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Molecular, morphological, and toxinological characterizations of an Argentinean strain of *Halamphora coffeaeformis* with potential biotechnological applications

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Abstract

Diatoms are a diverse group of unicellular microalgae with broad biotechnological and industrial applications. Recent studies have revealed that some species from the genus *Halamphora* accumulate high quantities of lipids, which make them potentially suitable for biodiesel production. However, due to their high phenotypic plasticity, molecular phylogenetic and ultrastructural analyses are usually needed for precise species identification, which in turn is crucial to define the cultivation strategies to obtain the main target compound. Moreover, the use of diatoms for nutrition, health, and aquaculture requires prior analysis of the potential toxicity of the selected species. In the present study we provide a detailed morphological, molecular, and toxinological evaluations of an Argentinian strain of *Halamphora coffeaeformis* with potential as feedstock of triglycerides adequate for biodiesel as well as a source of high-value metabolite production, in order to confirm its identification and to define the safety of its biomass in relation to domoic acid production. The obtained results will strengthen and diversify the use of this species in the microalgal market.

Keywords *Halamphora coffeaeformis* · 18S rDNA and *rbcL* sequences · Frustule ultrastructure · Toxicity

Introduction

Diatoms are a diverse group of unicellular microalgae found in marine and freshwater environments worldwide. In recent decades, diatoms have gained attention due to their broad biotechnological and industrial applications. Marketable products from diatoms include unsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5 ω-3) and arachidonic acid (ARA, C20:4 ω-6) for nutritional applications (Chauton et al. 2015), triglycerides (TAG) for biodiesel (Popovich et al. 2019); fucoxanthin with antioxidant, anticancer, antihypertensive, anti-inflammatory, and antiobesity effects (Zarekarizi et al. 2018) and anti-inflammatory peptides for cosmetics (Hayes et al. 2018). Other promising applications include the fields of nanomaterials, drug delivery vehicles, optical and immune-biosensors, and filters (Mishra et al. 2017). Moreover, diatoms are valuable indicators of ecological integrity, acidification, and eutrophication in aquatic ecosystems (Stoermer and Smol 2001).

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In order to allow an efficient exploitation of diatom species, a better understanding of their molecular, morphological, and toxinological characterizations is crucial to define the cultivation strategies necessary to obtain the main target compound. It is important to emphasize that these conditions are species and strain specific. The study of the shape and ultrastructure of the diatom cell walls or frustules represents the conventional taxonomic identification method (Round et al. 1990). However, in species difficult to identify from a morphological point of view, molecular phylogenetic analyses represent a key tool for a precise identification. This precision is important for commercial applications, new developments in biotechnology, genetic engineering, and patent development. Moreover, the use of diatoms for nutrition, health, and aquaculture requires prior analysis of the potential toxicity of the selected species (Hayes et al. 2018).

Among the diatom species studied for biotechnological applications are those belonging to the genus *Amphora sensu lato* (De la Peña 2007; Griffiths and Harrison 2009; Graham et al. 2012). In particular, recent studies demonstrated that some *Amphora* species accumulate high quantities of lipids (Scholz and Liebezeit 2013; Chtourou et al. 2015), which make them potentially suitable for biodiesel production. Recent taxonomical revisions suggest that most of these species correspond to the genus *Halamphora*, which was originally described by Cleve (1895) as a subgenus of *Amphora* and elevated to genus by Levkov (2009). Most *Halamphora* species live in marine or brackish water habitats, although they also occur in freshwater. The genus *Halamphora* comprises around 140 species (Kociolek et al. 2020), mostly defined by morphological characters, while only less than half of the *Halamphora* species have been characterized from a molecular point of view. The most extensive molecular analyses were held by Wang et al. (2014) and Stepanek and Kociolek (2019), and only the latter is associated with a detailed analysis of the valve morphology of the studied strains.

