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Halamphora oceanica (Catenulaceae, Bacillariophyta), a new species from the epipelagic region of the southwestern Gulf of Mexico

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Abstract

In the oceanic zones of the Gulf of Mexico a new species of diatom was found belonging to the genus *Halamphora*. Live cells were isolated using basic microbiological techniques for culturing and DNA analysis. Observations to determine morphological and morphometric characteristics were performed by both light and scanning electron microscopy. In addition, a phylogeny was built based on the expression and sequencing of the small subunit of the 18S gene. As a result of these observations, we conclude that this pennate diatom is a new species here proposed as *Halamphora oceanica*.

Key words: Catenulaceae, molecular analysis, Mexico, new species

Introduction

The genus *Halamphora* (Cleve) Levkov (Catenulaceae, Bacillariophyta**)** was recently established by Levkov (2009) to contain species with a single H-shaped plastid on the ventral girdle. Among the main characteristics that distinguish *Halamphora* from *Amphora* are: *Halamphora* species have a unilateral raphe ledge only on the dorsal side, and a medial helictoglossa fused into one compact structure which is elevated inwardly from the rest of the valve. Also, *Halamphora* species have some structure within the areola; internally, hymenes cover each areola, but some species have sieve plates within the areola, which are typically biseriate. In contrast, *Amphora,* species possess a raphe ledge on both sides of the raphe, uniseriate striae, and sometimes the external foramina of the areola are short and finger-like at the margins (Levkov 2009). According to Levkov (2009), most of the species from the genus *Halamphora* occur in brackish, saline inland or marine habitats, and a few species are found in freshwater habitats. Several authors have experienced problems with this genus due to the wide range of intra- and interspecific morphological variation. In such cases, molecular analysis could be a useful tool in taxonomic studies. Sato *et al*. (2013) tested the phylogenetic relations among different sections of *Amphora sensu lato* and suggested that *Halamphora* is probably monophyletic. Wang *et al*. (2014), based on morphological and molecular analysis of the small subunit rDNA, suggested that *Amphora sensu lato* species were not a monophyletic group. These analyses provide evidence that *Halamphora* Levkov is independent of *Amphora* Cleve. In the Gulf of Mexico, 66 *Amphora* species have been reported, mainly from bays and near shores (Krayesky *et al*. 2009).

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As a part of environmental monitoring program carried out by the Instituto Mexicano del Petróleo (México) in the oceanic waters of the Gulf of Mexico, a new diatom species was found belonging to the genus *Halamphora*, for which morphological characteristics distinguish it from other currently recognized species. Thus, in this research, we propose a new species collected in the oceanic waters of the Gulf of Mexico that is described by using detailed light microscope (LM) and scanning electron microscope (SEM). Additionally, we assessed the taxonomic position based on a nuclear-encoded small subunit (SSU) of the 18S gene, confirming the presence of a unique set of morphological and molecular characteristics that belong to the *Halamphora* clade.

Materials and methods

Sample collection and culture

The phytoplankton used in this research was collected in the Gulf of Mexico on October 18th, 2014 during an environmental monitoring study at 23° 0.0' 47.24" N, 95° 0.1' 0.98" W, at 3570 m of total depth, (Fig. 1). The phytoplankton sample was obtained by a horizontal trawl at sea surface level (0–5 m) using a CalCOFI net of 55 µm mesh, 20 cm in diameter at the mouth, and 90 cm long with a PVC collecting bucket and a General Oceanics flowmeter attached. Trawling was performed at a constant speed of 3 knots for one minute. The total volume of water filtered was 0.87 m^3. The sample was concentrated in the collecting bucket then transferred to a 125-mL sterile plastic bottle and kept at 4 °C in darkness.

