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An integrative taxonomy framework for the study of the genus *Ciona* (Ascidiacea) and the description of the new species *Ciona intermedia*

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Abstract

The genus *Ciona* Fleming, 1822 is an interesting "taxonomic case" since its evolutionary history and taxonomy have not been yet completely resolved. This study presents new findings, describing specimens of a non-identified *Ciona* species collected along the North-eastern coasts of Sardinia (Tyrrhenian Sea, Mediterranean Sea). Applying an integrative taxonomic approach, based on the joint examination of morphological and molecular traits, we identified these specimens as a new species, *Ciona intermedia*. Morphological comparisons and habitat peculiarities first revealed that these *Ciona* specimens have intermediate characters compared to those of other *Ciona* species. Then, the molecular characterization (based on three mitochondrial regions: two already used for discriminating *Ciona* cryptic species and a newly developed one) confirmed that our specimens could not be assigned to any previously molecularly-characterized species. Noteworthy, both molecular phylogenetic reconstructions and morphological data clearly indicated *C. intermedia* as a sister clade of *Ciona edwardsi* Roule, 1884. Our findings add further complexity to the *Ciona* taxonomy, underlying the importance of an integrative taxonomic approach for the study of this still enigmatic genus and of its evolutionary history.

Keywords: *Ciona*, Ascidian, new species, Mediterranean Sea, mitochondrial genes, DNA barcode

Introduction

The genus *Ciona* Fleming, 1822 includes several species used as model organisms in various research fields, from evolutionary developmental biology to chordate evolution (Millar, 1953; Dehal *et al.*, 2002; Cañestro *et al.*, 2003; Satoh *et al.*, 2003). Its suitability as model organism is due to anatomical and molecular features of this genus such as the large size, the transparency of the tunic, the macroscopic internal anatomy, the easily detected reactions to external stimuli (Millar, 1953) and the small compact genome as well as by the availability of powerful experimental tools for molecular studies (Lemaire, 2011). More recently, this genus has also become a case study for ecological studies (Procaccini *et al.*, 2011, Zhan *et al.*, 2015), notably because two of these members, namely *Ciona intestinalis* (Linnaeus, 1767) and *Ciona robusta* Hoshino & Tokioka, 1967 (formerly known as *C. intestinalis* type B and *C. intestinalis* type A, respectively) have been recognized invasive species in several regions at a worldwide level (see Supplementary Material in Bouchemousse *et al.*, 2016a). Nevertheless, this genus represents a real “taxonomic case”, since it is characterized by debated assignment at order level, strong difficulties in delineation and delimitation of the different species, and a complex evolutionary history. Indeed, the genus *Ciona* is currently placed in the order Phlebobranchia based on the presence of a large pharynx with inner longitudinal vessels (Monniot, 1991). In the past, it was included in the order Aplousobranchia (Kott, 1990, 2005) on the basis of its vanadium oxidation state (Hawkins *et al.*, 1983) and of the regenerative role of its epicardial tissue (Kott, 1990). More recently, some molecular phylogenetic reconstructions supported the sister relationship of the genus *Ciona* to Aplousobranchia (Turon & López-Legentil, 2004; Shenkar *et al.*, 2016), although sometimes with an unstable positioning (see discussion in Shenkar *et al.*, 2016). As for the difficulties in species delineation, for a long time (Berrill, 1950) several *Ciona* species were synonymized with the type species *C. intestinalis*, until, in the early 2000’s, molecular studies indicated the existence of a surprisingly high genetic divergence among specimens of *C. intestinalis* from distant geographic localities (Suzuki *et al.*, 2005; Caputi *et al.*, 2007; Iannelli *et al.*, 2007; Nydam & Harrison, 2007, 2010; Zhan *et al.*, 2010; Sato *et al.*, 2012). These findings led first to the description of *C. intestinalis sensu lato* as a complex of four cryptic species, named from A to D, and then to the in-depth morphological analyses revealing that *C. intestinalis* type A is *C. robusta*, while *C. intestinalis* type B corresponds to *C. intestinalis* (Brunetti *et al.*, 2015; Pennati *et al.*, 2015). In addition, in sympatric areas, no introgression has been reported between the two species, proving the existence of reproductive barriers between the two taxa (Bouchemousse *et al.*, 2016b, 2016c; Malfant *et al.*, 2018). Thus, the current knowledge indicates that *C. robusta* and *C. intestinalis* are clearly distinguishable by the morphology of both adults and larvae, as well as by their genetic background and global distribution. On the contrary, *Ciona* spC and spD still lack morphological diagnostic characters and can be currently identified only through molecular analyses (Nydam & Harrison, 2007, 2010; Zhan *et al.*, 2010; Brunetti *et al.*, 2015). Another peculiar case is represented by the *Ciona roulei* Lahille, 1890. As for the nomenclature, we need to clarify that the original binomial of this species was *Ciona roulii* Lahille, 1890, and that Brunetti *et al.*, (2015) considered the binomial *Ciona roulei* as an “Incorrect Subsequent Spelling” (ICZN, 1999 art 33.3) introduced by Hartmeyer (1909-1911) and Harant & Vernières (1933) without a clear justification. On the other hand, the *C. roulii* binomial was clearly created by Lahille to homage Roule (author of many ascidian species from 1883 to 1887), thus it can be considered an inadvertent misspelling (ICZN, 1999 art 32.5). Moreover, the *C. roulei* binomial was in prevailing usage by all subsequent authors (ICZN, 1999 art 33.3.1) (*i.e.*, Harant & Vernières, 1933). Therefore, here we choose to maintain the spelling *roulei*. Interestingly, mitochondrial phylogenetic analyses could not distinguish between *C. roulei* and *C. intestinalis* and

the two taxa hybridize with high rate of success in both directions, displaying survival and growth rates similar to those found in the respective intra-species crosses (Malfant *et al.*, 2018). Therefore, further in-depth investigations, integrating different types of data and different methodologies, are needed for confirming/excluding the taxonomic validity of *C. roulei*. It should be also noticed that, within the genus *Ciona*, the usage of the biological species concept should be carefully examined. For instance, *in vitro* crosses and first generation hybrids can be produced among accepted species, such as *C. intestinalis* and *C. robusta* (Sato *et al.*, 2014; Bouchemousse *et al.*, 2016c; Malfant *et al.*, 2018), two taxa that introgressed in the past (Roux *et al.*, 2013). Later on, studies of natural populations showed that hybridization is not followed by successful introgression (Bouchemousse *et al.*, 2016b), suggesting the existence of reproductive barriers between the two species in the wild. On the contrary, *Ciona edwardsi* Roule, 1884 showed a complete reproductive isolation from the species *C. intestinalis*, *C. roulei* and *C. robusta* (Lambert *et al.*, 1990; Malfant *et al.*, 2018). These case-studies show the complexity of the evolutionary history and speciation processes in this genus that can obscure complicated taxonomy and systematics studies. Altogether, these results point to the *Ciona* genus as a complex taxonomic group that needs to be studied according to an integrative taxonomy approach (Padial *et al.*, 2010). In fact, the intricate evolutionary history and the possible presence of still ongoing speciation events requires the combination of different types of data and the usage of different methodologies for the species delineation.

This study reports the identification along the North-eastern coasts of Sardinia of several specimens of the *Ciona* genus with unexpected (*i.e.*, not described previously) features. An integrative taxonomy approach has revealed that these specimens belong to a new species, *Ciona intermedia*, that is here described in detail by morphological, ecological and molecular traits. Furthermore, molecular phylogenetic reconstructions of the genus *Ciona* based on three mitochondrial regions, have allowed identifying the species *C. edwardsi* as the closest to *C. intermedia*. Altogether, our data provide an important contribution to the knowledge of the evolutionary history of this genus for which the species delineation still requires further clarifications.

Material and methods

Sampling and morphological analysis

Seven specimens of *Ciona* sp. were photographed and manually collected by SCUBA diving near Olbia (Sardinia, Tyrrhenian Sea, Italy) at a depth of 3-5 m in July 2014. The sampling area was mainly characterized by calcareous algae, large solitary ascidians belonging to the genera *Phallusia* and *Pyura*, and small colonial ascidians like *Symplegma brakenhielmi* (Michaelsen, 1904) (Mastrototaro *et al.*, 2019). Specimens of *Ciona* sp. were collected from shady sites, such as crevices of the rocks and under the pebbles. Five specimens were directly preserved in ethanol 99% for molecular investigations. The other specimens were narcotized with menthol crystals in seawater and then preserved in a 5% formaldehyde solution in seawater for morphological analyses. In the relaxed specimens the test was removed, and the body was coloured with Mayer's hemalum solution for detailed investigations. We considered numerous morphological characters (consistency of tunic, morphology of siphons, number and distribution of body muscles, structure of the pharynx, shape of stomach and gonads) as well as some ecological characteristics of the specimens (occurrence in light or shady sites).

Ciona sp. specimens from Olbia were morphologically compared to specimens of *C. edwardsi*, *C. intestinalis*, and *C. robusta* of the private collection of the Laboratory of Zoology of F. Mastrototaro (available on request; see Supplementary Table S1).

Molecular analyses

Total DNA of the three *Ciona* sp. specimens preserved in ethanol (labels ‘CR’, ‘Ca’ and ‘Cb’ in Supplementary Material Tables) was extracted from muscle tissue using a modified CTAB method (Hirose & Hirose, 2009). DNA from two *C. roulei* and three *C. edwardsi* specimens, sampled at Banyuls-sur-Mer (France), were also analysed. These *C. roulei* and *C. edwardsi* DNAs were obtained as part of a former study on experimental crosses within the *Ciona* genus (Malfant *et al.* 2018), during which the specimen identification was made by those authors based on morphological criteria, *i.e.*, the external morphology, the spermiduct and gonoduct features, and the larvae (see data in the Supplementary Material of Malfant *et al.*, 2018). The experimental crosses and the molecular analyses performed on those *C. roulei* and *C. edwardsi* specimens in Malfant *et al.*, (2018) and in an ongoing genetic (nuclear-based) study (M. Malfant, E. Pante, C. Daguin-Thiébaud, C. Roby & F. Viard, in preparation) matched the expectations based on the morphological identifications.

Using the primer pairs reported in Table 1, three mitochondrial (mt) regions were amplified:

- a fragment of the *cox1* gene about 1.2 kb- long, named COI-1.2kb, containing the 650 bp-long region widely used as a reliable DNA barcode;
- a fragment comprising the *cox2* (cytochrome oxidase subunit 2) and *cob* (cytochrome b) genes, named x2cb;
- a fragment, named x3n1, encompassing the three genes *cox3* (cytochrome oxidase subunit 3), *trnK* (tRNA-Lys) and *nad1* (NADH dehydrogenase subunit 1), and two non-coding spacers. This fragment was originally identified and used by Iannelli *et al.*, (2007) as a mt region able to distinguish *C. intestinalis* (formerly known as *C. intestinalis* type B) from *C. robusta* (formerly known as *C. intestinalis* type A), and then it was demonstrated to be able to distinguish also spC and spD (Zhan *et al.*, 2010).

The COI-1.2kb fragment was amplified in all specimens (three *Ciona* sp., two *C. roulei* and three *C. edwardsi*), while x3n1 was amplified in the three *Ciona* sp. specimens and in only one representative of *C. roulei* and *C. edwardsi*. The three analysed *Ciona* sp. had identical COI-1.2kb and x3n1 sequences. Finally, x2cb was amplified in one specimen for each of the three considered species (*Ciona* sp., *C. roulei* and *C. edwardsi*).

