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Un enfoque integrativo revela la identidad y la distribución interoceánica de la diatomea *Halamphora adumbratoides* Stepanek & Kociolek 2018 (Bacillariophyceae: Amphipleuraceae)

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ABSTRACT: *Halamphora adumbratoides* was described in 2018 from the Atlantic Ocean (Florida Bay, USA), the type locality of this species. In this report, we present the morphological characteristics and molecular identity of this taxon (strain CIBA 160) collected in the Gulf of California, Mexico (Pacific Ocean region). Cells of the strain CIBA 160 obtained from laboratory cultures showed morphological characteristics inconsistent with the original description, specifically the shape of the valve and the presence of pores at the end of the dorsal raphe ledge at each end of the raphe. Regarding size, the length (4.1–6.6 µm) of the CIBA 160 strain is much shorter than in the original description (10.0–18.0 µm). However, the valve width and number of striae do coincide with the ranges in that description. At the molecular level, DNA sequences of the CIBA 160 strain showed 100, 99.8, and 99.5% similarity, respectively, for SSU, *rbc*L, and *psb*C with the *H. adumbratoides* sequences from the Atlantic Ocean. Our study supports the first case of interoceanic distribution and the second overall recording of this species.
Key words: first recording, Gulf of California, Pacific Ocean, *psb*C, *rbc*L, SSU.

RESUMEN: *Halamphora adumbratoides* fue descrita en 2018 en muestras procedentes del Océano Atlántico, siendo Florida Bay, USA, la localidad tipo para esta especie. En esta publicación, presentamos las características morfológicas y la identidad molecular de este taxón (cepa CIBA 160) colectado en el Golfo de California, México (en la región del Océano Pacífico). Las células de la cepa CIBA 160, fueron obtenidas mediante cultivos de laboratorio y mostró características que no corresponden con la descripción original, específicamente la forma de la valva y la presencia de poros al final de cada extremo del margen dorsal del rafe. En relación al tamaño, la longitud (4.1–6.6 µm) de la cepa CIBA 160 es menor a la de la descripción original (10.0–18.0 µm). Sin embargo, el ancho de la valva y el número de estrías coinciden con los intervalos de la descripción. A nivel molecular, las secuencias del DNA de la cepa CIBA 160 mostraron 100, 99.8, y 99.5% de similitud, respectivamente con las secuencias de SSU, *rbc*L, y *psb*C de células de *H. adumbratoides* originarias del Océano Atlántico. Nuestro trabajo presenta el

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primer registro de la distribución interoceánica y el segundo registro para esta especie desde su descripción.
Palabras clave: primer registro, Golfo de California, Océano Pacífico, *psb*C, *rbc*L, SSU.

INTRODUCTION

Diatoms are an important and diverse group of organisms that fix almost 20% of global carbon (Armbrust, 2009; Malviya *et al.*, 2016; D. G. Mann, 1999; Onuma *et al.*, 2017). However, aside from the toxic diatoms, the taxonomy of the specious class of Bacillariophyceae is 'in a rather poor state' (Mann *et al.*, 2020). The taxonomic history of the genus *Halamphora* has been dynamic since its revival by Levkov (2009) produced a considerable number of inclusions via transfers of species new nomenclatural combinations, mostly from the genus *Amphora*, descriptions of new species, and taxonomic validations. The increased number of taxonomically accepted species and intraspecific names registered in Algaebase is remarkable, from 110 in 2018 to 163 in 2023 (Guiry & Guiry 2023), which reflects the degree of complexity that this genus still represents.

In diatom, monoclonal cultures form a vital part of an integrative taxonomic approach to identifying species using morphological and molecular data (Mohamad *et al.*, 2022). Also, electron microscopy of monoclonal cultures is commonly used to describe new diatom species with ultrastructural details. Stepanek & Kociolek (2018) described *H. adumbratoides* and complemented their description with molecular tools. Electron microscopy and molecular information have contributed significantly to the determination of new species of diatoms, taxonomic validations, and the transfer of taxa from one genus to another. For example, taxonomic relocations of taxa have been carried out with the closely related genera *Amphora*, *Cymbamphora*, and *Seminavis* (Levkov, 2009; Stepanek & Kociolek, 2013; 2015, 2018, 2019; Van de Vijver *et al.*, 2014).

