How do morphotypes and chemotypes relate to genotypes? The colonial ascidian *Cystodytes* (Polycitoridae)

Susanna López-Legentil & Xavier Turon

Accepted: 20 February 2004

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.

Intraspecies variability is widespread in marine invertebrates. Size, colour, texture, general shape and secondary chemistry can differ quite drastically from one individual to another. *Cystodytes dellechiajei* (Polycitoridae) is a cosmopolitan colonial ascidian with several morphotypes, most of which differ in colour and spicular composition. New molecular tools enable us to assess the taxonomic status of these morphotypes. To determine whether variation observed in Mediterranean *Cystodytes* has a genetic basis, we sequenced 45 specimens from eight locations of the western Mediterranean and one from Mayotte (Indian Ocean), and obtained a 617 bp fragment of the mitochondrial gene COI. Fifteen different colour morphs were recorded and four kinds of spicules were found: disk-shaped, sphere-shaped, star-shaped and discoidal, thick spicules with a toothed margin. Zooid morphology was remarkably uniform in the whole sample set. Different tree construction methods (distance-based, parsimony-based, and maximum-likelihood-based) yielded consistent results, and recognized six major clades, which had no correspondence with spicule shape and were only partially consistent with colour morphs. Results are discussed in the light of previous knowledge of the chemistry of blue, green, brown and purple colour morphs. In spite of the different colour patterns and spicular variability we concluded, on the basis of chemical and genetic data, that the morphological traits analysed were not consistent enough to be used to differentiate between *Cystodytes* species. We point out the importance of genetics and chemistry in assessing the taxonomic status of species with variable morphology.

Susanna López-Legentil & Xavier Turon, Department of Animal Biology (Invertebrates), Faculty of Biology, University of Barcelona, 645, Diagonal Ave. 08028 Barcelona, Spain. E-mail: slopez@bio.ub.es

Susanna López-Legentil, Laboratoire Chimie des Biomolécules et de l'Environnement (Centre de Phytopharmacie), University of Perpignan, 52, Paul Alduy Ave. 66860 Perpignan, France

Introduction

Intraspecies variability in benthic invertebrates has been a long-standing source of taxonomic and biological controversy. Species with a large range of distribution may show morphological variants, usually related to their geographical or bathymetric distribution. Thus, some degree of morphological differentiation within widely distributed or cosmopolitan species is to be expected. Colour variation is one of the most frequently reported (e.g. Aron & Solé-Cava 1991; Dalby 1997), but texture, general shape and other morphological characters may also change from one organism or one place to another, sometimes in response to specific environmental conditions (e.g. bryozoans, Harvell 1990). New molecular tools enable us to assess the genetic basis of such morphological changes (e.g. Miller *et al*. 2001; Howell *et al*. 2004; Mackenzie *et al*. 2004). Since the advent of molecular techniques, many cases of cryptic species have been uncovered in marine invertebrates (reviewed in Knowlton 2000). The correct assignment of species in benthic invertebrates is not merely a taxonomic issue, but has clear implications in applied aspects such as biodiversity management, tracing of invasions or determining sources of new pharmacologically active substances (Holland 2000; Sweijd *et al*. 2000; Féral 2002).

Ascidians, like other marine taxa, show some cases of high intraspecies variability, generally related to cosmopolitan species (e.g. *Botryllus schlosseri*, *Cystodytes dellechiajei*, *Ciona intestinalis*, Hoshino & Nishikawa 1985; Kott 1990; Monniot *et al*. 2001; Stoner *et al*. 2002) which in fact may be instances of speciation events. Aron & Solé-Cava (1991) found a genetic differentiation between two varieties of the Brazilian *Botryllus niger*. Dalby (1997, 2000) concluded that two Australian morphs of *Pyura stolonifera* were reproductively isolated. More recently,

Tarjuelo *et al*. (2001) studying the genetic structure of the ascidian *Clavelina lepadiformis* living inside and outside harbours suggested the existence of cryptic species.

We chose the genus *Cystodytes* (Polycitoridae) as a case study to assess the significance of morphological variation. Species of this genus, of which there are at least 24 (Sanamyan 2002), are found all over the world, including the Antarctic (Van Name 1945; Millar 1968; Monniot & Monniot 1974). Of these, the best known is undoubtedly *C. dellechiajei* Della Valle, 1877, a colonial soft-bodied ascidian widely distributed in tropical and temperate waters.

The taxonomic characters used in the assignment of species within this genus are unstable and largely influenced by authors' particular views, a problem derived from the morphological uniformity of the zooids and from their great contractility, which renders many characters difficult to observe (Van Name 1945; Monniot 1988; Kott 1990). On the other hand, colour and colony size and shape show an amazing degree of variability (e.g. Harant 1929; Kott 1990; Monniot *et al*. 2001). Therefore, authors either tended to accept a restricted number of species while recognizing the difficulty of finding trustworthy characters at the specific level (Van Name 1945; Ärnbäck-Christie-Linde 1950; Kott 1990) or tried, on the basis of careful morphological examination, to disentangle the specific status within the genus in local areas. For instance, Monniot (1988) described eight different species of *Cystodytes* in the lagoon of New Caledonia, seven of them new.

The assessment of spicular differences in ascidians is a reliable and common character used to differentiate between Didemnidae species (Lafargue & Laubier 1980), but its applicability to other families is dubious. Although Monniot (1970) concluded that spicular differences within the genus *Cystodytes* had no taxonomic value *per se*, they are still used in descriptions of new species, especially when the spicular features are distinctive (e.g. *C. ramosus* described by Kott 1992). Thus, the present specific diversity of the genus *Cystodytes* is largely a result of species assignment on the basis of colour, shape, zooid morphology and spicular composition, on which there is no general agreement. Some of the observed morphological differences may also be due to intraspecies variability, as a result of genetic diversity or as a consequence of habitat, diet, chemistry or some combination thereof. Intraspecies variability in *Cystodytes* is poorly understood; thus, molecular tools can undoubtedly cast some light.

Cystodytes dellechiajei is distributed around the world in both tropical and temperate littoral waters. Its distribution is also eurybathic, as some samples have been collected at a depth of 735 m (Monniot 1974). Colour, texture, spicular composition, shape and zooid size may vary without any clear pattern of distribution (Turon 1987; Méliane 2002). As so many forms have been attributed to this taxon, it might well be a group of species (Monniot 1988). All Mediterranean specimens of the genus have been traditionally included in the species *C. dellechiajei* (Pérès 1958; Turon 1987; Brunetti 1994), but there may be other species as well (Brunetti 1994; Méliane 2002).

