

Phylogeny of *Praestephanos* gen. nov. (Thalassiosirales, Bacillariophyceae) based on *Stephanodiscus suzukii*, and related freshwater thalassiosiroid diatoms

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Received 20 February 2014; Accepted 23 April 2014

Abstract: A new genus, *Praestephanos*, is described with its type species based on *Stephanodiscus suzukii*. In addition, two new combinations are described: *P. triporus* and *P. carconensis*. This genus is similar to the genus *Stephanodiscus*, however, the pattern of the areolae changes at the level of the marginal fultoportulae but not at the level of the rimoportulae or spines, and this differs from *Stephanodiscus*. Five clades, *Stephanodiscus*, *Cyclostephanos*, *Cyclotella ocellata*–*Handmannia* complex, *Praestephanos* and *Discostella* were detected by molecular analysis. The *Discostella* clade has the most basal position of these five clades. The *Praestephanos* clade holds a clearly basal position in relation to the *Stephanodiscus* and *Cyclostephanos* clades. However, the relationship between *Praestephanos* and *Cyclotella ocellata*–*Handmannia* was uncertain. Since these four clades separated with short branches in the phylogenetic tree, these four clades (excluding *Discostella*) may have evolved during a very short geological period.

Key words: post-Miocene type, *Praestephanos carconensis*, *Praestephanos triporus*, *Stephanodiscus carconensis*

Introduction

Stephanodiscus suzukii Tuji & Kociolek and *S. pseudosuzukii* Tuji & Kociolek from Lake Biwa, Japan were described by Tuji & Kociolek (2000). Both species are endemic to Lake Biwa, where several paleontological and evolutionary studies have been carried out using long geological cores (Mori 1974, Kuwae et al. 2007). Mori (1974) assumed that *S. niagarae* Ehrenb. is the origin of both species. However, more recent results (Kuwae et al. 2007) do not support this assumption.

Both species had previously been identified as *S. carconensis* Grunow described by Grunow (1878) and *S. carconensis* var. *pusilla* Grunow in Van Heurck (1882) and re-described by Skvortzow (1936) from Lake Biwa. The characteristics of *S. carconensis*, specifically, the long external opening of the rimoportula, the short pipe external opening of the fultoportulae, the depression of the valve face fultoportulae, and no relationship of pattern between the

costae and marginal fultoportulae, are shared with *S. suzukii* and *S. pseudosuzukii*. *S. carconensis* has been widely reported as fossils (Tuji & Kociolek 2000), and this species complex should be a key group for understanding the evolution of freshwater centric diatoms.

Alverson et al. (2007) carried out a phylogenetic analysis of thalassiosiroid diatoms, but without including the group discussed above.

Here, we report the results of our molecular analysis of *S. suzukii* and related Thalassiosiroid taxa, and discuss the evolution of freshwater diatoms with reference to the fossil records.

Materials and Methods

Cultured strains

The sampling localities, dates and strain numbers used in this study are presented in Table 1. Strains were isolated by the pipette washing method under an inverted microscope, and then maintained using 5–10 mL of D medium

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Table 1. Isolation information for new strains sequenced in this study. Voucher specimens are kept in the Department of Botany, National Museum of Nature and Science (TNS). PCR amplification of *Praestephanos suzukii* was performed directly from six cells in the field sample.

Species	Original strain Strain identifier	Locality	Isolation date	Voucher specimens	Other references or information
<i>Discostella woltereckii</i>	Ak506	Lake Kitaura, Japan	13-Mar-08	TNS-AL-56492	
<i>Discostella nipponica</i>	Ak669	Lake Biwa, Japan	12-Feb-11	TNS-AL-57776	
<i>Praestephanos suzukii</i>	su10	Lake Biwa, Japan	12-Feb-11	TNS-AL-57776	Six cells PCR
<i>Stephanodiscus hantzschii</i>	57172	Lake Teganuma, Japan	18-Jan-11	TNS-AL-57172	
<i>Stephanodiscus</i> cf. <i>akanensis</i>	Ak526	Lake Cyuzenji, Japan	29-Feb-08	TNS-AL-56510	Tuji (2010, nos.50)

contained in a screw cap test tube following the method described by Tuji (2000) as modified WC medium. The medium was adjusted to pH 7.7 by buffering with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The cultured strains were illuminated by red LED lamps, with a photon flux density of ca. $30 \mu\text{mol m}^{-2} \text{sec}^{-1}$, a photoperiod of L/D=12/12 hours or 24/0 hours, and a temperature of 5–20°C. Morphological observations were performed for the cultured strain by light microscopy (LM) (AxioPhot; Zeiss, Jena, Germany) and scanning electronic microscopy (SEM) (JSM-6390 with LaB6 gun and JSM-5600LV, JEOL).

DNA extraction and PCR amplification

DNA was extracted from cultured strains using GenomicPrep (Amersham Biosciences, NJ, USA) or the Chelex method (Walsh et al. 1991). Extraction of DNA using GenomicPrep was performed according to the manufacturer's protocol. Extraction of DNA using Chelex-100 (Bio-Rad Laboratories, CA, USA) was performed using 10% Chelex in sterile H₂O using the protocol outlined by Walsh et al. (1991).

Since we did not succeed in culturing a strain of *Stephanodiscus suzukii* during the present study, DNA was extracted using field specimens collected by A. Tuji on 12th February 2011 from Lake Biwa, Japan, using a plankton net with 20- μm mesh (TNS-AL-57776, Table 1). Six cells were selected from the specimen under an inverted microscope, and DNA was extracted using the Chelex method.

