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by

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Marine biodiversity: a multidisciplinary journey from genes to species

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TABLE OF CONTENTS

INTRODUCTION

The present dissertation is divided into an introduction and three different chapters. Its general organization is intended to facilitate future publications, and therefore Chapters 1, 2 and 3 are hereby reported in journal-like style and should lay the basis for independent papers to be submitted to international peer-reviewed journals as first author. On the contrary, the aim of this introduction is to focus and resume the work performed during my Ph.D. Thesis and discussed below. It starts with a description of the originally planned research project (partly covered by Chapter 1), analyzes technical problems that forced me to deviate from it, and introduces the subsequent Chapters 2 and 3. Finally, Chapter 4 resumes papers and short communications published or in press (first page only, due to copyright) during this Ph.D. programme.

One of the unsolved problems in evolutionary biology is which mutations generate evolutionarily relevant phenotypic variation. Understanding what kinds of molecular changes they entail, and what are the phenotypic magnitudes and frequencies of origin, may provide important insights into evolutionary processes (adaptive evolution, balancing selection, deleterious variation and genetic drift) that influence phenotypic variation within and among population and eventually lead to speciation processes. The marine tunicate *Ciona intestinalis* sp. A constitutes an excellent organism for linking genomics and developmental biology. A growing interest in population biology of *C. intestinalis*, prompted by the paucity of laboratory lines and the ease of sampling, has recently undermined extreme levels of heterozygosity and high rates of recessive mutations (13-20% wild individuals are heterozygote carriers of recessive mutations in developmental genes). When brought to homozygosity, these naturally occurring mutations often carry phenotypes that are similar to hypothesized evolutionary transitions among ascidian families and orders, such as reduction or absence of the tadpole larval tail (*e.g.* genus *Molgula*), proximal exhalent siphon (*e.g.* genus *Ascidia*) and absence of, or supernumerary, larval sensory organs (*e.g.* genus *Pycnoclavella*).

So far, results obtained in forward genetics of ascidians were mostly obtained from casual and random samplings, with no efforts in developing specific methods, such as for culturing and cryopreservation, and yet poor knowledge of several species-specific traits in the field on the other side, such as taxonomic status, demographic and reproductive dynamics. Therefore, these gaps need to be filled for optimal project design. The original aim of the present Ph.D. project was to investigate the developmental processes and genetic configurations of specific phylomimicking

mutations occurring within natural populations of the ascidian *Ciona intestinalis* sp. A by carrying out a naturally occurring mutation study. However, due to problems of technical and biological nature, I extended it as described below.

The first step was successfully achieved, and is partially explained in **Chapter 1**. This discusses the state-of-the-art of forward genetics in tunicates and review findings of research on naturally occurring mutants. In addition, it first reports the development and establishment of a comprehensive protocol for the study of genetic polymorphism in wild populations of *Ciona intestinalis* sp. A that includes newly developed procedures for sperm cryopreservation, culturing and screening, as well as the possibility of identifying subtle mutations by means of a multiple whole mount *in situ* hybridization with an automated system. I also show that the ecology and biology of natural populations at species-specific level holds traits that are relevant to correct project organization and conduction. Altogether, the effort hereby conduced lays the foundations for the identification of naturally occurring mutations in ascidian taxa, and may constitute an additional value when considering new marine model species for forward genetics purposes. However, after an initial survey of heterozygote carriers of developmental genes and the molecular characterization of the identified mutant larvae by ISH (*in situ* hybridization) and FISH (fluorescent *in situ* hybridization), unexpected cryobanking problems resulted in the loss of the mutants cryopreserved. This, together with field disappearance of the species, forced me to deviate from the project.

Therefore, since I had originally planned to acquire knowledge at gene and species level, I have spent the remaining part of my PhD working on two conceptually linked projects. One is concerned with an evolutionary developmental study of a specific gene in chordate evolution (**Chapter 2**). This chapter therefore consists of an EVO-DEVO study analyzing the *Magoh* (*Mago Nashi*) gene. In particular, starting by unpublished ISH and IHC data available in the laboratory on the tunicate species *Ciona intestinalis* sp. A, I re-organized previous data, performed an *in situ* hybridization study in the phylogenetically related species *Branchiostoma lanceolatum* (Pallas, 1774) and analyzed sequence conservation, gene duplication and synteny in Chordates. Here I show that, despite its long history, *Magoh* has become part of a conserved syntenic group only in vertebrates. Genetic and genomic analyses indicate that *Magoh* appears to have duplicated in the mammalian lineage. Whole mount *in situ* hybridization and immunohistochemistry demonstrate that, similar to all other bilaterians, *Magoh* is regionally expressed in mesodermal and endodermal

progenitor cells and tissues of the amphioxus *B. lanceolatum* and the tunicate *C. intestinalis* sp. A embryos. Further, ascidian *Magoh* protein undergoes early subcellular restriction and perinuclear localization in the fertilized egg, like in insects. Most importantly, *Magoh* is expressed in the brain of cephalochordate and ascidian larvae, where the protein accumulates in neurogenic and axonal structures. Thus, these data suggest that *Magoh* recruitment in neural cells is an innovation already present in the last common ancestor of Chordates.

Finally, I performed a multidisciplinary study concerning the identity of the molluscan gastropod *Bursa scrobilator* (Linné, 1758) by using independent (molecular and morphological) species concepts (**Chapter 3**). This species forms established populations in the Atlantic Ocean (mainly in the Azores and Canarians), but is only known from the Mediterranean area from single isolated specimens, therefore being heralded as one of the rarest inhabitants of the Mediterranean molluscan fauna. I explored the taxonomic identity and the intraspecific variability of the species by using a combination of morphological, anatomical and molecular analyses on specimens from the entire distributional range, as to test a possible speciation pattern in the Mediterranean Sea. No difference was observed when analyzing protoconch, teleoconch and anatomy. Conversely, radulae are highly variable in shape both between and within individuals. Sequence analysis of COI and 16S shows the absence of significant differentiation among sites of occurrence, as expected for a species with teleplanic larvae. In addition, the broad dispersal potential in the investigated family, as well as the absence of a genetic structure, would suggest that *B. scrobilator* does not constitute a resident species of the Mediterranean fauna, but that its Mediterranean presence may be consistent with a repetitive natural dispersal of single veliger through the Gibraltar Strait, therefore calling for a numerical re-assessment of resident Mediterranean biodiversity by molecular means.

CHAPTER 1 - MUTATION RESEARCH IN TUNICATES: TOOLS FOR STUDYING DEVELOPMENT AND EVOLUTION

Tunicates are a variegate group of marine invertebrates whose phylogenetic relatedness to vertebrates, witnessed by the larval body plan, has widely contributed to their use as model organisms (Satoh, 1994; Corbo et al., 2001; Dehal et al., 2002; Delsuc et al., 2006; Lemaire, 2011). These marine invertebrates possess simple embryology, small cell number and stereotyped development (Kumano & Nishida, 2007; Lemaire, 2009). In the class Ascidiacea, the capacity to self-fertilize as well as the growing availability of fully sequenced and annotated genomes (Dehal et al., 2002; Small et al., 2007; Denoeud et al., 2010) have allowed rapid progress in the study of mutations (Moody et al., 1999; Sasakura et al., 2003a, 2003b; Sordino et al., 2008). Covering fields once restricted to classical genetics, forward genetics has now become central in the identification of new genes and pathways, as well as to shade light on new functions of known genes.

Mutagenesis is the usual basic approach in forward genetics. Since decades, mutagenesis projects are performed in model organisms from bacteria to plants and animals (*e.g.* Charlesworth & Charlesworth, 1998; Rudall & Bateman, 2003; Chang & Shaw, 2003; Beutler et al., 2003; Naruse et al., 2004; Hintz et al., 2006; Parichy, 2006; Bégin & Schoen, 2007; Beutler et al., 2007). In the last 15 years, this approach has been extended to ascidians too, mostly by means of laboratory-induced mutations. During late '90s, two research groups developed a protocol for chemical mutagenesis of *Ciona savignyi* Herdman, 1882 and *Ciona intestinalis* sp. A. (*e.g.* Moody et al., 1999; Nakatani et al., 1999; Sordino et al., 2001). These methods were based on the use of N-ethyl-N-nitrosourea (ENU), a powerful point mutagen originally used in *Drosophila* and *Caenorhabditis*, and later applied to *Xenopus*, zebrafish and mouse (Justice et al., 1999; De Stasio & Dorman, 2001; Goda et al., 2006; Ashburner & Roote, 2007). Early findings of ENU mutagenesis in tunicates were encouraging, allowing the identification of remarkable phenotypes at different developmental stages. Subsequently, insertional mutagenesis was established by transposable elements *Minos* and I-*Sce*I (Sasakura et al., 2003a, 2003b; Deschet et al., 2003), and then by site-specific zinc finger nucleases (ZFN) (Kawai et al., 2012) and transcriptional activator-like effector nucleases (TALEN) (Treen et al., 2014).

The discovery of naturally occurring mutations in ascidian species has early flanked the study of genetic deletions induced in the laboratory. Large-scale screenings based on light/dark-induced self-fertilization showed a high rate of heterozygous alleles that disrupt embryonic traits (Deschet & Smith, 2004; Sordino et al., 2008). Naturally occurring mutations are not only an entry point to

molecular embryology, as they are also central to evolutionary questions of genetic variation and adaption. In the wild, natural selection may lead to the appearance of specimens with a genuine evolutionary potential, able to colonize distinct niches or conditions precluded to the founder, or compete with it in the same area (*e.g.* Drake et al., 1998; Rudall & Bateman, 2003; Chang & Shaw, 2003; Mitchell-Olds et al., 2007). Understanding the type of molecular changes that preside the origin of natural polymorphism is an exciting focus when studying phenotypic traits of evolutionary interest.

Besides promising results in forward genetics of naturally occurring mutations, yet a standardized methodology that integrates concepts and information from different areas of research (ecology, biogeography, population genetics, cryobiology, molecular biology) is needed. I hereby review main advances in the study of ascidian mutations. Further, I present a multidisciplinary procedure covering most methodological aspects that should be considered when planning and performing systematic studies of naturally occurring mutations. Following correct evaluation of details previously underestimated, other tunicate species may be amenable to the development of a similar methodology.

1. N-ETHYL-N-NITROSOUREA (ENU) MUTANTS

In *Ciona savignyi* and *Ciona intestinalis* sp. A classical F2 screenings, the point-mutagen N-ethyl-N-nitrosourea (ENU) has been used by the group of William Smith and at Stazione Zoologica of Naples to induce point mutations that affect the development of the ascidian brain, sensory organs and notochord (Nakatani et al., 1999; Moody et al., 1999; Sordino et al., 2001). In *chongmague* mutant embryos, the notochord failed to develop due to notochord progenitor cells that abnormally adopt a mesenchyme-like fate. By positional cloning, *chongmague* was later linked to the *C. savignyi* ortholog of the vertebrate alpha 3/4/5 family of laminins (Veeman et al., 2008). Like *aimless,* a mutation in the ortholog of the planar cell polarity (PCP) pathway component *Prickle*, *chongmague* suggests that PCP signaling has multiple distinct functions in notochord morphogenesis (Veeman et al., 2008). Genetic disturbance at distinct levels of sensory organ formation in *Ciona intestinalis* sp. A was observed in *big ocellus* and *albino,* with specific defects in organ shape and terminal differentiation, respectively (Sordino et al., 2001). *Spotless* is due to a point mutation in the *tyrosinase* gene of *C. savignyi,* that specifically disrupts sensory organs pigmentation, so that mutant larvae are unable to respond properly to gravity and illumination cues while settling (Jiang et al., 2005a). Loss of the *brachyury* gene resulted in stochastic fate

transformation (Chiba et al., 2009), while a null mutation in the *doublesex/mab3 related-1* gene caused severe abnormalities in the sensory vesicle (brain), palps and oral siphon primordium (Tresser et al., 2010). Besides these findings, ENU is now abandoned for mutagenesis screenings since, as previously suggested, seawater salinity appears to reduce the mutagenic activity, while *Ciona* genetic polymorphism hinders the identification of the exact genetic lesion (Hendrickson et al., 2004).

2. RANDOM INSERTIONAL MUTAGENESIS

DNA transposons are powerful tools for genetic analyses. First, the Tc1/*mariner* superfamily *Minos* was efficiently employed for transposon-based transgenesis and insertional mutagenesis in *Ciona savignyi* and *Ciona intestinalis* sp. A (Sasakura et al., 2003a, 2003b; Matsuoka et al., 2004). *Minos* activity in generating mutant and transgenic animals was found to be slightly weaker in *C. savignyi* than *C. intestinalis* (Matsuoka et al., 2004). These methodologies were further implemented by replacing mRNA and DNA microinjection of single cell embryos with electroporation of fertilized eggs (Matsuoka et al., 2005). Another method for generating stable transgenic lines of the ascidian *C. savignyi* involves co-injection of a green fluorescent protein (GFP) construct containing I-*Sce*I recognition sites with I-*Sce*I mRNA in fertilized eggs (Deschet et al., 2003). Evidence of *Minos* insertion in the Ci-*Musashi* gene, responsible for altered GFP expression, suggests an enhancer trap event (Awazu et al., 2004). Consequently, the enhancer trap approach utilizing transposable constructs yielded interesting insights about the molecular and cellular nature of embryonic development. For example, *swimming juvenile*, a *Minos*–induced mutation of the *cellulose synthase* gene (Ci-*CesA*), illustrates a new function of this gene in the coordination of morphogenetic events in the trunk and tail during metamorphosis (Sasakura et al., 2005). Then, *Minos* ability to transpose from DNA to DNA was observed in transposition assays in *C. savignyi* (Matsuoka et al., 2004). Using transposase-mediated remobilization of *Minos* in *C. intestinalis* sp. A chromosomes to generate new insertions, nearly 80% F1 families showed a changed pattern of GFP expression (Awazu et al., 2007).

3. TARGETED INSERTIONAL MUTAGENESIS

Yasunori Sasakura and collaborators have recently demonstrated that tunicate mutagenesis is amenable to methodological innovation. First, zinc finger nucleases (ZFN) were used to create DNA double-strand breaks (DSBs) at target sequences in *C. intestinalis* sp. A genome. Interestingly, adequate ZFN amounts induced insertional or deletional mutations specifically on the on-target site with less effect than the off-target sites (Kawai et al., 2012). Transcriptional activator-like effector nucleases (TALEN) allow routine gene targeting in a wide range of organisms (Porteus & Carroll, 2005) due to standard molecular cloning procedures (Cermak et al., 2011). TALEN electroporation of *Ciona intestinalis* sp. A eggs generates tissue-specific and ubiquitous gene knockouts and allows the rapid analysis of hundreds of TALEN-induced mutants (Treen et al., 2014; X. Bailly, personal communication). The study of a TALEN mutant revealed a new function of the Ci-*Fgf3* orthologous gene during ascidian metamorphosis (Treen et al., 2014).

4. NATURALLY OCCURRING MUTATIONS

Self-fertilization of the hermaphrodite *Ciona* species offers a rapid means for identifying recessive zygotic mutations (Deschet & Smith, 2004; Hendrickson et al., 2004; Sordino et al., 2008). Early results in *Ciona intestinalis* sp. A (Deschet & Smith, 2004) and *Ciona savignyi* (Jiang et al., 2005b) were based on the mutants *frimousse* and *aimless* and provided new insights into the anterior neural fate and anterior-posterior polarity of the notochord, respectively (Deschet & Smith, 2004; Jiang et al., 2005b). Yet, clear potential of naturally occurring mutations in developmental and evolutionary studies of *Ciona* species remains elusive. Therefore, implementations of standard methods (*e.g.* culturing and cryopreservation) as well as better knowledge of species-specific traits (*e.g.* taxonomic status, demographic and reproductive dynamics) are highly needed. A procedure for screening F1 progenies and for crossing mutant alleles by repeated spawning is available for *C. savignyi* (Hendrickson et al., 2004; Jiang et al., 2005b). However, sharp differences among cryptic species at genomic, developmental and ecological levels urge for tailor-made mutagenesis protocols (*e.g.* Hoshino & Nishikawa, 1985; Suzuki et al., 2005; Caputi et al., 2007, 2014; Nydam & Harrison, 2007, 2010; Vercaemer et al., 2011; Zhan et al., 2010, 2012). Next, I present and discuss a numbered step-by-step integrative methodology for large-scale study of naturally occurring mutations in *C. intestinalis* sp. A, including the development of new protocols.

4.1. TOWARDS A TOOLBOX FOR FORWARD GENETICS

4.1.1. Where, which and when of cryptic species and populations

C. intestinalis sp. A and B cryptic species are globally encountered in temperate areas of both hemispheres, where they occupy confined coastal environments, such as harbors and lagoons (*e.g.* Lambert & Lambert, 1998; Procaccini et al., 2011; Vercaemer et al., 2011). [1] These habitats definitively constitute best sites for collecting these species in high number.

Additionally, the strong anatomical conservation encountered among *Ciona* cryptic species prompts for molecular classification (Suzuki et al., 2005; Sordino et al., 2008; Sato et al., 2012; Caputi et al., 2014). [2] Therefore, perform sequence analysis of selected markers on populations of interest.

The wide distribution of *Ciona intestinalis* sp. A and sp. B reflects the tolerance of these species to environmental stressors (Lambert & Lambert, 1998; Caputi et al., 2007; Nydam & Harrison, 2007, 2010; Therriault & Herborg, 2008; Zhan et al., 2010, 2012; Procaccini et al., 2011). Population densities are mostly influenced by life style and environmental conditions at local geographical scale, with the appearance of dense but effimerous populations. Understanding distribution dynamics of the target species during a wide time lapse is valuable (Lo Bianco, 1909; Pérès, 1952; Sabbadin, 1958; Dybern, 1965, 1967; Brunetti & Menin, 1977; Marin et al., 1987; Petersen & Svane, 1995; Carver et al., 2003, 2006; Howes et al., 2007; Ramsay et al., 2009; Smale & Wernberg, 2012; Kanary et al., 2011; Vercaemer et al., 2011; Caputi et al., 2014). Of note, the strictness of self-sterility in ascidians appears to differ from one population to another, and may change seasonally within a population (Morgan, 1938, 1939, 1942, 1944; Murabe & Hoshi, 2002). [3] This highlights the importance of routine self-fertilization assays, that will confirm if the selected population may be used for "mutation" purposes.

4.1.2. From the field to the lab: sampling and maintainance

Concentrating sampling efforts and screening procedures during favourable periods, [1] *C. intestinalis* specimens with full oviducts and gonoducts are gently collected by hand picking. It is worth sampling nearly the exact number of specimens to be effectively analyzed within one week to avoid oversampling. Moreover, collecting distant specimens not belonging to the same cluster allows avoiding to resample the same mutations, due to local breeding and limited dispersal (F. Crocetta, personal observation). [2] Animals are kept in seawater tank during transport to laboratory

facilities, paying attention to avoid high densities. [3] Healthy and ripe (>6-7 cm) specimens are separated and acclimatized for >2-3 days with continuous water flow, checking their conditions on a daily basis. [4] Animals are fed with a solution of marine microalgae concentrates (Shellfish Diet 1800™ Instant Algae®: 0.5 ml in 1 liter of sea water): pour 500 ml of the solution into the tank, stopping the water flow for 5-6 hours to maximize the filtration rate of animals. [5] Place the animals under continuous illumination for at least 2-3 days, lighting the tank with a lamp from above (see below in self-fertilization procedures for the rationale).

4.1.3. From the field to the lab: self-fertilization procedures

Ripeness of *C. intestinalis* individuals is usually verified in transparency by the fullness of the gonoducts. An efficient method to prevent spontaneous spawning and to use animals with full gonoducts is to expose animals to continuous light, based on the observation that a number of ascidians spawn in response to light following darkness (see Lambert & Brandt, 1967). [1] After specimens have been kept under constant light to accumulate gametes, two different methods may be used to self-fertilize animals: light/dark-induced spawning and dissection.

[2] *Light/dark-induced spawning method*

[2.1] Place single *C. intestinalis* specimens in 500 ml dark chambers containing 0.22 μM MFSW (Millipore filtered natural sea-water). [2.2] Wait 30 minutes as a means of inducing spawning of both homotypic gametes. [2.3] Transfer gametes in a 9 cm Petri dish filled with 0.22 μM MFSW.

[2] *Dissection method*

[2.1] Dissect *C. intestinalis* specimens at the base of the atrial siphon with a sterile blade. [2.2] Collect eggs from the oviduct with a Pasteur pipette and transfer them in a 9 cm Petri dish filled with 10 mL of 0.22 μM MFSW. [2.3] Collect sperm (as dry as possible) from the spermiduct with a Pasteur pipette and transfer it into a 2 ml vial in ice (see cryopreservation). [2.4] Allow eggs to rest for a short time (5-10 minutes) before insemination to facilitate the expansion of the chorion (follicle cells), which makes the eggs float and thus improves fertilization. [2.5] Active sperm in a 1:1000 dilution (10 μL dry sperm in 10 mL MFSW: ~10^5 sperm/mL) in 15 mL Falcon tubes at 18°C. [2.6] Perform *in vitro* self-fertilization by further diluting sperm to final 1:1.000.000 in the 9 cm Petri dish.

Finally, [3] store Petri dishes in an incubator at 17-18°C. [4] One hour after insemination, separate embryos from unfertilized eggs and wash them with fresh 0.22 μM MFSW in order to remove surplus sperm. [5] Distribute eggs in more Petri dishes if the number is higher than 300. [6] Perform self-fertilization at around 2-3 PM in order to have tadpole larvae ready for morphological screening the morning after (see screening procedures).

4.1.4. Saving ascidian mutations by cryopreservation

Last ten years saw an increasing number of reports on the cryopreservation of sperm and embryos from a variety of aquatic organisms (*e.g.* Gwo, 2000; Suquet et al., 2000; Chao & Liao, 2001; Paredes & Bellas, 2009). Once confined to species of patrimonial importance, semen cryopreservation currently represents a fundamental tool for the storage and distribution of transgenic and mutant lines of model systems, including tunicates (*Ciona savigny*: Moody et al., 1999; Hendrickson et al., 2004; *Ciona intestinalis* sp. A: Sorrenti et al., 2014; *Oikopleura dioica*: Ouchi et al., 2011), where the use of dimethyl sulfoxide (DMSO) is uniformly considered as the best cryoprotective agent. However, cryopreservation for mutant purposes needs practical tools for standardized sperm cryopreservation protocol, able to provide repeatable good post-thaw fertility of *C. intestinalis* sperm. Here I present a newly implemented protocol for semen freezing in liquid nitrogen (see Crocetta et al., 2012). After collecting sperm into a 2 ml vial on ice (see dissection method): [1] depending on visual evaluation of sperm dryness, dilute 1:5/1:10 by adding pre-chilled 0.22 μM Millipore filtered natural sea-water (MFSW); [2] add 1:10 DMSO to diluted sperm, and after 15 minutes transfer the sperm to cryo-paillettes (max storage 150 μl) on ice; [3] chill the sperm by leaving cryo-paillettes for 15 minutes on a metallic grid (with an outer 6 cm-high polystyrene frame) floating on liquid nitrogen (N_2); [4] adapt sperm to freezing conditions by keeping cryo-paillettes into liquid N_2 for 10 minutes; [5] store cryo-paillettes into liquid N_2 in a cryo-container.

4.1.5. Sorting mutations by morphology

A simple F1 scheme based on artificial self-fertilization and morphology-based mutation screenings has been previously used to identify deleterious alleles acting during *C. intestinalis* sp. A embryogenesis and to isolate potentially important phenotypes in *C. intestinalis* (Sordino et al.,

2008; Crocetta & Sordino, 2012). After performing self-fertilization at around 2-3 PM (see self-fertilization laboratory procedures), F1 larvae will be fully developed at around 9:30-10 AM. Then, [1] carefully analyse alive F1 at the stereomicroscope for obvious phenotypes (with Mendelian frequencies around 20-30%) that affect gross anatomy. [2] Fix all F1 larvae for future *in situ* hybridization (ISH) analysis as following: [3] fix larvae 1/1.5 hour(s) in 2 ml vials in 1:1 8% PFA in 0.1 M MOPS pH 7.4:MFSW (PFA: paraformaldehyde; MOPS: 3-(N-morpholino)propanesulfonic acid; MFSW: Millipore filtered natural sea-water); [4] wash 3 x 10 minutes in 1 ml 1X PBT in DEPC H_2O (PBT: Phosphate-buffered saline and Tween 20; DEPC: diethylpyrocarbonate); [5] dehydrate in EtOH series (3 x 10 minutes in 1 ml 30%, 50% and 70% ethanol (EtOH) in DEPC H_2O); [6] store at -20°C after 70% EtOH in DEPC H_2O. According to the presence/absence of obvious mutation phenotypes, appropriate riboprobes are employed for manual or automated *in situ* hybridization (ISH) and fluorescent *in situ* hybridization (FISH) (protocols as in Christiaen et al., 2009) to look for mutations with subtle phenotypes (see below).

