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Morphology and plastid *psb*A phylogeny of *Zygnema* (Zygnemataceae, Chlorophyta) from Korea: *Z. insigne* and *Z. leiospermum*

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Zygnema is a conjugating filamentous green algal genus that is distributed in a broad range of freshwater habitats, from sea level to alpine summits. Although more than 150 species have been described worldwide, their taxonomy remains unclear, probably owing to their relatively simple morphology. We investigated the detailed morphology of Korean *Zygnema* species, combined with analysis of the plastid *psb*A gene from 22 specimens of the genus and putative relatives, in order to develope a key to their identification and isolation, and to determine their relationships. We recognized two species of *Zygnema*; *Z. insigne* and *Z. leiospermum*, based on morphological characters such as width of the vegetative cell, position of zygospores, dimensions and form of spores, shape of female gametangia, and color of mesospores. The analysis of *psb*A data was consistent with morphological comparison. The pairwise divergence between two species was 3.7-4.1% (34-38 bp) in *psb*A sequences. The phylogeny of *psb*A revealed the monophyly of *Z. insigne* and *Z. leiospermum* together with two isolates of *Z. circumcarinatum* from Germany and Scotland. This is the first report on the *psb*A gene phylogeny of *Zygnema*.

Key Words: green algae; morphology; *psb*A; systematics; *Zygnema insigne*; *Z. leiospermum*

INTRODUCTION

Zygnema J. Agardh (1824) is a conjugating unbranched filamentous green algal genus that is widely distributed in aquatic habitats, from sea level to alpine summits (Transeau 1951). The slimy masses of this alga, less slippery than the *Spirogyra*s but more slippery than the *Mougeotia*s, occur in small lotic or lentic bodies of water (Transeau 1951, Bold and Wynne 1985). However, their life cycle is completed in a few weeks, and reproductions are frequently found in temporary ponds and ditches (Transeau 1951). Non-flagellated amoeboid gametes, sexual reproduction by isogametes, and short cylindrical cells containing a pair of stellate chloroplasts are features that assign *Zygnema* to the family Zygnemataceae (Smith 1933, Transeau 1934).

Since the description of *Zygnema* by J. Agardh (1824), more than 150 species have been described by taxonomic studies (Randhawa 1959, Kadlubowska 1984, Rundina 1998, Novis 2004, Zarina et al. 2006). Morphological criteria such as vegetative cell size, details of sexual reproduction, shape, dimension and color of zygospores, and ornamentation of the median spore wall are important in the identification of species (Randhawa 1959, Kadlubowska 1984, Johnson 2002). However, features such as cell width, and size of the zygote are highly variable

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in both culture and nature. For example, the variation of cell width in *Z. circumcarinatum* clones was much higher than expected (Miller and Hoshaw 1974), and these ploidal variants in clonal culture and field collected materials have led to a proliferation of species diversity in Zygnemataceae (McCourt and Hoshaw 1990).

Molecular studies of *Zygnema* began with phylogeny of the family Zygnemataceae, in which plastid *rbc*L and nuclear small subunit of ribosomal gene (SSU rDNA) were analyzed for a few species (McCourt et al. 1995, 2000, Gontcharov et al. 2003, 2004, Hall et al. 2008). Recently, Stancheva et al. (2012) gave an intensive phylogeny of *Zygnema* from California, based on the *cox*3 and *rbc*L genes, recognizing two clades within the genus. However, the relationships within *Zygnema* remain unresolved due to limited taxon sampling.

To date, eleven species of *Zygnema* have been recorded by floristic studies on local populations in Korea (Chung 1968, 1970, Wui and Kim 1990, Kim and Kim 2009): *Z. carinthiacum* Beck, *Z. cruciatum* (Vaucher) Agardh, *Z. decussatum* (Vaucher) Agardh, *Z. insigne* (Hassall) Kützing, *Z. leiospermum* De Bary, *Z. pectinatum* (Vaucher) Agardh, *Z. peliosporum* Wittrock, *Z. shigaense* Yamagishi, *Z. stellinum* (Vaucher) Agardh, *Z. sterile* Transeau and *Z. vaucherii* Agardh. Of these, *Z. cruciatum* is the only species for which the morphology was studied by light microscopy and scanning electron microscopy (Kim and Kim 2009). There are no phylogenetic studies of Korean *Zygnema* probably owing to difficulties in their identification and limited sampling of fertile specimens.

