



Research Article

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Five phototrophic *Scrippsiella* species lacking mixotrophic ability and the extended prey spectrum of *Scrippsiella acuminata* (Thoracosphaerales, Dinophyceae)

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Mixotrophic dinoflagellates act as primary producers, prey, and predators in marine planktonic food webs, whereas exclusively autotrophic dinoflagellates are primary producers and prey. Species of the dinoflagellate genus *Scrippsiella* are commonly found in marine ecosystems and sometimes cause harmful red tides. Among the 28 formally described *Scrippsiella* species, *S. acuminata* has been found to be mixotrophic and two unidentified species have been found to be mixotrophic. To determine whether the other species in this genus are similarly mixotrophic, the mixotrophic ability of *S. donghaiensis* SDGJ1703, *S. lachrymosa* SLBS1703, *S. masanensis* SSMS0908, *S. plana* SSSH1009A, and *S. ramonii* VGO1053 was explored using 15 potential prey items, including 2-μm fluorescently labeled microspheres (FLM) and heterotrophic bacteria (FLB), the cyanobacterium *Synechococcus* sp., and various microalgal prey species. The ability of *S. acuminata* to feed on FLM and FLB was also investigated. We found that *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* did not feed on any potential prey tested in this study, indicating a lack of mixotrophy. However, *S. acuminata* fed on both FLM and FLB, confirming its mixotrophic ability. These results lowered the proportion of mixotrophic species relative to the total number of tested *Scrippsiella* species for mixotrophy from 100% to 29–38%. Owing to its mixotrophic ability, *S. acuminata* occupies an ecological niche that is distinct from that of *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii*.

Keywords: dinoflagellate; feeding; harmful algal bloom; protist; red tide; Thoracosphaeraceae; trophic mode

Abbreviations: FLB, fluorescently labeled heterotrophic bacteria; FLM, fluorescently labeled microspheres; LSU, large subunit; rDNA, ribosomal DNA

INTRODUCTION

Mixotrophy, a combination of autotrophy and heterotrophy, is observed in many marine flagellates, dinoflagellates, and ciliates (Stoecker et al. 1997, Burkholder et al. 2008, Esteban et al. 2010, Jeong et al. 2010a, Lee et al.

2014a). Mixotrophs play diverse roles as primary producers, prey, and predators in marine ecosystems, whereas exclusively autotrophic organisms play the roles of primary producers and prey (Burkert et al. 2001, Jeong et al.



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2012). Mixotrophy increases the growth rate of protists (Li et al. 2000, Smalley et al. 2003, Jeong et al. 2015, 2021, Ok et al. 2019, Kang et al. 2020, You et al. 2020), causes horizontal gene transfer, and is the main driving force in the evolution of photosynthetic organisms (Bhattacharya et al. 2004, Wisecaver et al. 2013, Hehenberger et al. 2019). Therefore, understanding mixotrophy is of ecological and evolutionary importance.

Dinoflagellates are ubiquitous protists in marine environments (Luo et al. 2021, Ok et al. 2021, Morquecho et al. 2022), and often form red tides or harmful algal blooms that can cause human diseases and massive mortality in shellfish, finfish, and mammals (Shumway 1990, Hallegraeff 1992, Flewelling et al. 2005, Jeong et al. 2017, Sakamoto et al. 2021). The trophic modes, growth and ingestion rates, and mortality rates due to predation of dinoflagellate species should be investigated to understand and predict the outbreak of dinoflagellate red tides (Franklin et al. 2006, Jeong et al. 2015). In the last three decades, many dinoflagellate species previously thought to be autotrophs were reclassified as mixotrophs (Bockstahler and Coats 1993, Stoecker et al. 1997), including many species that form red tides (Jeong et al. 2005a, 2005b, Burkholder et al. 2008, Park et al. 2013, Flynn et al. 2018). Approximately 90% of the dinoflagellates that form global red tides are mixotrophs (Jeong et al. 2021); however, less than 10% of the approximately 1,200 phototrophic dinoflagellates have been tested for mixotrophy (Stoecker et al. 1997, Jeong et al. 2005a, 2005b, Park and Kim 2010, Lee et al. 2014b, 2015, Lim et al. 2018, 2019). Therefore, understanding the ecological and genetic characteristics and red tide dynamics of a phototrophic dinoflagellate species requires an examination of their mixotrophic ability. Moreover, the prey species of mixotrophic dinoflagellates should be identified.

Since the description of the genus *Scrippsiella* by Balech (1959) with the type species *S. sweeneyae*, 28 species have been formally described (Hoppenrath et al. 2014, Guiry and Guiry 2023) (Supplementary Table S1). The species in the genus *Scrippsiella* have a global distribution; however, only *S. acuminata* (previously *S. trochoidea*) has caused red tides in the waters of many countries (Hallegraeff 1992, Pitcher et al. 2007, Pitcher and Joyce 2009, Soehner et al. 2012, Jeong et al. 2021). Red tides dominated by *Scrippsiella* spp. can cause fish mortality through hypoxia (Hallegraeff 1992); therefore, to minimize losses caused by *Scrippsiella* red tides, the eco-physiology and population dynamics of each *Scrippsiella* species should be understood (Jeong et al. 2015). Of the formally described and unidentified species in this ge-

nus, only *S. acuminata* and two unidentified *Scrippsiella* species have been confirmed to be mixotrophic (Jacobson and Anderson 1996, Jeong et al. 2005a, 2005b, Coats et al. 2020). Therefore, the mixotrophic abilities of other *Scrippsiella* species should be investigated. *S. acuminata* cells have been reported to feed on the cyanobacterium *Synechococcus* sp., prymnesiophyte *Isochrysis galbana*, cryptophytes *Rhodomonas salina* and an unidentified species, raphidophyte *Heterosigma akashiwo*, and phototrophic dinoflagellates *Amphidinium carterae* and *Prorocyclinium cordatum* (Jeong et al. 2005a, 2005b). Furthermore, an unidentified *Scrippsiella* sp., which was collected from the waters off Korea, has been reported to feed on the tintinnid ciliate *Helicostomella longa* using a feeding tube (Coats et al. 2020). Another unidentified *Scrippsiella* sp. isolated from West Boothbay Harbor, Maine, United States was reported to possess food vacuoles (Jacobson and Anderson 1996). Heterotrophic bacteria are common in almost all marine ecosystems (Caron et al. 1982, Kjelleberg et al. 1987, Van Wambeke et al. 2000, Seong et al. 2006, Sanz-Sáez et al. 2020, Vijayan et al. 2022) and serve as prey for many mixotrophic dinoflagellates (Seong et al. 2006, Jeong et al. 2010a, 2012, Lee et al. 2014b, Millette et al. 2017). Thus, the ability of each *Scrippsiella* species to feed on heterotrophic bacteria warrants investigation.

