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Prorocentrum rivalis sp. nov. (Dinophyceae) and its phylogenetic affinities inferred from analysis of a mixed morphological and LSU rRNA data set

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Abstract :

A new freshwater epiphytic *Prorocentrum* species, *Prorocentrum rivalis*, from the temperate region of the Haute-Vienne, France, is described. This species is the third freshwater species identified among approximately 60 marine *Prorocentrum* species. This new species is described using scanning electron microscope and phylogenetic analyses by a polyphasic approach (LSU rRNA sequences combined with 9 morphological characters). The phylogenetic analysis attests that *P. rivalis* is close to other planktonic freshwater species and the freshwater *Prorocentrum* clade is evolutionarily derived from an epiphytic freshwater prorocentroid ancestor. The unique marine species in the freshwater clade results from an ecophysiological reversion. *P. rivalis* differs from other epiphytic taxa by its rarity, its temperate distribution and its ecophysiological needs. The phylogeny confirms also that all planktonic *Prorocentrum* species are evolutionarily derived from epiphytic/benthic ancestors.

Keywords : *Prorocentrum rivalis* – epiphytic microalgae – LSU – phylogeny – polyphasic approach – SEM

1. Introduction

The dinoflagellate genus *Prorocentrum* Ehr. consists of about 60 validly published and identifiable species (Murray et al. 2009) and is usually considered to be exclusively marine, occurring in oceanic, neritic, and littoral environments (Hoppenrath & Leander 2008). However, two species (*P. playfairi* and *P. foveolata*) are described from Australian freshwaters and fresh coastal lagoons (Croome & Tyler 1987).

Only a few epiphytic *Prorocentrum* species have been described. All these species are marine and mainly associated with the production of okadaic acid or its analogues. Moreover they often constitute a significant part of water toxin producers. These epiphytic *Prorocentrum* species are cosmopolitan and the majority has been described from tropical and subtropical waters of the Caribbean Sea, Pacific and Indian Oceans (Aligizaki et al. 2009).

However, a new epiphytic *Prorocentrum* taxon has been recorded in the Vienne and Aurence rivers in France in 2009. Thus the aim of this study is to describe the morphology and the phylogeny of this rare species which has particular distribution and ecological needs.

2. Materials and methods

2.1. SEM preparation

Macrophyte samples covered with epiphytic microalgae were collected in the Vienne river near the Fournet bridge (45°42’42’’N 1°52’17’’E, Rempnat, Haute-Vienne, France) and in the Aurence river near the north industrial zone of Limoges (45°52’86’’N 1°16’77’’E, Limoges, Haute-Vienne, France). The biological material was fixed overnight in a Sorensen’s phosphate buffer (75% NaH_2PO_4 (v/v), 25% KH_2PO_4 (v/v), pH 7.2) containing 2.5% glutaraldehyde (v/v). Samples for scanning electron microscopy (SEM) were dehydrated in an ethanol graded series (10° ethanol (1x), 30° ethanol (1x), 50° ethanol (1x), 70° ethanol (1x), 90° ethanol (1x), absolute ethanol (4x)), sputter-coated with gold, and observed using a FEI Quanta 200 SEM.

2.2. Choice of the phylogenetic method

Despite of 99 investigations in nine Vienne and Aurence river stations on macrophytic and benthic substrates, and in the water column, only two populations were found. Only morphological characters could be observed on this new species because of their SEM preparation. Its place in the *Prorocentrum* genus phylogeny was done by applying the robust and widely used polyphasic method (Berrendero et al. 2008; Cameron & Williams 2003; Varga et al. 2007; Yli-Mattila et al. 2002): some morphological characters from 25 *Prorocentrum* species and from the new species were used to establish cladisitic relationships between taxa and then materialized in a cladogram. Some gene sequences of the 25 species were also used to put evolution quantifications in the same phylogenetic analysis in order to transform the precedent cladogram into a phylogram. The efficiency of this phylogenetic methodology combining morphological and molecular data, particularly in the case when some important data such as the genetic sequence of the studied species is missing, has been proved especially in palaeobiological studies where molecular data lack (Giribet 2010; Springer et al. 2001). Indeed, the question of missing data has been evaluated in detail by many authors who agree in that missing data do not affect the phylogenetic analysis (Kearney 2002; Norell & Wheeler 2003; Wenzel & Siddall 1999; Wiens 1998, 2003, 2005, 2006).

