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SHORT COMMUNICATION

Preliminary Analysis of Length and GC Content Variation in the Ribosomal First Internal Transcribed Spacer (ITS1) of Marine Animals

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Abstract Length and guanine–cytosine (GC) content of the ribosomal first internal transcribed spacer (ITS1) were compared across a wide variety of marine animal species, and its phylogenetic utility was investigated. From a total of 773 individuals representing 599 species, we only failed to amplify the ITS1 sequence from 87 individuals by polymerase chain reaction with universal ITS1 primers. No species was found to have an ITS1 region shorter than 100 bp. In general, the ITS1 sequences of vertebrates were longer (318 to 2,318 bp) and richer in GC content (56.8% to 78%) than those of invertebrates (117 to 1,613 bp and 35.8% to 71.3%, respectively). Specifically, gelatinous animals (Cnidaria and Ctenophora) were observed to have short ITS1 sequences (118 to 422 bp) with lower GC content (35.8% to 61.7%) than the other animal taxa. Mollusca and Crustacea were diverse groups with respect to ITS1 length, ranging from 108 to 1,118 and 182 to 1,613 bp, respectively. No universal relationship between length and GC content was observed. Our data indicated that ITS1 has a limited utility for phylogenetic analysis as obtaining confident sequence alignment was often impossible between different genera of the same family and even between congeneric species.

Keywords GC content · ITS1 · Length · Marine animals · Phylogenetic utility

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Introduction

Due to its noncoding structure and easy isolation by polymerase chain reaction (PCR), the first internal transcribed spacer (ITS1) of nuclear ribosomal DNA has become a popular element for molecular systematics studies in a wide variety of eukaryote taxa. Another advantage of using ITS1 is the rapid evolution and homogenizing forces of concerted evolution and molecular drive (Dover 1986), which are believed to act to magnify differences between species and to minimize the degree of intraspecific variation, respectively. However, the ITS1 database coverage of marine animals is far more incomplete than it is for fungi, the most extensively sequenced taxon for ITS1 (Croce et al. 2006). Therefore, querying the ITS1 database with nucleotide sequences isolated from unknown animal sources often fails to reveal any matches (Takeyama, personal communication). Furthermore, the phylogenetic utility of ITS sequences for higher taxa has not been completely investigated (Chu et al. 2001; Coleman and Vacquier 2004). We attempted to (1) amplify the ITS1 regions of a wide variety of marine animals and determined the partial or entire nucleotide sequences in order to expand the information in the ITS1 database, (2) compare the ITS1 nucleotide sequences between taxa, and (3) investigate the phylogenetic utility of ITS1 sequences for the classification of marine animals.

