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Morphological and Morphometric Description of a Novel Shelled Amoeba *Arcella gandalfi* sp. nov. (Amoebozoa: Arcellinida) from Brazilian Continental Waters

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Abstract. Arcellinida are free-living lobose amoebae that produce an outer shell (test). Here, we describe a conspicuous new species, *Arcella gandalfi* sp. nov, from Brazilian continental waters, along with a morphological and biometrical characterization. Test diameter and test height are on average 81 and 71 respectively. This new species has an apical conical extension, which differentiates it from other *Arcella* species. *A. gandalfi* seems to be closely-related to *A. brasiliensis*, due to the distinct marginal ring (test brim) present only in these two species. Since *A. gandalfi* is easily identified by morphological features and due to its apparent geographic restriction to South America, we discuss its possible use as a new flagship species.

Key words: Testate amoeba, new species, morphology, morphometry, flagship species.

INTRODUCTION

Arcellinida are free-living lobose amoebae that produce an outer shell (test). These organisms are currently classified in the Amoebozoa: Tubulinea (Adl *et al.* 2005) and are largest and most diverse group of testate amoebae, with an estimated 700 to 1,300 nominal species (Meisterfeld 2002, Kosakyan *et al.* 2016), but this number may increase significantly with recent discoveries of widespread cryptic/pseudo-cryptic species (Kosakyan *et al.* 2013, Oliverio *et al.* 2015). Thus, the real richness of testate lobose amoebae is difficult to estimate. This is mainly due to sparse sampling and unrevised nomenclature, with a large number of synonymous, homonymous and ambiguous taxa (Kosakyan *et al.* 2016). Furthermore, many species of testate amoebae show high intraspecific morphological variability, suspected to be induced by environmental conditions (Wanner and Meisterfeld 1994, Bobrov and Mazei 2004, Porfírio-Sousa *et al.* 2016).

Arcella Ehrenberg 1832 is one of the largest genera, with more than 130 described taxa (Tsyganov and Mazei 2006). Taxonomy in this genus is generally based on shell morphology and morphometry. *Arcella* has a hemispheric shell in side view, and circular in apertural view (Pearl and Dunbar 1903). Tests are

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made up with organic material, secreted by the organism and arranged in hexagonal alveolar units (Netzel and Grunewald 1977). Due to lack of morphological and morphometric data, as well as phenotypic plasticity, some *Arcella* species are difficult to distinguish (Tsyganov and Mazei 2006). They inhabit diverse freshwater environments such as lakes, rivers, streams, mosses, macrophyte roots, plankton and benthos (Dabés and Velho 2001). Many species are cosmopolitan, but others have a restricted geographical distribution (Beyens and Meisterfeld 2002).

Several geographic regions are poorly studied and numerous new species remain to be found around the world, especially in the Southern Hemisphere (Smith *et al.* 2007). Descriptions of new species as well as new occurrences of described species illuminate real diversity in different environments, helping in the assessment of cosmopolitan and endemic species (Nicholls 2005, Yang *et al.* 2005, Bobrov and Kosakyan 2015, Reczuga *et al*. 2015). Of special interest are easily recognizable, large species that can be found in the southern hemisphere, as these can be considered “flagship species” (Foissner 2006, Lahr and Souza 2011, Zapata and Fernandez 2008).

Here we describe a new *Arcella* morphotype (*Arcella gandalfi* sp. nov.), using morphological and biometrical tools. Since *A. gandalfi* is a large organism, widely distributed in Brazilian continental waters, and clearly defined by morphological features, we propose it as a new flagship species that is geographically restricted to South America.

MATERIALS AND METHODS

This new *Arcella* morphotype was found in widespread localities within Brazil, in the states of Minas Gerais (September, 1991), Tocantins (December, 2009), Paraná (December, 2011), Amapá (May, 2015) and Rio de Janeiro (December, 2015) (Table 1). The environmental parameters (Table 2) were measured *in situ* in each locality, excepted for Minas Gerais, where the parameters shown are obtained from Honorato and Pelli (2011).