We used two molecular approaches to analyse variation in Mediterranean *Cystodytes*: genetics and secondary chemistry. Genetic data can provide information about clade relatedness, patterns of evolution and indirect estimates of gene flow. Among the panoply of genetic tools used in ascidians, some are more informative at the phylogenetic level, e.g. 18 s rDNA sequences (Swalla *et al*. 2000; Stach & Turbeville 2002), while others, such as allozymes (Aron & Solé-Cava 1991; Yund & O'Neil 2000; Dalby 2000) or microsatellites (Stoner *et al*. 1997, 2002) have been used at the population level. Mitochondrial sequence data are a bridge between these two approaches (Avise *et al*. 1987). Analysis of mtDNA sequence data has advantages, such as maternal inheritance without recombination, higher mutational rate, shorter coalescence times and more sensitivity than nuclear genes in tracing population subdivision over large geographical scales (Avise *et al*. 1987; Palumbi *et al*. 2001). MtDNA is commonly used in intraspecific phylogeographical studies (Avise 2000) and has been applied in ascidians to address cryptic speciation problems (Tarjuelo *et al*. 2001; Turon *et al*. 2003).

Chemotaxonomy can also be used to assess inter- or intra-species variability (Miller *et al*. 2001). López-Legentil (2003) studied the major alkaloids present in four *C. dellechiajei* colour morphs (blue, green, brown and purple) from the north-western Mediterranean. Two major chemotypes were found. The first had sulphur-containing pyridoacridines, corresponding to the purple morph. The second had C_9 unsubstituted pyridoacridines, found in the blue and green forms. No major alkaloid was found in the brown morph.

The aim of the present study was to determine how variability in traditional taxonomic characters such as colour and spicular complement, relates to genetics and secondary chemistry in several *Cystodytes* morphotypes from the western Mediterranean. We examined the morphology and spicular composition of 46 colonies of different colours and/or localities. A fragment of 617 pb of the mitochondrial gene COI was sequenced to define the haplotypes and to perform phylogenetic analyses.

Materials and methods

Ascidian samples

For morphological and genetic analyses, 45 colonies of *C. dellechiajei* were collected from eight Mediterranean sites: North Catalonia (NE of Iberian peninsula), Cabo de Gata, Cabo de Palos and Alborán Island (Southern Iberian coast), Mallorca-Menorca and Ibiza-Formentera (Balearic Islands),

Table 1 List of haplotypes found, with sampling location, colour, major alkaloid known and spicular shape. GenBank accession numbers are also indicated.

Haplotype	Location	Colour	Major alkaloid known	Spicular shape	GenBank #
S1	Ibiza-Formentera	Brown	—	Disk	AY523042
S2	Catalonia	Purple	Shermilamine B	Disk + Sphere	AY523043
S3	Catalonia	White	—	Disk	AY523044
S4	Mallorca-Menorca	Green	—	Disk + Sphere	AY523045
S5	Ibiza-Formentera	Blue	Ascididemin	Disk	AY523046
S6	Catalonia	Blue	Ascididemin	Disk	AY523047
	Alborán I.	Blue	—	Disk	
	Alborán I.	White-grey	—	Disk	
S7	Catalonia	White, brown circles	—	Disk	AY523048
S8	Cabo de Gata	Green	Hydroxyascididemin	Disk + Sphere	AY523049
	Sicily	Pink	—	Disk + Star	
S9	Tunisia	Brown-Green	—	Disk + Sphere	AY523050
S10	Tunisia	Orange	—	Disk + Star	AY523051
	Catalonia	Purple	Shermilamine B	Disk + Sphere	
S11	Tunisia	Green, green circles	Hydroxyascididemin	Disk + Sphere	AY523052
S12	Tunisia	White, green circles	Hydroxyascididemin	Disk + Sphere	AY523053
S13	Tunisia	White, brown circles	—	Disk	AY523054
	Sicily	White, red circles	—	Disk	
S14	Sicily	Blue	—	Disk + Sphere	AY523055
	Sicily	White	—	Disk	
	Mallorca-Menorca	Blue	—	Disk	
	Mallorca-Menorca	Green	—	Disk + Sphere	
S15	Sicily	Yellow	—	Disk + Sphere	AY523056
S16	Catalonia	Green	—	Disk + Sphere	AY523057
	Mallorca-Menorca	Green	—	Disk + Sphere	
S17	Cabo de Palos	Green	—	Disk + Sphere	AY523058
S18	Mallorca-Menorca	Brown	—	Disk	AY523060
S19	Mallorca-Menorca	Brown	—	Disk	AY523061
S20	Catalonia	Purple	Shermilamine B	Disk + Sphere	AY523062
S21	Catalonia	Purple	Shermilamine B	Disk + Sphere	AY523063
S22	Catalonia	Purple	Shermilamine B	Disk + Sphere	AY523064
S23	Mallorca-Menorca	Green	—	Disk + Sphere	AY523065
S24	Catalonia	White, brown circles	—	Disk	AY523066
	Catalonia	White	—	Disk	
S25	Mallorca-Menorca	Blue	—	Disk	AY523067
	Mallorca-Menorca	Green	—	Disk + Sphere	
S26	Mallorca-Menorca	Blue	—	Disk	AY523068
S27	Mallorca-Menorca	Green	Hydroxyascididemin	Disk + Sphere	AY523069
S28	Ibiza-Formentera	Blue	Ascididemin	Disk	AY523070
S29	Ibiza-Formentera	Blue	Ascididemin	Disk	AY523071
S30	Ibiza-Formentera	Green	Hydroxyascididemin	Disk + Sphere	AY523072
S31	Ibiza-Formentera	Green	Hydroxyascididemin	Disk + Sphere	AY523073
S32	Cabo de Gata	Green	Hydroxyascididemin	Disk + Sphere	AY523074
S33	Cabo de Gata	Green	Hydroxyascididemin	Disk + Sphere	AY523075
S34	Mallorca-Menorca	Green	—	Disk + Sphere	AY523076
S35	Mayotte	Black	—	Disk + Toothed	AY523059

Tunisia and Sicily (Table 1, Fig. 1). A single black colony from Mayotte (Indian Ocean) was used for comparison. Samples were collected by SCUBA diving in 2001 and 2002. The original colour of the colony was recorded before fixation in absolute ethanol, and general colony and zooid morphology were examined in the fixed material. Some of the samples were anaesthetized by cold exposure as described elsewhere (Turon 1987) and fixed in formaldehyde for examination of zooids in a relaxed state.

