An ectosymbiosis-based mechanism of eukaryogenesis

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Abstract

The findings of a deep branching Microsporidia clade on the SSU rRNA tree, and diversity of sequence motifs in eukaryotic Hsp70s rendered invalid the endosymbiosis-first theory that mitosome- and hydrogenosome-containing amitochondriate eukaryotes (AMIs) arose from mitochondriate eukaryotes (MTEs) via reductive evolution. Instead, evidence of widespread ectosymbioses indicated that eukaryogenesis was started by an archaeal parent via its acquisition of archaeal proteins through ‘accelerated gene adoption’, and bacterial proteins from ectosymbionts including a clostridial ectosymbiont that supplied its [Fe] hydrogenase and pyruvate:ferredoxin oxidoreductase genes to the AMIs. Subsequent endosymbiosis with *Tistrella* gave rise to mitochondria with the participation of other alphaproteobacteria. The high frequencies of top similarity bitscores displayed by *Giardia*, *Edhazardia* and *Trichomonas* toward *Aciduliprofundum boonei* (Abo) pertaining to the enzymes of DNA biology, far surpassing the frequencies toward any Asgard or TACK archaeon, established Abo as the source of these enzymes in eukaryotes, and the archaeal parent of Eukarya.

For over a century endosymbiosis has been regarded as the basis for eukaryogenesis, with recent focus placed on an archaeal parent-alphaproteobacterial endosymbiosis¹. It was pointed out based on the SSU rRNA sequence of *Vairimorpha necatrix* that Microsporidia branched very early from the other eukaryotes on the SSU rRNA tree². The evolution of MTEs from the AMIs was postulated by the ‘Archezoa’ theory based on the autogenous evolution of a primitive phagotrophic archezoan³, and by the ‘crown’ theory based on the locations of MTE branches above the AMI branches on the SSU rRNA tree⁴. However, the discoveries of typical mitochondrial proteins in the AMIs gave rise to the ‘endosymbiosis first’ theory that the presence of mitochondrial proteins in AMIs implies the harboring of mitochondria or their endosymbiotic ancestors by the AMIs in the past^{5,6}. As a result, the proposal of Lokiarchaeota being a suitable archaeal parent was held up by the missing mechanism for importing an alphaproteobacterial endosymbiont⁷, and the proposal of the *Aciduliprofundum* archaeons being appropriate archaeal parents would not be implementable if these small archaeons are required to accommodate an upfront endosymbiont⁸. In addition, searches have not yielded a consensual phylogenetic location for the Last Eukaryotic Common Ancestor (LECA)⁹⁻¹², and autogenous development of eukaryotes has not given a better fit to omics data than endosymbiosis¹³. In view of these difficulties, the present study has been directed to an investigation of the possibility of LECA formation through ectosymbiosis instead of endosymbiosis.

Primitivity of Microsporidia

Rejection of the primitivity of AMIs despite their low branchings on SSU rRNA trees stemmed from the supposition that their long branches may be due to an accelerated rate of evolution, making them susceptible to the artifact of long branch attraction¹⁴. However, the tree in Figure 1, built using the maximum likelihood method that minimized long branch attraction artifacts¹⁵, not only positioned the AMIs as low branchers, but also grouped the Microsporidia species into a clade.

The branches of the Amoebozoa AMI species Ein and Enh (see Table 1 for abbreviations of species names), which reductively evolved from Amoebozoa MTE species¹⁶, were indeed much longer than the MTE branches of Asu, Ddi and Dpu. However, despite their long branches, Ein and Enh were included in the same Amoebozoa clade as Asu, Ddi and Dpu on the tree. Both Mon which lost the mitochondria or mitochondria-related organelles (MROs) it once possessed¹⁷, and Cpa which has degenerate mitochondria¹⁸ also did not move to low-branching positions on the tree on account of their experience of reductive evolution. The nucleomorphs with their vestigial genomes likewise stayed with their ancestral red or green algae on the tree¹⁹. Accordingly, there was little basis to assume that the clade of nine AMI Microsporidia species had moved to a low branching position on the tree because of their reductive evolution from fungi²⁰, away from the modern fungal Sce, Spo, Lbi, Ncr, Mci, Bde and Spu sitting at the top of the tree. Among the Microsporidia, Ecu with its 2.9 Mb genome was much more reduced in size compared to some of the other microsporidians²¹, but it was located within the same clade as other microsporidians on either the SSU rRNA tree (Figure 1) or a tree for 53 concatenated proteins²². Therefore, the evidence for Microsporidia being an independently evolved AMI group rather than products of reductive evolution, in contradiction to any theory that requires the derivation of all AMIs from MTEs, was strong.

The motifs of eukaryotic Hsp70 sequences also rendered uncertain the evidence for the endosymbiosis-first theory founded on the affinities between mitochondrial proteins and their counterparts in the AMIs²³. Figure 2 shows the Group-I EKP/DKP/GKP and Group-II NGDAWV motifs, which were both absent from the archaeal sequences of Abo, Lok and Hgi but resembled bacterial sequences: the EKP-motif resembled that of Aba, the DKP-motif resembled those of Cur and Kae, and the GKP-motif resembled those of Mca and Eco. The NGDAWV-motif was found in not only the alphaproteobacteria Tis, Atu and Rru, but also the gammaproteobacterium Eco and the deltaproteobacterium Pel. Such diversified motifs in both AMIs and MTEs were in keeping with the profuse variety of prokaryotic proteins found in the proteomes of eukaryotes^{8,24-27}, but neither the diversity nor the apparent paucity or lack of the EKP motif in the MTEs was consistent with the origin of all MTE and AMI Hsp70s from a single alphaproteobacterial endosymbiont.

