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Hidden generic diversity in desmids: description of *Pseudomicrasterias gen. nov.* (Desmidiaceae, Zygnematophyceae)

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ABSTRACT
The taxonomy and nomenclature of *Micrasterias arcuata* are very problematic because of a wide morphological variation and the large number of poorly circumscribed infraspecific taxa. This study aimed at evaluating the phylogenetic position of strains traditionally assigned to *M. arcuata* based on molecular and phylogenetic approaches, and morphometric analyses. Multigene analyses were performed using the nuclear SSU rDNA and both chloroplast *rbc*L and *psa*A markers. Genetic analyses revealed that all three investigated strains of *M. arcuata* formed reasonably well-supported lineages, separate from all species of the core *Micrasterias* lineage and closely related to the *Euastrum* 2 lineage. Morphometric analyses exhibited differences among strains revealing important trends for morphological differentiation and delimitation of infraspecific taxa assigned to *M. arcuata*. All three investigated strains are separated by their morphological features and represent different taxonomic entities from the molecular and phylogenetic points of view. Available data support the proposal of *Pseudomicrasterias gen. nov*. within the family Desmidiaceae. One infraspecific taxon is erected to species level and classified as new combination of *Pseudomicrasterias*, and two new species are recognized.

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INTRODUCTION

Desmidiaceae is the most species-rich family belonging to class Zygnematophyceae of the Streptophyta lineage (Brook 1981; Nemjová *et al.* 2011; Škaloud *et al.* 2011). Currently, the family comprises 49 genera and more than 2.500 validly described species, most of which were established during the 19th and 20th centuries, based exclusively on traditional taxonomic criteria, i.e. entirely on morphological grounds (Gontcharov 2008; Guiry & Guiry 2021).

Recent molecular studies led to major changes in the taxonomy of desmids (McCourt *et al.* 2000; Gontcharov 2008; Gontcharov & Melkonian 2004, 2008, 2010; Hall *et al.* 2008; Neustupa *et al.* 2010, 2011, 2014; Nemjová *et al.* 2011; Škaloud *et al.* 2011, 2012; Šťastný *et al.* 2013). Such advances raised questions about the taxonomic value of many morphological characters traditionally used for classification in the group, challenging species concepts in the family and questioning the large number of infraspecific taxa identified only by morphological criteria (Gontcharov & Melkonian 2008; Neustupa *et al.* 2010).

Phylogenetic relationships among genera and closely related species have been investigated by using multigene analyses, increasing phylogenetic resolution and solving some inconsistencies present in single-gene phylogenies, and therefore providing greater insight (Gontcharov & Melkonian 2004; Hall *et al.* 2008; Neustupa *et al.* 2010; Škaloud *et al.* 2011; Šťastný *et al.* 2013).

Micrasterias C. Agardh *ex* Ralfs includes unicellular individuals, sometimes forming pseudofilaments, *e.g.* in *Micrasterias foliacea* Bailey *ex* Ralfs (Sormus 1980). The cell consists of two identical and bilaterally symmetric semicells, with a single polar lobe and several lateral lobes and lobules. Semicells are connected by a narrow isthmus that contains the centrally positioned nucleus (Neustupa *et al.* 2010; Neustupa & Šťastný 2018).

According to traditional morphological classification, the first- to fourth-order lobes were among the most important features to define taxa within *Micrasterias* (Škaloud *et al.* 2011). The genus includes 3-lobed (3 lobes and 2 interlobular incisions) and 5-lobed specimens (5 lobes and 4 interlobular incisions) (Krieger 1939; Prescott *et al.* 1977). Diagnostic characteristics are often related to the dimensions and morphological complexity of the semicells (Sormus 1980). As for other desmid genera, many taxa in *Micrasterias* were described during the 19th century, including many species and several hundreds of infraspecific taxa (Krieger 1939; Růžička 1981). Presently, according to AlgaeBase, *Micrasterias* includes 220 species and 756 infraspecific names including taxonomic varieties and forms (Guiry & Guiry 2021).

Micrasterias is one of the most studied and well-known genera of Desmidiaceae, especially from the molecular phylogenetic point of view. Recent studies using the combination of molecular and morphological methods revealed the difference

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within closely related species and/or species complexes within the genus (Neustupa *et al.* 2010, 2011, 2014; Nemjová *et al.* 2011; Škaloud *et al.* 2011; Neustupa & Šťastný 2018). Previous phylogenetic studies revealed that the *Micrasterias* lineage comprises at least eight monophyletic lineages that were named A–H (Škaloud *et al.* 2011), and that the genus includes taxa with morphology ranging from relatively simple elliptic semicells without any marked incision, as is the case of *Micrasterias ralfsii* (Brébisson *ex* Ralfs) Škaloud, Nemjová, Veselá, Černá & Neustupa, to the very complex starlike shape (Neustupa 2016).

Micrasterias arcuata Bailey is a 3-lobed species described by Bailey (1851) from material collected in the State of Florida, USA (Prescott *et al.* 1977). The species has basal lobes curved upward, concave apical margin, and a transversely projected polar lobe (Bailey 1851; Prescott *et al.* 1977). Geographically, it has previously been documented from the USA, Canada, Cuba, West Africa, Asia and South America (Prescott *et al.* 1977; Sormus 1980; Fonseca *et al.* 2019), and in the former Soviet Union (Kossinskaja 1960).

The species displays a large amount of documented morphological variation (*e.g.* Nordstedt 1877; Borge 1899; Sormus 1980). In addition to the typical variety, 23 infraspecific taxa, both varieties and forms, have been described (Bailey 1851; Nordstedt 1877; West & West 1896, 1897; Borge 1899; Prescott & Scott 1943, 1952; Förster 1964, 1969; Guiry & Guiry 2021), suggesting that the species is a complex taxon worthy of interest from both taxonomic and nomenclatural points of view.

Micrasterias arcuata was already recorded in all Brazilian regions (Borge 1899, 1918; Förster 1964, 1969; Bittencourt-Oliveira & Mecenas 1994; Fonseca & Estrela 2015; Oliveira *et al.* 2017; Santos *et al.* 2018; Ramos *et al.* 2019), and seven varieties recorded for this species were described based on Brazilian material (Borge 1899; Förster 1964, 1969).

The majority of studies dealing with *M. arcuata* were classic α taxonomy. Only a few were dedicated to other aspects of the species, such as the taxonomic revision and distribution patterns (Sormus 1980), polymorphism and ecology (Gil-Gil & Bicudo 2000; Bicudo & Gil-Gil 2003), and the relationship between morphological features and ecology (Fonseca *et al.* 2019).

The present study aimed at evaluating the phylogenetic position of strains traditionally assigned to *M. arcuata*, in order to test the monophyly and phylogenetic differentiation of this species complex. In addition, morphological analyses using geometric morphometrics, traditional morphological methods and scanning electron microscopy (SEM) were performed. Furthermore, we focused on the morphological peculiarities of the phylogenetic lineages identified within this species.

MATERIAL AND METHODS

Sampling, isolation and cultivation of strains

Phytoplankton samples were collected in November 2018 using a 20 µm mesh plankton net in a small pond covered to a large extent by *Sphagnum* sp., located in the Reserva Experimental de Itirapina (22°13.836′S, 47°49.165′W), Itirapina Municipality, São Paulo State, southeast Brazil.

Clonal cultures identified as *M. arcuata* according to their morphology were established by micropipeting single cells and cultivating in tubes with approximately 15 ml of MES-buffered DY IV liquid medium (pH *c.* 5; Andersen *et al.* 1997). Cultures were maintained at 18–24°C under constant illumination of 40 µmol photons m^{-2} s^{-1} from 18 W cool fluorescent tubes (Philips TLD 18 W/33, Royal Philips Electronics, Amsterdam, Netherlands) for 8–10 weeks.

Strains were deposited at the Culture Collection of Freshwater Microalgae, Universidade Federal de São Carlos (CCMA-UFSCar, WDCM 835). In addition, strains were also fixed with formaldehyde and deposited in the Herbarium of the Instituto de Botânica, 'Herbário Científico do Estado Maria Eneyda P. Kauffmann Fidalgo – SP', São Paulo, São Paulo State, Brazil.