Scanning Electron Microscopy (SEM)

To obtain the calcareous spicules, small pieces of the tunic were removed and boiled in commercial bleach for several minutes until complete dissolution of the tissue. They were

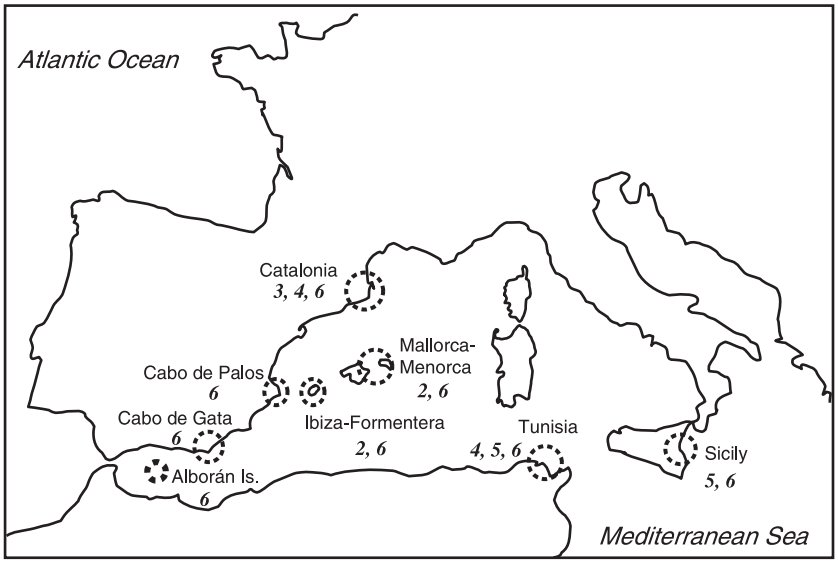

Fig. 1 Map showing the Mediterranean zones sampled: Catalonia, Cabo de Palos, Cabo de Gata, Alborán Island, Ibiza-Formentera, Mallorca-Menorca, Tunisia and Sicily. Numbers refer to the presence of the corresponding clade (code of clades as in Figure 5) in each area.

washed several times in water to remove organic remains and dehydrated in absolute ethanol. Randomly pipetted samples were deposited on a stub covered with bi-adhesive tape, sputter-coated with gold, and studied under a Hitachi H1200 SEM.

DNA extraction and sequencing

To maximize DNA extractions, we separated the zooids from the tunic and their spicular capsules by forceps under a binocular microscope. The zooids were then kept in absolute ethanol at –25 °C and later processed. Mitochondrial DNA was extracted using the Quiamp Mini Kit (Quiagen). Sequences were obtained for a segment of the Cytochrome *c* Oxidase subunit I (COI) mitochondrial gene. We used the universal primers HCO2198 and LCO1490 described in Folmer *et al.* (1994) to amplify the purple and brown forms and also to design more specific primers (AvA & AvB) for the blue and green morphs. The sequences of AvA (forward) and AvB (reverse) are 5′TTGGAATATGGTCCGCATTA3′ and 5′ATGGCTGCAGCTAAAACTGG3′, respectively.

Amplification was performed in a 20 µL total-reaction volume with 0.4 µL of each primer (25 µM), 0.5 µL dNTP's (10 mM), 2 µL 10× buffer containing 15 mM $MgCl_2$ (Promega), 1 U Taq polymerase (Promega) and 1 µL DNA. When using UniA & UniB primers, a single soak at 94 °C for 2 min was followed by 35 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 at 72 °C for 7 min, on a Perkin Elmer 840 DNA amplifier. The use of AvA and AvB primers followed an initial denaturation step at 94 °C for 2 min and 35 cycles (94 °C for 35 s, 45 °C for 45 s and 72 °C for 1 min), with a final extension at 72° for 5 min, on a Perkin Elmer 9700 DNA amplifier.

The sequencing reaction was carried out using the same primers used for the amplification step and the ABI Big-Dye Ready-Reaction kit of Perkin Elmer. Sequences were obained on an ABI Prism 377XL automated sequencer.

Phylogenetic analysis

Sequences were aligned with SeqPup version 0.6 and alignments were confirmed by eye. All sequences have been deposited in the GenBank (accession numbers listed in Table 1). Tajima's D statistic was calculated with DnaSP version 3.51 (Rozas & Rozas 1999) in order to test whether mutations were selectively neutral or subject to selective pressures.

Relationships between haplotypes were established by using three methods: neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). We used the COI sequence of another aplousobranch species, *Aplidium fuscum* (F. Polyclinidae), obtained by the authors in previous research, as an outgroup for the phylogenetic analyses.

The NJ algorithm, using the absolute number of nucleotide differences, was performed with the computer program MEGA version 2.1 (Kumar *et al.* 2001). The robustness of the NJ tree was evaluated by bootstrap analysis (Felsenstein 1985) with 1000 replicates.

MP cladograms were obtained using PAUP (version 4.0, Swofford 1998). Because of the large number of taxa, heuristic searches were performed (with TBR option). To minimize the risk of being trapped in local optima, searches were replicated 100 times with random addition of taxa. Bootstrap analysis was performed with 1000 replicates (Felsenstein 1985).

We used Modeltest 3.0 (Posada & Crandall 1998) to select the best-fit model of nucleotide substitution for our data set. Using PAUP, a haplotype tree was constructed under the ML criterion with the parameter estimates obtained under

the best-fit model. A heuristic search was made with 100 replicates of random stepwise addition and tree bisection-reconnection (TBR) branch-swapping. Confidence in the nodes was assessed by 1000 bootstrap replicates of the above procedure but with fast-stepwise addition to keep computer time within reasonable bounds.

Results

In all, 15 different colours or colour combinations appeared in our sample set (Table 1). Their relative abundances varied greatly and, in most cases, only one or two colour varieties were present at a single locality, although in some places three or even four colour varieties were found, with overlapping ecological and bathymetric distribution.

Morphology of the zooids

We did not find any clearcut difference in the organization of the systems of zooids in the colonies or in the zooids themselves. However, detailed observation of zooids was only possible on the three main colour morphs collected off the Catalan coast (purple, blue and green), for which we obtained relaxed material and observed ripe individuals and larvae. These forms were remarkably similar; the general colony shape varied only in thickness (from 4 to 8 mm, approximately), which correlated with certain characters of the zooids such as total length and the number of stigmata per row, while the remaining zooid characters were uniform. A short description of the zooids follows:

Thorax length in relaxed individuals from 1.15 mm (thin colonies) to 2.8 mm (thick colonies). Abdomen length from 1.15 mm to 3 mm, respectively. Siphons 6-lobed, the atrial one highly variable in length. Tentacles from 13 to 17 in small zooids and up to 35 in larger zooids. Four rows of stigmata, from 13 to 27 per half row as a function of zooid size (Fig. 2A). The first row bends anteriorly near the dorsal midline. The thorax musculature is strong and continues as a wide band over the right side of the abdomen. The band bifurcates at the level of the stomach and ends in two short protrusions at the end of the abdomen, possibly anchoring the zooid into the tunic (Fig. 2B). Short stolonic vessels arise from the bifurcation point in some zooids. A skirt of tissue emerges dorsally from the oesophagial region; this skirt is only visible in specimens carefully removed from the tunic (Fig. 2C). Abdomen twisted in most zooids, with stomach in a dorsal position. Stomach smooth, joined to a mid-intestine and a rectum. Anus opens between the third and four rows of stigmata. Testis formed by 6–8 pear-shaped follicles arranged radially on the left side of the abdomen (Fig. 2D). Sperm-duct originates in the centre of the testis and runs straight anteriorly, except when oocytes are present, in which case the proximal part of the sperm-duct curves around them. Ovary slightly above the testis, formed, when mature, by one large ovum and one or two small oocytes. One larva can be brooded on the left side of the top of abdomen, where it protrudes with the dorsal side of the larva facing downwards (Fig. 2E,F). Occasionally a second embryo, much less developed, may be present under the first. Larvae, when mature, have a typical ring-shaped ectodermal expansion around the three adhesive papillae.