The COI-1.2kb fragment was amplified with the high fidelity PrimeStar HS DNA polymerase (Takara Bio Inc.) in a 25 µl reaction volume containing: 1X reaction buffer with 1 mM final concentration of MgCl₂ (Takara Bio Inc.), 0.2 mM of each dNTP, 0.3 µM of each primer and 1.25 Units of PrimeStar HS DNA polymerase (Takara Bio Inc.). Amplification conditions were: 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 46-52°C (depending on the specimen), extension for 2 min at 72°C; a final elongation step of 5 min at 72°C.

Amplifications of the x2cb fragment were performed according to long-range PCR protocol, since the frequent gene order rearrangements typical of the ascidian mt genome (Gissi *et al.*, 2010) made it impossible to predict *a priori* the distance between the *cox2* and *cob* genes in the studied *Ciona* taxa. Therefore, amplification of the x2cb fragment was carried out with the high fidelity LA Taq DNA polymerase (Takara Bio Inc.) at the following conditions: an initial denaturation for 1 min at 94°C, then 30 amplification cycles with denaturation for 10 s at 98°C, annealing for 5 s at 50 °C, extension for 12 min at 68°C; a final elongation step of 10 min at 72°C. The reactions were carried out in a final

volume of 25 µl containing: 1X reaction buffer with 2.5 mM final concentration of MgCl₂ (Takara Bio Inc.), 0.4 mM of each dNTP, 0.2 µM of each of the two primers, and 1.25 Units of LA Taq DNA polymerase (Takara Bio Inc.).

The x3n1 fragment was amplified with the DreamTaq polymerase (Thermo Fisher Scientific) in a final volume of 25 µl containing: 1X reaction buffer with 2 mM final concentration of MgCl₂ (Thermo Scientific), 0.2 mM of each dNTP, 0.5 µM of each of the two primers, and 1.25 Units of DreamTaq polymerase. The amplification conditions were: an initial denaturation for 3 min at 95°C, then 30 amplification cycles (denaturation for 30 s at 95°C; annealing for 30 s at 50 °C; extension for 2 min at 72 °C) followed by a final elongation step of 5 min at 72°.

All obtained amplicons were purified with the DNA Clean&Concentrator kit (Zymo Research), and directly sequenced according to the Sanger method at the Microsynth AG (Switzerland) or Eurofins Genomics (Germany). Sequence quality check and assembly were carried out with Geneious ver. 5.5.7.2 (<http://www.geneious.com>; Kearse *et al.*, 2012). All sequences were deposited at the ENA database (European Nucleotide Archive), and their accession numbers are reported in Supplementary Tables S2, S3, S4.

For comparative analyses, homologous sequences of the genus *Ciona* were searched in the NCBI non-redundant nucleotide database (nr-nt db, at 1st February 2019) by Blastn (Altschul *et al.*, 1990), using as query our *Ciona* sp. sequences. Concerning *cox1*, we analysed a final dataset consisting of: representative sequences of *C. robusta* and *C. intestinalis* included in Malfant *et al.*, (2018); three sequences of *Ciona* spC and *Ciona* spD from another yet unpublished study (M. Malfant, E. Pante, C. Daguin-Thiébaud, C. Roby, F. Viard, in preparation); all other *Ciona* species whose sequences were available in nr-nt db. The analysed sequences of *cox1*, *x2cb* and *x3n1* are listed in Supplementary Table S2, S3 and S4, respectively.

Phylogenetic analyses were performed separately for the three mt regions. Sequences were aligned by hand or with MAFFT (Kato *et al.*, 2002), preserving the codon structure of the protein-coding genes. The *cox1* final alignment was 1575 bp long (with 737 ungapped sites in 87% of the sequences) and consisted of 54 *Ciona* sequences plus a sequence of *Clavelina lepadiformis* (Müller, 1776) used as outgroup (see Supplementary Table S2). The *x2cb* final alignment was 1127 bp long (with 1078 ungapped sites) and consisted of 9 *Ciona* sequences plus 5 Aplousobranchia species as outgroups (see Supplementary Table S3). The *x3n1* final alignment was 676 bp long (with 502 ungapped sites) and consisted of 19 *Ciona* sequences (see Supplementary Table S4). *C. savignyi* Herdman, 1882 *sensu* Roule, 1884 was not included in the *x3n1* alignment since in this species the *nad1* gene is not adjacent to *cox3-tnK*.

Phylogenetic reconstructions were performed with the Maximum Likelihood (ML) method and by Bayesian Inference (BI). For ML, we used the online PhyML-SMS v3.0 software, which includes the automatic model selection by Smart Model Selection (SMS) (Guindon & Gascuel, 2003; Lefort *et al.*, 2017) (<http://www.atgc-montpellier.fr/phyml-sms/>). The best-fit substitution model selected under the Akaike Information Criterion (AIC) was the GTR+I+G for both the *cox1* and the *x2cb* alignments, and the TN93+G for the *x3n1* alignment. The proportion of invariant sites (I) and the gamma shape parameter (alpha) for the 4 rate categories were estimated by the PhyML v3.0 software itself. Bootstrap values, indicating node reliability, were based on 100 replicates. Bayesian trees were inferred with MrBayes v. 3.2.7a (Ronquist *et al.*, 2012). The BI analyses were performed using the model already selected by PhyML-SMS. However, the more general GTR+G model was used for the *x3n1* alignment instead of the TN93+G, since this last model is not implemented in MrBayes. Two parallel analyses, each composed of one cold and three incrementally heated chains, were run for

1,000,000 generations. Trees were sampled every 100 generations and the results of the initial 250,000 generations were discarded (burn-in fraction of 25%), after verifying that stationarity of the lnL was reached. The PSRF (Potential Scale Reduction Factor) was also checked as convergent diagnostic, according to the indications reported in the MrBayes manual. Therefore, a total of 7,500 tree were used to calculate the Bayesian posterior probabilities (BPP) at the different nodes.

Species delimitation analyses were carried out with two methods based on completely different approaches: the Automatic Barcode Gap Discovery method (ABGD; Puillandre *et al.*, 2012), a sequence similarity clustering method, and the Poisson Tree Processes (PTP; Zhang *et al.*, 2013), a tree-based coalescence method. ABGD clusters sequences into partitions, consisting of hypothetical species, based on the statistical inference of the “barcode gap”, *i.e.*, the gap in the distribution of intra-species and inter-species pairwise distances. On the contrary, PTP infers putative species boundaries on a non-ultrametric input tree assuming the existence of two independent classes of Poisson processes, one describing speciation and the other coalescent events. The hypothetical species identified by the two methods are hereafter referred as Operational Taxonomic Units (OTUs). These methods were applied only to the *cox1* and the x3n1 datasets, since they consist of at least three sequences per most species, a number suitable for the species delimitation analyses.

ABGD analyses were performed on the web-based interface <http://wwwabi.snv.jussieu.fr/public/abgd/> (Puillandre *et al.*, 2012), initially using the default values for both the proxy of the minimum relative gap width ($X=1.5$) and the scanned range of prior intraspecific divergence ($P_{min} - P_{max}$: 0.001 – 0.1). Then, the robustness of the ABGD results was checked by changing the parameter value one at a time, in particular by increasing P_{max} (to 0.2 or 0.3) in order to take into account the fast substitution rate typical of ascidians (Yokobori *et al.*, 1999; Tsagkogeorga *et al.*, 2010; Rubinstein *et al.*, 2013), and by decreasing/increasing X (to 1, 2, 3) in order to verify the existence/avoid smaller local gaps. For each X and P_{max} value, the pairwise distances were calculated according to the three nucleotide substitution models available in ABGD: Jukes-Cantor (JC) (Jukes & Cantor, 1969), Kimura 2 parameter (K2P) (Kimura, 1980) and uncorrected p-distances (p-dist). This strategy allowed excluding possible bias of the selected evolutionary model on the OTU delimitation. Therefore, a total of 18 ABGD analyses were performed per alignment.

Since our *cox1* alignment contains several missing data, *i.e.*, gapped sites related to the different length of the sequences available in nr-nt db (see Supplementary Table S2), to exclude potential bias due to these sites, the ABGD analyses were carried out on the following four *cox1* alignments (without outgroup), consisting of:

- all the 1566 (gapped plus ungapped) sites of all 54 *Ciona* sequences (1566-All);
- the only 451 ungapped sites present in all 54 *Ciona* sequences (451-Nogap-All);
- 737 ungapped sites obtained after excluding the 7 shortest *Ciona* sequences (737-Nogap-47taxa) (see Supplementary Table S2 for the excluded taxa);
- 1084 sites, with only six gapped sites, obtained considering only the 12 longest *Ciona* sequences (1084-12taxa). Thus, this alignment includes only 1 or 2 sequences per species (see Supplementary Table S2 for the included taxa).

The x3n1 alignment was analysed as it is, including gapped sites, since in this case the gapped sites correspond to real insertion/deletions, not to missing data.

The PTP method (Zhang *et al.*, 2013) was applied to the *cox1* and x3n1 datasets using as input both the ML and the Bayesian tree, although PTP was demonstrated to be quite robust to different tree reconstruction methods (Tang *et al.*, 2014) The Bayesian implementation of the PTP (bPTP) was

performed through the web interface <http://species.h-its.org/ptp/> (Zhang *et al.*, 2013) removing the outgroup and using the following parameters: 500,000 MCMC generations, thinning every 100 generations and a burn-in fraction of 0.20. The convergence of the MCMC chains was confirmed by visual inspection of the likelihood plot, as reported in the PTP help (<https://species.h-its.org/help/>), and the maximum likelihood solution was recorded.

Results

Based on our integrative taxonomy approach, we can confidently hypothesize that the collected specimens belong to a new species, *Ciona intermedia*. As detailed below, in our approach we considered numerous morphological characters, some ecological characteristics, and three molecular markers: two mitochondrial regions (COI-1.2kb and x3n1) already successfully used for discriminating cryptic species within the so-called *Ciona intestinalis* species complex (Nydam & Harrison, 2007; Iannelli *et al.*, 2007; Zhan *et al.*, 2010), and the new fragment x2cb, here examined for the first time for reconstructing the phylogeny of the genus *Ciona*.

FAMILY CIONIDAE LAHILLE, 1887
GENUS *CIONA* FLEMING, 1822
CIONA INTERMEDIA NOV. SP. MASTROTOTARO

Etymology

The specific name refers to some anatomical features of this species which appear intermediate (*intermedius*, in latin) compared to the already known species belonging to the genus *Ciona*.

Type material

Holotype: MUZAC-6550, 7 cm in height, July 2014, collected by Mastrototaro F. and Chimienti G., type locality, 3-5 m depth, dissected, preserved in 4% formalin.

Paratypes: MUZAC-6551 and MUZAC-6552, two specimens preserved in 99% ethanol, collected by Mastrototaro F. and Chimienti G., type locality, 3-5 m depth, not dissected. The holotype and the paratypes have been deposited in the collection of the Zoological Museum of the University of Bari.

Type locality

Olbia, North-eastern coasts of Sardinia, Italy (40°54'55''N; 9°34'05''E).

Morphological description

Smooth and soft tunic, semi-transparent without tubercular prominences. The inner part of the animal is visible and characterized by a reddish anterior region. The red pigmentation is due to the red-orange spots present in the anterior part of the body wall, that becomes red intense near the siphons (Fig. 1A-C; Fig. 2A-C). In living specimens, the tunic does not follow the body during sudden contractions (Fig. 1A, 1C).

The oral and the atrial siphons have almost the same length (Fig. 2A-B), with the oral one slightly longer. The oral aperture has 8 lobes while the atrial has 6 lobes (Fig. 2E). One red-orange ocellus lies between each lobe (Fig. 2F). At the base of the oral siphon there are about 30 narrow tentacles of different length, the longest alternating with the shortest ones (Fig. 3A-B).

