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Genomic Approaches Reveal Unexpected Genetic Divergence Within *Ciona intestinalis*

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Abstract. The invertebrate chordate *Ciona intestinalis* is a widely used model organism in biological research. Individuals from waters ranging from arctic to temperate are morphologically almost indistinguishable. However, we found significant differences in whole genomic DNA sequence between northern European and Pacific *C. intestinalis*. Intronic and transposon sequences often appear unrelated between these geographic origins and amino acid substitutions in protein coding sequences indicate a divergence time in excess of 20 MYA. This finding suggests the existence of two cryptic species within the present *C. intestinalis* species. We found five marker loci which distinguish the two genetic forms by PCR. This analysis revealed that specimens from Naples, Italy, have the Pacific-type genome, perhaps due to human-mediated marine transport of species. Despite major genomic divergence, the two forms could be hybridized in the laboratory.

Key words: *Ciona intestinalis* — Cryptic species — North European specimens — Pacific specimens — Genetic diversity — Genome evolution

Introduction

Ascidians, or sea squirts, are invertebrate members of the phylum Chordata, which also includes all vertebrates. The ascidian *Ciona intestinalis* is a marine organism whose tadpole-like larval stage gives way to a sessile adult stage adapted for filter feeding. In the field of ecology, *C. intestinalis* has been an important animal to study coastal benthic ecosystems (e.g., Stachowicz et al. 1999). As a basal chordate, its unique evolutionary position has also attracted molecular biologists to use *C. intestinalis* as a model organism for studies of developmental biology (Satoh and Jeffery 1995; Corbo, Di Gregorio and Levine 2001; Satoh 2003). Its status as a favored model organism in biological research led to the sequencing of the *C. intestinalis* genome, which is about 17 times smaller than the human genome. It was estimated by genomic DNA sampling that the total gene number in *C. intestinalis* was approximately 16,000 (Simmen et al. 1998) and this was broadly confirmed by the draft genomic sequence of *C. intestinalis* (Dehal et al. 2002). In addition, analysis of the distinctive methylation patterns of 120 kb of *C. intestinalis* cosmids has been initiated (Simmen et al. 1999) and there have been large-scale EST analyses of developmental stages and adult tissues (Satoh et al. 2003).

Linné (1767) originally defined the species resident in European coastal waters as *Ascidia intestinalis*. Subsequently, *C. intestinalis* (Linnaeus 1767), as it was renamed, was found, based on its distinctive morphological features, to be represented in virtually all the seas of the world (see Hoshino and Nishikawa 1985). *C. intestinalis* has been regarded as a single cosmopolitan species, and the animals from different places have been used for biological studies. Within the *C. intestinalis* species, there is evident DNA se-

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quence polymorphism. The number of repeats in microsatellite sequences, which behave as selectively neutral rapidly evolving markers, has shown genetic variability within and between three local populations from the Southern Tyrrhenian coasts of Italy (Pricaccini et al. 2000). A significant level of allelic polymorphism (up to 1.2%) has also been observed in the whole-genome sequences of a single Californian individual, perhaps reflecting the enormous effective population size of the species (Dehal et al. 2002). These moderate levels of divergence were eclipsed, however, when the draft genome of Californian *C. intestinalis* showed major differences from the sequence of three cosmids from British *C. intestinalis* (Simmen et al. 1999). Here we have investigated the basis of this difference. We find that there are indeed extensive DNA sequence differences in the species *C. intestinalis*. In spite of these differences, *C. intestinalis* from the West coast of Scotland can hybridize with the Pacific form to generate apparently normal offspring in the laboratory. However, relatively inefficient fertilization was observed using a combination of Japanese eggs and British sperm.

Materials and Methods

Overall Identity Between Genomes

DNA Sequence Data. For the sequence comparison between British and Californian *C. intestinalis* genomes, we used 120 kb of British cosmid and Californian whole-genomic sequence. The three Cosmids of British *C. intestinalis*, cicos2, cicos46 and cicos41, were originally sequenced by Simmen et al. (1998, 1999) from specimens collected in Plymouth, UK. Their best hit sequences were obtained as orthologous sequence through a Blast search from the Californian *C. intestinalis* genome draft: http://genome.jgi-psf.org/ciona4/ciona4.home.html. For the sequence comparison between Japanese and Californian sequence, we used randomly selected Japanese *C. intestinalis* shotgun data because the sequence corresponding to the British cosmid is not available from the unassembled Japanese shotgun library, NIG1 (Dehal et al. 2002). The 70 shotgun sequences of ~600 bp which were used for the sequence comparison in Fig. 1D are available at http://mwsross.bms.ed.ac.uk/public/Ciona/shotgun.html. Their best-hit Californian sequences were also obtained from the Californian *C. intestinalis* genome draft through a Blast search using the shotgun sequences as query.

