# Cryptic speciation in a model invertebrate chordate

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**We applied independent species concepts to clarify the phylogeographic structure of the ascidian *Ciona intestinalis*, a powerful model system in chordate biology and for comparative genomic studies. Intensive research with this marine invertebrate is based on the assumption that natural populations globally belong to a single species. Therefore, understanding the true taxonomic classification may have implications for experimental design and data management. Phylogenies inferred from mitochondrial and nuclear DNA markers accredit the existence of two cryptic species: *C. intestinalis* sp. A, genetically homogeneous, distributed in the Mediterranean, northeast Atlantic, and Pacific, and *C. intestinalis* sp. B, geographically structured and encountered in the North Atlantic. Species-level divergence is further entailed by cross-breeding estimates. *C. intestinalis* A and B from allopatric populations cross-fertilize, but hybrids remain infertile because of defective gametogenesis. Although anatomy illustrates an overall interspecific similarity lacking in diagnostic features, we provide consistent tools for in-field and in-laboratory species discrimination. Finding of two cryptic taxa in *C. intestinalis* raises interest in a new tunicate genome as a gateway to studies in speciation and ecological adaptation of chordates.**

anatomy | *Ciona intestinalis* | cryptic species | gamete compatibility | phylogenetics

Correct classification of model species is a major component for corroborating conclusions drawn from comparative biology. When laboratory colonies are not correlated with species concepts, or experimental material is largely obtained from natural populations, preventing uncovered speciation requires the taxonomic assessment through a phylogeographic approach (1, 2). The tunicate *Ciona intestinalis* L. (Chordata: Ascidiacea) is a marine filter-feeding organism that commonly lives in shallow waters of temperate to boreal regions. Favored biotopes typically include natural and artificial substrates in moderately sheltered habitats with high nutrient content and low wave exposure, such as mussel farms, estuaries, bays, lagoons, and ports (3). *C. intestinalis* is raising ecological and economical concerns as an invasive pest that outcompetes for space and food, reduces native diversity, and influences entire ecosystems (4, 5). A chordate body plan, research amenability, worldwide distribution, and easy sampling are the main reasons for the success of *C. intestinalis* in biosciences (6, 7). In 2002, completion of the whole genome sequence marked an impressive transition in the history of *C. intestinalis*, transforming it into a model organism of prime importance in integrative genomics (8–10). Besides progress in culturing, line storage, and formal genetics, research with *C. intestinalis* is largely based on sampling in nature (like in the 18th century) under the view that natural populations globally belong to the linnean taxon (data not shown and refs. 11–15). Notably, the taxonomic identity of this ascidian has been exhaustively reviewed through synonymies, museum specimens, and geographical variation, highlighting the sensitivity of phenotypic characters to environmental conditions and intraspecific polymorphisms (i.e., tunic texture and color morphs) (16). However, recent reports of genetic divergence between Mediterranean–Pacific and Atlantic populations of *C. intestinalis* have provided clues about an ancient episode of speciation or subspeciation (17–19). Because deciphering the taxonomy of this organism is central to integrative research, we interpreted the identity of *C. intestinalis* in the framework of three species concepts, focusing on Mediterranean and North European populations (see *Materials and Methods* for collection sites). Initially, two types of intersterile populations were identified as part of an international screen for genetic polymorphisms, during crossing assays undertaken to create genetic linkage maps and laboratory strains (20).

## Results

**Gamete Compatibility.** According to Mayr's definition (21), species are "groups of interbreeding populations in nature, unable to exchange genes with other such groups living in the same area." Finding of both cryptic species of *C. intestinalis* in a well known transition area (English Channel, Plymouth, U.K.; Brest, France) (22), provides a unique opportunity to examine patterns of sexual isolation in a simultaneous hermaphrodite and to relate gamete compatibility with geographical distance and genetic partitioning.

