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FEBS Letters 341 (1994) 146–151

FEBS 13801

FEBS LETTERS

Hypothesis

The chaperone connection to the origins of the eukaryotic organelles

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Received 14 February 1994

Abstract

The heat-shock 60 proteins (Hsp60) constitute a subset of molecular chaperones essential for the survival of the cell, present in eubacteria as well as in eukaryotic organelles. Here, we have employed these highly conserved proteins for the inferences of the origins of the organelles. Hsp60s present in mitochondria from different eukaryotic lineages formed a clade, which showed the closest relationship to that of the *Ehrlichia/Rickettsia* cluster among the α-Proteobacteria. This, in addition to phenotypic characteristics, suggests that these obligate intracellular parasites and the lineage that generated the mitochondrion shared last common ancestry. In turn, Hsp60s present in chloroplasts from plants and a red alga, respectively, clustered specifically with those of the cyanobacteria, suggesting that all plastids derive exclusively from this eubacterial lineage.

Key words: Organelle phylogeny; Mitochondrial origin; Plastid origin; Molecular chaperone; Heat-shock protein; Evolutionary chronometer; Phylogeny

1. Introduction

Molecular data have played a fundamental role in supporting a xenogenous, eubacterial origin for mitochondria and chloroplasts, a hypothesis based on the morphological, biochemical and genetic characteristics of the eukaryotic organelles [1–5]. The actual debate resides over whether mitochondria or chloroplasts in the different eukaryotic lineages represent remnants of a single endosymbiotic event, or evolutionary mosaics of several, independent events [2–6]. This, in turn, is closely related to the issue of the specific eubacterial affiliation of these ancestors in the different eukaryotic lineages [2–6].

The comparison of conserved portions of mitochondrial rRNAs to their corresponding eubacterial homologs indicated an origin of these organelles among the α-subdivision of the purple bacteria (or α-Proteobacteria) [7,8], a result that supports previous inferences based on cytochrome *c* comparisons [1]. Moreover, the earlier branching of plants in mitochondrial rRNA trees (when compared to those of nuclear rRNAs) [7,8] has been interpreted as suggesting a separate, more recent origin for these genes in plant mitochondria with respect to other eukaryotes [3]. Nevertheless, a problem associated with the use of mitochondrial rRNAs that has complicated phylogenetic inferences consists in the radically distinct rates of evolution and the idiosyncrasies shown by these molecules in the different eukaryotic lineages [2,3,7–9], a situation that has resulted in the selection of only a limited portion of their sequences for the construction of evolutionary trees [7,8]. It is also relevant to this discussion that phylogenies derived from other molecular chronometers such as elongation factor Tu [10] are controversial with those of rRNAs [7,8], suggesting an affiliation of (at least) yeast mitochondria to the obligate intracellular parasite *Chlamydia trachomatis*.

In the case of chloroplasts, rRNA-derived trees point to their origin in all photosynthetic eukaryotes among the cyanobacterial lineage [1–6,8,11–13]. However, inferences based on ribulose bisphosphate carboxylase genes (*rbc*) indicate that plastids from non-green algae derive from the α- or β-Proteobacteria [5], again pointing to a scenario that includes ancestors from different eubacterial division lineages [3–6].

Explanations for some of these conflicting results include either lateral transfer of genes, paralogy, differential retention of duplicated operons present in a common ancestor of the endosymbionts, or limitations inherent to the assumptions on which phylogenetic methods are based [2–6]. Concerning the latter, inferences based on a macromolecule which has evolved differentially in the various organisms under study may not necessarily re-

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SSDI 0014-5793(94)00186-Y

flect the true phylogeny of these lineages [2–5,14–18]. Therefore, as pointed out by several authors [2–5,14,18–20], it is becoming increasingly evident that the resolution of the evolutionary history of organisms in general (and eukaryotic organelles in particular) will undoubtedly require of a comparative and critical analysis of data from different macromolecules, the fossil record (when available), and a correlation of the inferred phylogenies with the phenotypic traits of the organisms under consideration.

The Hsp60 heat-shock proteins (also known as chaperonins, or GroEL in bacteria), constitute a family of housekeeping proteins ubiquitously distributed among eubacteria and eukaryotic organelles [21–23]. We have used these remarkable (structural and functionally) conserved proteins [21] to study the phylogenies of mitochondria and chloroplasts, and propose that these macromolecules constitute useful tools for the study of the origin and evolution of the organelles.