Ectosymbiotic mechanism of eukaryogenesis

In view of the unlikelihood of endosymbiosis-first, and the intrinsic compatibility of ectosymbiosis with AMI primitivity and Hsp70 diversity, an ectosymbiosis-based mechanism of eukaryogenesis was examined in Figure 3.

In *Stage 1* of this mechanism, bacterial ectosymbionts were attached to an archaeal parent. Enwrapment of part or whole of the ectosymbionts by the archaeal parent led to the influx of bacterial genes, proteins and metabolic products, whereupon bacterial ester-lipids

(blue) infiltrated the archaeal ether-lipid (red) membranes to form initially chimeric and later fully bacterial membranes. The archaeal host and the ectosymbionts worked together in *Stage 2* to develop the mitosome, which contributed protein presequences for organellar targeting²⁸ and took part in Fe-S cluster assembly²⁹. Upon departure of the ectosymbionts from the archaeal host, a mitosome-containing LECA/MSO emerged. It was suggested that an early membrane-curving module might play a role in the origin of nuclear pore complex and coated vesicles³⁰, in which case extra curvable prokaryotic membranes such as those of Abo³¹ would merit extensive investigation. One of the incentives for the formation of cell nucleus might be that archaeal introns were spliced by an endoribonuclease at RNA bulge-helix-bulge motifs. Without a nucleus, some mRNAs from bacterial ectosymbionts would risk misrecognition as introns by the archaeal enzyme and erroneously excised.

At *Stage 3* the archaeal host collaborated further with the ectosymbionts to replace the mitosome with hydrogenosome, and generated hydrogenosome-containing AMIs. The bioenergetic resourcefulness of the hydrogenosome was illustrated by the amitochondriate hydrogenosome-containing animal phylum *Loricifera*³². Further archaeal-bacterial cooperation produced alphaproteobacterial endosymbionts at *Stage 4*, which led to the formation of mitochondria and the MTEs. Thus the ectosymbiotic mechanism gives rise to an LECA that is AMI in nature at *Stage 2*, and the many ectosymbionts participating in *Stages 1-4*, far exceeding the ones illustrated in Figure 3, would readily account for the multiple bacteria-derived Hsp70 motifs in eukaryotes in Figure 2.

Prokaryotic proteins in eukaryotic proteomes

The eukaryotic proteomes were enriched with prokaryote-derived proteins. The bacterial/archaeal protein ratios of 0.73/0.85/1.09/1.26/1.32 for Eae/Gla/Trv/Sce/Dme respectively pointed to the diminishing proportion of archaeal proteins as evolution of the MTEs proceeded, possibly due to the greater functional scope offered by bacterial proteins relative to archaeal proteins. The proteomes of Per, Bbo, Pfa and Bho were more primitive in character than those of other MTEs based on their higher proportions of archaeal proteins (Figure 4).

The entry of prokaryotic proteins into eukaryotes could be mediated by a number of different mechanisms. While horizontal gene transfers (HGTs) might be limited by low rates of acceptance by the gene recipients³³, it was found recently that some archaea displayed an ‘accelerated gene adoption’ (AGA) phenotype detectible by the elevated levels of bacteria-derived proteins in their proteomes⁸. Moreover, the genes of the bacterial species Rpr, Bap, Ctr, Mpn, and Tpa furnished relatively few protein coding genes that were adopted by the AGA-active archaeons, and their proteins were also depleted in the proteomes of both Gla and Trv, demonstrating the key role played by AGA in controlling influx of bacterial genes into the eukaryogenic lineage. Also the paucity of AGA activity in bacteria would also render them much less effective than archaea in hosting eukaryogenesis owing to the importance of collection of exogenous genes by the host in this regard. Anaerobic Abo and its facultatively anaerobic companion species Acf, on account of their top average similarity bitscores among archaeons toward Gla and Trv proteomes and the PEP-utilizing enzyme mobile domain of Gla, along with their outstanding AGA activity, emerged as leading candidate archaeal parents⁸. When the DNA-biology enzymes/proteins belonging to the Cluster of Ortholog Groups (COG) for replication, recombination and repair³⁴ were analyzed with respect to the similarity bitscores displayed by primitive AMIs toward Abo and various Asgard and TACK archaeons

which included some attractive candidate archaeal parents^{8,35}, the frequencies of top similarity bitscores received by Abo from the primitive AMIs pertaining to the DNA-biology enzymes, viz. for 8/21 enzymes from Gla, 6/20 from Eae, 7/29 from Trv and 7/20 from Gin, far surpassed those of any Asgard or TACK archaeon tested (Figure 5a and Supplementary Figure S1). These results identified Abo as the major source of the DNA-biology enzymes in the genomes of all of these primitive AMIs, and therefore Abo as the archaeal parent of Eukarya.