In the present study, the mixotrophic abilities of five *Scrippsiella* species (*Scrippsiella donghaiensis* SDGJ1703, *S. lachrymosa* SLBS1703, *S. masanensis* SSMS0908, *S. plana* SSSH1009A, and *S. ramonii* VGO1053) were identified after they were provided 2-μm fluorescently labeled microspheres (FLM), fluorescently labeled heterotrophic bacteria (FLB), *Synechococcus* sp., and 12 microalgal species as potential prey. Whether *S. acuminata* STKP9909 can feed on FLM and FLB was also investigated. The present study provides a basis for understanding the ecological roles of these *Scrippsiella* species in marine ecosystems and their evolution within the genus *Scrippsiella*.

MATERIALS AND METHODS

Preparation of experimental organisms

Scrippsiella acuminata STKP9909, *S. donghaiensis* SDGJ1703, *S. lachrymosa* SLBS1703, and *S. masanensis* SSMS0908 were isolated from the waters off Kunpho in September 1999, Gijang in March 2017, Busan in March 2017, and Masan in August 2009, respectively. A clonal culture of each of the four species was established using two serial single-cell isolations (Kim et al. 2019, Lee

et al. 2019) (Table 1). To isolate and culture *S. plana* SSSH1009A, surface sediment samples were collected from Shiwha Bay, Korea, in September 2010 using an Ekman Grab (Wildco; Wildlife Supply Company, Buffalo, NY, USA) (Table 1). The collected sediments were stored at 4°C in the dark and incubated as described by Jeong et al. (2014). A clonal culture of *S. plana* SSSH1009A was established using two serial single-cell isolations from the incubated sediment samples. A culture of *S. ramonii* VGO1053 that was originally collected from Ebro Delta in Spain was obtained from the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo (Table 1). All *Scrippsiella* cultures were transferred every two weeks in 250- or 800-mL flat culture flasks containing fresh F/2-Si medium (Guillard and Ryther 1962) and maintained in a 14 h : 10 h light : dark cycle under 50–100 µmol photons m⁻² s⁻¹ from a cool-white fluorescent light at 20°C.

The microalgal species selected as potential prey were maintained under the same light and temperature conditions as the *Scrippsiella* species (Table 2). The cyanobacterium *Synechococcus* sp. was maintained under 5–10 µmol photons m⁻² s⁻¹ from cool-white fluorescent light with a 14 h : 10 h light : dark cycle at 20°C.

Feeding occurrence test

Experiments 1–4 were designed to explore whether *S. donghaiensis* SDGJ1703, *S. lachrymosa* SLBS1703, *S. masanensis* SSMS0908, *S. plana* SSSH1009A, and *S. ramonii* VGO1053 can feed on the FLM (Expt 1: diameter = 2 µm; 18604 Fluoresbrite YG Microspheres; Polysciences Inc., Warrington, PA, USA), FLB (Expt 2), *Synechococcus* sp. (Expt 3), or microalgal prey species (Expt 4). Whether *S. acuminata* STKP9909 can feed on the FLM and FLB was also investigated.

Table 1. Culture conditions for the six *Scrippsiella* species used in this study

Organisms	Strain name	ESD	Location	Date	T	S
<i>Scrippsiella lachrymosa</i>	SLBS1703	17.7	Busan, Korea	Mar 2017	10.9	33.5
<i>S. donghaiensis</i>	SDGJ1703	19.4	Gijang, Korea	Mar 2017	13.2	33.9
<i>S. masanensis</i>	SSMS0908	22.0	Masan bay, Korea	Aug 2009	27.0	31.5
<i>S. acuminata</i> (= <i>S. trochoidea</i>)	STKP9909	22.8	Kunpho, Korea	Sep 1999	-	-
<i>S. plana</i>	SSSH1009A	24.9	Shiwha bay, Korea (Surface sediment)	Sep 2010	21.3	15.6
<i>S. ramonii</i>	VGO1053	25.5	Ebro Delta, Spain	-	-	-

ESD, equivalent spherical diameter (µm); T, water temperature (°C); S, salinity; -, not available.

Table 2. Culture conditions for the potential prey items offered to *Scrippsiella* species in feeding occurrence tests (Expts 1–4)

Organisms (strain name)	ESD	Origin	Date	T	S
Microsphere	2.0				
Bacteria					
Heterotrophic bacteria	0.5–1.0	Each <i>Scrippsiella</i> culture	-	-	-
<i>Synechococcus</i> sp. (N54-2)	0.5–1.0	East China Sea	Jul 2005	25.5	33.2
Prymnesiophyte					
<i>Isochrysis galbana</i> (IG)	4.8	-	-	-	-
Prasinophyte					
<i>Pyramimonas</i> sp. (PSSH1204)	5.6	Shiwha bay, Korea	Apr 2012	-	-
Cryptophytes					
<i>Teleaulax amphioxeia</i> (TSGS0202)	5.6	Gomso bay, Korea	Feb 2002	7.8	30.1
<i>Storeatula major</i> (SSSH1103)	6.0	Shiwha bay, Korea	Mar 2011	4.3	19.1
<i>Rhodomonas salina</i> (RS)	8.8	-	-	-	-
Raphidophyte					
<i>Heterosigma akashiwo</i> (HAKS9905)	11.5	Kunsan, Korea	May 1999	16.0	27.7
Phototrophic dinoflagellates					
<i>Heterocapsa rotundata</i> (HRSH1201)	8.2	Shiwha bay, Korea	Jan 2012	0.2	31.0
<i>Heterocapsa minima</i> (HMMJ1604)	9.5	Mijo Port, Korea	Apr 2016	12.9	30.3
<i>Amphidinium carterae</i> (SIO PY-1)	9.7	USA	Nov 1985	-	-
<i>Prorocentrum cordatum</i> (PMKS9906)	12.1	Kunsan, Korea	Jun 1999	21.1	30.1
<i>Prorocentrum donghaiense</i> (PDYS1407)	13.3	Yeosu, Korea	Jul 2014	-	-
<i>Prorocentrum micans</i> (PMSH0910)	26.6	Shiwha bay, Korea	Oct 2009	16.8	27.0
<i>Akashiwo sanguinea</i> (ASUSA)	30.8	-	-	-	-

ESD, equivalent spherical diameter (µm); T, water temperature (°C); S, salinity; -, not available.

In Expt 1, approximately 3×10^7 FLM were added to a 30-mL polycarbonate (PC) rounded bottle containing either *S. acuminata*, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, or *S. ramonii*. One experimental bottle (one *Scrippsiella* species + FLM), one prey control bottle (FLM only), and one predator control bottle (one *Scrippsiella* species only) were included in each experimental run. The bottles were incubated on a plankton wheel rotating at 0.9 rpm (0.00017 $\times g$) at 20°C under a 14 h : 10 h light-dark cycle (20 μmol photons $\text{m}^{-2} \text{s}^{-1}$). After 2 and 24 h of incubation, 3-mL aliquots were removed from each bottle and transferred to confocal dishes (SPL100350; SPL Life Sciences Co., Ltd., Pocheon, Korea). To observe *Scrippsiella* spp. feeding on the FLM, the protoplasm of 200 cells of each target *Scrippsiella* species was carefully observed under an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss Ltd., Göttingen, Germany) and photographs were taken using a digital camera (Zeiss Ax-

iocam 506; Carl Zeiss Ltd.) attached to the microscope at 1,000 \times magnification.