Polymerase chain reaction (PCR) of 18S rDNA (SSU), 28S rDNA (LSU), and the *rbcL*, *psbC* regions was performed using the primer sets shown in Table 2, employing a thermal cycler (iCycler) with Ex Taq DNA polymerase (Takara, Tokyo) or KOD FX Neo DNA polymerase (Toyobo, Osaka) according to the manufacturer's protocol. Annealing temperatures were 55, 58 or 60°C for SSU, 58 or 60°C for ITS and LSU, 47 or 50°C for *rbcL* and *psbC*. PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the instruction manual. The cycle sequencing samples were purified by ethanol precipitation. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, U.S.A.). The obtained sequences were assembled

using Chromas PRO (Technelysium Pty Ltd, Tewantin, Australia).

Phylogenetic reconstruction

Phylogenetic and molecular evolutionary analyses for the obtained sequences of *rbcL* and *psbC* were conducted using the MEGA 5 computer program (Tamura et al. 2011). Analyses of the combined sequences SSU+LSU and SSU+LSU+*rbcL*+*psbC* were conducted using the RAxML computer program (Stamatakis et al. 2008) with the default parameters.

Alignments were checked manually. A maximum likelihood (ML) tree was calculated using MEGA software with the best fit model determined by Bayesian information criterion (BIC) scores or Akaike Information Criterion (AIC) corrected scores, and the substitution nucleotide matrix parameters were calculated by the software. A tree using 1000 bootstrap replicates was generated. Each codon position was partitioned and analyzed for *rbcL* and *psbC*. All positions containing gaps and missing data were eliminated.

Results

Taxonomic description and morphological analysis

Praestephanos Tuji & M. Julius gen. nov.

Type species: *Stephanodiscus suzukii* Tuji & Kociolek, Phycol. Res. **48**: 238. f. 17–26. 2000.

The valve is rounded. One to several rimoportulae exist on the shoulder of the frustule, which is at the same level as the spines if spines are present. Marginal fultoportulae are close to the valve rim under the level of the rimoportulae, and each is often not on the extension of costae. The pattern of the areolae changes at the level of the marginal fultoportulae but not at the level of the rimoportulae or spines. The exterior openings of the marginal fultoportulae sometimes merge with the valve edge. The exterior opening of the rimoportula is stalked. Valve face fultoportulae are sometimes located in a deep depression.

Table 2. Primers used for PCR and sequencing in this study.

Gene	Primer	Sequence 5'-3'	Direction	Reference
SSU region	SR1	TACCTGGTTGATCCTGCCAG	Forward	Nakayama, T. et al. (1996)
	443r	RCGSRCTGCTGCTGCCTTCCTTG	Reverse	Beszteri et al. (2001)
	4XF	TAGGTCTGGCAATTGGAATGAG	Forward	
	SR5	ACTACGAGCTTTTAACTGC	Reverse	Nakayama, T. et al. (1996)
	997r	AAAACATCCTTGGWARATGCT	Reverse	Beszteri et al. (2001)
	SR9	AACTAAGAACGGCATGCAC	Reverse	Nakayama, T. et al. (1996)
	SR10	AGGTCTGTGATGCCCTTAGA	Forward	Nakayama, T. et al. (1996)
	SR12	CCTTCCGCAGGTTACCTAC	Reverse	Nakayama, T. et al. (1996)
	MedA	AACCTGGTTGATCCTGCCAGT	Forward	Medlin et al. (1988) as A
	ste-340f	CGGGTAACGAATTGTTAGGGCAAG	Forward	This study
ITS region	ste-345r	TCCGAAATCTTGCCCTAACAAATTC	Reverse	This study
	ITSd	AAGGTGAAGTCGTAACAAGG	Forward	This study
	ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	White et al. (1990)
LSU region	D1R	ACCCGCTGAATTTAAGCATA	Forward	Scholin et al. (1994)
	D1C	ACTCTCTTTTCAAAGTCCT	Forward	Scholin et al. (1994)
	D2C	CCTTGGTCCGTGTTTCAAGA	Reverse	Scholin et al. (1994)
	Euk34r	GCATCGCCAGTTCTGCTTACC	Reverse	Liu et al. (2009)
	LSU-R2	ATTCGGCAGGTGAGTTGTTAC	Reverse	Takano, Y. et al. (2005)
	ste-66f	AAATAAATGTCTCAWTCTGTATC	Forward	This study
<i>rbcL</i> region	dp7	AAASHDCCTTGTGTWAGTYTC	Reverse	Daughjerg and Andersen (1997)
	ste-66bf	GGAGAAATAAATGTCTCAWTCTGTATC	Forward	This study
	ste-rbcl-r	GTTTGCAGTAGCTGTTTCAG	Reverse	This study
	Alga-720f	AACCATTYATGCGTTGGAGAG	Forward	This study
	Alga-730r	TCTCTCCAACGCATRAATGG	Reverse	This study
	psbC-f	CACGACCWGAATGCCACCAAT	Forward	Alverson et al. (2007) as psbC+
<i>psbC</i> region	psbC-499f	ACGTGCCCAAGAGAATGGTTTTG	Forward	Alverson et al. (2007) as psbC499+
	psbC-587r	ATCTTGTGTGGTGGTCATATTTGG	Reverse	Alverson et al. (2007) as psbC587-
	psbC-r	ACAGGTTYGCTTGGTGGAGTGG	Reverse	Alverson et al. (2007) as psbC-

***Praestephanos suzukii* (Tuji & Kociolek) Tuji comb. nov.**
(Figs. 1, 2a, b)

Basionym: *Stephanodiscus suzukii* Tuji & Kociolek, Phycol. Res. **48**: 238. figs 17–26. 2000.