Thirty individuals were separated from the Rio de Janeiro sample to obtain the morphological and morphometric data (there were no living individuals to obtain molecular data). Individuals were embedded into glycerin and mounted on slides using coverslips with a small sphere of modeling clay on the edges to prevent smashing of specimens. This method allows slight movements in the coverslips to observe both apertural and lateral views while avoiding shell damage. The slides were observed under an AxioVert A1 inverted microscope (Zeiss Inc.), using 40 x and 63 x (oil) Differential Interference Contrast optics. We used DarkTable open source software (www.darktable.org) for photodocumentation. Images were then analyzed using open-source software FIJI (available from http://fiji.sc; Schindelin, 2012) with the plugin Wormbox (available in https://codeload.github.com/nelas/WormBox/legacy.zip/master) to obtain measurements from images. A few representative individuals were selected for scanning electron microscopy analyses, and prepared according to the protocol described in Lahr and Lopes (2006). Samples were then analyzed under a Sigma VP (Zeiss, Inc.) SEM, at IB-USP imaging facility (CAIMi).

Ten characters were measured; in lateral view (Figure 1A): test height (th), test top diameter (ttd), test top invagination (tti), aperture height (ah); in apertural view (Figure 1B): test diameter (td), aperture diameter (ad), test border (tb1 and tb2) and brim width (bw1 and bw2). Morphometric data were analyzed using the software Paleontological Statistics (PAST, version 3.02, available in http://folk.uio.no/ohammer/past/). The following statistics were calculated: arithmetic mean (X), median (M), standard deviation (SD), standard error of mean (SE), coefficient of variation in % (CV), extreme values (MIN and MAX); additionally we constructed histograms showing normal curves and the data curve calculated by Kernel Density Estimation, these analysis primarily describe the data from measurements obtained.

RESULTS

Taxonomic Description

Amoebozoa (Lühe 1913, emend. Cavalier-Smith 1998)
Tubulinea (Smirnov *et al.* 2005)
Arcellinida (Kent, 1880)
Sphaerothecina (Kosakyan *et al.* 2016)
Arcellidae (Ehrenberg 1843)
Arcella gandalfi sp. nov. Féres, Porfírio-Sousa, Ribeiro, Rocha, Sterza, Souza, Soares and Lahr

Shell morphology: Shell hemispherical with an apical cone in lateral view (Fig. 2 A–F). The top of this conical extension is closed and presents a minor invagination (Fig. 2 A–D and Fig. 3 A–D). The top invaginated region is not always circular (Fig. 3 A–D). These features give the shell a “funnel shape” which has not been reported in any other *Arcella*. Shell is circular in apertural view with a circular, central and invaginated aperture (pseudostome) with a small collar (Fig. 4 A–B). Distinct marginal ring (test brim) present (Fig. 4 A–B) similar to *Arcella brasiliensis* (Fig. 5; see Lahr and Lopes 2009 for a description of *Arcella brasiliensis*), this feature is distinctive from the other *Arcella* species. Scanning Electron Microscopy (SEM) observations showed that the chitinous shell is made of small hexagonal alveolar units, however these units are elongated on the conical extension (Fig. 6 A–C). Shell color varies from light yellow to brown.

Table 1. Geographical localities and characteristics of investigated samples. N/A – data not available, in these localities, the presence of the organism was recorded, but no counts were made as to numbers of individuals.

Locality	Coordinates	Collection date	N	Microhabitat
Gameleira River (Uberaba/MG)	20º00'50.00"S 47º52'52.00"W	September, 1991	N/A	Shallow river, with decomposition of many plant residues, under the influence of wetland areas (Varzea lagoon)
"Vereda" Bonfim (Conceição do Tocantins/TO)	11º46'40.27"S 47º43'27.69"W	December, 2009	N/A	Shallow-water environments, characterized by the presence of palm trees (buritis) and decomposition of organic matter
Paraná River (Ilha Porto Rico/PR)	22º45'17.52"S 53º15'28.62"W	December, 2011	105	River margin comprised mainly of grass and sedges. Average depth 1.2 m, approximate area 0.91 ha
Araguari River (Porto Grande/AP)	00º47'46.21"N51º19'32.16"W	May, 2015	2	Deep river, surrounded by forest fragments and pasture
Veiga Lagoon (São João da Barra/RJ)	21º49'45.54"S 41º0'24.74"W	December, 2015	110	Shallow fresh water coastal lagoon, surrounded by marginal vegetation (*Typha* sp.) and near a port complex

Table 2. Environmental parameters of investigated samples. Units: Electrical conductivity (μs/cm), total phosphorus (μg/L), dissolved oxygen (mg/L) and temperature (°C).