2.3. Morphological characters

All morphological characters used in this study are listed in Tables 1 and 2. These characters were commonly used in the taxa discrimination of the *Prorocentrum* genus (Cohen-Fernandez et al. 2006; Cortés-Altamirano & Sierra-Beltrán 2003; Faust 2008; Grzebyk et al. 1998) because of their relevance in the species determination. Thus they were of important advised interest here. They were coded as discrete with the exception of two characters treated as continue and measured with the biometric software Visilog 6.404 Viewer (Noesis 2005).

2.4. RNA sequences and alignment

The LSU rRNA gene sequences from 25 *Prorocentrum* species (Tables 1 and 2) were aligned using the CLUSTAL X algorithm (Thompson et al. 1997). The monophyly of the *Prorocentrum* genus has been proved several times (Hoppenrath & Leander 2008; Saldarriaga et al. 2004) so no outgroup is necessary. The open and extended gap penalties were set to 10 and 5, respectively.

2.5. Phylogenetic analyses

An unweighted maximum parsimony (MP) phylogenetic analysis of the aligned sequences was performed using the PHYLIP program (Felsenstein 1989). All parsimony analyses utilized branch and bound searches, unless otherwise indicated. MP tree was estimated for the combined nucleotide and morphological data sets. Gaps were coded as single characters, regardless of their length, and appended to the nucleotide data matrix. Bootstrap analyses implemented in PHYLIP (heuristic search, 500 replicates, branch swapping) were performed to provide measures of relative support for each node estimated in the maximum parsimony tree.

3. Results

3.1. *Prorocentrum rivalis* Delmail, Labrousse, Crassous, Hourdin, Guri et Botineau sp. nov. (Fig. 1 a-c).

Latin diagnosis. Cellula photosynthetica, ovata, 9.9-15.3 µm longa et 3.4-6.4 µm lata. Valvae convexae, poris numerosi. Area apical valvae dextra ovata. Porus flagellaris magnus et pori apicali parvuli et sine spina apicale. Balteus intercalaris horizontale striatus.

Type localities. Vienne river, Rempnat, Haute-Vienne, France (45°42’42’’N 1°52’17’’E) and Aurence river, Limoges, Haute-Vienne, France (45°52’86’’N 1°16’77’’E).

Holotype. Collected in nature in April 2009 and deposited at the LIMO Herbarium (Fig. 1 a-c).

Etymology. *rivalis* (Latin, “from river sites”) referring to the freshwater distribution of this species.

General morphology. Ovate cell, 9.9-15.3 µm long, 3.4-6.4 µm wide. Overall shape biconvex. Valve surface smooth with marginal pores. Pores uniformly small with smooth margin ranging in diameter from 0.07 to 0.010 µm (Fig. 1b). Periflagellar area like an ovoid depression in the anterior end of the right valve (Fig. 1c). Flagellar pore large and ovate. Auxiliary pores smaller, narrow and ovate. Periflagellar area without ornamentation such as an apical spine. Intercalary band horizontally striated.

Occurrence. This species was recorded in the Vienne and Aurence rivers in April 2009. The species does not co-occurred with other *Prorocentrum* species.

Habitat. The Vienne river is one of the most important rivers in south-western France, a significant left tributary of the lower Loire. The Aurence river is a 27-km tributary to the Vienne river which flows along the town of Limoges’ northern and western boundaries. These two rivers have very close chemical properties listed in Table 3. *P. rivalis* lives in a river environment, epiphytic on the macrophyte *Myriophyllum alterniflorum* (Haloragaceae). Only a mucilage allows the adhesion of *P. rivalis* on the macrophyte leaves. Other macrophyte taxa co-occur in this environment (*Callitriche hamulata* (Plantaginaceae), *Ranunculus peniciliatus* (Ranunculaceae), *Fontinalis squamosa* (Fontinalaceae), *Dermatocarpon weberi* (Verrucariaceae)). The lighting is shade due to the presence of many caducifoliate trees, the water column is shallow and the water flow is heavy.

