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ORIGINAL ARTICLE

Phylogeny and Genetic/Morphological Variation of *Strombidinopsis minima*-like Species (Ciliophora: Choreotrichia)

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ABSTRACT

Six isolates of mineral-enveloped *Strombidinopsis minima*-like species were collected from the coastal waters across several regions in Korea. Morphological observations and molecular analyses were performed. The ribosomal DNA sequences (including small subunit ribosomal DNA, internal transcriber spacer 1-5.8S ribosomal DNA-internal transcriber spacer 2; and part of large subunit ribosomal DNA) of these six isolates were compared. Their morphological characteristics were also compared with those of *S. minima* populations reported. The marked genetic differences (with a similarity range of 96.85–98.48%) in SSU rDNA among these *S. minima*-like entities suggest the existence of multiple species. This finding is also supported by morphological variations detected in this study and reported in the literature (e.g. 15–32 collar membranelles in different populations). In addition, *S. minima*-like species are clustered with *S. batos* and *S. sinicum*, and therefore, our SSU rDNA results support previous results suggesting that the genus *Strombidinopsis* is not monophyletic in origin. Further collection of morphological and molecular data may facilitate the determination of a new genus carrying mineral-enveloped *Strombidinopsis* species.

MEMBERS of the genus *Strombidinopsis* (Ciliophora, Choreotrichia) belonging to the aloricate choreotrichid ciliates have been frequently reported in marine and brackish water worldwide and can be an important part of the pelagic ciliate assemblage (Agatha 2011). *Strombidinopsis* is also a key phylogenetic taxon among the aloricate ciliates, representing a potentially ancestral lineage (Kim et al. 2005, 2010a). However, recent studies (Gao et al. 2016; Liu et al. 2016) have shown that *Strombidinopsis* is not monophyletic due to the discovery of a second clade belonging to this genus. Additionally, molecular studies have demonstrated the presence of genetically related species in this genus (Kim et al. 2010a).

Despite the comprehensive morphological studies related to these common taxa (Agatha 2003; Alekperov and Asadullayeva 1997; Lei et al. 1999; Liu et al. 2016; Lynn et al. 1991; Montagnes and Taylor 1994; Song and Bradbury 1998), the identification of distinct species is not facilitated due to similar and overlapping morphological characters. In recent years, sequencing strategies using molecular markers have enabled the identification and analysis of evolutionary relationships between lineages of ciliates. However, the small subunit ribosomal DNA (SSU rDNA) sequences are only available for four species of *Strombidinopsis* (Gao et al. 2009; Kim et al. 2005, 2010a; Liu et al. 2016).

Among the *Strombidinopsis* spp., *S. minima* is generally found in coastal and turbid waters (Agatha 2003), and is characterized by a mineral envelope comprising mineral particles and covering the whole body. However, the cell size (19–64 × 18–62 μm) and the number of collar membranelles (15–32) show significant differences among populations (Agatha 2003; Lei et al. 1999; Song and Bradbury 1998). Therefore, *S. minima* is either morphologically diverse, or distinct with species exhibiting established life stages. To establish the likelihood of either hypothesis, we collected the morphological characteristics and sequence data (SSU rDNA, ITS1-5.8S-ITS2, partial LSU rDNA) from six *S. minima*-like populations originating in three different locations in Korean coastal waters.

Table 1. Sample list and site information

Isolate	Collecting date	Water temp. (°C)	Salinity (psu)	Site
DG	Mar, 29. 2013	17.5	12.8	Donggeom Island (37°37′27″, 126°22′32″)
HS	May, 25. 2013	20	27.0–29.5	Hwangsan Island Pier (37°37′29″, 126°32′21″)
SC1	June, 18. 2015	30	30	Seocheon tidal flat (36°01′05″, 126°39′50″)
SC2	Oct. 8. 2015	20	30	Seocheon tidal pool 1 (36°01′05″, 126°39′50″)
SC3	Oct. 8. 2015	20	17.5	Seocheon tidal pool 2 (36°01′05″, 126°39′50″)

MATERIALS AND METHODS

Study site, sampling, cultivation, and isolation

Five samples were collected from three stations located in the tidal flats of western Korea during low tide (Table 1). The Hwangsan Island population was sampled using a 20-μm net. Other populations were collected directly from surface waters together with the bottom sediment using a culture flask. Samples (~40 ml each) were transferred to the laboratory and distributed into 10-cm Petri dishes. Autoclaved rice grains were added to the dishes to enrich the bacteria used as a food source for *Strombidium minima*. When the populations were abundant, the cells were isolated with a micropipette for taxonomic and molecular analyses. Each isolate was harvested for analysis within 15 d after sampling, with the exception of the Hwangsan Island population, which was harvested 2 mo after sampling.

Morphological observation

Cells were selected randomly from the Petri dishes under a dissecting microscope. The cells were visualized live using light microscopy at magnifications of 100X to 1,000X under bright field and differential interference contrast. Protargol staining was conducted following Wilbert's method (Wilbert 1975), and only the Hwangsan Island population successfully revealed infraciliature. Staining in other populations failed due to the low number of cells. To compare the morphological variation of *S. minima*-like species, the data reported in previous studies were used. We followed the terminology proposed by Agatha and Riedel-Lorjé (2006).

