Re-examination of Mereschkowsky’s genus *Tetramphora* (Bacillariophyta) and its separation from *Amphora*

JOSHUA G. STEPANEK* & J. PATRICK KOCIOLEK

Department of Ecology and Evolutionary Biology and Museum of Natural History, University of Colorado Boulder, Boulder, CO, USA

The raphid diatom genus *Amphora* has been subject to considerable revision over the previous 30 years, with many species transferred to newly created or existing genera. Despite these efforts, recent work has demonstrated that taxa presently assigned to the genus continue to be non-monophyletic and further revision is required. This investigation re-examines Mereschkowsky’s genus *Tetramphora*, which he described as including all *Amphora* species with four chloroplasts, arranged in pairs linked by large pyrenoids. Although largely ignored over the last 100 years, recent work has shown that taxa aligning with *Tetramphora* are monophyletic and distinct from *Amphora* sensu stricto. Nine species belonging to *Tetramphora* are illustrated and described, based on light and scanning electron microscope observations, as well as a preliminary molecular phylogeny for the group. From these observations, we designate Mereschkowsky’s *T. ostrearia* as the generitype of *Tetramphora*, recognize *T. lineolata*, and transfer the *Amphora* taxa, *A. decussata*, *A. sulcata*, *A. rhombica*, *A. intermedia* and *A. securicula*, and the *Halamphora* taxon *H. chilensis* to *Tetramphora*. In addition, three new species, *T. fontinalis*, *T. lineolatoides* and *T. robusta*, are described.

**Keywords**: *Tetramphora*, *Amphora*, *Mereschkowsky*, phylogeny, new species, diatom

**Introduction**

It has long been speculated that the genus *Amphora* Ehrenberg ex Kützing, characterized by a distinct asymmetry of the apical and valvar planes, is not monophyletic (Cleve 1895, Krammer 1980, Mann 1994). The morphological diversity in features, other than symmetry, has hampered efforts at a natural classification, as evidenced by many early, and largely unsuccessful, attempts (Smith 1873, Cleve 1895, Mereschkowsky 1903, Gusylakov 1985). Of these early attempts, Cleve’s (1895) was, and remains, the most comprehensive and influential classification system. Cleve (1895) divided the genus into nine subgenera, *Archiamphora*, *Amblyamphora*, *Amphora*, *Calamphora*, *Cymbamphora*, *Diplamphora*, *Halamphora*, *Oxyamphora* and *Psammamphora*, based on morphological characteristics of the valve and girdle. Cleve’s (1895) revision was noteworthy for another reason; he was the first to postulate that the genus was composed of several unrelated groups, and that amphoroid symmetry is likely a product of shared growth form rather than shared evolutionary history. Although he believed his subgenera should be separate genera, he was reluctant to make these changes at that time.

Of the early researchers, Mereschkowsky (1903) alone examined plastid morphology across the genus, and identified nine chloroplast types, based on their number, shape and position within the cell. Although not addressing all the chloroplast categories, from his observations Mereschkowsky proposed two new genera, *Clevamphora*, containing all of the species with a Type 1 chloroplast (single four-lobed ventrally appressed chloroplast) corresponding to Cleve’s *Amphora s.s.*, and *Tetramphora*, for species with a Type 8 chloroplast (two dorsally and two ventrally appressed chloroplasts connected in pairs by pyrenoids) that includes some, but not all, members of Cleve’s *Oxyamphora* (Mereschkowsky 1903). Because it refers to species within *Amphora s.s.*, *Clevamphora* is considered nomenclaturally superfluous (Fourtanier & Kociolek 2011) while *Tetramphora* has largely been ignored by later taxonomists, most of whom adopted Cleve’s (1895) nine subgenera within *Amphora*.

Although concerns surrounding the non-monophyly of the genus were first raised 120 years ago (Cleve 1895), only recently has there been an effort to revise the classification of *Amphora*. Recent changes have included the transfer of some taxa to existing or newly created genera such as *Undatella* Paddock & Sims (Paddock & Sims 1980), *Biremis* D.G. Mann & Cox (Mann & Cox in Round et al. 1990) and *Colliculoamphora* Williams & Reid (Williams & Reid 2006), as well as the transfer of Cleve’s...
subgenus *Cymbamphora* to the new genus *Seminavis* D.G. Mann (Mann in Round et al. 1990), and the elevation of the subgenus *Halamphora* to generic status (Levkov 2009).

Although a considerable number of taxa have been transferred out of *Amphora*, recent molecular phylogenetic work has demonstrated that the genus, as it is currently conceived, continues to be non-monophyletic, with taxa dispersed widely across raphid diatom lineages (Stepanek & Kociolek 2014). Although *Amphora* as a whole is non-monophyletic, Stepanek & Kociolek (2014) demonstrated that there are several monophyletic groups outside *Amphora s.s*. One such group corresponds to Mereschkowsky's *Tetramphora* and includes several species still placed within *Amphora*. We present a re-examination of *Tetramphora*, using light microscope (LM) and scanning electron microscope (SEM) observations of taxa collected from coastal and inland waters of the United States and Japan. In addition, a preliminary, molecular phylogeny is presented to show the position of *Tetramphora* within the raphid diatom lineages.

**Materials and methods**

**Taxon collection and isolation**

Material was collected from coastal and inland waters of Florida, New Jersey and Utah, USA, and Japan from 2011 to 2013 (Table 1). pH and conductivity measurements were taken near the shore at the time of collection using a YSI 556 multi-probe (YSI Incorporated, Yellow Springs, Ohio, USA) (Table 1). To remove the organic portions of samples for LM and SEM observations, samples were boiled in concentrated nitric acid 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 mounted onto permanent slides using Naphrax mounting medium (Brunel Microscopes, Chippenham, UK). All LM observations were conducted using an Olympus BX-51 LM (Olympus America Inc., Center Valley, Pennsylvania, USA) with a 100× oil immersion objective (N.A. 1.40). Light micrographs were captured with an Olympus DP 71 digital camera. For SEM observations, cleaned material was air-dried onto glass coverslips and mounted onto aluminium stubs. The stubs were coated with ca. 1.5 nm of gold using a Cressington 108 auto sputter coater (Cressington Scientific Instruments Ltd., Watford, UK). All SEM images were taken using a JEOL JSM 7401 field emission SEM at an acceleration voltage of 3 kV.