In the present study, we investigate the morphology and *psb*A (encoding the photosystem II thylakoid protein D1) sequences of two *Zygnema* species, *Z. insigne* and *Z. leiospermum*, in Korea, with the aim of clarifying their taxonomic identities. One species of *Zygnema* from the Culture Collection of Algae and Protozoa (CCAP) was included for a better understanding of phylogeny. Putative relatives, *Spirogyra* and *Mougeotia*, were included in the study as outgroup. This is the first report on the *psb*A phylogeny of *Zygnema*.

MATERIALS AND METHODS

Samples, culture and morphology

A total of 22 isolates of *Zygnema* and putative relatives were included in the present study (Table 1). The strains

Table 1. Taxa, collection site and date, and GenBank accession number of the *psb*A sequences of the Zygnemataceae used in the present study

Taxa	Voucher code	Collection site and date	GenBank accession No.
Mougeotia transeaui Collins	UTEXLB2496	UTEXLB2496	KC170329
Mougeotia sp. 1	KCH85	Nonsan, Korea; Apr 13, 2004	KC170325
Mougeotia sp. 1	KCH223	Yecheon, Korea; Oct 29, 2004	KC170327
Mougeotia sp. 2	KCH47	Changwon, Korea; Oct 31, 2003	KC170324
Mougeotia sp. 3	KCH258	Boeun, Korea; Apr 17, 2008	KC170328
Mougeotia sp. 4	KCH175	Donghae, Korea; Oct 28, 2004	KC170326
Spirogyra decimina (Müller) Kützing	KCH124	Boeun, Korea; Oct 22, 2004	KC170343
	KCH149	Goseong, Korea; Oct 13, 2004	KC170344
S. ellipsospora Transeau	KCH18	Sacheon, Korea; Oct 31, 2003	KC170342
	KCH177	Donghae, Korea; Oct 28, 2004	KC170345
Spirogyra sp.	KCH251	Taean, Korea; Oct 24, 2007	KC170340
Spirogyra sp.	KCH275	Nonsan, Korea; Apr 30, 2008	KC170341
Zygnema insigne (Hassall) Kützing	KCH77	Pohang, Korea; Mar 25, 2004	KC170330
	KCH87	Seocheon, Korea; Apr 13, 2004	KC170331
	KCH225	Gochang, Korea; Apr 24, 2005	KC170332
	KCH226	Gochang, Korea; Apr 24, 2005	KC170333
Z. leiospermum De Bary	KCH202	Yeongdeok, Korea; Oct 29, 2004	KC170334
	KCH254	Taean, Korea; Oct 25, 2007	KC170335
	KCH263	Buyeo, Korea; Apr 30, 2008	KC170337
	KCH277	Eumseong, Korea; May 9, 2008	KC170336
	KCH280	Jecheon, Korea; May 15, 2008	KC170338
Z. circumcarinatum Czurda	CCAP698/1A	CCAP698/1A	KC170339

of nine *Zygnema* were isolated from various freshwaters bodies across South Korea, and vegetative filaments containing 2-3 cells of each collection were used for unialgal culture. For the present study, six *Spirogyra* and five *Mougeotia* isolates from Korea were also included. In addition, one *Zygnema* and one *Mougeotia* isolate were obtained from CCAP and the Culture Collection of Algae at the University of Texas at Austin (UTEX), respectively. All isolates were grown in Wood Hole liquid medium buffered to pH 7.0 (Nichols 1973). Cultures were maintained at 20 ± 1°C on a 16 : 8-h light : dark cycle under 30-50 illumination with μmol m^{-2} s^{-1} with cool-white fluorescent lamps (Pringsheim 1967, Stein 1973). Morphological features were observed under a light microscope (Nikon Optiphot; Nikon, Tokyo, Japan) equipped with the Nikon UFX-II camera. Voucher specimens were deposited at the herbarium of Chungbuk National University (CBNU), Cheongju, Korea.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Live or air dried specimens from unialgal cultures of each strain were used for DNA extraction. Genomic DNA was extracted from approximately 0.01 g of algal powder, ground in liquid nitrogen, using the DNeasy Plant Mini Kit (Qiagen Gmbh, Hilden, Germany). Extracted DNA was dissolved in 150 μL of distilled water, stored at -20°C and was used to amplify the *psb*A gene.