It has been pointed out that, because different groups of present-day eukaryotes share few common features, the vertical inheritance between them remains an assumption³⁵. However, Figure 5b panels 1-8 show that a series of archaeal proteins including DNA-biology enzymes and eukaryotic signature proteins³⁶ (ESPs) maintained fairly steady similarity bitscores toward both AMIs and MTEs ranging from Microsporidia to Hsa, which attested to the vertical transmission of these proteins between eukaryotes, and their significance as part of the core of eukaryotic proteomes. Bho and its subtypes Bs1, Bs4, and Bs3 (no reported proteome and therefore not included in Figure 4), which were in possession of both hydrogenosome components and partial mitochondria³⁷, shared with the mitosome- and hydrogenosome-containing AMIs as well as the MTEs Bbo and Pfa the presence of Ssp-Tte-Hth proteins in their proteomes, thus pointing to a relationship between these firmicute species and the MRO-containing AMIs in keeping with the metabolic resemblance between hydrogenosome and *Clostridium*³⁸. This was confirmed in Figure 5b panels 9-10, where the MRO-containing AMIs other than Microsporidia exhibited substantial bitscores toward the [Fe] hydrogenase and pyruvate:ferredoxin oxidoreductase (PFO) of clostridial Tte. The low or zero bitscores exhibited by the Microsporidia could be the result of similarity erosion due to the extensive mutation of microsporidian proteins: Nbo was the only microsporidian in the 9-member clade on the SSU rRNA tree with any residual bitscore toward the PFO of Tte.

Previously, *Paracoccus* was found to branch near eukaryotes on the trees for the subunits of succinate dehydrogenase/fumarate reductase³⁹. The similarity bitscores of these subunits from Pde toward eukaryotes (Figure 5b panels 11-12) showed the expected absence of these subunits from the AMIs in contrast to their presence in a variety of MTEs. Comparison of panels 9-12 indicated that Ein and Enh exchanged their mitochondrial FrdB and SdhA for mitosomal [Fe] hydrogenase and PFO in their adaptation to anaerobiosis. The source of their [Fe] hydrogenase and PFO, be it AGA from *Clostridium*, or some ancestral AMI or Amoebozoa remains an open question.

When 151 different alphaproteobacteria together with some other bacteria were compared with respect to their similarity bitscores toward the mtDNA-encoded proteins of different eukaryotes including the bacteria-like Ram⁴⁰ (Figure 5c top panel; Supplementary Figure S2), the bitscores of Tis ranked the highest, followed by another Rhodospirillales Azo, suggesting that the endosymbiont precursor of mitochondria was either Tis or a close relative, in accord with the placement of the Tis branch next to the mitochondrial branch on the alphaproteobacterial tree⁴¹. However, a range of other alphaproteobacteria either contributed mtDNA-encoded proteins or showed overlapping similarities with Tis proteins; among the non-alphaproteobacteria, only Rso and Xca exhibited some modest bitscores. These findings were indicative of a shared genetic propensity of the alphaproteobacterial group for symbiosis with the eukaryotes, as in the case of the graded propensities of different strains of *Nostoc* for ectosymbiosis with *Anthoceros*⁴². Such shared propensity promoted symbioses between the eukaryotic lineage and multiple alphaproteobacterial species, thereby allowing entry of their genes into some eukaryotes to replace or recombine with the Tis-genes in mtDNA. Among human mtDNA-encoded proteins HM1-11, Ehrlichia and Rpr yielded the highest bitscores

toward HM2 and HM3 respectively (middle panel), suggesting that their proteins had replaced original Tis sequences. Since Ehrlichia and Rpr were both infectious for humans, the replacements might have occurred during infection. Replacements/recombinations also could be a factor in the exceptionally diverse sources of Blastocystis mtDNA-encoded proteins and host-related SSU rRNA subtypes⁴³.

Discussion

Because the 1.5 Mb archaeal parent genome of Abo was much smaller than for example the 11.2 Mb eukaryotic genome of Gla, recruitment of exogenous prokaryotic genes into the Abo genome was a top priority right from the start of eukaryogenesis. Too small to house an upfront endosymbiont, the archaial host had to depend on AGA and ectosymbiosis for acquisition of archaeal proteins from such prominent sources as Mco, Pfu, Mac, TACKs and Asgards into the AMIs (Figure 4). The modest numbers of bacterial proteins from Tis, Rru, Bja, Atu, Cvo, Cur, Rso, Aba, Xca, Pel, Dra, Tht, Bsu, Syn, Ssp, Tte, Hth, Cpo, Mau and Pmo detected in various AMIs pointed to gene acquisitions by the AMIs from these bacteria through ectosymbioses. The Eco proteins was a special case: they were present in modest numbers in the microsporidial Slo-Oco, but increased sharply from Ebi onward to Hsa. Thus Eco was likely an ectosymbiont with modest contribution of proteins at first, but became later either an endosymbiont, or an especially effective or sustained ectosymbiont with an enhanced influx. In any event, the presence of Eco proteins in both the AMIs and MTEs was consistent with the Eco-like *str-S10-spc-alpha* operons, as well as clustering and organization of *rps* and *rpl* genes in some mtDNAs⁴⁴.