Morphology of the spicules

The genus is characterized by the presence of a capsule formed by layers of overlapping discoidal calcareous spicules, which encases the abdominal region of each zooid. When contracted, the branchial portion also retracts inside the spicular capsule. Small differences can be observed between these spicules, which vary from shield-shaped forms (with the centre somewhat pointed, sometimes with a small hole in it) to flat, disk-shaped ones (Fig. 3A). In some cases, these spicules are also randomly distributed along the tunic, apparently 'left behind' during budding (Lambert 1979).

Three additional spicule types were observed. The first were spherical (Fig. 3B), found either abundantly throughout the tunic or restricted to a few spicules concentrated in a portion of it, in which case they were easily overlooked. This variation in abundance showed no relationship with colour. Within this spicular type, two morphologies were found: the first had concentric needles and the second was quite compact (Fig. 3C). As both types were found together in the same colony, no morphological importance is accorded to this difference, which may be the result of the preparation method. Although the spherical spicules were generally smaller than the discoidal ones, some small discoidal spicules were also observed (*c.* 100 μm, Fig. 3D). Therefore, the flattened discoidal spicules do not seem to be the result of a two-dimensional growth of the spherical ones.

The second type, star-shaped spicules, was present in two specimens: one pink colony from Sicily (Fig. 3E) and an orange one from Tunisia. Finally, discoidal thick spicules with a toothed margin were found in the black morph from Mayotte (Fig. 3F).

Genetic analyses

Forty-six sequences were obtained for the COI mitochondrial gene with a final alignment length of 617 bp. No gap was needed. Thirty-five haplotypes were identified (labelled S01 to S35) with a total of 160 (26%) variable sites (Table 1). Nucleotide variation was scattered across the entire sequenced region. As most variation was restricted to the third-base position of the codons, 157 (98.12%) of the nucleotide substitutions yielded synonymous changes. Replacement changes occurred only three times: two transversions affecting haplotypes S05 and S18, and one transition present in 14 of the haplotypes. Tajima's D was not significant ($D = 0.264$, $P > 0.10$)

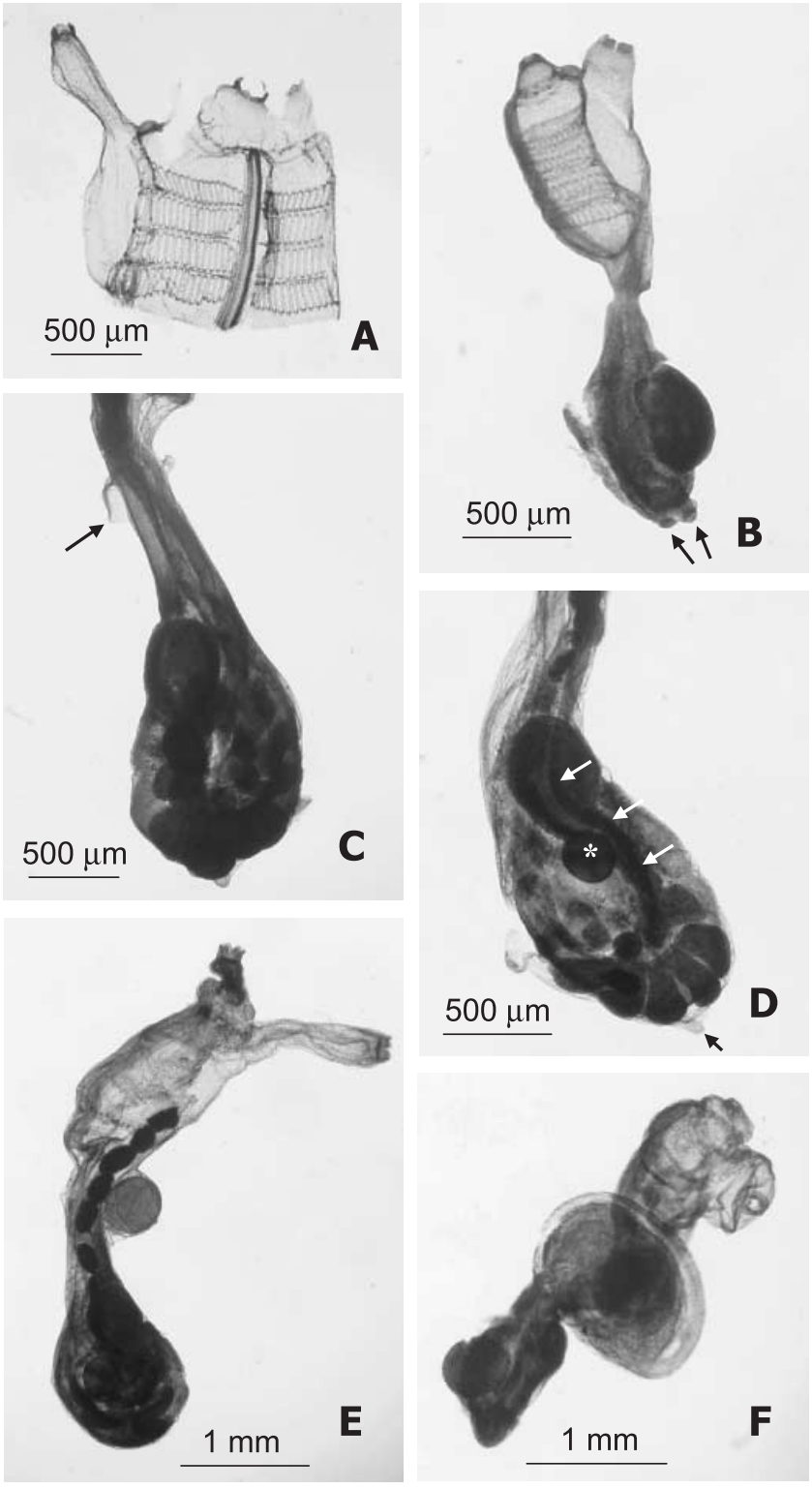

Fig. 2 A–F. Light micrographs of *C. dellechiajei*. Specimens from Catalonia. —A. Green form, dissected branchial sac showing the four rows of stigmata. —B. Green form, juvenile zooid. The arrows point to the protruding end of the muscular processes on the right side of the body. —C. Purple form, abdomen of a zooid with male gonads, showing the characteristic dorsal skirt in the esophagial region (arrow). —D. Blue form, abdomen of a mature zooid with testes and a developing oocyte (asterisk). White arrows mark the course of the sperm-duct. Black arrow points to end of muscular process. —E. Purple form, zooid brooding an embryo at a very early stage of development. —F. Green form, zooid brooding an almost fully developed larva.