Light and scanning electron microscopy (SEM)

Living cells of isolates were observed and photographed using a Zeiss Axioplan 2 light microscope equipped with a Zeiss AxioCam MRc digital camera and AxioVision SE64 Rel 4.9 software at 400× and 1000× magnifications, and an inverted microscope (Zeiss Axio Observer D1) with a 2.5× Optovar and image capture AxioCam MRc Rev. 3, Imaging Systems 4.7.2 and ZEN Zeiss software v. 2012, at 400× magnification. Picture analysis software (ZEN Zeiss software v. 2012 and the AxioVision SE64 Rel 4.9 software) was used to examine each individual morphological feature and to obtain photomicrographs.

For SEM analysis, acetone-washed glass coverslips were heated and coated three times with a poly-L-lysine solution (1:10 in deionized water) to ensure appropriate cell adhesion. A drop of the formaldehyde-fixed cell suspension was transferred to 30% acetone and dehydrated by using an acetone series. Subsequently, cells were critical-point-dried using liquid CO_2. Finally, they were sputter-coated with gold (Bal-Tec Sputter Coater SCD 050, Capovani Brothers Inc., Sconia, New York, USA) and examined with a JEOL 6380 LV (JEOL Ltd., Tokyo, Japan) scanning electron microscope (Šťastný *et al.* 2013). All photomicrographs were graphically adjusted using Adobe Creative Cloud Photoshop software v. 2013.

Morphometric analyses

For traditional morphometric analysis, and to obtain linear measurements, individual cells of each *M. arcuata* cultured strain were investigated and measured using software tools for image analysis (ZEN Zeiss software v. 2012 and the AxioVision SE64 Rel 4.9 software). Seven morphological traits were measured: maximum cell length (MCL), i.e. distance between the tips of polar lobes of opposite semicells; average cell length (ACL), i.e. distance between midpoints of polar lobes of opposite semicells; maximum cell width (MCW), i.e. distance between basal lobes of the same semicell; width of polar lobe or minimum cell width (BPL), i.e. distance between the tips of polar lobe of the same semicell; and basal lobes distance (BLD), i.e. distance between the tips of basal lobes of opposite semicells. All these measurements were proposed in

Gil-Gil & Bicudo (2000). In addition, we measured isthmus width (IW) and height of polar lobe (HPL), i.e. distance between the base of basal lobe and the base of lateral margin of polar lobe in the same semicell. A schematic figure of the seven morphological features analysed herein is provided in Fig. S1. Box-plot graphs were constructed in RStudio v. 1.4.1 (RStudio Team 2020) in the *ggplot* R-Package (Wickham 2016).

For geometric morphometric analysis, well-developed semicells of each *M. arcuata* cultured strain were investigated, and the photomicrographs were taken using the same software described above for the traditional morphometric analysis. In addition, landmarks were digitized on semicells of 30 taxa illustrated in some authoritative taxonomic monographs (Bailey 1851; Förster 1964, 1969; see Table S2). In the present study, illustrations of *M. arcuata* varieties recognized in classical literature and reproduced by Förster (1964, 1969) were included.

To conduct the geometric morphometric analysis, TpsUtil 1.61 software (Rohlf 2015) was used to transform photomicrographs into a TPS file format. Then, software TpsDig 2.32 (Rohlf 2005) was used to digitize landmarks on scaled images.

Forty-nine equidistant landmarks were depicted in the front view of the *M. arcuata* semicell (Fig. S2) according to a previous study concerning this species (Fonseca *et al.* 2019). One landmark was positioned at the bilateral symmetry axis of the semicell, and the other 48 were bilaterally symmetrically positioned on each side of the semicell. Thirteen landmarks were considered fixed points, as follows: points 1, 49 – margin of the isthmus; 7, 43 – basal lobes extremities; 13, 37 – base of basal lobes; 14, 36 – midpoint of polar lobe height; 15, 35 – base of lateral margins of polar lobe; 19, 31 – extremities of lateral margins of polar lobe; 25 – central incision of polar lobe (apical margin). The remaining 36 landmarks were positioned along the semicell outline to minimize Procrustes distances and any apparent shape difference (Zelditch *et al.* 2012).

Landmark data were imported into RStudio v. 1.4.1 (RStudio Team 2020) and opened in the Geomorph R-package (Adams & Otarolla-Castillo 2013). Data were aligned by Generalized Procrustes Analysis (GPA) and were symmetrized. Principal component analysis (PCA) was generated to visualize shape variation among individuals in the morphospace. The Thin-Plate Spline deformation grids (TPS-grids) were created and plotted considering the first two PCA axes to allow visualization of the shape changes of morphotypes studied. Linear discriminant analysis (LDA) was conducted using all non-zero PCA axes in the MASS R-Package (Venables & Ripley 2002) to evaluate the groups discrimination by reassignment probabilities (McLachlan 1992) that were analysed by leave-one-out cross-validation. The non-parametric MANOVA (Anderson 2001) was also performed to test significant differences between morphotypes on the matrix of Euclidean distances in Past v. 2.17 (Hammer *et al.* 2001).

DNA isolation, PCR amplification and sequencing

Total genomic DNA was extracted from liquid cultures of *M. arcuata* strains with InstaGene matrix (Bio-Rad Laboratories, Hercules, California, USA). Nuclear encoded SSU rDNA and chloroplast markers *rbc*L and *psa*A were amplified by PCR in 20 µl reaction volumes: 13.1 µl of sterile Mili-Q water, 2 µl of AmpliTaq Gold 360 Buffer 10x (Applied Biosystems, Life Technologies, Carlsbad, California, USA), 2.2 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP mix (10 mM), 0.25 µl of each primer (25 nM), 0.6 µl of 360 GC enhancer, 0.2 µl of AmpliTaq Gold 360 DNA polymerase and 1 µl of DNA template (not quantified) using the amplification primers listed in Table 1 and cycling conditions listed in Table 2. The PCR products were purified with AMPure (Beckman Coulter, Inc.) purification reagent and sequenced at Macrogen Inc. in Seoul, Korea.

Sequence analysis and phylogenetic tree construction

Individual chromatograms were read and edited using the SeqAssem program (Hepperle 2004). Nuclear encoded SSU rDNA, and plastid encoded *rbc*L and *psa*A sequences from this study and reference sequences of Desmidiales lineages published in previous studies (McCourt *et al.* 2000; Hall *et al.* 2008; Gontcharov & Melkonian 2010) were retrieved

Table 1. List of primers used for PCR amplification and sequencing.

Molecular marker	Primer	Primer sequence (5′–3′)	Reference
SSU rDNA	18S-F	AACCTGGTTGATCCTGCCAGT	Katana *et al.* (2001)
SSU rDNA	18S-R	TGATCCTTCTGCAGGTTCACCTACG	Katana *et al.* (2001)
*psa*A	*psa*A 2100R	GGCAATTCCACCCAGAAGG	Hall *et al.* (2008)
*psa*A	*psa*A IF	TTCCTTTGCCTCATGAATTC	Hall *et al.* (2008)
*rbc*L	ZygF	TATGTCAACCACAAAC	Pichrtová *et al.* (2018)
*rbc*L	1385R	GGAAAGAAATTAAATTTGAATT	McCourt *et al.* (2000)

Table 2. PCR cycling conditions.

Region	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final denaturation)
SSU rDNA	94°C (4 min)	94°C (1 min)	52°C (1 min)	72°C (2 min)	35×	72°C (10 min)
*rbc*L	95°C (10 min)	94°C (1 min)	48°C (1 min)	72°C (2 min)	35×	72°C (10 min)
*psa*A	94°C (5 min)	94°C (1 min)	52.5°C (1 min)	72°C (1 min)	35×	72°C (10 min)

from GenBank. Multiple alignment was created and automatically edited using MAFFT v7.3 web interface (Katoh *et al.* 2002) applying Q-INS-I algorithm strategy and trimmed using Trimal 1.2 (Capella-Gutiérrez *et al.* 2009). A concatenated matrix of 4.675 pb (1.705 pb of 18S rDNA, 1.335 pb of *rbc*L and 1.635 pb of *psa*A) containing 95 taxa was constructed for phylogenetic analyses. Four sequences of Peniaceae were selected as outgroup taxa. The GenBank accession numbers of all strains and sequences involved in the phylogenetic analyses are provided in Table S1.

The best-fit evolutionary models of nucleotides substitutions for SSU rDNA and the first, second and third codon positions for *rbc*L and *psa*A were determined in jModelTest2 based on the Bayesian Information Criterion (Darriba *et al.* 2012) as implemented in the CIPRES Science Gateway (Miller *et al.* 2010). This BIC-based model selected the General Time Reversible (GTR) substitution with Gamma distribution (G) and proportion estimate of invariable sites (I) for SSU rDNA, first, second and third codon position of *psa*A and first and third codon position of *rbc*L, whereas the Hasegawa, Kishino and Yano (HKY) model + G + I was selected for the second codon position of *rbc*L.