4.1.6. Searching for subtle mutants by automated whole mount ISH

Until now, ascidian mutagenesis projects have exclusively focused on events of visible disruption of developmental processes, easy to identify by stereomicroscopy. However, the identification of subtle phenotypes in developmental stages could allow a more complete understanding of mutation frequency. To this aim, a high-throughput protocol for a whole mount *in situ* hybridization by automation has been developed, able to screen simultaneously the activity of multiple genes with different, not overlapping, expression domains in *Ciona intestinalis* larvae. In particular, Ci-*Arrestin*, Ci-*Glyr* and Ci-*Six 3*, expressed in ocellus photoreceptor cells (*Arrestin*), retinal ganglion cells (*Glycine receptor*) and sensory vesicle (*Six homeobox 3*), were used. This approach is performed in 30-well plates with identical conditions for all antisense riboprobes, including hybridization temperature and comparable mRNA staining intensity. Protocol (notes: all steps at room temperature and using diethylpyrocarbonate (DEPC) water except where indicated, solutions as in Marino et al., 2012): [1] rehydrate larvae 2 x 10 minutes each in 250 μl of 50% and 30% ethanol (EtOH); [2] wash 3 x 7 minutes each in 250 μl of 1X PBT (Phosphate-buffered saline and Tween 20); [3] postfix 1 hour in 250 μl of 4% paraformaldehyde (PFA) in 1X Phosphate-buffered saline (PBS); [4] wash 3 x 7 minutes each in 250 µl of 1X PBT; [5] incubate 30 minutes in 250 µl of 1X PBT containing 4 µg/ml Proteinase K at 37°C water bath; [6] re-fix 1 hour in 250 µl of 4% PFA in 1X PBS; [7] wash 3 x 7 minutes each in 250 µl of 1X PBT; [8] wash 3 x 10 minutes in

250 µl of 0.25% acetic anhydride, 0.1 M triethanolamine; [9] wash 3 x 7 minutes each in 250µl of 1X PBT; [10] incubate 20 minutes in 250 µl of 1:1 hybridization solution and 1X PBT; [11] incubate for 30 minutes in 250 µl of hybridization solution; [12] incubate for 2 hours in 250 µl of hybridization solution at 55°C; [13] incubate around 18 hours in 250 µl of hybridization solution containing 0.3-0.6 ng/ml DIG-labelled riboprobes at 55°C (Note: DIG-labelled riboprobes concentration to be previously estimated by dot blot analysis); [14] wash at 55°C in: 2 x 15 minutes in 250 µl of washing buffer 1; 2 x 15 minutes in 250 µl of washing buffer 2; [15] wash 3 x 10 minutes in 250 µl of Solution A at 37°C; [16] incubate 30 minutes at 37°C in 250 µl of Solution A containing 20 µg/ml RNAseA; [17] wash 15 minutes at 37°C in 250 µl of Solution A; [18] wash at 55°C in: 1 x 20 minutes in 250 µl of washing buffer 2; 2 x 15 minutes in 250 µl of washing buffer 3; [19] wash 15 minutes in 250 µl of 1:1 1X SSC in PBT; [20] wash 4 x 7 minutes in 250 µl of 1X PBT in sterile H_2O; [21] incubate 1 hour in 250 µl of blocking buffer; [22] incubate 5 hours in 250 µl of fresh blocking buffer containing 1:2000 Alkaline Phosphatase anti-DIG antibody; [23] wash 11 x 20 minutes in 250 µl of 1X PBT in sterile H_2O; [24] wash 2 x 10 minutes in 250 µl of AP buffer. Then, perform manually the following two steps: [25] incubate in 1 ml AP buffer containing 4.5 µl of nitro blue tetrazolium chloride (NBT) and 3.5 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP); [26] stop the staining reaction with 1X PBT in sterile H_2O.

4.1.7. Back to facilities: raising the ascidian mutation

Worldwide research on *Ciona* species is based on wild-collected specimens (Caputi et al., 2014), as no isogenic or semi-isogenic line is available. Ascidians are raised and grown in open (Cirino et al., 2002; Hendrickson et al., 2004) and closed culturing systems (Joly et al., 2007). Since *Ciona* is sessile, an important aspect is to provide growing animals with a support, plastic Petri dishes being the most appropriate one, allowing the animals to grow downwards (a position closer to one adopted in the wild). Undisturbed ascidians filter water continuously at constant rates, so that continuous feeding is needed to ensure the constant presence of suspended food in the ascidian environment. Here, I present a new protocol for growing mutant *Ciona intestinalis* sp. A in open systems, based on a mix of live culture of 2 microalgae species (50% *Isochrysis galbana* and 50% *Tetraselmis suecica*). Other details on culturing systems are as in Cirino et al. (2002). Note that the swiftest way to verify the health of growing animals is to control their filtration efficiency, which is revealed by gut fullness and release of fecal strings from the atrial siphon. When raising lines from cryopreserved sperm, consider that some paillettes of *Ciona* spp. cryopreserved sperm are not able

to fertilize eggs due to variable cryopreservation efficiency (Hendrickson et al., 2004; F. Crocetta, A. Macina, personal observation). Therefore, it is advised to thaw at least two aliquots (paillettes) per batch. [1] Remove cryopaillettes from liquid nitrogen (N_2). [2] Hold them in hands for 30 seconds. [3] Cut with sterile forceps. [4] Perform *in vitro* self-fertilization by letting *C. intestinalis* sp. A sperm to drop into 15 cm Petri dishes containing ~300 eggs collected from at least 2 individuals in 0.22 μM Millipore filtered natural sea-water (MFSW). [5] Store Petri dishes in an incubator at 17-18°C. [6] One hour after insemination, separate embryos from unfertilized eggs and remove surplus sperm. [7] The day after, transfer swimming larvae to clean plastic Petri dishes (15 cm) containing fresh 0.22 μM MFSW: first set up a shallow layer of water into the dish; then add larvae that will attach mainly onto the bottom and not on the sides of the dish; wait 30 minutes before adding more water and finally incubate dishes at 18-22°C. [8] On the third day, transfer swimming larvae to new dishes since the contact with clean plastic induces settlement. [9] Renew 0.22 μM MFSW on a daily basis. [10] During next two days, check larval metamorphosis under the stereomicroscope. At the opening of the two siphons, the ascidians initiate feeding and therefore require an external food source: add 1-3 drops of a live *I. galbana* culture into each juveniles containing Petri dish. [11] Transfer dishes into tanks and add 100 ml live microalgal cultures per 20 liters of seawater (see above). [12] The following day, activate automatic feeding through a peristaltic pump (see Cirino et al. 2002 for further details): live cultures of microalgae should be added continuously to seawater, and food tank content should be prepared twice a week. When feeding animals, stop continuous water flow for 5-6 hours and add 100-200 ml of the solution directly to the tank. [13] Check daily at the stereomicroscope the health of the ascidians and their filtration efficiency, in order to adjust the food schedule and to remove abnormal individuals using a thin stick.

4.2. TESTING THE PROTOCOL

4.2.1. Field observations, sampling and self-fertilization data

Central Mediterranean populations of *Ciona intestinalis* sp. A were subjected to a strong demographic crisis during 2009/2010, followed by a partial population recovery from spring 2011 (Caputi et al., 2014). Therefore, field observations begun in September 2011. Of the four suitable sites taken in consideration for sampling, two (Fusaro Lagoon: 40°49’10’’N, 014°03’32’’E; Villaggio Coppola: 40°58’32’’N, 013°58’59’’E) were selected as the most appropriate locations for

performing a screening for naturally-occurring mutations. Concerning the others, the Pozzuoli (Pz) population totally disappeared during the whole period of investigation (F. Crocetta, personal observation), revealing to be an effimerous entity, whilst the Castellammare di Stabia (CdS) one was excluded because too far from the laboratory to be considered for weekly sampling, and because it was not possible to perform hand-sampling. A long-term demographic and reproductive study was recently carried out on *Ciona intestinalis* sp. A populations in the Gulf of Naples, allowing to reveal that self and non-self fertilization successes follow reverse trends during the year, but altogether are constantly high (usually >40%) as to allow whole-year studies. On the contrary, population size monthly averages show a bimodal pattern, usually with a robust peak in May-June, a drastic minimization in August and a second smaller peak in October/November, followed by a less pronounced die-off (Caputi et al., 2014). Demographic fluctuations drastically reduce the number of months available for mutagenesis. In this line, samplings and screening procedures hereby carried out were performed from October 2011 to May 2012 in the two selected sampling sites. Altogether, the amount of F1 progenies (generated by self-fertilization, and scored for heterozygote mutations affecting embryonic development) that were analyzed during this PhD project is 186. Of these, 102 (all coming from the Fusaro Lake) with the light/dark-induced spawning method, whilst 84 (coming from Fusaro Lake and Villaggio Coppola) with the dissection method. The first method has been previously applied with success both in *C. savignyi* and *C. intestinalis* sp. A in order to identify deleterious alleles (avoiding the sacrifice of potential carriers) (Moody et al., 1999; Hendrickson et al., 2004; Sordino et al., 2008). The rate of spawning hereby observed was 78 out of 102 specimens (76%), whilst that of self-fertility was 127 out of 186 (68%). Altogether, these results are in agreement with spawning (78.8-89.0%) and self-fertility rates (74%) observed by Sordino et al. (2008), as well as with the possibility to perform self-fertilization during the whole year, as suggested by long-term data by Caputi et al. (2014).

4.2.2. Searching for mutants: morphological and automated ISH screening procedures

Ciona intestinalis sp. A larvae has 6 developed organ systems (tunic, epidermis, notochord, tail musculature, adhesive organ and nervous system) and approximately 4 organ system rudiments (mesodermal pockets and pharynx, atrial and gut primordia) (Katz, 1983). Visual inspection of F1 larvae led to the identification of 23 possible mutations affecting larval morphology (listed in Table 1). Of these, 4 potential carriers showed phenotypes likely generated by multiple mutations (17%), whilst 19 accounted for single mutations (83%). Among single mutation carriers, different

phenotypic classes were recognized: 14 showed abnormalities affecting sensory organs (74%), 2 tail (10.5%), 2 trunk (10.5%) and 1 tunic (5%). Among these, the tunic mutation constitutes the first example of a previously unknown phenotypic class. Selected images of mutant larvae are reported in Figure 1. Obvious mutations resulted to be far easy to be identified because of the simple anatomy of *Ciona intestinalis* sp. A larvae, characterized by few cells and tissues (Katz, 1983). Frequency of mutation phenotypes was variable, but altogether around ~25% of F1 individuals (Table 1). That allowed to separate "mutants" (occurring in mendelian ratios) from "monsters", the latter mostly occurring due to poor gamete quality. Unfortunately, only 11 of these mutations were cryopreserved. In fact, following light/dark-induced spawning method, repetitive photoperiod alterations and induced spawning stressed the mutation carriers, thus leading to the absence of sperm production and the impossibility of cryopreserving a potential mutation (F. Crocetta, P. Cirino, P. Sordino, personal observation). Therefore, the dissection method followed by *in vitro* self-fertilization and early semen cryopreservation is strongly supported. Eventually, future screenings should include an additional concomitant outcross with w/t eggs as a further contribution to line preservation (in addition to frozen sperm) and to grow the F1 for inheritance test and phenotype characterization.

During my experimental procedures, I searched for subtle mutations by means of riboprobes against transcripts of genes expressed in specific organs or tissues of *Ciona* larvae. However, *in situ* hybridization (ISH) procedures may not constitute a reliable method when observing a possible reduction of gene transcription patterns. When loss of staining sites was detected, it was always coupled with clear morphological abnormalities (see below). Future screening for subtle phenotypes may utilize transgenic strategies and fine molecular and cellular markers, such as DAPI staining of nuclei.

4.2.3. Growing ascidian mutant lines

After sperm thawing, recovery of cryopreserved lines was successfully achieved, besides a rate of mortality higher than expected. Growth rate (as mean±standard deviation), based on measurements of 20 randomly selected specimens per week, is reported in Figure 2 together with explicative images. Male gamete production in growing *Ciona* was observed after 14 weeks (Figures 2A, 2F), and egg production after 17 weeks (Figures 2A, 2G). All cryopreserved mutations were validated by re-screen, therefore suggesting that visual screening constitutes a valid tool for detecting mutations in Mendelian ratio, even without re-screen validation.

4.2.4. ISH and FISH on sensory organ mutants

Appropriate riboprobes were selected for *in situ* hybridization (ISH) and fluorescent *in situ* hybridization (FISH) on mutant phenotypes. Sensory organ mutants at larval stage were subjected to five riboprobes involved in melanin pigmentation and photo-transduction processes: *Tyrosinase* (Ci-*Tyr*), *Tyrosinase Related Protein-1* (Ci-*Trp1/2a*), *Tyrosinase Related Protein-2* (Ci-*Trp1/2b*), *Opsin* (Ci-*Opsin*) and *Arrestin* (Ci-*Arrestin*).

General overview on sensory organs and selected riboprobes

- **Melanin pigments**: melanin pigments are heterogeneous macromolecules derived mainly from the oxidation of tyrosine (Plonka & Grabacka, 2006). Melanin biosynthesis is a multi-step process that comprises several biochemical reactions, mainly at the hand of three highly conserved enzymes: *Tyrosinase*, *Tyrosinase Related Protein-1* and *Tyrosinase Related Protein-2*. All together, they catalyze melanin biosynthesis, using the amino-acid L-tyrosine as initial substrate. In particular, *Tyrosinase* is considered the key enzyme of the pathway because, solely, it is responsible for the first two reaction steps, and also catalyzes one of the final steps of melanin biosynthesis. A survey of the *Ciona intestinalis* sp. A genome revealed the presence of three tyrosinase family genes, one *tyr* (Ci-*Tyr*) and two *tyrps* (Ci-*Tyrp1/2a* and Ci-*Tyrp1/2b*) (Esposito et al., 2012). All these genes have been demonstrated to be specific markers of *C. intestinalis* sp. A pigment cell lineage from late gastrula stage. In particular, Ci-*Tyrp1/2a* is the first to be expressed, from late gastrula stage, in the a9.49 blastomere pair, which corresponds to the pigment cell precursors and then inherited in both the a9.49 progeny (the a10.97 and a10.98 pairs) at middle and late neurula stages. The expression persisted up to the larva stage, where the posterior a10.97 cells differentiate into the otolith and ocellus pigment cells. Ci-*Tyr* and Ci-*Tyrp1/2b* expression territories were overlapping with that of Ci-*Tyrp1/2a*, the only difference being that their transcriptional activation was detected later than Ci-*Tyrp1/2a* at middle and late neurula stages (Esposito et al., 2012).

- **Photo-transduction process**: in vertebrates, the photo-transduction process is mediated by the visual *Opsins*, G-protein coupled receptors, and the visual *Arrestins*, small proteins needed to regulate *Opsin* signal transduction (Arshavsky, 2002; Blomhoff & Blomhoff, 2006). In tunicate genomes, including *Ciona intestinalis* sp. A, one *Arrestin* and three *Opsin* genes have been found, expressed mainly in ocellus photoreceptor cells. On the basis of sequence conservation, they

appeared closely related to vertebrate *Arrestin* and *Opsin* genes, expressed in photoreceptor cells of the retina and pineal organ (Kusakabe et al., 2001; Nakagawa et al., 2002; Nakashima et al., 2003).

Main results

The expression patterns of Ci-*Tyrp1/2a* and Ci-*Tyr* genes were investigated, at larval stage, in *mutant 16*, showing a pale ocellus pigment (Figure 1D). FISH experiments revealed that the two genes had normal expression patterns (localized in the pigment cells otolith and ocellus) in all the analyzed larvae, and that no differences occurred when compared to wild type control larvae (Figures 3A, B; data not shown). Although the mutant phenotype suggests disrupted expression of one of the master genes involved in the biosynthetic pathway of melanin, my results showed that these enzymes are not altered at a transcript level. Similar results were obtained in *mutant 17* with the same morphological abnormality. ISH experiment revealed no differences in the expression of the Ci-*Arrestin* gene when compared to wild type control larvae (Figure 3E; data not shown).

In *mutant 19*, showing no otolith and ocellus pigments, melanin pigments and photo-transduction process were analyzed by double FISH with Ci-*Arrestin* and Ci-*Tyrp1/2b* probes, at larval stage (Figure 1E). In mutant larvae, Ci-*Tyrp1/2b* (red) is expressed in the otolith pigment cells and ocellus region, whilst Ci-*Arrestin* (green) in the ocellus region only. However, endogenous transcripts were observed in photoreceptor and pigment cells with the same distribution and staining intensity as in wild type larvae (Figure 3D; data not shown).

Mutant 13 and *mutant 18* showed a clear duplication of the pigmented cell of the ocellus, even if it was not clear if all ocellus components were duplicated in these mutants. These mutant larvae were respectively subjected to ISH with Ci-*Arrestin* and to FISH with Ci-*opsin3*, as specific markers of photoreceptor cells. In this case, both the ocellus pigmented cells were surrounded by a clear hybridization signal with both Ci-*Arrestin* and Ci-*opsin3*, with the same distribution and staining intensity as in wild type larvae (Figures 3C, 3F; data not shown).

Taken altogether, the results obtained on sensory organ mutants would suggest that "pale" (*mutants 16, 17*) and "albus" (*mutant 19*) mutations were simply impaired in pigmentation biosynthesis, and that a limited alteration occurred in pathway leading to pigment formation and not in photoreceptor and pigment cell specification. Instead, results obtained on double ocellus mutants (*mutants 13, 18*) clearly suggest the duplication of pigmented cell and photoreceptors if not of the entire ocellus structure. Ocellus formation in the *Ciona* trunk is governed by left-right patterning processes, suggesting that mutation phenotypes with duplicated ocellus are due to heterozygote recessive alleles of the gene regulatory network that controls left-right asymmetry.

4.2.5. ISH on a trunk mutant

Appropriate riboprobes were selected for preliminary ISH on the trunk mutant. In particular, it was subjected to two riboprobes usually expressed in the endoderm, all tested at larval stage: Thyroid specific transcription factor 1 (Ci-*Titf1*) and Nuclear Receptor 1 (Ci-*NR1*).

General overview on endoderm and selected riboprobes

All the endodermal cells of ascidian larvae are derived from the two pairs of vegetal blastomeres of the eight-cell embryo. After three cell divisions, at the 64-cell stage, the endodermal fate is restricted to five blastomere pairs that give rise to approximately 500 endoderm cells of the larva, located in both the trunk and the tail (endodermal strand) (Nishida, 1987). *Ciona* larvae, as other ascidian species, do not feed since they are not provided of functional digestive organs. Trunk endoderm appears as a mass of undifferentiated cells rich in yolk granules. Only after metamorphosis, larval endodermal cells become differentiated into specialized endodermal organs of juveniles as endostyle, branchial sac, peribranchial epithelium, digestive organs, peripharyngeal band and dorsal tubercle. However, despite the lack of evident organogenesis, it has been demonstrated, in the ascidian *Halocynthia roretzi*, that the developmental fates of larval endodermal cells are almost fixed and a certain degree of regionalization in this tissue already exists before the metamorphosis. Indeed, a study centered on fate map of adult endodermal organs in *H. roretzi* larval endoderm showed that the anterior endoderm forms the branchial basket (branchial sac, endostyle, peripharyngeal band, dorsal tubercle, and peribranchial epithelium) and the posterior endoderm develops into the digestive tract, consisting of the esophagus, stomach, and intestine (Hirano & Nishida, 2000).

- Thyroid specific transcription factor 1: Ci-*Titf1* demarcates the endodermal blastomere cells since the earliest fate restriction of this territory (64-76 cell stage). Furthermore, from the middle tailbud and up to the larval stages, its expression is localized in the most anterior and ventral parts of head endoderm, regions which give rise, after metamorphosis, to the adult endostyle (Ristoratore et al., 1999).

- Nuclear receptor 1: Ci-*NR1* codes for an ortholog of vertebrate thyroid hormone receptors and is expressed in the endodermal territories, starting from neurula stage, being localized in the most posterior part of the trunk endoderm from tailbud up to the larval stages (Carosa et al., 1998).

Main results

The expression patterns of Ci-*Titf1* and Ci-*NR1* were investigated, at larval stage, in *mutant 23*, showing clear aberrations in the trunk which appears smaller, round shaped or somehow reduced and not well organized; also the palp formation seems to be affected. ISH experiments clearly showed that both genes are not expressed in the mutant larvae (Figures 3H, 3J) when compared to wild type control larvae (Figures 3G, 3I). It indicates that the pathways controlling endoderm differentiation are strongly affected during *mutant 23* embryos development.

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N Mutation	SS	SF	%	Phenotypic class	Description	Cryo	Additional validation	Figures
mutant 1	FuL	light/dark	25	sensory organs	pale ocellus pigment	---	---	---
mutant 2	FuL	light/dark	26	tail	shorter tail	---	---	---
mutant 3	FuL	light/dark	24	sensory organs	no pigments	---	---	---
mutant 4	FuL	light/dark	22	sensory organs	double ocellus	---	---	---
mutant 5	FuL	light/dark	26	multiple	External sensorial vesicle, shorter tail	---	---	---
mutant 6	FuL	light/dark	28	sensory organs	no pigments	---	---	---
mutant 7	FuL	light/dark	25	sensory organs	no pigments	---	---	---
mutant 8	FuL	light/dark	25	trunk	small and round trunk	---	---	---
mutant 9	FuL	light/dark	27	sensory organs	double ocellus	---	---	---
mutant 10	FuL	light/dark	23	multiple	double ocellus; shorter tail	---	---	---
mutant 11	FuL	light/dark	27	sensory organs	double ocellus	---	---	---
mutant 12	FuL	light/dark	27	sensory organs	no pigments	---	---	---
mutant 13	FuL	dissection	25	multiple	double ocellus; shorter tail	D. blue 07-03-2012	rescreen	Figs. 1A, 3F
mutant 14	VC	dissection	28	tail	shorter tail	Red 07-03-2012	rescreen	Fig. 1B
mutant 15	VC	dissection	27	sensory organs	double ocellus	Green 14-03-2012	rescreen	Fig. 1C
mutant 16	VC	dissection	26	sensory organs	pale ocellus pigment	Pink 19-03-2012	rescreen	Figs. 1D, 3A, B
mutant 17	VC	dissection	25	sensory organs	no ocellus pigment	Purple 19-03-2012	rescreen	Fig. 3E
mutant 18	VC	dissection	26	sensory organs	double ocellus	L. blue 11-04-2012	rescreen	Fig. 3C
mutant 19	FuL	dissection	27	sensory organs	no pigments	Yellow 23-04-2012	rescreen	Figs. 1E, 3D
mutant 20	VC	dissection	28	tunic	failed tunic development	Pink 02-05-2012	rescreen	Fig. 1F
mutant 21	VC	dissection	24	multiple	double ocellus; shorter tail	Grey 02-05-2012	rescreen	---
mutant 22	VC	dissection	27	sensory organs	double ocellus	Purple 02-05-2012	rescreen	---
mutant 23	VC	dissection	28	trunk	small and round trunk	Yellow 02-05-2012	rescreen	Figs. 1G, 3H, J

Table 1. Naturally occurring mutations identified during the October 2011-May 2012 screening. Abbreviations: N, number; SS, sampling site; FuL, Fusaro Lake; VC, Villaggio Coppola; SF, self-fertilization procedures; %, percentage of mutants larvae; Cryo, cryopreservation; L., light; D., dark.

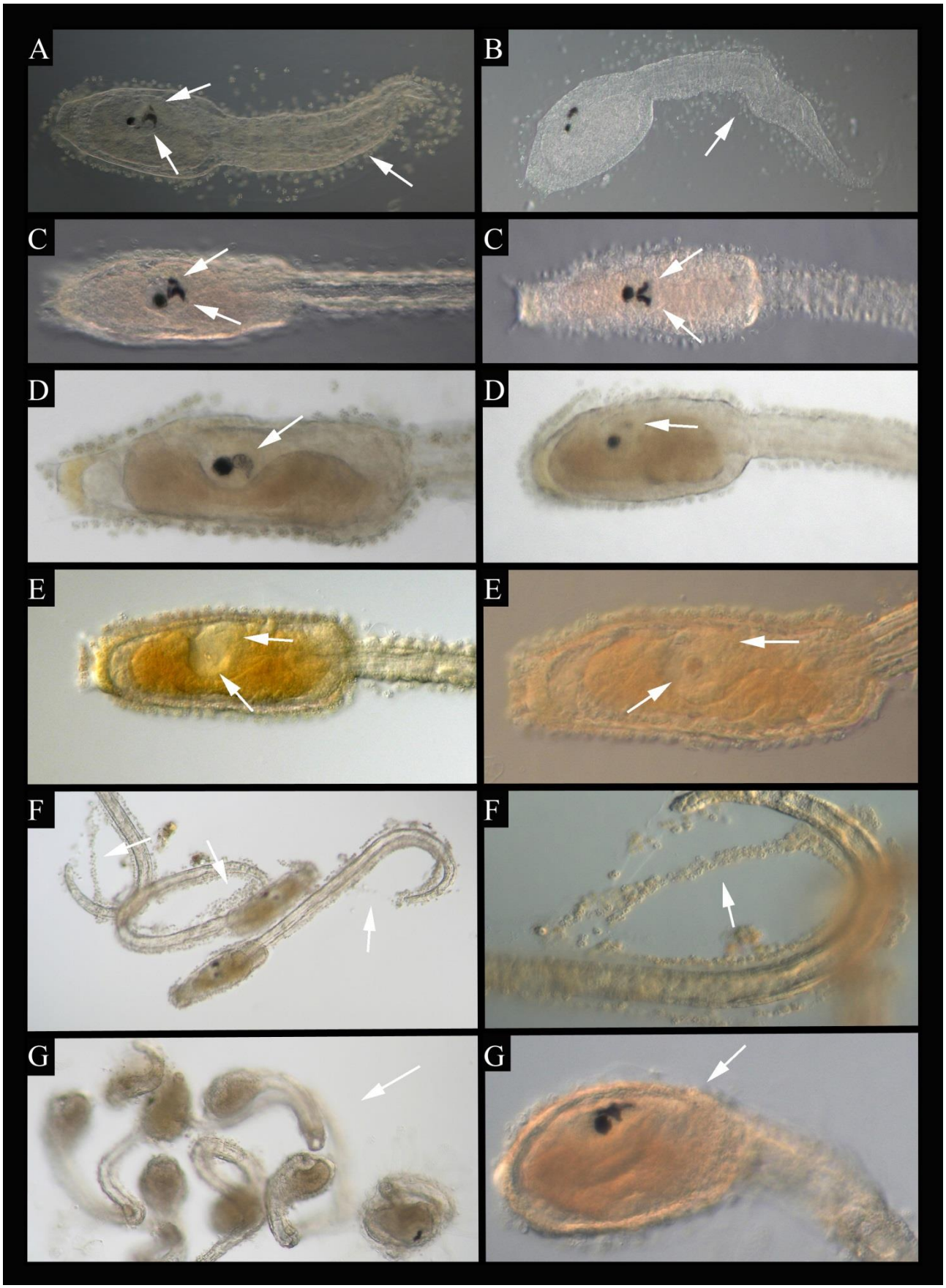

Figure 1. Photos of selected mutants larvae. Morphological abnormalities as described in Table 1. A. *mutant 13*. B. *mutant 14*. C. *mutant 15*. D. *mutant 16*. E. *mutant 19*. F. *mutant 20*. G. *mutant 23*.

