Ansanella granifera gen. et sp. nov. (Dinophyceae), a new dinoflagellate from the coastal waters of Korea

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A small dinoflagellate, Ansanella granifera gen. et sp. nov., was isolated from estuarine and marine waters, and examined by light microscopy, scanning electron microscopy, and transmission electron microscopy. In addition, the identity of the sequences (3,663-bp product) of the small subunit (SSU), internal transcribed spacer (ITS) region (ITS1, 5.8S, ITS2), and D1-D3 large subunit (LSU) rDNA were determined. This newly isolated, thin-walled dinoflagellate has a type E eyespot and a single elongated apical vesicle, and it is closely related to species belonging to the family Suessiaceae. A. granifera has 10-14 horizontal rows of amphiesmal vesicles, comparable to Biecheleria spp. and Biecheleriopsis adriatica, but greater in number than in other species of the family Suessiaceae. Unlike Biecheleria spp. and B. adriatica, A. granifera has grana-like thylakoids. Further, A. granifera lacks a nuclear fibrous connective, which is present in B. adriatica. B. adriatica and A. granifera also show a morphological difference in the shape of the margin of the cingulum. In A. granifera, the cingular margin formed a zigzag line, and in B. adriatica a straight line, especially on the dorsal side of the cell. The episome is conical with a round apex, whereas the hyposome is trapezoidal. Cells growing photosynthetically are 10.0-15.0 μm long and 8.5-12.4 μm wide. The cingulum is descending, the two ends displaced about its own width. Cells of A. granifera contain 5-8 peripheral chloroplasts, stalked pyrenoids, and a pusule system, but lack nuclear envelope chambers, a nuclear fibrous connective, lamellar body, rhizocysts, and a peduncle. The main accessory pigment is peridinin. The SSU, ITS regions, and D1-D3 LSU rDNA sequences differ by 1.2-7.4%, >8.8%, and >2.5%, respectively, from those of the other known genera in the order Suessiales. Moreover, the SSU rDNA sequence differed by 1-2% from that of the three most closely related species, Polarella glacialis, Pelagodinium bei, and Protodinium simplex. In addition, the ITS1-5.8S-ITS2 rDNA sequence differed by 16-19% from that of the three most closely related species, Gymnodinium cori, Pr. simplex, and Pel. bei, and the LSU rDNA sequence differed by 3-4% from that of the three most closely related species, Protodinium sp. CCM419, B. adriatica, and Gymnodinium sp. CCM425. A. granifera had a 51-base pair fragment in domain D2 of the large subunit of ribosomal DNA, which is absent in the genus Biecheleria. In the phylogenetic tree based on the SSU and LSU sequences, A. granifera is located in the large clade of the family Suessiaceae, but it forms an independent clade.

Key Words: new genus; new species; protist; Suessiaceae; Symbiodiniaceae; taxonomy; ultrastructure
INTRODUCTION


The order Suessiales was established by Fensome et al. (1993), with the two new families Suessiaceae and Symbiodiniaceae. Species in the family Suessiaceae comprised fossil dinosporin cysts with a 7-9 latitudinal paraplate series (i.e., arrangement of amphiesmal vesicles [AVs] in latitudinal series), whereas species in the family Symbiodiniaceae comprised extant symbionts with a 7-latitudinal plate series. However, the number of latitudinal series of AVs has since been extended to include species with more than 10 (Kremp et al. 2005). Moestrup et al. (2009a) merged Suessiaceae and Symbiodiniaceae, as the fossil species of the Suessiaceae are morphologically very similar to extant species such as Polarella (Montresor et al. 1999), and it seems unnecessary to establish two parallel taxonomic systems for extant and fossil species. Moestrup et al. (2009a) and Siano et al. (2010) included several genera in the family Suessiaceae, based on four major morphological characters: eyespot structure, morphology of the apical apparatus, structure of the resting cyst, and presence or absence of a nuclear fibrous connective, and also by molecular characterization. Siano et al. (2010) later proposed the number of latitudinal series of AVs as an additional morphological character for differentiating genera. Gómez (2012a) retained Symbiodiniaceae for the extant and Suessiaceae for the fossil species and included eight genera in this Symbiodiniaceae (Aureodinium Dodge, Biecheleria Moestrup, Lindberg and Daugbjerg, Biecheleriopsis Moestrup, Lindberg and Daugbjerg, Pelagodinium Siano, Montresor, Probert and de Vargas, Polarella Montresor, Procaccini and Stoecker, Piscinodinium Lom, Protopodinium Lohmann, and Symbiodinium Freudenthal). However, whether Aureodinium and Piscinodinium belong here need to be supported by additional studies (Lohmann 1908, Freudenthal 1962, Dodge 1967, Lom 1981, Montresor et al. 1999, Moestrup et al. 2009a, 2009b, Siano et al. 2010, Gómez 2012a). Aureodinium has not been examined since its original description by Dodge (1967) and its phylogenetic affinities are uncertain. A molecular study (single subunit [SSU]) of the parasite Piscinodinium, however, indicated phylogenetic relationship to the Suessiales (Levy et al. 2007), but additional information on amphiesmal plates and other morphological data are required to further evaluate the phylogenetic relationship.