The ripe specimen sampled is about 7 cm in length with the tunic, 5 cm extracted from the tunic (Fig. 2A-C). Thoracic wall is characterized by a strong musculature that consists of a longitudinal system of six well-defined bands of muscles running from the basal attachment to the siphons (Fig. 2C-E; Fig. 5B), while the circular system is composed by thin transverse strands, encircling the whole body and very evident on both siphons (Fig. 2G).

On each side, the oral siphon is crossed by 4 bands of longitudinal muscles that reach the margin of the oral lobes, while the atrial siphon is supplied by the other two longitudinal bands (Fig. 2E).

The prepharyngeal area (Fig. 3A) does not have papillae (Fig. 3B). The flat pharynx (Fig. 3H) occupies a wide part of the zooid, consisting of many longitudinal vessels and numerous stigmata (Fig. 3F). The stigmata are crossed by narrow parastigmatic vessels (Fig. 3F). At each intersection between transverse vessels and longitudinal vessels, is placed a ventrally-notched papilla (Fig. 3G). In the observed specimens the number of longitudinal vessels on each side of the pharynx is 36, and the stigmata per mesh in the middle portion of the animal is four (Fig. 3E); these characters are considered not fixed but variable with the size of the specimens in the other known *Ciona* species (Millar, 1953; Kott, 1990). No significant difference is noted in the meshes of the anterior and posterior part of the pharynx. The dorsal lamina is divided in numerous languets corresponding to the transverse vessels (Fig. 3D) with the endostylar appendix placed at its end (Fig. 3C).

The digestive system occupies about 1/4 of the whole size of the zooid, at its posterior end (Fig. 4A). The wide stomach is ovoid in shape and lies on the left side of the zooid. In particular, it is positioned slightly to the right of the middle line, beneath the pharynx. In living specimens, the alimentary canal is clearly visible, with the orange stomach having about 40 irregular broken folds on its surface (Fig. 4A-B). The intestine ends with a lobed anus that opens at the level of 1/3 of the upper part of the pharynx (Fig. 4A). The rectum is less long than the gonoducts (Fig. 4C).

The ovary, containing a large number of brown-yellowish oocytes of about 100 μm in diameter (Fig. 4C-D; Fig. 6A₁), is positioned in the inner part of the sinusoidal loop formed by the gut. The oviduct extends parallel to the rectum, projecting beyond the anus with the spermiduct running laterally to it (Fig. 4C). The walls and the end of the gonoducts are yellow-orange. The spermiduct is visible by the accumulation of white sperm (Fig. 4C) with four to eight white narrow papillae projecting from its distal end (four visible papillae in Fig. 4E and Fig. 6A). The branching system of tubular follicles forming the testis lays on both stomach and intestine, forming two major ducts which are joined at the level of the ovary (Fig. 4F); this single spermiduct runs parallel to the oviduct up to the genital aperture (Fig. 4C).

Molecular phylogenetic reconstructions

The identity percentage between *C. intermedia* and other *Ciona* species, including *C. roulei* and *C. edwardsi*, is lower than 94.28% for *cox1*, 90.14% for *x3n1*, and 91.89% for *x2cb*. The *cox1* value is very distant from the 2-3% divergence found in several taxonomic groups as maximum *cox1* intra-specific divergence (Hebert *et al.*, 2003; Hebert *et al.*, 2004; Smith *et al.*, 2005), thus providing a first clue that *C. intermedia* could not belong to any already molecularly characterized *Ciona* species (*i.e.*, *C. intestinalis sensu* Brunetti *et al.*, 2015, *C. robusta*, *C. savignyi*, *C. roulei*, *C. edwardsi*, *Ciona* spC and spD).

Figure 7 summarizes the results of the species delimitation analyses and the phylogenetic reconstructions carried out on *cox1*. The bPTP method consistently identifies *C. intermedia* as a distinct OTU, using both the Bayesian and the ML tree as input (see bPTP-Btree and bPTP-MLtree bars, respectively, in Fig. 7). The other recognized OTUs correspond to already described species (*C.*

edwardsi, *C. robusta* and *C. savignyi*), to a species so far defined only molecularly (*Ciona* spD), and to the cluster including both *C. intestinalis* and *C. roulei* (Fig. 7). Unlike what is expected, *Ciona* spC is not recognized as a single OTU but as 2-3 OTUs depending on the input tree (see differences between bPTP-Btree and bPTP-MLtree bars in Fig. 7). The ABGD results demonstrate the existence of the barcode gap and show a perfect match between the initial and the recursive partitions for prior intraspecific divergences ranging from 0.1-0.28% (depending on the used alignment and parameter options) to 5.99% (see Supplementary Table S5). The results of the 72 (*i.e.*, 18x4) ABGD analyses confirm the OTUs identified by bPTP, except for *Ciona* spC and few sporadic inconsistencies (Fig. 7 and Supplementary Table S5). Indeed, *Ciona* spC is recognized as a single OTU in all ABGD analyses, except for few partitions obtained with $X=1$, where even other species are incongruously split in several improbable OTUs (see red values in square brackets in Supplementary Table S5). *Ciona intermedia* is recognized as a single OTU in all ABGD analyses except in one case. Indeed, it is merged with *C. edwardsi* into a single OTU only in the initial and recursive partitions corresponding to a prior intraspecific divergence of 2.15%, obtained in the ABGD performed on the 1566-All alignment using the p-dist (see Supplementary table S5). Going into details of the 72 ABGD results (Supplementary Table S5), the 18 analyses performed on the same alignment with different parameters/substitution models give identical results, with only few exceptions mainly when using the p-dist (see 1566-All and 737-Nogap-47taxa alignments) or at the lowest prior intraspecific divergences for $X=1$ especially in the alignment with the lowest site number (see square brackets values for the 451-Nogap-All alignment in Supplementary Table S5). Even the results of the ABGD analyses performed on the four different *coxI* alignments (*i.e.*, 1566-All, 451-Nogap-All, 737-Nogap-47taxa and 1084-12taxa) are almost identical, since they identify OTUs with the same composition, except for some recursive partitions at the lowest prior intraspecific divergences for $X=1$ (see square brackets values in Supplementary Table S5). Remarkably, the 737-Nogap-47taxa and 1084-12taxa alignments give almost identical results in spite of the very different species representativeness (≥ 3 sequences per most species in the 737-Nogap-47taxa alignment; only 1-2 sequences per species in the 1084-12taxa alignment). All these results indicate that, at least for our *coxI* dataset, the ABGD method is quite robust to parameter variation, presence of missing data, and differences in species representativeness, thus making us quite confident on the obtained results.

Remarkably, the OTUs identified by the bPTP and ABGD analyses on *coxI* correspond to clades strongly supported in the *coxI* phylogenetic trees (Fig. 7). Indeed, the bPTP/ABGD OTUs consisting of more than one specimen form statistically significant clades that have both ML bootstrap support $\geq 90\%$ and BPP ≥ 0.99 (black dots in Fig. 7). Even the OTU mixing *C. roulei* and *C. intestinalis* sequences is identified as a well-supported clade (ML bootstrap: 95% and BPP: 1). Noteworthy, *C. intermedia* forms the sister group of *C. edwardsi*, and this relationship is again strongly supported (ML bootstrap: 99% and BPP: 1) (Fig. 7). Unfortunately, the basal nodes of Figure 7 remain unresolved. Therefore, our COI-1.2kb fragment is able to discriminate different *Ciona* species, demonstrating to be a robust DNA barcode for the *Ciona* genus, but it is unable to clarify the exact phylogenetic relationships among the species.

The species delimitation analyses and the ML/Bayesian phylogenetic reconstructions based on the x3n1 fragment (Fig. 8) confirms the results obtained with *coxI* (Fig. 7). Indeed, the bPTPs based on both the ML and the Bayesian trees give identical results and discriminate six OTUs, corresponding to: *C. intermedia*; two already described species (*C. edwardsi* and *C. robusta*); two molecularly-identified cryptic species (spC and spD); and a clade consisting of *C. intestinalis* plus *C. roulei* (Fig. 8). The ABGD analyses indicate the existence of a clear barcode gap and show a perfect match

between initial and recursive partitions at prior intraspecific divergences ranging from 0.77-1.29% (depending on distance metric) to 5.99% (see Supplementary Table S5). The results of the 18 ABGD analyses performed with different parameters/substitution models are mainly congruent (Supplementary Table S5) and identical to bPTP (Fig. 8). Few differences are observed only in the recursive partitions at the lowest intraspecies divergence (where *C. roulei* is recognized as a distinct OTU; see dashed bars in Fig. 8), and at prior intraspecific divergence of 0.77%-1.29% when using the p-dist (where *C. intermedia* is clustered with *C. edwardsi* in the same OTU) (see red values in Supplementary Table S5). The x3n1 phylogenetic reconstructions recognize as highly supported: the sister relationship between *C. intermedia* and *C. edwardsi* (maximum bootstrap and BPP values); two clades corresponding to single species (*i.e.*, *Ciona* spD, *C. robusta*); the clustering of *C. intestinalis* with *C. roulei* (see black dots in Fig. 8). The sister relationship of spC to the *C. intermedia*-*C. edwardsi* clade obtains only marginal support (ML bootstrap: 57%; BPP: 0.91), so it needs to be confirmed by additional data. As *cox1*, even x3n1 leaves unresolved the basal nodes of the *Ciona* tree. Figure 8 reports also the internal structure of the x3n1 mitochondrial fragment, highlighting that the gene order *cox3-trnK-nad1* is conserved in all reported *Ciona* species (except for *C. savignyi*, see Materials and Methods). On the contrary, the length of the non-coding spacers upstream and downstream *trnK* varies between species (see yellow and blue boxes in Fig. 8), thus causing the overall size variability of the x3n1 fragment between species.

The x2cb fragment is about 1.1 kb long and is characterized by a short overlap between the ORFs (Open Reading Frame) of the two genes in all three species *C. intermedia*, *C. edwardsi* and *C. roulei* here analysed for the first time. The same situation was found in all other published mt genomes of *Ciona* species (see Supplementary Table S3). The ML and Bayesian phylogenetic reconstructions based on this x2cb fragment are shown in Figure 9. Remarkably, all nodes of this phylogeny, including the basal ones, are fully resolved (ML bootstrap $\geq 93\%$ and BPP ≥ 0.97 ; see black dots in Fig. 9), thus providing clear information about the relationships between the *Ciona* species: *C. intermedia* is confirmed as the sister clade of *C. edwardsi*, while *C. robusta* is the sister group of the cluster mixing *C. intestinalis* and *C. roulei*. Finally, *C. savignyi* is basal to the remaining *Ciona* species.

Discussion

The family Cionidae Lahille, 1887 includes the three genera *Ciona*, *Araneum* and *Tantillulum*, characterized by solitary animals with a transparent tunic, stomach positioned under the pharynx and gonad lying in the gut loop (Brunetti & Mastrototaro, 2017). The genus *Ciona* is typical of shallow waters but also includes some abyssal species (Brunetti & Mastrototaro, 2017) and even *C. intestinalis* has been found below 100 m deep in Oslo fjord (Dybern, 1967). This genus can be distinguished for the presence of longitudinal and parastigmatic vessels with papillae in the pharynx; oral and atrial siphons with 8-10 and 6-8 lobes, respectively; and the presence of ocelli between the lobes. In some species the endostyle ends with an appendix, while the stomach lies on the left side of the zooid (Millar, 1953; Brunetti & Mastrototaro, 2017). Some characteristics of the papillae projecting from the spermiduct (*i.e.*, shape, number and pigmentation) and the oocyte size have been used as further features to allow specific identification. In particular, these characters have been previously described in four *Ciona* species (Hoshino & Nishikawa, 1985; Caputi *et al.*, 2007; Sato *et al.*, 2012; Supplementary Material in Malfant *et al.*, 2018) (Fig. 6).