Percentage Identity Plots and Alignments. Dynamic percentage identity plots showing Californian and British genomic sequence divergence were made by zPicture (http://zpicture.dcode.org/) with a default setting. ClustalX 1.83 (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) was used to align British and Californian sequence in Fig.1 E and calculate percentage identity between Japanese shotgun data and the best-hit Californian sequences.

Comparison of Gene Coding Sequences. For gene coding region comparisons, 24 alignments of nucleotide sequences were made using the orthologous British and Japanese cDNAs (Table 1). ORF regions were extracted from the British ESTs, which were randomly cloned and sequenced from adult *C. intestinalis* by Simmen et al. (1998). Their best-hit sequences were obtained by Blast search from the Japanese *C. intestinalis* cDNA database which includes almost all of the ESTs (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). After the ClustalX nucleotide alignment, gap-containing regions were eliminated from the alignment as unreliable, because the British ESTs were single reads and may possess slipped nucleotide readings, particularly near their ends. Amino acid sequence alignments were conducted with sequences translated from those used in the nucleotide comparison. Nucleotide N and amino acid X were excluded from alignments. The degree of nucleotide substitution was calculated using K-ESTIMATOR 6.0 (http://www.biology.uiowa.edu/comeron/).

Phylogenetic Reconstruction

Amino Acid Sequence Data. In order to infer the phylogenetic relationship between northern European and Pacific origins as well as to calculate their divergence time, a phylogenetic tree was constructed. Amino acid sequences of 13 genes (see Table 1) from British, Japanese, and Californian *C. intestinalis*, and of their best-hit relative genes in *C. savignyi*, *Drosophila melanogaster*, *Anopheles gambiae*, human, and *mus musculus* were concatenated and used for the tree construction. We included *C. savignyi* Herdman 1882 in the analysis, as this organism is also widespread in shallow coastal waters, though not in northern European seas. Both species occur in Californian and Japanese coastal waters (Hoshino and Nishikawa 1985). Recently, a 14-fold coverage of the *C. savignyi* genome has been completed and the resulting sequences are available. The amino acid sequences of the 13 genes were obtained from the following databases: *C. savignyi* genome draft (http://www-genome.wi.mit.edu/annotation/ciona/), the insect genomes (http://www.ncbi.nlm.nih.gov/BLAST/Genome/Insects.html), and the NCBI protein nr database (http://www,ncbi.nlm.nih.gov/). The aligned amino acid sequences are available at http://mwsross.bms.ed.ac.uk/public/Ciona/aa.html

Phylogenetic Reconstruction Method. Based on the aligned amino acid sequences, we determined the phylogenetic relationships among Californian and British *C. intestinalis*, using mouse, human, mosquito, and *Drosophila* as outliers. ProML (Phylip ver. 3.6) was used to calculate the distances and construct a tree by the maximum likelihood method (http://evolution.genetics.washington.edu/phylip.html).

Comparison of European and Pacific Samples by PCR Markers

Specimens were obtained from the following locations: Oban, Scotland; Plymouth, England; Naples, Italy; Fiskebackskil, Sweden; and Kyoto, Japan. Genomic DNA was extracted from seven individuals from each location with TRIzol reagent (GibcoBRL) and used as a PCR template. PCR was carried out in 50-μl reaction volumes for 35 cycles with fast start Taq DNA polymerase in accordance with the manufacturer's instructions (Roche). Primers used to amplify molecular markers were derived from British cosmid sequences (cicos 46 and cicos 41): marker 1, exon 1 to exon 2 of C46.3, 5′-TGTATTTACTATTTTCAACG and 5′-CTCCAC TGCTAGCAACTGC; marker 2, exon 4 to exon 5 of C41.2, 5′-GTGTCTTAGAAGAGGAGAAT and 5′-CTTTGTCCACTGA GAGCACT; marker 3, exon 1 to exon 2 of C41.5, 5′-AGCTTCCA TGCTCTTTTCCA and 5′-GGACACACCGAGGACAAAGT; marker 4, C41.1 to C41.8, 5′-GCTTTGCTATGGAGGACCAG and 5′-TCAAGAAGCGCTCCTAGCTC; and marker 5, 5′-TAGT TTAGTGCGACGGTTAG and 5′-GGTCACACCACCCGTAT