PCR amplification, cloning, and sequencing

We selected a single cell from each population cultured in the Petri dishes. Each cell was rinsed at least five times with autoclaved seawater to remove other organisms. One to two cells were transferred to individual PCR tubes containing 10 μl of distilled water. Without a DNA extraction step, the PCR mixture was transferred to a PCR tube containing a single cell in a total volume of 50 μl. TaKaRa LA Taq polymerase (Takara Bio Inc., Kusatsu, Japan) was used to amplify the ribosomal RNA genes using EuKA (5′-AAC CTG GTT GAT CCT GCC AGT-3′; Medlin et al. 1988) and ReV2 primers (5′-ACG ATC GAT TTG CAC GTC-3′; Sonnenberg et al. 2007) according to the manufacturer's instructions. The PCR product was purified, and directly sequenced from both ends without cloning, using a commercially available service (SolGent Co. Ltd, Daejeon, Korea). The sequencing primers used were those described by Kim et al. (2013). The PCR product of Seocheon's population (SC2) was cloned according to the procedures described by Kim et al. (2010b). Two SC2 sequences resulted from different PCR sequencing replicates. These sequences were not identical and were labeled SC2-1 and SC2-2 (Table 2).

Table 2. Morphometric characterization of *Strombidinopsis* from Hwangsan Island (upper line) and Seocheon (lower line) populations

	x	M	Min	Max	*n*
Cell, length	31.6	30	20	45	11
	19.8	21	16	21.5	6
Cell, width	32.7	30	25	45	17
	19.1	19	15	22	8
Macronucleus, width	13	14	7	15	17
	9.7	10	7	12	3
Macronucleus, length	5	5	3	9	15
	2.7	2.5	2.5	3	3
Macronuclei, number	2	2	2	2	11
	2	2	2	2	3
Micronucleus, number	1	1	1	1	11
	1	1	1	1	3
Collar membranelles, number	23	23	21	23	9
	–	–	–	–	–
Buccal membranelle, number	1	1	1	1	11
	–	–	–	–	–
Somatic kinety, number	21	21	19	22	7
	–	–	–	–	–
Kinetosome, number	17	17	16	19	7
	–	–	–	–	–

M = median; Max = maximum; Min = minimum; *n* = number of individuals investigated; *x* = arithmetic mean.
Data are based on protargol-impregnated specimens. Measurements in μm.

DNA sequence comparisons

Species diversity was determined by comparing the DNA sequences of *Strombidinopsis* in this study with sequences of congeners obtained from the National Center for Biotechnology Information (NCBI). The sequences were aligned using MAFFT v 7.017 (Katoh et al. 2002). Intra- and inter-specific similarities were investigated by comparing the DNA similarities of SSU rDNA, partial large subunit rDNA (LSU rDNA), the D2 domain of LSU rDNA, ITS1, and