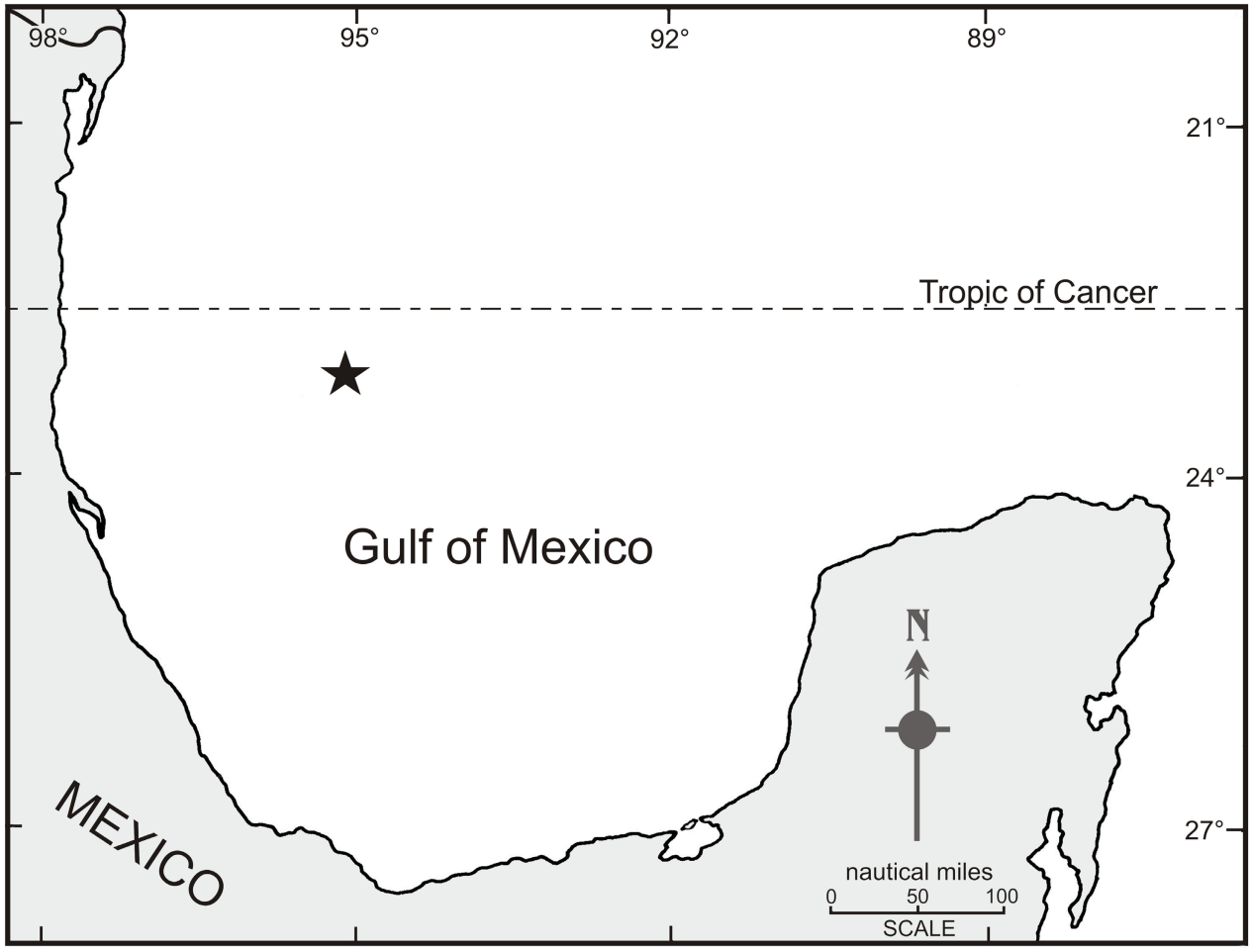


FIGURE 1. Location of the sampling site for the current study in the Gulf of Mexico.

Diatom species isolation was performed in f/2 medium/agar (2%) in new and sterilized Petri dishes using the stretchmark technique. Later, the diatoms were transferred to liquid medium f/2 (Guillard 1975) prepared with artificial seawater (Instant Ocean®, 35 psu) and sterilized using a membrane filter with a 0.22 µm pore diameter (EMD Millipore, Massachusetts, U.S.A., catalogue # GSWP04700). Cultures were grown in 4-L glass bottles at 22 ± 2 °C supplied with continuous air bubbling. Unialgal and axenic cultures (using antibiotic and antimitotic solution 100 ×, Sigma A5955)

were irradiated with fluorescent tubes (60 µmol photons m^{-2} s^{-1}) with a 12:12 h light-darkness cycle. The medium was exchanged once every third week. In exponential growth, a sample of 25 mL was fixed (glutaraldehyde 2%, Electron Microscopy Grade, 70% aqueous solution) for later microscopy studies. Samples were prepared by acid digestion with K_2MnO_4/HCl followed by 10 drops of hydrogen peroxide (30%), rinsed several times with distilled water, and mounted on glass slides using Naphrax®.

Microscopy

Live cells were observed to gather information on chloroplasts using a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen, Germany) equipped with a Canon PowerShot G6 digital camera. The images were digitized using the Axio Vision 4.8.2 program. Slides mounted with Naphrax were examined using a confocal microscope (Nikon A1R) with DIC optics and a plan-apo-100× oil immersion objective. Also, cleaned diatom samples were mounted directly on EM metal stubs and dried. The mounted samples were then coated with gold and observed using SEM (Hitachi SU1510). The terminology used in this study follows the website *Diatoms of the United States,* http://westerndiatoms.colorado.edu (Spaulding *et al*. 2010), and the systematic scheme follows Cavalier-Smith (2015) and Guiry & Guiry (2017). Liquid samples and permanent slides with their identification numbers (NI–1808, NI–1808A–C) were deposited in the National Herbarium of Mexico (MEXU).

DNA extraction and PCR amplification

Live cells were concentrated by filtration using a Millipore filtration system using fiberglass membranes with a pore size of 0.22 µm. Cells were dehydrated in a glass desiccator in the presence of silica for 2 days. Dehydrated cells were pulverized with a TissueLyser II (Qiagen) with 5 mm stainless steel beads in sterile conditions, using liquid nitrogen. DNA extractions were performed according to the 2-cetyltrimethylammonium bromide (CTAB) 2× protocol (Doyle & Doyle 1987) modified by adding 2% (w/v) polyvinyl pyrrolidone (PVP) to the extraction buffer. DNA extracts were purified by precipitation with chilled isopropanol with a subsequent wash in 70% ethanol. Quantization and measurement of the quality of obtained DNA were done in a Thermo Scientific NanoDrop ND 2000c spectrophotometer (Thermo Fisher Scientific, U.S.A.). Expressions of SSU of the 18S gene were performed using the following primers proposed by Wang *et al*. (2014): 18S-F53 (TTGTCTCAAAGATTAAGCCATG) and 18S-R1335 (CCTGTTATTGCCCCTATCTTCC), both from 5′ to 3′. The PCR reaction was prepared using a commercial kit (Taq Core PCR Kit; Qiagen, Hilden, Germany) following the manufacturer's protocols, adding 0.5 µl of a 0.4% aqueous solution of bovine serum albumin (BSA) to neutralize potential inhibitors (Kreader 1996). The amplification was carried out in 12.5 µl: 9.39 µL of H_2O, 1.25 µL of Buffer 10 ×, 0.25 µL of dNTP, 0.125 µL of 18S-F53, 0.125 µL of 18S-R1335, 0.5 µL of $MgCl_2$, 0.0625 µL of Taq polymerase, and 0.3 µL of DNA. The PCR cycling was conducted in a PCR System 9700 (Applied Biosystems; Foster City, California 94404, USA) under the following conditions: pre-denaturation at 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 following the procedure described by Wang *et al.* (2014). The PCR products were visualized by electrophoresis in an agarose gel at 1% with TBE 1× and GelRed Nucleic Acid Gel Stain (Biotium). PCR products that produced bright bands were sent to Laboratorio de Secuenciación de la Biodiversidad y la Salud (Ciudad de México, México) for purification and DNA sequencing in both directions on a 3100 Genetic Analyzer automated sequencer (Applied Biosystems Inc.), using the same primers that were used for the PCR.

