

Molecular Diversity of the Syndinean Genus *Euduboscquella* Based on Single-Cell PCR Analysis

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The genus *Euduboscquella* is one of a few described genera within the syndinean dinoflagellates, an enigmatic lineage with abundant diversity in marine environmental clone libraries based on small subunit (SSU) rRNA. The region composed of the SSU through to the partial large subunit (LSU) rRNA was determined from 40 individual tintinnid ciliate loricae infected with *Euduboscquella* sampled from eight surface water sites in the Northern Hemisphere, producing seven distinct SSU sequences. The corresponding host SSU rRNA region was also amplified from eight host species. The SSU tree of *Euduboscquella* and syndinean group I sequences from environmental clones had seven well-supported clades and one poorly supported clade across data sets from 57 to 692 total sequences. The genus *Euduboscquella* consistently formed a supported monophyletic clade within a single subclade of group I sequences. For most parasites with identical SSU sequences, the more variable internal transcribed spacer (ITS) to LSU rRNA regions were polymorphic at 3 to 10 sites. However, in *E. cachoni* there was variation between ITS to LSU copies at up to 20 sites within an individual, while in a parasite of *Tintinnopsis* spp., variation between different individuals ranged up to 19 polymorphic sites. However, applying the compensatory base change model to the ITS2 sequences suggested no compensatory changes within or between individuals with the same SSU sequence, while one to four compensatory changes between individuals with similar but not identical SSU sequences were found. Comparisons between host and parasite phylogenies do not suggest a simple pattern of host or parasite specificity.

The dinoflagellate lineage can be roughly divided into two major groups, one that includes most familiar dinoflagellates with the typical dinokaryotic nucleus, or the dinokaryotes, and a second, the syndineans, that includes parasitic species lacking a dinokaryon (15). Syndinean dinoflagellates are a diverse and poorly understood marine group and may not represent a monophyletic lineage. Known syndinean hosts range from single-celled plankton, including other dinoflagellates, ciliates, and radiolarians, to parasites of fish eggs, copepods, and commercially important crustaceans such as lobsters and blue crabs (5, 10).

Interest in syndinean dinoflagellates was rekindled when marine environmental clone libraries dramatically expanded the breadth of the syndinean clade in small subunit (SSU) rRNA trees from just a few sequences for the genus *Amoebophrya* (18) to a series of clades that ultimately included thousands of sequences, although very few can be attributed to described species (17, 33). Originally described as belonging to marine alveolate groups I and II, sequences attributed to syndineans can now be classified into seven major groups (called clades I to VII), with groups I and II containing the bulk of the sequences.

In the past several years, progress has been made connecting syndinean rRNA sequences with the data from environmental clone libraries. Based on data currently available in GenBank, there are 204 rRNA sequences from syndinean taxa described to at least the genus level, mostly from the genera *Hematodinium* (176 sequences) (35, 42) and *Amoebophrya* (22 sequences) (18, 25), with a few sequences attributed to *Duboscquella* (2 sequences) (12a, 20), *Ichthyodinium* (12 sequences) (41, 48), and *Syndinium* (40). Syndinean group I was shown to contain species of both *Ichthyodinium*, parasites of fish eggs, and *Duboscquella*, parasites most often described as isolated from tintinnid ciliates.

Recently, several typical dinokaryotes such as *Tintinnophagus* and *Duboscquodinium* have also been shown to parasitize tintinnid

ciliates (11), and the type species for the genus *Duboscquella* was also moved into the dinokaryotes, requiring the creation of a new genus, *Euduboscquella*, for the true syndinean parasites formerly in the genus *Duboscquella* (12a). Based on the SSU phylogeny, the two previously reported sequences for *Duboscquella* would represent syndineans assigned to *Euduboscquella* (12a, 20). Here we focus on the syndinean genus *Euduboscquella*, which, based on SSU rRNA phylogeny, belongs within clade I of the seven previously defined large-scale syndinean or marine alveolate clades (17, 20, 33).

The *Euduboscquella* life cycle involves a trophic intracellular growth phase emerging from the host cell and forming an elongating chain of rapidly dividing cells. After division is complete, different types of spores, including both motile and nonmotile spores, may be produced, although individual infections only produce one type of spore (4, 12a). For example, the recently described species *E. crenulata* produces two differently sized nonmotile spores and one motile spore, while *E. anisospora* produces two differently sized motile spores.

For the eight described *Euduboscquella* species, the most common hosts are tintinnid ciliates, although two species infect dinoflagellates and other protists may also act as hosts (4, 5, 10, 12a, 47). The tintinnid ciliates are attached to and surrounded by a lorica or shell, often with a wineglass- or bell-shaped outline (27).

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The lorica has distinctive features useful for host genus- or species-level taxonomy and also prevents the extracellular stages of the parasite life cycle from dispersing until late stages of infection. Here we describe the diversity and phylogeny of the genus *Euduboscquella* found within tintinnid ciliates as determined by the use of single-cell PCR for rRNA on field-collected material. A host phylogeny was simultaneously developed to compare host and parasite diversity characteristics.

MATERIALS AND METHODS

Sampling. Plankton samples were collected using a 35- μm -pore-size plankton net at the water surface and were kept cool ($\sim 15^\circ\text{C}$) during transport (see Table S1 in the supplemental material for site locations and dates). The samples were observed live with a dissecting or compound microscope for infected hosts and also fixed with Lugol's iodine to final concentrations of 0.04% (wt/vol) iodine and 0.06% (wt/vol) potassium iodide. Individual cells were picked with drawn glass capillaries and photographed with a digital camera mounted on a compound microscope at $\times 200$ to $\times 400$ magnification. Whenever possible, multiple replicate individual cells of the same morphotype were selected for amplification and sequencing. Host loricae were measured using Zeiss Axiocam and Axiovision software (Carl Zeiss Inc., Thornwood, NY). Whenever possible, 30 replicate host loricae from each ciliate morphotype were measured. Hosts were identified to the genus or species level, using the primary literature and assistance of S. Agatha (Fachbereich Organismische Biologie, Universität Salzburg).

Single-cell isolation. For ciliates with apparent *Euduboscquella* infections, the hosts could often be dislodged from the lorica by repeated pipetting, with the developing parasite spores remaining behind in the lorica. Individual ciliate loricae were prepared by transferring each lorica through at least five drops of 0.45- μm -pore-filtered water under a dissecting microscope using drawn glass pipettes. For live cells, filtered site water was used, and for Lugol's iodine-fixed cells, filtered deionized water was used. The individual loricae containing either the parasite or host were then placed into a 1.5-ml centrifuge tube in a minimal volume (1 to 3 μl) of water and kept frozen.

Cell disruption. Tubes containing individual host loricae were centrifuged at $10,000 \times g$ for 1 min, and 40 μl of deionized UV-treated water was added to each tube. The cells were disrupted using a probe-tipped sonicator set to a 30% duty cycle and a power level of three with 3 pulses over 5 s (model W-225R; Heat Systems Ultrasonics Inc., Plainview, NY). Between samplings, the probe was rinsed with a 10% bleach solution for 30 s, rinsed with UV-treated deionized water, and dried with a lint-free tissue. Blank samples containing only water were interleaved after every third experimental sample, sonicated, and carried through the PCR in parallel with the experimental samples as negative controls. The sonicate was used as a template for PCR without further purification and contained sufficient volume for ~ 8 individual reactions.

