

TIMESCALE LESSONS OF *DURINSKIA BALTICA* (KRYPTOPERIDINIACEAE, DINOPHYTA) FROM FRESHWATER THROUGH A MOLECULAR PHYLOGENY

LECCIONES SOBRE LA ESCALA TEMPORAL DE *DURINSKIA BALTICA* (KRYPTOPERIDINIACEAE, DINOPHYTA) DE AGUA DULCE A TRAVÉS DE UNA FILOGENIA MOLECULAR

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Abstract

Background: The evolutionary pattern of dinophytes is complex and requires comprehensive taxonomic studies addressing the species' development and morphology, ecology, evolution, and genetics.

Questions: How recent was the transition of *D. baltica* from Mexico to freshwater based on a calibrated phylogeny tree of the Kryptoperidiniaceae?

Studied species and data description: *Durinskia baltica*, Kryptoperidiniaceae and the known fossil record of dinotoms (endosymbiont diatom-host dinophytes).

Study sites and dates: Xochimilco, Mexico City. 2010 to 2019.

Methods: Phylogenies were constructed with Bayesian inferences and host sequences were calibrated with a relaxed clock method.

Results: We obtained genetic sequences of the 18S rDNA sector in the host and the *rbcL* sector in the endosymbiont. The Nitzschia-type endosymbiont is genetically distinct from endosymbionts of other dinotomes with the same diatom type. In contrast, considering the known freshwater species of *Durinskia*, the genetic relationship between hosts is robust and of recent origin. The antiquity of the Mexican sequence coincides with the geological origin of the Xochimilco basin, suggesting particularities in the evolutionary history of dinotomes linked to the geographic regions they inhabit.

Conclusions: Our evolutionary scenario with a calibrated phylogeny indicates that the transition of *Durinskia baltica* to freshwater in the Xochimilco basin is geologically contextualized. The historical environmental change of Lake Xochimilco with respect to salinity corresponds with an initial colonization of *D. baltica* in a brackish environment and its subsequent adaptation to freshwater.

Keywords: Algae, dinophytes, dinotoms, relaxed clock, taxonomy.

Resumen

Antecedentes: El patrón evolutivo de las dinofitas es complejo y requiere estudios taxonómicos exhaustivos, en desarrollo y morfología, ecología, evolución y genética de las especies.

Preguntas: ¿Qué tan reciente fue la transición de *D. baltica* de México hacia agua dulce con base en la filogenia calibrada temporalmente de las Kryptoperidiniaceae?

Especies estudiadas y descripción de los datos: *Durinskia baltica*, Kryptoperidiniaceae y el registro fósil conocido de dinotomos (dinofitas con diatomeas endosimbiontes)

Sitios y años de estudio: Xochimilco, Ciudad de México. 2010 a 2019.

Métodos: Se usaron inferencias bayesianas y secuencias de hospederos calibradas con un método de reloj relajado para generar las filogenias.

Resultados: Se obtuvieron secuencias genéticas del sector 18S rDNA en el hospedero y del sector *rbcL* en el endosimbionte. El endosimbionte es genéticamente distinto de otros endosimbiontes con el mismo tipo de diatomea. En cambio, considerando las especies de agua dulce conocidas de *Durinskia*, la relación genética entre hospedadores es robusta y de origen reciente. La antigüedad de la secuencia mexicana coincide con el origen geológico de la cuenca de Xochimilco, sugiriendo particularidades en la historia evolutiva de los dinotomos ligadas a las regiones geográficas que habitan.

Conclusiones: Nuestro escenario evolutivo con una filogenia calibrada indica que la transición de *Durinskia baltica* hacia agua dulce en la cuenca de Xochimilco está geológicamente contextualizada. El cambio ambiental histórico del lago de Xochimilco con respecto a la salinidad corresponde con una colonización inicial de *D. baltica* en un ambiente salobre y su posterior adaptación al agua dulce.

Palabras clave: Algas, dinofitas, dinotomos, reloj relajado, taxonomía

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D*urinskia baltica* (Levander) Carty & Cox has been identified in Mexico as a phytoplanktonic species in Xochimilco Lake (Lira *et al.* 2017). It belongs to the family Kryptoperidiniaceae, and it is a dinotom, i.e., a dinophyte possessing a tertiary diatom-like endosymbiont. To confirm the identity of the Mexican material, we compared it with the original description of the genus and the iconotype of *D. baltica*, which relied on a population from a drainage ditch in College Station, Texas, USA (Carty & Cox 1986, Figures 11-14, p. 202). The name *Durinskia baltica* is considered a heterotypic synonym of *Durinskia dybowskii* (Wolozinska) Carty (Molinari-Novoa 2022); for other authors and with plausible arguments, the legitimacy of the name *D. dybowskii* is in doubt (INA 2023, Lira *et al.* 2022). This controversy is one of the reasons why in this work, we maintain the name *D. baltica*.

Lira & Tavera (2019) provided life cycle knowledge for this species. This knowledge made evident this organism's particular developmental strategies in the freshwater environment, which explains its dominance in the phytoplankton of Xochimilco Lake (Lira *et al.* 2017). Throughout its morphological study and isolation in culture, it was necessary to analyze the controversial systematic history of the genus, which is related to an extensive ecological record (Lira *et al.* 2022).

