Description of Two Species of Early Branching Dinoflagellates, *Psammosa pacifica* n. g., n. sp. and *P. atlantica* n. sp

Noriko Okamoto1*, Aleš Horák1,2,3, Patrick J. Keeling1

1 Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada, 2 Laboratory of Molecular Taxonomy, Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic, České Budějovice, The Czech Republic, 3 Faculty of Sciences, Department of Evolutionary Protistology, University of South Bohemia, České Budějovice, The Czech Republic

Introduction

Alveolates are a major eukaryotic assemblage that includes three large and well-studied lineages, apicomplexans, dinoflagellates, and ciliates [1,2]. Each of these lineages has developed remarkable and complex innovations, sometimes taken to extremes, and alveolate evolution has consequently been a topic of considerable interest. In the case of apicomplexans and dinoflagellates, reconstructing the evolution of their unusual characteristics has been aided tremendously by the discovery of deep-branching relatives of both lineages. Interestingly in the case of the non-photosynthetic apicomplexans, where recently discovered photosynthetic relatives had led to several breakthroughs [3,4,5,6].

About half of the described species of dinoflagellates are photosynthetic, and the rest are grazers or parasites [7]. The well-studied “core” dinoflagellates, or the dinokaryotes, share distinctive features, i.e. dinokaryon (nucleus with permanently condensed chromosomes), the motile cell consists of two distinctive parts named epicone and hypocone, and an undulating transverse flagellum and a straight longitudinal flagellum [7]. Moreover, a number of unusual molecular innovations have also been found to be universal among dinokaryotes, including unusual organisation of organelle genomes [8,9,10,11,12,13,14], and messenger RNAs with spliced leaders [15,16,17]. How these features evolved and relate to the evolution of their apicomplexan relatives is, however, not clear from the study of dinokaryotes alone: to understand the evolution of such features, a greater knowledge of the whole dinoflagellate diversity is required.

Environmental sequence data reveal that there is substantial unexplored diversity within the dinokaryotes but even more so among the basal lineages of dinoflagellates, many of which remain unknown at the cellular level [18,19,20,21,22,23,24,25,26,27,28,29,30,31]. Those basal lineages were referred to as marine alveolate groups (MAG) in the early reports, and are now confirmed to include a handful of parasitic dinoflagellate groups historically called syndineans [32,33,34,35,36,37,38,39].

Syndineans are groups of intracellular parasitic dinoflagellates characterized by the absence of dinokaryotes or theca (cell wall of
the “armoured” dinoflagellates), the presence of alveoli vesicle underneath the plasma membrane. The motile stage of syndinians has epicone-hypocone architecture and laterally inserted flagella in most known cases [40]. Syndinians, as originally described, are polyphyletic, including some dinokaryotes such as Blastodinium spp. and others that branch paraphyletically at or near the base of dinokaryotes. These basal syndinians currently include seven genera, namely, Syndinium, Hematodinium, Amoebophrya, Endobiosquilla, Ichthyodinium, Ellobiopsis and Thalassomonas. Molecular phylogeny revealed that Syndinium, Hematodinium and Amoebophrya are part of MAG II [24,33,36,41,42,43,44], and that Endobiosquilla and Ichthyodinium are part of MAG I [34,37,39,45,46]. The ellobiopsids, consisting of Ellobiopsis and Thalassomonas, form an independent and fast evolving lineage that does not belong to either of these major clades [32,33]. Although the branching order of these early diverging lineages and the dinokaryotes has not been satisfactorily resolved, their early divergence from the ancestor of dinokaryotes is generally supported by molecular phylogenetic data and “syndinian-like” nuclear morphology sensu Leander & Hoppenrath [47] i.e., centrally located nucleus with peripherally condensed chromatins that are also found in some Perkinsids and colpodellids, as well as the intra nuclear spindle during nucleokinesis, which are shared with the apicomplexans [7,40,43,48,49].

The primary challenge is that we don’t have the information of MAG I and II at cellular level, except those eight genera. Secondarily, even among those parasitic genera, there’re limited cases of ultrastructural studies on the flagellate stage (Ichthyodinium chabelardi [44] in MAG I, Hematodinium sp. [50] and Amoebophrya spp. in MAG II [31,32,53]). This burdens a direct comparison between the dinokaryotes, Perkinsids, or the more distantly related apicomplexans. In addition to MAG I and II, there are several taxa that are argued to be early diverging dinoflagellates such as Ozyrhis marina, though their phylogenetic positions are yet to be determined.

In this study, we report a newly discovered free-living flagellate that branches among the early-diverging dinoflagellates, Psammosa pacifica n. g., n. sp. and P. atlantica n. sp. We discovered P. pacifica from Boundary Bay, British Columbia and P. atlantica from Blomidon Beach, Bay of Fundy, Nova Scotia, Canada. Psammosa cells are dorsoventrally compressed barley shape. The cell has a protrusion in the middle of dorsal face and grooves on the both side of the protrusion, where a shorter anterior and longer posterior flagellum separately emerge from distinct grooves. Cell division occurs along the transverse plane. Psammosa is a predator, feeding on other eukaryotes such as a heterotrophic stramenopile flagellates (P. pacifica) or diatoms (P. atlantica).

Only flagellate cells are observed for both species of Psammosa, which proliferate via transversal fission as is shown in O. marina. Ultrastructural observation of P. pacifica revealed that it possesses bipartite trichocysts and a syndinian-like nucleus, as found in the dinokaryotes, Perkinsids and some colpodellids; a flagellar transition region with an inclusion body as is found in some Syndinians and Perkinsids; two-dimensional flagellar scales and flagellar hairs as found in O. marina; and most remarkably an apical complex with pseudoconoid such as that found in Perkinsids and colpodellids. Small subunit (SSU) ribosomal DNA and heat shock protein 90 (Hsp90) sequence were characterised from Psammosa, and phylogenetic analysis, together with a unique insertion deletion character in Hsp90 [34], show that they form an independent lineage branching early in the tree of dinoflagellates. Altogether, we concluded that Psammosa represents the earliest lineage of dinoflagellates known to date, and as such has the potential to provide many insights into early alveolate evolution.