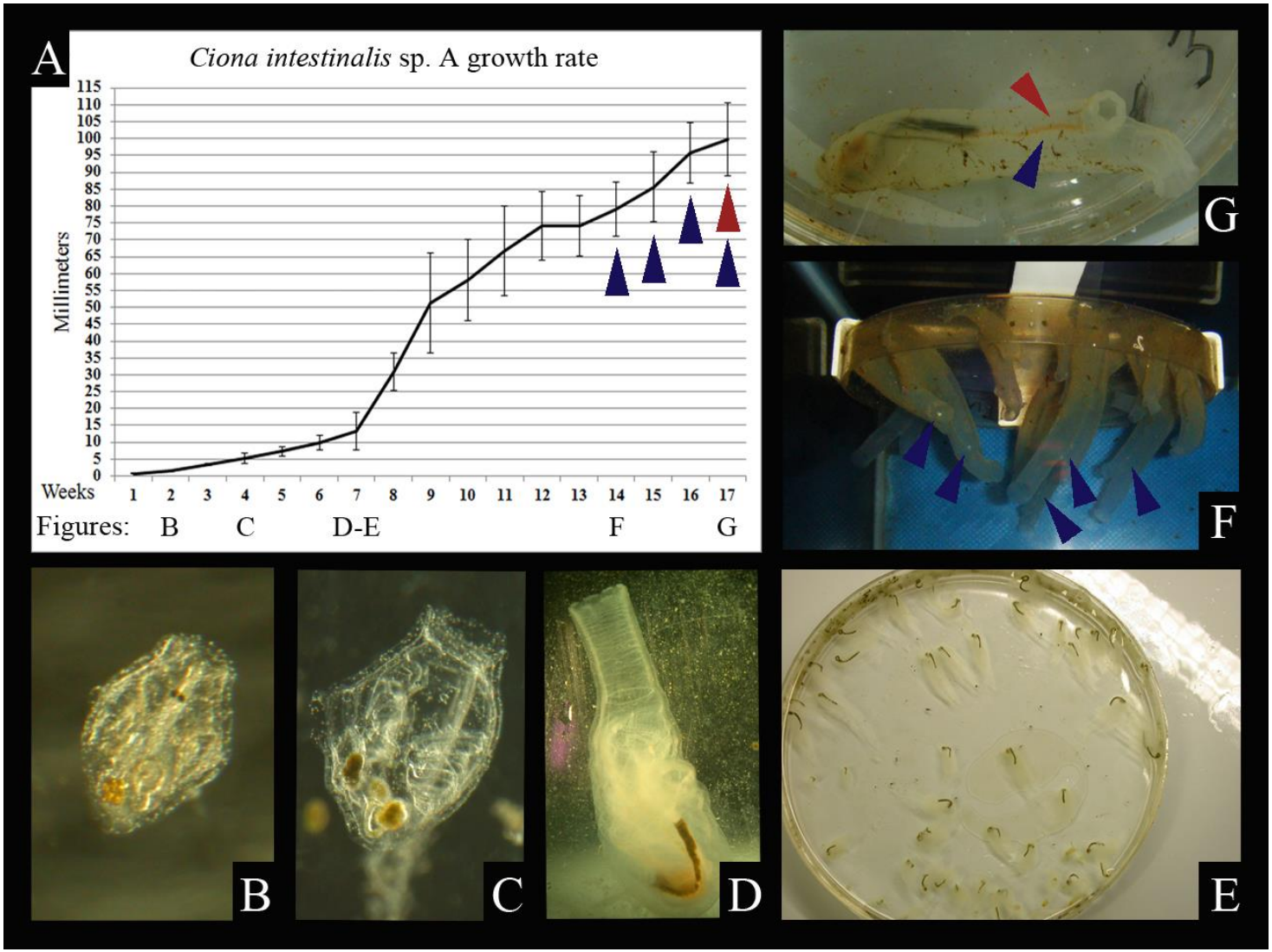

Figure 2. *Ciona intestinalis* sp. A growth rate and explicative photos. Blue arrow: presence of sperm. Red arrow: presence of eggs. A. Growth rate. Error bars: standard deviations. Weeks values (in millimeters) represent the average of 20 randomly selected specimens per week. Letters under weeks corresponding to explicative figures in plate. B. 2 mm. C. 7 mm. D-E. ~14 mm. F. ~8-9 cm. G. 10 cm.

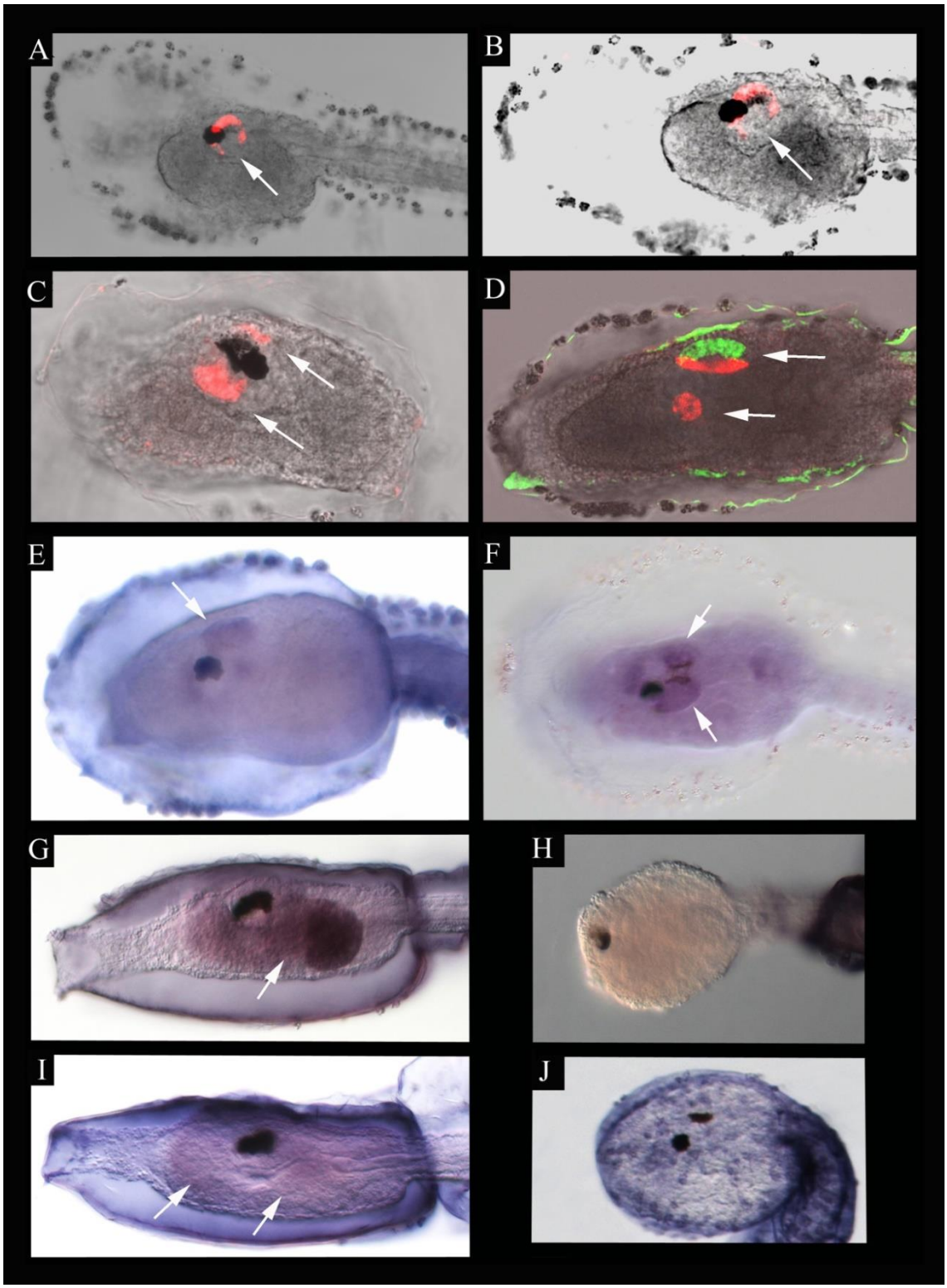

Figure 3. Preliminary ISH and FISH on selected mutants larvae. Morphological abnormalities as described in Table 1. Expression patterns highlighted by white arrows. A. *mutant 16*. FISH with Ci-*Tyrp1/2a*. B. *mutant 16*. FISH with Ci-*Tyr*. C. *mutant 18*. FISH with Ci-*opsin3*. D. *mutant 19*. Double FISH with Ci-*Arrestin* and Ci-*Tyrp1/2b*. E. *mutant 17*. Automated ISH with Intavis InsituPro system (including Ci-*Arrestin*). F. *mutant 13*. Automated ISH with Intavis InsituPro system (including Ci-*Arrestin*). G-I. *mutant 23* (H,J) and wild type larvae (G,I) from the same F1. G.H. ISH with Ci-*NR1*. I.J. ISH with Ci-*Titf1*.

CHAPTER 2 - *MAGOH* IN BASAL CHORDATES: INSIGHTS INTO THE EVOLUTION OF THE BRAIN

1. INTRODUCTION

The RNA-binding exon-junction complex (EJC) is a transient binding platform for many nuclear and cytoplasmic factors involved in mRNA processing like splicing, translation, localization and non-sense decay (Tange et al., 2004; Giorgi & Moore, 2007; Le Hir & Seraphin, 2008). The EJC consists of four core subunits, three of which (*Magoh*, *Y14* and *elF4A*) constitute the pre-EJC. The EJC, pre-EJC and EJC components system ensures tight control on spliced mRNA decisions throughout the life of eukaryotic organisms by exerting general and specific binding properties (Roignant & Treisman, 2010). Localization of specific mRNAs by means of EJC-mediated cytoskeleton rearrangements generates cell asymmetry for germ cell specification and for the anteroposterior (AP) and dorsoventral (DV) axes (Micklem et al., 1997; Newmark et al., 1997; Li et al., 2000; Hatchet & Ephrussi, 2001; Wiens et al., 2006). The role of *Magoh* (also known as *Mago Nashi*) in the initial establishment phase of axial patterning has been well characterized in *Drosophila*, where the orthologous protein *Mago Nashi* controls the transport of *oskar* mRNA at the posterior pole and the migration of the oocyte nucleus at the dorsal pole (Boswell et al., 1991; Newmark & Boswell, 1994; Micklem et al., 1997; Hatchet & Ephrussi, 2001).

Functional studies of *Magoh* orthologs in vertebrate cells and embryos reveal a distinct scenario when compared to protostomes. While this pre-EJC and EJC core factor is not required in axial patterning, it has newly evolved a role in cell differentiation and renewal in the central nervous system. In *Xenopus*, *Magoh* expression is restricted to neural tissues at neurula stage. Morpholino injection-mediated loss of *Magoh* results in a significant reduction of neural crest (NCC)-derived pigment cells in *Xenopus* tadpoles (Kenwrick et al., 2004; Haremaki et al., 2010). In mice, *Magoh* knockout is early lethal at E9.5. However, the heterozygote phenotype is partially penetrant, showing microcephaly and melanocyte reduction due to defective mitosis of neural stem (NSC) and NCCs, respectively (Silver et al., 2010, 2013). The EJC or EJC components interacts with nuclear actin and cytoplasmic *Lis-1*, a microtubule-associated protein essential for mitotic spindle integrity in vertebrates. Reorganizing the cytoskeleton, *Magoh* regulates the balance of asymmetric, proliferative and neurogenic neural stem cell (NSC) divisions (Silver et al., 2010). Moreover, *Magoh* is found within a 55-gene deletion on chromosome 1p32.3 that is associated with mental retardation and abnormalities in brain size (Brunetti-Pierri et al., 2008).

The creation of new splice forms typically changes protein structure more than the evolution of the protein after gene duplication, allowing a much larger number of proteins available for mediating new functions. It was suggested that the elaboration of additional transcripts was central to changes in size and internal organization during brain evolution (Holland & Short, 2008; Bae et al., 2014). The overall size of the brain plays an important part in neural adaptation and has significant implications for patterns of internal organization: as the absolute size of the brain increases, interacting neurons are located further apart and the brain is likely to become more modular in organization (Kaas, 2006).

The paucity of information on the evolutionary nature of EJC functions in Chordates can be addressed in simple prototypical ascidian and cephalochordate embryos. Protochordates are crucially important to comparative zoologists for the clues they provide concerning the course of early chordate evolution and the nature of ancestral vertebrates. Here, I present an *in silico* and *in vivo* analysis of *Magoh* in the cephalochordate *Branchiostoma lanceolatum* (Pallas, 1774) and the ascidian tunicate *Ciona intestinalis* sp. A. The ancestry and conservation of *Magoh* orthologs in Eukarya suggests that this EJC component serves basic cellular functions. *Magoh* mRNA processing in Amphioxus and *Ciona* is tightly regulated at the maternal and zygotic level, with transcriptional and translational domains revealing a combination of protostome and deuterostome conditions, consistent with a multipurpose function in different tissues and timings during development. My findings suggest that *Magoh* functions in the early establishment phase of axial patterning in tunicates, and that this role has been lost in cephalochordates and craniates. In turn, transient *Magoh* expression in endoderm precursors of *Ciona* and amphioxus embryos might be the vestige of a protostome condition or, alternatively, independent innovations. EJC or EJC components were therefore recruited in neural progenitors during early chordate line of descent. Finally, I discuss what sort of insights can be gained from *Magoh* expression and genomic organization in basal chordates in terms of evolution of the central nervous system.

2. MATERIALS AND METHODS

2.1. Bioinformatics

2.1.1. Phylogenetic analysis

Aminoacidic sequences used in my phylogenetic analysis were retrieved from the NCBI database. The protein set was aligned using ClustalW (Thompson et al., 1994) and Mega5 (Tamura et al., 2011) with default parameters. Phylogenetic tree reconstruction was carried out using the Bayesian method, using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Two independent runs of 1.2 million generations each were performed, and convergence was reached when the value for the standard deviation of split frequencies stayed <0.01. The bootstrap value for each node is reported, and a color code is used to group the phylogenetic taxa (Figure 1A).

2.1.2. Synteny conservation analysis in Chordates

The presence of synteny conservation was analyzed using the Genomicus (v74.01) (Louis et al., 2013) in addition to manual searches in amphioxus (*B. floridae*, JGI v2.0) and vase tunicate (*C. intestinalis*, JGI v2.0) genomes. In Figure 1B, I show the most conserved genes around the *Magoh* locus, therefore lineage-specific insertions of genes were discarded (the position is indicated by a vertical bar).

2.2. Biological material

2.2.1. Sampling and acclimatization procedures

Ciona intestinalis sp. A

Adult specimens were collected in the Fusaro Lagoon by hand picking at low depth, and kept in a seawater tank during transport up to the arrival at SZN (Stazione Zoologica Anton Dohrn, Naples) laboratory facilities. Animals were acclimatized for 2-3 days in open system tank setups at ~20°C. Ripe individuals were then exposed to continuous illumination for 2-3 more days in order to accumulate gamets or to prevent gamete spawning, and fed every day with a solution of marine microalgae concentrates (Shellfish Diet 1800™ Instant Algae®: 0.5 ml in 1 liter of sea water).

Branchiostoma lanceolatum

Adult specimens were collected in two different sites of the Gulf of Naples by ship trawling at

10-15 m depth, and kept in a seawater tank during transport up to the arrival at SZN laboratory facility. Animals were quarantined (observation and antibiotics treatment to prevent bacterial contamination) for 7 days, and then introduced in the open marine water system at ~17°C with a 10-14 h day-night light cycle in tanks containing sandy substrate from the original sampling sites. Adult animals were fed every day with 200 ml of fresh microalgae mixture (*Dunaliella tertiolecta*, *Isochrysis galbana* and *Tetraselmis suecica*) at SZN laboratory facility.

2.2.2. Gamete sampling and embryo collection

Ciona intestinalis sp. A

Ripe specimens were dissected at the base of the atrial siphon with a sterile blade in order to expose gonoducts. To avoid self-fertilization, only one kind of gamete was collected from each specimen. Sperm was collected from the spermiduct by using sterile Pasteur pipettes. Workable sperm samples were then pooled into pre-chilled 1.5-2 mL vials in ice. Eggs were collected with Pasteur pipettes and transferred in a 9 cm Petri dish filled with 0.22 μM Millipore filtered natural sea-water (MFSW) at 18°C and 38 practical salinity units (psu). Workable egg samples from different individuals were pooled in 9 cm Petri dishes where they were allowed to expand the chorion and the follicle cells, which make the eggs floating and improve fertilization. Fertilization assays were carried out in 9 cm Petri dishes filled with 10 mL 0.22 μM MFSW at 18°C and 38 psu, and different developmental stages of *C. intestinalis* sp. A were fixed overnight in 4% paraformaldehyde (PFA) in 3-(N-morpholino)propanesulfonic acid (MOPS) and further dehydrated to 70% ethanol (ETOH).

Branchiostoma lanceolatum

Single ripe specimens were induced to spawn by heat shock, as previously described (Fuentes et al., 2007). Eggs and sperms were collected with sterile plastic Pasteur pipettes and mixed in a 9 cm Petri dish filled with 0.22 μM MFSW at 19°C and 38 psu to obtain fertilization (final sperm dilution 1:1000). Embryos were reared in filtered seawater at 19°C in incubator. Desired developmental stages were fixed overnight at 4°C in 4% PFA in a buffer containing 0.1 M MOPS, 0.5 M sodium chloride (NaCl), 2 mM magnesium sulfate ($MgSO_4$), 1 mM ethylene glycol tetraacetic acid (EGTA) pH 7.4, and further dehydrated to 70% ETOH.

For both species, fixed developmental stages were then stored at –20ºC for subsequent *in situ* hybridization and immunohistochemistry analyses.

2.3. Magoh transcription and translation

2.3.1. Digoxigenin-labeled antisense and sense riboprobe synthesis

Ciona intestinalis sp. A

Two oligonucleotides (Forward: 5'-GGAGTTTGAATTTCGACCAG-3'; Reverse: 5'-CTCTCATTGGGCTGCATTTC-3') were designed to amplify a ~350 bp *Magoh* fragment from ovary cDNA, soon after the identification of the gene sequence in the *C. intestinalis* sp. A EST database (Satou et al., 2001, 2002).

Branchiostoma lanceolatum

The availability of the *Branchiostoma floridae* genome database (a sister species of the hereby analyzed amphioxus) (Putnam et al., 2008) allowed us to design two oligonucleotides (Forward: 5'-ATGGCTTCCAACGATTTCTATC-3'; Reverse: 5'-CTAGATGGGTTTAATCTTGAAG-3') to amplify a 441 bp *Magoh* fragment.

For both species, PCR amplifications were performed in 50 μL containing 5 μL 10× Roche PCR reaction buffer + Mg, 5 μL 10mM Roche PCR Grade Nucleotide (dNTPs) Mix, 5 μL each primer (10 mM), 5 μL 10× BioLabs Purified Bovine Serum Albumine (BSA), 0.5 μL Roche Taq DNA Polymerase and 1 μL cDNA. The cDNA fragment was amplified with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 60 s and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR-amplified cDNA fragment was run on a 1.5% (*C. intestinalis* sp. A) and 1% (*B. lanceolatum*) agarose gel, and the *Magoh* fragment was first purified with the Qiagen Gel Extraction Kit following the manufacturer's instructions and then cloned into Dual TOPO-TA cloning Kit (Invitrogen). The pCRII-TOPO vector, containing *Magoh*, was introduced into One Shot TOP10 chemically competent *Escherichia coli* cells by thermal shock. White-blue colony screening was performed on a LB/Amp plate supplied by IPTG/X-GAL, and white colonies were picked up and both transferred into PCR tubes to perform colony PCR using M13F and M13R primers and inoculated in LB medium with ampicillin (50 μg/mL). Plasmid DNA miniprepping (with the Qiagen QUIAprep), sequencing with Applied Biosystems Automated 3730 DNA Analyzer, and

subsequent maxiprepping (GenElute Plasmid Maxiprep Kit) were performed. Digoxigenin (DIG)-labeled antisense and sense riboprobes for *Magoh* were synthesized using a template linearized with *Xho*I and transcribed using SP6 RNA polymerase for the antisense probe, or linearized with *Kpn*I and transcribed using T7 RNA polymerase for the sense probe. Quantification of riboprobes was performed by DOT-BLOT analysis.

2.3.2. *In situ* hybridization (ISH) and immunohistochemistry (IHC) analyses

Ciona intestinalis sp. A

ISH - *In situ* hybridization analyses on developmental stages of *C. intestinalis* sp. A were hand-performed as previously described (Christiaen et al., 2009). Pre-larval stages were manually dechorionated. ISH experiments were all performed in triplicate with consistent results. The sense riboprobe was tested in order to confirm signal specificity.

IHC - Two synthetic peptides, NH_2-KIGSLVDVTDCKDSDG-COOH and NH2-AVLEELKRVIDDSEIMK-COOH, corresponding respectively to the predicted immunogenic Magoh epitopes 111-126 and 63-79, were used to immunize rabbits (PRIMM, Milan, Italy). Two specific anti-sera, C67 and D67, have been obtained and used for immunohistochemical studies in *C. intestinalis* sp. A as following: after rehydration in phosphate-buffered saline (PBS) 1X, pre-larval stages were manually dechorionated. Following, permeabilization was carried out in ice-cold acetone for 10 minutes at –20°C. Then, samples were incubated for 1 hour in IB solution (PBS 1X, 2% BSA, 1% dimethyl sulfoxide (DMSO), 0.5% TritonX-100) with 10% sheep serum (SS), followed by overnight incubation in IB solution with 1% SS with primary antibody diluted 1:1000 at 4°C. Hence, samples were washed (4 x 15 min, 1 x 1 h) in IB solution at room temperature, and then incubated overnight at 4°C with 1:200 secondary anti-rabbit antibody HRP-conjugated in IB solution with 1% SS. Unbound antibody was washed away with IB solution (4 x 15 min, 1 x 1 h) and PBS 1X (1 x 15 m) at room temperature. Controls were run in parallel by using the corresponding pre-immune rabbit IgG at the same dilution. Staining was performed using DAB tablets.

Branchiostoma lanceolatum

ISH - In situ hybridizations were performed essentially as previously described (Holland et al., 1996), with the following modifications. After rehydration, embryos were digested with 7.5 µl of Proteinase K for 5-30 min depending on the size and stage of development (Sky Yu & Holland,

2009). Pre-hybridization was conducted at 50-65°C with gentle shaking for at least 3 hours. Hybridization was performed overnight at 60°C with 50-200 ng of DIG-labeled probes. The embryos were blocked in 10% PBT (in phosphate buffered saline-Tween20 buffer) for at least 3 hours at room temperature, and then incubated in pre-absorbed 1:1500 anti-DIG-alkaline phosphatase (Roche) at 4°C overnight. After stopping the staining reaction, all developmental stages of both species were mounted in 80-100% glycerol and imaged using a Zeiss Axio Imager M1 microscope equipped with Axiocam digital camera (Zeiss).

3. RESULTS

3.1. Sequence conservation and gene duplication

Genetic and genomic databases analysis shows that *Magoh* is an ancient eukaryotic gene that duplicated once after the origin of vertebrates (Figure 1). The alignment of *Magoh* protein sequences revealed a very high aminoacidic identity that in some cases is the reason for poor resolution of the phylogenetic analysis. This is particularly true for protostomes (pink) and basal chordate (violet) sequences. On the other hand, all plant proteins grouped together and are highlighted by a green colour (Figure 1A). The overall conclusion of the phylogenetic analyses performed here is that *Magoh* has an extremely conserved structure in evolution and that the unique case of gene duplication is observed in Mammals. If this comes close to the truth it means that, for unknown reasons, *Magoh* is escaped from the vertebrate whole genome duplication, known as 3R (Ohno, 1970), and probably single events of gene duplication happened in Mammals (Figure 1A).

3.2. Synteny conservation in Vertebrates

Gene order on a chromosome of evolutionary distant species is often a symptom of a well established and conserved expression pattern in ontogenetic pathways (Kuraku & Meyer, 2012). For this reason I looked at the syntenic conservation of the *Magoh* gene locus being interested in finding an explanation for the duality of neural/non-neural expression patterns observed in evolution. Strikingly, I did not find any kind of conservation in neighbouring genes in the genomes of eukaryotic organisms including basal chordates, despite the fact that at least the amphioxus genome is considered very much static and hardly changed respect to the chordate's ancestor genome, one

example of which is the evolutionary conserved *Hox* cluster (Pascual-Anaya et al., 2008, 2012, 2013). Conversely, a high degree of syntenic conservation is detected in the genomes of terrestrial vertebrates (Tetrapods) (Figure 1B).