PCR methods. The amplification reaction mixtures contained bovine serum albumin (Sigma A2053) (500 mg/ml), 50 mM Tris-HCl (Sigma T5128) at a pH of 8.3, 3 mM MgCl_2 , 10 μM deoxynucleoside triphosphates (dNTPs) (Promega, Madison, WI) (2.5 μM each nucleotide), 0.2 μM of each primer, and 0.12 U of GoTaq DNA polymerase (Promega) in a total volume of 20 μl , of which 4 μl was template. After initial denaturation at 94°C for 2 min, the reactions were run for 40 cycles of 94°C denaturing for 15 s, 55°C annealing for 15 s, and 90 s of extension at 72°C . For rRNA amplification, three partially overlapping primer pairs were used (see Table S2 in the supplemental material) as follows: EukA was paired with EukB for the SSU; a modified dinoflagellate-specific primer, DinoQ, from the end of the SSU was paired with a general large subunit (LSU) primer (25R1) for the region composed of the internal transcribed spacer (ITS) to the LSU; and, finally, a third general primer pair, 25F1 and LSU2, was used to amplify the remaining LSU region. In several cases, taxon-specific primers were designed based on preliminary sequence data in order to obtain good-quality sequences (see Table S2 in the supplemental material). No nested PCR was done. PCR products were subjected to

electrophoresis using 1% (wt/vol) agarose gels and Tris-acetate EDTA buffer, stained with ethidium bromide, and visualized using a GelDoc UV transilluminator and imager (Bio-Rad, Hercules, CA). Successful reactions were purified using polyethylene glycol precipitation (34). Sequencing reactions were run using a 10- μl volume and BigDye Terminator master mix (version 3.1; Life Technologies, Carlsbad, CA) and an Applied Biosystems 3730XL DNA analyzer according to the manufacturer's directions. For SSU rRNA sequencing, 6 primers were used, and for the remaining regions, an additional 6 primers were used (see Table S2 in the supplemental material).

Cloning. For cloning of parasite ITS and other rRNA regions, high-fidelity *Pfu* Turbo (Stratagene, Hercules, CA) or Phusion (Fermentas, Glen Burnie, MD) polymerase was used with the reaction buffer supplied with the enzyme; the dNTP, primer, template, reaction volume, and thermocycling conditions were as described above. Cloning and transformation were done using ClonJET vector (Fermentas) and JM109 (Promega)- or Invitrogen TOP10 (Life Technologies, Carlsbad, CA)-competent cells according to manufacturer directions. The resulting individual bacterial colonies were added into 50 μl of 10 mM Tris (pH 8.0)–1 mM EDTA buffer, which was heated to 94°C for 5 min, followed by 5 min of centrifugation at $3,000 \times g$. Bacterial lysate (2 μl) was used as a template with pJET primers in a 20- μl PCR reaction mixture (Fermentas) for 30 cycles, with master mix, cycling conditions, PCR cleanup, and sequencing as described above.

The sequences were processed with Sequencher 4.10 software (Gene Codes, Ann Arbor, MI) to assemble and edit individual sequence reads into contiguous double-stranded sequences. The edited sequences were exported from Sequencher in FASTA format and manipulated in MacClade software (32). The nonredundant rRNA sequences from directly sequenced *Euduboscquella* spp., cloned *Euduboscquella* rRNA sequences, and novel ciliate SSU rRNA sequences have been deposited in GenBank (see below for accession numbers).

Host cell phylogeny. For host sequences, all 61 SSU rRNA sequences for 41 different "tintinnida" (as circumscribed by GenBank) were downloaded. The sequences were aligned with Clustal W software (29), and the alignment was further refined manually with MacClade software. Positions were removed using Gblocks at different stringency levels to observe the overall effect on the number of useable sites and support for specific branches (6) (data not shown). Only SSU rRNA sequences were used for analysis of host relationships. For phylogenetic analysis, RAxML in the general time-reversible model with gamma correction and 100 bootstrap replicates was used (44).

Parasite phylogeny determined using SSU rRNA. The large number of variable-quality environmental clone library sequences required a subsampling strategy. Analyses were performed using data sets with from 57 to 692 sequences from a starting group I syndinean SSU rRNA data set of 1,258 sequences (17). Pairwise comparisons of all the group I sequences were calculated using BLASTn. The BLAST results were parsed using a PERL script based on two criteria. First, only sequences that were redundant over 90% of the length were kept. Sequences were considered redundant at two stringency levels, allowing either two or five bases of difference. Second, three different size cutoffs (700, 1,000, and 1,400 bases) were used to exclude sequences. The two parameters created a matrix of six different nested subsampled data sets containing only sequences found at least twice in environmental clone libraries (3 sequence lengths \times 2 identity cutoffs). The individual data sets were then aligned using MUSCLE software (14) and analyzed with RAxML using 100 bootstrap replicates and the general time-reversible model with gamma rate correction (44).

A second set of analyses with a more inclusive approach included singleton (unique) sequences. In this larger data set, duplicate sequences within two bases were eliminated as redundant and all sequences greater than 690 bases in length were kept, producing a data set with 700 nonredundant sequences. For alignment calculation of this large data set, the sequences were initially adjusted for length at the 5' end. First, BLAST was used to align all sequences to the longest sequence (EU304548) as a reference and a PERL script was used to introduce the 5' gap needed to make an initial alignment of the 5' end to the reference sequence. Alignment with-

out this adjustment was otherwise hampered, probably due to large differences in sequence sizes (from 690 bases to 6,000 bases) as well as large internal gaps in some of the sequences. After the initial 5' alignment to the reference sequence, MUSCLE software was used to refine the alignment with 4 iterations, repeating the process three times and checking the alignment for changes after each repeat. The alignment was then iteratively checked using the RAxML tree as a guide to examine long branches for alignment artifacts. This was repeated four times. Eight sequences were excluded during this process due to extreme branch length, poor coverage across the alignment, or multiple unique insertions in the alignment. After the first alignment iteration, the data set was trimmed to include only the SSU region. The final data set was ultimately subjected to RAxML analysis with the general time-reversible model with gamma correction as described above but with a total of 500 bootstrap replicates.

ITS2 secondary structure for *Euduboscquella*. Comparing the relatively conserved 5.8S, SSU, and LSU regions on either side of the more divergent ITS regions provided a rough estimate of the beginning and end of ITS1 and -2. Secondary structures were generated using mfold software with default parameters and putative ITS2 regions (49). The exact borders of the ITS2 region were then identified based on the base pairing between the end of the 5.8S and the start of the LSU region flanking the ITS2 (16, 23). When several structures were generated by the use of mfold software, comparisons were made between structures from closely related taxa to determine a common structure for the ITS2 in that clade. A query against the ITS2 database using the hidden markov model from the ITS2 database was also used but did not return a match (23). Compensatory base changes were identified based on predicted structures, and the alignment was adjusted to reflect the secondary structure.

Nucleotide sequence accession numbers. The nonredundant rRNA sequences from directly sequenced *Euduboscquella* spp. were deposited in GenBank under accession no. JN934984 to JN934997, while cloned *Euduboscquella* rRNA sequences had the following accession numbers: JN934998 to JN935003 and JN966947 to JN966980. Novel ciliate SSU rRNA sequences were deposited under GenBank accession no. JN871720 to JN871726.

RESULTS

Host morphometry. Morphological data used to identify host species are presented in Table S1 in the supplemental material. Based on lorica attributes, hosts belonged to the genus *Eutintinnus* (two species), *Favella* (three species), or *Tintinnopsis* (four species) (27) (Fig. 1). The *Eutintinnus* and *Favella* species were identified to the species level. Two of the four *Tintinnopsis* morphospecies were from the Rhode River, MD; one was tentatively identified as *T. cf. subacuta* (a *Tintinnopsis* species that looks like *T. subacuta*), and a second (referred to here as *Tintinnopsis* sp. from the Rhode River) was identified only to the genus level. A third *Tintinnopsis* species from Annapolis harbor, MD, was identified as *T. major*, and the fourth *Tintinnopsis* morphotype based on a specimen from Villefranche-sur-Mer, France, was identified only to the genus level.