**Results**

**Taxonomic Summary**

**Assignment.** Eukaryota; Chromista; Alveolata; Dinozoa.

Psammosa n. g. N. Okamoto, A. Horák and Keeling, 2012.

Plasmens n. g. N. Okamoto, A. Hora and Keeling, 2012.

**Diagnosis.** The cell is biflagellate, dorsoventrally compressed barley shape with the round anterior end and acute posterior end, with a kink on the left ventral contour. It has a subapical diagonal ridge on the ventral face dividing anterior-left section and posterior-right section of the cell. The ventral side of the anterior-left section is concave towards the margin to make a wide groove on the right side of the ridge, where the anterior flagellum is inserted. The posterior-right section has a longitudinal depression in the middle, where the posterior flagellum is inserted. The cell is devoid of body scale. Both flagella bear two dimensional scales and mastigonomes. The cell contains a refractile body in the posterior section. The cell is colorless and devoid of any visible evidence of plastid. The cell proliferates by binary fission along the transverse plane at the margin of the anterior-left section and posterior-right section where the anterior flagellum emerge. The cell often rests on the bottom surface with the periodically beating posterior flagellum. The cell swim with rotation in the water column; while on the bottom surface, it swims in a tight circle pivoting around the cell apex without rotation. Both the anterior and the posterior flagella bears simple mastigonomes and two dimensional cobweb-shape flagellar scales.

**Type species.** Psammosa pacifica N. Okamoto, A. Horák and Keeling, 2011.

**Etymology.** Psammosa is derived from the greek psammon, meaning sand, the material from which both species of Psammosa were isolated.

Psammosa pacifica n. sp. N. Okamoto, A. Horák and Keeling, 2011.

urn:lsid:zoobank.org:act:E3F62250-F13C-4E2D-B7AB-7CC83F67C7D4.

**Diagnosis.** Cell is 7–8 μm in length and 4–5 μm in width, dorsoventrally compressed barley shape with a round anterior end and an acute posterior end. The posterior end of the anterior-right section and the posterior left section meet at c.a. two third of the entire cell length. Apical pore located at the anterior cell apex. Cell is eukaryore and feeds on Stramenopiles. Marine interstitious.

**Type locality.** The cells were collected from the intertidal water in the intertidal sandy beach at Boundary Bay, British Columbia, Canada (49.0086’N; −123.0228’W).

**Type figure.** Fig. 1a.

**Type sequence.** Partial small subunit ribosomal RNA gene of P. pacifica, JN873311.

**Type specimen.** TEM block deposited in Marine Invertebrate Collection, Beaty Biodiversity Museum, UBC: MI-PR110.

**Etymology.** Epithet refers to the ocean that type locality situates.

**General Morphology of Psammosa pacifica n. sp.**

Cell is 7–8 μm in length and 4–5 μm in width, dorsoventrally compressed barley shape with a round anterior end and an acute posterior end. The posterior end of the anterior-right section and the posterior left section meet at c.a. two third of the entire cell...
length. The cell is a eukaryovore, feeding on small flagellates such as *Spumella* sp. Prey is taken up at the apical end of the cell without changing the cell shape as would be seen in phagocytosis (Figure 1a).

**Cell division of *P. pacifica***. Cells undergo division transversally along the boundary between the anterior right and the posterior left sections of the cell (Fig. 1c–d). Flagellar duplication initiates prior to cell fission. Increasing numbers of the dividing cells are observed towards the end of the dark period under culture conditions.

**Swimming behaviour of *P. pacifica***. The cell swims with rotation in the water column; while on the bottom surface, it swims in a tight circle pivoting around the cell apex without rotation (Movie S1, S2, S3). Cells are sometimes observed resting on the surface of culture flasks with the posterior flagellum beating periodically.

**Surface morphology of *P. pacifica***. The cell lacks body scales. SEM preparation caused a slight shrinkage of the cell and revealed the pattern of alveoli beneath the cell membrane (Fig. 2a–d). The alveoli are arranged in polygonal pattern over the entire surface of the cell (Fig. 2c). Closer observation under SEM revealed that a small lobe of cytoplasm is situated on the right side of the anterior and posterior flagellar insertions (Fig. 2a–b). The anterior end of the lobe terminates with a pore (an opening of a narrow invagination near the cell apex) (Fig. 2e–f), which coincides with the point of contact with the food/prey cell (not shown).

---

**Figure 1. General morphology of *Psammosa pacifica* and *P. atlantica*.**

- a–c. *P. pacifica*;
- d–e. dividing cell of *P. pacifica*;
- g–i. *P. atlantica*.

Scales = 5 μm.

doi:10.1371/journal.pone.0034900.g001

---
Flagellar Scales, Mastigonemes and Flagellar Transition Region of *P. pacifica*

The cell has short anterior and long posterior flagella (Fig. 1a–b, 2a, d). The anterior flagellum is inserted transversally and no undulation of either flagellum is observed. When the cell is resting on the bottom of the culture vessel, the posterior flagellum beats periodically while the anterior flagellum shows little movement.

The posterior flagellum is ca. 380 µm in diameter, along the proximal half of the length, then bluntly reduce to ca. 250 µm in diameter along the distal half. The anterior flagella is consistent in its diameter, ca. 250 µm.

---

**Figure 2. Surface morphology of *Psammosa pacifica*. a–c.** Ventral view of *P. pacifica*. *b* and *c* is the same cell. The polygonal margins of the alveoli vesicles are indicated to facilitate the visualization. *d*. Dorsal view of *P. pacifica*. *e–f*. Close up view of the apical pore. Scales = 3 µm in a–d; 500 nm in e–f.

doi:10.1371/journal.pone.0034900.g002
Both flagella are covered by elliptical scales along their entire length (Fig. 2a–d, 3a–c). Preparation for SEM and TEM forced the scales to detach from flagella. The scale is ca. 210 nm in longer diameter and ca. 180 nm in shorter diameter, with a cobweb-like pattern consisting of 8 spokes radiating from the centre, and the 4–5 rings bridging the spokes (Fig. 3a–b). It is unclear in which subcellular compartment the scales are produced.

