

**The Three Domains of Life:**

**A Challenge to the concept of the Universal Cellular Ancestor?**

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## **Abstract:**

With the discovery of the uniqueness of Archaeobacteria in rRNA sequence and by comparative studies with well-characterized molecular systems, cell walls, lipid compositions and features of the transcriptional and translational machineries, the three domains of life, namely Archaea, Bacteria and Eukarya, has become the currently accepted paradigm in the field of molecular taxonomy. Sequence analyses based on functional proteins across the three domains also suggest each of the three domains as independent monophyletic lineage representing ribosomal, metabolic, biosynthetic proteins as well as the replicational, transcriptional and translational machineries. Current view suggests that the universal tree of life branched from the universal ancestor in separate lineages leading to Bacteria and Archaea, the latter then diverged into Eukarya. The search for the universal ancestor has led to postulating a universal communal gene pool (*progenotes*) in which lateral or horizontal gene transfer (HGT) played the most important role in diversification since the three domains of life are resistant to HGT after they have crystallized into cellular communities. This scenario challenges the concept of the Universal Cellular Ancestor and may be open to alternative views based on design.

## **Introduction:**

In the age of genomics when entire genome sequences of organisms are known, molecular taxonomy has become a dominant way by which phylogenetic relationship of organisms are analyzed. Under the prevailing Darwinian paradigm of Common Descent, sequence comparison is supposed to give evidence of the nature and history of evolutionary lineages. Similar protein sequences across taxa presumably are results of a common universal ancestor. Microscopic characteristics have classified the living world into the two primary domains of Eukaryotes (*Eukarya*) and Prokaryotes (*Bacteria*) (Murray, 1968).

Eukaryotes and Prokaryotes differ from each other mainly on the basis of the presence or absence of the membrane-bound nucleus as well as characteristics of other cellular organelles (Murray, 1968)(Table 1).

Woese and coworkers proposed a third domain of life based on the studies of a heretofore poorly known

group of prokaryotes, the archaeobacteria(*Archaea*). From the identification of signature sequences on the 16S ribosomal RNA, which are distinctive in eukaryotes, prokaryotes and archaeobacteria, the third domain *Archaea* was proposed (Woese, 1987, 1992)

Additional evidence of the *Archaea* distinctions was also found in comparative studies with well-characterized molecular systems, cell walls, lipid compositions and features of the protein synthesis machinery (Woese, 1987). A summary of the characteristics of the three domains of life can be seen in Table 2.

The Three Domain paradigm was challenged by other sequence analyses and the morphological characterization of cellular envelop of gram negative and gram-positive bacteria. The former are surrounded by an external and an internal membrane (*diderm*) and while the latter, one membrane (*monoderm*). (Gupta, 1998a, 1998b). From the universal ancestor, a lineage of monoderm prokaryotes (i.e. gram positive bacteria and archaeobacteria) and diderm prokaryotes (i.e. gram negative bacteria) diverged. Eukaryotic nucleus then developed by the fusion event between an archaeobacterium of the *Crenarachaeota* division and a gram-negative bacterium. Gupta's hypothesis has been criticized since it also requires gene transfer to explain conflicting phylogenies.(Doolittle, 1999a). One question that remains is whether lateral gene transfer is truly a major component of genome evolution. The examples of lateral gene transfer that are published in the literature often involve specific, isolated lineages, such as the occurrence of glutaminyl-tRNA synthetase in certain bacterial groups (Doolittle and Handy, 1998). In contrast to isolated incidents, rampant gene transfer should abolish our ability to recognize coherent evolutionary lineages. The present study will contribute to this discussion by evaluating the integrity of the three domains as coherent phylogenetic units. The monophyletic nature of the domains as well as relationships between the three domains within functional classes of proteins will be examined.

#### **Sequence Analysis of functional proteins across taxa:**

We have attempted to verify the three-domain hypothesis by examining the databases of available protein sequences for sequence alignment. Functional protein sequences instead of nucleic acid sequences were chosen because of its importance in the extant organisms to bypass the necessity to assign functions to obscure genomic sequences. The criteria being used for these analyses were: 1. The protein must be functional for the whole cell. Thus, with very few exceptions, we excluded putative sequences as well as proteins from mitochondria or chloroplasts. 2. Diverse proteins were selected with at least 4 species represented in each of the three domain. Gram positive and gram-negative bacteria, plants and animals were selected when available. 3. High degree of similarities as inferred from the BLAST scores (Altschul, et.al. 1997). Of these 68 protein families, Brown and Doolittle (1997) and Doolittle and Handy (1998) list 20 (30%) as families in which gene transfer is thought to have occurred. Thus, if gene transfer is widespread, we should observe those transfer events in at least 30% of our protein families. Since the complete genome of the Archaea *Methanococcus jannaschii* is known (Bult, et al), its entire protein database was evaluated for potential use. After examining closed to 5,000 and tracing thoroughly 250 proteins on the basis of similarities by using the search engine of BLAST, 68 proteins fulfilling the above criteria were further analyzed. The sequences were aligned using CLUSTALW (Thompson, et.al. 1994)) and the programs NEIGHBOR JOINING DISTANCE (NJ) (which is based on the number of estimated nucleotide differences separating two proteins) (Felsenstein, 1995) and the most recently updated version of PAUP (Swofford, 1996). Similar results were also obtained by using the program PROTEIN PARSIMONY (PROTPARS)(which is the exact number of nucleotide replacements that are minimally necessary to bring about the difference in amino acid sequence between 2 species) (Felsenstein, 1995).