Durinskia baltica has been observed in freshwater, marine, and brackish waters, and its geographic distribution covers most continents (Kretschmann *et al.* 2018). Such a wide geographic and ecological distribution is difficult to explain for a single species. In *Durinskia baltica*, when analyzing the ecological significance of its presence in such different environments, apparently worldwide, it is necessary to face the historical complexity of its systematic circumscription. This issue has been extensively addressed in previous studies (Lira *et al.* 2022). There is also a description of *Durinskia oculata* (Stein) Hansen & Flaim as a distinct freshwater entity, recently confirmed with a genetic study of its type material (Kretschmann *et al.* 2018). To complete a comprehensive characterization of the population recorded in Mexico, its genetic analysis and molecular phylogenetic comparison with *D. oculata* and other freshwater species of the genus for which sequences exist in databases was mandatory. This analysis also included brackish water and marine sequences available for dinotoms. We used the 18S rDNA sector to amplify the sequence of the dinophyte, and as it is a dinotom, we also considered the genetic analysis of the endosymbiont based on the sector coding for the large subunit of ribulose, 1-5-bisphosphatase carboxylase/oxygenase (*rbcL*). In parallel and based on database sequences, we explored the *rbcL* sector in a Bayesian inference of present-day diatoms associated with dinotom endosymbionts.

The temporal component that places events in a phylogeny plays an essential role in understanding relationships among dinophytes that have colonized freshwater, as is the case for the species of our interest (Žerdoner-Čalasan *et al.* 2018, 2019). Even with the extent of the recent literature spectrum (Table S1, Supplementary material), it is notable that calibrated molecular dating is scarcely addressed for the family Kryptoperidiniaceae. Thus, we aimed to base the phylogenetic analysis on the fossil record and a relaxed molecular clock. In the evolutionary context of the colonization of freshwater environments by dinophytes, the case of the Mexican population stood out because the molecular calibration for the host sequence coincides with the geological origin proposed for the Xochimilco basin, from which the specimens studied here originate (Arce *et al.* 2019).

Material and methods

Study area. *Durinskia* material was based on isolated populations from Xochimilco Lake from 2010 to 2019 (Figure 1); this lake is currently a system of natural freshwater canals, sometimes very wide, located in central Mexico (19° 16' N, 99° 00' W; 19° 16' N, 99° 04' W; 19° 16' N, 99° 05' W). Observations of the organisms were made using differential interference contrast (DIC) with a Nikon-Optiphot photon microscope (Tokyo, Japan) equipped with a Nikon Coolpix S10 camera.

Processing for scanning electron microscopy (Jeol 5410 LV microscope, Massachusetts, USA) followed standard protocols previously described in Lira *et al.* (2017). Dinokaryon observations were performed by staining single cells with 1 µl ml⁻¹ of 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) (Sigma-Aldrich™ D95242, Missouri, USA). Epifluorescence image was acquired using an upright confocal microscope (TCS-SP8, LEICA, Wetzlar, Germany). Cells were excited with a 405 nm laser and emission was collected between 410-480 nm with a hybrid detector. Samples were observed with 63x/1.40 oil immersion objective.

Dating of the molecular phylogeny of *Durinskia baltica*

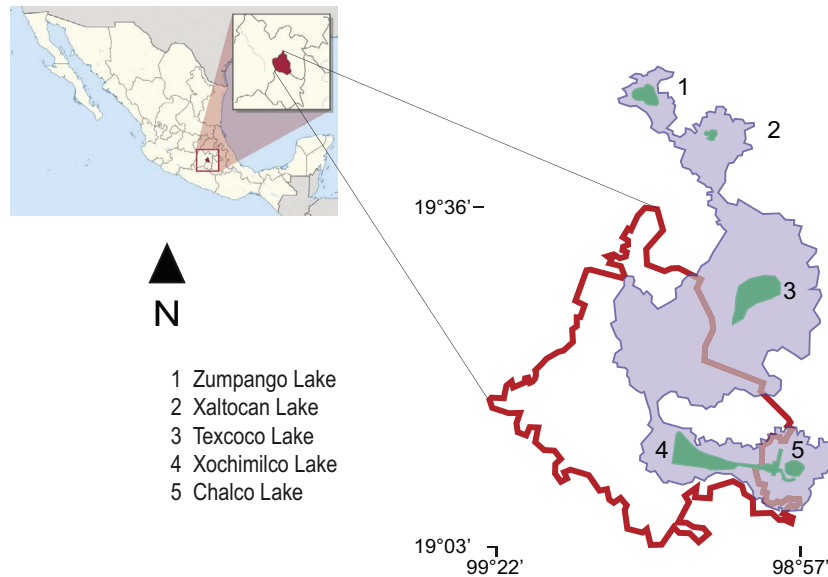


Figure 1. Map of Mexico showing (left) Mexico City (CDMX), filled in red. On the right, CDMX is bounded by a red line. The blue area delimits the original lake area (until the 12th century). Current lakes are shown in green. The number corresponds to the list of names. Map source: Commons license under GNU General Public <https://commons.wikimedia.org/w/index.php?curid=1192145> (public domain).