In addition to the flagellar scales, the entire anterior flagellum and the distal half of the posterior flagellum bears bipartite hairs composed of shaft and with 1–2 terminal hair(s) (Fig. 3b–d). Similar hair structures are observed between the inner and the outer nuclear membranes (Fig. 3e).

In the flagellar transition region, the central pair of microtubules terminate above the basal plate (Fig. 4a–c). Electron dense material was present on the outside of the central pair at termination. The centre of the basal plate is curved sharply toward the distal end of the transition region (Fig. 4b). Beneath the basal plate, lies an electron dense black globule in the posterior flagellar basal body (Fig. 4c).

Intracellular Ultrastructure of P. pacifica

The nucleus is situated in the mid-anterior region of the cell (Fig. 5a). The nucleus is not a dinokaryon, but rather is a syndinian-like [47], with dark stained materials lining the inner surface of the inner nuclear membrane and a spherical nucleolus situated in the centre of nucleus (Fig. 5a–b). The space between the outer and inner nuclear membranes is swollen and has fibrous material viable (Fig. 5a). Part of the outer nuclear membrane is studded with ribosomes and is associated with the rough endoplasmic reticulum (ER) (Fig. 5c). The Golgi body was not observed. Alveolar vesicles of various size are situated underneath of cell membrane and are found throughout the entire cell surface, except at the grooves where flagella are inserted. The alveoli were not observed to contain electron dense fibrils or theca (Fig. 5a; asterisks).

The Mitochondrion is often found anterior to the nucleus. Its morphology is as expected for an alveolate, with clearly tubular cristae (Fig 5d).

Bipartite trichocysts enclosed in a single membrane are present beneath the entire cell surface (Fig. 6a–b). A trichocyst is

Figure 3. Flagellar appendages of Psammosa pacifica. a. A flagellar scale showing cob-web pattern prepared by whole-mount method. b. A close up view of flagellum to show the flagellar hairs (mastigonema) and scales. c. A detached flagellar hair (mastigoneme) showing the shaft and fine hairs. d. The flagellar hairs (mastigonema) on a flagellum prepared by whole-mount method. e. The compartment between the inner and the outer nuclear envelops contains fine hairs similar to mastigonema on flagella. Scales = 100 nm in a; 500 nm in b–e. doi:10.1371/journal.pone.0034900.g003
composed of dark stained rod that has square profile (Fig. 6b), and lightly stained head of ca. 1 mm in length (Fig. 6a). In response to stimulation, such as chemical fixation of whole mounts for transmission electron microscopy, trichocysts discharge a ribbon-like structure (Fig. 6c) with a maximum width of ca. 10–17 nm and striations consisting of ca. 6 nm of dark and ca. 2 nm of light in an alternating pattern, in some views with an additional narrow dark band within the light band.

An apical complex is present at the anterior end of the cell (Fig. 7a). The complex consists of a pseudoconoid and two electron dense vesicles: longitudinally aligned rod-shaped rhoptries-like vesicles arranged in parallel to each other, and large spherical bodies situated posterior to the rhoptries (Fig. 7a–b). The pseudoconoid is composed of eight microtubules arranged in a slight curve convex toward the ventral side of the cell (Fig. 7a; arrowheads). The apical complex is associated with a gullet (Fig. 7b; G) that leads to the apical pore (Fig. 2e–f, 7a).

*Psammosa atlantica* n. sp. N. Okamoto, A. Horák and Keeling, 2012.

**Diagnosis.** Cell is 10–13 mm in length and 5–10 mm in width, dorsoventrally compressed barley shape with a round anterior end and an truncated posterior end. The posterior end of the anterior-right section and the posterior left section meet at c.a. one third of the entire cell length. Cell is eukaryvore and feeds on *Navicula* sp. (PRA-314, ATCC, VA) (Bacillariophyceae, Stramenopiles). Marine interstitials.

**Type locality.** The cells were collected from the interstitial water in the intertidal sandy beach at Blomidon Beach in the Bay of Fundy, Nova Scotia, Canada (45.25580°N; –64.34907°W).

**Type figure.** Fig. 1g.

**Type sequence.** Partial small subunit ribosomal RNA gene of *P. atlantica*: JN873310.

**Type specimen.** SEM deposited in Marine INvertebrate Collection, Beaty Biodiversity Museum, UBC:MI-PR111.

**Etymology.** Epithet refers to the ocean that type locality situate.

**General Behaviour of Psammosa atlantica n. sp**

Cells are often observed resting on the bottom surface of culture vessel and seldom swim up towards mid to top layer of water column. Cells started swimming vigorously upon addition of ubiquinone-ethanol solution. The cell is eukaryvore feeding on a small pennate diatom *Navicula* sp. The prey uptake was not directly observed, though the frustule of the prey was never observed within *P. atlantica* cells.

**Surface Morphology and Flagellar Appendages**

The cell lacks body scales. The pattern of alveoli vesicles was visible after a slight shrinkage during SEM preparation (Fig. 8a–c). The alveoli are arranged in polygonal pattern over the entire surface of the cell. The cell has short anterior and long posterior flagella (Fig. 1g–h, 8a–c). The anterior flagellum is inserted transversally and no undulation of either flagellum is observed. Both flagella are covered by elliptical scales along their entire length (Fig. 8d). The scales are c.a. 120 mm in length and 100 mm in width. The scales are arranged in longitudinal rows. In each row, the scale proximal to the flagellar insertion overlaps the distal one. Distance between the centre of the neighbouring scales is ca. 100 mm. The posterior flagellum is ca. 380 mm in diameter, along the proximal half of the length, then bluntly reduce to ca. 250 mm in diameter along the distal half. The anterior flagella is consistent in its diameter, ca. 250 mm. Mastigonemes are also present on the
Figure 5. Internal structure of *P. pacifica*. **a.** Longitudinal section of *P. pacifica*. **b.** Nuclear contents are condensed on periferal. Nucleolus is situated in the middle of the nucleus. **c.** Rough endoplasmic reticulum is connected to the outer nuclear envelop. **d.** Profile of mitochondrion shows the tubular cristae. D = dark stained vesicles; PNC = perinuclear compartment; MT = mitochondrion; N = nucleus; n = nucleolus; R = rhoptries; asterisks = alveolar vesicles. Scales = 1 μm in **a**; 500 nm in **b–d**.

doi:10.1371/journal.pone.0034900.g005
entire anterior flagellum, and the distal half of the posterior flagellum bears fine hairs. (Fig. 8e).