Phylogenetic analyses were performed using maximum likelihood (ML) analysis and Bayesian inference (BI) analysis. Maximum likelihood analysis (ML) was performed in RAxML_GUI (Silvestro & Michalak 2012) using 2 independent runs and 1.000 pseudoreplicates. For the concatenated dataset, SSU rDNA was analysed as a single partition, whereas *rbc*L and *psa*A were partitioned by codon. All partitions were analysed using a GTR + I + G model. Bayesian analysis was performed in MrBayes v.3.2.4 (Ronquist & Huelsenbeck 2003) on the CIPRES Science Gateway v.3.3 web portal (Miller *et al.* 2010). Two independent runs were carried out for 8×10^6 generations. Chains were sampled every 100th cycle and 25% of records were discarded as burn-in. Convergence of the MCMC algorithm was assessed by (1) examination of the potential scale reduction factor (PSRF, average = 1; maximum 1.012) and (2) examination of the average standard deviation of split frequencies <0.01. The resulting tree was visualized using FigTree v. 1.4.3 (Rambaut 2016). Sequence divergences between different lineages were calculated in MEGA 6.0 (Tamura *et al.* 2013) using *p*-distance estimation or number of base pairs (bp).

RESULTS

Morphometric analyses

Boxplot graphs of seven morphological attributes showed differences in the cell size and width of the three strains investigated (Fig. 1). Individuals of CCMA-UFSCar 717 and those of CCMA-UFSCar 718 presented the highest values of maximum cell length (MCL) and average cell length (ACL) as well as those of maximum cell width (MCW) and minimum cell width or width of polar lobe (BPL).

Individual specimens of CCMA-UFSCar 717 strain presented the highest values for the distance between basal lobes (BLD), and those of CCMA-UFSCar 718 strain the highest values of isthmus width (IW), this being the main difference observed between the two strains. Specimens belonging to CCMA-UFSCar 719 had proportionately smaller values of length and width, and therefore the highest values of length-to-width ratio. In addition, these individuals showed the lowest values of BLD and variable HPL (Fig. 1; Table 3).

Results obtained from NPMANOVA showed that CCMA-UFSCar 719 strain has statistically significant differences when compared to the other two strains evaluated ($F = 57.22$, $p < 0.0001$).

First principal component analysis (Fig. 2) of the symmetrized data spanned 63.3% of the total variation, and the second PC2 axis 17.4%. Specimens of CCMA-UFSCar 717 were plotted on the negative sides of PC1 and PC2 axes. These specimens had a relatively longer polar lobe, with the lateral projections slight to strongly curved downward and the apical margin retuse to slightly concave, with both sides flattened at the base (Figs 4–6). Basal lobes were conical and slender, with the upper margin almost straight at the base and the apices of the lateral projections strongly curved upward (Figs 5, 6). Some specimens had a longer polar lobe, with lateral projections shorter and apices slightly curved downward, and apical margin convex and basal lobes conical and straight (Fig. 4).

Specimens of CCMA-UFSCar 718 were plotted at the positive extreme of PC1 axis and to the negative side of PC2 axis (Fig. 2). These specimens were identified by their relatively shorter polar lobes with a retuse apical margin and both sides flattened at the base. Lateral extensions of polar lobes were shorter, faintly straight to slightly curved upward, and in some cases slightly curved downward, and basal lobes conical, rounded, swollen and straight at the base (Figs 7–9).

Individuals of CCMA-UFSCar 719 (Figs 10–15) presented a wide morphological variation and were plotted in the first, third and fourth quadrants of the PCA graph (Fig. 2). The shape of such specimens is characteristic by its compact cells with shorter polar lobes and retuse apex (Fig. 10) to slightly curved upward at the apices, in the latter case with the apical margin slightly concave (Figs 11–13). Some specimens showed conical basal lobes, swollen at the base and their apices slightly concave (Figs 11–13). Other specimens exhibited a short polar lobe, a concave apical margin with both sides flattened at the base, and polar lobe lateral projections slightly curved downward. Basal lobes were conical and straight at the base, the upper margin of these projections slightly curved upward, the bottom margin slightly subsigmoid (Figs 13–15).

The linear discriminant analysis (LDA) successfully discriminated strains (Fig. 3). Moreover, according to the correct classification/prediction, all of them had 100% correct classification (Table S2). Results obtained from NPMANOVA showed statistically significant differences among the three strains currently evaluated according to the geometric morphometric analyses ($F = 42.80$, $p < 0.0001$).

Taxonomic determination of investigated strains

To determine taxonomically the investigated strains, a PCA was performed based on the geometric morphometric data (PCA; Fig. S3), along with the 30 illustrations here taken as representative of *M. arcuata* varieties described so far. The first PC1 axis

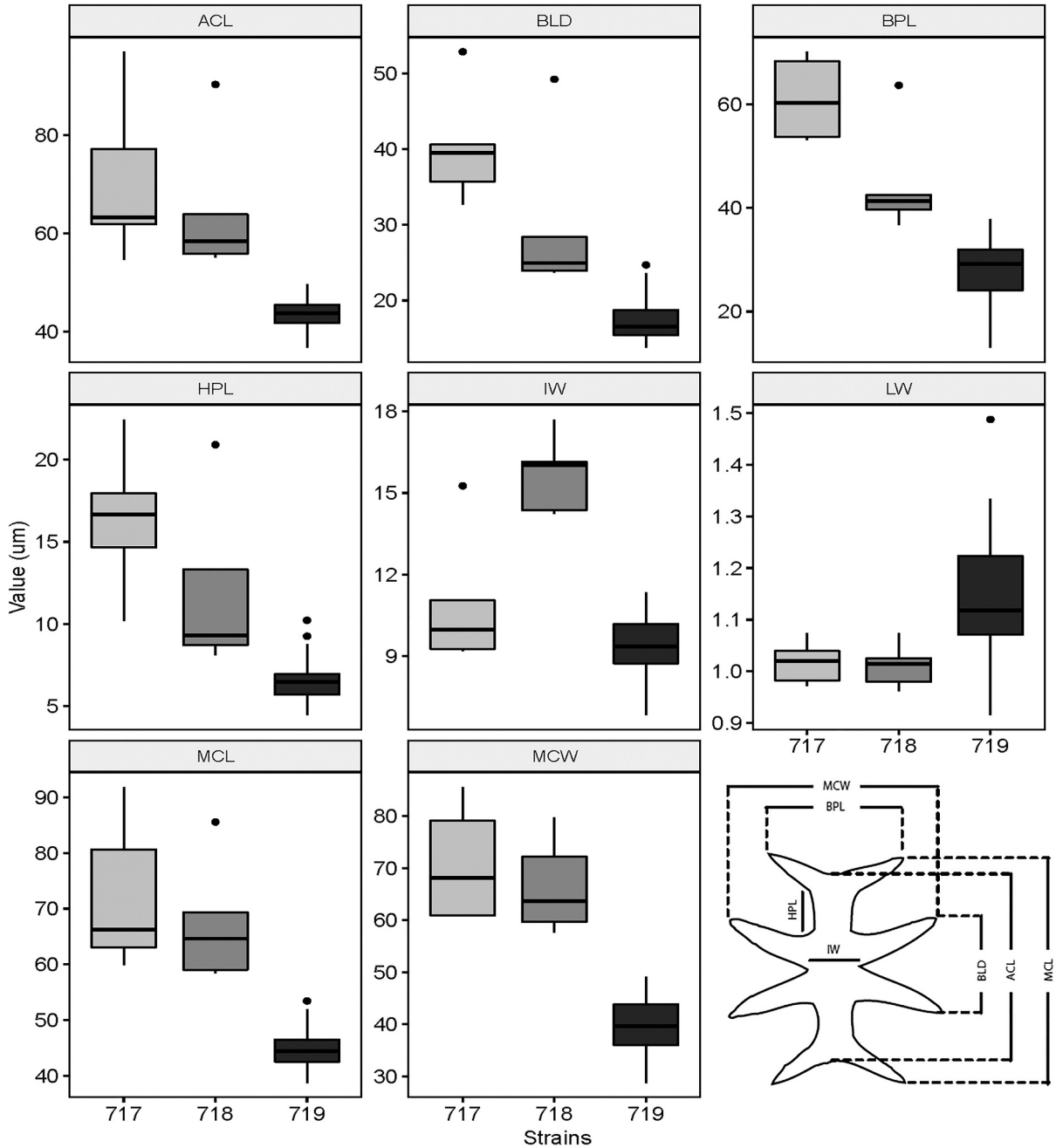


Fig. 1. Boxplot of the seven morphological attributes of *Pseudomicrasterias* strains analysed by traditional morphometric analysis: average cell length (ACL), distance between basal lobes (BLD), width of polar lobe or minimum cell width (BPL), height of polar lobe (HPL), isthmus width (IW), length to width ratio (LW), maximum cell length (MCL), maximum cell width (MCW).