For each protein analyzed, alignments comprised four sequences from the same domain, or a systematic aligning of 3 sequences from one domain (ingroup) with 1 sequence from a different domain (outgroup), such that every possible alignment and analysis of the eukaryotic, bacterial, and archaeal sequences was performed accordingly. A total of 99 permutations were carried out in the analysis of a single gene family, i.e. 3 ingroups only, 32 each of ingroup/outgroup using 3 sequences each of the 3 domains as ingroups and systematically assorting with one sequence each of the remaining 2 domains as outgroups. For statistical analysis of the ingroup vs. the outgroup results, we employed the power primer *effect size*

or Cohen's  $d$  (Cohen, 1992). It expresses the difference in units of population standard deviations. For example, in 3A1B, a NJ or PAUP tree of the sequences of each protein is generated from 3 Archaea and 1 representative of the Bacteria. The *differences* of the average distances or the parsimony steps between the outgroup and ingroup divided by their respective standard deviations are reported as Cohen's  $d$ . To evaluate the 68 proteins (70 assortments of sequences including two each of Inosine 5' monophosphate dehydrogenase and Argininosuccinate synthetase) for groupings,  $d$  of the parsimony and neighbor joining results of 0.5 and above (although most are much larger) are assumed to have practical significance. Several surveys suggested that Cohen's  $d$  of 0.5 approximates the average size of observed effects in the fields to establish separate lineages. (Kirk, 1999) Separate lineages are represented as hyphens (i.e. B-A-K). Ambiguities amongst the groupings are defined as those that have  $d$ 's of less than 0.5. Repeated analyses of the same genes using other species within the same domain yielded similar results. (i.e. Inosine 5' monophosphate dehydrogenase and Argininosuccinate synthetase)

Three subsets of analyses for each protein on NJ and parsimony were performed (see Tables 3 and 4 respectively). Of the 420 total analyses, 85.95% conformed to grouping into three monophyletic domains, B-A-K (Table 5). 9.29% of the analyses do not distinguish between the two domains of AK, BA and BK enough to group them into separate lineages, i.e. B-(AK), (BA)-K and (BK)-A. Only 4.76% of the analyses fail to distinguish the 3 domains individually (i.e. BAK). The NJ and parsimony methods yielded slightly varying results as expected (Table 6). Additionally, the parsimony and NJ results could yield conflicting relationships between the domains (i.e. Seryl-tRNA synthetase, RPS 13, RPS15, etc.) (Tables 3 and 4). However, with the Bonferroni adjustment with three planned comparisons, there are no significant differences amongst the  $d$ 's for all of the two ingroup/outgroup columns as estimated by the paired t-test ( $p > 0.017$ ) (See legend d in Tables 3 and 4). Repeated analyses of the same genes using other species within the same domains yielded similar results.

The usual assumption in molecular phylogenetic studies is descent from a common organismal ancestor. If correct, genetic lineages should converge in terms of protein similarity within protein classes depending on evolutionary divergence points. But in situations such as the endosymbiotic capture, lateral

gene transfer, or chimeric fusion, phylogenies of different genes should yield conflicting results. In our study, the proportion of groupings representing three monophyletic lineages was exceedingly high in comparison with all other combinations of groupings, implying that gene transfer after the divergence of the three domains is a low frequency phenomenon in comparison with inheriting genes from organismal ancestors. The robust nature of the grouping (both overall and within the various protein classes) representing three monophyletic domains suggests three independent lineages with little or no genetic transfer. Individual exceptions to this statement should and do exist (Brown and Doolittle, 1997, Doolittle and Handy, 1998), but the overall pattern is one of vertical inheritance rather than horizontal transfer.

Similar to the work of Jain et al. (1999) and others, our results show a slightly higher incidence of anomalous phylogenies among the metabolic genes than among the informational genes (see Table 6), indicating the possibility of an infrequent occurrence of inter-domain gene transfer after the origin of the three domains. The overall frequency of anomalous phylogenies is only 14%, and in no category does the frequency of anomalous phylogenies exceed 36.67%; thus, we infer that large-scale inter-domain transfers have not been a major factor in the evolution of the three domains. In contrast to the findings of Doolittle and Handy (1998), we found no significant anomalous phylogenies among the aminoacyl-tRNA synthetases. This is undoubtedly because the broader taxonomic sampling of Doolittle and Handy (1998) is more likely to reveal isolated examples of individual gene transfers.

Regarding the origin of the three domains themselves, the results of this study reflect positively on Jain et. al's (1999) complexity hypothesis albeit in a much smaller scale than originally proposed. Gene transfer has been continual throughout the history of the monophyletic evolution of the three domains, but the coherence of the domains argues that transfer cannot have played a major role in genomic evolution of most organisms. Alternatively, Woese's genetic annealing hypothesis (1998, 2000) cannot be ruled out using our present data. Gene transfer may well have been widespread prior to the emergence of the three domains. Our results would be consistent with that history. For Gupta's hypothesis, if the gram-positive bacteria/archaic clade represented a phylogenetic lineage distinct from the gram-negative

bacteria, our results should have clearly shown this, but this was not the case. We find that the domains Archaea, Bacteria, and Eukarya are monophyletic after their emergence from the universal ancestor. Similar results using large combined alignments of 23 orthologous proteins conserved across 45 species from all domains also strongly supports separate monophyletic domains. (Brown et al, 2001)