To prepare Expt 2, marine heterotrophic bacterial cells were obtained by filtering non-axenic cultures of *S. acuminata*, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii*. Aliquots of 300–2,000 mL from each *Scrippsiella* culture were serially filtered through 5.0- μm and 1.2- μm pore-sized filter papers (Merck Millipore, Burlington, MA, USA). The filtrates containing only bacterial cells were centrifuged at 3,000 rpm (2,063 $\times g$) for 30 min at 4°C (Labogene 1696R; Gyrozen Co., Gimpo, Korea) in Falcon tubes (Falcon; Corning, New York, NY, USA). The centrifuged bacterial cells were fluorescently labeled with 5-(4,6-dichlorotriazin-2-yl) amino fluorescein hydrochloride (D0531; Sigma-Aldrich, St. Louis, MO, USA) according to the method of Sherr et al. (1987) and then stored in the dark at 4°C until use. The FLB cells were resuspended in the culture medium using a sonica-

Table 3. Feeding occurrence results for the six *Scrippsiella* species tested

Potential prey	ESD	IPC	Potential predator					
			<i>S. acuminata</i>	<i>S. donghaiensis</i>	<i>S. lachrymosa</i>	<i>S. masanensis</i>	<i>S. plana</i>	<i>S. ramonii</i>
Microspheres	2.0	1,000	Y ^a	N	N	N	N	N
Bacteria								
Heterotrophic bacteria	0.5–1.0	700–1,000	Y ^a	N	N	N	N	N
<i>Synechococcus</i> sp.	1.0	1,000	Y ^{a,b}	N	N	N	N	N
Prymnesiophyte								
<i>Isochrysis galbana</i>	4.8	100–200	Y ^c	N	N	N	N	N
Prasinophyte								
<i>Pyramimonas</i> sp.	5.6	100	-	N	N	N	N	N
Cryptophytes								
Unidentified cryptophyte	5.6	100	Y ^c	-	-	-	-	-
<i>Teleaulax amphioxiae</i>	5.6	50–100	-	N	N	N	N	N
<i>Storeatula major</i>	6.0	50–100	-	N	N	N	N	N
<i>Rhodomonas salina</i>	8.8	30–50	Y ^c	N	N	N	N	N
Raphidophytes								
<i>Heterosigma akashiwo</i>	11.5	10–30	Y ^c	N	N	N	N	N
Phototrophic dinoflagellates								
<i>Heterocapsa minima</i>	8.2	60	-	-	-	N	N	N
<i>Heterocapsa rotundata</i>	9.5	50	-	N	N	-	-	-
<i>Amphidinium carterae</i>	9.7	30–60	Y ^c	N	N	N	N	N
<i>Procentrum cordatum</i>	12.1	13–30	Y ^c	N	N	N	N	N
<i>Procentrum donghaiense</i>	13.3	13–30	N ^c	N	N	N	N	N
<i>Procentrum micans</i>	26.6	2–5	N ^c	N	N	N	N	N
<i>Akashiwo sanguinea</i>	30.8	1–3	N ^c	N	N	N	N	N
References			Jeong et al. (2005a, 2005b), this study	This study	This study	This study	This study	This study

The heterotrophic bacteria used in this study and *Synechococcus* sp. for *S. acuminata* used in Jeong et al. (2005a) were fluorescently labeled. Initial *Scrippsiella* concentrations were approximately 2,000–7,000 cells mL^{-1} .

ESD, equivalent spherical diameter (μm); IPC, initial prey concentration ($\times 10^3$ cells or particles mL^{-1}); Y, the *Scrippsiella* species fed on prey; N, the *Scrippsiella* species did not feed on prey; -, not available.

^aRarely fed on the prey.

^bJeong et al. (2005a).

^cJeong et al. (2005b).

tor (Bransonic cleaner 5510E-DTH; Branson Ultrasonics, Danbury, CT, USA) for 10 min and then filtered through a 3- μm pore-sized filter paper (Merck Millipore) to remove aggregated bacterial cells. Each *Scrippsiella* spp. was tested using FLB collected from its own culture.

In Expts 2 and 3, approximately 3×10^7 FLB (Expt 2) or *Synechococcus* cells (Expt 3) were added to 30-mL PC rounded bottles containing each *Scrippsiella* species (Table 3). After 1 and 4 h of incubation, 3-mL aliquots were removed from each bottle, transferred into confocal dishes (SPL Life Sciences Co., Ltd.), and carefully observed as described above.

In Expt 4, dense cultures of *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* were added to the wells of 6-well plates containing each algal prey species. One experimental well (one *Scrippsiella* species + one microalgal prey species), one prey control well (microalgal prey species only), and one predator control well (one *Scrippsiella* species only) were established, and the plate was cultured in a 14 h : 10 h light : dark cycle ($20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 20°C. After 2 and 24 h of incubation, 3-mL aliquots were removed from each experimental well and transferred to confocal dishes (SPL Life Sciences Co., Ltd.). The protoplasm of 200 cells of target *Scrippsiella* spp. was observed using an inverted microscope (Carl Zeiss Ltd.) and photographed at 1,000 \times magnification.

DNA sequencing and phylogenetic analysis

The large subunit (LSU) rDNA sequences of *S. acuminata* STKP9909, *S. donghaiensis* SDGJ1703, and *S. plana* SSSH1009A were not previously reported and that of *S. ramonii* VGO1053 was previously partially reported. To obtain these sequences, 1 μL from each culture was removed and added into 0.2-mL polymerase chain reaction (PCR) tubes with 38.75 μL deionized sterile distilled water. Subsequently, a mixture of 1 μL dNTP mix, 0.25 μL F-StarTaq DNA polymerase, 5 μL 10 \times F-StarTaq buffer (BioFACT Co., Ltd., Daejeon, Korea), and 4 μL of each forward-reverse primer set needed for amplification of the LSU rDNA region (Set 1, ITSF2 and LSU500R; Set 2, D1R and 1483R) was added to the test tubes. The sequences of the primers used are as follows: ITSF2, 5'-TAC GTC CCT GCC CTT TGT AC-3'; D1R, 5'-ACC CGC TGA ATT TAA GCA TA-3'; LSU500R, 5'-CCC TCA TGG TAC TTG TTT GC-3'; 1483R, 5'-GCT ACT ACC ACC AAG ATC TGC-3' (Scholin et al. 1994, Daugbjerg et al. 2000, Litaker et al. 2003). PCR was conducted using an AllInOne Cycler (Bioneer, Daejeon, Korea) under the same thermal condi-

tions as described by You et al. (2023). The PCR products were purified using an AccuPrep DNA Purification Kit (Bioneer) and then sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and manually edited using Contig-Express (Infomax, Frederick, MD, USA).