The morphological and ecological traits of the specimens described in this study confirm their belonging to the *Ciona* genus but do not fit into any of the already described *Ciona* species reported so far in the Mediterranean as well as in other seas. In the genus *Ciona*, fourteen species are currently valid and here briefly described: *C. antarctica* Hartmeyer, 1911, *C. edwardsi*, *C. fascicularis* Hancock, 1870, *C. gelatinosa* Bonnevie, 1896, *C. hoshinoi* Monniot, 1991, *C. imperfecta* Monniot & Monniot, 1977, *C. intestinalis*, *C. longissima* Hartmeyer, 1899, *C. mollis* Ritter, 1907, *C. pomponiae* Monniot & Monniot, 1989, *C. robusta*, *C. roulei*, *C. savignyi*, and *C. sheikoi* Sanamyan, 1998.

Ciona antarctica was rarely collected and only in Antarctic waters from 300 to 500 m of depth (Hartmeyer, 1911; Monniot & Monniot, 1983; Ramos-Esplá *et al.*, 2005; Monniot *et al.*, 2011). This species is characterized by a peculiar organ on each side of the posterior part of the body, between the gut and the body wall, made of thick lobed lamellae of unknown function and by the gonoducts ending before the anus (Monniot, 1998). Also *C. gelatinosa*, species collected in the Arctic, North Atlantic (Van Name, 1945; Hoshino & Nishikawa, 1985) and North-eastern Pacific (Sanamyan & Sanamyan, 2007), is characterized by the gonoducts ending before the anus, as well as by a large muscular post-abdominal extension of the body wall, no endostylar appendix, and the absence of pigment spots around male papillae or on other parts of the body (Sanamyan & Sanamyan, 2007). *Ciona fascicularis* has been recorded mainly along the North-western Atlantic coasts and presents peculiar cylindrical rhizoids at the base of the body (Hancock, 1870; Monniot, 1963). *Ciona hoshinoi* is known from a single specimen from New Caledonia and another one collected in Palau, both of 1.5 cm in length, with the Palau specimen extremely contracted (Monniot, 1991; Monniot & Monniot, 2001). This species is mainly characterized by the presence of small round papillae between transverse vessels and longitudinal vessels and smaller papillae associated only to the parastigmatic vessels, as well as a smooth stomach, and anus ending at the level of the gonoducts aperture (Monniot, 1991). *Ciona imperfecta* is an abyssal species recorded at 4000 m depth. It lacks the endostylar appendix and has a pharynx consisting of only 4 rows of stigmata and 5-6 longitudinal vessels (Brunetti & Mastrototaro, 2017). *Ciona longissima* has a peculiar long stalk at the base of its body and has been recorded only in Arctic waters deeper than 1000 m. *Ciona mollis* was collected for the first time by Ritter in 1907 off California at 2000 m depth, and subsequently in the Mexican Pacific at 4400 m depth (Monniot, 1998). The musculature of this species is peculiar: six strong muscular bands on each side converge to a round area along the ventral side, with the bands stopping at the end of this area. Moreover, *C. mollis* has a bilobed anus ending near the gonoducts aperture, the spermiduct characterized by only one papilla, no endostylar appendix and no transverse body muscles. *Ciona pomponiae* was originally described on only one specimen collected between 300 and 800 m of depth off the Galapagos Islands (Monniot & Monniot, 1989), and subsequently it was recorded in the North-eastern Pacific (Sanamyan & Sanamyan, 2007) and from the Bering Sea as *Ciona gefesti* Sanamyan, 1998 (Sanamyan, 1998) (synonymized by Sanamyan & Sanamyan in 2007 with *C. pomponiae*). *Ciona pomponiae* has only four longitudinal muscles bands, and a stomach with folds not well defined and not clearly distinct from the intestine (Sanamyan & Sanamyan, 2007). Finally, the description of the species *C. sheikoi* is based on twelve specimens recorded in the North-western Pacific region, all characterized by thick longitudinal mantle muscles, long and remarkably curved gonads and well-defined cylindrical lobes around the anus. Moreover, this species presents papillae on the enlarged posterior part of the tunic, no endostylar appendix, and a smooth stomach with the anus placed near the gonoducts openings (Sanamyan, 1998). Each of the above-described species has peculiarities preventing their possible mis-identification with *C. intermedia*. The remaining five species are currently known in the Mediterranean Sea. In particular, *C. intestinalis* and

C. robusta are both characterized by a pleated branchial wall (like an accordion), 5-6 longitudinal muscles (usually 5 with the third divided in two bands) and transverse vessels of different sizes. Moreover, *C. robusta* can be distinguished from the very similar congeneric *C. intestinalis* also by the presence of several tubercles on the surface of the tunic, especially around the siphons, where they are usually arranged in longitudinal rows (Brunetti *et al.*, 2015). *Ciona edwardsi* is typically found in shady sites and in circalittoral-moderate deep waters and has a bright sulphur-yellow coloration (Fig.1D-E) (Mastrototaro & Relini, 2011). At morphological level, this species is characterized by 6 longitudinal muscles bands (Fig. 5C), and by a pharynx with a flat branchial wall and transverse vessels of about equal sizes (Copello *et al.*, 1981; Mastrototaro *et al.*, 2000; Mastrototaro & Relini, 2011; Brunetti & Mastrototaro, 2017). *Ciona roulei*, a poorly described species, is mainly characterized by the presence of four longitudinal muscle bands (with the third divided in two bands) and a reddish coloration (Lahille, 1890; Harant & Vernières, 1933; Brunetti & Mastrototaro, 2017). Moreover, the validity of this last species is yet questionable, as suggested by the results of crossing experiments (Malfant *et al.*, 2018), as well as by molecular phylogenetic reconstructions within the *Ciona* genus based on both nuclear and mitochondrial genes (Nydam & Harrison, 2007, 2010; See tree in Figure S1 in Malfant *et al.*, 2018). Finally, *C. savignyi*, firstly collected and described by Herdman (1882), is characterized by a whitish-grey tunic, large paddle-shaped papillae on the intersection between transverse and longitudinal vessels and no endostylar appendix (Hoshino & Nishikawa, 1985; Brunetti & Mastrototaro, 2017). However, the presence of this species in the Mediterranean Sea is doubtful (Brunetti & Mastrototaro, 2017).

As summarized in Table 2, *C. intermedia* sp. nov. shows intermediate characters between those of the above reported *Ciona* species, *i.e.*, a mixture of features, each one in common with only one/some of the other shallow-water *Ciona* species. In particular, *C. intermedia* has been found in shady sites as has *C. edwardsi* (Table 2); it has six longitudinal muscles bands like all other species except for *C. roulei* (Fig. 5); a flat pharynx as in *C. edwardsi*, instead of the accordion-shaped of *C. intestinalis* and *C. robusta* (Fig. 3H); transverse vessels of about equal sizes, as in *C. edwardsi* and *C. roulei* (Brunetti & Mastrototaro, 2017); a smooth tunic surface without the tubercles identified as diagnostic feature of *C. robusta* by Brunetti *et al.*, 2015 (Table 2). *Ciona intermedia* appears morphologically very similar to *C. edwardsi* (Table 2), but it has been found in shallower location (*i.e.*, littoral-shallow, at 3-5 m depth, whereas *C. edwardsi* is commonly present in circalittoral-moderate deep waters, at 20-40 m depth) and displays a different pigmentation. Although the coloration is generally not considered as a valid taxonomic character, it is important to point out that all *C. edwardsi* records showed a characteristic sulphur-yellow coloration (Copello *et al.*, 1981; Mastrototaro *et al.*, 2000; Mastrototaro & Relini, 2011) (Table 2). Concerning the number of papillae projecting from the spermiduct, the specimens of *C. intermedia* show from 4 to 8 narrow uncoloured papillae at the distal end of the spermiduct (Fig. 6A), a situation more similar to that of *C. intestinalis* (Fig. 6C), while *C. edwardsi* shows a turf of 15-30 thick white papillae projecting from all around the spermiduct, as reported by Hoshino & Nishikawa (1985) (Fig. 6B) (see also Table 2). *Ciona robusta* is characterized by ellipsoidal papillae usually orange-coloured, although their colour cannot be considered a true diagnostic character (Fig. 6G) (see also as *C. intestinalis* sp.A in Caputi *et al.*, 2007 and Sato *et al.*, 2012; Brunetti *et al.*, 2015; Malfant *et al.*, 2018). Finally, as for the oocytes size, the *C. edwardsi* oocyte diameter is almost twice that of the other three analysed species and they present a narrower follicular envelope (Fig. 6A₁, B₁, C₁, D₁). A larger size of the *C. edwardsi* oocytes has been also reported by Malfant *et al.*, (2018) in comparison with *C. robusta*, *C. intestinalis* and *C. roulei*.

The examination of a larger number of *C. intermedia* specimens is needed for further verifying the intra-species variability of the morphological traits here investigated, as well as for searching character(s) unique of this species, if any.

As for the molecular data, the species delimitation analyses have been carried out with a clustering (ABGD) and a tree-based (bPTP) method on two molecular markers: COI-1.2kb (*i.e.*, an elongation of the classical *cox1* DNA barcode), and x3n1. Both methods and markers consistently recognize *C. intermedia* as a distinct OTU and confirm that it cannot be assigned to any known described or undescribed *Ciona* species, including *Ciona* spC and spD currently defined only molecularly (Fig. 7-8). The congruency of these results increases the confidence in their validity. Indeed, the combined usage of two distinct methods/markers allows compensating for possible confounding factors differentially affecting the efficacy and/or sensitivity of each method/marker. As for the sample size and species representativeness, our datasets consist of at least three sequences per 50-70% of the expected species (for the x3n1 and *cox1* alignments, respectively), thus it is characterized by an uneven sampling similar to that observed in other species delimitation studies (see for example Kekkonen & Hebert, 2014; Kekkonen *et al.*, 2015). Based on simulation tests (Puillandre N., personal communication) and on the many papers that have used ABGD in combination with other methods, ABGD works well enough even when some species in the dataset include only 1-2 specimens (Ahrens *et al.*, 2016; Kekkonen & Herbert, 2014; Kekkonen *et al.*, 2015). Moreover, in our case, the unbalanced sampling is due to the fact that not only *C. intermedia* but also spC and spD have been found and sampled in only one locality (Nydam & Harrison 2007, 2010; Zhan *et al.*, 2010). Therefore, these three species belong to the called "singletons", estimated to account for about 30% of the formally described invertebrate species (Lim *et al.*, 2012). The commonness of rarity in nature and the consequent incomplete taxon sampling is a problem common to many DNA-based species delimitation studies (Lim *et al.*, 2012). However, recent analyses have shown that the shortcomings of poor and unbalanced sampling can be overcome including data from related lineages (the so called "subclade addition"), *i.e.*, by extending the study of a focal clade to a broader set of species (Talavera *et al.*, 2013; Ahrens *et al.*, 2016). Ahrens *et al.*, (2016) have also shown that this strategy has a different performance on the different species delimitation methods. In particular, in subclade analyses ABGD decreases, while PTP increases the number of recognized OTUs compared to the analysis of a total dataset consisting of all subclades, with consequent variation of the match to morpho-species (Ahrens *et al.*, 2016). Here, in order to at least mitigate the singleton issue in our datasets, we have analysed the sequences of all available *Ciona* species with the two methods, ABGD and PTP, that were shown to be differently affected by the uneven sampling and to have a different response to the subclade addition strategy (Ahrens *et al.*, 2016). Nevertheless, both ABGD and PTP methods gave consistent results, including the identification of *C. intermedia* as a distinct species.