Table 1. Comparison of gene coding sequences

British cDNA				Nucleotide					AA
				Length	Identity				Identity
5′	3′	Japanese cDNA	Best hit human gene name	(bp)	(%)	K_S	K_A	K_A/K_S	(%)
*AJ227619		CLSTR00675	Hypothetical protein FLJ35834	240	92.5	0.3704	0	0	100
*AJ227620	AJ627621	CLSTR03031	ATP synthase β-subunit	447	97.1	0.1342	0	0	100
*AJ227622	AJ627623	CLSTR04145	Karyopherin α 2	433	95.3	0.1138	0.0126	0.1103	98.0
*AJ227625		CLSTR02107	Ribosomal protein L24-like	353	96.9	0.1347	0.0034	0.0249	100
*AJ227626	AJ227627	CLSTR00060	Guanine nucleotide binding protein	535	95.2	0.1675	0.0083	0.0494	99.4
AJ227628		CLSTR05527	Proteasome 26S ATPase subunit 1	292	94.5	0.2498	0	0	100
AJ227630		CLSTR00326	No hit	270	97.8	0.0628	0	0	100
*AJ227633	AJ227634	CLSTR01271	CDC6 cell division cycle 6 homolog	549	94.2	0.2365	0.0137	0.0577	97.8
*AJ227635		CLSTR05254	Flotillin 1	241	95.0	0.2595	0	0	100
AJ227637		CLSTR03609	Alkylated DNA repair protein alkB	278	91.7	0.3836	0.0044	0.0114	98.9
*AJ227639		CLSTR05476	Hypothetical protein FLJ22313	357	96.6	0.0937	0.0103	0.1100	97.5
*AJ227640		CLSTR03116	ATP synthase coupling factor 6	339	96.5	0.1282	0.0072	0.0558	98.2
AJ227643		CLSTR12115	NEDD8-conjugating enzyme	221	94.6	0.1924	0.0179	0.0931	94.5
*AJ227644		CLSTR00094	Aldolase B, fructose-bisphosphate	287	92.7	0.2440	0.0253	0.1036	96.8
*AJ227651		CLSTR05824	Unnamed protein product	321	95.0	0.2185	0.0143	0.0655	98.1
AJ227652		CLSTR00555	Ribosomal protein L11	251	99.2	0.0360	0	0	100
AJ227653		CLSTR00396	ATP synthase α-subunit	376	97.6	0.1061	0	0	100
*AJ227657		CLSTR02447	Phosphoserine aminotransferase	256	95.0	0.1818	0.0102	0.0559	97.7
AJ227661	AJ227660	CLSTR05103	No hit	514	93.6	0.2880	0.0107	0.0371	97.7
AJ227662		CLSTR31898	No hit	369	95.9	0.0784	0.0284	0.3627	93.4
AJ227665		CLSTR00178	No hit	265	90.6	0.5342	0.0047	0.0087	98.9
AJ227666		CLSTR00046	β cytoskeletal actin	210	99.1	0	0.0074	NA	98.6
AJ227667		CLSTR01137	No hit	391	93.9	0.2221	0.0142	0.0638	97.4
*AJ227669		CLSTR00730	Ribosomal protein L10	324	94.1	0.2419	0.0109	0.0450	98.1
Average					95.2				98.4

Note. AA, amino acid; NA, not applicable for calculation.
*Sequences used for phylogenetic analysis in Fig. 3.

CTG. Amplified PCR markers were cloned and sequenced to make sure they are the specific products. Molecular markers 3, 4, and 5 were digested with 0.1 U of restriction enzymes, *Nco*I, *Xba*I, and *Hpa*II (New England Biolabs), respectivezly.