*Tetramphora chilensis* (from Blue Lake, UT) was isolated into monoculture via micro-pipette serial dilution. This taxon was grown in a modified f/2 artificial saltwater medium using the marine sea salts Instant Ocean (United Pet Group, Blacksburg, Virginia, USA). The salt concentration was decreased to a conductivity of 10 mS cm$^{-1}$ to match the measured conductivity at the collection site. The culture was maintained under fluorescent illumination, ca. 50 μmol m$^{-2}$, at ca. 25°C and a 12:12 light:dark cycle.

**Table 1.** List of material examined including locality information, geographical coordinates, pH and conductivity measurements.

| Taxon                | Accession number | Locality                                       | Latitude       | Longitude       | pH   | Conductivity mS cm$^{-1}$ |
|----------------------|------------------|------------------------------------------------|----------------|-----------------|------|--------------------------|
| *Tetramphora chilensis* | COLO JPK 8531  | Blue Lake warm spring, Tooele County, Utah, USA | 40.50257°N      | 114.03359°W     | 7.60 | 9.32                     |
| *Tetramphora fontinalis* | ANSP GC 65228   | Blue Lake warm spring, Tooele County, Utah, USA | 40.50257°N      | 114.03359°W     | 7.60 | 9.32                     |
| *Tetramphora lineolata* | COLO JPK 9655   | Jenkins Sound, Cape May County, New Jersey, USA | 39.06423°N      | 74.82346°W      | n.a. | n.a.                     |
| *Tetramphora lineolatoides* | ANSP GC 36356  | Sea of Japan, Wakasa Bay, Toyama, Japan        | 35.61063°N      | 135.90234°E     | n.a. | 50.36                    |
| *Tetramphora ostrearia* | COLO JPK 6914   | Gulf of Mexico, Marco Island, Collier County, Florida, USA | 25.93336°N      | 81.65008°W      | 7.64 | 61.24                    |
| *Tetramphora intermedia* | COLO JPK 6847   | Florida Bay, Plantation Key, Monroe County, Florida, USA | 24.96308°N      | 80.57083°W      | 8.34 | 61.90                    |
| *Tetramphora robusta*    | ANSP GC 59143   | Long Beach Backwater, Big Pine Key, Monroe County, Florida, USA | 24.64167°N      | 81.33877°W      | 7.00 | 53.70                    |
| *Tetramphora securicula*  | COLO JPK 6904   | US1 Canal, Dade County, Florida, USA            | 25.28829°N      | 80.44263°W      | 7.34 | 21.29                    |
| *Tetramphora sulcata*     | COLO JPK 6824   | Biscayne Bay, Bayfront Park, Miami-Dade County, Florida, USA | 25.46216°N      | 80.33791°W      | 8.39 | 65.79                    |
| *Mastogloia sp.*          | COLO JPK 9949   | Unnamed Playa, Hwy 6, Utah County, Utah, USA   | 39.95472°N      | 111.88512°W     | 8.39 | 10.50                    |

Notes: ANSP: Academy of Natural Sciences, Philadelphia, COLO: University of Colorado Kociolek collection, n.a.: not available.
DNA extraction, amplification and sequencing

DNA was extracted from *T. chilensis* monocultures using a Chelex100 extraction method (Richlen & Barber 2005). The nuclear encoded 18S small subunit rDNA (SSU) and the chloroplast encoded large subunit of RUBISCO (rbcL) and photosystem II chlorophyll-a binding protein psbC were chosen as phylogenetic markers to match previous published sequence data from this group (Stepanek & Kociolek 2014). Amplification and sequencing primers 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, USA) following the manufacturers’ protocol. The PCR profile used for all markers was a 94°C initial denaturation for 3 min 30 s, followed by 36 cycles of 94°C for 50 s, 52°C for 50 s, 72°C for 80 s, with a final extension period of 72°C for 15 min. The amplified PCR product was purified using ExoSap-it (Affymetrix, Santa Clara, California, USA) following the manufacturers’ protocol. Sequencing was performed by Functional Biosciences (Madison, Wisconsin, USA) and returned sequence files were assembled and edited in Geneious ver. 5.6 (Drummond et al. 2012).

Sequence alignment and phylogenetic analysis

Sequences were aligned in Geneious using a muscle alignment algorithm (Edgar 2004) and edited by hand as needed. Each marker was aligned individually and the ends were trimmed to minimize missing data before concatenating. Although trees inferred from individual markers may give conflicting results, alignment concatenation has been shown to be a successful strategy for returning the most accurate tree within diatoms (Theriot et al. 2015). Single marker alignment lengths were 1602, 1328 and 1059 bp for SSU, rbcL and psbC, respectively. Due to variable sequence availability between markers, SSU-rbcL-psbC and SSU-rbcL alignments were created to maximize taxon coverage. For the concatenated alignments, *Tetramphora* sequences were combined with sequence data for an additional 53 and 77 taxa obtained from GenBank (see supplemental Table S2 for a list of taxa included) for the three marker and SSU-rbcL alignments, respectively, and include taxa from most major lineages of raphid diatoms (sensu Round et al. 1990). Four araphid diatom species were chosen as outgroup taxa. The three marker and SSU-rbcL concatenated alignments used in this study are included in NEXUS format as supplementary materials Alignment S1 and Alignment S2.

