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Phylogenetic position of eight *Amphora sensu lato* (Bacillariophyceae) species and comparative analysis of morphological characteristics

Pengbin Wang[1], Bum Soo Park[1], Jin Ho Kim[1], Joo-Hwan Kim[1], Hae-Ok Lee[1] and Myung-Soo Han[1,2,*]

[1]*Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea*
[2]*Research Institute for Natural Sciences, Hanyang University, Seoul 133-791, Korea*

Amphora Ehrenberg ex Kützing *sensu lato* is a common and widespread benthic diatom genus with a taxonomy that has been under continual revision, particularly based on molecular analyses. Although *Amphora* species have been studied using modern microscopy in recent years, there has not been much progress on molecular characterization of the species, especially in Asia. In this study of *Amphora*, sampling was carried out from September 2009 to August 2010 in Korean coastal waters. The morphological and molecular characteristics of eight *Amphora sensu lato* were examined: *Amphora marina*, *A. proteus*, *Halamphora costata*, *H. coffeaeformis*, *H. eunotia*, *H. holsatica*, *H. terroris*, and *Halamphora* sp. Based on previous accounts, morphology suggested that *A. marina* and *A. proteus* belong to the subgenus *Amphora* Cleve, which have smooth girdle bands and rather coarse and very distinct areolae on the valve. The other species, *H. coffeaeformis*, *H. costata*, *H. eunotia*, *H. holsatica*, *H. terroris*, and *Halamphora* sp. belong to the subgenus *Halamphora* Cleve, which was recently elevated to generic status by Levkov 2009, have plicate girdle bands, puncta which do not form straight longitudinal lines, valves which have a narrow ventral portion and apices that are generally rostrate-capitate and recurved. In agreement with analysis based on morphological characteristics, phylogenetic analysis based on small subunit rDNA suggested that the eight *Amphora sensu lato* species were not a monophyletic group as the morphological classification. Also, the results of molecular work and statistical analysis on all these *Amphora sensu lato* combined with phylogenic analysis on our geographically representative samples give strong evidence that *Halamphora* Levkov is independent of *Amphora* Cleve. Furthermore, in this study, *Amphora terroris* was transferred *Halamphora* as *Halamphora terroris* (Ehrenberg) Wang comb. nov. and *Amphora marina* was recorded for the first time in Korea.

Key Words: diatom; molecular phylogeny; morphology; nuclear DNA; taxonomy

INTRODUCTION

Amphora sensu lato is widely distributed in marine, freshwater and estuarine environments, with few species in freshwater and many in marine environments (Lee and Round 1987, Sala et al. 2007). These species exhibits extensive diversity with around 350 species and intra-specific taxa distributed worldwide (Nagumo 2003). Van Heurck (1896) first gave a systematic overview of the entire genus approximately following Cleve's subgeneric classification system. Cleve-Euler (1953) made much progress on characterization using light microscopy. Cleve's subgeneric arrangement has also been followed by modern authors (Jin et al. 1982, Chin et al. 1992), although many diat-

Received October 2, 2013, **Accepted** March 27, 2014
***Corresponding Author**
E-mail: hanms@hanyang.ac.kr
Tel: +82-2-2220-0956, Fax: +82-2-2220-1171

omists recognized that this required revision (Round et al. 1990, Sala et al. 2006). However, species placed in these subgenera may be difficult to identify using light microscopy (LM) due to the similarity of their valve outline and dimensions. Krammer (1980) also commented that small forms of *Amphora*, such as *A. pediculus*, were difficult to identify using LM, and resulted in numerous taxonomic problems. However, there have been several studies of *Amphora* in the last few years.

Scanning electron microscopy (SEM) has facilitated reliable differentiation among species using fine cell structures (Sala et al. 2006). The first detailed account of some species (*A. normanii*, *A. pediculus*, and *Halamphora* sp.) using SEM was published by Schoeman and Archibald (1976). Krammer (1980) made additional important contributions to the study of *Amphora* using electron microscopy. *A. ovalis*, *A. libyca*, *A. pediculus*, *A. thumensis*, and two new species, *A. inariensis* and *A. aequalis* were examined by Lee and Round (1987), all of which are present in freshwater. Clavero et al. (2000) listed of important characteristics for species observation and identification. Although *A. tenerrima* and *A. tenuissima* have now been transferred to the genus *Halamphora* as *H. tenerrima* and *H. tenuissima*, these characteristics are still suitable for studying *Amphora sensu lato*. In this decade a large number of observations have been made from high quality figures (Nagumo 2003, Levkov 2009). Regardless, there is still insufficient information to fully understand the morphology and phylogeny of these organisms, from the perspective of the structure of girdle bands and gene sequences.

Nuclear-encoded small subunit (SSU) rDNA sequences are now available for representatives of most major diatom lineages (Medlin et al. 1993, 1995, 1996, Kooistra and Medlin 1996, Beszteri et al. 2001, Ki et al. 2009, Jung et al. 2010). Some previous studies have addressed diatom evolution and systematics using SSU rDNA (Damsté et al. 2004, Medlin and Kaczmarska 2004, Bruder and Medlin 2008), in which they analyzed some species of *Amphora*. However, there has not been a systematic study and analysis of *Amphora* as many species have not yet been grown in culture (Mann and Poulícková 2010). Although molecular phylogenetic investigations are growing, there are few available sequences that can be used when addressing the relationships between *Amphora* species. Even Sato et al. (2013) gave several SSU sequences of *Amphora sensu lato*, with unidentified species making it hard to give a strong conclusion regarding the phylogeny and its implications.

In this paper, we provide light and scanning electron microscope data for eight diatom species of *Amphora sensu lato* from Korean coastal waters: *Amphora marina*, *A. proteus*, *H. coffeaeformis*, *H. costata*, *H. eunotia*, *H. holsatica*, *H. terroris*, and *Halamphora* sp. Detailed descriptions are provided on morphology. In addition, SSU rDNA sequence data of these eight species were also included and analyzed because SSU rDNA is a common used gene for diatoms, especially amphoroid classification and systematics (Medlin and Kaczmarska 2004, Bruder and Medlin 2008, Sato et al. 2013). Furthermore, phylogenetic position of eight *Amphora sensu lato* (Bacillariophyceae) species were evaluated in the context of morphological characteristics.

MATERIALS AND METHODS

Field sampling and cultures

Samples were collected with an algae-harvesting tool (Fig. 1) or by brushing algae from stones at 17 coastal Korean sites (Fig. 2). Species of *Amphora* were successfully cultured from five sites: Uljin, Pohang, Gilcheon Port, Geoje, and Jeju, Korea. Single cells were isolated by the capillary method (Andersen 2005) under a Zeiss Axioplan 100 inverted microscope (Carl Zeiss, Jena, Germany) and cultured in 96-well plates containing 200 µL of f/2 medium (Guillard and Ryther 1962). The diatoms reached the exponential growth stage 7-10 days after inoculation (Katano et al. 2007), and cells showing good growth were further subcultured into glass tubes with 15 mL of fresh f/2 medium at 10 days. All cultures were kept at 20°C in a 12 : 12-h light : dark cycle at 100-150 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Strains were subcultured with fresh f/2 medium at about 20-day intervals to maintain healthy cultures.

Fixation and frustule-cleaning

Natural and cultured samples were fixed in Lugol's solution (Throndsen 1978) with a final content of c. 2%, for at least eight hours at 4°C. Before frustule-cleaning, the cells were rinsed with distilled water. Organic compounds were removed from fixed cells by concentrating samples to discard the supernatant, adding an equal amount of HNO_3 and three times the sample amount of H_2SO_4, and finally boiling for three minutes. Next, the samples were rinsed with distilled water to completely remove the acid (Ki et al. 2009).

Material from cleaned samples was mounted in Na-

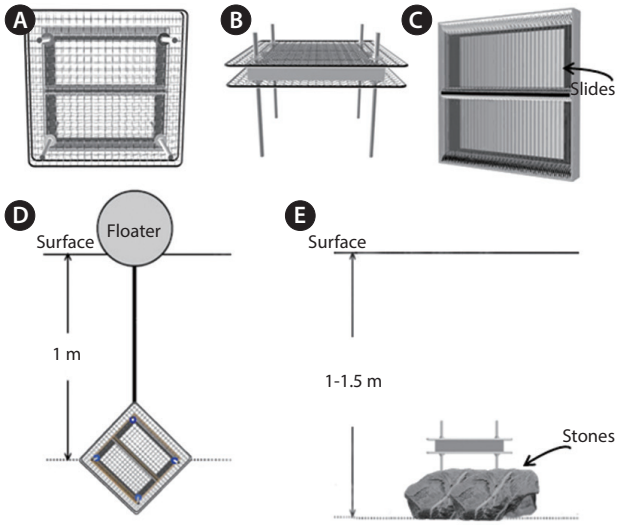

Fig. 1. Schematic views of attached algae harvesting tool and setting. (A) Harvesting tool's plan view. (B) Harvesting tool's lateral view. (C) Different substrate slides can be set in harvesting tool box. (D) Attached algae harvesting tool in floating setting. (E) Attached algae harvesting tool in sessile setting, stones were used for fixing the harvesting tool.

