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Intraspecific variations in the ITS region of Recent radiolarians

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Abstract

A molecular phylogenetic investigation of radiolarians has been done using ribosomal DNA sequences. We determined nucleotide sequences of 18S rDNA, 5.8S rDNA, 28S rDNA, and the internal transcribed spacer (ITS) regions of three spumellarians and one nassellarian specimens. Two sympatric individuals of the same species collected at the same time demonstrated that the ITS sequences evolve much more rapidly than the rDNA sequences, with the variation in 18S rDNA, 18S rDNA + 5.8S rDNA + 28S rDNA, and ITS1 + ITS2 being 0.5%, 0.5%, and 4.3%, respectively. This faster evolutionary rate is reflected in a longer branch length in molecular phylogenetic trees, allowing clearer discrimination of spongodiscinid species. Our results indicate that ITS sequences will be a key to have insight into lower-level relationships in radiolarians.

Key words: Internal transcribed spacer, intraspecific variation, molecular phylogeny, Radiolaria, Spongodiscidae, Spumellaria, 18S rDNA

Introduction

Molecular phylogeny has refreshed traditional views on evolution of virtually all organisms. Radiolarians are no exception, and following the pioneering framework study by Amaral Zettler *et al.* (1997), radiolarian relationships including those among acantharians and polycystines have been re-examined in recent years using 18S rDNA (Takahashi *et al.* 2004; Oka *et al.* 2005; Yuasa *et al.* 2005, 2006; Kunitomo *et al.* 2006). Since 18S rDNA evolves relatively slowly, it has been useful for the studies of higher-order relationships, but is not suitable for inferences of lower-order genealogies, such as at the species level and lower, because 18S rDNA sequences are invariant at these levels. In order to have insight into finer-grained phylogeny and genetic structures in relation to ecologies in radiolarians, it is desirable to have a molecular marker that evolves faster than 18S rDNA. We therefore embarked on to characterize the internal transcribed spacer (ITS) region sequences, which are known to evolve quickly, since they are trimmed out from the transcript that matures into 5.8S, 18S, and 28S ribosomal RNAs, thus are much less functionally constrained than the rRNA sequences.

In this study, we determined nucleotide sequences of 18S, 5.8S, and 28S rDNAs as well as the ITS regions for three spumellarian and one nassellarian specimens. Sequence comparisons indicated that the ITS regions indeed evolve more quickly than rRNA sequences, discriminating the two spumellarian species studied more clearly, and that the two individuals of the spumellarian *Dictyocoryne truncatum* can be distinguished by their nucleotide sequences of the ITS regions. This paper represents the first report for the ITS sequences in radiolarians.

Material and methods

Sample collection and DNA extraction

Live spumellarians and nassellarians were collected using a plankton net off the coast of Shimoda, Izu Peninsula, central Japan (34°36′83″N, 138°56′70″E–34°36′78″N, 138°56′38″E) on 6 December 2007. Three spumellarians and one nassellarian, for which nucleotide sequences were determined in this study, include two individuals of the spumellarian *Dictyocoryne truncatum* (Fig. 1a, b), one spumellarian individual of a spongodiscid species, and one individual of the nassellarian *Pterocorys* cf. *zancleus* (Fig. 1c).

After identification under an inverted microscope, each specimen was rinsed in MilliQ-water and transferred to a 1.5 ml sample tube. The tubes were then stored in liquid N_2 prior to extraction of genomic DNA, that was accomplished by addition of 30 μl of 10% (w/v) Chelex 100 resin (BioRad Laboratories) to each specimen, followed by incubation at 60°C for 30 min and 94°C for 3 min (Walsh *et al.* 1991).