## **The Search for a universal ancestor**

The reconstruction of the *Tree of Life* based on a common ancestor demands a universal ancestor (*cenancestor*). Analysis of 66 proteins gauging the interdomain relationship suggests Archaea and Eukarya were the nearest domains in 34 comparisons, Archaea and Bacteria were the nearest domains in 21 comparisons, and Bacteria and Eukarya were the nearest domains in the remaining 11. (Brown and Doolittle, 1997). A popular model in which two lineages emerged leading to Bacteria and Archaea which later diverged into Eukarya was proposed. Protein similarities or the lack thereof incongruent with this model were explained by lateral or horizontal gene transfer (HGT, or exchange of genetic information across major taxa) or specific gene losses which occurred after the development of the three domains. Woese (1998, 2000), while affirming the Darwinian universal genealogical tree of all life, traced a universal phylogenetic tree, not to a universal *cellular* ancestor, but rather, to a universal communal gene pool that he popularized, *progenotes* (Woese, 1998). In a way analogous to physical annealing while temperature is cooled, the rampant activities of mutation and HGT amongst this universal gene pools until the precursors of the individual domains are “crystallized” so that the components in each of them have little functional significance in another pool. Therefore HGT was a pervasive mechanism in this universal gene pool and it became restrictive to *within* each individual domain but not *between* different domains when the different organismic lineages emerged. This cellular complexity that became refractory to global HGT first appeared in the translational machineries especially in its RNA component and then followed by other systems such as metabolic enzymes and transmembrane proteins (Woese, 2000). Doolittle (2000) came close to admitting the possibility of the absence of a *cellular* ancestor because of the need to accommodate rampant HGT and opened up the possibility of a *polyphyletic*

origins of the three domains of life. In other words, the investigation of the early cellular evolution is in a state of serious confusion (Doolittle, 1999b).

### **Horizontal Gene transfer: A challenge to the concept of the Universal Cellular Ancestor?**

Mendelian genetics accounts for vertical inheritance from parents to offspring within the same taxon. However, HGT is only observed readily in prokaryotes and in some lower eukaryotes in the forms of transduction, conjugation, or transformation (Madigan, et.al. 2000). Although it is proposed to account for sequence similarities amongst the three domains of life (Gogarten et.al. 1999), it lacks mechanistic content in regards to *inter-domain* gene exchanges. Models of HGT are predicated on the Darwinian paradigm of a common ancestor in a universal tree of life. The recently published draft of the human genome raised the issue of role of HGT from prokaryotes to vertebrate eukaryotes although there is no consensus on this debate. (International Human Genome Sequencing Consortium, 2001, Celera Genomics, 2001). Since there is confusion in the investigation of early cellular evolution, it is far from settled whether a universal cellular ancestor existed. However, the implication of polyphyletic origins of the three domains of life from a universal pool of progenotes seems to demand a mechanism beyond the realm in which Darwinian natural selection can operate (Doolittle, 2000; Woese, 1998, 2000, 2002). Theories explaining the origin of life on the basis of the “RNA world”(Joyce and Orgel, 1993) and the deterministic processes of self organizing properties intrinsic to the primordial earth (DuDuve, 1995) have not solved the problem of the origin of information carried by the *sequence* of the nucleic acids (Meyer, 2000). A new theory accounting for origin of intermediary metabolism presupposes a pruning or constraining algorithm, which may be physical, chemical, biological, and informational, or a combination thereof. (Morowitz et.al., 2000). The thermodynamic constraints (Shuster, 2000) thus applied resulted in the selection of 153 organic molecules from *Beilstein*, the most comprehensive encyclopedia of organic chemistry, which contain all 11 members of the most basic network of intermediary metabolism, the reductive citric acid cycle. This is an example of a new way of scientific thinking, *emergence*, or *hierarchical reductionism*: the whole is greater than the sum of its parts. (Morowitz, 2002). Various pruning algorithms are applied to different hierarchy of emergences, from matter to spirit. However,

these constraints may also be consistent with *Complex Specified Information* as a design inference (Demski, 1998, 1999, 2001). They also seem to follow the criteria of design as involving “directly or indirectly, free, deliberate, intentional agent activity, aimed at generating some phenomenon typically embodying a mind-correlative pattern, which, if left to itself, nature would not (normally) produce.”(Ratzsch, 2001) The pruning algorithm (or agent) was selected deliberately to generate the 11 compounds of the reductive citric acid cycle (a mind-correlative pattern), when if left to itself, nature would not normally produce. Since natural selection cannot operate at the pre-cellular stage of the progenotes, might there be a specific pruning algorithm or constraint that can be applied to the emergence of the genetic communities from the universal gene pool such that they will be crystallized into the three domains of life?

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**Table 1: Comparison of Eucaryotic and Prokaryotic cells**

	PROCARYOTIC CELL	EUCARYOTIC CELL
GROUPS WHERE FOUND AS UNIT OF STRUCTURE	BACTERIA, BLUE-GREEN ALGAE	MOST ALGAE, FUNGI, PROTOZOA, HIGHER PLANTS AND ANIMALS
Nuclear Membrane	-	+
Mitotic Division	-	+
Chromosome Number	1(?)	Always Greater Than One
Cytoplasmic Streaming	-	+ or -
Mitochondria	-	+
Chloroplasts	-	+ or -
Contractile Locomotor Organelles	Bacterial Flagella } Axial Filaments } in Some	Multistranded Flagella or Cilia } in Some
Ameboid Movement	-	+ or -
Chromosomal Protein	-	+
Nucleolus	-	+
9 + 2 Structure in Cellular Appendages	-	+
Golgi Apparatus	-	+ or (-)
Endoplasmic Reticulum	-	+
Ribosomes	70S	80S (Cytoplasmic) 70S (Organelar)