Morphometric similarity between taxa of different genera is rare; however, it is not uncommon among species of closely related genera. For example, the morphology *of H. adumbratoides* is very similar to that of *Amphora adumbrata* in terms of the shape of the valve and the fine striae composed of a single elongated areola (Stepanek & Kociolek, 2018). The number of striae was a key characteristic for differentiating these taxa by traditional taxonomy (morphology) (Desianti et al., 2015; Stepanek & Kociolek, 2018). At the molecular level, SSU, *rbc*L, and *psb*C markers were used to identify *H. adumbratoides* (Stepanek & Kociolek, 2018).

In geographical terms, *H. adumbratoides* has been recorded only from the coasts of the Atlantic Ocean, in Florida Bay, Cotton Key, Monroe Country, FL, USA. In this publication, we present morphological characteristics and use molecular identity of this taxon (strain CIBA 160) to report its presence in the Pacific Ocean region from the coastal Balandra lagoon (La Paz Bay) in the Gulf of California, on the southern coast of the Baja California Peninsula, Mexico. It is the second recording but the first interoceanic registration of *H. adumbratoides*. We also register considerable morphological features and morphometry variations in the laboratory-cultured cells.

MATERIALS AND METHODS

Sample collection and morphological observation
Water samples of 50 mL were taken by triplicate from the Balandra lagoon (24°19′9.01″N 110°19′18.17″ W) near La Paz, Baja California Sur (Figure 1), in December 2018, and brought to the laboratory. The samples were filtered through a 30-µm Nitex mesh and f/2 medium with silicates added to the filtered samples (Guillard, 1975).

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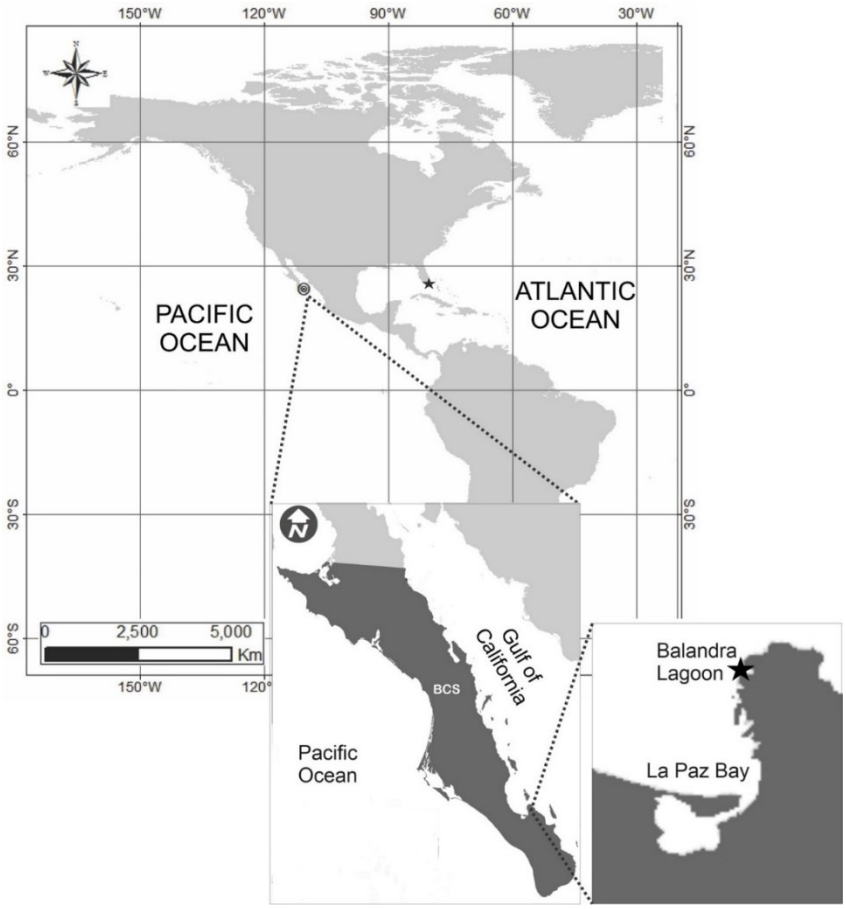

Figure 1. Current recordings of *Halamphora adumbratoides* in the Pacific and Atlantic Ocean (solid stars). Inset figures show an expanded map of the collection site in the Balandra Lagoon, Mexico.
Figura 1. Registros actuales de *Halamphora adumbratoides* en el Océano Pacífico y Atlántico (estrellas sólidas). Las figuras insertadas muestran un mapa amplificado del sitio de recolección en la Laguna de Balandra, México.