DNA amplification, cloning and sequencing

An aliquot of 1 μl each of the DNA preparations was used as template in polymerase chain reaction (PCR) to amplify a DNA region flanked by 18S and 28S rRNA sequences using 18S-Spumellaria or 18S-Nassellaria and ITS-a3 as the forward and reverse primers, respectively (Table 1). These forward primers were designed based on known spumellarian and nassellarian 18S rDNA sequences, respectively. The ITS-a3 primer was

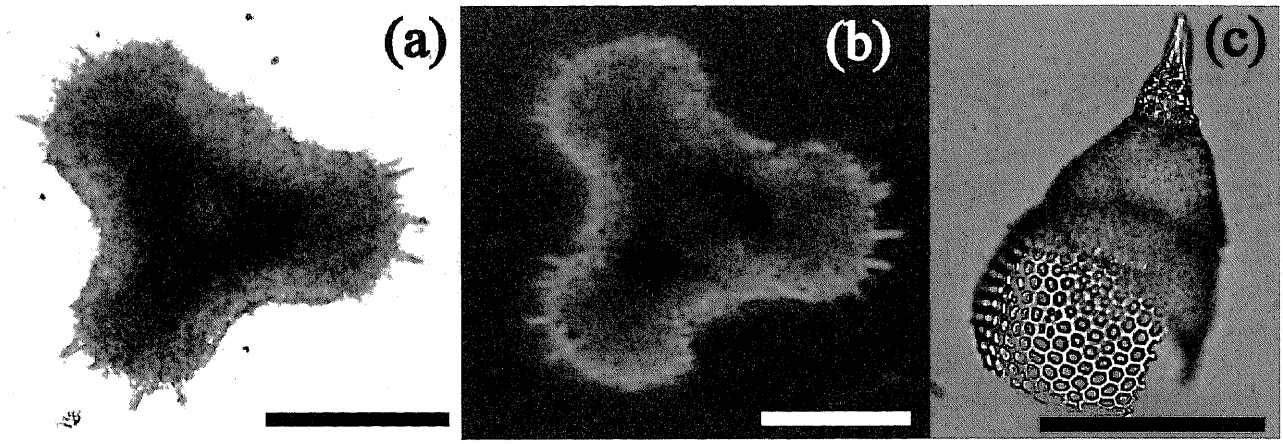

Fig. 1 Radiolarian specimens from which rRNA and ITS sequences reported in this study were obtained. (a) *Dictyocoryne truncatum* f28. (b) *Dictyocoryne truncatum* N. (c) *Pterocorys* cf. *zancleus* I. Scale bars indicate 100 μm.

Table 1 Oligonucleotide primers used in this study.

Name of primer	nucleotide sequence (5′ to 3′)	GC contents (%)	Forward (F) or Reverse (R)
18S-Spumellaria	AATACRTGCACKAAGGGGCGA	52	F
18S-Nassellaria	GCGTATCATTCAAGTTTCTGA	38	F
ITS-a3	TCACCATCTTTCGGGTCCCAACA	52	R
ITS-18S-Rad1	ACCGCCCGTCGCTCCTACCG	75	F
28S-Rad2	TAAGCGGAGGAAAAGAAA	39	F
ITS-28S-Rad1	CCCTCACGGTACTTGTTCGC	60	R
18S-riv-Rad1	CCACCAACTAAGAACGGCCA	55	R
18S-5.8S-Rad2	GCGTTCTTCATCGTTGCG	56	R

designed based on known 28S rDNA sequences of cercozoans, which are closely related to radiolarians. Each PCR reaction mixture (20 μl) contained 2.5 mM Tris-HCl, pH 8.3, 12.5 mM KCl, 0.375 mM $MgCl_2$, 50 pM each of dNTP, 50 pM each of primers and 2.5 units of ExTaq Hot Start Version (TAKARA BIO.). PCR amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems) with 1 min denaturation at 95°C prior to 35 cycles of 94°C for 30 sec, 52°C for 1 min, and 72°C for 3 min, followed by a final extension at 72°C for 5 min. Target PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN) after electrophoresis on agarose gels, and cloned into a TA plasmid pGEM-T Vector (Promega), which was used to transform *Escherichia coli* DH5α (TOYOBO). The insert sequences were amplified by colony direct PCR, and purified using ExoSAP-IT (USB). DNA sequencing was attained using BigDye Terminator v.3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with the vector specific T7 and SP6 primers as well as a set of internal primers ITS-18S-Rad1, 28S-Rad2, ITS-28S-Rad1, 18S-riv-Rad1, and 18S-5.8S-Rad2 listed in Table 1. Successful amplifications of radiolarian 18S rDNA sequences were verified by screening sequences stored in DDBJ databank (http://www.ddbj.nig.ac.jp/index-j.html) with BLAST (Altschul *et al.* 1997).