Reciprocal fertilization trials using individuals of the two species from Plymouth (PlyMBA sp. A and PlyS sp. B) always resulted in major dysfunctions within one to two cell cleavages, suggesting high levels of pre- and early postzygotic mechanisms against reproductive interactions (Fig. 1*A*). In contrast, heterospecific crossings between allopatric individuals (FuI sp. A × PlyS sp. B) displayed a higher degree of gamete compatibility, allowing development and growth of $F_1$ generations. As shown in Fig. 1*A*, only Mediterranean female gametes could be fertilized and grown to adulthood in homo- and heterospecific combinations. Conversely, fertilization assays performed by using Plymouth spp. A and B oocytes resulted in lethal embryos that died before the larval stage. Hybrids suffered from reduced fitness, and, most notably, they did not produce functioning gametes. Indeed, although mature oocytes were never observed in the oviduct throughout the hybrid lifespan, spermatozoa collected from the male duct (vas deferens) were unable to inseminate *C. intestinalis* A eggs. The hypothesis of sexual isolation is reinforced by lack of microsatellite-based genetic recombination within *C. intestinalis* spp. A and B from the English Channel and from geographically distant populations (Fig. 1*B* and refs. 23 and 24). Progenies generated by homospecific crosses with FuI and PlyMBA sp. A specimens were capable of reaching sexual maturity, giving rise to $F_2$ generations when backcrossed with field-collected Mediterranean individuals. If compared with sympatric individuals, fertilization success between allopatric *C. intestinalis* A was affected by early postzygotic

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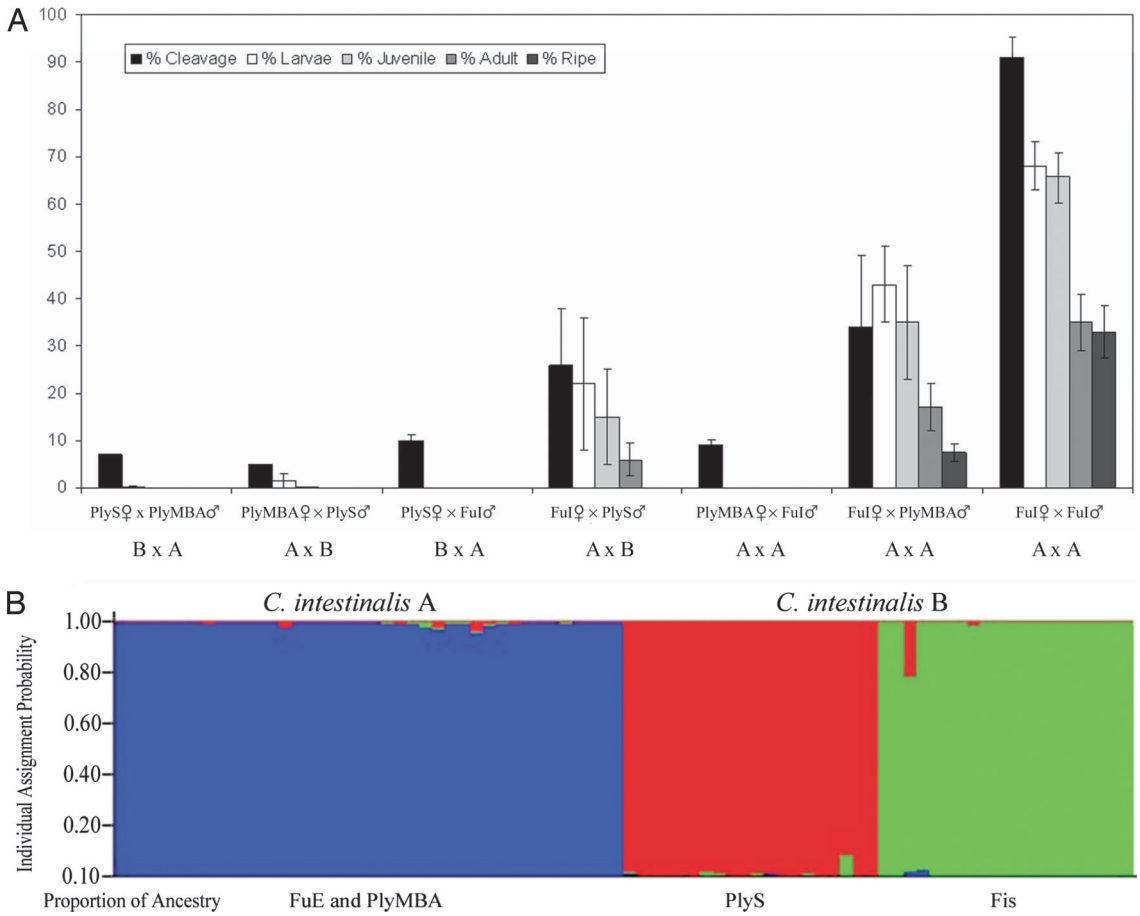