3.2. Phylogenetic relationships

Determining the ecological clades is the best way to process and understand the *Prorocentrum* phylograms (Hoppenrath & Leander 2008; Murray et al. 2009). The *Prorocentrum* species could be separated in two different ecological clades (Fig.2). A high bootstrap support value is noted for the planktonic clade (83.4). The studied species, except those in the previous planktonic clade, form a paraphyletic benthic/epiphytic clade. However three planktonic species (*P. foveolata*, *P. playfairi* and *P. arabianum*) are distributed in the paraphyletic clade. *P. rivalis* sp. nov. is epiphytic and is part of the paraphyletic clade. This new species has close relationships to *P. playfairi*, *P. foveolata* and *P. borbonicum*.

4. Discussion

P. rivalis sp. nov. is branched within paraphyletic clade with high statistical support (bootstrap of 100). The most closely related species to *P. rivalis* sp. nov. are the planktonic freshwater species (*P. playfairi* and *P. foveolata*) and the epiphytic marine *P. borbonicum* which form a distinct clade with high statistical support (bootstrap of 100). These three species are also the most similar to *P. rivalis* sp. nov. from a morphological perspective (Tables 1 and 2). All four species are small, have an ovate cell shape and an unornamented periflagellar area.

From an evolutionary point of view, the most parsimonious theory is to consider that the ancestor of the four-species clade was epiphytic and acquired freshwater ecophysiology. *P.*

borbonicum ancestors undergo later an ecophysiological reversion to become marine and ancestors of the two Australian freshwater species developed later the planktonic way of life. Due to the phylogenetic proximity of the three freshwater species, these previous common morphological characteristics could be typical of a freshwater *Prorocentrum* ecophenotypy.

Our analysis confirmed earlier reports that *Prorocentrum* species form two distinct ecological clades (Grzebyk et al. 1998; Hoppenrath & Leander 2008; Litaker et al. 1999; Murray et al. 2005; Saldarriaga et al. 2004): a benthic/epiphytic clade and a planktonic clade (Fig. 2).

Saldarriaga et al. (2004) have reported a weakly supported sister relationship between the two *Prorocentrum* ecological clades in phylogenetic analyses of LSU rDNA sequences which is highlighted in our study by the presence of three planktonic species (*P. foveolata*, *P. playfairi* and *P. arabianum*) in the benthic/epiphytic clade. These three species could be considered as homoplasies due to convergent ecological evolution with the planktonic clade which is polyphyletic. In further studies it would be important to increase the number of sequenced species and individuals to enhance the phylogenetic resolution of this sister relationship and to confirm it.

However, the Prorocentrales form a robust monophyletic group with no ecological differentiation from a morphological and cladisitical perspective, but phylogenetic studies do not refute the monophyly of this order and provide significant insights into the evolutionary history (Hoppenrath & Leander 2008; Saldarriaga et al. 2004). Indeed our polyphasic study suggests that planktonic *Prorocentrum* species are evolutionarily derived from epiphytic/benthic prorocentroid ancestors as the ecology of the less derived *Prorocentrum* species is epiphytic (*P. emarginatum*) and benthic (*P. sculptile*). However, the number of independent convergences/reversions from a benthic to a planktonic mode of life remains unknown and currently molecular phylogenetic data using ribosomal gene sequences cannot adequately address hypotheses of ecophysiological evolution within the *Prorocentrum* genus. Our knowledge of *Prorocentrum* diversity and the exploration of several different molecular phylogenetic markers, such as nucleus encoded protein genes must be improved (Hoppenrath & Leander 2008).

