RESEARCH ARTICLE

Genealogical relationships within and among shallow-water *Ciona* species (Ascidiacea)

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Abstract In spite of historical and current interest in *Ciona intestinalis* and its congeners, little is known about evolutionary relationships among the members of the genus *Ciona.* Here 744-bp sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene are used to examine phylogenetic relationships among three described species (*C. intestinalis, C. roulei, C. savignyi*) sampled from multiple coastal sites in the Northeast Pacific (CA, USA), Northwest Atlantic (from New Hampshire to Connecticut, USA), Northeast Atlantic (Sweden and The Netherlands), and Mediterranean (Banyuls-sur-Mer, France). The samples were collected in June–October 2005. The COI sequences of Northeast Pacific/Mediterranean (Type A) and Northwest Atlantic (Type B) *C. intestinalis* differ by ~12% and *C. roulei* is nested within Type B *C. intestinalis. Ciona savignyi* differs from all other haplotypes by 13–16%. A previously undescribed but morphologically distinct *Ciona* sp. found at the Banyuls-sur-Mer site was >10% divergent from all other haplotypes. Although these data arise from a single gene study, they indicate that further elucidation of species relationships within the genus and of the species' distributions will be needed if continuing invasions and potential reproductive isolation are to be investigated.

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Introduction

The ascidian *Ciona intestinalis* has served as a developmental model species for over a century (Chabry 1887; Morgan 1904; Minganti 1948; Sordino et al. 2000). Both *C. intestinalis* and its congener *C. savignyi* have invaded benthic communities throughout the temperate zone, necessitating expensive eradication programs (Lambert 2001; Lambert and Lambert 2003). Recently, the genome sequences of *C. intestinalis* and *C. savignyi* have become available (Dehal et al. 2002; Vinson et al. 2005), providing tools for comparative genomics and insights into vertebrate evolution (Boffelli et al. 2004; Johnson et al. 2004; Missal et al. 2005). Comparisons of the mitochondrial genomes of *C. intestinalis, C. savignyi*, and other tunicate species also have answered questions about chordate evolution (Gissi and Pesole 2003; Yokobori et al. 2003, 2005). Despite the historical and current interest in *C. intestinalis* and its congeners, we know very little about genealogical and phylogenetic relationships within and among members of this genus.

The genus *Ciona* comprises 12 known species, 4 found in shallow water and 8 found exclusively in deep water (Harant and Vernieres 1933; Van Name 1945; Monniot and Monniot 1977, 1983, 1989, 1990; Hoshino and Nishikawa 1985; Sanamyan 1998). Many of the deep-water species have been described from only one specimen and most are difficult to collect. Here we focus on relationships among the common shallow-water species. *Ciona intestinalis* is thought to be endemic to northeast Atlantic waters, where it was described (Linnaeus 1767). It is widely distributed in the temperate zone presumably through transport in ballast water and on ship bottoms and now has a range

that includes the Pacific Coasts of Japan and North America, New Zealand, Australia, and Chile, the Atlantic Coasts of North America, Europe, and South Africa, the Black Sea and the Mediterranean Sea (Van Name 1945; Kott 1952). *Ciona savignyi* (Herdman, 1882) is endemic to Japan, but has spread to the Pacific Coast of North America and the Atlantic Coast of Argentina (Hoshino and Nishikawa 1985). *Ciona roulei* (Lahille, 1887) is found only in the Mediterranean Sea, where it has only been described from five sites along the coasts of France and Spain (Harant and Vernieres 1933). The fourth shallow water species, *C. edwardsi* (Roule, 1886), is rare and was not included in this study. Although the morphology of several *Ciona* species has been compared (Hoshino and Nishikawa 1985), no study thus far has examined phylogenetic relationships among these species.

Within *C. intestinalis*, two "types" have been recognized (Suzuki et al. 2005). Type A inhabits the Pacific Ocean and the Mediterranean Sea, while Type B inhabits the Atlantic Ocean. Type A and Type B are working names agreed upon by the community of *Ciona* biologists. The two types of *C. intestinalis* are partially reproductively isolated. Crosses using British Type B *C. intestinalis* eggs and Japanese Type A *C. intestinalis* sperm result in normal rates of fertilization, but the reciprocal cross yields many fewer fertilized eggs (Suzuki et al. 2005). While *C. roulei* and *C. intestinalis* are clearly distinct on morphological grounds (Harant and Vernieres 1933), crosses between these two species are also known to produce viable offspring. In particular, Mediterranean Type A *C. intestinalis* sperm × *C. roulei* eggs produced viable F1 tadpole larvae. However, fertilization fails in the reciprocal cross (Lambert et al. 1990). *Ciona intestinalis* and *C. savignyi* are completely reproductively isolated (Byrd and Lambert 2000).

Here we use mitochondrial DNA (mtDNA) sequence data to investigate the genealogical and phylogenetic relationships within and among *Ciona* species. In particular, we focus on the relationships and amounts of divergence between Type A and B *C. intestinalis*, and among *C. roulei, C. savignyi*, and *C. intestinalis*.

