The emerging picture of the mitochondrial protein import complexes of Amoebozoa supergroup

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Abstract

Background: The existence of mitochondria-related organelles (MROs) is proposed for eukaryotic organisms. The Amoebozoa includes some organisms that are known to have mitosomes but also organisms that have aerobic mitochondria. However, the mitochondrial protein apparatus of this supergroup remains largely unsampled, except for the mitochondrial outer membrane import complexes studied recently. Therefore, in this study we investigated the mitochondrial inner membrane and intermembrane space complexes, using the available genome and transcriptome sequences.

Results: When compared with the canonical cognate complexes described for the yeast Saccharomyces cerevisiae, amoebozoans with aerobic mitochondria, display lower differences in the number of subunits predicted for these complexes than the mitochondrial outer membrane complexes, although the predicted subunits appear to display different levels of diversity in regard to phylogenetic position and isoform numbers. For the putative mitosome-bearing amoebozoans, the number of predicted subunits suggests the complex elimination distinctly more pronounced than in the case of the outer membrane ones.

Conclusion: The results concern the problem of mitochondrial and mitosome protein import machinery structural variability and the reduction of their complexity within the currently defined supergroup of Amoebozoa. This results are crucial for better understanding of the Amoebozoa taxa of both biomedical and evolutionary importance.

Keywords: Amoebozoa, Mitochondria, Mitosomes, Protein import, TIM22 complex, TIM23 complex, small Tims, MIA complex, PAM complex, OXA complex

Background

Currently, the division of eukaryotic organisms consists of six large supergroups, namely Chromalveolata, Excavata, Archaeplastida, Rhizaria, Amoebozoa, and Opisthokonta [1, 2]. As summarized in Fig. 1, the Amoebozoa, regarded as a sister clade to the Opisthokonta which involves animals and fungi, include some organisms that are known to have mitosomes, such as Entamoeba histolytica, an intestinal pathogen of humans [3, 4], but also organisms that have aerobic mitochondria, such as the slime mold Dictyostelium discoideum, a free-living organism that inhabits soil and compost [5], as well as the amoeba Acanthamoeba castellanii, a soil free-living amoeba also known as a facultative human parasite [6]. The organisms represent different subclades and subdivisions of the Amoebozoa [1]. A. castellanii represents the Lobosa, whereas D. discoideum and E. histolytica belong to the Conosa and are included in the Mycetozoa and Archamoebae, respectively (Fig. 1). Thus, the Amoebozoa encompass taxa of both biomedical and evolutionary importance, yet their genomic and transcriptomic diversity remains largely unsampled. The same applies to the mitochondrial protein import complexes. Accordingly, the data concerning the subunit organization of the mitochondrial protein import complexes for members of the
same supergroup classified into distinct subclades and divisions are not well known.

Studies of the mitochondrial protein import machinery have revealed that the machinery consists of complexes located in all mitochondrial compartments and have provided information about their organization, function, and interplay. The most commonly used model organism in the studies is the yeast *Saccharomyces cerevisiae*. Consequently, the *S. cerevisiae* protein import machinery, schematically shown in Fig. 2, is defined as a canon and applied as a reference model in studies of the machinery of other eukaryotic organisms, including plants and animals [7–9]. The canon includes the presence of at least three complexes in the outer membrane, four in the inner membrane, and three in the intermembrane space. The TOM (translocase of the outer membrane), TOB/SAM (topogenesis of the mitochondrial outer membrane β-barrel proteins/sorting and assembly machinery) and MIM (mitochondrial import machinery) complexes are located in the outer membrane. The TOM complex represents a general entry gate for most proteins imported into mitochondria, whereas the TOB/SAM complex and MIM complex enable the insertion of β-barrel proteins and proteins containing one or more transmembrane-spanning helices into the membrane, respectively. The TIM22 (translocase of the inner membrane 22), TIM23 (translocase of the inner membrane 23), PAM (presequence translocase-associated motor), and OXA (oxidase assembly factor) complexes are located in the inner membrane. The TIM22 and TIM23 complexes mediate the import of precursor proteins with targeting signals located within their sequence and at their N-terminus, respectively. The PAM complex cooperates with the TIM23 complex to drive protein translocation into matrix, whereas the OXA complex participates in the insertion of proteins from the matrix side into the inner membrane. The MIA (mitochondrial intermembrane space assembly) complex as well as small Tim chaperone-like protein complexes (small Tims), i.e. Tim8-Tim13 and Tim9-Tim10-Tim12 complexes, are located in the intermembrane space. The MIA complex is responsible for the import of small intermembrane space proteins with the multiple cysteine residues due to the thiol-disulfide exchange, whereas small Tims associate with the TOB/SAM and TIM22 complexes to protect precursor proteins from misfolding in the intermembrane space.