After two weeks, microalgal cells were isolated in a 1:50 dilution in f/2 medium and streaked on marine agar plates containing the same medium. Single-cell colonies were isolated under low magnification in a microscope, with a sterile hypodermic needle and then transferred to 24-well plates containing 2 ml of culture medium. Two weeks later, the cells were transferred to test tubes with 5 ml of culture medium, and the unialgal culture of isolated cells was examined under light microscopy. Once confirmed, the monoclonal cultures were deposited in CIBNOR's Microalgae Collection under catalog number CIBA 160 (https://www.cibnor.gob.mx/investigacion/colecciones-biologicas/coleccion-de-microalgas) after three reseeding periods. We have a collection of three fixed slides with the skeletons of the cells, that remains in the laboratory.

For light and electron microscopy and molecular analyses, cells were cultured in triplicate in 500-mL flasks with 250 mL of medium at 25±1°C, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 12:12 h, and 35 PSU. At the exponential stage (on the sixth day), separate samples were taken for each analysis. Live and cleaned cells were observed under a Zeiss Axio Lab A1 microscope, and photographs were taken with a Canon EOS Rebel T5i camera (Figure 2). The cells were prepared for scanning electron microscopy (SEM) as indicated previously by López-Fuerte *et al.* (2020). Briefly, the organic matter was eliminated by oxidation, heat-assisted with concentrated nitric acid. The cells were then washed in distilled water to reach a neutral pH. SEM images of the cells were taken with a

Hitachi SU3500 electron microscope operating at 10 kV and a 6-mm working distance. A coverslip holding the cells was attached to a 32-mm aluminum stub using conductive carbon tape and coated with around 15 nm of gold in a Hummer 6.2 sputtering unit.

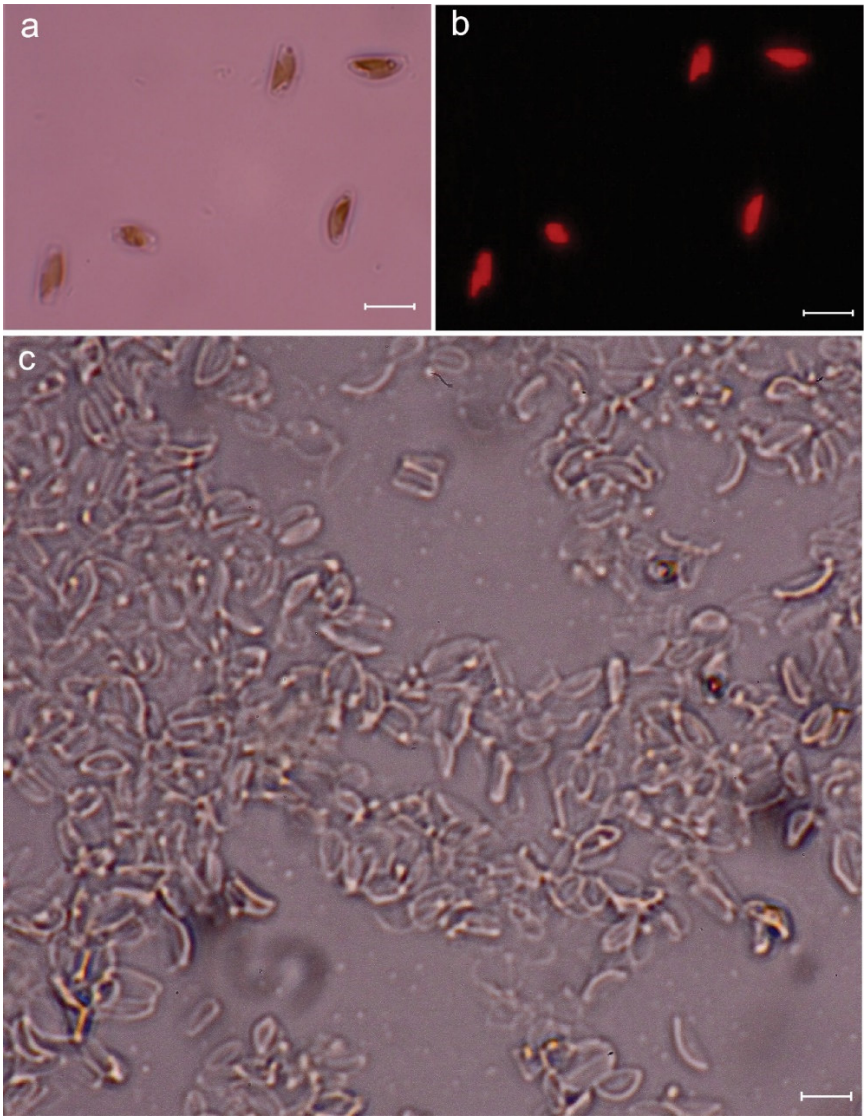

Figure 2. a–c. *Halamphora adumbratoides*, a. live cell showing plastids; b. fluorescence microscopy showing plastids; c. cleaned specimens. Scale bars represent 5 µm.
Figura 2. a–c *Halamphora adumbratoides*, a. células vivas que muestran los cloroplastos; b. microscopía de fluorescencia que muestra los cloroplastos; c. especímenes limpios. Las barras de escala representan 5 µm.