Holotype: —Japan. Shiga Pref.: Lake Biwa, Slide TujiLB001a (TNS!).

Praestephanos suzukii has one stalked rimoportula positioned at the valve face on the costa, and a T-shaped elongated external opening of the rimoportula (Fig. 1). The spines are often absent, and otherwise are present on the level of the rimoportula (Fig. 3). One or sometimes two valve fuloportulae with three or four satellite pores are located in a deep depression near the center of the valve (Tuji & Kociolek 2000). Marginal fuloportulae with three satellite pores are positioned at the valve margin and the ends of the mantle costae (Tuji & Kociolek 2000), and the exterior openings of the marginal fuloportulae form small pipes and merge with the valve edge (Fig. 1c, d). The position of the marginal fuloportulae is not related to the position of the costae (Fig. 4). Spines are not evident or traces. The pattern of areolae changes at the level of the marginal fuloportulae but not at the level of the rimoportulae or spines (Fig. 3).

Stephanodiscus pseudosuzukii Tuji & Kociolek (2000: 239) is similar to *S. suzukii*. Small spines exist on the valve

face and at the level of the rimoportulae, which are sessile and not elongated. Kato et al. (2003) considered *S. pseudosuzukii* a synonym of *S. suzukii*. Further study is required to definitively accept or reject that hypothesis.

***Praestephanos carconensis* (Grunow) Tuji comb. nov.**

Basionym: *Stephanodiscus carconensis* Eulenstein ex Grunow, Naturwissenschaftliche Beiträge zur Kenntniss der Kaukasusländer, auf Grund seiner Sammelbeute. p. 128. 1878.

Holotype: —U.S.A. Carcon: slide 1468 in Grunow collection (W!).

Praestephanos carconensis has one stalked rimoportula positioned at the valve face with the external opening of the rimoportula at the level of the spines on the costa. Several valve face fuloportulae with three satellite pores are located in a deep depression near the center of the valve (Tuji & Kociolek 2000). Marginal fuloportulae with three satellite pores are positioned near the valve rim. The exterior openings of the marginal fuloportulae form small pipes (Tuji & Kociolek 2000). The pattern of the areolae changes at the level of the marginal fuloportulae, and not at the level of the rimoportulae or spines (Fig. 3).

***Praestephanos triporus* (Genkal & G.V. Kuzmin)
Tuji & J.-S. Ki comb. nov.**

(Figs. 7–8)

Basionym: *Stephanodiscus triporus* Genkal & G.V. Kuzmin, Botanicheskii Zhurnal 63: 1310. pl. 2. figs 1–8. 1978.

Holotype: —Russia: aquatio Volgogradskoje, vii. 1969, S. I. Genkal.

Synonym: *Stephanodiscus vestibulis* Håk., E.C. Ther. & Stoermer, Nord. J. Bot. 6: 504. 1986.

Praestephanos triporus has one sessile rimoportula positioned at the valve face with a pipe-like external opening (Fig. 2c, d). The spines are at the level of the rimoportula on the costa at the shoulder. One valve fuloportula with two or three satellite pores is located near the center of the valve in a shallow depression. Marginal fuloportulae with two satellite pores are positioned at the valve margin and the ends of the mantle costae, and the exterior openings of the marginal fuloportulae form small pipes and merge with the valve edge, and are partly surrounded by an arch, with a porch-like structure resembling a vestibule (Fig. 2c, d, Tuji & Houki 2001: pl. 20). Marginal fuloportulae are located near the end. The pattern of the areolae changes near the rim at the level of the marginal fuloportulae (Fig. 3).

The valve of *Praestephanos* is rounded, and it is very difficult to distinguish a mantle area from a valve surface. The level of the shoulder (Sh) and the level at which the

pattern of the areolae changes (CA), both can be at the valve-mantle junction, are the same in *Stephanodiscus*, but different in *Praestephanos*. The CA level, not the shoulder, is held to be the valve-mantle junction for *Praestephanos* (Fig. 3). Kiss et al. (2013) consider *S. vestibulis* to be a synonym of this taxon. *Praestephanos triporus* is distributed worldwide (Kiss et al. 2013).

Phylogenetic analysis

In the present study, we analyzed 23 sequences, including five newly obtained from the SSU and LSU regions, and six newly obtained from the *rbcL* and *psbC* regions (Table 3). The selected models for *rbcL* and *psbC* were the Tamura-Nei model (Tamura & Nei 1993) using a discrete Gamma distribution (+G) and the Hasegawa-Kishino-Yano model +G (Hasegawa et al. 1985). The phylogeny trees of SSU+LSU, *rbcL*, *psbC* and SSU+LSU+*rbcL*+*psbC* (all-combined tree) are shown in Fig. 5.