Cross-Breeding Experiments

We used British specimens collected in Oban, Scotland, which have the northern European-type genome (see Results), in addition to showing more than 99% identity to the British cosmid sequence originating from specimens obtained from Plymouth (M. Suzuki, unpublished). British and Japanese *C. intestinalis* adults were held at 13° and 18°C, respectively, with light until full maturation of gametes. Eggs and sperm were removed from the gonoducts by dissection and the eggs from each individual were washed four times in 15 ml of filtered seawater. Sperm collected from five individuals from one geographic origin were mixed and diluted with filtered seawater just before use. Fertilizations were performed at 18°C using heterologous and homologous inseminations with eggs from one individual and the sperm mixture. By in vitro fertilization, we could obtain synchronized cleaving eggs. The first cleavage normally happened 60 min after the fertlization at 18°C. All of the eggs which had not cleaved by this time failed to reach the two-cell stage later, indicating that they were unfertilized eggs. We assessed the first cleavage frequency at 60 min as fertilization efficiency. Embryos were kept at 18°C in filtered seawater and development was observed. After metamorphosis, animals were fed Brine shrimp food (NT laboratories, UK) daily and reared at 13°C for 1 month.

Results

Comparative Overview of Genetic Diversity Between British and Pacific C. intestinalis

To compare large genomic sequences and derive an overview of genetic diversity within *C. intestinalis*, three well-studied datasets of genomic sequence were used: British, Californian and Japanese. Regions of the Californian genome that corresponded to the sequences of British cosmids, cicos2, cicos46, and cicos 41, were found in scaffolds 6:234856–196887, 3:559408–599636, and 140:127601–10056 of the Californian dataset, respectively. These related nucleotide sequences were uninterrupted by unrelated sequence, indicating no major structural rearrangements between the two genomes in these regions. Comparison of the nucleotide sequence divergence by percentage identity plots (Figs. 1A–C) showed more than 90% identity in some regions, but most of the sequence fell between 50 and 90% identity and some showed no obvious relationship. In marked contrast, sequence divergence between Californian and Japanese genomes was low. All of the randomly selected Japanese unassembled shot-