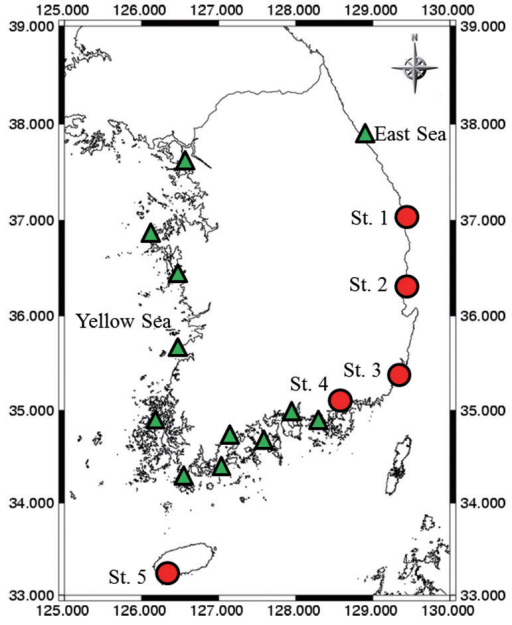

Fig. 2. Location of sampling stations and sampling date in the Korean coastal waters. Red circles: Sites of *Amphora* species isolatations were successed. St. 1 is Uljin, Korea (March 2010, 37°06′21 N, 129°22′39 E). St. 2 is Pohang, Korea (October 2010, 36°12′54 N, 129°23′08 E). St. 3 is Gilcheon Port, Korea (May 2010, 35°19′30 N, 129°17′05 E). St. 4 is Geoje, Korea (November 2009, 34°53′41 N, 128°36′54 E). St. 5 is Jeju, Korea (June 2010, 33°14′38 N, 126°24′51 E). Green triangles: Sites were also sampled but the *Amphora* species isolatations were not successed.

phrax for morphometric analysis; the slides are preserved in the Laboratory for Water Environmental Ecology and Restoration at Hanyang University, with accession numbers AM001-AM008. Light microscope observations were made on slides from exponentially-growing cultures and / or natural samples using an Axioplan microscope (Carl Zeiss) equipped with Nomarski differential interference contrast optics. Light micrographs were taken using a cooled CCD camera (Sensys Photometrics, München, Germany) and analyzed with Image-pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA). Diatom cells in the exponential growth stage were examined for cell length, chloroplast shape and nucleus position. Average cell length and width were calculated from measurement of more than 30 cells. We used the striae-counting method outlined in Schoeman and Archibald (1976).

Scanning electron microscopy (SEM)

To determine the actual shape and fine structure of the diatom frustules, both the fixed specimens and the specimens with organic compounds removed were observed in the SEM. The specimens were dehydrated in a graded ethanol series (30, 50, 70, 90, and 100%; each stage for 30 min). Next, 50-μL of dehydrated specimens were mounted on a 0.1% w/v (in water) poly-L-lysine solution (Sigma, St. Louis, MO, USA)-treated glass cover-slip (25 μL of poly-L-lysine solution on an 18-mm-diameter glass cover-slip, uniformly coated and dried at room temperature). Or dehydrated specimens were directly mounted onto a 0.2-μm GTTP Millipore Filter Membrane (Millipore Filter Corporation, Cork, Ireland). Both treated glass and filter membranes were glued onto SEM stubs before specimen mounting. The mounted specimens were dried at room temperature for 12 h. Finally, specimens were coated with gold for 200 s with a 25-mA current (BAL-TEC SCD 005 Super Coater; BAL-TEC, Liechtenstein, Germany) and examined with the SEM (Hitachi S-2380n; Hitachi, Tokyo, Japan and JSM-6300; Jeol, Tokyo, Japan).

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

Samples of clonal cultures (3 mL) in the mid-logarithmic growth phase were harvested by centrifugation at 8,000 ×g for 5 min. The concentrated cells were transferred to 1.5-mL Eppendorf tubes with 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM ethylenediaminetetraacetic acid) and stored at -20°C until DNA extraction (Ki and Han 2007). Genomic DNA was isolated from the

stored cells using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

For getting the SSU rDNA sequences, primers shown in Table 1 were employed in the PCR amplification. PCR reactions were performed with 50-μL reaction mixtures containing 33.5 μL sterile distilled water, 5 μL 10× *EX* PCR buffer (TaKaRa, Tokyo, Japan), 4 μL dNTP mixture (2.5 mM each) (TaKaRa), 3 μL of each primer (10 pmol), 0.5 μL of *EX* Taq polymerase (5 unit μL^{-1}) (TaKaRa) and 1 μL of template. PCR cycling was carried out in a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA) as follows: pre-denaturation 94°C for 4 min, 37 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 50 s, and a final extension at 72°C for 5 min. The resulting PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ultraviolet transillumination after 10 min of gel staining in ethidium bromide. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA-sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA, USA) with primers listed in Table 1. Labeled DNA fragments were analyzed by capillary electrophoresis on an ABI 3730xl Genetic Analyzer (Applied Biosystems). Editing and contig assembly of rDNA sequence fragments were carried out with Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA).

DNA sequence comparisons

Full multiple alignment of our nuclear SSU rDNA sequences with NCBI (Table 2) sequences were performed with the Clustal W1.8 (Thompson et al. 1994) portion of the Bioedit program v7.0.9.0 (North Carolina State University). The aligned nuclear SSU rDNA sequences were trimmed to the same length at each end by Bioedit. In addition, all the SSU rDNA sequences of eight species sequenced here together with other *Amphora* species (Table 2) obtained from the NCBI databases were analyzed using similarity measures and genetic distance (p-distances). The DNA similarities were calculated using Bioedit. The corrected p-distances analysis (Oh et al. 2010), employing a bootstrap method with 1,000 replicates and a Kimura 2-parameter model, were calculated in MEGA5.0 software (Tamura et al. 2011). The ANOVA test of similarity of different species was conducted by SPSS for Windows (version 18.0; SPSS Inc., Chicago, IL, USA) based on the similarity data in Table 3 (marked as three different colours), excluding the data of *Amphora* sp. (AM501957) and *Amphora* sp. (AB183590). The three groups were independent and significant ($p < 0.001$).

The alignment matrix was analyzed using the maximum likelihood (ML) method. To determine the optimal model of nucleotide substitution, hierarchical likelihood ratio tests (hLRTs) and calculated Akaike information criterion (AIC) values (Posada and Buckley 2004) in MrModelTest 2.3 (Nylander 2004), assisted by PAUP*4.0b10 (Swofford 2003), were used. The best-fit model (GTR + I + G) was selected from the 24 tested models for the PhyML 3.0 (Guindon et al. 2010) settings. Bootstrap values (branch support) were obtained with 1,000 replicates. Bootstrap values greater than 50 are indicated at each branch node.

For the Bayesian inference (BI) analysis, the optimal model of nucleotide substitution was determined as the method in ML analysis. The best-fit model (GTR + I + G) was selected from 24 tested models for the MrBayes 3.2.1 (Ronquist et al. 2012) settings. The Markov Chain Monte Carlo (MCMC) process was set at two chains, and 1,000,000 generations were conducted. The sampling frequency was arranged as occurring with every 10 generations. After analysis, making sure the standard deviation of frequencies occurred below 0.01, the first 25% trees were deleted as burn-in, and a consensus tree was constructed. Bayesian posterior probabilities (BI) greater than 0.50 were indicated at each branch node.

Table 1. Primers used for amplifications and sequencing of the nuclear SSU rDNA in this study

Primer	Nucleotide sequence (5′ to 3′)	Reference
18S-F53	TTGTCTCAAAGATTAAGCCATG	This study
AT18R02	GTTTCAGCCTTGCGACCATACTCC	Ki et al. (2007)
18S-F549	GCTCGTAGTTGGATTTGTG	This study
18S-R1335	CCTGTTATTGCCCCTATCTTCC	This study
18S-F934	CTATGCCGACAAGGGATTGG	This study
AT18R01	GCTTGATCCTTCTGCAGGTTCACC	Ki et al. (2009)

SSU, small subunit; F, forward primer; R, reverse primer.