Ciona intermedia is identified as the sister group of *C. edwardsi* with high statistical support in all ML and Bayesian phylogenetic reconstructions based on all three analysed mt fragments (Fig. 7-9), thus corroborating the close similarity already observed at morphological level. Interestingly, inter-specific crosses between *C. edwardsi* and any of the three species *C. intestinalis*, *C. robusta* and *C. roulei* revealed strong reproductive isolation (Lambert *et al.*, 1990; Malfant *et al.*, 2018). Therefore, given the strong genetic similarity between *C. intermedia* and *C. edwardsi*, we can hypothesize that *C. intermedia* might also be reproductively isolated from other *Ciona* species. Of course, this hypothesis needs to be experimentally tested. The use of nuclear markers could also be helpful to better delineate these two species and investigate their past demographic history, as it was done for *C. intestinalis* and *C. robusta* (Roux *et al.*, 2013; Bouchemousse *et al.*, 2016b).

Finally, the phylogeny of the genus *Ciona* inferred from x2cb gives for the first time a well-resolved picture of the relationships within this genus, even at basal level (Fig. 9). Indeed, the basal nodes of the *Ciona* tree are unresolved in all previously published phylogenetic reconstructions of this genus, that were based on a *cox1* fragment of only 750-bp (Nydam & Harrison, 2007; Malfant *et al.*, 2018), on nuclear genes (Nydam & Harrison, 2010) or on combined nuclear and mt sequences (Zhan *et al.*, 2010). Noteworthy, even these previous phylogenetic reconstructions do not include one or two of the following rarely molecularly sampled *Ciona* species: spC, spD and *C. edwardsi* species (lack of spD in Nydam & Harrison, 2007, 2010; lack of *C. edwardsi* in Zhan *et al.*, 2010; lack of spC and spD in Malfant *et al.*, 2018), so they analyse an incomplete species dataset exactly as our x2cb tree. Therefore, the better resolution observed in our x2cb phylogenetic tree cannot be an artefact related to the analysis of a reduced taxon sampling (*i.e.*, lack of spC and spD). Furthermore, our results show that x2cb has a resolving power even higher than the *cox1* fragment of about 1.2 kb, since it is able to resolve all nodes that instead remain unresolved in our COI-1.2kb tree (compare Fig. 9 to Fig. 7). Moreover, even the COI-1.2kb ML and Bayesian trees reconstructed using exactly the same taxon sampling of x2cb (*i.e.*, excluding spC and spD from the *cox1* dataset) are not fully resolved (data not shown), supporting the conclusion that the low resolution of *cox1* is not related to the presence of spC and spD. Thus, although the x2cb sequences of spC and spD are essential to further confirm our observations, we strongly encourage using the x2cb fragment as a new molecular marker in future analyses on the *Ciona* species.

In conclusion, our results point to an integrative taxonomy approach, involving the analysis of morphological, ecological and molecular characters, as a fundamental requirement for the delineation and the description of new *Ciona* species. This is in accordance with previous studies of the *Ciona* genus, where molecular data were crucial for addressing morphological re-analyses and taxonomic revisions (Suzuki *et al.*, 2005; Caputi *et al.*, 2007; Iannelli *et al.*, 2007; Nydam & Harrison, 2007, 2010; Zhan *et al.*, 2010; Sato *et al.*, 2012; Brunetti *et al.*, 2015; Pennati *et al.*, 2015; Gissi *et al.*, 2017; Malfant *et al.*, 2018). In view of these results, the "*Ciona* case" is still far from being resolved.

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Supplementary Tables S1

Specimens of *C. edwardsi*, *C. intestinalis*, and *C. robusta* of Mastrototaro F. private collection examined in this study. Number of samples, collection site and identifiers are also reported.

Supplementary Tables S2

List of the aligned *cox1* sequences. Red colour indicates sequences produced in this study. Sequences excluded and included in the alignment “737-Nogap-47taxa” and “1084-12taxa”, respectively, are also reported.

Supplementary Tables S3

List of the *cox2* and *cob* sequences included in the alignment of the x2cb fragment. Red colour indicates sequences produced in this study. ORF: Open Reading Frame

Supplementary Tables S4

List of the sequences included in the alignment of the x3n1 fragment. Red colour indicates sequences produced in this study.

Supplementary Tables S5

Results of the ABGD analyses on the four *cox1* alignments and on the x3n1 alignment using the default values for parameters X and Pmin-Pmax. The few differences to results obtained with X=1, 2 or 3 are also reported within brackets. The "n° of expected OTUs" includes one OTU for *C. intermedia*, and one OTU for the clade joining *C. intestinalis* with *C. roulei*. Red: values different from those expected; round brackets: content of unexpected OTUs merging several species; square brackets: different results obtained with X=1; curly brackets: different results obtained with X=2 and X=3; Init: initial partitions; Rec: recursive partitions; nd: not determined.

Table 1.

Mitochondrial regions and primers used in this study.

Fragment name	Primer pair	kb ^a	Primer name	Sequence (5'→3')	Notes/ Reference
COI-1.2kb	dinF/ConsR1	1.2	dinF	CGTTGRTTTATRTCTACwAATCATAARGA	Brunetti <i>et al.</i> , 2017
			consR1 ^b	CATTGATYYCCTCTTTTACTAGATTAAGTTTT	This study
x2cb	ux2F/ucbR	1.1	ux2F	GYAGTTRGDCAYCARTGATATTG	Iannelli <i>et al.</i> , 2007
			ucbR	GGAATASAYCGTAAAATVGCATARGC	Iannelli <i>et al.</i> , 2007
x3n1	tx3F/tn1R	0.6-0.7	tx3F	GAGTGTGCKATTTGGTATTGAC	Iannelli <i>et al.</i> , 2007
			tn1R	ATYTGAGCYACTCCTCGAATTC	Iannelli <i>et al.</i> , 2007

a: average range for the known *Ciona* species

b: consR1 is a modification of the “consensus R” primer of Nydam & Harrison (2007), obtained by deleting three nucleotides at the 5’end, and the last nucleotide at the 3’ end, corresponding to a third codon position of *cox1*.

Table 2.Comparison of *Ciona intermedia* to other four Mediterranean *Ciona* species.

	<i>C. intestinalis</i> ^a	<i>C. robusta</i> ^b	<i>C. roulei</i>	<i>C. edwardsi</i>	<i>C. intermedia</i>
Habitat ^c	light sites	light sites	-	shady sites	shady sites
Colour in vivo	various: whitish, reddish or yellowish	from whitish to yellowish	red	sulphur- yellow	Transparent tunic, siphons and upper part of the body orange-red
Depth	littoral, shallow water	littoral, shallow water	littoral, shallow water	circalittoral, mesophotic water	littoral, shallow water
Size (height)	about 20 cm	about 20 cm	about 10 cm	about 20 cm	about 7 cm
Test surface	smooth	with tubercles	smooth	smooth	smooth
n. of oral lobes	8	8	-	8-10	8
n. of atrial lobes	6	6	-	6-8	6
Muscle bands (per side) ^d	5-6	5-6	4	5-6	6
Branchial wall	pleated	pleated	-	flat	flat
Transverse vessels (observed on the external surface)	of different size	of different size	of about equal size	of about equal size	of about equal size
Spermiduct papillae	5-20, ellipsoidal, variable in colour, usually white	5-20, thick, variable in colour, usually orange-red	5-10, white	15-30, thick white-yellow	4-8, narrow and white
Oocytes diameter	about 100 µm	about 100 µm	about 100 µm	about 200 µm	about 100 µm

a: according to Brunetti *et al.*, (2015), formerly known as *C. intestinalis* type Bb: according to Brunetti *et al.*, (2015), formerly known as *C. intestinalis* type Ac: differences probably due to the different phototropism of the larvae (positive in *C. intestinalis* and *C. robusta*, probably negative in *C. edwardsi*) (Brunetti & Mastrototaro, 2017)

d: variable character the third band sometimes is divided in two bands

Figures

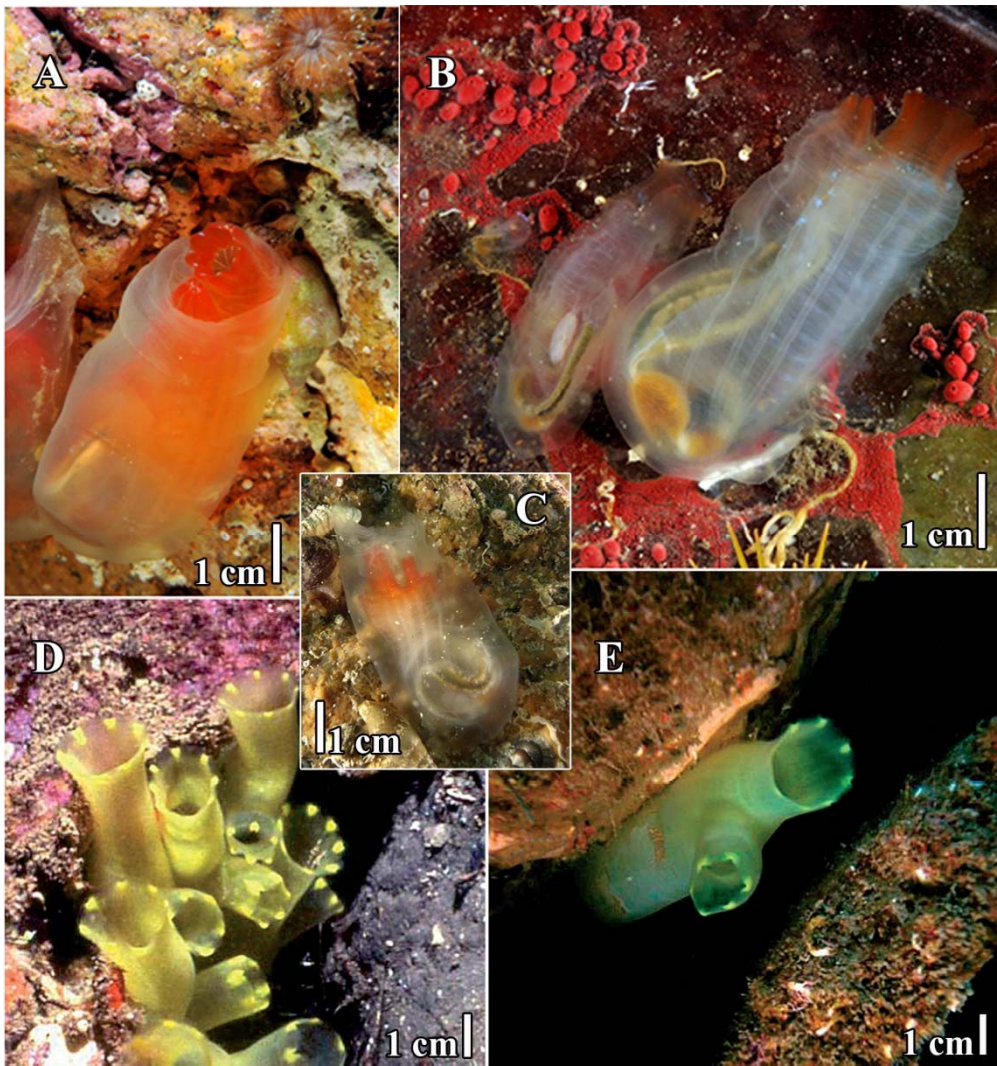


Figure 1. (A-C) Several living specimens of *C. intermedia* photographed and collected along the coasts of Olbia (Sardinia, Tyrrhenian Sea, Italy). (D-E) Living specimens of *C. edwardsi* with the typical sulphur-yellow appearance, photographed along Ligurian coasts (Mastrototaro & Relini, 2011).