Molecular Phylogeny of *Psammosa* spp

Bayesian phylogeny based on SSU rRNA (Fig. 9) recovered the main lineages as expected: (1) MAG I, which includes *Eudubosquella* and *Ichthyodinium*; (2) MAG II, which includes *Syndinium*, *Hematodinium* and *Amoebophrya*; (3) the ellobiopsids clade, which includes *Ellobiopsis* and *Thalassomyces*; and (4) the dinokaryotes. Although *Oxyrrhis marina* is a taxon of interest for this part of the tree, it was excluded from analysis due to its notoriously long branch. It was confirmed overall tree topology did not change significantly whether it was included or excluded (not shown). The *Psammosa* lineage in this tree branches independently of any other major group at the base of whole dinoflagellate lineage (including perkinsids and syndineans s.l.), though without any support. An environmental sequence (AB505506) from the deep sea [55] was also found to fall within the *Psammosa* lineage. Maximum likelihood (ML) analysis yielded somewhat different topology with *P. atlantica* and *O. marina* forming clade at the base of dinokaryotes (see dashed topology at Fig. 10). Neither hypotheses are rejected by AU test. Similarly to SSU dataset, we have also created alternative topologies by ML and tested them again. To our surprise, another topology that was not rejected was the one with *P. atlantica* branching at the base of apicomplexan s.l. clade (i.e. including colpodellids and chromerids), although with marginal probability (0.063).

We also have created alternative dataset including partial sequence of *Amoebophrya* sp. ex *Karlodinium veneficum*. Here, the *P. atlantica* again forms clade with *O. marina* and branch between *P. marinus* and *Amoebophrya* (dotted topology at Fig. 10). The post-perkinsid origin of *Psammosa* lineage is further supported by deletion of an amino acid in Hsp90 that are characteristic of dinoflagellates and *O. marina*, but are absent in other eukaryotes including *P. marinus* and apicomplexans.

**Discussion**

Novelty and Distribution of *Psammosa*

In this study, we reported two new species of a novel lineage of early dinoflagellates, *Psammosa pacifica* and *P. atlantica* from an intertidal sandy beach environment. Based on their unique phylogenetic position and morphology, we have erected a new
Figure 7. The apical complex of *P. pacifica*. a–e. A series of longitudinal section along dorsoventral axis of the apical complex showing 8 microtubules of pseudoconoid (arrowheads) associated with elongated rhomboid structure of rothories (R) near a gullet (G). f. Profile of pseudoconoid microtubules. g. A longitudinal section along sinistrodextral axis of the apical complex showing a cluster of rothories, dark stained vesicles (D) and gullet (G). MT = mitochondrion; N = nucleus; T = trichocyst; Asterisk = Alveoli vesicles; Double arrowheads = cortical microtubules underneath the alveoli vesicles. Scales = 500 nm.

doi:10.1371/journal.pone.0034900.g007
Figure 8. Surface morphology of *P. atlantica*. a–c. dorsal view. d. A close up view of the middle of the posterior flagellar showing a blunt reduction in the thickness. Rows of flagellar scales covers the entire surface of the anterior and the posterior flagella in an overlapping manner. The flagellar hairs (mastigonema) are present, though the special association with the scales is not clear e. A close up view of the anterior flagellar showing flagellar scales and hairs (mastigonema). Scales = 5 μm in a–c; 500 nm in d–e.
doi:10.1371/journal.pone.0034900.g008
genus for these two species: *P. pacifica* and *P. atlantica*. They share general cell structure, i.e. compressed barley shape body with a subapical diagonal ridge, while the difference in cell length (i.e., *P. pacifica* is 7–8 μm; *P. atlantica* is 10–13 μm) and the contour of the posterior part of the cell (*P. pacifica* has acute end; *P. atlantica* is truncated end). In addition to the morphological difference, their prey preferences are different. *P. atlantica* feeds on *Navicula* sp., while *P. pacifica* does not feeds on *Navicula* sp. or other diatoms, but on *Spumella* sp. Molecular sequences of ribosomal SSU are not identical between two species. Based on those similarity and dissimilarity, we concluded that two organisms are members of the same genus *Psammosa*, but different species.

Although compressed barley shaped cell with laterally inserted flagella is not necessarily defining characters among protists, the centrally located protrusion and cell architecture separated into right anterior and left posterior parts, presence of the refractile body and two flagella inserted in two separate grooves delineate the genus *Psammosa* from non-alveolate colourless flagellated protists such as katablepharids (lacking protrusion and refractile body; both flagella emerge from a single depression; possessing light microscopically recognizable ejectosomes) or developayellids (lacking protrusion and refractile body; both flagella emerge from a single depression; bac teriovores).

Some alveolate flagellate especially colpodellids are light microscopically similar to *Psammosa*. In fact we found one species, *Colpodella unguis* resembles most to *Psammosa*. *Colpodella unguis* is originally described solely based on light microscopy from the shallow sandy sediment of Shark Bay, Western Australia, though it was not used to genus *Colpodella* is tentative [57]. Light micrographs from the original description of *C. unguis* share some similar characteristics with *Psammosa pacifica*; specifically, (1) two laterally inserted mastikonts, and (2) elongated reniform cell of similar size range (7–8 μm for *P. pacifica*; 7–10 μm for *C. unguis*) with a protrusion in the middle. However, the proportion of anterior left part is different: the left anterior part of *P. pacifica* is about two thirds of the cell length, whereas that of *C. unguis* is about one third, and thus forms an acute end described as “rostrum” in the original description [57].