Ciliate phylogeny. Amplification and sequencing of ribosomal DNA from single-host ciliate cells yielded unambiguous sequences with good matches to ciliates in BLASTn searches, although apparent prey sequences were amplified in a few cases (data not shown). In this study, all SSU rRNA sequences from replicate individuals of the same ciliate morphotype were identical. For example, *Favella panamensis* yielded a single specific SSU rRNA sequence when different individuals were sampled from the same location and when individuals from different locations such as Masan Bay, Republic of Korea (Fig. 1H), or the U.S. Atlantic coast (Fig. 1D to G) were compared; overall, the SSU rRNA sequences for nine individual *F. panamensis* isolates from 5 sites

were identical. In addition, two *Tintinnopsis* morphotypes from a single sample, one tentatively identified as *T. cf. subacuta* (Fig. 1K) and the second as *Tintinnopsis* sp. (Fig. 1J), yielded identical SSU rRNA sequences from eight individual cells. Only one ciliate host, identified as *Tintinnopsis major*, was not subjected to molecular analysis due to an insufficient sample.

Comparison of the ciliate host sequences to GenBank sequences by the use of BLASTn often yielded very close matches, although an exact identity between novel and GenBank sequences was found only in the case of *Favella ehrenbergii* and *F. panamensis* (AY143572), which may be synonymous (26). In other cases, such as that of *E. pectinis*, very similar sequences (4 base differences across 1,685 sites) attributed to the same species were found in GenBank. In one case, for the host identified here as *Favella arcuata*, collected from Assawoman Bay, MD, the most similar sequences in GenBank were associated with three genera, *Rhabdonella* (AY143566), *Metacylis*, (AY143567 and AY143568), and *Favella* (AF399162 and FJ196073) (30, 43, 46).

For host phylogenetic analysis, the 61 SSU rRNA sequences from tintinnida in GenBank were reduced to 39 nonredundant sequences by eliminating redundant identical or nearly identical sequences from the same species and by including the six novel sequences from this study. The ciliate host phylogeny clearly distinguishes between four well-supported clades: *Eutintinnus* (100% bootstrap), *Tintinnidium* (and others; 100%), *Favella* (pro parte; 100%), and a large, less-well-supported "*Tintinnopsis*" clade (87%) that includes sequences attributed to 7 or 8 genera (Fig. 2). There was poor support within the broadly defined *Tintinnopsis* clade, with multiple polyphyletic genera, and at least one sequence attributed to the genus *Tintinnopsis* (DQ487200) was outside this clade (30, 43, 45, 46).

Basic morphology of parasites. The parasites exhibited development typical of the genus *Euduboscquella*, with parasites often found after emergence from the host or in late stages of sporogenesis, sometimes with the host remaining (Fig. 1). The characteristic chain of dividing cells (Fig. 1D, F, G, J, and L) or the mass of spores (Fig. 1A to C, E, H, and I) was readily identified as representing *Euduboscquella* parasites. The species *E. crenulata* was identified based on both trophont morphology and sporogenic products. Similarly, the complete host engulfment as well as sporogenic division patterns of *E. cachoni* were features diagnostic of the species. The remaining parasites were identified only to the genus level as *Euduboscquella*.

Syndinean group I SSU phylogeny. Sequences attributed to syndineans have been classified into seven major groups (I to VII), and *Euduboscquella* was placed within syndinean group I, which was in turn divided into clades 1 to 8 in a previous study (17) (Fig. 3). Within group I, the only clades with sequences attributed to described species or genera were of clade 3 with *Ichthyodinium* and clade 4 with *Euduboscquella*.

Here, a total of nine different group I data sets were analyzed, subsampling the total available environmental clone library data. In six of the nine data sets, only duplicate sequences were used to avoid artifacts (Table 1, Fig. 3). Generally, as more sequences were included, most of the clades were well supported and consistent across the different analyses. Clades 2 and 4 consistently had 100% bootstrap support in all 9 different data sets. Clades 1 and 5 were generally well supported (94 to 100% support), and within both clades, two well-supported clades were found. Similarly, when multiple sequences were present, clades 6, 7, and 8 had high sup-