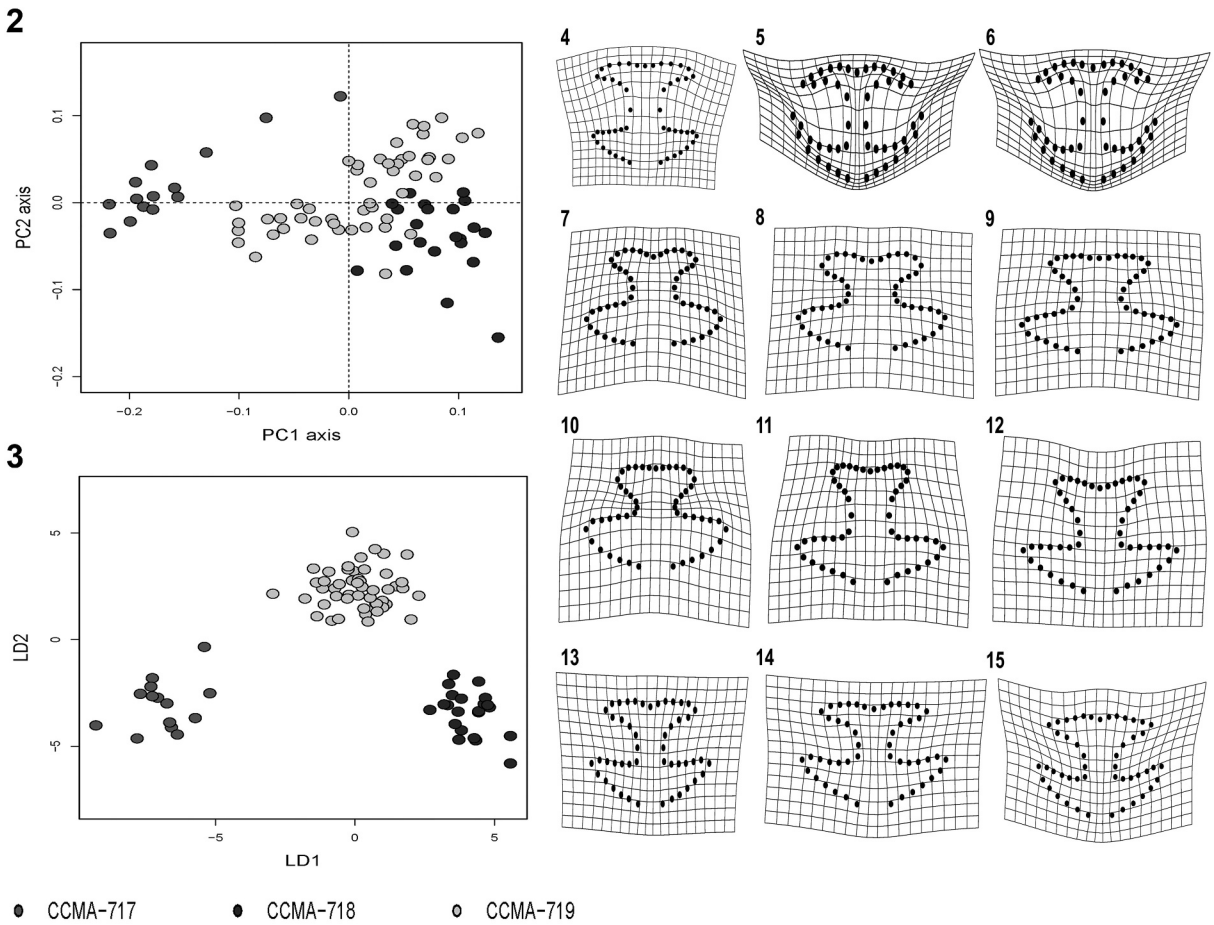
spanned 61% of the total variation and the second PC2 axis 17.5%. PCA ordination plot (Fig. S3) was highly similar to the plot in Fig. 2, with the majority of the illustrations of *M. arcuata* taxa spread outside the morphospace of the three investigated strains. Yet, some of these illustrations were morphologically highly similar to the strains.

According to the classification analyses (Table S3), the cells of strain CCMA-UFSCar 717 were clustered with *Micrasterias arcuata* var. *arcuata* or *M. arcuata* var. *gracilis* West & G.S. West. Individual specimens of strain CCMA-UFSCar 718 clustered with *M. arcuata* var. *perforata* Kurt Förster & Eckert, *nom. inval.*, *M. arcuata* var. *borgei* Kurt Förster, *M. arcuata* var. *robusta* Borge and *M. arcuata* [var. *robusta*] f. *goyazensis* Kurt Förster & Eckert. Finally, specimens of strain CCMA-UFSCar 719 clustered with *M. arcuata* var. *cornuta* Kurt Förster & Eckert, *M. arcuata* f. *goyazensis*, *M. arcuata* var. *subcornuta* Kurt Förster & Eckert, *Micrasterias arcuata* var. *subparallela* Kurt Förster (≡*M. arcuata* [var. *subpinnatifida*] f. *gracilis* Kurt Förster & Eckert, *nom. illeg.*), *M. arcuata* [var. *subpinnatifida*] forma Borge (1918, p. 65), *M. arcuata* [var. *subpinnatifida*] forma Förster (1964, p. 375) and *M. arcuata* var. *arcuata*.

Table 3. Comparison between *Pseudomicrasterias* species based on traditional morphometric analysis (linear measurements) and analyses of the shape of semicell by geometric morphometric and SEM images.

Feature	*P. arcuata* (CCMA-717)	*P. perforata* (CCMA-718)	*Pseudomicrasterias* sp. (CCMA-719)
MCL (µm)	72(–60–92)*	67(–58–86)*	45(–41–53)*
ACL (µm)	71(–55–97)*	65(–55–90)*	44(–39–50)*
BLD (µm)	40(–33–53)*	30(–24–49)*	18(–14–25)*
MCW (µm)	71(–61–86)*	67(–58–80)*	41(–33–49)*
BPL (µm)	61(–53–70)*	45(–37–64)*	30(–21–38)*
IW (µm)	11(–9–15)*	16(–14–18)*	10(–8–11)*
HPL (µm)	16(–10–22)*	12(–8–21)*	7(–4–10)*
L:W ratio	1.0	1.0	1.1
Basal lobes	Slender to very slender, arcuate upward	Swollen, straight to slightly arcuate upward	Swollen, slightly arcuate upward
Polar lobe	Slender, arcuate downward	Swollen, shorter, straight to slightly arcuate upward	Stout, shorter, straight, oriented upward (sometimes) to slightly arcuate downward
Apical margin	Straight to slightly concave, elevated on either side	Straight to strongly concave, elevated on either side	Straight to slightly concave, elevated on either side
Cell wall punctation	Finely punctate	Strongly punctate, thick pores	Finely punctate
Teeth	Conical and short	Conical, short and robust	Conical and short

*Mean (min-max).



Figs 2–15. Graphic representation of *Pseudomicrasterias* strains across morphospace and the thin-plate splines illustrating the changes in the shape of strains. Grid deformations were magnified 1.5× in order to better illustrate the shape changes.

Fig. 2. Principal component analysis (PCA) showing *Pseudomicrasterias* strains across the morphospace.

Fig. 3. Linear discriminant analysis (LDA) of *Pseudomicrasterias* strains.

Figs 4–6. Grids of deformation obtained from the analysis of individuals of CCMA-717 *(P. arcuata)*.

Figs 7–9. Grids of deformation obtained from the analysis of individuals of CCMA-718 *(P. perforata)*.

Figs 10–15. Grids of deformation obtained from the analysis of individuals of CCMA-719 *(Pseudomicrasterias sp.)*.

Considering present specimens examined using light microscopy and SEM, as well as the original descriptions of taxa, we were able to assign strain CCMA-UFSCar 717 to *M. arcuata* var. *arcuata* (Fig. 17; see also Bailey 1851) and strain CCMA-UFSCar 718 to *M. arcuata* var. *perforata* (Fig. 18).