Locality	Conductivity	Total phosphorus	Dissolved oxygen	pH	Temperature
Gameleira River (MG)	116.3	4700.0	2.7	5.6	21.0
"Vereda" Bonfim (TO)	65.5	70.0	1.9	6.87	27.9
Paraná River (PR)	56.4	19.4	9.21	7.37	27.1
Araguari River (AP)	21.4	< 50	5.36	5.17	26.8
Veiga Laggon (RJ)	941.0	61.0	0.37	7.38	24.6

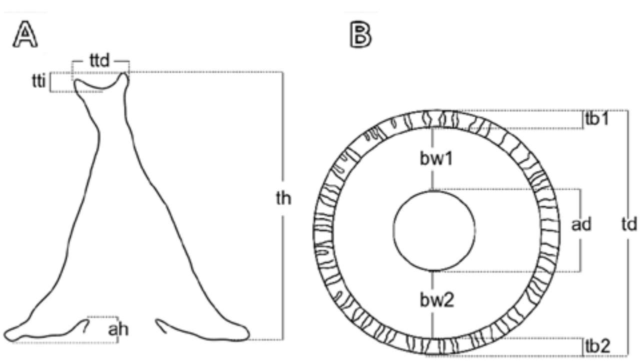

Fig. 1. Representation of measured characters. **A** – *Arcella gandalfi* in lateral view, showing aperture height (ah), test height (th), test top invagination (tti) and test top diameter (ttd). **B** – *Arcella gandalfi* in apertural view, showing the test diameter (td), aperture diameter (ad), test border 1–2 (tb1–2) and brim width 1–2 (bw1–2).

Shell morphometry: Test diameter on average 81 μm, test height 71 μm, test border (1–2) 7 μm, test top invagination 3 μm, test top diameter 17 μm, brim width (1–2) 21 μm, aperture diameter 24 μm and aperture height 5 μm. Test height has the largest range compared to others characters, ranging from 52 to 91, with standard deviation of 11.8 (Table 3). The test diameter and brim width are the least variable measures (CV 6–8.9, Table 3) compared to all other characters (CV 11–49.2, Table 3), with standard error of the mean ranging from 0.3 to 0.89 (Table 3). Test top invagination and test top diameter are the most variable measures (CV 28.8 and 36.2, respectively, Table 3), with standard error of the mean 0.2 and 0.9, respectively. The aperture height (ah), test diameter (td) and test top diameter (ttd) present a distribution similar to a bimodal distribution (Fig. 7), test height (th) shows a different distribution,