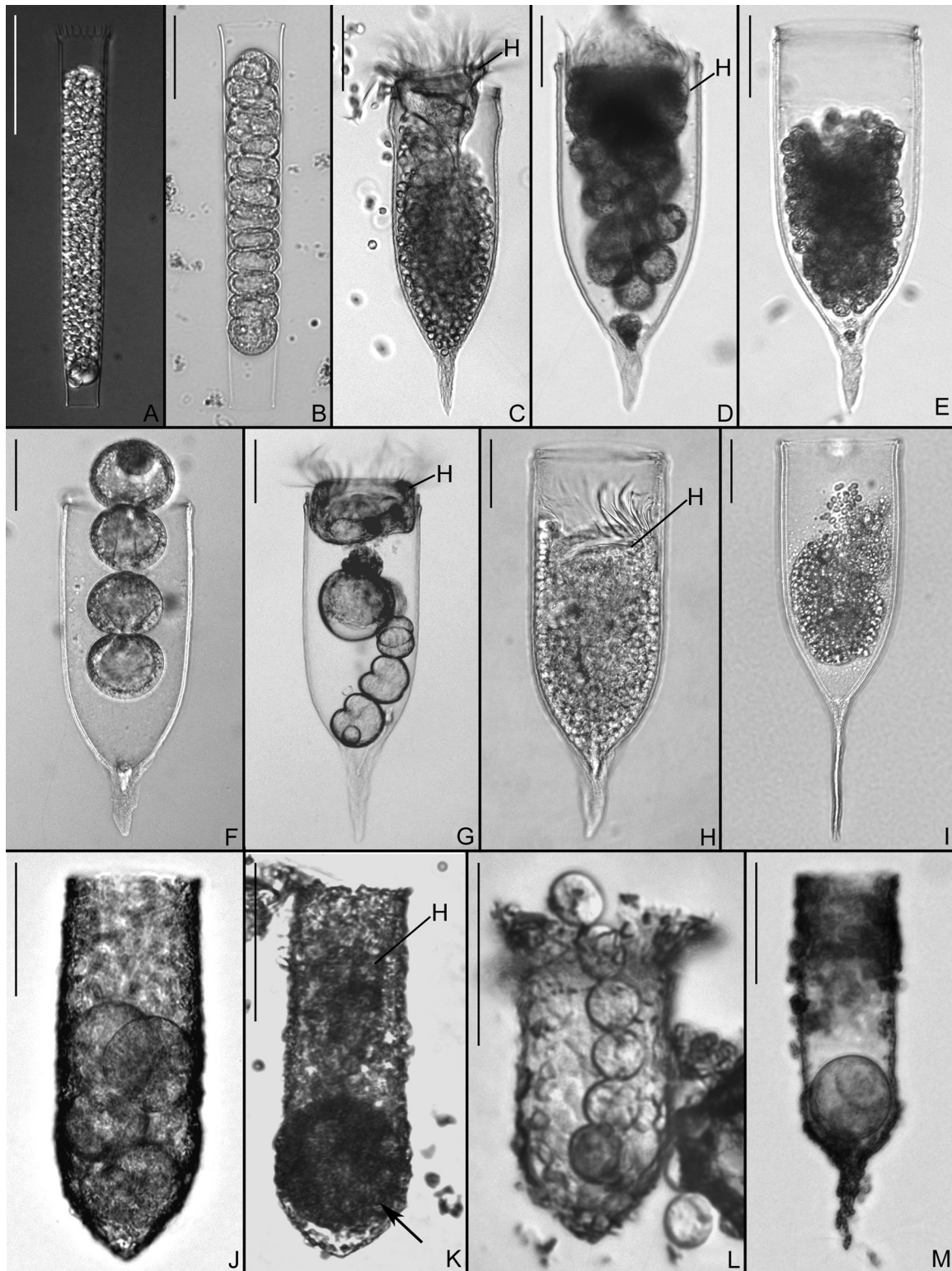


FIG 1 Representative images of host species and parasites for which rRNA sequences were obtained. Bars, 50 μm . (A) *Eutintinnus pectinis* lorica filled with sporocytes of *Euduboscquella cachoni*. (B) Lorica of *Eutintinnus tenuis* containing *Euduboscquella cachoni* in early sporogenesis. (C) *Favella arcuata* lorica, with sporocytes of *Euduboscquella* sp. located below host cell (H). (D) *Favella panamensis* from Assawoman Bay, MD, with host cell (H) positioned above sporocytes of *Euduboscquella crenulata*. (E) Lorica of *F. panamensis* from Indian River Lagoon, FL, housing sporocytes of *Euduboscquella* cf. *crenulata*. (F) *Favella panamensis* lorica from Solomons Island, MD, with *Euduboscquella* cf. *crenulata* in a second sporogenic division. (G) Host cell (H) and sporocytes of *Euduboscquella* cf. *crenulata* in *F. panamensis* lorica from York River, MD. (H) *F. panamensis* from Masan Bay, Republic of Korea, with numerous sporocytes of *Euduboscquella* cf. *crenulata* posterior to host cell (H). (I) Lorica of *Favella markusovszkyi*, with a mass of sporocytes from *Euduboscquella* sp. (J) *Tintinnopsis* sp. lorica from Rhode River, MD, containing four sporocytes of *Euduboscquella* sp. (K) Lorica of *Tintinnopsis* cf. *subacuta* from Rhode River, MD, with a tomont of *Euduboscquella* sp. (arrow) located below host cell (H). (L) *Tintinnopsis major* lorica from Annapolis Harbor, MD, containing *Euduboscquella* sp. sporocytes. (M) Lorica of *Tintinnopsis* sp. from the Bay of Villefranche-sur-Mer, France, harboring a tomont of *Euduboscquella* sp.

Host phylogeny

Parasite phylogeny

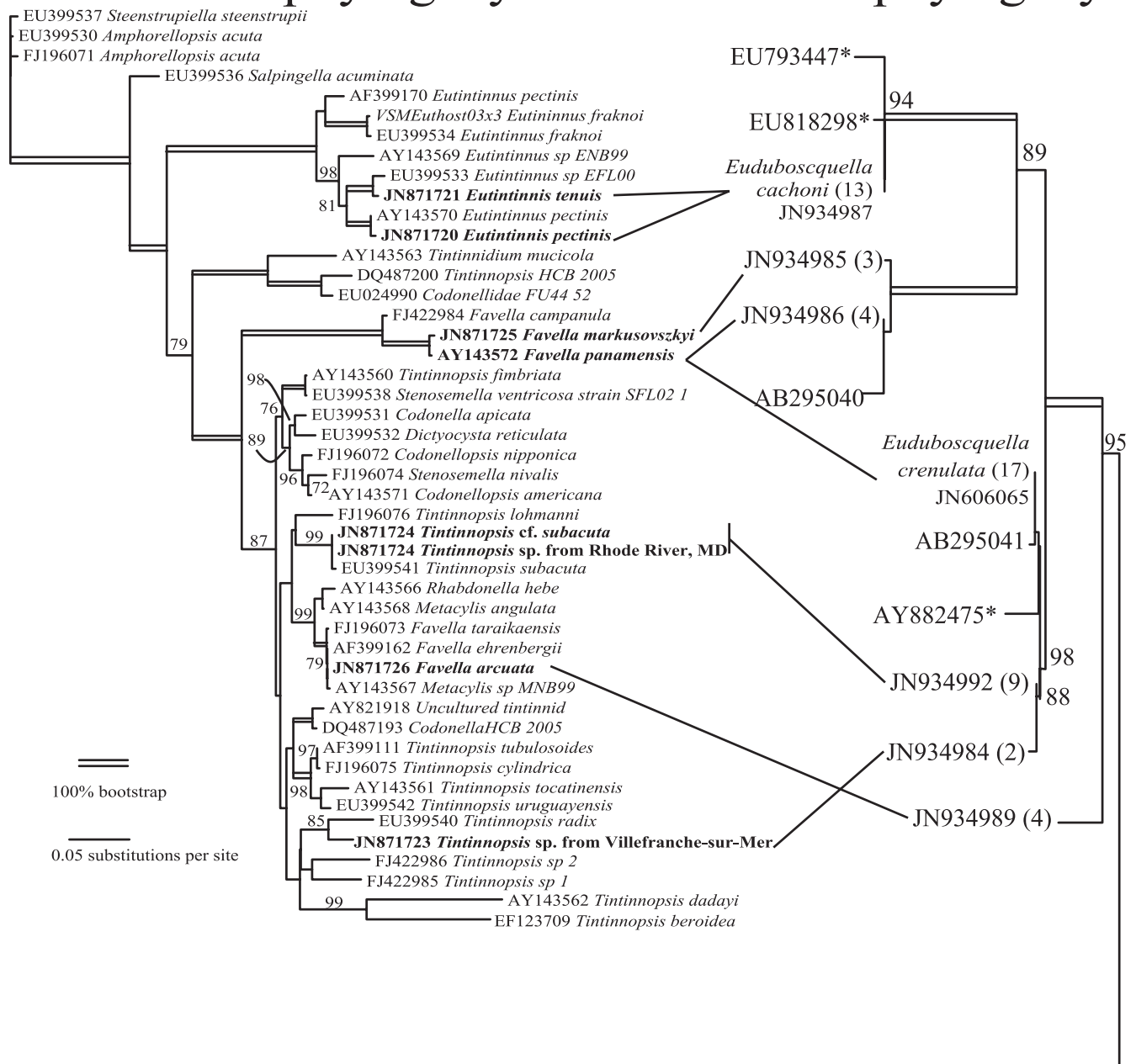


FIG 2 Comparison of host and parasite SSU rRNA phylogenies. On the left, the maximum-likelihood phylogeny for the tintinnid hosts is shown, with the novel sequences in boldface type. On the right is a portion of the corresponding phylogeny for the parasite, showing only the *Euduboscquella* clade (see Fig. 3 for the complete phylogeny), with lines indicating the hosts. Next to the parasite accession numbers are the numbers of cells for which identical SSU sequences were determined. Bootstrap values are shown above the branches when greater than 70%.

port (100%). Clade 3 was consistently poorly supported (maximum of 63% bootstrap) and was not monophyletic in some analyses, as was previously reported (17). More broadly, clades 3, 4, 6, and 8 together consistently formed a supported monophyletic group (79 to 100% bootstrap), although the relationships between clades 1, 2, 5, and 7 and the clade combining 3, 4, 6, and 8 were not consistent. In the largest, most inclusive data set, with 694 sequences of at least 690 bases, clades 1 and 5, each containing over 200 sequences, were the most common, followed by clade 4 with

85 sequences and clade 3 with 41. Clades 2, 6, and 7 were much smaller, containing at most 8 to 11 sequences (Fig. 3; see also Table S1 in the supplemental material).

Clade *Euduboscquella*. The *Euduboscquella* clade was consistent but often not well supported in the different analyses (55 to 91% bootstrap) and was always embedded within clade 4 (Fig. 2 and 3). Within the *Euduboscquella* clade, there were four different groups. First, the *Euduboscquella* parasite of *F. arcuata* from Assawoman Bay (Fig. 3) was most distant, even