2. Methods

Since widely different base composition in the organisms under study may mislead phylogenetic inferences when nucleotide comparisons are employed [4,17], in this study we have compared protein sequences of Hsp60 rather than their corresponding nucleotide sequences, following the suggestion of some authors [17]. To calculate the matrices of evolutionary distances, the amino acid conversion table compiled by Dayhoff et al. [24] was used, from 537 aligned positions after removal of transit peptides from eukaryotic Hsp60s, and a C-terminal portion of ca. 20 amino acids from all sequences. Alignments were done as described [21], and refined by visual inspection. For the construction of phylogenetic trees, we have employed the neighbor-joining (NJ) distance method [15]. This procedure has been shown in model studies to be relatively consistent even in the presence of unequal rates of evolution among the molecules under comparison [15,16]. Confidence limits to the inferences obtained by NJ were placed by the bootstrap procedure [14].

The programs PROTDIST, NEIGHBOR, SEQBOOT, CONSENSE, and PROTPARS, present in the PHYLIP package [14] (version 3.5, kindly provided by Dr. J. Felsenstein, University of Washington, Seattle, Washington) were employed for this work. The Hsp60 protein sequences were obtained from the National Center for Biotechnology Information (NCBI). Peptide database searches were performed at NCBI by using the BLAST network service [25]. The organisms from which Hsp60 sequences were obtained, as well as the respective databases accession numbers are provided in the legends to figures. Amino acid alignments and calculated evolutionary distances were provided for the reviewing process, and are available from the authors on request.

3. Organelle phylogenies

3.1. Mitochondrial origins

As reported previously [21], a close similarity exists between Hsp60-derived phylogenetic inferences and those of eubacterial 16S rRNAs [19], a situation that reinforces phylogenetic relationships based on these molecules. These results are particularly relevant to the concerns posed on phylogenetic inferences based on nucleotide comparisons of organisms showing widely different base compositions in their genomes [4,17], as is the case in this work.

We have extended the phylogenetic analysis to the origins of the eukaryotic organelles, by including the available Hsp60 sequences from a larger number of eubacteria as well as from mitochondria and chloroplasts. Fig. 1 shows the evolutionary relationships between Hsp60 proteins from mitochondria and species of the Proteobacteria. The separation of the latter in two major groups (α- and γ-, respectively) indicates that the resemblance of Hsp60 to 16S rRNA phylogenetic trees of the Proteobacteria [19] is extended to its subdivision levels. In turn, and also in agreement with rRNA-based trees [26–28], the α-subdivision of the Proteobacteria appears in the figure as clearly separated into two defined groups (Fig. 1). One of these groups includes Hsp60s from the obligate intracellular pathogens *Ehrlichia chaffeensis* and *Rickettsia tsutsugamushi* (the *Ehrlichia/Rickettsia* cluster), and the other (the *Agrobacterium/Rhizobium* cluster [26]) is composed of Hsp60s from other species assigned to the α-Proteobacteria which include, among others, *Agrobacterium tumefaciens*, different *Rhizobium* and *Bradyrhizobium* species, animal pathogens such as *Bartonella bacilliformis* and *Brucella abortus*, etc., respectively (Fig. 1). It is worth mentioning the existence in 2 (*R. meliloti* and *B. japonicum*, respectively) of the proteobacterial species analyzed (19 in total) of more than one copy of *hsp60* genes (Fig. 1). Nevertheless, the omission of Hsp60 sequences from these species from the analysis did not change the outcome of the phylogenetic tree depicted in the figure. These results are specially relevant to the ensuing discussion about the origins of mitochondria.