Sequence alignment and phylogenetic analysis

Obtained sequences were edited and aligned (Seaview Version 4.6.1; Gouy *et al*. 2010) with regard to other sequences of the 18S gene belonging to other members of the diatom genus *Amphora* and *Halamphora,* which were found by running the Basic Local Alignment Search Tool (BLAST) by NCBI. In addition to these, other sequences such as *Asterionella formosa* Hassall (1850: 9–10), *Rhizosolenia hyalina* Ostenfeld in Ostenfeld & Schmidt (1902: 160), *Helicotheca tamesis* (Shrubsole) M. Ricard (1987: 75), *Thalassiosira rotula* Meunier (1910: 264), and *Chaetoceros rostratus* Ralfs in Lauder (1864: 79) were chosen as members of families that conform to outgroups based on previous reports (Wang *et al*. 2014).

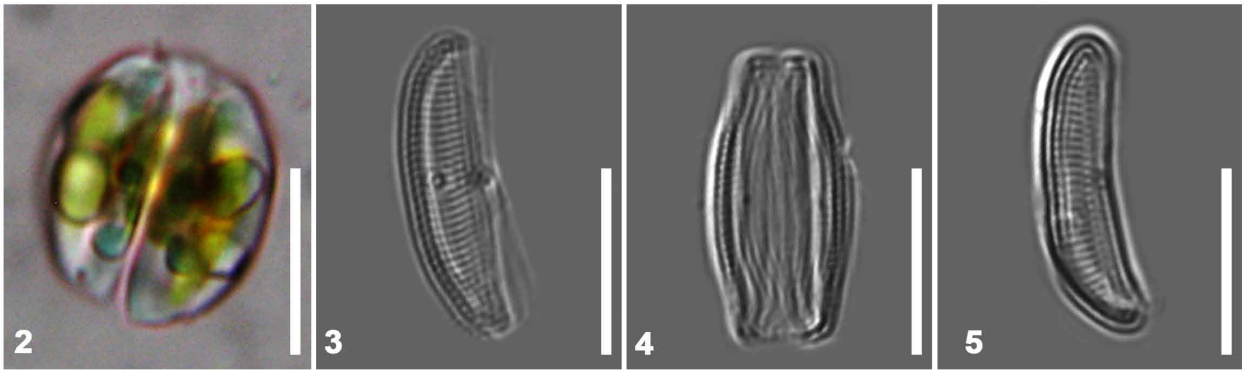
The alignment matrix was analyzed according to the maximum likelihood method (MLM) using MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets (Kumar *et al*. 2016) and following the protocol proposed by Hall (2013) for MEGA 5. The obtained model of the best nucleotide substitution performed by MEGA7 was the Tamura 3-parameter model with discrete Gamma distribution to model evolutionary rate differences among sites. The sequences of *Halamphora oceanica* (1 and 2) were deposited in Genbank obtaining their access numbers respectively (MF362225, MF362226).

Results

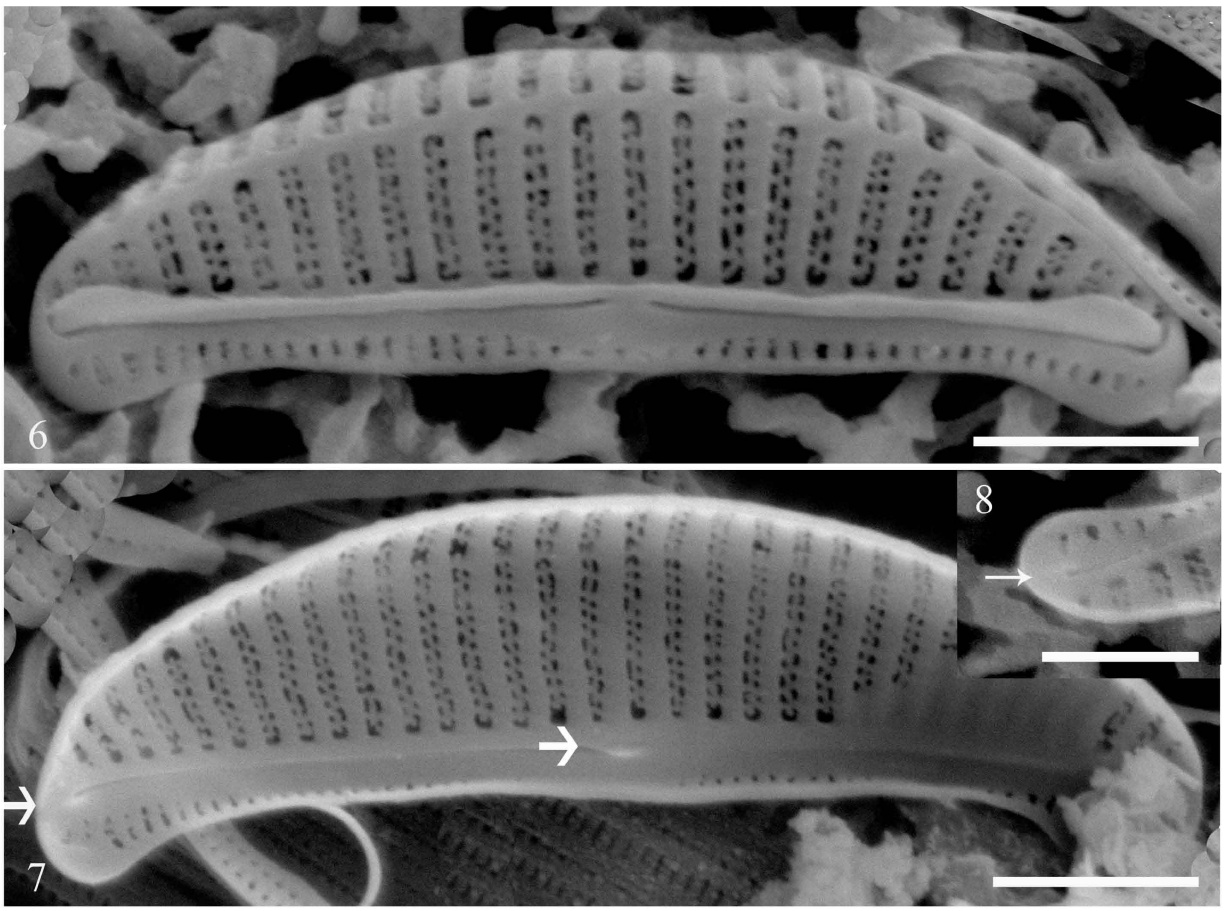
A new species is proposed, hereafter referred to as *Halamphora oceanica* H.F. Olivares-Rubio, L.I. Cabrera, J.L. Godínez-Ortega, L. Salazar-Coria & A. Vega-López *sp. nov*.