Interestingly, Myl’nikov [58] re-isolated *C. unguis* from a different locality and performed ultrastructural observations, which also revealed a similar transition plate (though lacking the proximal dark stained inclusion body we observed), and an apical complex composed of a pseudoconoid (though with more than eight microtubules) as well as ‘micronemes’ and ‘rhoptries’ (see discussion below). The nuclear morphology of the Russian *C. unguis* is also distinct from that of *P. pacifica* in that it is characterized by a fibrous nuclear content, which may be due to different preparation conditions. The Russian strain of *C. unguis* is also shown to proliferates via “oblique-transversal” cell division, rather than via cyst development. Based on the light microscopical features, its habitat, life history and ultrastructures, it is possible that *C. unguis* may well be another member of the lineage. It is interesting the correct position of *C. unguis* in relation to *Psammosa* spp. In this study, however, we don’t have any direct evidence of their correct phylogenetic position, such as SSU rDNA sequence. Thus, we will refrain from proposing any taxonomic changes to *C. unguis*.

**Habitat and Trophic Strategy**

Both species of *Psammosa* from different location opposite side of the continent but under very similar condition, i.e., in the top interstitial layer of a dissipative beach. *Colpodella unguis*, the suspected member of this lineage is also found from shallow marine benthic habitat [57,58,59]. This newly recognised lineage would be associated to interstitial/benthic habitat and is not included in the water column. This would explain why there is only one environmental sequence that belongs to the *Psammosa* clade. We recognised a previously unidentified environmental sequence from coastal marine sediment of about 1000 m depth [53] that is also a member of the *Psammosa* clade, although it is unclear if the sequence is from an actively growing cell, or a dormant cyst that drifted into the sediments [60]. The revolution of our knowledge of protist diversity and distribution has been restricted to particular environments such as coastal marine water [19,24,28], open ocean [19,24,28,29], deep sea beds [20,21,24,26], anoxic/oxygen deplete marine environments [10,22,23,24,30], arctic ocean [24,25], or freshwater [24,61]. Although our view of protist biodiversity has been greatly improved by those environmental survey, there are different conventional surveys and the frequency of novel taxa discoveries. *Psammosa* spp. are heterotrophic eukaryvores, while all the basal dinoflagellates and the sister group, perkinsids are parasitic, except *Oxyrrhis marina* that is also a heterotrophic eukaryvore. Considering perkinsids, the immediate outgroup of *Psammosa*, are also parasitic, it may appear predation would be extraordinary cases. However, it may also be the case that only parasitic lineages have been described so far and that vast majority of diversity demonstrated by environmental surveys may include organisms of various trophic strategies. It is not necessarily appropriate to assume that MAG lineages to be all parasitic, considering the diversity included in those clades are somewhat equivalent to that of the dinokaryotes, which include various trophic modes ranging from photosynthesis, predation, symbiosis and parasitism. Cellular level investigation of unobserved MAG I and MAG II, as well as yet to be discovered novel lineages will be required to fill the gap of our knowledge.

**Molecular Phylogeny**

Molecular phylogeny based on small subunit ribosomal RNA (SSU rRNA) and Hsp90 recovered monophyly of dinoflagellates and perkinsids, but the branching order within the clade is not resolved with any support by bootstrap value or Bayesian post probability. Although topologically equivalent, the support of those branching is lower than some of the previous studies [32,33,34,35,36,37,38,39,44,61,62,63,64,65]. This may be partly because our trees retain more taxa including fast evolving clades, such as the ellipsoidis in SSU rRNA tree, which artifically attracts nothulicoid clades in the ML tree. The best ML trees of SSU rRNA placed *Psammosa* either basal to dinoflagellates after divergence of perkinsids. AU test also did not exclude the possibility that (b) *Psammosa* branches from common ancestor of perkinsids and dinoflagellates, or (c) *Psammosa* is basal to *Amoebophrya* clade within MAG II (never observed as the best topology). In Hsp90 tree, topology (b) was consistently rejected; instead, topology (a) was supported regardless whether with or without a short Hsp90 fragment of *Amoebophrya* in the analyses. The inconsistency among Hsp90 analyses is in the affinity between *Psammosa* and *O. marina*. *Psammosa* branches after divergence of Perkinsids but before that of *O. marina* in a Bayesian tree without a short *Amoebophrya* sequence, whereas they form a monophyletic clade of a Bayesian tree that includes partial *Amoebophrya* sequence and a ML tree. As both sequences of *P. atlantica* and *O. marina* are rather divergent and their relationship can be a result of so called long-branch artifact. This close affinity between *Psammosa* and *O. marina* is consistent with the ultrastra-
Figure 9. Small subunit ribosomal RNA (SSU rRNA) maximum likelihood phylogeny (ML) of two *Psammosa* species in context of recently described biodiversity of perkinsids and ‘lower’ dinoflagellates. The tree was inferred using RAxML 7.2.8 under the GTR model.
with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.
doi:10.1371/journal.pone.0034900.g009

Novel Early Branching Dinoflagellates Psammosa sp.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.
doi:10.1371/journal.pone.0034900.g009

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.
doi:10.1371/journal.pone.0034900.g009

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

with gamma correction from at total 1297 nucleotides. Numbers at nodes represent branching support as inferred in RAxML (500 replicates). Second number represents bayesian inference posterior probability, as calculated using Phylobayes 3.2 under the empirical admixture model C40 combined with LG exchange rate matrix. Only posterior probability higher than 0.94 and bootstrap support of 50 and more is shown. Branch lengths of ellobipsids (shaded clade) were reduced as indicated on figure. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.
Figure 10. Phylogenetic position of *Psammoa atlantica* as revealed by maximum likelihood (ML) analysis of amino acid sequences of Hsp90 gene (532 aa included). The tree was inferred using PhyML-CAT software under the empirical admixture model C40. Numbers at nodes.
represent branching support with the first number being result of a approximate likelihood-ratio test (a-LRT) computed in PhyML-CAT (under the above specified model). The second number shows bayesian posterior probability as inferred with Phylobayes 3.2 software under the empirical admixture model C40 combined with LG exchange rate matrix. The third number represents non-parametric bootstrap support of maximum likelihood (ML) analysis as inferred by RAxML (LG matrix, 500 replicates). Posterior probability and a-LRT higher than 0.94 and bootstrap support of 50 and more is shown. Dotted lines reveal ML and BI topology of alternative Hsp90 dataset with syndinian Amoebophrya sp. included. Dots by nodes point to alternative topologies of Psammosa spp. as tested by AU test (empty represent rejected and black not-rejected). Uncultured/environmental sequences are represented only by GenBank accession number. Accession numbers of sequences with known taxonomy are shown in brackets. See text for more details.