The *psb*A region was amplified with *psb*A-F and *psb*A-R2 primers as described by Yoon et al. (2002). PCRs were carried out in a 25 μL reaction volume containing 10× Ex *Taq* buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 pmole of each primer, 5 U *Taq* polymerase (Takara Ex Taq; Takara Bio Inc., Tokyo, Japan), 25-50 ng DNA template, and distilled water. Amplification was performed using a modified protocol of McCourt et al. (1995). The PCR started with an initial denaturation cycle at 95°C for 4 min, followed by 33 cycles of denaturation at 95°C for 1 min, primer annealing at 47°C for 1 min, and an extension at 72°C for 2 min. The amplification was terminated with a final extension at 72°C for 6 min. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The sequences of the forward and reverse strands were determined for all strains using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The electropherogram output for each specimen was edited using the program Sequence Navigator v. 1.0.1 (Applied Biosystems).

Phylogenetic analyses

Twenty-three *psb*A sequences, including previously published sequence of *Zygnema circumcarinatum* SAG698-1a (Turmel et al. 2005), consisting of eleven *Zygnema*, six *Spirogyra*, and six *Mougeotia*, were collated using the multisequence editing program, SeqPup (Gilbert 1995) and were aligned by eye. There were no gaps in our alignments of the *psb*A region.

Phylogenetic trees were reconstructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses (BA). MP trees were constructed with PAUP* 4.0b.10 (Swofford 2002) using a heuristic search algorithm with the following settings: 100 random sequence-addition replicates, tree bisection-reconnection (TBR) branch swapping, MulTrees, all characters unordered and unweighted, and branches with a maximum length of zero collapsed to yield polytomies. Bootstrap values (BS) for nodes in the tree were obtained from 1,000 bootstrapping replicates (Swofford 2002).

For ML and BA, a likelihood ratio test was carried out with ModelTest 3.08b (Posada and Crandall 1998). The general time reversible (GTR) model, with a gamma correction for rate variation across sites (G) and proportion of invariable sites (I), was chosen as the best model for our data. ML analysis was conducted using PAUP* with GTR + I + G model. To find the best tree, we used a heuristic search with 100 random sequence-addition replicates, TBR branch swapping, and MulTrees options. The ML BS for each branch was estimated by performing 1,000 replicate ML searches, with two random addition sequence replicates.

BA were performed using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Each analysis was initiated from a random starting tree, and the program was set to run four chains of Markov chain Monte Carlo iterations simultaneously for 1,000,000 generations, with tree sampling every 100th generation. The likelihood scores stabilized at approximately 110,000 generations, and thus the first 1,100 trees were burned.

RESULTS

Morphology

We collected nine isolates of *Zygnema insigne* and *Z. leiospermum* from Korean water systems. All isolates

were cultured in the laboratory and the cultured materials were compared with field-collected specimens. However, most of quantitative characters were based on the field specimens. A comparative morphology of two species is given in Table 2.

Zygnema insigne (Hassall) Kützing (Fig. 1)

Kützing 1849, p. 444; Czurda 1932, p. 127, Fig. 131; Jao 1935, p. 567, Pl. 1, Fig. 6; Transeau 1951, p. 35; Randhawa 1959, p. 234, Fig. 176; Kadlubowska 1984, p. 201, Fig. 297.

Basionym. *Tyndaridea insignis* Hassall 1843.

Specimens examined. Rice field, Pohang, Korea (Mar 25, 2004); Ditch water, Seocheon, Korea (Apr 13, 2004); Ditch water, Gochang, Korea (Apr 24, 2005); Rice field, Gochang, Korea (Apr 24, 2005).

World distribution. Australia, China, Europe, India, Japan, Pakistan, and the United States of America.

Morphology. Plants are unbranched filamentous of short cylindrical cells with plane end wall (Fig. 1A). Cells have two stellate chloroplasts with a central pyrenoid. Vegetative cells are 29-30 μm in width, 30-50 μm in length. Sexual reproduction is scalariform (Fig. 1B). Zygospores are formed in receptive (female) gametangia that remain cylindrical or slightly enlarged on the conjugating side (Fig. 1C). Zygospores are spherical or ellipsoid, 25-35 μm wide, and 30-50 μm long. Median spore wall is smooth and yellow-brown at maturity.

Remarks. *Tyndaridea insignis* Hassall was transferred to the genus *Zygnema* by Kützing (1849) based on the vegetative cell size and the shape of zygospores. Jao (1935) provided characteristics of additional features such as lateral or scalariform conjugation, shape of fertile cells, shape and size of zygospores, ornamentation and color of median spore wall. Transeau (1951) observed the occurrence of aplanospores.