Table 2. Strains in this study and GenBank accession numbers for DNA sequences

Species	Strain	Taxonomic position[a]	Isolation locality	GenBank accession No.
Achnanthes brevipes	CCMP100	Achnanthales	-	AY485476
Achnanthidium minutissimum	AT-196Gel02	Achnanthales	Germany	AM502032
Amphora cf. *fogediana*	AT-212.06	Thalassiophysales	Germany	AM502022
Amphora cf. *proteus*	-	Thalassiophysales	Antarctica	AJ535147
Amphora lybica	AT-117.10	Thalassiophysales	Germany	AM501959
Amphora pediculus	AT-117.11	Thalassiophysales	Germany	AM501960
Amphora sp.	MBIC10098	Thalassiophysales	-	AB183590
Amphora sp.	AT-221.04	Thalassiophysales	Germany	AM501957
Asterionella formosa	AT-67-2b	Fragilariales	Germany	AM712617
Bellerochea malleus	-	Hemiaulales	-	AF525671
Biddulphiopsis titiana	-	Biddulphiales	-	AF525669
Bolidomonas mediterranea	MINB11E5	Bolidophyceae	-	AF123596
Bolidomonas pacifica	OLI31SE3	Bolidophyceae	-	AF123595
Chaetoceros rostratus	-	Chaetocerotales	-	X85391
Cylindrotheca closterium	MGB0402	Bacillariales	-	AY866418
Cylindrotheca fusiformis	CCMP339	Bacillariales	-	AY485457
Detonula confervacea	-	Thalassiosirales	-	AF525672
Ditylum brightwellii	CCAP 1022/2	Lithodesmidales	-	X85386
Eucampia antarctica	3/a	Hemiaulales	-	X85389
Entomoneis alata	CCAP1003/3	Surirellales		AY485497
Entomoneis cf. *alata*	-	Surirellales	-	AY534908
Entomoneis cf. *alata*	-	Surirellales	-	AJ535160
Entomoneis ornata	14A	Surirellales	-	HQ912411
Entomoneis sp.	CCMP1693	Surirellales	-	EF585586
Fragilaria crotonensis	-	Fragilariales	-	AF525662
Fragilariopsis sublineata	-	Bacillariales	-	AF525665
Grammatophora gibberula	WK48	Striatellales	-	AF525656
Grammatophora oceanica	CCMP410	Striatellales	-	AY485466
Halamphora cf. *capitellata*	P363	Thalassiophysales	Antarctica	AJ535158
Halamphora montana	TSA2	Thalassiophysales	Hungary	AJ243061
Halamphora normanii	AT-105Gel05	Thalassiophysales	Germany	AM501958
Helicotheca tamesis	CCAP 1076/1	Lithodesmidales	-	X85385
Hyalosira delicatula	-	Striatellales	-	FM164375
Lampriscus kittonii	-	Triceratiales	-	AF525667
Licmophora juergensii	P367	Licmophoraceae	-	AF525661
Lithodesmium undulatum	-	Lithodesmidales	-	Y10569
Navicula capitatoradiata	AT-212Gel07	Noctilucales	Germany	AM502012
Navicula cryptotenella	AT-210Gel05	Noctilucales	Germany	AM502015
Navicula phyllepta	HP	Noctilucales	-	AY485456
Odontella sinensis	-	Eupodiscales	-	Y10570
Papiliocellulus elegans	-	Cymatosirales	-	X85388
Pleurosira laevis	-	Triceratiale	-	AF525670
Rhabdonema sp.	LM-2002	Rhabdonematales	-	AF525660
Rhaphoneis belgicae	-	Rhaponeidales	-	X77703
Rhizosolenia robusta	-	Rhizosoleniales	-	AY485481
Staurosira construens	D-121	Fragilariales	-	EF465467
Tabularia tabulata	CCMP846	Fragilariales	-	AY216907
Thalassionema nitzschioides	CCAP1084 /1	Thalassionemales	-	X77702
Thalassiosira eccentrica	-	Thalassiosirales	-	X85396
Thalassiosira rotula	CCAP 1085/4	Thalassiosirales	-	X85397

[a]Taxonomic position following the Medlin and Kaczmarska's molecular systematics of diatoms (Medlin and Kaczmarska 2004).

Maximum-parsimony (MP), with branch and bound searches, was done using PAUP*4.0b10 (Swofford 2003) with the hLRTs setting. Bootstrap values (branch support) were obtained with 1,000 replicates. Bootstrap values greater than 50 were indicated at each branch node.

RESULTS

Comparisons of rDNA sequences

The nuclear SSU rDNA sequences from eight *Amphora* species ranged from 1,651 to 1,667 bp in length; the sequences have been deposited into NCBI databases (Table 3). Nucleotide frequencies were as follows: A, 25.5-26.1%; T, 27.8-29%; C, 18.6-19.6%; and G, 26.6-27.3%. Based on the G + C content, which was 45.4-46.8% (mean 46.16%), the differences in nucleotide composition among the eight species were not significant. The results of genetic distance and sequence similarity are presented in Table 3. The results of similarity scores showed that the highest similarity among our eight species, which was also the highest similarity score in all 17 analyzed species, was 99.4% between *A. pediculus* and *A.* cf. *fogediana* in the subgenus *Amphora* group. The genetic distance (p-distance) was 0.4% between these two species (Table 3). In subgenus *Amphora*, the lowest similarity (95.9%) was between *A. proteus* and *A.* cf. *proteus*, between which the genetic distance was 3.5%. In the *Halamphora* group, the highest similarity (98.1%) was between *A. normanii* and *A.* cf. *capitellata*, while the lowest similarity (94.7%) was between *H. eunotia* and *H. terroris*, which had a genetic distance of 4.5%. The lowest genetic distance in the *Halamphora* group was 1.5% between *H. costata* and *H.* cf. *capitellata*. The range of similarity between species from *Amphora* and *Halamphora* was 92.7-95.8%, while the range of genetic distances was 3.0-6.2%. *Amphora proteus* and *H. montana* both got the lowest similarity (92.7%) and p-distance (0.4%) of all the *Amphora* species analyzed. The range of similarity of *Amphora* sp. (AM501957) and other analyzed species was 93.3-99.2%, with genetic distances ranging from 0.5 to 5.8%. According to the high similarity (96.9-99.2%) within group *Amphora*, *Amphora* sp. (AM501957) is likely a species close to *Amphora lybica*. The other *Amphora* sp. (AB183590) has a range of similarity of 93.7-98.3%, with a genetic distances range of 1.5-5.1%. Based on the high similarity (95.4-98.3%) with group *Halamphora*, *Amphora* sp. (AB183590) is likely a species close to *H. costata*.

The results of ANOVA tests of similarity and genetic distance are displayed in Fig. 3. The three groups were independent and significantly different ($p < 0.001$). Group

Table 3. Similarity score and genetic distance of 17 pairs aligned *Amphora sensu lato* nuclear SSU rDNA sequence (1,667 bp)

	Species	GenBank accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
			[Similarity]																
1	*Amphora marina*	KC222329		98.4	97.9	97.7	97.4	96.1	93.6	94.0	93.3	94.6	95.6	94.5	92.9	93.7	94.4	97.4	93.9
2	*Amphora proteus*	KC222324	1.1		97.4	97.4	97.2	95.9	93.4	93.7	93.1	94.3	94.8	94.3	92.7	93.4	94.2	96.9	93.7
3	*Amphora lybica*	AM501959	1.8	2.1		99.0	99.1	97.1	94.1	94.6	93.8	95.2	95.2	95.4	93.7	94.0	95.0	99.2	94.6
4	*Amphora pediculus*	AM501960	1.7	2.2	0.6		99.4	97.5	94.4	94.8	93.9	95.5	95.4	95.6	94.0	94.4	95.3	98.6	94.7
5	*Amphora* cf. *fogediana*	AM502022	2.0	2.3	0.5	0.4		97.4	94.2	94.8	93.8	95.5	95.3	95.8	94.2	94.3	95.3	98.6	94.8
6	*Amphora* cf. *proteus*	AJ535147	3.3	3.5	2.4	2.2	2.2		94.5	94.9	93.7	95.6	95.0	95.4	94.2	94.6	95.1	96.6	94.7
7	*Halamphora coffeaeformis*	KC222325	5.3	5.5	4.7	4.6	4.7	4.5		97.1	96.9	96.6	95.9	96.5	96.3	96.7	97.7	93.7	96.7
8	*Halamphora costata*	KC222327	4.8	5.1	4.3	4.2	4.2	4.1	2.8		95.7	97.7	96.9	97.0	97.6	97.2	98.0	94.2	98.3
9	*Halamphora eunotia*	KC222326	5.7	6.0	5.3	5.3	5.3	5.3	2.6	3.7		95.1	94.7	95.0	94.9	95.2	96.0	93.3	95.4
10	*Halamphora holsatica*	KC222331	4.1	4.5	3.5	3.3	3.3	3.0	2.9	1.7	4.1		97.5	97.8	96.7	96.8	97.7	94.9	97.0
11	*Halamphora terroris*	KC222330	3.3	4.1	3.7	3.4	3.6	3.8	3.6	2.6	4.5	1.9		97.4	95.9	96.3	97.1	94.8	96.7
12	*Halamphora* sp.	KC222328	4.3	4.5	3.4	3.3	3.1	3.4	3.2	2.6	4.3	1.6	2.2		96.2	96.8	97.7	94.9	96.7
13	*Halamphora montana*	AJ243061	6.0	6.2	5.2	4.9	4.7	4.9	3.6	2.2	4.5	2.7	3.7	3.4		96.6	97.3	93.4	97.2
14	*Halamphora normanii*	AM501958	5.1	5.4	4.8	4.4	4.5	4.3	3.2	2.6	4.3	2.6	3.1	2.9	3.3		98.1	93.8	97.0
15	*Halamphora* cf. *capitellata*	AJ535158	4.3	4.5	3.7	3.4	3.4	3.6	2.0	1.5	3.4	1.6	2.2	1.9	2.3	1.6		94.6	97.7
16	*Amphora* sp.	AM501957	2.1	2.5	0.5	0.9	0.9	2.7	5.3	4.7	5.8	3.8	4.0	3.9	5.5	5.0	4.1		94.1
17	*Amphora* sp.	AB183590	4.9	5.1	4.2	4.2	4.0	4.2	3.1	1.5	3.9	2.4	2.9	2.9	2.6	2.7	1.9	4.7	
			[% p-distance]																

