

Single cell genomics of uncultured Marine Alveolates (MALVs) shows paraphyly of basal dinoflagellates

Running Title: Paraphyly of Marine Alveolates

Jürgen F. H. Strassert^{1*}, Anna Karnkowska^{1**}, Elisabeth Hehenberger¹, Javier del Campo¹, Martin Kolisko^{1,2}, Noriko Okamoto¹, Fabien Burki^{1*}, Jan Janouškovec^{1***}, Camille Poirier³, Guy Leonard⁴, Steven J. Hallam⁵, Thomas A. Richards⁴, Alexandra Z. Worden³, Alyson E. Santoro⁶, Patrick J. Keeling¹

¹ Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada

² Institute of Parasitology, Biology Centre CAS, 37005 České Budějovice, Czech Republic

³ Monterey Bay Aquarium Research Institute, Moss Landing, California 95039, USA

⁴ Biosciences, University of Exeter, Geoffrey Pope Building, Exeter, EX4 4QD, UK

⁵ Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

⁶ Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, California 93106, USA

Correspondence: JFH Strassert or PJ Keeling, Department of Botany, University of British Columbia, 3529–6270 University Boulevard, Vancouver, BC, V6T 1Z4, Canada

E-mail: strassert@protist.eu or pkeeling@mail.ubc.ca

* Current address: Science for Life Laboratory, Program in Systematic Biology, Department of Organismal Biology, Uppsala University

** Current address: Department of Molecular Phylogenetics and Evolution, Faculty of Biology, Biological and Chemical Research Centre, University of Warsaw

*** Current address: Department of Genetics, Evolution and Environment, University College London

The authors declare no conflict of interest.

Abstract

Marine Alveolates (MALVs) are diverse and widespread early-branching dinoflagellates, but most knowledge of the group comes from a few cultured species that are generally not abundant in natural samples, or from diversity analyses of PCR-based environmental SSU rRNA gene sequences. To more broadly examine MALV genomes, we generated single-cell genome surveys from seven individually isolated cells. Genes expected of heterotrophic eukaryotes were found, with interesting exceptions like presence of proteorhodopsin and vacuolar H⁺-pyrophosphatase. Phylogenetic analysis of concatenated SSU and LSU rRNA gene sequences provided strong support for the paraphyly of MALV lineages. Dinoflagellate viral nucleoproteins (DVNPs) were found only in MALV groups that branched as sister to dinokaryotes. Our findings indicate that multiple independent origins of several characteristics early in dinoflagellate evolution, such as a parasitic life style, underlie the environmental diversity of MALVs, and suggest they have more varied trophic modes than previously thought.

Introduction

The Marine Alveolate (MALV) lineages were discovered in the first large molecular surveys of SSU (18S) rRNA from marine eukaryotes (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001). Subsequent environmental SSU PCR-based surveys have consistently shown them to be diverse and widespread, often representing up to 50% of eukaryotic sequences (Chambouvet *et al.*, 2008). SSU rRNA phylogeny has also shown that several “syndinian” dinoflagellates branch with MALVs; these species have been investigated directly, and are all parasites infecting a broad range of host organisms such as cnidarians, fish, crustaceans, and protists (Guillou *et al.*, 2010). Because of this, MALVs are generally assumed to be parasites, and by extension that their abundance in small size fractions may reflect large numbers of infectious cells (perhaps blooms following host blooms), which are thought to have ecological importance in controlling host populations. This is reinforced by recent surveys that show MALVs make up approximately 90% of putative parasites in the piconanoplankton globally (de

Vargas *et al.*, 2015). However, most MALVs are only known from these environmental SSU rRNA gene sequences, and those that have been characterized further are relatively rare and do not represent all MALV subgroups. Since only SSU rRNA is generally known, the phylogenetic position of MALVs is also partially uncertain: they are typically basal to dinokaryotes (i.e., dinoflagellates with permanently condensed chromosomes), but may be monophyletic or paraphyletic with dinokaryotes branching among them, and in either case the phylogenies lack support (e.g., Guillou *et al.*, 2008; Skovgaard *et al.*, 2009; Horiguchi, 2015). Overall, most MALV diversity is unexamined and how MALV groups are related to one another and to dinokaryotes remains poorly resolved.