Five clades were distinguished in the phylogenic trees generated in the present study. Clade a consists of *Stephanodiscus* Ehrenb. The support values for this clade were low for SSU+LSU and *rbcL*, but high for *psbC* (99) and the all-combined tree (100). Clade b consists of *Cyclostephanos* E.C. Ther. et al.; this is a monophyletic group with relatively high support values (77 for SSU+LSU, 90 for *rbcL*, 99 for *psbC* and 100 for the all-combined tree). Clade c consists of the new genus *Praestephanos*. It has only two members, *P. suzukii* and *P. triporus*, and shows high sup-

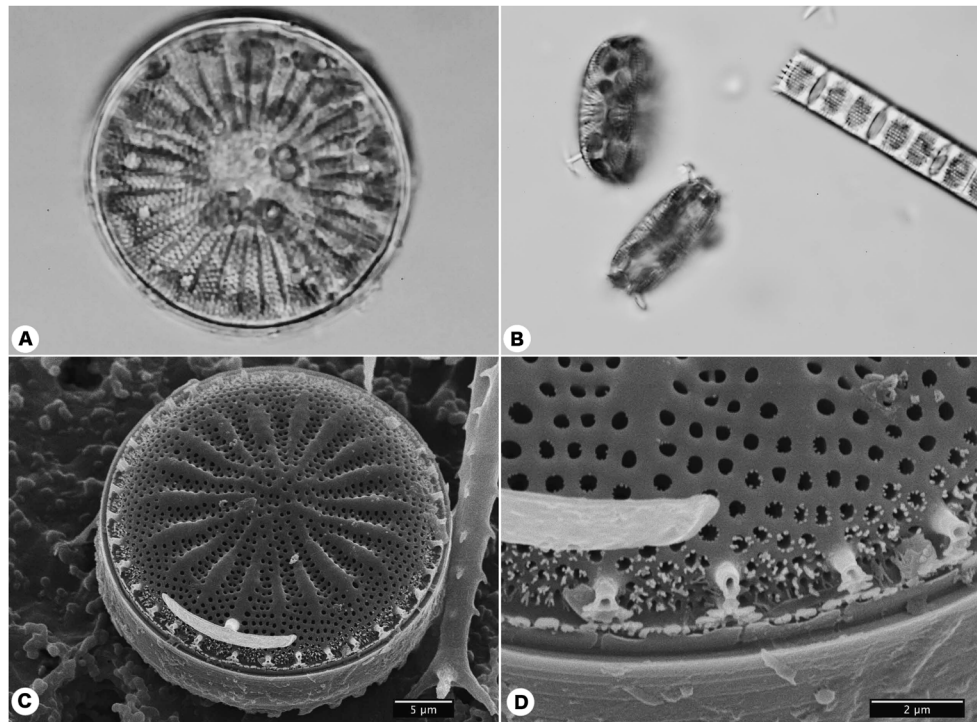


Fig. 1. *Praestephanos suzukii*. A, B: LM images of living individuals showing chloroplasts and the T-shaped external opening of a rimoportula. C: SEM image of outer face of valve showing T-shaped external opening of a rimoportula. D: Enlargement of SEM image showing exterior openings of marginal fuloportulae forming small pipes and merging with the valve edge.

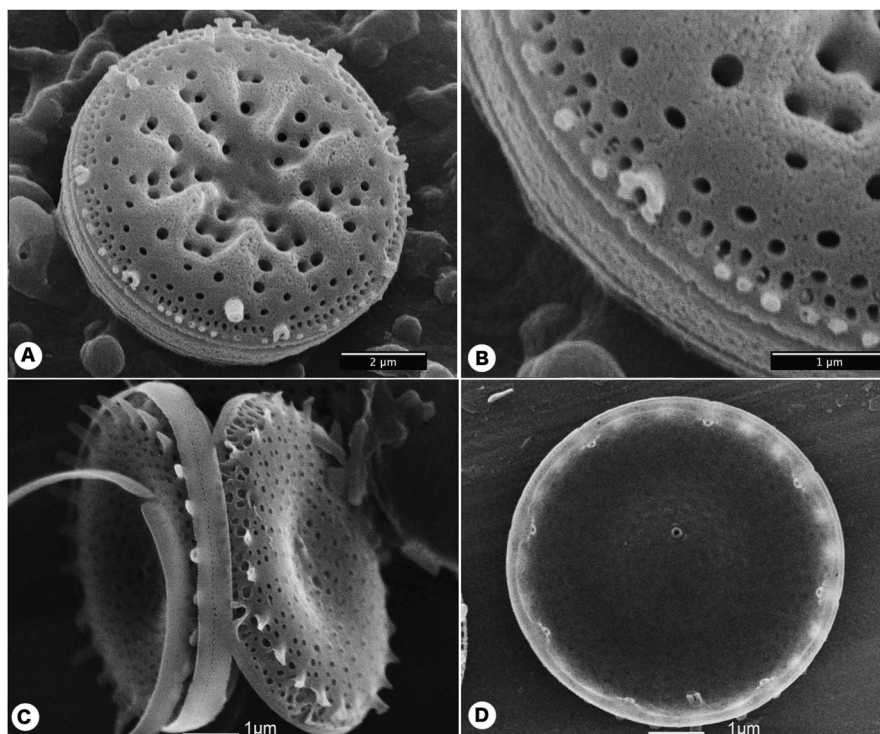


Fig. 2. SEM images of the genus *Praestephanos*.