Phylogenetic analysis

For phylogenetic reconstructions based on 18S rDNA, a total of 19 radiolarian sequences including three spumellarian sequences obtained in this study, as well as one outgroup acantharian and 15 spumellarian sequences obtained from the DDBJ databank were analyzed (Table 2). Sequences were aligned using CLUSTAL X (Thompson *et al.* 1997) and refined manually using MacClade 4 (Sinauer Associates). Evolutionary models were optimized using MrModeltest 2.2 (Nylander 2004), and phylogenetic trees based on maximum likelihood (ML) and Bayesian methods were reconstructed using PAUP* version 4.0b10 (Swofford 1998) and MrBayes version 3.1.1 (Huelsenbeck and Ronqutst 2001), respectively. Consensus trees for ML analysis were drawn based on 50% majority rule. Bayesian analyses were carried out for 300000 generations, and trees sampled every 100 generations.

Table 2 List of nucleotide sequences used in this study. Sequences determined in this study are indicated in boldface.

	Species name	Accession number	length (bp)
Acantharea	*Hexaconus serratus*	AB178587	1770
Spumellaria	*Dicranastrum furcatum*	AB179733	1727
	Dictyocoryne profunda	AB101540	1726
	Dictyocoryne truncatum	AB101541	1727
	***Dictyocoryne truncatum* f28**	AB430757	2934
	***Dictyocoryne truncatum* N**	AB430758	2949
	Didymocyrtis tetrathalamus	AB193605	1723
	Euchitonia elegans	AB179732	1728
	Spongaster tetras	AB101542	1726
	Spongodiscidae gen. et sp. indet. D	AB430760	2952
	Spongodiscus biconcavus	AB246695	1722
	Spongodiscus resurgens	AB246696	1724
	Spongopyle osculosa	AB246689	1605
	Stylodictya sp. 8037	AB246698	1712
	Styptosphaera sp. 2013	AB246684	1801
	Styptosphaera sp. 2022	AB246686	1798
	Tetrapyle octacantha	AB246680	1667
	Tetrapyle sp. 2008	AB246688	1680
	Triastrum aurivillii	AB179734	1726
Nassellaria	***Pterocorys* cf. *zancleus* I**	AB430759	2747

For the ITS sequences obtained in this study, nucleotide sequences were aligned for each pair of sequences, and a simple sequence similarity was calculated after removing indels. Bayesian tree was reconstructed for each of the datasets of 18S, 18S+5.8S+28S, and ITS1+ ITS2 sequences.

Results and Discussion

Radiolarian ITS sequences

Nucleotide sequences spanning from a posterior part of the 18S rDNA to an anterior part of the 28S rDNA were determined for three spumellarian (*Dictyocoryne truncatum* f28, *D. truncatum* N, and Spongodiscidae gen. et sp. indet. D = spongodiscid D) and one nassellarian (*Pterocorys* cf. *zancleus* I) specimens (Fig. 1; Table 2). Sequence analysis indicated that the 18S rDNA, ITS1, 5.8S rDNA, ITS2, and 28S rDNA are arranged in tandem on the chromosome, therefore the entire ITS sequences were obtained for the above four individuals. The sizes of the ITS1 sequences of *D. trunctum* f28, *D. truncatum* N, spongodiscid D, and *Pterocorys* cf. *zancleus* I are 191, 195, 162, 201 base pairs (bp) respectively, and the sizes of the ITS2 are 173, 185, 217, 185 bp, respectively (Figs. 2, 3). It was evident from this alignment that ITS sequences are variable even between two individuals of the same species collected from the same locality (Figs. 2, 3).