Results and Discussion

Using manual single-cell isolation (or isolation of infected hosts) and Fluorescence-Activated Cell Sorting (FACS), we surveyed heterotrophic eukaryotes and identified cells corresponding to MALVs by SSU rRNA phylogeny, resulting in seven distantly related species from the Northeastern Subarctic Pacific Ocean (Line P) and from Monterey Bay out to the edge of the North Pacific Subtropical Gyre (Line 67; for collection and screening details, see Supplementary Table S1 and Supplementary Material and Methods). Two manual isolations were morphologically identifiable hosts (Figure 1); a copepod (L67-3) in the process of bursting to release spores identified as the dinoflagellate *Chytriodinium*, and a tintinnid ciliate (L67-6) that yielded two distinct MALV phylotypes (93.8% similarity, likely from two different MALV II species). Manually isolated cells L67-1 and L67-4 are inferred to be host-free and fit within size fractions that previously yielded MALV sequences: L67-1 is spherical-shaped with a diameter of 13.8 μm and with greenish inclusions, L67-4 is colourless, spindle-shaped, and 14.5 \times 6.3 μm (Figure 1). One isolation (L67-5) was large and inferred to be a host, which could not be identified, and the remaining two cells (L67-2 and LP-1) were isolated by FACS.

Genomic data from each isolation was generated, assembled, and screened for contamination (e.g. from bacteria or host genomes) using a combination of BLAST-based and phylogenetic

approaches (i.e., phylogenetic trees were inferred from gene alignments of a broad range of taxa including the MALVs *Hematodinium* and *Amoebophrya*; for details see Suppl. Methods). Because dinoflagellate genomes are large and gene-sparse (Wisecaver and Hackett, 2011), we expected to recover partial genomes and focused only on regions encoding a recognizable gene, to avoid including non-coding regions from contaminants. This resulted in 0.5 to 3.1 Mb per cell confidently assigned to MALVs, encoding between 97 to 917 predicted genes, 30% to 60% of which corresponded to KEGG hits (Figure 1; Supplementary Tables S2, S3; Supplementary Figure S1). Estimated coverage of eukaryotic conserved single copy genes using BUSCO showed 86% to 99% were missing. The genomic coverage was therefore low, as expected, but an improvement over only SSU rRNA.

The proteins that were identified matched the expectations for heterotrophic eukaryotes since no plastid-related protein was found, as observed in *Hematodinium* (Gornik *et al.*, 2015), but mitochondrial proteins were detected. Interestingly, L67-5 encoded a photoactive proton pump, proteorhodopsin, most similar to homologues from *Oxyrrhis* and *Alexandrium*, and five samples encoded a vacuolar H⁺-pyrophosphatase (L67-1–L67-3, L67-5, LP-1). These two proteins have been hypothesized to function together in dinoflagellates to generate energy in the form of pyrophosphate (Slamovits *et al.*, 2011). The presence of light-driven proton pumps in MALVs suggests an early origin of this system in the dinoflagellate lineage, and that some MALVs may be able to use light for energy (despite the absence of plastids), as this is the suspected function of these proteins in other non-photosynthetic dinoflagellates.

Assemblies from all seven cells include the rRNA operon, generally complete. Mapping *Tara* Oceans amplicon data to their SSU V9 region demonstrated that two cell types were moderately abundant (>27,000 and >77,000 reads for L67-2 and L67-4, respectively; Figure 1) and widespread in pico- and nano-plankton size fractions (Figure 2A, B). This distribution is beyond *Tara*'s sampling representation bias (i.e., mostly pico- and nano-plankton were sampled; Figure 2C), so if MALVs are parasites, then they predominantly exist as free-living spores rather than host-associated (consistent

with the image of L67-4; Figure 1). Four other phylotypes were rare (<5 reads; Figure 1) in the *Tara* Oceans data. Notably, however, North Pacific data from *Tara* are unavailable, so these four phylotypes may be endemic to the North Pacific (e.g., parasites of North Pacific hosts).