**Fig. 1.** Sexual interactions and Bayesian clustering. (*A*) Heterotypic and homotypic crosses between allopatric and sympatric individuals of *C. intestinalis* A and B. Gamete compatibility is represented as key developmental steps from fertilization to sexual maturation. Each stage is expressed as percentage of the previous one (*y* axis). (*B*) Absence of natural hybridization between *C. intestinalis* A and B is uncovered by Bayesian-based clustering analysis of 12 microsatellite loci. Data were normalized. Error bars indicate standard deviations.

barriers and low fitness, in support of genetic isolation by distance. Variability (see error bars in Fig. 1) reflected seasonal effects, with higher rates of fertilization and growth occurring in winter.

These results demonstrate the existence of two reproductively isolated entities within *C. intestinalis* according to the criteria of the biological species concept (21). Yet hybridization sensitivity related to behavioral differences in spawning and/or to ecophysiological limits will need to be addressed in detail.

**Phylogenesis.** Genetic relationships inferred from 2,578 bp of concatenated nuclear (n; internal transcribed spacer 2, ITS-2; EPIC *Hox5* and *Gsx* introns; *Hox13* exon) and mitochondrial (mt; *cytochrome oxidase I*, COI) sequences under differential selective pressure (see *Materials and Methods* for details), using *Ciona savignyi* as the outgroup, generated two highly supported clades. One includes specimens from the English Channel, the Mediterranean Sea, and the Pacific Ocean, and the other includes those from North European coasts (Fig. 2*A*). Twenty individuals from four European localities were genotyped in 12 microsatellite loci, revealing a sharp break between phylogenetic subdivisions (see *Materials and Methods* for details). The two populations sampled within each clade of *C. intestinalis* were also distinct, unveiling two levels of genetic differentiation: (*i*) cryptic genetic diversity at the interspecies level and (*ii*) genetic structuring within each lineage denoted by the presence of two populations in each clade sharing distinct alleles (Figs. 1*B* and 2*B*). A low intralineage genetic diversity was observed between the two populations assayed [Table 1 and supporting information (SI) Table 3]. Genetic structure and distinct levels of gene flow between geographically predefined populations within lineages are further corroborated by the number of migrants among them (Table 2). The Bayesian-based clustering method demonstrated that the only model able to explain partitioning of genetic variability within the data set was that with $K = 3$ clusters. Each geographic population within *C. intestinalis* sp. B belongs only to one cluster, indicating distinct levels of population structure. Conversely, populations of *C. intestinalis* sp. A are equally distributed in a single cluster, indicating lower population distinction. Microsatellites clearly resolve *C. intestinalis* A and B populations and lineages, whereas sequence markers fail to discriminate populations at the intralineage level despite the geographical distance (Figs. 1*B* and 2*B*). We hypothesize that more subgroups may exist within *C. intestinalis* A and B at worldwide scale. Here they may have been missed owing to limited sampling coverage.