For phylogenetic analysis, LSU rDNA sequences from 19 *Scrippsiella* spp. were obtained from this study and GenBank and aligned using MEGA v4 (Tamura et al. 2007). A LSU rDNA phylogenetic tree was constructed using Bayesian (the default GTR + G + I model in MrBayes v3.1) (Ronquist and Huelsenbeck 2003) and maximum likelihood analyses (the default GTRGAMMA model in RAxML 7.0.3 program) (Stamatakis 2006). The assumed empirical nucleotide frequencies of the LSU rDNA comprised a substitution rate matrix where A-C = 0.0344, A-G = 0.1778, A-T = 0.0562, C-G = 0.0163, C-T = 0.6361, and G-T = 0.0792. The rates were assumed to follow a gamma distribution with a shape parameter of 0.4747 for the variable sites. The proportion of the sites that were assumed to be invariable was 0.2041.

RESULTS

Feeding occurrence of the *Scrippsiella* spp.

In Expt 1, the 2- μm FLM were found in the protoplasm of *S. acuminata* STKP9909 cells, although they were rarely observed (Table 3, Fig. 1). However, no FLM were observed in the protoplasm of observed *S. donghaiensis* SDGJ1703, *S. lachrymosa* SLBS1703, *S. masanensis* SSMS0908, *S. plana* SSSH1009A, or *S. ramonii* VGO1053 cells (Table 3, Fig. 2). Similarly, in Expt 2, FLBs were found in the protoplasm of *S. acuminata* STKP9909 cells, although they were rarely observed (Table 3, Fig. 3A & B); however, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* did not feed on FLB (Table 3, Fig. 3C-L). Moreover, in Expt 3, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* did not feed on *Synechococcus* sp. (Table 3).

In Expt 4, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* did not feed on any of the microalgal prey species, which included the prymnesiophyte *Isochrysis galbana*, prasinophyte *Pyramimonas* sp. (PSSH1204), cryptophytes *Teleaulax amphioxeia* (TSGS0202), *Storeatula major* (SSSH1103), and *Rhodomonas salina*, raphidophyte *Heterosigma akashiwo* (HAKS9905), and phototrophic dinoflagellates *Heterocapsa rotundata* (HRSH1201), *Heterocapsa minima*

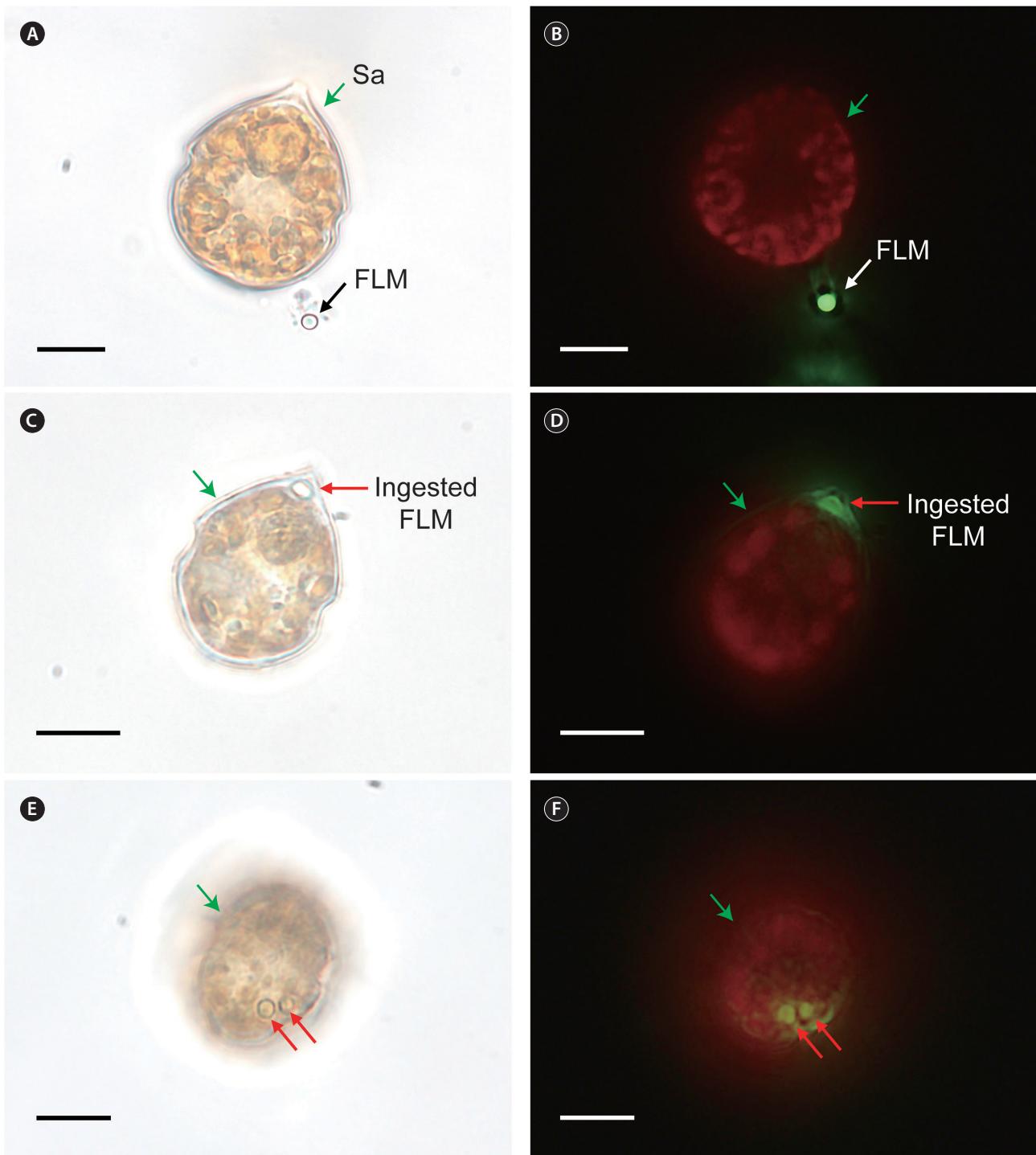


Fig. 1. *Scrippsiella acuminata* STKP9909 (Sa) not fed (A & B) or fed (C–F) fluorescently labeled microspheres (FLM). Micrographs A, C, and E were taken under a light microscope and B, D, and F under an epifluorescence microscope. Green arrows indicate Sa cells, black and white arrows indicate not ingested FLM, and red arrows indicate ingested FLM within Sa cells. Scale bars represent: A–F, 10 μ m.