3.3. Early polarization and neural restriction of *Magoh* mRNA and protein in *Ciona intestinalis* sp. A

ISH - In ascidian, *Magoh* mRNA is both maternal and zygotic and shows dynamic regulation throughout development. Transcript labeling is quite uniformly distributed in the cytoplasm of the unfertilized egg (Figure 2A), but is rapidly restricted to the vegetal pole after fertilization (Figure 2B), as clearly seen at 2-cell stages (Figures 2C-E). At 4-cell, *Magoh* mRNA remains polarized in the cytoplasm of all blastomeres (Figures 2F-I). Then, transcript staining is observed in the nuclei of all 8-cell blastomeres (Figures 2H-I). From 16-cell to 76-cell stages, *Magoh* transcript is found in nuclei or cytoplasm of A8.5, A8.6 and B8.6 (notochord), A7.6 (trunk lateral cells, TLC), B8.5 and B7.7 (mesenchyme) blastula cells (Figures 2J-N). At 110-cell stage, the spatial profile of *Magoh* expression is similar to that observed in 76-cell stage (Figures 2O-P). In addition, a transient signal is found in endoderm progenitor cells A7.2 that will give rise to the oesophagus (Hirano & Nishida, 1997, 2000) (Figure 2O). Subsequently, *Magoh* transcription is present only in mesenchymal cells at gastrula (Figures 2Q-R), and is activated in the nervous system from neurula to larval stage (Figures 2S-X), followed by a second, transient domain of expression in the posterior notochord at tailbud (Figures 2U-V). Tadpole larvae show *Magoh* expression in palps, sensory vesicle, spinal cord and TLCs (Figures 2W-X).

IHC - The spatial-temporal analysis of *Magoh* protein synthesis during ascidian ontogenesis is mostly in line with mRNA patterns described above, adding interesting insights on protein function. Before fertilization, Magoh protein distribution shows a marked confinement (Figure 3A), unlike mRNA, that is further clear following sperm entry (Figure 3B). During the ooplasmic segregation that follows egg fertilization, immunochemical staining of *Magoh* protein shows a perinuclear signal around the female nucleus, in correspondence with the future posterior pole (Figure 3C). Prior any sign of cell division, the perinuclear domain splits presumably because of ongoing mitosis (Figure 3D). Then, the two signals separate and are positioned at the vegetal hemisphere (Figure 3E). At 2-cell stage, *Magoh* distribution is polarized to the vegetal pole to less extent than the mRNA pattern. In addition, a stripe of high protein signal at the vegetal pole

suggests the future plan of the second cell division (Figure 3F). Then, protein localization from 4-cell stage to tailbud stage is highly reminiscent of mRNA distribution (Figures 3G-P). At larval stage, *Magoh* protein is observed in specific anatomical domains (Figures Q-R), including the posterior sensory vesicle (Figures 3Q-R), the edge of syphon primordia, anterior and posterior to the sensory vesicle (Figure 3Q), and two axonal bundles elongating from the sensory vesicle into the spinal cord down to the tail end (Figure 3R) (white arrows). After metamorphosis, *Magoh* immunolocalization occurs in the oesophagus, in a group of ciliated epithelial cells abutting where gonads will form (Okada & Yamamoto, 1999; Yamamoto & Okada, 1999) (Figures 3S-U).

3.4. *Magoh* mRNA expression in *Branchiostoma lanceolatum*

Magoh is a maternal gene in amphioxus as it is already expressed in unfertilized eggs (Figure 4A) and persists during fertilization as well (Figure 4B), where it is possible to see the fertilization membrane clearly. During the first phases of embryonic cleavage, *Magoh* is equally distributed in the two, four and following blastomeres (Figures 4C-F). During gastrulation and neurulation, *Magoh* is extensively diffused in the whole embryo except for epidermis (Figures 4G-H), while few hours of development later it becomes clearly confined to the ventral part from the rostral to the most posterior regions (Figures 4I-J). This is presumably the precise developmental stage in which there is a switch of the maternal to the zygotic expression. As development follows, *Magoh* is strongly expressed in the region of the embryo where the mouth opening will soon be formed (red arrow), along the forming gut until the tailbud, with a faint expression in the brain vesicle (white arrow), that constitute the most rostral structure of the nervous system, in connection with the nerve cord (Figure 4K). Later in development the expression becomes more faint, but still visible: it is presumably expressed in the club shaped gland (green arrow), in the endostyle (red arrow) and in the preo-oral pit (white arrow) (Figure 4L). Overall the *Magoh* expression pattern in amphioxus during embryonic development is therefore ventral, a part from the brain vesicle.

4. DISCUSSION

Living members of phylum *Chordata* are divided into three groups: the *Cephalochordata* (e.g. amphioxus), the *Tunicata* (e.g. *Ciona*) and the *Craniata* (e.g. vertebrates). These animals are

united by a common body plan, a key component of which is the development of a neural tube dorsal to a notochord. Studying the genetics and embryology of these animals allows evolutionary comparison to be made between the mechanisms controlling the development of homologous body parts in different taxa.

This paper focuses specifically on the comparative analysis of *Magoh*, an exon-junction complex (EJC) component that plays a central role in mRNA processing events such as nonsense-mediated decay, translation, splicing and transportation. The interaction of the EJC with a wealth of peripheral partners allows specific functions in basic cellular functions and in developmental processes. For example, *Magoh* is essential in the initial establishment phase of dorso-ventral patterning in insects, and regulates proliferation and expansion of neural stem cells and neural-crest cells in mammals. Besides the fundamental importance of the EJC in mRNA processing, little is known about the activity of *Magoh* orthologs in animals other than the classical model organisms in genetics, such as *Caenorhabditis elegans* (*Nematoda*), *Drosophila melanogaster* (*Insecta*) and mouse (*Mammalia*).

The ancestry of the *Magoh* gene in organismal evolution can be traced back to yeast, and is reflected in a striking degree of sequence conservation, as noticeable when plotting the phylogenetic tree of *Magoh* proteins onto classical eukaryotic taxonomy. Mostly as a single copy gene, *Magoh* appears to have duplicated in the mammalian lineage, with lineage-specific events of paralogue gene loss. An extra duplication probably occurred, witnessed by a *Magoh* pseudogene, and appears to be a primate-specific event as it is found only in man and chimpanzee, and not in other mammals as mouse or dog. Gene order organization in the chromosomal region encompassing the *Magoh* locus reveals lack of syntenic conservation in all non-tetrapods genomes, suggesting that *Magoh* gene recombination may have provided new regulatory functions depending on the chromosomal context. Altogether, evolutionary conservation of the *Magoh* sequence suggests this protein has served an important role in mRNA processing throughout eukaryotic evolution, and was increasingly optimized in vertebrates by synteny and gene duplication.

Before fertilization, *Magoh* mRNA and protein are found in the entire ooplasm, except for a narrow subcortical region, likely towards the animal pole. Soon after fertilization, *Magoh* products become initially restricted toward the vegetal pole (future dorsal), co-localizing with myoplasm, and then move to the future posterior pole following ooplasmic segregation. Subcellular restriction of the *Ciona Magoh* ortholog since first cell divisions supports a conserved role in the polarisation of the oocyte cytoskeleton during the initial establishment phase of anterior–posterior axis patterning of tunicates. The way maternal dorsalizing activities are localized to the future dorsal side of the embryo differs between protostomes and deuterostomes. While a localized cascade of transforming

and epidermal growth factors, kinases and proteases activates a Toll pathway on the ventral side of the protostome *Drosophila* (Le Mosy et al., 1998), cortical rotation transports dishevelled protein dorsally where it triggers the downstream portions of a Wnt signalling pathway in the deuterostome *Xenopus* (Miller et al., 1999). Here, restriction of *Magoh* activity toward the future dorsal side of the tunicate embryo is in contrast with the expected dorsal-ventral inversion of anatomical topographies at the transition from protostomes to deuterostomes (Arendt & Nübler-Jung, 1994, 1997; Holley et al., 1995; De Robertis & Sasai, 1996). To be clarified, such discrepancy will need to be the subject of functional approaches. Interestingly, subcellular mRNA segregation during early development is absent in cephalochordates, likely due to a secondary change of the regulatory context.

At the two cells-stage, *Ciona Magoh* immunostaining in a vegetal stripe corresponding to the prospective plane of the second blastomere division suggests an association between Magoh activity and cell division mechanisms. Subsequently, *Magoh* mRNA and protein are observed in muscle/mesenchyme and neural precursors. The mesodermal pattern changes at 64-cells stage, when the two muscle and mesenchyme differentiation fates separate. From now on, *Magoh* expression persists only in the lineages that give rise to the dorsal neuroectoderm and mesenchyme, including trunk lateral cells (TLC) that will give rise to blood and tunic (Hirano & Nishida, 1997). Further, the detection of *Magoh* protein in a region of the oesophagus adjacent to the area of gonad formation lends support to a role in gonadogenesis (Boswell et al., 1991; Newmark & Boswell, 1994; Micklem et al., 1997; Mohr et al., 2001). *Magoh* expression in mesoderm and endoderm precursors of ascidian and amphioxus larvae is in line with data from insects to man. In human, *Magoh* is transcribed in gastric epithelial progenitor cells but with no apparent function in differentiation (Micklem et al., 1997; Newmark et al., 1997; Mills et al., 2002). However, there are preliminary indications that the genetic program initiating ventral mesoderm formation in amphioxus may not be involved in the specification of the amphioxus ventral mesoderm as a whole (Panopoulou et al., 1998).

The key features of organization across nervous systems and their surprisingly common genetic bases such as many genes involved in patterning the nervous system are common to all bilaterally symmetrical animals and have been inherited from a common ancestor. The transition to the new craniate head was a sudden event of considerable complexity. Large brains with elaborate architecture are a classic example of complex biological structures that are thought to have evolved independently within various groups of protostomes and within craniates (hagfishes and vertebrates). Among deuterostomes, craniates likewise have large, elaborate brains with diverse peripheral sensory systems. The evolutionary expansion of the cerebral cortex in the lineage leading

to humans has been interpreted as the result of variation in neurogenesis later in development, when cells in preestablished compartments proliferate, die, and/or differentiate into mature neurons and glia cells. According to the radial unit hypothesis, simply altering the first of the three phases of cell division that produce cortical excitatory neurons can scale the size of the cortex (Rakic, 1995). In contrast, the intermediate progenitor hypothesis supports more neurogenesis during the final phase of proliferation (Kriegstein et al., 2006). In this context, recruitment of *Magoh* expression in the central nervous sytem of chordates is perhaps better understood for the EJC role in the creation of alternative splice forms to generate a much larger number of proteins available for mediating new functions.

Here, genetic, genomic and developmental data provide unique insights into possible EJC functions in the evolution of the chordate brain. Understanding the evolutionary path connecting the EJC function to NSC regulation might illuminate the evolution of the central nervous system in chordates and the etiology of brain pathologies like microcephaly and depression (Segman et al., 2005).

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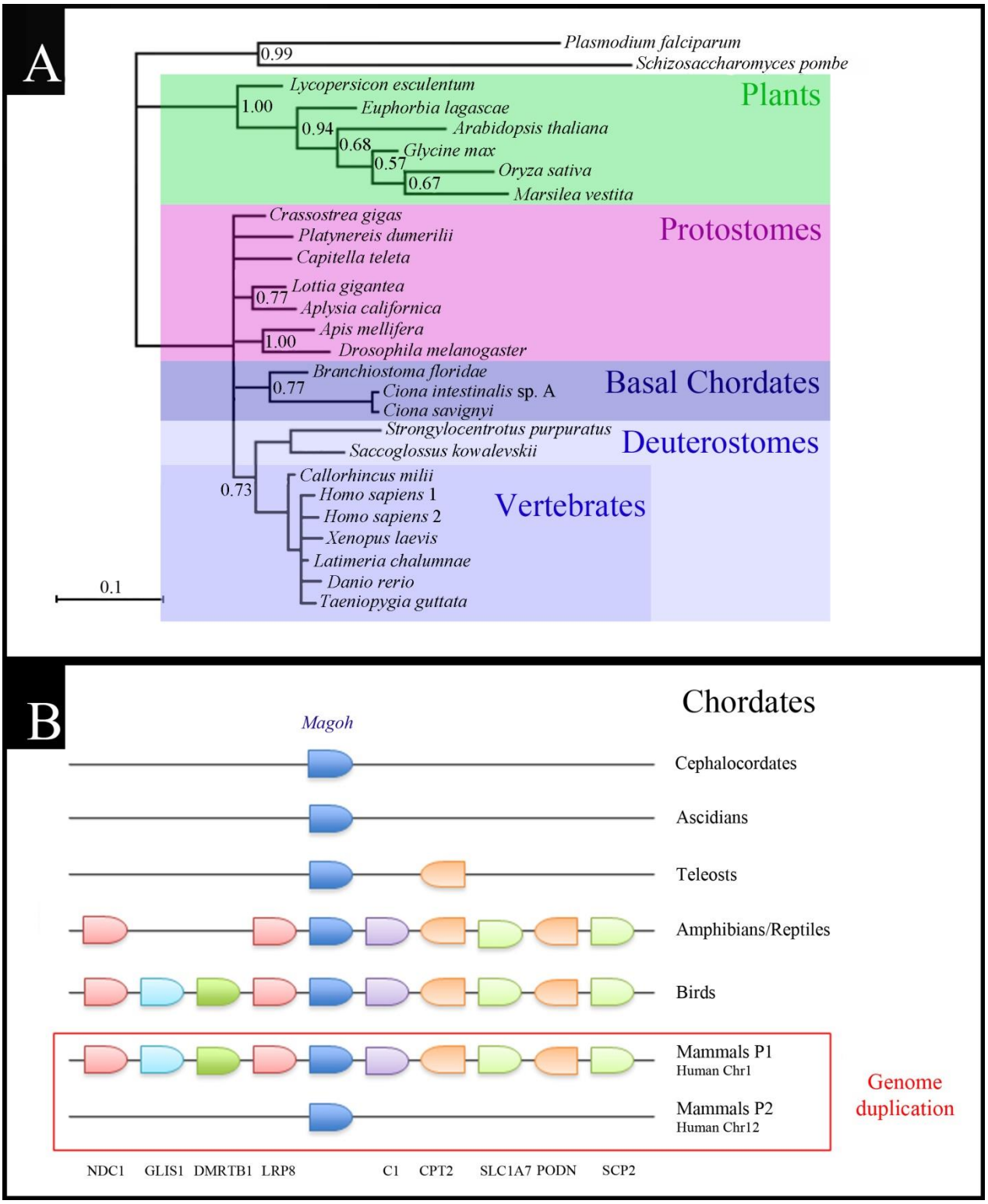

Figure 1. Molecular phylogeny and genomic organization of chordate *Magoh* orthologous genes. A. Phylogenetic tree based on Bayesian Inference method. B. Syntenic conservation in vertebrates and duplication event in Mammals. Abbreviations used: P, paralogons; Chr, chromosome.

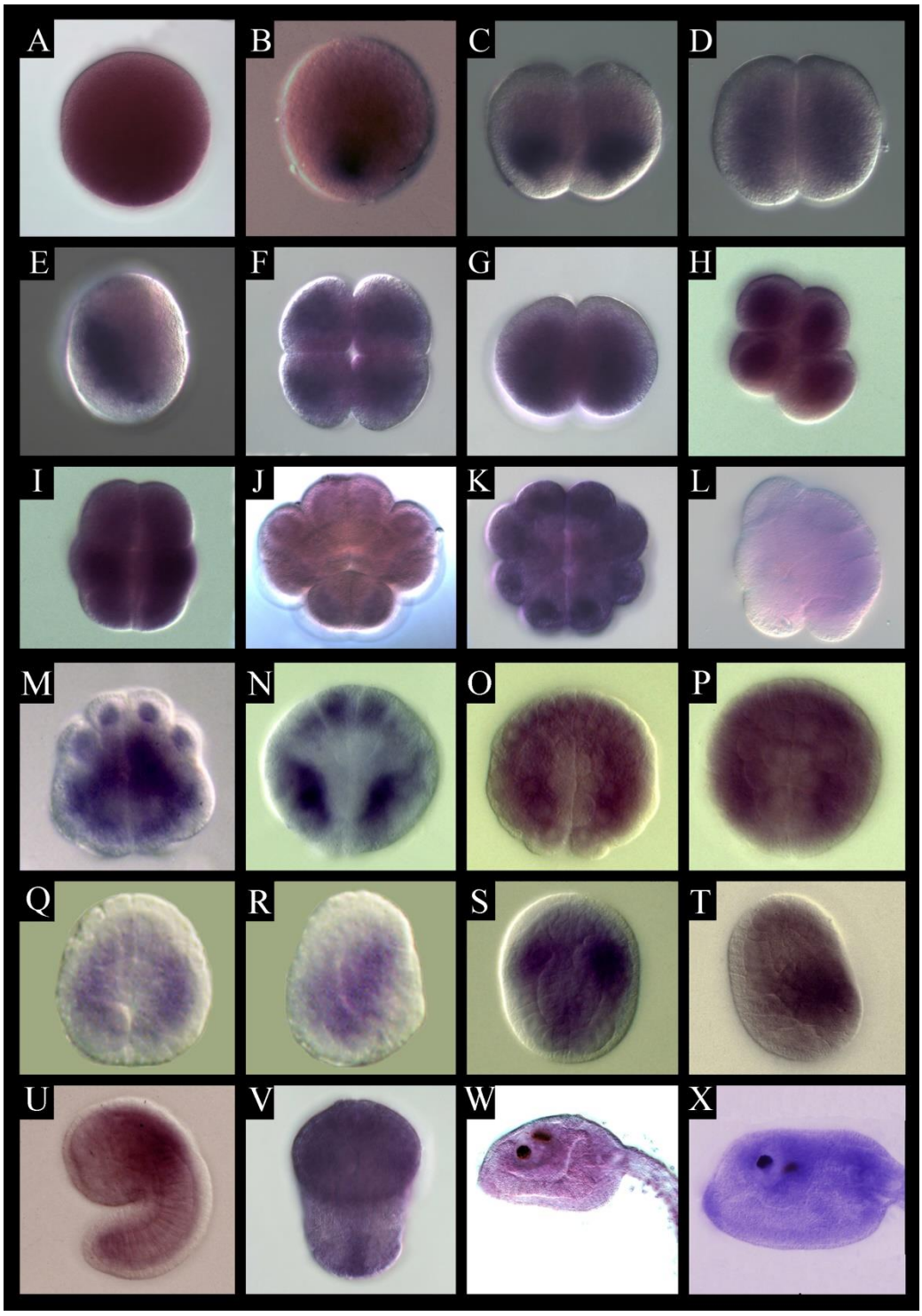

Figure 2. Spatial expression of *Magoh* transcript. A. Unfertilized egg. B. Animal pole at the upper side. Fertilized egg: frontal view. C. Animal pole at the upper side. 2-cell stage: frontal view. D. 2-cell stage: vegetal view. E. Animal pole at the right corner. 2-cell stage: lateral view. F. 4-cell stage: animal pole view. G. Animal pole at the upper side. 4-cell stage: lateral view. H. Ventral pole at the upper side. 8-cell stage: lateral view. I. 8-cell stage: posterior-animal pole view. J. 8-16-cell stage: vegetal pole view. K. 16-cell stage: vegetal pole view. L. 16-cell stage: lateral view. M. 32-cell stage: vegetal view. N. 76-cell stage: vegetal view. O-P. 110-cell stage: vegetal view. Q. Gastrula: dorsal view. R. Gastrula: lateral view. S. Neurula: dorsal view. T. Neurula: lateral view. U. Middle tailbud: lateral view. V. Middle tailbud: ventral view. W-X. Larva: lateral view.

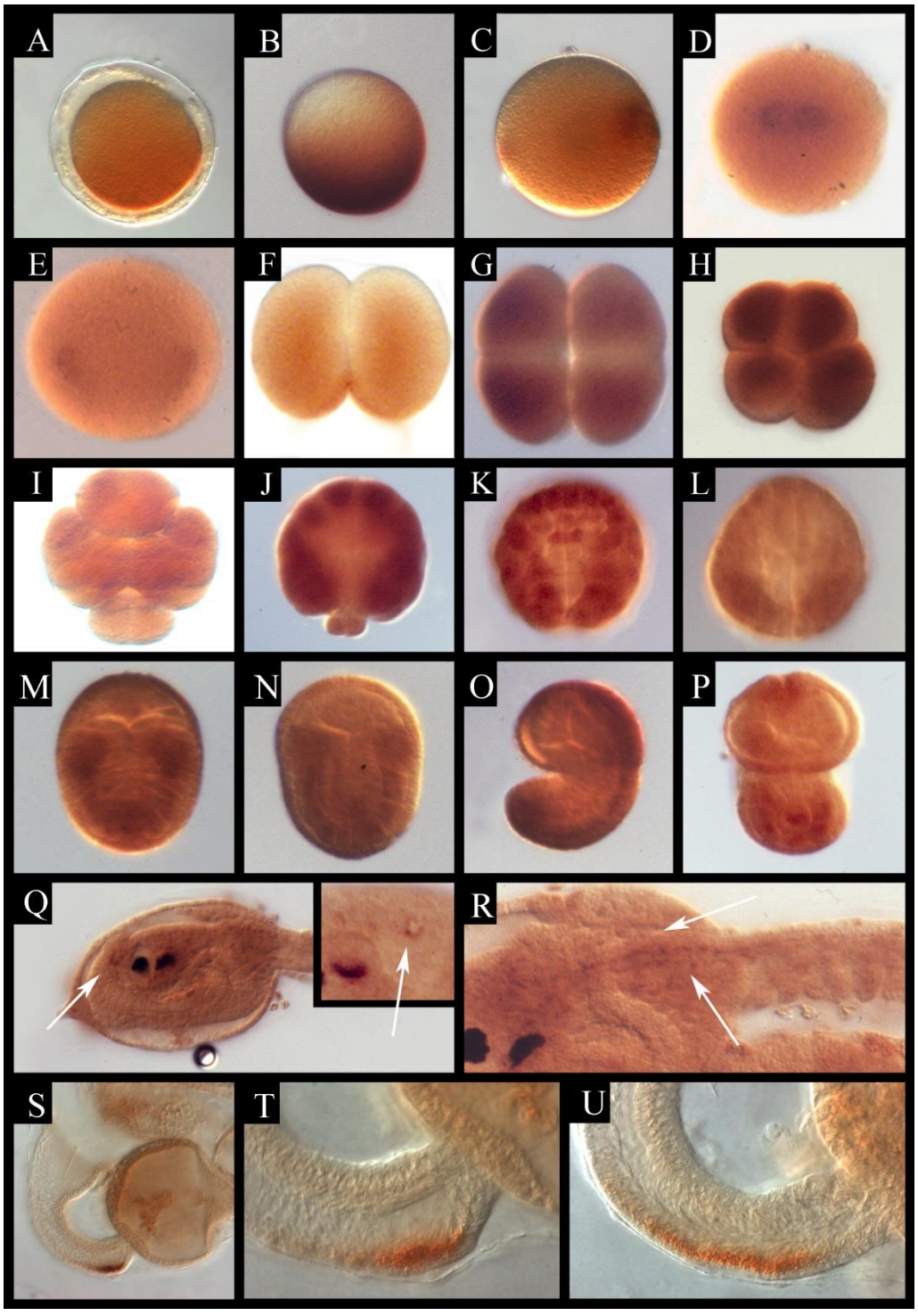

Figure 3. Spatial expression of *Magoh* protein. A. Unfertilized egg. B. Animal pole at the upper side. Fertilized egg: frontal view. C. Animal pole at the upper side. Fertilized egg: lateral view. D-E. Animal pole at the upper side. Fertilized egg: frontal view. Ongoing mitosis. F. Animal pole at the left corner. 2-cell stage: frontal view. G. 4-cell stage: animal pole view. H. Ventral pole at the upper side. 8-cell stage: lateral view. I. 8-16-cell stage: vegetal pole view. J. 32-cell stage: vegetal view. K. 110-cell stage: vegetal view. L. Gastrula: vegetal view. M. Neurula: dorsal view. N. Neurula: ventral view. O. Middle tailbud: lateral view. P. Middle tailbud: ventral view. Q-R. Larva: lateral view. S-U. Young adult: lateral view.