For tree inference, maximum likelihood (ML) estimation was performed in RAxML ver. 7.3.2 (Stamatakis 2006) with the graphical user interface raxmlGUI ver. 1.2 (Silvestro & Michalak 2012) using the general time reversible model of sequence evolution with a gamma rate distribution (Γ) and a proportion of invariable sites (I). For the ML estimation, three alignment partitioning strategies were employed, partition by marker, partition by marker and third codon position for the protein coding genes (rbcL and psbC), and partition by marker and all codon positions for the protein coding genes. For all alignment partitions, the final tree was chosen as the most likely tree from 100 independent tree searches and node support was estimated on the most likely tree with 500 bootstrap (BS) replicates. Bayesian inference was conducted using MrBayes ver. 3.2.1 (Ronquist et al. 2012). The Bayesian estimation was run on an alignment partitioned by marker for 15 million generations, with a burn-in of 3 million generations, using two runs of four chains sampled every 1000 generations. MCMC run and posterior probability convergence was evaluated using AWTY (Nylander et al. 2008).

Results

An amended description of *Tetramphora* based on LM and SEM observations follows. *Tetramphora ostrearia* is designated as the generitype, with an expanded description of this and of *T. lineolata*. Four new combinations and three new species are described, and two other species are transferred into this genus based on published data.

### Tetramphora Mereschkowsky emend. Stepak & Kociolek

**Generitype** (here designated) *Tetramphora ostrearia* (Brébisson) Mereschkowsky

**Basionym** *Amphora ostrearia* Brébisson ex Kützing 1849, p. 94. Schoeman & Archibald 1986, figs 57–65; Levkov 2009, pl. 275, figs 5–11.

**Description**

Cells elliptical with strong valvar asymmetry creating a frustule with both valve faces in a single plane. Valves semi-elliptical to semi-lanceolate and moderately to strongly dorsiventral. The raphe is more or less biarcuate. The distal raphe ends are dorsally deflected, the proximal ends are either ventrally deflected or completely obscured by a prominent hyaline area or siliceous flap at the dorsal central area. In LM, this hyaline area gives the appearance of highly dilated to abruptly undulate proximal raphe ends. The striae are typically composed of small apically or transapically oriented areolae with thin slit-like external openings. Internally, the raphe slits terminate distally in helictoglossae and are often continuous through the central area. Internally, the raphe branches are flanked by more or less developed axial costae, often becoming a distinct siliceous projection in the dorsal central area. The girdle area is composed of numerous narrow girdle bands. Each band has a single row of large areolae along one margin, a feature that is clearly visible in the LM but often obscured in SEM by band overlap. Living cells
exhibit Mereschkowsky’s type 8 amphoroid chloroplast, containing two pairs of plate-like plastids, one of each pair dorsally appressed, the other ventrally appressed, bridged by large pyrenoids. Species are only known from marine, brackish and high conductivity inland waters.

**Tetramphora ostrearia** (Brébisson) Mereschkowsky (Figs 1–8)

*Basionym*

*Amphora ostrearia* Brébisson ex Kützing 1849, p. 94.

*Description*

Valves dorsiventral and semi-elliptical. The dorsal margin is smoothly arched, becoming nearly flat at the valve centre in some views, the ventral margin is straight to concave. Valve length 58.0–66.0 μm, valve breadth 7.0–8.0 μm. Valve ends are narrowly rounded and deflected ventrally. The raphe is highly biarculate and is difficult to view in a single focal plane due to the curvature of the valve face. The axial area is narrow throughout.

Although no dorsal fascia is present, thickened central virgae are observable in LM. Striae are parallel near the valve centre, becoming radiate near the apices on the dorsal side, convergent on the ventral side. Stria density 13–14 in 10 μm.

In SEM, the striae are separated by thin internal virgae (Figs 6–8) and are crossed by fine vimines to form areolae. The areolae open externally by narrow longitudinally oriented slits. Internally, the central dorsal virgae are distinctly thickened (Fig. 7, arrow) and a small protuberance is visible on the dorsal side of the central area (Fig. 7, thin arrow). The raphe appears continuous through the internal central area and is bordered dorsally and ventrally by raised axial costae creating a narrow trough in which the raphe lies (Figs 7–8). Although difficult to see on the imaged specimens, the external distal raphe ends appear to terminate on the dorsal mantle of the valve (Figs 6–8, arrows). The girdle area is composed of many narrow, open girdle bands, with the open ends of adjacent bands alternating at each valve apex. A single row of large areolae

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**Figs 1–5.** *Tetramphora ostrearia* whole frustule and valves showing size range, LM. Fig. 1. Whole frustule. Figs 2–5. Single valves. Scale bar = 10 μm.
run along one margin of each band, although these are often obscured by the adjacent overlapping girdle band (Fig. 8).

**Taxonomic notes**

Although Mereschkowsky did not designate a type for *Tetramphora* when he proposed the genus, *A. ostrearia* is one of the two species originally transferred (along with *A. lineolata*) into *Tetramphora*. Of these, *T. ostrearia* is the only taxon for which the type material has been extensively investigated and illustrated (Schoeman & Archibald 1986, figs 57–65; Levkov 2009, pl. 275, figs 5–11). For this reason, we designate *T. ostrearia* as the generitype of *Tetramphora*. Schoeman & Archibald (1986) reported lengths 58–95 μm, breadths 9–16 μm and 12–14 stria in 10 μm for the population on the type slide (BM 18948). Our population, although agreeing in length and stria densities, has narrower valves, 7.0–8.0 μm wide. It is worth noting that Schoeman & Archibald (1986) cautioned that the shape of the valve in this taxon ‘varies considerably depending on the plane of viewing’ and several images illustrated by Levkov (2009, pl. 275, figs 5–6), also from BM 18948, seem to correspond well to the proportions and valve angle of our specimens.

**Figs 6–8.** *Tetramphora ostrearia*, SEM. Fig. 6. Internal whole valve showing distal raphe ends. Fig. 7. Detail of the internal central valve showing thickened central virgae (arrow), protruding central thickening (thin arrow), and axial ribs. Fig. 8. Dorsal view showing distal raphe ends (arrow) and girdle bands. Scale bars = 10 μm (Figs 6, 8), 1 μm (Fig. 7).
Tetramphora lineolata (Ehrenberg) Mereschkowsky
(Figs 9–21)
Basionym
Amphora lineolata Ehrenberg 1838, p. 189.