Available data indicate that the subunit organization of the complexes of animals and plants is more or less similar to those depicted in Fig. 2 for *S. cerevisiae* (for reviews, see e.g. [7, 9–11] although some new subunits have recently been identified for animals e.g. [10] and plants [11]. Nevertheless, in other eukaryotes, the differences are more pronounced. Consequently, the commonly occurring subunits of the TOM, TOB/SAM, and TIM22 complexes are their channel-forming subunits, Tom40, Sam50/Tob55, and Tim22, respectively, whereas the common presence of Tim50, Tim17, and Tim23 is reported for the TIM23 complex, Erv1 for the MIA complex, and Tim9 and Tim10 for the small Tim protein complexes [12, 13]. In the case of the MIM and OXA complexes, data are still missing for this kind of analysis. The data concerning the protein import machinery are also strongly limited in mitosome-carrying organisms. As summarized by Heinz and Lithgow [12] as well as by Makiuchi and Nozaki [13], they are available for *Cryptosporidium parvum* (Chromalveolata), *Encephalitozoon cuniculi* (Opisthokonta), *Giardia intestinalis* (Excavata), and *E. histolytica* (Amoebozoa). The organisms carry Tom40 and Sam50/Tob55, although the

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*Fig. 1* Schematic representation of the phylogenetic position of the studied organisms in the eukaryotic tree. Based on [1, 2, 46]
latter was not detected for *G. intestinalis*, and only two receptor subunits of the TOM complex, Tom70 and Tom60, were predicted for *E. cuniculi* and *E. histolytica*, respectively. Moreover, *C. parvum* and *E. cuniculi* have Tim17 and Tim23 (the TIM23 complex) as well as Tim22 (the TIM22 complex), but it is difficult to distinguish the proteins at the level of their sequences. Other subunits of the complexes, Tim50 (the TIM23 complex) and Tim18 (the TIM22 complex), were found for *C. parvum* and *E. cuniculi* with the exception of *G. intestinalis* Tim18. In the case of small Tims, only Tim8 and Tim13 seem to be expressed by *C. parvum*. As far as the PAM complex subunits are concerned, mtHsp70 was found for all the mentioned organisms, Mge1 for *C. parvum*, *G. intestinalis*, and *E. histolytica*, while Tim44 and Pam16 were detected only for *C. parvum*.

Our previous study, based on searching of the available genome and transcriptome data concerning amoebozoan subunits of the TOM and TOB/SAM complexes, indicated that the complexes may display structural variability in the Amoebozoa lineage and their subunit number reduction, as compared with the corresponding canonical complexes of *S. cerevisiae* [14]. Accordingly, the predicted number of subunits of the TOM and TOB/SAM complexes were different for *A. castellanii*, and representatives of slime molds and entamoebas as well as within the slime molds and entamoebas. Importantly, entamoebas are proposed to contain mitosomes and *E. dispers*, and *E. nuttalli* are now recognized as separate species from the well-known pathogenic *E. histolytica* and *E. invadens*, although their pathogenicity is still a matter of debate [15–17]. To study the issue further, we extended our research on the transcriptome and genome analysis of the complexes located in the inner membrane and intermembrane space of the amoebozoan mitochondria. As mentioned above, we include the TIM22, TIM23, PAM, and OXA complexes located in the inner membrane as well as the MIA complex and the complexes of small Tim proteins forming Tim8-Tim13, and Tim9-Tim10-Tim12 complexes, all located in the intermembrane space. The predicted subunits indicate that for amoebozoans with aerobic mitochondria, organization variability of the complexes may be less pronounced than in the case of the mitochondrial outer membrane complexes. Nevertheless, the subunits predicted for the inner membrane and the intermembrane
space complexes display different levels of diversity in regard to phylogenetic position and some of them have isoforms. Moreover, elimination of the complexes in the putative mitosome-bearing amoebzoans is predicted to be much more conspicuous than in the case of the outer membrane complexes. The results appear to be important for discussion on the complex localization and functions as well as their contribution to the protein import and functions of mitochondrial membranes.

Methods
Species of amoebzoans
The studied organisms included Acanthamoeba castellanii (Lobosa; Discosea), Dictyostelium discoideum, D. purpureum, D. fasciculatum, and Polysphondylium pallidum (Conosa; Mycetozoa), as well as Entamoeba dispar and E. nuttalli (Conosa; Archamoebae). Additional file 1: Table S1 summarizes data concerning their genome and transcriptome sequences.

Acanthamoeba castellanii strain Neff used in this study was delivered from American type Culture Collection (ATCC) with the number 30010.

Acanthamoeba castellanii cell culture and isolation of total RNA
Cells of Acanthamoeba castellanii (strain Neff) were cultured at 28 °C, in an axenic environment in the standard medium described by Neff [18] with some modifications: 1.5% proteos-peptone, 0.15% yeast extract, 30 mM MgCl2, 30 mM FeSO4, 27 mM CaCl2, 1.5% glucose, 2.5 mg/l vitamin B12, 1 mg/l vitamin B1, and 0.2 mg/l vitamin H. Cells in the trophozoite stage were collected in the intermediary phase after 48 h and next were frozen in liquid nitrogen and homogenized in TRizol reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instructions (Invitrogen). DNase I was added to eliminate remaining genomic DNA. The absence of DNA was confirmed by PCR and agarose gel electrophoresis.

Acanthamoeba castellanii cDNA preparation, sequencing, and transcriptome assembly
CDNA was prepared using an mRNA-Seq Sample preparation Kit (Illumina), according to the manufacturer's instructions. Sequencing of the cDNA, i.e. mRNA-Seq of A. castellanii was performed on the HiSeq 2000 platform (Illumina) with 36-bp single-end reads. AC_RNASeq data are available in DDBJ database; the accession number DRA006231 (BioSample number SAMD00097225). The obtained raw reads were subjected to quality control analysis. After removal of poor-quality sequences, short reads were assembled using Trinity RNA-Seq [19] with -SS_lib_type F (AC_RNASeq) and min_contig_length 300.

