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Morphological variability of choanocyte kinetids supports a novel systematic division within Oscarellidae (Porifera, Homoscleromorpha)

Choanocyte kinetids in Oscarellidae

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Abstract

Homoscleromorpha is a recently recognized class within the phylum Porifera. While molecular analyses support monophyly of Homoscleromorpha, morphological data are largely lacking. Here we investigate and comparatively analyze the detailed morphology of kinetids (flagellar apparatus) in choanocytes of ten Oscarella species. The kinetid is known to be a phylogenetically important trait that can possibly reflect relationships of sponges at the level of orders. We also conduct a phylogenetic analysis based on previously published sequences. We found the family Oscarellidae is subdivided into two large groups, and choanocyte kinetids structure strongly supports this division. The first group includes O. lobularis, O. tuberculata, O. bergenensis, O. viridis, O. rubra, O. malakhovi, O. microlobata, O. carmela, O. filipoi and O. zoranja. These species possess nuclei located in a basal position without any connection to the kinetosome. The second group includes O. balibaloi, O. nicolae, O. kamchatkensis, O. pearsei, O. imperialis, and Pseudocorticium jarrei, whose nuclei are connected with the kinetosome. In addition, this second group also shares mesohylar spherulous cells harboring paracrystalline inclusions. The choanocyte kinetid structure seems to be an essential phylogenetic and diagnostic morphological trait, and we propose to include it in future species descriptions. Our results imply assignation of the second group to the genus *Pseudocorticium.* This taxonomic suggestion, however, needs additional data on the remaining Oscarella species..

Introduction

In the last fifteen years, the taxonomy and systematics of the phylum Porifera underwent tremendous changes. The three sponge classes, Demospongiae, Calcarea and Hexactinellida, was complemented by a fourth group, the Homoscleromorpha. It was elevated to the rank of class according to molecular systematics' studies and re-examination of cytological traits such as flagellated exo- and endopinacocytes, a basement membrane lining both choanoderm and pinacoderm, and the presence of peculiar flagellated apopylar cells (Gazave et al. 2012). Integrative systematics justified the subdivision of the single order of this class into two families (Gazave et al., 2010; Ivanišević et al., 2011; Boury-Esnault, Lavrov, Ruiz, & Pérez, 2013), namely the Plakinidae with a spiculate skeleton and the Oscarellidae without skeleton. Currently, this very simple subdivision has become a matter of debate, especially after the discovery of several members of Plakinidae without skeleton (Ruiz et al., 2017). Additional morphological characters could help clarifying Homoscleromorpha systematics.

The flagellum with its basal intracellular part (is called kinetid or flagellar apparatus) has been shown to be an important source of phylogenetic information in a number of taxa (Barr, 1981; Melkonian, 1982; Moestrup, 1982, 2000; O'Kelly, & Floyd, 1983; Karpov, 1988, 2000, 2016; Andersen et al., 1991; Baldauf, 1999; Cavalier-Smith, 2000, 2013; Dewel, Connell, & Dewel, 2003; Letcher et al., 2005; Yubuki, & Leander, 2013). The kinetid of sponge choanocyte consists of three main parts: (1) a free part, or flagellum, containing an axoneme; (2) a basal intracellular part; and (3) the transition zone connecting the free and basal parts. The basal intracellular part normally comprises a flagellar basal body (= kinetosome), nine transition fibers attaching the distal part of the kinetosome to the plasma membrane, a basal foot (conical shaped body), lateral microtubules radiating from the foot, a centriole (non-flagellar kinetosome) and fibrillar roots (bundles of fibrils of different lengths) deriving from the kinetosome or, more rarely, from the centriole (Fig. 1). In sponges, the choanocyte kinetid is regarded as a potentialmorphological phylogenetic marker, as it includes evolutionary conservative structures along with variable ones (Pozdnyakov, Sokolova, Ereskovsky, & Karpov, 2017, 2018).

At the end of the 19th century, Minchin (1896) and Bidder (1898) proposed a classification of calcareous sponges based on the cytological traits of their choanocytes, in particular the position of the nucleus and its relation to the flagellum (connected or not connected). This morphology-based classification was confirmed a century later by molecular analyses (Manuel et al., 2003; Manuel,

2006). The relevance of these two features related to the kinetid structure was discussed for Demospongiae (Pozdnyakov, & Karpov, 2016a, 2016b; Pozdnyakov et al., 2017, 2018), but so far has not been extended to the Homoscleromorpha, although cytological observations are essential to describe the diversity of Oscarellidae.