The key morphological characteristics –length, width, and density of the striae– were measured by light and electron microscopy and compared to those reported for *Halamphora adumbratoides* (Stepanek & Kociolek, 2018). The validity nomenclatural status of the name was verified on the Algaebase website (Guiry & Guiry 2023).

DNA extraction, PCR amplification, and sequencing
Cells of the CIBA 160 strain obtained from the exponential stage cultures were utilized to extract genomic DNA by the CTAB method (Doyle, 1991; Herrera *et al.*, 2014). Four regions of DNA were analyzed: fragment of two nuclear genes, the small sub-unit of nuclear gene 18S ribosomal

RNA (SSU), and the large subunit of nuclear gene 28S ribosomal RNA (LSU) were amplified following the PCR conditions described in López-Fuerte *et al.* (2020). A partial sequence of the ribulose bisphosphate carboxylase/oxygenase gene (*rbc*L) from the chloroplast was amplified according to the published protocols (López-Fuerte *et al.*, 2020). Also, the photosystem II CP43 protein gene (*psb*C) was amplified with 30 picomoles of the *psb*C+ and *psb*C– primers (Alverson *et al.*, 2007) in 40 µL of a PCR reaction mixture containing genomic DNA, 0.3 mM dNTPs, 2 mM $MgCl_2$, 4 µL of 10X PCR buffer, and 2 units of platinum *Taq* polymerase (Invitrogen™). The PCR conditions for *psb*C amplification were denaturation at 94°C for 3.30 min, then 35 cycles of 30 s at 94 °C, 50 s at 52 °C, and 80 s at 72 °C. The final extension was for 10 min at 72 °C. Both strands of all markers were sequenced with the amplification primers and an internal primer for the SSU and chloroplast genes (18S 962R, *rbc*L1255, *psb*C857).

We edited the four sequences (SSU, LSU, *psb*C, and *rbc*L) in DNA Baser 4.5 program (http://www.dnabaser.com) and compared the SSU, *rbc*L, and *psb*C sequences to those of *H. adumbratoides* in the GenBank database. The phylogenetic relationship of the CIBA 160 strain was inferred based on the concatenated nucleotide sequences of SSU, LSU, *psb*C, and *rbc*L with 23 *Halamphora* species (Table 1) using the maximum likelihood (ML) method in PAUP 4.0a166 (Swofford, 2002) and Bayesian inference (BI) in MrBayes 3.2.7a (Ronquist *et al.*, 2012). The best fit nucleotide model TIM3+I+G selected according to the BIC criterion in jModeltest 2.1.10 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) for the concatenated nucleotide sequences was implemented in both analyses. Maximum likelihood analysis was performed with 500 pseudoreplicates under the option of heuristic tree-searching with tree bisection-reconnection branch swapping to generate a majority rule ML consensus tree. Bayesian inference was run for 10 million generations, and a majority rule BI consensus tree was obtained after eliminating 25% of the initial trees using the burn-in option.

Table 1. *Halamphora* and *Amphora* sequences used in the phylogenetic analyses.
Tabla 1. Secuencias de *Halamphora* y *Amphora* utilizadas en los análisis filogenéticos.

Taxa	18S	rbcL	psbC	28S	Reference
Halamphora adumbratoides AMPH041	MG027270	MG027434	MG027514	NA	Stepanek & Kociolek, 2019
Halamphora adumbratoides CIBA 160	ON714546	ON736839	ON736840	ON714544	This study
Halamphora pellicula AMPH134	MG027316	MG027481	MG027561	MG027401	Stepanek & Kociolek, 2019
Halamphora pellicula AMPH153	MG027320	MG027486	MG027566	MG027407	Stepanek & Kociolek, 2019
Halamphora elongata AMPH001	MG027259	MG027423	MG027503	MG027337	Stepanek & Kociolek, 2019
Halamphora aponina AMPH049	MG027275	MG027439	MG027519	MG027355	Stepanek & Kociolek, 2019
Halamphora aponina AMPH102	MG027296	MG027461	MG027541	MG027381	Stepanek & Kociolek, 2019
Halamphora pseudoholsatica AMPH165	MG027327	MG027493	MG027573	MG027414	Stepanek & Kociolek, 2019