Specimens of strain CCMA-UFSCar 719 displayed some wide-ranging morphological variation, and these specimens were similar to several illustrations of some infraspecific taxa, even if they were different in other aspects of their cell morphology, especially the shape of basal lobes. However, as such differences were not consistent and not enough to clearly discriminate these specimens from several *M. arcuata* taxa, no taxonomic conclusion concerning this strain and its morphological relationship with other infraspecific taxa of *M. arcuata* is proposed.

Molecular data and phylogeny

The phylogenetic analyses based on the concatenated dataset of SSU rDNA, *rbc*L and *psa*A sequences are exhibited in Fig. 16. Phylogenetic analyses using Bayesian Inference (BI) and maximum likelihood (ML) generated similar topologies in which 20 well-defined lineages and eight single branch sequences were recognized including the outgroup and the lineage corresponding to the sequences generated in this study.

Sequences of investigated *M. arcuata* strains were clustered within a lineage with high support in all analyses; this lineage is here referred as *Pseudomicrasterias gen. nov*. (Fig. 16). Sequences were distantly related to the lineage consisting of all other *Micrasterias* species (*p*-distance 2.6% for SSU rDNA (Table S4) and 7.0% for *rbc*L (Table S5).

Pseudomicrasterias was closely related to *Euastrum* 2 lineage (*p*-distance 1.6% for SSU rDNA and 6.0% for *rbc*L; Tables S4, S5), composed of *Euastrum intermedium* Cleve and the lineage of *E. oblongum* Ralfs, *E. crassum* Ralfs, *E. affine* Ralfs and *E. pinnatum* Ralfs.

Based on the results, *M. arcuata* var. *arcuata* is transferred to the new genus as *Pseudomicrasterias arcuata* (Bailey) C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *comb. nov*.; the specimens resembling the description and illustration of '*M. arcuata* var. *perforata* Kurt Förster & Eckert', *nom. inval*., are described as *Pseudomicrasterias perforata* C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *sp. nov*.; and specimens of strain CCMA-UFSCar 719 are referred as *Pseudomicrasterias* sp. (see taxonomy treatment below).

Taxonomic treatment

Pseudomicrasterias C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *gen. nov.*

DESCRIPTION: Single, bilaterally symmetrical cells. Semicells 3-lobed, polar lobe T-shaped, base subcylindrical, expanded into lateral projections on each side, apical margin concave or slightly retuse, basal lobes conical, divergent, straight or curved upward, median constriction deep, sinus open, acute. Chloroplast axial, following the shape of the semicell, with several to many pyrenoids. Semicell fusiform in vertical view. Both basal and polar lobes ending in a conical tooth.

TYPE SPECIES: *Pseudomicrasterias arcuata* (Bailey) C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *comb. nov.*

ETYMOLOGY: Greek *pseudo*, false, and *Micrasterias*, the genus of Desmidiaceae to which all currently recognized species, including the type species of the new genus, were previously assigned.

Pseudomicrasterias arcuata (Bailey) C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *comb. nov.*

Figs 17, 19–22, 31–33

BASIONYM: *Micrasterias arcuata* Bailey 1851, *Smithsonian Contributions to Knowledge* 2 (8): 37, pl. 1, fig. 6.

HOMOTYPIC SYNONYM: *Tetrachastrum arcuatum* (Bailey) W. Archer in Pritchard (1861, p. 725).

HOLOTYPE: [icon] pl. 1, fig. 6 in Bailey (1851). Since no original material is known to exist, the single original illustration published with the protologue is regarded as the type (Art. 9, Note 1, Turland *et al*. 2018).

TYPE LOCALITY: Six miles from Pilatka Lake, Florida, USA.

DESCRIPTION: Vegetative cell as long as broad; median constriction deep, sinus acute to widely open; polar lobe subcylindrical, lateral projections slightly arcuate downward, apical margin flat, often somewhat elevated on either side of the middle, sometimes retuse, basal lobes conical, slender to very slender, sometimes straight at the base, arcuate upward towards the lateral projection of the polar lobe, both basal lobes and the polar lobe projections ending in a conical, short tooth. Axial chloroplast following the shape of the semicell, with several pyrenoids. Vertical view narrow and fusiform. Zygospore not observed, but other sexual reproduction stages such as germling cells and pre-vegetative cells were observed. Asexual reproduction commonly observed. Cell wall with fine pores, visible in SEM.

GENBANK ACCESSION: MW678848 for nuclear encoded SSU rDNA, MW686753 for plastid encoded *rbc*L and MW686756 for plastid encoded *psa*A. Phylotype differs from *P. perforata* and *Pseudomicrasterias* sp. regarding 18S rDNA, *rbc*L and *psa*A phylogenies.

REMARKS: A formaldehyde fixed sample of strain CCMA-UFSCar 717 is deposited in the Herbário Científico do Estado 'Maria Eneyda P. Kauffmann Fidalgo – SP', at Instituto de Botânica, São Paulo, São Paulo State, Brazil, under access number SP400043. Authentic strain CCMA-UFSCar 717 is maintained at the Culture Collection of Freshwater Microalgae, Universidade Federal de São Carlos (CCMA-UFSCar), São Carlos, São Paulo State, Brazil.

Pseudomicrasterias perforata C.B. Araújo, C.E.M. Bicudo, Šťastný & Škaloud *sp. nov.*

Figs 18, 23–26, 34–36

PUBLISHED ILLUSTRATIONS OF THE SPECIES: The following designation, arguably not validly published for lack of indication of type, and its subordinate taxa, are considered to represent this species – '*Micrasterias arcuata* var. *perforata* Kurt Förster & Eckert', *nom. inval.* in Förster (1964, p. 373, pl. 13, figs 14, 15; pl. 44, fig. 10); *Micrasterias arcuata* [var. *perforata*] f. *magniperforata* Kurt Förster & Eckert in Förster (1964, p. 373, pl. 13, fig. 16, pl. 43, fig. 10); '*Micrasterias arcuata* [var. *perforata*] f. *robustior* Kurt Förster & Eckert', *nom. inval.* in Förster (1964, p. 373, pl. 13, figs 17–20, pl. 53, figs 1–3).

DESCRIPTION: Vegetative cells as long as broad, median constriction deep, sinus acute, sublinear to open; polar lobe subcylindrical, relatively short and robust, lateral projections of polar lobe subconical, sometimes slightly directed upwards, sometimes parallel to the basal lobes, apical margin concave in the mid region, basal lobes broadly conical, lateral projections extending horizontally, rare slightly curved upward, both polar and basal lobes ending in a conical, short and robust tooth. Axial chloroplast following the shape of the semicell, with several pyrenoids. Vertical view fusiform. Although zygospores were not observed, some