We provide here a detailed description of the choanocyte kinetid structure of two *Oscarella* species, *Oscarella lobularis* (Schmidt, 1862) and *Oscarella balibaloi* Pérez, Ivanišević, Dubois, Pedel, Thomas, Tokina, & Ereskovsky, 2011 (Homoscleromorpha, Oscarellidae), accompanied by data on the nucleus position within the choanocyte in eight other *Oscarella* species. Comparing morphological and genetic data, we aim to assess the relevance of this cytological trait to support the current inter-specific relationships among the family Oscarellidae.

Materials and methods

Material collection

Sponge specimens were sampled by SCUBA diving. *Oscarella balibaloi* Pérez, Ivanisevic, Dubois, Pedel, Thomas, Tokina & Ereskovsky, 2011, *O. lobularis* (Schmidt, 1862) and *O. tuberculata* (Schmidt, 1868) were collected in the NW Mediterranean Sea at Maïre Island (France), *O. viridis* Muricy, Boury-Esnault, Bézac, & Vacelet, 1996, *O. microlobata* Muricy, Boury-Esnault, Bézac, & Vacelet, 1996 and *Pseudocorticium jarrei* Boury-Esnault, Muricy, Gallissian, & Vacelet, 1995 in the Jarre Cave, Marseille (France), *Oscarella rubra* (Hanitsch, 1980) near Roscoff (NW France), *O. carmela* Muricy, & Pearse, 2004 and *O. pearsei* Ereskovsky, Richter, Lavrov, Schippers, & Nichols, 2017 at Long Marine Laboratory of the University of California (Santa Cruz, USA), *O. malakhovi* Ereskovsky, 2006 in the Sea of Japan, Vostok Bay (Russia), and *O. kamchatkensis* Ereskovsky, Sanamyan, & Vishnyakov, 2009 in Avacha Gulf, Pacific Coast of the Kamchatka Peninsula (Russia). *Oscarella rubra* is currently not accepted in the World Porifera Database where it is classified as a member of the genus *Aplysilla* (Van Soest et al. 2019). However, this species was included in this study because our morphological (our non-published date) and genetical analysis clearly shows that it belongs to the genus *Oscarella* (Gazave et al. 2013), which is in accordance with the original description of this species by Hanitsch (1890).

Morphological analysis

For ultrastructural studies of *O. balibaloi* and *O. lobularis*, sponge fragments (8-10 mm³, about 15-20 fragments for each species) were fixed according to the following protocol: 1) the buffer used in all fixatives was prepared by mixing cacodylate 0.2 M with seawater (1:2); 2) prefixation was carried out for 3 min by the addition of 1 ml of osmium tetroxide 1% to 3 ml of seawater with a sponge fragment; 3) then, 4 ml of glutaraldehyde 4% were added, and the sponge fragment was maintained in this mixture for 15 min, on ice and in darkness; 4) then the fixative mixture was replaced by 4 ml of glutaraldehyde 2% for 1 h on ice; 5) samples were then rinsed twice in the buffer and post-fixed with osmium tetroxide 1% for 1 h. Finally, the samples were washed twice in the buffer for 10 min, dehydrated through graded concentrations of ethanol and embedded in Epon Araldite. After polymerization, the resin blocks containing the sponge fragments were trimmed with a Leica EM UC6 ultramicrotome using a glass knife and Leica UCT ultramicrotome equipped with a Drukkert 45° diamond knife. The ultrathin sections were stained with 2% uranyl acetate in 70% ethanol and Reynolds' lead citrate, and then observed using a JEM 1400 transmission electron microscope (TEM) equipped with an Olympus Veleta digital camera, and a Zeiss-1000 TEM. In total, about 50-70 choanocytes of each species were analized during this study. For most of them, serial longitudinal or transverse sections were obtained.

