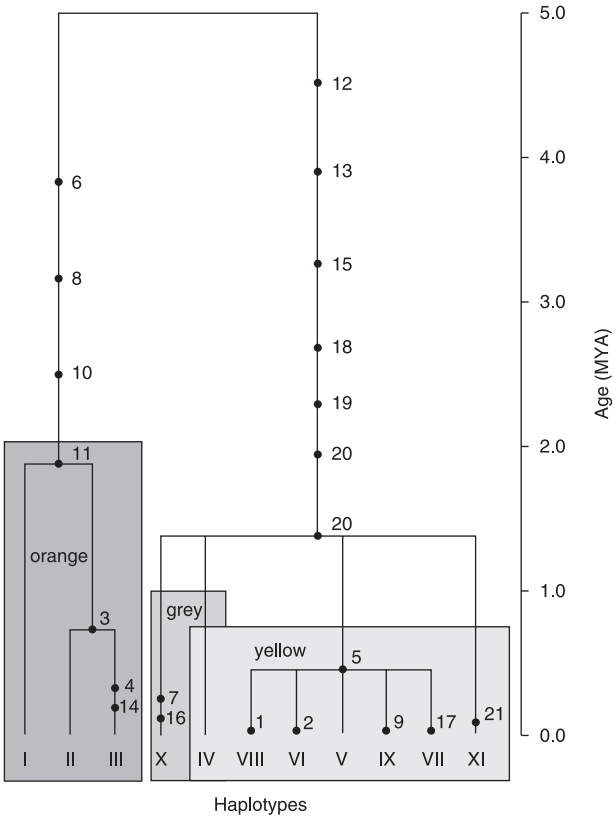

Fig. 6 Age of mutations in the fragment of mtCOI gene analysed. Mutational changes are represented by a solid dot. The time scale is in millions of years (MYA).

Discussion

Speciation

Using Templeton's Cohesion Species Concept, our phylogenetic analyses indicate the existence of two lineages within *P. crucigaster*, corresponding to the orange and the yellow–grey clades. We reject the first hypothesis in Templeton's framework, that organisms are derived from the same evolutionary network. The second hypothesis that lineages are genetically and/or ecologically exchangeable is also rejected. Our genetic data support the notion of genetic inexchangeability between these populations. In addition, the spatial partitioning of colour morphs (see below) allows us to reject the notion of ecological exchangeability. Although the degree of genetic divergence is moderate (2.12%), the inferred fragmentation event suggests an ancient isolation (5 MY) between these two clusters of *P. crucigaster*. The populations of both morphs are interspersed geographically, they occupy the same habitat in the study area (rocky bottoms) and they show the same bathymetrical distribution (approximately from 2 to 20 m, see Turon 1987). In one locality inhabited by the orange morphotype (Tossa de Mar), a few specimens of the yellow form have been observed occasionally, but *c*. 50 m away from the orange colonies (Turon, personal observation), so sympatry at the microgeographical level has never been found. Restricted as it is, the potential time span of the larval period (several hours based on laboratory observations, Tarjuelo 2001), should allow with some frequency for dispersal events of several km, enough to permit colonization along the Iberian coast and, probably, occasional jumps to and from the Balearic Islands. Therefore, although we acknowledge that a mosaic distribution of haplotypes can be the result of a strongly phylopatric dispersal mode, the phylogeographical pattern of both forms is most probably the result of some kind of reproductive isolation mechanism. Accordingly, we suggest that the orange and the yellow–grey clades are in fact two different species.

Differences in morphology, secondary chemistry, symbionts or biology often emerge in cases where genetic analyses suggest speciation phenomena (e.g. Miller *et al*. 2001; De Caralt *et al*. 2002; McGovern & Hellberg 2003). In our case, careful morphological studies did not substantiate any significant difference (Turon 1987, 1993). Cross-fertilization studies are not feasible in this species because they are hermaphrodite and brooders, and they do not survive well in aquaria. The biological cycles are well known in the orange form (Turon 1988; Turon & Becerro 1992) and it would be instructive to monitor populations of the other morphotypes for comparison. In this sense, it would be particularly desirable to compare the information gleaned from mtDNA with that supplied by codominant nuclear genes to verify the pattern observed.

The results of the neutrality tests also support the view that the two clades represent distinct species. The positive Tajima's *D*-value in synonymous substitutions is indicative of the subdivision between both clades, while the negative values in the yellow–grey clade might be the result of a recent expansion, supported by the R_2 test and Fu's F_S. In essence, the McDonald–Kreitman test revealed an excess of amino acid variation (nonsynonymous changes) within clades (species) compared to the fixed differences between clades, which were all synonymous. This pattern agrees with the nearly neutral theory, whereby weakly deleterious amino acid variants may accumulate within species, but not persist long enough to contribute to interspecific divergence (Nachman *et al*. 1994).

Almost all nonsynonymous changes are found in the Blanes population and they appear to be very recent (less than 0.45 MY) in our coalescence analysis (cf. mutations 1, 2, 5, 9 and 17 in Fig. 6). Moreover, when all populations within the yellow–grey clade were analysed separately by the Ramos–Onsins and Rozas test, only in Blanes did we detect the signal of an expansion ($R_2 = 0.1147$, $P < 0.05$). Thus the scenario suggested by our analyses for the origin of the Blanes population is a relatively recent bottleneck

and subsequent expansion followed by a relaxation of selection that has not been able to eliminate nonsynonymous (possibly mildly deleterious) changes that have appeared. An alternative explanation is that a mutation in the nuclear proteins that are necessary for the function of the respiratory enzymes may have led to a rapid accumulation of adaptive changes (Gerber *et al*. 2001; Schmidt *et al*. 2001; Rawson & Burton 2002) in the mitochondrial genes coding for respiratory enzymes in this population. To explore further the influence of natural selection on the divergence of this population subsequent studies will examine nuclear genes, especially those candidate loci associated with colour determination.