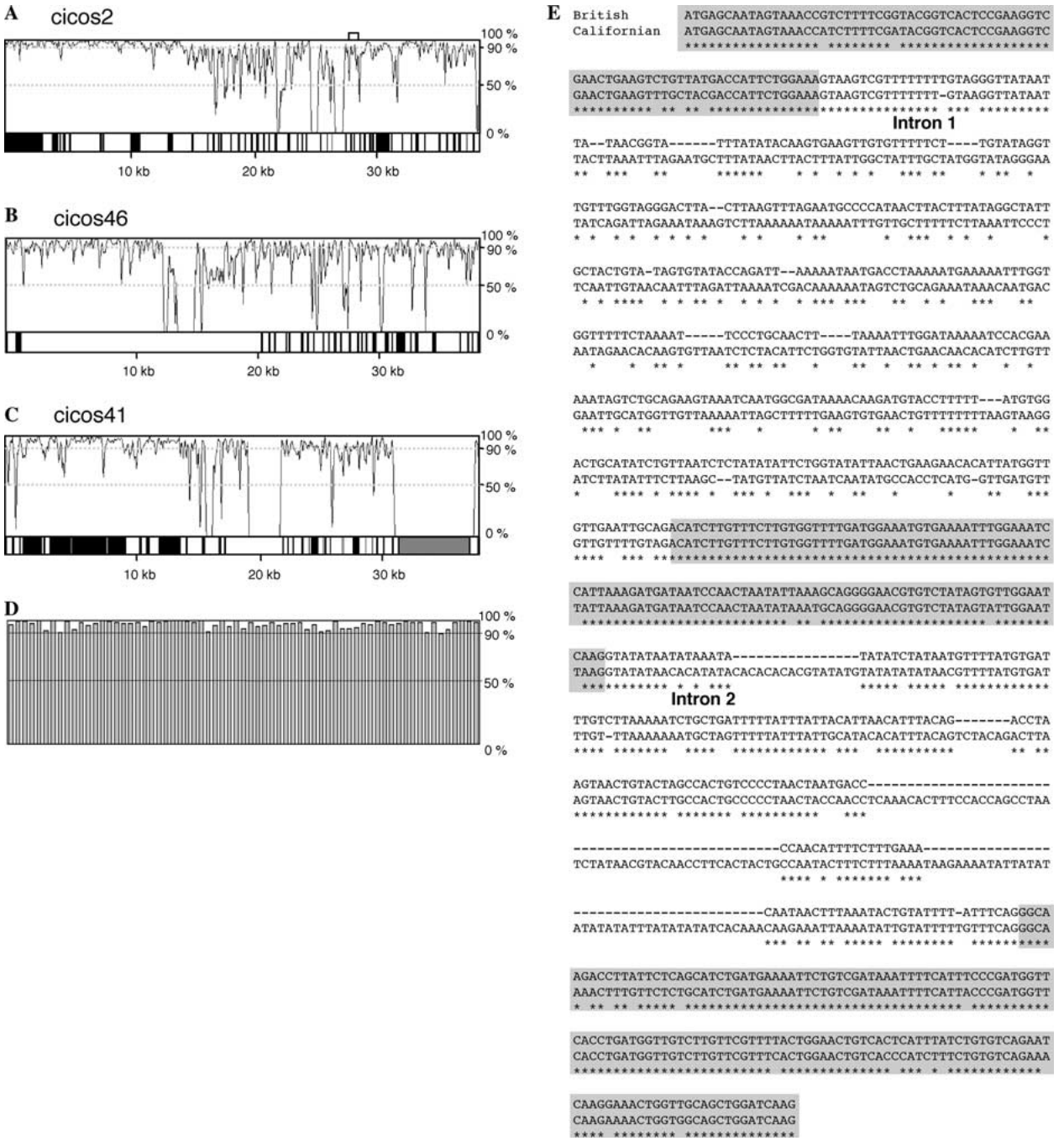

Fig. 1. Genetical diversity between British and Pacific *C. intestinalis* as shown by a percent identity plot. **A, B** British cosmid sequences are on the *x*-axis. Nucleotide sequence identity over a sliding 19-bp window is shown on the *y*-axis. Predicted coding regions are shown by black boxes under the bars. Transposon *Cigr-1* is shown as the gray box in **C**. **D** Moderate levels of DNA sequence diversity of shotgun genomic sequence between Japanese and Californian *C. intestinalis*. Bars show sequence identity of randomly selected 600-bp shotgun sequences. **E** Example of ClustalX alignment of the exon and intron region. The corresponding region is indicated by the bracket in **A**. Exon regions are shaded.

gun sequences, representing 42 kb in total, showed 90–100% identity to the Californian sequence (Fig. 1D).

Predicted gene coding regions were always conserved more than 90% between British and Pacific *C. intestinalis* (Figs. 1A–C), whereas non-protein coding regions were highly divergent. In particular, intronic regions often showed no significant relationship, although 15 to 30 bp of intronic sequence near exon boundaries remained conserved (Fig. 1E). In addition to nucleotide substitutions, as seen in intron 1 of the C2.3 gene (Fig. 1E), divergence was often caused by insertions and deletions of DNA leading to frequent gaps in the alignments (e.g.,

Fig. 1E; intron 2). In the analyzed 120-kb region, gap frequencies were similar in British and Pacific *C. intestinalis*. A region of more than 9 kb in cicos41 that does not show any apparent sequence identity (Fig. 1C) is due to the presence of the Gypsy Ty3 retrotransposon *Cigr-1* (Simmen et al. 1999; Simmen and Bird 2000) in British but not Californian *C. intestinalis*.

It was previously shown that there are about 75 copies of *Cigr-1* in the British genome (Simmen et al. 1999). Restriction digests indicated that the element was homogeneous and transpositionally active, as individuals from the same location showed evidence of variable positions of the elements within their genomes. We asked whether a *Cigr-1*-like element was also present in the Californian *C. intestinalis* genome draft. The results of a Blast search using *Cigr-1* as query detected some sequences related to the extremities (*Cigr-1* long terminal repeats; E-value, $<1e{-}10$) in the Californian genome. The major internal domain of *Cigr-1*, however, did not show any alignment with the Californian genome (E-value, >0.08). We conclude that Pacific forms of *C. intestinalis* do not contain an obvious equivalent element to *Cigr-1*. This strongly supports the view that the two forms have been reproductively isolated for an evolutionarily significant period of time.

Comparison of Gene Coding Sequences

We next analyzed the degree of conservation of coding sequences between the two geographic origins. Randomly chosen ESTs corresponding to 24 genes of the British form found their orthologous counterparts in the Japanese cDNA database (Table 1). Alignments showed a mean identity at the nucleotide level of 95.2% with a standard deviation of 2.2%. This was consistent with the identity plot, which showed more than 90% sequence identity in exon regions. Nucleotide substitutions in the protein coding regions were divided into two classes: those that changed the coding amino acid (nonsynonymous) and those that did not (synonymous). Normalized values of K_A, the number of nonsynonymous substitutions per nonsynonymous site, and K_S, the number of synonymous substitutions per synonymous site, were calculated and are listed in Table 1. K_S reflects the opportunity to change nucleotides independently in the two populations in the absence of selection. The ratio K_A/K_S has been found to be significantly less than 1 for genes that have experienced purifying selection (Kimura 1983). In this set of genes, all had K_A/K_S values $<<1$, indicating strong selective pressure on the gene coding sequences, which is likely to be the reason for the relatively high conservation. As expected, sequence identity was higher (98.4% on average) when the nucleotide sequences were conceptually translated into amino acids.