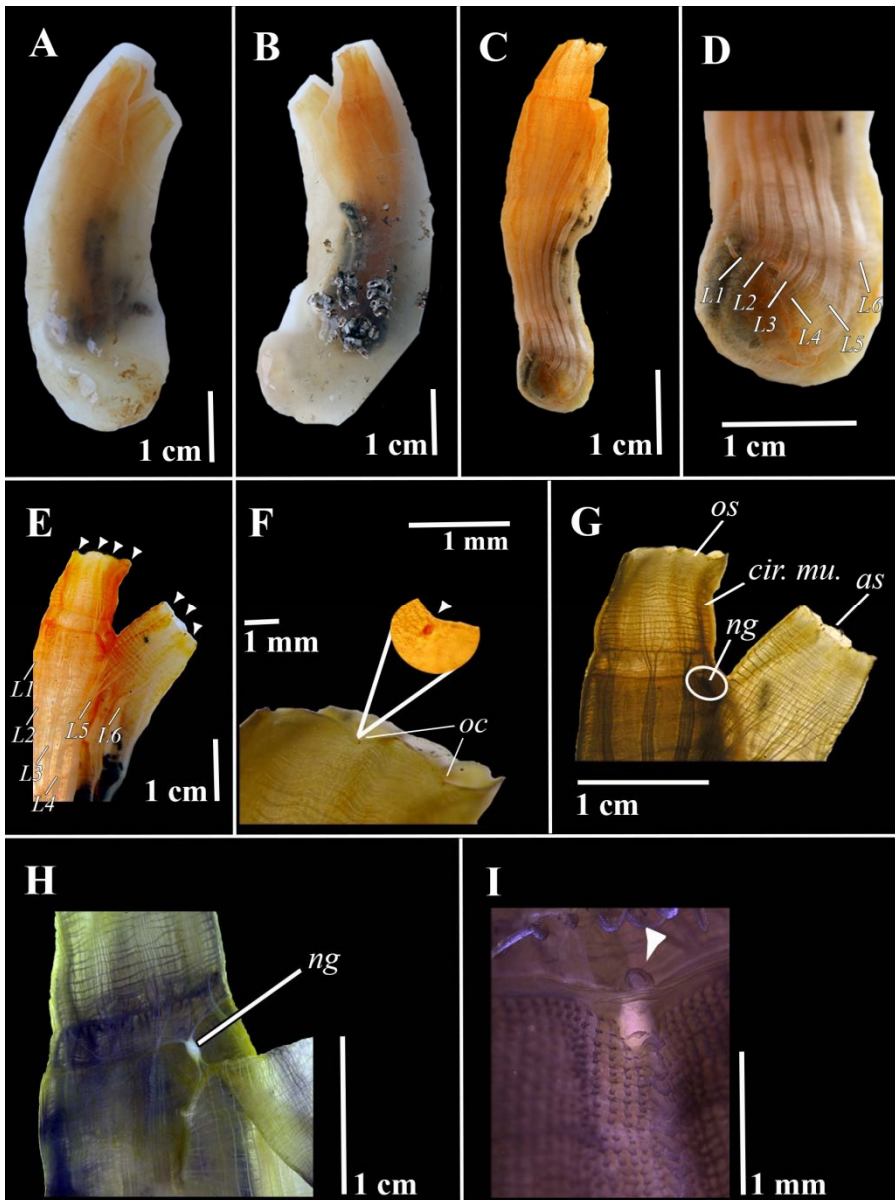


Figure 2. *Ciona intermedia*. (A) Left side of a specimen with the tunic. (B) Right side of a specimen with the tunic. (C) Specimen without the tunic, showing the six longitudinal muscle bands. (D) Magnification of the base of a specimen, white bars indicate the six longitudinal muscle bands (L1-L6). (E) Oral and atrial siphons with eight and six lobes, respectively (white arrows) and longitudinal muscle bands at the level of the siphons (L1-L6). (F) Red ocelli at the base between each lobe with the magnification of one red ocellus. (G) Oral and atrial siphons, with circular muscle systems, and the neural ganglion encircled in white. (H) Neural ganglion lying at the base of the oral and atrial siphons. (I) Magnification of the anterior region with dorsal tubercle (arrow). Abbreviations: as, atrial siphon; cir. mu., circular muscles; ng, neural ganglion; oc, ocellus; os, oral siphon.

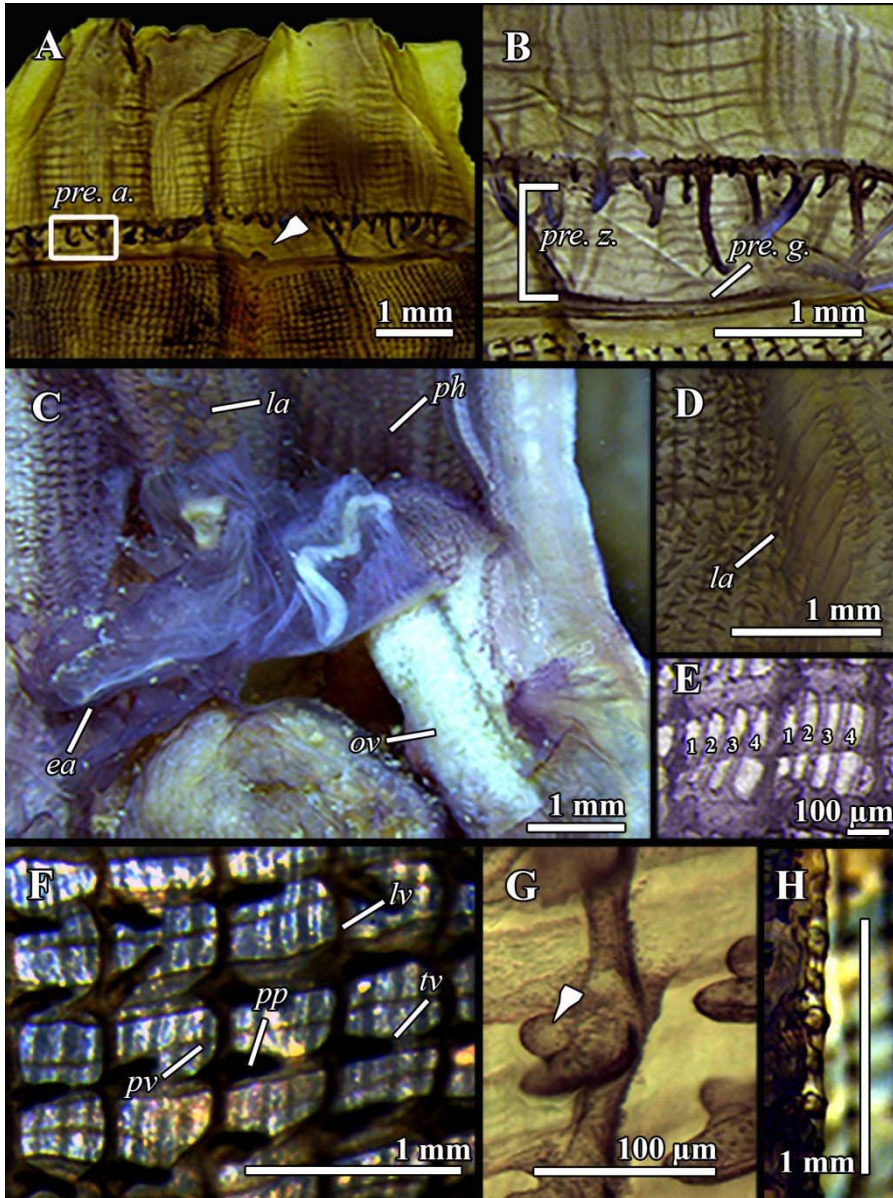


Figure 3. *Ciona intermedia*. (A) Prepharyngeal area (pre. a.), the white arrow indicates the dorsal tubercle. (B) Magnification of the prepharyngeal area with a wide prepharyngeal zone (pre. z.) and a narrow prepharyngeal groove (pre. g.), oral tentacles are clearly visible. (C) Bottom of the pharynx of a dissected specimen, with the white bar indicating the endostylar appendix. (D) Dorsal lamina with languets. (E) Magnification of the mesh of stigmata, with 4 stigmata per mesh (1-4). (F) Magnification of the branchial wall with the papillae placed at the intersection between the transverse vessels and the longitudinal vessels. Stigmata crossed by a narrow parastigmatic vessel. (G) Magnification of the papillae, white arrow showing the notch. (H) Section of the flat branchial wall. Abbreviations: ea, endostylar appendix; la, languet; lv, longitudinal vessel; ov, ovary; ph, pharynx; pre. a., prepharyngeal area; pre. g., prepharyngeal groove; pre. z., prepharyngeal zone; pp, papilla; pv, parastigmatic vessel; tv, transverse vessel.

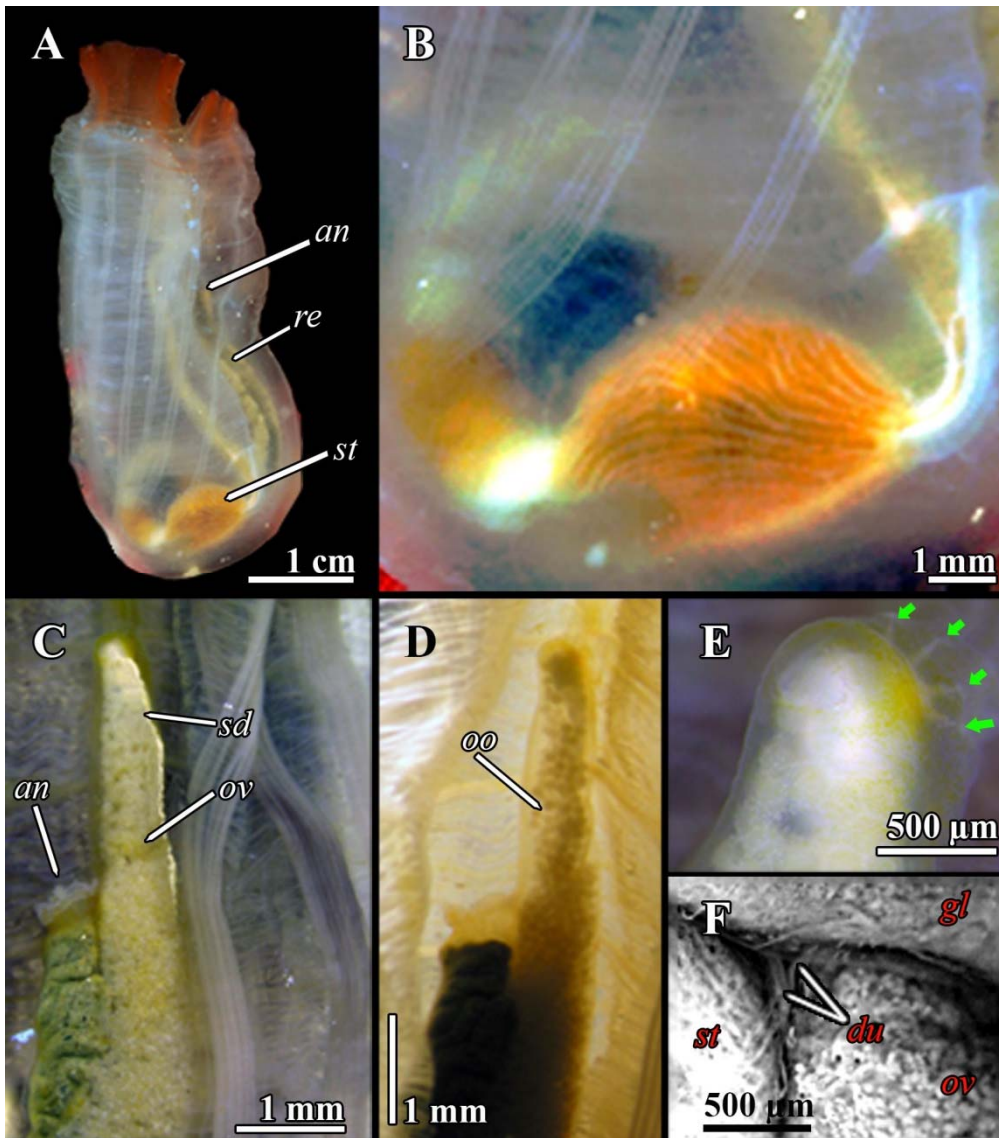


Figure 4. *Ciona intermedia*. (A) Living specimen with orange stomach. (B) Stomach with about 40 irregular broken folds on its surface. (C) Magnification of the ovary and lobed anus. (D) Ovary with numerous oocytes. (E) Magnification of the end of the gonoduct apertures showing the orange-yellow pigments and the four visible papillae at the distal end of the spermiduct (green arrows). (F) Magnification of the abdominal region showing testis follicles between stomach and the posterior gut loop forming two ducts that join in a single spermiduct at the level of the ovary. Abbreviations: an, anus; du, collecting ducts of the testis; gl, gut loop; oo, oocytes, ov, ovary; re, rectum; sd, spermiduct; st, stomach.