Molecular phylogenies. We extracted genomic DNA from cultured cells using the Power Biofilm DNA Isolation Kit (MoBio Laboratories, California, USA) according to the manufacturer's specifications. For the amplification of the 18S rDNA region, four sets of primers were used (Tavera & Díez 2009, Lira 2018), and for the sector coding *rbcL*, we used specific diatom primers according to Tamura *et al.* (2005) (Table 1), following the protocols proposed by DNA polymerase Taq Master Mix (Ampliqon, Odense, Denmark) for both regions. For 18S rDNA, PCR amplification conditions were as follows: 95 °C / 15 min, followed by 40 cycles at 95 °C / 30 s; 60 °C / 30 s; 72 °C / one min, and a final extension at 72 °C / five min, to end with four °C / ∞. For *rbcL*, the conditions were: initial temperature of 94 °C / three min, followed by 35 cycles of 94 °C / one min; 50 °C / one min; 72 °C / one min, 72 °C / five min, and four °C / ∞.

Sequences (Sanger) were obtained through MacroGen Sequencing Services (MacroGen Inc. Korea) and manually cleaned and edited using the BioEdit editor (Hall 1999). Multiple sequence alignment methods were SINA for 18S rRNA sequences and MUSCLE for *rbcL* sequences available in the GenBank database (Edgar 2004, Priesse *et al.* 2012, Sayers *et al.* 2022). Forty-one partial sequences of 1,300 bp, including the one in our study, supported the alignment of the 18S rRNA and 30 partial sequences of 1,400 bp supported the alignment of the *rbcL* gene. BEAST v. 2.6.7 software was the frame for Bayesian inference analysis of sequences and estimation of divergence times (Aleksyenko *et al.* 2022). The analysis in BEAST focused on a relaxed molecular clock with a log-normal distribution for interbranch rate variation and an assumption of independent rates between branches (gamma). In addition, we selected the GTR model of sequence evolution and a Yule-calibrated prior on evolutionary rates. Divergence dates were obtained from the dinophyte literature and from the fossil record calculated in the evolutionary Timetree of life, founded on diversification studies with multigene molecular clocks (Fensome *et al.* 1996, Berney & Pawlowski 2006, Parfrey *et al.* 2011, Heled & Drummond 2012, Kumar *et al.* 2017, Žerdoner-Čalasan *et al.* 2019). A series of 6'000,000 generations, with a 10 % burn-in, was completed on the BEAST dataset. Trees from BEAST analyses were summarized with Tree Annotator v. 1.7.1 and visualized with FigTree v. 1.4.4 (Drummond & Rambaut 2007, Rambaut 2018).

Table 1. A) Primer sequences used to amplify the 18S rRNA and *rbcL* regions (specific for diatoms). B) Obtained sequences and size.

A		
Gene	Primer	Sequence
18S rRNA	DbalF	5'-AACCTGGTTGATCCYGCCAG-3'
18S rRNA	DbalR	5'-TGATCCTTCTGCAGGTTACCTAC-3'
18S rRNA	EUKA	5'-AACCTGGTTGATCCTGCCAGT-3'
18S rRNA	EUKB	5'-TGATCCTTCTGCAGGTTACCTAC-3'
<i>rbcL</i>	DiatrbcL2F	5'-ACAGTAAAACCTAAATTAGG-3'
<i>rbcL</i>	DiatrbcL5R	5'-ATTTGACCACAGTGGATACC-3'
B		
Obtained sequence	Base pairs	
EUKA (18S)	1014	
EUKB (18S)	1223	
DbalF (18S)	600	
DbalR (18S)	600	
CONTIG EUKA-EUKB (18S)	1165	
DiatrbcL2F (<i>rbcL</i>)	407	
DiatrbcL5R (<i>rbcL</i>)	659	
DiatrbcL2F- DiatrbcL5R (<i>rbcL</i>)	560	

Results

Morphology. In the Mexican material, vegetative cells were spherical to ellipsoidal. Dorsoventrally, cells were slightly flattened, with 24.0-34.0 µm long dimensions and 18.0-22.0 µm wide. Generally, the epitheca and hypotheca were similar in size. The cingulum presented a downward planozone orientation, with a wide groove not reaching the antapex. A prominent red stigma locates within the hypotheca; it is hook-shaped, according to Lira *et al.* (2017). The epitheca presented the typical red accumulation bodies. The cell presented numerous golden, discoid chloroplasts scattered throughout (Figure 2A). The dinokaryon (nucleus) was located in the center of the cell and presented the condensed chromosomes characteristic of dinophytes; its dimensions were 16.0-24.0 µm long and 10.0-12.0 µm wide (Figure 2A, B). The thecal formula consisted of the following arrangement: Po, x, 4', 2a, 6''', 5c, 4s, 5''''', 2'''''' (Figures 3, 4). Thecal plates were asymmetrical. Plate 1' was rhomboidal and exceptionally large (Figure 3A, B). The plates showed fine, scattered pores and pores parallel to the suture lines (Figure 4A). The cell presented an inconspicuous ridge surrounding the apical pore (Figure 4B) and presented two intercalary plates located just above the 3'' and 4'' plates, one followed by the other: plate 1a, was smaller than plate 2a and diamond-shaped (Figures 3B, 4B). Through transmission electron microscopy, Lira *et al.* (2017) showed that the nucleus of endosymbiotic origin, associated with chloroplasts, is surrounded by a simple membrane, thus isolating it from the dinokaryon and host cytoplasm.

