PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL OUTER MEMBRANE ß-BARREL CHANNELS

Westfälische Wilhelms-Universität Münster

Marcin Jąkalski and Wojciech Makałowski

Institute of Bioinformatics, Faculty of Medicine, University of Muenster, Muenster, Germany

Introduction

The biogenesis and proper function of mitochondria require an import of 90-99% of mitochondrial proteins as well as a metabolite exchange with cytoplasm. These transport processes are initiated by proteins from the mitochondrial outer membrane that display channel activity, namely **VDAC**, **Tom40**, and **Sam50/Tob55** (Figure 1). VDAC (voltage-dependent anion-selective channel), known also as mitochondrial porin, supports the flux of metabolites. Sam50/Tob55 is a crucial component of the SAM/TOB complex (sorting and assembly machinery/topogenesis of the mitochondrial outer membrane ß-barrel proteins), essential for insertion of ß-barrel integral proteins into the mitochondrial outer membrane. Tom40 is a core component of the TOM complex (translocase of the mitochondrial outer membrane) that is regarded as a general entry gate for mitochondria and as the complex responsible for decoding of targeting signals, translocation of imported proteins across or into the outer membrane, and their subsequent sorting.

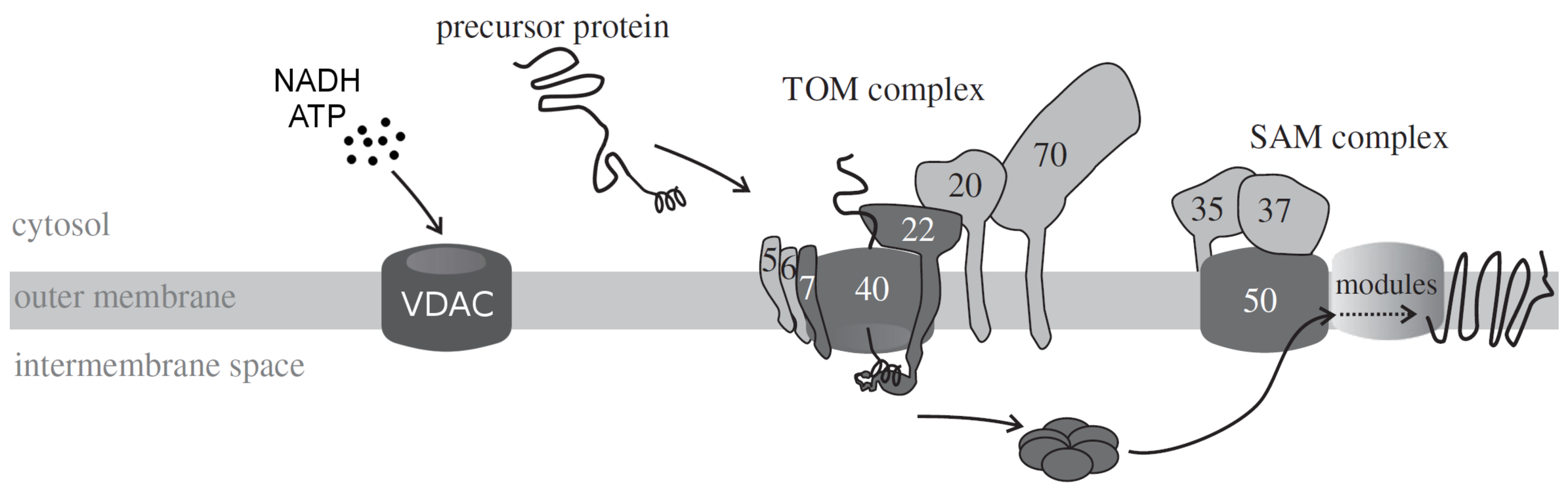

Fig. 1: Mitochondrial outer membrane import machinery (model from yeast). Protein substrates translated on cytoplasmic ribosomes are targeted to the TOM complex and subsequently sorted and directed to the outer membrane, inner membrane or matrix. Ions and small molecules like ATP and NADH cross mitochondrial outer membrane through the VDAC channel.