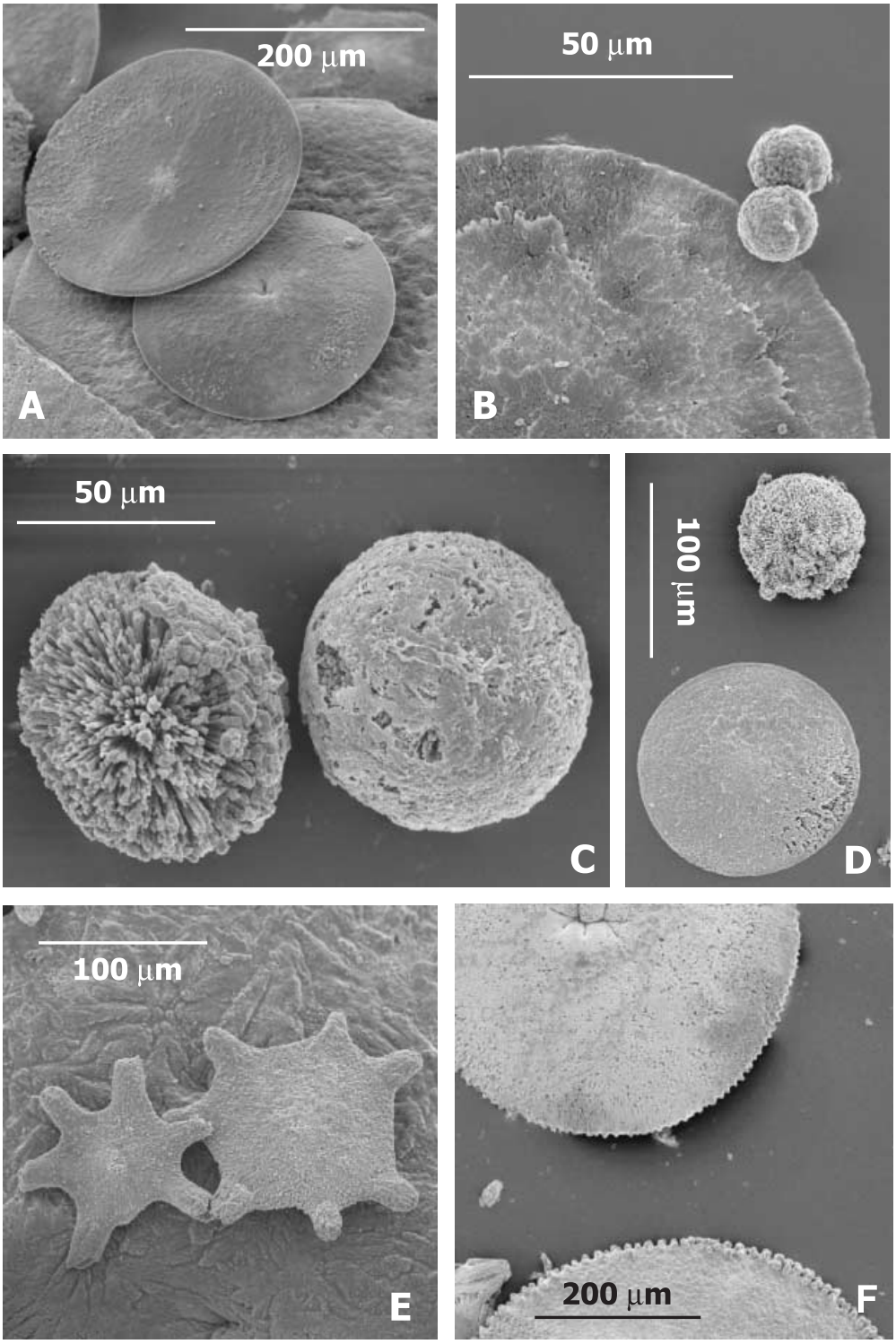

Fig. 3 A–F. SEM observations of *Cystodytes* spicules. —A. Blue specimen from Catalonia. Disk-shaped spicules characteristic of the genus. —B. Purple specimen from Catalonia. Two sphere-shaped spicules along with a disk-shaped one. —C. purple specimen from Catalonia. Two different morphologies of sphere-shaped spicules: one constituted of concentric needles and the other compact. —D. Green specimen from Catalonia. A disk-shaped spicule of small size and a sphere-shaped spicule. —E. Yellow specimen from Sicily. Star-shaped spicules. —F. Black specimen from Mayotte. Discoidal spicules with a toothed margin, typical of *C. aucklandicus*.