Originally, MALVs were divided into two major groups: MALV I including *Ichthyodinium* and *Duboscquella* (Harada *et al.*, 2007; Skovgaard *et al.*, 2009), and MALV II, including *Syndinium*, *Hematodinium* and *Amoebophrya* (Skovgaard *et al.*, 2005). Both groups were subsequently subdivided further, but the relationships between subgroups remained uncertain (Guillou *et al.*, 2008; Skovgaard *et al.*, 2009; Horiguchi, 2015; see also Supplementary Figure S2). The concatenated SSU/LSU tree (Figure 1) recovers four strongly-supported subgroups: MALV Ia, Ib, II, and IV (following the names of Guillou *et al.* 2008, but distinguishing two phylogenetically distant subgroups of MALV I). Most interestingly MALVs are paraphyletic, with MALV II/IV branching as sister to dinokaryotes (“core dinoflagellates”). Currently, no synapomorphy for a dinokaryote-MALV-II/IV group is known, but one possibility worth investigation is the presence of DVNPs (dinoflagellate viral nucleoproteins). Within MALVs, DVNPs are found in *Hematodinium* (MALV IV), *Amoebophrya*, L67-3, and LP-1 (MALV II; Gornik *et al.*, 2012; Marinov and Lynch, 2015; Supplementary Table S3), but so far, no evidence for their presence in MALV I has been obtained.

The phylogeny raises interesting questions about the distribution of parasitism in early alveolate evolution. In particular, the paraphyletic relationship of seemingly aplastidal parasites at the base of dinoflagellates could suggest a parasitic ancestral state. However, this scenario leaves unexplained many specific shared similarities in the plastids of dinokaryotes, apicomplexans, and chrompodellids (Janouškovec *et al.*, 2015), and is inconsistent with the apparent ancestral function of the apical complex as a feeding apparatus (Okamoto and Keeling, 2014a, b). Alternatively, a mixotrophic ancestry that included both the plastid and the apical complex for feeding is possible; in this case, one must explain an apparently strong predilection to losing photosynthesis and transitioning to parasitism by modifying the feeding apparatus. A recent analysis of metabolic redundancies within the

apicomplexan/dinoflagellate clade also argued for a recent plastid gain, before the divergence of these groups but after they diverged from ciliates (Waller *et al.*, 2015), which is also consistent with this view.

Despite their abundance, distribution, and inferred ecological significance, MALVs remain mysterious. They are assumed to be parasites, and the microscopic and genomic data described here are consistent with this, and in some cases identify new hosts. Their exact evolutionary relationship to dinokaryotes has also been controversial (e.g., Guillou *et al.*, 2008; Massana *et al.*, 2008; Horiguchi, 2015), and our data provide strong support for their paraphyly. These insights impact how we reconstruct the evolution of plastids and parasitism in MALVs and dinoflagellates. Finally, surprising aspects of gene content and potential endemism, reveal possible complexities in trophic modes and ecology that serve as a springboard for future investigations.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

We thank the captain and crew of the R/V *Western Flyer*, M. Blum, F. Chavez, V. Jimenez, J. T. Pennington, J. M. Smith, S. Sudek, J. Swalwell, C. Wahl, and S. Wilken, for logistical assistance prior to and during the cruises. This work was supported by a grant from the Gordon and Betty Moore Foundation (GBMF3307) to P.J.K., T.A.R., A.Z.W., and A.E.S. Sequencing for L67-2 was performed through a JGI Technology Development Program grant to T.A.R., A.Z.W., P.J.K., and A.E.S. Ship time and sorting methods were supported by a grant from the David and Lucile Packard Foundation through MBARI and GBMF3788 to A.Z.W. J.dC. was supported by a Marie Curie International Outgoing Fellowship grant (FP7-PEOPLE-2012-IOF – 331450 CAARL), and A.K., N.O., M.K., and

J.dC. were supported by a grant from the Tula Foundation to the Centre for Microbial Biodiversity and Evolution at UBC.