The wide ranging influxes of prokaryotic genes through AGA and ectosymbiosis enabled the AMIs to expand in genome size, and in cell volume akin to the development of giant cell size by the archaeon *Gigantothaum karukerense* through ectosymbiosis with a gammaproteobacterium⁴⁵. The convergence of enlarged cell volume, an expanded genome with ESPs from Asgards, TACKs and other prokaryotes, and an exceptionally flexible cell membrane that could enwrap symbionts with its blebs³¹ equipped the AMIs for the development of phagocytosis^{46,47}. This resulted in endosymbioses with Tis marked by a flood of Tis proteins that greatly exceeded the protein influxes from various ectosymbionts; and still later with a cyanobacterium marked by a comparable flood of Syn-like proteins into the algae Tps, Esi, Bpr, Gth and Cme. These endosymbioses gave the MTEs the twin biofuel engines of oxidative phosphorylation and photosynthesis that drove their relentless expansion for the next two giga years. In addition, the Rru, Bja, Atu, Cvo, Pde, Cur, Rso, Aba, and Xca proteins all grew to pronounced levels in the MTEs that signaled their endosymbioses or highly effective/sustained ectosymbioses with the eukaryotes. Notably, the mtDNA-encoded proteins of numerous eukaryotes notched higher bitscores toward Tis than Pde, Bja, Bsu, Filomicrobium and Halocynthiibacter (Figure 5c top panel), whereas the nuclear encoded PycA, NirB, AceF, AHP1 and FMR2 of some eukaryotes notched higher bitscores toward Pde, Bja, Bsu, Filomicrobium or Halocynthiibacter than Tis (bottom panel). Therefore the eukaryotes plausibly formed separate symbioses with Tis, Pde, Bja, Bsu and others; while the Tis-endosymbiont served as precursor to mitochondria, the symbioses with Pde, Bja, Bsu and others introduced a variety of useful proteins into the eukaryotes.

With respect to the ectosymbiosis-based mechanism (Figure 3), the role of Abo as archaeal parent and the presence of numerous ectosymbionts detected in Figure 4 confirmed the postulated interactions of archaeal parent with ectosymbionts in various stages starting from

Stage 1. The contributions of [Fe] hydrogenase and PFO by Tte confirmed the postulate of archaeal-ectosymbiont collaborations to build mitosome and hydrogenosome in *Stage 2* and *Stage 3* respectively. Identification of a Tis-endosymbiont as precursor of mitochondria confirmed the postulate of archaeal-alphaproteobacterial endosymbiosis in *Stage 4*. Clearly, a horde of micron-size prokaryotes generated the Eukarya domain with their genes through accelerated gene adoption, ectosymbiosis and endosymbiosis, and all four stages of the ectosymbiotic mechanism were verified by fluxes of prokaryotic protein-coding genes into the eukaryotes⁴⁸.

The unique crown-capped structure of the SSU rRNA tree⁴ stands as a monument to the cataclysmic effects of oxygen on eukaryotic evolution. That Abo and the lowest branching AMIs were anaerobic suggests that eukaryogenesis started in the anoxic Archean Eon. Oxygen-producing cyanobacteria evolved at or before 2.7 Gya, providing an atmosphere containing a trace amount of free oxygen that persisted up to 2.4 Gya. It made possible sterol biosynthesis in eukaryotes, leaving sterane footprints of eukaryotes in 2.7-Gya rocks even though it fell far short of supporting aerobic metabolism⁴⁹. The increased levels of oxygen during the Great Oxidation Event at about 2.4 Gya in early Proterozoic Eon was a grave hazard to the anaerobic bacteria, archaea and eukaryotes, for the incongruence between enzyme distribution in aerobic metabolic pathways and organismal speciation also indicated that all three domains of organisms were already formed at that juncture. In response to the rising oxygen, all the surviving species from that era built oxic metabolic networks in addition to their earlier anoxic ones⁵⁰. Among the species on the eukaryotic SSU rRNA tree, which was by necessity an all-AMI tree stemming from an amitochondriate LECA, the lowest branching species with their limited resources probably built oxic networks that were little more than 3-4% their anoxic networks as exemplified by the anaerobic prokaryotes Tma, Mka and Tte. As a result, these lowest branchers remained as AMIs, were saddled with reduced competitiveness, and had to choose between parasitism and confinement to anaerobic niches. Their long branches on the present-day tree could be indicative of their arduous evolutionary journeys to stay alive. Higher up on the tree, Pfa built an oxic network equal to about 5% of its anoxic one and managed to possess mitochondria, but still ended up as a parasite. In contrast, the elaborate species at the top of the tree acquired mitochondria and built large oxic networks greater than 20% of the anoxic network in Sce, 30% in Cel, 40% in *Arabidopsis thaliana*, or 50-60% in the mammals. Endowed with large oxic networks and plentiful ATP from oxidative phosphorylation, they launched into adaptive radiation to form the spikes on the crown of the tree. The accomplishments of the Hsa lineage began when it became one of the spikes.

Methods

Source of Data and Materials

Protein sequences were retrieved from NCBI GenBank release 231 (ftp://ftp.ncbi.nlm.nih.gov/genomes/)⁵¹. Assemblies of species analyzed were given in Supplementary Table S1. Quality checked SSU rRNA sequences were downloaded from the SILVA database release 132 (<https://www.arb-silva.de/>)⁵². The accession numbers of SSU rRNAs analyzed were available in Supplementary File S1. Eukaryotic mitochondrial DNA-encoded protein sequences were retrieved from the RefSeq mitochondrial reference genomes in the NCBI Protein database (<https://www.ncbi.nlm.nih.gov/protein>)⁵³.

265 ***Maximum-likelihood SSU rRNA tree***

266 The SSU rRNAs of 47 eukaryotes and 3 archaeons (Abo, Mja, Lok) as outgroup were aligned
267 in Clustal Omega⁵⁴, and the tree was constructed using RAXML with an GTR+GAMMA+I
268 model, with 1000 bootstraps⁵⁵. The final maximum-likelihood tree with bootstrap support
269 values is available in Supplementary File S2.

