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ORIGINAL ARTICLE

# Description of *Phaeobola aeris* gen. nov., sp. nov (Rhizaria, Cercozoa, Euglyphida) Sheds Light on Euglyphida's Dark Matter

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## ABSTRACT

The majority of Euglyphida species are characterised by shells with imbricated silica scales. Environmental surveys indicate a large unexplored diversity and recent efforts hinted at a certain diversity of yet undescribed, inconspicuous, scale-lacking Euglyphida. Here we describe *Phaeobola aeris* gen. nov., sp. nov. that shows a variety of morphological characters typical for the Euglyphida but lacks silica scales-instead, this species bears an agglutinated test. Neither its morphology nor phylogenetic placement allows its assignment to any currently described family. We erected the yet monospecific genus *Phaeobola gen.* nov., which with yet available data remain Euglyphida *incertae sedis*.

EUGLYPHIDA are an abundant order of testate amoebae in soil litter, mosses, and freshwater habitats, with some taxa being also found in brackish and marine environments (Heger et al. 2010; Todorov et al. 2009). They secrete a characteristic test (shell), which in most described species is reinforced by silica scales whose size, shape, and arrangement are taxonomically informative (Chatelain et al. 2013; Heger et al. 2010; Kosakyan et al. 2016). Phylogenetic analyses suggested a close relationship of several cercozoan taxa, that is Euglyphida, Thaumatomonadida, Spongomonadida, and others that are able to build silica scales, which were accordingly grouped within the class Imbricatea (Cavalier-Smith and Chao 2003; Scoble and Cavalier-Smith 2014). Scale shape, size, and arrangement vary within Imbricatea. Many Euglyphida bear single-tiered scales agglutinated to a shell with a yellowish to brownish organic cement, while most Thaumatomonadida bear interlocking two-tiered scales with much higher complexity and variation in shape (Dumack and Siemensma 2020; Dumack et al. 2018; Scoble and Cavalier-Smith 2014). The ancestor of Imbricatea is presumed to have possessed more or less oval single-tier scales, which subsequently diversified in shape (Scoble and Cavalier-Smith 2014). The recent discovery of *Kraken*, which bears such scales and branches weakly at the base of Imbricatea and its sister class Thecofilosea, supports this hypothesis (Cavalier-Smith et al. 2018; Dumack et al. 2016, 2017).

However, not all Imbricatea bear scales. Increasing reports of scale-lacking Imbricatea, which do not necessarily have to be closely related, indicate a frequent loss of scales in Imbricatea (Dumack et al. 2019; Howe et al. 2011; Scoble and Cavalier-Smith 2014; Shiratori et al. 2014). Although scales have been considered an important synapomorphy defining the Euglyphida (Meisterfeld 2002), novel findings of scaleless taxa in Euglyphida cast doubt on the validity on the taxonomical value of this trait. The Paulinellidae, for example, include two shelled but scaleless taxa, that is the small marine *Ovulinata parva* and an even smaller terrestrial *Micropyxidiella edaphonis* (Howe et al. 2011; Tarnawski and Lara 2015). These diminutive

euglyphids (8–15 μm) have probably gone unnoticed until recently due to their inconspicuous appearance.

Findings of small inconspicuous taxa in an otherwise well-studied group raise the question about a hidden diversity of minute euglyphids that do not exhibit silica scales, especially since metabarcoding studies showed a wealth of Euglyphida-assigned environmental sequences that may represent new families (Lara et al. 2016). One of these sequences even became an indicator taxon of late decomposition stages when dating decay of corpses in a forensic study (Seppey et al. 2016). The identity of these "dark matter" euglyphids remains an open question, as most genera of Euglyphida still have not been sequenced, but can be well placed within known families based on their morphology (Kosakyan et al. 2016). It is well-proven phenomenon that taxonomists tend to describe large species earlier than smaller species (Gaston 1991), a relationship so general that it is used to estimate levels of undescribed arthropod diversity (Stork et al. 2015). A logical conclusion is that Euglyphida-assigned environmental sequences are likely composed of inconspicuous forms, probably small-sized and scaleless. If this hypothesis holds true, a large part of Euglyphid diversity could be composed of these cryptic forms (Lara et al. 2016).

With this study, we add a further element to this debate by describing *Phaeobola aeris,* a new small-sized euglyphid species (about 17 μm in diameter) that lacks silica scales. We discuss its phylogenetic position and the extent of euglyphid "dark matter" that may make these cercozoans the most diverse group of testate amoebae.