doi:10.1371/journal.pone.0034900.g010

Figure 11. Character matrix of Psammosa and the flagellate stage of dinoflagellate lineages, perkinsids and colpodellids.

doi:10.1371/journal.pone.0034900.g011
posterior and relatively distant from the pseudoconoid. We have chosen to refer the elongated rhomboid structure of *P. pacifica* as a "rhoptry" based on its appearance and association with the pseudoconoid, though again its homology to rhoptries in other myozoaons must be tested. The profile of the pseudoconoid in *P. pacifica* typically shows eight microtubules, though there seems to be additional microtubules running posteriorly towards the basal bodies (data not shown). This would suggest another parallel with *Perkinus marinus*, where some of pseudoconoid microtubules extend to the posterior [89], but to address this question, and the overall state of this interesting structure, a detailed three dimensional reconstruction of the apical complex would be required.

Interestingly, a recent ultrastructural investigation of *Amoebo-

phyra* sp. from *Akashiwo sanguinea* revealed the presence of elongated “electron-dense bodies” in the anterior part of the infectious flagellate cell [53] that seemingly used for host invasion, similar to the rhoptries and micronemes of the apicomplexan parasites, [90]. It will be interesting to investigate the apical complex of *Psammosa* and possibly “electron-dense bodies” of *Amoebo-

phyra* to the well-studied apicomplexans’ apical complex in detail, e.g. three dimensional architecture or molecular sequence level to test the possible homology and evolutionary link.

**Concluding Remarks**

In this study, we report a new lineage of early-diverging dinoflagellates, which we name *Psammosa*, with description of two new species of *P. pacifica* and *P. atlantica*. *Psammosa* displays a range of interesting morphological characteristics further supporting its divergence early in the evolution of dinoflagellates. Specifically, it retains a number of characters likely to be ancestral to the group, most importantly the apical complex, which is common among the apicomplexans, colpodellids and Perkinsids but never before observed in dinoflagellates. Based on its potential to illuminate the origin of a number of strange and unique characteristics that appeared early in the evolution of apicomplexans and dinoflagellates, further study of the *Psammosa* apical complex and its genomics and gene expression systems seem likely be of particular interest.

**Materials and Methods**

**Collection and Culture Conditions**

No specific permits were required for the described field studies. *Psammosa pacifica* was isolated from a sand sample collected at Boundary Bay, British Columbia, Canada (49.00863°N; -123.02281°W) on 15th April 2010. *Psammosa atlantica* was isolated from a sand sample collected at Blomidon Beach in the Bay of Fundy, Nova Scotia, Canada (45.25580°N; -64.34907°W) on 30th July 2008. Notably, both beaches are characterised by an extremely flat beach face due to the macrotidal range and to the very sheltered nature of the beach, respectively. The surface layer of wet sand was collected from the intertidal zone, samples vigorously shaken with K-Si medium [91], and suspensions incubated at 18°C under the cycle of Light:Dark = 6 h:18 h. Subsequently, single cells were isolated by micropipetting. *Psammosa pacifica* was incubated in modified K-Si medium with addition of 1 ml of 95% ethanol saturated with ubiquinone per 1000 ml, with a strain of bacterivorous streptomycete (Tofino-D66Ga) and *P. atlantica* was incubated with *Navicula* sp. (PRA-314, ATCC, VA) as a food source. Prey organisms were pre-cultured separately either in a polystyrene culture plate or a polystyrene culture flasks. *Psammosa* spp. were added whenever the prey cells were fully consumed in the previous inoculation. The *P. atlantica* strain was no longer viable in culture after 6 months.

**Microscopy**

Live cells were observed using differential interference contrast (DIC) microscopy using an Axioplan2 compound microscope (Zeiss, Germany) equipped with an XL H1s camcorder (Cannon, Japan) mounted using a PROHDVC adaptor (Micro Tech Lab, Austria) with an additional 6 mm ring.

Scanning electron microscopy (SEM) was carried out by fixing cell cultures of *P. pacifica* and *P. atlantica* in K-Si medium containing 2.5% Glutaraldehyde (final concentration) on coverslips coated with poly-L-lysine or polyethyleneimine at room temperature for 30 min. The cells were rinsed three times with distilled water, then dehydrated through a graded series of ethanol and critical point dried with CO2 using a Tousimis Samdri 795 CPD (Rockville, MD). Dried coverslips were mounted on aluminum stubs and then sputter coated with gold (5 nm thickness) using a Gressington high-resolution sputter coater (Gressington Scientific Instruments Ltd, Watford, UK). The coated cells were viewed under a Hitachi S4700 scanning electron microscope (Hitachi, Japan).

Whole-mount transmission electron microscopy (TEM) was performed using actively growing *Psammosa pacifica* culture isolated by micropipetting, fixed with 2% Uranyl acetate (final concentration) for 5 mins on a formvar coated mesh grid and rinsed with distilled water. The grid was viewed under a Hitachi H7600 transmission electron microscope (Hitachi, Japan).

Serial ultrathin and thin section TEM was also performed on actively growing *Psammosa pacifica* culture harvested by centrifugation at 1000 g for 15 minutes that was semi-simultaneously fixed [92] with 2.5% Gutarardehyde and with 0.01% Osmium tetroxide in sea water (final concentration, respectively) for 1 hour at room temperature. Cells were then rinsed once with distilled water, dehydrated through ethanol series, then embedded in SciPon resin in beam capsules. Serial ultrathin sections (50 nm thickness) were collected on Formvar-coated slot grids. Ultrathin sections were post stained with uranyl acetate for 15 minutes and lead citrate for 5 minutes [93]; then observed under a Hitachi H7600 electron microscope (Hitachi, Japan), and post processed on a Photoshop CS5 software (Adobe, CA).