Prediction of proteins
To find the best annotated amino acid sequences for subunits of the TIM22, TIM23, PAM, OXA, small Tim and MIA complexes, keyword searches against the NCBI (http://www.ncbi.nlm.nih.gov/) and Pfam (http://pfam.sanger.ac.uk) databases were performed. First, sets of well-known sequences from different species representing various eukaryotic lineages including subunits identified for S. cerevisiae as well as detected specifically for plant or animal mitochondrial protein import complexes (Additional file 1: Table S2) were used as queries in tBLASTn searches [20] against the transcriptome of A. castellanii with variable e-values (from 10^{-3} to 1). For the proteins that were not predicted by tBLASTn, a HMMER search based on Hidden Markov Model was performed [21]. In the case of multiple-protein hits, sequences giving the highest coverage of transcripts were selected. To translate the transcripts into protein sequences, the ExPASY server was used [22]. The amino acid sequences of putative proteins were subjected to a BLASTp [20] search in order to compare the sequences with available protein datasets of A. castellanii [6]. To find previously un-annotated proteins, a tBLASTn search against the available genome of A. castellanii was performed.

Subsequently, proteins predicted for A. castellanii were used in a tBLASTn search against the protein datasets of Dictyostelium purpureum, D. discoideum, P. pallidum, E. nuttalli, E. dispar (Additional file 1: Table S1). For the proteins that were not predicted by the analysis, the tBLASTn algorithm was used against the available genomes of D. discoideum, D. purpureum, P. pallidum, E. dispar, and E. nuttallii. Finally, reference sequences from various eukaryotic lineages (Additional file 1: Table S2) were used to predict proteins not found by the previously applied methods.

Phylogenetic inference
Protein sequences were aligned using CLUSTAL W [23] and phylogenetic trees were inferred using the Neighbor-Joining method [24] with the amino acid substitution model using Poisson correction as implemented in MEGA (version 7) software [25, 26]. The data were bootstrapped by 1000 replicates [27].

Results
We investigated the presence of genes encoding subunits of the mitochondrial protein import complexes located in the intermembrane space and inner membrane using available genome and transcriptome sequences. As summarized in Table S1 (Additional file 1), among the studied amoebzoans the transcriptome data are not available for the slime mold Dictyostelium fasciculatum.
and both the entamoebas, i.e. *Entamoeba nuttalli* and *Entamoeba dispar*. Moreover, in the case of the amoeba *Acathamoeba castellanii* we applied two transcriptome datasets, one already available at GenBank [6] and the second (AC_RNaseq) assembled by us (see Methods). The reason is that we noticed some differences between the transcriptome datasets reflected in amino acid sequences of proteins predicted for the mitochondrial outer membrane [14]. The involvement of transcriptome data supplies more information regarding gene isoforms and gene structure giving more opportunity in prediction process of examined genes.

**Subunits predicted for A. castellanii**

The application of the available GenBank and AC_RNASeq enabled prediction of 17 *A. castellanii* possible subunits of mitochondrial protein import complexes located in the intermembrane space and the inner membrane (Table 1; for the complex organization see Fig. 2). Importantly, four of the subunits appeared to have isoforms (Tim9, Tim10, Pam16, and Pam18). All the predicted proteins displayed a high level of amino acid sequence identity to the GenBank data but also some differences were observed when AC_RNASeq was taken into account. The differences are shown in Additional file 1: Figures S1 and S2.

The amino acid sequences predicted for Tim21, Tim44, Erv1 and Oxa1 have been already specifically annotated so the existing accession numbers were added to Table 1. The sequences predicted for Tim9C, Tim10A, Sdh3, Tim17, Tim23, Tim15 and Pam18A have been named specifically (Table 1, underlined letters; Tim15 is under the process of annotation). To check the accuracy of the predicted *A. castellanii* Tim9 and Tim10 isoforms the phylogenetic analysis was performed (Fig. 3a). In the obtained phylogenetic tree, two clusters representing Tim9 and Tim10 families can be easily distinguished. Although bootstrap values were not very high for the internal branches within two clusters, they were separated by the middle branch, which was supported by the bootstrap value of 98%. Thus, the isoforms were predicted properly and they appeared to be orthologs. Accordingly, to estimate amino acid similarity between *A. castellanii* isoforms of Pam16 and Pam18 and their phylogenetic relationships, phylogenetic trees were built (Fig. 3b–c). The obtained results indicated that the slime mold clades of Pam16 and Pam18 grouped with *A. castellanii* clades of these proteins. Thus the *A. castellanii* proteins appeared to be orthologs.

For the rest of the predicted proteins some differences were observed between AC_RNaseq and the GenBank data (Additional file 1: Figure S1). Consequently, we proposed some corrections of sequences predicted for Tim9A, Tim10B, Tim22, Tim50, Mgr2, Pam16A, Pam16B, and mtHsp70. In the case of the predicted Tim9A, the protein already stored in the GenBank (XP_004340603) was mis-annotated, as it contained only part of the functional domain of Tim9 proteins and was fused with EF-hand Ca^{2+}-binding domain that was removed from the corrected Tim9A version. The predicted Tim10B, Tim22, and Pam16A were longer than cognate annotated proteins (XP_004336134, AA1766174 and XP_004335678, respectively), whereas Tim50 and Mgr2 were longer than hypothetical proteins (XP_004344840 and XP_004333275, respectively). The protein XP_004368030.1 appeared to be encoded by an erroneously fused gene that was eliminated from the corrected Pam16B version. In the case of predicted mtHsp70, the correction concerned two amino acid substitutions at the C-terminus, namely alanine to serine and leucyne to methionine. Finally, the genes encoding Tim9B, Pam18B, and Mge1 were not present in the GenBank data but were supported by hits found in tBLASTn alignment against AC_RNASeq (Additional file 1: Figure S2).