Concerning the ecophysiology, *P. rivalis* sp. nov. is found epiphytically in freshwater habitats. Studies on microorganisms attest that predominantly marine species could also occasionally survive in low-salt environments (Rengefors et al. 2008). However, the localities where this new species was found are not in the vicinity of any marine or brackish water inputs. Indeed these stations are located at 183 kms for the Aurence locality and at 245 kms for the Vienne locality from the nearest salt waters of the Atlantic Ocean. Thus *P. rivalis* sp. nov. appears to be a truly freshwater species with no marine affinities.

As said previously, the genus *Prorocentrum* is predominantly present in marine waters and tolerate high intensities of photosynthetically active radiation (PAR) spanning most of the spectrum and so have pigment adaptations (Helbling et al. 2008). However, *P. rivalis* sp. nov. is present in shallow waters where PAR is restricted to the red end of the spectrum (Luria & Kinney 1970). It would be useful to study the photosynthetic pigment characteristics of *P. rivalis* sp. nov. and to compare them with the two other freshwater *Prorocentrum* species. Moreover it will be interesting to see if biochemical pathways present in *P. rivalis* sp. nov. lead to the production of toxins like their epiphytic marine relatives.

Thus it would be essential to multiply *ex situ* investigations to find many *P. rivalis* sp. nov. specimens to develop their *in vitro* culture in order to sequence parts of its genome for phylogeny and to analyze its biochemical components like eventual toxins. It would be also important to make ecological studies about natural populations of *P. rivalis* sp. nov. (e.g. dynamics, reproduction) to evaluate the rarity degree of this species because it is only present in 2% of the 99 studied localities.

5. Acknowledgments

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7. Tables

Table 1. LSU rRNA GenBank accession numbers and morphological characters of the 13 planktonic *Prorocentrum* species used in the polyphasic phylogenetic analysis. The number in brackets below the discrete/continue value of the morphological character corresponds to the article which references the data: (1) Grezbyk and Sako, 1998; (2) Cortés-Altamirano and Sierra-Beltrán, 2003; (3) Hernández-Becerril et al. 2000; (4) Steidinger and Tangen, 1996; (5) Fukuyo, 2004; (6) Krakhmalnyy, 2005; (7) Lu and Goebel, 2001; (8) Lu et al. 2005; (9) Morton et al. 2002; (10) Croome and Tyler, 1987; (11) Cohen-Fernandez et al. 2006.

		Cell morphology			Valve surface				Periflagellar area	
	LSU rRNA	Length (µm)	Width (µm)	Cell shape	Concave vs. convex	Intercalary band	Ornament	Pore pattern	Shape in apical view	Ornament
P. arabianum Morton & Faust	EF566752	45.0 (9)	37.5 (9)	Ovate (9)	Concave (9)	Horizontally striated (9)	Depressions (9)	Scattered (9)	V-shaped (9)	None (9)
P. balticum (Lohmann) Loeblich III	AF042816	9.5 (7)	13.5 (7)	Ovate (4)	Convex (4)	Transversally striated (4)	Spines (4)	Scattered (4)	V-shaped (4)	Spine (4)
P. compressum (Bailey) Abé ex Dodge	EF517256	40.0 (4)	27.5 (4)	Ovate (4)	Convex (4)	Smooth (4)	Depressions (4)	Scattered (4)	V-shaped (4)	Spine (4)
P. dentatum Stein	AY863006	18.5 (3)	9.0 (3)	Pyriform (3)	Convex (3)	Smooth (6)	Spines (3)	Marginal (3)	Oblong (6)	None (6)
P. donghaiense Lu	EU586259	19.0 (7)	11.8 (7)	Pyriform (7)	Convex (7)	Horizontally striated (8)	Spines (7)	Scattered (7)	V-shaped (7)	None (8)
P. foveolata Croome & Tyler	AY259173	27.5 (10)	20.5 (10)	Ovate (10)	Concave (10)	Transversally striated (10)	Depressions (10)	Scattered (10)	Ovoid (10)	None (10)
P. gracile Schütt	EF517251	50.0 (11)	24.0 (11)	Pyriform (11)	Convex (11)	Smooth (11)	Depressions (11)	Radial lines (11)	Oblong (11)	Spine (11)
P. mexicanum Osorio Tafall	AF260378	35.0 (1)	22.5 (1)	Ovate (1)	Convex (2)	Smooth (2)	Depressions (1)	Radial lines (1)	Ovoid (1)	Spine (1)
P. micans Ehrenberg	EF517257	52.5 (1)	35.0 (1)	Pyriform (1)	Convex (4)	Smooth (4)	Depressions (1)	Radial lines (1)	Oblong (1)	Spine (1)
P. minimum (Pavillard) Schiller	EF517247	16.5 (1)	15.5 (1)	Ovate (1)	Convex (5)	Smooth (5)	Spines (1)	Scattered (1)	Ovoid (1)	Spine (1)
P. playfairi Croome & Tyler	AY259175	45.0 (10)	33.5 (10)	Ovate (10)	Concave and convex (10)	Smooth (10)	Depressions (10)	Scattered (10)	Ovoid (10)	None (10)
P. sigmoides Bohm	EF566746	72.0 (11)	21.5 (11)	Pyriform (11)	Convex (11)	Smooth (11)	Spines (11)	Radial lines (11)	Oblong (11)	Spine (11)
P. triestinum Schiller	AY863010	21.5 (3)	11.5 (3)	Pyriform (3)	Convex (3)	Smooth (3)	Depressions (3)	Scattered (3)	V-shaped (3)	None (3)