The type species of the genus *Halamphora*, *Halamphora coffeaeformis* (C. Agardh) Levkov, previously *Amphora coffeaeformis* (C. Agardh) Kützing, is frequently mentioned in brackish waters of Europe, South Africa and South America (Guiry and Guiry 2020). However, a detailed re-examination of *H. coffeaeformis* material from different locations revealed that this species has been misidentified based on morphological characters (Archibald and Schoeman 1984; Levkov 2009; López-Fuerte et al. 2020). A large diversity of *Halamphora* species morphologically resembling *H. coffeaeformis* has been detected from field samples in Argentina, leading to misidentifications (Sala et al. 1998). Sala et al. (1998) found six different species when comparing materials reported in different regions of the country with the type material studied by Archibald and Schoeman (1984).

Furthermore, some strains of *H. coffeaeformis* were found to produce the neurotoxin domoic acid (DA) (Shimizu et al. 1989; Maranda et al. 1990) that is associated with amnesic shellfish poisoning in humans, birds, and marine mammals (Lelong et al. 2012). However, this finding has been debated and currently the production of DA in diatoms is mainly associated to the marine planktonic genus *Pseudo-nitzschia*, which includes at least 26 toxigenic species (Bates et al. 2018).

Recently studies on growth, lipid accumulation and bioproducts of a strain morphologically identified as *H. coffeaeformis* isolated from Bahía Blanca Estuary (Argentina) suggest its potential as feedstock of triglycerides adequate for biodiesel (Martín et al. 2016, 2018), as well as a source of high-value metabolites (Popovich et al 2020; Scodelaro Bilbao et al. 2020). In this context, the present work presents a detailed morphological, molecular and toxinological evaluation of the Argentinian strain of *H. coffeaeformis* grown in a photobioreactor, used in all the above mentioned researches, in order to confirm its identification and to define the safety of its biomass in relation to DA production. These results will strengthen and diversify the use of this species in the microalgal market.

Materials and methods

Strain isolation and culture conditions

Halamphora coffeaeformis was isolated from Bahía Blanca Estuary (38° 45′ S, 62° 22′ W), Argentina, South America, in March 2015. The strain is maintained in stock cultures in f/2 medium (McLachlan 1973) at the Laboratorio de Estudios Básicos y Biotecnológicos en Algas (LEBBA), CERZOS-CONICET, Bahía Blanca, Argentina. The culture medium was prepared with natural seawater from Bahía Blanca Estuary at a salinity of 33 ppt. The cells were cultured in a 25-L cylindrical photobioreactor (FIGMAY S.R.L, Córdoba, Argentina) at 20 ± 1 °C with continuous bubbling of air (500–700 cm^3 min^{-1}) enriched with CO_2 (1%). Light was supplied by a panel of LED tubes in a 12:12-h light–dark photoperiod at an average of 100 μmol photons m^{-2} s^{-1}.

For molecular and morphological analyses, samples of 250 mL were harvested at the exponential growth phase (184 × 10^3 cells mL^{-1}), and for toxinological analyses, samples of 250 mL were harvested at the exponential (184 × 10^3 cells mL^{-1}) and stationary phases (134 × 10^3 cells mL^{-1}).

Morphological analysis

For morphological studies, live cells were observed under a Leica DM 2500 light microscope (LM) with phase contrast, equipped with a Leica DFC 420 digital camera. The material was then treated to eliminate organic matter following the method described in CEN/TC 230 (2002). Clean subsamples were mounted on permanent slides with Naphrax mounting medium, and the observations were conducted with a

differential interference contrast (DIC) Leica DM 2500 LM, equipped with a Leica DFC 420 digital camera. Morphometric data are based on the measurement of 70 specimens. The permanent slides of this strain were deposited in the diatom collection of the Herbarium of the División Ficología del Museo de La Plata with the acronym LPC 15909. For scanning electron microscopy (SEM), subsamples were mounted on glass stubs and coated with gold in a JEOL JFC-1100 fine coat ion sputter and observed using a LEO, EVO-40XVP (CC-CONICET, Bahía Blanca) and a Carl Zeiss NTS SUPRA 40 (Centro de Microscopías Avanzadas, Universidad de Buenos Aires). Terminology is according to Levkov (2009).