Materials and Methods

Extracted DNA, tissue, and whole body samples of a wide variety of marine animals (Annelida, Arthropoda, Chaetognatha, Chordata, Cnidaria, Ctenophora, Echinodermata, and Mollusca) were donated by the research personnel of the Fisheries Research Agency, Japan. Sequences of the universal primer pair, ITS1 and 5.8S, used to amplify the ITS1 region were obtained from Duke University (http://www.biology.duke.edu/fungi/mycolab/primers.htm). The forward primer [(ITS1) 5′-TCCGTAGGTGAACCTGCGG-3′] was designed to anneal near the 3′ end of 18S rDNA, and the reverse primer [(5.8S) 5′-CGCTGCGTTCTTCATCG-3′] was designed to anneal near the 5′ end of 5.8S rDNA. Usually, strong amplification of a single fragment was observed using this pair of primers under the following PCR conditions: 30 cycles of denaturation at 96°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min with LA *Taq* DNA polymerase (Takara, Japan). In cases where multiple PCR products were obtained (probably due to ITS paralogs or nonspecific amplification), the species or individuals were excluded from further analysis. Direct nucleotide sequence analysis was performed for the PCR products using an ABI Big-dye Ready Reaction kit (Perkin-Elmer Cetus, Norfolk, VA, USA). Occasionally, primer walking was performed to determine longer sequences and/or PCR products were cloned to obtain better resolution. The boundaries between 18S rDNA and ITS1 and between ITS1 and 5.8S rDNA were determined according to Hsueh et al. (2001) and Gottschling and Plötner (2004), respectively. Since full-length sequences may be preferentially determined for shorter fragments, comparisons of ITS1 lengths between taxa using full-length sequence data may have potential bias. Therefore, fragment lengths estimated by agarose gel electrophoresis were also incorporated after subtracting the number of nucleotides (82 nucleotides: from the forward priming site to the 3′ end of 18S rDNA and from the reverse priming site to the 5′ end of 5.8S rDNA) from the gel-estimated fragment lengths. Guanine–cytosine (GC) content was calculated for both partial- and full-stretch sequences. When several individuals per species were analyzed, the averages of ITS1 length and GC content were used as representative estimates for the species. Length (in base pairs) and GC (in percent) data were natural log transformed and subjected to analysis of variance; a Tukey test was performed to test significant differences between taxa. Alignment of the nucleotide sequences was done with the ClustalW algorithm and calculation of unweighted percent nucleotide differences between sequences, including gaps as substitution, were performed using Lasergene (DNA STAR). A single sequence per species was arbitrarily selected for comparison between species.

Results and Discussion

A total of 773 individual samples derived from 599 animal species were subjected to PCR amplification of ITS1 (Table 1). Of those, we failed to generate a PCR product in only 87 individuals, indicating the overall universal utility of the ITS1-specific primer pair used. Failure of amplification was usually individual- or species-related, which was probably due to the quality of template DNA or variation in priming site sequence, respectively. However, some differences in the efficiency of PCR amplification may exist among higher taxa since no ITS1 amplification was achieved in any of the five mammal (cetacean) and two reptile (marine turtle) species; a relatively high proportion of individuals from Chondrichthyes, Echinodermata, Cephalopoda, Gastropoda, and Cnidaria failed to generate a PCR product. We determined entire or partial ITS1 sequences of 316 individuals from 272 species, including full-length sequences from 232 individuals of 185 species. Adding these to the ITS1 sequences that already existed in the database for marine animal species resulted in a data set comprising 468 sequences from 424 species, 354 sequences of which are full-length (Table 2). Furthermore, the ITS1 length data of an additional 367 individuals from 338 species estimated from gel electrophoresis (available at http://fsf.fra.affrc.go.jp/chow/ITSlength.htm) were incorporated into the length comparison between taxa. Table 3 summarizes the length and GC content of ITS1 among major taxa. Sharks and rays (Chondrichthyes) were observed to have the longest ITS1 sequence (average 1,494.3 bp). Specifically, among 23 Chondrichthyes species, three (*Bathyraja abyssicola*, *Hydrolagus barbouri*, and *Urolophus aurantiacus*) had a 878 to 1,004 bp ITS1 while all others were longer than 1,200 bp and the maximum ITS1 length (2,318 bp) was observed in the oceanic whitetip shark (*Carcharhinus longimanus*). ITS1 length of bony fishes (Osteichthyes) varied widely (318 to 1,518 bp), but the average (635.1 bp) was significantly larger than invertebrates. Thus, in general, fishes may possess larger ITS1 sequences than invertebrates. Only a few invertebrates in the present data set were observed to have ITS1 sequences longer than 1,000 bp; a squid species (*Nototodarus sloanii*) and a common clam (*Meretrix lusoria*) in Mollusca, and snapping shrimp (*Alpheus* sp.) and anomuran and brachyuran crabs (*Dardanus arrosor*, *Scylla serrata*, and *Thalamita prymna*) in Crustacea. No representatives of either the gelatinous animals (Cnidaria and Ctenophora) or Prochordata were observed to have an ITS1 sequence longer than 500 bp, and Cnidaria and Ctenophora had significantly shorter ITS1 sequences (averages 253.9 and 243.2 bp, respectively) than the other animal taxa except for Prochordata (average 311.8 bp). Eukaryotes typically have ITS1 regions shorter than