270 ***Hsp70-type Sequences***

271 The proteome of each species analyzed was searched against the database of Clusters of
272 Orthologous Groups of proteins (COG)³⁴ using RPSBLAST⁵⁶ at a threshold set by the ‘-evalue’
273 flag at 0.01. Potential Hsp70 sequences in each species were identified as those mapped to
274 COG0443 (Molecular chaperone DnaK or Hsp70). Among the potential Hsp70 sequences, one
275 sequence was manually selected, if available, for each of the Group I _KP motif and the Group
276 II NGDAWV motif. See Supplementary Table S2 for sequence accession numbers.

277 ***Estimation of Nuclear or Mitochondrial Proteome Similarity Bitscores***

278 When comparing proteome similarities for Figures 4 and 5c top panel, the proteomes of all
279 subject species were used to construct a local BLAST database using makeblastdb⁵⁶, and every
280 query proteome was searched against the local database using BLASTP with a BLOSUM62
281 matrix and thresholds setting to e-value < 1x10⁻⁵, percent identity > 25% and query coverage >
282 50%. Only the query and subject sequences that were the best-match of each other, viz. when
283 query sequence *n* from species 1 exhibited the highest bitscore toward subject sequence *m*
284 among all proteins of species 2 and vice versa, were included in the estimation of inter-
285 proteome similarity, which was given by the sum of BLASTP bitscores of all such best-
286 matched proteins between the two proteomes. The data for nuclear and mitochondrial proteome
287 similarities is given in Supplementary Tables S3 and S4a respectively. The bacterial/archaeal
288 protein ratio (*R*) was estimated as $R = \sum_1^i N_b / \sum_1^j N_a$, where *i* was the number of proteins in a
289 eukaryotic proteome that could be blasted to the proteins of any of the 33 bacterial species
290 analyzed in Figure 4; and *N_b* was the highest bitscore so obtained. Likewise, *j* was the number
291 of proteins in the same eukaryotic proteome that could be blasted to any of the also 33 archaeal
292 species; and *N_a* was the highest bitscore so obtained.

293 ***Similarity of COGs between eukaryotes and prokaryotes***

294 Best-matched query and subject sequences passing BLASTP thresholds of e-value < 1x10⁻⁵,
295 percent identity > 25% and query coverage > 50% that were mapped to the same COG using
296 RPSBLAST at a threshold of e-value < 0.01 furnished data for Figures 5a, 5b and 5c middle
297 and bottom panels. For Figure 5b, the bitscores of 10 out of 196 protein pairs that did not pass
298 the percent-identity or query-coverage thresholds but showed BLASTP e-values < 1x10⁻¹⁰ were
299 also included (see Supplementary Table S5 for the e-values, percent identities and query
300 coverages for Figure 5b).

301 ***Data availability***

302 All the data generated in this study were included in the supplementary information files.

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Acknowledgements

The authors would like to acknowledge the University Grant Council for funding support to Applied Genomics Center [No. 5112-703-0110D-42002].

Author contributions

J.T.W. and H.X. conceived the study; X.L. collected the data and performed computational analysis; and J.T.W., X.L. and H.X wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that there is no conflict of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Partial list of species analysed.

