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Description and phylogenetic position of *Amphora aliformis* (Bacillariophyta), a new species from Tokyo Bay

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Abstract: The genus *Amphora* is a large and widely distributed group of raphid diatoms, frequently found in fresh, brackish, and salt water environments from around the world. Presented here is the description and phylogenetic position of *Amphora aliformis*, a new species collected from the Banzu Flats, Tokyo Bay, Japan. This species has a unique morphology, with features seldom or never before observed within the genus *Amphora*, such as a dorsal marginal wing and ventral apical pores, as well as a unique phylogenetic position as an early diverging lineage within the genus. As with many large and diverse groups of diatoms, new evidence has called into question the validity of the long standing classification system for the genus *Amphora*. Within the previous system, *A. aliformis* would be prescribed to the subgenus *Diplamphora* based on morphological valve features. However, molecular phylogenetic analysis demonstrates that *Diplamphora* is not monophyletic and furthers evidence that *Amphora* classification is in need of revision. Included here is a description of *A. aliformis* based on light microscope, scanning electron microscope, and molecular phylogenetic data, as well as a re-examination and discussion of the current state of *Amphora* classification.

Key Words: *Amphora*, *Diplamphora*, Japan, New species, Phylogeny, Tokyo Bay

INTRODUCTION

The genus *Amphora* Ehrenberg ex Kützing *sensu lato* (*s.l.*) is a large and diverse group of raphid diatoms commonly found in fresh, brackish, and salt water environments (Stoermer & Yang 1971; Wachnicka & Gaiser 2007; Levkov 2009; Stepanek & Kociolek 2013). This morphologically diverse group has traditionally been separated into nine subgenera based on morphological features such as a 'simple' or 'complex' connecting zone (girdle area), the presence or absence of 'longitudinal lines', as well as several raphe and striae features (Cleve 1895). Although this classification stood for over 100 years, recent molecular phylogenetic data have shown the group to be non-monophyletic (Sato *et al*. 2013; Stepanek & Kociolek 2014; Wang *et al*. 2014), with members distributed across several orders of raphid diatoms (Stepanek & Kociolek 2014). Despite the polyphyly of the taxa currently assigned to *Amphora*, a classification based on monophyly, which does not necessarily correspond to the subgenera of Cleve (1895), is beginning to be developed (Stepanek & Kociolek 2014). One such clade includes taxa from the subgenera *Amphora sensu stricto* (*s.s.*) (including the generitype *Amphora ovalis* (Kützing) Kützing), *Diplamphora*, and *Oxyamphora* (Stepanek & Kociolek 2014).

The subgenus *Diplamphora* was described by Cleve (1895) to include forms that share morphological similarities with *Amphora s.s.* in having linear to semi-lanceolate valves, a dorsal valve face with one or two longitudinal lines, and striae composed of transverse costae or rows of puncta (Cleve 1895). These taxa differed from *Amphora s.s.* in what Cleve termed a 'complex connecting zone' (areolate girdle bands), as opposed to the 'simple' or unornamented girdle bands in *Amphora s.s.* This rather broad definition has led to a group with a remarkable variety of forms, an issue addressed by Cleve when he remarked the subgenus *Diplamphora* 'comprises a number of species, very different in appearance but agreeing in the complex connecting zone and the longitudinal line on the dorsal side' (Cleve 1895, p. 107). Although Cleve was unsure whether the difference in girdle structure was sufficient to consider *Diplamphora* as a separate genus, he remained confident in his assertion that the group was very closely allied to *Amphora s.s.* (Cleve 1895).

Recent molecular phylogenetic studies by Sato *et al*. (2013) and Stepanek & Kociolek (2014) have supported the close association between the subgenera *Amphora s.s.* and *Diplamphora*. Although both demonstrate this close association, these studies differed in the taxa included in the analyses and the relationship between *Diplamphora* and *Amphora s.s.* Stepanek & Kociolek (2014) found taxa of *Diplamphora* and *Oxyamphora* forming a monophyletic group sister to *Amphora s.s.*; whereas, the analyses of Sato *et al*. (2013) showed *Diplamphora* taxa as a paraphyletic grade into a monophyletic *Amphora s.s.*

Presented here is the description and phylogenetic placement of *Amphora aliformis*, a new species from the genus *Amphora* collected from the Banzu Flats in Tokyo Bay, Japan, based on morphological and molecular phylogenetic data. Although exhibiting several unique morphological features, *A. aliformis* conforms to the broad concept of Cleve's (1895) subgenus *Diplamphora*, and a discussion of the systematic implications of this new species in the evolving concept of the genus *Amphora* follows.

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MATERIAL AND METHODS

Taxon collections

Materials containing *Amphora aliformis* were collected from the Banzu Flats in Tokyo Bay, Tokyo Prefecture, Japan (35.44081°N, 139.91078°E). Collections of low tide sand samples from the flats were made by Stepanek and Mayama in June 2013.

Amphora aliformis was isolated into monoculture through micropipette serial dilution into Daigo's Artificial Seawater medium (Nihon Pharmaceutical Co., Tokyo, Japan). Owing to the epipsammic growth form of *A. aliformis*, collected sand grains were first placed into Petri dishes containing growth medium, cells were allowed to divide and grow off of the sand grains onto the Petri dish, and these cells were then isolated into monoculture. All cultures were maintained at *c.* 25°C under a 12:12 light regime with an irradiance of *c.* 50 μmol min^{-2} sec^{-1}.