## MATERIALS AND METHODS

The amoebae were sampled in August 2018 from sediments of a quarry pond surrounded by reeds, tall grass, and deciduous trees in Cologne Pesch, Germany. The samples were repeatedly screened for filose amoebae with a light microscope (Nikon Eclipse TS100; Ph1; up to 400X). Single cells were isolated and individually transferred into a new well of a 24-well plate filled with Waris-H + Si (Mcfadden and Melkonian 1986) and a mixture of *Nitzschia communis* and *Characium* sp. as prey. Photographs were taken with a Nikon Eclipse 90i (DIC, up to 600X). Unfortunately, the culture died before the time of publication. For electron microscopy, individual cells were washed in distilled water and then in ethanol (70%). The cells were subsequently transferred onto SEM stubs and left overnight in a desiccator. Cells were coated with gold (ca. 8 nm thick film) using a Balzers SCD004 Sputter Coater and observed with a Hitachi S3000N microscope at a tension of 15 kV. For sequencing, single individuals were starved overnight. Approximately 1 μl medium containing a single cell was transferred into a PCR tube containing 4 μl $ddH_2O$. Subsequently, 4.6 μl PCR mixture was added, including 1.7 μl $ddH_2O$, 1 μl Thermo Scientific Dream Taq Green Buffer, 1 μl of 10 μM forward and reverse primers each, 0.2 μl 10 μM dNTPs, and 0.1 μl DreamTaq polymerase (Thermo Fisher Scientific, Dreieich, Germany). The SSU rDNA was amplified with the general eukaryotic primers EukA & EukB (Medlin et al. 1988). Using 1 μl of the first PCR as a template, semi-nested reamplifications were conducted with the primer pairs EukA + 963R_Cerco and S616F_Cercomix + EukB (Fiore-Donno et al. 2017). Eight μl of the PCR products were purified by adding 0.15 μl of exonuclease, 0.9 μl FastAP and 1.95 μl $ddH_2O$ and heating the mixture for 30 min at 37 °C, and subsequently for 20 min at 85 °C. The Big dye Terminator Cycle sequencing Kit (Thermo Fisher Scientific) and an ABI PRISM automatic sequencer were used for the sequencing. The sequence (1,510 bp length) was submitted to the NCBI database as accession number MW186810. The sequence was screened for close relatives via the BLASTn algorithm (V2.3.0) against the NCBI nucleotide database, which indicated a relationship to Euglyphida. Phylogenetic trees of the Euglyphida using several species of Thecofilosea and Sarcomonadea as outgroup (GenBank accession numbers: AF411276, AF411275, AF411265, DQ211597, AF411270, AY496046, AJ418794, DQ303924, AF411283) were constructed using the RAxML v.8.2.10 (Stamatakis 2014) and MrBayes (Ronquist and Huelsenbeck 2003) as implemented on the CIPRES Portal (Miller et al. 2010). The RAxML analysis was conducted using The GTR + GAMMA model with default settings and 1,000 bootstraps. The MrBayes analysis was conducted using The GTR + GAMMA model with default settings on two independent runs sampled every 100 generations. The analysis was automatically stopped when convergence was reached after 1,065,000 generations resulting in 21,300 trees of which 25% were discarded as the burn-in.

## RESULTS AND DISCUSSION

### Observations and phylogeny

The amoebae bore a roundish to oval test with 17.6 ± 3.4 μm in length and 15.9 ± 2.9 μm in width and an average mean length:width ratio of 1.1 ± 0.2 ($n$ = 27). The test was colourless to amber-coloured and covered with organic cement with a rough surface that exhibited attached xenosomes (Fig. 1A, C). The ellipsoid nucleus (mean length: 7.3 ± 0.8 μm; mean width: 5.7 ± 0.8 μm; $n$ = 15) with its central and spherical nucleolus (mean length: 3.1 ± 0.7 μm; mean width: 2.7 ± 0.6 μm) was located at the apical end of the cell (opposite to the aperture, Fig. 1D). Central to the cell, a layer of granules formed which surrounded the nucleus in a pattern typical for Euglyphida (Kosakyan et al. 2016). Close to the aperture, contractile and food vacuoles could be observed (Fig. 1D). The filose pseudopodia were used to creep over the surface. They were readily branching and sometimes anastomosing (Fig. 1B). The amoebae fed on both offered species of algae. No cysts were observed.