The genetic divergence based on all mitochondrial and nuclear markers is strictly comparable to that observed between either *C. intestinalis* cryptic species and the congeneric *C. savignyi* (13% and 15%, respectively). This should not to be confused with individual (1.2%) (8) or interpopulation (3.0% in the Gulf of Naples) polymorphisms. The resolved network inferred from COI demonstrates the existence of two distinct sister haplogroups separated by 58 substitution steps. Each haplogroup

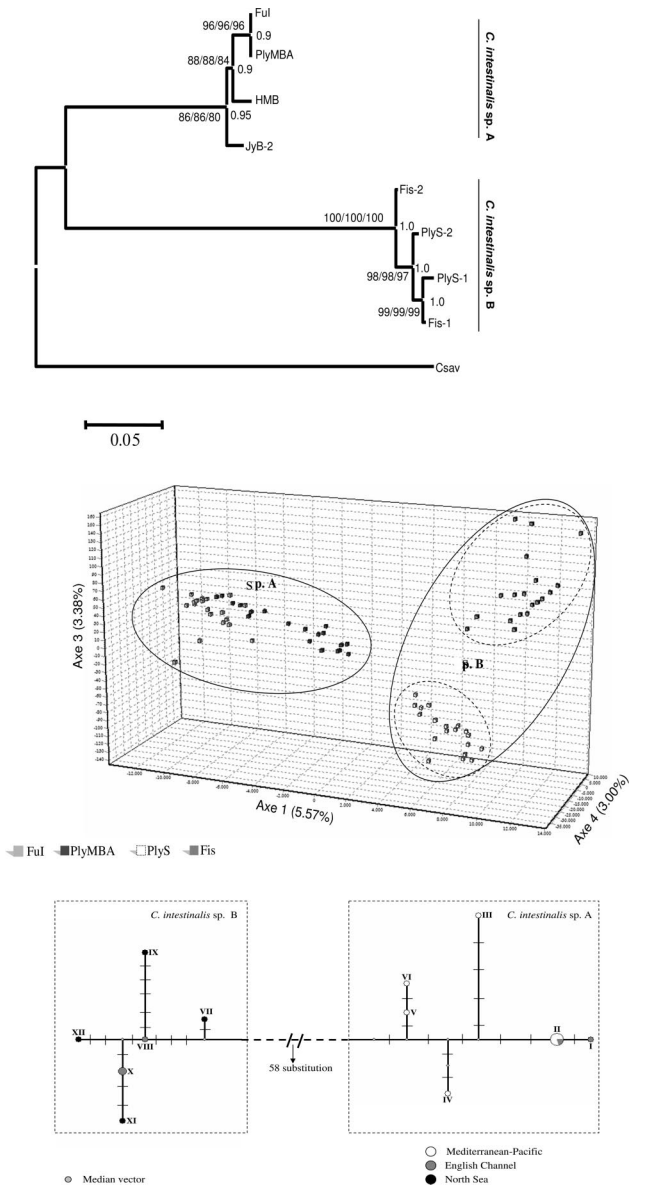


**Fig. 2.** Genetic divergence. (*A*) A single maximum parsimony tree (1,148 steps of length, confidence interval = 0.96) inferred from 2,578-bp concatenated nuclear (*Hox5*, *Gsx*, *Hox13*, and ITS-2) and mitochondrial (COX1) sequences is shown (192 parsimony informative sites, 7.44%). Maximum likelihood/maximum parsimony/neighbor joining bootstrap support (1,000 iterations) and Bayesian posterior probability values are shown, respectively, on the left and right of each node. Numbers after locality abbreviations indicate distinct haplotypes. (*B*) Microsatellite genotype-based factorial correspondence analysis in three-dimensional representation. Color codes: black, FuI; light gray, PlyMBA; dark gray, Fis; white, PlyS. (*C*) Median joining haplotype network based on 515-bp COI alignment. Haplotypes are shaded according to minimum–maximum latitudes. I, PlyMBA; II, FuI, FuE, CdS, VC, Ve, Ta, LT, Al, and Br-1; III, HMB; IV, JyB-2; V, OnM; VI, JyB-1; VII, Fis-2; VIII, PlyS-2; IX, BH; X, PlyS-1 and Br-2; XI, Fis-1; XII, Ed.

includes several private alleles and some frequent ones, all of which have disjoint geographic origin. *C. intestinalis* A specimens form an assemblage consisting of a dominant Mediterranean/English Channel haplotype and two distinct Pacific ones. Specimens from the aforementioned localities are clearly separated by six equally frequent *C. intestinalis* B haplotypes (Fig. 2*C*).