Table 1 The number of individuals and species of marine animal taxa used for ITS1-specific PCR and the results of amplification

Phylum	Class	No. of		PF[a]	NF[b]
		Individuals	Species		
Vertebrata	Agnatha	2	1	0	0
	Chondrichthyes	48	26	2	7
	Mammalia	5	5	0	5
	Osteichthyes	311	253	3	15
	Reptilia	2	2	0	2
Prochordata	Ascidiacea	4	3	0	0
Echinodermata	Asteroidea	6	6	1	1
	Echinoidea	10	9	0	1
	Holothuroidea	10	3	0	7
	Ophiuroidea	1	1	0	1
Arthropoda	Crustacea	56	38	5	3
	Pycnogonida	1	1	0	0
Annelida	Polychaeta	4	4	0	0
Mollusca	Bivalvia	82	76	5	5
	Cephalopoda	35	22	7	8
	Gastropoda	65	56	5	12
	Polyplacophora	12	11	3	0
Cnidaria	Anthozoa	4	3	0	0
	Cubozoa	1	1	0	0
	Hydrozoa	60	40	0	13
	Scyphozoa	29	15	0	4
	unidentified	5	5	0	0
Ctenophora	Cyclocoela	10	8	0	2
	Typhlocoela	4	4	0	1
Chaetognatha	Sagittoidea	6	6	0	0
Total		773	599	31	87

[a] Number of individuals showing plural fragment amplification
[b] Number of individuals showing no fragment amplification

1,100 bp (von der Schulenburg et al. 2001), and our observations in marine animals are consistent with that trend; however, Chondrichthyes is a notable exception. Mammals are also known to have long (<1,000 bp) and GC-rich (<70%) ITS1 sequences (Subrahmanyam et al. 1982; Goldman et al. 1983; Gonzalez et al. 1990). However, a longer ITS1 sequence is not restricted to vertebrates since ladybird beetles have been observed to have relatively long ITS1 sequences (<700 bp) and one species' ITS1 exceeds 2,500 bp (von der Schulenburg et al. 2001).

The ITS1 sequences of vertebrates analyzed in this study (Chondrichthyes and Osteichthyes) were more GC-rich (averages 67.3% and 68.0%, respectively) compared with those of the invertebrate taxa, which ranged from 35.8% to 69.5% GC content (Table 3). Among the invertebrates, gelatinous animals, Cnidaria and Ctenophora, with significantly shorter ITS1 regions, also had the lowest GC content, from 35.8% to 61.7% (average 45.6%) and from 45.6% to 55.2% (average 49.9%), respectively. Crustacea and Mollusca are diverse groups in both length and GC content where the ITS1 lengths ranged from 182 to 1,613 bp for the former and from 117 to 1,118 for the latter and GC content from 42.8% to 66.5% for the former and from 43.4% to 69.5% for the latter. Thus, it would seem that the length of an ITS1 region is predictive of its GC content. Coefficients of correlation between the ITS1 lengths and GC content for taxa overall (only full-length sequences were sampled) were positive and significant (R^2=0.345, p<0.01, n=307). However, the relationships were confounded since significant positive correlations between ITS1 length and GC content were observed only in Mollusca (R^2=0.41, p<0.01, n=85) and Crustacea (R^2=0.38, p<0.01, n=30). The ladybird beetles have long ITS1 regions that are not GC-rich (48% to 53%) (von der Schulenburg et al. 2001) while the ITS1 sequences of frogs are not long (500 to 560 bp) but are extremely GC-rich (78% to 84%) (Hall and Maden 1980; Furlong and Maden 1983; Sumida et al. 2004), pointing to a negative correlation between ITS1 length and GC content. ITS1 length variation may be better correlated with genome size, since according to the animal genome size database (http://www.genomelength.com/index.php), Cnidaria and Urochordata have genomes with a small C value (0.07 to