Samples were cleaned of organic matter for light microscope (LM) observations by boiling in concentrated sulphuric acid with the addition of potassium permanganate, followed by repeated rinses with distilled water until a neutral pH was reached. For LM observations, cleaned material was air dried onto glass coverslips, and permanent slides were prepared using Mount Media® (Wako Pure Chemical Industries, Osaka, Japan). All LM observations were performed using an Olympus BX-51 light microscope (Olympus America Inc., Center Valley, Pennsylvania) with ×100 oil immersion objective (numerical aperture 1.40). Light micrographs were taken with an Olympus DP71 digital camera. For scanning electron microscope (SEM) preparation, culture material was cleaned of organic matter using a low temperature plasma treatment, previously shown to keep frustules intact for observation (Watanabe *et al.* 2010). Culture material was air dried onto glass coverslips and plasma processed for 40 minutes in a Diatom Asher Model II (Kyotodensikeisoku Co., Kyoto, Japan). For SEM images featuring cell interiors, individual valves of cultured cells were separated and cleaned of organic matter under an inverted microscope using a drop of dilute bleach solution and a fine probe. After the cleaning procedure, the separated valves were rinsed with drops of distilled water and air dried onto glass coverslips. For SEM observations, processed glass coverslips were coated with *c.* 5 nm Osmium using an OPC40 Osmium Plasma Coater (Filgen, Nagoya, Japan). Electron micrographs were taken with a Hitachi S-4500 field emission SEM at an acceleration voltage of 15 kV.

Valve structure terminology follows that of Cox & Ross (1981) and Nagumo (2003).

DNA extraction, amplification, and sequencing

Extraction of DNA from *Amphora aliformis* was performed from pelleted culture material using a Chelex 100® extraction method (Richlen & Barber 2005). For the phylogenetic analysis, two nuclear markers, the nuclear encoded small subunit (SSU) ribosomal DNA and the D1–D2 region of the nuclear encoded large subunit (LSU) ribosomal DNA, and two chloroplast markers, the large subunit of RUBISCO (*rbc*L) and the photosystem II Chlorophyll *a* binding protein *psb*C, were amplified. These markers were chosen to coincide with the recently published molecular phylogeny of *Amphora s.l.* (Stepanek & Kociolek 2014) with the addition of LSU, a region that has previously been shown to resolve species level associations within diatoms (Alverson *et al.* 2007; Hamsher *et al.* 2011). Primers used for the amplification and sequencing of these markers are listed in supplementary Table S1.

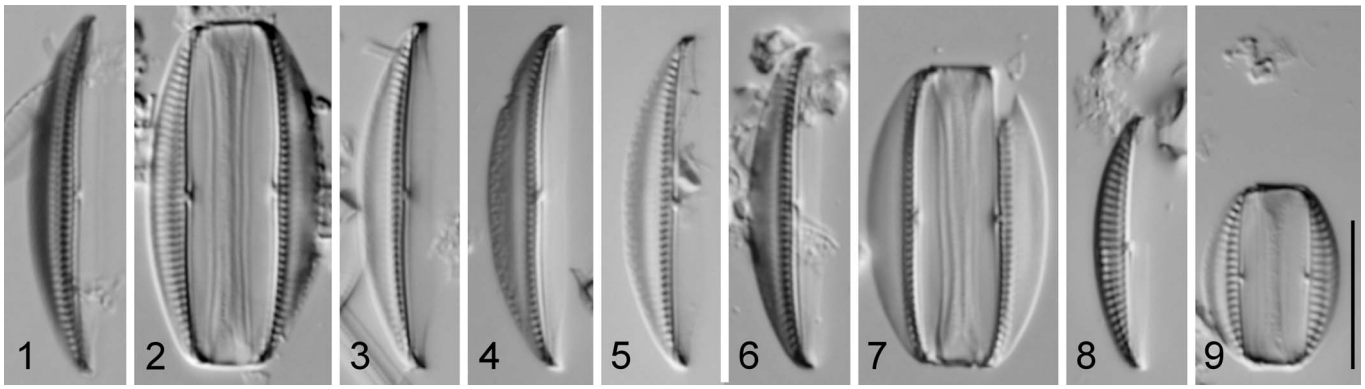
All markers were amplified by polymerase chain reaction (PCR) using GE healthcare Illustra Ready-To-Go™ PCR beads (GE Healthcare Biosciences, Pittsburgh, Pennsylvania) following the manufacturer's protocol. The PCR profile used for each molecular marker was 94°C initial denaturation for 3 minutes 30 seconds, followed by 36 cycles of 94°C for 50 seconds, 52°C for 50 seconds, 72°C for 80 seconds, with a final extension at 72°C for 15 minutes. Amplified PCR product was purified using ExoSap-it (Affymetrix, Santa Clara, California) following the manufacturer's protocol. Sequencing of the purified PCR product was performed by Functional Biosciences (Madison, Wisconsin) and returned raw sequence files were edited and assembled using Geneious ver. 5.6 (Drummond *et al.* 2012). All newly created sequence data have been submitted to GenBank (see Table S2 in supplementary materials for individual accession numbers).

Sequence alignment and phylogenetic analysis

Recent findings have shown members of the genus *Amphora* distributed widely across raphid diatom lineages (Stepanek & Kociolek 2014); therefore, it was important to test whether *Amphora aliformis* belonged to the clade containing members of *Amphora s.s.* before moving forward with the analysis. To test this, sequence data for *A. aliformis* was added to the three marker (SSU, *rbc*L and *psb*C) concatenated alignment used by Stepanek & Kociolek (2014) that includes a broad sampling of raphid diatom lineages. The ML phylogram inferred from this dataset showed, with strong support (BS 99), that *A. aliformis* is part of the *Amphora s.s.* + *Diplamphora* + *Oxyamphora* clade (supplementary Fig. S1). Because of this finding, and to allow for the inclusion of additional sequence data (LSU), all subsequent alignments and analyses included only taxa from the *Amphora s.s.* + *Diplamphora* + *Oxyamphora* clade, with members of the genus *Halamphora* (from the sister clade to *Amphora*) used as outgroup taxa.