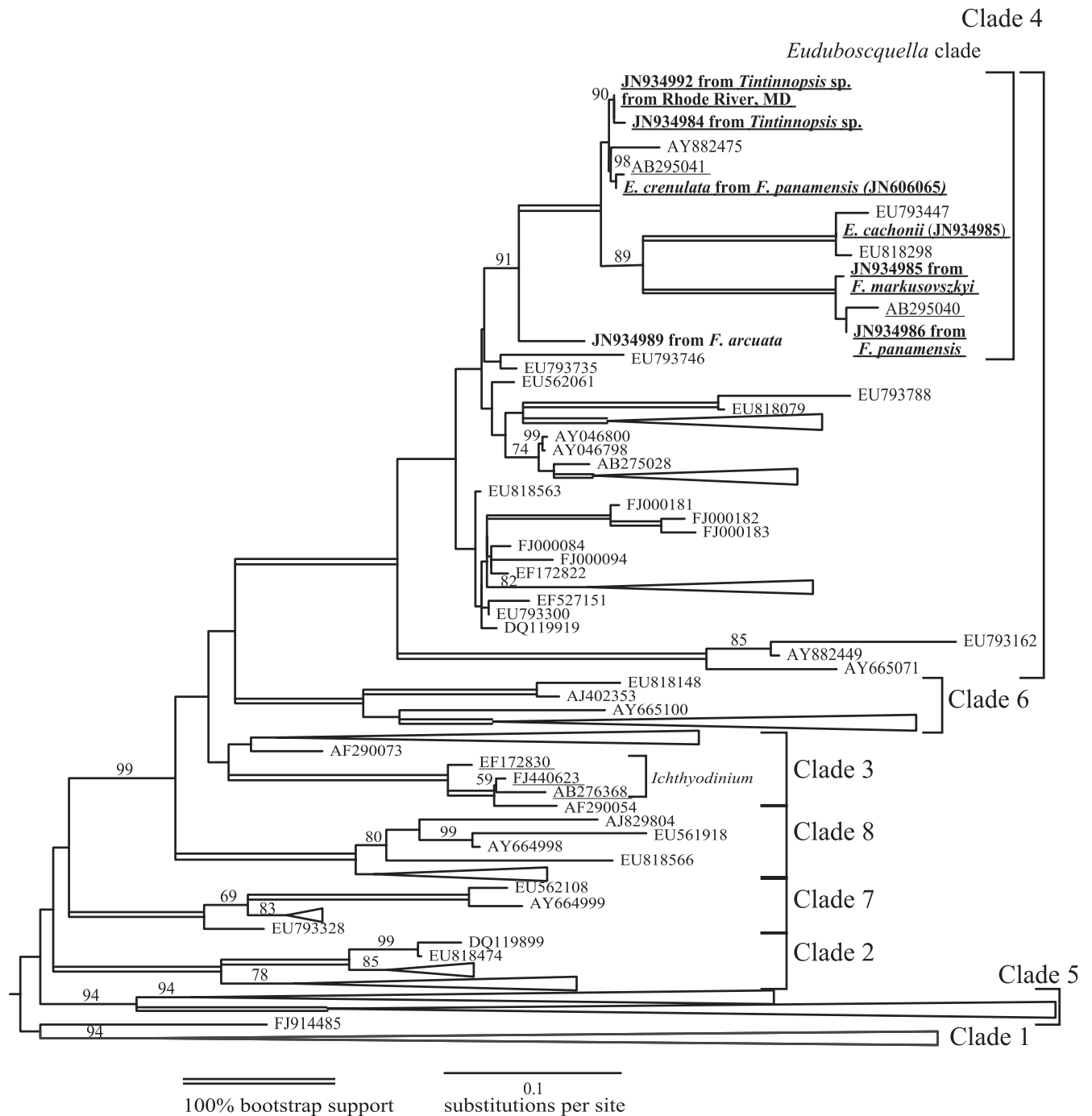


FIG 3 Maximum-likelihood phylogeny of syndinean group I based on SSU rRNA. This tree contains 694 SSU sequences, of which 593 were from environmental clone libraries. These sequences were selected from a total of 1,258 based on a length of >690 bases, and redundant sequences (within two bases) were removed. Clade labels were based on data from reference 17. The two clades containing sequences attributed to *Eudubosquella* and *Ichthyodinium* were the only sequences from identified taxa, and those GenBank numbers are underlined. Bootstrap values greater than 60% are shown above the branches. See the supplemental material for an uncompressed version of the tree in Newick format.

branching with 20 environmental sequences in one analysis but most often forming an outgroup to the *Eudubosquella* clade. The second *Eudubosquella* clade comprised a cluster of five very similar sequences, three from this study (SERC39, VSM21, and *E. crenulata* from *F. panamensis*), one attributed to *Dubosquella* in a previous study, and a single environmental se-

quence. The third and fourth clades each had three very similar members; one included *E. cachoni* (Fig. 1A and B) and two environmental sequences and the second two novel sequences and a sequence attributed to *Dubosquella* in a previous study. The relationships between these four *Eudubosquella* clades were well supported in most SSU analyses (Table 1).

TABLE 1 Bootstrap support for different syndinean group I SSU rRNA datasets with maximum likelihood analysis

Minimum sequence size (bp) ^a	Redundancy ^b	Total no. of sequences	Clade 1		Clade 2		Clade 3		Clade 4		Clade 5		Clade 6		Clade 7		Clade 8		Unassigned	<i>Euduboscquella</i> (% support)	% bootstrap support (clades 3, 4, 6, and 8)
			% support	No. of sequences	% support	No. of sequences	% support	No. of sequences	% support	No. of sequences	% support	No. of sequences	% support	No. of sequences	% support	No. of sequences	% support	No. of sequences			
700	2	113	100	25	100	3	10	5	100	13	100	10	NA	1	NA	0	NA	0	0	78	100
100	2	73	100	31	100	4	NA	6	100	19	100	12	NA	1	NA	0	NA	0	0	55	100
1,400	2	57	100	49	100	5	42	6	100	22	100	30	NA	1	NA	1	NA	0	0	56	100
700	5	170	100	65	100	9	31	7	100	30	100	52	100	3	100	2	NA	1	1	81	95
1,000	5	110	100	39	100	7	16	7	100	16	100	23	100	3	NA	1	NA	1	0	65	79
1,400	5	87	100	29	100	7	22	6	100	21	100	20	100	2	NA	1	NA	1	0	74	92
1,400	NA ^c	200	100	88	100	10	NA	19	100	24	100	49	100	7	NA	1	100	2	1	100 ^d	99
690	NA	696	81	282	100	24	63	41	100	85	95	224	100	10	100	8	100	11	11	73	100
690	NA	692	94	279	100	24	13	41	100	85	94	223	100	10	100	8	100	11	11	91	99

^a Sequences shorter than the cutoff were excluded.
^b Data represent the number of allowable nucleotide differences allowed between redundant sequences.
^c NA, only sequences differing by 2 or more bases were retained.
^d Data include 20 environmental clone sequences.

Sequence variability between and within individual parasite cells.

Sequence variability within and between individuals was present. Replicate rRNA sequences between parasites from the same host species and from same location often contained ambiguous sites in the ITS1 (0 to 15 sites), ITS2 (1 to 10), and LSU (0 to 5) regions (Table 2; see also the supplemental material). As an example of a parasite with little variation, two parasitized *Tintinnopsis* spp. were found in a single sample from Villefranche-sur-Mer, France (Fig. 1M), and there was variation between the sequences from these two individuals. In the pairwise comparison, there were a total of 7 differences: none in the SSU, four in ITS1, three in ITS2, and none in the partial LSU. However, two of the ITS1 differences were ambiguous bases (double peaks) from one individual, suggesting a degree of sequence polymorphism within an individual.

Overall, cloning of rRNA amplicons was used to estimate polymorphism or sequence variation within an individual for 8 different individual infected hosts from five different ciliate hosts. Polymorphic sites were considered only when sequences from two or more clones or direct sequences shared a nucleotide change; variation found in only a single cloned sequence was not considered. Using rRNA from the *Euduboscquella* sp. found in *Favella arcuata* from Assawoman Bay (JN934989 in Fig. 2 and 3), a 2,955-base amplicon from the SSU to the LSU was generated and cloned from two individual loricae from the same site collected 1 week apart. Six clones were sequenced from each individual. The sequences differed by zero bases or one base in ITS1, one to six bases in ITS2, and two bases in the LSU, with a total of nine and six polymorphic sites in ITS-to-LSU sequences from these two individuals. Compared to results determined for two directly sequenced individual

TABLE 2 Nucleotide changes in different regions based on comparisons of individual sequences and cloning