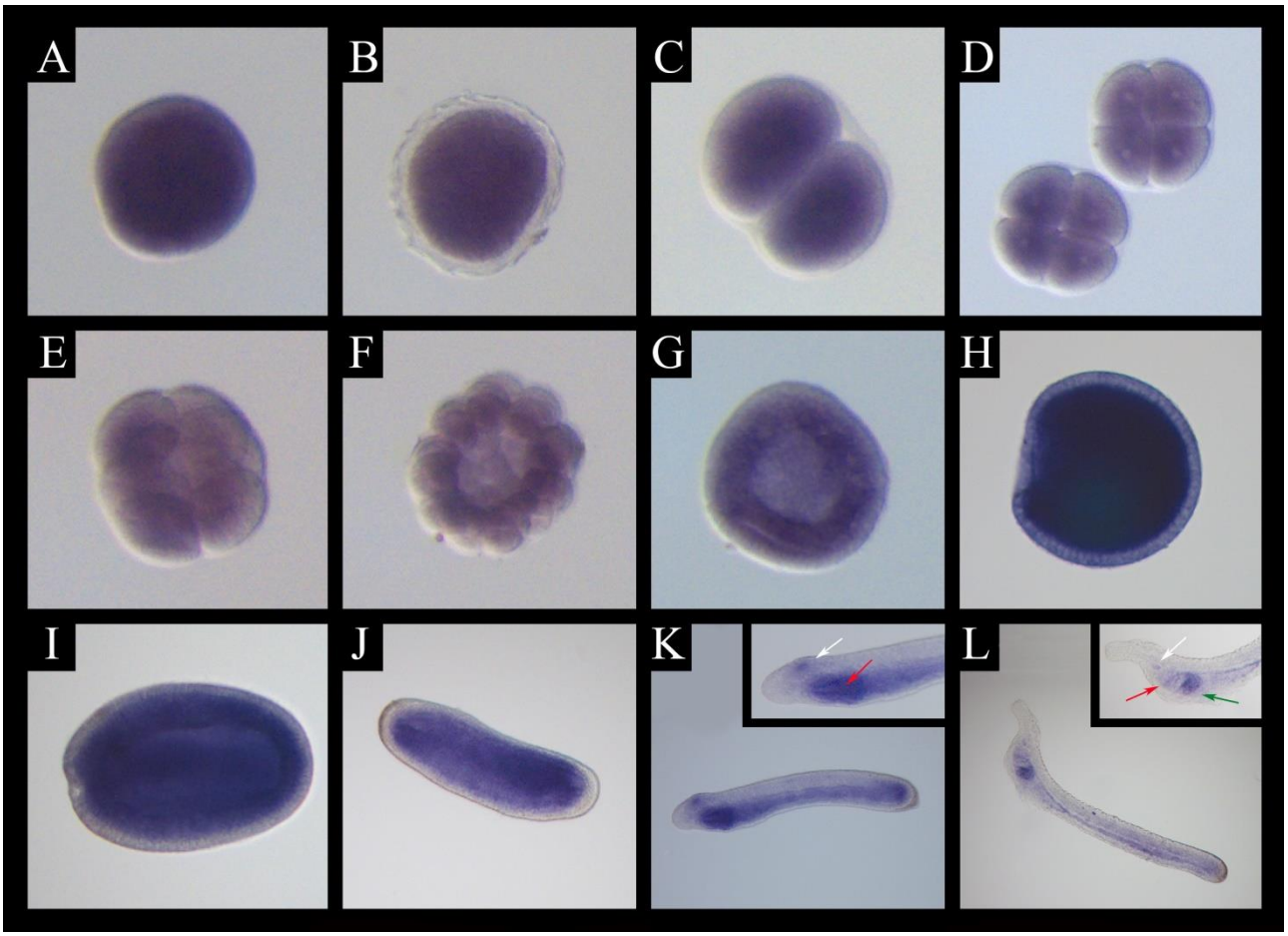

Figure 4. Spatial expression of *Magoh* transcript. A. Unfertilized egg. B. Fertilized egg. C. 2-cell stage. D. 4-cell stage. E. 8-cell stage. F. Morula. G. Early gastrula. H. Late gastrula (anterior part to the left). I. Early neurula (anterior part to the left): dorsal view. J. Late neurula (anterior part to the left and dorsal part to the top): lateral view. K. Pre-mouth larva (anterior part to the left and dorsal part to the top): lateral view. L. Larval stage (anterior part to the left and dorsal part to the top): lateral view.

CHAPTER 3 - FLAGGING A MEDITERRANEAN ICON: *BURSA SCROBILATOR* (LINNÉ, 1758)

1. INTRODUCTION

The present-day Mediterranean fauna and flora is mostly an Atlantic province (Briggs, 1974). The Messinian Salinity Crisis minimalized the stenoecious marine biota, leaving some paleoendemic survivors. Then, the early Pliocene re-establishment of an Atlanto-Mediterranean connection allowed massive recolonization of the Mediterranean Sea through Gibraltar from the neighbouring eastern Atlantic (Bouillon, 2004; Coll et al., 2010; Sabelli & Taviani, 2014). The subsequent 5 million years of evolution within the complex framework of the basin have led to a peculiar and variegate Mediterranean assemblage, dominated by temperate organisms with a few subtropical elements, and a main transitional zone (the Alboran Sea) hosting several endemisms (Maldonado & Uriz, 1995; Gofas, 1998; Bianchi & Morri, 2000; Oliverio, 2003, Coll et al., 2010; Sabelli & Taviani, 2014). Besides the physical connection between Atlantic Ocean and Mediterranean Sea, the cold Canary Current, the Saharan upwelling (Vermeij, 2012) and the Almería-Oran Front (Patarnello et al., 2007) are robust barriers to dispersal that have contributed to genetic differentiation between species and populations (*e.g.* Patarnello et al., 2007; Borrero-Pérez et al., 2011; García-Merchán et al., 2012; Barco et al., 2013), producing also a large number of Mediterranean endemics (Boudouresque, 2004).

These patterns are particularly well exemplified by molluscs, whose Mediterranean biota is considered the best known in the world (Oliverio, 2003). New molluscan taxa are discovered every year from the Mediterranean, with 103 species only during the last decade (2001-2010) (Crocetta et al., 2012). However, it should be noted that molluscan taxonomy is still commonly based on shell characters despite polymorphisms due to adaptation to environmental pressures and the existence of cryptic species (*e.g.* Boisselier-Dubayle & Gofas, 1999; Calvo et al., 2009; Sá-Pinto et al., 2010; Barco et al., 2013; Modica et al., 2013; Crocetta et al., submitted). Among the less known Atlanto-Mediterranean molluscan species, *Bursa scrobilator* (Linné 1758) needs a special mention. There are four species of the genus *Bursa* Röding, 1798 in the Eastern Atlantic/Mediterranean area: *Bursa corrugata* (Perry, 1811), *Bursa ranelloides* (Reeve 1844), *Bursa rhodostoma* (G.B. Sowerby II, 1835) and *B. scrobilator* (review in Beu, 2010). Bursidae are well known as having teleoplanic veliger larvae that remain in the plankton up to one year, suggesting a high potential to colonizing new areas (Scheltema, 1972). *B. scrobilator* is the only species of the genus whose distributional

range extends into the Mediterranean Sea (Templado & Villanueva in Coll et al., 2010). This species has always attracted the attention of Mediterranean malacologists as an iconic inhabitant of the Mediterranean molluscan fauna, likely because of its elusive nature despite its large shell height (up to ~10 cm) and relatively easy-to-access bathymetric range (not deeper than ~20 m).). The known information on this species are scanty, and largely scattered in several papers. Most reports are concerned with records of living specimens or dead shells (*e.g.* Micali, 1975; Ghisotti, 1977; Barletta, 1980; Trillò, 2001; Lòpez Soriano & Tarruella Ruestes, 2002; Tarruella Ruestes & Lòpez Soriano, 2004). Recently, a living specimen (Russo, 1981) and radular features (Melone, 1975) were illustrated. Additionally, the hypothesis of Mediterranean clines or subspecies distinct from the Atlantic one(s) is currently debated, so far only on the basis of external macro-shell features (Barberini, 1985; Verdejo Guirao, 2001; Beu, 2010).

Therefore, a careful multidisciplinary re-assessment of this molluscan taxon is desirable. Here, I study the taxonomic status of Atlantic and Mediterranean specimens of *B. scrobilator* (Linné, 1758) using morphology (protoconch and teloconch), anatomy (soft parts) and mitochondrial genetic markers (cytochrome oxidase I and 16S rRNA). To test possible morphological and genetic differentiation at species level, I analyzed a representative set of individuals from two Atlantic sites (Canary and Azores) and five central Mediterranean sites. Further, I discuss Mediterranean records in the light of the hypothesis of casual, but repetitive, larval transport across the Gibraltar Strait.

2. MATERIAL AND METHODS

2.1. Sample data and morphological/anatomical features

Atlantic *Bursa scrobilator* (Linné, 1758) specimens used in this work have been recently collected by scuba diving during research (Azz_1-Azz_4) and recreational activities (Can_1-Can_6), and fixed in 70-100% ethanol upon collection. Specimens from the Mediterranean Sea analyzed herein were searched in local museums and private collections. They consist of two recently sampled specimens, fixed in 70-100% ethanol (Med_1-Med_2), and three “old” specimens, one fixed in denatured-alcohol (Med_3), one < year 1911 ethanol-preserved museum sample (Med_4 - Stazione Zoologica Anton Dohrn - Naples: MOL 052) and one dried sample (Med_5). The latter is the only available specimen with soft parts among those published from the Mediterranean Sea (*in* Trillò, 2001). Sample localities are summarized in Table 1 with their tissue-

processing codes, whilst representative Atlantic and Mediterranean specimens/shells are reported in Figure 1.

Radulae were extracted from dissected buccal masses after tissues had been partly dissolved in a 10% sodium hydroxide, then rinsed in distilled water and observed under a stereomicroscope. Protoconchs were also observed under stereomicroscope. After comparison, selected radulae and protoconchs were air dried, mounted on SEM stubs and gold-palladium coated in an SC7640 Sputter coater for SEM examination with a Jeol JSM-6700 F microscope.

Anatomical studies were performed on recently collected specimens only. Soft parts were carefully extracted from their shells, elongated against a metal bar, the total length measured with a millimetric ruler, and then dissected under a Leica MZ6 stereomicroscope. Two individuals showing the best conservation status were used for the anatomical description: a 75 mm female and a 79 mm male. The animals were dissected dorsally, starting from the anterior edge of the mantle, which was lifted to show the dark muscular foregut. Dissections were performed on a paraffin-filled Petri dish, with the aid of a scalpel, ophthalmic surgery scissors and metal pins to fix anatomical parts. After dissection, excised parts were collected and conserved in vessels filled with 70% alcohol. Various stages of the dorsal dissections, proceeding ventrally towards further layers and organs, were documented by taking photos under a Leica Z16 APO microscope. A photo of the ruler was taken as well. The photos obtained were then opened in Adobe Photoshop, rotated and fitted each other to reconstruct the whole anatomy, and a new layer was created, as to draw the outlines of visible organs. Finally, the layer of the photos was deleted to obtain anatomical drawings.

2.2. DNA sequencing and alignment

Museal and long-time preserved Mediterranean samples were first transferred to fresh 100% ethanol and let to rest for one week. A piece of tissue was then dissected from the foot of each specimen for DNA extraction. Soon after rehydration, DNA extraction was performed after tissue digestion in proteinase K using a phenol-chloroform protocol, with slight modifications as described in Oliverio & Mariottini (2001). A fragment of the mitochondrial cytochrome oxidase I (COI) was amplified using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994), whilst a fragment of the mitochondrial 16S was obtained with the primers 16Sar-L and 16Sbr-H (Palumbi et al., 2002). Multiple attempts to amplify regions of the nuclear ribosomal cluster (in particular the ITS1-5.8S-ITS2 fragment), using different primers, were unsuccessful. Polymerase chain reaction (PCR) amplifications were performed in 50 μL containing 5 μL of 10× Roche PCR reaction buffer + Mg, 3 μL of 10mM Roche PCR Grade Nucleotide (dNTPs) Mix, 1 μL of each

primer (10 mM), 5 μL of 10× BioLabs Purified BSA, 0.5 μL of Roche Taq DNA Polymerase and ~50 ng of genomic DNA. DNA fragments were amplified with an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 60 s (COI) and an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 40 s and extension at 72 °C for 60 s (16S). These cycles were followed by an extension at 72 °C for 10 min. PCR products were purified with the GenElute Gel Extraction Kit following the manufacturer's instructions, and the amplicons were sequenced using the Applied Biosystems Automated 3730 DNA Analyzer, using the same PCR primers. COI and 16S fragments were aligned and visually edited using Bioedit v7.0.9.0 (Hall, 1999).

2.3. Phylogenetic Analyses

For phylogenetic purposes, 51 (plus three outgroups) sequences of Bursidae were retrieved from the GenBank and added to the *Bursa scrobilator* (Linné, 1758) COI alignment. COI nucleotide and haplotype diversity were computed using the software DNAsp v.5 (Librado & Rosas, 2009). A neighbour-joining (NJ) tree was derived using MEGA v.6 (Tamura et al., 2013), under the Kimura 2-parameter model (Kimura, 1980). The robustness of the NJ tree was evaluated by bootstrap analysis with 10000 replicates.

Maximum likelihood (ML) trees were inferred with PAUP*4.0b10 (Swofford, 2002), using the best-fit models (HKI+G+I) of sequence evolution selected under the Akaike Information Criterion (AIC), as implemented in MODEL TEST version 3.06 (Posada & Crandall, 1998). A heuristic search with 1000 replicates of random stepwise addition and tree-bisection-reconnection (TBR) was performed. Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985).

Bayesian inference (BI) was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Analyses were run for 10 million generations, sampling one tree every 100 generations. Two independent Bayesian analysis runs with four differentially heated chains were performed simultaneously.

ARLEQUIN 3.1 was used to estimate molecular variance (AMOVA) (Excoffier et al., 2005) and to test the following two hypotheses: a) non-genetic differentiation between Mediterranean, Canaries and Azores individuals; b) genetic differentiation between Mediterranean and extra-Mediterranean individuals.

A statistical parsimony analysis was finally performed to estimate genealogical relationships among all COI haplotypes using TCS v. 1.21 (Clement et al., 2000). The connection limit of parsimony was set at 95% and gaps were treated as missing characters.

3. RESULTS

3.1. Morphological analyses

3.1.1. Protoconch-teleoconch features

No significant differences were observed in the protoconch size, both among different geographic areas and among specimens within the same area. Larval shells are tall-mammillate and light brown in color, of 3.75 convex whorls (Figure 2A-C). Finding of one juvenile specimen (Azz_4: Figure 1C) with well preserved protoconch sculpture (Figure 2A, D-E) allows its description: protoconch I of 0.5-0.75 whorl, with a dense chaotic sculpture ending in 3-4 axial riblets (Figure 2D). Protoconch II with a cancellate sculpture of 3 spiral and several axial riblets, extending for 2.25 whorls (Figure 2E). Four major spiral cords are present since the early teleoconch whorls, presumably corresponding to the knobs on the outer lip (Figure 2F-H).

3.1.2. Radular features

Typical taenioglossate radula, with a central rachidian tooth flanked on each side by a lateral tooth and two marginal teeth (Figure 3A-C). Despite the general organization was the same in all the specimens hereby analyzed, a significant intraspecific variability is observed within tooth typology. Rachidian tooth broad and usually with a long and slender, elongated, median cusp, flanked by a variable number of secondary denticles (from two to four, varying also in the same tooth) (Figure 3 D-H). Two short and inner teeth are always present (Figure 3 D-I). Sickle-shaped lateral tooth, with a broad base and one-two inner tooth/teeth and two-five outer secondary teeth (Figures 3 J-K). Sickle-shaped marginal teeth, with one-two inner secondary tooth/teeth (Figure 3 M-O). One specimen showed thick central rachidian teeth with no secondary denticles, thick lateral teeth with no inner teeth, and thick lateral teeth with no inner and outer teeth along the entire radular ribbon (Figure 3I,L,P). Additionally, the ribbon size was nearly doubled with respect to all other specimens hereby analyzed (see scale bars in Figure 3).

3.1.3. Gross anatomy

Body massive, whitish, brownish, yellowish and bluish patchily colored, with small yellow-orange spots (Figure 1A). Tentacles and eyes are evident in the anterior part of the body, disposed along the upper profile of the proboscis, showing dark concentric rings adorned by a pattern of small yellow-orange spots (Figure 1A). The eyes are small, dark and round shaped. Proboscis dark blue, with an electric blue ring at the end (Figure 1B). Light brown operculum (Figure 1A), directed towards the lower side of the animal and partially covered by the foot, from a dorsal point of view (Figure 1G,H). Males are characterized by the presence of a large, muscular penis. It is tongue-shaped, with depressed lineages in the dorsal side, and is situated on the right side, just below the inhalant siphon (Figure 1H). When dissecting the inhalant siphon, the wide gill chamber appears in its rear position (Figure 1G). In females, gonads are compacted in the posterior part of the body, just over the muscular stomach (Figure 1G). A small ovary was found in the female individual hereby drawn, apparently not mature (Figure 1G). In males, two large testes (flattened disk-shaped) are compacted over the posterior part of the stomach, and their collection allows the observation of two long and convoluted *vas deferens*, conducting to the right part of the body (Figure 1H). The digestive tract starts with the large flattened elliptical muscular proboscis, followed by a solid foregut (forming a C-shaped structure) and ending into a caecum, in turn followed by a short thin duct and the muscular stomach (Figure 1G, H). This was empty in all the examined samples. The intestine completes the structure and continues with the rectum, folded toward the right anterior part of the body. The excision of the digestive tube allows seeing various nervous ganglia disposed under the foregut. The largest one, the pedal ganglion, was clearly visible under the anterior part of the muscular stomach.

3.2. Molecular analyses

Eleven COI and eight 16S *Bursa scrobilator* (Linné, 1758) sequences were aligned and visually edited using Bioedit v7.0.9.0 (Hall, 1999). The COI alignment included eleven *B. scrobilator* samples, two from the Azores Islands, five from the Canaries Island and four from the Mediterranean Basin. The amplified fragment consisted of 566 base pairs, with 557 conserved positions and 4 parsimony-informative sites. The overall dataset consisted of 9 different haplotypes (over 11 sequenced individuals), with haplotype diversity (h) of 0.96364 and nucleotide diversity (π) of 0.00556 (Table 2). Of the four Mediterranean samples, two showed the same haplotype

(Table 2). The B. scrobiculator sequences once included in a bursid dataset and analyzed by neighbour-joining (NJ) and maximum likelihood (ML) methods, as well as Bayesian inference (BI), proved consistently a highly supported monophyletic clade (Figure 4). Hierarchical analyses of molecular variance (AMOVA) for the COI fragment rejected genetic differentiation between Mediterranean and Extra-Mediterranean samples, and strongly support the existence of a single group of *B. scrobilator* sequences (Table 3). The COI statistical parsimony network confirmed the absence of geographical differentiation between samples, including any Mediterranean *vs.* Atlantic separation. The most frequent haplotype (MFH) was represented by 3 individuals (two from the Mediterranean Sea and one from the Canary Islands) and was also the most ancestral haplotype among those encountered (Figure 5).

The 16S alignment included eight *B. scrobilator* individuals: two from Azores Islands, four from Canary Islands and two from the Mediterranean Sea. The total alignment was 365 base pairs long, with no Parsimony Informative sites. I found only a single variable nucleotide, namely a T to C transversion at position 90 of one Azores sample (Azz_4) (Supplementary Material 1), and therefore no further analyses were performed on this gene.

4. DISCUSSION

As usual for molluscan species, the taxonomy of *Bursa scrobilator* (Linné, 1758) has been studied on the basis of shell characters, resulting in the objective synonymy of three (*Murex scrobilator* Linné, 1758, *Bufonaria pesleonis* Schumacher, 1817 and *Apollon quercina* Mörch) of the four binomial names available for recent specimens ascribed to this taxon (Beu, 2010). Aiming to extend this analysis to *B. scrobilator* specimens from the entire distributional range (Eastern Atlantic and Mediterranean), and from its as close as possible designed type locality (Palermo, Sicily - Italy), I used a combination of morphological and molecular analyses in the most comprehensive review of the taxon. The main conclusion of the present study is the conspecificity between Mediterranean and Atlantic specimens from Canary and Azores. In fact, although based on few samples due to the rarity of the species (particularly from the Mediterranean Sea: *see* Beu, 2010), my data clearly demonstrated that a single species is involved, excluding the presence of a Mediterranean cline/subspecies, as sometimes proposed in the past. In fact, no morphological difference was observed when analyzing protoconch, teleoconch and anatomical features. Conversely, radular intraspecific (and intra-individual) variability was high, but with no geographic

pattern of varoiation. This variability is further illustrated by a previously described radula, that differs from all those analyzed in this study except for that of Med_5 (Melone, 1975) (Figures 3A-P). A few radulae of Bursidae have been illustrated to date and no study exists on radular intraspecific variation in this molluscan family (Beu, 1981; Ekawa & Toki, 2005), making of interest a study of ecophenotypic plasticity in Bursidae. Ontogenetic changes and sexual dimorphisms are able to modulate radular features in molluscs (*e.g.* Maes, 1966; Bertsch, 1976; Nybakken & Perron, 1988; Meirelles & Matthews-Cascon, 2003; Mutlu, 2004; Matthews-Cascon et al., 2005; Warén, 2005; Martínez-Pita et al., 2006). However, my observations do not support intraspecific variation due to sex (evaluated as presence/absence of penis) and age (evaluated as total shell height).

On the other hand, my work also lays the basis for reviewing the relationship of *B. scrobilator* with the fourth taxon involved in its synonymy, *Ranella coriacea* (Reeve, 1844) from the West Africa, and with *Talisman parfaiti* de Folin, 1884 from southeastern Europe or western Africa. *R. coriacea*, subjective synonym of *B. scrobilator* (Linné, 1758), has been often considered in the past as a separate species, subspecies or form (with respect to *B. scrobilator*) due to its finely granulose sculpture and fine to prominent shoulder nodules, usually absent in most Atlantic and Mediterranean specimens (Cossignani, 1994; Ardovini & Cossignani, 2004; Beu, 2010). Unfortunately, no specimens belonging to *R. coriacea* were available for DNA extraction, at least at this point of the study (F. Crocetta, unpublished data). The correct identity of *T. parfaiti* is still elusive, as this species was originally described on a larval shell (*see* de Folin, 1884) that may belong to *B. scrobilator*, *Bursa corrugata* (Perry, 1811) or *Aspa marginata* (Gmelin, 1791) (discussions *in* Warén & Bouchet, 1990; Beu, 2010). Among them, protoconch whorls and microsculpture are known in both *B. corrugata* (*see* Laursen, 1981) and *A. marginata* (*see* Warén & Bouchet, 1990) only. On the contrary, the only available description of *B. scrobilator* protoconch morphology belongs to a worn Pliocene specimen from Estepona (Landau et al., 2004). Although no differences were noted in protoconch whorls with respect to recent specimens, this fossil was devoid of any kind of protoconch microsculpture. This may have prevented past authors from the correct taxonomic attribution of *T. parfaiti*. Type material, preserved in Travailleur & Talisman collection (Muséum national d'Histoire naturelle - Paris, France) is unfortunately temporarily unavailable till March 2014, due to mantainance work (Virginie Héros, personal communication). While *B. scrobilator* protoconch differs from *A. marginata* in having a more reticulate Protoconch II, more accurate analysis is needed to state differences with respect to *B. corrugata*.

The genetic homogeneity among the analyzed specimens suggested by the almost complete lack of nucleotide variability detected in the B. scrobilator 16S fragments is further supported by the

AMOVA and the COI statistical parsimony network. The absence of significant genetic differentiation among sites of occurrence is commonly attributed to a gene flow strong enough to prevent genetic drift or selective actions. Teleplanic larvae such as those of *B. scrobilator* can overcome zoogeographic barriers and colonize new areas, maintaining genetic continuity between populations that otherwise would be separated by seemingly insurmountable obstacles such as ocean basins (Scheltema, 1972). My results indicate a negligible level of genetic differentiation among sites, which may be suggestive of a strong genetic connettivity among the analyzed samples/populations. The extreme rarity of this species in the Mediterranean, despite it being relatively common in the Atlantic, may be due to ecological constraints which limit the demography in the Mediterranean because of either reduced food sources (echinoderms), or limiting environmental condition for reproduction, or a combination of both. Especially factors affecting reproduction may result in the extreme case of non-reproductive Mediterranean pseudopopulations due to casual, but repetitive, events of larvae transport through the Gibraltar Strait, as it has been previously supposed for some deeper sea species in the Mediterranean Sea (Bouchet & Taviani, 1992). However, testing this hypothesis will require a deeper study of the genetic variation and diversity, possibly including the use of multiple genetic markers and a larger sample sizes.

My results, however, raise intriguing questions concerning the overall biodiversity of the Mediterranean Sea. This basin is commonly considered as a marine biodiversity hot spot, hosting around 17.000 marine species, of which 11.500 animals. Among them, Mollusca account for more than 2100 species, of which around 200 are human-introduced, and constitute the most speciose phylum after crustaceans (2250 species) (Coll et al., 2010). Several species currently included in the resident Mediterranean fauna are still known from infrequent and scattered specimens. This type of multidisciplinary analysis, enriched by multiple genetic markers and a larger sample sizes, may serve to validate molluscan taxa in the frame of a qualitative and quantitative re-assessment of the Mediterranean biodiversity.

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ID code	*Legit*	Sampling locality	Geographic region	GenBank	COI	16S
Azz_1	Marco Oliverio	Fajã Grande - Flores Island - 12 m - 08/2008	AO - Azores Islands	XXXXX	X	-
Azz_2	Marco Oliverio	Caloura - São Miguel Island - 8 m - 09/2008	AO - Azores Islands	-	-	-
Azz_3	Marco Oliverio	Fajã Grande - Flores Island - 12 m - 08/2008	AO - Azores Islands	XXXXX - XXXXX	X	X
Azz_4	Marco Oliverio	Fajã Grande - Flores Island - 12 m - 08/2008	AO - Azores Islands	XXXXX	-	X
Can_1	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	XXXXX	X	-
Can_2	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	XXXXX - XXXXX	X	X
Can_3	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	XXXXX - XXXXX	X	X
Can_4	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	XXXXX - XXXXX	X	X
Can_5	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	XXXXX - XXXXX	X	X
Can_6	Javier Martin Barrios	Las Eras - Tenerife Island - ~20 m - 02/2010	AO - Canary Islands	-	-	-
Med_1	F.C., Water Renda, Angelo Vazzana	Reggio Calabria - Altafiumara - 5 m - 08/2009	Mediterranean Sea	XXXXX - XXXXX	X	X
Med_2	Marco Oliverio	Marettimo - Punta Bassana - 11 m - 07/2011	Mediterranean Sea	XXXXX - XXXXX	X	X
Med_3	Pasquale Micali	Messina - Pace - 5-7 m - ~1980	Mediterranean Sea	XXXXX	X	-
Med_4	Carlo Praus Franceschini	Naples - <05/1911 (SZN MOL 052)	Mediterranean Sea	XXXXX	X	-
Med_5	Piergiorgio Trillò	Ustica - 10 m - 08/1999	Mediterranean Sea	-	-	-

Table 1. Sampling localities, with ID code, *legit* and GenBank accession number. Abbreviations used: m, meters; AO, Atlantic Ocean.

COI nucleotide and haplotype diversity				
	N	**Nh**	**π**	**h**
Complete dataset	11	10	0.00556	0.96364
Azores Islands	2	2	0.00883	1.00000
Canary Islands	5	5	0.00604	1.00000
Mediterranean	4	3	0.00442	0.83333

Table 2. COI nucleotide (π) and haplotype (h) diversity, and number of haplotypes for the three populations examined. Abbreviations: N, overall number of individuals; Nh, overall number of haplotypes.