Molecular analysis

For DNA extraction, approximately 250 mL of culture was concentrated by centrifugation and flash frozen in liquid nitrogen. DNA was extracted with DNeasy Plant Mini kit (Qiagen). The nuclear gene 18S rDNA was amplified by PCR using the primers 18S-F53 (5′TTGTCTCAAAGATTAAGCCATG3′) and 18S-R1335 (5′CCTGTTATTGCCCCTATCTTCC3′) reported by Olivares-Rubio et al. (2017). The chloroplast gene *rbcL* was amplified by PCR using the primers rbcL-F (5′ GGACTCGAATYAAAAGTGACCG3′) and rbcL1444 (5′ GCGAAATCAGCTGTATCTGTWG3′) reported by Jiang et al. (2015). PCR conditions included an initial denaturing step at 95 °C for 2 min, followed by 36 cycles of 94 °C for 40 s, 56 °C for 50 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. The PCR products were sequenced by Sanger sequencing and edited in Sequencher 5.2 (Gene Codes Corp., USA). Homologous sequences reported by Stepanek and Kociolek (2019) were obtained by BLAST from NCBI databases, and multiple sequence alignments for each gene were constructed using Aliview v.1.26 (Larsson 2014). ML phylogenetic analyses were performed with RAxML v.8.0.0 (Stamatakis 2014) with 500 rapid bootstrap replicates. The 18S rDNA and *rbcL* sequences from the strain of *H. coffeaeformis* described here were deposited in GenBank under accession numbers MT982423 and MT982424, respectively.

Domoic acid production

For toxinological analyses samples were harvested at the exponential and stationary phases on days 3 and 7, respectively. Twenty milliliter (28×10^5 cells approximately) was filtered through GF/F filters (Munktel MGF) and stored at − 20 °C until analysis. DA was extracted from the filters by homogenization with extraction solvent (methanol:water; 50:50). The concentration of DA was carried out by HPLC with diode array detection following Quilliam (2003). Detection limit was 50 ng mL^{-1}. The system was calibrated using a DA standard (CNRC-NRC National Research Council, Canada). To increase analytical sensibility, a 500 μL aliquot of methanol extract was concentrated using vacuum centrifugation to dryness and resuspended in 200 μL of chromatographic eluent (water:acetonitrile:TFA; 900:100:1).

Results and discussion

Morphological characterization

Cells solitary with a single H-shaped chloroplast appressed to the ventral girdle; plastid contains several pyrenoids usually 4 (Fig. 1a, b). Frustules in girdle view broadly elliptical, 12–17.7 μm in width, with short rostrate ends (Fig. 1a, b). Valves semi-lanceolate, with convex dorsal margin and straight to slightly concave ventral margin; valve ends subcapitate except some smaller specimens with undifferentiated ends (Fig. 1c–i, l, m). Length 9.5–42 μm, valve breadth 4.5–7 μm. Dorsal mantle deep, with a marginal ridge along the junction of the valve face (Fig. 1i–k); ventral mantle shallow. Raphe lying near the ventral margin, weakly arched with straight raphe branches (Fig. 1c–h); dorsal raphe ledge moderately developed (Fig. 1h–k); externally proximal raphe endings straight (Fig. 1j) and distal raphe endings deflected towards the dorsal margin (Fig. 1k); internally proximal raphe ends straight with fused central helictoglossae (Fig. 1n); terminal ends deflected ventrally in poorly developed helictoglossae (Fig. 1o). Axial area narrow dorsally, slightly expanded at the ventral valve center (Fig. 1j, n). Dorsal striae biseriate, slightly radial throughout (Fig. 1h–o), 17–25 in 10 μm at the center, with 65–72 areolae in 10 μm. Ventral striae short, uniseriate, 35–40 in 10 μm (Fig. 1m–o) interrupted at valve center (Fig. 1j–n). Girdle composed of several open bands (Fig. 1p), with two rows of poroids elongated on the dorsal side of the frustule and one row on the ventral side, 40–45 areolae in 10 μm (Fig. 1q).