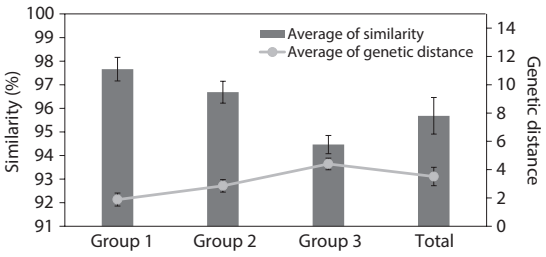

Fig. 3. ANOVA test based on similarity and genetic distance of different species, the three groups were independently and significantly ($p < 0.001$). Group 1: The similarity between species in *Amphora*. Group 2: The similarity between species in *Halamphora*. Group 3: The similarity relationship between species in *Amphora* and *Halamphora*. Total was analyzed result of whole data on similarity and genetic distance, but without ANOVA test.

1 is the similarity between species in *Amphora* with an average of similarity 97.66% and an average genetic distance of 1.89. Group 2 is the similarity between species in *Halamphora* with an average of similarity 96.68% and an average genetic distance of 2.86. Group 3 is the similarity relationship between species in *Amphora* and *Halamphora* with an average of similarity 94.46% and an average genetic distance of 4.40. Total was analyzed result of whole data on similarity and genetic distance, but without ANOVA test. The genetic distance was also consistent with these being three independent groups from statistical analysis (Fig. 3).

Phylogenetic analysis

Phylogenetic relationships among the eight species of *Amphora* sampled with 46 diatoms of differing relationships were investigated based on the nuclear SSU rDNA sequences. *Bolidomonas mediterranea* and *B. pacifica* were designated as outgroups. After alignment and elimination of all positions containing gaps and missing data, a total of 1,470 positions were used for ML, Bayesian, and MP phylogenetic analysis.

Based on an analysis with MrModelTest, the model GTR + I + G was selected for the main results of the comparison of the negative log likelihood (-ln*L* = 10,328.3496), the number of estimated (free) parameters (K = 10), and the AIC (20,676.6992). Base frequencies were as follows: freq A = 0.2703, freq C = 0.1828, freq G = 0.2656, and freq T = 0.2812.

On the basis of the best-fit model, a phylogenic tree was constructed (Fig. 4). ML scores of the ML tree was calculated to be -ln*L* = 10,285.78 by the program PhyML 3.0. The tree size is 1.73958. Bayesian likelihood scores (using MrBayes 3.1.2) was calculated to be -ln*L* = 10,324.97.

The topology was similar for the phylogenetic trees obtained by ML, Bayesian, and MP phylogenetic analysis methods. With respect to the tree in Fig. 4, the genus *Amphora sensu lato* is not monophyletic; however, genus *Entomoneis* is a sister group to Group A. *Amphora sensu lato* forms two separate groups. The first monophyletic group (Group A in Fig. 4) consists of nine species, *H. terroris*, *Halamphora* sp., *H. holsatica*, *H. coffeaeformis*, *H. montana*, *H. eunotia*, *H. costata*, *A.* cf. *capitellata*, and *H. normanii*. These species were classified in *Amphora* subgenus *Halamphora* (Cleve 1895), and the complex was recently elevated to generic status by Levkov (2009). The second monophyletic group (Group B in Fig. 4) consists of six species, *A.* cf. *proteus*, *A. proteus*, *A. marina*, *A. pediculus*, *A.* cf. *fogediana*, and *A. lybica*, and has been classified in *Amphora* as the subgenus *Amphora*. Subgenus *Amphora* is sister to *Entomoneis* plus *Halamphora*.

Morphological characteristics

Amphora marina Smith 1857 (Fig. 5A-D)

Smith 1857, p. 7, Pl. 1, Fig. 2; Van Heurck 1896, p. 129, Pl. 1, Fig. 14; Péragallo and Péragallo 1908, Pl. XLIV, Figs 15-17; Cleve-Euler 1953, p. 92, Fig. 671; Nagumo 2003, p. 32, Pls 71-75; Levkov 2009, p. 470, Fig. 7.

Cells are solitary and chloroplasts are attached to the inner side of the frustule (Fig. 5A). The pyrenoid can be observed clearly at the center of the cell (Fig. 5A). Cells are sessile, but they are usually motile, almost always lying in girdle view. The frustule is elliptical to widely lanceolate with truncated ends (Fig. 5A & B). The girdle bands are not plicate and four girdle bands can be observed (Fig. 5B). Valves are small and lunate, with a convex dorsal margin, a ventral margin that is slightly concave, and with rounded apices (Fig. 5C & D). The valve length is 15.0 to 19.2 µm, and the valve width is 5.2 to 7.9 µm (Table 4). The raphe is well-marked and slightly deflected at the median (Fig. 5C & D). The marginal ridge is not pronounced. The ventral striae are composed of single areolae. The size of the areolae increases towards the mid-valve and they converge toward the ends (Fig. 5C & D). Striae are rather coarsely punctate on the dorsal side and radiate throughout the dorsal side (Fig. 5C), with 17-19 striae present in 10 µm (Table 4). Striae are interrupted by the central area, which is conspicuous at the center of the valve (Fig. 5C & D).

Distribution. Jeju, Korea.

Remark. *Amphora marina* is recorded for the first time in Korea.