Table 2. . LSU rRNA GenBank accession numbers and morphological characters of the 13 benthic/epiphytic Prorocentrum species used in the polyphasic phylogenetic analysis. The number in brackets below the discrete/continue value of the morphological character corresponds to the article which references the data: (1) Cortés-Altamirano and Sierra-Beltrán, 2003; (2) Hoppenrath and Leander, 2008; (3) Murray et al. 2007; (4) Grezbyk and Sako, 1998; (5) Faust, 2008; (6) Faust, 1993; (7) Morton et al. 2002; (8) Faust, 1990; (9) Ten-Hage et al. 2000; (10) Aligizakia et al. 2009; (11) Faust, 1994; (12) Morton, 1998.

		Cell morphology			Valve surface				Periflagellar area	
	LSU rRNA	Length (µm)	Width (µm)	Cell shape	Concave vs. convex	Intercalary band	Ornament	Pore pattern	Shape in apical view	Ornament
P. arenarium Faust	EF566747	31.0 (5)	39.0 (5)	Ellipsoid (5)	Convex (5)	Smooth (5)	Smooth (4)	Marginal (4)	V-shaped (4)	None (5)
P. belizeanum Faust	DQ238042	57.5 (6)	52.5 (6)	Ovate (6)	Concave (6)	Horizontally striated (7)	Areolae (6)	Scattered (6)	V-shaped (6)	None (6)
P. borbonicum Ten-Hage, Turquet, Quod, Puiseux-Dao & Couté	AJ567466	21.0 (9)	18.0 (9)	Ovate (9)	Convex (10)	Horizontally striated (10)	Depressions (9)	Scattered (9)	V-shaped (9)	None (9)
P. concavum Fukuyo	EF566751	52.5 (4)	41.5 (4)	Ovate (4)	Concave (8)	Horizontally striated (7)	Areolae (4)	Scattered (4)	V-shaped (4)	None (4)
P. emarginatum Fukuyo	EF566750	35.0 (4)	29.5 (4)	Ovate (4)	Concave (5)	Transversally striated (5)	Smooth (4)	Radial lines (4)	V-shaped (4)	None (4)
P. faustiae Morton	EF566744	46.0 (12)	40.0 (12)	Ovate (12)	Concave (12)	Horizontally striated (7)	Depressions (12)	Scattered (12)	V-shaped (12)	None (12)
P. fukuyoi Murray & Nagahama	EU196416	35.0 (3)	24.0 (3)	Oblong (3)	Convex (3)	Horizontally striated (3)	Smooth (3)	Radial lines (3)	V-shaped (3)	Spine (3)
P. hoffmannianum Faust	EU196415	50.0 (8)	42.5 (8)	Ovate (8)	Concave (8)	Smooth (8)	Areolae (8)	Scattered (8)	V-shaped (8)	None (8)
P. levis Faust, Kibler, Vandersea, Tester & Litaker	DQ238043	42.0 (5)	38.5 (5)	Ellipsoid (5)	Concave (5)	Smooth (5)	Smooth (5)	Scattered (5)	V-shaped (5)	None (5)
P. lima (Ehrenberg) Dodge	EF566748	39.0 (4)	31.0 (4)	Ellipsoid (5)	Concave (5)	Smooth (5)	Smooth (4)	Marginal (4)	V-shaped (4)	None (5)
P. rhathymum Loeblich III, Sherley & Schmidt	EF566745	40.8 (1)	25.5 (1)	Ovate (1)	Convex (1)	Smooth (1)	Depressions (1)	Radial lines (1)	V-shaped (2)	None (1)
P. rivalis sp. nov. Delmail, Labrousse, Crassous, Hourdin, Guri & Botineau	-	12.6	4.9	Ovate	Convex	Horizontally striated	Smooth	Marginal	Ovoid	None
P. sculptile Faust	EF566749	34.5 (11)	31.0 (11)	Ovate (11)	Concave (11)	Smooth (11)	Depressions (11)	Scattered (11)	V-shaped (11)	None (11)