**Molecular Methods and Phylogenetic Analyses**

100 cells of *P. pacifica* and *P. atlantica* were isolated using micropipette to avoid the contamination of the prey cells. DNA samples were prepared from the manually isolated cells using MasterPure™ Complete DNA & RNA Purification Kit (Epi-

centre Biotechnologies, WI). Small subunit (SSU) rRNA and Hsp90 genes were amplified by nested PCR using primers listed in table S1. PCR reactions were performed following Okamoto et al for SSU rRNA [94] and Kim et al for Hsp90 [95]. Although template DNA has a minimum contamination of the prey cells, sequences were determined after subcloning of PCR products to avoid the possible contamination. Sequences were deposited in GeneBank under accessions (SSU of *P. pacifica*: JN873311; *P. atlantica*: JN873310; Hsp90 of *P. pacifica*: JN873312).

Sequences of *Psammosa* were aligned with other alveolate sequences using arb-aligner (http://www.arb-silva.de/aligner/) for SSU rRNA and Mafft 6.86 [96,97] for Hsp90. Ambiguous parts of alignments were deleted using SeaView 4 [98]. Maximum likelihood topology was calculated using RAxML 7.2.8 [99] under the GTR (SSU rRNA) and LG (Hsp90) models of evolution. The phylogeny with highest likelihood score was chosen from 200 independent runs each starting with different starting topology. Non-parametric bootstrap support was estimated from 500 replicates. Bayesian posterior probabilities were calculated using Phylobayes 3 [100] under the CAT admixture model (limited to
40 rate categories) combined with GTR (SSU rRNA) and/or LG (Hsp90) exchange rates. Approximately unbiased topology test was performed using Consel [101].

Nomenclatural Acts

The electronic version of this document does not represent a published work according to the International Code of Zoological Nomenclature (ICZN), and hence the nomenclatural acts contained in the electronic version are not available under that Code from the electronic edition. Therefore, a separate print-only edition is available on request from PLoS by sending a request to PLoS ONE, Public Library of Science, 1160 Battery Street, Suite 100, San Francisco, CA 94111, USA along with a check for $10 (to cover printing and postage) payable to “Public Library of Science”.

In addition, this published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the ICZN. The ZooBank LSID (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub:D2BDEF96-E795-47508BB1-4D213DFC952A.

Supporting Information

Table S1  List of primers used in this study.  (DOC)

Movie S1  Movie clip showing Psammosa pacifica resting on a bottom surface.  (MP4)

Movie S2  Movie clip showing swimming behaviour of Psammosa pacifica at low magnification. Psammosa switches between two modes of swimming, namely, “swimming with rotation” and “spiral swimming without rotation”.  (MP4)

Movie S3  Movie clip showing Psammosa pacifica swimming at high magnification. The cell is before division, with posterior flagella duplicated.  (MP4)

Acknowledgments

We are thankful to Dr. Juan Saldarriaga for discussion and UBC Bioimaging Facility for technical assistance.

Author Contributions

Conceived and designed the experiments: NO PJK. Performed the experiments: NO. Analyzed the data: AH. Contributed reagents/materials/analysis tools: NO AH. Wrote the paper: NO AH PJK.

References

1. Cavalier-Smith T (2004) Only six kingdoms of life. P Roy Soc Lond B Bio 271: 1251–1262.
2. Leander BS, Keeling PJ (2003) Morphostasis in alveolate evolution. Trends in Ecol Evol 18: 393–402.
3. Moore RB, Obornik M, Janoutové J, Chudimuký T, Vancová M, et al. (2008) A photosynthetic alveolate closely related to apicomplexan parasites. Nature 451: 959–963.
4. Oborník M, Janoutové J, Chudimuký T, Lukáˇc J (2009) Evolution of the apicoplast and its hosts: From heterotrophy to autotrophy and back again. Int J Parasitol 39: 1–12.
5. Janoutové J, Horák A, Oborník M, Lukáˇces J, Keeling PJ (2010) A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. PNAS 107: 10949–10954.
6. Botta CZ, Yamaryo-Botteé Z, Janoutové J, Keeling PJ, et al. (2011) Identification of Plant-like Galactooligos in Chromera velia, A Photosynthetic Relative of Malaria Parasites. J Biol Chem 286: 29893–29903.
7. Taylor MJR, Hoppenrath M, Saldarriaga JP (2006) Dinoflagellate diversity and distribution. Biodivers Conserv 17: 407–418.
8. Liu S, Zhang H, Spencer DF, Norman JE, Gray MW (2002) Widespread and extensive editing of mitochondrial mRNAs in dinoflagellates. J Mol Biol 320:727–39.
9. Jackson CJ, Norman JE, Schnare MN, Gray MW, Keeling PJ, et al. (2007) Broad genomic and transcriptional analysis reveals a highly derived genome in dinoflagellate mitochondria. BMC Biol 5: 41.
10. Stanovits CH, Saldarriaga JP, Laroche A, Keeling PJ (2007) The highly reduced and fragmented mitochondrial genome of the early-branching dinoflagellate Oxyrrhis marina shares characteristics with both apicomplexans and dinoflagellate mitochondrial genomes. J Mol Biol 372: 356–368.
11. Waller RF, Jackson CJ (2009) Dinoflagellate mitochondrial genomes: stretching the rules of molecular biology. Bioscience 51: 237–245.
12. Zhang ZD, Green BR, Cavalier-Smith T (1999) Single gene circles in dinoflagellate chloroplast genomes. Nature 400: 155–9.
13. Green BR (2004) The chloroplast genome of dinoflagellates – a reduced instruction set? Protop 155: 23–31.
14. Liu S (2011) Genomic understanding of dinoflagellates. Res Microbiol 162: 551–569.
15. Zhang H, Hou Y, Miranda L, Campbell DA, Sturh NR, et al. (2007) Spliced leader RNA trans-splicing in dinoflagellate. PNAS 104: 4618–4623.