Recently, we isolated thin-walled dinoflagellates from Shiwha Bay, Korea, and established two clonal cultures. The morphology of the cells from the cultures was almost identical, and there was only one base pair difference in the sequences of their SSU, internal transcribed spacer (ITS) region (ITS1, 5.8S, ITS2), and D1-D3 large subunit (LSU) rDNA. On the basis of morphological characters (in particular, the structure of the eyespot, thylakoids, and apical apparatus) and molecular analyses, these dinoflagellates are classified as a species of the family Suessiaceae, of which Symbiodiniaceae is considered to be a synonym. However, there is no other genus or species matching the characteristics of these dinoflagellates within the family. Therefore, in this study, we propose classifying these thin-walled dinoflagellates in the new genus Ansanella.

This study describes the morphological features of Ansanella granifera gen. et sp. nov., using light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The molecular sequences of the SSU, ITS region (ITS1, 5.8S, ITS2), and D1-D3 LSU rDNA from cultured cells are also described, as well as pigment data obtained using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Collection and culturing

Samples of surface sediment were collected from Shiwha Bay, Korea (37°18′N, 126°36′E) in 2010 and 2012, using an Ekman grab (Wildco; Wildlife Supply Company, Buffalo, NY, USA). Samples were stored in the dark at 4°C until processed further. The sediments were sieved consecutively through 100-μm and 15-μm Nitex meshes. The material on the 15-μm mesh was then transferred to a 50-mL beaker containing filtered seawater. A manual vortex was applied and the suspended sediment fraction was recovered. The sediment fraction was incubated in f/2 me-

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dium without Si (Guillard and Ryther 1962), in a growth chamber at 20°C under 20 μmol m⁻² s⁻¹ illumination, in a 14-h light, 10-h dark cycle. The incubated sediments were observed regularly for the presence of motile cells, using a stereomicroscope (SZX-12; Olympus, Tokyo, Japan).

In 2013, one clonal culture of *A. granifera* from sediments collected in September 2010 (*A. granifera* AGSW10) was established using two serial single-cell isolations, and another clonal culture was established from sediments collected in December 2012 (*A. granifera* AGSW12). The temperature and salinity of the ambient waters during collection of the sediments were 21.3°C and 15.6, respectively, in 2010, and 2.0°C and 28.0, respectively, in 2012. When the concentration of *A. granifera* had increased sufficiently, aliquots of cells were transferred to 32-mL, 270-mL, and 500-mL polycarbonate bottles containing fresh f/2 medium. The bottles were placed on a shelf, and incubated under the same conditions as those used for the sediments.

**Morphology**

The morphology of *A. granifera* was examined using light microscopy, SEM, and TEM. An image analysis system was used to measure the length and width of live vegetative flagellated cells in images captured using a compound microscope (Zeiss Axiosvert 200M; Carl Zeiss Ltd., Göttingen, Germany).

For SEM, a 20-mL aliquot of a dense culture of *A. granifera* was fixed for 10 min in 1% osmium tetroxide (final concentration). The fixed cells were collected on a 3-μm pore size, polycarbonate membrane filter without rinse. DNA extraction, polymerase chain reaction (PCR) amplification, sequencing, and data analysis

Three to five cells of *A. granifera* were transferred to a 1.5-mL tube containing 10-μL distilled water. The tube was then stored at -72°C for 1-3 min to completely disrupt the cells. DNA extraction, amplification of the SSU, ITS1, ITS2, and D1-D3 LSU rDNA regions, PCR reactions, sequencing, and alignment were performed according to protocols used previously by Kang et al. (2011a).

**Sequence availability and phylogenetic analysis**

Phylogenetic analyses of the SSU and LSU rDNA regions of *A. granifera* were conducted using MEGA v.4 (Tamura et al. 2007) and Clustal X2 (Larkin et al. 2007), including sequences obtained from GenBank. Maximum-likelihood (ML) analysis of the two regions was conducted using the RAxML 7.0.3 program (Stamatakis 2006), with a default GTR + G + I model. Tree likelihoods were estimated using a heuristic search with 100 random additional sequence replicates, and tree bisection and reconnection branch swapping. ML bootstrap was also conducted. Bayesian analyses were performed using MrBayes v.3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) with the default GTR + G + I mode, to determine the best available model for the data of each region. For all sequence regions, four independent Markov Chain Monte Carlo runs were performed according to methods used previously by Kang et al. (2010).

**Pigment analysis**

Approximately 100,000 cells of *A. granifera* were transferred to a 50-mL tube. The 50-mL aliquot was filtered onto a 1.2-μm pore-sized GF/C filter. A total of 3 mL of
95% methanol was used for extraction, and a Waters C8 column (150 mm × 4.6 mm, 3.5-μm particle size, 0.01-μm pore size) was used for separation. Analysis of pigments was performed using HPLC (LC-10A system; Shimadzu Co., Kyoto, Japan) according to methods used previously by Zapata et al. (2000). Pigments were identified and quantified by comparing retention times and absorption spectra with those of authentic standards. All standards were purchased from DHI Water and Environment (Horsholm, Denmark).

RESULTS

Phylum Dinoflagellata Bütschli 1885 sensu Fensome et al. 1993
Class Dinophyceae Pascher 1914
Order Suessiales Fensome et al. 1993
Family Suessiaceae Fensome et al. 1993

Ansanella H. J. Jeong, S. H. Jang, Moestrup and N. S. Kang gen. nov.

