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Short communication

Lack of genetic variation in mtDNA sequences over the amphiatlantic distribution range of the ascidian *Ecteinascidia turbinata*

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1. Introduction

The colonial ascidian *Ecteinascidia turbinata* Herdman 1880 (Phlebobranchiata; Perophoridae) is a widespread tunicate found in tropical and subtropical waters on both sides of the Atlantic and in the Mediterranean Sea (Berrill, 1932). This species has often been reported to foul floats and other human-made structures (e.g. WHOI, 1952). Intercontinental dispersion of this species is attributable to maritime transport as the larvae have short planktonic life-spans (Bingham and Young, 1991) and colonies can attach to ship hulls (Calder et al., 1966). Although introductions of benthic invertebrates may originate in marginal marine habitats (such as marinas), they can eventually spread to open habitats (Turon et al., 2007). In addition, this species has recently elicited interest because one of its secondary metabolites, Ecteinascidin-743, displayed remarkable antitumor activities in *in vitro* and *in vivo* models (Rinehart et al., 1990; Sakai et al., 1992; Valoti et al., 1998; Izbicka et al., 1998). In order to obtain the amount required for clinical trials, aquaculture of *E. turbinata* was intensively developed in Formentera (Balearic Islands, Spain) by Pharmamar S. A. (Spain) and in Long Key (Florida, USA) by CalBioMarine Technologies (Carlsbad, CA) (Carballo et al., 1997; Pomponi, 1999).

Analysis of mtDNA sequence data has proven to be a useful tool for tracing recent evolutionary history, such as founder events, population bottlenecks and phylogeography in marine organisms (Gopurenko et al., 1999; King et al., 1999; Wilke and Davis, 2000). The mitochondrial gene cytochrome *c* oxidase subunit I (COI) has been a molecule of choice to conduct intra-specific phylogeographic studies due to its high degree of variability (e.g. Avise, 2000; Hamm and Burton, 2000; Castilla et al., 2002; Duran et al., 2004b; Reuschel and Schubart, 2005). Among marine invertebrates, the only prominent examples of almost negligible levels of intra-specific variation in COI have been reported for basal Phyla such as sponges and anthozoans (Shearer et al., 2002; Frances and Hoover, 2002; Duran et al., 2004a). In ascidians, the COI gene has proven to be highly polymorphic (Tarjuelo et al., 2001, 2004; Castilla et al., 2002; Turon et al., 2003; Turon and López-Legentil, 2004; López-Legentil and Turon, 2006; López-Legentil et al., 2006).

The objective of this study was to analyze a fragment of the mtDNA gene COI in an attempt to determine the intra-specific genetic structure and phylogeography of the ascidian *E. turbinata* over its amphiatlantic distribution range.

2. Material and methods

2.1. Samples

A total of eight populations of *E. turbinata* were collected from the Mediterranean, the Caribbean and the Atlantic (Table 1): two from the western Mediterranean (Pollença and Alcúdia; Balearic Islands); four from the Florida Keys (Key Largo, Long Key, Islamorada and Rodriguez Key); one from the USA Atlantic coast (Fort Pierce, FL); and one from the Spanish Atlantic coast (Cádiz). Sampling was undertaken in 2006 by SCUBA diving or snorkelling. The specimens were identified as *E. turbinata* based on Berrill (1932) and Van Name (1945).

2.2. DNA extraction and sequencing

In order to optimize DNA extractions, we separated the branchial sac from the tunic and digestive system under a

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Table 1
Ecteinascidia turbinata populations sampled with their geographical location, sample size (*N*), and color morph observed

Sea	Population	GPS position	*N*	Color morph
Atlantic	Fort Pierce	27:30:08N; 80:18:48W	18	White
	Cádiz	36:23:49N; 6:12:16W	15	White
Caribbean	Key Largo	25:04:10N; 80:27:45W	17	Orange, white
	Long Key	24:49:04N; 80:48:07W	16	Orange
	Islamorada	24:53:88N; 80:39:63W	17	Orange, white
	Rodriguez Key	25:03:12N; 80:27:09W	18	Orange
Mediterranean	Pollença	39:54:34N; 3:05:57E	31	White
	Alcúdia	39:49:35N; 3:08:50E	31	White