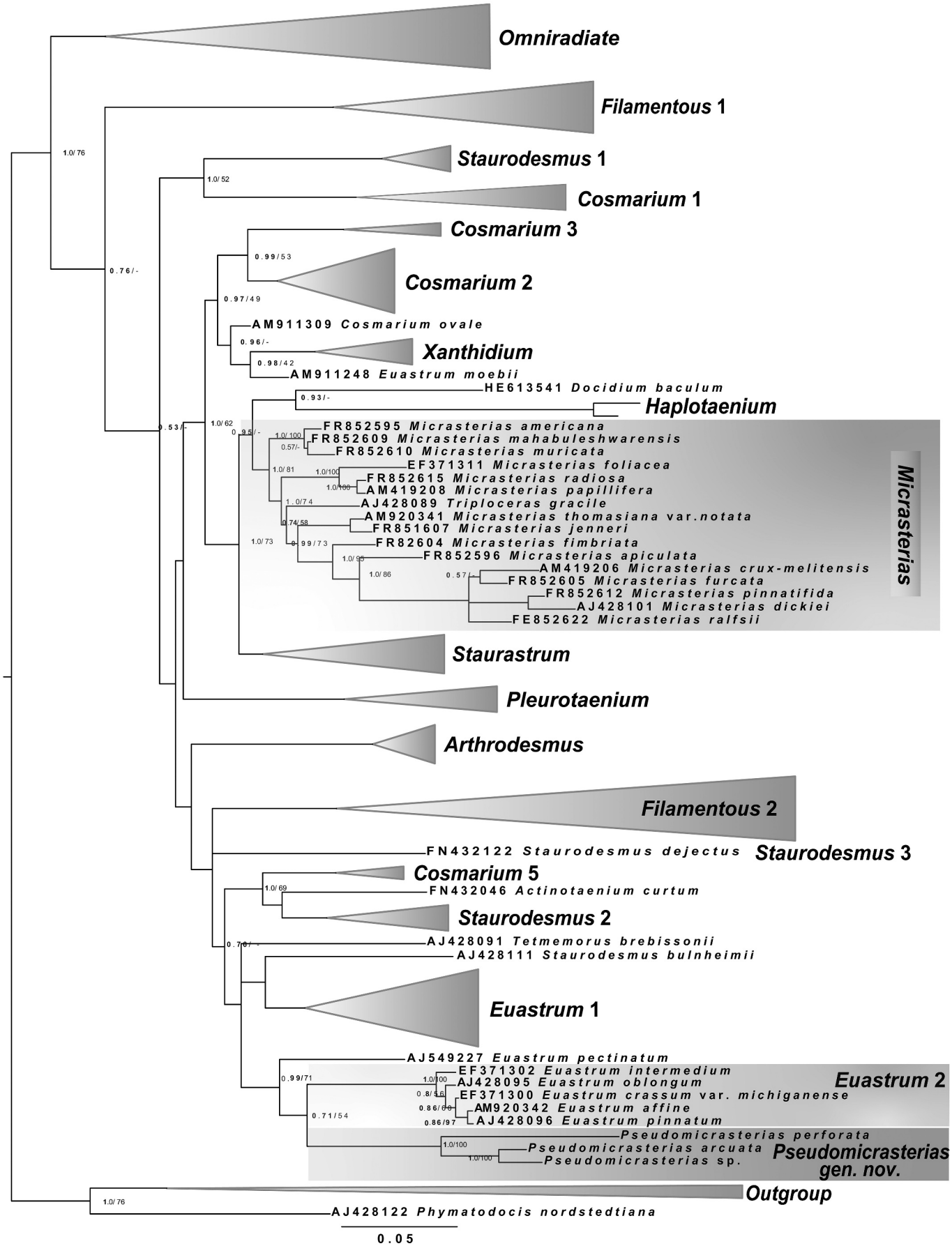
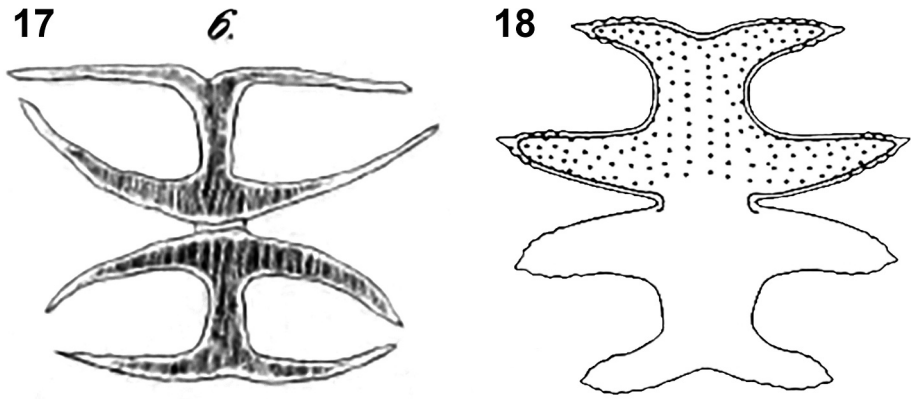


Fig. 16. Phylogenetic tree constructed based on concatenated data set of SSU rDNA, *rbc*L and *psa*A sequences. Nineteen lineages and eight single branch sequences of Desmidiaceae *sensu* Gontcharov & Melkonian (2010) are indicated. The tree was rooted using sequences of *Penium* spp as outgroup. Values at nodes indicate statistical support estimated by two methods: Bayesian Inference: posterior probability (left) and maximum likelihood: bootstrap (right). Posterior probabilities <0.70 and bootstrap values <50% are omitted. Scale bar = estimated number of substitutions per site. Sequences generated in this study and nomenclatural changes proposed are identified as *Pseudomicrasterias gen. nov.*



Figs 17, 18. Original illustrations of infraspecific taxa of *Micrasterias arcuata*. No scale bars provided.
Fig. 17. *Micrasterias arcuata* var. *arcuata*, reproduced from Bailey (1851, pl. 1, fig. 6).
Fig. 18. *Micrasterias arcuata* var. *perforata*, reproduced from Förster (1964, pl. 13, fig. 15).

sexual reproduction stages were detected, namely, the germination phase with the release of a germination vesicle containing two sister gones, and pre-vegetative cells. Cell wall strongly punctate, with thick pores visible in SEM. There is a series of 10 thick pores above the isthmus.

HOLOTYPE: SP400044, a formaldehyde fixed sample of strain CCMA-UFSCar 718 is deposited in the Herbário Científico do Estado 'Maria Eneyda P. Kauffmann Fidalgo – SP', at the Instituto de Botânica, São Paulo, São Paulo State, Brazil.

TYPE LOCALITY: 22°13.836′S, 47°49.165′W; a small pond covered by *Sphagnum* sp., located in the Reserva Experimental de Itirapina, Itirapina Municipality, São Paulo State, southeast Brazil.

ETYMOLOGY: From Latin *perforatus, -a, -um*, pierced with holes, referring to strongly punctate cell wall, with tick pores.

GENBANK ACCESSION: MW678849 for SSU rDNA and MW686754 for *rbc*L. Phylotype differs from *P. arcuata* and *Pseudomicrasterias* sp. according to 18S rDNA and *rbc*L phylogenies.

REMARKS: Material of the authentic strain CCMA-UFSCar 718 is maintained in the Culture Collection of Freshwater Microalgae, Universidade Federal de São Carlos (CCMA-UFSCar), São Carlos, São Paulo State, Brazil. Figure 26 (present study) is taken as a representative illustration of *Pseudomicrasterias perforata sp. nov.*

Pseudomicrasterias sp.
Figs 27–30, 37–39

DESCRIPTION: Vegetative cells longer than broad, median constriction deep, sinus acute, open, polar lobe shortly subcylindrical, lateral projections of polar lobe subconical, retuse to slightly curved downward, apical margin flat, often somewhat elevated on either side of the middle, basal lobes conical, swollen, straight at the base, upper margin of basal lobes slightly curved upward, bottom margin slightly subsigmoid, both basal and polar lobes ending in a conical, short tooth. Axial chloroplast following the shape of the semicell, with several pyrenoids. Vertical view oblong to fusiform. Although zygospores were not observed, some sexual reproduction stages were detected, viz. pre-vegetative cells. Cell wall with fine pores, visible in SEM.

GENBANK ACCESSION: MW678850 for SSU rDNA and MW686755 for *rbc*L. Phylotype differs from *P. arcuata* and *P. perforata* according to 18S rDNA and *rbc*L phylogenies.

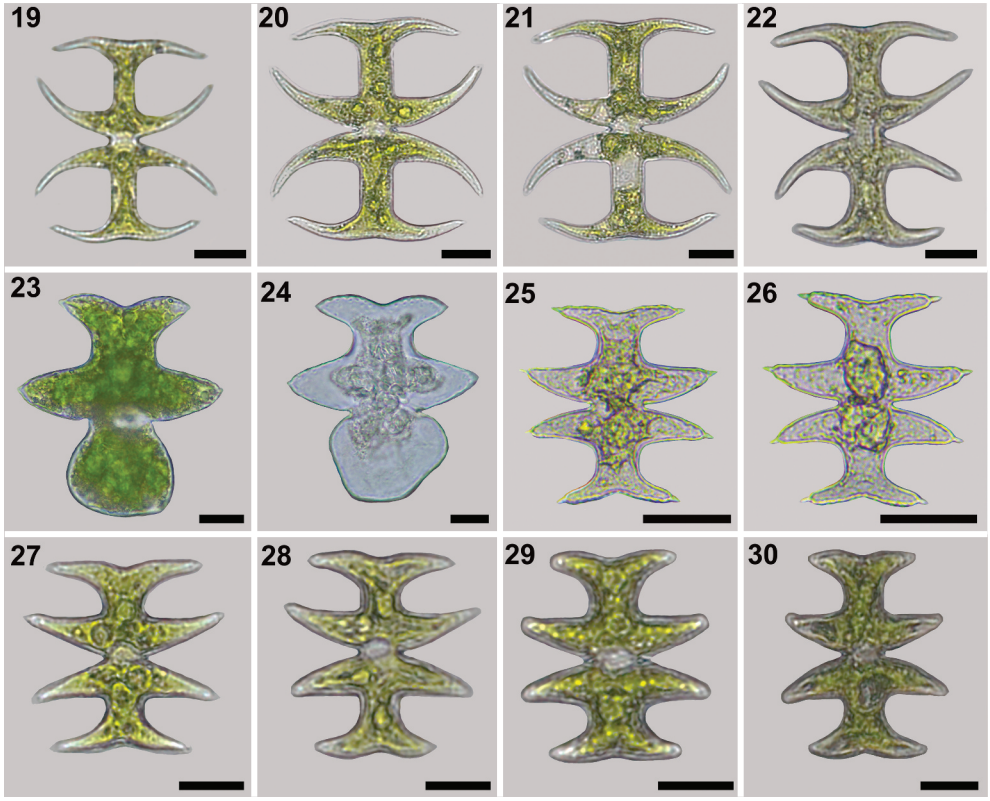
REMARKS: Figure 27 (present study) is taken as a representative illustration of *Pseudomicrasterias* sp. Further studies are necessary to conclude the species circumscription and know the relationship of this material with other infraspecific taxa of *M. arcuata*. A formaldehyde fixed sample of strain CCMA-UFSCar 719 is deposited in the Herbário Científico do Estado 'Maria Eneyda P. Kauffmann Fidalgo – SP', at the Instituto de Botânica, São Paulo, São Paulo State, Brazil, with access number SP400045. Material of the authentic strain CCMA-UFSCar 719 is maintained in the Culture Collection of Freshwater Microalgae, Universidade Federal de São Carlos (CCMA-UFSCar), São Carlos, São Paulo State, Brazil.