Data Accessibility

Raw reads have been submitted to GenBank under accession numbers SRR5145189 and SRR5177625–SRR5177630, and the assembled contigs can be accessed via the Dryad Digital Repository ###.

Supplementary information is available at ISME Journal's website.

References

- Chambouvet A, Morin P, Marie D, Guillou L. (2008). Control of toxic marine dinoflagellate blooms by serial parasitic killers. *Science* **322**: 1254–1257.
- Gornik SG, Febrimarsa, Cassin AM, MacRae JI, Ramaprasad A, Rchiad Z, *et al.* (2015). Endosymbiosis undone by stepwise elimination of the plastid in a parasitic dinoflagellate. *Proc Natl Acad Sci U S A* **112**: 5767–5772.
- Gornik SG, Ford KL, Mulhern TD, Bacic A, McFadden GI, Waller RF. (2012). Loss of nucleosomal DNA condensation coincides with appearance of a novel nuclear protein in dinoflagellates. *Curr Biol* **22**: 2303–2312.
- Guillou L, Alves-de-Souza C, Siano R, González H. (2010). The ecological significance of small, eukaryotic parasites in marine ecosystems. *Microbiol Today* **37**: 92–95.
- Guillou L, Viprey M, Chambouvet A, Welsh RM, Kirkham AR, Massana R, *et al.* (2008). Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). *Environ Microbiol* **10**: 3349–3365.
- Harada A, Ohtsuka S, Horiguchi T. (2007). Species of the parasitic genus *Duboscquella* are members of the enigmatic Marine Alveolate Group I. *Protist* **158**: 337–347.

- Horiguchi T. (2015). Diversity and phylogeny of marine parasitic dinoflagellates. In: Ohtsuka S, Suzuki T, Horiguchi T, Suzuki N, Not F (eds). *Marine Protists: Diversity and Dynamics*. Springer Japan: Tokyo, pp 397–419.
- Janouškovec J, Tikhonenkov DV, Burki F, Howe AT, Kolísko M, Mylnikov AP, *et al.* (2015). Factors mediating plastid dependency and the origins of parasitism in apicomplexans and their close relatives. *Proc Natl Acad Sci U S A* **112**: 10200–10207.
- López-García P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D. (2001). Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* **409**: 603–607.
- Marinov GK, Lynch M. (2015). Diversity and divergence of dinoflagellate histone proteins. *G3 (Bethesda)* **6**: 397–422.
- Massana R, Karniol 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.
- Moon-van der Staay SY, de Wachter R, Vaultot D. (2001). Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**: 607–610.
- Okamoto N, Keeling PJ. (2014a). The 3D structure of the apical complex and association with the flagellar apparatus revealed by serial TEM tomography in *Psammosa pacifica*, a distant relative of the apicomplexa. *PLoS One* **9**. e-pub ahead of print, doi: 10.1371/journal.pone.0084653.
- Okamoto, N, Keeling, PJ. (2014b). A comparative overview of the flagellar apparatus of dinoflagellates, perkinsids, and colpodellids. *Microorganisms* **2**: 73–91.
- Skovgaard A, Massana R, Balagué V, Saiz E. (2005). Phylogenetic position of the copepod-infesting parasite *Syndinium turbo* (Dinoflagellata, Syndinea). *Protist* **156**: 413–23.
- 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**: 2030–2041.
- Slamovits CH, Okamoto N, Burri L, James ER, Keeling PJ. (2011). A bacterial proteorhodopsin proton pump in marine eukaryotes. *Nat Commun* **2**: 183.

de Vargas C, Audic S, Henry N, Decelle J, Mahe F, Logares R, *et al.* (2015). Ocean plankton.

Eukaryotic plankton diversity in the sunlit ocean. *Science* **348**: 1261605.