Sequences were aligned in Geneious using a muscle alignment algorithm (Edgar 2004), visually inspected, and edited by hand as needed. Sequences for each of the four molecular markers were aligned individually and trimmed to minimize missing data before concatenation into an alignment consisting of the nuclear encoded markers (SSU and LSU), an alignment consisting of the chloroplast encoded markers (*rbc*L and *psb*C), and a four marker concatenated alignment (Alignment S1). For all alignments, 15 ingroup (including *Amphora aliformis*) taxa and four outgroup taxa from the genus *Halamphora* were included (Table S2). The included markers had a final trimmed length of 1604, 559, 1369, and 1112 bps for SSU, LSU, *rbc*L, and *psb*C, respectively.

Using jModelTest ver. 0.1.1 (Guindon & Gascuel 2003; Posada 2008), the general time reversible (GTR) model with a gamma distribution (Γ) and a proportion of invariable sites (I) was chosen as the best model of sequence evolution for the individual markers as well as for the concatenated alignments.



Figs 1–9. Light micrographs of whole frustules and valves of *Amphora aliformis* showing observed size range. Cleaned collection material, JPK 10105. Scale bar = 10 μm.

For all alignments, maximum likelihood (ML) estimation was performed using PhyML version 3.0 (Guindon *et al.* 2010) implemented in SeaView version 4.3.4 (Gouy *et al.* 2010), and branch support was estimated using 500 bootstrap replicates. Bayesian estimation was conducted using MrBayes version 3.2.1 (Ronquist *et al.* 2012). The Bayesian estimation was run for 15 million generations, with a burn-in of 3 million generations, using two runs of four Markov chain Monte Carlo chains sampled every 1000 generations.

RESULTS

Morphological analysis

Amphora aliformis Stepanek, Mayama & Kociolek *sp. nov.*
Figs 1–24

DESCRIPTION: Frustule elliptical with bluntly truncated ends. Valves semi-elliptical, moderately dorsiventral, with a smoothly arched dorsal margin and a straight to slightly convex ventral margin. Valve length 12.0–24.0 μm, valve breadth 3.5–5.5 μm; although, in the LM the apparent breadth can vary depending on the angle at which the valve is lying. Valve ends are narrowly rounded and slightly deflected ventrally. The raphe is nearly centrally placed on the valve, straight to slightly arched, with straight proximal raphe ends and distal raphe ends that are difficult to observe in the LM. The axial area is narrow throughout dorsally. Dorsal striae are uninterrupted, nearly parallel at the valve centre and becoming radiate near the apices. Dorsal striae number 19–20 in 10 μm. In the LM, depending on the valve angle, the dorsal striae are often obscured near the dorsal margin by a distinct hyaline area. Ventral striae are absent; although, a broad unornamented ventral area is present.

GENBANK SEQUENCES: KP229525 (SSU), KP229544 (LSU), KP229546 (*rbc*L), KP229548 (*psb*C).

HOLOTYPE: TNS-AL-56400s, slide deposited at the National Museum of Nature and Science, Tsukuba, Japan (TNS). Collected 23 June 2013.

ISOTYPES: TNS-AL-56400m, cleaned material deposited at the National Museum of Nature and Science, Tsukuba, Japan (TNS). JPK10169, slide and material deposited at the University of Colorado Boulder, Kociolek collection at COLO. Collected 23 June 2013.

TYPE LOCALITY: 35.44081°N, 139.91078°E, epipsammic, Banzu Flats, Tokyo Bay, Japan.

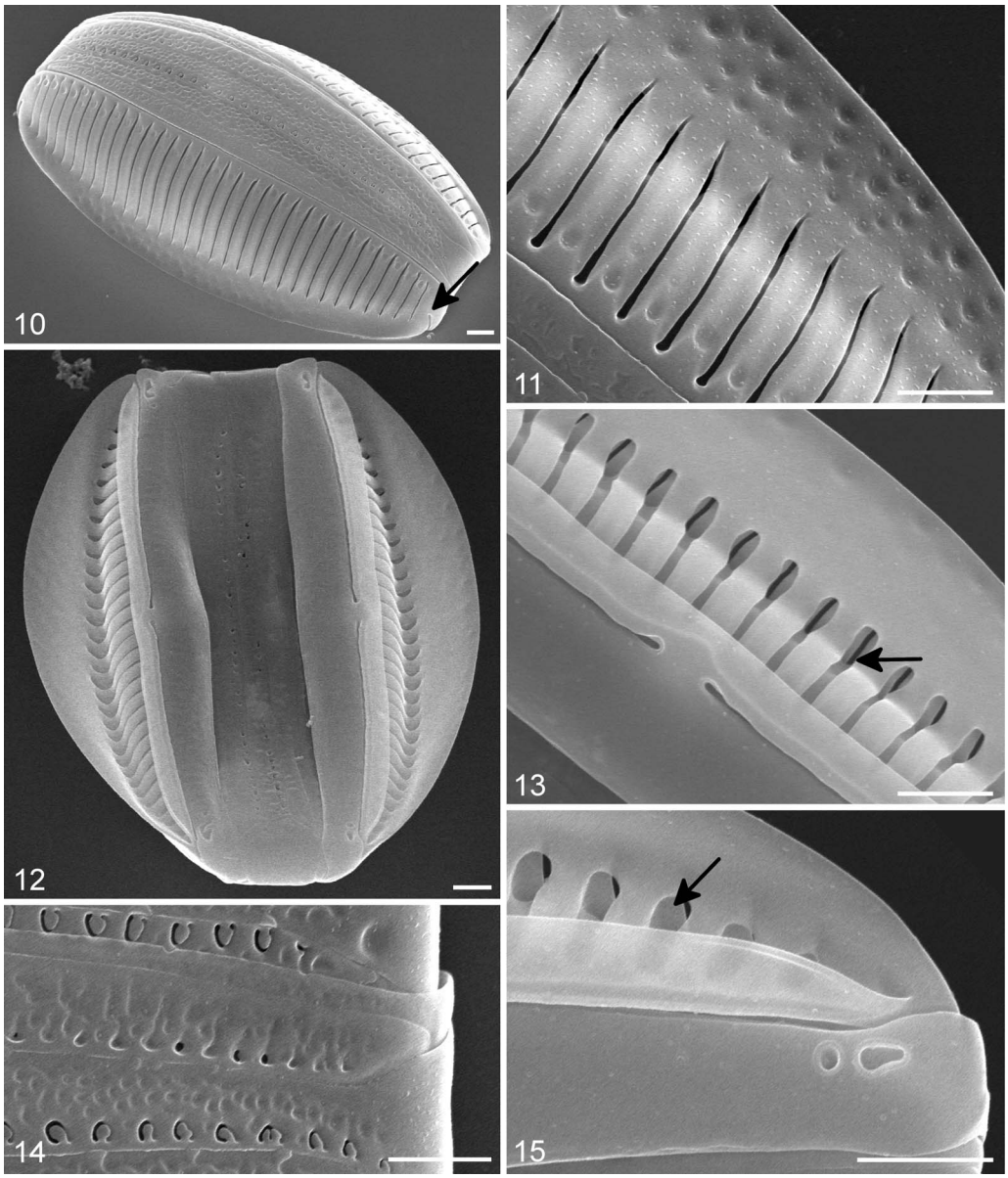
ETYMOLOGY: *aliformis* (Latin = wing-shaped) refers to the prominent dorsal marginal wing exhibited by this taxon.