Description
Valves semi-elliptical and strongly dorsiventral. The dorsal margin is smoothly arched, the ventral margin straight to slightly convex. Valve ends are rounded and slightly ventrally deflected. Valve length 25.0–48.0 μm, valve breadth 5.5–8.0 μm. The raphe, which is largely visible in a single focal plane, lies near the ventral margin and is weakly biarcuate. The axial area is narrow, although there is a slight dilation at the central area. The striae are fine and not obviously areolate, parallel at the valve centre, becoming slightly radiate near the apices. Stria density 21–23 in 10 μm.

In SEM, the valves are much less flexed than in other members of the genus making observations of the raphe easier in both the LM and SEM. Externally, the raphe is weakly biarcuate with distal ends hooking dorsally and proximal ends dipping ventrally near the central area and terminating, closely, in simple pores (Figs 18–19). Although a small, unornamented area is present dorsal to the proximal raphe ends, this structure is highly reduced when compared to the large siliceous flap that often obscures the proximal raphe ends in other Tetramphora species. The areolae open externally by narrow, longitudinally oriented slits. Internally, the raphe is continuous through the central area (Fig. 20) and is bordered by well-developed axial costae (Fig. 21). Near the central area the dorsal axial costa diminishes before forming a small dorsally positioned siliceous projection (Figs 20–21). The striae are separated by thin virgae, more or less crossed by weakly developed vimines.

Taxonomic notes
Tetramphora lineolata is easily identified by its distinctly flat valve face (nearly the entire raphe system is visible in a single focal plane), and fine parallel striae (21–23 in 10 μm). It should be noted that the current concept of T. lineolata differs from Ehrenberg’s (1838), who describes a larger cell than is currently reported for this taxon (Jahn & Kusher 2004). Here we follow the concept adopted by Cleve (1895) and Peragallo & Peragallo (1897–1908), in which they present a length of 32–45 μm and a stria count of 20–23 in 10 μm (Peragallo & Peragallo 1897–1908 give a stria count of 19–23 in 10 μm) for T. lineolata. This is the same concept of T. lineolata that is currently accepted (Krammer & Lange-Bertalot 1986, Witkowski et al. 2000).

Tetramphora sulcata (Brébisson) Stepanek & Kociolek, comb. nov. (Figs 22–34)
Basionym
Amphora sulcata Brébisson 1854, p. 256; pl. 1, fig. 8.

Description
Valves semi-elliptical and dorsiventral. The dorsal margin is smoothly arched, becoming flat over the central
portion of the valve in some specimens, the ventral margin is biarcuate. Valve apices are acute, narrowly rounded and ventrally deflected. Valve length 35.0–58.0 μm, valve breadth 5.5–7.5 μm. The raphe is highly biarcuate and the proximal ends are difficult to view due to the valve curvature. The axial area is narrow throughout. The striae are distinctly areolate and nearly parallel throughout. Stria number 18–19 in 10 μm.

In SEM, externally, the raphe is biarcuate with distal ends deflected dorsally and proximal ends dipping towards the ventral margin before terminating, close together, in simple endings (Figs 31, 32). An unornamented area is present on the dorsal central area but is not extended into a flap (Fig. 32). The areolae open externally as narrow longitudinally oriented slits. Internally, the raphe slit is continuous through the central area (Fig. 33) and is bordered by axial costae. Near the central area the ventral axial costa is thickened and the dorsal costa is reduced to a small protuberance (Figs 33, 34). The striae are separated by thin virgae crossed by weakly developed vimines.

**Taxonomic notes**

Although Cleve (1895) treats *A. sulcata* as a variety of *A. arcus* Gregory, he notes that this variety is akin to *A. lineolata* with a stria count of 14–20 in 10 μm. *Tetramphora sulcata* is similar to *T. lineolata* and *T. lineolatoides* Stepanek & Kociolek sp. nov. in having more or less parallel striae with longitudinally oriented external areolar slits, and lacking a central raphe flap. It is distinguished from *T. lineolata* and *T. lineolatoides* by its distinctly flexed and biarcuate valve shape.
**Tetramphora intermedia** (Cleve) Stepanek & Kociolek, comb. nov., stat. nov. (Figs 35–44)

**Basionym**
*Amphora rhombica* var. *intermedia* Cleve 1895, p. 127.

**Description**
Valves broadly semi-lanceolate and strongly dorsiventral. The dorsal margin is arched, the ventral margin is nearly straight. Valve ends are acutely rounded and weakly ventrally deflected. Valve length 80.0–105.0 μm, valve breadth 17.0–19.0 μm. The raphe is weakly biarcuate and lies near the ventral margin. The proximal and distal raphe ends are difficult to view due to the valve curvature. The axial area is narrow throughout. The dorsal and ventral striae are areolate and radiate throughout. Stria number 14–17 in 10 μm.

In SEM, the external raphe path is biarcuate with dorsally deflected distal ends and proximal ends partially obscured by a large flap of unornamented silica (Fig. 41). A thin, weakly developed rib runs along either side of the raphe (Fig. 41, arrow), but is more prominent near the apices. The striae are areolate and open externally by narrow, transapically oriented slits. A weakly developed marginal ridge appears to interrupt the striae as they continue onto the dorsal margin (Fig. 41, thin arrow). Internally, the striae are separated by very thin virgae, vimines are not well developed (Fig. 42). The raphe system is bordered by moderately developed axial costae, ending distally at a helictoglossae (Figs 43–44). A small protuberance is present at the internal central area (Fig. 42).

**Taxonomic notes**
This taxon is easily distinguished from other *Tetramphora* species by its large broad valves. It is separated from *T. rhombica* by its relatively smaller size and finer striaation (Cleve 1895 gives length 130–260 μm, breadth 21–50 μm, and 10–12 striae in 10 μm for *A. rhombica* Kitton).