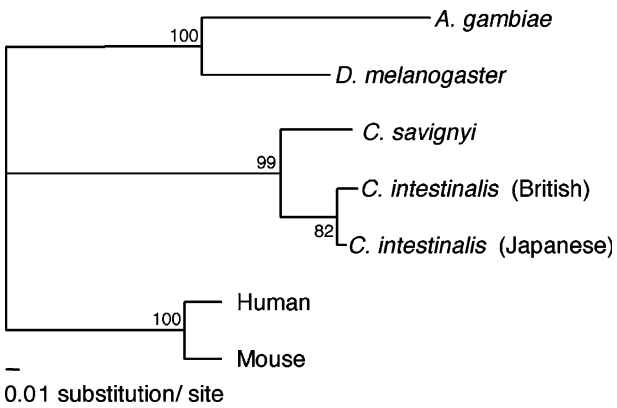

Fig. 2. Phylogenetic relationships of the *Ciona* genus inferred from a maximum-likelihood analysis using 1,051 amino acid residues. Local bootstrap confidence values are shown on the branches. The horizontal branch lengths are proportional to the extimated number of amino acid substitutions per site. This tree is rooted using *D. melanogaster* and *A. gambiae*.

Phylogenetic relationship within C. intestinalis

Two possible explanations could be considered to account for the difference between the draft genome of Californian *C. intestinalis* and the cosmids from British *C. intestinalis* . Either there is unprecedented DNA sequence divergence between British and Pacific origins or the cosmid DNA was derived from a species other than *C. intestinalis*. To clear up this point, a molecular phylogenetic tree was constructed from 1051 amino acids of well-aligned concatenated sequence from 13 genes (Fig. 2). The tree supports a close relationship between British and Californian *C. intestinalis* with a high bootstrap value (82%). The Japanese and Californian subgroups of *C. intestinalis* did not branch from one another because only one amino acid difference was detected in the aligned sequences. British and Californian forms are in the same clade, but not grouped with the other *Ciona* species, *C. savignyi*. From these results, we conclude that British and Pacific specimens are both *C. intestinalis* rather than *C. savignyi*, and the major differences in their genomic sequences have arisen within the species.

Genetic Species Diagnosis by PCR Marker

The widely different intron length between the two *C. intestinalis* forms, as shown in Fig. 1E, suggested that a PCR assay could rapidly assign individuals to one or other group. Primer pairs were designed in exon sequences to amplify the intervening intron or intergenic region. Most of the primer pairs that we tested on British and Japanese DNA samples ob-

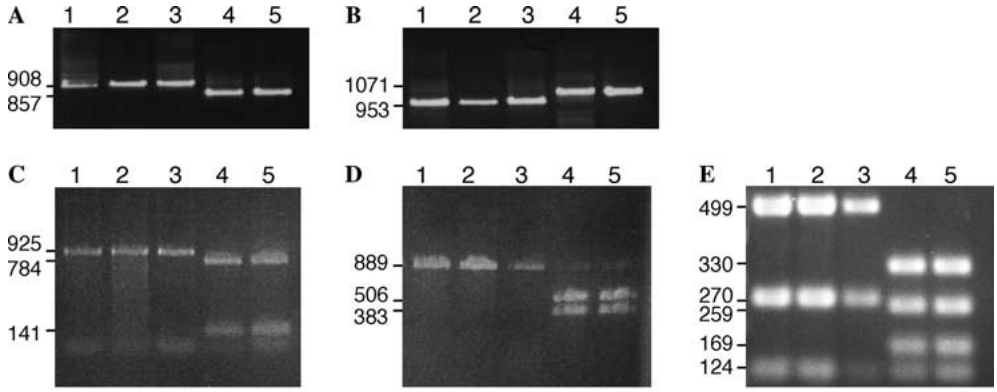

Fig. 3. Genetic typing of *C. intestinalis* populations by diagnostic molecular markers. PCR markers tested against five populations: 1, Scotland; 2, South England; 3, Sweden; 4, Japan; and 5, Italy. Numbers show lengths of amplified markers, in base pairs. **A** Marker 1. **B** Marker 2. **C** Marker 3 PCR products digested with *Nco*I. **D** Marker 4 digested with *Xba*I. **E** Marker 5 PCR digested with *Hpa*II.