In the SEM, the most prominent feature of *Amphora aliformis* is a dorsal marginal ridge that extends into a broad 'wing' running the length of the dorsal margin (Figs 10–13). When the whole frustule is viewed from the dorsal margin side, this wing lies flush against the substrate (Fig. 10). The wing is broad and unornamented except for small depressions or dimples irregularly spaced throughout the structure (Fig. 11). When viewed from the ventral mantle side, the dorsal valve face quickly falls away from the valvar plane before meeting the dorsal wing (Fig. 12). The wing then comes back to the valvar plane creating a deep depression or valley between the axial area and the dorsal edge of the wing (Fig. 12). The steep curvature of the dorsal valve face combined with the broad unornamented dorsal wing creates the obscured dorsal striae seen in the LM (Figs 1–9).

The striae are alveolate with the external areolae openings consisting of a narrow slit interrupted only by the dorsal marginal wing (Figs 10–13). The external areolae openings extend some way onto the protracted wing, past the internal occlusions that make up the internal valve structure (Fig. 13, arrow). This feature is seen in both the valve face and marginal striae, creating a hollow chamber at the base of the wing that is open via the external striae but is closed to the valve interior by the internal striae coverings (Figs 13, 15, arrows). In cross-section this chamber is open, connecting the dorsal and marginal alveoli but is partitioned between each stria by virgae and does not continue the longitudinal length of the valve (Fig. 16).

Externally, the raphe is weakly arched with proximal ends slightly deflected dorsally. The distal raphe ends are sharply deflected dorsally past the distal end of the raphe ledge and dorsal marginal wing, continuing onto the dorsal margin (Fig. 10, arrow). The raphe ledge is continuous on the dorsal side, not apparent on the ventral side (Figs 12, 13, 15). The ventral valve is broad and unornamented aside from several large pores positioned near the valve apices (Fig. 15).

The girdle bands are somewhat irregularly areolated, varying between small pores to more complex crescent and