Description. Free-living dinoflagellates with very thin transparent polygonal AVs arranged in longitudinal rows. The AVs are further arranged in 10-14 horizontal rows: 4-6 rows on the episome, 3-4 rows in the cingulum, and 3-4 rows on the hyposome. A single straight elongated apical vesicle, containing a single row of globular knobs, is present on the cell apex. Grana-like thylakoids in the chloroplasts and an eyespot of Type E are present. The peridinin-containing chloroplasts are lined by three membranes. A 51-base pair fragment of domain D2 of the peridinin-containing chloroplasts are lined by three membranes. A 51-base pair fragment of domain D2 of the large subunit rDNA, which is not found in genus Biecheleria, is present. Nuclear fibrous connective, nuclear envelope chambers, rhizocysts and lamellar body are absent.

Type species. A. granifera H. J. Jeong, S. H. Jang, Moestrup and N. S. Kang.

Etymology. The generic name “ansanella” refers to the name of the city, Ansan, enclosing Shiwha Bay where this species was collected.

Ansanella granifera H. J. Jeong, S. H. Jang, Moestrup and N. S. Kang sp. nov.

Description. Episome conical with a round apex, larger than the trapezoidal hyposome. The cell has a wide and distinctive cingulum, which is displaced about its own width. The length and width of living cells are 10-15 μm and 9-12 μm, respectively. The ratio of cell length to cell width of living cells is 1.1-1.3. The nucleus is oval and located in the anterior to central part of the cell. Chloroplasts are yellowish-brown with the lobes arranged mainly near the surface of the cell; three or four starch-enveloped pyrenoids are penetrated by thylakoids; pusule present. Peduncle and trichocysts absent. Estuarine and marine dinoflagellate.

Holotype. A holotype slide labeled USNM slide 1231539, of a culture fixed in 1% (final concentration) osmium tetroxide, was deposited in the Protist Type Specimen Slide Collection, US Natural History Museum, Smithsonian Institution, Washington, DC, USA.

Molecular characterization of Ansanella granifera: The rDNA gene sequence (SSU, ITS1-5.8S-ITS2, and D1!-D3 LSU rDNA), GenBank accession Nos. HG529978-HG529980 (3,663-bp product) and HG792066 (3,663-bp product).

Type locality. Shiwha Bay, Korea (37°18’ N, 126° 36’ E).

Etymology. The specific name “granifera”, indicates the characteristic chloroplast grana-like structures of this organism.

Observations

The morphologies of all the observed cells of Ansanella granifera AGSW10 and Ansanella granifera AGSW12 were almost identical. The episome was conical with a round apex, and was larger than the trapezoidal hyposome (Fig. 1A-F, Appendix 1A-C). There was a distinct elongated apical vesicle (EAV) (Figs 2A, C, 3C, D, 4A & C, Appendix 1F). The cingulum was displaced about its own width (Figs 2A & 4A, Appendix 1A). The sulcus became wider toward the antapex (Figs 1B, 2A, 3F & 4A, Appendix 1A). The nucleus was oval and located in the anterior to central part of the cell (Fig. 1C-E, Appendix 1B & C). Five to eight yellowish-brown chloroplasts were arranged in bands near the cell periphery (Fig. 1C & E, Appendix 1B). A bright red eyespot was located ventrally near the sulcus (Fig. 1B, Appendix 1A). One to three round pyrenoids were often visible in the light microscope (data not shown). The ranges (mean ± standard error, n = 40) of the length and width of living cells were 10.0-15.0 μm (12.6 ± 0.2 μm) and 8.5-12.4 μm (10.3 ± 0.2 μm), respectively. The ratio of cell length to cell width was 1.1-1.3 (1.2 ± 0.01 μm).

Under the SEM, the elliptical to round episome appeared larger than the trapezoidal hyposome (Fig. 2A-C). Cells were covered with hexagonal or pentagonal AVs. The length of each side of a hexagonal or pentagonal AV was 1.1-2.5 μm. The AVs were arranged in 10-14 rows (mostly 11, n = 100), as follows: 4-6 rows on the episome (mostly
Fig. 1. Cells of *Ansanella granifera* AGSW10 gen. et sp. nov. Micrographs taken by light microscopy (A–D) and epifluorescence microscopy (E & F). (A) Cells of various sizes and shapes. (B) Ventral view showing an eyespot (ES) and sulcus (SU). (C) Dorsal view showing the large nucleus (N) and yellowish-brown chloroplasts (C). (D) Lateral view. (E) Ventral view showing the nucleus extending from the anterior to the central part of the cell. (F) Dorsal view showing the chloroplasts located at the cell periphery. Scale bars represent: A, 20 µm; B–F, 5 µm.