**Table 2: Summary of major differentiating features among Bacteria, Archaea, and Eukarya<sup>a, b</sup>**

Characteristic	Bacteria	Archaea	Eukarya
Prokaryotic cell structure	Yes	Yes	No
DNA present in covalently closed and circular form	Yes	Yes	No
Histone proteins present	No	Yes	Yes
Membrane-enclosed nucleus	Absent	Absent	Present
Cell wall	Muramic acid present	Muramic acid absent	Muramic acid absent
Membrane lipids	Ester-linked	Ether-linked	Ester-linked
Ribosomes	70S	70S	80S
Initiator tRNA	Formylmethionine	Methionine	Methionine
Introns in most genes	No	No	Yes
Operons	Yes	Yes	No
Capping and poly-A tailing of mRNA	No	No	Yes
Plasmids	Yes	Yes	Rare
Ribosome sensitivity to diphtheria toxin	No	Yes	Yes
RNA polymerases	One (4 subunits)	Several (8-12 subunits each)	Three (12-14 subunits each)
Transcription factors required	No	Yes	Yes
Promoter structure	-10 and -35 sequences (Pribnow box)	TATA box	TATA box
Sensitivity to chloramphenicol, streptomycin, and kanamycin	Yes	No	No
Methanogenesis	No	Yes	No
Reduction of $S^0$ to $H_2S$ or $Fe^{3+}$ to $Fe^{2+}$	Yes	Yes	No
Nitrification	Yes	No	No
Denitrification	Yes	Yes	No
Nitrogen fixation	Yes	Yes	No
Chlorophyll-based photosynthesis	Yes	No	Yes (in chloroplasts)
Chemolithotrophy ( $Fe$ , $S$ , $H_2$ )	Yes	Yes	No
Gas vesicles	Yes	Yes	No
Synthesis of carbon storage granules composed of poly- $\beta$ -hydroxyalkanoates	Yes	Yes	No
Growth above 80 °C	Yes	Yes	No

<sup>a</sup> Note that for many features only particular representatives within a domain show the property.

<sup>b</sup> Adapted with permission from Table 12.3, *Biology of Microorganisms*, by Madigan, Martinko and Parker, Prentice Hall, 2000

**TABLE 3: 4 taxa analyses of Neighbor Joining distances as measured by  $d$  values obtained from pairwise comparisons between species within the same domain (ingroup) and between species of two different domains (ingroup/outgroup)<sup>a</sup>**

		<b>3 A 1 B</b>	<b>3 A 1 K</b>	<b>3 B 1 A</b>	<b>3 B 1 K</b>	<b>3 K 1 A</b>	<b>3 K 1 B</b>
DNA synthesis	Topoisomerase I	2.425865	2.669138	4.365063	7.046429	2.015413	4.694234
Transcription	RNA Polymerase subunit A	8.550514	6.499713	6.296476	11.19691	1.258097	4.662997
	RNA Polymerase subunit B	8.333718	11.05004	6.54597	8.01662	2.869581	12.15119
Translation	Alanyl-tRNA synthetase	10.48018	14.35547	6.932123	1.9045	12.34256	4.988126
(aminoacyl-tRNA synthetases	Aspartyl-tRNA synthetase	10.07547	13.89807	4.397734	12.57243	5.60298	8.99439
and elongation factors)	Histidyl-tRNA synthetase	5.400502	5.625913	6.309885	11.58169	4.576737	7.10963
	Isoleucyl-tRNA synthetase	5.884458	3.654536	3.840167	6.443964	10.50844	11.68186
	Lysyl-tRNA synthetase	5.801957	6.378536	12.24134	1.572325	11.75166	4.813168
	Methionyl-tRNA synthetase	3.030693	3.875524	-0.47733 <sup>+</sup>	0.053119 <sup>+</sup>	4.489012	3.366238
	Seryl-tRNA synthetase	0.564095	0.751593	0.902697	2.090663	0.934657	1.141011
	Threonyl-tRNA synthetase	10.95221	10.60724	10.4773	3.352697	14.73694	7.052162
	Tryptophanyl-tRNA synthetase	3.458452	1.641817	3.233482	6.36127	3.261609	5.449539
	Tyrosyl-tRNA synthetase	4.946913	2.637877	7.217573	9.162542	4.130168	9.006909
	Valyl-tRNA synthetase	6.944471	6.577877	5.622093	2.086238	10.07869	3.495736
	EFG	10.02705	12.56153	13.25872	22.00327	7.374463	10.80744
	EFTU	8.283279	3.019429	16.43295	18.86874	4.541272	12.64965
Translation(ribosomal proteins)	RP L2	8.033114	4.183989	7.783404	16.26909	2.800233	5.850763
	RP L3	8.346263	4.731174	9.169058	11.21214	11.11747	11.32213
	RP L5	4.832026	2.299371	3.811596	6.031977	6.715524	12.44565
	RP L6	6.032922	3.635271	8.90574	10.63961	4.675371	4.195127
	RP L11	2.02601	3.397762	9.755808	12.05037	7.231043	8.972034

RP L13	3.875092	4.339421	3.484009	4.248353	7.676802	5.065983
RP L14	4.717789	3.201212	5.759651	11.73742	10.37709	17.09212
RP L15	4.678269	3.597705	5.817614	5.700324	5.182529	5.519944
RP L22	2.280295	2.566729	3.436419	5.765489	4.96957	5.784356
RP S3	7.094445	6.587102	8.37842	12.80231	8.227666	13.85075
RP S4	10.45883	5.755453	7.027778	5.359134	4.705402	6.903423
RP S5	5.221323	4.531926	4.458939	3.980542	9.339481	8.375188
RP S7	3.967652	1.383791	5.179935	5.235539	5.763569	8.105104
RP S8	5.764757	2.770651	4.305347	5.552367	9.588174	11.96894
RP S9	4.377231	2.519016	2.808853	3.099939	5.519817	8.163763
RP S10	6.982472	8.075887	5.384902	3.735241	8.583352	8.896095
RP S11	9.571898	3.633321	2.708759	4.391194	5.159107	14.45112
RP S12	6.966376	2.781513	9.282328	12.7398	5.092316	10.73942
RP S13	2.350179	0.896496	5.389141	4.990714	6.551423	6.625785
RP S15	4.508114	1.360359	7.366926	9.213613	5.85749	8.513674
RP S17	4.228876	3.329663	3.775771	5.266656	9.395147	3.498226
RP S19	3.362313	3.343713	5.148133	7.394784	7.5393	9.184511
Biosynthesis (pyrimidines and purines) Adenylosuccinate synthase	10.34674	9.312978	1.580922	6.545712	6.545712	4.983417
Argininosuccinate lyase	2.077386	2.64909	2.592517	1.80623	2.596425	1.207013
Aspartate transcarbamoylase	3.172877	2.537617	-0.15323*	0.71388*	0.116314 <sup>++</sup>	2.292724 <sup>++</sup>
CTP synthase	2.20526	3.875606	-0.48925*	2.205425*	1.059454	1.682138
Dihydro-orotase	2.482885	2.275617	4.060343	3.188276	-0.00381 <sup>+</sup>	0.059053 <sup>+</sup>
Inosine 5' monophosphate dehydrogenase <sup>b</sup>	2.966254	6.069944	1.916606	5.503693	5.959759	4.810327
Inosine 5' monophosphate dehydrogenase <sup>b</sup>	3.001449	5.444483	3.604289	7.240405	3.307368	2.800365