Korean specimens correspond well with the previous descriptions of the species, except aplanospores and lateral conjugation. Korean *Z. insigne* has a vegetative cell width of 25-30 μm, zygospores which are formed in receptive gametangia, mostly cylindrical female gametangia, spherical or ellipsoid zygospores, and a smooth median spore wall. It is common in the fresh water systems in Korea.

Z. insigne has a similar vegetative cell width to *Z. stellinum* (Vaucher) Agardh and *Z. vaginatum* (Vaucher) Agardh (Randhawa 1959, Kadlubowska 1984), as well as similar scalariform conjugation and zygospores formed in receptive gametangia, and color of mesospores. However, *Z. stellinum* has enlarged female gametangia, ovoid zygospores and a scrobiculate median spore wall, and *Z. vaginatum* contains slightly enlarged female gametangia, globose to ovoid zygospores and a verrucose-tuberculate median spore wall. This species was previously described

Table 2. Comparisons of morphological characteristics for *Zygnema* isolates investigated in the present study

Taxa	Vegetative cell		Conjugation	Zygospore			Median spore wall	Shape of female gametangia
	Width (μm)	Length (μm)		Width (μm)	Length (μm)	Shape		
Zygnema insigne								
KCH77	29-30	-	Scalariform	-	-	-	-	-
KCH87	29-30	50	Scalariform	25-35	30-50	Spherical to ellipsoid	Smooth	CT, ET
KCH225	29-30	-	Scalariform	30-35	30-35	Spherical to ellipsoid	Smooth	CT
KCH226	29-30	30-35	Scalariform	30-35	30-40	Spherical to ellipsoid	Smooth	CT
Z. leiospermum								
KCH202	27-28	30	Scalariform	32-35	32-35	Spherical	Smooth	CT, ET
KCH254	22-25	50-100	Scalariform	-	-	-	-	-
KCH263	23-27	-	Scalariform	33-35	33-35	Spherical	Smooth	CT, ET
KCH277	27-30	27-30	Scalariform	27-35	27-35	Spherical	Smooth	ET
KCH280	27-30	-	Scalariform	40-43	40-43	Spherical	Smooth	ET
Z. circumcarinatum	20-22	-	Scalariform	24-29	24-29	Spherical to compressed spherical	Scrobiculate with pits	-

CT, cylindrical type; ET, enlarged type.

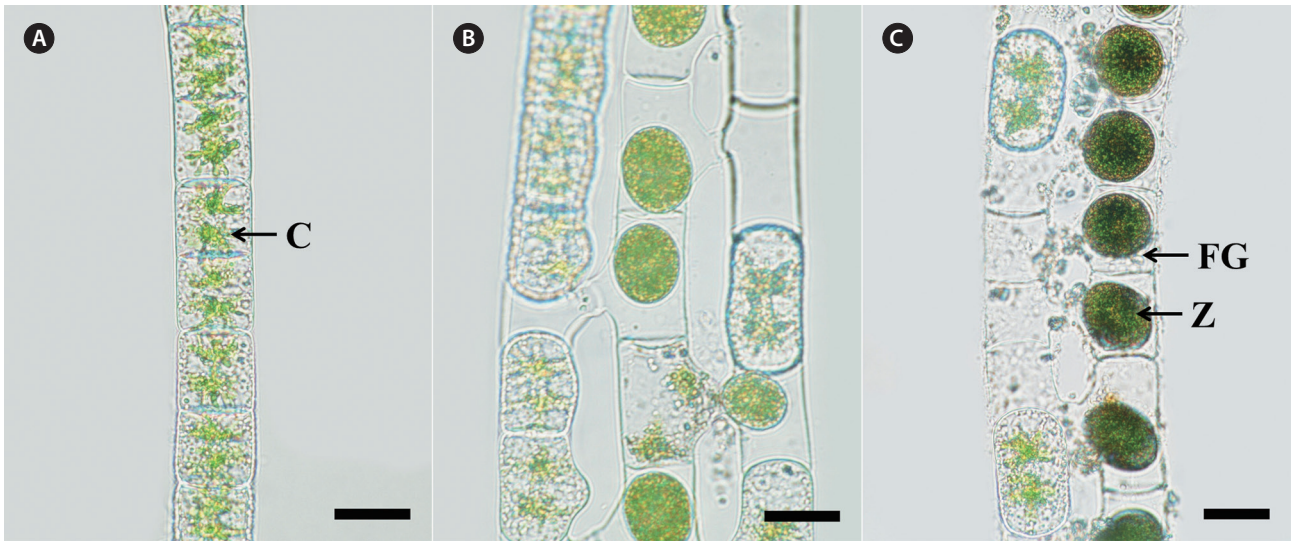

Fig. 1. *Zygnema insigne* (Hassall) Kützing. (A) Vegetative filament with plane septa and two stellate chloroplasts per cell. (B) Triple scalariform conjugation showing the fusion of amoeboid gametes and the formation of zygospores. (C) Cylindrical or slightly enlarged female gametangia and spherical to ellipsoid zygospores. C, chloroplast; FG, female gametangium; Z, zygospore. Scale bars represent: A-C, 30 μm.