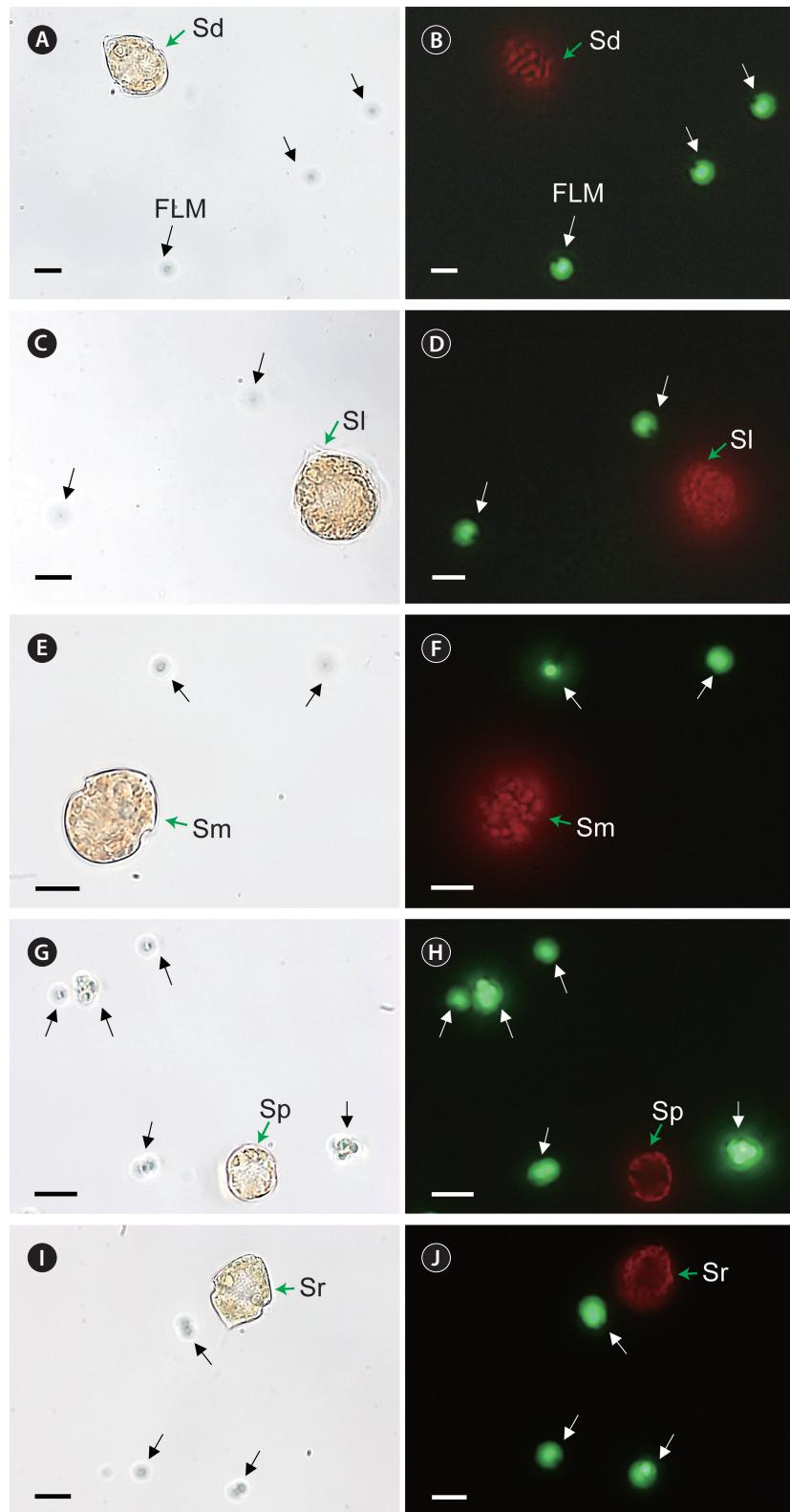


Fig. 2. *Scrippsiella donghaiensis* SDGJ1703 (Sd; A & B), *S. lachrymosa* SLBS1703 (Sl; C & D), *S. masanensis* SSMS0908 (Sm; E & F), *S. plana* SS-H009A (Sp; G & H), and *S. ramonii* VGO1053 (Sr; I & J), not fed fluorescently labeled microspheres (FLM). Micrographs A, C, E, G, and I were taken under a light microscope and those in B, D, F, H, and J under an epifluorescence microscope. Green arrows indicate *Scrippsiella* cells; black and white arrows indicate not ingested FLM. Scale bars represent: A–J, 10 µm.