ABBR.	SPECIES NAME	ABBR.	SPECIES NAME
ARCHAEA		BACTERIA (CONTINUED)	
Abo	<i>Aciduliprofundum boonei</i>	Spn	<i>Streptococcus pneumoniae</i>
Acf	<i>Aciduliprofundum</i> sp. MAR08-339	Ssp	<i>Sporanaerobacter</i> sp. NJN-17
Afu	<i>Archaeoglobus fulgidus</i>	Syn	<i>Synechocystis</i> sp. PCC 6803
Aia	<i>Acidilobus</i> sp. 7A	Tht	<i>Thermobaculum terrenum</i>
Alt	<i>C.Altiarchaeales archaeon</i>	Tis	<i>Tistrella mobilis</i>
Ape	<i>Aeropyrum pernix</i>	Tma	<i>Thermotoga maritima</i>
Bat	<i>C.Bathyarchaeota archaeon</i>	Tte	<i>Thermoanaerobacter tengcongensis</i>
Csu	<i>C.Caldiarchaeum subterraneum</i>	Xca	<i>Xanthomonas campestris</i>
Csy	<i>Cenarchaeum symbiosum</i>	EUKARYA	
Fac	<i>Ferroplasma acidiphilum</i>	Aal	<i>Anncaliia algerae</i>
Ffo	<i>Fervidococcus fontis</i>	Acc	<i>Acanthamoeba castellanii</i>
Hal	<i>Halobacterium salinarum</i>	Ago	<i>Andalucia godoyi</i>
Hei	<i>C.Heimdallarchaeota archaeon</i>	Asu	<i>Acytostelium subglobosum</i>
Hgi	<i>Haloferax gibbonsii</i>	Bbo	<i>Babesia bovis</i>
Hla	<i>Halobiforma lacisalsi</i>	Bde	<i>Batrachochytrium dendrobatidis</i>
Kcr	<i>C.Korarchaeum cryptofilum</i>	Bho	<i>Blastocystis hominis</i>
Lok	<i>Lokiarchaeum</i> sp. GC14 75	Bpr	<i>Bathycoccus prasinos</i>
Mac	<i>Methanosarcina acetivorans</i>	Bs1	<i>Blastocystis</i> sp. subtype 1
Mbo	<i>Methanoregula boonei</i>	Bs3	<i>Blastocystis</i> sp. subtype 3
Mco	<i>Methanocella conradii</i>	Bs4	<i>Blastocystis</i> sp. subtype 4
Mes	<i>C.Methanosuratus</i> sp.	Cel	<i>Caenorhabditis elegans</i>
Min	<i>C.Methanomassiliicoccus intestinalis</i>	Cme	<i>Cyanidioschyzon merolae</i>
Mja	<i>Methanocaldococcus jannaschii</i>	Cne	<i>Cryptococcus neoformans</i>
Mka	<i>Methanopyrus kandleri</i>	Cpa	<i>Cryptosporidium parvum</i>
Mnt	<i>Methanonatronarchaeum thermophilum</i>	Ddi	<i>Dictyostelium discoideum</i>
Mph	<i>Methanophagales archaeon</i>	Dme	<i>Drosophila melanogaster</i>
Mte	<i>C.Methanoplasma termitum</i>	Dpu	<i>Dictyostelium purpureum</i>
Nca	<i>C.Nitrosocaldus cavascurensis</i>	Dre	<i>Danio rerio</i>
Nga	<i>C.Nitrososphaera gargensis</i>	Eae	<i>Edhazardia aedis</i>
Odi	<i>C.Odinarchaeota archaeon</i>	Ebi	<i>Enterocytozoon bienersi</i>
Pae	<i>Pyrobaculum aerophilum</i>	Ecu	<i>Encephalitozoon cuniculi</i>
Pfu	<i>Pyrococcus furiosus</i>	Ein	<i>Entamoeba invadens</i>
Psy	<i>C.Prometheoarchaeum syntrophicum</i>	Enh	<i>Entamoeba histolytica</i>
Sso	<i>Saccharolobus solfataricus</i>	Esi	<i>Ectocarpus siliculosus</i>
Tac	<i>Thermoplasma acidophilum</i>	Gin	<i>Giardia intestinalis</i>
Tho	<i>C.Thorarchaeota archaeon</i>	Gla	<i>Giardia lamblia</i>
Tvo	<i>Thermoplasma volcanium</i>	Gth	<i>Guillardia theta</i>
Woa	<i>C.Woesearchaeota archaeon</i>	Has	<i>Homo sapiens</i>
BACTERIA		Imu	<i>Ichthyophthirius multifiliis</i>
Aae	<i>Aquifex aeolicus</i>	Lbi	<i>Laccaria bicolor</i>
Aba	<i>Acinetobacter baumannii</i>	Mbr	<i>Monosiga brevicollis</i>
Atu	<i>Agrobacterium tumefaciens</i>	Mci	<i>Mucor circinelloides</i>
Azo	<i>Azospirillum</i> sp. M2T2B2	Mon	<i>Monocercomonoides</i> sp. PA203
Bap	<i>Buchnera aphidicola</i>	Mpa	<i>Marchantia paleacea</i>
Bja	<i>Bradyrhizobium japonicum</i>	Nbo	<i>Nosema bombycis</i>
Blo	<i>Bifidobacterium longum</i>	Ncr	<i>Neurospora crassa</i>
Bsu	<i>Bacillus subtilis</i>	Ngr	<i>Naegleria gruberi</i>
Cex	<i>Caldisericum exile</i>	Oco	<i>Ordospora colligate</i>
Cje	<i>Campylobacter jejuni</i>	Per	<i>Perkinsella</i> sp. CCAP 1560/4
Cpo	<i>Cloacibacillus porcorum</i>	Pfa	<i>Plasmodium falciparum</i>
Cur	<i>Curvibacter</i> sp.	Pte	<i>Paramecium tetraurelia</i>
Cvo	<i>Chelativorans</i> sp. BNC1	Ram	<i>Reclinomonas Americana</i>
Dra	<i>Deinococcus radiodurans</i>	Sap	<i>Saprolegnia parasitica</i>
Dth	<i>Dictyoglomus thermophilum</i>	Sce	<i>Saccharomyces cerevisiae</i>
Eco	<i>Escherichia coli</i>	Slo	<i>Spraguea lophii</i>
Hth	<i>Hungateiclostridium thermocellum</i>	Spo	<i>Schizosaccharomyces pombe</i>
Kae	<i>Klebsiella aerogenes</i>	Spu	<i>Spizellomyces punctatus</i>
Kol	<i>Kosmotoga olearia</i>	Sra	<i>Strongyloides ratti</i>
Mau	<i>Mahella australiensis</i>	Ssa	<i>Spiroplasma salmonicida</i>
Mca	<i>Macroccoccus caseolyticus</i>	Tbr	<i>Trypanosoma brucei</i>
Mtu	<i>Mycobacterium tuberculosis</i>	Tgo	<i>Toxoplasma gondii</i>
Pde	<i>Paracoccus denitrificans</i>	Tps	<i>Thalassiosira pseudonana</i>
Pel	<i>Pelobacter</i> sp. SFB93	Trh	<i>Trachipleistophora hominis</i>
Pmo	<i>Petrotoga mobilis</i>	Trv	<i>Trichomonas vaginalis</i>
Rpr	<i>Rickettsia prowazekii</i>	Tth	<i>Tetrahymena thermophila</i>
Rru	<i>Rhodospirillum rubrum</i>	Ttr	<i>Thecamonas trahens</i>
Rso	<i>Ralstonia solanacearum</i>	Vcu	<i>Vavraia culicis</i>

432 Note: C. in front of species name stands for Candidatus. Detailed species information is given in
433 Supplementary Table S1.