Materials and methods

Sampling

Individuals from three described species (*C. intestinalis, C. roulei* and *C. savignyi*) and one unknown species (*Ciona* sp.) were collected by SCUBA or from docks (Table 1). *Ciona intestinalis* individuals were collected from five sites on the California Coast of North America, 12 sites on the Atlantic Coast of North America, and 1 site in the Northwestern Mediterranean (Banyuls-sur-mer, France). Additional individuals were obtained from two sites in the Northeast Atlantic (Fiskebäckskil, Sweden and Breskens Harbor, The Netherlands). *Ciona roulei* individuals were sampled from one site in the Northwestern Mediterranean (Banyuls-sur-mer, France). Individuals of a previously unidentified species (*Ciona* sp.) were also collected in the harbor at Banyuls-sur-mer. *Ciona savignyi* individuals were sampled from four sites on the California Coast of North America. Specimens of *C. roulei* and *Ciona* sp. were deposited at the American Museum of Natural History. A sequence from *Halocynthia roretzi* (GenBank Accession No. NC_002177; Yokobori et al. 1999) was used as the outgroup. Sequence data were obtained from 106 of the collected individuals, and the data set contained 107 sequences including the outgroup.

DNA extraction, amplification and sequencing

Ovaries were dissected from freshly-collected individuals, cut into several pieces, immediately preserved in dimethyl sulfoxide (DMSO), and ultimately (within 12 days) stored at −80°C until needed. We used ovaries because they are easily removed from the body and yield high-quality DNA. Samples from Sweden and The Netherlands were preserved and shipped in ethanol soon after collection. Upon arrival, the ovaries from these individuals were dissected and preserved in DMSO. Total DNA was extracted from the ovaries using the Qiagen DNeasy® Tissue Kit (Qiagen). Primers to amplify 856 bp of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene were developed from the consensus sequence of the published *C. savignyi* and *C. intestinalis* mitochondrial genomes (Consensus F: 5′GA GTAAGAACTGGRTGRACAGTTAYCCTCC 3′, Consensus R: 5′ATTAAAACTTAATCTAGTAAAA AGAGGRRATCAATGG 3′). PCR amplification was performed in a 20-μl total reaction volume with 2 mM MgCl$_2$, 0.2 mM dNTPs, 2 μl of 10× buffer, 0.2 μM of each primer, 0.8 U of *Taq* Polymerase (Gibco-BRL) and 2 μl of template DNA. The PCR protocol was as follows: 35 cycles (95°C for 50 s, 48°C for 1 min and 72°C for 1 min) and a final extension step at 72°C for 7 min, on an OmniGene (Hybaid) thermal cycler. PCR products (diluted up to 1:20 depending on the intensity of the PCR band on an agarose gel) were incubated with 1 μl each of Exonuclease I and Antarctic Phosphatase at 37°C for 30 min, followed by 90°C for 10 min. The

Table 1 Collection sites for *Ciona* species for which COI sequences were determined

Species	Region/state or country	Site	Number of individuals
Ciona intestinalis Type A	Northeast Pacific/California	Newport Harbor	6
		Alamitos Bay	2
		Santa Barbara Harbor	4
		Half Moon Bay	7
		Sausalito	1
Type B	Mediterranean/France	Banyuls-sur-Mer	7
	Northwest Atlantic/New Hampshire to Connecticut	Newcastle	2
		Gloucester	2
		Salem	2
		Beverly	3
		Winthrop	2
		Quincy	3
		Sandwich	1
		Buzzards Bay	3
		Woods Hole	4
		Westport Point	3
		Newport Shipyard	2
		Mystic	3
	Northeast Atlantic/Sweden	Fiskebäckskil	8
	Northeast Atlantic/The Netherlands	Breskens	15
C. roulei	Mediterranean/France	Banyuls-sur-Mer	6
C. savignyi	Northeast Pacific/California	San Diego Bay	8
		Newport Harbor	1
		Santa Barbara Harbor	3
		Sausalito	6
Ciona sp.	Mediterranean/France	Banyuls-sur-Mer	2

products were purified on Sephadex® columns (Sigma-Aldrich). The purified product was sequenced with a Big Dye Terminator Cycle sequencing kit and an ABI-3700 automated sequencer (Applied Biosystems) using the primers listed above. All unique haplotypes (56 sequences) have been submitted to GenBank (Accession Numbers EF209056–EF209110).

Alignment

Sequences were edited and trimmed to 744 bp with SeqMan (DNASTAR) and aligned using the Clustal-W algorithm in MEGA 3.1 (Kumar et al. 2004). Alignments were confirmed visually, and no gaps were present in the final alignment. There is no evidence for nuclear pseudogenes in our dataset. Direct sequencing of PCR products yielded clean sequence and no stop codons were found in the inferred amino acid sequence.

Phylogenetic analysis

Phylogenetic trees were constructed using Maximum Parsimony (MP), Maximum Likelihood (ML), and Neighbor Joining (NJ) methods. Analyses were performed using a data set comprised only of the unique haplotypes (56 sequences).

Parsimony analyses were performed using PAUP* 4.0 (Swofford 2003). Tree space was explored using a heuristic search, with 1,000 replicates of random stepwise addition and TBR branch swapping. A strict consensus was obtained from this heuristic search. Support was assessed using bootstrap analysis (1,000 replicates) with 10 replicates of random sequence addition for each of the bootstrap replicates.

The best-fit model of nucleotide substitution, determined by the hierarchical Likelihood Ratio Test in Modeltest 3.7 (Posada and Crandall 1998), was TIM+G. The TIM+G model was used to generate a ML tree in PAUP* 4.0 (Swofford 2003). Bootstrap support was determined with 250 replicates of a heuristic search strategy, with an as-is addition sequence and TBR branch swapping.

PAUP* 4.0 (Swofford 2003) was also used to perform NJ analyses. Distances based on the TIM+G model were used in the analysis, and confidence in the tree was assessed using 1,000 bootstrap replicates.