Description

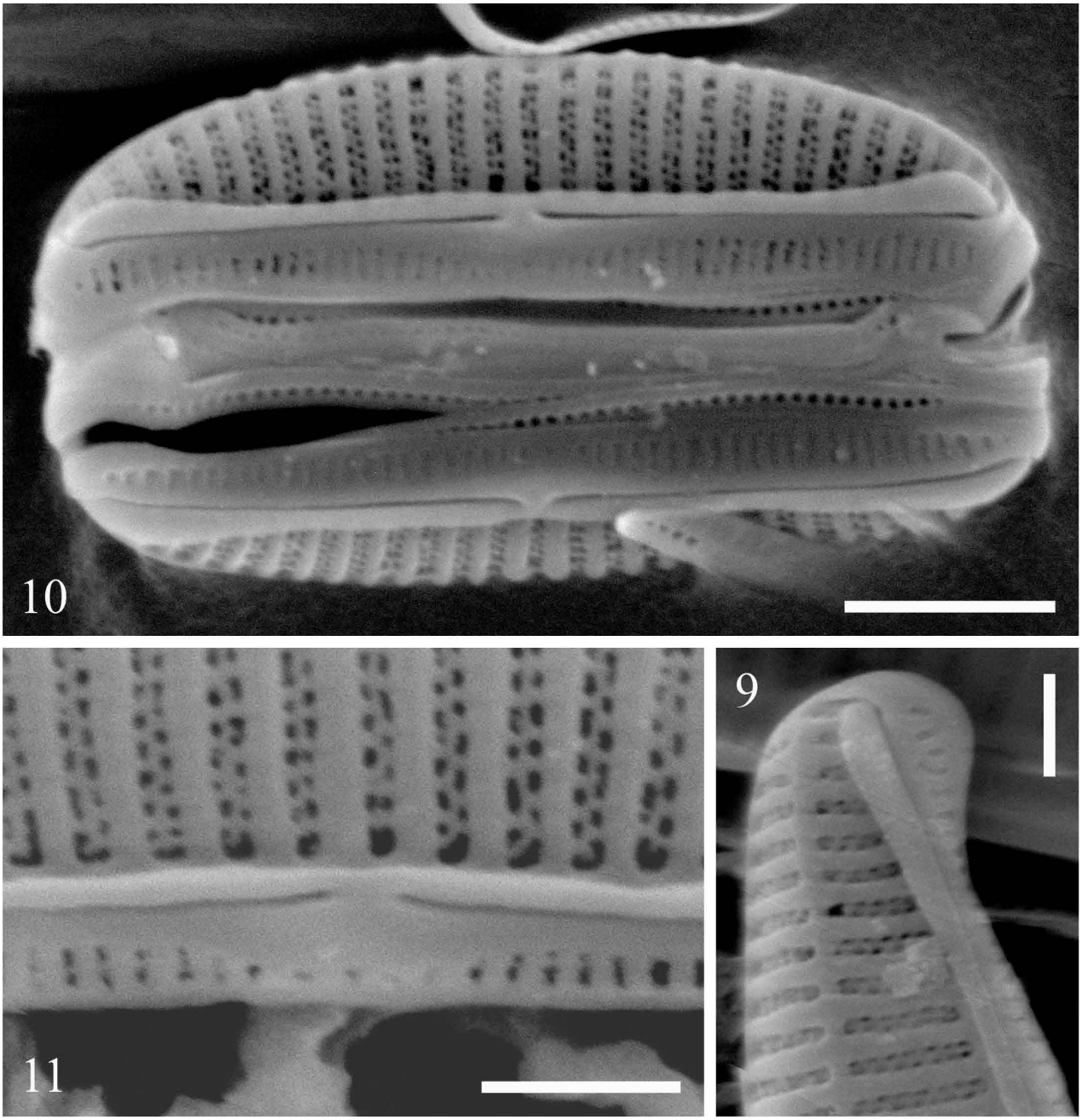
Halamphora oceanica H.F. Olivares-Rubio, L.I. Cabrera, J.L. Godínez-Ortega, L. Salazar-Coria & A. Vega-López. *sp. nov.* (Figs 2–14).