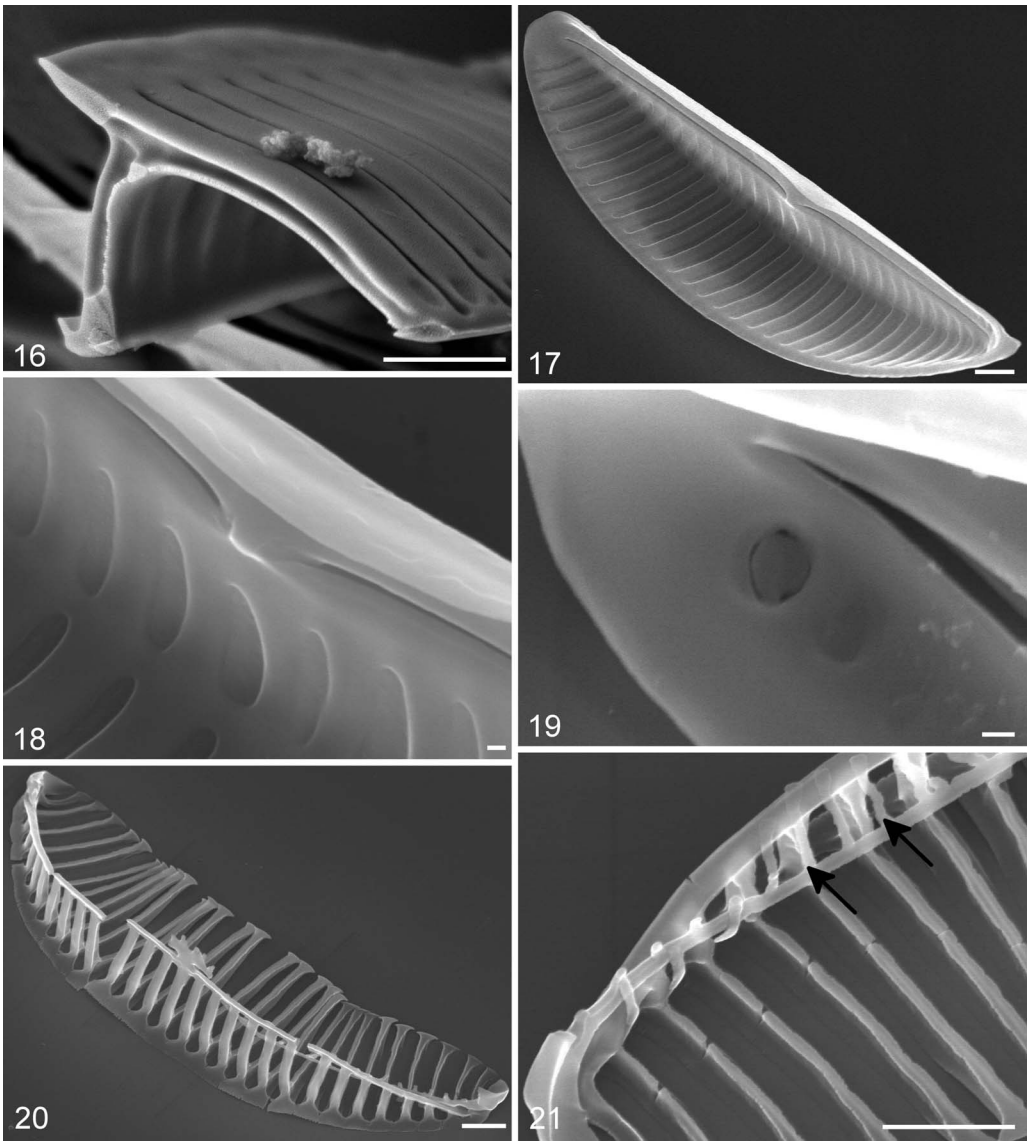


Figs 10–15. Scanning electron micrographs of *Amphora aliformis*. Cleaned culture material, JPK culture 10105-AMPH177.
Fig. 10. External dorsal view of the whole frustule, showing marginal wing, areolate girdle bands, and distal raphe end extending onto the dorsal mantle (arrow). Scale bar = 1 μm.
Fig. 11. Detail of the external dorsal mantle showing irregularly spaced depressions on the marginal wing. Scale bar = 1 μm.
Fig. 12. External ventral view of the whole frustule showing sharply curved dorsal valve face, marginal wing, and absence of ventral striae. Scale bar = 1 μm.
Fig. 13. Detail of the external valve centre showing raphe ledge and hollow chamber created by the marginal wing (arrow). Scale bar = 1 μm.
Fig. 14. Detail of the areolate girdle bands. Scale bar = 1 μm.
Fig. 15. Detail of the external valve end showing the ventral apical pores and hollow chamber created by the marginal wing (arrow). Scale bar = 1 μm.

horseshoe shaped openings. Along with the pores, many irregular depressions are visible throughout the girdle area (Figs 10, 12, 14).

Internally, the striae are composed of a single elongate areolae interrupted only at the valve margin by a band of silica (Figs 17, 18). The internal raphe terminates proximally at a weakly developed central helictoglossae (Fig. 18). The ventral apical pores are occluded internally by a sieve plate anchored in several places to the ventral valve (Fig. 19). Although in the fully developed cell the ventral striae are absent, in early valve development ventral virgae are initially formed and must be filled in later development (Figs 20, 21, arrows).

Living cells of *Amphora aliformis* contain a single ventrally appressed chloroplast consisting of four lobes extending up the valve sides towards the dorsal girdle area (Figs 22–24). This chloroplast arrangement is consistent with Meresch-kowsky's (1903) type 1 *Amphora* chloroplast, shown by

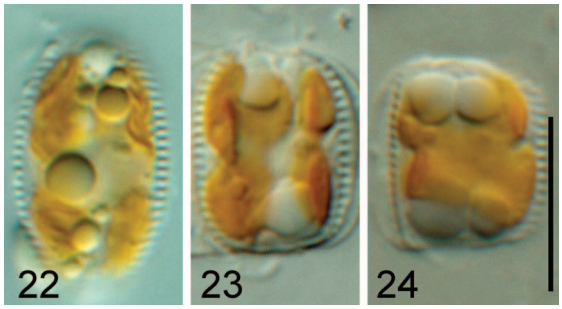


Figs 16–21. Scanning electron micrographs of *Amphora aliformis*. Cleaned culture material, JPK culture 10105-AMPH177.
Fig. 16. Valve cross-section showing alveolate dorsal and marginal striae extending into the marginal wing. Scale bar = 1 μm.
Fig. 17. Internal view of single valve showing internal striae. Scale bar = 1 μm.
Fig. 18. Detail of internal valve centre showing internal marginal ridge and weakly developed central helictoglossae. Scale bar = 100 nm.
Fig. 19. Detail of internal valve end showing ventral apical pore occluded by a sieve plate anchored to the valve with struts. Scale bar = 100 nm.
Fig. 20. Developing valve. Scale bar = 1 μm.
Fig. 21. Detail of developing valve showing early development of ventral virgae (arrows). Scale bar = 1 μm.

Stepanek & Kociolek (2014) to be shared within the *Amphora s.s.* + *Diplamphora* + *Oxyamphora* clade.

Taxonomic remarks

Amphora aliformis is similar in valve outline and stria features to other members previously placed in the subgenus *Diplamphora* such as *Amphora graeffeana* Hendey (Schoeman & Archibald 1986; Levkov 2009), *Amphora beaufortiana* Hustedt (Hustedt 1955), and *Amphora australiensis* John (John 1981). However, *A. aliformis* is easily distinguished from *A. graeffeana* and *A. beaufortiana* in the LM by the distinct hyaline area created by the dorsal marginal wing and its lack of ventral striae. *Amphora australiensis* (considered a synonym of *Amphora polita* Krasske by Levkov 2009), along with sharing valve outline striae features with *A. aliformis*, has been shown to have a distinct siliceous flap extending from the dorsal marginal ridge (John 1981). *Amphora aliformis* can be distinguished from *A. australiensis* by its lack of ventral striae and broad dorsal axial area, both evident in *A. australiensis*. Cleve (1895) reports *Amphora truncata* Cleve, a member of the subgenus *Diplamphora*, as having a structureless ventral side; however, *A. truncata* is larger (50–55 μm) and has a broad dorsal axial area, and no mention is made of a dorsal hyaline area.



Figs 22–24. Light micrographs of living cells of *Amphora aliformis* showing chloroplast structure. Live culture material, culture JPK 10105-AMPH177. Scale bar = 10 μm.