Table 2 Accession numbers for marine animal taxa included in the ITS1 sequence analysis

Phylum	Class	Accession numbers
Vertebrata (120, 116, 60)	Chondrichthyes	AB375546–AB375554
	Osteichthyes	AB127400, AB127404, AB193474, AB193567, AB212029, AB212043, AB276984, AB277029, AB375555–AB375656, DQ678697
Prochordata (8, 7, 8)		AB377708, AF158724–AF158726, EF035103, EF035105, EF035106, X53538
Echinodermata (19, 18, 15)	Asteroidea	AB377704, AF212169, AF346609, AF346611, AF346617
	Crinoidea	AY275906–AY275912, AY278740
	Echinoidea	AB377703, AB377705–AB377707, AJ457832, X00350
	Holothuroidea	DQ320502
Arthropoda (61, 50, 49)	Crustacea	AB193500, AB426490–AB426518, AF253520, AF253521, AF288000, AF316395, AF463509–AF463512, AJ549194, AM083342, AM114421, AY074918, AY074920, AY234857, AY275451, AY275452, AY275456–AY275458, AY297725, AY315658, AY331590, AY496261, AY500279, AY599492, DQ328766, DQ466197, EF035109, EF035130, EF035133, EF532820, EF532821
Annelida (16, 16, 14)	Polychaeta	AB377709, AF212165, AF332152, AF332156, AF332167, AY099478, DQ172767, DQ172771, DQ172810, DQ172812, DQ470300, DQ470372, EF117908, EF545150–EF545152
Mollusca (132, 126, 95)	Bivalvia	AB041760, AB377611, AB377613, AB377614, AB377619, AB377623–AB377625, AB377629–AB377631, AB377636, AB377638, AB377660–AB377675, AB377679–AB377690, AB377695–AB377699, AB377701, AB377702, AF093838, AY172341, AY172342, AY172345, AY230083, AY230084, AY230088, AY825242, DQ060188, DQ132789, DQ190444–DQ190446, DQ220290, DQ346656, DQ389106, DQ389108, DQ785895, DQ873892, DQ873893, DQ873902, DQ873908, DQ873910, DQ873911, DQ873913, DQ885589, DQ924556, DQ924558, DQ924559
	Cephalopoda	AB377640–AB377642, AF034565, AF034844
	Gastropoda	AB377609, AB377610, AB377612, AB377615–AB377617, AB377620–AB377622, AB377626–AB377628, AB377632–AB377635, AB377637, AB377639, AB377643–AB377652, AB377676–AB377678, AB377688, AB377691, AB377692, AB377700, AF296849, AF296858, AY146404–AY146406, AY319420, DQ076088, DQ225169
	Polyplacophora	AB377618, AB377653–AB377659, AB377693, AB377694
Cnidaria (89, 69, 86)	Anthozoa	AB090910, AB214161, AB377598, AB377599, AF038906, AF050211, AF262345, AF262346, AM404318, AM404320–AM404322, AM404326, AM404328, AM404329, AY948135, DQ645392, DQ645394, U60605
	Cubozoa	U65477
	Hydrozoa	AB377538, AB377541, AB377542, AB377544–AB377546, AB377549, AB377550, AB377555–AB377584, AB377592, AB377593, AB377596, AY513614, AY513619, AY513620, AY513634, AY513636, AY513637, AY730678, U65483, U65484
	Scyphozoa	AB377535, AB377536, AB377539, AB377543, AB377548, AB377551–AB377589, AB377594, AB377595, AB377597, AF461405, AF461408, U65481
	Unidentified	AB377537, AB377540, AB377547
Ctenophora (25, 24, 25)	Cyclocoela	AB377600–AB377606, AB377608, AF293688, AF293691, AF293693–AF293695, AF293698, EF175463, EF175465, U65480
	Typhlocoela	AB377607, AF293673, AF293674, AF293676, AF293677, AF293683, AF293684
	Unidentified	AF293686

Numbers in parenthesis indicates numbers of sequence (*left*), species (*center*), and full-length sequence (*right*)

1.85 pg), while Chondrichthyes have large *C* values (2.8 to 13 pg), analogous to what we observed for ITS1 length. In contrast, it is completely unclear why the GC content is so high in this noncoding region of certain animal taxa.