The morphology of the specimens observed during the present study coincides with the description of the type material of *H. coffeaeformis* provided by Archibald and Schoeman (1984) and the more recent morphological and molecular revision provided by Stepanek and Kociolek (2018). However, the studied materials have more ventral striae compared with the type material (35–40 vs. 21–36), and some specimens presented shorter valves when compared with previous descriptions (Table 1). The occurrence of small forms of *H. coffeaeformis* (5–9 μm) has been previously observed in cell cultures growing on solid media (Garduño et al. 1996).

The only morphological studies associated with strains analyzed from molecular point of view are in Stepanek and Kociolek (2018, 2019), and the fine morphology of the studied strain coincides with our materials. Based on these results, we can assign the studied material to *H. coffeaeformis* with certainty and also corroborate the description made for this strain by Martín et al. (2016).

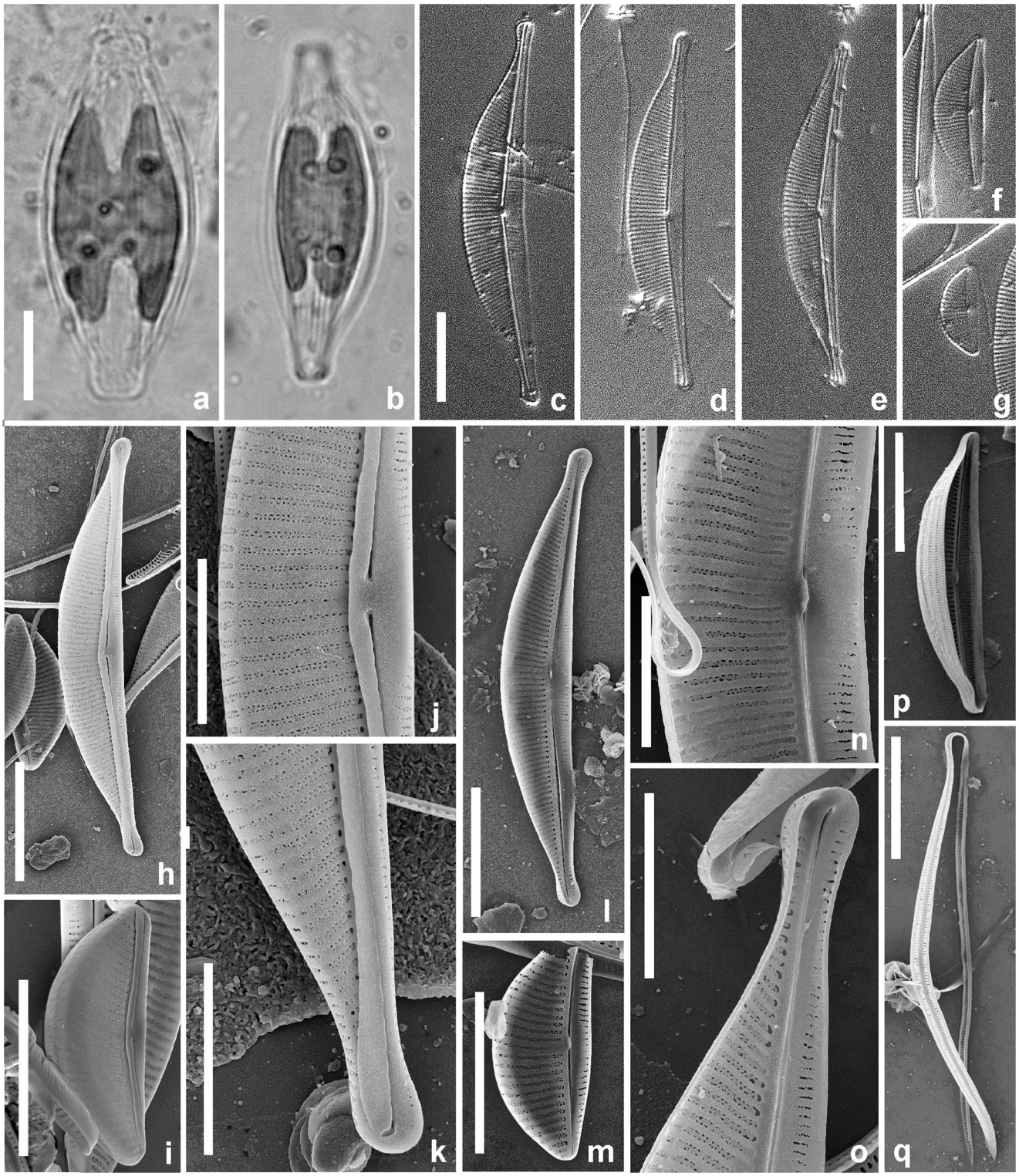

Fig. 1 *Halamphora coffeaeformis*: **a**–**g** LM; **a**, **b** cells *in vivo* observed under phase contrast, showing the H-shape chloroplast and the pyrenoids; **c**–**f** valve views observed under DIC, showing size and shape variation; **h**–**q** SEM; **h**–**k** external valve views; **h**, **i** whole valves; **j** detail of the central area and proximal raphe ends; **k** detail of the valve end and distal end of raphe; **l**–**o** internal valve views; **l**, **m** whole