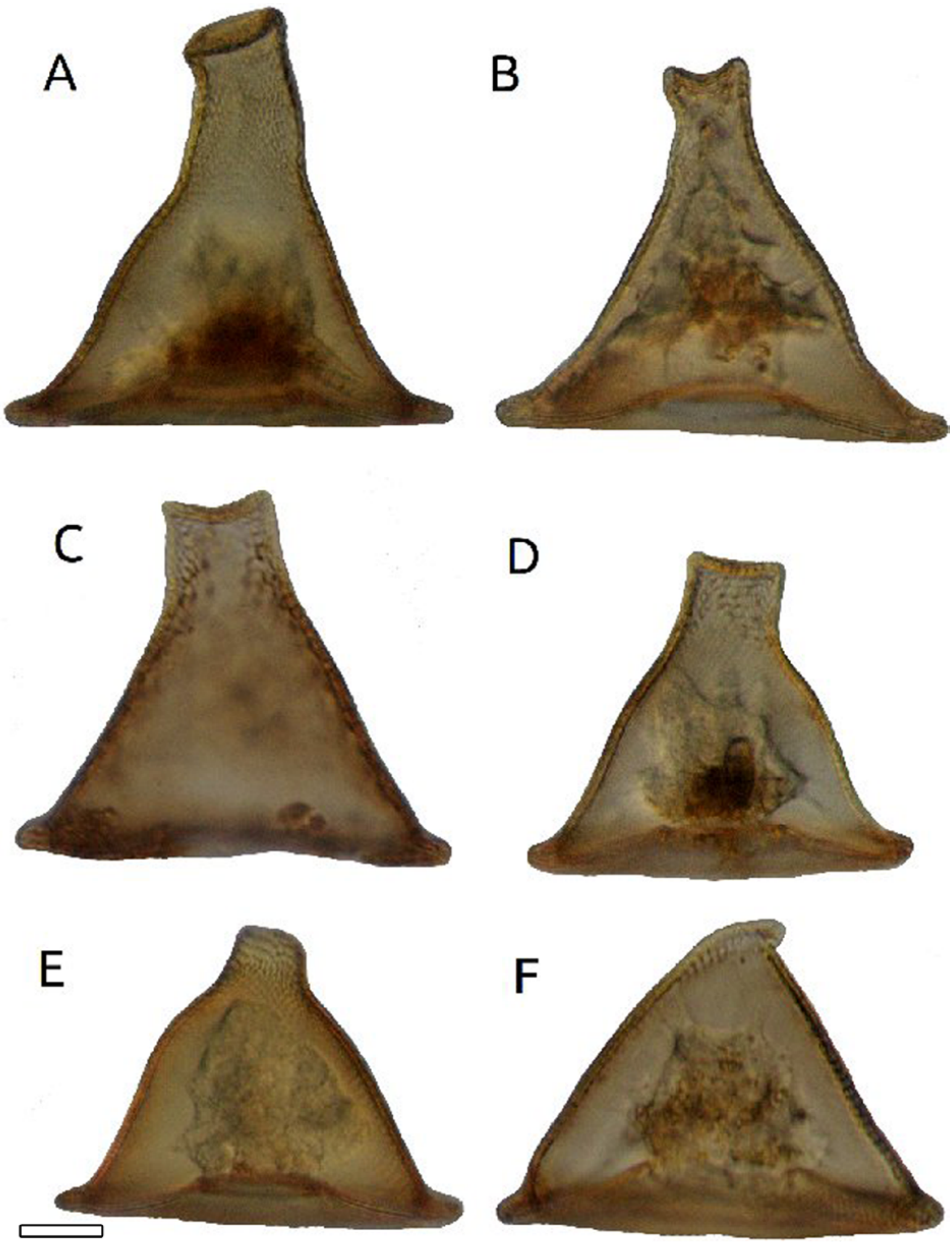

Fig. 2. Lateral view of six individuals of *Arcella gandalfi*. **A** and **B** – individuals with highest shell, top invagination is easily seen; **C** and **D** – individuals with intermediate shell height, top invagination easily seen; **E** and **F** – individuals with lowest shell height. Scale bar: 20 μm.

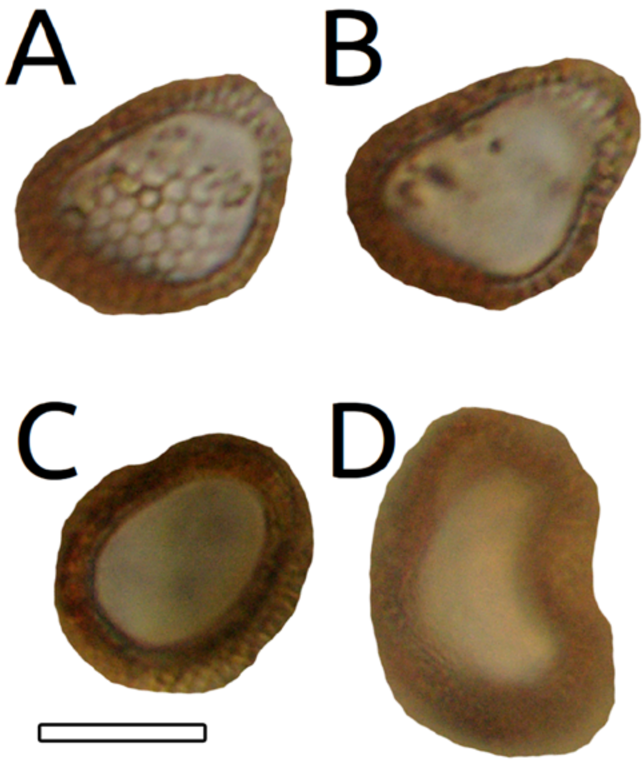

Fig. 3. Top view of the *Arcella gandalfi* shell showing the top invagination region. **A** – top of the shell with alveolar units, showing that this region is closed; **A–D** shows that the top is not always circular. Scale bar: 20 µm.

while all the other characters show a nearly normal distribution (Fig. 7).