Fig. 2. Micrographs of *Ansanella granifera* AGSW10 gen. et sp. nov. taken by scanning electron microscopy. (A) Ventral view of the cell showing five rows (E1–E5) of amphiesmal vesicles (AVs) in the episome, and the elongated apical vesicle (EAV). (B) Left side view of the cell showing three rows (E3–E5) of AVs on the episome, three rows (C1–C3) in the cingulum, and four rows (H1–H4) on the hyposome. (C) Dorsal view of *A. granifera* showing five AV rows (E1–E5) including a small vesicle (asterisk). (D) Right-side view. FLP, finger-like protrusion; LF, longitudinal flagellum; TF, transverse flagellum. Scale bars represent: A–D, 2 µm.
Fig. 3. Micrographs of Ansanella granifera AGSW10 gen. et sp. nov. taken by scanning electron microscopy (SEM). (A) Apical view. (B) Antapical view. (C) Apical view showing the elongated apical vesicle (EAV) (dashed box). (D) Drawing of the EAV-the ventral part of the EAV showing the long and narrow central plate ornamented with knobs. (E) Cingulum view showing a cell in which the outer membrane of some amphiesmal vesicles were removed (asterisks), showing the zigzag line of the lower cingular margin (arrows). (F) Ventral view showing 6-7 rows of amphiesmal vesicles in the sulcus. (G) SEM figure enlarged from Fig. 3F, showing the longitudinal flagellum (LF) and transverse flagellum (TF). FLP, finger-like protrusion. (H) Drawing of the sulcus. Scale bars represent: A, B & E, 2 µm; C & F-H, 1 µm; D, 0.5 µm.
Thin TEM sections showed the main features of the cell, such as the chloroplasts, eyespot, fibrous vesicles, golgi body, mitochondria, nucleus, pyrenoid, and starch (Figs 5A-C & 6A). Longitudinal TEM serial sections showed the nucleus to contain many chromosomes, and its length and width were approximately half of the cell length (data not shown). The nucleus lacked nuclear chambers and a nuclear fibrous connective. Mitochondria were present in both central and peripheral parts (Fig. 5A-C). The golgi apparatus, composed of 6-10 stacked cisternae and fibrous vesicles, was located near the nucleus (Fig. 5A & B). Chloroplasts with complex lobes were located...
predominantly near the cell surface. These chloroplasts
typically had three thylakoids per lamella (Fig. 6C & F),
but arrangements of five, six, or more thylakoids per la-
mella (i.e., grana thylakoid associations) were also seen
(Fig. 6D-F; Appendix 2B & C). All sectioned cells showed
extensive “grana” formation (Fig. 6D-F; Appendix 2B & C).
Three evenly spaced membranes enclosed each chloro-
plast (Fig. 6C). The chloroplasts formed a peripheral net-
work that comprised 3-4 pyrenoids, each surrounded by
a hemispherical starch grain (Fig. 6A & B, Appendix 2A).
Underneath the sulcus, there was a large and conspicu-
ous eyespot comprising approximately ~7 cisternae with
brick-like material (Fig. 7A-C). The length and width
of the crystalline bricks were 100-230 nm and 50-120 nm,
respectively. The eyespot was located near the flagellar apparatus (Fig. 7B). A single row of microtubules (i.e., Root 1, R1) was observed in the narrow space between the outermost cisterna and the cell membrane of the sulcal region (Fig. 7B). The flagellar apparatus of several cells was examined in TEM serial sections (Figs 8-12), and a diagrammatic 3D-reconstruction was generated (Fig. 13). The transverse basal body (TB) and longitudinal basal body (LB) formed an angle of approximately 135° (Fig. 8A), as estimated from the serial sections. A striated collar surrounded the exit aperture of each flagellar canal (longitudinal striated collar and transverse striated collar [TSC]) (Figs 8 & 12). In the flagellar root system, a large multi-membered microtubular root 1 (R1, longitudinal

Fig. 7. Micrographs of Ansanella granifera AGSW10 gen. et sp. nov. take by transmission electron microscopy. (A) Transverse section through the cell showing the eyespot (ES) in the gap between the chloroplasts. (B) Transverse section of a cell showing the eyespot vesicles (ES) containing crystalline bricks and the R1 flagellar root. (C) The ES consists of seven layers of brick-containing cisternae. Scale bars represent: A, 1 µm; B & C, 0.2 µm.
Fig. 8. *Ansanella granifera* AGSW10 taken by transmission electron microscopy. (A) Longitudinal section of the cell showing the basal bodies (longitudinal basal body [LB], transverse basal body [TB]) and the eyespot (ES). (B-E) Flagellar apparatus. Non-adjacent, nearly longitudinal serial sections proceeding from left to right. The encircled numbers are section numbers. Micrograph showing relative positions of the LB, TB, Root 1 (R1), putative Root 2 (R2), Root 4 (R4), striated root connective (SRC), basal body connectives (bbc), and longitudinal striated collar (LSC). Scale bars represent: A-E, 0.2 µm.

A mcotubular root) was located to the left of the LB (Figs 8-10 & 12) and extended underneath the sulcus toward the antapex (not shown). A single-stranded microtubular root (R2) was associated obliquely with the right side of the LB (Figs 8D, 9C, 10C & D). The transverse microtubular root (R3) was attached to the right side of the TB (Fig. 10). The transverse microtubular root extension structure was located near the TB and R3 (Fig. 10C & D). The transverse striated root and the transverse striated root microtubule of root 4 were located near the TB (Figs 8E, 11 & 12A). The two basal bodies were connected by a small striated connective, the basal body connectives (Fig. 8D). A number of fibrous connectives or fibres were associated with the various roots. Thus, the R1 root was attached to the LB by
two fibrillar connectives, the $C_{1,LB/R1}$ and $C_{2,LB/R1}$, respectively (Fig. 9). The $C_{1,LB/R1}$ was situated slightly posterior to $C_{2,LB/R1}$ and attached to the two rightmost microtubules of the R1 and one of the LB triplets. It was relatively short and only visible in two consecutive sections. The $C_{2,LB/R1}$ showed a distinct striation pattern and was intimately associated with the ventral surface of the R1 (Fig. 9C). The R1 and R4 roots were interlinked by a striated root connective (Fig. 8D & E). A dorsal fibre, with almost the same striation pattern as the striated fibre of R4, was located on the dorsal side of the R1 (Figs 10B, C, E & 12C). Transverse TEM serial sections showed the pusule system (PU) (Fig. 11). In the right-antapical side view, the PU was located next to the TBs and TSC (Fig. 11). We serially sectioned 5 whole cells transversally and longitudinally, but peduncle, lamellar body and rhizocysts were not observed.