valves; **n** detail of the central area; **o** detail of the valve end; note distal end of raphe and helictoglossa; **p** valve with cingular bands in dorsal view; **q** open cingular band, note the dorsal side with two rows of areolae and the ventral side with one row of areolae. Scale bar: **a**–**i**; **l**–**m**; **p**, **q** 10 μm; **j**, **k, n**, **o** 5 μm

Table 1 Comparison of morphometric data of *H. coffeaeformis* from the literature and this study. *Archibald and Schoeman (1984)

H. coffeaeformis	Length (μm)	Width (μm)	Dorsal stria/ 10 μm	Ventral stria/10 μm	Areolae/10 μm (dorsal striae)	Girdle band pores/10 μm
This study (n = 70)	9.5–42	4.5–7	17–25	35–40	65–72	40–45
Type material*	14–55	3.5–7.3	16–26	21–36	51–93	37–45
Stepanek and Kociolek (2018)	25–40	5.5–7.5	16–19	30–32	nd	nd

Molecular characterization

Although *H. coffeaeformis* has a wide distribution and has been occasionally mentioned as a potentially toxigenic species, molecular information of this taxon has only recently become available. The complete sequences of the chloroplast and mitochondrial genomes of a single strain of *H. coffeaeformis* (Hamsher et al. 2019) and a few molecular markers of a handful of strains have been reported (Ruck et al. 2016; Saba et al. 2016; Stepanek and Kociolek 2019). Recently, morphological and ecological features were reevaluated based on an extensive molecular study of the genus *Halamphora* including 77 taxa from fresh, brackish, and salt water habitats in USA (≈ 40–48° N) (Stepanek and Kociolek 2019).