Etymology: The specific epithet refers to the shape of the shell that resembles a wizard's hat, similar to that of Gandalf the Grey, a central character in J. R. R. Tolkien's fantasy novel The Lord of the Rings.

Type specimen: Fig. 2 A.

Ecology: *Arcella gandalfi* was found in both still and running freshwater environments, presenting a range of ecological characteristics (Table 1) and environmental parameters (Table 2). Electrical conductivity, total phosphorus and dissolved oxygen show a large range comparing all localities, ranging from 21.4 to 116.3 µs/cm, 19.4 to 4700.0 µg/L and 0.4 to 9.2 mg/L, respectively. Although, pH and temperature were less variable, ranging from 5.2 to 7.4 and 21.0 to 27.9°C, respectively (Table 2). Therefore it seems that *A. gandalfi* may occur in various trophic conditions, preferably in acid to neutral pH and in relatively high temperatures.

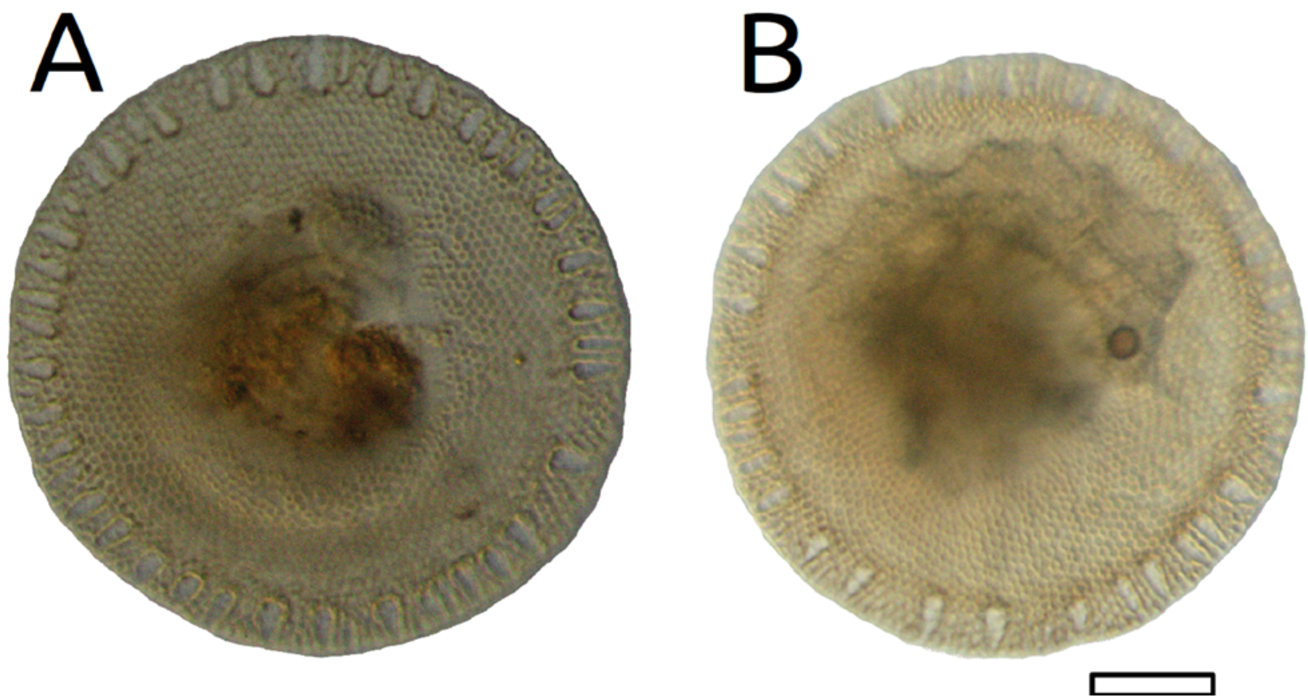

Fig. 4. Apertural view of two individuals of *Arcella gandalfi*. **A** and **B** – distinct marginal ring (test brim) similar *to Arcella brasiliensis* easily seen. Scale bar: 20 µm.