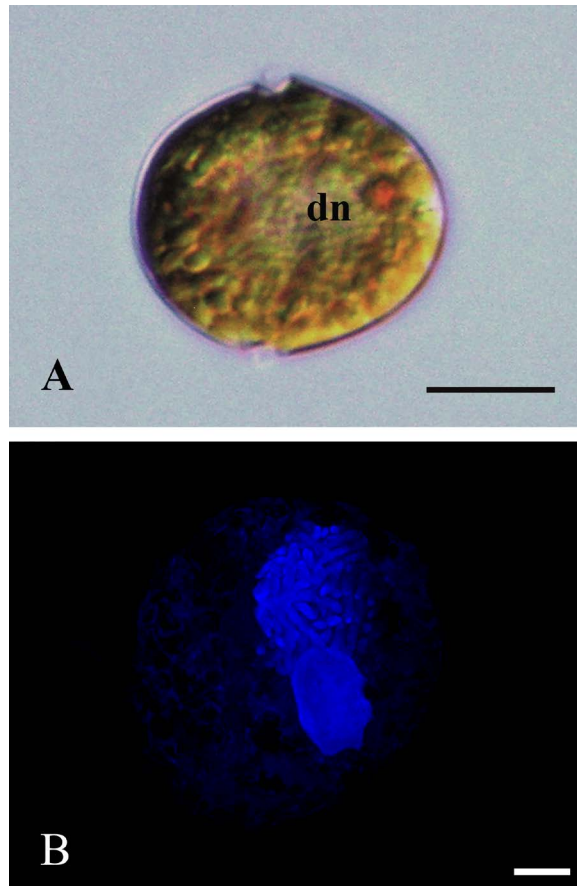


Figure 2. A): ML photograph of a *D. baltica* cell (sample from Xochimilco Lake) showing the typical dinophyte dinokaryon (dn) with condensed chromosomes and a red accumulation body in the epitheca; some chloroplasts towards the periphery of the cell. Bar = 10 μ m. B): Epifluorescence photograph highlighting with DAPI staining the nucleus from *D. baltica*, and the nucleus from its endosymbiont (sample from Lago Xochimilco). Condensed chromosomes stand out in dinokaryon, besides the smaller endosymbiont nucleus. Bar = 5 μ m.

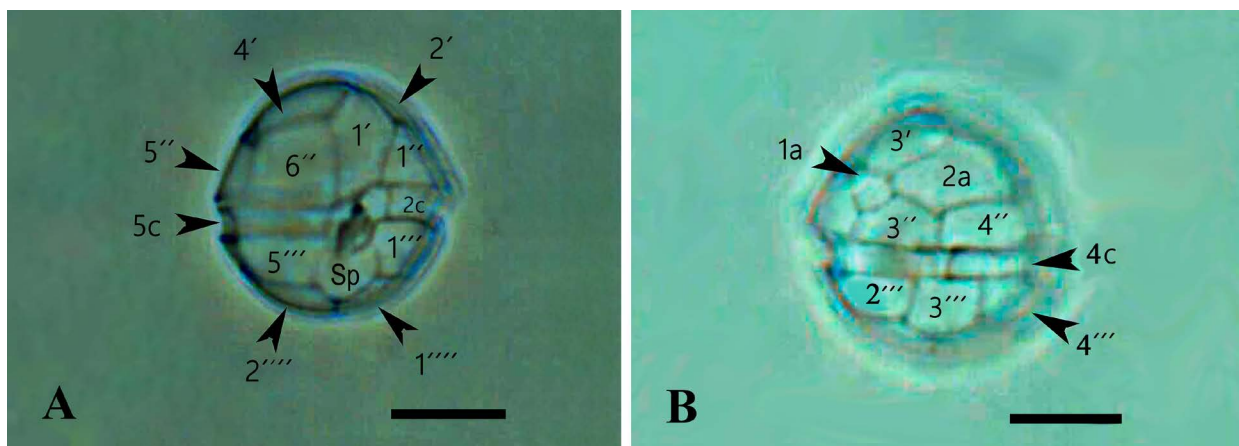


Figure 3. A): ML photograph of a *D. baltica* theca (sample from Xochimilco Lake) showing thecal plate nomenclature in ventral view. Bar = 10 μ m. The posterior sulcal plate (Sp) reaches the antapical region just between the suture of antapical plates 1'''' and 2'''''. Over Sp is visible the folding of the left sulcal plate (Sd). B): ML photograph of a *D. baltica* theca (sample from Xochimilco Lake) showing thecal plate nomenclature in dorsal view. Visible the two intercalary plates, 1a and 2a that characterize *Durinskia* species. Bar = 10 μ m.