Table 3. Water-column characteristics of the oligotrophic localities where *Prorocentrum rivalis* was observed (J. Jaouen, com. pers. 2009). Values are given with mean ± standard deviations calculated from 3 monitoring measurements.

Property	Units	
Conductivity	(µS.cm^{-1})	49,0 ± 3,0
pH		6,8 ± 0,2
Total chlorides	(mg.l^{-1})	5,0 ± 0,9
Total sulfates	(mg.l^{-1})	2,0 ± 0,0
Nitrates	(mg.l^{-1})	4,2 ± 0,6
Nitrites	(mg.l^{-1})	0,0 ± 0,0
Total fluorides	(mg.l^{-1})	0,1 ± 0,0
Total phosphorus	(mg.l^{-1})	0,1 ± 0,0
Total organic carbon	(mg.l^{-1} C)	3,8 ± 0,6
Chemical oxygen demand	(mg.l^{-1} O_2)	0,0 ± 0,0
Turbidity	(FNU)	3,9 ± 1,6
Suspended matter	(mg.l^{-1})	8,0 ± 4,4

8. Figures

Figure 1. *Prorocentrum rivalis* sp. nov. (a) Cell in right valve view is ovate and presents many marginal pores (scale bar = 5 µm). (b) Valve pores are uniformly small and round with a smooth margin (scale bar = 500 nm). (c) Unornamented periflagellar area sets in an ovoid depression (scale bar = 5 µm).