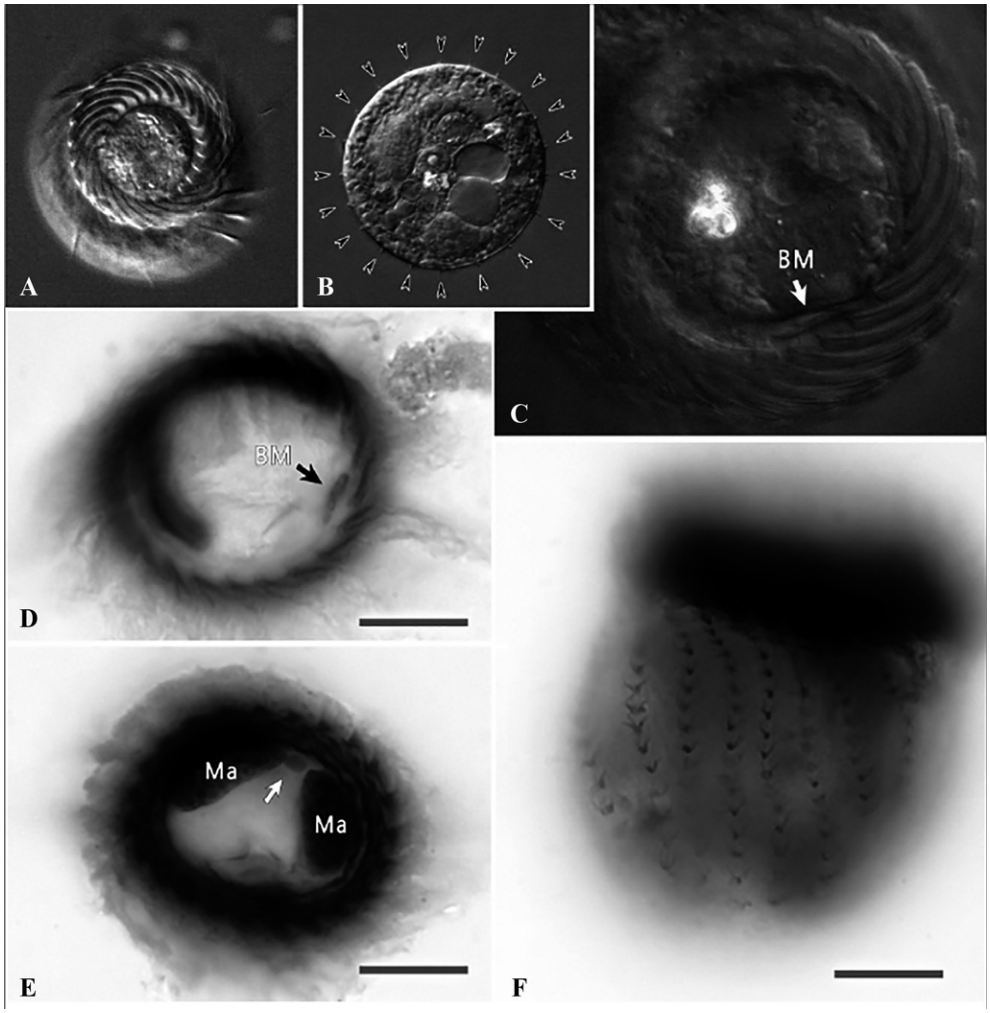

Figure 1 Photomicrographs of *Strombidinopsis minima*-like species from Hwangsan Island of Korea, live (**A**–**C**) and after protargol impregnation (**D**–**F**). A, C. Apical view shows collar and buccal membranelles. Arrow indicates buccal membranelle. B. Posterior view focuses on somatic cilia. Arrowheads mark somatic kineties. D. Top panel shows collar and buccal membranelles. Arrow marks buccal membranelle. E. Top panel displays macronuclei and micronucleus. Arrow marks micronuclei. F. Lateral view shows somatic kineties. Scale bars: 20 μm.

ITS1-5.8S rDNA-ITS2 using the Geneious program v. 9 (http://www.geneious.com). The genetic divergence was calculated using MEGA X (Kumar et al. 2018) based on the model of p-distance. The V4 region of SSU rDNA and D2 domain of LSU rDNA were identified following the guidelines for *Tetrahymena canadensis* and Engberg et al. (1990), respectively. For sequences obtained from clone SC2, chimerism was evaluated in the sequence alignment de novo. No chimerism was found.

Phylogenetic analysis

Ninety SSU rRNA gene sequences of choreotrichs were retrieved from the NCBI database (Table S1). Most sequences of choreotrichs were included, although partial sequences shorter than 1,537 bp were excluded. The data set was aligned using MAFFT v 7.017 (Katoh et al. 2002). After both ends of the alignments were trimmed, separate phylogenetic analyses were performed for SSU rRNA (1,585 bp). The following six species derived from subclasses Oligotrichia and Stichotrichia were used as the outgroups: *Lynnella semiglobulosa* (FJ876965), *Halteria grandinella* (AF508759), *Hemiurosomoida longa* (AF508763), *Sterkiella histriomuscorum* (AF508770), *Urostyla grandis* (AF508781), and *Strombidium rassoulzadegani* (AY257125). The MrModeltest v. 2 program (Nylander 2004) selected GTR + I (0.6076) + G (0.4608) as the best model using Akaike information criterion. A Bayesian tree was constructed based on an output of 5,000 trees generated by MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) using 5,000,000 cycles for the Markov chain Monte Carlo algorithm and sampling at every 1,000th generation. Stationary likelihood scores were determined by plotting the –lnL against the generation. The first 500 trees below the observed stationary level were discarded for burn-in. A maximum likelihood (ML) tree was constructed with RAxML version 7.2.8 (Stamatakis et al. 2008) installed in