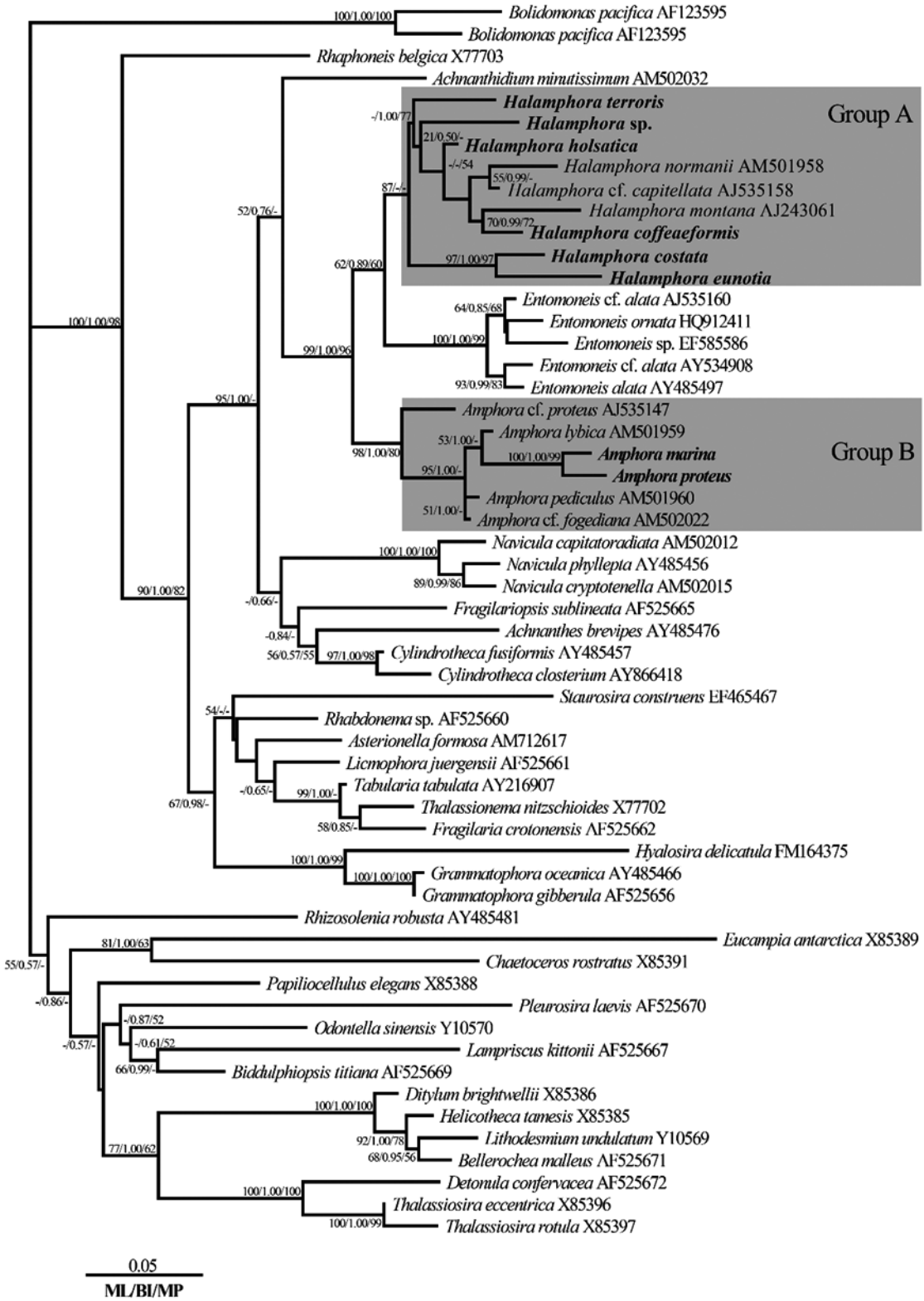

Fig. 4. Maximum likehood (ML) phylogenetic tree based on the partially complete nuclear small subunit rDNA sequences showing the relationships of eight *Amphora sensu lato* species (in bold) and other diatoms. The phylogeny is rooted with *Bolidomonas mediterranea* and *Bolidomonas pacifica*. Bootstrap tests involving 1,000 resamplings were performed and bootstrap values greater than 50% are given in front of the relevant nodes for ML and maximum parsimony (MP) analyses. Bayesian posterior probabilities were more than 0.5 using Bayesian analysis. BI, Bayesian inference.

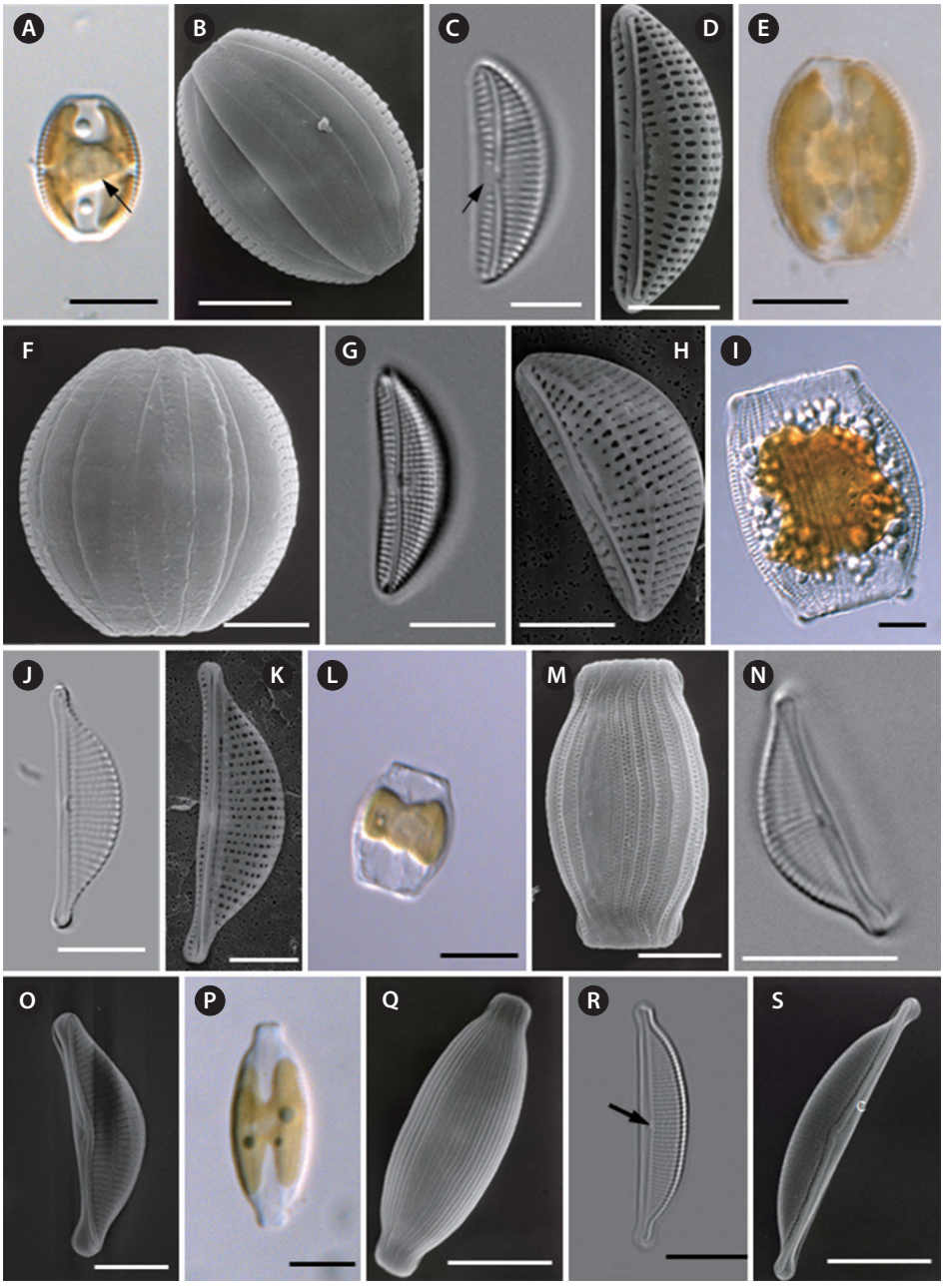

Fig. 5. (A-D) Cells of *Amphora marina* with light microscopy (LM) and scanning electron microscopy (SEM) micrographs. (A) LM. Frustule in dorsal view. Note a rounded pyrenoid (arrow) at the central of cell and chloroplast adherent at the internal cell wall. (B) SEM. Frustule in dorsal view. Note girdle area of the frustule is not plicate. (C) LM. General appearance of the internal valve. Note central nodule conspicuously (arrow). (D) SEM. Another specimen photographed at a different tilt. Note the distribution of the ventral striae. (E-H) Cells of *Amphora proteus* with LM and SEM micrographs. (E) LM. Frustule in dorsal view. Note the truncate ends of the cell. (F) SEM. Frustule in dorsal view. Note girdle area of the frustule is not plicate. (G) LM. General appearance of the valve. Note the dorsal punctate. (H) SEM. Valve in external view, showing the narrow conopeum, the pattern of striation and marginal ridge. (I-K) Cells of *Halamphora eunotia* with LM and SEM micrographs. (I) LM. Frustule in dorsal view. Girdle area of the frustule is wide and plicate for the punctate girdle bands. (J) General appearance of the valve. Note the dorsal punctate striae, the raphe system and a conspicuous central nodule. (K) SEM. Frustule in ventral view. Note the raphe central terminals slightly bent to the dorsal side and then fold back. (L-O) Cells of *Halamphora terroris* with LM and SEM micrographs. (L) LM. Frustule in dorsal view. Note the broad girdle area. (M) SEM. Frustule in dorsal view. (N) LM. General appearance of the valve. Note the dorsal punctate striae, the raphe system and a conspicuous central nodule. (O) SEM. Frustule in ventral view. (P-S) Cells of *Halamphora holsatica* with LM and SEM micrographs. (P) LM. Frustule in dorsal view. Note the H-shape chloroplast. (Q) SEM. Frustule in dorsal view. Note the valve mantles and girdle bands. (R) LM. General appearance of the valve. Note the dorsal punctate striae, the raphe system and a conspicuous central nodule (arrow). (S) Valve in external view showing the narrow conopeum (c). Scale bars represent: A, E, G, J, L, N & P-S, 10 μm; B-D, F, H, I, K, M & O, 5 μm.