For more basic TEM observations of choanocytes including the shape and position of the nucleus, samples of *O. tuberculata*, *O. viridis*, *O. microlobata*, *O. carmela*, *O. pearsei*, *O. malakhovi*, *O. kamchatkensis*, *O. rubra* and *Pseudocorticium jarrei* were fixed in glutaraldehyde 2.5%, prepared with cacodylate buffer 0.2 M and filtered seawater, during 2 h, and post-fixed in OsO₄ 2% in seawater for 2 h at room temperature. After fixation, samples were successively washed in 0.2 M cacodylate buffer and distilled water, and finally dehydrated with a graded series of ethanol and embedded in Epon Araldite. Ultrathin sections were cut and stained in the same way as described above. *Oscarella imperialis* Muricy, Boury-Esnault, Bézac, & Vacelet, 1996, *O. filipoi* Pérez, Ruiz, 2018, and *O. zoranja* Pérez, Ruiz, 2018 were analyzed on the basis of original descriptions.

Phylogenetic reconstruction

To assess the possible phylogenetic value of the studied morphological characters we conducted a phylogenetic analysis, based on molecular data from previously published papers (Gazave et al., 2013; Ereskovsky et al., 2017; Pérez, & Ruiz, 2018). For most oscarellid diversity, the two markers sequenced most often, were the nucear 28S rRNA gene (28S) and 18S rRNA gene (18S) and it was

shown that phylogenetic reconstructions, based on these markers, are compatible to those from mtDNA (Gazave et al., 2013; Ereskovsky et al., 2017). However, for two species included in the morphological analysis (*O. filipoi* and *O. zoranja*), only mitochondrial *cytochrome c oxidase subunit 1* (COI) sequences (I3-M11 fragment) (Pérez, & Ruiz, 2018) were used. Therefore, to improve the resolution of phylogenetic reconstructions, the final dataset included all three loci, even if only COI was available for some species. In the case of *O. pearsei*, a preliminary analysis showed that all GenBank 28S and 18S sequences under its species name (acc. numbers EF654526, EF654519, KY513286) are identical to corresponding *O. carmela* sequences. As a result, *O. pearsei* grouped together with *O. carmela*, contradicting phylogenetic hypotheses of Ereskovsky et al. (2017). Therefore, to avoid possible misidentification and wrongly interpreted results, for *O. pearsei* only the COI sequence was included in the final dataset. Species from the family Plakinidae and *Leucaltis clathria* were chosen as outgroups (Ereskovsky et al., 2017). GenBank accession numbers for studied specimens are given in Table 1. Both alignments and original tree files have been deposited in Zenodo (https://zenodo.org/record/3706996#.XmoBq-oueUI).

Sequences were aligned with MUSCLE (Edgar, 2004) in MEGA7 (Kumar, Stecher, & Tamura, 2016). Indel-rich regions of the 28S and 18S markers were identified and removed with Gblocks (Talavera and Castresana, 2007). We determined blocks using a less stringent selection by allowing gap positions within the final blocks and less strict flank positions. The length of resulting alignments was of 492 bp for COI, 1630 bp for 18S and 1120 bp for 28S, and after using Gblocks it consisted of 492 bp for COI, 1582 bp for 18S and 979 bp for 28S. Since only publicly available sequences were used, and single-gene trees for each marker have been already published (Gazave et al., 2013; Ereskovsky et al., 2017; Pérez, & Ruiz, 2018), only an analysis based on concatenated sequences was conducted. Sequences were concatenated by a simple biopython script (Chaban, Ekimova, Schepetov, & Chernyshev, 2019). The best-fitting nucleotide evolution model was tested in the MEGA7 toolkit based on the Bayesian information criterion (BIC) for each partition. The best-fitting model for COI partition was HKY+G, for 28S partition – GTR+G, and for 18S partition the best model was K2+G. Phylogenetic reconstructions were performed by applying evolutionary models for partitions separately. The Bayesian estimation of posterior probability was performed in MrBayes 3.2 (Ronquist, & Huelsenbeck, 2003). Markov chains were sampled at intervals of 500 generations. The analysis was started with random starting trees and 3×10^6 generations. Maximum Likelihood-based phylogeny inference was performed in HPC-PTHREADS-AVX version of RAxML (Stamatakis,

2014) with ultrafast bootstrapping (UFBoot approximation approach) (Minh, Nguyen, & von Haeseler, 2013) in 1000 pseudoreplicates under the GTRCAT approximation of nucleotide evolution. Bootstrap values were placed on the best tree found, with SumTrees 3.3.1 from DendroPy Phylogenetic Computing Library V 3.12.0 (Sukumaran, & Holder, 2010). Final phylogenetic tree images were edited in FigTree 1.4.0. and further colored using Adobe Illustrator CC2015.