Mobile elements are good candidates as molecular tools for mapping the geographic distribution of the two *C. intestinalis* species. For example, the LTR Gypsy Ty3 retrotransposon Cigr-1 and the MITE Cimi-1 retrotransposon are located, respectively, in intergenic and intronic positions of English but not Californian or Neapolitan individuals (18, 25). Herein we report a *C. intestinalis* A-specific 210-bp insertion in the second intron of the *Hox5* gene, with 85% similarity to a Cili-2 non-LTR retrotransposon element (26) [SI Fig. 4]. This length polymorphism is easily recognizable on agarose gels and provides a fast molecular approach to distinguish between the two organisms (see *Materials and Methods*). In addition, a species-specific gene order was identified in the mitochondrial genome, and it can be also applied as a fast PCR screening method (58).

**Table 1. Pairwise *F* statistics**

| Population (type) | FuE (A) | PlyMBA (A) | PlyS (B) | Fis (B) |
|---|---|---|---|---|
| FuE (A) | — | **1.176** | **0.868** | **0.657** |
| PlyMBA (A) | 0.176 | — | **0.774** | **0.668** |
| PlyS (B) | 0.224 | 0.244 | — | **0.806** |
| Fis (B) | 0.276 | 0.272 | 0.237 | — |

A higher interspecific rather than intraspecific genetic differentiation shows that *C. intestinalis* A is clearly separated from *C. intestinalis* B. A lower genetic distinction between populations provides evidence of genetic structure within each species at different spatial scales ($P < 0.001$ based on 9,999 permutations). 0.25[(1/Fst) − 1] = number of migrants, shown in bold in the upper right part of the matrix, where F*st* is the proportion of the total genetic diversity that separates the populations.

**Morphology.** Taxonomy of *Ciona* spp. is currently formulated upon different anatomical characters, including presence of endostylar appendage, spermiduct papillae and postabdominal extension of mantle, position of pharyngeoepicardiac openings, and gill structure (3, 16, 27). An accurate analysis of external and internal anatomy in two populations per cryptic species confirmed the absence of diagnostic morphological characters (16) (SI Table 4). However, the two forms can be told apart by looking at spermiduct pigmentation. In *C. intestinalis*, the vas deferens opens well inside the atrial siphon, just below the anus, where it ends with a variable number of genital apertures in the shape of ellipsoidal papillae. With one exception (see below), characteristic bright orange pigmentation of these papillae is typical of *C. intestinalis* A populations analyzed in this study (149 individuals from 13 localities), while the duct itself is always uncolored. In contrast, pigmentation in *C. intestinalis* B (57 individuals from six localities) is confined to the duct, where it ranges from subdistal spots to more proximal extension. Remarkably, *C. intestinalis* A from South England lack orange pigmentation on both spermiduct and papillae, a condition observed also in sp. B populations from European and Canadian coasts of the North Atlantic Ocean (19) (Fig. 3). Although pigmentation of the genital openings is deprived of taxonomic value, this character may serve as an appropriate element for

**Table 2. Molecular variance**

| Levels of variance | df | % |
|---|---|---|
| Among species | 1 | 6 |
| Among populations within species | 2 | 19 |
| Within populations | 156 | 75 |
| F*rt* | 0.060 | |
| F*sr* | 0.207 | |
| F*st* | 0.254 | |

AMOVA was calculated by using GenAlEx 6 software ($P < 0.010$). F*rt*, the proportion of the variance among species; F*sr*, the proportion of the variance among populations within species; F*st*, the proportion of the total genetic diversity that separates the populations.