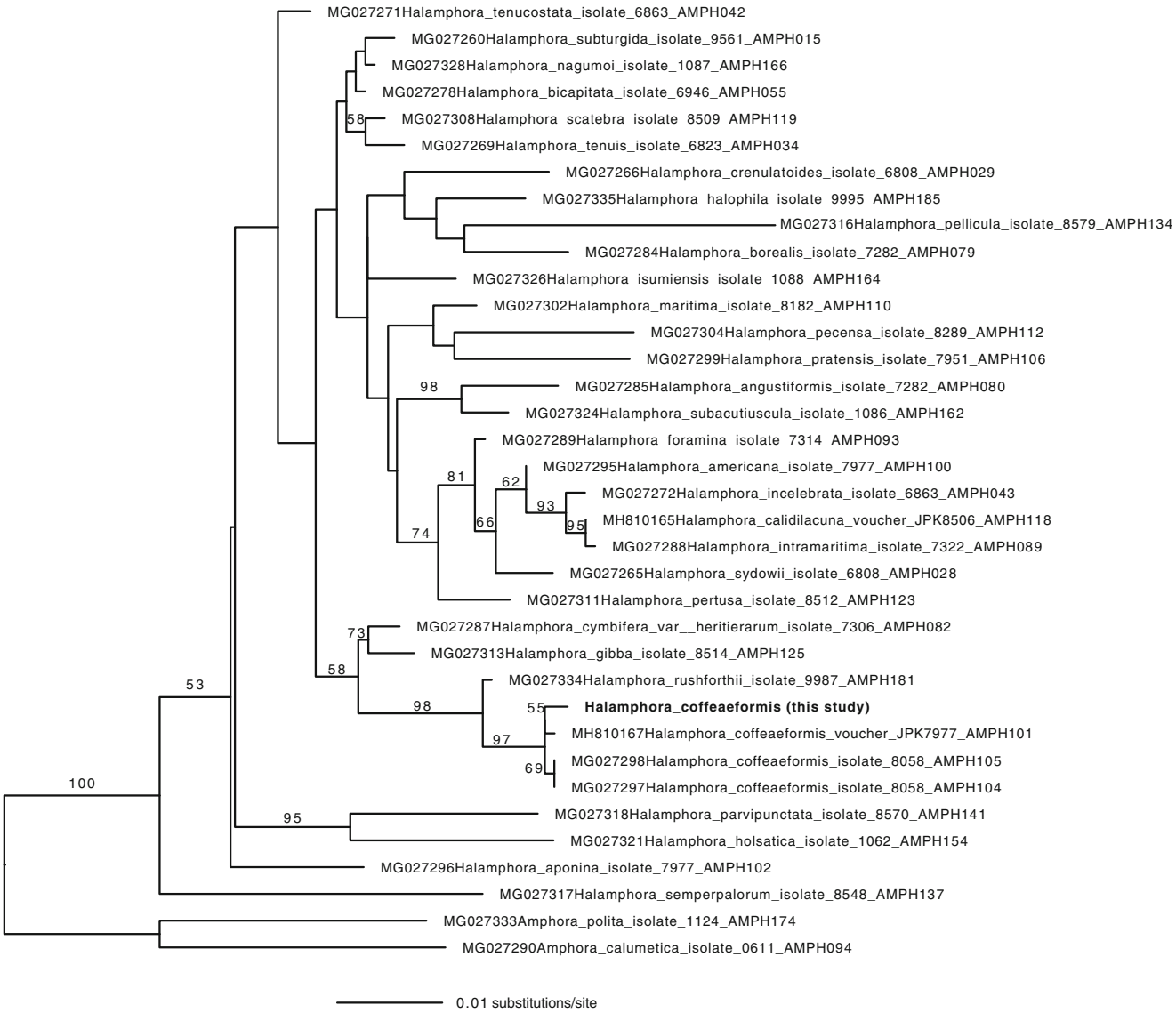

Fig. 2 Maximum likelihood phylogenetic tree of species of the genus *Halamphora* based on the nuclear gene 18S rDNA. Numbers above branches represent support values > 50% based on 500 bootstrap replicates