Results

Kinetid structure of Oscarella lobularis choanocytes

Choanocytes of *O. lobularis* are elongated in an apical-basal direction (Fig. 2 A). The flagellum has a typical eukaryotic axoneme (9 + 2). After fixation, the flagellum often became squeezed at its base (Figs. 2A; 3B). Central microtubules start approximately 120-200 nm above the level of the cell surface (Figs. 3C; 4J); therefore, the transition zone (area between the proximal ends of the central microtubules and kinetosome) can be classified as moderately long. At high magnification we distinguished latticed bodies in the transition zone (Fig. 4 I). Slightly more distal from these bodies, the flagellum is filled with electron-dense material (Figs. 3 B; 4 H; I). The kinetosome consists of nine microtubular triplets (Figs. 3 B, C; 4 C-E). An axial granule (the dense body usually found in sponge choanocytes in this location (Pozdnyakov and Karpov, 2015; Pozdnyakov et al., 2017)) is attached by filaments to microtubules in the distal part of the kinetosome (Figs. 3 C; 4 B, F; I-K). In longitudinal sections, the axial granule is revealed as a three-layered structure: two dense plates separated by an electron translucent layer (Figs. 3 C; 5 J).

Nine thick transitional fibrils, nearly 130 nm long, radiate from the distal end of the kinetosome (Figs. 3 H; 4 B, C, F), each of them has a shape of an elongated triangle with the apex connecting to the plasma membrane (Fig. 4 C, F; K). Small electron-dense bodies mark the place where the transitional fibrils connect to the cell membrane (Figs. 3 E; 4 C-F).

The basal foot is 150-170 nm long, has a wide base, and is located on the lateral side of the distal part of the kinetosome (Figs. 3 E; 4 D, E). It consists of two or three stalks attached to the kinetosome and a spherical head (Fig. 4 D), which serves as a microtubule organizing center (MTOC). Numerous microtubules radiate from the head in different directions (Figs. 3 D; 4 D-E). Some of them reach the plasma membrane, whereas others pass towards the nucleus or along the lateral sides of the

kinetosome. We also observed lateral microtubules starting directly from the kinetosome surface, but we did not find pronounced additional MTOCs here (Fig. 4 D, E).

A non-flagellar centriole is present in choanocytes of *O. lobularis* (Fig. 3 A-J). It is located in a more basal position than the basal foot (Fig. 3 D, E), at a short distance from the kinetosomal edge (Fig. 3 B, G, H). The longitudinal axis of the centriole is perpendicular to the basal foot, as well as to the kinetosome (Fig. 3 A-E). A fibrillar bridge connects the kinetosome and centriole (Fig. 3 C, D). A thick non-striated fibrillar root of arcuate shape projects from the centriole in a lateral direction (Fig. 3 G-I).

The kinetosome of the *O. lobularis* choanocyte has no fibrillar root. The nucleus occupies a basal position in the cell and is separated from the kinetosome by almost the entire cytoplasm (Fig. 2 A). Additionally, the cisternae of the Golgi apparatus and large vacuoles filled with fibrillar material are located between the kinetosome and the nucleus (Fig. 4 L). Therefore, the nucleus is disconnected to the flagellar apparatus. A schematic drawing (Fig. 5) summarizes the structure of choanocyte kinetid in *O. lobularis*.

Kinetid structure of Oscarella balibaloi choanocytes

Choanocytes of *O. balibaloi* have a flagellum with a typical axoneme (9 + 2). Usually, it becomes squeezed near its base after fixation (Figs. 2 B; 6 F, G). The flagellar transition zone is moderately long (Figs. 2 A; 6 F).

The kinetosome consists of nine microtubular triplets (Figs. 6 D-F, I; 7 A-E). It contains an axial granule connected by thin filaments with the inner surface of the kinetosome (Fig. 6 I). The axial granule is elongated in an apical-basal direction and its shape is irregular. Transitional fibers project radially from the distal end of the kinetosome forming a star-like structure (Figs. 6 F; 7 A). The kinetosome surface bears a triangle-shaped basal foot with lateral microtubules (Figs. 6 D-F; K; 7 B-C). We did not find additional MTOCs, but observed microtubules starting directly from the kinetosome (Fig. 7 C).