	Nucleoside diphosphate kinase	2.600565	2.910789	-0.11253 <sup>+</sup>	-0.36813 <sup>+</sup>	3.473889	2.253486
	Serine hydroxymethyl-transferase	7.639994	8.042556	11.20126	4.377509	8.947983	3.681123
Biosynthesis (amino acids)	Argininosuccinate synthetase <sup>b</sup>	3.676644	5.981398	2.282775	4.876388	40.07526	31.15312
	Argininosuccinate synthetase <sup>b</sup>	3.601791	5.784803	2.109738	4.512754	2.999101	3.121791
	Chromate synthase	1.047015	2.184124	0.255894 <sup>+</sup>	0.422024 <sup>+</sup>	1.264819	0.741218
	Glutamine synthetase	1.910787	4.624558	0.88194	3.937935	5.506518	4.1325
	hisD product	0.938341	1.027258	0.464528 <sup>+</sup>	0.150131 <sup>+</sup>	2.144213	1.477558
	Methionine aminopeptidase	7.43407	5.961264	3.968743	6.843636	5.100095	8.412203
	Ornithine transcarbamoylase	1.393398	4.125869	-0.59833 <sup>*</sup>	1.484952 <sup>*</sup>	-0.06913 <sup>+</sup>	-0.01365 <sup>+</sup>
	trpB product	0.566339 <sup>++</sup>	0.347684 <sup>++</sup>	0.226153 <sup>+</sup>	-0.33282 <sup>+</sup>	0.35406 <sup>+</sup>	-0.09892 <sup>+</sup>
	trpC product	0.880984	1.566121	-0.24955 <sup>+</sup>	0.309951 <sup>+</sup>	6.549307	1.81533
Biosynthesis (porphyrins)	5'Aminolevulinic acid dehydratase	3.44493	5.269262	0.566539	2.110579	0.548359 <sup>**</sup>	0.482805 <sup>**</sup>
	Glutamyl-tRNA reductase	12.32934	2.911192	8.69422	1.758346	-0.26637 <sup>+</sup>	-0.40601 <sup>+</sup>
Central metabolism	Acetyl-CoA synthase	1.100762	1.900968	0.969107 <sup>**</sup>	0.268286 <sup>**</sup>	2.695585 <sup>**</sup>	0.356196 <sup>**</sup>
	Adenylyhomocysteinase	1.227794	5.670255	-0.76481 <sup>*</sup>	0.176183 <sup>*</sup>	7.067481	2.912659
	Enolase	-0.15191 <sup>+</sup>	0.350178 <sup>+</sup>	1.919142	4.380932	2.923901	7.095949
	Glyceraldehyde 3-phosphate dehydrogenase	13.27622	8.627617	7.667763 <sup>**</sup>	-0.5135 <sup>**</sup>	8.411295	2.059158
	Peptidylprolyl-cis-trans isomerase	2.374129	6.037126	0.67996	4.526522	0.259228 <sup>++</sup>	0.781774 <sup>++</sup>
	Phosphoglycerate kinase	2.267047	3.96564	2.657607	0.488554	8.880773	5.396645
	Protein-L-isoaspartate-O-methyltransferase	3.931223	4.490642	-0.12623 <sup>*</sup>	0.623949 <sup>*</sup>	4.768992	4.305801
	Ribosephosphate pyrophosphokinase	2.956937	3.772016	4.671677	1.570705	3.502506 <sup>**</sup>	0.339275 <sup>**</sup>
	Superoxide dismutase (Mn)	3.616171	1.13814	5.125609	3.173079	4.861756	2.833747
	Triose phosphate isomerase	4.974146	6.365248	4.262532 <sup>**</sup>	0.064757 <sup>**</sup>	10.07005	5.764093
Membrane	Preprotein translocase secY	11.6246	4.472734	5.861304	8.710552	5.427163	9.661261

Chaperon	Heat shock protein 60	13.44378	11.07189	15.08218	26.78237	5.440546	12.71524
paired t test		.481 <sup>c</sup>	.1369 <sup>d</sup>	-1.025 <sup>c</sup>	0.0281 <sup>d</sup>	-0.333 <sup>c</sup>	0.4867 <sup>d</sup>

<sup>a</sup>The differences of Neighbor Joining distances are statistically significant at the  $p < 0.05$  level using student t test with equal or unequal variances. Proteins are grouped into the three domains (B-A-K) based upon an analysis on the *effect size* ( $d$ , the difference between independent means of ingroups and outgroups divided by the standard deviation which is the degree to which the null hypothesis is false) of Neighbor Joining distances. Statistically insignificance or negative differences in  $d$ 's ( $< 0.5$ ) are indicated by the *italics* and lack of hyphens between the groups: i.e. *BAK*<sup>+</sup>, *B-(AK)*<sup>++</sup>, *(BA)-K*<sup>\*</sup>, *(BK)-A*<sup>\*\*</sup>.