Cell category and site	Host	No. of cells	No. of sequences	No. of nucleotide changes ^b			
				ITS1	ITS2	LSU	Total
Variability between individual parasite cells							
U.S. Atlantic coast	<i>Favella panamensis</i>	17	17	3	2	1	6
Villefranche-sur-Mer, France	<i>Favella markusovszkyi</i>	3	3	0	2	3	5
Masan Bay, Republic of Korea	<i>Favella panamensis</i>	4	4	1	1	1	3
Villefranche-sur-Mer, France	<i>Tintinnopsis</i> sp.	2	2	4	3	0	7
Rhode River, MD	<i>T. cf. subacuta</i>	4	4	6	9	4	19
Rhode River, MD	<i>Tintinnopsis</i> sp.	4	4	5	6	5	16
Assawoman Bay, MD	<i>Favella arcuata</i>	4	14	1	6	3	10
Rhode River, MD	<i>Eutintinnus tenuis</i>	5	35	15 (5)	10 (5)	3	28 (10)
Annapolis harbor, MD	<i>Eutintinnus pectinis</i>	2	27	8 (4)	9 (3)	0	17 (7)
Variability within individual parasite cells							
Assawoman Bay	<i>Favella arcuata</i>	1	6 ^a	1	6	2	9
Assawoman Bay	<i>Favella arcuata</i>	1	6 ^a	0	1	2	3
Rhode River	<i>T. cf. subacuta</i>	1	24 ^a	1	0	3	4
Rhode River	<i>Eutintinnus tenuis</i>	1	24 ^a	12 (4)	8 (4)	3	20 (4)
Annapolis Harbor	<i>Eutintinnus pectinis</i>	1	22 ^a	3 (2)	9 (2)	0	12 (4)

^a Number of clones sequenced.
^b Numbers in parentheses represent inferred insertions and deletions in the alignment.

TABLE 3 Compensatory and total nucleotide changes (above and below diagonal line, respectively) in the ITS2 region for a selected *Euduboscquella* clade

Host	Total no. of individual cells with identical ITS2 ribotypes in parasite from indicated host ^a												
	<i>F. panamensis</i> ^b		<i>Tintinnopsis</i> sp. from Villefranche-sur-Mer, France ^b		<i>Tintinnopsis</i> sp. from Rhode River, MD			<i>T. cf. subacuta</i>				<i>T. major</i>	
	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.	accession no.
	JN606065 (n = 10) ^b	JN606065 (n = 1)	JN606065 (n = 1)	JN934984 (n = 1)	JN934984 (n = 1)	JN934994 (n = 1)	JN934992 (n = 1)	JN934996 (n = 1)	JN934990 (n = 1)	JN934997 (n = 2)	JN934995 (n = 1)	JN934991 (n = 1)	JN934993 (n = 1)
CBC7	0	0	3	3	4	4	4	4	4	4	4	4	4 (2)
OC30	2	0	3	3	4	4	4	4	4	4	4	4	4 (2)
Well10N	1	1	3	3	4	4	4	4	4	4	4	4	4 (2)
VFSM22	24	26	25	0	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
VFSM21	24	26	25	4	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
SERC30	29	31	30	11	11	0	0	0	0	0	0	0	1
SERC39	29	31	30	15	15	8	0	0	0	0	0	0	1
SERC65	30	32	31	12	12	7	11	0	0	0	0	0	1
SERC62	27	29	28	11	11	6	8	9	0	0	0	0	1
SERC37	28	30	29	11	13	8	10	11	8	0	0	0	1
SERC66	25	27	26	11	11	6	10	9	4	10	0	0	1
SERC53	28	30	29	16	16	10	11	12	13	15	11	0	1
Ann30	25	27	26	11	11	4	8	7	6	8	6	11	

^a Compensatory and total nucleotide changes are shown above and below the diagonal line, respectively. Numbers in parentheses represent compensatory changes in the “stem” between 5.8S and LSU.

^b Single nonredundant sequences with ambiguous bases as needed were deposited in GenBank.

cells (Table 2), across all sequences from four individuals (two by direct sequencing and 12 cloned sequences), a total of 10 polymorphic sites were observed, eight of which were polymorphic according to comparisons of the two directly sequenced cells, and only two additional sites were found in the sequences from clones (Table 2). The sequence variation was restricted to single nucleotide polymorphism, and no insertions or deletions were detected. None of these base changes were compensatory across base-paired “stems” in the ITS2 or LSU regions.

E. cachoni in *Eutintinnus tenuis* and *E. pectinis* had a larger degree of sequence variation in the ITS region within an individual cell. Direct sequencing of SSU, ITS, and LSU PCR products was attempted using parasites from 10 individual parasitized loricae, and only two from *E. tenuis* produced interpretable contiguous sequences across the ITS region. However, for all 10 individuals, SSU and partial LSU sequences were obtained, and the sequencing chromatograms implied length variation in the ITS regions. In *E. cachoni*, there was a single ambiguous base in the SSU region, with 6 sequences having a G and four having an ambiguous G or T at the site, although there was not a correlation with host species.

Sequences for cloned PCR products for *E. cachoni* revealed differences across the problematic ITS region from the end of the SSU rRNA to the LSU. Multiple sequences were obtained by cloning for five different host loricae containing *E. cachoni*: three from *E. tenuis* (Fig. 1A) and two from *E. pectinis* (Fig. 1B). For the five different loricae, 3, 4, 6, 24, and 22 clones were sequenced and compared to the two directly sequenced ITS PCR products from *E. tenuis*. Comparisons of only the 22 and 24 more deeply sequenced clones representing the two different hosts are presented in Table 2. Although the two host species, *E. tenuis* and *E. pectinis*, were clearly distinct, as determined on the basis of SSU comparisons and morphology (Fig. 1A and B, 2, and 3), for the

parasite, the sequence variation within an individual cell was as broad as the variation between individuals isolated from the two host species. In phylogenies determined using ITS regions as a whole or in part, sequences from parasites from the two different host species intermingled and did not form distinct clades (see Fig. S1 and S2 in the supplemental material). Variation occurred both between and within individual loricae, with up to 20 variable sites found in comparisons of clones from one individual, including two to four insertion deletion sites. Preliminary rarefaction (data not shown) suggested incomplete sampling of overall diversity. Across all 62 ITS-to-LSU regions for *E. cachoni* from 7 infected hosts found at two sites in two host species, the ITS1 region contained 5 instances of one to four base insertion deletions, as well as 16 polymorphic sites, and the ITS2 region contained 5 distinct insertion deletions and 16 polymorphic sites, while the LSU region contained 3 polymorphic sites. When mapped onto a provisional ITS2 structure, the sequence variation in the ITS2 region was mostly centered on the two “loops” at the end of stems III and IV; none of the sequence variations caused compensatory changes on both sides of a base-paired stem in ITS2.

In contrast to *E. cachoni*, the parasites from *Tintinnopsis* sp. (Fig. 1J) and *T. cf. subacuta* (Fig. 1K) from the Rhode River, MD, had little apparent sequence variation within an individual cell but relatively large variation between individuals (Table 2 and Table 3). These two *Tintinnopsis* host morphotypes yielded identical parasite SSU rRNA sequences in all 8 cases (3 from *Tintinnopsis* sp. and 5 from *T. cf. subacuta*), as well as identical host SSU sequences (6 from *T. cf. subacuta* and 2 from *Tintinnopsis* sp.). The parasite SSU sequences were also identical to the sequence amplified from a single *T. major* isolated from a nearby site about a month later, for which only a single parasitized host was found. Within the four parasite sequences from *T. cf. subacuta*, there were a total of 19