**Tetramphora securicula** (Peragallo & Peragallo) Stepanek & Kociolek, comb. nov. (Figs 45–54)

**Basionym**
*Amphora securicula* Peragallo & Peragallo 1897–1908, p. 224; pl. 50, fig. 2.

**Description**
Valves semi-elliptical and dorsiventral. The dorsal margin is smoothly arched, the ventral margin is straight to biarcuate. Valve ends are acutely rounded and ventrally...
Deflected. Valve length 55.0–75.0 μm, valve breadth 8.0–10.0 μm. The raphe is strongly biarcuate and the proximal and distal endings are obscured by the curvature of the valve face. The axial area is narrow throughout. The dorsal striae are areolate, almost parallel near the valve centre, becoming radiate towards the apices. Ventral striae are obscured near the valve centre, radiate throughout. Stria number 16 in 10 μm.

In SEM, the external raphe path is biarcuate and bordered by a weakly developed axial rib (Fig. 51). The distal raphe ends are deflected dorsally and the proximal raphe ends are obscured by a large, unornamented siliceous flap (Fig. 52). The areolae open externally in small transapically oriented slits. Internally, the striae are separated by thin virgae crossed by weakly developed vimines. Internally, the raphe is bordered by axial costae and a small siliceous protuberance is present at the dorsal central area (Figs 53–54).

**Taxonomic notes**

This taxon can be distinguished by its large size, coarse striae and prominent, strongly biarcuate raphe. Although this taxon has not been extensively illustrated, the population reported here conforms well to Wachnicka & Gaiser’s (2007) observations of *A. securicula* from southern Florida. They reported valve metrics of their specimens as length 55–59 μm, breadth 9–10, and 16–17 striae in 10 μm. In SEM, our specimens correspond to the taxon illustrated by Levkov (2009, pl. 282, figs 1–6) as *A. securicula*, based on material from the Natural History Museum (BM). Both populations agree with ours in overall valve shape, the prominent central siliceous flap, and the transapically oriented external areolar slits.

**Tetramphora chilensis** (Hustedt) Stepanek & Kociolek, comb. nov. (Fig 55–67)

_Basionym_  
_Amphora chilensis_ Hustedt 1927, p. 239; pl. 7, figs 8–10.

_Synonym_  
_Halamphora chilensis_ (Hustedt) Levkov
Description
Valves are narrowly semi-elliptical and strongly dorsiventral. The dorsal margin is smoothly arched, the ventral margin is straight to slightly concave. The valve ends are narrowly rounded to rostrate and ventrally deflected. Valve length 20.0–42.0 μm, valve breadth 4.5–6.0. The raphe is straight to slightly arched, lying near the ventral margin. The distal raphe ends are dorsally bent and the proximal raphe ends sharply and distinctly ventrally deflected as they near the central area. The striae are areolate and weakly radiate throughout. Dorsal stria number 20–23 in 10 μm, ventral striae are finer, 26–30 in 10 μm.

In SEM, the external raphe path is nearly straight with dorsally deflected distal ends (Fig. 64). The proximal raphe ends dip towards the ventral margin as they approach the central area before terminating close together (Figs 64–65). A broad unornamented dorsal central area is present but not extended into a flap (Fig. 65). The external areolar openings consist of small transapically oriented slit-like openings. Internally, the raphe is straight with each distal end terminating as a helictoglossa and the proximal ends straight, terminating closely (Figs 66–67). Both the highly developed axial costae and dorsal central swelling seen in other Tetramphora taxa are absent from T. chilensis (Fig. 67). The striae are separated by relatively thick virgae crossed by weakly developed vimines.

Taxonomic notes
Tetramphora chilensis is unique and easily identified within the genus given its small flat valves and nearly straight raphe. Although Levkov (2009) noted that A. chilensis lacked several common features of Halamphora taxa (raphe ledge and internal central helictoglossa), he (Levkov 2009) nevertheless transferred this taxon to Halamphora based on girdle band and areola structure. While the overall valve shape, stria and areola structure in this taxon are quite similar to taxa such as Halamphora veneta (Kützing) Levkov, the external central hyaline area, and resulting deflected proximal raphe ends, as well as molecular data, place this species within Tetramphora.
Tetramphora lineolatoides Stepanek & Kociolek sp. nov. (Figs 68–82)

Description
Valves strongly dorsiventral and semi-elliptical. The dorsal margin is rounded, nearly flat through the central portion of the valve, the ventral margin is nearly straight to slightly convex. The valve ends are narrowly rounded and ventrally deflected. Valve length 28.0–45.0 μm, valve breadth 5.0–8.0 μm. The raphe is moderately biarcuate and lies near the ventral margin. The valve face is weakly flexed, obscuring the proximal raphe endings in some views. The axial area is narrow, although a slight inflation is visible at the central area in some orientations. The striae are areolate and nearly parallel throughout. Stria number 18–19 in 10 μm.

In SEM, the valves are only moderately flexed. The raphe is biarcuate with dorsally deflected distal raphe ends and proximal ends dipping towards the ventral margin before terminating in simple endings (Figs 77–79). An unornamented area is present on the dorsal side of the proximal raphe ends, but it does not extend into a flap (Fig. 79). The
Figs 45–50. *Tetramphora securicula* comb. nov., single valves showing size range, LM. Scale bar = 10 μm.

External areola openings consist of narrow longitudinally oriented slits. Internally, the raphe is continuous through the central area, bordered by weakly developed axial costae and ending distally in helictoglossae (Figs 80–82). A small protuberance is present on the dorsal central area (Fig. 82). The striae are separated by thin virgae crossed by very weakly developed vimines.

**Holotype**
Slide ANSP GC 36356, deposited at the Academy of Natural Sciences, Philadelphia, USA. Holotype specimen illustrated in Fig. 70.

**Isotypes**
Cleaned material ANSP GCM 24052, deposited at the Academy of Natural Sciences, Philadelphia, USA. Slide and cleaned material JPK 10929, deposited at the University of Colorado Boulder, Kociolek Collection at COLO.