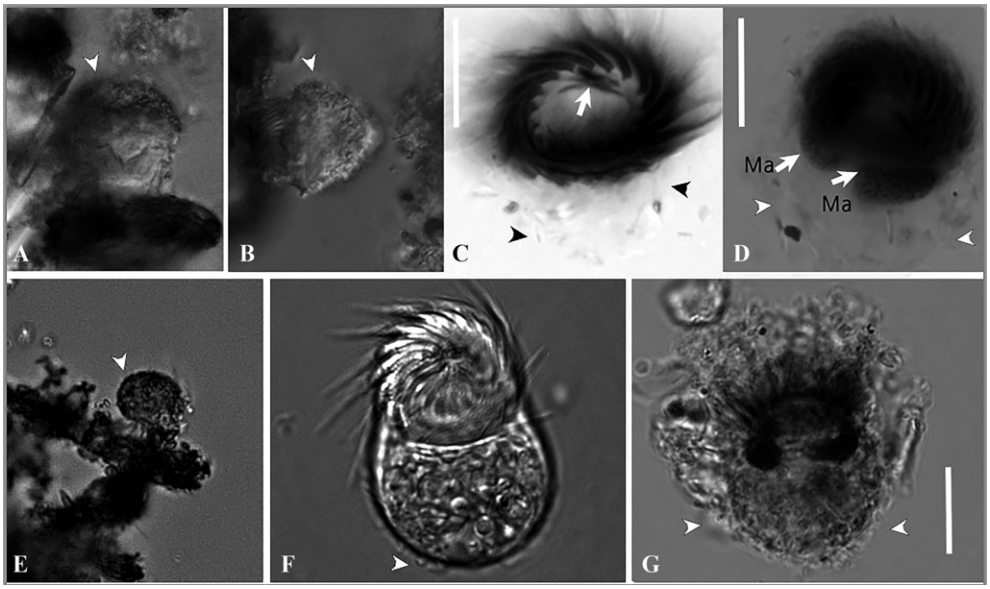

Figure 2 Photomicrographs of *Strombidinopsis* species derived from Donggeom Island of Korea (**A–D**) and Seocheon in June 2013, (**E–G**) live (A, B, E, F) and after protargol impregnation (C, D, G). A, B. Lateral view of a cell attaching to substrate. C. Top view shows collar and buccal membranelles (arrows) and mineral envelope (arrowheads). D. Top panel displays collar membranelles and macronuclei (arrows). E. Cell attached to substrate in the lateral view. F. Lateral view shows collar membranelles. G. Lateral view shows macronuclei. Arrowheads in (D–G) indicate mineral envelope. Scale bars: 10 μm.

the Geneious program (v.9.0.5). The nucleotide model used was GTR GAMMA I and the algorithm involved rapid bootstrapping and search for the best-scoring ML tree. The number of bootstrap replicates was 10,000. TreeView v. 1.6.6 (Page 1996) and MEGA v. 4.0 (Tamura et al. 2007) were used to visualize tree topology.

Deposition of slides

Four voucher slides of Hwangsan Island were deposited at the National Institute of Biological Resources with the following registration numbers: KOSPPR0000104835–KOSPPR0000104838.

RESULTS

Species identification

The Hwangsan Island specimens (HS) were identified based on morphological characteristics, including infraciliature (Table 2 and Fig. 1A–F). Other populations failed to reveal infraciliature and were identified using distinct morphological characteristics of a mineral envelope (Agatha 2003; Fig. 2A–G). The cell size and number of collar membranelles were only observed in a few cells due to poor protargol impregnation of the populations in Seochoen (SC1) and Donggeom Island (DG; Table 2). Because morphological characteristics could not be observed in the other Seocheon populations (SC2, SC3), these populations were not explicitly assigned to *S. minima*. Nonetheless, these populations were affiliated to *S. minima-like* species because of their mineral envelopes, which are the defining characteristic of *S. minima*.

Morphological observation

Hwangsan Island population

After protargol impregnation, the cell size of the Hwangsan Island population was determined to be 25–45 × 20–45 μm. The body shape was ellipsoid (Tables 2,3 and Fig. 1A–F). Two round-to-elongate macronuclei, measuring 7–15 × 3–9 μm in size, were present (Fig. 1E). A single ovoid micronucleus, approximately 1.5–3.0 μm in diameter, was present between the two macronuclei (Tables 2,3 and Fig. 1E). Nearly 21 longitudinal somatic kineties, each consisting of 16 to 19 ciliated dikinetids, with ~2 μm long cilia protruding outward in different directions (right and left; Tables 2,3 and Fig. 1B, F). Twenty-three collar membranelles and one buccal membranelle were observed in this population (Tables 2,3 and Fig. 1A, C, D).

Donggeom Island population

After protargol impregnation, the cell size was 15–16 × 15–16 μm (data not shown). Both live and preserved cells carry mineral envelopes (Fig. 2A–D). Approximately 18 collar membranelles and one buccal membranelle were observed (Fig. 2C).

Seocheon population (SC1)

Mineral envelopes were observed in both live and protargol-stained specimens (Fig. 2E–G). After protargol impregnation, the cell size was 15–22 × 16–21.5 μm. The body shape changed from an ellipsoid to an elongated structure

Table 3. Morphological comparison of *Strombidinopsis* species and related species