*Phaeobola aeris* branched robustly within the Euglyphida (bootstrap = 93%, posterior probability = 0.99), and could not be assigned to any of the already described families. Instead, it branched weakly (B = 29%, PP = 0.96%)

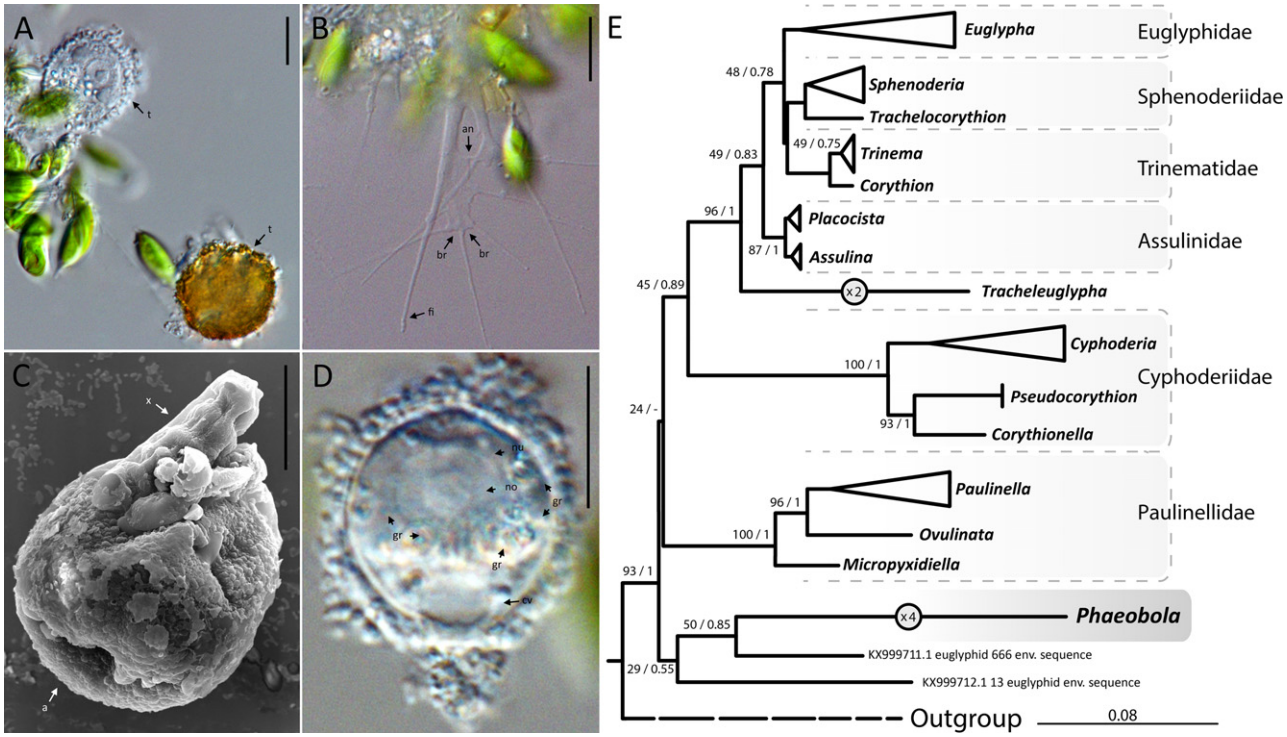


**Figure 1** Morphological overview of *Phaeobolaaeris* (**A**–**D**) and phylogenetic placement (**E**). Scale bars indicate 10 μm; pictures A, B, and D were taken with DIC; picture C was taken with a scanning electron microscope. (A): two differently coloured individuals with xenosomes on the surface of their test, colour difference of individuals is shown, (B): branching and anastomosing network of pseudopodia, (C): surface features are shown, including a coated diatom as xenosome, (D): cellular features with zoning and granular layer, (E): Maximum likelihood phylogenetic tree of the 18S rDNA sequences available on GenBank with the consensus sequence of *P.aeris*. Bootstrap values and Bayesian posterior probabilities, respectively, are indicated along with the nodes. The minus indicates that this split was recovered as a polytomy with the parent node on the Bayesian phylogeny. As every genus appeared as monophyletic, their branches were collapsed. The branches leading to *Tracheleuglypha* and *Phaeobola* have been shortened for clarity. a = aperture; an = anastomosing; br = branching; cv = contractile vacuole; fi = filopodia; gr = granules; nu = nucleus; no = nucleolus; t = test; x = xenome.

together with unidentified environmental sequences retrieved from forest soil (Seppey et al. 2016). However, the phylogenetic position of *P. aeris* is still uncertain because of its long branch.