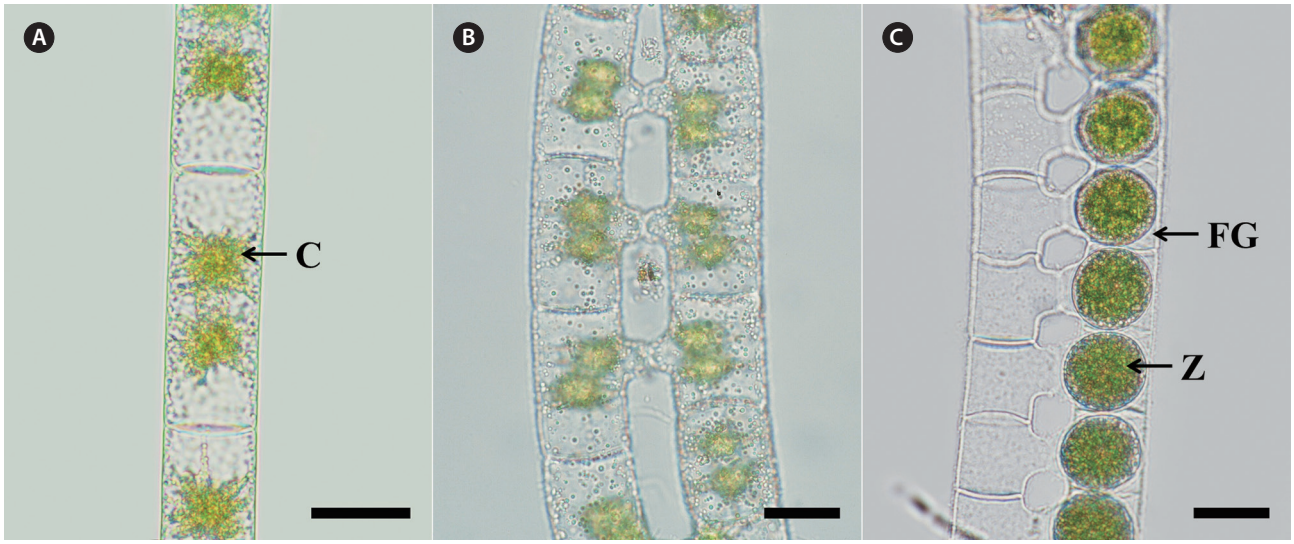

Fig. 2. *Zygnema leiospermum* de Bary. (A) Vegetative filament with plane end wall and two stellate chloroplasts per cell. (B) Early conjugation between two filaments. (C) Inflated female gametangia on the inner side and spherical zygospores. C, chloroplast; FG, female gametangium; Z, zygospore. Scale bars represent: A-C, 30 μm.

in Korea (Chung 1968, 1993), and has also been reported in Japan (Yamagishi 1965) and China (Jao 1935).

Zygnema leiospermum de Bary (Fig. 2)

De Bary 1858, p. 77, Pl. I, Figs 7-14; Czurda 1932, p. 119, Fig. 123; Transeau 1951, p. 32; Randhawa 1959, p. 234, Fig. 175; Kadlubowska 1984, p. 174, Fig. 240.

Specimens examined. Stream, Yeongdeok, Korea (Oct 29, 2004); Ditch water, Taean, Korea (Oct 25, 2007); Gungnam pond, Buyeo, Korea (Apr 30, 2008); Rice field, Eumseong, Korea (May 9, 2008); Rice field, Jecheon, Korea (May 15, 2008).

World distribution. British Isles, China, Europe, Greenland, Iceland, Japan, and the United States of America.

Morphology. Plants are unbranched filamentous of cylindrical cells with plane end wall (Fig. 2A). Cells have two star-shaped chloroplasts and each chloroplast has a cen-

tral pyrenoid. Vegetative cells are 22-30 μm in width, and 27-100 μm in length. Sexual reproduction is scalariform (Fig. 2B). Zygospores are formed in only one of the gametangia that remain enlarged on the conjugating side (Fig. 2C). Zygospores are spherical, 24-43 μm wide and 24-43 μm long. The median spore wall is smooth and yellow-brown at maturity.