FIGURES 2–5. LM. *Halamphora oceanica sp. nov*. 2. Live material showing a single H-shaped chloroplast. 3. Valve view showing the size and shape, a longitudinal line runs through the striae near the dorsal margin. 4. Whole frustule. 5. Internal valve view. Scale bar = 5 µm.



FIGURES 6–8. SEM *Halamphora oceanica sp. nov*. 6. External dorsal valve view. 7. Internal whole valve view showing central nodule (arrow) and distal raphe (left arrow). 8. Detail of internal-valve end showing poorly developed helictoglossa. Scale bars: 2 µm (6, 7), 1 µm (8).



FIGURES 9–11. SEM *Halamphora oceanica sp. nov.* 9. Detail of valve apex showing raphe endings curved toward the dorsal side of the valve. 10. Frustule in ventral view. The central area is expanded on the ventral side only and closed with striae near the valve margin. 11. The dorsal striae presented biseriate areola. Scale bars: 2 µm (10), 1 µm (9, 11).

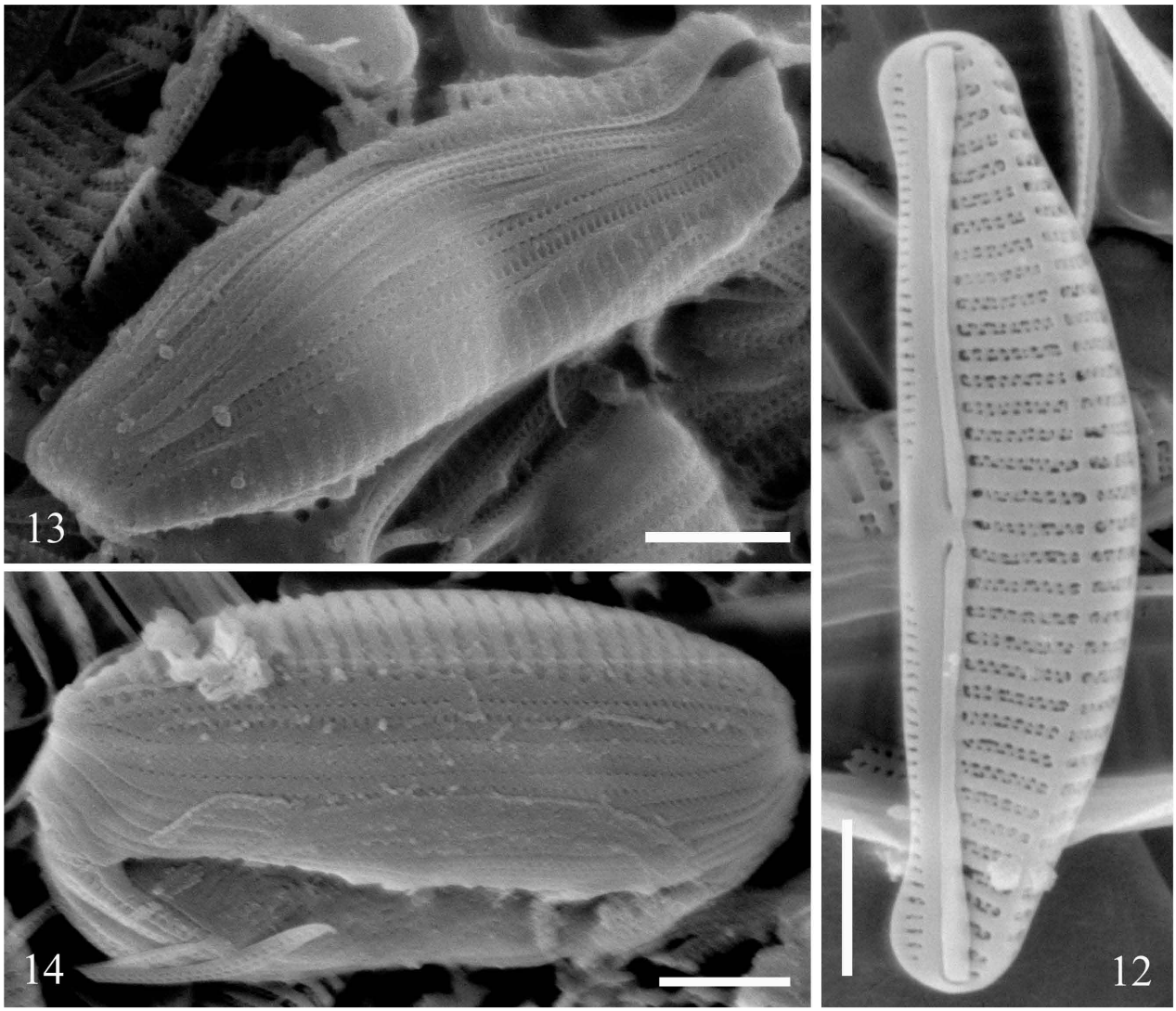
Cells are solitary, with a single H-shaped chloroplast (Fig. 2). The frustule is shaped dorsi-ventrally, semi-elliptical, or semicircular. Valves are semielliptical and the dorsal margin is arched, slightly concave at the ventral margin, and with rounded ends; the valve length is 9–17 µm, and the width is 2.5–5.0 µm (n = 30; Figs 3–5, Table 1); the end of the valves are slightly protracted and rounded. The external central nodule is conspicuous, small, and not raised (Fig. 3); the internal nodule is raised (LM, Fig. 5; SEM, Fig. 7). The dorsal striae are uninterrupted and nearly parallel at the valve center, becoming radiate near the poles, 28–34 in 10 µm; the ventral striae are hard to define in light microscopy. In SEM, the raphe branches are straight and the proximal endings are slightly bent with an axial zone that is very narrow, but slightly wider near the valve center (Fig. 6). The raphe endings are curved toward the dorsal side of the valve (Figs 6, 9); the distal raphe ends terminate before the valve apices, and poorly developed helictoglossae are present at the internal distal raphe ends (Figs 7, 8). The conopeum (or raphe ledge) is well developed and linear on the dorsal side of the valve with broadened poles truncated at the ends (Fig. 9). The central area is expanded on the ventral side only and