Phylogenetic analysis

The results from the ML estimation of the four marker concatenated alignment are shown in Fig. 25. Both the ML and Bayesian estimation returned identical topologies with log likelihood values of −15500.69 and −15517.67 for the ML and Bayesian estimations, respectively. The resulting tree strongly supports (100/100) *Amphora aliformis* sister to a clade containing the remaining *Amphora* taxa, including representatives from the subgenera *Amphora s.s.*, *Diplamphora*, and *Oxyamphora*. In this tree *A. aliformis* and *Amphora commutata* Grunow in Van Heurck represent early branching lineages within the genus *Amphora*; however, there was not congruence in the position of *A. aliformis* between the trees inferred from the nuclear encoded markers (Fig. 26) and the chloroplast encoded markers (Fig. 27). The ML and Bayesian estimation of the SSU–LSU alignment returned a strongly supported (100/100) position of *A. aliformis* identical in topology to the four marker tree with *A. aliformis* positioned sister to the remaining *Amphora* representatives (Fig. 26). The *rbc*L–*psb*C tree, however, placed *A. aliformis* in a moderately supported (85/100) position sister to the *Amphora s.s.* clade, with *A. commutata* sister to the resulting *A. aliformis* + *Amphora s.s.* clade (Fig. 27). In this tree the remaining *Diplamphora* taxa form a separate clade sister to the *Oxyamphora* taxa (Fig. 27).

DISCUSSION

Cleve (1895) described the subgenus *Diplamphora* as being most closely related to *Amphora s.s.*, differing only in the 'complex connecting zone' seen in *Diplamphora* taxa. Morphologically, *Amphora aliformis* fits this definition in terms of general valve outline, raphe ledge, and chloroplast structure that ally this taxon with the *Amphora s.s.* clade. Conversely, the areolate girdle bands, slit like alveolate dorsal striae, infilling of the ventral striae, and the highly reduced central helictoglossae (see images in Sato *et al.* 2013; Stepanek & Kociolek 2014), all point to its inclusion in the subgenus *Diplamphora*.

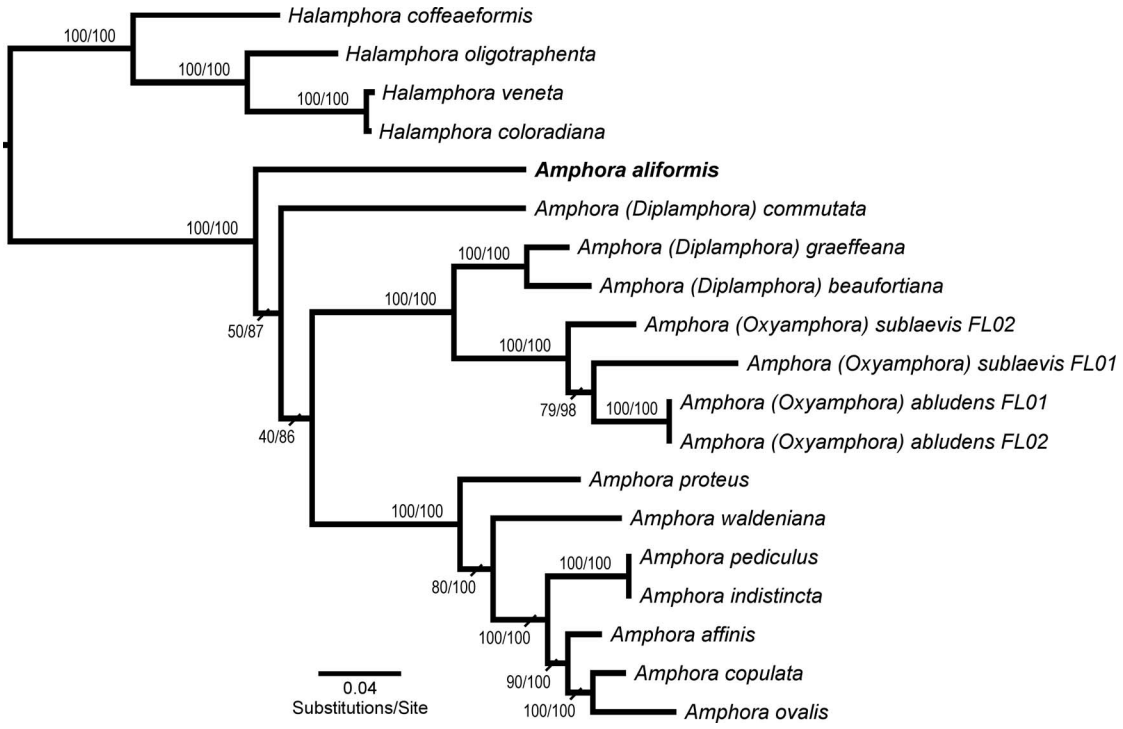


Fig. 25. Maximum likelihood phylogram inferred from a concatenated four molecular marker alignment, including the nuclear markers SSU rDNA and LSU rDNA, and the chloroplast markers *rbc*L and *psb*C. Node support is given on the branches as maximum likelihood bootstrap values (500 replicates)/Bayesian posterior probability (as a percentage). Cleve's (1895) subgenus names are in parentheses where applicable.