Summing up, as can be concluded from data shown in Table 1, organization of the *A. castellanii* TIM23 and PAM complexes may be identical to cognate complexes of *S. cerevisiae* whereas in the case of small Tims, TIM22, OXA and MIA complexes some subunits described for cognate *S. cerevisiae* complexes appear not to be present.

**Subunits predicted for slime molds**

Altogether 18 subunits were predicted for the intermembrane space and inner membrane import complexes (Fig. 2) of the studied slime molds, i.e. *D. discoideum*, *D. fasciculatum*, *D. purpureum* and *Polysphondylium pallidum* (Table 1). Most of the proteins were identical to sequences stored in the GenBank as specifically annotated or hypothetical proteins (the latters are marked as underlined letters in Table 1). The isoforms were predicted only for Oxa1. To check their accuracy phylogenetic analysis was performed and the obtained phylogenetic tree supported the notion that the slime mold Oxa1A and Oxa1B were paralogs (Fig. 3d). Moreover, for *A. castellanii* Oxa1 a higher similarity to the slime mold Oxa1B was observed. The only correction concerned *P. pallidum* Tim9, detected by the genome data analysis. The sequence turned out to be a part of a sequence stored in the GenBank under accession number EFA81367 (Additional file 1: Figure S1B).

Summing up, it appears that organization of the slime mold TIM23, PAM and MIA complexes may be identical to cognate complexes of *S. cerevisiae* whereas in the case of small Tims, TIM22 and OXA complexes some subunits are probably not present. Interestingly, with
Table 1 The predicted subunits of Tim9-10-12, TIM22, TIM23 complexes of the studied Amoebozoa

| Subunit       | Tim9       | Tim10      | Tim22       | Sdh3       | Tim17       | Tim21       | Tim23       | Tim50       | Mgr2       |
|---------------|------------|------------|-------------|------------|-------------|-------------|-------------|-------------|------------|
| A. castellanii| A: XP_0043400603 MF045879 | A: XP_004339376 MF045875 | A: XP_004367486 XP_004347782 | XP_004340873 | XP_004336406 | XP_00433275 | XP_004344840 | XP_00435867 | XP_00435874 |
|               | B: XP_004336134 MF045868 | C: XP_004340763 MF045880 | B: XP_004340873 XP_004336406 | MF045865 | MF045865 | MF045865 | MF045865 | MF045865 | MF045865 |
| D. discoideum | XP_644845 XP_639927 | XP_643757 XP_637129 | XP_629896 XP_637678 | XP_646611 XP_637862 | XP_644845 XP_639927 | XP_643757 XP_637129 | XP_629896 XP_637678 | XP_646611 XP_637862 | XP_644845 XP_639927 | XP_643757 XP_637129 | XP_629896 XP_637678 | XP_646611 XP_637862 |
| D. fasciculatum| XP_004354109 XP_004358579 | XP_004361430 XP_004362434 | XP_00435582 XP_004350055 | XP_004361430 XP_004354259 | XP_004354109 XP_004358579 | XP_004361430 XP_004362434 | XP_00435582 XP_004350055 | XP_004361430 XP_004354259 | XP_004354109 XP_004358579 | XP_004361430 XP_004362434 | XP_00435582 XP_004350055 | XP_004361430 XP_004354259 |
| D. purpureum  | XP_003293366 XP_003286712 | XP_003289095 XP_003290414 | XP_003289186 XP_003287691 | XP_003287004 XP_003284507 | XP_003293366 XP_003286712 | XP_003289095 XP_003290414 | XP_003289186 XP_003287691 | XP_003287004 XP_003284507 | XP_003293366 XP_003286712 | XP_003289095 XP_003290414 | XP_003289186 XP_003287691 | XP_003287004 XP_003284507 |
| P. pallidum   | EFA81367 EFA78336 | EFA79918 EFA81038 | EFA79679 EFA84293 | EFA78450 EFA75083 | EFA81367 EFA78336 | EFA79918 EFA81038 | EFA79679 EFA84293 | EFA78450 EFA75083 | EFA81367 EFA78336 | EFA79918 EFA81038 | EFA79679 EFA84293 | EFA78450 EFA75083 |
| E. dispar/nuttalli | Pam16 Pam18 | Tim44 mHsp70 | Tim15 Mge1 Mia40 AIF Env1 Oxa1 | A: XP_004335678 MF045873 | A: XP_0043435808 MF045895 | A: XP_004339581 MF045872 | A: XP_0043335035.1 MF045870 | A: XP_004367724 XP_004344684.1 |
| A. castellanii| A: XP_0043435808 MF045895 | A: XP_004339581 MF045872 | A: XP_0043335035.1 MF045870 | A: XP_004335678 MF045873 | A: XP_0043435808 MF045895 | A: XP_004339581 MF045872 | A: XP_0043335035.1 MF045870 | A: XP_004367724 XP_004344684.1 |
| D. discoideum | Q54SV6 Q54QNI | EAL64247 Q8OH7 | XP_638912 XP_643501 | XP_636815.1 XP_645302 | EAL64247 Q54QNI | EAL64247 Q54QNI | EAL64247 Q54QNI | EAL64247 Q54QNI | EAL64247 Q54QNI | EAL64247 Q54QNI | EAL64247 Q54QNI |
| D. fasciculatum| XP_004356846 EG324887 | XP_004367372 XP_004360938 | XP_004362148 XP_004357088 | XP_004360441 EG321561.1 | XP_004356846 EG324887 | XP_004367372 XP_004360938 | XP_004362148 XP_004357088 | XP_004360441 EG321561.1 |
| D. purpureum  | XP_003293668 EGC31346 | XP_003293823 XP_003283943 | XP_003294616 XP_003291222 | EGC31346 | XP_003293668 EGC31346 | XP_003293823 XP_003283943 | XP_003294616 XP_003291222 | EGC31346 |
| P. pallidum   | EFA78034 EFA86354 | EFA81857 EFA85858 | EFA77076 EFA83431 | EFA83584 A: EFA84401.1 |
| E. dispar     | XP_001736890 | XP_008855870 | |
| E. nuttalli   | XP_008855870 | |