Genetic diversity

Average *p* distances (total number of differences between two sequences divided by total length of sequence compared) within and between sites were calculated using PAUP* 4.0 (Swofford 2003). In addition,

distances were estimated based on the TIM+G model. The number of haplotypes and haplotype diversity for each site were determined using DnaSP 4.10.4 (Rozas and Rozas 1999).

Population structure

We characterized population structure within *Ciona* species and sites using an analysis of molecular variance (AMOVA) in Arlequin 3.0 (Schneider et al. 2000). Pairwise Φ_{ST} values between sites were calculated by AMOVA and *P*-values were obtained by a 110-replicate permutation test.

Results

With the exception of Mediterranean Type A, for which all individuals had the same haplotype, haplotype diversities within collection sites of *C. intestinalis* were high, ranging from 0.916 to 0.956 (Table 2). Haplotype diversity was similarly high in *C. roulei* (0.933), and only slightly lower in *C. savignyi* (0.752). Analyses based on AMOVA suggest that most variation was within sites, and that there was relatively little differentiation among sites within a region (Table 3; Northeast Pacific Type A *C. intestinalis* $\Phi_{ST} = -0.073$ ($P > 0.05$), Northwest Atlantic Type B *C. intestinalis* $\Phi_{ST} = 0.103$ ($P > 0.05$), *C. savignyi* $\Phi_{ST} = -0.099$ ($P > 0.05$)). But within both Type A and Type B *C. intestinalis*, differentiation existed between regions [Table 3; Type B $\Phi_{ST} = 0.226$ ($P < 0.0001$), Type A $\Phi_{ST} = 0.476$, ($P < 0.002$)].

In contrast to the limited variation among sites of the same species/type, there was substantial differentiation between Type A and B *C. intestinalis* and between *C. intestinalis* and *C. savignyi*. Within *C. intestinalis*, Type A and B differed in mtDNA sequence by ~12%. *Ciona savignyi* exhibited 13.5–16.2% mtDNA

Table 2 *Ciona* spp. haplotype diversity [calculated in DnaSP 4.10.4 (Rozas and Rozas 1999)] and number of haplotypes in each species or region within species

Species	Region	Haplotype diversity (±SD)	Number of haplotypes
C. intestinalis	Mediterranean	0	1
Type A	Northeast Pacific	0.916 ± 0.041	11
Type B	Northeast Atlantic	0.937 ± 0.033	14
	Northwest Atlantic	0.956 ± 0.026	21
C. roulei		0.933 ± 0.122	5
C. savignyi		0.752 ± 0.075	5
Ciona sp.		0	1

Table 3 Distribution of variation within and among sites and among regions resulting from AMOVA tests performed in Arlequin 3.0 (Schneider et al. 2000)

Type A *C. intestinalis*	% of variation
Among regions	45.49
Among sites within regions	2.13
Within sites	52.38
Northeast Pacific Type A *C. intestinalis*	% of variation[a]
Among sites	−7.27
Within sites	107.27
Type B *C. intestinalis*	% of variation
Among regions	5.37
Among sites within regions	17.24
Within sites	77.39
Northwest Atlantic Type B *C. intestinalis*	% of variation
Among sites	10.27
Within sites	89.73
C. savignyi	% of variation[a]
Among sites	−9.91
Within sites	109.91

Regions and sites as in Table 1

[a] Negative covariances can result from an absence of genetic structure

sequence divergence from either of the other *Ciona* species (Table 4).

Maximum Parsimony and Maximum Likelihood methods produced bootstrap majority-rule consensus trees with identical topologies. Both the 50% majority rule bootstrap MP tree (1,000 replicates; Fig. 1) and the 50% majority rule bootstrap ML tree (250 replicates; not shown) support the monophyly of *C. savignyi* and place it sister to a *C. intestinalis/C. roulei* clade. The *C. intestinalis/C. roulei* clade is divided into two well-supported sub-clades in both trees (Fig. 1). One sub-clade, with 100% bootstrap support, defines Type A *C. intestinalis* and includes haplotypes from both the Northeast Pacific and the Mediterranean. A second sub-clade, also with 100% bootstrap support, includes Type B *C. intestinalis* from the Northeast and Northwest Atlantic, together with *C. roulei*. Thus Type B *C. intestinalis* is paraphyletic with respect to *C. roulei*. The NJ tree defines the same major clades revealed by the MP and ML analyses, and clearly shows that interspecific (or inter-type) differences dwarf intraspecific differences (Fig. 2).
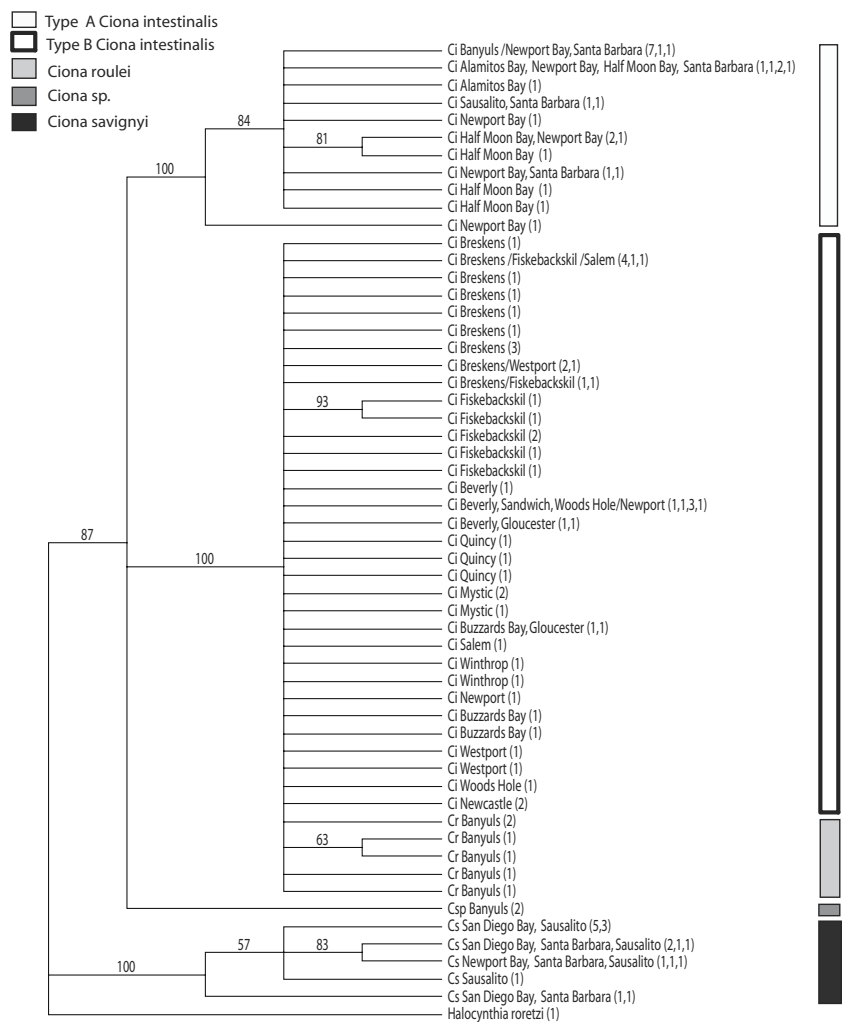
Ciona sp.