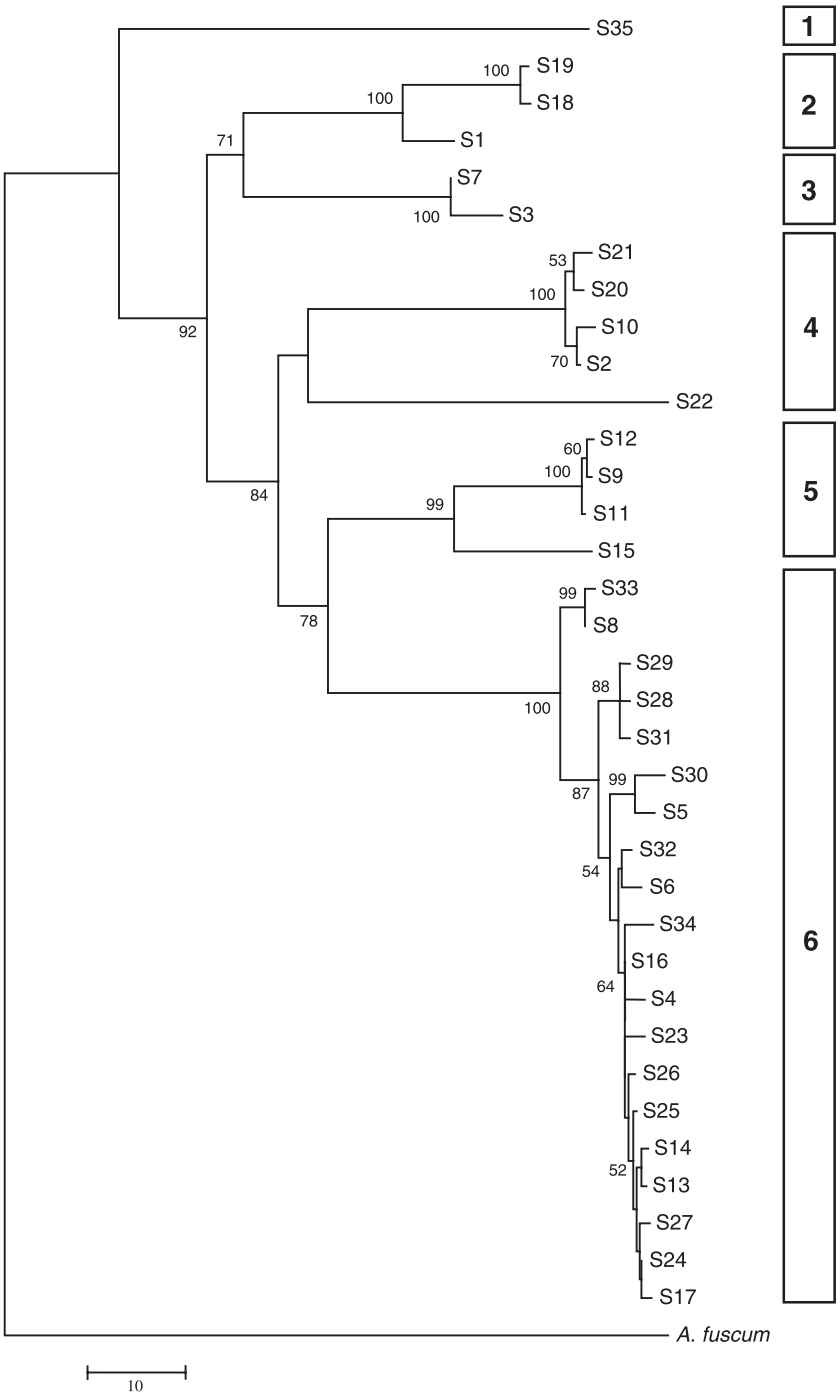

Fig. 4 Haplotypic neighbour-joining tree. Bootstrap values are shown for the branches with more than 50% support. The six recognized clades are signalled. Haplotype codes as in Table 1. Scale in distance units.

for our total data set, showing no evidence for selection in this gene and indicating that a neutral model cannot be rejected.

For the phylogenetic analyses we used the 35 haplotypes obtained (Table 1) with the sequence of *A. fuscum* as outgroup. The three different tree-construction methods used: neighbour-joining, maximum parsimony and maximum likelihood gave similar results. In Fig. 4 the NJ cladogram is shown, with the support values obtained from bootstrap. The topology of MP and ML trees was essentially the same, so only the former is shown in Fig. 5, which depicts the majority-rule consensus MP tree, as more than 60 best trees were obtained. Bootstrap support values from the MP and ML analyses are also shown in Fig. 5. The comparisons between the different likelihood scores for each model of evolution showed that the

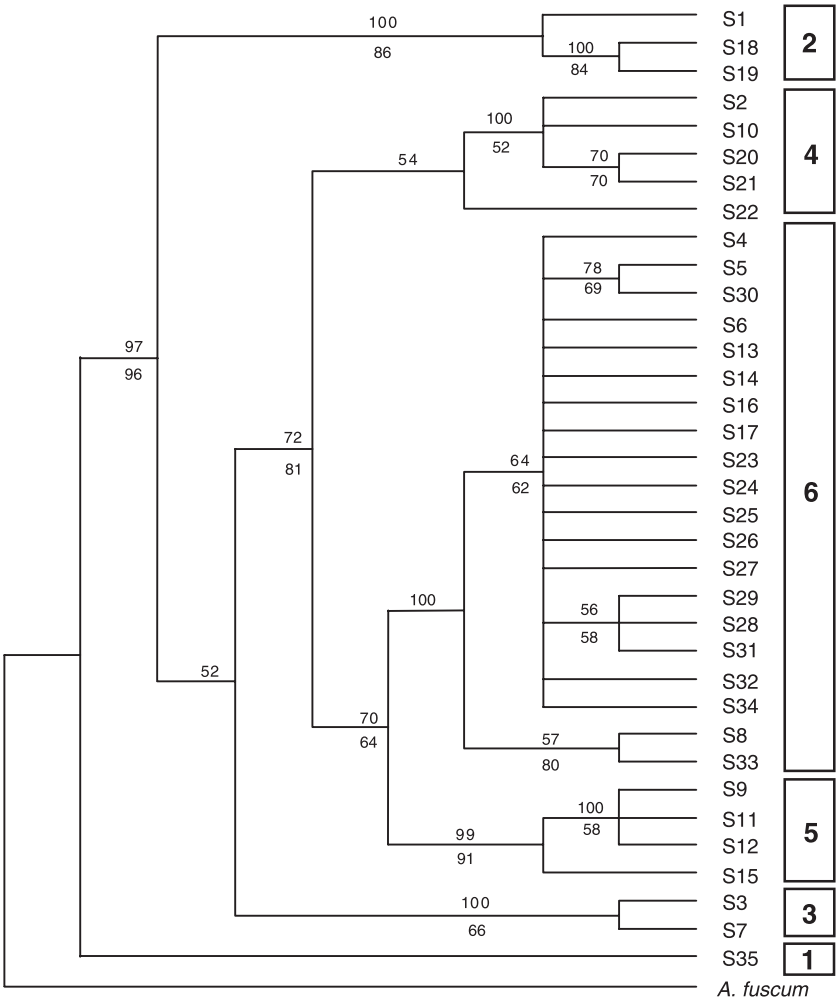

Fig. 5 Majority-rule consensus tree of the maximum-parsimony analysis. Bootstrap values are shown above the branches. Below the branches is indicated the support (if higher than 50%) obtained in the maximum likelihood analysis. The six recognized clades are signalled. Haplotype codes as in Table 1.

K81uf + I model (K81 of Kimura 1981; but with unequal base frequencies) was the best-fit model for our data. It incorporates a substitution model and a proportion of invariable sites (I = 0.678).

Six major clades appeared in all trees (indicated in Figs 4 and 5), differing only in the position of clade 3 (that clusters with clade 2 in the NJ tree and with clades 4, 5 and 6 in the MP and ML trees). The first clade (labelled 1 in the Figures) is formed by the single sequence obtained from the Mayotte black form (S35) and stands in all analyses as outgroup to the remaining *Cystodytes* sequences. The second clade (2) groups three brown morphs from the Balearic Islands (with haplotypes S1, S18, S19), while the third (3) has only two haplotypes (S3 & S7), both white forms from Catalonia. Clade 4 contains the haplotypes from all the purple forms from Catalonia and one orange morph from Tunisia. It can be noted than in the ML tree the haplotype S22 stands outside this clade, as the sister group to the rest of clade 4 plus clades 5 and 6. Clade 5 is composed of haplotypes from three morphs from Tunisia featuring either green circles or some green pigmentation and one yellow morph from Sicily (S15). Finally, clade 6 contains haplotypes coming from a mixture of different forms, colours and localities but is mainly dominated by green and blue morphs.

Discussion

Morphological and genetic differences were found for *Cystodytes* inhabiting the Mediterranean Sea. Zooids, however, appear quite similar in most morphological characters. In particular, the muscle arrangement with two bands on the right side is the same as that found by Brunetti (1994) in Mediterranean specimens, but different from that found (one band at each side) in other areas (e.g. Van Name 1945; Kott 1990).

The mitochondrial gene studied is highly variable, with 35 haplotypes in 46 individuals. The different phylogenetic analyses reveal the same basic genetic structure, from which we determined the existence of six clades. The biggest genetic differentiation was found with the black morph from Mayotte,

the only non-Mediterranean specimen analysed. This single colony also had a specific kind of spicule (thick, disk-shaped, with a toothed margin), originally described in *C. aucklandicus* Nott, 1892, and later found several times by Monniot (1988) and Monniot & Monniot (1996, 2001). Our data therefore support the validity of this species, to which our specimen is assigned. However, the black colour of our sample seems an unreliable character, as Monniot (1988) and Monniot & Monniot (1996) found grey and brown *C. aucklandicus*, and many black *C. dellechiajei* were described by Brewin (1948).