The Hsp60 proteins present in mitochondria from different eukaryotic lineages, including different species of mammals and the arthropod *Heliothis virescens*, those of several monocot and dicot plant species, the yeast *Saccharomyces cerevisiae* and the deuteromycete fungus *Histoplasma capsulatum*, as well as the flagellate *Trypanosoma cruzi*, respectively, formed a clearly defined clade (support of 100/100 trees by the bootstrap test, Fig. 1). Moreover, this mitochondrial Hsp60 cluster appeared as distant yet specifically related to the aforementioned *Ehrlichia/Rickettsia* group (support of 85/100 trees by the bootstrap test, Fig. 1). The specific affiliation of mitochondria to the second major cluster of the α-Pro-

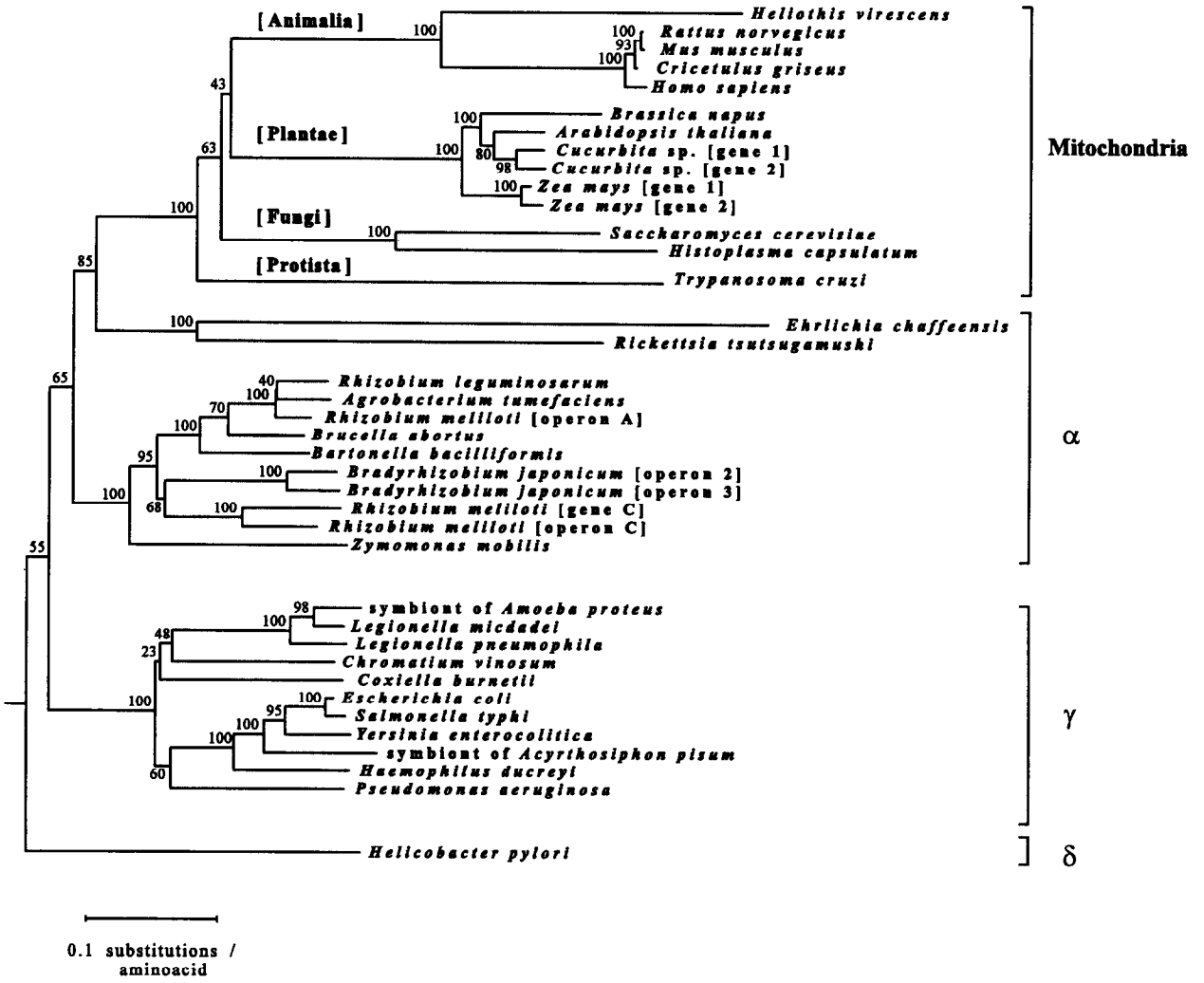

Fig. 1. Evolutionary relationships between species of the Proteobacteria and mitochondria from distinct eukaryotic lineages as inferred from Hsp60 comparisons. An unrooted phylogenetic tree was constructed from 66 Hsp60 protein sequences by using the NJ procedure [15], as described in the section 2, and the subtree indicating the relationships between Proteobacteria and mitochondria is depicted in the figure. The values on the nodes represent the number of bootstrapped trees (from 100 resamplings) that support a respective affiliation. The evolutionary distance scale (number of substitutions per site) is indicated at the bottom of the figure. The existence of different genes coding for Hsp60 mitochondrial proteins, as well as the presence of the distinctly encoded subunits that compose land plants plastid Hsp60s (α or β), respectively, are indicated. In bacteria bearing different *hsp60* (*groEL*) copies, their presence either in isolated form (gene) or in an operon linked to *groES* genes (operon) is also indicated. The organism's source of the Hsp60 used in this study, their affiliation (as indicated by 16S rRNA analysis [31]), as well as GenBank database accession numbers are as follows. **Mitochondria**: *H. sapiens*, M34664; *R. norvegicus*, X54793; *M. musculus*, X55023; *C. griseus*, M22383; *H. virescens*, X56034; *T. cruzi*, L08791; *S. cerevisiae*, M33301; *H. capsulatum*, L11390; *B. napus*, Z27165; *A. thaliana*, Z11547; *Cucurbita* sp. gene 