Divergence times

Although the coalescent estimates for the age of mutations should be taken with caution (Graur & Martin 2004), especially in the light of a single gene, the estimates obtained here seem to make sense. The differentiation of the two main clades, according to our coalescence analysis, took place right after the Messinian crisis (5–6 MY) (Maldonado 1985), when the Mediterranean Sea connected again with the Atlantic and was colonized by new species coming from that sea. Due to its low dispersal capability, habitat fragmentation would be responsible for population differentiation and accumulation of a large number of mutations along with chromatic differentiation. Indeed, sporadic dispersal events could have shaped the present day distribution of the morphotypes afterwards. In the yellow–grey clade, the presumably ancestral haplotype IV was found both in the Iberian coast and the Balearic Islands. Aside from two mutations in the populations of the islands, the main change within this clade is the diversification of haplotypes in the Blanes population, as explained above. For the orange morph, Cala Ratjada contains the highest number of haplotypes and therefore could represent the ancestral population. More recently this orange form could have colonized the Iberian coast through long-range dispersal events. Due to the founder event, one of the alleles present in the original population would have become fixed in Tossa de Mar (haplotype I). The low levels of genetic variability could also suggest a recent selective sweep.

Life history

Molecular tools have revealed strong genetic structure and/or heterozygote deficiency in colonial ascidians (Grosberg 1991; Yund & O'Neil 2000; Ben-Shlomo *et al*. 2001; Stoner *et al*. 2002). In the present study we found a high degree of population structure in *P. crucigaster*. To allow for comparison with published data, we have computed *Nm* (number of migrants) from γ_{ST} values within clades, assuming an island model (Wright 1951, 1978). *Nm* values should be taken only as indicative, because the underlying assumptions are too stringent and unlikely to be met in most natural situations (Bossart & Prowell 1998). The mean values of *Nm* within the yellow–grey and orange clades are 3.16 and 1.05, respectively. They are lower than the gene flow estimates (again indirect) for solitary ascidians, with higher dispersal capabilities than colonial forms (Svane & Young 1989): gene flow for *Pyura gibbosa* $Nm = 125$ over 215 km of coastline (Ayre *et al*. 1997). Our estimates are, on the other hand, comparable to gene flow values among populations of other compound ascidians (*Stolonica australis*, $Nm = 1$ over a distance of 90 km and *Botrylloides magnicoecum*, $Nm = 1$ over 150 km, Ayre *et al*. 1997).

In the work conducted by Tarjuelo *et al*. (2001), gene flow estimates were obtained for the colonial ascidian *Clavelina lepadiformis* for the same gene, and in the same stretch of coast as the present study, thus allowing for direct comparisons. When we look at the gene flow between sympatric populations of both species (in our case they would be Cadaqués and Blanes) the value is six to seven times greater in *C. lepadiformis* ($Nm = 4.54$) than in *P. crucigaster* ($Nm = 0.70$). The results correlate well with the life history of both species. *C. lepadiformis* is a compound ascidian with a relatively high fecundity among colonial forms (66 larvae/zooid, Tarjuelo 2001; Tarjuelo & Turon 2004) and has small and undifferentiated larvae with a larval lifespan longer than than *P. crucigaster* (they survive for some hours to days in the laboratory before settling: personal observation). Dispersal capabilities in *C. lepadiformis* are therefore potentially greater than in *P. crucigaster* and isolation among populations of the latter is greater than among *C. lepadiformis* populations.

Genetic structure of *P. crucigaster* in the area studied is complex. A past fragmentation event seems the most probable explanation for the existence of two separate clades corresponding to the yellow–grey and the orange morphotypes. The strong genetic isolation found between these clades, which couple with their ecological differentiation, supports our contention that these are indeed distinct species. A pattern of highly structured populations within clades, even at short spatial scales, has been found, indicating poor dispersal capabilities and strong phylopatry in these forms. Broadening the sampling with more localities and nonmitochondrial genes is necessary before establishing the role of dispersal and selection in the shaping of the present-day distribution of these clades. As our case study indicates, genetic studies are a valuable tool to understand the relationships between geographical distribution, population structure and life-cycle parameters in speciation studies.

Our results demonstrate how an analytical framework can be used effectively to test species boundaries, not only in terms of genetic differentiation but also in terms of ecological differentiation, and the correlation of these

differences. In our study, we have shown that colour morphology partitions well with genetic differentiation rejecting the null hypothesis of a single species using the Cohesion Species Concept. Our results suggest further that natural selection may have played a significant role in this speciation event. Future studies will attempt to develop molecular markers associated with the colour differences and experimental studies aimed at determining the evolutionary significance of these colour differences.

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This study was part of Isabel Tarjuelo's PhD research project on reproductive strategies and dispersal of colonial ascidians. Marta Pascual's current research focuses on the study, with molecular markers, of species that have recently invaded new habitats. David Posada is interested in statistical phylogeography and population genetics. Keith Crandall has a general interest in addressing questions in speciation and conservation genetics, and in testing population genetic methodology through computer simulation and comparing methodological approaches using both simulation and empirical data. Xavier Turon works on ascidian taxonomy and on biology of benthic invertebrates, including chemical ecology, reproductive biology and population genetics.