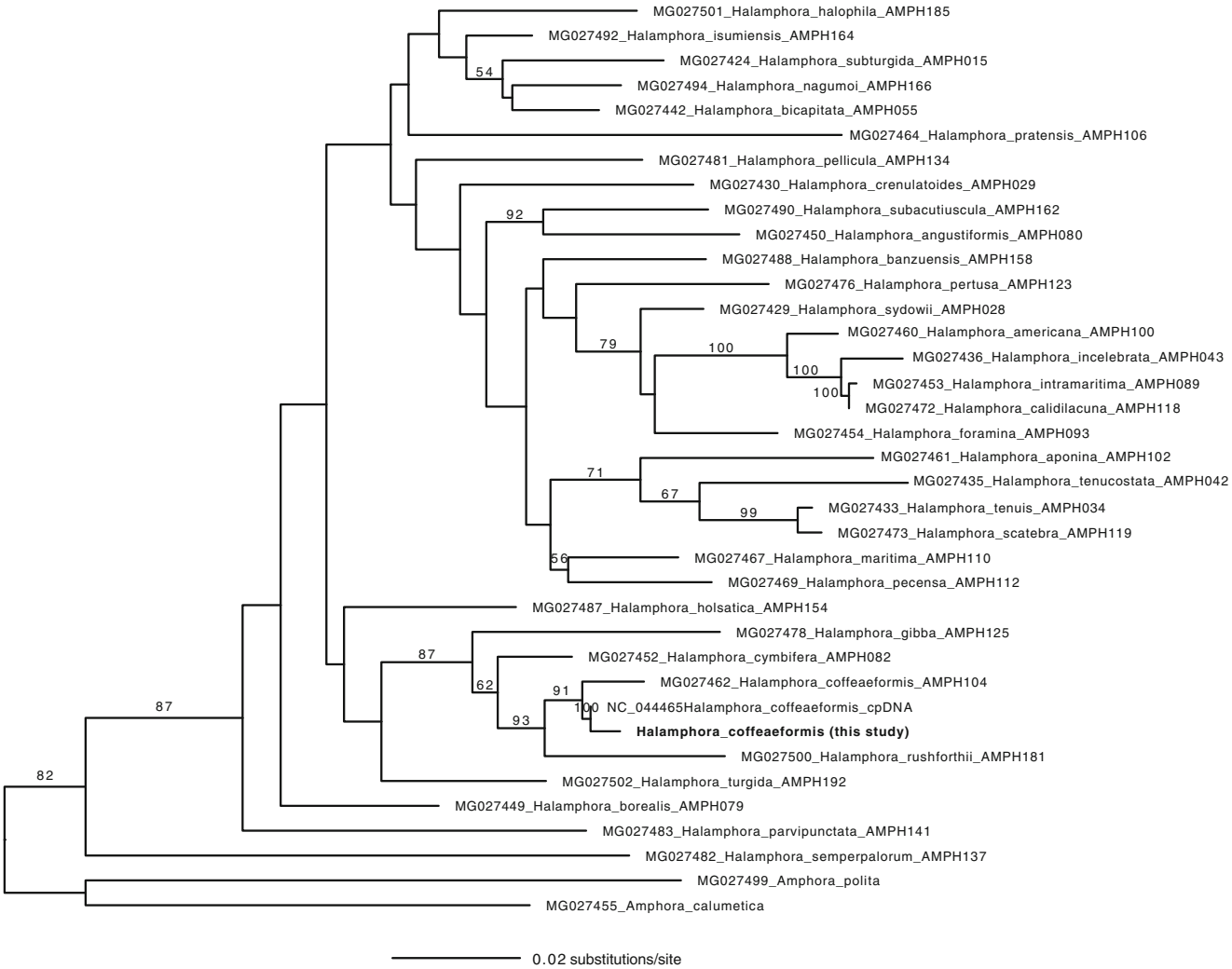

Fig. 3 Maximum likelihood phylogenetic tree of species of the genus *Halamphora* based on the chloroplast gene *rbcL*. Numbers above branches represent support values > 50% based on 500 bootstrap replicates