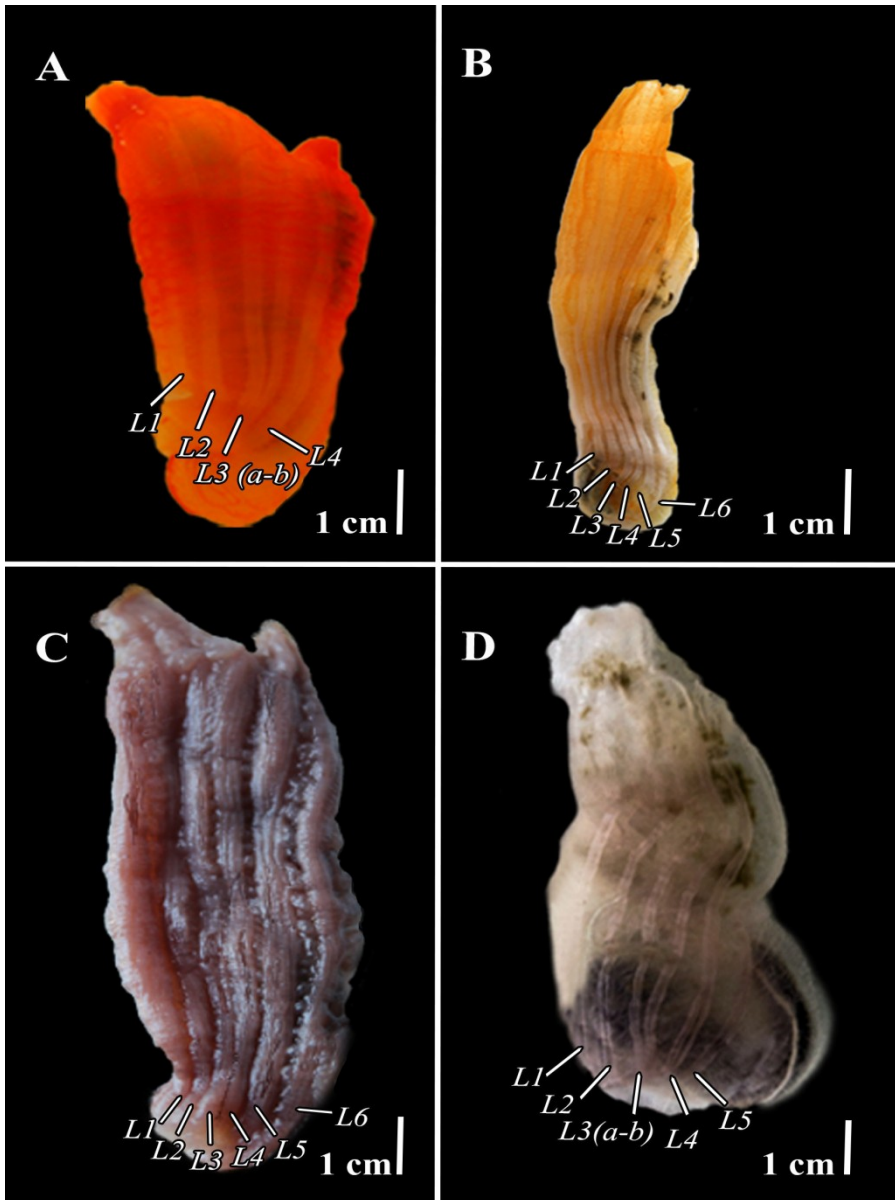


Figure 5. Comparison plate between four *Ciona* species, with bars highlighting the number of muscle bands. (A) *Ciona roulei* (courtesy of Marie Nydam) (B) *Ciona intermedia* collected in Olbia (Sardinia, Italy). (C) *Ciona edwardsi* collected in Sorrento, Tyrrhenian Sea by Mastrototaro F. (D) *Ciona robusta* collected along the coasts of Taranto, Ionian Sea (Mastrototaro *et al.*, 2008).

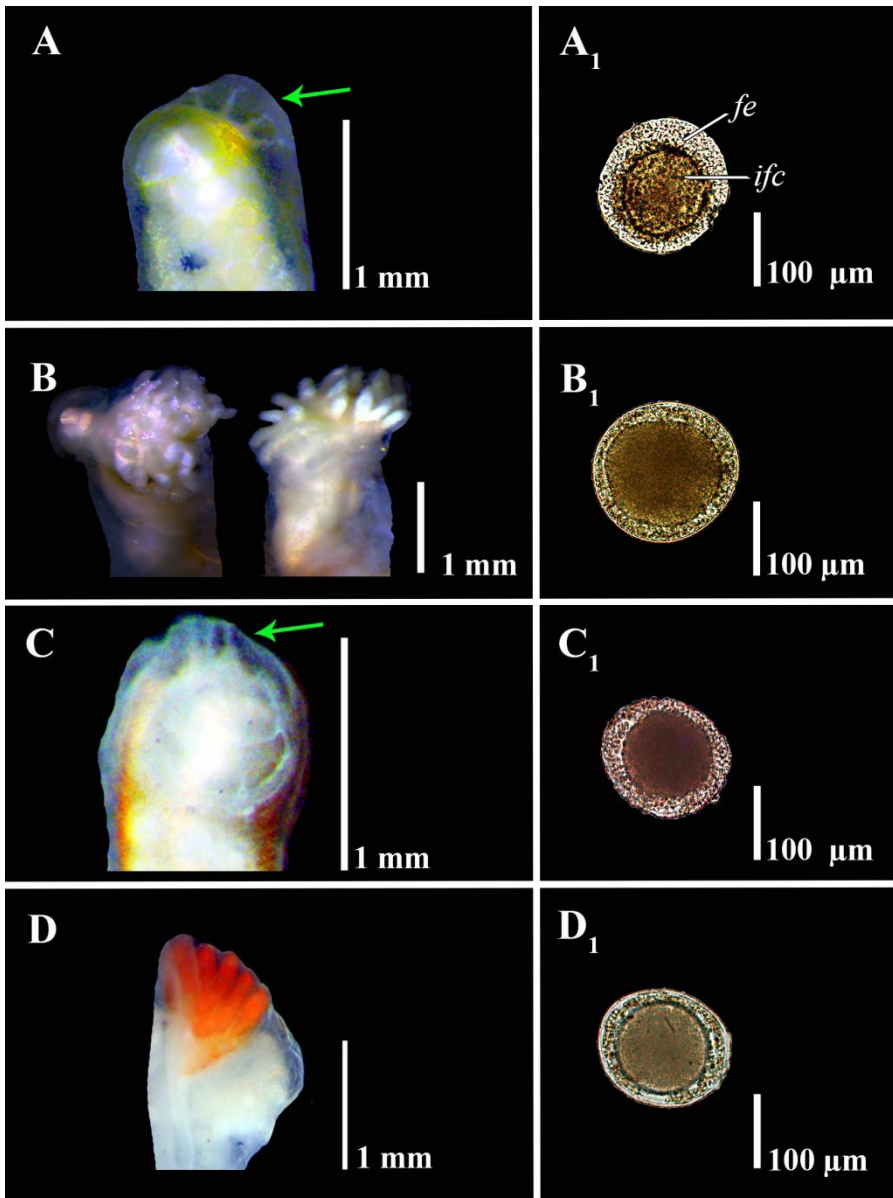


Figure 6. Comparison plate between spermiduct ends and oocytes of four *Ciona* species: (A-A₁) *Ciona intermedia*, spermiduct with four visible papillae (green arrow), oocyte of about 100 μm and a large outer follicular envelope. (B-B₁) *Ciona edwardsi*, lateral and back view of the spermiduct with numerous papillae and oocyte larger in size than the oocytes found in the other three species. (C-C₁) *Ciona intestinalis*, spermiduct with white papillae (green arrow), oocyte of about 100 μm. (D-D₁) *Ciona robusta*, spermiduct with ellipsoidal red papillae, oocyte of about 100 μm. Abbreviations: fe, follicular envelope; ifc, inner follicle cells.