A–B: *P. suzukii* (Tuji & J.P.Kociolek) Tuji '*pseudosuzukii*' type. A: Whole valve showing the relationship between the rimoportula, spines and marginal fultoportulae. B: Enlargement of the exterior openings of marginal fultoportulae merged with valve edge.

C–D: *P. triporus*, strain KHR001. C: Whole valve showing exterior openings of marginal fultoportulae merged with valve edge, and partly surrounding by an arch, the structure porch-like or resembling a "vestible". D: Whole valve showing the inner openings of the rimoportula and marginal fultoportulae.

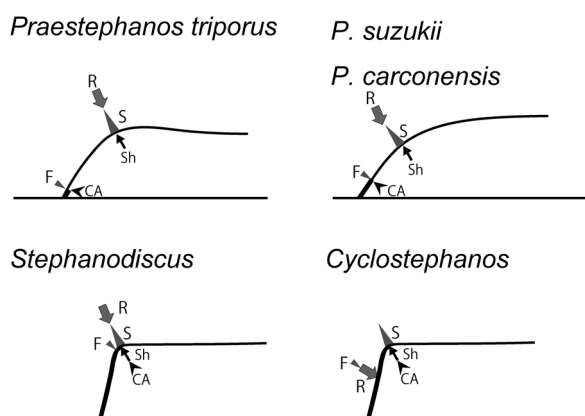


Fig. 3. A schematic drawing of levels of the rimoportula (R), fultoportula (F), spine (S), shoulder (Sh) and the level of changes in the pattern of areolae (CA) in *Praestephanos*, *Stephanodiscus* and *Cyclostephanos*.

port values (91 for SSU+LSU, 99 for *rbcL*, 100 for *psbC* and 100 for the all-combined tree). Clade d, which includes *Cyclotella bodanica* Eulenstein ex Grunow (= *Handmannia* Perag.) and *C. ocellata* Pant., has a long branch length, and relatively high support values (99 for SSU+LSU, 85 for

rbcL, 100 for *psbC* and 100 for the all-combined tree). Clade e consists of *Discostella* Houk & Klee and has consistently high support values (100 for SSU+LSU, 100 for *rbcL*, 99 for *psbC* and 100 for the all-combined tree). We used this clade as an out-group as per Alverson et al. (2007).

Discussion

The position of rimoportulae and marginal fultoportulae are important characters in the defining Thalassiosiroid genera. The rimoportulae of *Praestephanos* exist at the same level as the spines, and marginal fultoportulae are close to the valve rim under the level of the rimoportulae. These characters are shared with the genus *Stephanodiscus* but differ from the genus *Cyclostephanos* and other related genera. However, the pattern of the areolae changes at the level of the marginal fultoportulae but not at the level of the rimoportulae or spines, and the marginal fultoportulae of *Praestephanos* are often not on the extension of costae. These characters differ from the genus *Stephanodiscus*.

The molecular information gained in the present study suggests the unique position of the newly described genus

Praestephanos, which should therefore not be assigned to other previously described genera such as *Stephanodiscus*, *Cyclostephanos*, *Cyclotella* (Kütz.) Bréb., *Handmannia* or *Discostella*. Since *Stephanodiscus niagarae* is positioned

in a different clade from *P. suzukii*, it should not be considered basal to *P. suzukii*. The morphology of the *Stephanodiscus* species (sp. A–sp. E) presented in Kuwae et al. (2007) from the 400 ka core, support the present results; they share characteristics, such as the valve face fultoportula located in a deep depression near the center of the valve, and the exterior openings of the marginal fultoportulae forming small pipes and merging with the valve edge. The marginal fultoportulae of most *Stephanodiscus* species (sp. A–sp. D) with the exception of sp. E., are not always on the extension of the costae. Because of a lack of information and photographs on *Stephanodiscus* species (sp. A–sp. D), further study is required for the confirmation of these characters.

In the present study, we found that *Stephanodiscus* sp. E (Kuwae et al. 2007: figs. 45–47) appeared at a depth of approximately 128.3 m (about 370 ka), and has a ridged rim without areolae. This part may correspond to the mantle area, which may have been lost in the evolution of this group. This new knowledge on the genus *Praestephanos* is useful for understanding the evolution of the *P. suzukii* group at Lake Paleo-Biwa.

Since the four clades, *Stephanodiscus*, *Cyclostephanos*, *Praestephanos* and *Handmannia-C. ocellata* were separated with short branches in the combined tree, these clades are assumed to have appeared and diversified within a very short period of time. The species in these four

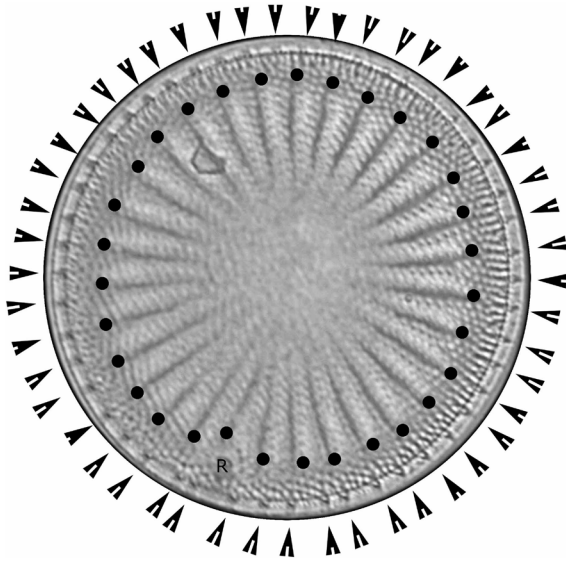


Fig. 4. *Praestephanos suzukii* showing the lack of a relationship between costae (filled circles) and marginal fultoportulae (arrow heads). Holotype slide.