The centriole of *O. balibaloi* is located near the kinetosome, in the same position as in the choanocytes of *O. lobularis*. The fibrillar bridge connects the kinetosome with the centriole (Figs. 6 D, E; 7 E). It merges with the non-striated fibrillar lateral root starting from the centriole (Fig. 6 J). The choanocyte of *O. balibaloi* has an apical nucleus, and the broad and short (about 120 nm length) non-striated fibrillar root projecting from the proximal end of the kinetosome attaches to the nuclear

surface (Figs. 2 B; 6 C, D, I, J). Cross sections reveal the fibrillar ring connecting the kinetosome to the conical nuclear projection (Fig. 7 F). The Golgi apparatus is located slightly more basally than the centriole, being situated aside the flagellar apparatus and nucleus (Fig. 6 A, B). A schematic drawing illustrating the choanocyte kinetid of *O. balibaloi* is shown in figure 8.

Kinetid structure of choanocytes in other Oscarellidae species

The analysis of longitudinal sections of different *Oscarella* species reveals that the choanocyte nucleus is located in a basal position without any connection to the kinetosome in *O. rubra* (Fig. 9 A), *O. bergenensis* (Fig. 9 B), *O. tuberculata* (Fig. 9 C), *O. carmela* (Fig. 9 D), *O. viridis* (Fig. 9 E), *O. microlobata* (Fig. 9 F), *O. malakhovi* (Fig. 9 G). As in *O. lobularis*, the Golgi apparatus and large vacuoles are located between the kinetosome and the nucleus. Additionally, the kinetosomes of these species do not possess fibrillar roots. Therefore, the general scheme of their kinetid structure corresponds to the flagellar apparatus of *O. lobularis*.

In the choanocytes of *O. nicolae* (Fig. 9 H), *O. kamchatkensis* (Fig. 9 I) and *O. pearsei* (Fig. 9 J), the nucleus occupies an apical position and connects to the kinetosome by fibrillar roots. Thus, the choanocytes of these three species have a flagellar apparatus similar to *O. balibaloi*. Moreover, the same scheme of the kinetid structure was also found in the choanocytes of *Pseudocorticium jarrei*, the only species of Oscarellidae that does not belong to the genus *Oscarella* (Fig. 9 K).

Phylogenetic tree of the family Oscarellidae

The sequence alignment of concatenated 28S, 18S, and COI markers included a total of 3264 positions before GBlocks applied, and 3053 position after using GBlocks. The topology of the trees resulting from the Bayesian Inference (BI; Fig. 10) and the Maximum Likelihood analysis (ML) of the concatenated alignments were largely congruent in both topology and nodes support. The BI tree, however, contained more polytomies corresponding to poorly supported nodes in the ML tree. No conflict in supported nodes in the single-gene trees (28S, 18S, and COI; Gazave et al., 2013; Ereskovsky et al., 2017; Pérez, & Ruiz, 2018) and the trees found in analyses of the concatenated sequences (both pre- and post-GBlocks) was identified. The family Oscarellidae was monophyletic and highly supported (PP (posterior probability from the Bayesian Inference) = 1; ML (bootstrap values from the Maximum Likelihood) = 100). The genus *Oscarella* was recovered paraphyletic due to *Pseudocorticium jarrei* nested within it (PP = 1; ML = 99). Within the *Oscarella* +

Pseudocorticium clade there are two highly supported large species groups: the first one comprises *Oscarella lobularis, O. bergenensis, O. lobularis, O. rubra, O. carmela, O. malakhovi, O. viridis, O. filipoi* and *O. zoranja* (PP = 1; ML = 100). The second group includes *O. balibaloi, O. pearsei, O. kamchatkensis, O. nicolae* and *Pseudocorticium jarrei* (PP = 1; ML = 99). *Oscarella microlobata* represents a singleton that is recovered as sister to the first group (forming a common group with them), however the node support is moderate or low in this case (PP = 0.97; ML = 36). Within two mentioned groups of species some supported clades of closely related species can be found: *O. bergenensis* is sister to *O. tuberculata* (PP = 1; ML = 97) and both form a single clade together with *O. lobularis* and *O. rubra* (PP = 1; ML = 98); *O. carmela* is sister to *O. malakhovi* (PP = 1; ML = 100) and *O. balibaloi* is sister to *O. nicolae* (PP = 1; ML = 91). Recovered relationships between other species received moderate or low support.

