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Report of the thesis of Binnypreet Kaur 'Genetic modifications and functional analyses of Diplonemids'

The thesis of Binnypreet Kaur concerns a poorly studied group of protists known as Diplonemids that are now appreciated as the most diverse group of eukaryotes in the ocean, being particularly abundant in the deep ocean. The topic is therefore important and the thesis is expected to reveal novel and pioneering information. Overall, I was very impressed with the work, which presents the results of five years of study. The quality of the reported studies is amply evidenced by it being the subject of several original research articles and reviews that have already been published, as detailed below. The candidate is first author on three of these papers that have been published in good quality journals, and is mid author on others in which she has participated, including two reviews. One other paper is in preparation. Overall the written English is of high quality and the content of the thesis is clearly presented.

The introduction presents an overview of Diplonemid diversity and cell ultrastructure, as well as discussing their free-living vs commensal vs parasitic lifestyles, and their preferences for abiotic factors (temperature, light, pressure, and oxygen concentration). A typical feature is phagotrophy, as evidenced by the large apparent vacuole within Diplonemid cells, which are additionally characterized by a single highly reticulated peripheral mitochondrion with lamellar cristae. Knowledge of Diplonemid biology is limited by the lack of species in culture and of any genome sequences. Although no nuclear genomes have yet been reported, their mitochondrial genomes have been studied extensively by others prior to this thesis work, and they are known to display

extensive post-transcriptional processes such as trans-splicing and RNA editing. The introduction is well referenced but is quite poor in figures. It should include a figure to summarize data about Diplonemid distributions and diversity from *Tara* Oceans data as well as additional figures presenting more details about the extensive RNA editing that has been described in these unusual organisms. Furthermore, the Figure 1 legend is not accurate, and the source of the information is not indicated in detail, in particular with respect to the unconventional TSAR nomenclature which should be explained.

In the first results chapter of the thesis, the discovery of five new Diplonemid species and three new genera from surface marine water samples is described. Axenic cultures of these strains were also established, allowing their life cycles under cultivation conditions to be described by light and electron microscopy. The chapter also describes phylogenetic analysis based on 18S rRNA gene sequences. Binnypreet Kaur is joint first auther with three others on the paper which was published in Protist describing this work, with a total of twelve authors.

The second results chapter of the thesis describes the successful genetic transformation of *Diplonema papillatum*, the first documented case of transformation in a euglenozoan protist outside the well-studied kinetoplastids. The method utilizes electroporation to introduce foreign genes, using puromycin as a selectable marker. Nuclear transformation is demonstrated by Southern blotting, and expression/protein translation are confirmed by qPCR and western blotting, respectively. Although the fluorescent marker mCherry was inserted as a foreign gene, fluoresecence was however not observed in the transformed cells. Notwithstanding, these first results are highly encouraging and further indicate that homologous recombination may be exploitable in future attempts of Diplonemid gene manipulation, which is explored later in the thesis. The candidate is first out of eight authors on the article describing the work, which was published in Environmental Microbiology. This work can be considered a breakthrough for Diplonemids, although it is somewhat disconcerting that the species transformed here is unconventional in terms of its mitochondrial ultrastructure and its extremely complex mitochondrial genome. I wonder what are the implications of this for using *D. papillatum* as a model for the entire group.

Two other results chapters deal with mitochondrial genome structure and RNA editing in diverse Diplonemid species. RNA editing is seen to be pervasive and to be extremely prominent. One of these chapters is presented in the form of a review, published as a book chapter, whereas the second is an original research article published in Nucleic Acids Research. Binnypreet Kaur is joint first author with two others and a total of seven authors. In this paper, a comparative study of mitochondrial genome architecture, gene structure and post-transcriptional processing in six recently isolated, phylogenetically diverse Diplonemid species is reported. They are seen to display such high complexity that they challenge all other currently known systems.

A further chapter is in the form of a paper currently in press in Nature Methods. This is an extensive paper describing a wide range of new model systems for using reverse genetics to explore microbial eukaryotes and the candidate is one of multiple authors. Although some results relate to Diplonemids, the specific contribution is not noted and so this should be clarified, or the chapter should be removed.

The final results chapter presents quite extensive results showing the deployment of homologous recombination to manipulate Diplonemid nuclear genes. The results appear convincing and I was encouraged to finally see fluorescence from the mCherry reporter protein. The chapter appears to

be mature for formal publication and I expect that the results will be of interest to a wide research community.

My overall impression of the thesis of Binnypreet Kaur is very positive and I congratulate the candidate for these excellent studies that have extended significantly our knowledge of Diplonemids. In addition to the minor comments indicated above, I would like to see more explicit descriptions of the role of the candidate in each of the studies, and I look forward to the formal presentation of the thesis on Feb 24.

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Review report on PhD thesis entitled

'GENETIC MODIFICATIONS AND FUNCTIONAL ANALYSES OF DIPLONEMIDS'

by

Binnypreet Kaur

Binnypreet Kaur submitted a PhD thesis which represents a complex insight into the diversity and genomics of diplonemids, one of yet poorly known but ecologically highly important group of protists.

The introduction is well-written, summarizing the background information related to the diversity, evolution, and mitochondrial genome architecture of diplonemids, as well as transformation methods to introduce foreign gene constructs into unrelated organisms. The main body of the work consists of five journal articles or manuscripts and one book chapter, in two of them B. Kaur is the first author. Me personally, I highly value the paper describing a unique gene fragmentation and RNA editing of diplonemid mitochondrial genome, published in Nucleic Acid Research. All contributions are of a very high scientific standard and many aspects of the thesis are novel and exciting.

In general, I consider this thesis as a great piece of work, and I have no significant reservations to any of its parts. The introduction is well organized, carefully prepared, and properly accompanied by illustrations. In conclusion, this thesis is of excellent quality and highly deserving to be awarded a PhD title.

Questions for the PhD candidate:

1. I am wondering why diplonemids were overlooked for such a long time, despite their high abundance, ecological significance, and quite large cell sizes enabling their morphological observation.
2. Diplonemids are considered as the 3rd most diverse group of oceanic eukaryotes, even morphologically highly diverse dinoflagellates. However, diplonemid phenotypic diversity is strikingly low. What if we are confused by mere focusing on molecular data when estimating their species diversity? High genetic diversity revealed by the TARA Oceans expedition may be simply caused by the high mutation rate in the V9 18S rDNA, leading to the accumulation of neutral mutations within a few coherent species units.
3. The modular structure of mitochondrial genes and RNA editing is very well characterized and understood. However, in the introductory part I miss any explanation why such complex and energetically costly processes evolved. A neutral evolutionary ratchet is probably the most parsimony explanation, however, are there any alternative explanations? Are Hemistasiidae, showing an obscuring level of gene fragmentation, specific in any way?

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