DISCUSSION

The information presently available on the phylogeny of Desmidiaceae is consistent with previous studies that indicated that the number of lineages exceeded the number of genera currently recognized for the family (Gontcharov & Melkonian 2010). Our results showed that the three taxa previously regarded as infraspecific of *M. arcuata* form a distinct lineage within the Desmidiaceae.

Species of *Pseudomicrasterias* have cell walls with fine to quite prominent pores and without spines or any other kind of ornamentation. In contrast, species of *Micrasterias* usually have ornamented cell walls, some species showing prominent spines in the apical margin of semicells (*e.g. Micrasterias fimbriata* Ralfs; Neustupa *et al.* 2011) or more than one spine or tooth at the apices of basal and polar lobes (Prescott *et al.* 1977).

Micrasterias arcuata was transferred by Archer (in Pritchard 1861) to the genus *Tetrachastrum* R.V. Dixon, published a short time earlier with three included species (Dixon 1859): *T. mucronatum* R.V. Dixon, *T. oscitans* (Ralfs) R.V. Dixon and *T. pinnatifidum* (Ralfs) R.V. Dixon. Archer (in Pritchard 1861) divided the genus into two groups: (1) species with extremities of lobes entire, mucronate or acute and (2) species with extremities of lobes bidentate. *Tetrachastrum arcuatum* (Bailey) W. Archer and *T. expansum* (Bailey) W. Archer (= *M. expansa* Bailey) were classified in group 1 together with *T. mucronatum*, whereas *Tetrachastrum* group 2



Figs 19–30. *Pseudomicrasterias* species, LM.
Figs 19–22. *Pseudomicrasterias arcuata*, CCMA-UFSCar 717 strain. Scale bar = 25 µm.
Figs 23–26. *Pseudomicrasterias perforata*, CCMA-UFSCar 718 strain. Scale bar in Figs 23, 24 = 10 µm. Scale bar in Figs 25, 26 = 25 µm.
Figs 27–30. *Pseudomicrasterias* sp., CCMA-UFSCar 719 strain. Scale bar = 25 µm.

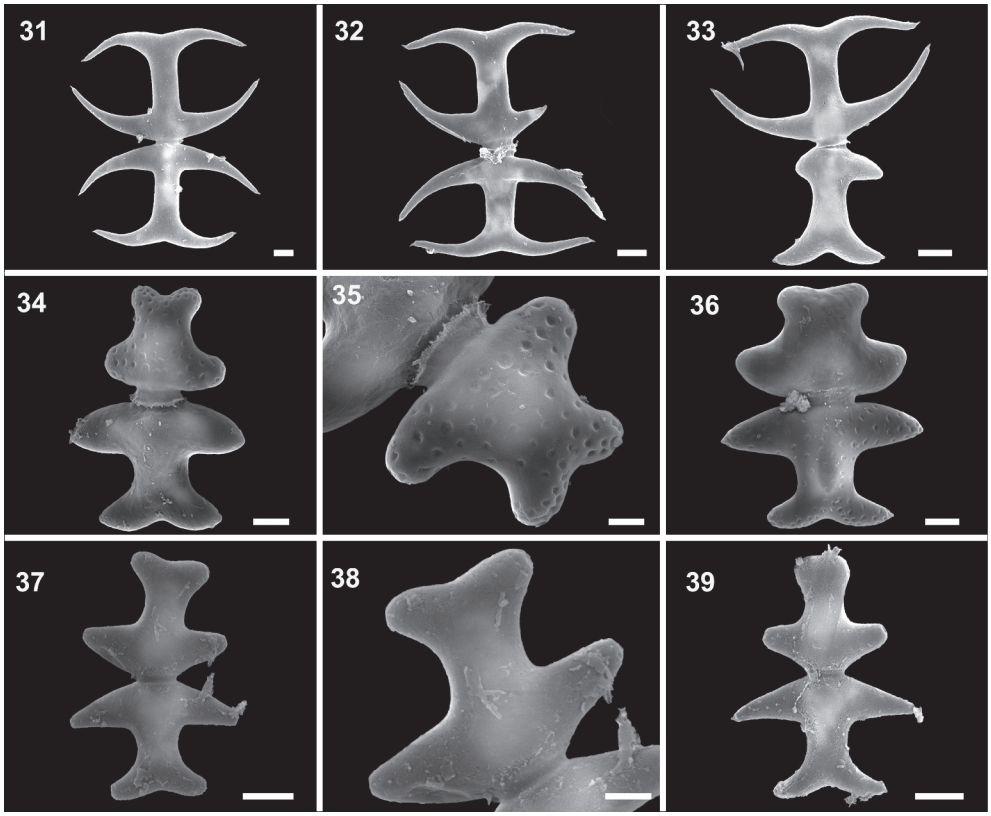
included *T. oscitans, T. pinnatifidum* and three other species (Pritchard 1861). The genus *Tetrachastrum* was treated by Krieger (1939) and all subsequent monographers as a synonym of *Micrasterias* (Guiry 2013).

Despite a similarity in general cell shape, a comparison between *Micrasterias arcuata* and the species included in *Tetrachastrum* by Dixon (1859) reveals some morphological differences. *Tetrachastrum mucronatum* has the polar lobe with a short lateral projection tipped with a mucro, and broadly round, bluntly triangular basal lobes, with one, two or three mucronate spines at the margin on each side (Dixon 1859). Both *T. oscitans* and *T. pinnatifidum* include individuals with the extremities of all lobes bidentate (Dixon 1859; Pritchard 1861). Of the three species, *T. pinnatifidum* (= *M. pinnatifida*) is the most morphologically similar to *M. arcuata*, but differs in having the apical margin straight to slightly convex, the lateral lobes tumid and especially in the presence of two teeth/spines at the end of both polar and basal lobes (Ralfs 1848). Moreover, in the phylogenetic reconstructions available *M. pinnatifida* clustered with *Micrasterias dickiei* (Ralfs) Škaloud, Nemjová, Veselá, Černá & Neustupa, within subclade A4 together with *Micrasterias furcata* C. Agardh *ex* Ralfs, the type species of the genus *Micrasterias* (Guiry 2013). Therefore, although phylogenetic data on *T. mucronatum* and *T. oscitans* are not yet available due to the inexistence of strains in culture collections, present evidence suggests that *Micrasterias arcuata* belongs in a genus different from *Tetrachastrum*, which we describe herein as *Pseudomicrasterias gen. nov.*

Our results illustrate the closely related phylogenetic position of *Pseudomicrasterias* and *Euastrum* 2 lineage (*e.g. Euastrum affine, Euastrum oblongum*). Species belonging to the *Euastrum* 2 lineage have 3- or 5-lobed (*Euastrum pinnatum*) pyramidal semicells, with rather deep incisions separating the basal, lateral and apical lobes. The basal lobes are arcuate and show round, entire or sinuate-emarginated lobe apices (Pritchard 1861), with a deep, closed invagination in the middle region of the apical semicell (Prescott *et al.* 1977).