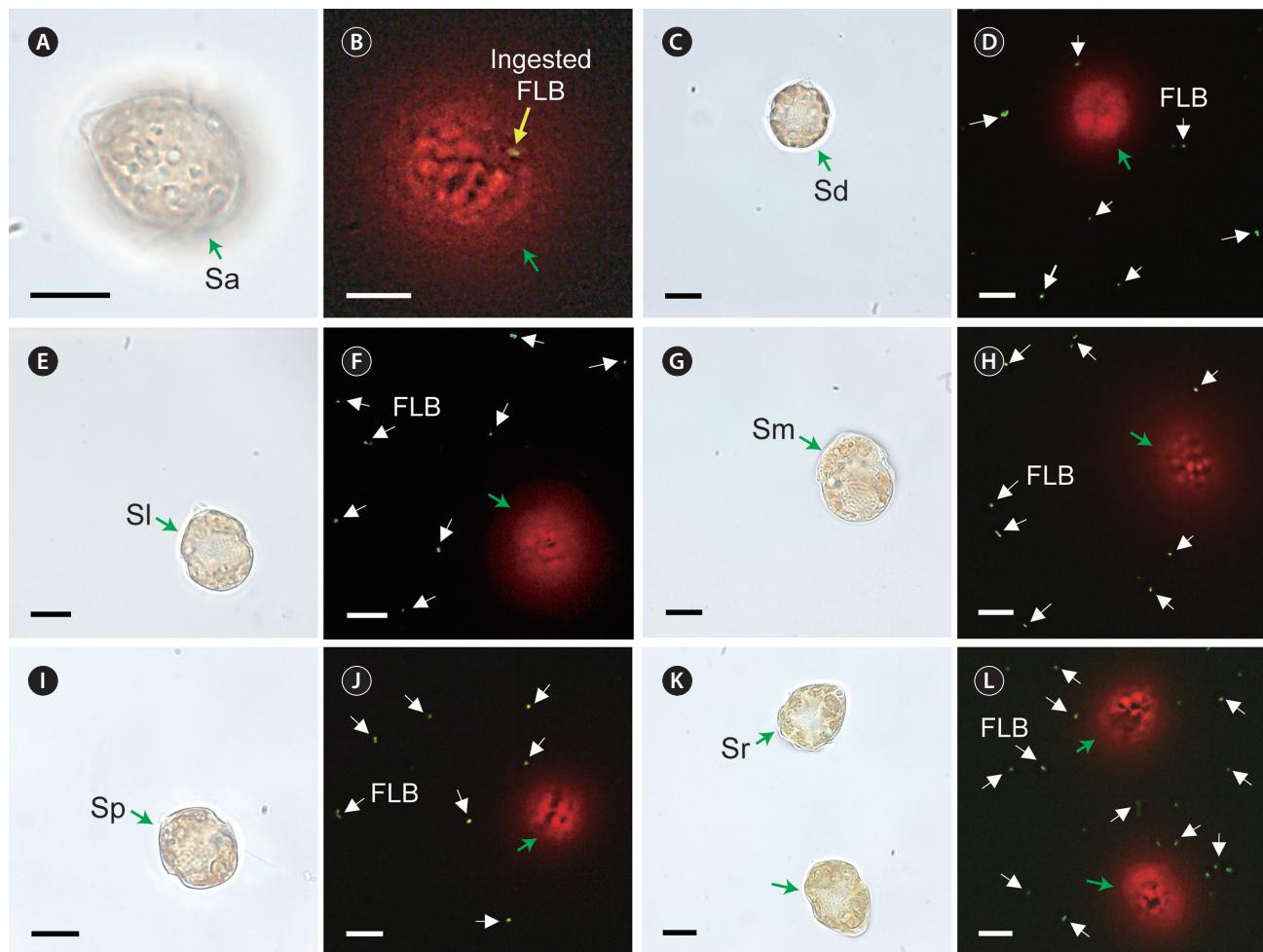


Fig. 3. A *Scrippsiella acuminata* STKP9909 (Sa; A & B) cell feeding on the fluorescently labeled heterotrophic bacteria (FLB) and *S. donghaiensis* SDGJ1703 (Sd; C & D), *S. lachrymosa* SLBS1703 (Sl; E & F), *S. masanensis* SSMS0908 (Sm; G & H), *S. plana* SSSH1009A (Sp; I & J), and *S. ramonii* VGO1053 (Sr; K & L) not feeding on the FLB. Micrographs A, C, E, G, I, and K were taken under a light microscope and B, D, F, H, J, and L under an epifluorescence microscope. Green arrows, *Scrippsiella* cells; a yellow arrow, ingested FLB within a *Scrippsiella* cell; white arrows, not ingested FLB. Scale bars represent: A–L, 10 μ m.

(HMMJ1604), *Amphidinium carterae* (SIO PY-1), *Procentrum cordatum* (PMKS9906), *Procentrum donghaiense* (PDYS1407), *Prorocentrum micans* (PMSH0910), and *Akashiwo sanguinea* (ASUSA) (Table 3, Fig. 4). Furthermore, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* did not show any attack behaviors toward the prey items, inhibit their swimming, or lyse the prey.

Phylogenetic analysis of *Scrippsiella* spp.

We obtained four novel LSU rDNA sequences from *S. acuminata* STKP9909, *S. donghaiensis* SDGJ1703, *S. plana* SSSH1009A, and *S. ramonii* VGO1053 and deposited them in GenBank under the accession numbers

OQ266790, OQ266882, OQ266885, and OQ275008 for *S. acuminata* STKP9909, *S. donghaiensis* SDGJ1703, *S. plana* SSSH1009A, and *S. ramonii* VGO1053, respectively. A phylogenetic tree generated using LSU rDNA showed that *S. acuminata*, *S. lachrymosa*, and *S. ramonii* belong to a large clade along with *S. spinifera*, *S. kirschiae*, *S. trifida*, *S. bicarinata*, *S. erinacea*, *S. sweeneyae*, *Scrippsiella* sp. JKG47-2, *S. precaria*, and *S. irregularis* (Fig. 5). This clade included not only the two mixotrophic species, *S. acuminata* STKP9909 and *Scrippsiella* sp. JKG47-2, but also *S. lachrymosa* and *S. ramonii*, which lack mixotrophic abilities. The clades that included *S. donghaiensis* SDGJ1703, *S. masanensis* SSMS0908, and *S. plana* SSSH1009A, which lack mixotrophic abilities, were divergent in the phylogenetic tree.