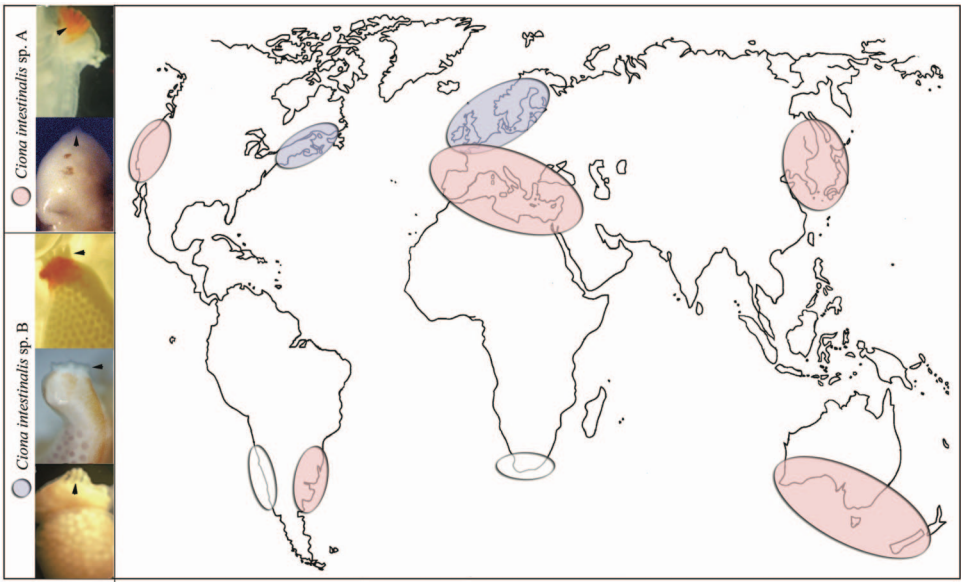


**Fig. 3.** Geographical distribution of *C. intestinalis* sp. A (pink) and sp. B (pale blue) and corresponding patterns of spermiduct pigmentation. See *Morphology* for detailed information on pattern distribution. Empty ovals indicate uncharacterized records of *C. intestinalis* populations. Arrowheads point to the ellipsoidal papillae at the end of the spermiduct.

rapid discrimination by light microscopy. Pigmentation has been considered in the evaluation of speciation events in the colonial ascidians *Pseudodistoma crucigaster* and *Cystoydes dellechiajei* and in the assignment of *C. intestinalis* var. *sydneiensis* to the proper taxon (16, 28, 29), yet this phenotypic trait may be influenced by selective adaptation to environmental parameters (e.g., ref. 30). Herein the Mediterranean pattern is dominantly inherited in offspring of both homo- and heterospecific crosses (data not shown). We therefore conclude that in *C. intestinalis* this is a genetic and not environmentally controlled character with species-specific segregation.

## Discussion

Is *C. intestinalis* a single cohesion taxon, or does it embody species- or subspecies-level divergence? According to reproductive and molecular data, the taxonomic classification of this invertebrate model chordate must be redrawn with the description of two morphologically cryptic species. Because of the poor detail of the original descriptions, the taxonomic status of the two cryptic species should be neatly dealt with through a PCR-assisted revision of anatomy, synonymies, and type specimens.

*C. intestinalis* spp. A and B inhabit largely disjoint geographical regions that overlap in the English Channel. We cannot exclude an accidental introduction of *C. intestinalis* sp. A along the southern coasts of England, where the only available record consists of volunteer individuals growing in the water facility of the Laboratory of the Marine Biological Association in Plymouth. However, an expansion of this taxon toward northeastern Atlantic is supported by the identification of populations in Brest (France) and Vigo (Spain). At this juncture we did not explore large sectors of the worldwide distribution of *C. intestinalis sensu* Linneus, including some areas of invasive introduction (e.g., refs. 31 and 32). In this respect, microsatellites lend themselves as informative markers for phylogeography, migration, and recruitment studies (23, 24).