Three individuals from Banyuls-sur-Mer Harbor, France, had haplotypes that were distinctive from those seen in all other individuals (Table 4, Figs. 1, 2). Sequence divergences between these haplotypes and haplotypes found in *C. intestinalis* and *C. savignyi* ranged from 10.8 to 14.0% (Table 4). These individuals

Table 4 Sequence divergence within and between *Ciona* species/regions within species

Species	Region	Mediterranean	Northeast Pacific	Northeast Atlantic	Northwest Atlantic	*C. roulei*	*C. savignyi*	*Ciona* sp.
		Type A	Type A	Type B	Type B			
C. intestinalis	Mediterranean	**0.000**	0.005	0.199	0.200	0.208	0.267	0.185
Type A	Northeast Pacific	0.005	**0.005**	0.201	0.201	0.210	0.267	0.190
Type B	Northeast Atlantic	0.122	0.123	**0.006**	0.007	0.010	0.324	0.216
	Northwest Atlantic	0.123	0.123	0.007	**0.007**	0.011	0.324	0.214
C. roulei		0.125	0.126	0.010	0.008	**0.004**	0.328	0.217
C. savignyi		0.136	0.135	0.161	0.161	0.162	**0.002**	0.276
Ciona sp.		0.108	0.109	0.127	0.125	0.126	0.140	**0.000**

Above the diagonal: divergences corrected using the TIM + G model. Below the diagonal: uncorrected *p* distances

Fig. 1 Bootstrap majority rule Maximum Parsimony tree generated from the 56 unique haplotypes found in 107 individuals (106 ingroup sequences and 1 outgroup sequence, listed in Materials and methods). Numbers along branches indicate bootstrap percentages. Nodes with bootstrap percentages <50% have been collapsed. Numbers in parentheses beside each site refer to the number of individuals sequenced from each site

(hereafter referred to as *Ciona* sp.) were also morphologically distinct from *C. intestinalis* and *C. roulei* found in Banyuls-sur-mer. Furthermore, they did not match morphological or ecological descriptions of *C. edwardsi* (the fourth shallow-water species) from the region (Fiala-Medioni 1974; Copello 1981). The tunics of these individuals were reddish-orange and opaque,

and the body clear. In contrast, the *C. roulei* individuals had clear tunics and deep red bodies, while *C. intestinalis* individuals (from this region) had clear tunics and milky-white, opaque bodies. The tunics of *Ciona* sp. were very thin, well-attached, and difficult to remove, in contrast to the tunics of *C. intestinalis* and *C. roulei*. The three individuals assigned to *Ciona* sp.

Fig. 2 Neighbor Joining phylogram generated from the 56 unique haplotypes found in 107 individuals (106 ingroup sequences and 1 outgroup sequence, listed in Materials and methods). *Numbers along each branch* indicate the number of changes along that branch and (*in parentheses*) the bootstrap percentages. Bootstrap percentages <50% not shown. Numbers in parentheses beside each site refer to the number of individuals sequenced from each site