Underlined letters denote sequences determined in this study. Not-underlined letters denote sequences stored in the GenBank as specifically annotated proteins.
exception of Mia40, the missing subunits are the same as for *A. castellanii*.

**Subunits predicted for entamoebas**
The only subunit predicted for *E. dispar* and *E. nuttalli* was mtHsp70. The proteins have been already annotated (Table 1).

**Phylogenetic position of the predicted proteins**
To assess the phylogenetic position of the predicted proteins we estimated the similarity of their amino acid sequences to cognate subunits (Table 2). The predicted subunits displayed mostly the highest similarity to cognate proteins of Opisthokonta (fungi and animals) although the highest similarity to Archaeplastida (plants), Chromalveolata, and Excavata proteins was also observed in some cases. The least diverse in terms of similarity to reference proteins was the MIA complex, as the predicted subunits displayed the highest similarity to Opisthokonta proteins (animals and fungi) with the exception of *D. fasciculatum* Mia40 (Archaeplastida, plants). The prevailing similarity of the predicted subunits to Opisthokonta proteins (animals and fungi) was also observed for TIM23 complex subunits as the similarity to Archaeplastida proteins was only observed for the predicted *A. castellanii* Tim23 and *P. pallidum* Tim21. In the case of TIM22 complex as well as Tim9 and Tim10 proteins, the majority of the predicted proteins also displayed the highest similarity to the cognate Opisthokonta proteins (animals and fungi). However, the highest similarity to Chromalveolata cognate proteins was observed for the predicted *D. fasciculatum* Sdh3 as
| Subunit     | Tim9  | Tim10 | Tim22 | Sdh3  | Tim17 | Tim21 | Tim23 | Tim50 | Mr2   |
|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| **A. castellanii** | A. C. neoformans | POCR7 | Opisthokonta | (Fungi) 9.00E-18 | B. C. neoformans | POCR7 | Opisthokonta | (Fungi) 1.00E-15 | C. G. CAJT365 | 551 Chromalveolata 9.00E-16 |
| **D. discoideum** | P. trehalosi CD96 | 6.00E-12 | Chromalveolata 6.00E-13 | N. parvum 2.00E-24 | H. sapiens 1.00E-26 | | | | |
| **D. fasciculatum** | G. CAJT365 | Excavatea 2.00E-12 | | | | | | | |
| **D. purpureum** | P. trehalosi CD96 | 6.00E-12 | Chromalveolata 6.00E-13 | N. parvum 2.00E-24 | H. sapiens 1.00E-26 | | | | |
| **P. pallidum** | B. stichosporon 0.00E-14 | 8.00E-14 | Chromalveolata 6.00E-13 | N. parvum 2.00E-24 | H. sapiens 1.00E-26 | | | | |
| **E. gossypii** | Pam18 | | | | | | | | |
| **A. castellanii** | A. Plutai EBH38935 | 2.00E-27 | | | | | | | |