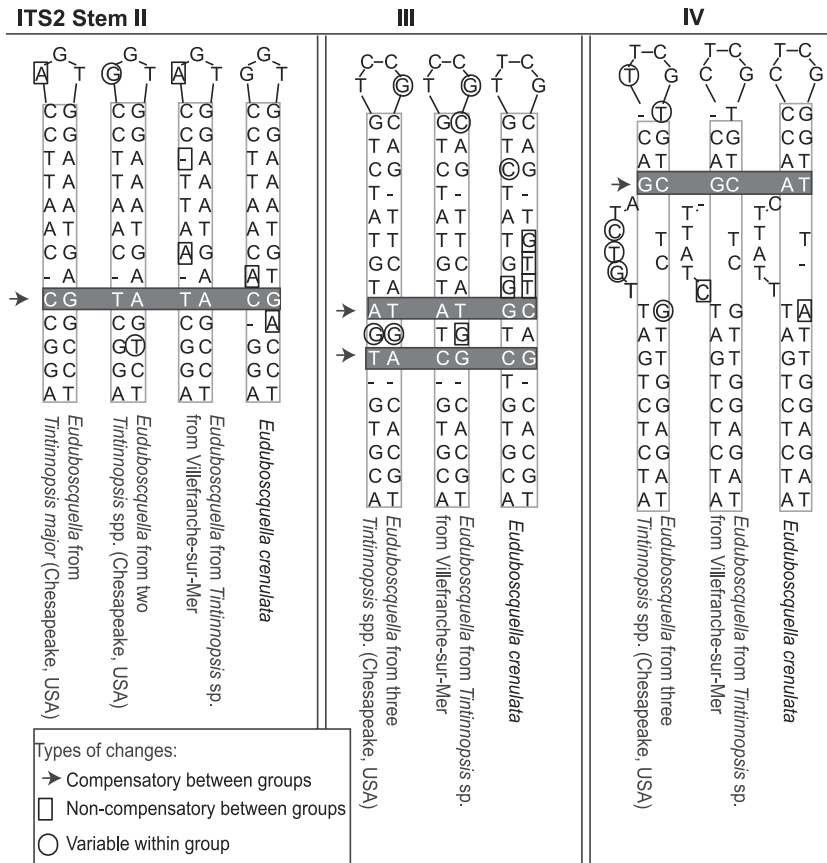


FIG 4 Alignments of homologous ITS2 regions of different *Euduboscquella* sequences. Compensatory changes in three different base-paired stems from the ITS2 of selected *Euduboscquella* species define four different parasite ITS2 ribotypes. Only stem II from the parasite from *Tintinnopsis major* isolated from Annapolis Harbor, MD, is shown. The sole ITS2 compensatory change between the sequence from the parasite from *T. major* isolated from Annapolis Harbor, MD, and the sequences from the other two *Tintinnopsis* host morphotypes isolates from the Rhode River, MD, was found in stem II. No compensatory changes between parasites from these three host morphotypes in stems III or IV were found. There was also a single compensatory change in stem III found for the parasites from the three *Tintinnopsis* hosts from the Chesapeake Bay, MD (*T. major*, *T. cf. subacuta*, and *Tintinnopsis* sp. from the Rhode River), and the parasite isolated from a fourth *Tintinnopsis* sp. from Villefranche-sur-Mer.

variable sites, while the four sequences from *Tintinnopsis* sp. from the Rhode River contained 16 variable sites (Table 2). Across all 9 individuals from the three different host morphotypes, there were a total of 27 variable sites in the parasite ITS-to-LSU regions over 1,415 bases. One individual from *T. cf. subacuta* was selected for cloning, and the 24 cloned ITS sequences showed little within-cell variation, with one variant site found in the ITS1, none in the ITS2, and three in the LSU region. While the variation within an individual cell was small, the variable sites discovered in cloning were novel compared with variable sites from single-cell sequencing. Overall, the variable ITS sequences from this parasite did not sort with host morphotype.

Compensatory base changes in the ITS2 region. The parasites from all four *Tintinnopsis* hosts formed a tight cluster with *E. crenulata* from *F. panamensis* in the SSU tree, making comparisons of provisional ITS2 alignments and structures within the group possible (Fig. 4). The typical base-paired stem between the end of the 5.8S and beginning of the LSU rRNA was clearly distinguishable, and the provisional ITS2 structures followed the general four-stem pattern (23). The ITS2 differences were sorted into compensatory and noncompensatory changes. Compensatory changes were seen when both nucleotides in base-paired stem re-

gions changed compared to noncompensatory changes occurring in single-stranded loops or on only one side of a stem (Table 3 and Fig. 4). Within this clade, there were 13 unique ITS2 ribotypes, 3 associated with *E. crenulata* in *F. panamensis*, 2 with the *Tintinnopsis* sp. host from Villefranche-sur-Mer, 7 from *Tintinnopsis* sp. and *T. cf. subacuta* hosts from the Rhode River, and 1 from *T. major* from Annapolis harbor. Overall, there were up to 30 ITS2 changes across all ribotypes, but most of the changes were non-compensatory. Between one and four compensatory base changes distinguished four groups of sequences, with each group having some noncompensatory variation: (i) *E. crenulata* with 2 noncompensatory changes across 3 ribotypes; (ii) the parasite of *Tintinnopsis* sp. from Villefranche-sur-Mer with 4 noncompensatory changes in the two ribotypes; (iii) the parasites from *T. cf. subacuta* and *Tintinnopsis* sp. from Rhode River, MD, with 4 to 15 noncompensatory changes among the 8 ribotypes; and (iv) the single *T. major* parasite that differed from the other *Tintinnopsis* species from the Rhode River by a single compensatory change in stem II. Overall, *E. crenulata* was more distantly related to the other parasites. Among these 13 ITS2 ribotypes, the unpaired loops at the end of the stem regions were most variable, with many noncompensatory changes.

There were a number of differences between the Masan Bay parasite from *F. ehrenbergii* and the parasite from *F. markusovszkyi* found in Villefranche-sur-Mer with respect to the ITS2 regions. Between these two parasites, there were only seven SSU differences (JN934985 versus JN934986) (Fig. 2 and 3), but there was a large 34-base extension of stem IV in the ITS2 from *F. ehrenbergii* in the Masan Bay parasite, and there were four compensatory changes in stems I to III among the *Euduboscquella* isolates from these two sites and hosts.

DISCUSSION

Syndinean group I phylogeny based on SSU rRNA. The reported diversity of syndinean dinoflagellates is largely based on data from environmental clone libraries, and here we compare results from directed sequencing of a specific syndinean genus, *Euduboscquella*. Sequences from environmental clone libraries may suffer from a number of potential artifacts, including amplification or sequencing errors leading to single nucleotide changes and chimeric amplicons combining partial sequences from two or more distinct organisms (3). Within the dinokaryotes, the divergence of rRNA genes has a broad range. In some dinoflagellates, multiple SSU rRNA sequences are present in a single individual such that multiple environmental clone sequences could be attributed to a single species (38). At the other extreme, there is an example of identical rRNA sequences (SSU through LSU) for two dinokaryote species (31). Determining whether a particular environmental clone represents the sequence from an organism in the environment can be difficult, particularly when reference sequences from described species are not present, as was often the case for syndineans.

Here we employed a graduated strategy using only duplicate sequences to estimate the phylogeny of syndinean group I and took advantage of a simple heuristic. Since a chimeric breakpoint would probably not happen at the same place twice, duplicated or redundant sequences would be less likely to be chimeric (28). Similarly, using differently sized sequences in the analysis might also alter the tree topology or support. However, the results from analysis performed with increasing numbers of sequences indicated that inclusion of unique and shorter sequences did not decrease bootstrap support or alter the tree topology. The phylogenies were quite stable across the range of sequence parameters. Overall, these results suggest that sequence artifacts have not created misleading clades in this group and, instead, that the environmental clone libraries represent genuine undiscovered species diversity in the ocean.

The syndinean group I SSU rRNA tree contains a number of diverse clades, often with well-supported branches, although the larger-scale relationships between clades 1, 2, 5, and 7 and the monophyletic group that includes clades 3, 4, 6, and 8 remain ambiguous. The SSU tree for syndinean group I strongly contrasts with the poorly resolved and unsupported relationships among dinokaryotic dinoflagellates based on SSU rRNA sequences (21, 36, 39). Within the *Euduboscquella* clade, there were very few environmental clone sequences and few overlaps between the two sampling strategies, and more individual cell sequencing and environmental clone libraries would be needed to exhaustively sample overall diversity of the group.