1, X70867; *Cucurbita* sp. gene 2, X70868; *Z. mays* gene 1, Z12114; *Z. mays* gene 2, Z12115; **α-Proteobacteria**: *B. bacilliformis*, M98257; *B. abortus*, L09273; *A. tumefaciens*, X68263; *R. leguminosarum*, L20775; *R. meliloti* operon A, M94192; *R. meliloti* gene C, M94190; *R. meliloti* operon C, M94191; *B. japonicum* operon 2, Z22604; *B. japonicum* operon 3, Z22603; *Z. mobilis*, L11654; *E. chaffeensis*, L10917; *R. tsutsugamushi*, M31887; **γ-Proteobacteria**: *C. burnetii*, M20482; *P. aeruginosa*, M63957; *H. ducreyi*, M91030; *S. typhi*, U01039; *E. coli*, X07850; *A. pisum* symbiont, X61150; *Y. enterocolitica*, X68526; *C. vinosum*, M99443; *L. pneumophila*, M31918; *L. micdadei*, X57520; *A. proteus* symbiont, M86549; **δ-Proteobacteria**: *H. pylori*, X73840; **Chlamydia**: *C. trachomatis*, M31739; *C. pneumoniae*, M69217; *C. psittaci*, X51404; **Spirochaetes**: *L. interrogans*, L14682; *B. burgdorferi*, X54059; *T. pallidum*, X54111; **Firmicutes (low G+C)**: *C. perfringens*, X62914; *C. acetobutylicum*, M74572; thermophilic bacterium PS-3, P26209; *B. stearothermophilus*, L10132; *B. subtilis*, M81132; *L. lactis*, X71132; *S. aureus*, S62126; **Firmicutes (high G+C)**: *S. albus* gene, M76658; *M. leprae* gene, M14341; *M. bovis*, M17705*; *S. albus* operon, M76657; *S. coelicolor*, X75206; *M. tuberculosis*, X60350; *M. leprae* operon, S25181; **Cyanobacteria**: *Synechococcus* sp., M58751; *Synechocystis* sp. D12677; **Chloroplasts**: *C. caldarium*, X62578; *T. aestivum* (α), X07851; *R. communis* (α), X07852; *B. napus* (α), M35599; *B. napus* (β), M35600; *A. thaliana* (β), JT0901*. Asterisks indicate PIR accession numbers.

teobacteria, i.e. *Agrobacterium/Rhizobium* (Fig. 1) was found in 5/100 bootstrapped trees in our analysis (results not shown). In turn, the specific affiliation of mitochondria to the three chlamydial Hsp60 was found in only 1/100 bootstrapped trees (results not shown). It is worth mentioning here that the above described clustering of all mitochondrial Hsp60 sequences, and their specific affiliation to the *Ehrlichia/Rickettsia* cluster among the

Proteobacteria, was also obtained by parsimony analysis using PROTPARS [14] (not shown).