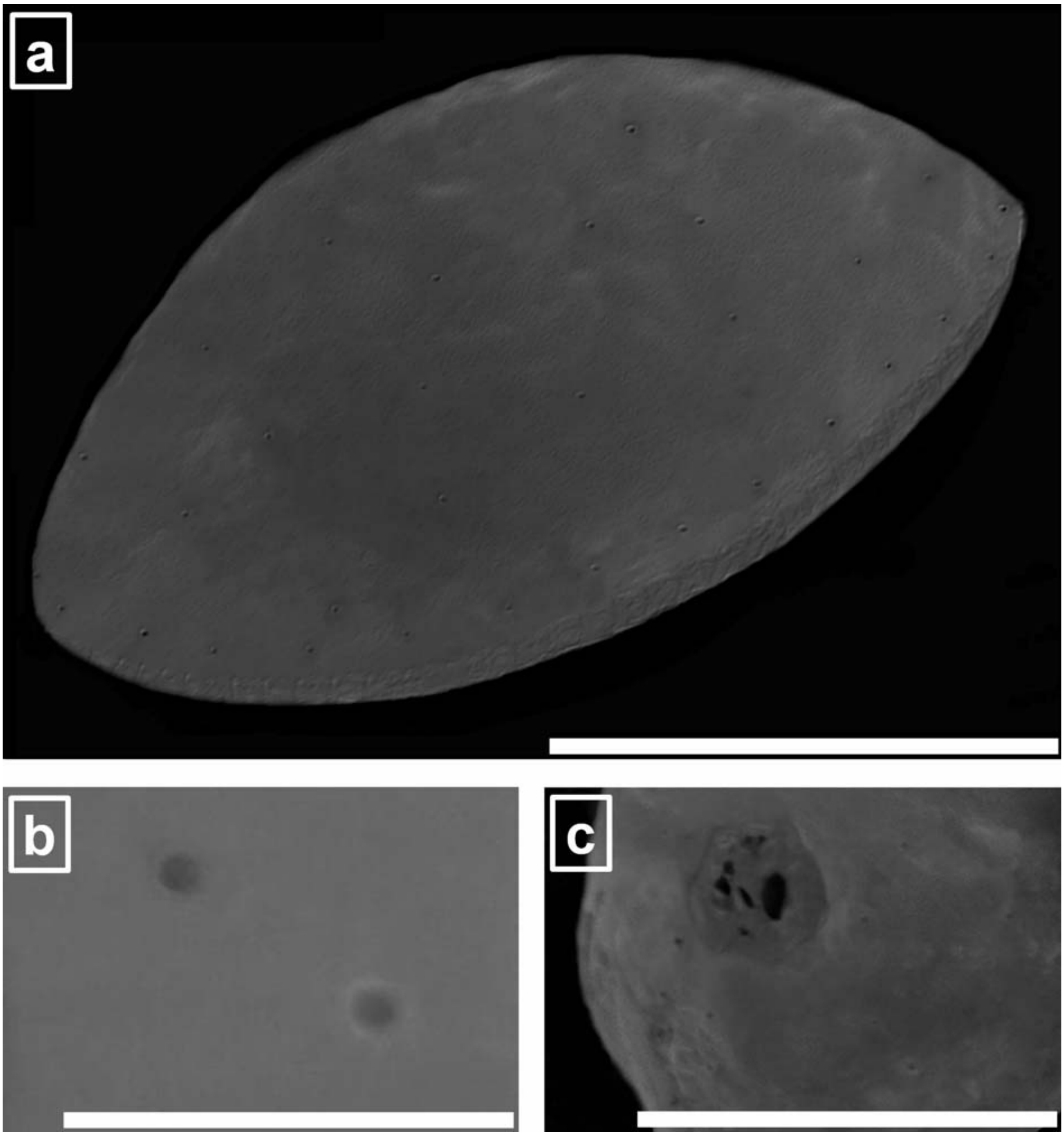
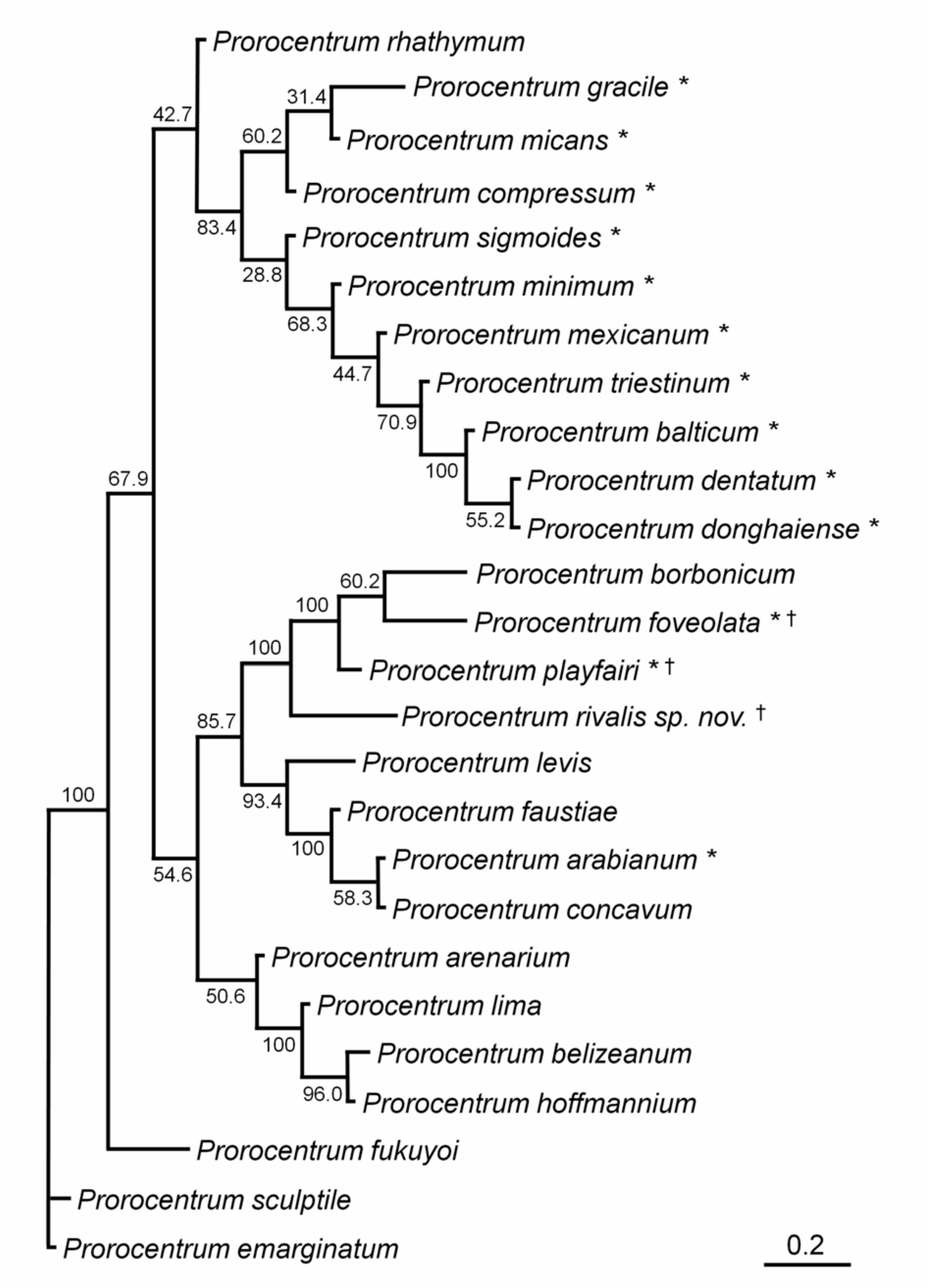