closed with striae near the valve margin (Fig. 10). The ventral striae have a uniseriate form with 44–52 in 10 μm and are composed of one or two round- to irregularly shaped areolae; proximally, the ventral striae are interrupted near the central nodule (Fig. 10). The dorsal striae are composed of biseriate areolae with irregular contours. The areolae are round to ovoid. Some areolae are transapically elongated, the puncta do not form straight longitudinal lines, and the basal poroids close to the raphe ledge are frequently uniseriate (Fig. 11). A prominent longitudinal line runs through the striae near the dorsal margin of the valve face (Fig. 12). Girdle bands are numerous, with two rows of ovoid pores both on the dorsal and ventral sides. There are 50–70 areolae in 10 μm on the dorsal side (Fig. 13). Both ends of the same frustule show the epicingulum and the association of the valve with four girdle bands (Fig. 14).

Type:—MEXICO. The epipelagic region of the Gulf of Mexico, 23° 0.0' 47.24'' N, 95° 0.1' 0.98'' W, collected by Lucia Salazar-Coria (IMP) in October 2014. Holotype slide MEXU 4808! (represented by the valve in Fig. 3), isotype slides MEXU 4809!, 4810!, and 4811!, and type material specimens preserved in 100% ethanol (NI–1808D).

Etymology:—The species is named *Halamphora oceanica* because it is the first species of the *Halamphora* genus that was collected from the surface of oceanic waters (3569 m deep).

Ecology:—This new taxon was found in assembly with Oscillatoriales (cyanobacteria), *Pseudanabaena* cf. *frigida* (F.E. Fritsch) Anagnostidis (2001: 360). The water conditions were 6.15 mg/L OD, 28.8 °C water temperature, 36.4 psu salinity, and conductivity of 5.9 S/m.



FIGURES 12–14. SEM *Halamphora oceanica sp. nov*. 12. Longitudinal line exists through the striae near the dorsal margin of the face of the valve. 13. Whole frustule in dorsal girdle view showing girdle bands with two rows of poroids. 14. The same frustule, showing the epicingulum and the association of valve and 4 girdle bands. Scale bars: 2 μm.

BLAST

The lengths of the edited and aligned SSU sequences of 18S for *Halamphora oceanica* PCR products (by duplicated product) were 1260 pb. *H. oceanica* 1(MF362225) and *H. oceanica* 2 (MF362226) were almost identical (100%). Through BLASTN searches in the GenBank database, the two sequences under study, *H. oceanica* 1(MF362225) and *H. oceanica* 2 (MF362226), were in the same group as *Halamphora*. The sequences under study showed an identity of 99% and a query cover of 100% with Bacillariophyta (GenBank access number AB183592), followed by *H. coffeiformis* (C. Agardh) Levkov (2009: 179; HM805019) and *H. montana* (Krasske) Levkov (2009: 207; KC736615), both with an identity of 97%, a query cover of 100%, and an E value of 0.

Phylogenetic analysis

The analysis included 1260 characters and 39 samples. The constructed tree is based on 18S rDNA with clade support from the bootstrap analysis (Bootstrap Percentages, BP) (Fig. 15). The most divergent group consisted of the *Helicotheca-Chaetoceros-Thalassiosira* and *Rhizosolenia* clades. The following clade represented members of *Frustulia* Rabenhorst (1853), within the Amphipleuraceae family; the next clades were all members of *Amphora* and *Halamphora* (Catenulaceae), both supported by a BP of 99%.

The *Halamphora* clade was composed of *H. coffeiformis*, *H. coloradiana* J.G. Stepanek & J.P. Kociolek (2013: 73), *H. costata* (W. Smith) Levkov (2009: 181), *H.* cf. *fluminensis* (*nom. prov*.), *H. eunotia* (Cleve) Levkov (2009: 187), *H. holsatica* (Hustedt) Levkov (2009: 196), *H. montana*, *H. normanii* (Rabenhorst) Levkov (2009: 208), *H. oligotraphenta* (Lange-Bertalot) Levkov (2009: 213), *H. terroris* (Ehrenberg) P. Wang in P. Wang *et al*. (2014: 67), and *H. veneta* (Kützing) Levkov (2009: 242). *H. coffeiformis* and *H. veneta* were not monophyletic groups. The clade of *Halamphora montana* (BP 99%) was the sister group of the clade formed by the two *Halamphora* samples collected in the Gulf of Mexico (BP 99%)**.**