— 5 changes

Discussion
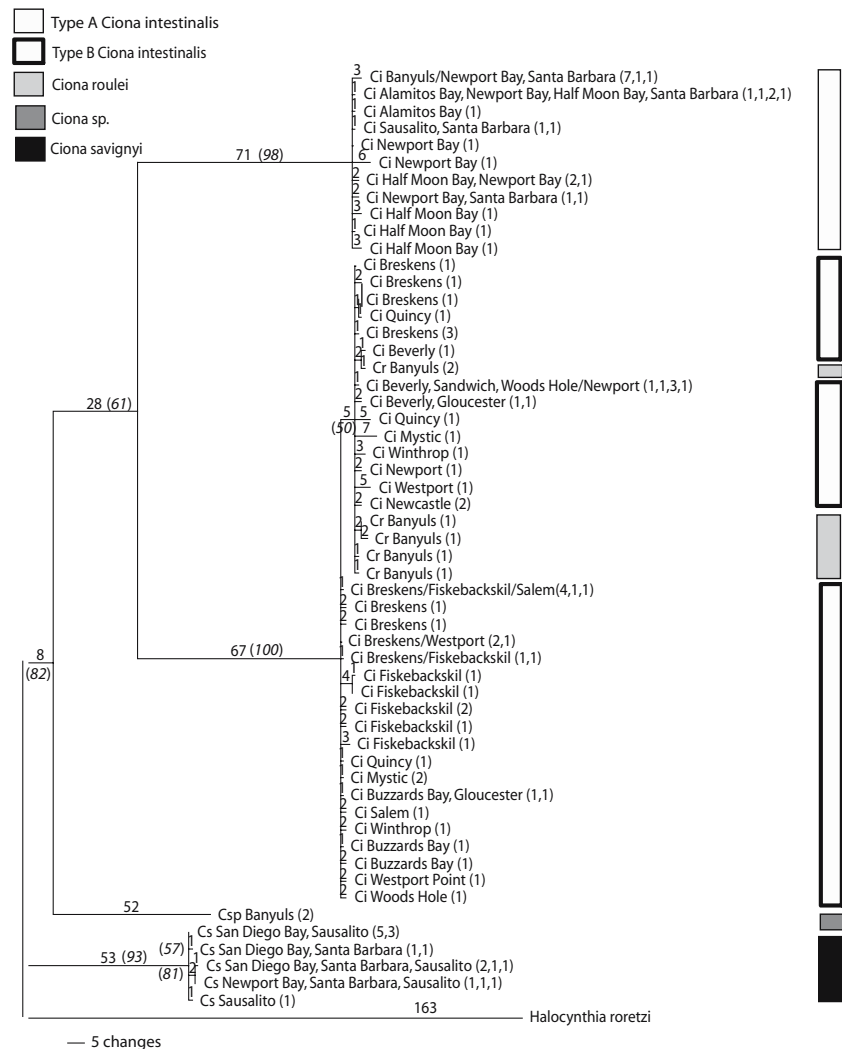
Mitochondrial DNA sequence data confirm that *C. intestinalis* and *C. savignyi* exhibit substantial genetic divergence. The mtDNA phylogeny also shows *C. roulei* haplotypes to be embedded within and not clearly distinct from haplotypes found in Type B *C. intestinalis*. Furthermore, Type A and Type B *C. intestinalis* are exclusive groups (Figs. 1, 2), with sequence diver-

were 3–4 cm long fully extended with tunic intact, which was considerably smaller than *C. intestinalis* and *C. roulei* from this region. The buccal and atrial siphons were proportionally longer compared to those of *C. intestinalis* and *C. roulei*, and extended half the length of the body. The atrial siphon had six red pigment dots, and the buccal siphon had at least six. Photos of *Ciona* sp. have been placed on the Dutch Ascidians website: http://www.ascidians.com.

gence that suggests a most recent common ancestor in the Pliocene, assuming a molecular clock rate of about $3\%^{/my}$ [estimated from mtDNA clocks in three echinoderm genera: Lessios et al. (1999), McCartney et al. (2000), Lessios et al. (2001)].

Suzuki et al. 2005 compared 120 kb of cosmid sequence from British Type B *C. intestinalis* with the whole genome sequence from a Northeast Pacific Type A *C. intestinalis*. This revealed a broad range of sequence divergence, with predicted coding regions showing the least divergence. Twenty-four randomly chosen ESTs from British Type B *C. intestinalis* showed 95.2% average sequence identity to orthologues found in the Japanese Type A *C. intestinalis* cDNA database (Suzuki et al. 2005). Unfortunately, the selection of genomic regions in these analyses does not allow comparisons with patterns of divergence in other marine invertebrate taxa, because of the difficulty in identifying homologous gene regions. However, mitochondrial COI data allow us to compare

distances between Type A and B *C. intestinalis* to distances between other closely related marine taxa.

We searched the literature for studies that estimated amounts of interspecific and intraspecific mtDNA COI sequence divergence between congeneric marine invertebrate species. These comparisons used a variety of distance corrections, depending on what authors judged to be the most appropriate model of molecular evolution. Only a very few studies provided uncorrected sequence divergence. We calculated an average distance, regardless of correction method. Based on 464 comparisons of 101 nominal species of cnidarians, annelids, arthropods, bivalves, and echinoderms, the average interspecific divergence is 0.159 ± 0.002 (data from (Arndt et al. 1996; Hart et al. 1997; O'Foighil et al. 1998; Hellberg 1998; Metz 1998; Bucklin et al. 1999; Lessios et al. 1999; McCartney et al. 2000; Dawson and Jacobs 2001; Hill et al. 2001; Hurtado et al. 2002; Biermann et al. 2003; Landry et al. 2003; Zigler et al. 2003; Holland et al., 2004; Zigler and Lessios 2004). The average intraspecific difference is 0.109 ± 0.01, based on 80 comparisons of 45 nominal species of cnidarians, arthropods, annelids, bivalves, echinoderms and urochordates (van Syoc 1994; Burton 1995; Edmands et al. 1996; Hoeh et al. 1996; Palumbi et al. 1997; Hart et al. 1997; O'Foighil et al. 1998; King et al. 1999; Medina et al. 1999; Lessios et al. 1999; Lee 2000; Tarjuelo et al. 2001; Hill et al. 2001; Holland et al., 2004; Hurtado et al. 2004; Tarjuelo et al. 2004; Glover et al. 2005; Jolly et al. 2005). The average TIM+G corrected distance between Type A and B *C. intestinalis* (0.2, Table 4) is twice as high as the average corrected intraspecific distance for marine invertebrates, and is even higher than the average corrected interspecific distance. Although the intraspecific comparisons from the literature may be biased towards species with substantial differentiation between sites, correcting such a bias would only increase the disparity between *C. intestinalis* and other marine invertebrates. These data highlight the substantial differentiation between Type A and B *C. intestinalis* compared with intraspecific differentiation in other marine invertebrates.