VDAC, Tom40, and Sam50/Tob55 are predicted to have a ß-barrel topology that is characteristic for proteins present in outer membranes of Gram-negative bacteria and in the organelles of endosymbiotic origin (*i.e.* mitochondria and chloroplasts), so their presence in the organellar outer membrane reflects the evolutionary origin of mitochondria and chloroplasts from endosymbiotic bacteria. In this study we focused on the amoeba *Acanthamoeba castellanii*'s and the slime mold *Dictyostelium discoideum*'s outer membrane ß-barrels. Till now only Tom40 and VDAC of D. discoideum have been identified.The aim of this study was to identify Sam50/Tob55 of *D. discoideum*, as well as VDAC, Tom40, and Sam50/Tob55 of *A. castellanii* and use them to perform comprehensive analysis of the evolutionary history of mitochondrial outer membrane channels of a predicted ß-barrel topology. Both microorganism are currently classified as amoebozoans - a sister group of fungi and Metazoa, which diverged from the animal/fungal line after its split from plants. They present common features to both lineages, alike in their mitochondrial physiology as well as protein repertoire. For these reasons, results of our phylogenetic studies might aid the verification of the position of *A. castellanii*, *D. discoideum*, and other amoebozoans on the eukaryotic tree of life.

Methods

Identification of mitochondrial outer membrane protein import and metabolite exchange machinery in *D. discoideum*

- similarity searches against *Dictyostelium*'s genome (TBLASTN, BLASTP)
- Hidden Markov Models searches for protein domains (HMMER package)
- validation of candidates, secondary structure prediction (InterProScan, PSIPRED, CDD)

Construction of VDAC, Tom40, and Sam50/Tob55 protein sequences datasets

- keyword searches with NCBI Entrez and EBI SRS
- sequence similarity searches against the RefSeq, SwissProt, nr, JGI, TBestDB databases (various BLAST flavours and parameters variants)

Phylogenetic analysis of mitochondrial outer membrane ß-barrels

- multiple sequence alignments (MUSCLE)
- phylogenetic relationships reconstruction
  - Neighbor Joining - (PHYLIP package, JTT matrix, no gamma rates-across-sites model, 1000 bootstrap replicates)
  - Maximum Likelihood - (RAxML, RETRV matrix, best tree topologies determined under PROTCAT with 25 per site rate categories. Support values for trees assessed with 1000 bootstrap replicates. Likelihood values of final trees evaluated and optimized under PROTGAMMA with four gamma-distributed discrete rate categories.