Spumellarian phylogeny based on 18S rDNA

BLAST searches indicated, and phylogenetic analyses based on a total of 1300 bp sequences confirmed, that the 18S rDNA sequences obtained in this study came from the radiolarian genomes rather than from any extraneous sources (Figs. 4, 5). The ML tree (Fig. 4) and the Bayesian tree (Fig. 5), both rooted with the acantharian outgroup, showed generally the same topology, with pyloniids, *Stylodictya* + *Spongopyle*, ethmosphaerids (two individuals of *Styptosphaera* sp.), and three individuals of *D. truncatum* forming a monophyletic group, respectively. The overall patterns of relationships are much the same as those reported by Kunitomo *et al.* (2006). In the present study, the group of the coccodiscid + spongodiscids, except for *Stylodictya* and *Spongopyle*, also formed a clade, and the group of *Stylodictya* and *Spongopyle* was suggested to be closer to pyloniids than to other spongodiscids, but the statistical support for this branch is rather low (a bootstrap value of 61% in the ML tree, and a posterior probability of 0.79 in the Bayesian tree), therefore the relations remain inconclusive.

Evolutionary rates of rDNA and ITS sequences

Simple calculations of nucleotide similarities of 18S, 18S+5.8S+28S, and ITS1+ITS2 sequences among the four individuals studied indicated that the similarities between spumellarians and the nassellarian are much

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      5         15         25         35         45         55
f28              GACACACAAA C-G--TTGGT TCTTAGTTGA ACCAACA--C AAAACATTTC CACCGCGTAT
N                GACACACAAA CTG--TTGGC TCTT--CTGA GCTGACAACC AAAACATTTC CACCGCGTAT
D                GACACACAAA GTGGTTTGGC TTCTAAAGCT GGCCACA-CC AAAACATTTC CACCGCGTAT
                 **********   *  ****  *  *          ***  * ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      65         75         85         95        105        115
f28              CCACA-AGCT TGAATACTTA TCCGCGTTGT CTGA-GCCGG CTTC----AA ---CATTTAT
N                CCACA-AGCT TGAATACTAA AACGCGTTGT CTGA-GCCGG CTTC----AA TAACATTTCT
D                TAACACAGCT TAAATACTAA ACTGCGTTGT CTGAAGCCGG CTCTTTAAAA AAACTTTTCT
                   *** **** * ****** *    ******* **** ***** **      **    * *** *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     125        135        145        155        165        175
f28              TTTTTCACCC AAACCACCAC AACA-AACGC CTGAAGCGC- ATACTAAACA TAT-GCGCAA
N                TTTTTCACCC AAACCACCAC AACA-AACGC CTGAAGCGC- ATACCAAAAA TAT-GCGCAA
D                GTTTACACCC AAACCACCAA AACTCAACGA TTGAAGCGCG ATGATATAAT CATCACGCAA
                 *** ***** *********  ***  ****   ********  **   * *    **  *****

                 ....|....| ....|....| ....|...
                     185        195        205
f28              GGATGACAAA ACAAAAACCA AAAGAATT
N                GGATTCAAAA ACAAAAACCA AAAGAATT
D                ---TATCAA- --AAATACAA AAAGATTT
                    *   **    *** ** * ***** **
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Fig. 2 An alignment of the spumellarian ITS1 sequences. *Dictyocoryne truncatum* f28 (f28), *Dictyocoryne truncatum* N (N), Spongodiscidae gen. et sp. indet. D (D).