**Type locality**
35.61063°N, 135.90234°E. Epiphytes, Wakasa Bay, Sea of Japan, Fukui Prefecture, Toyama, Japan.

**Etymology**
*lineolatoides* refers to the close morphological affinity to the species *T. lineolata*.

**Taxonomic notes**
*Tetramphora lineolatoides* shares several features with *T. lineolata*, including a relatively flat valve face, lack of a central raphe flap, and the orientation of the external areolar slits. However, *T. lineolatoides* can be easily distinguished by its coarser striae and, in SEM, its more widely spaced proximal raphe ends. Its valves are less flexed and biarcuate than *T. sulcata*, with which it shares a similar size, stria counts, and SEM features.
Tetramphora fontinalis Stepanek & Kociolek sp. nov. (Figs 83–95)

Description
Valves dorsiventral and narrowly semi-elliptical becoming linear through the central portion of the valve. The dorsal margin is arched, becoming flat over the valve centre, the ventral margin is more or less straight. Valve ends are narrowly rounded and ventrally deflected. Valve length 38.0–60.0 μm, valve breadth 6.0–8.0 μm. The raphe is highly biarcuate and both the proximal and distal ends are difficult to view due to the valve curvature. The striae are distinctly areolate, near parallel at the valve centre, weakly radiate across the rest of the valve. Stria number 17–21 in 10 μm.

In SEM, the external raphe path is biarcuate with dorsally bent distal raphe ends and appears continuous through the central area (Figs 90–92). The unornamented, dorsal, central area is well developed into a siliceous flap obscuring the proximal raphe ends (Fig. 91). The external areola openings consist of small transapically oriented slits. Internally, the raphe is bordered by well-developed axial costae (Fig. 93). The raphe terminates distally in helictoglossae and is continuous through the central area (Figs 94–95). A protruding central thickening is present on the dorsal side of the central area (Figs 93, 95). The striae are separated by virgae crossed by weakly developed vimines.

Holotype
Slide ANSP GC 65228, deposited at the Academy of Natural Sciences, Philadelphia, USA. Holotype specimen illustrated in Fig. 83.

Isotypes
Cleaned material ANSP GCM 5701, deposited at the Academy of Natural Sciences, Philadelphia, USA. Slide
and cleaned material JPK 8504, deposited at the University of Colorado Boulder, Kociolek Collection at COLO.

**Type locality**
40.50257°N, 115.03359°W. Periphyton, Blue Lake warm spring, Tooele County, Utah, USA.

**Etymology**
*fontinalis* (Latin = growing in a spring) refers to the type locality of this taxon, Blue Lake warm spring, Utah, USA.

**Taxonomic notes**
This taxon, collected from Blue Lake, Utah, appears very closely allied to the coastal marine *T. securicula*. Both exhibit valves that are transapically flexed near the valve centre with a highly biarcuate raphe. In SEM, they share a prominent central raphe flap and areola orientation. *Tetramphora fontinalis* differs from *T. securicula* in its narrow distinctly linear valves (valve breadth 8.0–10.0 μm in *T. securicula*, 6.0–8.0 μm in *T. fontinalis*), and finer dorsal striae (16 in 10 μm in *T. securicula* and 17–21 in 10 μm in *T. fontinalis*). *Tetramphora fontinalis* shares a similar valve outline, size and stria count with *T. sulcata*, but can be distinguished from this taxon under LM by its more distinct areolae and radiate striae. Under SEM, *T. fontinalis* is distinguished from *T. sulcata* by its prominent central siliceous flap obscuring the proximal raphe ends and by its transapically oriented areola slits.

**Tetramphora robusta Stepanek & Kociolek sp. nov.**

*(Figs 96–105)*

**Description**
Valves broadly semi-elliptical and strongly dorsiventral. The dorsal margin is smoothly arched, the ventral margin appears straight to slightly convex depending on the orientation of the specimen. The valve ends are narrowly rounded. Valve length 45.0–65.0 μm, valve breadth 8.0–10.0 μm. The raphe is weakly biarcuate and positioned near the ventral margin. The valve is highly flexed near the apices and central area making it difficult to image the entire raphe system in a single focal plane. The axial area is narrow throughout. The striae are coarse and distinctly areolate, parallel near the valve centre and becoming radiate near the apices. Stria number 13–14 in 10 μm.

In SEM, externally, the raphe is biarcuate with dorsally deflected distal ends and proximal ends that dip towards the ventral margin before terminating close together in simple endings (Figs 101–103). An unornamented area is present at the dorsal central area, but is not extended into a flap (Fig. 103). The external areolar openings consist of small transapically oriented slits. Internally, the raphe is bordered by well-developed axial costae (Figs 104–105). The distal raphe ends terminate in helicoglossae. In the imaged specimens the central area is obscured by the flexure of the valve. Although the majority of the central area is obscured, a protruding central thickening is visible (Figs 104–105). The striae are separated by thin virgae, the virmenes are not well developed.
Figs 64–67. *Tetramphora chilensis* comb. nov., SEM. Fig. 64. Whole frustule dorsal view showing girdle bands. Fig. 65. Detail of external valve centre showing transapically oriented dash-like areolae, central hyaline area and closely spaced proximal raphe ends. Fig. 66. Detail of internal valve centre showing closely spaced proximal raphe ends. Fig. 67. Internal whole valve. Scale bars = 1 μm.

**Holotype**
Slide ANSP GC 59143, deposited at the Academy of Natural Sciences, Philadelphia, USA. Holotype specimen illustrated in Fig. 97.

**Isotypes**
Cleaned material ANSP GCM 4859, deposited at the Academy of Natural Sciences, Philadelphia, USA. Slide and cleaned material JPK 8566, deposited at the University of Colorado Boulder, Kociolek Collection at COLO.

**Type locality**
24.64167°N, 81.33877°W. Benthos, Long Beach Backwater, Big Pine Key, Florida Keys, Florida, USA.

**Etymology**
*robusta* refers to the large, coarsely striated valves of this species.

**Taxonomic notes**
*Tetramphora robusta* is distinguished from *T. securicula* with which it shares similar length and breadth by the
more ventral position of its weakly biarcurate raphe, and its
coarser striae (\textit{T. robusta} 13–14 striae in 10 μm, \textit{T. securi-
cula} 16 striae in 10 μm). \textit{Tetramphora robusta} is similar in
valve outline and raphe position to the large-celled \textit{T.
rhombica} and \textit{T. intermedia}. It is distinguished from both
by its smaller size (Cleve 1895 gives lengths of 130–260
μm and 90–100 μm for \textit{A. rhombica} and \textit{A. intermedia},
respectively) and more flexed valves.