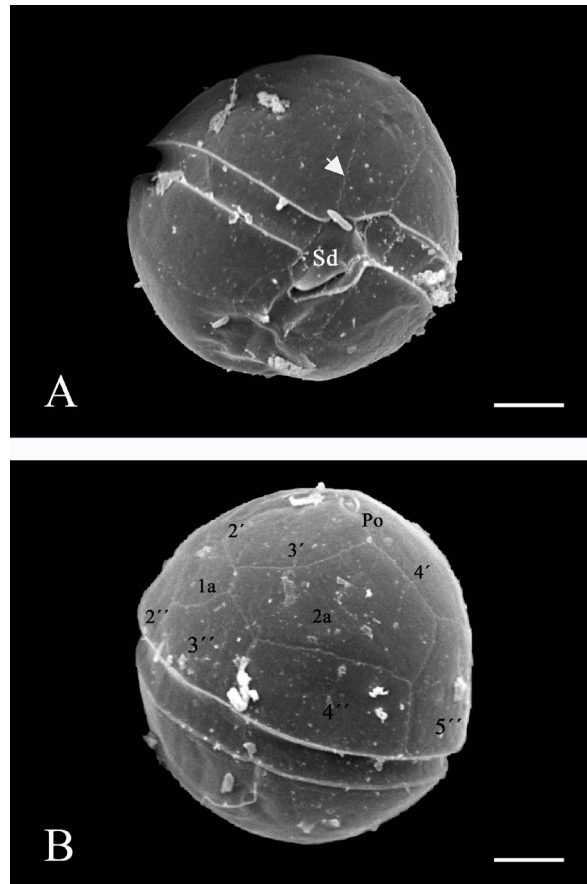


Figure 4. A): In ventral view, a SEM micrograph with backscattered electrons for 10 kV of a *D. baltica* theca (sample from Lago Xochimilco). The cingulum shows the Sd plate forming a fold over the sulcal area, the arrow points to the pores parallel to the suture lines. Bar = 5 μ m. B): SEM micrograph with backscattered electrons for 10 kV of a *D. baltica* theca (sample from Lago Xochimilco) in dorsal view, with plate nomenclature in the epitheca. The apical pore (Po) and plate 1a, characteristic of the genus, stand out. Bar = 5 μ m.

Genetic analysis. 18S rRNA sequences of populations isolated from cultures of *Durinskia*, selected from Xochimilco, Mexico, were examined using GenBank datasets for the same marker in this and other genera from several families of Dinophyta (41 sequences). This dataset was analyzed using Bayesian criteria. The monophyly of the family Kryptoperidiniaceae revealed a high support value (BPP 0.94) (Figure 5). *Peridinium quinquecorne* Abé was basal and formed the most senior group together with *Perkinsus marinus* (Mackin, Owen & Colliere) Levine, representing Alveolates (outgroup), which designated *P. quinquecorne* (circa 750 mybp) as one of the earliest dinophytes to diverge from Alveolata (about 1300 mybp, Parfrey *et al.* 2011). Diversifying in the Mesozoic era distinguished the Dinophyta group, probably initiated in the late Permian (Fensome *et al.* 1996, Žerdoner-Čalasan *et al.* 2019). Our results indicated that the ancestors of marine, brackish, and freshwater dinophytes appeared around the Ordovician-Silurian boundary (443.8 mybp). In the case of *Durinskia*, freshwater species were grouped with brackish-water species, sharing a common ancestor. The origin of these species was among the most recent. In the marine sequences, only that representing dinotoms with temporary endosymbionts such as *D. kwazulunatalensis* Yamada, Sim & Horiguchi and *D. capensis* Pienaar, Sakai & Horiguchi (Yamada *et al.* 2017, 2019), showed a recent origin (Figure 5).

In our results, several freshwater species in Kryptoperidiniaceae appear to have originated less than 50 mybp ago (Figure 5). The freshwater *Durinskia* species, *Durinskia oculata* (Europe) and *Durinskia baltica* (Mexico), originated 20 mybp. The origin calculated for the Mexico sequence was very close to the geological origin of the lake basin (Arce *et al.* 2019).

Thirty chloroplast sequences were analyzed with Bayesian criteria; *rbcL* was the marker used for the Mexican population and a GenBank dataset for the same marker in dinotoms and extant diatoms (Figure 6). The genetic analysis determined that the *rbcL* sequence from Xochimilco distinguished a tertiary endosymbiont of the *Nitzschia* type. Most of the endosymbiont sequences were associated with freshwater Bacillariophyceae diatom sequences, mainly with *Nitzschia* Hassall species, regardless of whether or not the endosymbiont corresponded to a brackish, marine, or freshwater host. Only the endosymbionts of *Peridinium quinquecorne* and *Peridiniopsis* Lemmermann species corresponded environmentally with freshwater or marine diatom sequences of the class Mediophyceae. In no case was the sequence of an endosymbiont associated with that of another endosymbiont from different host species.

Discussion

Morphology. The cell shape in dinotoms of the genus *Durinskia* from continental environments varies, ranging from globular to slightly dorsoventral. In the literature, this feature is considered necessary for describing the species (Hansen & Flaim 2007, Cavalcante *et al.* 2017, Lira *et al.* 2017, Kretschmann *et al.* 2018, Moestrup & Calado 2018, Lira & Tavera 2019). In our material, there are specimens with spherical cells and ellipsoidal cells, both slightly dorsoventrally flattened.