Taking into account that, although limited, very different eukaryotic lineages are represented in the analysis, these results suggest that these mitochondrial Hsp60s have a common origin in the α-Proteobacteria, and point to the species that compose its *Ehrlichia/Rickettsia* group as sharing the last common ancestor with the mitochondrion.

Parasitic and/or symbiotic bacteria that form the *Ehrlichia/Rickettsia* cluster [26–28] share some characteristics [27–30] which are relevant and tend to support the above proposed relationships, such as:
(i) obligate intracellular parasitic behavior, including the ability to escape the phagolysosome action and reproduce inside of a variety of eukaryotic cells;
(ii) a highly reduced or no detectable lipopolysaccharide or peptidoglycan layer in some species of the group (notably *R. tsutsugamushi* and most ehrlichiae);
(iii) aerobic metabolism, a functional tricarboxylic acid cycle, no detectable glycolytic pathway;
(iv) an ADP/ATP translocator in their plasma membranes;
(v) smaller, AT-rich genomes, showing a high degree of heterogeneity when compared to free-living species of the Proteobacteria.

It is worth mentioning here that in the comparisons present in a major rRNA database [31], maize mitochondrial 16S-like rRNA also clusters with its homologs of the *Ehrlichia/Rickettsia* group. However, rRNA sequences other than that of maize mitochondria were not included in these comparisons [31].

The branching order observed in Fig. 1 merits comments, since it closely resembles those obtained from other nuclear-encoded molecules [3,5,8,20,32–34]. The earlier branching of *Trypanosoma cruzi* agrees with that of these flagellates observed in nuclear rRNA- [3,5,20,33] as well as glyceraldehyde-3-phosphate dehydrogenase- [32] derived trees, respectively. Therefore, the result shown in Fig. 1 is particularly noteworthy given that the extreme idiosyncrasies of trypanosome mitochondrial rRNAs have precluded phylogenetic studies based on these molecules [10].

The exact order of branching between the plant, fungi, and animal lineages, respectively, still remains a matter of controversy [2,5,20,33–36], and a similar situation occurs in our analysis (Fig. 1). Although a closer relationship between the plant and animal lineages (with the fungal sequences as outgroups) was obtained in Fig. 1 (in agreement with inferences based on other molecules [36]), the low confidence limits obtained for these particular relationships cannot exclude other affiliations. In fact, the specific relationship between the fungi and animal lineages (as in [2,20,33–35]) was obtained in 37/100 trees by the bootstrap test (data not shown). In turn, the lowest support was found for a specific affiliation between the fungi and plant lineages (10/100 trees by the bootstrap test, data not shown).

3.2. Plastid origins

By the use of distance or parsimony methods (Fig. 2 and data not shown), the Hsp60 sequences analyzed which included the plastid-encoded protein from the red alga *Cyanidium caldarium* (*Galderia sulphuraria*), and the α- and β-subunits of chloroplast Hsp60 from several land plants, respectively, formed a clade which included their homologs present in *groESL* operons of cyanobacterial species (this specific affiliation was obtained in 98/100 trees by the bootstrap test, not shown). Therefore, our results point to an origin for all plastids among the cyanobacterial lineage, in agreement with rRNA-derived trees [11–13]. This result is specially relevant in the case of *C. caldarium*, since inferences derived from the plastid-encoded *rbc* genes from rhodophytes indicate an origin of these genes among the α- or β-Proteobacteria [4–6]. Moreover, these results (Fig. 2) support a closer relationship of the rhodophyte Hsp60 to its cyanobacterial homologs than to its plant counterparts (100/100 trees by the bootstrap test). This particular result is supported by amino acid sequence compari-