Waller RF, Gornik SG, Koreny L, Pain A. (2015). Metabolic pathway redundancy within the apicomplexan-dinoflagellate radiation argues against an ancient chromalveolate plastid. *Commun Integr Biol* **9**: e1116653.

Wisecaver JH, Hackett JD. (2011). Dinoflagellate genome evolution. *Annu Rev Microbiol* **65**: 369–387.

Figure Legends

Figure 1. Morphology and phylogenetic relationships of Marine Alveolates (MALVs).

Tree topology is based on maximum likelihood analysis of concatenated SSU and LSU rRNA gene sequences (>3,900 nucleotide positions; 7.1% gaps). Node support is shown by RAxML bootstraps (non-parametric) and MrBayes posterior probabilities. Black circles indicate a maximum support in both analyses. Sequences obtained in this study are printed in bold. Numbers in polygons refer to the number of grouped taxa, roman numerals signify the different MALV groups (compare with Guillou *et al.*, 2008), and the arrows indicate parasitic clades. Removal/addition of the long-branching sequence (dotted line) did not change the tree topology. Micrographs show the organisms (including hosts) whose genomic DNA has been sequenced (not available for FACS-derived samples LP-1 and L67-2). L67-3 represents a disrupted copepod (identified by the extremities) with an attached cyst containing spores of the parasitic dinoflagellate *Chytriodinium* (*Ch*). The copepod was isolated after the cyst burst and released the spores, but it is not clear if the MALV is associated with the animal or the dinoflagellate. The micrograph of L67-6 shows a tintinnid ciliate. Since two non-related MALV II 18S rRNA gene sequences (with 93.8% similarity; Supplementary Figure S2) and one 28S rRNA gene sequence were obtained from three different contigs of this sample, its phylogenetic position is not shown in the SSU/LSU rRNA gene tree. L67-1 and L67-4 are assumed to represent host-free MALV cells. L67-4 is represented by two phlotypes with a SSU and a LSU sequence similarity of 97.9% and 98.6%, respectively. Length of the cleaned assembly, number of redundant/non-redundant KO homologs, number of hits to *Tara* Oceans V9 data (not available for L67-6, for which the V9 region was not sampled), and collection depth are presented beside each photo.

Figure 2. Distribution of Marine Alveolates (MALVs) in SSU rRNA amplicon data.

(A) Geographical distribution of the two most abundant cells, L67-4 and L67-2, based on *Tara* Oceans SSU V9 data using a sequence similarity cutoff of 98%. Dot sizes are proportional to the total number of reads in each location for the two phylotypes. (B) Size fraction, depth, and temperature distributions of L67-4 and L67-2. The abundances are based on normalized numbers of *Tara* Oceans V9 reads. (C) Percentages of all *Tara* samples obtained from different size fractions, depths, and water temperatures. N/A – information on size or temperature was not available; polar: <10 °C, temperate: 10–19 °C, tropical: >19 °C.

Figure S1. Venn diagram showing the proportion of shared KEGGs (i.e., genes with same KEGG annotation) among the Marine Alveolates (MALVs).

The relationships are shown for MALVs belonging to the same clade (MALV Ia, Ib, or II) and between each of those clades. K10408 (DNAH; dynein heavy chain, axonemal) could be found in all samples whereas K00933 (creatine kinase) could be found in all three groups but not in all samples (L67-1, L67-4, and L67-6). For details, see Supplementary Table S3).

Figure S2. Maximum likelihood tree of SSU rRNA gene sequences illustrating the relationships between Marine Alveolates (MALVs).

Tree topology is based on 1,441 sequences, >1,750 nucleotide positions; 3.4% gaps. Sequences obtained in this study are highlighted and environmental sequences are in grey. Node support is shown by RAxML standard bootstraps ($\geq 50\%$). Numbers in polygons refer to the number of grouped taxa. The SILVA taxonomy is shown to indicate discrepancies between the taxonomic classification and the non-monophyletic clustering of the MALV groups. In SILVA MALVs are equated to Syndiniales based on SSU rRNA (see Guillou *et al.*, 2008).