\textbf{Tetramphora rhombica (Kitton in Schmidt) Stepanek &
Kociolek comb. nov.}

\textit{Basionym}
\textit{Amphora rhombica} Kitton in Schmidt et al. 1874–1959, pl.
40, fig. 39.

Schmidt et al. 1874–1959, pl. 40, fig. 39; Peragallo & Peragallo
1897–1908, pl. 50, fig. 4; Cleve 1895, pl. 4, figs 10, 11; Wachnicka & Gaiser 2007,
fig. 87; Lobban et al. 2012, pl. 1, figs 7–9; pl. 54, fig. 5; pl.
55, figs 1–3.

\textit{Taxonomic notes}
Although not extensively imaged, the type drawing in
Schmidt et al. (1874–1959) clearly illustrates features aligning \textit{A. rhombica} with \textit{Tetramphora}. These include
overall valve shape, transapically oriented slit-like areo-
lae and characteristic flexure near the central area. Navarro
(1982) illustrated a taxon that agrees well with the descrip-
tion of \textit{A. rhombica} (Cleve 1895 gives length 130–260
μm, breadth 21–50 μm and 10–12 striae in 10 μm; Navarro
1982 gives length 198 μm, breadth 36 μm and 10 striae in
10 μm for his taxon). These images show the characteris-
tic unornamented dorsal central area, transapically oriented
areolae and internal axial costae.

\textbf{Tetramphora decussata (Grunow) Stepanek & Kociolek
comb. nov.}

\textit{Basionym}
\textit{Amphora decussata} Grunow 1867, p. 23.

Peragallo & Peragallo 1897–1908, pl. 49, figs 23–25;
Cleve 1895, pl. 4, figs 10, 11; Wachnicka & Gaiser 2007,
fig. 87; Lobban et al. 2012, pl. 1, figs 7–9; pl. 54, fig. 5; pl.
55, figs 1–3.

\textit{Taxonomic notes}
As previously illustrated, \textit{Tetramphora decussata} is both
easily identifiable and morphologically unique. In LM, the
striae are parallel and run diagonally across the valve face
converging on a thin semi-stauros. In SEM images pre-
sented by Lobban et al. (2012), these diagonal striae are
crossed internally by thin vimines, although the vimines
are not visible externally. Although this stria structure and
orientation appears unique within the genus, the chloro-
plast shape and arrangement (Lobban et al. 2012, pl. 1,
figs 7–9), girdle bands, unornamented dorsal central area
and internal axial costae (Lobban et al. 2012. pl. 55,
figs 1–3) clearly place this species within \textit{Tetramphora}.

\textit{Phylogenetic analysis}
The results of independent tree searches utilizing
three alignment partitioning strategies returned identical
topologies with the degree of alignment partition having
no effect on the inferred position of \textit{Tetramphora}. All
presented phylograms are inferred from alignments parti-
tioned by marker. Both the ML and Bayesian estimations
Taxonomy and phylogeny of *Tetramphora*

Figs 77–82. *Tetramphora lineolatoides* sp. nov., SEM. Fig. 77. External whole valve. Fig. 78. Detail of external valve end showing dorsally deflected dorsal raphe ends and longitudinally oriented areolae. Fig. 79. Detail of external valve centre showing central hyaline area and proximal raphe ends. Fig. 80. Internal whole valve. Fig. 81. Detail of internal valve end showing distal helictoglossa. Fig. 82. Detail of internal valve centre showing continuous raphe and protruding central thickening. Scale bar = 1 μm.

Conducted on the three marker concatenated alignment returned a weakly supported (41/57, BS/BPP) position for *Tetramphora*, sister to *Mastogloia* Thwaites in Smith, within a larger clade containing taxa from the Cymbellales, D.G. Mann and Naviculales Bessey (Fig. 106). The ML estimation conducted on the larger SSU-rbcL alignment returned an identical position for *Tetramphora* as sister to *Mastogloia* (BS 25) within a larger clade containing
members of the Cymbellales and Naviculales (Fig. 107). The Bayesian estimation conducted on the SSU-rbcL alignment was the only analysis to return a tree with Tetramphora not sister to Mastogloia (Fig. 108). In this case, Mastogloia was sister to Phaeodactylum tricornutum Bohlin (BPP 83), which was part of a polytomy with a monophyletic Tetramphora and a clade containing taxa from the Cymbellales and Naviculales (Fig. 108). These analyses support earlier findings that Tetramphora is distantly related to Amphora s.s. (Stepanek & Kociolek 2014) and lends strong support for the transfer of H. chilensis as part of an early branching lineage within this genus (100/100).

**Chloroplast arrangement**

Live cells of T. lineolatoides (Figs 109–111) and Mastogloia sp. (Figs 112, 113) (see Table 1 for collection details for Mastogloia sp.) were imaged to illustrate the number and arrangement of the chloroplasts. Tetramphora lineolatoides exhibits Mereschkowsky’s type 8 Amphora chloroplast with two pairs of plastids linked by large pyrenoids (Figs 109–111). This arrangement was interpreted by Mereschkowsky (1903) as four chloroplasts per cell, although more recent works describe this arrangement as two chloroplasts each with two plates connected by a pyrenoid-containing isthmus (Round et al. 1990). Mastogloia sp. exhibits a similar plastid structure when viewed in girdle view (Figs 112, 113), with pairs of lobed chloroplasts linked by large pyrenoids.