**Fig. 9.** Non-adjacent transverse serial sections of the flagellar apparatus taken by transmission electron microscopy. Sectioning is from anterior to posterior and the cell is seen from the anterior end. The encircled numbers are section numbers. (A) Micrograph showing the longitudinal basal body (LB) and Root 1 (R1). (B-D) The $C_{1, LB/R1}$ and $C_{2, LB/R1}$ interconnect the R1 root and one of the LB triplets. The dorsal fibre (DF) is present on the dorsal side of the R1. Scale bars represent: A-D, 0.2 µm.
Fig. 10. *Ansanella granifera* AGSW10 taken by transmission electron microscopy. Nonadjacent longitudinal serial sections of the flagellar apparatus. The cell is seen from the outside, and the sectioning proceeds from right to left. The encircled numbers are section numbers. (A-D) Micrograph showing relative positions of the longitudinal basal body (LB), transverse basal body (TB), Root 1 (R1), putative Root 2 (R2), Root 3 (R3), Root 4 (R4), dorsal fiber (DF), and microtubular extension (transverse microtubular root extension [TMRE]). The striation pattern of the DF is very distinct. Notice also flagellar root R3 and its microtubular extension (TMRE). (E) Enlargement from Fig. 10C. Scale bars represent: A–D, 0.2 µm.
Fig. 11, *Ansanella granifera* AGSW10 taken by transmission electron microscopy. (A-F) Adjacent longitudinal serial sections. The cell is seen from the outside, and the sectioning moves from posterior to anterior. The encircled numbers are section numbers. The striated pattern of transverse striated root (TSR) is evident. Notice the Root 4 (R4) root with its single microtubule transverse striated root microtubule (TSRM). Pusule vesicles (PU) and the transverse striated collar (TSC) are also visible. TB, transverse basal body. Scale bars represent: A-F, 0.2 µm.
Fig. 12. *Ansanella granifera* AGSW10 taken by transmission electron microscopy. Nonadjacent serial sections. The sulcus region in transverse section, the sectioning moves from posterior to anterior. The encircled numbers are section numbers. (A-D) Micrograph showing relative positions of the longitudinal basal body (LB), transverse basal body (TB), Root 1 (R1), Root 4 (R4), transverse striated root (TSR) + transverse striated root microtubule (TSRM), dorsal fiber (DF), transverse striated collar (TSC), and eyespot (ES). The R1 flagellar root is located in the narrow space between the eyespot and the cell surface. Scale bars represent: A-D, 0.2 µm.
Pigments

Pigment analysis of Ansanella granifera AGSW10 showed chlorophyll a as the major pigment, and chlorophyllide a, chlorophyll c2, peridinin, diadinoxanthin, dinoxanthin, pheophytin a, and beta-carotene as accessory pigments (Fig. 14).

Genetic variability

DNA sequences of A. granifera gen. et sp. nov. The SSU, ITS1, 5.8S, ITS2, and D1-D3 LSU rDNA sequences of A. granifera AGSW10 (GenBank accession Nos. HG529978-HG529980, 3,663-bp product) were only 1 base pair different from those of A. granifera AGSW12 (HG792066, 3,663-bp product). When properly aligned, the SSU rDNA sequence of A. granifera differed by 1.2-7.4% from those of the other genera included in the order Suessiales (i.e., Biecheleria, Biecheleriopsis, Polarella, Protodinium [it should be noted that strains such as CCMP 419 and CCMP 420 appear to belong to Biecheleriopsis adriatica, not to Protodinium], Pelagodinium, Symbiodinium, Piscinooidinium, Baldinia, Cystodinium, and Phytodinium). In addition, the ITS1, 5.8S, and ITS2 rDNA sequences of A. granifera differed by >8.8% from those of the other genera included in the family Suessiaceae (i.e., Biecheleria, Biecheleriopsis, Polarella, Protodinium, and Pelagodinium). A. granifera had a 51-base pair fragment in domain D2 of the large subunit of ribosomal DNA, which has not been found in genus Biecheleria. The D1-D3 LSU rDNA sequences of A. granifera differed by >2.5% from those of other genera included in the order Suessiales (i.e., Biecheleria, Biecheleriopsis, Polarella, Protodinium, and Pelagodinium) (Table 1).

When properly aligned, the SSU rDNA sequence of A. granifera differed by 1-2% from those of the 3 most closely related species—Polarella glacialis (EF417317), Pelagodinium bei (U37406) (to be spelled with a single i, commemorating A.W.H.Bé), and Protodinium simplex (U41086). The ITS1-5.8S-ITS2 rDNA sequence differed by 16-19% from those of the 3 most closely related
species—Gymnodinium corii (AF318226), Protodinium simplex (AY688651), and Pelagodinium bei (DQ195358). Furthermore, the D1-D3 LSU rDNA sequence differed by 3-4% from those of the 3 most closely related species—“Protodinium sp.” CCMP419 (EF205015), Biechelerosipis adriatica (EU857537), and Gymnodinium sp. CCMP425 (EF205006).