Discussion

Based on the comparative morphologic characteristics of species within *Halamphora* (Table 1), *H. oceanica* is one of the smallest (9–17 µm), along with *H. montana* (12–20 µm) and *Halamphora coloradiana* (12–25 µm), but they differ in the shape of the striae. One characteristic of *H. oceanica* is its semi-elliptical form very slight protracted and rounded apices, which is unlike the other diatoms included in Table 1 that are semi-lanceolate with protracted and capitated valve apices. A concave ventral margin in *H. oceanica* differs from convex, tumid, or straight margins in *H. normanii*, *H. holsatica*, *H. montana*, *H. oligotraphenta*, *H. eunotia*, *H. hybrida* (Grunow) Levkov (2009: 198) and *H. terroris*. The apical rib is a relevant characteristic of *H. oceanica*; *H. hybrida*, *H. coloradiana*, and *H. coffeiformis* also possess this feature, but the main differences between *H. oceanica* (ratio 3.3–3.8) and *H. hybrida* (ratio 8–9.2) are the sizes (length/width ratio) and frustule shape, which, in fact, is longer than it is wide. In *H. coloradiana* the dorsal marginal ridge continues to the ventral margin. *Halamphora oceanica* is similar to *H. oligotraphenta* in the number of striae (26–30 in 10 µm) but *H. oligotraphenta* has uniseriate striae. *Halamphora oceanica* has ventral striae composed of one or two round of irregularly shaped areolae. In contrast, *H. hybrida* has uniseriate ventral striae composed of elongated areolae which are interrupted at the central nodule. In *H. coloradiana*, the dorsal striae are interrupted by many irregularly spaced intercostal ribs forming elongated areolae of various sizes (Stepanek & Kociolek 2013). *Halamphora coffeiformis* reported in the study of Pniewski *et al*. (2010) has a different position of the hyaline rib, more equidistant to the raphe and to the dorsal margin. In addition, the conopeum in *H. coffeiformis* is flat. In *H. oceanica* it is elevated, and the distal raphe end is dilated. In *H. oceanica*, the conopeum broadens at the poles, which is not observed in *H. holsatica*.

Halamphora coffeiformis sensu lato has problems with its morphological, phylogenetic, and ecological information (e.g. Archibald & Schoeman 1984). For example, specimens and populations have been associated with this species despite the fact that there is a great difference in morphology and morphometry (e.g., Sala *et al*. 1998; Pniewski *et al*. 2010; Wang *et al*. 2014; Rochín-Bañaga *et al*. 2015). This is also observed through the current analysis (Fig. 15) and probably indicates a misinterpretation of the species. In addition, this species has been found around the world in different environments; therefore, it could be considered cosmopolitan (Witkowski *et al.* 2000). Probably, several of the specimens identified as *H. coffeiformis* would be different species in the genus *Halamphora*. In the current topology

(Fig. 15), the *Halamphora* genus is considered monophyletic (BP 100%), as was also found in the study of Stepanek & Kociolek (2014) and Jiang *et al*. (2015) based on analysis of sequences of the nuclear region (SSU rDNA and 18S rDNA) and the chloroplast (*rbc*L and *psb*C). The results showed that the 18S sequences of *H. oceanica* were associated with two sequences of *H. montana* (KC736615 & AJ243061). *Halamphora montana* is a fossil and recent species from freshwater environments and cold mountain places whose records have been documented worldwide (Levkov 2009; Guiry & Guiry 2017). *Halamphora montana* and *H. oceanica* are only similar in size (Table 1). *Halamphora oceanica* can be distinguished from *H. montana* based on morphological characteristics. The difference is that *H. montana* possesses a stauros at the central area, which is not observed in *H. oceanica*. Interestingly, the topology of the *Halamphora* clade shows a low BP among taxa, probably because only a few 18S sequences of this species exist in GenBank.