Several other lines of evidence also point to substantial differentiation between Type A and B *C. intestinalis*. Hoshino and Nishikawa (1985) recognized differences between *C. intestinalis* from Naples and Japan (Type A) and the Northeast Atlantic (Type B) in the rate of change with growth of the number of inner longitudinal vessels. In addition, British Type B *C. intestinalis* and Japanese Type A *C. intestinalis* are partially reproductively isolated. Although crosses between British Type B *C. intestinalis* eggs and Japanese Type A *C. intestinalis* showed normal fertilization

rates (as estimated by the frequency of "normal two-cell stage embryos"), the reciprocal cross yielded many fewer fertilized eggs (Suzuki et al. 2005).

Given this genetic, morphological and reproductive evidence, we tentatively advocate the separation of *C. intestinalis* into two species according to the phylogenetic species concept. This cryptic speciation in *C. intestinalis* is consistent with recent studies of other marine taxa in which molecular data uncover cryptic species previously hidden by anthropogenic transport or morphological similarity (Dawson and Jacobs 2001; Holland et al. 2004).

Ciona roulei is not a monophyletic group with respect to mtDNA haplotypes; some *C. roulei* haplotypes are more closely related to Type B *C. intestinalis* haplotypes than to other *C. roulei* haplotypes. Crosses between *C. roulei* and Mediterranean Type A *C. intestinalis* are successful in one direction (Lambert et al. 1990), leading the authors to suggest that *C. roulei* and *C. intestinalis* diverged more recently than *C. intestinalis* and *C. savignyi* (between which hybrids have not been produced). This distinctness of *C. savignyi* is strongly supported by the mitochondrial COI data. The position of *C. roulei* nested within the larger Type B *C. intestinalis* clade suggests that *C. roulei* was recently derived from Type B *C. intestinalis*. Phylogenetic analyses lend support to a hypothesis that Northeast Atlantic Type B *C. intestinalis* individuals moved into the Mediterranean Sea, where they diverged from the ancestral Type B *C. intestinalis*, giving rise to *C. roulei*. Reproductive compatibility between *C. roulei* and Type B *C. intestinalis* has not been investigated; successful crosses between *C. roulei* and Type B *C. intestinalis* would reinforce the Northeast Atlantic origin of *C. roulei*'s ancestors.

The substantial intraspecific divergence in *C. intestinalis*, the placement of *C. roulei* within the Type B *C. intestinalis* clade, and the phylogenetically distinct *Ciona* sp. highlight the evolutionary and taxonomic ambiguities within the genus *Ciona*. Type A and B *C. intestinalis* are 12% divergent at the mitochondrial COI locus, and yet they exhibit partial reproductive compatibility. These two types are sympatric in southern England, and exhibit pre-zygotic reproductive isolation at this site (P. Sordino, personal communication). Similarly, mtDNA COI sequences differ by 12.5% between the sympatric *C. roulei* and Mediterranean Type A *C. intestinalis*, but viable F1 hybrids are produced from one of the two reciprocal crosses. Although they occur along the same coastline, this pair of species may be ecologically isolated. Nothing is known about reproductive isolation between *C. roulei* and Type B *C. intestinalis*. Differences in compatibility between individuals from allopatric and sympatric sites of these two species

will help clarify the evolutionary processes generating reproductive isolation in *Ciona*.

While successful crosses between other pairs of ascidian species have been well documented in the laboratory (Lambert et al. 1981; Jeffery and Swalla 1990), hybrids between species have never been identified in nature. This lack of natural hybridization suggests that pre-zygotic isolating mechanisms may have a large role in the maintenance of reproductive isolation in ascidians. Even so, genetically distinct ascidian species such as *C. intestinalis* and *C. savignyi* have been confused historically (Hoshino and Nishikawa 1985), raising the possibility that hybrids could be morphologically cryptic as well. These uncertainties necessitate further study of species level relationships within *Ciona* in the context of improved understanding of intrinsic barriers to gene exchange.