<sup>b</sup>Repeated analyses using different species of the same domains.

<sup>c</sup>Mean differences of  $d$ 's in the comparison between all of the sequences in two ingroup/outgroup columns by paired t test analysis.

<sup>d</sup> $p$  values of paired t test analysis for null hypothesis of no difference of  $d$ 's in the comparison between all of the sequences in two ingroup/outgroup columns. The Bonferroni adjustment with three planned comparisons (3A1B/3A1K; 3B1A/3B1K; 3K1A/3K1B) can reduce the significance level to 0.017 (3 times 0.017 gives .05). (Aron and Aron, 1999)

**Table 4: 4 taxa analyses of parsimony steps as measured by *d* values obtained from pairwise comparisons between species within the same domain (ingroup) and between species of two different domains (ingroup/outgroup)<sup>a</sup>**

		3 A 1 B	3 A 1 K	3 B 1 A	3 B 1 K	3 K 1 A	3 K 1 B
DNA synthesis	Topoisomerase I	1.3706018	2.7821192	0.049484*	0.856147*	0.7335481	0.8740844
Transcription	RNA Polymerase subunit A	3.4533153	7.0811108	1.5866542	4.6651135	-0.96719 <sup>++</sup>	1.3413819 <sup>++</sup>
	RNA Polymerase subunit B	3.0068864	2.5887286	1.5725337	5.2827012	1.0238204	4.698922
Translation	Alanyl-tRNA synthetase	8.4767845	9.2020544	4.3048616	1.6788991	3.9788592	3.3666902
(aminoacyl-tRNA synthetases and elongation factors)	Asparyl-tRNA synthetase	7.5286684	10.922658	2.0212973	4.8931258	1.8685671	3.5349373
	Histidyl-tRNA synthetase	3.2958297	9.0710193	4.1038739	6.2658704	4.2754518	11.72631
	Isoleucyl-tRNA synthetase	2.2364377	5.2883643	2.6632756	3.6544159	3.3524667	6.4596799
	Lysyl-tRNA synthetase	5.4760961	6.1843668	9.94454	1.0883617	4.6218736	3.4439812
	Methionyl-tRNA synthetase	3.4184336	2.2448813	1.5260945	1.283383	0.9264348	0.9958263
	Seryl-tRNA synthetase	0.8938277	0.9640493	-0.35709*	1.2131787*	0.123302 <sup>++</sup>	4.210477 <sup>++</sup>
	Threonyl-tRNA synthetase	11.394439	13.730879	19.314739	6.6853665	6.3727957	4.0486328
	Tryptophanyl-tRNA synthetase	1.5601568	2.2732348	2.0710589	4.3730477	1.9122434	2.2271063
	Tyrosyl-tRNA synthetase	2.7839589	2.0944372	3.0489991	8.1766015	2.2171276	5.3488794
	Valyl-tRNA synthetase	5.8743962	5.0433621	3.8319439	1.0929649	1.6954226 <sup>**</sup>	0.475683 <sup>**</sup>
	EFG	4.0110771	3.4042815	10.01793	12.851717	2.7210027	3.3517673
EFTU	1.3816495	0.847901	16.064015	21.889732	3.8736395	22.241935	
Translation(ribosomal proteins)	RP L2	14.374802	5.6880138	6.168864	8.8700864	2.6402564	4.0608454
	RP L3	24.647583	29.845748	9.8194401	11.068913	6.8289129	5.3079458
	RP L5	3.6089364	2.6644636	2.184888	3.0451008	4.3300445	6.2858377
	RP L6	4.2638673	3.8867907	9.8550648	12.716044	4.1827366	6.9171334
	RP L11	1.3056855	1.3548938	7.4257214	10.314508	7.2635624	9.8634719

RP L13	1.5395078	2.4784743	2.421705	3.2173369	2.0608115	3.9839973
RP L14	16.79486	15.335453	25.640438	37.042956	14.494523	19.471683
RP L15	1.4540532	1.8475153	4.1128688	3.7569592	3.510433	2.4967106
RP L22	0.218432*	1.3371741*	2.2765623	2.6271959	2.3619642	1.90689
RP S3	2.1780101	2.4926616	5.0190034	10.653103	3.3469061	3.6018405
RP S4	24.556843	18.095433	14.313725	13.339527	1.5652588	4.2797566
RP S5	1.7328153	1.4457166	3.3067086	3.4964117	4.847476	3.7989966
RP S7	2.6769275	2.6610044	3.3905234	4.6943053	2.7470913	5.6108849
RP S8	3.6940492	2.2758899	2.9971494	3.4029337	6.8595112	11.793564
RP S9	3.7822969	2.3644667	1.7206778	1.7684166	4.3343352	5.3295484
RP S10	7.204636	9.3399526	4.3766773	5.860812	3.7379647	4.819793
RP S11	9.0838476	5.5557331	1.755411	2.4760084	3.3659266	6.2214618
RP S12	3.2779731	2.0775059	5.3511409	5.6848071	5.0494731	8.9561084
RP S13	0.003435*	0.5818534*	3.6339176	4.7575129	3.7856631	3.8421613
RP S15	0.7537964 <sup>++</sup>	-1.38558 <sup>++</sup>	7.0735294	14.31886	3.7179416	3.7070497
RP S17	1.1752336	2.5349031	3.3976593	3.1715374	3.6009259	1.682321
RP S19	1.0738405	3.0726012	4.4243405	4.4584283	2.5848626	2.5988892
Biosynthesis(pyrimidine and purine) Adenylosuccinate synthase	13.628533	9.120675	0.679126	1.2555184	2.3315799	3.4538153
Argininosuccinate lyase	1.3901582	1.7436943	2.0760491	1.4937131	1.8060808	0.9422392
Aspartate transcarbamoylase	2.5612447	1.0191553	-1.69549 <sup>+</sup>	-0.38619 <sup>+</sup>	0.338168 <sup>++</sup>	0.9597468 <sup>++</sup>
CTP synthase	2.2606056	1.7988628	-0.61723 <sup>*</sup>	0.9817685 <sup>*</sup>	0.8065065	1.1494777
Dihydro-ototase	1.7857473	1.0524691	2.9066398	1.8598842	4.1433039	2.7579809
Inosine 5' monophosphate dehydrogenase <sup>b</sup>	2.339885	3.9106072	1.7042835	3.3694149	3.5771179	3.0253698
Inosine 5' monophosphate dehydrogenase <sup>b</sup>	1.6799224	3.446211	0.679203	1.9656599	2.0323751	2.3150474
Nucleoside diphosphate kinase	0.328157 <sup>*</sup>	3.1341506 <sup>*</sup>	-0.04494 <sup>+</sup>	0.00 <sup>+</sup>	2.1129706	1.477563