While ITS1 regions may be used for inferring phylogenetic relationships below the genus level (Harris and Crandall 2000; Chu et al. 2001), the possibility of using this region to reconstruct phylogenies at generic or even higher taxonomic levels has been proposed (Coleman and Vacquier 2004). In our data set, we sampled 109 full-length sequences from 81 species belonging to 62 genera and 26 families, and the percent nucleotide differences between sequences were estimated for the different taxonomic levels (Fig. 1). Intra-individual variation was investigated in the Japanese spiny lobster (*Panulirus japonicus*) and a copepod (*Paracalanus parvus*) where two to three clones from an individual were sampled and sequenced. Nucleotide sequence differences between clones obtained from three individuals of *Panulirus japonicus* and two *Paracalanus parvus* ranged from 0.6% to 0.9% and from 0.0% to 7.9%, respectively. Full-length sequences of several individuals in a species were

Table 3 Average and range of rDNA ITS1 length and GC content in nine marine animal taxa

Phylum	Class	bp length[§]		Percent GC	
		Average±SD	Range	Average±SD	Range
Vertebrata	Chondrichthyes	1,494.3±299.4[a*] (23)	878–2,318	67.3±4.6[ab] (9)	57.3–72.7
	Osteichthyes	635.1±159.3[b] (254)	318–1,518	68.0±4.2[a] (107)	56.8–78.0
Prochordata		311.8±84.8[de] (8)	187–486	55.8±10.6[deg] (8)	38.1–66.6
Echinodermata		401.2±72.6[c] (22)	300–639	62.5±4.9[bcd] (18)	57.9–71.0
Arthropoda	Crustacea	549.0±285.0[c] (56)	182–1,613	55.1±7.1[ef] (50)	42.8–66.5
Annelida	Polychaeta	467.5±110.8[cd] (16)	272–650	58.6±6.5[ce] (16)	50.0–64.9
Mollusca		492.4±190.2[c] (161)	117–1,118	55.9±5.7[ef] (124)	43.4–69.5
Cnidaria		253.9±65.2[e] (82)	118–422	45.6±6.2[h] (66)	35.8–61.7
Ctenophora		243.2±37.0[e] (27)	148–307	49.9±3.0[g] (23)	45.6–55.2

[*] Averages not sharing the same letter are significantly different ($p<0.05$, Tukey test). In parenthesis is the number of species
[§] ITS1 length data were from full-length sequences and from size estimation by gel electrophoresis (data available at http://fsf.fra.affrc.go.jp/chow/ITSlength.htm)

obtained in 17 species where confident sequence alignments were readily obtained and nucleotide sequence differences between individuals within species were observed to range from 0.0% to 7.9% (Fig. 1a). The largest differences within species were observed in a copepod (*Paracalanus parvus*) and a squid (*Ommastrephes bartrami*) (7.9% and 6.1% difference, respectively) in which gaps due to repetitive elements were primarily responsible for the relatively large differences. Sequence differences arising from repetitive elements were also observed in a spiny lobster (*Panulirus japonicus*), while singleton gaps and/or substitutions were responsible for the nucleotide differences between sequences from other species. Much wider ranges and higher estimates of sequence differences were observed in a comparison between congeneric species (0.4% to 40.4%) and between species belonging to different genera of the same family (0.5% to 68.6%) (Fig. 1b,c). Sequence comparisons between species within the same genus and between species of different genera within the same family indicated that the repetitive elements responsible for ITS1 length and nucleotide sequence differences were observed only in lobster species of the genus *Panulirus*. von der Schulenburg et al. (2001) has suggested that