Halamphora holsatica AMPH154	MG027321	MG027487	MG027567	MG027408	Stepanek & Kociolek, 2019
Halamphora rushforthii AMPH117	MG027306	MG027471	MG027551	MG027391	Stepanek & Kociolek, 2019
Halamphora coffeaeformis AMPH104	MG027297	MG027462	MG027542	MG027382	Stepanek & Kociolek, 2019
Halamphora pratensis AMPH106	MG027299	MG027464	MG027544	MG027384	Stepanek & Kociolek, 2019
Halamphora isumiensis AMPH164	MG027326	MG027492	MG027572	MG027413	Stepanek & Kociolek, 2019
Halamphora tenucostata AMPH042	MG027271	MG027435	MG027515	MG027350	Stepanek & Kociolek, 2019
Halamphora tenuis AMPH034	MG027269	MG027433	MG027513	MG027348	Stepanek & Kociolek, 2019
Halamphora scatebra AMPH119	MG027308	MG027473	MG027553	MG027393	Stepanek & Kociolek, 2019
Halamphora subturgida AMPH015	MG027260	MG027424	MG027504	MG027338	Stepanek & Kociolek, 2019
Halamphora bicapitata AMPH055	MG027278	MG027442	MG027522	MG027359	Stepanek & Kociolek, 2019
Halamphora nagumoi AMPH166	MG027328	MG027494	MG027574	MG027415	Stepanek & Kociolek, 2019
Halamphora oligotraphenta AMPH009	KJ463451	KJ463481	KJ463511	KP229528	Stepanek & Kociolek, 2014; Stephanek *et al.* 2015 (28S)
Halamphora veneta AMPH005	KJ463452	KJ463482	KJ463512	KP229530	Stepanek & Kociolek, 2014; Stephanek *et al.* 2015 (28S)
Halamphora venetoides AMPH017	KJ463453	KJ463483	KJ463513	NA	Stepanek & Kociolek, 2014, 2018
Halamphora coloradiana AMPH025	KJ463450	KJ463480	KJ463510	KP229529	Stepanek & Kociolek, 2014; Stephanek *et al.* 2015 (28S)
Amphora allanta AMPH129	MG027314	MG027479	MG027559	MG027399	Stepanek & Kociolek, 2019
Amphora commutata AMPH126	KP229526	KP229547	KP229549	KP229545	Stepanek *et al.*, 2015

RESULTS

The length and width recorded for the valves of the laboratory cultured cells of the strain CIBA 160 (*Halamphora adumbratoides*) are 4.1–6.6 and 1.6–2.4 µm, respectively. The number of dorsal and ventral striae for the CIBA 160 strain are 57–60 and 60–70 in 10 µm, respectively. The valve shape is semi-elliptical, and the ends are broadly rounded (Figure 3a–j). The raphe is straight with straight proximal ends, while the distal raphe ends are deflected to hooked dorsally (Figure 3a–b). Externally, two pores are visible at the end of the dorsal raphe ledge at each end of the raphe (Figure 3a–d black arrow), or they may appear fused (Figure 3d white arrow), while internally two, only a single pore is observed (Figure 3e–g, j). Internally, the proximal raphe ends terminated in a broad, fused central helictoglossa (Figure 3e–f, h, white asterisk). The dorsal raphe ledge is nearly absent, especially through the valve center (Figure 3a–d). A reduced dorsal raphe ledge is present, more accentuated towards the distal end of the valve (Figure 3a–b, black asterisk). The dorsal and ventral striae are continuous through the valve center, slightly radiate (Figure 3a–d), and not resolved in the LM (Figure 2b). Figure 3e–j shows internal views of the deformed valves and other deformed structures, like the raphe, which is in a different position than in the undeformed valves.