Table 4. Sizes and striae densities in valves of eight *Amphora sensu lato* species and records in other literatures

Species	*Amphora marina*	*Amphora proteus*	*Halamphora eunotia*	*Halamphora terroris*	*Halamphora holsatica*	*Halamphora costata*	*Halamphora* sp.	*Halamphora coffeaeformis*
In this study								
No. of observations	33	37	33	56	32	48	48	34
Length (μm)	15.0-19.2	12.2-22.7	20.7-27.6	10.5-18.3	26.1-29.6	17.3-22.8	9.1-15.1	14.2-18.6
Width (μm)	5.2-7.9	5.6-8.4	6.8-8.6	4.0-6.3	5.1-7.0	6.0-9.2	3.9-5.7	4.5-7.4
Striae density in 10 μm	17-19	14-16	13-14	22-24	18-20	8-11	14-20	14-18
In other literatures								
Length (μm)	40-83[a-c]	15-150[a,c-i]	50-120[a-c,f,g,j]	26-70[g,i]	25-50[c,k]	20-80[c,d,f,g,i,j,l]	-	13-60[c,g,i,k]
Width (μm)	9-23[b,c]	7-60[a,c,d,g,i]	8-50[a-c,j]	4-30[g,i]	7-25[c,k]	9-55[c,d,g,i,j]	-	5-19[c,i,k]
Striae density in 10 μm	11-19[a-c,e,g,m]	9-22[a,c,d-g,i,n]	6-11[a-c,f,g]	6-9[g,i]	11-19[c,k]	8-16[c,d,f,g,i,j]	-	13-22[c,i]

[a]Van Heurck (1896), [b]Chin et al. (1992), [c]Levkov (2009), [d]Gregory (1857), [e]Péragallo and Péragallo (1908), [f]Boyer (1927), [g]Cleve-Euler (1953), [h]Wood and Crosby (1959), [i]Jin et al. (1982), [j]Hendey (1964), [k]Krammer et al. (1991), [l]Smith and West (1853), [m]Rabenhorst (1868), [n]Boyer (1916).

Amphora proteus Gregory 1857 (Fig. 5E-H)

Gregory 1857, p. 518, Pl. 13, Fig. 81; Van Heurck 1896, p. 129, Pl. 24, Fig. 671; Boyer 1916, p. 65, Pl. 15, Figs 5, 6 & 19; Boyer 1927, p. 254; Cleve-Euler 1953, p. 92, Fig. 673; Levkov 2009, p. 548, Figs 1-9, p. 824, Figs 4-6.

Synonyms. *Amphora affinis* var. *proteus* (Gregory) Tempère and Peragallo 1908, Pl. 41, Figs 76 & 77.

Cells are solitary and chloroplasts are attached to the inner side of frustule (Fig. 5E). Cells are sessile, but they are usually motile, almost always lying in girdle view (Fig. 5E). The frustule is elliptical to widely lanceolate with truncated ends (Fig. 5E & F). The girdle area of the frustule is not plicate (Fig. 5F). Valves are small and lunate, with a very convex dorsal margin, slightly concave ventral margin and rounded apices (Fig. 5H). Valve length is 12.2 to 22.7 μm, and valve width is 5.6 to 8.4 μm (see Table 4). Raphe are well marked and slightly inflexed at the median (Fig. 5G). The marginal ridge is pronounced (Fig. 5H). The ventral margin forms a row of elongate areolae, increasing in length toward the mid-point of the valve (Fig. 5G) and converging toward the ends. Striae with very distinct robust puncta radiate on the dorsal side, with 14-16 striae per 10 μm (Table 4). The central nodule is rather large, with no conspicuous central area (Fig. 5G).

Distribution. Gilcheon Port, Korea.

Halamphora eunotia (Cleve) Levkov 2009 (Fig. 5I-K)

Cleve 1873, p. 21, Pl. 3, Fig. 17; Van Heurck 1896, p. 129, Pl. 24, Fig. 684; Péragallo and Péragallo 1908, Pl. L, Fig. 17; Cleve-Euler 1953, p. 99, Fig. 687; Levkov 2009, p. 502, Figs 1-12, p. 814, Figs 1-4.

Synonym. *Amphora eunotia* Cleve 1873, p. 21, Pl. 3, Fig. 17.

Cells are solitary. Cells are usually motile, almost always lying in girdle view and then appearing sub-rectangular, with the two sides having an arching bulge (Fig. 5I). The girdle area of the frustule is wide and plicate for the punctate girdle bands (Fig. 5I). Valves are semi-lanceolate with a convex ventral margin, and the poles are ventrally deflected with protracted apices (Fig. 5J & K). Valves are 20.7 to 27.6 μm long and 6.8 to 8.6 μm wide (Table 4). Raphe are straight and lie along the ventral edge (Fig. 5J & K). The central terminals of the raphe are slightly bent to the dorsal side and then fold back (Fig. 5K). The central nodule is conspicuous (Fig. 5K). Striae are parallel at the central part and become curved and more divergent toward the ends. Dorsal striae are distinctively punctate (Fig. 5J), with 13-14 striae per 10 μm (Table 4). Ventral striae are hardly visible under LM. Striae are not interrupted by the

central nodule area.

Distribution. Geoje, Korea.

Halamphora terroris (Ehrenberg) Wang comb. nov. 2014 (Fig. 5L-O)

Ehrenberg 1853, p. 526; Cleve-Euler 1953, p. 99, Fig. 689.

Synonym. *Amphora terroris* Ehrenberg 1853, p. 526.

Cells are solitary (Fig. 5L). Cells are usually motile, almost always lying in girdle view and then appearing subrectangular with the two sides having an arching bulge (Fig. 5L & M). The girdle area of the frustule is wide and plicate for the punctate girdle bands (Fig. 5M). In each girdle band, there are two rows of punctae. Valves are semi-lanceolate with a convex ventral margin, and poles are ventrally deflected with protracted apices (Fig. 5N). Valve are 10.5 to 18.3 µm long and 4.0 to 6.3 µm wide. From the valve view, the raphe slit is slightly curved and lies along the ventral edge (Fig. 5N & O). The central nodule is conspicuous (Fig. 5N). Externally, the proximal fissures of the raphe are dilated in the central part of the cell and dorsally bent (Fig. 5O). The striae are nearly parallel at the central valve and become divergent toward the ends. Striae are not interrupted by the central nodule area at the dorsal side, and there are 22-24 striae per 10 µm (Table 4).

Distribution. Jeju, Korea.

Remark. *Halamphora terroris* is transferred from *Amphora* Ehrenberg to *Halamphora* Levkov in this study as a new combination species of *Halamphora*.

Halamphora holsatica (Hustedt) Levkov 2009 (Fig. 5P-S)

Hustedt 1925, p. 115, Fig. 4; Hustedt 1930, p. 345, Fig. 633; Cleve-Euler 1953, p. 99, Fig. 688; Sar et al. 2003, Figs 1-13; Levkov 2009, p. 522, Figs 1-11, p. 782, Figs 1-6.

Synonym. *Amphora holsatica* Hustedt 1925.

Cells are solitary, usually motile, and almost always lying in girdle view (Fig. 5P). The frustule is elliptical to widely lanceolate, with subrostrate-truncated ends (Fig. 5P & Q). The girdle area of the frustule is plicated for the punctate girdle bands (Fig. 5Q). Valves are semi-lanceolate with a convex dorsal margin and straight ventral margin (Fig. 5R). Valves are 26.1 to 29.6 µm long and 5.1 to 7.0 µm wide (Table 4). The raphe are straight and lie along the ventral edge (Fig. 5R). Viewed under SEM, the raphe at the valve lies near the ventral margin, and both branches of the raphe are straight or nearly straight, forming an obtuse angle of nearly 180° (Fig. 5S). The conopeum is well-developed on the dorsal side of the valve, straight with a slight widening at the center, and its ends embrace the valve poles (Fig. 5S). Along the transition between the dorsal valve face and mantle, there is no costa (hyaline bar) interrupting the striae (Fig. 5R & S). The central nodule is conspicuous (Fig. 5R). The dorsal striae are parallel at the central part and become slightly divergent at the two ends of the valve (Fig. 5R). Striae are not interrupted by the central nodule area (Fig. 5R), and there are 18-20 striae per 10 µm (Table 4).

Distribution. Pohang, Korea.

Halamphora costata (W. Smith) Levkov 2009 (Fig. 6A-D)

Smith and West 1853, p. 20, Pl. 30, Fig. 253, Figs 15-17; Wolle 1894, Pl. IX, Fig. 3; Van Heurck 1896, Pl. 24, Fig. 679; Péragallo and Péragallo 1908, Pl. L, Fig. 20; Cleve-Euler 1953, p. 99, Fig. 690.