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      5         15         25         35         45         55
f28              TTTGCAAACC AAAAAAGTTT TCATAGACGA AGCTGGGCGT TGCAGAAAT- ---TCTGGAT
N                TTTGCAAACC AAAAAAGTTT TCATAGACGA AGCTGGGCGT TGCAGAAATA AATTCTGGAT
D                TTTGCAAAAT AAAAATGCAT ATAAAAACGA ACATGGACGT TGCAACAATC AATGTTGGAT
                 ********   ***** *  *   * * **** *  *** *** ****  ***       *****

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      65         75         85         95        105        115
f28              CGCCTCAAGA GCTTGCTTGG TCTGGTGGAC AAATCGATTT --GCCGGCAC CGAGGTAGAG
N                CGCCTCAAGA GCTTGCTTGG TCTGGCGGAC AAATCACATA CGGCCGGCAC CGAGGTAGAC
D                CGTCTCAAGA TAGAGCTTTG CTTGGGCCAC GTGCAAACTC GTGCCGGCAC CGAGGTA-AC
                 ** *******     **** *   ***   **         *    ******** ******* *

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     125        135        145        155        165        175
f28              CA---ATCAA CCGACCGTGT TAGCTAGATG CGTCCGATAT CCAAACTAAT CACAA----C
N                TACAAATCGA CCGAACGTGT TAGCTAGATG CGTCCGA-AC CCAAACTAAA AAAAAATCTT
D                GATTATTCTT ATCAACGTGT TCGAAAAGAG TTTCACAAAA AGTGTTTGCC TTCTTTCTTC
                 *    **      * ***** * *  *   *   **  * *       *

                 ....|.
                     185
f28              TATTGC
N                TATTGC
D                TAATAC
                 ** * *
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Fig. 3 An alignment of the spumellarian ITS2 sequences. *Dictyocoryne truncatum* f28 (f28), *Dictyocoryne truncatum* N (N), Spongodiscidae gen. et sp. indet. D (D).