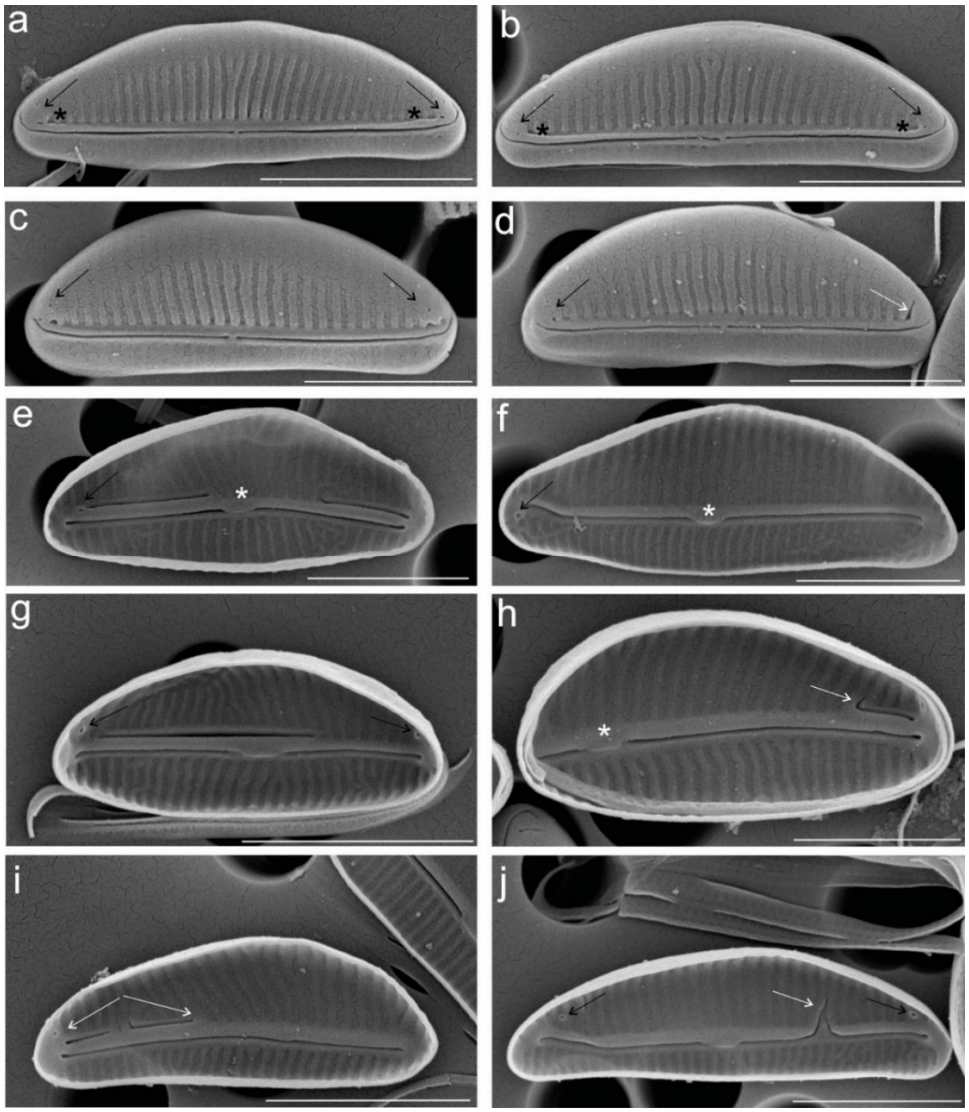

Figure 3. a–d. External view of the valve. a–d showing two distal pores (black arrows). The external and internal proximal raphe ends are not deflected, but the external distal raphe ends are dorsally deflected, a–d, e. Externally, the dorsal and ventral raphe ledges are small but continuous along the length of the valve, e–j. Internal view of the valve.

e, f, h. Internally, the proximal raphe ends terminate in a broad, fused central helictoglossa (white asterisks), e. f, g, h, j. One distal pore is visible (black arrows). f, g, h, i, j. White arrows show the abnormal dorsal raphe ledge. Scale bars correspondence: a, 3 µm, b–j, 2 µm.

Figura 3. a–d Vista exterior de la valva. a–d. se muestran dos poros distales (flechas negras). Los extremos proximales externo e interno del rafe no están desviados, pero los extremos distales externos del rafe están desviados dorsalmente, a-d. e. Externamente, los bordes dorsal y ventral del rafe son pequeños pero continuos a lo largo de la valva, e – j. Vista interna de la valva. e, f, h. Internamente, los extremos proximales del rafe terminan en una helictoglosa central amplia y fusionada (asteriscos blancos), e. f, g, h, j. Se observa un poro distal (flechas negras). f, g, h, i, j. Las flechas blancas muestran el borde dorsal anormal del rafe. Las barras de escala corresponden a: a, 3 µm, b – j, 2 µm.

Molecular analysis

We obtained 4,004 bp nucleotide sequences (SSU 1375 bp, *rbc*L 1444 bp, *psb*C 1185 bp; GenBank accession numbers: ON714546, ON736839, ON736840) for the CIBA 160 strain (*H. adumbratoides*) from the Pacific Ocean. Comparison of our sequences to those of the type species from the Atlantic Ocean in GenBank showed 100, 99.8, and 99.5% similarity for SSU, *rbc*L, and *psb*C, respectively. The BI and ML phylogenetic analyses placed the *H. adumbratoides* strains in one clade with *H. pellicula* as a sister clade (Figure 4). This node was supported strongly by the BI (100%) and ML (92%) analyses.