Species name	Population	Cell, length	Cell, width	Collar membranelles, number	Buccal membranelle, number	Somatic kinety, number	Kinetid, number in kinety	Mineral envelope	Reference of gene sequence	References
S. minima	Korea (HS)	20–45 (31.6)	25–45 (32.7)	21–23 (23)	1	19–22 (21)	16–19 (17)	Absence	This study	This study
S. minima	Korea (SC1)	16–21.5 (19.8)	15–22 (19.1)	~16	–	–	–	Presence	This study	This study
S. minima	Korea (DG)	~15–16 (–)	~15–16	~18	–	–	–	Presence	This study	This study
S. minima	China	40–64 (49)	43–62 (47)	26–32 (29)	1	20–29 (24)	12–18 (15)	Presence	–	Song and Bradbury (1998)
S. minima	China	36–60 (45.5)	35–60 (47.7)	28–31	–	24–26	17–20	Presence	–	Lei et al. (1999)
S. minima	China	20–30 (25)	24–32 (28)	16–18	–	15–17	5–6	Presence	–	Lei et al. 1999; identified as *S. sphaira*; synonymized by Agatha (2003)
S. minima	Italian population	19–35 (25)	18–30 (22)	15–17 (16)	1	13–18 (17)	7–14 (10)	Presence	–	Agatha (2003)
S. minima	Venezuela	25–37 (34)	24–38 (32)	17–19 (18)	1	23–30 (27)	13–26 (21)	Presence	–	Agatha (2003)
S. azerbaijanica		15–20	15–20	15–16	0	18–20	–	Absence		Alekperov and Asadullayeva (1997)
S. batos	Gulf of Alaska	17 (12–20)	14 (10–17)	16 (14–17)	1	13 (10–16)	~6	Absence	Gao et al. (2016)	Lynn et al. (1991)
S. cercionis	Caribbean Sea	58 (48–65)	30 (24–40)	(13–14)	–	(11–14)	10–14	Absence	–	Lynn et al. (1991)
S. cheshiri		62 (35–82)	38 (29–49)	(15–16)	4–5	14 (13–19)	20–40	Absence	–	Montagnes and Taylor (1994)
S. cheshiri		34–110	32–60	14–16	4	(12–15)	18–32	Absence	–	Snyder and Ohman (1991)
S. chilorhax	Maine, USA	29 (24–35)	24 (17–29)	17 (15–18)	1	18 (15–18)	~10	Absence	–	Lynn et al. (1991)
S. elegans		29 (27–31)	31 (27–35)	(26–27)	1	22 (19–24)	8 (6–11)	Absence	–	Song and Bradbury (1998)
S. sphaira	Caribbean	22 (18–25)	21 (16–28)	14 (13–15)	1	14 (13–15)	~6	Absence	–	Lynn et al. (1991)
S. sinicum		40.2 (33–46)	42.3 (37–46)	16 (15–18)	1	23 (20–26)	12 (9–14)	Absence	Liu et al. (2016)	Liu et al. (2016)
S. spiniferum	Barents Sea	76 (62–90)	49 (36–57)	15 (14–15)	3	18 (17–21)	28–58	Absence	–	Lynn et al. (1991)
S. acuminatum	Gulf of Mexico	94 (70–124)	35 (29–50)	15	3	15 (15–16)	20–52	Absence	Kim et al. (2010a)	Lynn et al. (1991)
S. elongata		92.4 (78–103)	52.1 (46–61)	21 (19–24)	1–2	13 (11–15)	41 (33–56)	Absence	–	Song and Bradbury (1998)
S. multiauris		95 (42–140)	45 (32–64)	14–15	4–5	18 (14–25)	50–60	Absence	–	Montagnes and Taylor (1994)
S. spiniferum		76 (62–90)	49 (36–57)	15 (14–15)	3	18 (17–21)	28–58	Absence	–	Lynn et al. (1991)
S. jeokjo		149 (100–190)	78.5 (66–105)	16 (15–17)	5 (2–8)	28 (26–28)	34 (23–44)	Absence	Jeong et al. (2004)	Jeong et al.(2004)

–, No data. Data are based on protargol-impregnated specimens. Measurements in μm.

when two macronuclei were observed, resulting in a round-to-elongated shape. No micronuclei were observed. About 16 collar membranelles were observed. Buccal membranelles and somatic ciliary patterns were not detected due to poor impregnation. Morphological data obtained from the Seocheon populations collected in October (SC2, SC3) are not presented because only a few cells were observed, and the impregnation was poor, without any pictures of live samples. However, SC2 and SC3 carried clear mineral envelopes and were used for molecular analyses.

Genetic distance of rDNA sequences among *Strombidinopsis minima*-like isolates

Table 4 summarizes the sequence data of the six isolates obtained in this study. The total length of these sequences was ~3,000 bp, including the D2 domain, known to be a highly variable region in the LSU rDNA. The SSU rDNA in the Korean populations of *S. minima*-like species showed a similarity of 96.85–98.48% (88.69–97.29% in the V4 region of SSU rDNA) and a p-distance of 0.014–0.029 (0.027–0.109 in the V4 region of SSU rDNA; Table 5).

We also found high variations in ITS1-5.8S-ITS2 (with 87.62–94.03% similarity and a p-distance of 0.056–0.102), ITS1 (71.72–89.90% similarity and 0.082–0.211 p-distance), partial LSU rDNA (92.67–95.93% similarity and 0.037–0.069 p-distance), and the D2 domain of LSU rDNA (80.28–88.26% similarity and 0.105–0.186 p-distance), among the populations of *S. minima*-like species (Tables S2,S3).