**Table 2: The highest similarity of the amino acid sequences of the predicted subunits of Tim9-10-12, TIM22, TIM23, PAM, MIA, and OXA complexes to the applied reference sequences.**
| Taxon | Species | Genbank Accession | Lineage | E-Value |
|-------|---------|-------------------|---------|---------|
| Dd discoideum | M. truncatula EH 2915 | A. m. isis spp. XP_006266995 Opisthokonta (Animalia) 4.0 E-29 | Opisthokonta (Animalia) | 1.00 E-32 |
| | | Phymat atus EE B14183 Opisthokonta (Animalia) 1.00E-03 | Blastocystis sp. AFL03352 Chromaleolata 0.0 | S. cerevisiae A0A023ZHL4 Opisthokonta (Fungi) 2.0E-42 |
| | | | | S. solare AI69535 Opisthokonta (Animalia) 6.00E-08 |
| | | | | R. microsporus ORE05664.1 Opisthokonta (Fungi) 1.00E-13 |
| | | | | C. capitata JA887019 Opisthokonta (Animalia) 1.00E-39 |
| | | | | A. Ch. cucubnit. OB281755 Archaeplastida (Plantae) 7.00E-39 |
| | | | | B. P. brassicae (CE094839.1) Rhizaria 4.00E-24 |
| Dfasciculatum | O. sativa Q5JN36 Archaeplastida (Plantae) 2.00E-23 | Ch. reinhardtii EDPO2285 Archaeplastida (Plantae) 2.00 E-29 | L. mira nitina SCV03152.1 Opisthokonta (Fungi) 1.00E-39 |
| | | | | Tetraselmis sp. IAC74447 Archaeplastida (Plantae) 3.00E-12 |
| | | | | L. oculatus XP_015207227.1 Opisthokonta (Animalia) 2.0 E-121 |
| | | | | C. capitata JA887019 Opisthokonta (Animalia) 2.00E-40 |
| | | | | A. queenslalndica XP_001782477.1 Opisthokonta (Animalia) 2.00E-39 |
| | | | | B. P. patens XP_001782477.1 Archaeplastida (Plantae) 3.00E-30 |
| Dpurpureum | M. truncatula KEH2 5915 Archaeplastida (Plantae) 9.00E-19 | A. m. isis spp. XP_006266995 Opisthokonta (Animalia) 1.0 E-29 | T. angelli AGN32911 Excavata 6.00E-33 |
| | | | | H. sapiens NP_653237.1 Opisthokonta (Animalia) 5.00E-09 |
| | | | | S. formosus XP_018602154.1 Opisthokonta (Animalia) 1.00E-113 |
| | | | | A. mellifera XP_001120016 Opisthokonta (Animalia) 1.00E-39 |
| | | | | A. Ch. cucubnit. OB281755.1 Opisthokonta (Fungi) 8.00E-38 |
| | | | | B. S. lent*a WP_028771001.1 Bacteriacea 3. 00E-16 |
| Ppurpureum | T. caro EOYOH940 Archaeplastida (Plantae) 3.00E-15 | A. carolinensis XP_003218148 Opisthokonta (Animalia) 4.00E-28 | D. reio Q6DE18 Opisthokonta (Animalia) 4.00E-43 |
| | | | | S. mansoni CCC79254 Opisthokonta (Animalia) 5.00E-02 |
| | | | | C. anguillulae ORZ33374.1 Opisthokonta (Fungi) 1.00E-113 |
| | | | | A. mellifera XP_001120016 Opisthokonta (Animalia) 2.00E-40 |
| | | | | A. M. elongata OA286652.1 Opisthokonta (Fungi) 2.00E-39 |
| | | | | B. S. kowalevskii XP_006817147.1 Opisthokonta (Animalia) 4.00 E-28 |
| Edsper/ Erutallii | Blastocystis sp. AFL03352 Chromaleolata 0.0 | | | |
well as A. castellanii Tim9C and the slime mold Tim9 with exception of P. pallidum Tim9, which appeared to be the most similar to the Archaeplastida cognate proteins.

In the case of the predicted subunits of the PAM complex and OXA complex, the phylogenetic origin of proteins regarded as the best match appeared to be more variable. For the PAM complex subunits the similarity concerned the cognate proteins of Opisthokonta (fungi and animals), Archaeplastida, Chromalveolata and Excavata. In the case of the predicted Pam16, the highest similarity was observed for Opisthokonta (fungi) proteins (A. castellanii both isoforms) and Archaeplastida proteins (slime molds). For the predicted Pam18, the highest similarity was observed for Archaeplastida and Opisthokonta (animals) cognate proteins (A. castellanii Pam18A and Pam18B, respectively) whereas the slime mold Pam18 predicted proteins displayed the highest similarity to Opisthokonta (animals) cognate proteins with exception of D. fasciculatum (Archaeplastida). The predicted Tim44 proteins were the most similar to Opisthokonta (fungi) protein (A. castellanii) and Opisthokonta (animal) cognate proteins (all slime molds). Interestingly, all the predicted mtHsp70 proteins displayed the highest similarity to Chromalveolata cognate proteins, with the exception of D. fasciculatum, for which the predicted mtHsp70 appeared to be the most similar to an Opisthokonta (animal) protein. The predicted Mge1 appeared to be the most divergent in regard to phylogenetic relationships, as we observed the best match for Opisthokonta (fungi, animal) proteins (slime molds: D. discoideum, P. pallidum, and D. fasciculatum, respectively), Chromalveolata proteins (A. castellanii), and Excavata (D. purpureum). The same applies to the slime mold predicted Oxa1B isofrom that appeared to be a homolog of the predicted A. castellanii Oxa1 (Fig. 3d). The latter was the most similar to an Archaeplastida (plants) cognate protein but the slime mold predicted Oxa1B was the most similar to the cognate proteins of Bacteriaceae, Rhizaria, Archaeplastida (plants), and Opisthokonta (animals), depending on the studied species. However, the second predicted slime mold Oxa1 isoform; i.e. Oxa1A displayed the highest similarity to the Opisthokonta (fungi and animals) and Archaeplastida proteins.

Summing up, the phylogenetic position of the predicted subunits indicated a comparable level of diversity between A. castellanii and the slime molds as well as between the slime molds themselves. In the latter, the differences concerning the best match were the most pronounced for D. fasciculatum. However, this observation could not be verified by the involvement of the transcriptome dataset into analysis. Moreover, the obtained results revealed a higher level of diversity in the case of the PAM and OXA complexes. Nevertheless, the predicted subunits were mostly similar in their amino acid sequences to the cognate proteins of Opisthokonta (fungi and animals).