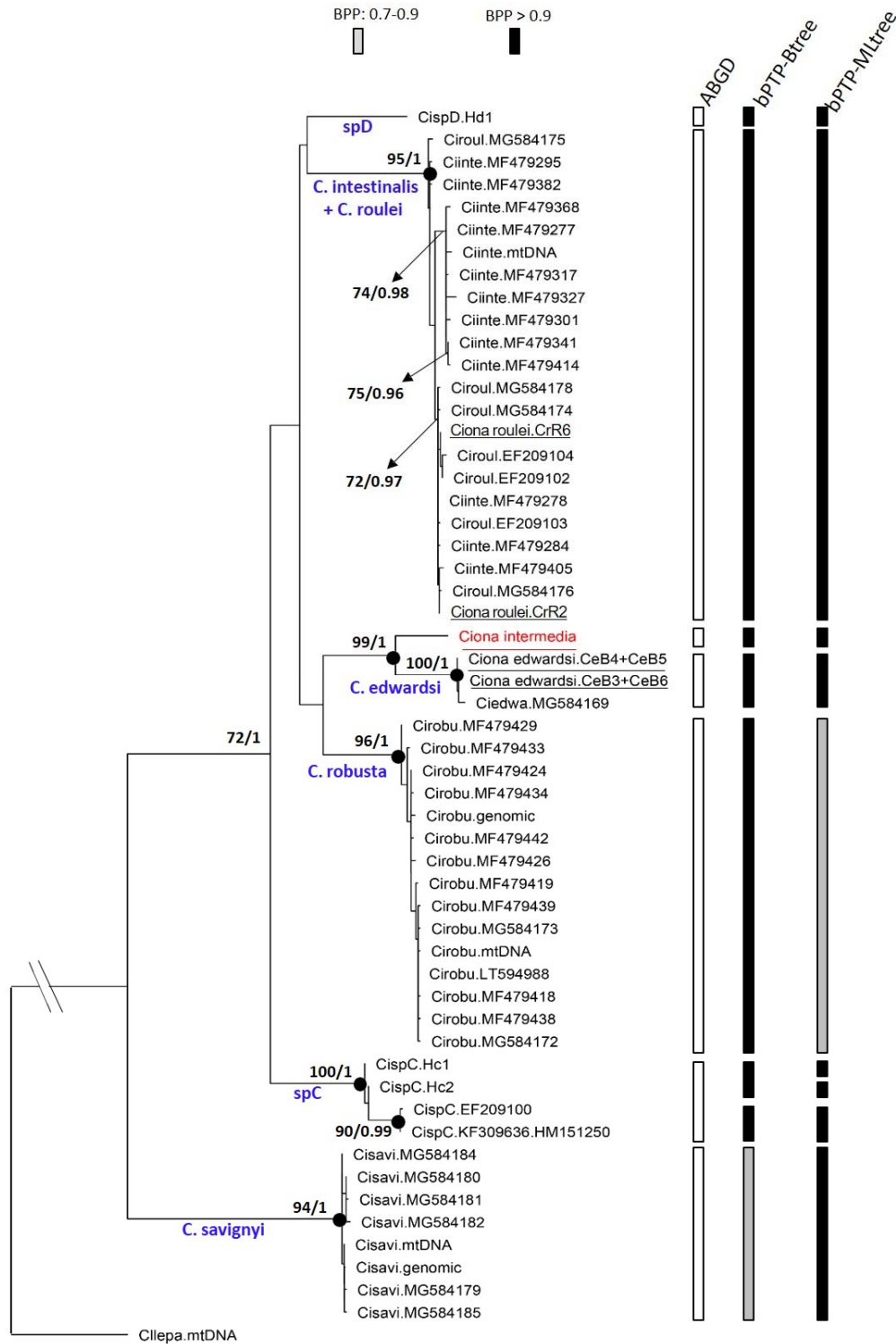


Figure 7. Maximum Likelihood tree based on the COI-1.2kb fragment, with mapping of the OTUs identified by the ABGD and bPTP analyses (vertical bars). Branch length is proportional to the number of substitutions per sites. Values close to the nodes are ML bootstrap percentage/BPP, and are shown only when both bootstrap $\geq 70\%$ and BPP ≥ 0.90 . Black dots: nodes having both bootstrap support $\geq 90\%$ and BPP ≥ 0.99 ; underlining: sequences obtained in this study; ABGD: OTUs consistently identified by the 72 ABGD analyses detailed in Supplementary Table S5; bPTP-Btree: bPTP performed on the Bayesian tree; bPTP-MLtree: bPTP performed on the ML tree. *Clavelina lepadiformis* (Cllepa.mtDNA) was used as outgroup. Acronyms: Ciinte = *Ciona intestinalis*; Ciroubu = *Ciona robusta*; Cisavi = *Ciona savignyi*; Ciroul = *Ciona roulei*; Ciedwa = *Ciona edwardsi*; CispC = *Ciona* spC; CispD = *Ciona* spD. Analyzed sequences are listed in Supplementary Table S2.

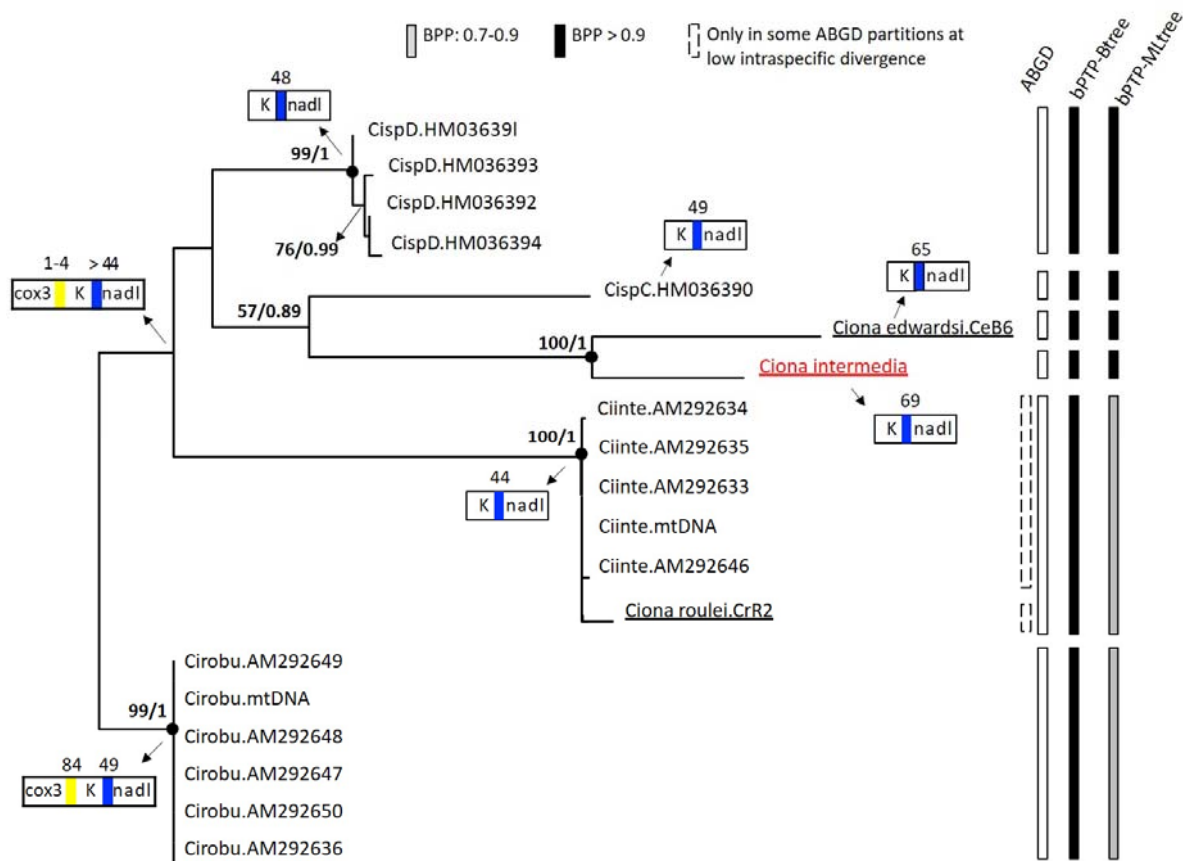


Figure 8. Maximum Likelihood tree based on the x3n1 fragment, with diagram of the gene content of the x3n1 fragment, and mapping of the OTUs identified by ABGD and bPTP analyses (vertical bars). Branch length is proportional to the number of substitutions per sites. Values close to the nodes are ML bootstrap percentage/BPP and are reported only when $> 50/0.50$. Blue and yellow boxes in the gene content diagram: non coding spacers, with length in bp; K: gene *trnK*; black dots: nodes having both bootstrap support $\geq 90\%$ and BPP > 0.99 ; underlining: sequences obtained in this study; ABGD: OTUs identified by the 18 ABGD analyses detailed in Supplementary Table S5, with dashed bars indicating some recursive partitions at the lowest intraspecific divergence; bPTP-Btree: bPTP performed on the Bayesian tree; bPTP-MLtree: bPTP performed on the ML tree. Species codes as in Figure 7. Analyzed sequences are listed in Supplementary Table S4.

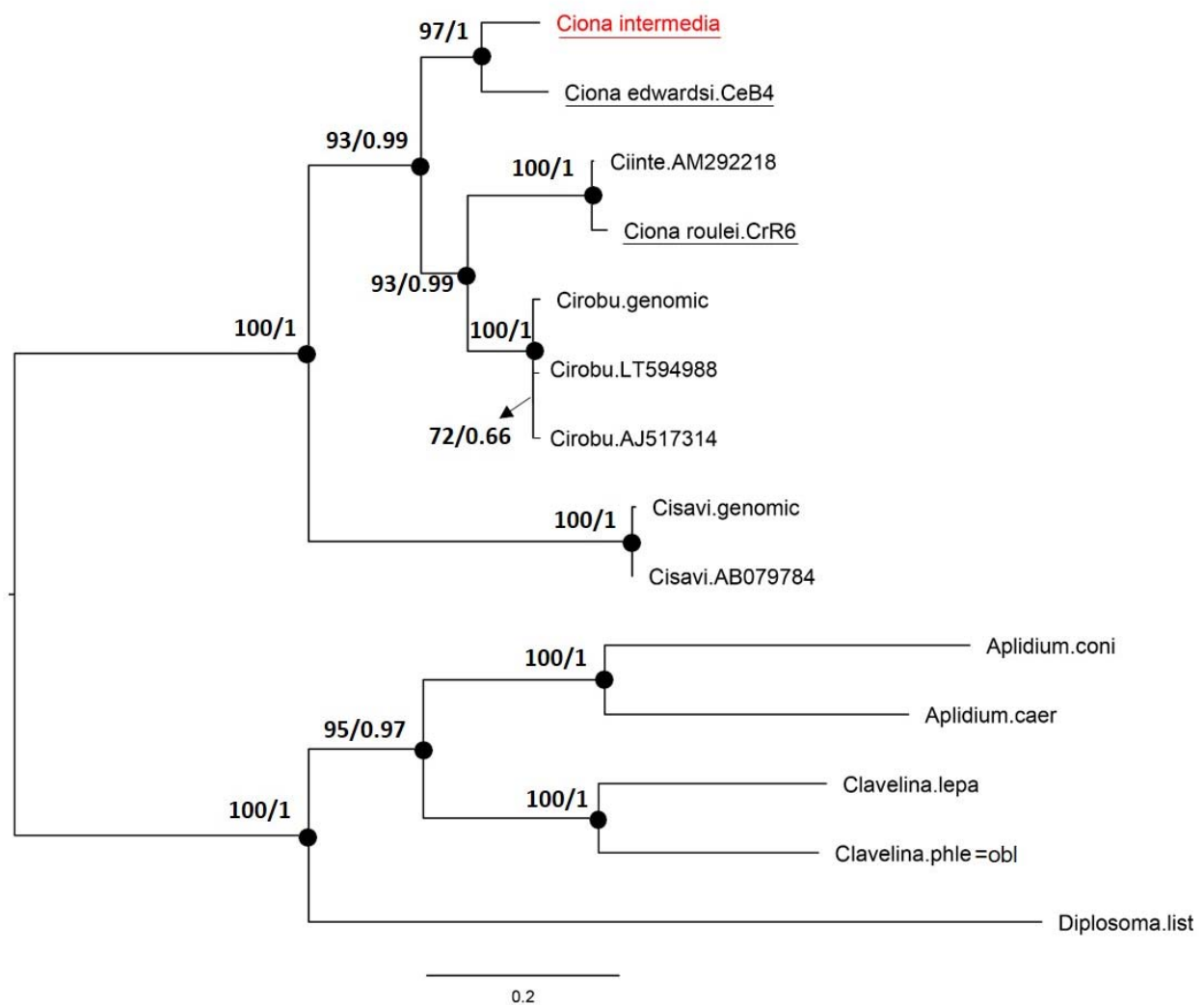


Figure 9. Maximum Likelihood based on the x2cb fragment, consisting of the partial sequences of the *cox2* and *cob* gene. Branch length is proportional to the number of substitutions per sites. Values close to the nodes are ML bootstrap percentage/BPP. Black dots: nodes having both bootstrap support $\geq 90\%$ and BPP ≥ 0.99 ; underlining: sequences obtained in this study. Five Aplousobranchia species were used as outgroups: *Aplidium conicum* (*Aplidium.coni*), *Aplidium coeruleum* (*Aplidium.caer*), *Diplosoma listerianum* (*Diplosoma.list*), *Clavelina lepadiformis* (*Clavelina.lepa*) and *Clavelina phlegraea*, a junior synonym of *Clavelina oblonga* (*Clavelina.phle=obl*) (Ordóñez *et al.*, 2016). Other species codes as in Figure 7. Analyzed sequences are listed in Supplementary Table S3