Grouping	**Source of variation**	**df**	**Sum of squares**	**Variance components**	**Percentage of variation**
One population	Among populations	2	3.045	-0.04770 - Va	-2.91
	Within populations	8	13.500	1.68750 - Vb	102.91
Mediterranean / Atlantic	Among groups	1	2.045	0.26183 - Va	15.32
	Among populations within groups	1	1.000	-0.24062 - Vb	-14.08
	Within populations	8	13.500	1.68750 - Vc	98.76

Table 3. Analyses of molecular variance within and among populations (AMOVA) based on COI. Abbreviations: df, degrees of freedom.

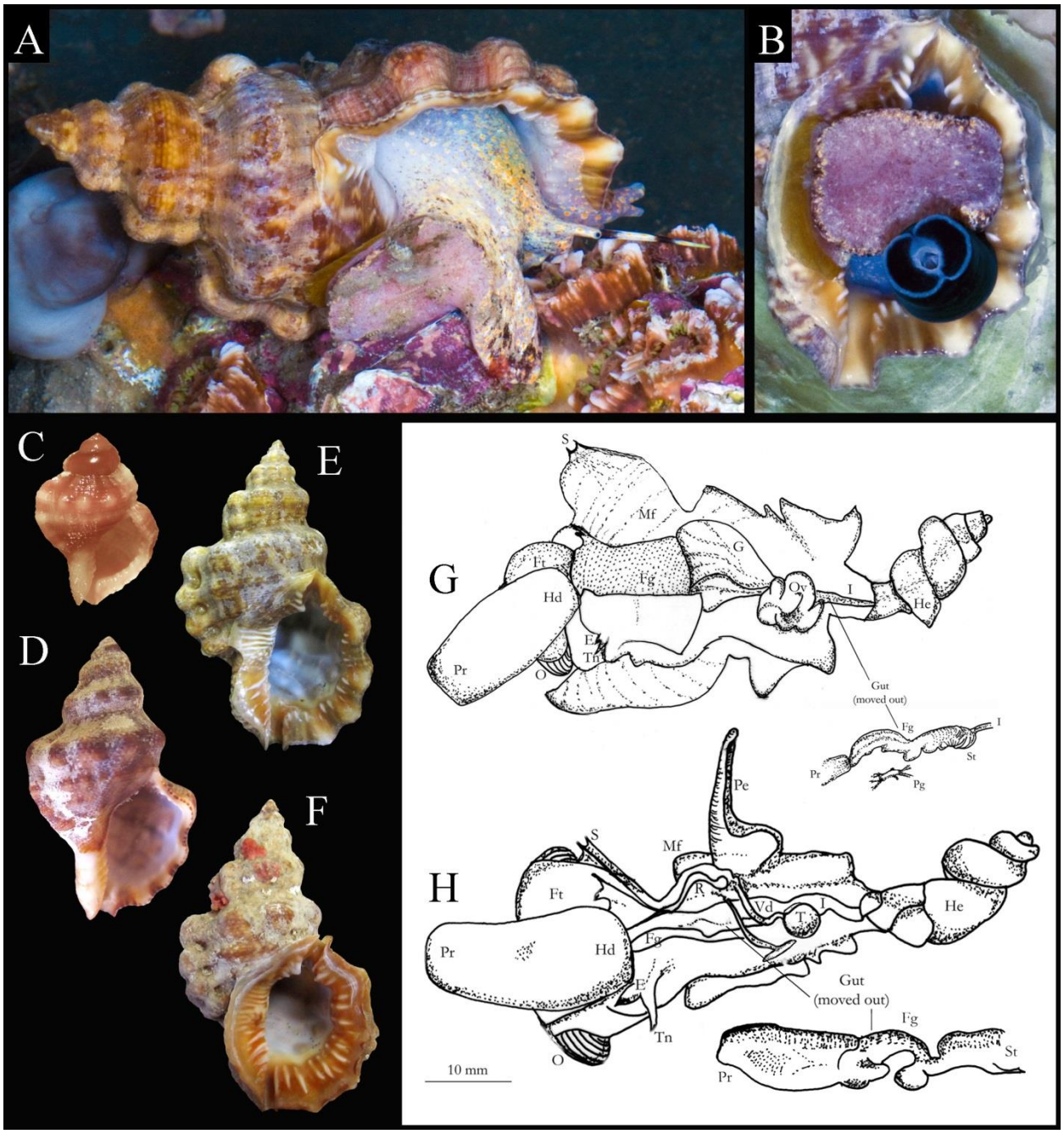

Figure 1. *Bursa scrobilator* (Linné, 1758): alive and dead (shells and anatomy). A. Living individual from Altafiumara (Med_1). B. The same specimen with protruded proboscis. C-F. Representative specimens of different growth stage which were used in this study. C. Azz_4 (total height: 0.9 cm). D. Can_6 (total height: 3.2 cm). E. Med_1 (total height: 7 cm). F. Can_5 (total height: 6.2 cm). G-H. Anatomy revealed by dissection. In the female, the anterior right half of the mantle has been removed to show the muscular foregut (Fg) covered by a dark epithelium. In the male, the testes were moved in the rear body to show the *vas deferens* (Vd), of which the left one is laterally displaced to highlight its flattened shape. G. A *B. scrobilator* female individual of 75 mm total length (elongated soft body), from which the digestive tract has been moved out (low, right) along with the pedal ganglion (Pg). H. A *B. scrobilator* male individual of 79 mm total lenght, from which the digestive tract has been moved out (low, right). G-H. Abbreviations used: E, eye; Fg, foregut; Ft, foot; G, gill; Hd, head; He, digestive gland; I, intestine; Mf, mantle flap; O, operculum; Ov, ovary; Pe: penis; Pg: pedal ganglion; Pr, proboscis; R, rectum; S, inhalant siphon; St, stomach; T, testes; Tn, tentacle; Vd, *vas deferens*.

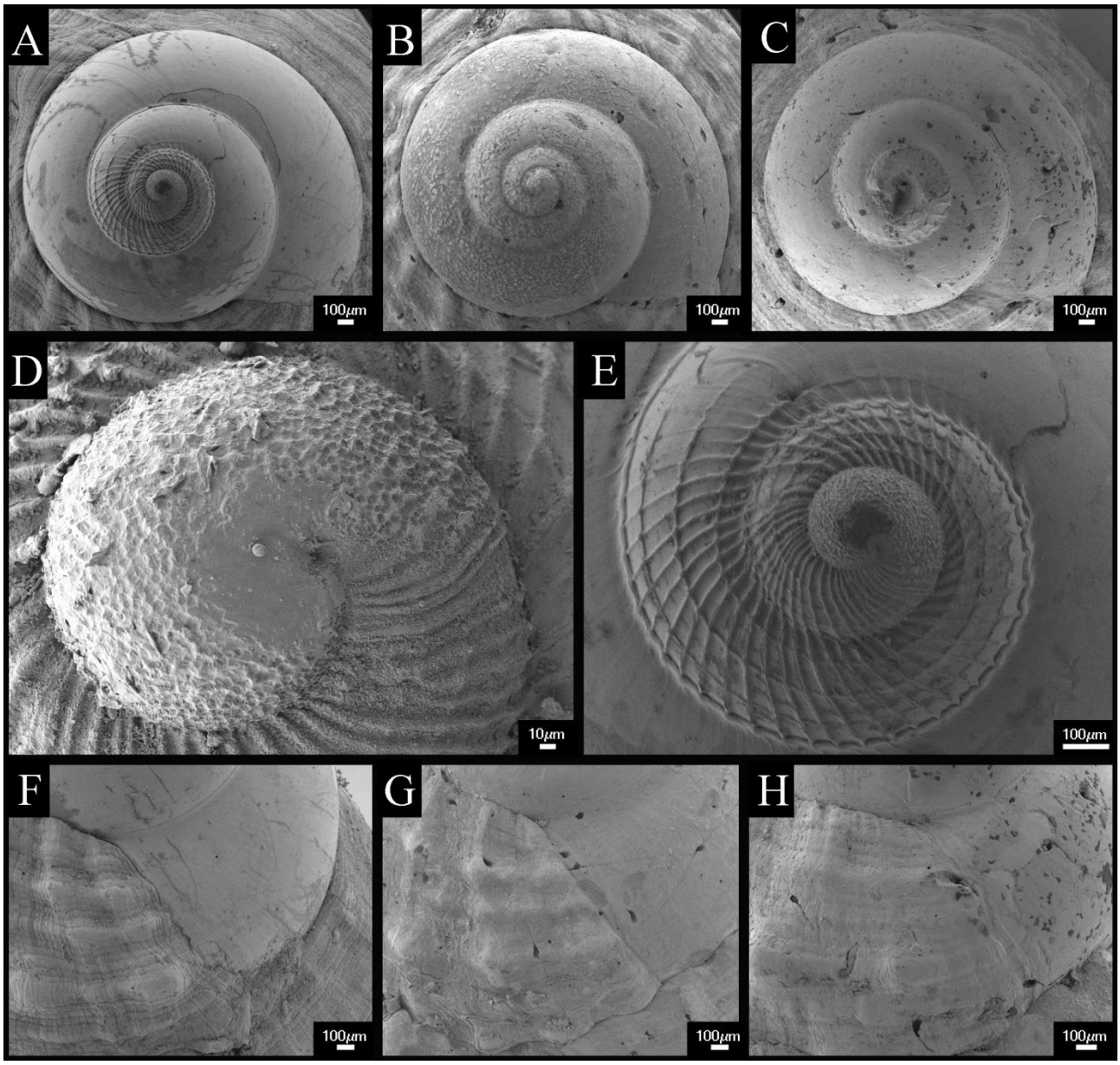

Figure 2. *Bursa scrobilator* (Linné, 1758): protoconch and early teleoconch features. A-C. Protoconchs. A. Azz_4. B. Can_6. C. Med_1. D. Detail of Protoconch I of Azz_4. E. Detail of Protoconch II of Azz_4. F-H. Early teleoconch features. F. Azz_4. G. Can_6. H. Med_1.

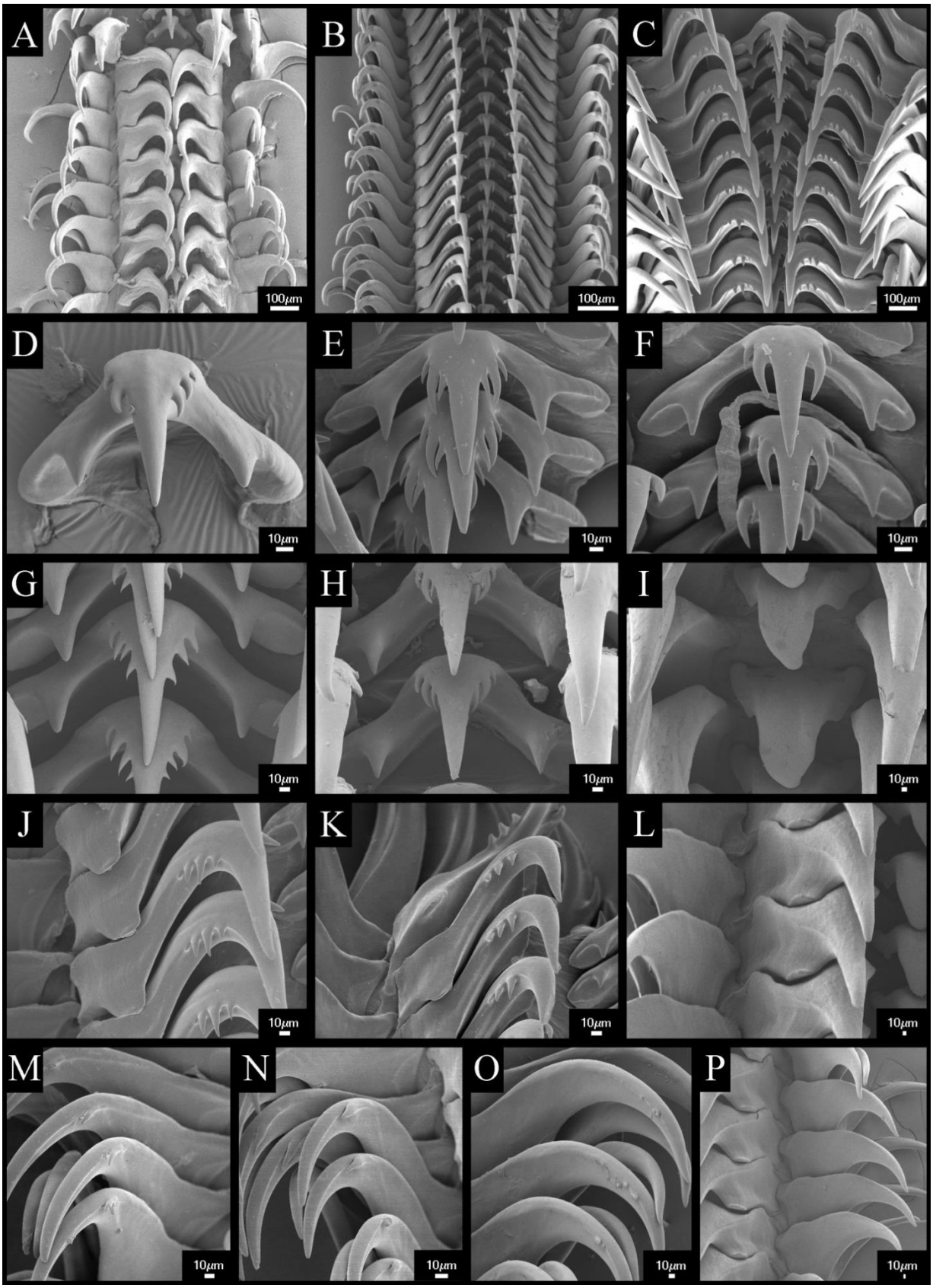

Figure 3. *Bursa scrobilator* (Linné, 1758): radular features. A-C. Tenioglossate radula. A. Azz_3. B. Can_6. C. Med_2. D-I. Rachidian teeth. D. Azz_3. E. Can_5. F. Can_6. G. Med_2. H. Med_3. I. Med_5. J-L. Lateral teeth. J. Can_5. K. Can_6. L. Med_5. M-O. Marginal teeth. M. Can_5. N. Can_6. O. Med_3. P. Med_5.

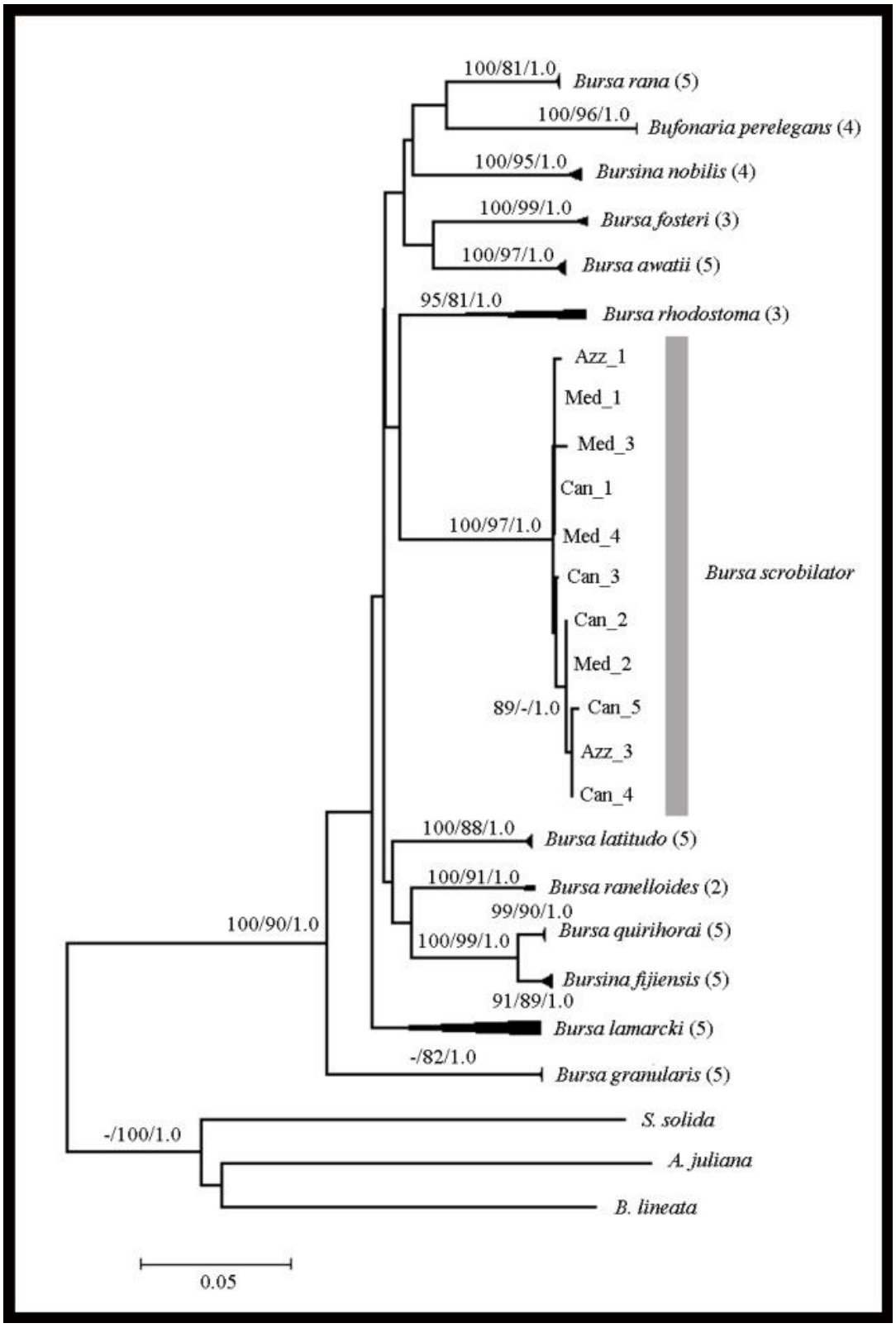

Figure 4. Genealogical relationships (NJ,ML,BI) among *Bursa scrobilator* (Linné, 1758) COI fragments and selected Bursidae COI sequences. Numbers at each node indicate NJ and ML bootstrap supports and Bayesian posteriors for that node. *Salinator solida*, *Aplysia juliana* and *Bullina lineata* COI sequences have been used as outgroups. Where present, numbers within parentheses indicate the number of distinct sequences used for each species.

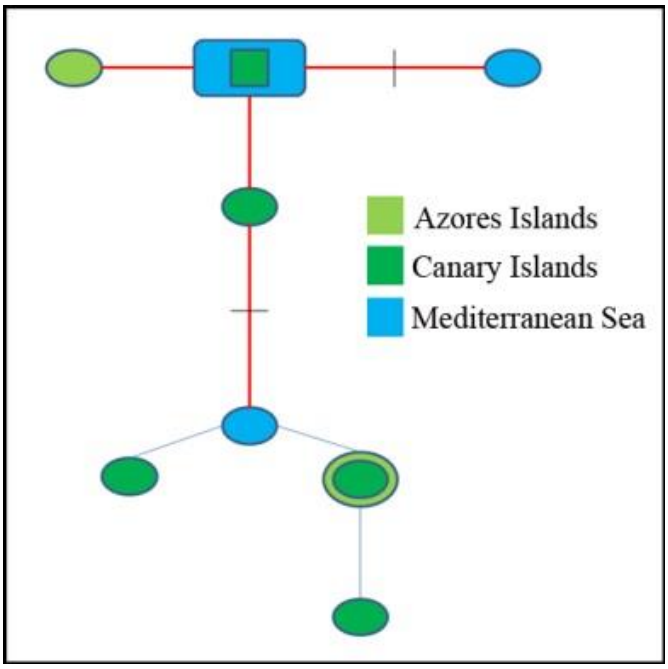

Figure 5. *Bursa scrobilator* (Linné, 1758) COI network. Sampled haplotypes are indicated by circles/rectangles and missing/unsampled haplotypes by hashes. Circle/rectangle sizes proportional to the observed haplotype frequency.

Supplementary Material 1. Alignment of *Bursa scrobilator* (Linné, 1758) 16S fragment sequences.

CHAPTER 4 - Appendix: papers and short communication published (or in press) during the Ph.D. programme (chronological order by 2014 to 2011)

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ORIGINAL ARTICLE

Long-term demographic and reproductive trends in *Ciona intestinalis* sp. A

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Keywords
Ciona intestinalis sp. A; demographic crisis; long-term recording; reproductive strategy; seawater temperature.

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Abstract

Ciona intestinalis sp. A (Tunicata, Ascidiacea) is a marine invertebrate with a widespread distribution and high invasive potential, yet little is known about its ecology. Here, the macrodynamics of demographic and reproductive trends were first determined using archive data covering 2002–2012. Species abundance in the central Tyrrhenian Sea (Campania, Italy - NW Mediterranean) displayed Poisson distribution across 2002–2008, massive die-off in 2009/2010 and population recovery in 2011/2012. We defined the seasonal ranges of sea surface temperature in which sampling size, animal size and reproductive status reached their highest values. In 2003/2004, self and non-self fertilization appeared to follow reverse trends across the year. The long-term recording run here on *Ciona intestinalis* sp. A provides novel insights into its population biology and ecology, and represents a valuable tool for sampling management and research planning.

Introduction

The benthic tunicate *Ciona intestinalis* (Linné, 1767) is a simultaneous hermaphrodite that usually forms dense clusters in sheltered subtidal environments (Lambert & Lambert 1998). Due to its resistance to pollutants (Bellas *et al.* 2004; Gallo *et al.* 2011), this filter feeder species is considered a possible bioindicator of habitat conditions in coastal biotopes (Naranjo *et al.* 1996). Given the limited dispersal of *C. intestinalis*, recruitment is mainly local and human vectors commonly mediate the colonization of new areas (Petersen & Svane 1995; Kanary *et al.* 2011; Zhan *et al.* 2012). Worldwide distribution of *C. intestinalis* is widening through invasions along the coasts of the USA, Chile, Western Australia, New Zealand, Canada and South Africa (Millar 1966; McDonald 2004; Blum *et al.* 2007; Ramsay *et al.* 2008, 2009; Dumont *et al.* 2011), where the alteration of the sessile community composition and the depression of species diversity sometimes has a severe ecological and economic impact on the local biota (Blum *et al.* 2007; Therriault & Herborg 2008). Although the growing genetic and genomic toolkit has prompted interest in this species (Dehal *et al.* 2002; Delsuc *et al.* 2006; Kano *et al.* 2006; Lemaire *et al.* 2008; Cutter & Agrawal 2010; Matsumoto *et al.* 2010; Ferrier 2011; Satou *et al.* 2012; Tsagkogeorga *et al.* 2012), *C. intestinalis* culturing is not straightforward (Cirino *et al.* 2002; Joly *et al.* 2007; Mita *et al.* 2012). Thus laboratory research is strongly dependent upon sampling and maintenance of wild specimens. Therefore, natural population studies of this species may be relevant to different branches of biology.

The species described by Linné consists of a complex of cryptic taxa that informally bear the names sp. A, B, C and D (Caputi *et al.* 2007; Nydam & Harrison 2007, 2010; Zhan *et al.* 2010, 2012; Sato *et al.* 2012). *Ciona intestinalis* sp. A and sp. B present a widespread, mostly disjoint, distribution that respectively reflects adaptation to warm and cold temperate waters (reviewed in Procaccini *et al.* 2011). *Ciona intestinalis* sp. A is the taxon for

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Investigating sperm cryopreservation in a model tunicate, *Ciona intestinalis* sp. A ☆

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ABSTRACT

In cryopreservation procedures, the capacity to protect the cells from freezing and thawing processes is sensitive to the choice of the cryoprotective agent (CPA) and to its optimal concentration. The advancement of research on Tunicate model species has raised interest in liquid nitrogen cryopreservation for the storage and distribution of genetic resources. *Ciona intestinalis* (Linnè, 1767) consists of a complex of cryptic taxa that are central to several areas of investigation, from comparative genomics to invasive biology. Here we investigated how five CPAs, three chilling rates and two freezing rates influence semen cryopreservation in *C. intestinalis* sp. A. By using larval morphology and motility as endpoints, we estimated that long term semen storage requires 10% dimethyl sulfoxide as a protective agent, −1 °C/min chilling rate (18 °C to 5 °C) and −13 °C/min freezing rate (5 °C to −80 °C), followed by immersion in liquid nitrogen.

Introduction

Ciona intestinalis sp. A (Tunicata: Ascidiacea) is a benthic hermaphrodite that inhabits temperate coastal environments, where it usually forms dense clusters in euryhaline areas with nutrient enrichment [8,34]. This species is a model organism that satisfies several requirements in molecular biology of development, comparative genomics and population biology, due to the phylogenetic position relative to Vertebrata, genome sequencing and high potential of colonization [7,8,12,13,44,48,61]. Knockout and transgenic methodologies have prompted interest in successful protocols for culturing, storage and distribution of genetic resources [45–52]. Not only long-term cryopreservation in liquid nitrogen allows low requirements for space/labor, but it also provides out of season supplies for the genetic studies in *C. intestinalis* sp. A.

In aquatic organisms, cryopreservation of eggs and larvae has been investigated with varying degrees of success [4,23,35,41], so that sperm freezing still represents the major preservation method for the maintenance of biological resources [5,21]. Semen cryopreservation was originally confined to species of patrimonial importance (threatened or commercially valuable) [20,32,33,53,54], and has now become central to research in genetics [24,30].