Phylogeny

In the phylogenetic trees based on the SSU and LSU rDNA sequences of dinoflagellates, *A. granifera* belonged to the large clade of the family Suessiaceae (Figs 15 & 16). In the tree based on SSU rDNA sequences, *A. granifera* formed an independent clade with other genera in the family Suessiaceae (Fig. 15). In the tree based on D1-D3 LSU rDNA sequences, *A. granifera* also formed a subclade with *Symbiodinium* spp. (Fig. 16). This relationship was supported in terms of posterior probability (66%). However, it was not significantly supported in terms of ML bootstrap values. Also, the clade containing *A. granifera* strains was clearly divergent from the clades containing the other genera in the family Suessiaceae (Fig. 16).

Remarks on culturing and behavior

The newly isolated cells moved in straight lines or with a helicoidal mode. They sometimes stopped suddenly, and then resumed movement very quickly, with a jump-like action. They grew at salinities >10 or <35, but died in fresh water. Thus, this dinoflagellate is an estuarine and marine species.

**DISCUSSION**

*Ansanella granifera* gen. et sp. nov. belongs to the family Suessiaceae of the order Suessiales; it has 10-14itudinal series of AVs, thus meeting the criteria for inclusion in the order Suessiales (Fensome et al. 1993, Kremp et al. 2005). In addition, *A. granifera* has eyespot type E (i.e., a series of cisternae, with brick-like contents) and a single elongated apical vesicle, which are key characters of the family Suessiaceae (Moestrup and Daugbjerg 2007, Moestrup et al. 2009a), whereas members of the family Borghiellaceae, with the two genera *Baldinia* and *Borghiella* have eyespot of type B (i.e., eyespots containing carotenoid globules located within the chloroplast) (Hansen et al. 2007, Moestrup and Daugbjerg 2007, Moestrup et al. 2008, 2009a).

The morphology of *Ansanella* gen. nov. is different from that of other genera in or possibly related to the family Suessiaceae (i.e., *Biechelerosipis, Pelagodinium, Polarella, Protodinium, Symbiodinium, perhaps Aureodinium and Piscinooidinium*) (Lohmann 1908, Freudenthal 1962, Dodge 1967, Lom 1981, Montresor et al. 1999, Moestrup et al. 2009).

**Table 1.** Comparison of the sequences of *Ansanella granifera* AGSW10 (GenBank accession Nos. HG529978-HG529980) with other genera included in the order Suessiales

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<th>A. granifera</th>
<th>Bie</th>
<th>Bieo</th>
<th>Pol</th>
<th>Pro</th>
<th>Pel</th>
<th>Sym</th>
<th>Pis</th>
<th>Bal</th>
<th>Bor</th>
<th>Cys</th>
<th>Phy</th>
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</thead>
<tbody>
<tr>
<td>SSU</td>
<td>26(1.5)-34(2.0)</td>
<td>26(1.5)</td>
<td>20(1.2)-28(1.6)</td>
<td>26(1.5)-51(2.9)</td>
<td>24(1.4)-46(2.7)</td>
<td>&gt;46(2.8)</td>
<td>87(5.0)-92(5.3)</td>
<td>86(5.4)</td>
<td>NA</td>
<td>127(7.4)</td>
<td>127(7.4)</td>
</tr>
<tr>
<td>ITS1-5.8</td>
<td>26(8.8)-87(17.9)</td>
<td>116(17.1)-113(17.7)</td>
<td>64(17.2)-127(20.3)</td>
<td>23(14.6)-68(17.8)</td>
<td>34(11.6)-124(21.1)</td>
<td>No similar sequence found</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>S-ITS2</td>
<td>30(2.5)-24(4.4)</td>
<td>36(2.8)-41(6.6)</td>
<td>47(3.7)-43(7.5)</td>
<td>34(2.7)-32(4.4)</td>
<td>44(4.9)-52(12.9)</td>
<td>&gt;166(13.0)</td>
<td>NA</td>
<td>87(8.2)</td>
<td>81(7.3)-82(7.4)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>LSU</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

The numbers are base pairs different from each other. The numbers in parenthesis are dissimilarity (%) including gaps.

Bie, Biechelerosipis; Bieo, Biechelerosipis sp.; Pol, Polarella; Pro, Protodinium; Pel, Pelagodinium; Sym, Symbiodinium; Pis, Piscinooidinium; Bal, Baldinia; Bor, Borghiella; Cys, Cystodinium; Phy, Phytoodinium; SSU, small subunit; NA, not available; ITS, internal transcribed spacer; LSU, large subunit.