No clear pattern of association between the various morphological parameters analysed (colour, spicular complement) or with any geographical area could be substantiated. Colours vary even between neighbouring colonies at the same site. Blue, green, white and brown morphs may be found together at the same locality (e.g. Blanes, northern Catalonia), and have several kinds of spicular composition. Conversely, the same spicular type can be found in several chromatic varieties (Table 1).

In addition, no relationship was found between spicule morphology and genetic clades. For instance, sphere-shaped spicules, which may be indicative of the species *C. philippinensis* Herdmann, 1886 (Kott 1990, 2002), were found in individuals from three different clades (4, 5 and 6). The two colonies with star-shaped spicules were found in two separate clades (4 and 6), and the colonies with only discoidal spicules, which are the spicular complement of the typical *C. dellechiajei* (see Kott 1990), appeared in clades 2, 3 and 6.

Colour varieties may have a sounder genetic basis, although there are many exceptions. The main regularities observed are: the purple morph appears in our phylogram as a distinct group (clade 4, albeit this group includes also an orange form), while green and blue morphs constitute most of clade 6 and all the brown colonies from the Balearic Islands form clade 2. The lack of consistent correspondence between colours and genetic clades may simply reflect the fact that mtDNA can be carried across chromatic varieties as a result of hybridization, thereby suggesting that we are dealing with a single species. In another colonial ascidian, *Botryllus schlosseri*, polychromatism is the result of intraspecies variability controlled by a few nuclear *loci* (Sabbadin 1982; Yund & O'Neil 2000).

López-Legentil (2003) points out two major chemotypes within north-western Mediterranean *C. dellechiajei*. A genetic basis for these two chemotypes seems substantiated. The purple morph (most of clade 4) was characterized by the presence of sulphur-containing pyridoacridines: shermilamine B (Carroll *et al*. 1989) and kuanoniamine D (Carroll & Scheuer 1990). The blue and green morphs (most of clade 6) presented the C_9 unsubstituted pyridoacridines: ascididemin (Kobayashi *et al*. 1988) and 11-hydroxyascididemin (Schmitz *et al*. 1991), respectively.

Our results show that groupings established from spicular composition or colour alone are not supported by genetic or chemical information. Thus neither spicular composition nor colour by itself is a sufficient criterion to discern between *Cystodytes* species. Some caution is necessary as we have only considered one mitochondrial gene and the study of others, especially nuclear genes, may yield different results. However, the COI gene seems well suited for discriminating between closely related species (Hebert *et al*. 2003).

Taken together, our results indicate that, if any distinction is to be made, it could be on the basis of the concordance of some genotypes with chemotypes that, in some cases, correlate with the colour morph. For instance, specimens of the purple morph clustered together in the genetic analysis (clade 4) and feature sulphur-containing pyridoacridines. The association between secondary chemistry and colour is not surprising in this case, as shermilamine B, the major alkaloid present in this form, is a purple compound (Banaigs & Bontemps-Subielos, pers. comm.). However, we do not know the chemistry of all the varieties of *Cystodytes* studied; all other characters are interspersed in our phylogram and there are no clear differences in zooid morphology. For the time being, therefore, the taxonomic status of the Mediterranean varieties of *Cystodytes* cannot be definitely assessed. The study of the genetic structure of selected populations of Mediterranean *Cystodytes*, coupled with knowledge of their reproductive cycles and biological parameters, could provide valuable information about gene flow between populations and their degree of reproductive isolation.

Acknowledgements

We thank Miguel Pozo and Emma Cebrian for providing samples from Sicily, Alborán and Menorca. Imene Méliane provided the Tunisian samples and valuable comments. Drs Isabel Tarjuelo and Sandra Duran helped with the sequencing work and phylogenetic analyses. Drs Bernard Banaigs and Nataly Bontemps-Subielos gave helpful comments on the secondary chemistry. The Scientific and Technical Services of the University of Barcelona provided automatic sequencer and SEM facilities. This project was funded by project REN2001-2312 of the Spanish Government and by the Interreg IIIA program of the EU. Pharmamar S.A. (Madrid) sponsored a collecting trip to the Balearic Islands.

References

Ärnbäck-Christie-Linde, A. (1950). *Ascidiacea. Part II. Further Zoological Results of the Swedish Antarctic Expedition, 1901–1903*, *4*, 26–28.

Aron, S. & Solé-Cava, A. M. (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, J. C. (2000). *Phylogeography: the History and Formation of Species*. Cambridge: Harvard University Press.

Avise, J. C., Arnold, J., Ball, R. M. Jr, Bermingham, E., Lamb, T. & Neigel, T. (1987). Intra specific phylogeography: the mitochondrial DNA bridge between populations genetics and systematics. *Annual Review of Ecology and Systematics*, *18*, 489–522.

Brewin, B. I. (1948). Ascidians of the Hauraki Gulf. Part I. *Transactions of the Royal Society of New Zealand*, 77, 115–138.

Brunetti, R. (1994). Ascidians of the northern Adriatic Sea. Aplousobranchia I. *Bollettino Zoologico*, *61*, 89–96.

Carroll, A. R., Cooray, N. M., Poiner, A. & Scheuer, P. J. (1989). A second shermilamine alkaloid from a tunicate *Trididemnum* sp. *Journal of Organic Chemistry*, *54*, 4231–4232.

Carroll, A. R. & Scheuer, P. J. (1990). Kuanoniamines A, B, C and D: Pentacyclic alkaloids from a tunicate and its prosobranch mollusc predator *Chelynotus semperi*. *Journal of Organic Chemistry*, *55*, 4426–4431.

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

Dalby, J. E. Jr (2000). Reproductive and electrophoretic evidence for genetic maintenance of dimorphism in the ascidians *Pyura stolonifera* near Melbourne, Australia. *Ophelia*, *47*, 227–243.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783–791.

Féral, J. P. (2002). How useful are the genetic markers in attempts to understand and manage marine biodiversity? *Journal of Experimental Marine Biology and Ecology*, *268*, 121–145.

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.

Harant, H. (1929). *Ascidies provenant des croisières du Prince Albert 1er de Monaco*. Monaco: Résultats des Campagnes Scientifiques accomplies sur son yacht par Albert 1er, Fascicule LXXV.

Harvell, C. D. (1990). The ecology and evolution of inducible defenses. *Ecology*, *65*, 323–340.

Hebert, P. D. N., Ratnasingham, S. & deWaard, J. R. (2003). Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society, London*, *270 (Suppl.)*, S96–S99.

Holland, B. S. (2000). Genetics of marine invasions. *Hydrobiologia*, *420*, 63–71.