tained from seven individuals from each location amplified multiple bands, presumably because of the polymorphism within each geographic origins (e.g., Fig. 3A, lane 1) Two primer pairs, however (marker 1 and marker 2), identified two loci of low polymorphism and were tested against DNA samples from Scotland, southern England, Sweden, Japan, and Italy. From this analysis, we could assign *C. intestinalis* from Japan, California, and Naples to the same group, as they exhibited alleles for markers 1 and 2 that were predicted by the Californian genome sequence (Figs. 3A and B). The other alleles for these two markers were found in Scottish, English, and Swedish samples (Figs. 3A and B). We also found that markers 3, 4, and 5 are able to discriminate based on the length of DNA fragments after restriction enzyme digestion (Figs. 3C and D). These results indicate that the northern European animals represent one isolated form of *C. intestinalis*, whereas the Pacific populations belong to a genetically distinct group. The genomic samples obtained from Naples showed that they are Pacific-type for all of five markers, although their location is within Europe.

Reciprocal Crosses Between the British and Japanese C. intestinalis *Yielded F_1 Offspring*

Are the two genetically distinct forms of *C. intestinalis* reproductively separated? To examine this question, we attempted to generate hybrids between them using gametes of Japanese and British origin. Homologous controls with eggs from five individuals of each population gave nearly 100% efficiency of fertilization (Table 2). Heterologous crosses between British eggs and Japanese sperm also gave a very high frequency of normal two cell–stage embryos. Many viable embryos were again produced from Japanese eggs and British sperm, but fertilization was considerably less efficient (Table 2). Hybrid embryos from each reciprocal cross developed into apparently normal tadpoles and grew to give young adults in our laboratory (Fig. 4).

Table 2. Efficiency of reciprocal cross fertilization

	Number of eggs		
Egg–sperm	Total	Fertilized	%
British–British	81	80	98.8
	43	41	95.3
	42	42	100
	86	86	100
	68	65	95.6
Japanese–Japanese	29	28	96.6
	39	38	97.4
	34	24	70.6
	47	45	95.7
	16	16	100
British–Japanese	52	52	100
	32	31	96.9
	48	48	100
	41	41	100
	29	29	100
Japanese–British	53	35	66.0
	27	19	70.4
	57	71	2.3
	47	91	9.1
	45	20	44.4

Discussion

The genus *Ciona* was long considered to comprise only the species *C. intestinalis* as a cosmopolitan shallow-water species but has been found to include more than 10 valid species, many of which are morphologically very similar. Hoshino and Nishikawa (1985) recognized two species among specimens referred to as *C. intestinalis* collected from arctic and warm temperate waters of the world. Morphological differences in the presence of an endostylar append-

Fig. 4. Homologous and heterologous hybrids of Scottish and Japanese *C. intestinalis* grown to adults. **A** Homologous Japanese hybrid. **B** Heterologous hybrid between a Scottish egg and Japanese sperm. **C** Heterologous hybrid between a Japanese egg and Scottish sperm.

age and the ventral location of the pharygeo-epicardiac openings were identified as diagnostic features that distinguished *C. intestinalis* from *C. savignyi*. Besides their sympatry, attempts to generate hybrids between *C. intestinalis* and *C. savignyi* were unsuccessful, indicating that they are distinct species (Lambert et al. 1990). In this study, we show a close phylogenetic relationship between British and Pacific specimens of *C. intestinalis*, and we establish that neither form is closely related to *C. savigny*. Despite the close phylogenetic relationship and absence of the morphological differences between British and Pacific forms, significant genetic diversity was apparent. This suggests that there are at least two unrecognized cryptic species that possibly arose by allopatric speciation within *C. intestinalis*.