References

Arndt A, Marquez C, Lambert P, Smith MJ (1996) Molecular phylogeny of eastern Pacific sea cucumbers (Echinodermata: Holothuroidea) based on mitochondrial DNA sequence. Mol Phylogenet Evol 6:425–437

Biermann CH, Kessing BD, Palumbi SR (2003) Phylogeny and development of marine model species: strongylocentrotid sea urchins. Evol Dev 5:360–371

Boffelli D, Weer CV, Weng L, Lewis K, Shoukry MI, Pachter L, Keys DN, Rubin EM (2004) Intraspecies sequence comparisons for annotating genomes. Genome Res 14:2406–2411

Bucklin A, Guarnieri M, Hill RS, Bentley AM, Kaartvedt S (1999) Taxonomic and systematic assessment of planktonic copepods using mitochondrial COI sequence variation and competitive species-specific PCR. Hydrobiologia 401:239–254

Burton R (1995) Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus*. Mar Biol 123:821–827

Byrd J, Lambert CC (2000) Mechanism of the block to hybridization and selfing between the sympatric ascidians *Ciona intestinalis* and *Ciona savignyi*. Mol Reprod Dev 55:109–116

Chabry L (1887) Contribution a l'embrologie normale teratologique des ascidies simples. J Anat Physiol Norm Path 23:167–319

Copello M (1981) *Ciona edwardsi* (Roule, 1886) Espece littorale de Mediterranee distincte de *Ciona intestinalis* (Linne, 1767). Vie Milieu B Oceanog 31:243–253

Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). Biol Bull 200:92–96

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A et al (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298:2157–2167

Edmands S, Moberg PE, Burton RS (1996) Allozyme and mitochondrial DNA evidence of population subdivision in the purple sea urchin *Strongylocentrotus purpuratus*. Mar Biol 126:443–450

Fiala-Medioni A (1974) Ascidians of the rocky benthos of Banyuls-sur-Mer. Faunistic inventory and ecology notes. Vie Milieu B Oceanogr 24:193–207

Gissi C, Pesole G (2003) Transcript mapping and genome annotation of ascidian mtDNA using EST Data. Genome Res 13:2203–2212

Glover AG, Goetze E, Dahlgren TG, Smith CR (2005) Morphology, reproductive biology and genetic structure of the whale-fall and hydrothermal vent specialist, *Bathykurila guaymasensis* Pettibone, 1989 (Annelida: Polynoidae). Mar Ecol P S Z N I 26:223–234

Harant H, Vernieres P (1933) Tuniciers. Faune de France 27:1–93

Hart MW, Byrne M, Smith MJ (1997) Molecular phylogenetic analysis of life history evolution in Asterinid starfish. Evolution 51:1848–1861

Hellberg ME (1998) Sympatric sea shells along the sea's shore: the geography of speciation in the marine gastropod *Tegula*. Evolution 52:1311–1324

Hill RS, Allen LD, Bucklin A (2001) Multiplexed species-specific PCR protocol to discriminate four N. Atlantic *Calanus* species, with an mtCOI gene tree for ten *Calanus* species. Mar Biol 139:279–287

Hoeh WR, Stewart DT, Sutherland BW, Zouros E (1996) Cytochrome *c* oxidase sequence comparisons suggest an unusually high rate of mitochondrial DNA evolution in *Mytilus* (Mollusca: Bivalvia). Mol Biol Evol 13:418–421

Holland BS, Dawson MN, Crow GL, Hofmann DK (2004) Global phylogeography of *Cassiopea* (Scyphozoa: Rhizostomeae): molecular evidence for cryptic species and multiple invasions of the Hawaiian Islands. Mar Biol 145:1119–1128

Hoshino ZI, Nishikawa T (1985) Taxonomic studies of *Ciona intestinalis* (L.) and its allies. Publ Seto Mar Biol Lab 30:61–79

Hurtado LA, Lutz RA, Vrijenhoek RC (2004) Distinct patterns of genetic differentiation among annelids of eastern Pacific hydrothermal vents. Mol Ecol 13:2603–2615

Hurtado LA, Mateos M, Lutz RA, Vrijenhoek RC (2002) Molecular evidence for multiple species of *Oasisia* (Annelida: Siboglinidae) at eastern Pacific hydrothermal vents. Cah Biol Mar 34:377–380

Jeffery WR, Swalla BJ (1990) Anural development in ascidians: evolutionary modification and elimination of the tadpole larva. Sem Dev Biol 1:253–261

Johnson DS, Davidson B, Brown C, Smith W, Sidow A (2004) Noncoding regulatory sequences of *Ciona* exhibit strong correspondence between evolutionary constraint and functional importance. Genome Res 14:2448–2456

Jolly MT, Jollivet D, Gentil F, Thiebaut E, Viard F (2005) Sharp genetic break between Atlantic and English Channel populations of the polychaete *Pectinaria koreni*, along the North coast of France. Heredity 94:23–32

King TL, Eackles MS, Gjetvaj B, Hoeh WR (1999) Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): conservation implications of range discontinuity. Mol Ecol 8:S65

Kott P (1952) The Ascidians of Australia. Aust J Mar Fresh Res 3:206–233

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Lambert G (2001) A global overview of ascidian introductions and their possible impact on the endemic fauna. In: Sawada H, Yokosawa H, Lambert CC (eds) Proceedings of the first international symposium on the biology of ascidians. Springer, Tokyo, pp 249–257

Lambert CC, Lambert G (2003) Persistence and differential distribution of nonindigenous ascidians in harbors of the Southern California Bight. Mar Ecol Prog Ser 259:145–161

Lambert G, Lambert CC, Abbott D (1981) *Corella* species in the American Pacific Northwest: distinction of *C. inflata* Huntsman, 1912 from *C. willmeriana* Herdman 1898 (Ascidiacea, Phlebobranchiata). Can J Zool 59:1493–1504

Lambert CC, LaFargue F, Lambert G (1990) Preliminary note on the genetic isolation of *Ciona* species (Ascidiacea, Urochordata). Vie Milieu B Oceanogr 40:293–295