Table 3. GenBank accession numbers for the species and strains analyzed. Accession numbers for sequences obtained in this study are shown in *italic*.

Species taxonomic name	Strain identifier strain name	GenBank accession No.			
		SSU	LSU	<i>rbcL</i>	<i>psbC</i>
<i>Cyclostephanos invisitatus</i>	FHTC26	DQ514899.1	DQ512455.1	DQ514827.1	DQ514745.2
<i>Cyclostephanos</i> sp.	WTC18	DQ514913.1	DQ512469.1	DQ514841.1	DQ514759.2
<i>Cyclostephanos tholiformis</i>	FHTC15	DQ514898.1	DQ512454.1	DQ514826.1	DQ514744.2
<i>Cyclostephanos</i> sp.		KC284714	KC284706	KC284710	KC284702
<i>Cyclotella bodanica</i>	J98-1	DQ514901.1	DQ512457.1	DQ514829.1	DQ514747.2
<i>Cyclotella ocellata</i>	LB8	DQ514904.1	DQ512460.1	DQ514832.1	DQ514750.2
<i>Praestephanos triporus</i>	su10	<i>AB829519.1</i>	<i>AB831881.1</i>	<i>AB831886.1</i>	<i>AB831886.1</i>
<i>Praestephanos suzukii</i>	KHR001	GQ844873.1	GQ844873.1	<i>KF429741</i>	<i>KF429742</i>
<i>Stephanodiscus agassizensis</i>	CHTC1	DQ514895.1	DQ512451.1	DQ514823.1	DQ514741.2
<i>Stephanodiscus binderanus</i>	ESB2	DQ514896.1	DQ512452.1	DQ514824.1	DQ514742.2
<i>Stephanodiscus hantzschii</i>	WTC21	DQ514914.1	DQ512470.1	DQ514842.1	DQ514760.2
<i>Stephanodiscus hantzschii</i>	57172	<i>AB831892.1</i>	<i>AB831892.1</i>	<i>AB831882.1</i>	<i>AB831887.1</i>
<i>Stephanodiscus</i> cf. <i>akanensis</i>	Ak526	<i>AB831893.1</i>	<i>AB831894.1</i>	<i>AB831883.1</i>	<i>AB831888.1</i>
<i>Stephanodiscus minutulus</i>	J95-21	DQ514900.1	DQ512456.1	DQ514828.1	DQ514746.2
<i>Stephanodiscus neoastreae</i>	Sneo4	DQ514906.1	DQ512462.1	DQ514834.1	DQ514752.2
<i>Stephanodiscus niagarae</i>	OKA-A	DQ514908.1	DQ512464.1	DQ514836.1	DQ514754.2
<i>Stephanodiscus yellowstonensis</i>	Y7	DQ514910.1	DQ512466.1	DQ514838.1	DQ514756.2
<i>Stephanodiscus</i> sp.	FHTC11	DQ514897.1	DQ512453.1	DQ514825.1	DQ514743.2
<i>Stephanodiscus oregonicus</i>	Y98-1	DQ514916.1	DQ512472.1	DQ514844.1	DQ514762.2
<i>Discostella stelligera</i>	L1360	DQ514903.1	DQ512459.1	DQ514831.1	DQ514749.2
<i>Discostella nipponica</i>	Ak669	<i>AB831889.1</i>	<i>AB831889.1</i>	<i>AB831879.1</i>	<i>AB831884.1</i>
<i>Discostella woltereckii</i>	Ak506	<i>AB831890.1</i>	<i>AB831891.1</i>	<i>AB831880.1</i>	<i>AB831885.1</i>
<i>Discostella woltereckii</i>	L435	DQ514902.1	DQ512458.1	DQ514830.1	DQ514748.2
<i>Discostella pseudostelligera</i>	ROR01-1	DQ514905.1	DQ512461.1	DQ514833.1	DQ514751.2