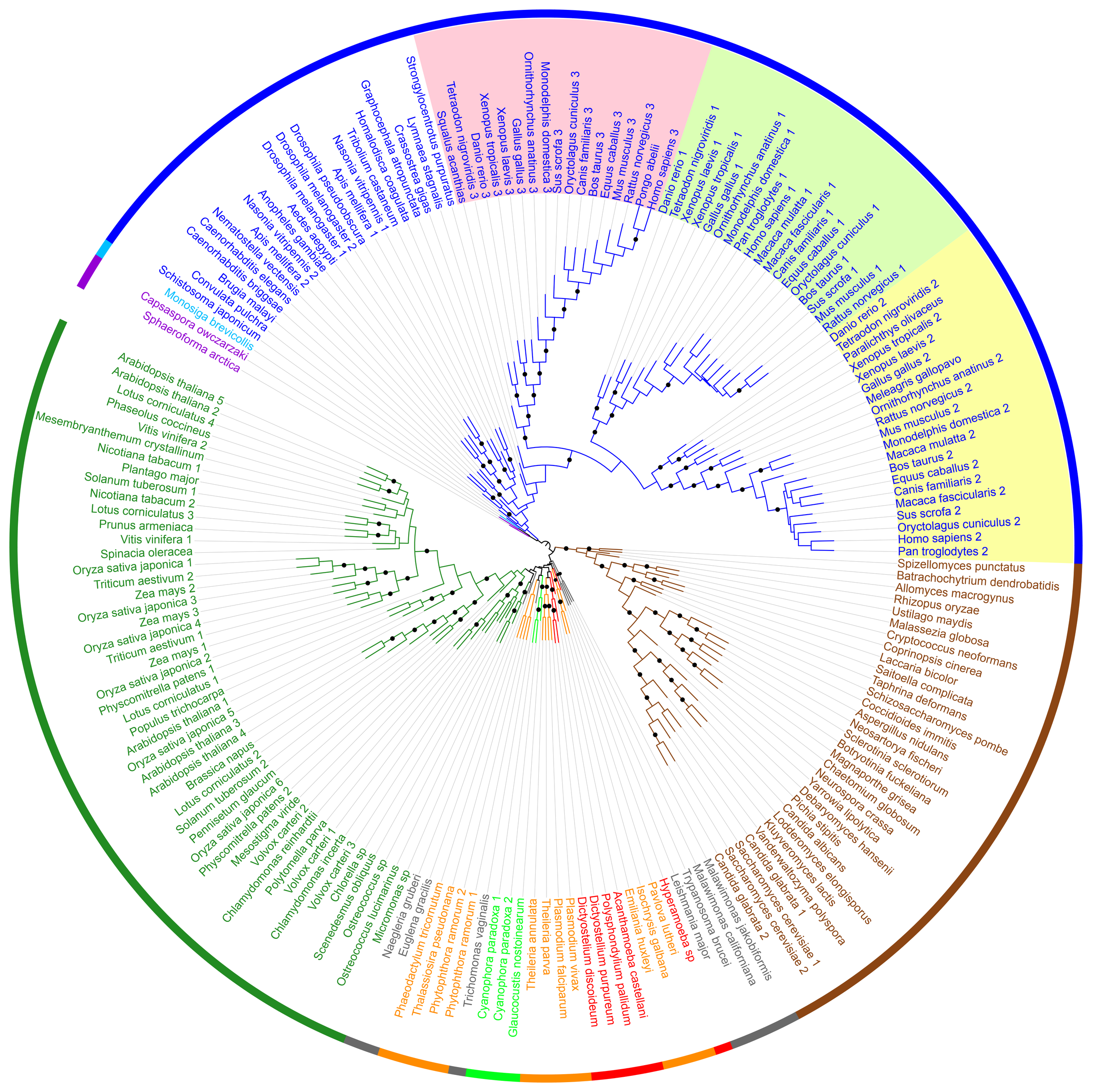

Fig. 2: Evolutionary relationships of 184 VDAC protein sequences based on Maximum likelihood analysis. Colors key: red – Amoebozoa, blue – Metazoa (Ophistokonta), light blue – Choanomonada (Ophistokonta), violet – Mesomycetozoa (Ophistokonta), brown – Fungi (Ophistokonta), green – Chloroplastida (Archeplastida), light green – Glaucophyta (Archeplastida), orange – Chromalveolata, grey – Excavata, yellow – Rhizaria. Colored leaf ranges denote three subclades created by three vertebrate VDAC isoforms. Filled black circles represent bootstrap values of 70% and above.