Discussion

The differences in the subunit organization of the mitochondrial protein import complexes have been mainly reported for members of different phylogenetic lineages, while for members of the same clade the issue is poorly studied. Thus, the data are missing to address the evolutionary aspects of the mitochondrial protein import machinery. As mentioned in the Introduction, the Amoebozoa includes taxa of both biomedical and evolutionary importance. We have recently shown comprehensive analyses of the mitochondrial outer membrane import complexes, i.e. the TOM and TOB/SAM complexes, of members of this supergroup, based on searching of the available genome and transcriptome data [14], but the mitochondrial protein import machinery located in the inner membrane and intermembrane have not been addressed.

Accordingly, the PubMed data searching for the Amoebozoa protein import complexes located in these mitochondrial compartments presents only results of a hidden Markov-model-based analysis performed for the D. discoideum and E. histolytica Tim9-Tim10-Tim12, TIM22, TIM23, and PAM complexes [12, 13, 28]. They predict the presence of the following subunits of the complexes for D. discoideum: TIM22 and undefined small Tim proteins (the TIM22 and Tim9-10-12 complexes), Tim17, Tim21, and Tim23 (the TIM23 complex) as well as Pam16, Pam18, and mtHsp70 (the PAM complex), whereas in E. histolytica, only mtHsp70 and a possible homolog of Mge1 have been predicted. Moreover, AIF was identified for D. discoideum [29]. Thus, we performed the analysis of genome and transcriptome sequences available for different amoebozoans to obtain a more coherent picture. The analysis includes the Tim9-Tim10-Tim12, TIM22, TIM23, PAM, MIA, and OXA complexes (Fig. 2) of organisms representing different subclades and subdivisions of the Amoebozoa (Fig. 1). In general, all the predicted subunits are conserved between S. cerevisiae and the studied amoebozoans.

For E. dispar and E. nuttalli only one subunit of all the studied complexes was predicted, namely mtHsp70, a member of the PAM complex. It is assumed that all Entamoeba species have mitosomes, although convincing data are still missing (Graham Clark, personal communication). Similarly, in contrast to the well-known pathogenic E. histolytica and E. invadens, the pathogenicity of E. dispar and E. nuttalli is still a matter of debate [15–17]. Nevertheless, it cannot be concluded that the predicted extreme reduction of the protein import
Moreover, the display similarity to canonical import complex subunits. histolytica mitosomes [4, 17]. These proteins do not of unknown function has been shown to be unique to E. barrel protein and other two inner membrane proteins possibility of protein prediction. datasets of E. dispar ones. On the other hand the lack of the transcriptome sequence similarity to any of the six previously identified serves seventh MBOMP subclass lacking any recognizable S. cerevisiae the canonical one described for A. castellanii putative genes, with the exception of genes encoding Tim50, Mgr2, Tim44, mtHsp70, and Erv1 (Additional file 1: Table S3). Accordingly, the encoding genes of the slime molds contain similar numbers of predicted exons, being not distinctly different from the numbers predicted for A. castellanii putative genes, with the exception of genes encoding Tim50, Mgr2, Tim44, mtHsp70, and Erv1 (Additional file 1: Table S3).

However, a distinct difference is apparent when the predicted subunits of the A. castellanii and slime mold Tim9-Tim10-Tim12, PAM, and OXA complexes are compared. Namely, the predicted A. castellanii Tim9, Tim10, Pam16, and Pam18 and the slime mold Oxa1 appear to have isoforms encoded by separate genes. Interestingly, searching of available databases indicates the presence of Tim9, Tim10, and Pam protein isoforms in mitochondria of other organisms, mainly animals and humans [7, 9]. Isoforms were also found for human Tim17 [32] and Tim8 [33–35]. In the case of the OXA complex, two genes encoding Oxa1 and Oxa2 have been reported for Opisthokonta (animals and fungi) and more than two for Archaeplastida (plants) [36]. On the other hand, members of the Excavata seem to have isoforms of Oxa2, while Oxa1 is not present, whereas only one gene encoding Oxa protein and a lack of the protein isoforms have been shown for Chromalveolata [7, 36, 37]. Thus the diversity of the OXA complex organization is still to be explained. Accordingly, the precise roles of the paralogs, including Oxa1 and Oxa2, have yet to be clarified [7]. The difference concerning the presence of small Tims, Pam16, Pam18, and Oxa1 isoforms between the studied amoebazoans constitutes an interesting observation from the point of view of the supergroup phylogeny, as the Amoeboza are regarded as a sister clade to the Opisthokonta [1, 2]. While the putative expression of the A. castellanii Tim9, Tim10, Pam16, and Pam18 isoforms resembles Opisthokonta cognate proteins, the possible presence of the slime mold Oxa1 isoforms is much more difficult to explain. Nevertheless, the differences may reflect the evolutionary history of the subclade formation.