Sequences of the 18S rDNA and *rbcL* genes from the species under study grouped with *H. coffeaeformis* strains isolated from benthonic and epiphytic samples by Stepanek and Kociolek (2019) with strong bootstrap support, confirming the identification of the strain (Figs. 2 and 3). The mean identity of the 18S rDNA and of the *rbcL* sequences of the strain under study with other strains of *H. coffeaeformis* were 99.6% and 99.34%, respectively. The clade of *H. coffeaeformis* was sister to *H. rushforthii* with high bootstrap support in both phylogenetic trees, and these two species were found in a moderately supported clade with the species *H. gibba* and *H. cymbifera* (Figs. 2 and 3), as previously reported (Stepanek and Kociolek 2019). The phylogenetic relationships based on the nuclear 18S rDNA and plastid *rbcL* genes generally agree with no strongly supported incongruences (Figs. 2 and 3). Both phylogenies found low support for most relationships among species of *Halamphora*. Even a study based on four molecular markers could not recover a fully resolved phylogeny of the genus (Stepanek and Kociolek 2019).

Domoic acid production

The analyzed strain of *H. coffeaeformis* showed no production of the neurotoxin DA (Fig. 4) in both culture conditions: exponential and stationary growth phases.

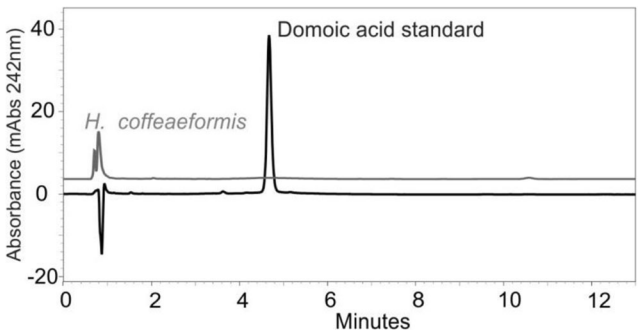

Fig. 4 HPLC chromatograms of toxins (absorption at 242 nm) from a domoic acid standard (black) and *H. coffeaeformis* concentrated extracts (gray)

Several species of the diatom genus *Pseudo-nitzschia* are known to produce DA, being the first record in Argentina in the year 2000 associated with *Pseudo-nitzschia australis* (Negri et al. 2004; Almandoz et al. 2017). In addition to *Pseudo-nitzschia* and a few *Nitzschia* species, DA has also been mentioned to be produced by *H. coffeaeformis* (Maranda et al. 1990). However, the last finding was later questioned by Bates (2000), given that other strains from the same area (Prince Edward Island, Canada and Nivå Bay, Denmark) were found to be non-toxic. Neither in Argentina toxic strains of *H. coffeaeformis* was detected. In line with this, the strain isolated in Bahía Blanca Estuary, tested negative for DA. The fact that DA was not detected in cultures of *H. coffeaeformis* makes it reasonable to continue developing biotechnological applications of this strain.

Conclusions

Biorefinery approaches can be classified based on the value of the main target compound, which can vary from low-value compounds (oils for biodiesel) to high-value ones (pigments, polyunsaturated fatty acids, essential amino acids, sterols). A strain of *H. coffeaeformis* isolated from Bahía Blanca Estuary (Argentina) and cultured under different culture strategies was recently found as a potential feedstock of marketable products, such as triglycerides for biodiesel production, as well as of ω-3 eicosapentaenoic fatty acid (EPA), sterols, essential amino acids, fucoxanthin, and chrysolaminarin. Our results allow assigning with certainty the studied material to the species *H. coffeaeformis*, showing a strong molecular affinity and high sequence identity with other strains of this species isolated from the USA and only minor morphological differences with the type material. Furthermore, the lack of toxin production in the strain studied is a remarkable feature considering its potential applications oriented to feed, food, and health.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interest.

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