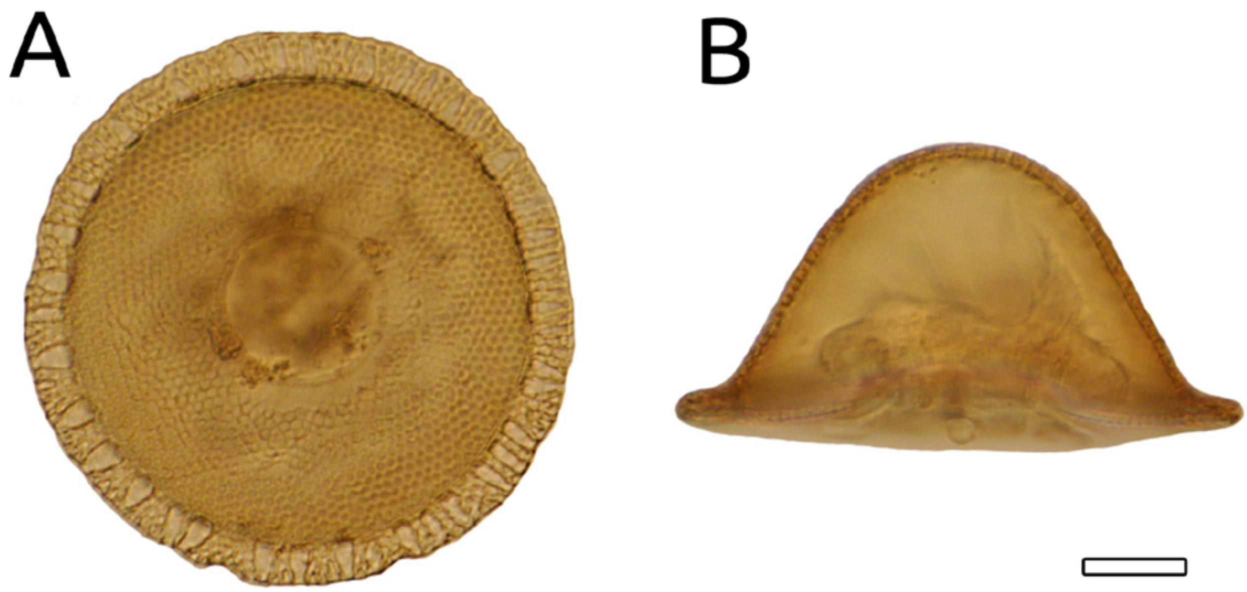

Fig. 5. Representative images of an *Arcella brasiliensis* individual. **A** – apertural view, showing the distinct marginal ring (test brim); **B** – lateral view showing the rounded dome. Scale bar: 20 µm.