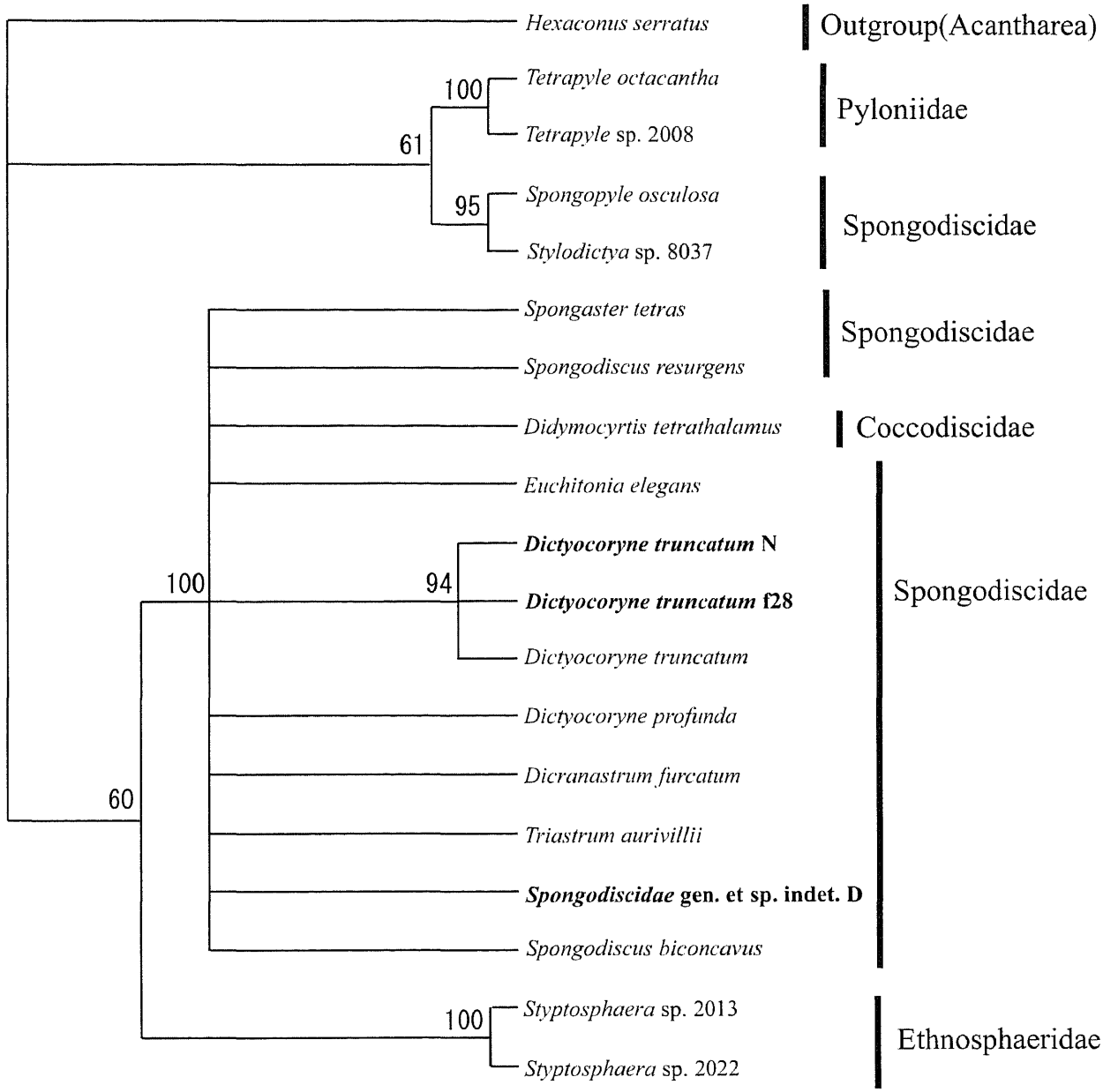

Fig. 4 Phylogenetic relationships of spumellarians based on 18S rDNA (ML tree). Bootstrap probability is indicated at each node.

lower in the ITS sequences (0.495–0.516) than in the 18S (0.818–0.820) and 18S+5.8S+28S (0.790–0.792) sequences (Fig. 6). Among the three spumellarians, the similarities between the two spongodiscid species are substantially lower in the ITS sequences (0.756–0.764) than in the 18S (0.986–0.989) and 18S+5.8S+28S (0.972–0.973) sequences (Fig. 5). The faster evolutionary rate of ITS sequences is also manifest in the comparison between the two sympatric individuals of the same species (*Dictyocoryne truncatum* f28 and *D. truncatum* N), with the variation in 18S, 18S+5.8S+28S, and ITS1+ITS2 being 0.5%, 0.5%, and 4.3%, respectively (Fig. 6).

Figure 7 shows Bayesian trees of the four individuals studied, reconstructed based on 18S rDNA (1458 bp), 18S rDNA + 5.8S rDNA + 28S rDNA (2293 bp), and ITS1 + ITS2 (238 bp) sequences, and rooted with the nassellarian species. The branch length is proportional to the estimated number of nucleotide substitutions, and the trees are drawn to the same scale. The length of the branch leading to the two individuals of *D. truncatum* is clearly longer in the ITS1+ITS2 tree than in the rDNA trees, corroborating the faster evolutionary rate of the ITS sequences and their utility in