binocular microscope. Each piece of branchial sac was then kept in absolute ethanol at −25 °C until used. Mitochondrial DNA was extracted using the Puregene kit (Gentra Systems). The universal primers LCO1490 and HCO2198, described in Folmer et al. (1994), were used to amplify a segment of the COI mitochondrial gene. Amplification was performed in a 25 μL total-reaction volume with: 1.25 μL of each primer (10 μM), 2.5 μL dNTP's (2mM), 2.5 μL 10× buffer, 2 μL $MgCl_2$, 0.2 μL *Taq* polymerase 5 U, and 1 μL DNA. A single soak at 95 °C for 5 min was followed by 35 amplification cycles (denaturation at 95 °C for 1 min; annealing at 55 °C for 1 min; and extension at 68 °C for 2 min), and a final extension at 72 °C for 10 min, in a Peltier PTC-200 gradient PCR.

PCR products were purified using the Qiagen PCR purification kit. The sequencing reaction was carried out with the BigDye TM terminator v. 3.1 using the same primers as in the amplification step. Sequences were obtained on an ABI Prism 3100 automated sequencer.

3. Results and discussion

The COI gene of 163 colonies of the ascidian *E. turbinata* was analyzed resulting in nucleotide sequences of 621 bp in length. From these colonies, which include individuals from the entire current distribution range of the species, only one haplotype was identified (GenBank Accession No. EF643374). The lack of haplotype diversity is remarkable given the fact that the COI gene has been found to be variable at the intra-species level in all ascidian species investigated to date (Tarjuelo et al., 2001, 2004; Castilla et al., 2002; Turon et al., 2003; López-Legentil and Turon, 2005, 2006; López-Legentil et al., 2006). Indeed, for colonial ascidians, the proportion of variable sites found in the same COI fragment ranged from 3.8% (*Pseudodistoma crucigaster*; Tarjuelo et al., 2004) to 25.8% (*Botryllus schlosseri*; López-Legentil et al., 2006). In addition, we found a 23.6% nucleotide variation between *E. turbinata* and *Ecteinascidia herdmanni*, which is the only available sequence for this genus (GenBank Accession No. AY600968; Turon and López-Legentil, 2004). Our results suggest, therefore, a very slow evolutionary rate of the cytochrome *c* oxidase gene in *E. turbinata*.

In addition several other factors may contribute to explain the lack of haplotype diversity found in this study. For instance, a recent origin of this species, a selective sweep or genetic drift associated with historical events, such as a recent population bottleneck or a founder event from an unknown source population. Aquaculture of *E. turbinata* has been developed in both the Mediterranean and the Caribbean Seas (Carballo et al., 1997; Pomponi, 1999). Re-seeding with colonies of the previous generation was regularly conducted; so that colonies with faster growth rates may have been unnoticeably selected to increase production. It is conceivable that fast-growing genotypes become fixed and thereafter spread to nearby populations, greatly reducing the original genetic variability. However, interest in *E. turbinata* dates only from the 1990s and larval dispersal is highly localized in this species (Bingham and Young, 1991; Carballo, 2000). Therefore, it is extremely unlikely that all native genotypes had disappeared from all sampled populations in such a short time. A founder effect or a recent bottleneck are also improbable as we sampled the whole distribution range of this species and yet found no variability in the studied COI fragment.

Moreover, some degree of intra-species variability was found among Atlantic and Mediterranean populations of *E. turbinata*. For instance, two different color morphs were observed during this study (Fig. 1). The orange morphotype (Fig. 1a) was observed in all the Florida Keys locations, whereas the white morphotype (Fig. 1b) was only rarely observed in Islamorada and Key Largo, but was the only one present in Fort Pierce, the Balearic Islands and Cádiz. In addition, we used non-specific degenerated primers (Borchiellini et al., 1998) to analyze the *hsp70* gene of 14 Florida colonies. We obtained four sequences that resulted in two different haplotypes. The first haplotype was found in Fort Pierce, Islamorada and Key Largo samples (GenBank Accession No. EF643375). The second haplotype was amplified for a colony from Rodriguez Key (GenBank Accession No. EF643376). Both haplotypes presented great genetic variability (360 polymorphic sites out of a 593 bp sequence).