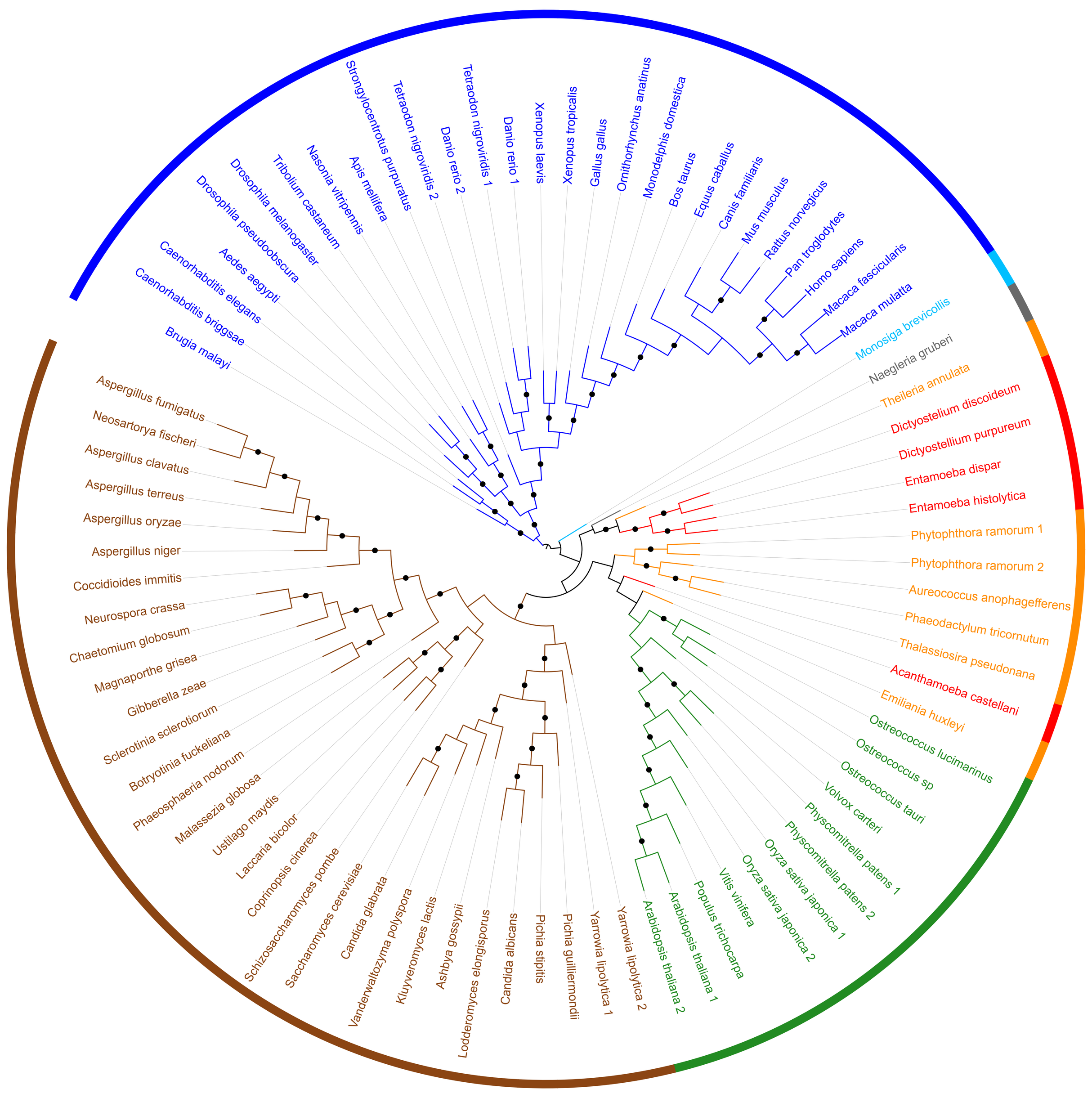

Fig. 3: Maximum likelihood tree of 84 Sam50/Tob55 protein sequences. Colors as described for Figure 2. Filled black circles represent bootstrap values of 70% and above.