In populations of *Durinskia* isolated from freshwater environments, including those in this study, there are smooth plates with few delicate and minute pores, scattered without order, and some aligned parallel to the plate margin (Hansen & Flaim 2007, Lira *et al.* 2017, Kretschmann *et al.* 2018, Lira & Tavera 2019). In the genus systematics, size, shape, and dorsoventral flattening are variable (Moestrup & Calado 2018). Suppose the presence of delicate pores were the diacritic character. In that case, there is no clear morphological difference between *D. baltica* from Mexico and *D. oculata* from Prague (Moldava River, the type locality of its basionym *Glenodinium oculatum* Stein) (Kretschmann *et al.* 2018). Regarding the genetic basis, the similarity of their sequences in the 18S rRNA phylogeny (Figure 5, Table S2, Supplementary material) would certainly support their synonymy. Their endosymbionts, on the other hand, do not appear to be so clearly related (Figure 6, Table S3, Supplementary material), so if the endosymbiosis occurred with different species of diatoms and perhaps in different regions and environments, there would not be a solid basis for using a single name at the moment. As we explained before, we decided to retain the name *D. baltica* for the population of Mexico.

Genetic Analysis. Analysis of the host sequences of the *Durinskia* population from Mexico generated an 18S rRNA phylogeny for dinotoms that is consistent with those presented by several authors, including the character of monophyly for Kryptoperidiniaceae (Horiguchi & Takano 2006, Moestrup & Daugbjerg 2007, Yamada *et al.* 2017, Kretschmann *et al.* 2018). The morphology of our population supports monophyly by showing that it possesses the apomorphic characters that distinguish it, i.e., the endosymbiont core, the hooked stigma, and the presence of only two intercalary plates (Pienaar *et al.* 2007, Takano *et al.* 2008, Lira *et al.* 2017, Kretschmann *et al.* 2018). The possible time of origin of dinotoms through molecular calibration of sequences has yet to be fully explored in Kryptoperidiniaceae. There are time estimates for Gymnodiniaceae and Peridinales, made to date marine to freshwater transitions. Since the sample from Mexico corresponds to an epicontinental environment, the molecular dating of the phylogenetic trees for the sequences was consistent with the information known so far (Žerdoner-Čalasan *et al.* 2019).

The phylogeny calibrated in our study also supports the postulate that, within each family, brackish and freshwater species had a more recent origin than marine species (Figure 5). The location of the ancestors of marine, brackish, and freshwater dinophytes that calibrated phylogeny suggests near the Ordovician-Silurian boundary (443.8 mybp) supports the transitions that have been proposed from secondary to tertiary endosymbiosis as having an essential role in the history of dinotomes and other dinophytes (Pienaar *et al.* 2007, Hehenberger *et al.* 2014, Ishida & Green 2022). As far as is known, dinotomes with a tertiary endosymbiont evolved from an ancestor with secondary endosymbiosis containing peridinin. The only remnant of the ancestral secondary endosymbiosis is preserved in the characteristic stigma they possess. Fucoxanthin, as in diatoms, is the carotenoid produced in the present-day chloroplasts of dinotoms (Pienaar *et al.* 2007, Hehenberger *et al.* 2014, Bai *et al.* 2022, Ishida & Green 2022).

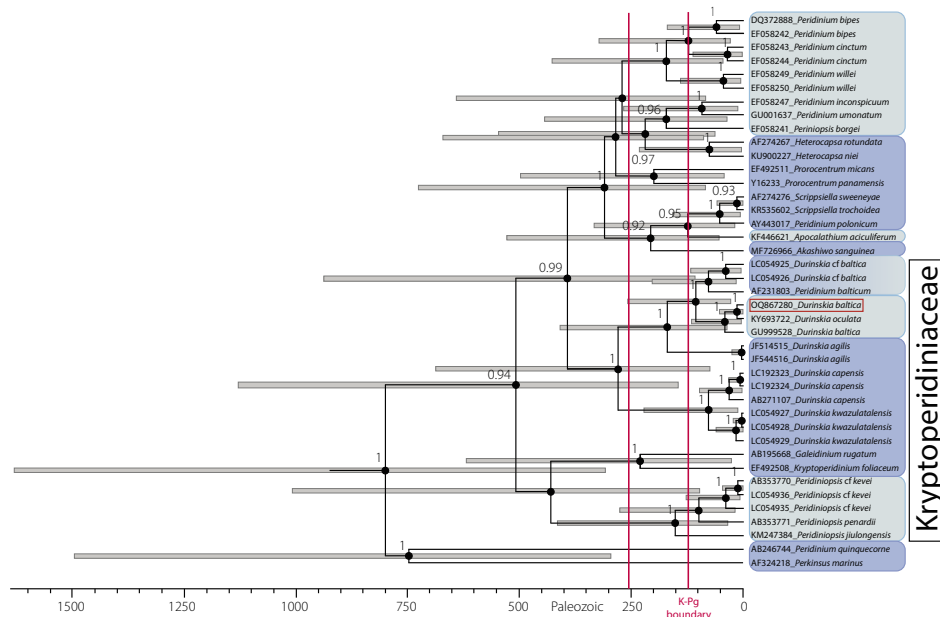


Figure 5. Dated phylogeny of Dinophyta obtained from BEAST analysis. Numbers at each node correspond to Bayesian posterior probability values. Gray bars represent 95 % confidence intervals for the height posterior density (HPD) in the estimates of divergence dates. The time scale is in millions of years before the present (mybp). Bayesian posterior probabilities < 90 are not shown, as the ages they represent are imprecise. The Mesozoic era is represented between the red vertical lines, indicating the K-Pg boundary. Light blue distinguishes freshwater taxa, dark blue marine taxa, and degraded blue brackish taxa. *Durinskia baltica* from Mexico framed in red.