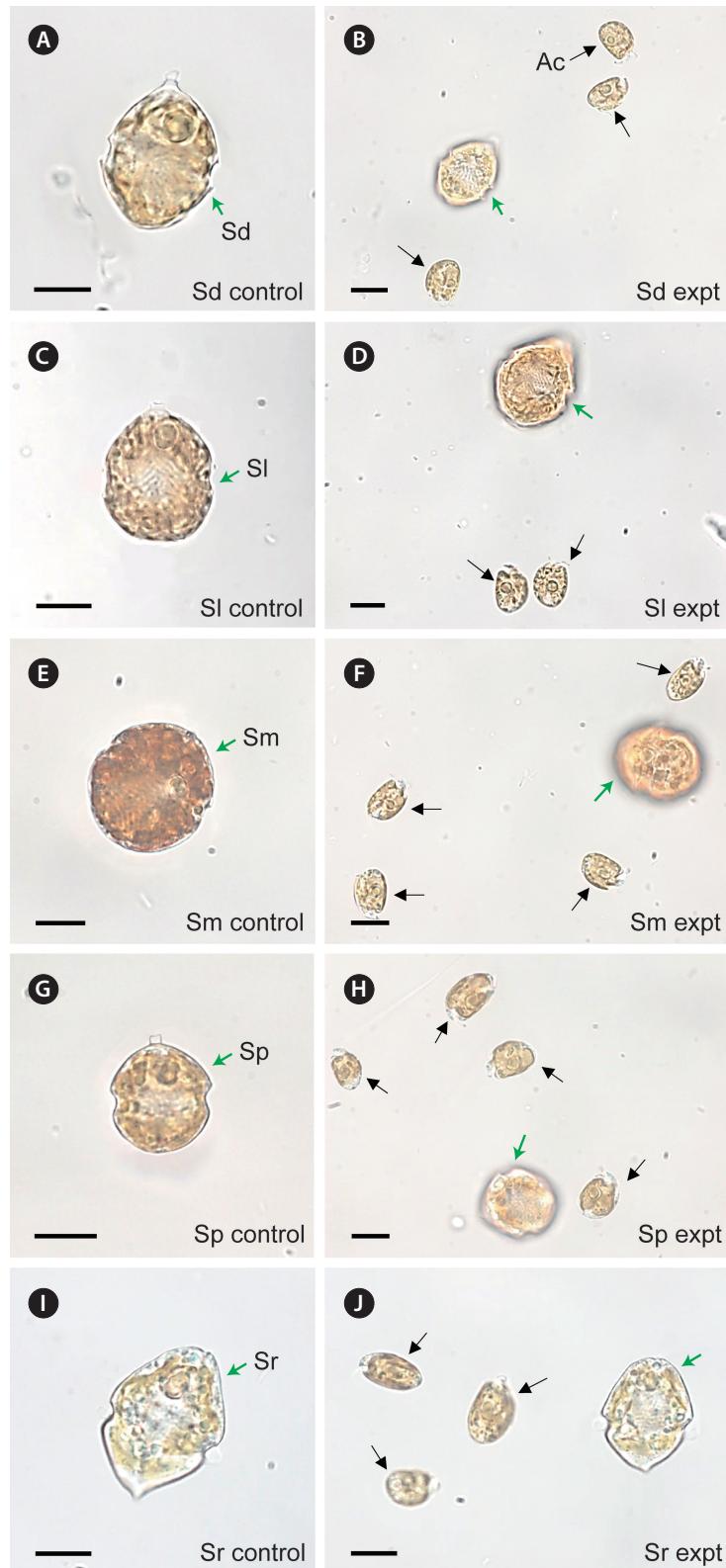


Fig. 4. Cells of *Scrippsiella donghaiensis* SDGJ1703 (Sd; A & B), *S. lachrymosa* SLBS1703 (Sl; C & D), *S. masanensis* SSMS0908 (Sm; E & F), *S. plana* SSSH1009A (Sp; G & H), and *S. ramonii* VGO1053 (Sr; I & J), not feeding on the dinoflagellate *Amphidinium carterae* (Ac). A, C, E, G, and I, *Scrippsiella* cells without Ac (control). B, D, F, H, and J, *Scrippsiella* cells with Ac (expt). Green arrows indicate *Scrippsiella* cells and black arrows indicate Ac cells. Scale bars represent: A–J, 10 µm.

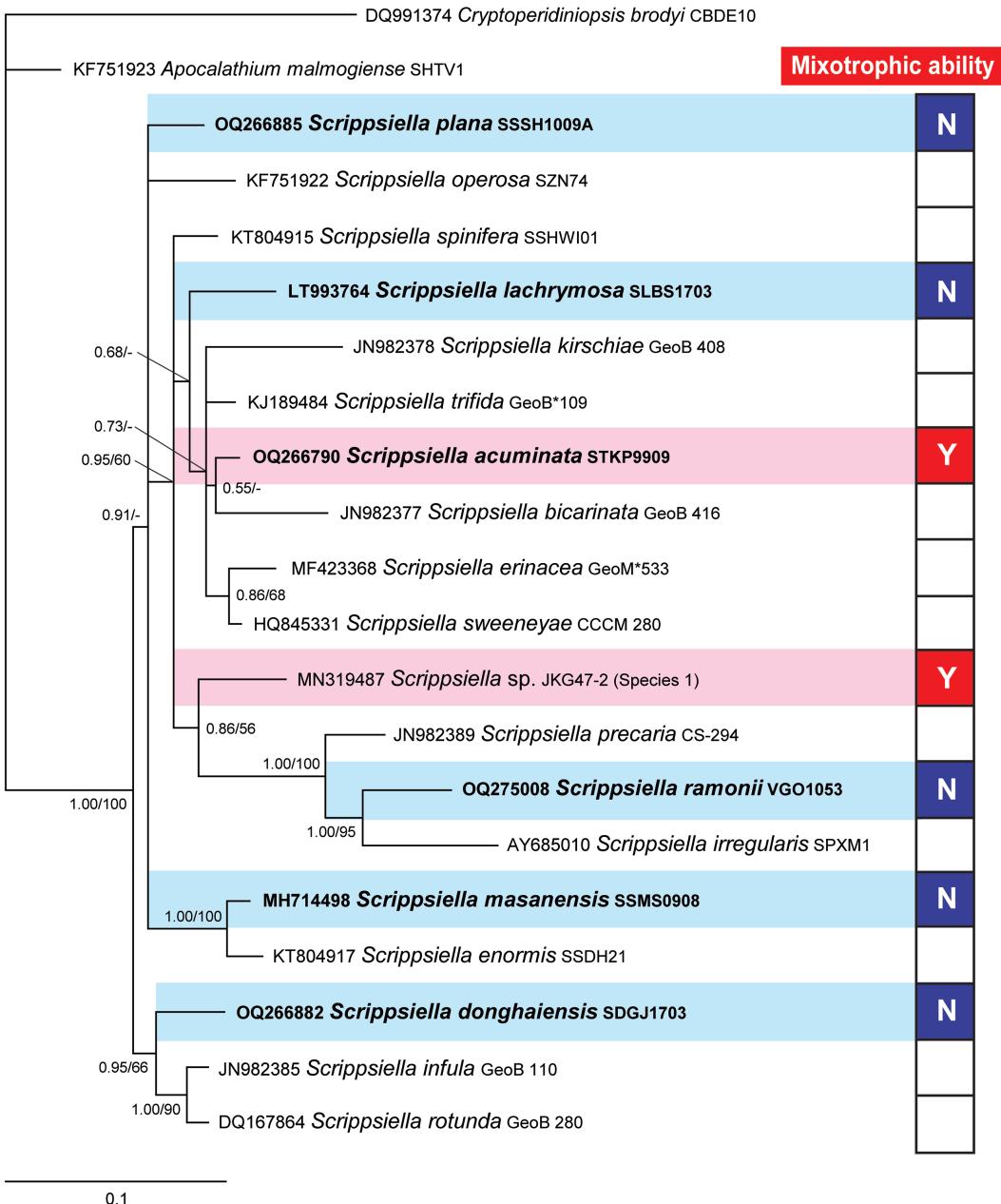


Fig. 5. Consensus Bayesian tree based on 601-bp aligned positions of the large subunit regions from 19 species within the genus *Scrippsiella*. Sequences from *Cryptoperidiniopsis brodyi* and *Apocalathium malmogiense* were used as an outgroup. The number of character changes are proportional to branch lengths and indicate the maximum likelihood bootstrap values (right) and Bayesian posterior probability (left); posterior probabilities ≥ 0.5 are shown; the species names are followed by the strain names of each species. The species tested in this study are shown in bold. Red boxes (Y) indicate mixotrophic *Scrippsiella* species; blue boxes (N) indicate *Scrippsiella* species without mixotrophic ability; and white boxes represent *Scrippsiella* species that were not tested for mixotrophy. Data of the presence or absence of mixotrophic ability for species within the genus *Scrippsiella* were obtained from this study, Jeong et al. (2005a, 2005b), and Coats et al. (2020).