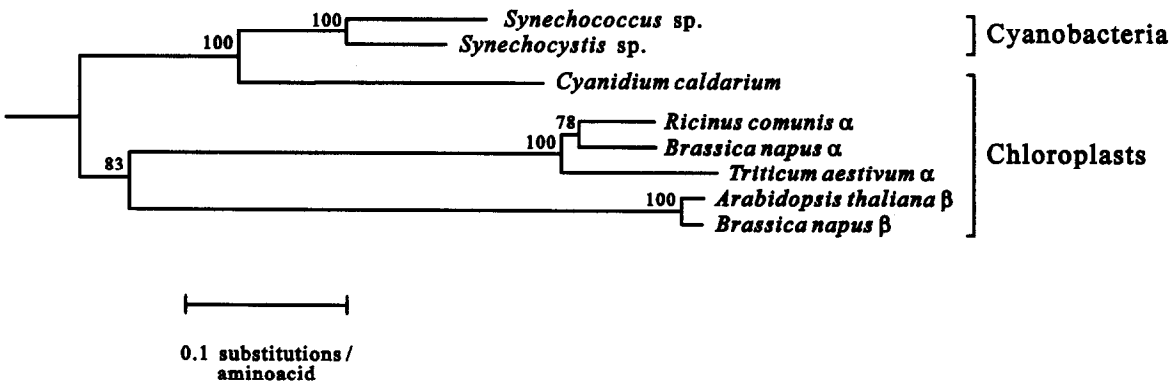

Fig. 2. Evolutionary relationships between cyanobacteria and chloroplasts as inferred from Hsp60 comparisons. An unrooted tree was constructed as described in the legend to Fig. 1, and the subtree corresponding to the cyanobacteria/chloroplasts cluster is shown. See legend to Fig. 1 for details.

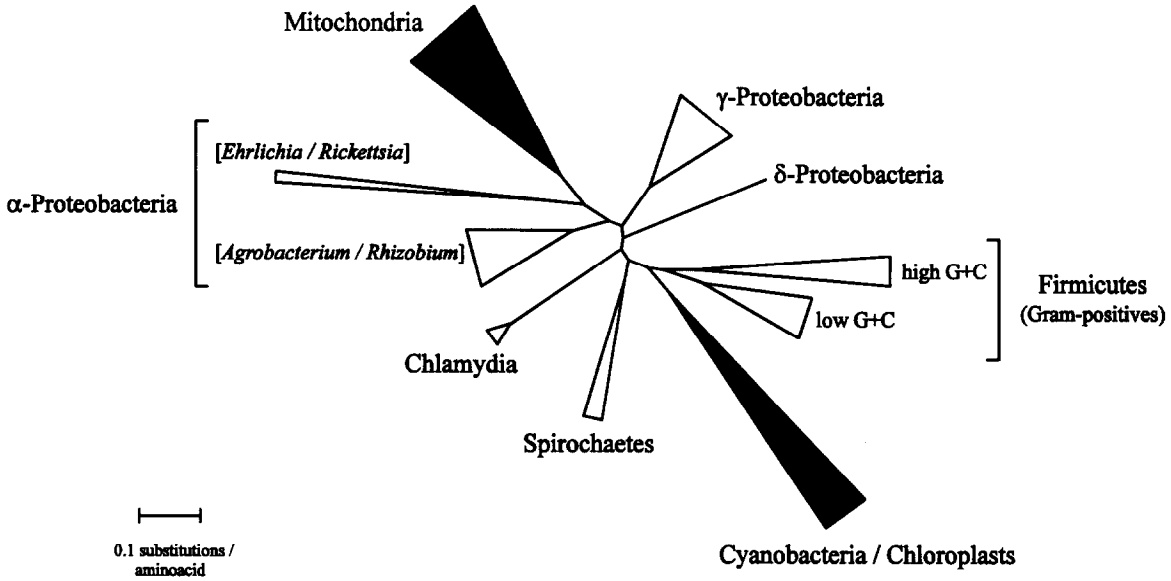

Fig. 3. Evolutionary relationships between Eubacteria and eukaryotic organelles inferred from Hsp60 proteins. An unrooted phylogenetic tree was constructed from 66 Hsp60 sequences as described in the legend to Fig. 1. The distinct eubacterial clusters are depicted as triangles in which the base is proportional to the number of sequences analyzed, and the height represents the average distance separating the terminal nodes from the deepest branching point within the cluster, respectively. The classification followed for each major cluster is essentially that of refs. [26,31]. The evolutionary distance scale is indicated at the bottom of the figure.

sons: there exist a particular deletion at the equivalent *E. coli* Hsp60 position 337, as well as a Trp residue at position 430, which are only observed among the homolog proteins from *C. caldarium* and the cyanobacterial species depicted in the figure, respectively, but are not observed among other Hsp60s including those from land plant plastids (not shown). It is worth mentioning here that this closer affiliation between cyanobacteria and rhodophyte plastids has been proposed earlier based on their similar pigment systems [4], and received support from some rRNA inferences [13] (although other rRNA-derived trees do not indicate such a specific relationship [11,12]).