**ACKNOWLEDGMENTS**

The authors are grateful to the reviewers for their valuable comments on the manuscript. This work was supported by a grant from the National Natural Science Foundation of China (No. 40676109) and the National Key Basic Research Program of China (No. 2011CB950602).
Fig. 15. Consensus Bayesian tree of the order Suessiales based on 1,534 aligned positions of nuclear small subunit rDNA. Cystodinium phaseolus and Phytodinium sp. comprised the outgroup. The parameters were as follows: assumed equal nucleotide frequency; substitution rate matrix with A-C substitutions = 0.0750, A-G substitutions = 0.2798, A-T substitutions = 0.0934, C-G substitutions = 0.0403, C-T substitutions = 0.4600, and G-T substitutions = 0.0515; proportion of sites assumed to be invariable = 0.5026; and rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.0929. The branch lengths are proportional to the amount of character change. The numbers above the branches indicate the Bayesian posterior probability (left) and maximum-likelihood bootstrap values (right). A filled black circle is used to indicate the highest possible support value for the two phylogenetic methods applied. Posterior probabilities ≥0.5 are shown.
Fig. 16. Consensus Bayesian tree of the order Suessiales based on 676 aligned positions of nuclear large subunit rDNA (including the highly divergent domain D2). *Baldinia anauniensis* was chosen as the outgroup taxon. The parameters were as follows: assumed equal nucleotide frequency; substitution rate matrix with A-C substitutions = 0.0551, A-G substitutions = 0.2047, A-T substitutions = 0.0829, C-G substitutions = 0.0452, C-T substitutions = 0.5090, G-T substitutions = 0.1031; proportion of sites assumed to be invariable = 0.2291; and rates for variable sites assumed to follow a gamma distribution with shape parameter = 1.0076. The branch lengths are proportional to the amount of character change. The numbers above the branches indicate the Bayesian posterior probability (left) and maximum-likelihood bootstrap values (right). A filled black circle is used to indicate the highest possible support value for the two phylogenetic methods applied. Posterior probabilities ≥0.5 are shown.
et al. 2009a, 2009b, Siano et al. 2010, Gómez 2012a): Cells of *Ansanella* gen. nov. possess a pusule, while a pusule has not been reported in *Aureodinium* (Dodge 1967); *Ansanella* lacks a nuclear fibrous connective, which is present in *Biecheleriopsis* (Moestrup et al. 2009b). Further, *B. adriatica* and *A. granifera* showed other morphological difference in the shape of margin in cingulum. In *A. granifera*, the cingular margin formed a zigzag line, while in *B. adriatica* it forms a straight line, especially on the dorsal side; *Ansanella* has a single elongated apical vesicle, which is absent in *Polarella* (Montresor et al. 1999); *Ansanella* has a larger number of latitudinal plate series (10-14) than *Pelagodinium*, *Polarella*, *Protodinium*, and *Symbiodinium* (7-9); *Ansanella* also has a larger total number of AVs (>50) than *Pelagodinium*, *Symbiodinium* (<50); *Ansanella* lacks trichocysts, which are present in *Protodinium* (Dodge 1974, however, the identity of the organism illustrated in the paper needs to be confirmed); *Ansanella* is a free-living form, and lacks rhizocysts, while *Piscinoodinium* is parasitic with rhizocysts (Lom and Schubert 1983); the morphology of *Ansanella* is similar to that of *Biecheleria* (Moestrup et al. 2009b). However, *Ansanella* has a 51-base pair fragment in domain D2 of the LSU rDNA, which is absent in the genus *Biecheleria* (Moestrup et al. 2009b, Takahashi et al. 2014).

*A. granifera* has grana-like thylakoids. In the Suessi-ales sensu lato, these have only been reported previously in the parasite *Piscinoodinium* (Dodge 1968, Lom and Schubert 1983), whereas grana-like thylakoids are present in other orders, including Gonyaulacales (*Alexandrium pseudogonyaulax, Pyramidodinium atrofuscum, Pyrocystis lunula, Gonyaulax spinifera*) and Thoracosphaerales (*Theleodinium calcisporum*) (Dodge 1975, Hansen et al. 1996, Horiguchi and Sukigara 2005, Craveiro et al. 2013).

In the family Suessiaceae, the Type E eyespot has been reported previously in *Biecheleriopsis adriatica* Moestrup, Lindberg and Daugbjerg; *Symbiodinium natans* Hansen and Daugbjerg; *Symbiodinium voratum* Jeong, Lee, Kang and Laeuneseus; *Polarella glacialis* Montresor, Proccacini and Stocker; *Pelagodinium bei* Siano, Montresor, Probert and de Vargas; *Biecheleria pseudopalustris* Moestrup, Lindberg and Daugbjerg; *Biecheleria baltica*

### Table 2. Comparison of the morphology of *Ansanella granifera* and other genera included in the family Suessiaceae

<table>
<thead>
<tr>
<th>Eyespot type</th>
<th>Ag</th>
<th>Bie</th>
<th>Bieo</th>
<th>Pel</th>
<th>Pol</th>
<th>Pro</th>
<th>Sym</th>
<th>Pis</th>
<th>Aur</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>?</td>
<td>E</td>
<td>E</td>
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<tr>
<td>Apical furrow</td>
<td>EAV</td>
<td>EAV</td>
<td>EAV</td>
<td>EAV</td>
<td>Absent</td>
<td>EAV</td>
<td>EAV</td>
<td>EAV</td>
<td>EAV</td>
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<tr>
<td>Lps</td>
<td>10-14</td>
<td>8-29</td>
<td>11-13</td>
<td>7</td>
<td>Absent</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>NA</td>
</tr>
<tr>
<td>Episome series</td>
<td>4-6</td>
<td>3-14</td>
<td>4-5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Cingulum series</td>
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<td>2-4</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hyposome series</td>
<td>3-4</td>
<td>3-11</td>
<td>4-5</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Total amphimembranous vesicle number</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&lt;50</td>
<td>?</td>
<td>?</td>
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<tr>
<td>Lmc</td>
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<td>Zigzag</td>
<td>Straight</td>
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<td>Zigzag</td>
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<tr>
<td>Pusule</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
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<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
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</tr>
<tr>
<td>Parasite</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Trichocysts</td>
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<td>Absent</td>
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<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>?</td>
</tr>
</tbody>
</table>