Other studies on dinophyte antiquity posit the marine-freshwater transition of several species (Fensome *et al.* 1996, Žerdoner-Čalasan *et al.* 2019). Also, consistently, our analysis presented significant diversification for freshwater species after the Cretaceous-Paleogene boundary (66 mybp) indicated as the K-Pg boundary (Figure 5). Branches representing freshwater sequences formed a strongly supported sister relationship with branches from marine sequences, which agrees with previous literature (Gottschling *et al.* 2017, Žerdoner-Čalasan *et al.* 2019). As mentioned by Kretschmann *et al.* (2018), the isolated arrangement of the freshwater sequence groups in the phylogenies suggests that there is little relationship between them; at the same time, their closest relatives within each family are marine or brackish origin (Figure 5).

The sample from Mexico belongs to a freshwater environment, and the freshwater dinotom species in Kryptoperidiniaceae, Peridiniaceae, and Peridiniopsidaceae have endosymbionts whose sequences are similar to diatoms such as *Discostella*, *Thalassiosira*, and *Cyclotella*, or *Nitzschia*-like, all from freshwater (Figure 6).

The genetic distance between *Durinskia* sequences from saline and freshwater environments was robust (BPP = 1.0), and they could have diverged by more than 250 mybp (Figure 5). The most recent origin for freshwater dinophytes of this genus was indicated by the sequences of *D. oculata* from Prague (type locality, sequence KY693722) and *D. baltica* from Xochimilco (sequence OQ867280), both 20 mybp. They showed a close genetic relationship and shared a common ancestor with other freshwater sequences, such as sequence GU999528 of *D. baltica* from China (Figure 5, Table S2, Supplementary material).

The results suggest that, by contrast, their endosymbionts have accumulated a different rate of change, implying a different degree of differentiation (Figure 6, Table S3, Supplementary material). Other studies have mentioned this observation (Zhang *et al.* 2011, Kretschmann *et al.* 2018), but further research is needed to establish how many alleles their genomes share and how much synteny exists, as in diatoms it is highly variable (Crowell *et al.* 2019). In this study, it was clear from the BPP values that the Bayesian phylogeny result was not informative enough to support a molecular calibration for endosymbionts (Figure 6). Also, exploring the *rbcL* sector of extant diatoms associated with dinotome endosymbionts revealed that genetic relationships among *Nitzschia* species are

unclear (Figure S1, Supplementary material), which may have contributed to weakening the endosymbiont analysis (Sorhannus 2007).

As pointed out by Žerdoner-Čalasan *et al.* (2018), “endosymbionts of Kryptoperidiniaceae found their closest relatives in free-living diatoms and not in other algal hosts.” Sequences from *Durinskia baltica* from Mexico and *D. oculata* from Europe showed the same (Figures 5, 6, Tables S2, S3, Supplementary material). Their endosymbionts do not share a close relationship (Zhang *et al.* 2011, Kretschmann *et al.* 2018). A comparable case was indicative in the marine dinotoms *D. capensis* and *D. kwazulunatalensis*, which have similar morphology and phylogenetic relationship and harbor distantly related endosymbionts. However, we do not regard it with equal importance because, in these two species, the endosymbionts have a different degree of incorporation with the host; in *D. capensis*, it is a temporary endosymbiont incorporated through kleptoplastidy, and in *D. kwazulunatalensis* the host incorporation is incomplete because it possesses several endosymbiont nuclei, not just one (Yamada *et al.* 2017, 2019). According to Moestrup & Daugbjerg (2007), those species with incomplete endosymbionts might be uninformative for phylogenies.

Our results support the proposed diversity of endosymbionts occurring in dinotoms, which belong to at least five diatom genera, according to Yamada *et al.* (2017). Although some host-endosymbiont genetic integration has not been proved in *Durinskia*, differences at the specific level of putative endosymbionts (*Nitzschia* type) may be considered in the systematics of this genus concerning the cytosolic activity of hosts that is not yet sufficiently studied (Hehenberger *et al.* 2016, Janouškovec *et al.* 2016).