## Taxonomic assignation of *Phaeobola aeris*

*Phaeobola aeris* exhibits both an unusually distinct morphology and distinct SSU rDNA sequence thus complicating its taxonomic placement. Based on the morphology of this testate amoeba (size, shape, pseudopodia morphology, and zonation of the cell body), it is quite clearly cercozoan and potentially may resemble a distinct lineage in Imbricatea or even its sister group Thecofilosea. Recently, Dumack and Siemensma (2020) discussed extensively differences in the organic cement of thecofiloseans and imbricateans. In brief, imbricatean species may appear often yellowish to brownish, while thecofilosean species are colourless. *Phaeobola aeris* shows a conspicuous gradient of shell colours ranging from colourless over yellowish to brownish specimens, exactly what can be expected for the organic cement of Imbricatea. Based on its morphological appearance, a closer relationship to the Euglyphia may be inferred (Siemensma and Dumack, 2020), and due to its size and shape, it fits well to the diversity of Euglyphida. However, it must be mentioned that *P. aeris* incorporates xenosomes into its shell, unlike any other described Euglyphida species, not even the scaleless genera *Micropyxidiella* and *Ovulinata* (Anderson et al. 1996; Dumack et al. 2018; Howe et al. 2011; Tarnawski and Lara 2015; Fig. 1C). Moreover, the shell of *P. aeris*, which is covered with rough organic cement, is unlike the smoothly covered shells of other Euglyphida (Fig. 1C).

*Phaeobola aeris* branches robustly within the Euglyphida in our phylogenetic analysis based on SSU rDNA data (support 93% bootstraps, 1 posterior probability). However, it cannot be placed with confidence in any of the described families; indeed, while other Euglyphid families are defined by the complex shape and arrangement of siliceous scales (Chatelain et al., 2013; Lara et al., 2007), *P. aeris* does not possess such structures but has characteristics on its own, that is a shell of rough organic cement and xenosomes. The long branch it produces in

SSU rRNA-based trees does not clarify its placement either, at least using a single-gene phylogeny. Based on its morphology, unique amongst known Euglyphida, and on its phylogenetic position, we justify the erection of the genus *Phaeobola* to accommodate *P. aeris*.

### Euglyphida's dark matter?

Environmental DNA surveys revealed considerable numbers of Euglyphida-related sequences that could not yet be assigned to any known family (Bass and Cavalier-Smith 2004; Kosakyan et al. 2016; Seppey et al. 2016). It is likely that these organisms, like *P. aeris,* are small and inconspicuous. Lara et al. (2016) defined four environmental clades of Euglyphida in forest litter and mosses (65 OTUs in EEC1-4) that could not be directly assigned to any known group. *Phaeobola aeris* did not branch convincingly within any of them (not shown)—our phylogenetic analysis shows only a weak relationship with two environmental clones obtained in another study (Seppey et al. 2016). The long branch at the base of the *P. aeris* sequence in the SSU rDNA tree suggests that related sequences are difficult to retrieve from the environment with broad-spectrum eukaryotic primers. This indicates that in addition to a large fraction of Euglyphida that have been likely overlooked by protistologists due to their inconspicuousness, even more sequences probably escaped molecular detection because of their divergent SSU rDNA gene sequences. Altogether, this suggests the existence of a taxonomically diverse and hitherto unseen majority ("dark matter") of euglyphids, with *P. aeris* being the first identified representative.

## TAXONOMIC ACTS

Taxonomic summary:
Euglyphida Copeland, 1956.
Euglyphida *incertae sedis*

### *Phaeobola* gen. nov

**Diagnosis:** Cells roundish to oval shaped. Shell colourless to amber-coloured, rough surface, few xenosomes included.

**Etymology:** *Phaeobola* (feminine)—derived from the Greek words phaios (=φαιός, brown) and obolos (=ὀβολός, a coin in ancient Greece; referring to the shape of the organism); the created name was feminised since most shelled amoeba taxa are by tradition feminine. The specific epithet refers to the amber-coloured shell that reminds of a bronze ("aes" in latin) coin.

**Type Species:** *Phaeobola aeris* sp. nov.

### *Phaeobola aeris* sp. nov.

**Diagnosis:** Cells exhibit characters as *Phaeobola*. Roundish to oval test, 17.6 ± 3.4 μm in length and 15.9 ± 2.9 μm in width, average mean length:width ratio of 1.1 ± 0.2 μm, ellipsoid nucleus, mean length: 7.3 ± 0.8 μm and width: 5.7 ± 0.8 μm; $n = 15$, central spherical nucleolus, mean length: 3.1 ± 0.7 μm; mean width: 2.7 ± 0.6 μm. Filopodia branching and anastomosing, sometimes reminding of a reticulose network.

**Remarks:** The genus is so far monotypic. Algivorous, probably bacterivorous as well; thus we consider it as omnivorous.

**Type:** Specimens depicted in Fig. 1 constitute the type, as illustrations can constitute a type in testate amoebae (Lara et al. 2020); in addition, a stub has been deposited at the Royal Botanical Garden of Madrid RJB-MA-Algae 11249.

**Type locality:** Quarry pond in Cologne Pesch, Germany; Coordinates: 50.995234, 6.866434

**ZooBank registration number:** urn:lsid:zoobank.org:pub:6B3F10E1-D014-412C-B8A3-1810AF87E45C

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