The single-cell PCR techniques used here were quite laborious, particularly compared to the bulk high-throughput sequencing of environmental clone data sets, and required infection diagnosis of

individual cells. However, the single-cell PCR technique allowed amplification of a large contiguous section of rRNA from the spore products of a single infected host. The variation within and between individuals could be estimated by comparing cloned sequences from an individual to sequences from multiple individuals parasitizing the same host. Simultaneously, diagnostic features could be used for genus or species level identification of both host and parasite.

Syndinean dinoflagellates have been distinguished from the other major dinoflagellate lineage, namely, the dinokaryotes, based primarily on nuclear features, with the dinokaryotes having high DNA content and chromosomes condensed during the interphase (15). Recently, the type for the genus *Duboscquella* was found to have dinokaryotic features, and so the remaining, truly syndinean tintinnid parasites were moved into the new genus *Euduboscquella* (12a). Although *Euduboscquella* has been most often described in studies of isolates from tintinnids, other dinoflagellate parasites of tintinnids include species of the genera *Duboscquodinium*, *Duboscquella*, and *Tintinnophagus*. In those three genera, extracellular growth, cell division, motile spores, and available rRNA sequence data indicated that these genera were dinokaryotic dinoflagellates and not syndineans (11, 12a).

All the parasite sequences described here can be assigned to the genus *Euduboscquella*. The genus *Euduboscquella* includes intracellular parasitic dinoflagellates that divide only after exiting the host cell and show characteristic features during the trophic growth stage (4, 8, 12a). The division products from a single infection produce either motile dinospores or nonmotile spores, although the full morphological variation within species remains poorly characterized (4, 9, 12a). In comparison, the syndinean genus *Amoebophrya*, found in syndinean clade II, produces a single type of spore and undergoes nuclear division inside the host cell (4, 5). Discrimination between different *Euduboscquella* species has often been based on specific trophont features found only during the intracellular life stage—features not accessible in many of the specimens seen here. Based on trophont and sporogenic stages, two species, *E. cachoni* and *E. crenulata*, were identified to the species level, although we hesitate to attribute species epithets to the remaining parasites. We chose to instead focus on describing the molecular diversity of the genus based on rRNA sequences.

Comparison of ITS2 changes. The very short branch lengths between individual parasite sequences based on SSU phylogenies required additional sequence data from the more rapidly evolving ITS regions to clearly resolve relationships. Although multiple different SSU rRNA gene copies are found in some dinoflagellate genera (38), in the genus *Euduboscquella*, SSU rRNA variation within an individual was not found in this study. However, it was first necessary to quantify and estimate the ITS diversity of different copies within individuals and then compare the differences between individuals.

In three of the SSU rRNA clades, including (i) *E. cachoni* infecting *Eutintinnus tenuis* and *E. pectinis*, (ii) the parasites of *Tintinnopsis* spp. and *E. crenulata* from *F. panamensis*, and, finally, (iii) the parasites of *F. panamensis* and *F. markusovszkyi*, there were parasite sequences attributed to two or more host species. The fourth, most distant SSU clade contained only the parasite from *F. arcuata*. Within these clades, ITS2 sequence variation could be mapped onto the secondary structure to apply the compensatory base change model to these data. This model views complementary changes to both sides of a base-paired stem as an

indication of a species barrier, based on the sexual incompatibility of individuals with different ITS2 structures (13) In the genus *Euduboscquella*, tests of the biological species concept based on sexual incompatibility and comparison to the compensatory model would be premature. However, the ITS2 compensatory change model is useful for describing molecular diversity within the genus and agrees well with the SSU-based tree.

In the case of *E. cachoni*, there was extensive sequence diversity (up to 20 sites) within individuals but no compensatory base changes between or within individuals were observed, suggesting that each individual contains multiple distinct rRNA gene copies. The sequence diversity within an individual was also greater than the diversity between individuals and between different host species, suggesting the presence of a single parasite species infecting two hosts.

Within the parasites of *Tintinnopsis* spp. and *E. crenulata* from *F. panamensis*, there were one to four compensatory base changes between (i) *E. crenulata* from *F. panamensis*, (ii) parasites of *Tintinnopsis* sp. from Villefranche-sur-Mer, (iii) parasites from *Tintinnopsis* sp. and *T. cf. subacuta* from the Rhode River, and (iv) *T. major* (a singleton sequence). The ITS2 compensatory base changes support the SSU phylogeny that defined three different subclades of *Euduboscquella* with very similar SSU sequences (*E. crenulata*, JN934984, and JN934992 on Fig. 2). The sequences attributed to *E. crenulata* were generated from multiple individuals isolated over 3 years from four distinct sites on the U.S. Atlantic coast, and yet, based on the rRNA sequence data, there was little variation in these parasite populations. In contrast, the parasite sequences from *Tintinnopsis* sp. and *T. cf. subacuta* were all generated from a single sample from the Rhode River, MD, and there was substantial ribosomal sequence diversity between individual cells, rather than variation within an individual, based on comparison to the cloned sequences. Since only two identical ITS2 sequences were found within this group, additional undescribed diversity in ITS ribotypes was likely, suggesting that the overall diversity was only partially sampled. Despite this variability, none of the changes were compensatory, again agreeing well with the compensatory base change model. Interestingly, there were no SSU sequence differences between the different host morphotypes. These two host morphotypes were here considered to represent two different species based on morphology, but the identical SSU sequences suggested that more molecular data would be required to distinguish the host species. Alternatively, the two host morphotypes may indeed belong to the same host species.

Similarly, according to the results of comparisons of the parasites of *F. panamensis* from Masan Bay, Republic of Korea, with the parasites from *F. markusovszkyi*, there were a number of distinct compensatory changes. The compensatory model seems to fit the data well; whether applied to variation within or between individuals, compensatory changes agreed well with subtle SSU sequence differences.

Comparing host and parasite phylogenies. Considering host and parasite phylogenies, the species and generic level taxonomy of both host and parasite may continue to change in the future (1, 2). However, comparing the existing host and parasite molecular phylogenies suggests three discordant patterns in the data. First, some parasites, such as *E. cachoni*, appear to infect multiple host species, although host preferences and prevalences were not explored. Second, a single host species, *F. panamensis*, was infected by two distinct parasites, *E. crenulata* from multiple sites in the

U.S. Atlantic coast and a different parasite found in the same host from Masan Bay, Republic of Korea. Finally, the similarity between *F. markusovszkyi* and *F. panamensis* hosts was mirrored by their parasites and might represent an example of host parasite coevolution. Model-based tests of coevolution would be premature, given the sparse sampling of diversity. Overall, these data suggest that discriminating *Euduboscquella* parasite species solely on the basis of host identification should be discouraged.

Comparison of syndinean clades I and II. The results for *Euduboscquella* from syndinean group I can be compared with the results for the genus *Amoebophrya* from syndinean group II. While some *Euduboscquella* seem to infect more than one host and more than one *Euduboscquella* may infect the same host species, very little work has been done on the genus *Euduboscquella* in comparison to *Amoebophrya*. Within the genus *Amoebophrya*, environmental and culture studies have suggested host parasite specificity in some cases (7, 12, 19, 22). However, there also seem to be cases where *Amoebophrya* may not be host specific (24, 37). In addition, while *Euduboscquella* species formed a specific clade within clade 4 of group I, sequences attributed to *Amoebophrya* were found in a large (in terms of both diversity and number) fraction of syndinean clade II. However, it remains to be seen whether these preliminary results truly capture the diversity of these different parasite genera or, more broadly, what features distinguish the organisms in syndinean clades I and II. Future work should help to reveal whether some of the more abundantly represented clades from environmental clone libraries include many unique parasite species.

However, estimating the overall diversity of syndineans, or of *Euduboscquella* and *Amoebophrya*, can only be described as a work in progress. The results presented here agree well with the idea that even subtle differences in SSU sequence may be correlated with distinct species at the ITS2 and ultimately at the morphological level, suggesting that the diversity of environmental clone sequences represents a diverse array of potential parasite species.

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