	Serine hydroxymethyl-transferase	4.2699826	2.6314899	9.486284	1.3564222	3.4609851	1.8621938
Biosynthesis (amino acids)	Argininosuccinate synthetase <sup>b</sup>	5.039236	7.626859	1.4399372	5.1590811	29.981093	34.619241
	Argininosuccinate synthetase <sup>b</sup>	5.2916013	8.0963171	1.2873666	4.3379435	1.9863247	2.3458244
	Chrysmate synthase	1.3863395	3.8298901	0.6352095	0.826114	-0.16487 <sup>+</sup>	-0.19101 <sup>+</sup>
	Glutamine synthetase	0.9187298	1.4742233	1.2468115	2.4871121	3.0371112	2.5091879
	hisD product	2.3516255	2.2220597	0.7350024	0.9622571	0.246397 <sup>+</sup>	0.209246 <sup>+</sup>
	Methionine aminopeptidase	1.1941248	4.4015488	1.986549	4.0793473	1.4108313	1.4699611
	Ornithine transcarbamoylase	1.207012	5.0462724	0.247345 <sup>*</sup>	1.2979743 <sup>*</sup>	0.006971 <sup>+</sup>	0.304284 <sup>+</sup>
	TrpB product	0.6135051	0.9095615	0.252801 <sup>+</sup>	-0.33078 <sup>+</sup>	-0.26722 <sup>+</sup>	-0.34712 <sup>+</sup>
	TrpC product	0.6749084	2.5727465	-1.41766 <sup>*</sup>	1.5190318 <sup>*</sup>	-0.38947 <sup>+</sup>	0.385957 <sup>+</sup>
Biosynthesis (porphyrins)	5'Aminolevulinic acid dehydratase	2.8311148	5.8701946	0.9055461	3.1449444	0.9272816	1.0154576
	Glutamyl-tRNA reductase	1.0120861	6.6077202	9.8922639	1.1085231	0.6688658 <sup>**</sup>	-0.1939 <sup>**</sup>
Central metabolism	Acetyl-CoA synthase	0.393795 <sup>*</sup>	1.116999 <sup>*</sup>	1.6709451	0.951743	1.3711129 <sup>**</sup>	-0.12298 <sup>**</sup>
	Adenylhomocysteinase	0.9672576	5.5396294	0.9709534 <sup>**</sup>	0.283225 <sup>**</sup>	4.7640435	1.9172425
	Enolase	0.396459 <sup>*</sup>	0.7884533 <sup>*</sup>	1.2443963	4.7901716	2.3614725	5.6413383
	Glyceraldehyde 3-phosphate dehydrogenase	14.723487	7.4311361	-1.71328 <sup>*</sup>	1.5563298 <sup>*</sup>	6.2686058	1.5118321
	Peptidylprolyl-cis-trans isomerase	0.310463 <sup>*</sup>	3.4058275 <sup>*</sup>	0.400398 <sup>*</sup>	6.0964981 <sup>*</sup>	-1.14182 <sup>+</sup>	-1.67242 <sup>+</sup>
	Phosphoglycerate kinase	1.2681259	3.560687	1.9008338 <sup>**</sup>	0.430519 <sup>**</sup>	5.85926	6.1965526
	Protein-L-isoaspartate-O-methyltransferase	1.0491945	3.9117577	0.4714942 <sup>*</sup>	0.9682615 <sup>*</sup>	6.0062509	0.9108006
	Ribosephosphate pyrophosphokinase	3.4207233	3.3265766	2.2780167	1.255728	3.5931077	2.5149622
	Superoxide dismutase (Mn)	4.1311269	3.425056	4.1003164	2.7153223	2.5725835	1.9159015
	Triose phosphate isomerase	3.4353892	4.1532853	2.9755709 <sup>**</sup>	-0.28349 <sup>**</sup>	4.7036455	5.0778748
Membrane	Preprotein translocase secY	7.448576	4.4098105	2.4137986	3.1824063	3.0562447	3.5479375
Chaperon	Heat shock protein 60	8.4506664	6.3408001	11.391225	11.662757	2.2842679	4.0457032
<b>Paired t test</b>		<b>-0.413<sup>c</sup></b>	<b>0.2011<sup>d</sup></b>	<b>1.301<sup>c</sup></b>	<b>0.0655<sup>d</sup></b>	<b>-0.868<sup>c</sup></b>	<b>0.0195<sup>d</sup></b>

<sup>a</sup>The differences of Parsimony Steps are statistically significant at the  $p < 0.05$  level using student t test with equal or unequal variances. Proteins are grouped into the three domains (B-A-K) based upon an analysis on the *effect size* ( $d$ , the difference between independent means of ingroups and outgroups divided by the standard deviation which is the degree to which the null hypothesis is false) of Parsimony Steps. Statistically insignificance or negative differences in  $d$ 's ( $< 0.5$ ) are indicated by the *italics* and lack of hyphens between the groups: i.e.  $BAK^+$ ,  $B-(AK)^{++}$ ,  $(BA)-K^*$ ,  $(BK)-A^{**}$ .