Remarks. *Z. leiospermum* was described by De Bary (1858) based on the vegetative cell size and features of zygospores. Czurda (1932) added features such as lateral conjugation, shape of fertile cells, shape and size of zygospores, ornamentation and color of median spore wall.

Korean specimens agree well with the description of *Z. leiospermum* except aplanospores. It is distinguished by the formation of zygospores in receptive gametangia, enlarged female gametangia, ovoid or spherical zygospores, and a smooth median spore wall. This species showed remarkable variations in the size of vegetative cells and zygospores in Korean populations. Genetic variation of Korean specimens, despite the conserved *psb*A gene in freshwater algae (Boo et al. 2010), is an interesting result that warrants further study.

Z. leiospermum is comparable to *Z. hausmannii* (De Notaris) Czurda and *Z. luteosporum* Czurda in terms of the width of the vegetative cells, scalariform conjugation, formation of zygospores in receptive gametangia, and yellow-brown mesospore (Transeau 1951, Kadlubowska 1984). *Z. hausmannii* has enlarged female gametangia, and scrobiculate mesospores with large (7-9 μm) diameter of pits, and *Z. luteosporum* contains cylindrical or slightly enlarged female gametangia, ovoid zygospores and a scrobiculate median spore wall with pits of less than 2 μm diameter. This species was previously listed in Korea without description or illustration (Yi 1980, Kim and Chung 1982), and has also been reported in Japan (Yamagishi 1965).

Sequence and phylogenetic relationships

The 923 nucleotides of the *psb*A gene were determined for 22 isolates of *Zygnema* and its relatives. Of these, 34 positions (3.7%) were variable, and 178 positions (19.3%) were parsimoniously informative. Nucleotide substitutions for *Zygnema* consisted of 2.22 times more transitions than transversions.

Within ingroup (Table 3), intraspecific pairwise divergence ranged from 0 to 0.1% within *Z. insigne* and to 0.8% within *Z. leiospermum*. However, two isolates of *Z. circumcarinatum*, one from SAG698-1a and the other from CCAP698/1A, differed by 43 bp (4.7% sequence divergence, not shown here). Interspecific pairwise divergence ranged from 3.7 to 4.1% (34-38 bp) between *Z. insigne* and *Z. leiospermum*. The p-divergence between *Z. circumcarinatum* CCAP698/1A and *Z. leiospermum* was slightly greater (5.3-5.9%, 49-54 bp) than that between *Z. circumcarinatum* CCAP698/1A and *Z. insigne* (4.6-4.7%, 42-43 bp). Outside from *Zygnema*, intraspecific pairwise divergence was 0 within *Spirogyra decimina*, *S. ellipsospora*, and *Mougeotia* sp. 1. The sequence divergence for *psb*A gene within *Spirogyra* ranged from 1.3% (between *S. decimina* and *Spirogyra* sp. KCH275) to 3.9% (between *S. ellipsospora* and *Spirogyra* sp. KCH251), and within *Mougeotia* ranged from 2.8% (between *Mougeotia* sp. 2 and *Mougeotia* sp. 1) to 10.9% (between *M. transeaui* and *Mougeotia* sp. 1). Sequence divergence between *Zygnema* and outgroup ranged from 7.5% between *Z. insigne* and *Spirogyra* sp. KCH275 to 12.3% between *Z. insigne* and *Mougeotia transeaui*.

The ML tree (Fig. 3) was identical to the single most parsimonious tree (tree length = 1,182 steps, consistency index = 0.589, and retention index = 0.828). The genus *Zygnema* formed a well-resolved clade (100% for MP and 97% for ML, and 1.0 Bayesian posterior probability for BA), being subdivided into four lineages: *Z. insigne*, *Z. leiospermum*, and two lineages of *Z. circumcarinatum*. *Z. insigne* and *Z. leiospermum*, both from Korea, were clearly distinct within the clade.