Phylogenetic divergence and genetic homogeneity at population level are indicators of ancient and distinct evolutionary trajectories, as also supported by broad differences in the structure of the mitochondrial genome (F. Iannelli and C. Gissi, personal communication). Microsatellite and mtDNA analyses delineate separate scenarios: *C. intestinalis* sp. A is genetically homogeneous, suggestive of recent spread, whereas *C. intestinalis* sp. B is represented by fixed haplotypes and spatial genetic structure, compatible with an ancient origin. No episode of gene flow was detected where distribution of the two species intersects. The fact that *C. intestinalis* A and B lack significant and obvious differences in adult morphology, despite the low level of gene flow, implicates stabilizing selection on their phenotypes (33, 34). The two taxa colonize similar habitats; hence a careful approach to ecology, physiology, and life histories is needed to understand the circumstances that molded species divergence.

We analyzed sexual isolation by homo- and heterospecific cross-breeding of sympatric and allopatric individuals, following offspring viability until age of sexual maturation. The two cryptic species do not interbreed in the laboratory, probably as a result of effective pre- and postzygotic mechanisms of reproductive isolation. Adult hybrids generated by crossing gametes of allopatric specimens remained infertile until their senescence. The intensity of the above compatibility barriers appears to be related to the phylogenetic and geographic distances uncovered in this work. Altogether, sexual interaction assays point to selective reinforcement of gamete incompatibility in the absence of physical isolation between the two *C. intestinalis* cryptic species (2, 35), and they may explain why genetic hybrids were not detected. Our knowledge of the sexual behavior in *C. intestinalis* spp. does not allow inferences on the gamete bias observed between the two sibling taxa. Studies of one-way cross-infertility in *C. intestinalis* support the notion that a multilocus mechanism controls sexual interactions (18, 36–42).

Demonstration of a phylogenetic split in the invertebrate model chordate, *C. intestinalis*, clarifies a previous hypothesis of subspeciation beyond any possible ambiguities (17–19). This result provides new views for a large number of research fields and points to the importance of careful taxonomic definitions in the comparative approach. Furthermore, it widens the genomic toolbox for studying processes and functions in the evolutionary history of chordates.

## Materials and Methods

**Biological Material.** We analyzed specimens or sequences of *C. intestinalis* sp. A and sp. B from 21 geographical sites. Following is the locality list per species with abbreviations.

*C. intestinalis* sp. A: two populations in the Fusaro Lagoon, Naples, Italy (FuI and FuE); Villaggio Coppola, Naples, Italy (VC); Castellamare di Stabia, Naples, Italy (CdS); Venice, Italy (Ve); Taranto, Italy (Ta); Lago Faro, Messina, Italy (LF); Alicante, Spain (Al); Lake Timsah, Egypt (LT); Brest, France (Br); Marine Biological Association, Plymouth, England (PlyMBA); Half Moon Bay, California (HMB); Santa Barbara, California (SB); Yokohama Bay, Japan (JyB); Onagawa, Miyagi, Japan (OnM); and Chungmu Port, South Korea (KCP).

*C. intestinalis* sp. B: Plymouth Sound, England (PlyS); Edinburgh, Scotland (Ed); Breskens Harbor, The Netherlands (BH); Fiskebäckskil, Sweden (Fis); Brest, France (Br); and Mahone Bay, Canada (MB).

Animals from FuI, FuE, VC, CdS, PlyMBA, PlyS, and Fis were maintained in the animal facility at the Stazione Zoologica until ready for experimental use in fertilization assays. These were integrated with specimens kindly sent from several localities to our laboratory.

**Culturing and Crossings.** Wild animals and hybrids were maintained according to standard procedures (11), with some modifications. To avoid cross-contaminations and for a correct classification, all parental and $F_1$ animals have been genotyped and kept in separate tanks. Hetero- and homospecific crossing experiments were performed in different years (2003–2006). Experiments consisted of five trials per gamete combination and were conducted at 14°C, 16°C, and 18°C (11).

**Morphology.** Five to 10 individuals from FuI, Fis, PlyMBA, and PlyS were anesthetized with menthol in filtered seawater, fixed in 4% formaldehyde in filtered seawater, and analyzed by using binocular stereomicroscopy before and after dissection. To examine spermiduct pigmentation, 5–10 individuals from each population were dissected, analyzed, and photographed.