Discussion

The choanocyte kinetids of *Oscarella lobularis* and *O. balibaloi* closely resemble the kinetids of previously studied sponge species (Pozdnyakov, & Karpov, 2013, 2016a, 2016b; Pozdnyakov et al., 2017, 2018). However, two features found in Oscarella choanocyte kinetids have not been found in the choanocyte kinetids of other sponges: these are the single microtubule organizing center (MTOC =basal foot) on the kinetosome without any additional ones, and a centriole with its own lateral fibrillar root. The basal or apical position of the nucleus in choanocytes of *O. lobularis* and *O. balibaloi* correlates well with corresponding absence or presence of a nuclear fibrillar root in their kinetosomes (Figs. 5; 8). Based on these traits, all investigated species of the genus *Oscarella* can be divided into two groups.

The "lobularis-group" (Figs. 2 A; 9 A-G) includes *O. lobularis*, *O. tuberculata*, *O. bergenensis*, *O. viridis*, *O. rubra*, *O. malakhovi*, *O. microlobata*, *O. carmela*, *O. filipoi* and *O. zoranja*. Their choanocyte nuclei are located in a basal position without any connection to the kinetosome. The Golgi apparatus is located between the kinetid and a vacuole containing fibrillar material. The kinetosomes of choanocytes in these species do not possess fibrillar roots.

The "balibaloi-group" (Figs. 2 B; 9 H-K) includes *O. balibaloi*, *O. nicolae*, *O. kamchatkensis*, *O. pearsei* and *Pseudocorticium jarrei*. Their choanocytes have apical nuclei connected to the kinetosomes with fibrillar roots.

The two groups described in our phylogenetic analysis are strongly congruent with recent comprehensive studies based on multi-locus analysis (Gazave et al., 2013), and with additional studies that grouped *O. filipoi* and *O. zoranja* with the members of "lobularis-group", and *O. pearsei* with the species of the "balibaloi-group" (Ereskovsky et al., 2017; Pérez, & Ruiz, 2018). The position of *O. microlobata* was conflicting in different phylogenies. It fell into the same group either with *O. lobularis* (Gazave et al., 2013, Gloeckner, Hentschel, Ereskovsky, & Schmitt, 2012), or with *O. balibaloi* (Ivanišević et al., 2011; Ereskovsky et al., 2017; Perez, & Ruiz 2018), or was recovered as sister to all other oscarellids (Gazave et al., 2010; Ereskovsky et al., 2017). Our results further support the close relationships of this species with the remaining members of the "lobularis-group", and thus confirm the monophyly of this group as a whole. The unstable position of *O. microlobata* could be a result of low substitution rates and a resulting unclear phylogenetic signal in the case of single-gene analyses.

The representatives of Plakinidae, another family of Homoscleromorpha, possess a choanocyte kinetid structure similar to that found in the "balibaloi-group" (Riesgo, Maldonado, & Durfort, 2007). Therefore, we can assume that the choanocyte kinetid of the "balibaloi-group" retained the plesiomorphic condition of homoscleromorph kinetid, in contrast to the ones found in the "lobularis-group" where the kinetid structure is apomorphic.

There is an additional cytological trait supporting the proposed subdivision of Oscarellidae into two clades: particular mesohylar spherulous cells containing paracrystalline inclusions are characteristic of all *Oscarella* species in the "balibaloi-group" (Muricy, Boury-Esnault, Bézac, & Vacelet, 1996; Pérez et al., 2011; Ereskovsky, Sanamyan, Vishnyakov, 2009; Ereskovsky et al., 2017; Gazave et al., 2013). These cells are ovoid, rarely spherical, with an anucleolated nucleus. Their paracrystalline inclusions are usually ovoid or cylindrical elements consisting of fibrils arranged in a cross striated pattern. This trait is also known for *Pseudocorticium jarrei*. Interestingly, it has never been observed in neither Plakinidae, nor in species of the "lobularis-group" with the exception of *O. microlobata*. This latter species possesses superficially similar cells with inclusions, but in comparison to the "balibaloi-group", their paracrystalline elements are less numerous and dispersed within the dense matrix of the cell spherules. According to our suggestion, the "balibaloi-group" should also include *Oscarella imperialis*, because according to its original description this species has an apical choanocyte nucleus and typical spherulous cells with paracrystalline inclusions (Muricy et al., 1996).

The documented distribution of cells with paracrystalline inclusions within the family Oscarellidae corresponds to the phylogenetic relationships of the two species groups. This cell type might be an ancestral feature for Oscarellidae, since it is not found in any other sponges except of species in the "balibaloi-group" and *O. microlobata*. It was retained in the "balibaloi-group" while in the "lobularis-group" it could be reduced, with *O. microlobata* demonstrating partial reduction of this character.