Genetic diversities of rDNA sequences among *Strombidinopsis* species

Among the *Strombidinopsis* species, the highest similarity in SSU rDNA was 99.94% (0.001 p-distance) between *S. batos* and *S. minima*-like species (SC3 population), while the lowest similarity in SSU rDNA was 91.44% (0.081 p-distance) between *S. acuminata* and *S. minima*-like species (SC1 population; Table 5). The similarity in the V4 region of SSU rDNA was 79.64–100% (Table 5), with the highest degree of similarity (100%; no genetic divergence) between *S. batos* and the SC3 population and between *S. jeokjo* and *S. acuminata*. The lowest similarity was found between the DG population and *S. jeokjo* (79.64%, 0.2 p-distance) and between the DG population and *S. acuminata*.

The similarities and p-distances between *S. batos*, *S. sinicum,* and *S. minima*-like species in the Korean populations (96.28–99.94% and 0.001–0.035 in SSU rDNA and 88.69–100% and 0–0.109 in the V4 region of SSU, respectively) almost overlapped with those of *S. minima*-like species (96.85–98.48% and 0.014–0.029 in SSU rDNA, 88.69–97.29% and 0.027–0.109 in the V4 region of SSU rDNA, respectively). Despite the low genetic diversity in SSU, the similarity and p-distances were only 61.22% and 0.240, respectively, for the ITS1-5.8S-partial ITS2 between *S. batos* and the SC3 population (data not shown). The intraspecies variation between the other *Strombidinopsis* in 5.8S, LSU, ITS1, and ITS2 could not be compared due to lack of prior studies.

Phylogenetic analyses

In the phylogenetic tree, the sequences of the genus *Strombidinopsis* were split into two clades. *S. minima*-like species were clustered together with *S. batos* and *S. sinicum* with high support in BI (1.0) and moderate support in ML (81%) analysis (Fig. 3). This clade clustered together with other Strobilidiidae species with rather moderate support in the BI analysis (0.93, Fig. 3). The second clade, composed of *S. acuminata*, *S. jekjo*, and three other *Strombidinopsis* spp., also secured a high degree of support, but did not constitute the sister clade in the *Strombidinopsis* clade mentioned above (Fig. 3), rendering *Strombidinopsis* polyphyletic in origin.

DISCUSSION

Morphological variation of *Strombidinopsis minima*-like species

To date, 13 *Strombidinopsis* species have been described using protargol staining methods, to elucidate their infraciliature, the key characteristic of species identification (Table 3). A few characteristics overlapped within the genus. The morphological characteristics of *S. minima*-like species varied among the different populations. Specifically, they showed significant variation in cell size (19–64 × 18–62 μm), numbers of collar membranelles (ranging from 15 to 32), and somatic kineties (ranging from 13 to 30; Table 3). Gruber (1884) described the length of *S. minima* as an average of 30 μm, with small mineral particles

Table 4. Lengths of SSU, ITS1-5.8S- ITS2, and partial of LSU rDNA sequences from different samples

Sample list	Total length (bp)	SSU rDNA (V4 region)	ITS 1-5.8S-ITS 2 (ITS 1)	Partial of LSU rDNA (D2 domain)	Accession number
DG	2,947	1,646 (221)	703 (99)	598 (212)	MK585219
HS	3,046	1,746 (220)	703 (99)	597 (210)	MK585220
SC1	3,029	1,747 (220)	686 (90)	596 (209)	MK585221
SC2-1	3,049	1,749 (221)	702 (97)	598 (211)	MK585222
SC2-2	3,050	1,749 (221)	703 (99)	598 (211)	MK585223
SC3	3,044	1,748 (221)	697 (95)	599 (212)	MK585224

DG = Donggeom population; HS = Hwangsan population; SC = Seocheon population.

Table 5. Similarities (first line) and p-distance (second line) of SSU rDNA and V4 regions of SSU rDNA sequences among *Strombidinopsis* species