Hoshino, Z. & Nishikawa, T. (1985). Taxonomic studies of *Ciona intestinalis* (L.) and its allies. *Publications of the Seto Marine Biology Laboratory*, *30*, 61–79.

Howell, K. L., Rogers, A. D., Tyler, P. A. & Billett, D. S. M. (2004). Reproductive isolation among morphotypes of the Atlantic seastar species. *Zoroaster Fulgens* (Asteroidea: Echinodermata). Marine Biology, *144*, 977–984.

Kimura, M. (1981). Estimation of evolutionary distances between homologous nucleotide sequences. *Proceedings of the National Academy of Sciences of the USA*, *78*, 454–458.

Knowlton, N. (2000). Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, *420*, 73–90.

Kobayashi, J., Cheng, J., Nakamura, H., Ohizumi, Y., Hirata, Y., Sasaki, T., Ohta, T. & Nozoe, S. (1988). Ascididemin, a novel pentacyclic aromatic alkaloid with potent antileukemic activity from the okinawan tunicate *Didemnun* sp. *Tetrahedron Letters*, *29*, 1177–1180.

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

Kott, P. (1992). The Australian ascidiacea (Suppl. 2). *Memoirs of the Queensland Museum*, *32*, 621–655.

Kott, P. (2002). Ascidiacea (Tunicata) from Darwin, Northern Territory, Australia. *Beagle, Records of the Museums and Art Galleries of the Northern Territory*, *18*, 19–55.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA 2—*Molecular Evolutionary Genetics Analysis*, Version 2.1. Tempe, Arizona: Arizona State University.

Lafargue, F. & Laubier, L. (1980). Lignée évolutive chez les Didemnidae des côtes de France. Valeur systématique des spicules. *Annales de l'Institut Océanographique*, *56*, 21–44.

Lambert, G. (1979). Early post-metamorphic growth, budding and spicule formation in the compound ascidian *Cystodytes lobatus*. *Biology Bulletin*, *157*, 464–477.

López-Legentil, S. (2003). *Assessment of chemotypes in the colonial ascidian cystodytes: Relationships with morphological characters and genetics*. MSc Thesis. University of Barcelona.

Mackenzie, J. B., Munday, P. L., Willis, B. L., Miller, D. J. & Van Oppen, M. J. H. (2004). Unexpected patterns of genetic structuring among locations but not colour morphs in *Acropora nasuta* (Cnidaria; Scleractinia). *Molecular Ecology*, *13*, 9–20.

Méliane, I. (2002). *Contribution to the Knowledge of the ascidian fauna in the south east of Tunisia*. MSc Thesis. University of Alicante.

Millar, R. H. (1968). Ascidians collected during 1928–30 by the Norwegian Antarctic expeditions. *Avhandlinger Utgitt av det Norske Videnskaps-Akademi I Oslo, Ny Serie*, *10*, 3–25.

Miller, K., Alvarez, B., Battershill, C., Northcote, P. & Parthasarathy, H. (2001). Genetic, morphological, and chemical divergence in the sponge genus *Latrunculia* (Porifera: Demospongiae) from New Zealand. *Marine Biology*, *139*, 235–250.

Monniot, F. (1970). Les spicules chez les tuniciers Aplousobranches. *Archives de Zoologie Experimentale and Générale*, *111*, 303–311.

Monniot, F. (1974). Ascidies littorales et bathyales récoltées au cours de la campagne Biaçores: Aplousobranches. *Bulletin du Muséum National d'Histoire Naturelle, 3e Série*, *251*, 1287–1325.

Monniot, F. (1988). Ascidies de Nouvelle-Calédonie V. Polycitoridae du lagon. *Bulletin du Museum d'Histoire Naturelle, 4e Série*, *10*, 197–235.

Monniot, C. & Monniot, F. (1974). Ascidies de la XXIIe expédition antarctique chilienne. *Boletín Sociedad Biológica de Concepción*, *48*, 365–383.

Monniot, C. & Monniot, F. (1996). New Collections of Ascidians from the Western Pacific and Southeastern Asia. *Micronesica*, *29*, 133–279.

Monniot, F. & Monniot, C. (2001). Ascidians from the tropical western Pacific. *Zoosystema*, *23*, 201–383.

Monniot, C., Monniot, F., Griffiths, C. L. & Schleyer, M. (2001). South African ascidians. *Annals of the South African Museum*, *108*, 1–141.

Palumbi, S. R., Cipriano, F. & Hare, M. P. (2001). Predicting nuclear gene coalescence from mitochondrial data: the three-times rule. *Evolution*, *55*, 859–868.

Pérès, J. M. (1958). Origine et affinités du peuplement en ascidies de la Méditerranée. *Rapports et Procés Verbaux des Réunions du Commité International Pour l'Exploration Scientifique de la Mer Méditerranéenne*, *14*, 493–502.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics*, *14*, 817–818.

Rozas, J. & Rozas, R. (1999). DnaSP, Version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, *15*, 174–175.

Sabbadin, A. (1982). Formal genetics of ascidians. *American Zoologist*, *22*, 765–773.

Sanamyan, K. (2002). ZooBase 2.20. Ascidians. Available via http://lithopssoft.com/zoo.

Schmitz, F. J., DeGuzman, F. S., Hossain, M. B. & Van der Helm, D. (1991). Cytotoxic aromatic alkaloids fom the ascidian *Amphicarpa meridiana* and *Leptoclinides* sp. Meridine and 11-Hydroxyascididemin. *Journal of Organic Chemistry*, *56*, 804–808.

Stach, T. & Turbeville, J. M. (2002). Phylogeny of tunicata inferred from molecular and morphological characters. *Molecular Phylogenetics and Evolution*, *25*, 408–428.

Stoner, D. S., Ben-Shlomo, R., Rinkevich, B. & Weissman, I. L. (2002). Genetic variability of *Botryllys schlosseri* invasions to the east and west coasts of the USA. *Marine Ecology Progress Series*, *243*, 93–100.

Stoner, D. S., Quattro, J. M. & Weissman, I. L. (1997). Highly polymorphic microsatellite loci in the colonial ascidian *Botryllus schlosseri*. *Molecular Marine Biology and Biotechnology*, *6*, 163–171.

Swalla, B. J., Cameron, C. B., Corley, L. S. & Garey, J. R. (2000). Urochordates are monophyletic within the deuterostomes. *Systematic Biology*, *49 (1)*, 52–64.

Sweijd, N. A., Bowie, R. C. K., Evans, B. S. & Lopata, A. L. (2000). Molecular genetics and the management and conservation of marine organisms. *Hydrobiologia*, *420*, 153–164.

Swofford, D. L. (1998). *PAUP*. Phylogenetic Analysis Using Parsimony and Other Methods*, Version 4. [Computer software and manual]. Champaign, Illinois: Illinois Natural History Survey.

Tarjuelo, I., Posada, D., Crandall, K. A., 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.

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.

Van Name, W. G. (1945). The North and South American ascidians. *Bulletin of the American Museum of Natural History*, *84*, 1–463.

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