We attempted to estimate the length of time that these cryptic species have been separated by comparing the degree of amino acid sequence divergence with that of other animal lineages. Based on a divergence time between the insects *D. melanogaster* and *A. gambiae* of approximately 235 MYA (see Gaunt and Miles 2002) and assuming no gene flow between the two groups, the divergence time between British and Pacific genomes from amino acid substitutions is estimated to be 37 MYA (Fig. 2). This could be an overestimate if the molecular clock of *C. intestinalis* runs faster than average, as suggested by the longer branch length compared to the other animals (e.g., Leveugle et al. 2004). We also estimated evolutionary distance by comparison with the divergence time between humans and *M. musculus* of 81.5 MYA (Tavere et al. 2002). The decreased rate of molecular evolution in vertebrates, which has been reported previously (Peterson et al. 2004; see Fig. 2), made it impossible to compare mammalian and urochordate molecular data directly. We therefore made the simplifying assumption that mammals and urochordates have had different but constant rates of molecular evolution since their separation. In this case, the divergence time between urochordates and ancestral vertebrates would be 486 MYA, which is consistent with the consensus that the phylum chordata arose more than 550 MYA (Dehal et al. 2002). The divergence time between Pacific and European *C. intestinalis* would now be 28 MYA. Unfortunately, it is impossible to test the accuracy of these estimates by paleontology because of the poor tunicate fossil record. Although the estimates of 28 MYA and 37 MYA are relatively similar, uncertainty concerning the absolute rates of molecular evolution within *C. intestinalis* leaves the true divergence time in doubt. In the unlikely event that our minimum estimate of 28 MYA is twofold greater than the true value, which we consider very unlikely, 14 MYA would still represent a remarkable degree of genetic separation within what has hitherto been classified as a single species.

Surprisingly, in view of their extensive genomic divergence and ancient isolation from one another, the British and Pacific forms could be hybridized in the laboratory. To our knowledge, there is no other example of successful hybridization between forms that are so divergent at the genomic level. This result

raises the possibility that these are not distinct species according to the biological species concept but, rather, can be considered subspecies. The result suggests that *C. intestinalis* may be an organism with a low reproductive barrier to fertilization. The idea that a single species can harbor extreme genetic divergence yet retain the ability to hybridise challenges the conventional concept of species as genetically homogeneous interbreeding groups of organisms. An alternative view is that the Pacific and British forms represent different species whose divergent genomes may be able to collaborate because the morphological end result of their developmental programmes is so similar.

Although Pacific and British *C. intestinalis* hybridized readily in the laboratory, weak reproductive barriers could be discerned that may be relevant in the wild. The percentage of eggs fertilized in reciprocal crosses between eggs from the Japanese specimens and sperm from the British ones was significantly lower than that seen when sperm and eggs came from the same form, which typically gave over 95% fertilization. Crosses between British eggs and Japanese sperm were as efficient as within-form crosses. In addition, a likely premating barrier to interbreeding of the two forms was seen in the laboratory. Unlike Japanese *C. intestinalis*, British *C. intestinalis* embryos did not grow well at 18°C, prefering a water temperature of 13°C. The requirement for different habitats may be a significant contributor to the isolation of these two forms. In the future, it will be necessary to study how these two subspecies are distributed worldwide. An important consideration is human-mediated transport of marine specimens by vessels. The phenomenon is known to have affected the geographical distribution of other marine species (e.g., Carlton 1992; Geller 1997) including ascidians (Eno et al. 1997). PCR marker analysis showed that the specimens from Naples are members of the Pacific subspecies. Because the Mediterranean sea is known to be one of the most heavily invaded (Galil 2000), this form might have been introduced recently from the Pacific area. Alternatively, Pacific forms might have spread from a Mediterranean origin. The observation that the Pacific and Napolitan specimens have less polymorphism at the nucleotide level than the North European group, regardless of their broad geographic distribution (data not shown), argues that they have become distributed recently.

C. intestinalis is a favored model organism for studies of embryology, physiology, evolutionary developmental biology, ecology, and other disciplines. The finding of extensive genetic divergence between northern European and Mediterranean/Pacific *C. intestinalis* therefore has significant practical implications. Comparative genomics is facilitated by the high levels of divergence between intergenic DNA sequences of the two forms. For example, the recognition of conserved motifs will aid definition of regulatory regions of the genome. Annotation of genes will also be assisted by use of comparative DNA sequence data. Another potential advantage concerns *C. intestinalis* genetics, which would benefit from hybrids of the two forms of *C. intestinalis*. Previous attempts to construct inbred lines of Japanese *C. intestinalis* have foundered, apparently due to homozygosity of deleterious alleles caused by repeated backcrossing (Kano et al. 2001). In this study we show that hybridization between the two populations yields viable animals. The extra degree of polymorphism may therefore allow backcrossing of the two forms to generate individuals that can be used as a resource for gene mapping in this organism.

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