As mentioned above, the difference is also observed for the MIA complex as only Erv1 protein is predicted for the A. castellanii MIA complex, whereas Erv1, Mia40 and AIF are predicted for all the studied slime molds. Interestingly, as in the case of plant and mammalian but not S. cerevisiae mitochondria [38], the predicted slime mold Mia40 appears to be a soluble protein (not shown). Therefore, one can suggest that AIF can serve as Mia40 receptor not only in mammalian mitochondria [39–41]. On the other hand, the apparent absence of Mia40 regarded as an essential protein (e.g. [38]) in some eukaryotes has also been reported. For example, the protein does not seem to be present in brown algae [31] and parasitic protists, such as Plasmodium falciparum, Leishmania tarentolae, and Trypanosoma cruzi [42]. Accordingly, A. castellanii is the only proven parasite among the studied amoebazoans, defined as an opportunistic pathogen responsible for amoebic keratitis and granulomatous amoebic encephalitis in humans [43, 44]. Yet, the conclusions in regard to the correlation between the lack of Mia40 and pathogenicity are at present unjustified. Importantly, the presence of small Tim proteins which undergo oxidative folding, indicate that an oxidative folding machinery exists in A. castellanii mitochondria [45]. It suggests that other protein(s) can replace the subunit. Accordingly, for the
**Conclusions**

Here we present results of a comprehensive bioinformatic analysis of the protein import complexes located in the mitochondrial inner membrane (the TIM22, TIM23, PAM, and OXA complexes) and intermembrane space (the Tim9-Tim10-Tim12 and MIA complexes) of selected species of the Amoebozoa. This analysis was based on searching of the available genome and transcriptome sequences. Our results indicate that in *A. castellanii* and slime molds, the complex organization appears to be quite similar to the canonical ones described for *S. cerevisiae*. However, distinct differences are observed in amino acid sequences of some of the predicted proteins and in the numbers of some of the protein isoforms. Moreover, the performed analysis for entamoebas, i.e. *E. dispar* and *E. nuttalli*, indicates the absence of any subunit of all the studied complexes except mtHsp70 (the PAM complex), and that further supports the suggestion that all entamoebas have mtosomes. Importantly, the reduction of the protein import apparatus of entamoebas is much more pronounced than in the case of the outer membrane. It seems to be a rule for entamoebas, including *E. histolytica*, but not for all organisms possessing mtosomes.

This constitutes an interesting issue from the evolutionary and biomedical perspective, as it addresses the problem of mitochondrial and/or mitosome protein import machinery variability within the currently defined supergroup of eukaryotes. On the other hand, it is becoming clear that the knowledge of mitochondrial protein import of model organisms cannot be generally transferred to all other eukaryotes.

**Additional files**

**Additional file 1: Table S1.** Availability of the genome and transcriptome data of the studied amoebozoans: *Acanthamoeba castellanii* (Ac), *Dictyostelium discoideum* (Dd), *D. fasciculatum* (Df), *D. purpureum* (Dp), *Entamoeba nuttalli* (En), and *Entamoeba dispar* (Ed). Table S2. Reference sequences from various eukaryotic lineages for subunits of the small Tims, TIM22, TIM23, PAM, MIA, and OXA complexes obtained by BLAST search. Table S3. Numbers of exons in genes encoding the predicted subunits for the studied complexes of selected Amoebozoa: *Acanthamoeba castellanii* (Ac), *D. discoideum* (Dd), *D. fasciculatum* (Df), *D. purpureum* (Dp), *Polysphondylium pallidum* (Pp), *Entamoeba dispar* (Ed), and *E. nuttalli* (En). Figure S1. Alignment of the predicted subunits of the studied complexes displaying differences in amino acid sequences as compared to their counterparts deposited in the GenBank: (A) *Acanthamoeba castellanii* Tim5A; (B) *Polysphondylium pallidum* Tim9; (C) A. castellanii Tim10B; (D) *A. castellanii* Tim22; (E) A. castellanii Tim50; (F) *A. castellanii* Mgr2; (G) A. castellanii Pam16A; (H) A. castellanii Pam16B; and (I) A. castellanii mtHsp70. Figure S2. *Acanthamoeba castellanii* sequences predicted by transcriptome analysis. Amino acid and mRNA assembly correction for the mitochondrial inner membrane and intermembrane space proteins in *A. castellanii* based on transcriptome and genome assemblies. (DOCX 334 kb)

**Abbreviations**

AIF: Apoptosis inducing factor; MIA: Mitochondrial intermembrane space assembly; MIM: Mitochondrial import machinery; OXA: Oxidase assembly factor; PAM: Presequence translocase-associated motor; small Tims: i.e. Tim8-Tim13 and Tim9-Tim10-Tim12 intermembrane space complexes; TIM22: Translocase of the inner membrane 22; TIM23: Translocase of the inner membrane 23; TOB/SAM: Topogenesis of the mitochondrial outer membrane ß-barrel proteins/sorting and assembly machinery; TOM: Translocase of the outer membrane

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**Availability of data and materials**

*Acanthamoeba castellanii* strain Neff used in this study was delivered from American type Culture Collection (ATCC) with the number 30010.
The datasets generated and analyzed during the current study (alignments and trees) are available in the TreeBASE repository http://purl.org/phylo/treebase/phylows/study/TB2:521918
AC_RNAseq with the accession number DRA006231 are available in the DDBJ database http://trace.ddbj.nig.ac.jp/BSSE/search/
biosample?acc=SAHHDD00097225
All other data generated and analyzed during this study are included in this published article and its supplementary information files.

Authors’ contributions
HK, MW and WM came up with research ideas. HK and WM supervised the performed analyses. MW, HK and WM wrote the final version of the manuscript. DB carried out the material preparation for the sequencing procedure and bioinformatics analyses. MW and VS performed some of the bioinformatics analyses and MW provided the A. castellani total RNA. YS conceived the transcriptome sequencing. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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