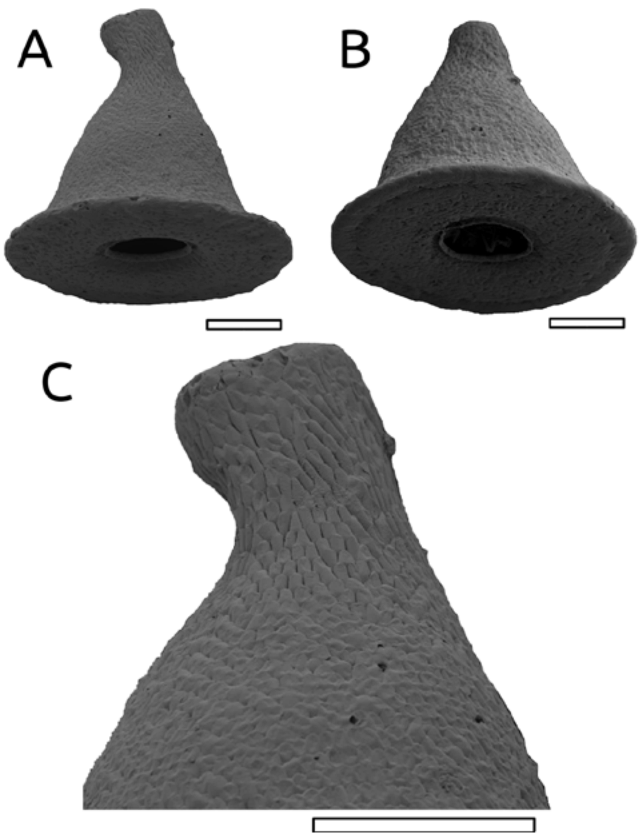

Fig. 6. Morphology of *Arcella gandalfi* under Scanning Electron Microscopy (SEM). **A** and **B** – lateral view showing the aperture region; **C** – shell detail showing elongated shape of the alveolar units on the conical extension of the shell. Scale bars: 20 µm.

DISCUSSION

Arcella gandalfi is a novel morphospecies, with a characteristic funnel-shaped shell. This shape, a conspicuous conical extension on the abapertural surface, is the main feature distinguishing *A. gandalfi* from any other *Arcella*. However, *A. gandalfi* shares morphological similarities with *A. brasiliensis*, like the distinct marginal ring (test brim), present in both species (see Lahr and Lopes 2009 for a description of *Arcella brasiliensis*). This distinct marginal ring was an exclusive feature of *A. brasiliensis* until now, which suggests that these species are closely related taxa. Future molecular analyses will provide a better understanding of this evolutionary relationship.

The structural hexagonal alveolar units are also observed in other *Arcella* species (Lahr and Lopes 2009). However, in *A. gandalfi*, these units exhibit an elongated shape on the conical extension of the shell that can be easily seen under SEM (Fig. 6 C). Such findings raise the question of how *A. gandalfi* shapes its conical extension. Some studies (Netzel 1971, Mignot and Raikov 1990) reveal the mechanism of cell division and daughter test construction of *Arcella*, as well as formation of the hexagonal alveoli. The size frequency distribution analyses indicate that *A. gandalfi* is a size-polymorphic species. Test height and test diameter distributions curves are not normal, with a high frequency on extreme values (Fig. 7).

Table 3. Morphometric characterization of individuals of *Arcella gandalfi*. X – arithmetic mean, M – median, Min – minimum, Max – maximum, SD – standard deviation, SE – standard error of the mean, CV – coefficient of variation in %, n – number of investigated specimens. Measures in µm.

Measures	X	M	Min	Max	SD	SE	CV	n
td	81	80	73	92	4.9	0.89	6	30
ad	24	24	16	30	2.7	0.5	11.4	30
ah	5	5	3	7	1.2	0.22	20.7	28
tb1	7	7	4	9	1.1	0.2	16.2	30
tb2	7	7	4	10	1.1	0.2	15.8	30
bw1	21	21	16	24	1.9	0.3	8.9	30
bw2	21	21	17	25	1.8	0.3	8.4	30
th	71	70	52	91	11.8	2.1	16.7	30
tti	3	3	1	7	1.3	0.2	36.2	28
ttd	17	15	9	30	5	0.9	28.8	30