The two groups recovered in our molecular phylogenetic analysis strongly support our morphological results and suggest a phylogenetic and, probably, taxonomic importance of the choanocyte kinetid structure within the family Oscarellidae. According to our findings, the kinetid structure of choanocytes seems to be a valid diagnostic trait distinguishing the two main clades of Oscarellidae, and we propose to include this cytological character in future species descriptions.

Our results highlight the need to revise the family Oscarellidae and at the current state of knowledge imply assignment of the "balibaloi-group" to the genus *Pseudocorticium*. However, missing genetic and cytological information on few other *Oscarella* species calls for additional research and does not allow us to propose a formal taxonomic redefinition of the family.

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Figures and legends

Fig. 1. General scheme of structures of the choanocyte flagellar apparatus.

A) Schematic overview of choanocyte. Area marked by red rectangle is magnified in B. B) The flagellar apparatus (= kinetid) magnified (see area marked by red rectangle in A).

Abbreviations: I – flagellum; II – basal intracellular part; III – transition zone.

bf – basal foot; c – centriole; cmt – central microtubules; fl – flagellum; fr – fibrillar root; Ga – Golgi apparatus; k – kinetosome; lmt – lateral microtubules; mv – microvilli; n – nucleus; pdm – peripherical duplets of microtubules; pm – plasma membrane; tf – transition fibril.

Fig. 2. Overviews of choanocytes of *Oscarella lobularis* (A) and *O. balibaloi* (B) in longitudinal sections.

Abbreviations: fl – flagellum; k – kinetosome; n – nucleus; vfm – vacuole with fibrillar material.

Fig. 3. Transmission electron micrographs of the flagellar apparatus in Oscarella lobularis.

A-E) series of consecutive longitudinal sections through the kinetosome; F) section through the longitudinal axis of the kinetosome of another choanocyte; G-I) series of consecutive sections through the kinetosome of another choanocyte.

Abbreviations: ag – axial granule; bf – basal foot; c – centriole; ecm – ends of central microtubules; edm – electron-dense material; fb – fibrillar bridge between centriole and kinetosome; fr – fibrillar root; Ga – Golgi apparatus; k – kinetosome; lfr – lateral fibrillar root; lmt – lateral microtubules; n – nucleus; pc – places of connection (of transition fibers to the plasmalemma); tf – transition fiber.

Fig. 4. Transmission electron micrographs of the flagellar apparatus in Oscarella lobularis.

A-E) series of consecutive transverse sections through the transition zone and kinetosome from distal to proximal end (view from inside the cell); F-H) series of consecutive transverse sections through the transition zone of another choanocyte from proximal to distal direction (view from outside the cell); I, J) longitudinal sections through the transition zone and axial granule of different choanocytes; K) transverse section through the axial granule of another choanocyte; L) longitudinal section through the flagellar apparatus of one more choanocyte.

Abbreviations: ag – axial granule; bf – basal foot; c – centriole; ecm – ends of central microtubules; edm – electron-dense material; Ga – Golgi apparatus; hbf – head of basal foot; k – kinetosome; lmt – lateral microtubules; lb – lattice body; pd – peripheral doublet of microtubules; sbf – stem of basal foot; tf – transition fiber; tz – transition zone; vfm – vacuole with fibrillar material.

Fig. 5. Schematic representation of Oscarella lobularis kinetid in lateral view.

Abbreviations: ag – axial granule; bf – basal foot; c – centriole; edm – electron-dense material; fb – fibrillar bridge between centriole and kinetosome; fl – flagellum; Ga – Golgi apparatus; k – kinetosome; lfr – lateral fibrillar root; lmt – lateral microtubules; n – nucleus; lb – lattice body; tf – transition fiber; vfm – vacuole with fibrillar material.

Fig. 6. Transmission electron micrographs of the flagellar apparatus of Oscarella balibaloi.

A-H) series of consecutive longitudinal sections through the kinetosome; F) section through the longitudinal axis of the kinetosome of another choanocyte; J) lateral fibrillar root; K) structure of basal foot; L) pattern of lateral microtubules originating in the basal foot (J, K, L – different choanocytes).