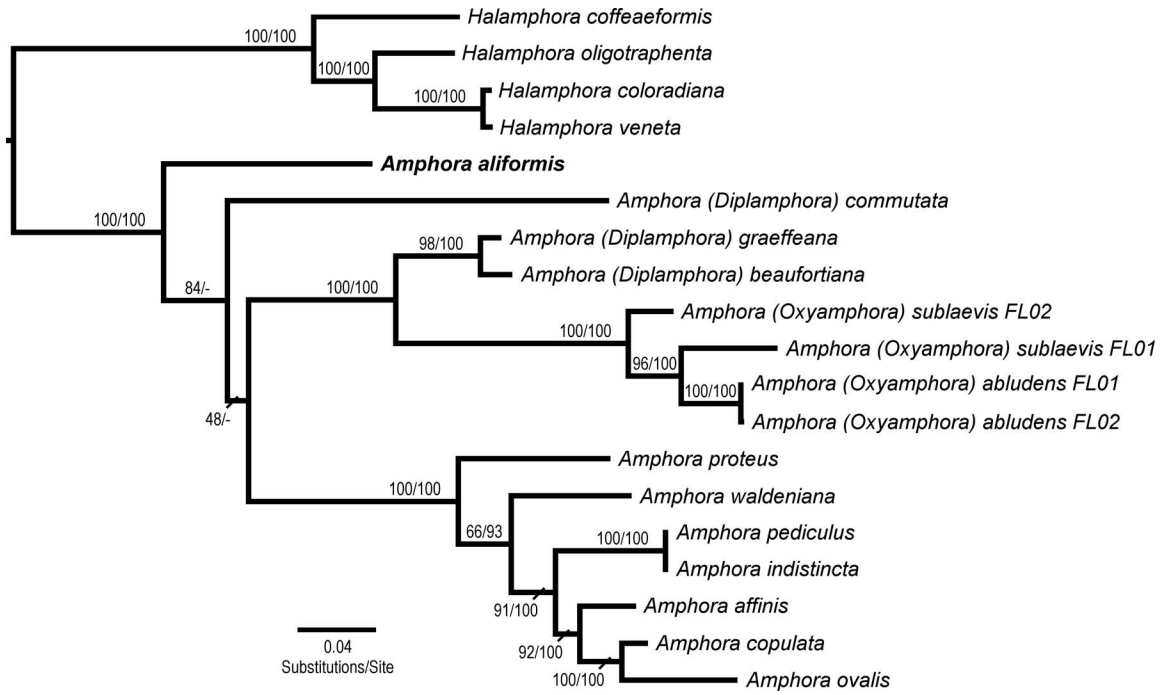


Fig. 26. Maximum likelihood phylogram inferred from a concatenated alignment of the nuclear encoded markers SSU rDNA and LSU rDNA. Node support is given on the branches as maximum likelihood bootstrap values (500 replicates)/Bayesian posterior probability (as a percentage). Posterior probabilities (shown as hyphens) indicate unresolved relationships in the Bayesian phylogram. Cleve's (1895) subgenus names are in parentheses where applicable.

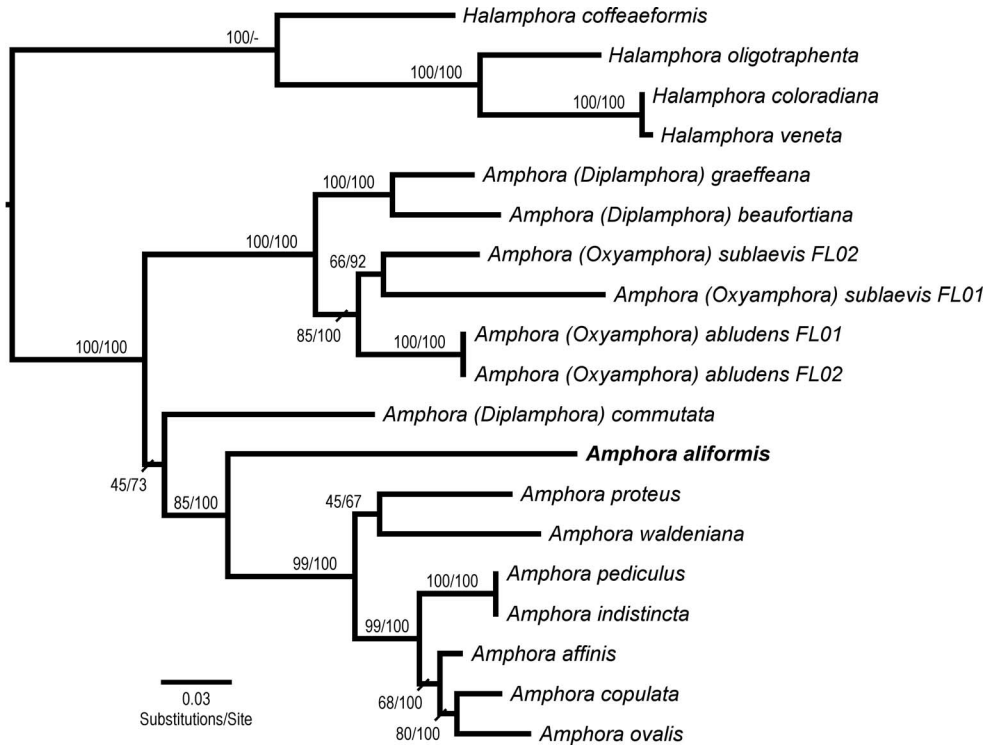


Fig. 27. Maximum likelihood phylogram inferred from a concatenated alignment of the chloroplast encoded markers *rbc*L and *psb*C. Node support is given above the branches as maximum likelihood bootstrap values (500 replicates)/Bayesian posterior probability (as a percentage). Cleve's (1895) subgenus names are in parentheses where applicable.

Additionally, *Amphora aliformis* shares several morphological features with the unusual *Diplamphora* taxon *Amphora commutata* (see Sato *et al.* 2013 for a detailed morphological treatment). The presence of the distinct pore found near the ventral apices has only previously been reported in *A. commutata* (Sato *et al.* 2013); although, *Amphora micrometra* Giffen shows a small pore near the dorsal apices (Ács *et al.* 2011). Although the distal pores in *A. aliformis* and *A. commutata* differ externally, with *A. aliformis* much larger and more prominent, internally the sieve plate occlusion suspended by what Sato *et al.* (2013) termed 'marginal struts', may indicate these are related features, and would suggest an affinity between these two species.