DISCUSSION

The present study reports, for the first time, that *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii* lack mixotrophic abilities. Previous studies reported that three *Scrippsiella* species, *S. acuminata* and two unidentified *Scrippsiella* spp. were mixotrophic (Jacobson and Anderson 1996, Jeong et al. 2005a, 2005b, Coats et al. 2020). The results of the present study lower a proportion of mixotrophic species relative to the total *Scrippsiella* species tested for mixotrophy. In the phylogenetic tree based on LSU rDNA, the unidentified *Scrippsiella* species isolated from Korean waters was divergent from *S. acuminata*, indicating a distinct species. If the two unidentified *Scrippsiella* species from the United States and Korean waters are distinct from each other and from *S. acuminata*, the proportion of mixotrophic species relative to that of the total *Scrippsiella* species tested for mixotrophy is 38% (3 of 8 tested species). However, if the unidentified *Scrippsiella* species from the United States is *S. acuminata* or the unidentified *Scrippsiella* species from Korean waters, the proportion of the number of mixotrophic species relative to that of the total *Scrippsiella* species tested for mixotrophy is 29% (2 of 7 tested species). The mixotrophic abilities of species in the dinoflagellate genera *Alexandrium*, *Paragymnodinium*, and *Karenia* have also been reported (Lim et al. 2019, Yokouchi et al. 2022, Ok et al. 2023) (Table 4). The proportion of mixotrophic species relative to the total species tested for mixotrophy was 44% (7 of 16 tested species) in the genus *Alexandrium*, 60% (3 of 5 tested species) in the genus *Paragymnodinium*, and 40% (2 of 5 tested species) in the

genus *Karenia* (Table 4). Thus, the proportion of mixotrophic species in the genus *Scrippsiella* was slightly lower than that in the genera *Karenia* or *Alexandrium* and considerably lower than that in the genus *Paragymnodinium*. Among the formally described 34, 10, and 28 species in the genera *Alexandrium*, *Karenia*, and *Scrippsiella*, respectively, the mixotrophic abilities of 16, 5, and 6 species have been explored. Thus, the proportion of mixotrophic species relative to the total species tested for mixotrophy may be changed.

In the phylogenetic tree based on the LSU rDNA sequences of 19 *Scrippsiella* species, a large clade included *S. acuminata* and *Scrippsiella* sp. JKG47-2, which have mixotrophic abilities, and *S. lachrymosa* and *S. ramonii*, which lack mixotrophic abilities. However, three clades, *S. donghaiensis* SDGJ1703, *S. masanensis* SSMS0908, and *S. plana* SSSH1009A, that lack mixotrophic abilities diverged from the large clade. Thus, the ancestral species of *Scrippsiella* may have lacked feeding ability and acquired it later through evolution. However, to confirm this hypothesis, the mixotrophic abilities of other *Scrippsiella* spp. need to be examined.

S. acuminata was able to feed on the cyanobacterium *Synechococcus* sp., a prymnesiophyte, cryptophytes, raphidophytes, and phototrophic dinoflagellates that were $\leq 12.1 \mu\text{m}$ in equivalent spherical diameter (Jeong et al. 2005a, 2005b). The results of the present study expand the range of prey items of *S. acuminata* to include FLB. Heterotrophic bacteria are ubiquitous (Caron et al. 1982, Seong et al. 2006, Sanz-Sáez et al. 2020, Vijayan et al. 2022); thus, the ability of *S. acuminata* to feed on heterotrophic bacteria may be critical for the survival of this

Table 4. Number of species having or lacking the mixotrophic ability in the dinoflagellate genera *Scrippsiella*, *Alexandrium*, *Karenia*, and *Paragymnodinium*

Genus	No. of the species tested for mixotrophic ability	No. of the species having mixotrophic ability	No. of the species lacking mixotrophic ability	References
<i>Scrippsiella</i>	7 or 8 ^a	2 or 3 ^a	5	Jacobson and Anderson (1996), Jeong et al. (2005a, 2005b), this study
<i>Alexandrium</i>	16	7	9	Jacobson and Anderson (1996), Jeong et al. (2005a, 2005b), Yoo et al. (2009), Blossom et al. (2012, 2017), Lim et al. (2015, 2019), Lee et al. (2016)
<i>Karenia</i>	5	2	3	Jeong et al. (2005a), Glibert et al. (2009), Zhang et al. (2011), Ok et al. (2023)
<i>Paragymnodinium</i>	5	3	2	Yoo et al. (2010), Yokouchi et al. (2022)

^aIf the unidentified *Scrippsiella* species from United States is *S. acuminata* or the unidentified *Scrippsiella* species isolated from Korean waters, the number of *Scrippsiella* species tested for mixotrophic ability and those having the ability changes from 8 to 7 and from 3 to 2, respectively.

dinoflagellate species under conditions of inorganic nutrient depletion. Heterotrophic bacteria usually have high phosphorus : nitrogen ratios (Vadstein et al. 1988, Tezuka 1990) and some cyanobacteria can conduct nitrogen fixation (Mitsui et al. 1987, Zehr 2011). Therefore, *S. acuminata* may obtain phosphorus and nitrogen for their growth by feeding on heterotrophic bacteria and cyanobacteria in offshore or oceanic waters (Jeong et al. 2010b).

S. acuminata has a global distribution and can cause red tides in many countries (Moncheva et al. 2001, Pitcher et al. 2007, Gárate-Lizárraga et al. 2009, Jeong et al. 2021, Tsikoti and Genitsaris 2021). However, *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, or *S. ramonii*, which lack mixotrophic abilities, has caused few or no red tides (Jang et al. 2022). Thus, the mixotrophic ability of *S. acuminata* may allow it to cause red tides in several marine ecosystems.

S. acuminata is preyed upon by the common heterotrophic dinoflagellates *Oxyrrhis marina*, *Gyrodinium dominans*, *Polykrikos kofoidii*, *Oblea rotunda*, and *Pfiesteria piscicida*, ciliates *Tiarina fusus* and *Strombidinopsis* sp., copepods *Acartia omorii*, *Calanus helgolandicus*, *Calanus pacificus*, and *Temora longicornis*, and larvae of the mussel *Mytilus galloprovincialis* (Gill and Harris 1987, Hassett and Landry 1990, Jeong et al. 2002, 2004, Shin et al. 2003, Kim et al. 2019). Thus, *S. acuminata* plays an ecological role as a primary producer and predator of heterotrophic bacteria, cyanobacteria, and diverse microalgae, and serves as a suitable prey item for many heterotrophic protists in marine ecosystems. *S. donghaiensis*, *S. lachrymosa*, and *S. masanensis* are also consumed by *O. marina*, *G. dominans*, *P. kofoidii*, *O. rotunda*, *P. piscicida*, and *Strombidinopsis* sp. (Kim et al. 2019). Thus, *S. donghaiensis*, *S. lachrymosa*, and *S. masanensis* may play ecological roles as primary producers and prey for heterotrophic protists in marine ecosystems, and owing to its mixotrophic ability, *S. acuminata* has a different ecological niche from that of *S. donghaiensis*, *S. lachrymosa*, *S. masanensis*, *S. plana*, and *S. ramonii*.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. List of the 28 formally described species of the genus *Scrippsiella* (<https://www.e-algae.org>).

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