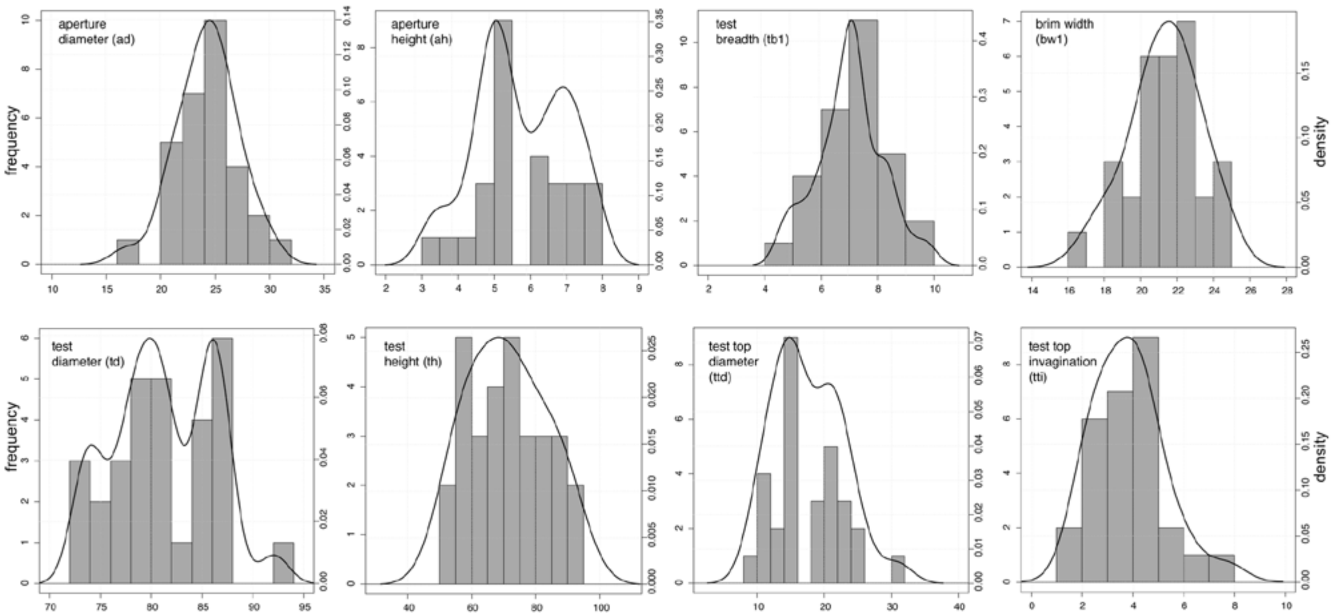

Fig. 7. Histograms depicting distribution of data for characters analyzed in this study. The black lines represent density curves that fit our data. Aperture height (ah), test diameter (td) and test top diameter (ttd) present a distribution similar to a bimodal distribution, while all the other characters show a nearly normal distribution. Characters tb2 and bw2 are not shown since they are equal to tb1 and bw1, respectively.

The conspicuous morphology defines *A. gandalfi* as a new example of flagship species: easily identified by morphological features and beyond any possible taxonomic misidentification, with a possibly restricted geographic distribution (Foissner 2006). We have found *A. gandalfi* widespread in the Brazilian territory, which was the only country sampled, but there is no reason to believe that such a widespread organism would not be present in other South-American countries. Flagship species are a proposed tool in biogeographic studies of microbial eukaryotes, designed to mitigate the effects of under-sampling (see Foissner 2006 for a review). The rationale is that very conspicuous, large organisms found in the Southern hemisphere should be endemic, because the Northern hemisphere is better studied (Smith and Wilkinson 2007). Opposite findings (i.e., large conspicuous organisms restricted to the Northern hemisphere) would not carry as much weight, since a large amount of basic information is still not known in the Southern hemisphere. The conclusion is that conspicuous organisms should have been recorded in the North if they occurred there (Mitchell and Meisterfeld 2005). In testate amoeba biogeographic studies, *Pseudonebela africana* and *Apodera vas* are two well-known proposed flagship species (Mitchell and Meisterfeld 2005, Smith and Wilkinson 2007, Zapata and Fernández 2008).

The "flagship species" strategy may have an Achilles heel. Even though the Northern hemisphere is better studied, microbial eukaryotes are still far more undersampled when compared to macroorganisms, especially when it comes to records in natural environments (Heger *et al.* 2014). A telling example is the testate amoeba *Pseudonebela africana*, recorded for Africa and South America, and tentatively proposed as having a Gondwanan distribution (Lahr and Souza 2011). More detailed, careful observations have revealed *P. africana* is also present in the Northern hemisphere, and maybe even a common species, as it is very abundant in samples from Tirol (Siemensma and Opitz 2014). Previous researchers in Europe had overlooked the presence of this species. Thus, although the logic behind the use of flagship species is elegant, care must be taken when making broad inferences, as is the case generally with microbial eukaryotes. At the moment, *Arcella gandalfi* seems biogeographically restricted to the South American continent, and stands as one more piece of evidence against the 'everything is everywhere: but the environment selects' paradigm (Wit and Bouvier 2006, O'Malley 2008). However, deeper observations in similar environments, especially in Southern Asia and Africa, where data are scarce (Qin *et al.* 2011, Schwind *et al*. 2013) need to be made to specifically test this hypothesis.

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