DISCUSSION

This is the first *psb*A report on the phylogenetic relationships of *Zygnema* in the family Zygnemataceae. We recognized only two species from Korea, *Z. insigne* and *Z. leiospermum*, despite the collection of samples from many water systems over more than three years (2007-2010). However, 11 species of *Zygnema* were reported in previous floristic studies in Korea (Chung 1970, Yi 1980, Wui and Kim 1990). The species not collected through the present study are *Z. carinthiacum*, *Z. decussatum*, *Z. pectinatum*, *Z. peliosporum*, *Z. shigaense*, *Z. stellinum*, *Z. sterile*, *Z. vaucherii*, and *Z. cruciatum*. Most of these species were described in morphology, but they were only reported for one or two locations. The stark discrepancy between our results, with extensive sampling and only a limited species collection, and the previous reports, may be due, at least in part, to misidentification of field-collected materials in previous floristic studies, or due to a reduction in the survival and reproduction of *Zygnema* species in Korean water bodies.

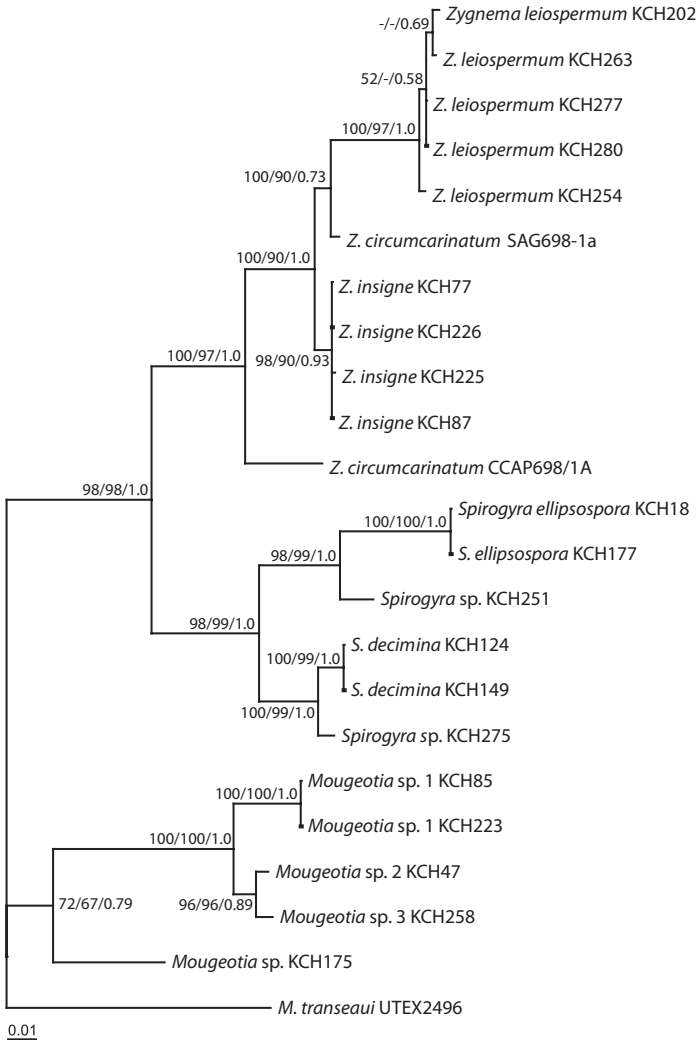

Fig. 3. Maximum likelihood (ML) tree for the Zygnemataceae estimated from the *psb*A sequences (GTR + I + G model, -ln L = 2,534.12854). The numbers above or under the branches are maximum parsimony and ML bootstrap values and Bayesian posterior probabilities. A dash indicates bootstrap support of <50%, and a posterior probability <0.5.

Table 3. Pairwise divergence in *psb*A sequences between specimens of *Zygnema* used in this study

		1	2	3	4	5	6	7	8	9	10
1	*Z. insigne* KCH77	-	0	0.001	0	0.040	0.038	0.037	0.038	0.038	0.046
2	*Z. insigne* KCH87	0	-	0.001	0	0.040	0.038	0.037	0.038	0.038	0.046
3	*Z. insigne* KCH225	1	1	-	0.001	0.041	0.039	0.038	0.039	0.039	0.047
4	*Z. insigne* KCH226	0	0	1	-	0.040	0.038	0.037	0.038	0.038	0.046
5	*Z. leiospermum* KCH202	37	37	38	37	-	0.007	0.003	0.004	0.004	0.054
6	*Z. leiospermum* KCH254	35	35	36	35	6	-	0.008	0.004	0.004	0.059
7	*Z. leiospermum* KCH263	34	34	35	34	3	7	-	0.003	0.003	0.053
8	*Z. leiospermum* KCH277	35	35	36	35	4	4	3	-	0	0.056
9	*Z. leiospermum* KCH280	35	35	36	35	4	4	3	0	-	0.056
10	*Z. circumcarinatum* CCAP698/1A	42	42	43	42	50	54	49	52	52	-

Numerals below the diagonal indicate absolute distances and those above diagonal indicate uncorrected p-distances.