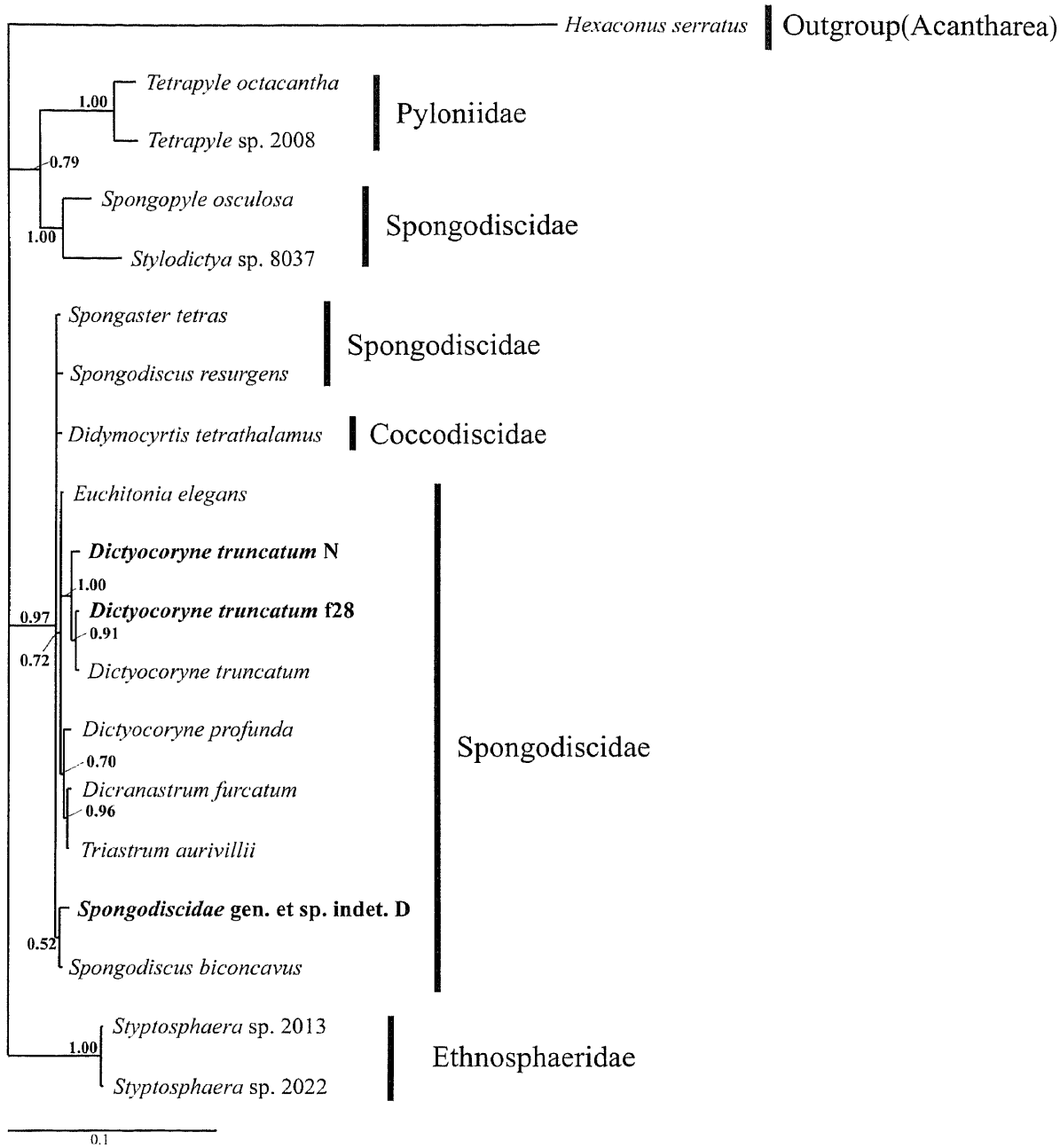

Fig. 5 Phylogenetic relationships of spumellarians based on 18S rDNA (Bayesian tree). Posterior probability is indicated at each node.

discriminating even between conspecific and sympatric individuals.

Our results demonstrated that the ITS sequences have accumulated enough variations to allow phylogenetic inferences at the species-level and lower in spongodiscid radiolarians. This new molecular marker should be useful for radiolarian biology in various ways, not least in unveiling speciation events and finer-order genetic structures in relation to ecology and geographic distributions.