References

This study: Biecheler (1952), etc.; Moestrup et al. (2009b), Takahashi et al. (2014); Siano et al. (2010); Montresor et al. (1999); Dodge (1974), Siano et al. (2009); Loeblich and Sherley (1979), etc.; Lom and Schubert (1983), Gómez (2012a); Dodge (1968), Gómez (2012a)


*Some strains named Protodinium in the GenBank (CCMP419 and 420) appear to be Biecheleriopsis adriatica. Thus, further morphological and genetic analyses are needed. Not mentioned, but seen in figures. Not mentioned but measured from figures.*

Hansen and Daugbjerg (2009), Jeong et al. (2014).
Moestrup, Lindberg and Daugbjerg; Biecheleria natalensis (Horiguchi and Pienaar) Moestrup; Biecheleria cincta (Siano, Montresor and Zingone) Siano; and Biecheleria brevisulcata Takahashi & Iwataki (Table 2). The eyespot of A. granifera consists of 7 layers of cisternae, identical or similar to those of B. brevisulcata (4), P glacialis (5), P. bei (6), B. adriatica (6), S. natans (6), S. voratum (6), B. natalensis (6), Prosoaulax lacustris (6), B. pseudopalustris (7), B. cincta (7), and B. baltica (9) (Horiguchi and Pienaar 1994b, Calado et al. 1998, Montresor et al. 1999, Kremp et al. 2005, Hansen and Daugbjerg 2009, Moestrup et al. 2009a, 2009b, Siano et al. 2010, Fig 15 in Kang et al. 2011a, Jeong et al. 2014, Takahashi et al. 2014). Whether Type E eyespot occurs in other species of the family Suessiaceae requires further investigation. It is a very unusual type of eyespot, and it has been suggested that the crystalline layers may function as a quarter-wave receptor (Foster and Smyth 1980, Horiguchi and Pienaar 1994b). Thus, the function of the eyespot in A. granifera merits further investigation. The actual photoreceptor has never been identified in any dinoflagellate.

The flagellar apparatus of Ansanella consists of typical dinoflagellate components, that is, R1-R4 flagellar roots and fibrous collars around the flagellar canals. It is basically similar to that described for Symbiodinium natans Hansen and Daugbjerg, with respect to the various connectives interlinking the flagellar roots and the basal bodies, with one notable difference, the presence of peduncle microtubules near the basal bodies in Symbiodinium natans (Hansen and Daugbjerg 2009). Also, the flagellar apparatus of A. granifera differs from S. natans by the presence of a ventral fiber. The possible presence of a peduncle and ventral fiber in the other species in the family Suessiaceae needs to be determined.

The SSU, ITS1, 5.8S, ITS2, and D1-D3 LSU rDNA sequences of the genus Ansanella clearly differ from those of any other genus in the family Suessiaceae. In the SSU and LSU phylogenetic trees, A. granifera belongs to the family Suessiaceae. However, in the LSU phylogenetic tree, the two strains of A. granifera formed a clade that is divergent from clades containing other genera in the family Suessiaceae. This clear divergence shows concordance between morphological and genetic characteristics. Therefore, on the basis of the morphological and molecular data, we propose that A. granifera to be a new species within a new genus.

There are indications that the order Suessiales may comprise not only the fish parasite Piscinoodinium but also species of the order Phytodiniales, members of which are coccoid freshwater dinoflagellates. Thus Logares et al. (2007) found both Cystodinium phaseolus and Phytodinium sp. of the Phytodiniales to be phylogenetically related to the Suessiales. Studies are underway to examine the ultrastructure of Cystodinium to determine whether Phytodiniales should be included in the Suessiales or retained as a separate order (Moestrup, unpublished). If the former turns out to be the case, the International Code of Nomenclature states that the priority rule is not mandatory for names above the level of family and the most suitable order name can therefore be applied (McNeill et al. 2012, Art. 16.4. Note 2).

ACKNOWLEDGEMENTS

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Appendix 1. Micrographs of Ansanella granifera AGSW12 gen. et sp. nov., showing whole cells. Micrographs taken by light microscopy (A & B), epifluorescence microscopy (C), and scanning electron microscopy (D–F). (A) Ventral view showing eyespot (ES) and sulcus (SU). (B) Dorsal view showing the large nucleus (N) and yellowish-brown chloroplasts (C). (C) Ventral view showing the nucleus located in the anterior to central part of the cell. (D) Apical view of the cell showing four rows (E1–E4) of amphiesmal vesicles (AVs) on the episome, and the EAV. (E) Right-side view of the cell showing three rows (C1–C3) of AVs in the cingulum, and three rows (H1–H3) on the hyposome. (F) Apical view showing the elongated apical vesicle (EAV) (dashed box). (G) Drawing of the EAV the ventral part of the EAV showing the long and narrow central plate ornamented with knobs. Scale bars represent: A–C, 5 µm; D & E, 2 µm; F, 1 µm; G, 0.5 µm.
Appendix 2. Micrographs of *Ansanella granifera* AGSW12 gen. et sp. nov. taken by transmission electron microscopy (TEM). (A) Transverse section showing profiles of four pyrenoids (PY). (B) Chloroplast lobe showing the grana-like thylakoids (GLT, dashed box). (C) A TEM figure enlarged from Appendix 2B, showing the GLT. (D) Transverse section showing eyespot structure (ES). Scale bars represent: A, 2 µm; B, 1 µm; C & D, 0.5 µm.