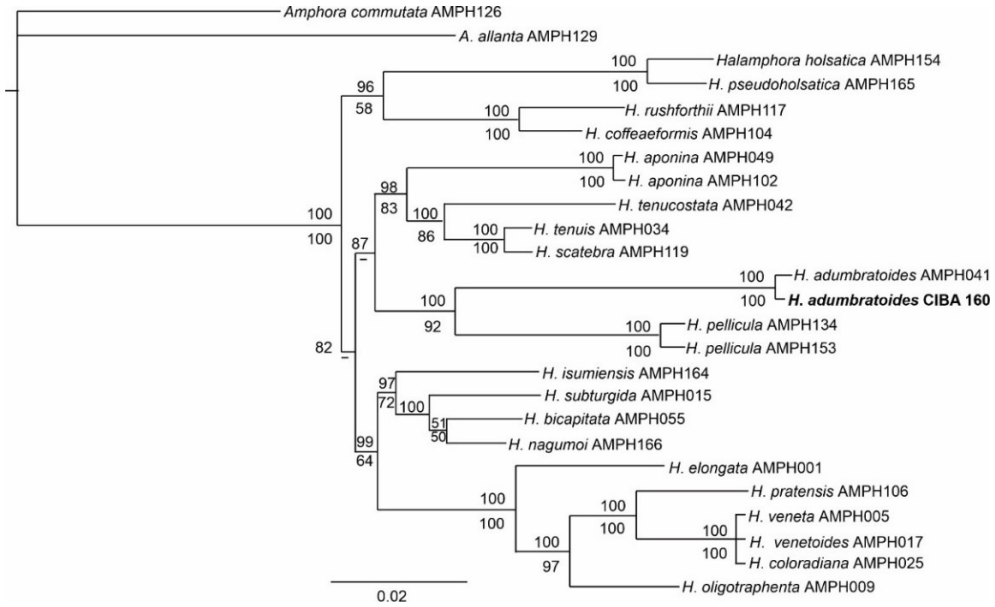

Figure 4. Bayesian tree of the *Halamphora* species based on the partial sequences of SSU, LSU, *psb*C, and *rbc*L. Bootstrap values of maximum likelihood analysis are given below, and posterior probabilities of Bayesian inference are given above the nodes. Nodes supported by less than 50% of Bootstrap values are indicated with the symbol `–´

Figura 4. Árbol Bayesiano de las especies de *Halamphora* basado en las secuencias parciales de SSU, LSU, *psb*C y *rbc*L. Los valores Bootstrap del análisis de máxima verosimilitud se dan debajo, y las probabilidades posteriores de la inferencia Bayesiana se dan arriba de los nodos. Los nodos soportados por menos del 50% de los valores de Bootstrap se indican con el símbolo `–´

DISCUSSION

Accurate identification of a species depends, in principle, on the methods' reliability. Historically, and even today, the identification of a diatom species is based on morphometric aspects observed under light microscopy, which requires extensive taxonomic experience (Mora *et al.*, 2019). The

characterization of certain diatom species is impossible only with this tool because of their small size and inconspicuous diagnostic structures (Li *et al.*, 2018; D. Mann *et al.*, 2020). Fortunately, this issue can be resolved with electron microscopy. However, not all diatom species can be delineated using morphology, especially species with subtle morphological differences (Beszteri *et al.*, 2007; Evans *et al.*, 2007; Malviya *et al.*, 2016). To aid in determining species boundaries among diatoms, molecular tools, such as DNA sequences are applied (An *et al.*, 2017; Beszteri *et al.*, 2007; Evans *et al.*, 2007; Guillard, 2005; D. Mann *et al.*, 2010, 2020; Medlin, 1991). However, this methodology has limitations as it may be unable to resolve the species identity (Malviya *et al.*, 2016) without conspecific and/or closely related species sequences in the nucleotide database. Therefore, morphological, and molecular methods are important in diatoms and should be considered as elements of a synergistic approach to species identification challenges.