Figure 2. Maximum parsimony (MP) polyphasic phylogram based on the phenotypic and genetic relationships (9 morphological endpoints and LSU rRNA gene sequences) between 26 *Prorocentrum* species. An asterisk following the species name which belong to the polyphyletic planktonic clade. Other species belong to the paraphyletic epiphytic/benthic clade. A cross following the freshwater-species name while other species are marine. Bootstrap analyses (heuristic search, 500 replicates, branch swapping) are performed to provide measures of relative support for each node estimated in the maximum parsimony tree. GenBank accession numbers and morphological characters values of the species used for this analysis are listed in tables 1 and 2.


Prorocentrum rhathymum
Prorocentrum gracile *
Prorocentrum micans *
Prorocentrum compressum *
Prorocentrum sigmoides *
Prorocentrum minimum *
Prorocentrum mexicanum *
Prorocentrum triestinum *
Prorocentrum balticum *
Prorocentrum dentatum *
Prorocentrum donghaiense *
Prorocentrum borbonicum
Prorocentrum foveolata * †
Prorocentrum playfairi * †
Prorocentrum rivalis sp. nov. †
Prorocentrum levis
Prorocentrum faustiae
Prorocentrum arabianum *
Prorocentrum concavum
Prorocentrum arenarium
Prorocentrum lima
Prorocentrum belizeanum
Prorocentrum hoffmannium
Prorocentrum fukuyoi
Prorocentrum sculptile
Prorocentrum emarginatum
42.7
31.4
60.2
83.4
28.8
68.3
44.7
70.9
100
55.2
67.9
60.2
100
100
85.7
93.4
100
58.3
54.6
50.6
100
96.0
100
0.2