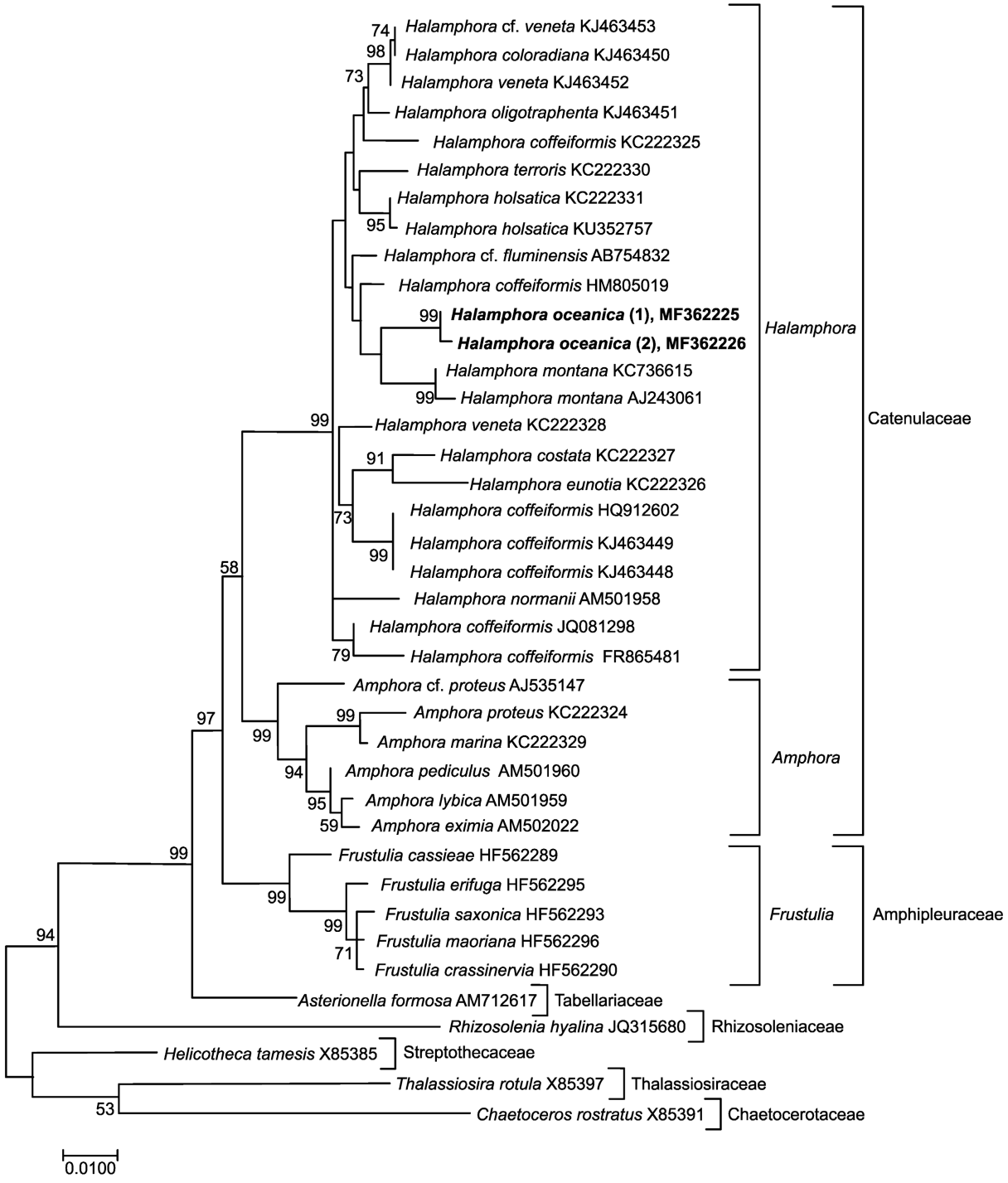


FIGURE 15. Molecular phylogenetic tree based on 18S rDNA sequences (1296 positions included). The tree shows the alignment matrix using the maximum likelihood method. Outgroup taxa were included. Nodal supported values greater than 50% are shown under the branches. Families are according to the taxonomy of Cavalier-Smith (2015).

TABLE 1. Morphometric data of *Halamphora oceanica* compared with related species (*cf*. Fig. 15).

Taxa	Valve length (µm)	Valve width (µm)	Dorsal striae (10 µm)	Ventral striae (10 µm)	Apical rib	Habitat	Reference
Halamphora oceanica	9–17	2.4–5.1	26–30, biseriate	48–62	Present	Marine	This study
Halamphora coffeiformis	23–35 (?14–55)	(3.5) 5–7.2	19–22, without puncta	ND	ND	Brackish	Levkov 2009
Amphora coffeiformis	30–53	5–6.5	17–26	20–24	ND	Brackish	Wachnicka & Gaiser 2007
Halamphora normanii	22–42	4–6.4	16–20	25–28	Absent	Freshwater	Levkov 2009
Halamphora costata	45–80	9–10	8–9	11	Absent	Brackish/ marine	Levkov 2009; Wachnicka & Gaiser 2007
Halamphora veneta	17–35	4–6.5	18–22, without puncta	24–30	Absent	Freshwater/ brackish	Levkov 2009
Halamphora holsatica	26–47	7–9	14–15, uniseriate	16–18	Absent	Brackish/ marine	Levkov 2009
Halamphora montana	12–20	3–4.6	40–45, uniseriate	40–45	Absent	Freshwater	Levkov 2009
Halamphora terroris	10.5–18.3	4–6.3	ND	22–24	Absent	Marine	Wang *et al*. 2014
Halamphora oligotraphenta	17–39	3.2–4.5	26–30, uniseriate	ND	Absent	Freshwater	Levkov 2009
Halamphora eunotia	55–75	8.5–10.5	9–11, uniseriate	11–14	Absent	ND	Levkov 2009
Halamphora hybrida	32–60	4–6.5	20–25, without puncta	ND	Present	Brackish	Levkov 2009
Halamphora coloradiana	12–25	2.5–4.5	23–24	ND	Present	Freshwater	Stepanek & Kociolek 2013

ND: not documented.

Through SEM, it is possible to determine other relevant characters for the identification of *H. oceanica*. The external raphe endings are curved toward the dorsal side of the valve and the helictoglossae is poorly developed at the internal raphe. Also, the dorsal striae present biseriate areolae with irregular contours, the puncta do not form straight longitudinal lines, and the basal poroids close to the raphe ledge are frequently uniseriate. Additionally, a prominent longitudinal flat line (hyaline rib) exists through the striae near the dorsal margin of the face of the valve. In accordance with our review based on phylogenetic analysis, three species of *Halamphora* possess a hyaline rib: *H. hybrida*, *H. coloradiana*, and *H. coffeiformis*. The specimen of *H. coffeiformis* reported in the study of Pniewski *et al*. (2010) has a different position of the hyaline rib compared to *H. oceanica*; however, the size and shape of the frustule of both diatoms are similar. *Halamphora oceanica* is notably wider than *H. hybrida*; in addition, the striae of *H. hybrida* are uniseriate and occluded (Levkov 2009). With regard to the number of striae on the dorsal side of the valve (20–36 in 10 µm), it is the same for *H. oceanica*, and *H. oligotraphenta*; however, the dorsal striae of this species are uniseriate, and the areolae have different contours which are located near to the raphe, and are longitudinally elongated (Levkov 2009). *Halamphora hybrida*, which also possesses a hyaline rib in the dorsal face, along with *H. oceanica*, has a similar number of dorsal striae; however, other differences have been mentioned above.

Conclusion

Few species of *Halamphora* or *Amphora* have been reported from oceanic surface and epipelagic waters (Krayesky *et al*. 2009; Guiry & Guiry 2017). Although several studies have reported the phytoplanktonic diversity of these regions, more studies are necessary to increase knowledge about this topic.

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