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18S	*D. truncatum* f28	*D. truncatum* N	Spongodiscid D	*Pterocorys* cf. *zancleus* I
D. truncatum f28	1			
D. truncatum N	0.995	1		
Spongodiscid D	0.989	0.986	1	
Pterocorys cf. *zancleus* I	0.819	0.819	0.820	1

18S+58S+28S	*D. truncatum* f28	*D. truncatum* N	Spongodiscid D	*Pterocorys* cf. *zancleus* I
D. truncatum f28	1			
D. truncatum N	0.995	1		
Spongodiscid D	0.973	0.972	1	
Pterocorys cf. *zancleus* I	0.791	0.792	0.790	1

ITS1+ITS2	*D. truncatum* f28	*D. truncatum* N	Spongodiscid D	*Pterocorys* cf. *zancleus* I
D. truncatum f28	1			
D. truncatum N	0.957	1		
Spongodiscid D	0.756	0.764	1	
Pterocorys cf. *zancleus* I	0.512	0.516	0.495	1

Fig. 6 Similarities of 18S, 18S+5.8S+28S and ITS1+ITS2 sequences among radiolarians studied.

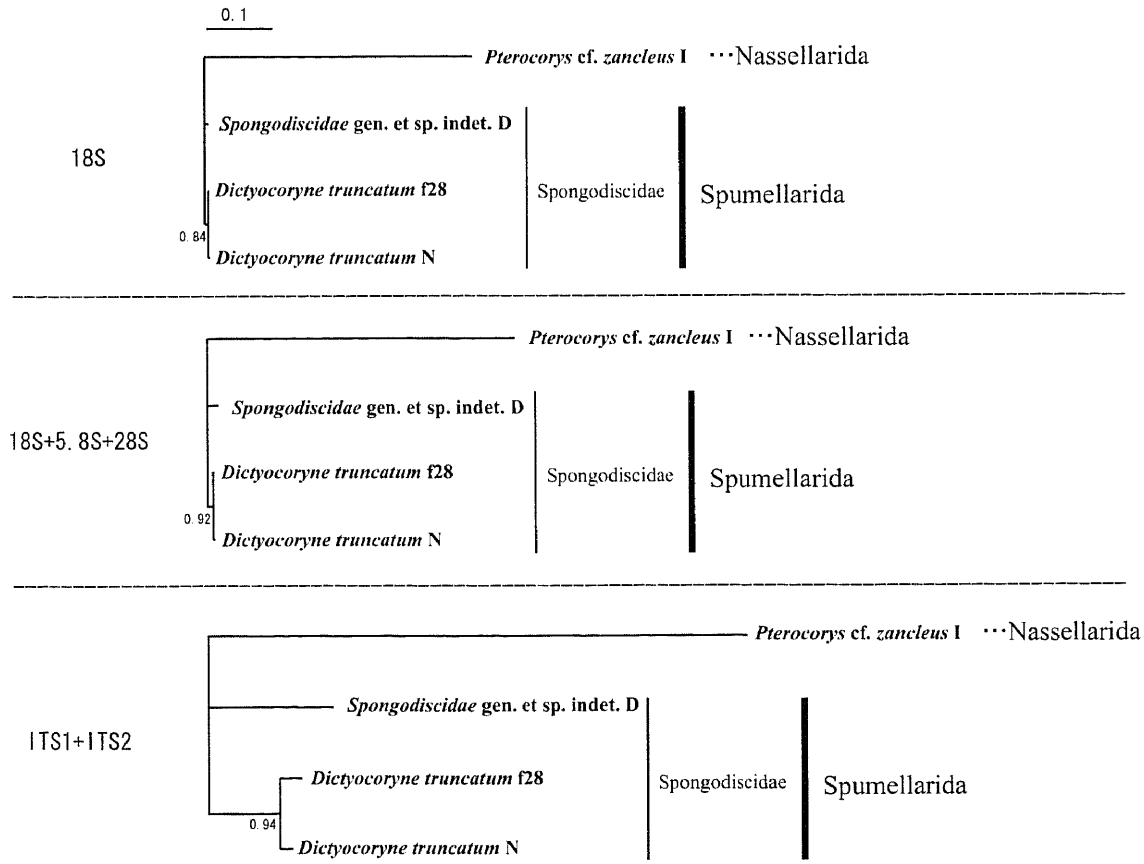

Fig. 7 Comparisons of evolutionary rates between rDNA and ITS sequences. The values at the node between the two individuals of *D. truncatum* indicate posterior probabilities.

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