After a morphometric review of the images of the laboratory cultured cells of the CIBA 160 strain obtained with both light and electron microscopy, it was not possible to identify this strain with any previously described taxon. However, approximations were made with *Amphora adumbrata* and *Halamphora adumbratoides*. Nevertheless, the morphometric analyses did not allow the taxonomic determination of the CIBA 160 strain as *H. adumbratoides* because the morphology of this strain does not exactly match the original description reported by Stepanek & Kociolek (2018), specifically due to the shape and size of the valve. In the original description of *H. adumbratoides*, the valves were narrow and semi-lanceolate, and the valve ends narrowly rounded to weakly subcapitate in larger specimens. The dorsal and ventral margins, however, do coincide with the original description, as they are arched and straight, respectively (Figure 3a–d). The presence of pores at the end of the dorsal raphe ledge at each end of the raphe was not mentioned in the original description. The axial area, not easily distinguishable in the original description, was not observed in the specimens of the CIBA 160 strain analyzed.

For the length and width of the valves, Stepanek & Kociolek (2018) reported ranges of 8.0–10.0 and 1.5–2.5 µm, while the cells (N=24) of the CIBA 160 strain recorded valve lengths and widths of 4.1–6.6 and 1.6–2.4 µm. Only the length range differed, as it was much shorter than in the original description. The number of dorsal and ventral striae in the CIBA 160 strain almost coincided with the original description, with 56–57 dorsal striae and *ca*. 70 ventral striae in 10 µm in the original description, while the striae range in 10 µm for the CIBA 160 strain were 57–60 and 60–70 for the dorsal and ventral striae, respectively.

A possible explanation for the size differences and morphological changes could be the effect of laboratory culturing on the cells of CIBA 160. In diatoms Rose & Cox (2013), Petrova *et al.* (2020) and Mohamad *et al.* (2022) have reported morphological changes in laboratory cultured cells. Diatoms tend to produce small-sized cells over time due to asexual reproduction (Mohamad *et al.*, 2022). This phenomenon has been demonstrated in 15 monoclonal pennate diatoms, but the width and number of striae did not differ from the original description (Mohamad *et al.*, 2022). Our results support the findings of Mohamad *et al.* (2022), and their proposal that the number of striae could be a useful character for species discrimination.

Our molecular analyses confirmed that the CIBA 160 strain belongs to *H. adumbratoides* by showing very high similarity to the sequences of this species from the Atlantic Ocean: 100, 99.8, and 99.5% similarity for SSU, *rbc*L, and *psb*C, respectively. The present study corroborates the results of Mohamad *et al.* (2022) that molecular data remain consistent irrespective of morphological changes in laboratory-cultured diatoms. Phylogenetic analyses of this study were also congruent with the relationship presented by Stepanek & Kociolek (2019) for the *H. adumbratoides* species, which is a sister group to *H. pellicula*.

We have registered morphological abnormalities in the cells of CIBA 160. The helictoglossa (Figure 3h white asterisk) is displaced to the left side of the valve. In contrast, the dorsal raphe ledge is very short and located only to the right of the valve (white arrow) (Figure 3h). The exact

opposite configuration is visible in Figure 3i (white arrow), where the structure is seen to the left of the valve. These abnormalities could be associated with long-term culture of cells in the laboratory (Estes & Dute, 1994; Falasco *et al.*, 2009; Petrova *et al.*, 2020).

In the genera *Amphora* and *Halamphora*, most taxonomic determinations and descriptions of new species are based on morphological analyses of specimens from natural populations (Levkov, 2009; Wachnicka & Gaiser, 2007). However, laboratory-cultured cells were also used for integrative taxonomic studies, combining morphological and molecular data (López-Fuerte *et al.*, 2020; Stepanek *et al.*, 2013, 2015a, 2015b, 2018).

It is important to mention that regardless of the origin of diatoms (natural or cultivated), the morphological description of a species must be accurate and informative, based on as much information as possible. This is because diatoms may present morphological variations throughout their life history, whether in the natural or artificial environment (Rose & Cox, 2013).

In our study, using morphological and molecular data has resulted in the reliable taxonomic identification of the CIBA 160 strain. Our results support using an integrative approach for fine-grained integrative taxonomic studies and the importance of laboratory cultures in the diatom taxonomy (Mohamad *et al.*, 2022).

CONCLUSIONS

The taxonomic identification of the CIBA 160 strain as *H. adumbratoides* was not possible based only on morphometric characteristics. This was due mainly to differences in the valve leaflet length and the presence of pores at the extremes of the valve on the raphe channel. Molecular analyses, however, allowed us to determine species identity with reference DNA sequences deposited in GenBank. Using DNA sequence data is important for cryptic, pseudo-cryptic diatom species and diatoms in general. Integrated approaches can play a vital role in discovering species with broad morphological variations.

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