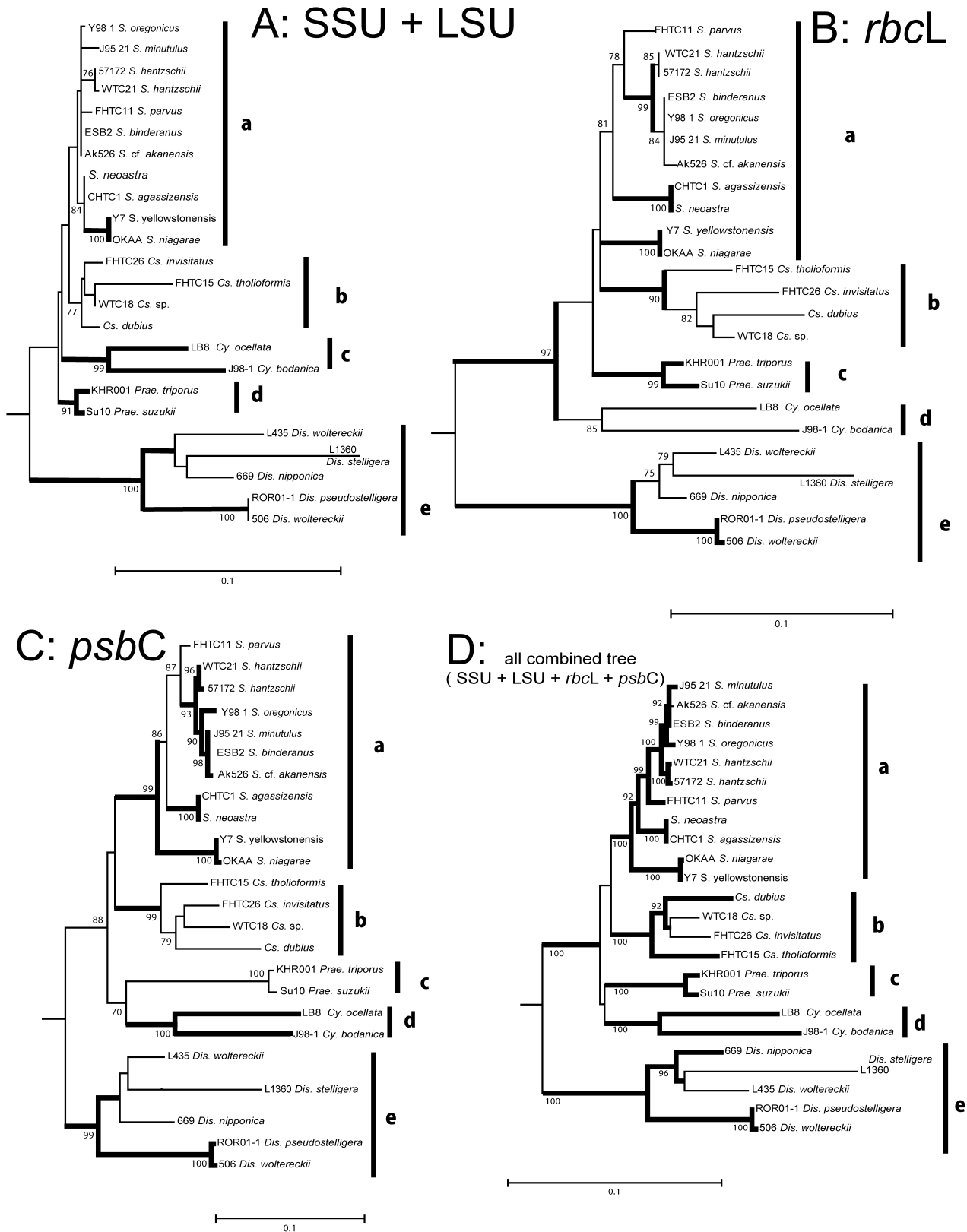


Fig. 5. Phylogeny of *Praestephanos* and related freshwater thalassiosiroid diatoms based on sequences and constructed with a Maximum Likelihood method (ML) analysis. Bootstrap values derived from 1,000 replicates are given at respective nodes as percentages (values less than 75% are not shown). Thick lines indicate high bootstrap value more than 90%. A: Combined analysis for SSU and LSU. 12 *rbcL*. B: *psbC*. C: Combined analysis of SSU, LSU, *rbcL* and *psbC*. Generic abbreviations are: *Stephanodiscus* (S.), *Cyclostephanos* (Cys.), *Praestephanos* (Prae.), *Discostella* (Dis.).

clades are dominant in recent freshwater lakes and ponds. Krebs et al. (1987) report that the genera *Cyclostephanos* and *Stephanodiscus* arose around 6 Ma (late Miocene to early Pliocene). The fossil record and the appearance of cyclostephanoid diatoms have been discussed in several papers (see Julius 2007, Khursevich & Kociolek 2012). Wolfe & Siver (2009) reported *Cyclotella*, *Handmannia* (as *Puncticulata*), and *Discostella* in Middle Eocene lacustrine sediments from the Giraffe Pipe locality in the Northwest Territories, Canada, and the fossil record of the family Stephanodiscaceae extended by 20 Ma to 40 Ma (Wolfe & Siver 2009). Then Khursevich & Kociolek (2012) described the paleo-age range of the genus *Handmannia* as 'Middle Eocene–Recent', and there is a 30 Ma gap from Krebs et al. (1987). Alverson (2014) discussed this gap problem using molecular clocks, and he found no support for the Eocene fossil dates. Since a record from the Eocene is only one, more data is needed. Molecular data for the genus *Handmannia* (as *Puncticulata*) is also limited, and this might lead to errors in paleo-age estimation using molecular clock.

The sudden appearance of various post-Miocene modern-type cyclostephanoid diatoms (eg. these four clades) occur around the Messinian Age and they exist in the modern era. However, further data from both molecular analyses and fossil records are essential to achieve a greater understanding of this event.

Acknowledgements

We want to extend our special thanks to Prof. Shuichi Endoh and Dr. Toshiyuki Ishikawa, Shiga University for sampling at Lake Biwa, Dr. Andrew Alverson for providing molecular data, and Dr. Yumiko Hirayama, National Museum of Nature and Science, for her part in the molecular analysis. We also thank two anonymous reviewers for kind and fruitful suggestions for the enhancement of this paper. This work was supported by JSPS KAKENHI Grant Number 23510021 to A.T., and the research project 'Biological Properties of Biodiversity Hotspots in Japan' by the National Museum of Nature and Science to A.T.