Abbreviations: ag - axial granule; bf - basal foot; c - centriole; fb - fibrillar bridge between centriole and kinetosome; fr - fibrillar root; fm - fibrillar material; Ga - Golgi apparatus; k kinetosome; lfr - lateral fibrillar root; lmt - lateral microtubules; lmt(lv) - lateral microtubules (longitudinal view); lmt(tv) - lateral microtubules (transverse view); n - nucleus; pc - places of connection (of transition fibers to the plasmalemma); tf - transition fiber.

Fig. 7. A-H) Transmission electron micrographs of consecutive transverse sections through the flagellar apparatus of *Oscarella balibaloi*.

Abbreviations: bf – basal foot; c – centriole; fm – fibrillar material; k – kinetosome; lmt – lateral microtubules; tf – transition fiber.

Fig. 8. Schematic representation of Oscarella balibaloi kinetid in lateral view.

Abbreviations: ag- axial granule; bf – basal foot; c – centriole; edm – electron-dense material (proposal); fb – fibrillar bridge between centriole and kinetosome; fl – flagellum; fr – fibrillar root; Ga – Golgi apparatus; k – kinetosome; lfr – lateral fibrillar root; lmt – lateral microtubules; n –

nucleus; lb – lattice body (proposal); tf – transition fiber.

Fig. 9. Transmission electron micrographs from series of longitudinal sections through the flagellar apparatus in different Oscarellidae species. A) *Oscarella rubra*; B) *O. bergenensis*; C) *O. tuberculata*; D) *O. carmela*; E) *O. viridis*; F) *O. microlobata*: G) *O. malakhovi*; H) *O. nicolae*; I) *O. kamchatkensis*; J) *O. pearsei*; K) *Pseudocorticium jarrrei*.

Abbreviations: fr – fibrillar root; Ga – Golgi apparatus; k – kinetosome; mv – microvilli; n – nucleus.

Fig. 10. Bayesian phylogenetic tree of the family Oscarellidae based on concatenated dataset (28S, 18S and COI loci). Numbers above branches indicate the posterior probabilities (>0.9), numbers below branches indicate bootstrap values (>70). Each clade with similar kinetid structure is highlighted in color.

	GenBank accession number				
Species	28S	18S	COI	References	
				<u> </u>	
Oscarella balibaloi	-	JX462763	KY682865	Gazave et al., 2013;	
				Ereskovsky et al., 2017	
Oscarella bergenensis	JX462771	JX462768	-	Gazave et al., 2013	
				Lavrov et al., 2008	
Oscarella carmela	KY513287	EU702422	EF081250	Wang, Lavrov, 2008	
				Ereskovsky et al., 2017	
Oscarella filipoi	-	-	MG009508	Pérez and Ruiz, 2018	
Oscarella kamchatkensis	JX462781	JX462762	-	Gazave et al., 2013	
Oscarella lobularis	HM118549	HM118536	KY682864	Gazave et al., 2010;	
				Ereskovsky et al., 2017	
Oscarella malakhovi	HM118550	HM118537	HQ269364	Gazave et al., 2010	
Oscarella microlobata	HM118551	HM118538	HQ269355	Gazave et al., 2010	
Oscarella nicolae	JX462770	JX462769	-	Gazave et al., 2013	
Oscarella pearsei	_*	_*	KY682864	Ereskovsky et al., 2017	
Oscarella rubra	JX462773	JX462765	-	Gazave et al., 2013	
Oscarella tuberculata	JX462777	JX462761	JX963640	Gazave et al., 2013	
Oscarella viridis	-	JX462764	HQ269358	Gazave et al., 2010, 2013	
Oscarella zoranja	-	-	MG009501	Pérez and Ruiz, 2018	

Table 1. Sequences used for the phylogenetic analysis with information on GB accession numbers

Pseudocorticium jarrei	HM118552	HM118539	HQ269357	Gazave et al., 2010
Corticium candelabrum	HM118553	HM118540	HQ269363	Gazave et al., 2010
Plakortis halichondrioides	HM118557	HM118543	KP972554	Gazave et al., 2010;
				Vicente et al., 2014
Plakinastrella onkodes	HM118562	HM118548	KU674372	Gazave et al., 2010;
	1111110302			Ruiz et al., 2017
I avagleig algebrig	10070200	AF452016	-	Manuel et al., 2003;
Leucanis ciainria	JQ272302			Voigt et al., 2012

*Sequences EF654526 (28S), EF654519, KY513286 (18S) under the name *Oscarella pearsei* in GenBank belong to *O. carmela* (see page 6).