Figure 1. Maximum-likelihood SSU rRNA tree. Eukaryotes with mitochondria, and also Mon, are labeled in blue; Microsporidia in pink; non-Microsporidia eukaryotes with mitosome in red; eukaryotes with hydrogenosome in orange; eukaryotes with MRO and part of mitochondria in green; and an archaeal outgroup in black. Nodes with > 90% bootstrap support are represented by black circles, > 70% grey circles, > 50% open circles, and ≤ 50% without circle.

Hsp70 I	KRLIGR	-KP	KMK
Gin I	DAKRLIGRRFNDPEVQADLKHFPPKVICGPD	EKPQIQVYKGETKTF	SPEEISSMVLTKMK 90-150
Gla I	DAKRLIGRRFNDPEVQADLKHFPPKVICGPD	EKPQIQVYKGETKTF	TPEEISSMVLTKMK 69-129
Aba	DAKRLIGRRFSDPTVQNDMLKWP	PKVISGPAEKP	MIVVTKGEEKQFAAEEISSMVLTKMK 3-63
Ein I	DAKRLIGRRFSDPAIQEDMKHWSFTVVDN	GNDKPLIQVEYKGETKKFT	TPEEISSMVLTKMK 71-131
Enh I	DAKRLIGRRFSDPAIQNDMKHWSFKVIDD	GHDKPLIEVEYKGEVKKFT	TPEEISSMVLTKMK 71-131
Cpa I	DAKRLIGRKFDQAVQSDMTHWPFKVV	RGPDKDPIISVNYLGEKKEFHAE	EISSAMVLQKMK 80-140
Ssa I	DAKRLIGRRFSEADVQDDMKHFPFTVVG	-VDDKPMIEVVYKGETKRF	TPEEISSMILLKMK 68-127
Ngr I	DAKRLIGRKFTDSSVQSDMKHWP	FKVITKGGDKPYIQVEYKNETHT	FTTPEQVSSMVLTKMR 71-131
Ddi I	DAKRLIGRKFSDSVQSDMKHWP	FKVITNDDKPLIQVDFKGETKTF	SPEEISSMVLTKMK 66-127
Acc I	DAKRLIGRNFSPIVQADMKHWP	FKVVQKPGDKPIISVEYKGETKEF	SPEEISSMVLTKMK 69-129
Lma I	DAKRLIGRKFNDSVQSDMKHWP	FKVITKGGDKPVISVQYRGEEK	FTTPEEISSMVLTKMK 69-129
Bsl I	DAKRLIGRHFSQIVQNDLKHWPF	FKLIADKNDKPIIEVTFMGKTQOYT	CEQISSMVLTKMK 74-134
Bs4 I	DAKRLIGRKFSDAIVQNDLKHWPF	FKLIPDKFDKPIIEVQYLGKTKQF	TCEQISSMVLTKMK 74-134
Pte I	DAKRLIGRKFSSTVQDILKLWPF	FKVEAGADKPIIVVKYKGENKKFH	PTEEISSMVLTKMK 69-129
Cur	DAKRLIGRKFSDSVQADAKHWP	FKVITRGGDKPLIEVTFKGETKQF	APEEVSSMVLTKMK 74-134
Kae	DATRLIGRRFTDASVQSDMKLWPF	FKVLPGPGDKPMIVVNYKGEKQF	SAEEISSMVLTKMR 1-61
Bho I	DAKRLIGRKFDASVQSDMKHWP	FTLKRGP	EGKPVIEVEYKGERKQFSPEEISSMVLTKMK 72-132
Esi I	DAKRLIGRKFTDPAVASDKHWP	FSVVAGTGGKPTIEVSFKGDKKQF	APEEISSMVLTKMK 119-179
Hsa I	DAKRLIGRKFDATVQSDMKHWP	FRVVS-EGGKPKVQVEYKGETKTF	FPTEEISSMVLTKMK 70-129
Mca	DAKRLIGRKFDATVQADMKHWP	FEVVS-DGGKPKIKVSYKGEDKTF	FPTEEISSMVLTKMK 71-130
Eco	DAKRLIGRKFDGSGVQSDMKHWP	FKVVNAGGGKPKVQVEYKGETKTF	FPTEEISSMVLTKMK 68-128