The molecular phylogenetic analyses revealed two lineages comprising three taxa previously ranked as infraspecific taxa of *M. arcuata*. These three taxa can be recognized on the basis of morphological analyses through careful light microscopical observations and morphometric analyses, and by SEM examination. The width of the polar lobe, the depth of the apical margin incision and the curve formed by the basal lobe were the most important features for the morphological differentiation and delimitation of the strains. The traditional morphometric features MCL, BLD, IW and HPL were found to be important, and in line with some classical attempts to characterize infraspecific taxa of *M. arcuata* (West & West 1897; Borge 1899; Förster 1964). A similar agreement between phylogenetic relationships and the results of accurate identification of infraspecific taxa by morphological features has previously been documented in desmids studies (Neustupa *et al.* 2010; Nemjová *et al.* 2011; Šťastný *et al.* 2013).

Attempts to analyse and morphologically correlate the strains reported herein with previously published



Figs 31–39. *Pseudomicrasterias* species, SEM.
Figs 31–33. *Pseudomicrasterias arcuata*, CCMA-UFSCar 717 strain. Scale bar = 10 µm.
Figs 34–36. *Pseudomicrasterias perforata*, CCMA-UFSCar 718 strain. Scale bar in Figs 34, 36 = 10 µm. Scale bar in Fig. 35 = 5 µm.
Figs 37–39. *Pseudomicrasterias* sp., CCMA-UFSCar 719 strain. Scale bar in Figs 37, 39 = 10 µm. Scale bar in Fig. 38 = 5 µm.

morphological data on *M. arcuata* and its infraspecific taxa (especially from the illustrations of classical literature) produced an interesting discriminative framework that allowed for delimitation and differentiation. Although these illustrations represent material collected under diverse field conditions and the accuracy of the drawings may be somewhat uncertain, our photomicrographs accurately revealed cell morphology and circumscription, in such way that all strains investigated fit well into the taxonomic definition and circumscription of *M. arcuata*.

In addition to the type species *P. arcuata*, we describe as new a second species, *P. perforata*. The latter species resembles *M. arcuata* var. *perforata* as described in Förster (1964). However, holotypes of new taxa were not designated by Förster (1964) and the indication of two different altitudes associated with the description of the locality from which the material originated raises doubts on whether 'a single gathering' was used as the source of the specimens described (Turland *et al.* 2018, Art. 40.2). To avoid the nomenclature instability caused by different views on whether *M. arcuata* var. *perforata* was validly published (*e.g.* see Förster 1981) we describe our material as a new species, rather than a new combination and status, with the advantage of associating the new species name with modern-type material and a DNA sequence, which are expected to reduce ambiguity in the future use of the name.

Two further taxa were described in Förster (1964) and associated with *M. arcuata* var. *perforata*: '*M. arcuata* f. *robustior*' was recorded along with var. *perforata* in Serra das Almas, Bahia State, Brazil (however, the indication of two sampling sites rendered the name invalidly published); *M. arcuata* f. *magniperforata* was a rare taxon recorded from material collected in Rio das Fêmeas, Goiás State, Brazil. The main difference between the two forms and var. *perforata* was in the shape of the polar lobes, which were straight in var. *perforata*, curved upward in f. *magniperforata*, and short and robust in the smaller (or juvenile), compact specimens of 'f. *robustior*'. All these morphological variations were observed in specimens of strain CCMA-UFSCar 718, suggesting that both forms should be considered synonyms of *Pseudomicrasterias perforata*.

Pseudomicrasterias sp. is morphologically similar to several infraspecific taxa of *M. arcuata* (Förster 1964, 1969). The main difference observed between our specimens and those of *M. arcuata* var. *robusta* f. *goyazensis* is the shape of the basal lobes, which in this form were uniformly rounded, open at an acute angle and straighter at the isthmus region (Förster 1964). Several other infraspecific taxa presented retuse basal lobes, whereas they varied from retuse to slightly arcuate in *Pseudomicrasterias* sp.

The geographic distribution of *Pseudomicrasterias* strains may be inferred through the analysis of all published records

of *M. arcuata* and its infraspecific taxa. *Pseudomicrasterias arcuata* seems to be widely distributed on all continents since the end of the 19th century, occurring more frequently in aquatic ecosystems of the USA (Bailey 1851; Prescott & Scott 1943, 1952; Prescott *et al.* 1977) and in Brazil (Förster 1964, 1969; Sormus 1980; Bicudo & Gil-Gil 2003). Interestingly, *P. perforata sp. nov.* was reported only for Brazil, near the border of Bahia and Minas Gerais States (Förster 1964), Bahia State (Förster 1964; Ramos *et al.* 2019), Goiás State (Förster 1964), Rio Grande do Sul State (Bittencourt-Oliveira & Mecenas 1994, as *M. arcuata* var. *subpinnatifida*) and São Paulo State (present study). *Pseudomicrasterias* sp. is currently reported only for the Itirapina municipality, São Paulo State.

All *Pseudomicrasterias* species present a wide morphological variation during cell development and some reproductive features were observed. Asexual reproduction occurred by cell division and sexual reproduction by conjugation, as in all Zygnematophyceae (Calderón & Tavera 2020). Although the architecture and structural differences of zygospores are not available for the three species presently investigated, some sexual reproductive stages were recorded, namely the two germling cells or the two sister gones that were released from the germination vesicle, and pre-vegetative cells. The zygospore of *M. arcuata* was illustrated by Förster (1964, 1969); we take the irregularly rounded to ellipsoid zygospore with a dark brown thick wall illustrated by Förster (1964) under the name *M. arcuata* var. *gracilis* as representing the zygote of *Pseudomicrasterias arcuata*. The zygospore of *Pseudomicrasterias perforata* illustrated and described by Förster (1964) as ellipsoid with brown wall from specimens identified as *M. arcuata* [var. *perforata*] f. *robustior*. Ramos *et al.* (2019) have illustrated a zygospore of *M. arcuata* var. *perforata* identical to the one reported by Förster (1964) from material collected in Chapada Diamantina, Bahia State, Brazil.

We observed many juvenile specimens or pre-vegetative cells of *Pseudomicrasterias perforata* in groups that probably divided before completing their development. According to Calderón & Tavera (2020), it corresponds to an important reproductive feature designated as pleomorphic variation, during which cell division occurs in juvenile forms before their full development. This has been observed in several genera of Desmidiaceae (Kallio 1951, 1963; Licalli 1973) and has recently been recorded in cultivated strains of *Staurastrum gracile* Ralfs (Calderón & Tavera 2020). Pleomorphism may affect morphological variability and has implications for taxonomy, as different development stages end up being unduly described as different, often infraspecific, taxa (Calderón & Tavera 2020). This was perhaps the case with the taxonomic forms of *M. arcuata* var. *perforata* described by Förster (1964).

The isolates (three strains) we report on came from a bog located in São Paulo State, Brazil. Considering the broad geographical distribution of *M. arcuata* and its documented morphological variability, it is possible that the diversity within the *M. arcuata* species complex is much higher than presently known, perhaps including both presently undescribed species and infraspecific taxa.

Although 738 different taxa of Zygnematophyceae (including infraspecific ranks) are registered or deposited in the six different microalgae public collections available worldwide (Zhou & von Schwartzenberg 2020), no cultures of *M. arcuata* are presently available in these collections. The current isolates are the first to be reported for *M. arcuata* in a public culture collection; they will be designated from now on as *Pseudomicrasterias* species. The DNA sequences associated with these strains will provide an important base for comparison with other species and strains.

Studies based on polyphasic approaches are important for a better understanding of biodiversity and the correct biological delimitation of numerous species complexes that include taxonomic and nomenclatural inconsistencies (Neustupa *et al.* 2010, 2011, 2014; Nemjová *et al.* 2011). The present results will also be of interest for taxonomical, nomenclatural and ecological studies, including metabarcoding approaches. This is particularly relevant for Desmidiaceae, which include a large number of taxa and/or ecomorphae in need of taxonomic clarification.

The present study demonstrated the usefulness of SSU rDNA, *rbc*L and *psa*A markers for determining the infraspecific diversity of *M. arcuata* species complex. The use of combined morphological analyses as linear measurements in a standardized way, or the use of geometric morphometric methods, appear to be powerful tools to differentiate species, even within a complicated species complex, and supported the recognition of *Pseudomicrasterias* and the discrimination of the three strains investigated.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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