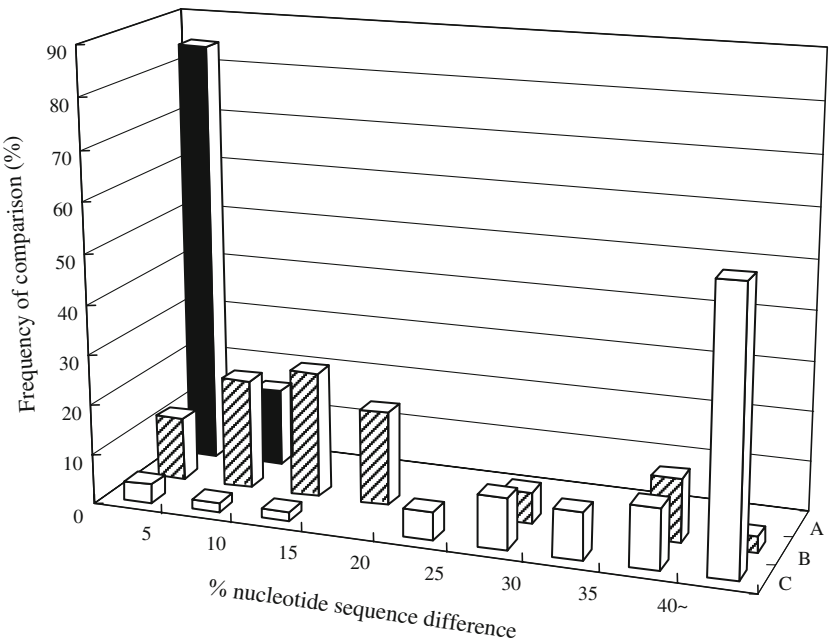

Fig. 1 Percent nucleotide sequence differences between sequences at different taxonomic levels: *A* between individuals within a species (16 species), *B* between congeneric species (28 species, and ten genera), *C* between species of different genera within the same family (65 species, 55 genera, and 18 families)

length variation in the ITS1 region may be attributed to the presence of repetitive elements, but our results indicate that repetitive elements make a relatively minor contribution to sequence divergence, including length differences.

Very small differences (0.4%) were observed between congeneric gastropod species (*Chlorostoma lischkei* vs. *C. xanthostingma*). In contrast, large differences (29.3% to 40.4%) were observed between some other congeneric species of Mollusca (*Acanthopleura* spp.) and Ctenophora (*Beroe* spp.), making confident sequence alignment troublesome. Obtaining confident sequence alignments became even more difficult between different genera within the same family, which is consistent with previous reports on flat worms and insects (von der Schulenburg et al. 1999, 2001). However, small nucleotide differences between species of different genera were observed in the northern king crabs (*Lithodes aequispinus* vs. *Paralithodes* spp.; 0.5% to 5.5%), snails (*Chlorostoma* spp. vs. *Omphalius pfeifferi*; 0.3% to 0.7%), and chitons (*Acanthopleura tenuispinosa* vs. *Liolophura japonica*; 0.7% to 0.9%), suggesting that reevaluation of the categorization of these genera may be necessary. Indeed, most of the other comparisons between genera had much larger values (≥25%) of sequence differences (Fig. 1c). We have tentatively determined that a 30% difference may be the lower limit for obtaining confident sequence alignments, and many comparisons between species of different genera exceed this value. Although secondary structure models have been proposed to be useful for phylogenetic analyses of higher taxa (Coleman and Vacquier 2004; Gottschling and Plötner 2004), results from the present study indicate that ITS1 sequences should be restricted to phylogenetic studies below the genus level. Nevertheless, the characteristic low intraspecific variation but large difference between species may be suited to the design of species-specific oligonucleotide probes for DNA microarrays that have recently been investigated to quickly and correctly identify marine animal species (Kochzius et al. 2008).

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