The topologies of the *psb*A trees are basically congruent in MP, ML, and BA. The monophyly of *Zygnema* was strongly supported (100% for MP, 97% for ML, and 1.0 for BA), as was in previous *rbc*L (McCourt et al. 1995, 2000), SSU rDNA (Gontcharov et al. 2003), both *rbc*L + SSU datasets (Gontcharov et al. 2004, Hall et al. 2008), and *rbc*L + *cox*3 datasets (Stancheva et al. 2012).

In our *psb*A sequence analyses, taxa of *Zygnema* consisted of four groups, in which two Korean species were included: *Z. insigne* and *Z. leiospermum*. The intraspecific divergence of *Z. insigne* was up to 0.1%. However, *Z. leiospermum* was variable in filament width and length, and in *psb*A sequences (up to 0.8%). The high pairwise divergence may be reflected either from morphological variation within the *Zygnema* species (Stancheva et al. 2012) and / or different ecological niches (Randhawa 1959).

Z. insigne and *Z. leiospermum* shared apomorphic characters such as scalariform conjugation, zygospores formed in receptive gametangia, and yellow-brown and smooth mesospores at maturity. However, they differed chiefly in their vegetative cell size, shape of female gametangium, and shape and dimensions of zygospore. *Z. insigne* is characterized by having cylindrical or slightly enlarged female gametangia, spherical to ellipsoid zygospores, and a broad vegetative cell width of 29-30 μm. *Z. leiospermum* is characterized by having enlarged female gametangia, spherical zygospores, and a narrow filament of 22-24 μm (Kadlubowska 1984).

Z. circumcarinatum CCAP698/1A and the two Korean species were similar in terms of their scalariform conjugation, spherical zygospores, and yellow-brown mesospores at maturity. Despite their similarity to the two species from Korea, *Z. circumcarinatum* is characterized by having 20-22 μm vegetative cells, zygospores formed in the conjugating tube, spherical or compressed-spherical zygospores, pitted and yellow-brown mesospore at maturity (Transeau 1951, Kadlubowska 1984).

Z. circumcarinatum SAG698-1a (Turmel et al. 2005) and CCAP698/1A analyzed in the present study markedly differed by 43 bp (4.7% p-distance) and hence, are not grouped together (Fig. 3). Based on the comparisons of *rbc*L sequences among four strains of *Z. circumcarinatum* from the Microalgae and Zygnemophyceae Collection of Hamburg (MZCH), UTEX, and SAG698-1a, Stancheva et al. (2012) suggested that the strain SAG698-1a (Turmel et al. 2005) is not *Z. circumcarinatum*, but is another species of the genus.

A single monophyletic *Spirogyra* clade was sister to *Zygnema* clade (98% for MP and ML and 1.0 for BA), as was present in previous *rbc*L (McCourt et al. 1995, 2000), and both *rbc*L + SSU datasets (Hall et al. 2008). In our *psb*A analyses, taxa of *Spirogyra* were divided into two major clades (98% for MP, 99% for ML, and 1.0 for BA), one containing *S. ellipsospora* and *Spirogyra* sp. KCH251, and the other consisting of *S. decimina* and *Spirogyra* sp. KCH275. A previous study on the phylogeny of 15 *Spirogyra* species from Korea, based on plastid *rbc*L gene, revealed four major clades within the genus (Kim et al. 2006). The four clades are morphologically supported, although the inter-cladal relationships have not been resolved (Kim et al. 2006).

In addition, four *Mougeotia* species were confirmed to occur in Korea. The results reveal the molecular taxonomy of Korean *Mougeotia* in which six morphological species of the genus have been reported (Chung 1993). Only one species of the genus was investigated for *rbc*L in Korea (Kim et al. 2006). Taxonomic revision of *Spirogyra* and *Mougeotia* from Korea are the next aim of this study group.

In conclusion, we confirmed two species of *Zygnema*, *Z. insigne* and *Z. leiospermum* from Korea, based on combined study of morphology and *psb*A gene sequences. Although vegetative filaments were variable in Korean *Zygnema* species, morphological features such as the size of vegetative cells, details of sexual reproduction, shape and dimensions of spore, and ornamentation and color of spore proved to be useful characteristics for identifying *Zygnema* species. Further taxon sampling may confirm more *Zygnema* species in Korea, and analyses of the fast evolving genes such as *tuf*A may be needed for a better understanding of the genus.

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