Hsp70 II	KRLIGR	NGDAWV	KMK
Gin II	ASKRLIGRRFTDAEVKRAAKHVS	YDIVQGPSGEAMINVPNL--QKAI	SPIEVGSEVLKYIK 77-135
Gla II	ASKRLIGRRFTDAEVKRAAKHVS	YDIVQGPNGEAMINVPNL--HKS	VSPIEVGEILKYIK 75-133
Ecu II	ASKRLIGRKFGDPEVEKYVGLP	FDTMSHCNGDVWIRV-D---	GKKYSPAQIGAFVLSKMK 78-134
Nce II	ASKRLIGRKFGDVELKDYLSL	PDYDTTKHCNGDIWIKI-D---	NKKYSPAQIGAFVLSKMK 73-129
Enh II	GVKRMIGCQYKNVEQ---	QRKPYKIVEGRNGEGWIYI-N---	GKTYSPSEISSFILKMK 74-126
Ein II	HAKRLIGIKYDQVTP---	FMRQGYTIKGPNDAWVEV-N---	GVKYSPOQISAEVLSKMK 74-127
Cpa II	ATKRLIGRRYEEEAIEKEQ	GILPYKIVRADNGDAWVEA-R---	GERYSPSQIGAFVLEKMK 117-173
Trv II	ATKRLIGRSFEDEMTKKCREM	VPYQIIKAKNGDAWVKDEK---	GNEYSPAQIGAFVLSKMK 88-145
Ngr II	AIKRLIGRRFDDPMTKRDME	IVPYDIIRGPNDAWVRA-G---	GKDLSPSQVGAFLQKMK 93-149
Ddi II	ATKRLIGRRFDDPMTKDMTM	VPYKIVKGNDAWVEV-K---	GKMISSPEAGAMVLQKMK 96-152
Lma II	AVKRLIGRRFEDEHIQDKIK	NVPYKIVRAGNGDAWVDGN---	GKQYSPSQIGAFVLEKMK 92-149
Acc II	AVKRLIGRDFNDPMTQDKLM	VPYKIVRHSNGDAWLEDSE---	GKKYSPSEISAFITLTKMK 107-164
Bho II	AAKRLIGRSYDDPEVAIKIK	LVPYIEIKGNDGDAWVSA-R---	GQKMSPSQIGSMVLTKMK 73-129
Bsl II	AAKRLIGRSYDDPEVAIKIK	LVPYIEIVKGNDAWVQV-R---	GQKMSPSQIGSMVLTKMK 98-154
Bs4 II	AAKRLIGRPSYDPEVEIKIK	LVPYIEIVKGNDAWVSV-R---	GQKMSPSQIGSMVLTKMK 98-154
Esi II	ATKRLIGRRFKDKEVQGVQ	KLVPYKIVESQDGAAWVEV-Q---	GNKMSPSQVGSMLTKMK 113-169
Pte II	ATKRLIGRRFDDPNVQDKI	HLSSVVRQNGDAWVSLKS---	GQTYSPSQIGAFVLSKMK 94-151
Hsa II	ATKRLIGRRYDDPEVQDKIK	NVPYKIVRASNGDAWVEA-H---	GKLYSPSQIGAFVLSKMK 119-175
Tis	AVKRLIGRTFNDPTQKDAK	MVPYRIVKADNGDAWVEV-R---	GKKQSPSQISAFILKMK 68-124
Atu	AVKRLIGRRYEDPTVEKD	KALVPYIEIVKGNDAWVKA-Q---	DKNYSPSQISAMILQKMK 68-124
Rru	AIKRLIGRRYSDPTVEKD	KLVPYRIVKGNDAWVQT-R---	DEKYAPSQSAFILQKMK 68-124
Eco	AIKRLIGRRFQDEEVQ	RDVSIMPFKIIAADNGDAWVEV-K---	GQKMAPPQISAEVLSKMK 68-124
Pel	AIKRLVGRKFDSDAVRK	DIETSPFKIINADNGDAWVEA-R---	GKKYSPPEISAMILQKMK 68-124

Archaea DnaK			
Abo	AFKRKMGTDYKY-----	KIY---	GKEYTPQQLSAFLLQKIK 73-105
Lok	AIKRKMGTSYKV-----	KID---	GKEYTPQELISAMILRKIK 72-104
Hgi	SIKRHMGE-----	GYSVDIE---	GEEYTPQELISAMILQKIK 70-103

Figure 2. Hsp70 sequence motifs. Group-I sequences with EKP/DKP/GKP motifs and Group-II sequences with NGDAWV motif in eukaryotic Hsp70 sequences and prokaryotic DnaK sequences were aligned using the KRLIGR and KMK motifs at the 5' and 3' ends of segment as positional markers. The numbers on the right indicate the positions of amino acid residues of each segment within its Hsp70 or DnaK sequence. Names of eukaryotes are colored as in Figure 1, and names of prokaryotes are colored black. The arrow indicates the position of an additional amino acid residue on the Ddi I sequence.

Figure 3. Ectosymbiotic mechanism of eukaryogenesis. Stages 1-4 are developmental stages. Ellipse enclosed by dashed line represents cell nucleus. The membranes of archaeal parent are colored red and bacterial membranes colored blue. Solid triangles are mitosomes, and solid squares hydrogenosomes. MSO, HYD and MTE represent mitosome-containing, hydrogenosome-containing and mitochondria-containing eukaryotes. The letters T, E, U, B, J, P, R and S stand for Tte, Eco, Rru, Bsu, Bja, Pde, Rso and Tis respectively, representing a small fraction of the bacteria taking part as ectosymbionts in the mechanism. Solid ellipses denote endosymbionts, and ellipses with cristae mitochondria.

Figure 4. Heat map of inter-proteome similarity bitscores between eukaryotes (y-axis) and prokaryotes (x-axis). For each eukaryotic proteome, its similarity bitscores toward different prokaryotes were scaled linearly from 0 to 1.0 on the thermal scale, with zero (blue) marking the minimum, and 1.0 (red) the maximum bitscores. The names of different eukaryotes are colored as in Figure 1; and the colour bands on the x-axis indicate different groupings of prokaryotic species. The raw bitscores for the heat map are shown in Supplementary Table S3.