The most prominent morphological feature of *Amphora aliformis* is the conspicuous dorsal marginal wing. Many *Amphora* taxa, including *Amphora absoluta* Levkov, *Amphora rotunda* Skvortzow, *Amphora serrataeformis* Levkov, and *Amphora sibirica* Skvortzow & Meyer exhibit a distinct marginal ridge (see Nagumo 2003; Levkov 2009), and in some taxa, such as *Amphora australiensis*, *Amphora calumetica* (Thomas in Thomas & Chase) Peragallo, *Amphora calumeticoides* Cocquyt, and *Amphora dentata* Edlund & Levkov in Levkov, siliceous flaps may extend from the marginal ridge (see John 1981; Levkov 2009). In contrast to these marginal features, the degree to which the marginal wing is developed in *A. aliformis* has not been previously illustrated in the SEM. Cleve (1895) observed several taxa with a 'broad hyaline limbus' that may correspond to a dorsal marginal wing structure, including *Amphora limbata* Cleve & Grove that Cleve assigned to the subgenus *Calamphora*; *Amphora schleinitzii* Janisch that was assigned to the subgenus *Amphora*; *Amphora weissflogii* Schmidt that was assigned to the subgenus *Diplamphora*; and *Amphora wittsteinii* Schmidt that Cleve was unable to assign to any of his nine newly created subgenera (see Schmidt *et al.* 1874–1959 and Peragallo & Peragallo 1897–1908 for illustrations). Their classification across many subgenera make it difficult to make any comparisons between these taxa and *A. aliformis*, and to our knowledge no modern morphological treatment has been performed on any of these potentially winged taxa.

Although not extended into a wing, *Amphora commutata* exhibits a broad marginal ridge (Sato *et al.* 2013). Interestingly, Sato *et al.* (2013, fig. 39) show that this ridge is not a solid band of silica but is chambered, a structure that may be homologous with the chambered wing of *Amphora aliformis*. How widespread this chambered dorsal marginal ridge is within the genus is difficult to say, as, with the exception of Nagumo (2003), SEM investigations of *Amphora* taxa seldom examine valves in cross-section.

Phylogeny and systematic implications

Although *Amphora aliformis* shares several morphological features with *Amphora commutata*, none of the phylogenetic analyses performed indicate they form a monophyletic group. Instead, the nuclear and full concatenated trees indicate that *A. aliformis* and *A. commutata* represent early branching lineages within the clade of *Amphora* + *Diplamphora* + *Oxyamphora*, and the chloroplast tree shows *A. aliformis* and *A. commutata* as early branching lineages grading into the *Amphora s.s.* clade. Although the inferred position of *A. aliformis* and *A. commutata* was not congruent between the nuclear encoded and plastid encoded markers, nor between individual marker trees (data not shown), it is important to note that no individual marker (SSU, LSU, *rbc*L or *psb*C) or concatenation of markers (nuclear, chloroplast, or four marker) returned a tree in which *Diplamphora* or *Diplamphora* + *Oxyamphora* was monophyletic. In addition, these results indicating the non-monophyly of *Diplamphora* taxa support the findings of Sato *et al.* (2013), which was based on SSU sequence data and included *Amphora graeffeana*, *A. commutata*, and an unnamed *Diplamphora* species. In their analysis Sato *et al.* (2013) also found the subgenus *Diplamphora* non-monophyletic, and instead were early branching lineages grading into *Amphora s.s.*

The non-monophyly of *Diplamphora*, with *Amphora aliformis* and *Amphora commutata* as either sister to *Amphora s.s.* (Fig. 27) or as sister to the entire *Amphora* + *Diplamphora* + *Oxyamphora* clade (Figs 25, 26), has implications for our changing concept of *Amphora s.l.* classification. First, it is becoming clear that Cleve's (1895) subgenera are largely inappropriate for continued use in *Amphora* systematics (Sato *et al.* 2013; Stepanek & Kociolek 2014; this study). Although *Amphora s.s.* continues to be recovered as monophyletic, the non-monophyly of the taxa previously placed in the subgenera *Diplamphora* and *Oxyamphora* (Sato *et al.* 2013; Stepanek & Kociolek 2014; this study) requires a re-examination of the defining features of the genus *Amphora* as it now stands.

A majority of the commonly cited characteristic features of *Amphora*, such as 'amphoroid' symmetry, dorsiventral valve shape, presence of a raphe ledge, central helictoglossae, and a single ventrally appressed chloroplast, have been shown to be symplesiomorpic (Stepanek & Kociolek 2014). Our results indicate areolate girdle bands (Cleve's 1895 important 'complex connecting zone') were likely the ancestral condition within the *Amphora* clade, shared by the taxa of *Diplamphora* and *Oxyamphora*, and were subsequently lost in the *Amphora s.s.* clade. Additionally, the distinctly separate central helictoglossae seen in most *Amphora s.s.* taxa (Stepanek & Kociolek 2014) appears to be a synapomorphy of the *Amphora s.s.* clade and not the genus as a whole (Fig. 18).

The remaining commonly cited *Amphora* traits that are synapomorphic for the genus are relatively few. Mereschkowsky's (1903) type 1 chloroplast remains the best feature unifying the group (Sato *et al.* 2013; Stepanek & Kociolek 2014; this study). Others could loosely include a dorsal marginal ridge and a biarcuate raphe; although, these features are quite variable across this morphologically diverse group. Certainly, as our understanding of how the remaining uninvestigated taxa of '*Diplamphora*' and '*Oxyamphora*' fit into this new view of the genus increases, it may be possible to further differentiate these groups based on shared derived features. This remains a daunting task, with the vast majority of *Amphora* diversity not currently included in any analysis, morphological or molecular. This study highlights the importance of including the greatest taxonomic breadth

possible in future studies, as the addition of new taxa continues to change our view of the systematics of *Amphora s.l.*

SUPPLEMENTAL DATA

Supplemental data associated with this article can be found online at http://dx.doi.org/10.2216/14–081.1.s1

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