Species name (accession no.)	*S. minima* DG (MK585219)	*S. minima* HS (MK585220)	*S. minima* SC1 (MK585221)	*S. minima* SC2-1 (MK585222)	*S. minima* SC2-2 (MK585223)	*S. minima* SC3 (MK585224)	FJ881862	KR263893	AJ628250	FJ790207
	SSU rDNA (1,665 bp)									
S. minima DG (MK585219)	–	97.27 (0.026)	97.27 (0.025)	97.45 (0.024)	97.63 (0.022)	98.48 (0.014)	98.42 (0.015)	97.03 (0.028)	91.72 (0.077)	91.65 (0.078)
S. minima HS (MK585220)	90.50 (0.091)	–	96.96 (0.028)	96.85 (0.029)	97.02 (0.027)	97.65 (0.021)	97.60 (0.022)	97.14 (0.029)	92.07 (0.077)	92.01 (0.079)
S. minima SC1 (MK585221)	89.59 (0.100)	89.59 (0.096)	–	97.08 (0.029)	97.20 (0.027)	97.31 (0.026)	97.25 (0.027)	96.57 (0.032)	91.51 (0.080)	91.44 (0.081)
S. minima SC2-1 (MK585222)	90.50 (0.095)	90.05 (0.095)	89.14 (0.105)	–	98.00 (0.021)	97.77 (0.021)	97.71 (0.022)	96.28 (0.035)	91.79 (0.078)	91.73 (0.079)
S. minima SC2-2 (MK585223)	93.21 (0.068)	92.76 (0.068)	91.40 (0.082)	97.29 (0.027)	–	97.83 (0.021)	97.77 (0.021)	96.45 (0.033)	91.90 (0.077)	91.84 (0.078)
S. minima SC3 (MK585224)	95.93 (0.041)	90.05 (0.095)	88.69 (0.109)	93.67 (0.063)	95.48 (0.045)	–	99.94 (0.001)	96.80 (0.030)	91.96 (0.076)	91.90 (0.077)
S. batos (FJ881862)	95.93 (0.041)	90.05 (0.095)	88.69 (0.109)	93.67 (0.063)	95.48 (0.045)	100 (0)	–	96.74 (0.030)	92.02 (0.075)	91.96 (0.077)
S. sinicum (KR263893)	90.95 (0.090)	92.76 (0.068)	90.05 (0.095)	90.05 (0.100)	92.76 (0.072)	90.50 (0.095)	90.50 (0.095)	–	92.30 (0.075)	92.24 (0.077)
S. jeokjo (AJ628250)	79.64 (0.200)	82.27 (0.177)	79.64 (0.196)	82.35 (0.173)	82.81 (0.168)	80.09 (0.195)	80.09 (0.195)	81.00 (0.186)	–	99.60 (0.004)
S. acuminata (FJ790207)	79.64 (0.200)	82.27 (0.177)	79.64 (0.196)	82.35 (0.173)	82.81 (0.168)	80.09 (0.195)	80.09 (0.195)	81.00 (0.186)	100 (0)	–
	V4 region of SSU (221 bp)									
References	This study						Gao et al. (2016)	Liu et al. (2016)	Jeong et al. (2004)	Kim et al. (2010a)

DG = Donggeom population; HS = Hwansan population; SC = Seocheon population.

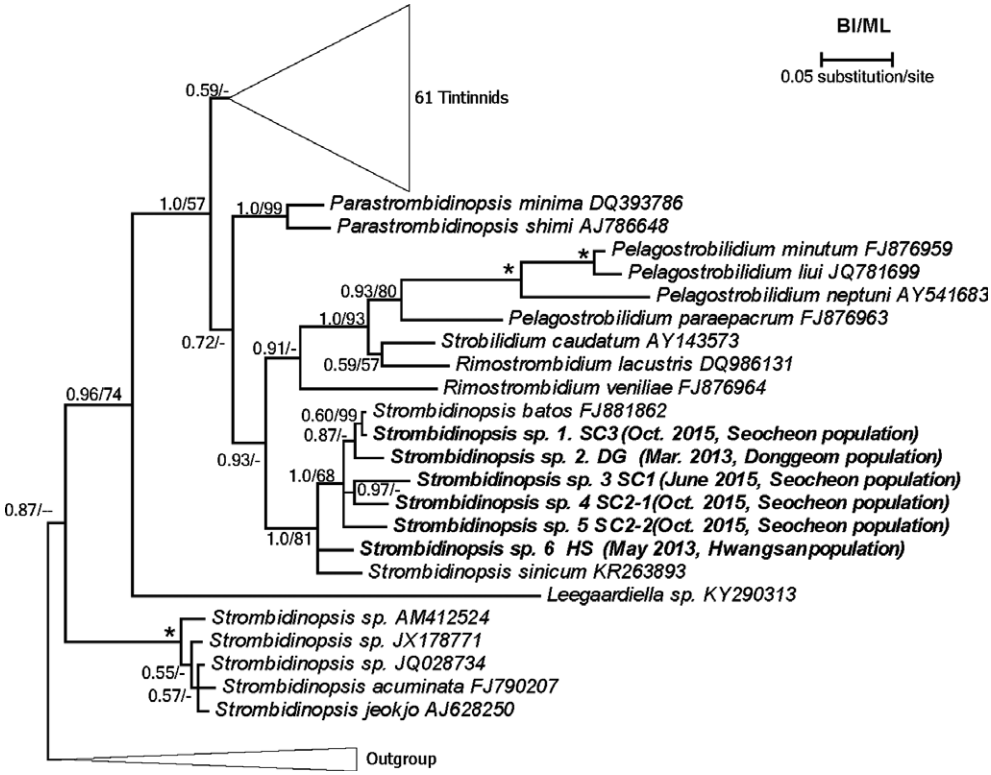

Figure 3 Bayesian tree based on small subunit rRNA gene sequences showing the relationships between *Strombidinopsis* species and other choreotrichid ciliates. New sequences are shown in bold. Numbers at the nodes represent support values in the following order: Bayesian posterior probabilities using the MrBayes algorithm (BI) and bootstrap values from maximum likelihood (ML) analyses as a percent of 1,000 replicates. Asterisk (*) denotes nodes with full bootstrap support in all algorithms. A hyphen (-) represents support values < 50% and disagreement between BI and ML at a given node.

attached to the cell, sometimes fully covering the entire cell surface. Since then, the identification of *S. minima* has appeared to depend primarily on its mineral envelope, although it does not always carry this feature (Gruber 1884).