Ascidian spermatozoa possess an asymmetric head that contains a single mitochondrion and a poorly developed acrosome [6,18,19,38]. Sperm motility starts upon dilution in seawater, and becomes more active following release of factors by unfertilized eggs [55]. In *C. intestinalis* sp. A, sperm motility and viability lasts at least 12 hours after activation [5]. In the process of *C. intestinalis* fertilization, acrosomal reaction occurs when the male gamete crosses the vitelline coat, a layer of follicle cells that surround the egg, by means of a glycoside–glycosidase binding mechanism [reviewed by 38].

During cryopreservation, the spermatozoon faces various stresses that may lead to functional impairment or death [59,60]. Systematic studies were conducted in order to describe the damage suffered by the sperm at the time of freezing-thawing [36]. Even in presence of cryoprotective agents (CPA), damages to the integrity of spermatozoa are visible at mitochondrial, chromatin or membrane levels [10]. Mitochondrial damage mainly affects intensity, percentage and duration of sperm motility after thawing; irreversible chromatin alterations may impede the process of fertilization and affect embryonic development; modifications of the plasma membrane may influence the ability to fuse with the egg cells [2,10,11,17,36,42,49]. As a consequence, post-thaw processes of sperm motility and fertilization success are not tightly

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Open Access

Research Article

The origin and dispersal pathway of the spotted sea hare *Aplysia dactylomela* (Mollusca: Opisthobranchia) in the Mediterranean Sea

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Abstract

The spotted sea hare *Aplysia dactylomela* Rang, 1828 is a large and conspicuous opisthobranch sea slug that since 2002 has rapidly colonized the eastern Mediterranean, establishing populations in numerous localities. The source of the Mediterranean populations has been the subject of debate, with two main hypotheses considered (Atlantic and Red Sea origin). A recent study on the taxonomy of *A. dactylomela* has shown that the spotted sea hare is a complex of at least two genetically distinct species (*A. dactylomela* in the Atlantic and *A. argus* in the Indo-Pacific), facilitating the correct identification of Mediterranean specimens by molecular means. We used sequence data from the mitochondrial cytochrome oxidase I gene to identify the Mediterranean individuals for the first time and to infer their origin. Our results confirmed that all the specimens collected in the Mediterranean belong to *A. dactylomela* and therefore have an Atlantic origin. The limited sample size does not allow identification of the dispersal pathway of *A. dactylomela* into the Mediterranean, but the colonization sequence is consistent with a "natural" dispersal event. This hypothesis is evaluated in light of local surface circulation patterns. Possible causes for the recent and rapid invasion of the eastern Mediterranean by *A. dactylomela* are discussed.

Key words: Aplysiidae; Atlantic Ocean; population genetics; haplotype network

Introduction

Aplysia dactylomela Rang, 1828 is a large species of sea hare (Opisthobranchia: Aplysiidae) reaching up to 200 mm in length with a distinctive pattern of dark rings on a yellowish-cream background color. This species was considered to have a world-wide native range in tropical and subtropical regions, including: the tropical Indo-Pacific from the Red Sea and South Africa to the Hawaiian Islands and Panama (Gosliner et al. 2008); and both the eastern and western Atlantic Ocean (Ortea and Martínez 1990; Cervera et al. 2004; Valdés et al. 2006). However, in a recent paper Alexander and Valdés (2013) revealed that the pan-tropical *A. dactylomela* is comprised of at least two genetically distinct species, *A. argus* (Rüppell and Leuckart, 1828) distributed in the tropical Indo-Pacific Ocean, and *A. dactylomela* found in the Atlantic Ocean. The two species are similar externally, thus genetic information is the most reliable tool to distinguish them (Alexander and Valdés 2013).

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Molecular data reveal cryptic lineages within the northeastern Atlantic and Mediterranean small mussel drills of the *Ocinebrina edwardsii* complex (Mollusca: Gastropoda: Muricidae)

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We used a molecular phylogenetic approach to investigate species delimitations and diversification in the mussel drills of the *Ocinebrina edwardsii* complex by means of a combination of nuclear (internal transcribed spacer 2, ITS2) and mitochondrial [cytochrome oxidase subunit I (COI) and 16S] sequences. Our sample included 243 specimens ascribed to seven currently accepted species from 51 sites. Five of the samples were from either the type locality of a nominal species or a close nearby locality (*O. edwardsii* from Corsica, *O. carmelae* and *O. piantonii* from the Kerkennah Islands, *O. hispidula* from the Gulf of Gabès and *O. leukos* from the Canary Islands), one from the inferred original locality (*O. ingloria* from Venice Lagoon), and specimens assigned in the recent literature to *O. nicolai*. We used a combination of distance- and tree-based species delimitation methods to identify Molecular Operational Taxonomic Units (MOTUs) to compare with the *a priori* species identifications. The consensus tree obtained by BEAST on the COI alignment allows the recognition of several distinct clades supported by the three species delimitation methods employed. The eight-MOTUs scenario, shared by the Automatic Barcode Gap Discovery (ABGD) and Generalized Mixed Yule-Coalescent (GMYC) methods, comprises the following major clades: clade A contains the south Tunisian species *Ocinebrina piantonii* Cecalupo, Buzzurro & Mariani from which the sympatric taxon *O. carmelae* Cecalupo, Buzzurro & Mariani (new synonym) cannot be separated; clades B and C bring together all populations from the Aegean Sea and some from the Ionian Sea, respectively; clade D groups, on the one hand, the south Tunisian samples morphologically assigned to *O. hispidula* Pallary and, on the other, Atlantic and Alboran Sea samples (including the Canarian taxon *O. leukos* Houart); clade E includes a sample from the type locality of *O. edwardsii* and several samples from the Tyrrhenian Sea; clades F and G correspond to a few samples from the Venice Lagoon and the Tyrrhenian Sea, respectively; clade H groups the bulk of samples from the Adriatic Sea, including samples from the Venice Lagoon morphologically identified as *Ocinebrina ingloria* (Crosse), and some from the Ionian Sea. No final conclusions could be reached to reconcile the currently recognized morphological taxa with the clades suggested by the COI data. The geographical structure proposed by the mitochondrial markers is similar to that found in other marine invertebrates and partially corresponds to the species defined by shell characters. We propose here a framework for the revision of the *Ocinebrina edwardsii* species complex, suggesting a geographical pattern for the diversification of this group in the studied area.

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ADDITIONAL KEYWORDS: ABGD – cytochrome oxidase I – DNA-barcoding – GMYC – Mediterranean Sea – species delimitation.

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RESEARCH ARTICLE

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Alien molluscan species established along the Italian shores: an update, with discussions on some Mediterranean "alien species" categories

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Abstract

The state of knowledge of the alien marine Mollusca in Italy is reviewed and updated. *Littorina saxatilis* (Olivi, 1792), *Polycera hedgpethi* Er. Marcus, 1964 and *Haminoea japonica* Pilsbry, 1895 are here considered as established on the basis of published and unpublished data, and recent records of the latter considerably expand its known Mediterranean range to the Tyrrhenian Sea. COI sequences obtained indicate that a comprehensive survey of additional European localities is needed to elucidate the dispersal pathways of *H. japonica*. Recent records and interpretation of several molluscan taxa as alien are discussed both in light of new Mediterranean (published and unpublished) records and of four categories previously excluded from alien species lists. Within this framework, ten taxa are no longer considered as alien species, or their records from Italy are refuted. Furthermore, *Trochocochlea castriotae* Bellini, 1903 is considered a new synonym for *Gibbula albida* (Gmelin, 1791). Data provided here leave unchanged as 35 the number of alien molluscan taxa recorded from Italy as well as the percentage of the most plausible vectors of introduction, but raise to 22 the number of established species along the Italian shores during the 2005–2010 period, and backdate to 1792 the first introduction of an alien molluscan species (*L. saxatilis*) to the Italian shores.

Keywords

Alien Mollusca, natural entries, translocations, state of knowledge, Italy

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Biogeographical homogeneity in the eastern Mediterranean Sea - I: the opisthobranchs (Mollusca: Gastropoda) from Lebanon

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Abstract

A review of opisthobranch species from Lebanon (eastern Mediterranean Sea), based on literature records (scattered throughout various papers published over a period of more than 150 years) and recently collected material (1999-2002 within the CEDRE framework and other samples), is presented, yielding a total number of 35 taxa identified to species level. Special emphasis has been placed on alien species, for which scattered notes are also given. The known opisthobranch biota is composed of 22 native (~ 63%), 12 alien (~ 34%) and one cryptogenic (~ 3%) taxa. Eleven of these (*Berthella aurantiaca*, *Berthella ocellata*, *Aplysia fasciata*, *Felimare picta*, *Felimida britoi*, *Felimida luteorosea*, *Felimida purpurea*, *Phyllidia flava, Dendrodoris grandiflora*, *Dendrodoris limbata* and *Aeolidiella alderi*) constitute new records for the Lebanese fauna, whilst the examined material of a further seven species (*Elysia grandifolia*, *Pleurobranchus forskalii*, *Aplysia dactylomela*, *Bursatella leachii*, *Syphonota geographica*, *Goniobranchus annulatus*, *Flabellina rubrolineata*), anecdotally cited from Lebanon on the basis of the samples studied here, is explained for the first time. One additional taxon belonging to the genus *Haminoea* has been identified to genus level only.

Keywords: Mediterranean Sea, Lebanon, Mollusca, Opisthobranchia, alien species, cryptogenic species.

Introduction

During the last decades, due mostly to the opening of the Suez Canal, aquaculture and ship transport, hundreds of alien species have established themselves in the Mediterranean Sea (Galil, 2009; Zenetos *et al.*, 2012). To date, the bulk of the introduced organisms in the basin (> 70%) are thermophilic species of Indo-Pacific origin, coming from the Red Sea either stepwise through the Suez Canal ("Lessepsian migration" in the most restricted sense) or as one-jump larvae or adults (Gofas & Zenetos, 2003). Mostly confined to the easternmost Levantine shores for decades, several Erythrean species are currently spreading further to its western and northern parts, encouraged by the general warming of the Mediterranean Sea (Occhipinti-Ambrogi, 2007).

The number of alien species invading the Mediterranean Sea is now continuously increasing, particularly in the eastern basin. After the first general review published by Zibrowius (1992), a growing literature on the subject has been published in recent years (e.g.: Galil, 2009; Zenetos *et al.*, 2012). Molluscs are one of the major groups in the marine fauna worldwide and the first contributors to the alien fauna in the Mediterranean Sea (Zenetos *et al.*, 2010, 2012). Among them, opisthobranchs are a diverse group of specialized gastropod molluscs and important components of benthic marine ecosystems, exhibiting a wide range of food and defensive strategies (Cervera *et al.*, 2004). To date, more than 500 species of opisthobranchs are listed as recorded from the Mediterranean Sea (Gosliner *et al.*, 2008; Templado & Villanueva in Coll *et al.*, 2010), of which ~ 30 species are exotic. Despite the Mediterranean molluscan fauna being commonly considered as the best known in the world (Oliverio, 2003), our general knowledge of the Levantine area still remains considerably poor due to lack of recent comprehensive studies, especially on opisthobranchs, and most of the current knowledge on the opisthobranch fauna of the Levant Sea originates from very sparse records. A comprehensive list of the opisthobranchs from Lebanon is currently missing, and data on distribution, taxonomy and ecology are scattered throughout various papers published over a period of more than 150 years. In fact, with the exception of a few papers from the early XIX and XX centuries (e.g. Puton, 1856; Pallary, 1912, 1919, 1938; Gruvel & Moazzo, 1929; Moazzo, 1931), during the last eighty years hardly a dozen papers, notes, abstracts and non peer-reviewed articles marginally cited opisthobranch species from

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Biogeographical homogeneity in the eastern Mediterranean Sea. II. Temporal variation in Lebanese bivalve biota

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ABSTRACT: Lebanon (eastern Mediterranean Sea) is an area of particular biogeographic significance for studying the structure of eastern Mediterranean marine biodiversity and its recent changes. Based on literature records and original samples, we review here the knowledge of the Lebanese marine bivalve biota, tracing its changes during the last 170 yr. The updated checklist of bivalves of Lebanon yielded a total of 114 species (96 native and 18 alien taxa), accounting for ca. 26.5% of the known Mediterranean Bivalvia and thus representing a particularly poor fauna. Analysis of the 21 taxa historically described on Lebanese material only yielded 2 available names. Records of 24 species are new for the Lebanese fauna, and *Lioberus ligneus* is also a new record for the Mediterranean Sea. Comparisons between molluscan records by past (before 1950) and modern (after 1950) authors revealed temporal variations and qualitative modifications of the Lebanese bivalve fauna, mostly affected by the introduction of Erythraean species. The rate of recording of new alien species (evaluated in decades) revealed later first local arrivals (after 1900) than those observed for other eastern Mediterranean shores, while the peak in records in conjunction with our samplings (1991 to 2010) emphasizes the need for increased field work to monitor their arrival and establishment. Finally, the scarce presence (or total absence) in the most recent samples of some once common habitat-forming species, as well as of some other native taxa, confirmed their recent rarefaction (or local extinction), possibly related to their replacement by the aliens *Brachidontes pharaonis*, *Spondylus spinosus* and *Chama pacifica*.

KEY WORDS: Mediterranean Sea · Lebanon · Mollusca · Bivalvia · Alien species · Faunal changes

INTRODUCTION

The present-day autochthonous Mediterranean marine fauna is mostly of Atlantic origin, having originated with the re-establishment of the Atlanto-Mediterranean connection (5.33 million years ago), after the Messinian Salinity Crisis (from 5.971 to 5.33 million years ago; Manzi et al. 2013) had probably nearly exterminated the stenoecious marine biota (Sabelli & Taviani in press). The subsequent 5 million years of evolution within the framework of the complexity of the basin gave rise to the peculiar and variegate Mediterranean assemblage, with the co-occurrence of temperate and subtropical organisms and a main transitional zone between the 2 seas (the Alboran Sea), hosting a mix of Mediterranean and Atlantic species (Bianchi & Morri 2000, Oliverio 2003, Coll et al. 2010).

The opening of the Suez Canal in the south-eastern corner of the Mediterranean in 1869 contributed to changes in the local biodiversity and provided an additional human-induced transitional zone. The spreading of alien species (used here as defined by

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Slipping through the Cracks: The Taxonomic Impediment Conceals the Origin and Dispersal of *Haminoea japonica*, an Invasive Species with Impacts to Human Health

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Abstract

Haminoea japonica is a species of opisthobranch sea slug native to Japan and Korea. Non-native populations have spread unnoticed for decades due to difficulties in the taxonomy of *Haminoea* species. *Haminoea japonica* is associated with a schistosome parasite in San Francisco Bay, thus further spread could have consequence to human health and economies. Anecdotal evidence suggests that *H. japonica* has displaced native species of *Haminoea* in North America and Europe, becoming locally dominant in estuaries and coastal lagoons. In this paper we study the population genetics of native and non-native populations of *H. japonica* based on mt-DNA data including newly discovered populations in Italy and France. The conclusions of this study further corroborate a Northeastern Japan origin for the non-native populations and suggest possible independent introductions into North America and Europe. Additionally, the data obtained revealed possible secondary introductions within Japan. Although non-native populations have experienced severe genetic bottlenecks they have colonized different regions with a broad range of water temperatures and other environmental conditions. The environmental tolerance of this species, along with its ability to become dominant in invaded areas and its association with a schistosome parasite, suggest *H. japonica* could be a dangerous invasive species.

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Introduction

Existing gaps in taxonomic knowledge and the shortage of trained taxonomists to fill the need to identify living organisms are collectively known as the taxonomic impediment [1,2]. The taxonomic impediment has implications for invasion biology [3]. Because alien species can come from all over the world, proper identification of newly arrived species is a major challenge [3]. Misidentification of these species can have serious consequences. For example, early detection is critical to manage and control alien species [4], thus confusing newly arrived aliens with native species can delay early intervention and exacerbate the problem. When those alien species impact human health and economies or harm native populations [5,6], early detection and control become even more critical.

In this paper we study the spread of *Haminoea japonica*, an opisthobranch sea slug native to Japan and Korea with a non-native range including the west coast of North America, Spain, Italy and France [7,8]. Documentation of the spread of *H. japonica* has been hampered by the taxonomic impediment. For example, the first record of this species in North America was published as a new species, *Haminoea callidegenita* Gibson and Chia, 1989 [7]. Additionally, the first COI sequence of *H. japonica* published in GenBank (DQ238004) was misidentified as belonging to *Haminoea hydatis* (Linnaeus, 1758), a morphologically similar European species.

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The evolution of the molluscan biota of Sabaudia Lake: a matter of human history

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SUMMARY: The evolution of the molluscan biota in Sabaudia Lake (Italy, central Tyrrhenian Sea) in the last century is hereby traced on the basis of bibliography, museum type materials, and field samplings carried out from April 2009 to September 2011. Biological assessments revealed clearly distinct phases, elucidating the definitive shift of this human-induced coastal lake from a freshwater to a marine-influenced lagoon ecosystem. Records of marine subfossil taxa suggest that previous accommodations to these environmental features have already occurred in the past, in agreement with historical evidence. Faunal and ecological insights are offered for its current malacofauna, and special emphasis is given to alien species. Within this framework, *Mytilodonta* Coen, 1936, *Mytilodonta paulae* Coen, 1936 and *Rissoa paulae* Coen in Brunelli and Cannicci, 1940 are also considered new synonyms of *Mytilaster* Monterosato, 1884, *Mytilaster marioni* (Locard, 1889) and *Rissoa membranacea* (J. Adams, 1800). Finally, human-driven environmental changes and cumulative anthropogenic pressures proved to be the whole driver of the constitution of the human-induced malacofauna studied, casting doubts on the correct use of the definition of "native fauna".

Keywords: Sabaudia Lake, Tyrrhenian Sea, Mollusca, alpha diversity, human-driven environmental changes.

RESUMEN: Evolución de la comunidad de moluscos del lago Sabaudia: causas antropogénicas. – Se hizo un seguimiento de la evolución de la comunidad de moluscos en el lago Sabaudia (Italia, región central del mar Tirreno), considerando datos del siglo pasado y recurriendo a consultas bibliográficas, material tipo en museos y muestreos de campo realizados entre Abril de 2009 y Septiembre de 2011. Nuestro análisis biológico reveló la existencia de fases bien diferenciadas que muestran de modo claro el cambio definitivo de esta laguna inicialmente de aguas dulces que posteriormente se transformó en un ecosistema de influencia marina debido a la acción humana. El registro de taxones de subfósiles marinos sugiere un contexto ambiental similar en una época anterior, en concordancia con datos históricos. Este trabajo proporciona detalles faunísticos y ecológicos de la malacofauna del lago Sabaudia, dando especial importancia a las especies invasoras. Dentro de este amplio contexto *Mytilodonta* Coen, 1936, *Mytilodonta paulae* Coen, 1936 y *Rissoa paulae* Coen en Brunelli y Cannicci, 1940 son considerados nuevos sinónimos de *Mytilaster* Monterosato, 1884, *Mytilaster marioni* (Locard, 1889) y *Rissoa membranacea* (J. Adams, 1800). Por último, los cambios ambientales de génesis humana y la presión antropogénica a lo largo del tiempo determinaron la composición de la malacofauna estudiada, levantando dudas sobre el uso correcto del concepto "fauna nativa".

Palabras clave: lago Sabaudia, mar Tirreno, moluscos, diversidad alfa, presión antropogénica.

INTRODUCTION

Worldwide confined coastal environments have a long history as human-dominated areas (e.g. Mannino and Thomas 2002, Davenport and Davenport 2006). They also play a key role within the homogenization of the global biota, being hotspots for the introduction and secondary spreading of alien species (e.g. Occhipinti-

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The alien spreading of *Chama pacifica* Broderip, 1835 (Mollusca: Bivalvia: Chamidae) in the Mediterranean Sea

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Abstract: A detailed Mediterranean synonymy (= list of published records) and distributional map is offered for the Pacific jewel-box *Chama pacifica* Broderip, 1835, an alien mollusk invasive in the deep eastern Mediterranean Sea. A more detailed examination of the single specimen published from the Thermaikos Gulf led us to consider it as a juvenile of the native congeneric *C. gryphoides* Linnaeus, 1758. Concomitantly, the presence of *C. pacifica* along the Greek shores is here first recorded on the basis of 4 adult live specimens sampled at Fokià Bay (Kàrpathos Island). Dates of first record in the Mediterranean for each country in which the species was recorded are reviewed with respect to the recent bibliography. On the basis of the specimens directly examined or reported in the literature, the first record date for Turkey and Lebanon is 1999, and for Greece, 2011. Finally, a discussion regarding the Mediterranean spreading of *C. pacifica* is offered.

Key words: *Chama pacifica*, Chamidae, alien Mollusca, Mediterranean Sea, Greece

1. Introduction

Biological pollution is a common phenomenon worldwide (Molnar et al., 2008) and although marine invasions have been widely documented all over the world (Molnar et al., 2008), they are very conspicuous in the Mediterranean Sea, as pointed out in several recent contributions (e.g., Zenetos et al., 2010). The large Pacific jewel-box *Chama pacifica* Broderip, 1835 is a very peculiar species usually considered as one of the most variable cosmopolitan chamids (Huber, 2010) recently included in the *CIESM Atlas of Exotic Species in the Mediterranean* (Zenetos et al., 2004). Its shell shows a strongly irregular outline from suboval to subcircular, including reports of narrower or elongated forms. It is a quite large bivalve species (shells growing up to about 100 mm in length or width), solid and inequivalve, with the lower valve (left valve, LV) usually bigger and deeper than the upper one (right valve, RV), which is usually flat. The ligament is external and the umbones are spirally coiled prosogyrate. The shell sculpture is typically different on each valve, with short- to medium-sized spines that are often more pronounced on the left side (seen from the external) of each valve. Internally with a hinge plate thick with characteristic pachyodont teeth and finely crenulated margins (sometimes not fully visible). Two large, subequal, ovate, and dorsoventrally elongate adductor muscles scars. Pallial line entire and with no sinus. External color highly variable, from white to pink-red, spines often white. Internal color usually half white and half rose red (half rose red on the right side in the RV and on the left side in the LV, seen from the internal).

In the present paper, the pattern of geographical and temporal spread of *Chama pacifica* Broderip, 1835 in the Mediterranean is reexamined following assembly of additional records based on published and as of yet unpublished data, offering a detailed Mediterranean synonymy (= list of published records) and distributional map, reviewing first record dates and reporting the first record in Greece on the basis of sampled specimens and reevaluation of the sole previous record from the area.

2. Materials and methods

Chama pacifica Broderip, 1835 is considered an alien in accordance with the definition proposed by the International Union for Conservation of Nature (IUCN) (http://www.iucn.org/themes/pbia/wl/docs/biodiversity/cop6/invasives.doc).

Literature was surveyed for Mediterranean records and first record dates of *C. pacifica*, particularly that concerning faunistics, taxonomy, and biogeography. Gathered data were used to build an updated Mediterranean distribution and synonymy.

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The spread of an Atlantic fish species, *Pomadasys incisus* (Bowdich, 1825) (Osteichthyes: Haemulidae), within the Mediterranean Sea with new additional records from the French Mediterranean coast

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Abstract
For the first time three specimens belonging to the species *Pomadasys incisus* (Bowdich, 1825), the bastard grunt, were caught in the French Ligurian Sea. This subtropical species, which naturally entered the Mediterranean Sea through the Straits of Gibraltar in the first half of the nineteenth century, and is currently colonizing the whole Mediterranean coast except the Adriatic Sea, was until now unknown for this region. We reviewed its distribution after a compilation of the available literature, and tried to explain the spreading and the establishment of the bastard grunt as a Mediterranean species in light of the prevailing Mediterranean currents and the possible climate changes.

Keywords: *Atlantic Ocean, Mediterranean Sea, Haemulidae,* Pomadasys incisus, *spreading*

Introduction

Four native and one non-native fish species belonging to Haemulidae were recently recorded in the Mediterranean Sea (Froese & Pauly 2011). Among them two belong to the genus *Pomadasys*, *P. incisus* (Bowdich 1825) and *P. stridens* (Forsskål 1775); the latter is the non-native species (CIESM 2009).

P. stridens is a Lessepsian species first observed before 1888 in Port-Saïd (see Carus 1893: 617) [and not by Torchio (1969) as usually stated (e.g. Golani et al. 2002)]. It is mainly present along the Libyan and Egyptian coasts (Golani et al. 2002) and has been recently found in Turkey too (Bilecenoglu et al. 2009).

P. incisus is a coastal demersal species inhabiting marine and brackish waters, usually near sandy or muddy substrate, at depths from 10 to 100 m but often not far from 50 m (Kapiris et al. 2008). It is also frequent in rocky habitats and seagrass meadows (Golani et al. 2006) and sometimes can live in very large shoals. This subtropical species naturally entered the Mediterranean Sea through the Straits of Gibraltar. Its main distribution encompasses the eastern part of the Atlantic Ocean, from Angola (Osorio 1909) to Gibraltar (Palacky 1895), including the Canary Islands (Vinciguerra 1883; Murray et al. 1912; Bianchi 1984), Madeira (Günther 1859; Andrade & Albuquerque 1935; Ribeiro et al. 2005) and the Cape Verde Islands (Osorio 1909). In fact, it was censused in Morocco (Bauchot 1963), Mauritania (Pellegrin 1914; Maigret & Ly 1986), the Ivory Coast and Senegal (Séret & Opic 1990) and the Gulf of Guinea (Fowler 1936; Blache et al. 1970), where it would inhabit brackish waters of several islands (Osorio 1895). However, it is also considered common in South Africa (Gilchrist & Wardlaw-Thompson 1908–1910), in the province of the Cape of Good Hope (Wardlaw-Thompson 1914) and in the Kwazulu-Natal (Fowler 1931).