The existence of intra-species variability in both color and nuclear genetic levels suggests that the absence of mitochondrial variability is not the result of genetic uniformity or a recent origin of this species. We suggest two main alternative hypotheses to explain the pattern found. First, a favorable mutation arose in the mitochondrial genome of *E. turbinata* and then spread to fixation through a selective sweep of the maternally inherited mtDNA. Thermal adaptation has been suggested to influence mitochondrial respiratory genes in poikilotherms (Ballard and Whitlock,

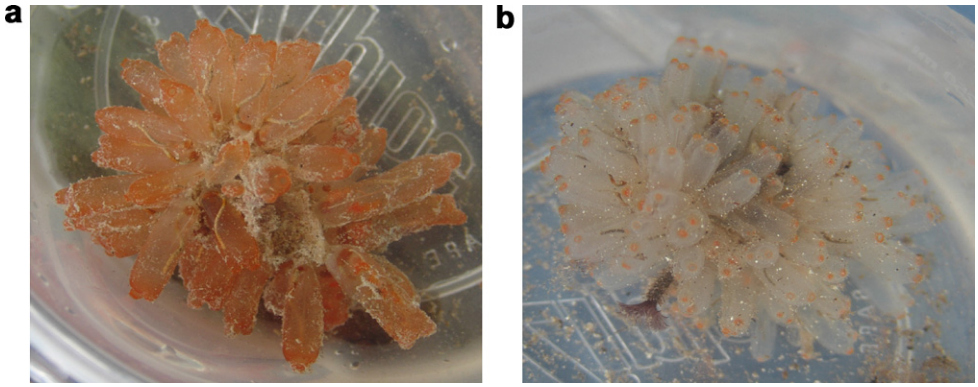

Fig. 1. (a) Orange morph and (b) white morph, of the ascidian *Ecteinascidia turbinata* found on mangrove roots in Key Largo (Florida).

2004). In a species living in shallow waters such as *E. turbinata* it is conceivable than a mutation related to temperature tolerance had a strong effect on fitness and could reach fixation. Although larvae have restricted dispersal, occasional long-range dispersal events through rafting or fouling may have spread the new variant across the Atlantic. Evidence for sporadic amphiatlantic dispersal has been found for another colonial ascidian (López-Legentil et al., 2006). Clonal reproduction may also have enhanced the rate of proliferation once individuals with the favorable mtDNA had arrived to pre-existing populations. However, unless this favorable mutation originated recently and spread amazingly fast, such a process fails to satisfactorily explain the lack of mutations for the third base pair position of the codon and seems, therefore, unlikely in our case.

A second and favored hypothesis is that this gene may be unusually conserved in this species. A possible explanation would be the presence of an mtMSH gene (=mitochondrial MutS homolog), which codes for a mitochondrial DNA mismatch-repair system. So far, from all eukaryotes examined, the mtMSH has been only found in the subclass Octocorallia (Cnidaria). Pont-Kingdon et al. (1998) suggested that the mtMSH gene was originally in the nucleus and was transferred to the mitochondrion in octocorals after their divergence from hexacorals. However, these authors also pointed out that this gene could have been acquired from another organism, most likely an endosymbiont which was only present in Octocorallia. In *E. turbinata*, three bacterial strains were characterized by Moss et al. (2003). The most abundant one (>50%), named "*Candidatus* Endoecteinascidia frumentensis", was probably an endosymbiotic bacteria in both adult and larval tissue. Although the mtMSH gene was found to be highly variable in Octocorallia (Frances and Hoover, 2001), we designed some primer sets targeting two "conserved" regions of this gene, but unfortunately no amplification was obtained. Further research to test the hypothesis of a repair system mechanism should consider sequencing the whole mitochondrial genome of *E. turbinata* and screen it for a possible mtMSH homolog gene.

In summary, this is the first time that a lack of variability in the mitochondrial COI gene has been reported in an ascidian. *E. turbinata* has been broadly studied because of the interesting pharmaceutical activities of its secondary metabolites. However, the presence of endosymbionts and a lack of COI divergence make this species unusual and suggest a different evolution pattern from other ascidians. Studies with more mitochondrial and nuclear genes (e.g. internal transcribed spacers or microsatellites) are necessary to complete the picture of evolutionary mechanisms and to assess phylogeographic patterns and population structure in this species.

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