Morphological variations have been reported among different populations of *S. minima* in previous studies (Agatha 2003; Lei et al. 1999; Song and Bradbury 1998, Table 3). In the Korean population of *S. minima*-like species, the morphological characteristics of *S. minima* overlapped with *S. batos* and *S. sinicum* (Table 3). In this context, whether or not a species had a mineral envelope, *S. batos, S. sinicum,* and *S. minima*-like species in the Korean population were closely related based on similarities in SSU rDNA and were placed in the same clade of the phylogenetic tree based on the SSU rDNA.

Here, five populations of *S. minima* were collected from the coastal waters of Korea, three of which were examined using the protargol staining method, although only partial infraciliatures were available for two populations. The cell size and number of collar membranelles differed between distinct isolates (Table 3). Results of our study suggest that the mineral envelope is not always present, and therefore, cannot be a defining characteristic of the species. The Hwangsan Island population did not exhibit a typical mineral envelope. However, other morphological characteristics (e.g. cell size, number of somatic kineties, and oral membranelles) of the Hwangsan population matched well with those of populations belonging to this species described in earlier studies (Table 3). Therefore, we grouped the Hwangsan population with *S. minima* (Fig. 3). We, therefore, conclude that the Hwangsan population is *Strombidinopsis minima*. Notably, the Hwangsan population was harvested 2 mo after sampling while the other populations were investigated within 15 d. The mineral envelope may protect cells from "scratches" due to environmental detritus to adapt to the benthic, detritus-rich lifestyle (Agatha 2003). The mineral envelope may not develop in culture. Thus, the presence of a mineral envelope is not a specific characteristic of *S. minima*. Although detailed morphological data of specific isolates were unavailable, the molecular data and observations of the presence or absence of a mineral envelope in five populations of *S. minima*-like species provided an opportunity to evaluate morphological variation across *S. minima*-like species.

Genetic variation in *Strombidinopsis* species

In *Strombidinopsis*, the sequences of SSU rDNA are only available for *S. batos, S. sinicum, S. acuminata,* and

S. jeokjo, which were identified at the species level (Gao et al. 2009; Kim et al. 2005, 2010a; Liu et al. 2016). Because these sequences of *S. acuminata* and *S. jeokjo* are almost identical, it is not easy to define intra- or interspecies variation in molecular diversities of *Strombidinopsis* using SSU rDNA (Kim et al. 2010a). Comparison with other molecular loci is currently not possible as only the sequences of ITS1, 5.8S rDNA, and partial ITS2 of *S. batos* have been analyzed. In addition, SSU rRNA gene dissimilarities of *S. mimina*-like species ranged from 0.06% to 3.15% (0.00–11.31% for V4 regions). A 1% cutoff is normally used in the molecular surveys of environmental samples via high throughput screening (HTS) to separate different ciliate operational taxonomic units (OTUs). When this cutoff value was used for *S. minima*-like species, it was difficult to estimate species diversity.

Here, both Donggeom (DG) and Seocheon (SC) populations had mineral envelopes. However, they showed varying similarities and p-distances in the SSU rRNA region (Table 5 and Tables S2,S3). Furthermore, strong dissimilarities were found between the Korean populations in the D2 domain of the LSU rDNA and ITS regions. These molecular regions have been used to analyze cryptic species in ciliates (Kim et al. 2013; Nanney et al. 1998; Xu et al. 2012). The high genetic variation of the D2 domain in the LSU rDNA and ITS regions of *S. minima*-like species implies that this species is currently ill-defined morphologically and consists of several genetically and morphologically distinct species.

CONCLUSION

Strombidinopsis minima manifests typical morphological characteristics of the genus, such as dikinetidal somatic kineties and two macronuclei. The SSU rDNA sequences of the various *S. minima*-like populations investigated in the present study were recovered in the clade with *S. batos* and *S. sinicum* (Gao et al. 2016; Liu et al. 2016). The current results further support the hypothesis by Liu et al. (2016) that *Strombidinopsis* is nonmonophyletic because all the species added in this study including the true, *S. minima*, were recovered in the clade with *S. batos* and *S. sinicum*. Apparently, a species found in one of the *Strombidinopsis* clades was assigned to a new genus. Notably, Agatha (2003) observed that the cell division of *S. minima* differed from that of *S. spinifera* and *S. acuminata*, that is, the oral primordium above two anteriorly shortened dorsal kineties in *S. minima* and between two dorsal kineties of ordinary left in *S. spinifera* and *S. acuminata*. However, at this stage, the essential morphological information to separate the two clades is unavailable to provide a comprehensive morphological description of the two species, or modify the description of the genus *Strombidinopsis*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Species list of Tintinnids used for phylogenetic analyses.
Table S2. Similarities (in %; first line) and genetic distance (second line) of ITS1-5.8S rDNA-ITS2 and ITS1 sequences from *Strombidinopsis* minima-like species.
Table S3. Similarities (in %; first line) and genetic distance (second line) between partial of LSU rDNA and D2 domain sequences from *Strombidinopsis* of Korean population.