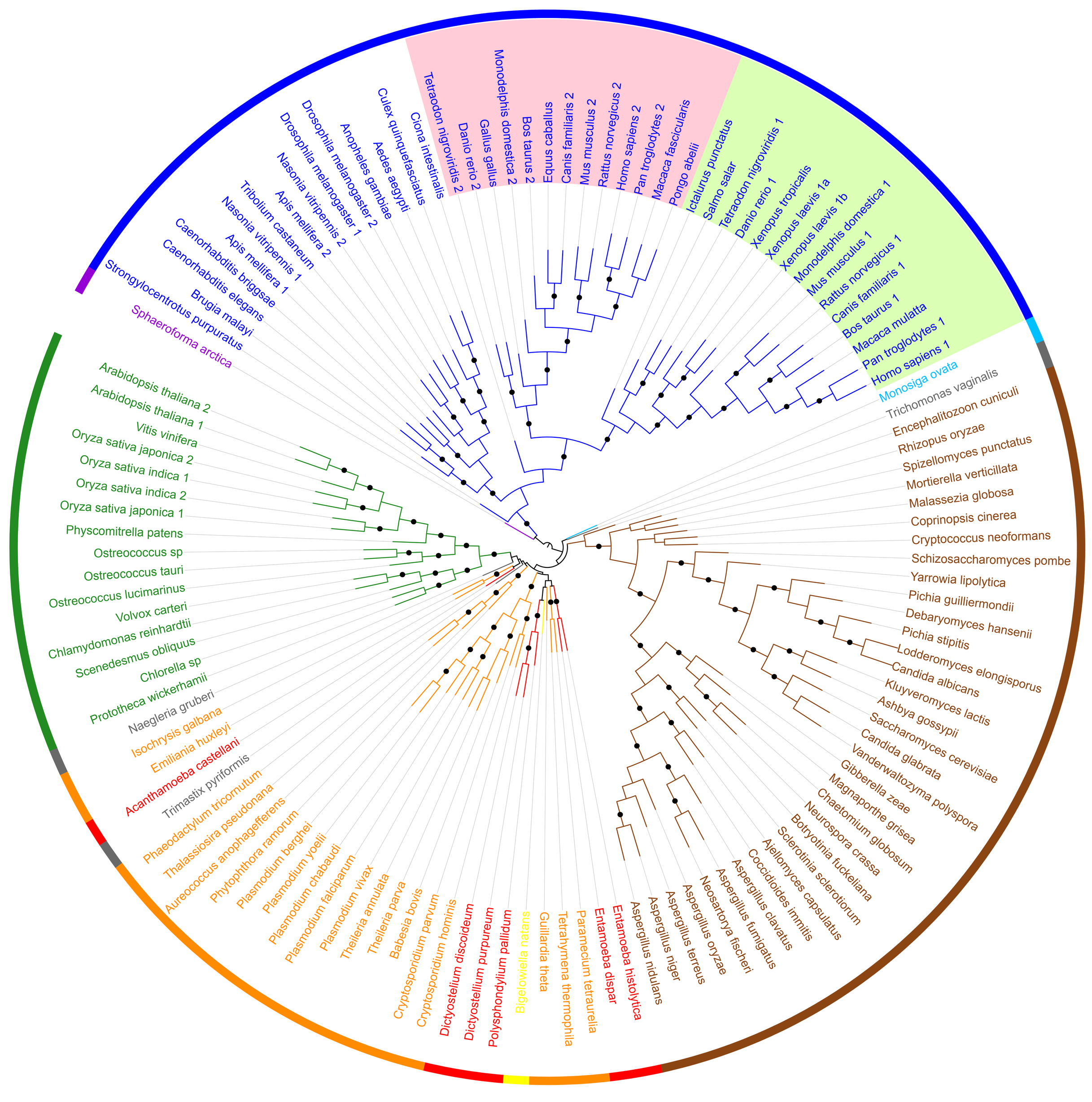

Fig. 4: Maximum likelihood tree of 124 Tom40 amino acid sequences. Colors as described for Figure 2. Colored leaf ranges denote two subclades created by two vertebrate Tom40 isoforms. Filled black circles represent bootstrap values of 70% and above.

Results

We managed to verify the previously discovered genes for VDAC and Tom40 in the *D. discoideum*'s genome and identified the remaining Sam50/Tob55 sequence that encoded for a 396 amino acid long protein. In the amino acid sequence it presented greater similarity to animal (e.g. human, chicken) than to fungal or plant counterparts. Search for protein domains detected the Bac_surface_Ag protein domain and because it was identified in all well annotated animal, fungal or plant Sam50/Tob55 sequences, the finding provided additional support that the identified sequence is a true ortholog. Protein was also predicted to be able to form transmembrane ß-strands, which is a crucial feature of membrane ß-barrel proteins.

The dataset collected for phylogeny contained the mitochondrial outer membrane ß-barrels from 157 species and it consisted of 84, 124, and 184 protein sequences of Sam50/Tob55, Tom40, and VDAC, respectively (see the Table below).

Table 1. Distribution of the analyzed mitochondrial outer membrane β-barrels among the six eukaryotic supergroups.

Supergroup	# of species	Sam50	Tom40	VDAC
Amoebozoa	7	5	6	5
Ophisthokonta	89	59	79	110
Archeplastida	32	12	16	51
Chromalveolata	20	7	19	11
Excavata	8	1	3	7
Rhizaria	1	0	1	0
	157	84	124	184

As shown in Figure 2, **VDAC**s from *A. castellanii* and *D. discoideum*, and other representatives of Amoebozoa group together, however, we do not observe a well defined monophyletic clade - they also cluster together with apicomplexans and haptophytes (Chromalveolata group). **Sam50/Tob55** sequence of *A. castellanii* identified in this study clusters to plants in a basal position (Figure 3). It is grouped between representativesof plants and haptophytes and stramenopiles (Chromalveolata). Other individuals from the Amoebozoa supergroup, including *D. discoideum*, are located between fungal and animal lineages and grouped with representatives of Excavata and Alveolata (Chromalveolata). Amoebozoan **Tom40** sequences set between plant and Chromalveolata/Excavata lineages (Figure 4). Tom40 of *A. castellanii* apears basally to the plant node and grous together with sequences of haptophytes. *Dictyostelium's* sequence is clustered with Tom40 sequences of Rhizaria and Cryptophycae (Chromalveolata). The obtained arrangement of clades appears to support only two eukaryotic supergroups - Ophisthokonta and Archeplastida. In all trees the internal nodes, that bear on validity of the supergroups, present low bootstrap values, while the external ones that group closely related species are rather well supported.

Conclusions

In contrast to animals or plants, where many duplication events took place during the evolution of mitochondrial outer membrane ß-barrels, among the analyzed Amoebozoa species, including *A. castellanii* and *D. discoideum*, no duplication occured and only single genes for VDAC, Tom40, and Sam50/Tob55 evolved, indicating no need for for a transport function innovation.

Moreover, despite the strong structure-function conservation, VDAC, Tom40 and Sam50/Tob55, diverged widely in the amino acid sequences between different eukaryotic lineages and also within them. This is probably caused by and coupled with the evolution of lineage specific, additional components of the import machinery and metabolite transport systems, which partially took over some functions of the very first simple transporters (eg. receptors like metaxins, Sam35, or Sam37).

Additionally, the observed differences in mitochondrial ß-barrels can sometimes, be driven by a secondary loss of the appended components of the complexes. As previously described for some parasitic organisms (e.g. amoebas, microsporidians), they seem to miss almost all the additional components of the TOM and SAM/TOB complexes what is caused by the reductive evolution affecting their mitochondrion-related organelles termed mitosomes.

Acknowledgements

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