<sup>b</sup>Repeated analyses using different species of the same domains.

<sup>c</sup>Mean differences of  $d$ 's in the comparison between all of the sequences in two ingroup/outgroup columns by paired t test analysis.

<sup>d</sup> $p$  values of paired t test analysis for null hypothesis of no difference of  $d$ 's in the comparison between all of the sequences in two ingroup/outgroup columns. The Bonferroni adjustment with three planned comparisons (3A1B/3A1K; 3B1A/3B1K; 3K1A/3K1B) can reduce the significance level to 0.017 (3 times 0.017 gives .05) (Aron and Aron, 1999)

**Table 5. Total number of grouping in the NJ and PAUP analyses as percentages out of 420<sup>a</sup>**

<b>Grouping</b>	<b>B-A-K</b>	<b><i>BAK</i></b>	<b>B-(<i>AK</i>)</b>	<b>(<i>BA</i>)-K</b>	<b>(<i>BK</i>)-A</b>
frequencies	361	20	7	19	13
percentage	85.95	4.76	1.67	4.52	3.10

<sup>a</sup>See Tables 3 and 4 for definition of groupings.

**Table 6. Summary of groupings in NJ and PAUP analyses in individual classes of proteins<sup>a</sup>**

		<b>B-A-K</b>	<b>BAK</b>	<b>B-(AK)</b>	<b>(BA)-K</b>	<b>(BK)-A</b>
<b><u>RNA Polymerase a and b/ Topoisomerase I</u></b>						
	<b>NJ (9)</b>	9	0	0	0	0
	<b>% of NJ</b>	100%	0.00%	0.00%	0.00%	0.00%
	<b>PARS (9)</b>	7	0	1	1	0
	<b>% of PARS</b>	77.78%	0.00%	11.11%	11.11%	0.00%
	<b><i>total=18</i></b>	<i>16</i>	<i>0</i>	<i>1</i>	<i>1</i>	<i>0</i>
	<b><i>% of total</i></b>	<i>88.88%</i>	<i>0.00%</i>	<i>5.56%</i>	<i>5.56%</i>	<i>0.00%</i>
<b><u>Aminoacyl tRNAsynthetases and Elongation Factors</u></b>						
	<b>NJ (39)</b>	38	1	0	0	0
	<b>% of NJ</b>	97.44%	2.56%	0.00%	0.00%	0.00%
	<b>PARS (39)</b>	36	0	1	1	1
	<b>% of PARS</b>	92.31%	0.00%	2.56%	2.56%	2.56%
	<b><i>total=78</i></b>	<i>74</i>	<i>1</i>	<i>1</i>	<i>1</i>	<i>1</i>
	<b><i>% of total</i></b>	<i>94.87%</i>	<i>1.28%</i>	<i>1.28%</i>	<i>1.28%</i>	<i>1.28%</i>
<b><u>Ribosomal Proteins</u></b>						
	<b>NJ (66)</b>	66	0	0	0	0
	<b>% of NJ</b>	100%	0.00%	0.00%	0.00%	0.00%
	<b>PARS (66)</b>	63	0	1	2	0
	<b>% of PARS</b>	95.45%	0.00%	1.52%	3.03%	0.00%
	<b><i>total=132</i></b>	<i>129</i>	<i>0</i>	<i>1</i>	<i>2</i>	<i>0</i>
	<b><i>% of total</i></b>	<i>97.73%</i>	<i>0.00%</i>	<i>0.76%</i>	<i>1.52%</i>	<i>0.00%</i>

**Biosynthetic Proteins**

<b>NJ (60)</b>	45	9	2	3	1
<b>% of NJ</b>	75.00%	15.00%	3.33%	5.00%	1.67%
<b>PARS (60)</b>	46	8	1	4	1
<b>% of PARS</b>	76.67%	13.33%	1.67%	6.67%	1.67%
<b><i>total=120</i></b>	<i>91</i>	<i>17</i>	<i>3</i>	<i>7</i>	<i>2</i>
<b><i>% of total</i></b>	<i>75.83%</i>	<i>14.17%</i>	<i>2.50%</i>	<i>5.83%</i>	<i>1.67%</i>

**Metabolic Proteins**

<b>NJ (30)</b>	20	1	1	2	6
<b>% of NJ</b>	66.67%	3.33%	3.33%	6.67%	20.00%
<b>PARS (30)</b>	19	1	0	6	4
<b>% of PARS</b>	63.33%	3.33%	0.00%	20.00%	13.33%
<b><i>total= 60</i></b>	<i>39</i>	<i>2</i>	<i>1</i>	<i>8</i>	<i>10</i>
<b><i>% of total</i></b>	<i>65.00%</i>	<i>3.33%</i>	<i>1.67%</i>	<i>13.33%</i>	<i>16.67%</i>

**HSP60 and SecY**

<b>NJ (6)</b>	6	0	0	0	0
<b>% of NJ</b>	100%	0.00%	0.00%	0.00%	0.00%
<b>PARS (6)</b>	6	0	0	0	0
<b>% of PARS</b>	100%	0.00%	0.00%	0.00%	0.00%
<b><i>total=12</i></b>	<i>12</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>
<b><i>% of total</i></b>	<i>100%</i>	<i>0.00%</i>	<i>0.00%</i>	<i>0.00%</i>	<i>0.00%</i>

\*See Tables 3,4 and 5 for explanation.