References

Alverson AJ (2014) Timing marine-freshwater transitions in the diatom order Thalassiosirales. *Paleobiology* 40: 91–101.
 Alverson AJ, Jansen RK, Theriot EC (2007) Bridging the Rubicon: Phylogenetic analysis reveals repeated colonizations of marine and fresh waters by thalassiosiroid diatoms. *Mol Phylogenet Evol* 45: 193–210.
 Beszteri B, Ács E, Makk J, Kovács G, Márialigeti K, Kiss T (2001) Phylogeny of six naviculoid diatoms based on 18S rDNA sequences. *Int J Syst Evol Microb* 51: 1581–1586.
 Daugbjerg N, Andersen RA (1997) A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded *rbcL* sequence data. *J Phycol* 33: 1031–1041.
 Grunow A (1878) Algen und Diatomaceen aus dem Kaspischen

Meere. In: *Naturwissenschaftliche Beiträge zur Kenntniss der Kaukasusländer, auf Grund seiner Sammelbeute. Sitzungsberichte der Naturwissenschaftlichen Gesellschaft Isis zu Dresden* (ed Schneider O). Dresden, pp. 99–133, pls. 3–4.
 Hasegawa M, Kishino H, Yano T (1985) Dating the human-ape split by a molecular clock of mitochondrial DNA. *J Mol Evol* 22: 160–174.
 Julius ML (2007) Perspectives on the evolution and diversification of the diatoms (ed Starratt S). *Pond Scum to Carbon Sink: Geological and Environmental Applications of the Diatoms. Paleontological Society Short Course, 27 October 2007. Paleontological Society Paper* 13: 25–36.
 Kato M, Tanimura Y, Fukusawa H, Yasuda Y (2003) Intraspecific variation during the life cycle of a modern *Stephanodiscus* species (Bacillariophyceae) inferred from the fossil record of Lake Suigetsu, Japan. *Phycologia* 42: 292–300.
 Khursevich G, Kociolek, JP (2012) A preliminary, worldwide inventory of the extinct, freshwater fossil diatoms from the orders Thalassiosirales, Stephanodisciales, Praliales, Aulacoseirales, Melosirales, Coscinodisciales, and Biddulphiiales. *Nova Hedwigia, Beiheft* 141: 315–364.
 Kiss KT, Genkal SI, Ector L, Molnár L, Duleba M, Biró P, Ácsa É (2013) Morphology, taxonomy and distribution of *Stephanodiscus triporus* (Bacillariophyceae) and related taxa. *Eur J Phycol* 48: 363–379.
 Krebs WN, Bradbury JP (1987) Neogene and Quaternary lacustrine diatom biochronology, western USA. *Palaos* 2: 505–513.
 Kuwae M, Yoshikawa S, Tsugeki NK (2007) Long-term species and morphological changes for *Stephanodiscus* in Lake Biwa for the last 400 ka. *Diatom* 23: 71–81.
 Liu H, Proberta I, Uitz J, Claustred H, Aris- Brosoue S, Fradab M, Not F, Vargasa C (2009) Extreme diversity in noncalcifying haptophytes explains a major pigment paradox in open oceans. *Proc. Natl. Acad. Sci. U.S.A.* 106: 12803–12808.
 Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491–499.
 Mori S (1974) Diatom succession in a core from Lake Biwa. *Paleolimnology of Lake Biwa and the Japanese Pleistocene* 2: 247–254.
 Nakayama T, Watanabe S, Mitsui K., Uchida H, Inouye I (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycol Res* 44: 47–55.
 Scholin CA, Herzog M, Sogin M, Anderson DM (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *J. Phycol* 30: 999–1011.
 Skvortzow BW (1936) Diatoms from Biwa Lake, Honshu Island, Nippon. *Philipp J Sci* 61: 253–96, 8 pls.
 Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the raxml web servers. *Syst Biol* 57: 758–771.
 Takano Y, Horiguchi T (2005) Acquiring Scanning Electron Microscopical, Light Microscopical and Multiple Gene Sequence Data from a Single Dinoflagellate Cell. *J Phycol* 42: 251–256.
 Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in

- humans and chimpanzees. *Mol Biol Evol* 10: 512–526.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *J Mol Evol* 28: 2731–2739.
- Tuji A (2000) The effect of irradiance on the growth of different forms of freshwater diatoms: Implications for succession of attached diatom communities. *J Phycol* 36: 659–661.
- Tuji A (2010) *Algae Aquae Dulcis Japonicae Exsiccatae*. Fasc. III. nos. 41–60. National Museum of Nature and Science, Tsukuba, 17 pp.
- Tuji A, Houki A (2001) Centric diatoms in Lake Biwa. *Lake Biwa Study Monographs* 7: 1–90.
- Tuji A, Kociolek JP (2000) Morphology and taxonomy of *Stephanodiscus suzukii* sp. nov. and *S. pseudosuzukii* sp. nov. from Lake Biwa, Japan, and comparison with the *S. carconensis* Grunow species complex. *Phycol Res* 48: 231–239.
- Van Heurck H (1882) *Synopsis des Diatomées de Belgique*. Atlas. Ducaju & Cie., Anvers. pls 78–103.
- Walsh SP, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10: 506–516.
- White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. (eds. Innis MA, Gelfand DH, Sninsky JJ, and White TJ). *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., New York, pp. 315–322.
- Wolfe AP, Siver PA (2009) Three extant genera of freshwater thalassiosiroid diatoms from Middle Eocene sediments in northern Canada. *Am J Bot* 96: 487–497.