The aim of our study is to: (1) provide evidence of the occurrence of the bastard grunt along the French Ligurian Sea, (2) to compile all the available records of *P. incisus* in the Mediterranean to provide a synthesis and update of its distribution in the entire basin, and (3) to discuss the hypotheses regarding the spreading and the establishment of the

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The status of the northeastern Atlantic and Mediterranean small mussel drills of the *Ocinebrina aciculata* complex (Mollusca: Gastropoda: Muricidae), with the description of a new species

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SUMMARY: The northeastern Atlantic and Mediterranean small mussel drills of the *Ocinebrina aciculata* complex are here revised and consist of at least 3 species. The type species, *Ocinebrina aciculata* (Lamarck, 1822), characterized by a slender shell with rounded whorls and primary and secondary spiral cords of approximately similar size, lives throughout the northeastern Atlantic and Mediterranean Sea at depths usually ranging between 0 and 105 m. Its synonymy is here stabilized by a neotype selection for *Murex corallinus* Scacchi, 1836. *Ocinebrina corallinoides* Pallary, 1912 (=*Ocinebrina buzzurroi* Cecalupo and Mariani, 2008, new synonymy), characterized by a strongly elongate and weakly convex shell and primary and secondary spiral cords of approximately similar size, is endemic to the Gulf of Gabès and is here considered a distinct species, pending genetic studies. *Ocinebrina reinai* n. sp. is here described from the central Mediterranean Sea (where it is sympatric with *O. aciculata*) on the basis of morphological diagnostic features of shell (rarest presence of labral tooth, commoner presence of infrasutural apertural denticle, dark spots on the ribs and spiral sculpture with differently sized primary and secondary cords and smaller threads) and radula, confirmed by genetic data. Divergence in COI sequences with sympatric samples of *O. aciculata* (>7%), confirm their status as a distinct species. A comparative table reporting diagnostic features of the congeneric species of the complex and those with which the new species was previously misidentified is offered.

Keywords: Mollusca, Muricidae, *Ocinebrina aciculata* complex, *Ocinebrina reinai*, new species, Mediterranean Sea.

RESUMEN: EL ESTATUS DE LAS ESPECIES DEL GRUPO DE *OCINEBRINA ACICULATA* (MOLLUSCA: GASTROPODA: MURICIDAE) DEL ATLÁNTICO NORESTE Y DEL MEDITERRÁNEO, CON LA DESCRIPCION DE UNA NUEVA ESPECIE. – Se revisa la taxonomía de las pequeñas especies de gasterópodos murícidos del grupo de *Ocinebrina aciculata* (Lamarck, 1822). Se consideran válidas tres especies, de las que una se describe como nueva. *O. aciculata* es la especie tipo del género, está caracterizada por una concha alargada, espiras redondeadas, cordones primarios y secundarios aproximadamente del mismo tamaño, y está distribuida en el área del Atlántico noreste y del Mediterráneo, entre 0 y 105 m de profundidad. Para estabilizar su sinonimia se ha seleccionado un neotipo para *Murex corallinus* Scacchi, 1836. *O. corallinoides* Pallary, 1912 (=*Ocinebrina buzzurroi* Cecalupo and Mariani, 2008, nueva sinonimia) es endémica del Golfo de Gabès, y se distingue por su concha muy alargada y poco convexa, con cordones primarios y secundarios aproximadamente del mismo tamaño. Se considera una especie distinta, a la espera de estudios genéticos. *O. reinai* n. sp. se describe aqui, y se ha encontrado en Mediterráneo central, donde vive simpátrica con *O. aciculata*, de la que se distingue por caracteres morfológicos de la concha (rara presencia del diente labral, diente apertural infrasutural más frecuente, manchas oscuras en las costas axiales, cordones primarios y secundarios de tamaño diferente) y de la rádula, confirmados por datos genéticos. Las secuencias del gen mitocondrial COI indican una distancia genética con *O. aciculata* en simpatría de más de 7%, confirmando su estatus de especie distinta. Se presenta una tabla comparativa de las diferencias entre las especies más similares.

Palabras clave: Mollusca, Muricidae, *Ocinebrina aciculata* complex, *Ocinebrina reinai*, nueva especie, mar Mediterráneo.

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Marine alien Mollusca in Italy: a critical review and state of the knowledge

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The state of the knowledge about the marine alien molluscan species from Italy is provided based on a critical review of records compiled from an extensive literature survey and from unpublished data obtained from 2005 to 2010. Based on the IUCN definition of 'alien', 35 molluscan taxa (18 Gastropoda, 16 Bivalvia and 1 Cephalopoda) are reported here, for each of which the following data (collected up to December 2010) are provided: published and unpublished records from the coastal and offshore territorial seawaters of Italy, including lagoons, within the 14 biogeographical sea divisions covering the Italian shores, date of first record, most plausible vector(s) of introduction and establishment status. The southern Ionian Sea, the northern Adriatic Sea and the eastern-central Tyrrhenian Sea resulted to be the areas most affected by alien molluscan introductions. The rate of records of new alien species (evaluated on the basis of live findings) is quite uniform over five decades, with six to eight species recorded per decade. The analysis of the vectors showed shipping/maritime transport to be the most common vector of introduction (40%), followed by trade (24%). Nineteen alien molluscan species (54%) were considered as established in Italy.

Keywords: Mediterranean Sea, Italy, marine Mollusca, alien species, biological pollution

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INTRODUCTION

Biological pollution is a common phenomenon worldwide (Molnar *et al.*, 2008). The alien species can negatively affect the native ones through predation and direct and indirect competition, can contaminate the native gene pool by the introduction of exotic genes (e.g. through hybridization), can diffuse parasites and pathogens and may modify ecosystem functioning and abiotic features of the environments (Strayer *et al.*, 2006). Thus, the prevention and control of alien species are a priority for conservation of biodiversity. Although marine introductions have been widely documented all over the world (Molnar *et al.*, 2008), they are very conspicuous in the Mediterranean Sea, where the native fauna is more susceptible to alien pressure due to the higher number of vectors of introductions: transport on ship hulls and/or with ballast water; intentional (aquaculture) and accidental introductions accompanying intentionally introduced species; escape from aquaria; trade, market discards and, mainly, lessepsian migration through the Suez Canal, a pathway peculiar to the Mediterranean and one that has resulted in several hundred introduced species from practically all marine phyla (Galil, 2009; Zenetos *et al.*, 2010). Invasions seem to be also locally amplified by the effects of global warming, that favours the occurrence and establishment of warm water species in the Mediterranean Sea and increases the possibility of unexpected changes in faunal assemblages including the decline and even collapse of certain marine ecosystems (Bianchi, 2007; Occhipinti-Ambrogi, 2007; Raitsos *et al.*, 2010).

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Given the difficulties in stopping invasions, a careful analysis of the present situation may help to correctly understand the ongoing phenomenon. From 2005 to 2010, field data collected by the author, along with an extensive literature survey, revealed three new alien species from Italy (Crocetta, 2005; Crocetta & Vazzana, 2008; Crocetta & Turolla, 2011), reviewed the date of the first Italian record for three species (Crocetta *et al.*, 2009; Crocetta & Turolla, 2011) and defined more accurately the distribution of twelve species along the Italian territorial seawaters (Crocetta & Soppelsa, 2006; Crocetta *et al.*, 2008, 2009, 2010; Crocetta & Colamonaco, 2010, 2011; Crocetta & Turolla, 2011).

The aim of the present paper is to review information on alien marine Mollusca collected up to December 2010 in order to enumerate the species recorded from the territorial seawaters of Italy, to report their occurrence (or absence) in the 14 biogeographical sea divisions covering the Italian shores, to establish the date of their first record and their most plausible vector(s) of introduction and to assess their establishment status, therefore giving the state of knowledge as regards the alien molluscan species recorded from Italian marine waters.

MATERIALS AND METHODS

The field survey of the Italian shores during the period 2005–2010, from the supralittoral zone down to a depth of 50 m, provides the main source of results reported here. In addition, SCUBA diver portfolios have been checked in search of alien species and a significant help was offered by researchers and shell collectors of the Società Italiana di Malacologia (S.I.M.), who communicated new findings of alien species and allowed the study of material housed in their private

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THE INVASIVE SPOTTED SEA HARE *APLYSIA DACTYLOMELA* (MOLLUSCA: GASTROPODA: APLYSIIDAE) – NEW RECORDS AND SPREAD PATTERN IN THE MEDITERRANEAN

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APLYSIA DACTYLOMELA
APLYSIIDAE
MOLLUSCA
MEDITERRANEAN
ALIEN

ABSTRACT. – Recent and overlooked unpublished records (2005-2011) of the invasive alien sea hare *Aplysia dactylomela* from Israel, Cyprus, Turkey, Greece, Malta and Italy are presented. We confirm the presence of established populations in Israel, Malta and the Gulf of Taranto (Italy, Ionian Sea), where hitherto the species was known from single records. In addition, new records from Cosenza and Vibo Valentia provinces point to a spread northwards along the Tyrrhenian coast of Italy. Finally, the possible pathway of introduction to the Mediterranean is discussed taking account of a previously unreported record off Israel.

INTRODUCTION

The spotted sea hare *Aplysia dactylomela* Rang, 1828 (Mollusca: Gastropoda: Aplysiidae) has a circumglobal distribution in shallow tropical and warm temperate waters, and is present along the central west African coast, Cape Verde, Canary and Madeira islands and in the Red Sea (see Eales 1960, Bebbington 1974, 1977, Wirtz 1998, Dekker & Orlin 2000). It is a large opisthobranch species, yellowish-purplish-brownish in color, with distinctive black rings superimposed on a reticulate pattern. The high parapodial lobes are joined low down posteriorly, and the anterior cephalic tentacles and rhinophores are broad and blunt (Yokeş 2006). It is considered an alien in the Mediterranean in accordance with the definition proposed by the International Union for Conservation of Nature (IUCN) (http://www.iucn.org/themes/pbia/wl/docs/biodiversity/cop6/invasives.doc).

Pasternak & Galil (2010) summarized the species distribution in the Mediterranean Sea: it was first recorded from Lampedusa Island in 2002, but has since spread to Sicily, Calabria and the Gulf of Taranto. In 2004 it was noted on the northern coast of Cyprus, and a year later in Akrotiri on the southern coast. It was collected in 2005 along the Mediterranean coast of Turkey, as well as off Rhodes and in Messiniakos Gulf, Greece; in 2006 off Crete, Paros Island and the Gulf of Saronikos, and in 2007 off Zakynthos. In 2006 it was recorded in Croatia and in 2008 a specimen was photographed off Malta. But only in 2010 it was recorded off the Israeli coast. The recorded presence of *A. dactylomela* in the Mediterranean has been limited so far to the central and eastern basins, but absence of early records from the SE Levant cast doubt on the provenance of these populations as Erythrean aliens. In the present paper, the pattern of geographical and temporal spread of *A. dactylomela* in the Mediterranean is clarified.

MATERIALS AND METHODS

A survey of published, grey literature and yet unpublished records for Mediterranean records and first record dates of *A. dactylomela* was conducted and information concerning its faunistics was noted. Unpublished data were obtained from analysis of preserved specimens, photographs and personal communications obtained from researchers and members of the public.

RESULTS

Aplysia dactylomela Rang, 1828

Published records

Aplysia dactylomela, Trainito 2003: 20, fig. 35; Trainito 2005: 22-23, fig. 34; Scuderi & Russo 2005: 338-341, figs 1-2; Çinar *et al*. 2006: 86-87, 90, fig. 5; Greco 2006: 125-128, figs 1-2; Yokeş 2006: 1-3, fig. 1; Zenetos *et al*. 2007: 3; Despalatović *et al*. 2008: 54-55; Schembri 2008: 111-114, fig. 1; Crocetta *et al*. 2009: 19, 21, fig. 2, F-G; Yokeş 2009: 17; Poursanidis *et al*. 2009: 175; Zenetos *et al*. 2009: 1-2, 6; Crocetta & Colamonaco 2010: 1-4, fig. 3; Delongueville & Scaillet 2010: 3-7, fig. 16; Yokeş *et al*. 2010: 701; Giacobbe *et al*. 2010: 528; Pasternak & Galil 2010: 437-440, fig. 1; Crocetta & Colamonaco 2011: 1-2, fig. 1; Katsanevakis 2011: 133-137; Turk & Furlan 2011: 7-8, fig. 5; Crocetta, 2012: 5.

80 µm in diameter, 200 µm long. Gametangia were only observed in more richly branching specimens in December 2011.

The species was reported for the first time in the Mediterranean Sea from Israel by Silva (1960) followed by Gallardo *et al*. (1993). It was also recorded from Alexandria (Egypt) by Aleem (1993), while until now it was not known to occur in the Aegean Sea (Zenetos *et al.*, 2009). Cormaci *et al.* (2004) assumed that it was probably introduced into the Mediterranean Sea by fouling, ballast water, anchors and fishing tools. Most probable vectors responsible for the introduction in Izmir Gulf include spreading from the population established in the Eastern Mediterranean, ship fouling or ballast waters.

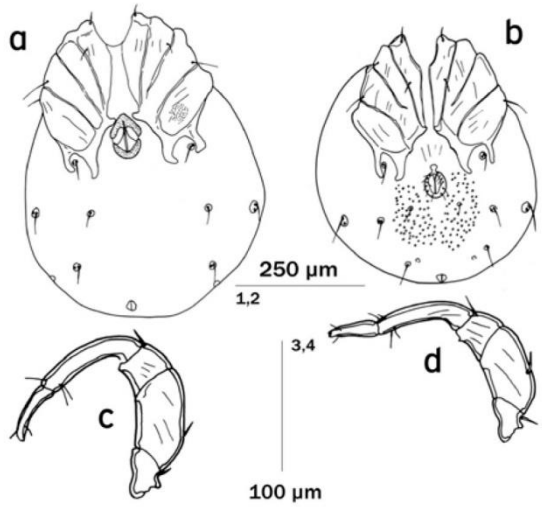
2. Animals

2.1. The marine mite genus *Litarachna* (Acari: Hydrachnidia): first record for the Turkish fauna

By Y.Ö. Boyaci and F. Durucan

The water mite family Pontarachnidae is the only Hydrachnidia family occurring in the marine environment, and is represented by 3 genera, namely, *Pontarachna* Philippi, 1840, *Litarachna* Walter, 1925 and *Paralitarachna* Cook, 1958 (Smit, 2012).

Two specimens (one male and one female) of *Litarachna divergens* Walter, 1925 were collected from the Mediterranean coast of Turkey in Side (Antalya) (36°45'56 N; 31°23'27 E) by hand netting among coralline algae and submerged macroalgae on shallow sublittoral rocks at a depth of < 2 m. This is the first record of the genus *Litarachna* from Turkey.



Figs 2 a-d: *Litarachna divergens* Walter: (a) idiosoma, ventral view, female; (b) idiosoma, ventral view, male; (c) Pedipalp, female; (d) pedipalp, male.

Based on the description by Schaub (1889) using material from Trieste, Walter (1925) established *Litarachna divergens* as a new species. The general morphology and palps of our specimens agree with Schaub's description. The characteristics of the specimens are given below (measurements in µm):

Female - Soft bodied, dorsum without sclerits. Suture lines of second and third coxal plates and of third and fourth coxal plates incomplete. Eyes in pair. Coxae directed posteriorly, first coxal plate fused, fourth coxae widely separated. Idiosoma L/W 586/464. Genital field L/W 86/65 (Fig. 2a). Palp total L 330 (Fig. 2c). PI – PV length (and as % of total L): 43 (13), 87 (26.3), 35 (10.6), 109 (33), 56 (16.9). PIV/PV ratio 1.94. PIV and PV very slender (Fig. 2c).

Male - Idiosoma L/W 486/429. Genital field L/W 64/42. Many small setae around genital field (Fig. 2b). Palp total L 303 (Fig. 2d). PI-PV length (and % of total L): 36 (12), 76 (25), 33 (11), 100 (33), 58 (19). PIV/PV ratio 1.74.

2.2. *Dyspanopeus sayi* (Brachyura: Xanthoidea: Panopeidae) in Lago Fusaro (SW Italy)

By F. Crocetta, P. Sordino and F. Toscano

Dyspanopeus sayi (Smith, 1869) is a euryhaline species, a native of the NW Atlantic coast, from Nova Scotia to Florida Keys. It is one of the most common crab species living in ports and estuaries, being able to thrive in polluted environments.

Since 1960, the species has begun to colonize new areas, unintentionally introduced by human vectors along the European Atlantic shores of Britain, France and The Netherlands (see Micu *et al.*, 2010; Schubart *et al.*, 2012 for references). Since 1978-1979, *D. sayi* has spread to the Mediterranean Sea along the Adriatic shores, where it is now established (Froglia and Speranza, 1993; Mizzan, 1995; Florio *et al.*, 2008). While very recent sightings from the Black Sea (Micu *et al.*, 2010) and the western Mediterranean Sea (Ebro River mouth: Schubart *et al.*, 2012) point to a relentless spreading towards both basins, records from intermediate locations are lacking.

During a field survey in Lago Fusaro (Campania, southern Italy), a brackish lagoon north of Naples, carried out from March to September 2011 on a weekly basis, the occurrence of *D. sayi* was recorded in serpulid reefs and *Mytilaster lineatus* beds (including ovigerous females and juveniles), with sporadic specimens walking on the predominantly muddy bottom. *D. sayi* was the most abundant crab species along the lagoon shores, accounting for hundreds of sighted specimens. Voucher specimens are preserved in the private collection of the first author (Naples, Italy) (Fig. 3).

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Marine alien Mollusca in the Gulf of Trieste and neighbouring areas: a critical review and state of knowledge (updated in 2011)

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The state of knowledge on marine alien molluscan species from the Gulf of Trieste and neighbouring areas is presented based on a critical review of records compiled from an extensive literature survey and from unpublished data obtained from 2006 to 2011, and enriched by older material preserved in private collections. Based on the IUCN definition of 'alien', 13 valid alien molluscan taxa (3 Gastropoda and 10 Bivalvia) are reported here, for each of which the following information (collected up to August 2011) is provided: published and unpublished records from the coastal and offshore territorial seawaters of the Gulf of Trieste and neighbouring areas, including lagoons; establishment status; vector(s) of introduction. The area was characterized by the presence of eight established alien species, while another four were considered as casual and one was, with caution, considered not established. Specimens of Anadara transversa *(Say, 1822) and* Limnoperna securis *(Lamarck, 1819) are first reported here from the Gulf of Trieste, thus reaching the extreme northern point of the Mediterranean Sea. Old distribution data on* Mercenaria mercenaria *(Linnaeus, 1758) and* Mya arenaria *Linnaeus, 1758 are considered unreliable, also according to the recent literature. Accurate analysis of bibliographic data as well as re-identification of specimens preserved in private collections or collected from the same published sampling sites led to the exclusion of A*ssiminea grayana *Fleming, 1828 and* Conomurex persicus *(Swainson, 1821) from the resident fauna of the Gulf of Trieste. Concerning* Arcuatula senhousia *(Benson in Cantor, 1842), its first record from Slovenia came from bibliographic misreading, so that the original record of this bivalve from the area is considered more recent. Finally, natural dispersal accounts for 46% of the plausible vectors of introduction, while shipping/maritime transport and aquaculture for 23%.*

Key words: Mediterranean Sea, Adriatic Sea, Gulf of Trieste, Marine Mollusca, Alien species

INTRODUCTION

Biological pollution is recognized worldwide as one of the main threats to biodiversity, the economy and human health (ELLIOTT, 2003). The opening of the Suez Canal in 1869 has led to the colonization of the Mediterranean Sea by a large number of tropical/subtropical species that have established viable populations along the Levantine coast and subsequently spread into the central Mediterranean Sea, in part by natural dispersal and in part by the transport of larvae, juveniles or adult specimens by shipping. The increase in human activities, aquaculture and leisure boating in the past century has contributed to the introduction of alien species in the Mediterranean Sea. Natural and/or anthropic introduction of alien species contributes to the

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Research Article

Marine alien species in Greek Seas: Additions and amendments by 2010

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Abstract

An update of the inventory of alien marine species from the coastal and offshore waters of Greece is presented. Records were compiled based on the existing scientific and grey literature, including the HCMR database of Greek alien species (ELNAIS), technical reports, scientific congresses, academic dissertations, websites, and unpublished/personal observations. 47 species were added to the inventory, including 34 invertebrates, one vertebrate (fish), three plants, eight protozoa, and one cyanobacterium. With the new records, the inventory of alien marine species of Greece now includes a total of 237 species (33 macrophytes, 131 invertebrates, 42 vertebrates, two bacteria and 29 protozoans). Among these, the presence of the gastropod *Hypselodoris infucata,* the bivalves *Dendrostrea frons* and *Septifer forskali* and the chondrichthyan *Rhizoprionodon acutus* is reported here for the first time. Based on molecular analysis, the occurrence of *Bulla arabica* in Greek waters is confirmed, and the suggestion that previous records of *Bulla ampulla* in the Mediterranean should be considered as misidentification of *B. arabica* is further supported. The acclimitization status of earlier records was revised in the light of new data, and thus the fish *Enchelycore anatina, Seriola fasciata* and *Tylerius spinosissimus,* the red algae *Hypnea cornuta* and *Sarconema scinaioides,* the scyphomedusa *Cassiopea andromeda,* the cephalopod *Sepioteuthis lessoniana,* the nudibranch *Chromodoris annulata* and the bivalves *Gastrochaena cymbium* and *Pseudochama corbieri* were upgraded from casual records to established populations. The increased rate of introductions of warm water species confirms previous findings, which link the rate of introduction in the eastern Mediterranean to climate change.

Keywords: Marine aliens; Greece; *Hypselodoris infucata; Rhizoprionodon acutus; Dendrostrea frons; Septifer forskali; Bulla arabica.*

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Research Article

New records of the genus *Pachygrapsus* (Crustacea: Decapoda) from the central Mediterranean Sea with a review of its Mediterranean zoogeography

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Abstract

The occurrence of *Pachygrapsus maurus* and *Pachygrapsus transversus* is reported from the Maltese Islands for the first time on the basis of one specimen of *P. maurus* collected in 1990 and numerous recent specimens, and the distribution of the two species is mapped. The controversial presence of *P. maurus* in Italy is confirmed and two new sites for this species are reported, including the first for the mainland of Italy. The examination of the historical specimen of *P. maurus* from the Genova area revealed a misidentification of *P. transversus*; this record could be a result of ship-mediated transport. First notes on the habitat of *P. maurus* in the central Mediterranean Sea are given. Updated maps of the distribution of *P. maurus* and *P. transversus* in the Mediterranean are provided and the zoogeography of these species is revisited.

Keywords: *Pachygrapsus marmoratus*; *Pachygrapsus maurus*; *Pachygrapsus transversus*; Zoogeography; Mediterranean Sea.

Introduction

The grapsid genus *Pachygrapsus* Randall, 1840 has been recently revised by Poupin *et al.* (2005) who recognized at least 12 valid species. Subsequently, Schubart *et al.* (2005), on the basis of morphological and genetic differences between populations of *Pachygrapsus transversus* sensu Rathbun (1918) and subsequent authors up to 2005, reinstituted the taxon *P. socius* Stimpson, 1871 for Eastern Pacific populations, raising to 13 the number of valid species in the genus.

Three species included in *Pachygrapsus* occur in the Mediterranean Sea: *P. marmoratus* (Fabricius, 1787), *P. maurus* (Lucas, 1846) and *P. transversus* (Gibbes, 1850); although these species can be readily distinguished on the basis of morphological features (Zariquiey Alvarez, 1968) (Fig. 1),

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— SHORT COMMUNICATION —

Mya arenaria Linné, 1758 (Mollusca: Bivalvia) in the Mediterranean Sea: its distribution revisited

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The alien soft-shell clam *Mya arenaria* Linnè, 1758 is first reported in the central Mediterranean Sea, while its presence in the eastern Mediterranean Sea is definitively confirmed by living specimens. There are no data to assess a certain origin of the Italian specimens, but its presence, after two years from oral communications and initial records, confirms that the species costantly occurs in the area. The Mediterranean distribution and local impact dates of observation are reviewed and an updated distributional map is given.

Key words: *Mya arenaria*, mollusc, alien species, Mediterranean Sea, distribution.

INTRODUCTION

The opening of the Suez Canal in 1869 linked two different zoogeographical regions, removing natural geographic barriers and causing the colonization of the Mediterranean Sea by tropical-subtropical species of Indo-Pacific origin. Furthermore, the increase in human activities, aquaculture and leisure boating in the past century, dramatically enhanced the introduction of non-indigenous species in the Mediterranean Sea (Zenetos *et al.*, 2008; Galil, 2009). The natural migration and human-facilitated introduction of non-native species contribute to the alteration of autochthonous communities and could disrupt the dynamic stability between native biota and their physical and biological environments (Boudouresque & Verlaque, 2002; Occhipinti-Ambrogi, 2007; Galil, 2007). We add new data clarifying the Mediterranean distribution of *Mya arenaria* and first reporting live specimens from the Central and the Eastern Mediterranean Sea.

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MATERIALS AND METHODS

Description of the area

Sacca di Goro (44°47′-44°50′ N and 12°15′-12°20′ E, Fig. 1, station 1) is an Adriatic coastal lagoon situated at the southern edge of the Po river delta. Water exchange with the sea is guaranteed both by a natural opening and by an anthropic cut of the "scanno". The main tributary of the Sacca di Goro Lagoon is the Po di Volano river, with other freshwater inlets located along the Po di Goro river and Canal Giralda, Bianco and Bonello. The fresh water inflow can cause marked changes in salinity, varying from 5 to 33‰. Due to its high trophic level, the Sacca di Goro Lagoon is one of the largest clam-farming grounds in Europe and about 1300 fishermen, associated in cooperatives, exploit about 10 km^2 of the aquatic surface, with an annual production, mainly of *Ruditapes philippinarum*, that reaches around 12,000-15,000 tons (Turolla, 2008).

The overall biodiversity, the composition and structure of the molluscan assemblage and the presence of clam seed production are seasonally assessed in the Sacca di Goro Lagoon by C.Ri.M. (Cen-

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