16. Leite KB, van Dolah FM (2007) Spliced leader RNA-mediated trans-splicing in a dinoflagellate, Karentia brevis. J Euk Microbiol 54: 427–435.
17. Zhang H, Campbell DA, Sturh NR, Lin S (2009) Dinoflagellate spliced leader RNA genes display a variety of sequences and genomic arrangements. Mol Biol Evol 26: 1757–1771.
18. Stoeck T, Zuerdorf A, Breiner H-W, Behnke A (2007) A molecular approach to identify active microbes in environmental eukaryote clone libraries. Microbial Ecol 53: 328–339.
19. Massana R, Kamili B, Pommier T, Bodaker I, Beja O (2008) Metagenomic retrieval of a ribosomal DNA repeat array from an uncultured marine alveolate. Environ Microbiol 10: 1335–1343.
20. Alexander E, Stock A, Breiner H-W, Behnke A, Bunge J, et al. (2009) Microbial eukaryotes in the hypersaline anoxic L’Atalante deep-sea basin. Microbial Ecol 55: 328–339.
21. Lopez-Garcia P, Rodriguez-Valera F, Pedroso-Alco G, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409: 603–607.
22. Stoeck T, Epstein SS (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. Appl Environ Microbiol 69: 2657–2663.
23. Stoeck T, Taylor G, Epstein SS (2003) Novel eukaryotes from the permanently anoxic Carasco Basin (Caribbean sea). Appl Environ Microbiol 69: 5656–5663.
24. Grosiškis A, Massana R, Valentínt V, Vaulot D, Guillou L (2006) Genetic diversity and habitats of two enigmatic marine alveolate lineages. Aquat Microb Ecol 42: 277–291.
25. Lovejoy C, Massana R, Pedroso-Alco G (2006) Diversity and distribution of marine microbial eukaryotes in the Arctic Ocean and adjacent seas. Appl Environ Microbiol 72: 3085–3093.
26. Lopez-Garcia P, Vereshchaka A, Moreira D (2007) Eukaryotic diversity associated with carbonates and fluid-seawater interface in Lost City hydrothermal field. Environ Microbiol 9: 546–554.
27. Cavelier ML, Ortiz A, Kim E, Moehlig H, Richardson DE, et al. (2008) Widespread distribution of a unique marine protistan lineage. Environ Microbiol 10: 1621–1634.
28. Massana R, Pedroso-Alco G (2008) Unveiling new microbial eukaryotes in the surface ocean. Curr Opin Microbiol 11: 213–218.
29. Brown MV, Philip GK, Bunge JA, Smith MC, Bissett A, et al. (2009) Microbial community structure in the North Pacific ocean. ISME J 3: 1574–1586.
30. Stocke T, Behnke A, Christen R, Amaral-Zettler L, Rodriguez-More MJ, et al. (2009) Massively parallel tag sequencing reveals the complexity of anoxic marine prokaryote communities. BMC Biol 7: 72.

31. Stern RF, Horak A, Andrew RL, Collefroth MA, Andersen RA, et al. (2010) Environmental barcoding reveals massive dinoflagellate diversity in marine environments. PLoS ONE 5: e13991.

32. Silberman J, Collins A, Gershwin L, Johnson P, Roger A (2004) Eubiotopsis of the genus Thalassosoma are alveolates. J Eukaryot Microbiol 51: 246–252.

33. Skovgaard A, Massana R, Balague V, Saiz E (2005) Phylogenetic position of the copepod-infecting parasitoid tentatively assigned to Duboscquella. Protist. 156: 413–423.

34. Harada A, Ohtsuka S, Horikuchi T (2007) Species of the parasitic genus Duboscquella are members of the enigmatic Marine Alveolate Group I. Protist. 158: 347–357.

35. Gómez F, Lopez-Garcia P, Nowaczyk A, Moreira D (2009) The crustacean parasites Eubiotopus Caudaull, 1910 and Thalassosoma Niezabitowski, 1913 form a monophyletic divergent clade within the Alveolata. Syst Parasitol 74: 65–74.

36. Skovgaard A, Milne J, Angelico MM (2006) Identifying the lethal fish egg parasite Ichthyodinium vorax as a member of Marine Alveolate Group I. Environ Microbiol 11: 2030–2041.

37. Cachon J, Cachon M (1987) Parasitic dinoflagellates. In: Taylor F, editor. The Biodiversity of Dinoflagellates. Oxford, UK: Blackwell Scientific Publications. 571-610.

38. Janson S, Gisselson L, Salomon P, Graneli E (2000) Evidence for multiple species within the endoparasitic dinoflagellate Amoebophrya aurea as inferred from hsp90 and actin phylogenies. J Phycol 36: 610.

39. Skovgaard A, Meneses I, Angelico MM (2009) Identifying the lethal fish egg parasite Ichthyodinium chabelardi as a member of Marine Alveolate Group I. Environ Microbiol 11: 65–74.

40. Janssen PH (2009) Dormant microbes: scouting ahead or plodding along? FEMS Microbiol Rev 33: 627–635.

41. Janssen PH (2009) Dormant microbes: scouting ahead or plodding along? FEMS Microbiol Rev 33: 627–635.
90. Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23: 633–638.
91. Tippit DH, Pickett-Heaps JD (1977) Mitosis in the pennate diatom *Santella ovalis*. J Cell Biol 73: 705–727.
92. Reynolds ES (1963) Use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212.
93. Okamoto N, Chantangsi C, Horák A, Leander BS, Keeling PJ (2009) Molecular phylogeny and description of the novel katatablepharid *Roombia truncata* gen. et sp. nov., and establishment of the Hacrobia taxon nov. PLoS ONE. pp. e7080.
94. Kim E, Simpson AGB, Graham LE (2006) Evolutionary relationships of apusomonads inferred from taxon-rich analyses of 6 nuclear encoded genes. Mol Biol Evol 23: 2455–2466.
95. Katoh T (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. BMC Bioinform 9: 212.
96. Katoh T (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9: 286–298.
97. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27: 221–224.
98. Stamatakis A (2006) RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic Analyses with Thousands of Taxa and Mixed Models. Bioinformatics 22(21):2688–2690.
99. Lartillot N, Lepage T, Blanquart S (2009) PhyloBayes 3, a Bayesian software package for phylogenetic reconstruction and molecular dating. Bioinform 25: 2286–2288.
100. Shimodaira H, Hasegawa M (2001) CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17, 1246–1247.