**DNA Extraction and Amplification.** Total DNA was extracted from fresh or 95% ethanol-preserved animal body wall. Fresh specimens were sustained for 2 days without feeding and with continuous change of 0.22 $\mu$M-filtered seawater to avoid stomach content contamination. DNA extraction was performed as previously described. A detailed list of primers and PCR conditions used to amplify mtDNA and nDNA markers is shown in SI Table 5 for Mt-haplotypes and sequence AA, see SI Table 6.

**Genome Databases.** Californian sequences were downloaded from the Joint Genome Institute *C. intestinalis* database (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) (8), Japanese sequences were from the Ghost database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) (43), and *C. savignyi* sequences were from the Ensembl genome browser (www.ensembl.org/Ciona_savignyi/index.html) (44) after BlastN with *C. intestinalis* sequences.

**Network.** Networks were generated by Network version 4.1.1.2 (www.fluxus-engineering.com/sharenet.htm) on ClustalW-aligned COI sequences using the Median Joining algorithm (45) and the parsimony postprocessing calculation option. Five sequences from each collection locality were used in the alignment.

**Population Genetic Analysis.** Eighty *Ciona* spp. individuals (20 from each locality per population) were genotyped with four previously reported (23) and eight newly selected microsatellite markers (24). Allele detection was conducted by using an automated capillary sequencer (CEQ 2000XL DNA Analysis system; Beckman Coulter, Fullerton, CA), and electropherograms were analyzed with Beckman CEQ 2000 version 3.0 software. Quality of the allelic matrices and postgenotypic errors by population and locus were assessed in MICRO-CHECKER version 2.2.1 (46). Allelic richness per locus per population, and a factorial correspondence analysis of the microsatellite data, were calculated in GENETIX version 4.05.02 (47). Deviation from Hardy–Weinberg equilibrium was estimated in GENPOP version 3.4 (48). The partition of the genetic diversity, variation, gene flow, *F* statistics, number of migrants, and analysis of molecular variance were explored as implemented in GENALEX version 6 (49) from 12 microsatellite allelic frequency data among FuE, PlyMBA, PlyS, and Fis. Levels of significance were Bonferroni-corrected. A Bayesian-based clustering analysis, as implemented in STRUCTURE version 2.1 (50), was also applied to the microsatellite data set. This analysis computes the number of clusters of genotypes or subpopulations ($K$) that best fits the genetic variability encountered in the data set and interprets it without having prior information on the number of predefined sampled geographical populations. Under the admixture model, five independent runs were performed of $K = 1$–5 at $10^6$ Markov chain–Monte Carlo repetitions and a burn-in period of 50,000 iterations without prior information and assuming correlated allele frequencies. The posterior probability was then calculated for each value of $K$, and the optimal number of clusters was chosen.

**Sequence Analysis and Tree Reconstructions.** Sequences were aligned with BioEdit version 4.8.5 (51). The alignment was refined by eye. Maximum parsimony and maximum likelihood phylogenies were inferred in PAUP* 4.0b10 for Windows (52). Hierarchical likelihood ratio tests were performed by using MODELTEST version 3.06 (53) to find the best-fitting parameters for the maximum likelihood analysis given the alignment. Bootstrap support for individual clades (54) was calculated on 1,000 replicates by using the same methods, options, and constraints as in the tree inferences but with all identical sequences removed. Bayesian posterior probabilities of individual clades were calculated by using a variant of the Markov chain Monte Carlo algorithm as used in MrBayes version 3.1.2 (55). To increase the resolving power associated with additional informative sites, concatenated phylogenies were inferred from a final alignment of 2,578 bp and five genes. Before the combined analysis, the significance of the incongruence length difference test (56) was evaluated as evidence of congruence among all of the gene regions involved in the final alignment as partitions.

**Cili-2 Retrotransposon.** The Neapolitan *Hox5* sequence was screened against the reference collection of repeats and masks homologous portions by using CENSOR (57) (www.girinst.org/censor/index.php). Five to 10 individuals from each population were tested via PCR amplification for Cili-2 presence/absence.

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