Several authors point out that in most dinotoms, especially marine dinotoms, the tertiary endosymbiont is of *Nitzschia* type and suggest that the replacement of the endosymbiont (and hence the current diversity) linked to the evolutionary history of dinophytes (Horiguchi & Takano 2006, Takano *et al.* 2008, Imanian *et al.* 2010, Hehenberger *et al.* 2016, Yamada *et al.* 2017). In the case of *D. baltica* from Mexico, endosymbiosis may have occurred in a brackish continental environment and subsequently transited to freshwater because Xochimilco was part of a large

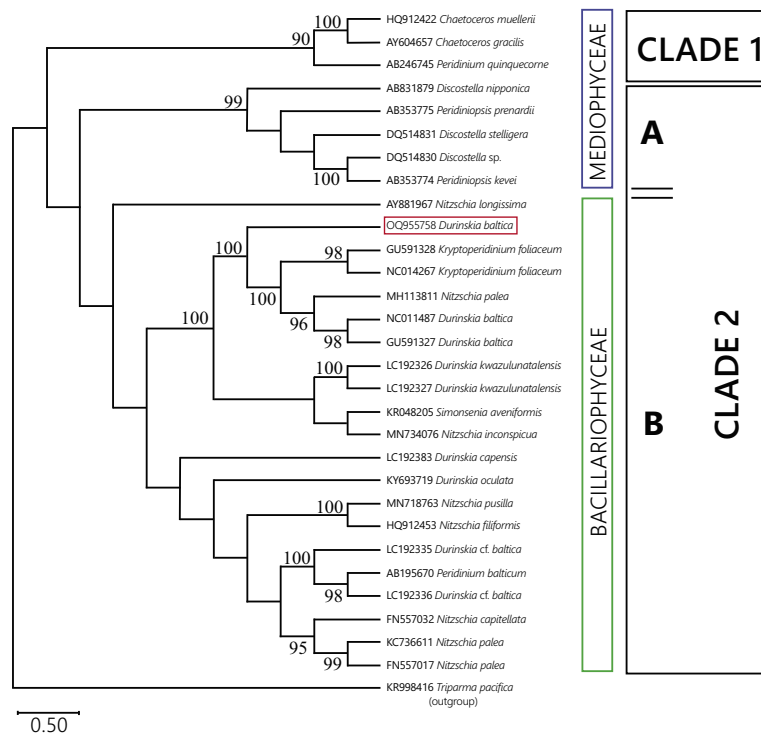


Figure 6. Bayesian phylogeny of *rbcL* sequences of dinotome endosymbionts and diatoms. Endosymbionts sequences are named after the host to which they belong. Numbers next to nodes correspond to Bayesian posterior probability values. For each clade, the class-level membership of the current diatom sequences is indicated. Clade 1 assembled diatom and endosymbiont sequences from marine habitats. Clade 2 assembled sequences from freshwater (Mediophyceae) and marine or freshwater (Bacillariophyceae) diatoms and endosymbionts. Endosymbiont of *Durinskia baltica* from Mexico framed in red.

brackish lacustrine area (Figure 1). The current freshwater condition appears to have begun about 8,000 years ago (Ortega-Guerrero *et al.* 2018). In our calibrated phylogeny (Figure 5), the origin of the Mexican dinotom places 20 mybp, and the geological origin of the Valley of Mexico basin to which Xochimilco belongs is 26 mybp (Arce *et al.* 2019). It is not precludable that some replacement in the endosymbiont diatom type would have occurred in the evolutionary history of *D. baltica* from Xochimilco. According to Yamada *et al.* (2017), there are records of *Nitzschia* species replacements in several dinotoms.

One observation that stands out in that evolutionary history is that currently, *Durinskia baltica* from Mexico corresponds with a dinotom that controls the karyokinesis of its guest and inherits it to daughter cells, *i.e.*, it is not the kleptoplastid or incomplete model proposed in the literature (Yamada *et al.* 2017, Kretschmann *et al.* 2018, Yamada *et al.* 2019). The character of the endosymbiont as definitive is strongly supported by previous studies of the Mexican population in culture, with light microscopy, epifluorescence, and transmission electron microscopy observations, which confirmed that a single membrane surrounds the single nucleus, chloroplasts, and ribosomes of the endosymbiont, permanently separating it from the host's cytoplasm; this structure is not modified during the life cycle and inherits by daughter cells (Lira *et al.* 2017 [Fig. 3], Lira & Tavera 2019).

We conclude that the calibrated phylogeny reflected the proposed general times of origin for dinotomes and allowed us to temporally place the proposed evolution of the group about the acquisition of tertiary endosymbionts. For example, *Durinskia*, *Peridinium*, *Peridiniopsis*, and *Kryptoperidinium* possess diatom-like endosymbionts whose evolutionary line separates them from those with a haptophyte-like endosymbiont. Therefore, it was crucial to place the Mexican species in the dating context that has been proposed in the literature for marine and freshwater dinotomes. Notably, the focus of this study highlighted that the evolutionary transition of marine dinophytes to brackish or freshwater proposed in the literature is geologically contextualized in the case of the Xochimilco basin, suggesting the occurrence of colonization of *D. baltica* in a brackish environment and its posterior adaptation to freshwater was consequent to the environmental change of Xochimilco Lake for salinity.

Likewise, the calibrated phylogeny indicates that freshwater *Durinskia* populations share a common ancestor and could have diverged before colonizing freshwater around the K-Pg boundary as proposed in the literature; this also applies to other freshwater dinotomes of *Peridinium* and *Peridiniopsis* (Figure 5). The differences between the endosymbiont diatom types are a product of each particular environment colonized by the dinotoms and is an exciting line of research going forward.

Supplementary material

Supplemental data for this article can be accessed here: <https://doi.org/10.17129/botsci.3259>

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