Our results (Fig. 2) also suggest that the α- and β-subunits that compose the plant chloroplast Hsp60 [23] are likely to be the products of an early gene duplication that occurred in the lineage that led to land plants.

4. Conclusions and perspectives

The fossil record provides evidence for the existence of aerobic, photosynthetic eukaryotes as early as 2,000 million years ago [33,37]. Therefore, the establishment of the symbiotic processes that eventually led to the eukaryotic organelles probably occurred well before this date [37]. Being exclusively located in the organelles, and most probably from eubacterial origin, the Hsp60 proteins known as chaperonins [21–23] may well provide clues as to the aboriginal eubacterial lineages that gave rise to mitochondria and chloroplasts. An advantage of their use for phylogenetic inferences results from the relocation of *hsp60* genes in the nucleus, a situation that would have freed them from possible events emerging from multiple endosymbiotic scenarios (such as multiple recombinations, composite organellar genomes, etc. [2–6]).

We have summarized the evolutionary relationships between eubacteria and eukaryotic organelles discussed in this work in the scheme shown in Fig. 3. Concerning the mitochondrion, Hsp60-based inferences agree with those indicating an origin of this organelle among the α-Proteobacteria [1–3], and suggest that the putative endosymbiont (at least in the eukaryotic lineages analyzed in this work) shared last common ancestry with that of the *Ehrlichia/Rickettsia* group. Interestingly, this cluster is exclusively composed of either obligate parasites or endosymbionts of eukaryotic cells [26–31], that show phenotypic traits (summarized above) which appear quite convenient to be originally present in a putative mitochondrial ancestor. The inclusion of a larger number of Hsp60 sequences from the *Ehrlichia/Rickettsia* cluster [31] as well as from mitochondria from different aerobic protists [2,3,20], respectively, may well contribute to elucidate the affiliation of the putative mitochondrial ancestor(s), and the controversies on whether or not these organelles represent genetic mosaics in the different eukaryotic lineages [2,3].

Concerning chloroplasts, Hsp60-based inferences agree with others [2–6,11–13] supporting evidence for a common origin of all plastids present in photosynthetic eukaryotes among the cyanobacterial lineage (Fig. 3). No evidence was found in our analysis for a closer rela-

tionship between the plastid-encoded Hsp60 from the red alga *C. caldarium* to those of the Proteobacteria, as inferred in the case of *rbc* genes [5], despite the fact that both *hsp60* and *rbc* genes are encoded in the plastid genome in rhodophytes [2–6,38,39]. Moreover, the rhodophyte Hsp60 appeared in our analysis more related to its extant cyanobacterial counterparts than to those of plants (Fig. 2), a situation that suggests in principle that the plastids in these eukaryotic lineages derive from separate events of endosymbiosis (from distinct lineages among the cyanobacteria). It is difficult to evaluate at this stage whether the discrepancies between the results presented here (or those based in rRNA analysis [11–13]) with *rbc*-based sequence comparisons [5] represent either a differential retention of duplicated genes originally present in the ancestor of the plastids, lateral transfer/replacement events (i.e. mosaic genomes) [2–6], or problems associated to current phylogenetic methods [2–4]. In any case, the inclusion of sequences from other photosynthetic eukaryotes, as well as from the cyanobacteria/prochlorophyte lineage may help to illuminate the origins and evolution of plastids.

Acknowledgements: We are indebted to Dr. J. Felsenstein for the generous gift of the PHYLIP 3.5 package, to Dr. Tetsuko Takabe for generous help, to Drs. K. Fukami-Kobayashi and M. Go for help and advice on the use of Fujitsu's SINCA program during preliminary work, and to Dr. H. Gramajo for his patient help in the use of computer systems. We are also grateful to Drs. E. Weiss and G. Dasch for providing us information on the rRNA phylogenetic analysis of *R. tsutsugamushi*. A.V. is a member of the Argentine Research Council (CONICET), and A.A. a graduate student of the School of Biotechnology of the National University of Rosario. This work was supported by grants from CONICET, the International Foundation for Science (Sweden), and the Third World Academy of Sciences (Italy).

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