Synonym. *Amphora rostrata* Van Heurck 1896, p. 133, Pl. XXIV, Fig. 679. *Amphora costata* Smith and West 1853, p. 20, Pl. 30, Fig. 253.

Cells are solitary, usually motile and almost always lying in girdle view (Fig. 6A). The frustule is elliptical to widely lanceolate, with ends broadly protracted into the subrostrate to truncate poles (Fig. 6A). The girdle area of the frustule is plicate for the punctate girdle bands (Fig. 6B). There is a double row of areolae at the girdle band. Valves are semi-lanceolate, with a convex dorsal margin and a nearly straight ventral margin (Fig. 6C). Valves are 17.3 to 22.8 µm long and 6.0 to 9.2 µm wide (Table 4). Raphe are straight and lie along the ventral edge (Fig. 6C). The proximal fissures of the raphe are dilated in the central pores and are dorsally slightly bent (Fig. 6D). The conopeum is well-developed on the dorsal side of the valve (Fig. 6D). The central nodule is conspicuous (Fig. 6C). The striae are parallel at the centrally and become slightly divergent toward the ends (Fig. 6C). Dorsal striae are distinctively punctate and spaced at 8-11 striae per 10 µm, and are not interrupted by the central nodule area (Fig. 6C). Ventral striae may be visible, and are interrupted by the central nodule area (Fig. 6C). The conopeum is fairly well developed on the dorsal side of the valve (Fig. 6D).

Distribution. Gilcheon Port, Korea.

Halamphora sp. (Fig. 6E-I)

Cells are solitary (Fig. 6E) and usually motile. They are almost always lying in girdle view and appear elliptical with truncated ends (Fig. 6E). Chloroplasts are attached to the inner side of frustule (Fig. 6E). The girdle area of the frustule is plicate for the punctate girdle bands (Fig. 6F). Valves are small and lunate, with rounded apices

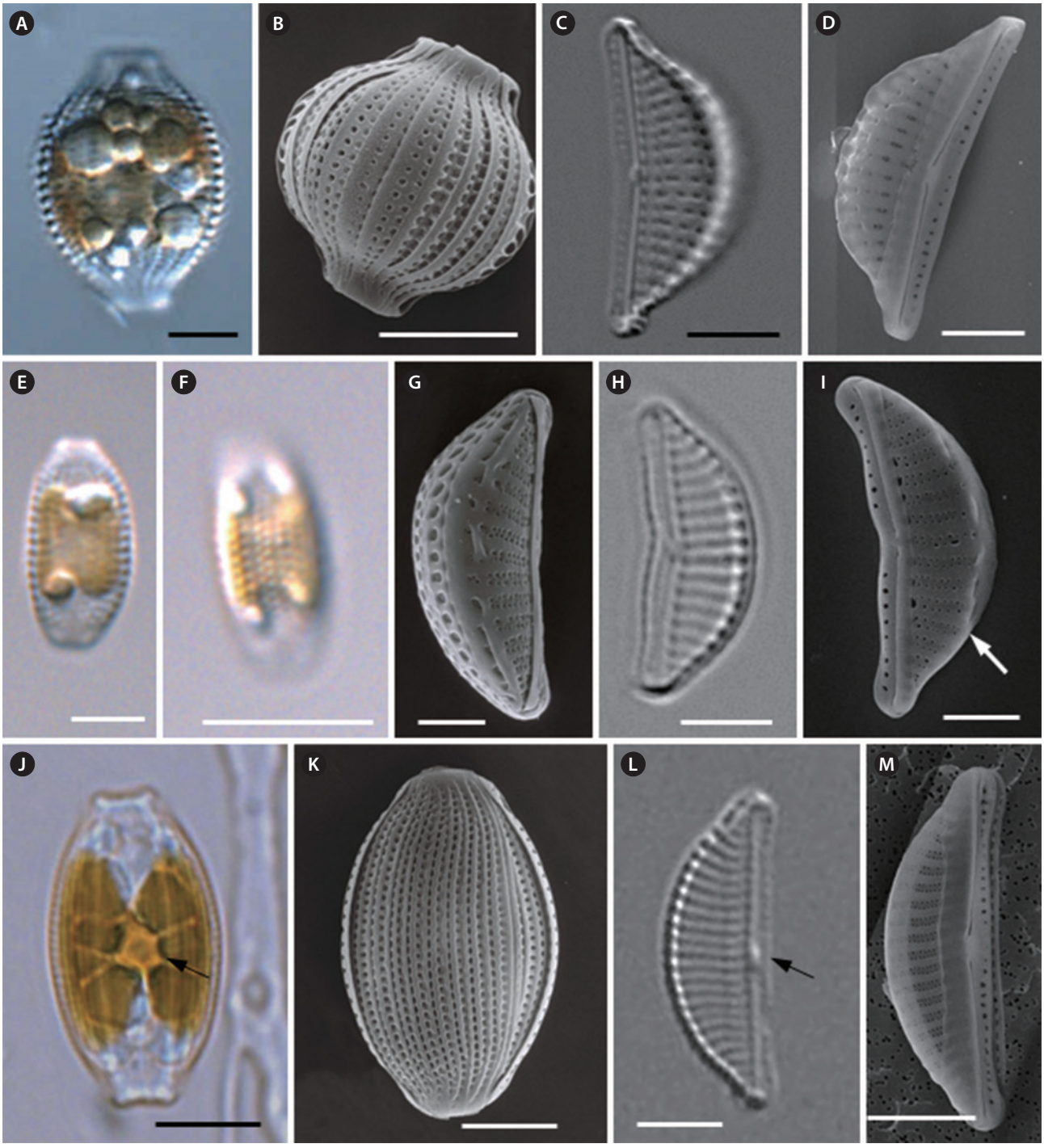

Fig. 6. (A-D) Cells of *Halamphora costata* with light microscopy (LM) and scanning electron microscopy (SEM) micrographs. (A) LM. Frustule in dorsal view. (B) SEM. Frustule in dorsal view, showing girdle area of the frustule is plicate for the punctate girdle bands. (C) LM. Valve in inside view showing the conspicuous arealae. (D) SEM. Valve view. (E-I) Cells of *Halamphora* sp. with LM and SEM micrographs. (E) LM. Frustule in dorsal view. (F) LM. Frustule in dorsal view, punctae of girdle bands. (G) SEM. Valve in external lateral view, showing the intermittent hyaline bar and the gap between conopeum and valve. (H) LM. General appearance of the valve. (I) SEM. Frustule in ventral view. Showing the puncta in double rows spaced in pairs (arrow). (J-M) Cells of *Halamphora coffeaeformis* with LM and SEM micrographs. (J) LM. Frustule in dorsal view. Note a pyrenoid (arrow) at the central of cell. (K) SEM. Frustule in dorsal view. (L) LM. General appearance of the valve. Note the dorsal costae, the raphe system and a conspicuous central nodule (arrow). (M) SEM. Frustule in ventral view. Scale bars represent: A, C-E, G, H & K-M, 5 μm; B, F & J, 10 μm; I, 2 μm.

(Fig. 6G & H). Valves are 9.1 to 15.1 µm long and 3.9 to 5.7 µm wide (Table 4). The raphe slit is nearly straight and lies along the ventral edge (Fig. 6H & I). Both branches of the raphe are slightly inflexed at the median, the raphe has a straight end at the center valve and a curve to the dorsal side at the apices of the valve (Fig. 6I). Along the transition between the dorsal valve face and mantle there is a costa (hyaline bar), but this is not continuous in all cells (Fig. 6I). The costae and striae are observed very clearly and are parallel throughout (Fig. 6H). Ventral striae are poorly visualized under light microscopy in the study specimens. Striae are not interrupted by the central nodule area on the proximal side, and are spaced with 14-20 striae per 10 µm (Table 4). The puncta are alternately spaced in double rows to form striae (Fig. 6G & I). The conopeum is fairly well-developed (Fig. 6I).

Distribution. Gilcheon Port, Korea.

Halamphora coffeaeformis (C. Agardh) Levkov 2009 (Fig. 6J-M)

Kützing 1844, p. 108, Pl. 5, Fig. 37; Wolle 1894, Pl. IV, Figs 19 & 20; Van Heurck 1896, Pl. 24, Fig. 671; Boyer 1916, p. 65, Pl. 15, Figs 8 & 18; Cleve-Euler 1953, p. 97, Fig. 685; Archibald and Schoeman 1984, p. 86, Figs 1-24, p. 87, Figs 25-29, p. 91, Figs 100-109, p. 92, Figs 110-122, p. 93, Figs 123-134, p. 96, Figs 135-146, p. 97, Figs 147-159, p. 98, Figs 160-162; Krammer and Lange-Bertalot 1986, p. 744, Pl. 151, Figs 1-6.