**Discussion**

With the possible exception of T. chilensis, the taxa currently included in Tetramphora share the following general features: strong ‘amphoroid’ frustule symmetry; a moderately to strongly dorsiventral valve with a biarcuate raphe; a central hyaline area that creates undulate proximal raphe ends; slit-like external areola openings; axial costae; a protruding dorsal central thickening; and Mereschkowsky’s (1903) type 8 chloroplast with two pairs of linked plastids (Figs 109–111). The taxa fall into two groups based
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Figs 90–95. Tetramphora fontinalis sp. nov., SEM. Fig. 90. External whole valve showing transapically oriented areolae. Fig. 91. Detail of external valve centre showing central siliceous flap and continuous raphe. Fig. 92. Detail of external valve end showing dorsally deflected distal raphe end. Fig. 93. Internal whole valve. Fig. 94. Detail of internal valve end showing distal helictoglossa. Fig. 95. Detail of internal valve centre showing continuous raphe and protruding central thickening. Scale bars = 10 μm (Figs 90, 93), 1 μm (Figs 91–92, 94–95).

on stria and raphe structure. Several of the taxa presented here (T. fontinalis, T. intermedia, T. robusta and T. securicula) share transapically oriented areolar slits and a highly developed central hyaline area that extends into a prominent siliceous flap. Conversely, the taxa sharing longitudinally oriented areolar slits (T. lineolata, T. lineolatoides, T. ostrearia and T. sulcata) have a reduced central hyaline area with clearly visible proximal raphe ends and, typically, a less flexed valve through the central area.

Although morphological similarities suggest these groups, they are not supported by the molecular phylogeny as currently sampled (Figs 106–108). In the three marker concatenated analysis, T. lineolatoides was more closely related to T. securicula (84/91) than to the morphologically more similar T. sulcata. This discrepancy between the morphological and molecular evidence may be the result of the small number of taxa included in the molecular phylogeny, or, as is often the case, these features
Figs 96–100. *Tetramphora robusta* sp. nov., whole frustule and single valves showing size range. Fig. 96. Whole frustule. Figs 97–100. Single valves. Fig. 97. Holotype specimen. Scale bar = 10 μm.

are not synapomorphies, but rather symplesiomorphic or homoplastic within the genus.

How additional, morphologically diverse, taxa such as *T. decussata* will fit into this classification remains to be seen. *Tetramphora decussata* differs dramatically from the taxa illustrated here in stria and areola features (see Lobban et al. 2012 for live LM, cleaned LM and SEM illustrations of *T. decussata*). Although these features differ greatly, typical *Tetramphora* plastid structure and a prominent central hyaline area extended into a siliceous flap (Lobban et al. 2012) place this taxon within *Tetramphora*. It is likely, with further observations, that other taxa included in this genus will expand the morphological range presented here.

Although there are likely other described species that may need to be transferred to *Tetramphora*, at the present time we only transfer taxa that have been directly observed or illustrated well by others. Taxa such as *Amphora acuta* Gregory, *Amphora arcus* Gregory and *Amphora aspera* Petit, although classified within Cleve’s (1895) subgenus *Oxyamphora*, and whose original illustrations would seem to ally them with *Tetramphora*, have upon further analysis been shown to exhibit features aligning them more closely with *Halophora* (Levkov 2009, Sato et al. 2013). Moving forward, Mereschkowsky’s type 8 chloroplast and undulate proximal raphe ends produced by a distinct central hyaline area will be important features for inclusion in *Tetramphora*.

**Systematic placement of Tetramphora**

Although monophyletic and distinct from *Amphora* (no marker or analysis has placed *Tetramphora* within *Amphora* or *Halophora*) where these species were originally placed, the systematic placement of *Tetramphora* based on the molecular phylogenetic analysis is far from conclusive, with node support of 41/57 and 25 for the
sister relationship with *Mastogloia* in the three marker and SSU-*rbcL* ML trees, respectively. This low support may be indicative of the overall poor taxon sampling across the diverse raphid diatom lineages, an issue that has led to low backbone support in many diatom molecular systematic studies. This issue is particularly acute when working with marine raphid lineages, which are severely underrepresented in sequence archives like GenBank. Although the relationship with *Mastogloia* does not have strong support, the molecular evidence gives little indication for an alternative placement. Within the Bayesian estimation of the SSU-*rbcL* alignment, although not sister to *Mastogloia*, *Tetramphora* is in an unresolved relationship with the other members of the *Mastogloia*–Cymbellales–Naviculales clade in which it has consistently been placed (Fig. 108). In addition, when a similar analysis was conducted without the inclusion of *Mastogloia* taxa (Stepanek & Kociolek 2014), the *Tetramphora* taxa remained associated with this clade, sister to *P. tricornutum* (Stepanek & Kociolek 2014, fig. 1).
Although frustule morphology differs greatly between *Tetramphora* and *Mastogloia*, the distinct axial costae seen in most *Tetramphora* taxa is a feature also observed in many *Mastogloia* and *Decussata* (Patrick) Lange-Bertalot species (see Lee et al. 2014, figs 12, 13 for an illustration of this feature in *Mastogloia* and Edlund et al. 2006, fig. 56 for *Decussata*). Plastid morphology provides additional support for this grouping. In Mereschkowsky’s (1903) treatment of diatom plastid types, in which he proposed the genus *Tetramphora*, he placed this genus within a larger group of diatoms containing four plastids, the Tetraplactae, in which *Mastogloia* was also included (Mereschkowsky 1903, p. 149). A comparison between the chloroplast structure of *Tetramphora*
and *Mastogloia* (Figs 109–113) demonstrates the similarity in structure, with both exhibiting two plastids, each with two oppositely appressed plates linked by large pyrenoids. Additionally, a very similar arrangement was observed by Edlund et al. (2006) in *Decussata* and was, in part, cited as evidence for its placement within the Mastogloiales D.G. Mann. Given the phylogenetic, chloroplast and structural evidence available, it seems appropriate to place *Tetramphora* within the Mastogloiales.
The early history of the genus *Amphora* saw it defined by overall frustule symmetry alone, creating a non-monophyletic group that persisted for 150 years. With modern imaging and molecular techniques employed over the last 30 years, this situation is slowly being resolved by the transfer of taxa, historically classified within *Amphora,*
to other genera. The recognition of *Tetramphora* as a genus is one more step in this effort to create a natural classification system for the ‘amphoroid’ diatoms.

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**Supplemental data**

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