Landry C, Geyer LB, Arakaki Y, Uehara Y, Palumbi SR (2003) Recent speciation in the Indo-West Pacific: rapid evolution of gamete recognition and sperm morphology in cryptic species of sea urchin. Proc R Soc Lond B 270:1839–1847

Lee CE (2000) Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate 'populations'. Evolution 54:2014–2027

Lessios HA, Kessing BD, Robertson DR, Paulay G (1999) Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. Evolution 53:806–817

Lessios HA, Kessing BD, Pearse JS (2001) Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. Evolution 55:955–975

Linnaeus C (1767) Systema Naturae. Vindobonae: Typis Ioannis Thomae, pp 1767–1770

McCartney MA, Keller G, Lessios GA (2000) Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. Mol Ecol 9:1391–1400

Medina M, Weil E, Szmant AM (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. Marine Biotechnol 1:89–97

Metz E (1998) Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia*. Mol Biol Evol 15:185–195

Minganti A (1948) Interspecific fertilization in ascidians. Nature 161:643–644

Missal K, Rose D, Stadler P (2005) Non-coding RNAs in *Ciona intestinalis*. Bioinformatics 21:i77–i78

Monniot C (1990) Ascidies de Nouvelle-Caledonie. 8. Phlebobranches (suite). B Mus Natl Hist Nat 12:491–515

Monniot C, Monniot F (1977) Tuniciers benthiques profonds du Nord-Est Atlantique. Resultats des campagnes Biogas. B Mus Natl Hist Nat 323:695–720

Monniot C, Monniot F (1983) Ascidies antarctiques et subantarctiques: morphologie et biogeographie. Mem Mus Natn Hist Nat Ser A 125:1–168

Monniot C, Monniot F (1989) Ascidians collected around the Galapagos Islands using the Johnson Sea Link research submersible. P Biol Soc Wash 102:14–32

Morgan TH (1904) Self-fertilization induced by artificial means. J Exp Zool 1:135–178

O'Foighil D, Gaffney PM, Wilbur AE, Hilbish TJ (1998) Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*. Mar Biol 131:497–503

Palumbi SR, Grabowsky G, Duda T, Geyer L, Tachino N (1997) Speciation and population genetic structure in tropical Pacific sea urchins. Evolution 51:1506–1517

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

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

Sanamyan K (1998) Ascidians from the north-western Pacific region. 5. Phlebobranchia. Ophelia 49:97–116

Schneider S, Roessli D, Excoffier L (2000) Arlequin: a software for population genetics data analysis, ver 2.000. Genetics and Biometry Lab, Department of Anthropology, University of Geneva

Sordino P, Heisenberg CP, Cirino P, Toscano A, Giuliano P, Marino R, Pinto MR, De-Santis R (2000) A mutational approach to the study of development of the protochordate *Ciona intestinalis* (Tunicata, Chordata). Sarsia 85:173–176

Suzuki M, Nishikawa T, Bird A (2005) Genomic approaches reveal unexpected genetic divergence within *Ciona intestinalis*. J Mol Evol 61:627–635

Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony and other methods), version 4.0b 10. Sinauer Associates, Sunderland

Tarjuelo I, Posada D, Crandall KA, Pascual M, Turon X (2001) Cryptic species of *Clavelina* (Ascidiacea) in two different habitats: harbours and rocky littoral zones in the northwestern Mediterranean. Mar Biol 139:455–462

Tarjuelo I, Posada D, Crandall KA, Pascual M, Turon X (2004) Phylogeography and speciation of colour morphs in the colonial ascidian *Pseudodistoma crucigaster*. Mol Ecol 13:3125–3136

Van Name W (1945) The north and south American ascidians. B Am Mus Nat Hist 84:1–476

van Syoc RJ (1994) Genetic divergence between subpopulations of the eastern Pacific goose barnacle *Pollicipes elegans*: mitochondrial cytochrome *c* subunit 1 nucleotide sequences. Mol Mar Biol Biotech 3:338–346

Vinson J, Jaffe D, O'Neill K, Karlsson E, Stange-Thomann N, Anderson S, Mesirov J, Satoh N, Satou Y, Nusbaum C, Birren B, Galagan J, Lander E (2005) Assembly of polymorphic genomes: algorithms and application to *Ciona savignyi*. Genome Res 15:1127–1135

Yokobori S-i, Ueda T, Feldmaier-Fuchs G, Paeaebo S, Ueshima R, Kondow A, Nishikawa K, Watanabe K (1999) Complete DNA sequence of the mitochondrial genome of the ascidian *Halocynthia roretzi* (Chordata, Urochordata). Genetics 153:1851–1862

Yokobori S-i, Watanabe Y, Oshima T (2003) Mitochondrial genome of *Ciona savignyi* (Urochordata, Ascidiacea, Enterogona): comparison of gene arrangement and tRNA genes with *Halocynthia roretzi* mitochondrial genome. J Mol Evol 57:574–587

Yokobori S-i, Oshima T, Wada H (2005) Complete nucleotide sequence of the mitochondrial genome of *Doliolum nationalis* with implications for evolution of urochordates. Mol Phylogenet Evol 34:273–283

Zigler KS, Raff EC, Popodi E, Raff RA, Lessios HA (2003) Adaptive evolution of bindin in the genus *Heliocidaris* is correlated with the shift to direct development. Evolution 57:2293–2302

Zigler KS, Lessios HA (2004) Speciation on the coasts of the new world: phylogeography and the evolution of bindin in the sea urchin genus *Lytechinus*. Evolution 58:1225–1241