Synonyms. *Frustulia coffeaeformis* (C. Agardh) Agardh 1827, p. 627. *Amphora coffeaeformis* (C. Agardh) Kützing 1844, p. 108, Pl. 5, Fig. 37.

Cells are solitary (Fig. 6J), usually motile, and almost always lying in girdle view (Fig. 6J). Single H-shaped chloroplasts are contained in each cell with a conspicuous pyrenoid at the center of the cell (Fig. 6J). The frustule is elliptical to widely lanceolate, with ends somewhat broadly protracted into the subrostrate to truncated poles (Fig. 6J). The girdle area of the frustule is plicate for the punctate girdle bands (Fig. 6K). Valves are semi-lanceolate, with rounded apices (Fig. 6L). Valves are 14.2 to 18.6 µm long, and 4.5 to 7.4 µm wide (Table 4). The central nodule is conspicuous (Fig. 6L). The raphe are straight and lie along the ventral edge (Fig. 6L & M). The costae and striae can be observed very clearly and are parallel at the centrally, becoming curved and more divergent toward the ends (Fig. 6L). Striae are not interrupted by the central nodule area at the proximal side, and are spaced with 14-18 striae per 10 µm (Table 4). SEM images show that the puncta are alternately spaced in double rows to form striae at the valve. Between the striae, the costae are very conspicuous. The ventral striae are denser and consist of only a single row of small linear to circular areolae, which are not uniform in shape, size or arrangement (Fig. 6M).

Distribution. Uljin, Korea.

DISCUSSION

All eight species studied are clearly distinct from one another, both morphologically and by sequencing analysis. All eight of these species' morphological characteristics have been compared with previous morphological descriptions; each species is identified.

The *Amphora* species in this study were smaller than those described previously (Table 4). As is commonly known, cell size decreases during cell division (Tomas 1996, Wehr and Sheath 2003). Sexual reproduction also contributes to size variation in diatoms (Mann 1993). However, our cultures did not contain any full-sized cells resulting from sexual reproduction, an observation consistent with the small average size of the cells. In this study, the observations were made on cultured cells. To acquire more accurate data on the size of *Amphora*, natural samples will need to be tested. However, many studies (Gregory 1857, Van Heurck 1896, Péragallo and Péragallo 1908, Cleve-Euler 1953, Levkov 2009) have shown that the same species from different locations can have various sizes. For example, the 70-150 µm cell lengths of *A. proteus* in England, Belgium, Germany, and Norway (Van Heurck 1896) were much larger than the 15-60 µm cell lengths in the Firth of Clyde and in Loch Fine (Gregory 1857). Therefore, size differences might be the result of cell division, sexual reproduction, life cycle and different environments (Veselá et al. 2009, Davidovich et al. 2012).

Differences in densities of stria observed in our study and previously may be attributable to measurement methods used by different researchers. Archibald and Schoeman (1984) also discussed this issue. In most publications, the actual site is not given, and one is left to assume that counts were made across the central parts of the valve. To obviate this problem, we emphasize that the striae counts herein are always made along the raphe, as done by Schoeman and Archibald (1976). Striae counts designated as being near the center were made on either side of the central nodule or central area and not across the central nodule.

With regard to *Amphora* ultrastructure, we found that the girdle area of different *Halamphora* species had different forms, although some were very similar or the same type. The *Halamphora* species had greater morphological

diversity in the girdle area. This contrasts with most diatoms which usually lack special morphological characteristics. In this study, *H. terroris* had two rows of punctae in a girdle band. *H. holsatica* and *H. coffeaeformis* had punctae in small linear girdle bands. *Halamphora costata* and *Halamphora* sp. had relatively large punctae in the girdle band. However, the sketches in earlier literature did not accurately illustrate the girdle area types for species definition. Examples of this can be seen in the girdle area type pictures of *A. granulata* (Gregory 1857, Fig. 96b) and *A. proboscidea* (Gregory 1857, Fig. 98b) shown in Gregory 1857. Although the sketches were relative clear, as shown in Plates 25-28 of Schmidt and Fricke (1875), the detail of the girdle area was not as sharp as that seen in SEM images. Also, with the acid used during the SEM pretreatment process, the girdle area was frequently lost. From previous literature, it was difficult to distinguish the different forms of the girdle area without application SEM or the loss of girdle bands after acid treatment. Consequently, confirming features of the girdle area of every *Amphora sensu lato* species is not easy, as is clear from the existing literature. Actually, more study is required before the connecting band structure can be used as a taxonomic feature. Punctae size, shape, and arrangement mode in every girdle band need to be examined in future *Amphora sensu lato* studies. Regarding the diversity of girdle bands, their structure may be of substantial use in *Amphora sensu lato* species taxonomy and also may be important diagnostic features for identification.

In this study, *Amphora terroris* is described for the first time since Cleve-Euler (1953), and it can now be assigned to *Halamphora* as *Halamphora terroris* (Ehrenberg) Wang comb. nov. In addition, *Amphora marina* is described for the first time from Korean coastal waters.

ANOVA of similarity and genetic distances showed that the species in subgenus *Amphora* were more similar to each other and had a closer genetic distance than *Halamphora* species. The *Halamphora* species considered had low interspecific similarity, though this should be confirmed by analyzing more species. Also, the Group 3 analysis demonstrated that Group 1 and Group 2 were markedly dissimilar, as seen in the similarity and genetic distance data for the groups in Fig 3. Furthermore, the significant interspecific differences in nuclear SSU rDNA of *Amphora sensu lato* can be used as a tool or molecular tag to differentiate species in *Amphora sensu lato* and to distinguish species of *Amphora* and *Halamphora* at generic level. All of these molecular characteristics accompany many differences in morphology. This is despite the fact that SSU rDNA is not a gene related to diatom frustule formation and cannot be the equivalent of an identification key. Regardless, all these SSU rDNA genes co-evolved with other structural gene for millions years.

From our phylogenetic tree, we found that species of *Amphora sensu lato* to some extent reflect the fact that diatoms migrated from marine, to brackish, to freshwater environments, as indicated by Wasell and Håkansson (1992). However, it was difficult to positively distinguish between marine, brackish, and freshwater species because *Amphora sensu lato* sequence information is limited.

Due to the fact that *Amphora sensu lato* was divided into two groups, Group A and Group B (Fig. 4), and Group A and the genus *Entomoneis* were monophyletic with the support of 62 / 0.89 / 60 (ML / BI / MP) bootstrap values, *Amphora sensu lato* was paraphyletic with *Halamphora* sister to the genus *Entomoneis* and subgenus *Amphora* sister to *Halamphora* adding *Entomoneis*. Bruder and Medlin (2007) studied the genus *Placoneis* using large subunit rDNA. They included five species of *Amphora* which divided into two groups, a pattern similar to that seen in our analysis (Fig. 4). However, Bruder and Medlin gave no explanation for this and further discussion is on the two-group division of *Amphora*. Comparison of the morphological structures of these two groups of *Amphora* in this study revealed that the species in Group A all belonged to *Halamphora* (Cleve) Levkov, which has a plicate girdle area and rostrate or capitate end valves (Cleve 1895, Van Heurck 1896). The species in Group B belonged to the subgenus *Amphora* Cleve, which has a smooth girdle area and a more or less lunate valve, while the *Amphora* cf. *proteus* was annotated as a species from the subgenus *Diplamphora* in Sato et al. (2013), which requires further study. To confirm the correctness of the above results in the phylogenetic tree, the ML Bayesian estimation and MP methods were used to analyze the evolutionary relationships among these eight *Amphora* species and other diatoms. Since the ML is based on a different statistical principle than Bayesian estimation (Beerli 2006, Klipp et al. 2009), the congruence of phylogenic trees give us confidence in the result. We conclude that *Amphora* is not a single monophyletic group as previously showed by Sato et al. (2013).

More definitive conclusions regarding the phylogenetic taxonomy of *Amphora* will require more DNA data from different species of *Amphora sensu lato* (cf. Mann and Poulícková 2010, Sato et al. 2013). Thus molecular data on species in the remaining subgenera of *Amphora* (i.e., subgenera *Amblyamphora*, *Archiamphora*, *Calamphora*, *Diplamphora*, *Oxyamphora,* and *Psammampho-*

ra) are needed to resolve the evolutionary relationships in the complex as a whole. Molecular phylogenies can determine whether these subgenera are independent groups, like the subgenera *Amphora* and *Halamphora*, or have other relationships. Based on a more accurate phylogeny, the morphology can then be evaluated to arrive at a comprehensive taxonomic assessment of the assemblage. This may necessitate the recognition of additional genera.

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