

# The chloroplastic protein translocation channel Toc75 and its paralog OEP80 represent two distinct protein families and are targeted to the chloroplastic outer envelope by different mechanisms

Kentaro Inoue\* and Daniel Potter

Department of Pomology, University of California, One Shields Avenue, Davis, CA 95616, USA

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\*For correspondence (fax +1 530 752 8502; e-mail kinoue@ucdavis.edu).

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## Summary

Toc75 is postulated to form the protein translocation channel in the chloroplastic outer envelope membrane. Proteins homologous to Toc75 are present in a wide range of organisms, with the closest homologs occurring in cyanobacteria. Therefore, an endosymbiotic origin of Toc75 has been postulated. Recently, a gene encoding a paralog to Toc75 was identified in *Arabidopsis* and its product was named atToc75-V. In the present study, we characterized this new Toc75 paralog, and investigated extensively the relationships among Toc75 homologs from higher plants and bacteria in order to gain insights into the evolutionary origin of the chloroplastic protein translocation channel. First, we found that the native molecular weight of atToc75-V is 80 kDa and renamed it (AtOEP80) *Arabidopsis thaliana* outer envelope protein of 80 kDa. Second, we found that AtOEP80 and Toc75 utilize different mechanisms for their targeting to the chloroplastic envelope. Toc75 is directed with a cleavable bipartite transit peptide partly via the general import pathway, whereas AtOEP80 contains the targeting information within its mature sequence, and its targeting is independent of the general pathway. Third, we undertook phylogenetic analyses of Toc75 homologs from various organisms, and found that Toc75 and OEP80 represent two independent gene families that are most likely derived from cyanobacterial sequences. Our results suggest that Toc75 and OEP80 diverged early in the evolution of plastids from their common ancestor with modern cyanobacteria.

**Keywords:** chloroplastic protein targeting, envelope membrane, evolutionary origin, protein translocation channel, Toc75, AtOEP80.

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## Introduction

It is widely believed that the chloroplast in higher plants is derived from an ancestral free-living photosynthetic prokaryote, and its direct descendants are represented by the modern cyanobacteria (McFadden, 1999). During evolution, most genes of the endosymbiont are transferred to the host nucleus, and systems that direct the gene products back to the organelle are established (Martin, 2003). As a result, currently, more than 95% of chloroplastic proteins are believed to be encoded in the nucleus (Abdallah *et al.*, 2000), and thus post-translational protein import is a prerequisite for the biogenesis of the organelle. In the last couple of decades, proteinaceous components responsible for this process have been identified from pea chloroplasts by

chemical cross-linking with precursor proteins, solubilization of the membranes using mild detergents, and/or immunoprecipitation. Their functions have been elucidated by biochemical, cell biological, and genetic approaches (for review, see Jarvis and Soll, 2001; Keegstra and Cline, 1999). However, their evolutionary origin remains unclear.

Three translocon at the outer envelope membrane of chloroplasts (TOC) components, psToc159, psToc34, and psToc75, were identified as constituents of the core protein translocation complex in the pea chloroplastic outer envelope (Schleiff *et al.*, 2003). Among them, psToc75 is the best characterized component in its function. First, it was identified as a protein import component (Perry and Keegstra,

1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995). It was then reconstituted into liposomes and was shown to form a beta-barrel structure with a cation-selective channel activity (Hinnah *et al.*, 1997, 2002). These results support the hypothesis that Toc75 forms part of the protein translocation channel. In *Arabidopsis*, there are three genes identified to encode proteins closely related to psToc75, but only one of them, *atTOC75-III*, appears to be expressed (Jackson-Constan and Keegstra, 2001). Finally, proteins that cross-react with psToc75 antisera are present in different plastid types and various plant species (Dávila-Aponte *et al.*, 2003).

The genome of a cyanobacterium, *Synechocystis* PC6803, was found to encode proteins homologous to four chloroplastic protein import components, Toc75, Tic20, Tic22, and Tic55, but no cyanobacterial genes were identified that encoded proteins related to other components, such as Toc159, Toc34, and Tic110 (Reumann and Keegstra, 1999). Based on this observation, it was suggested that the protein import apparatus may have originated from two sources, the endosymbiont and the host cell (Reumann and Keegstra, 1999). The cyanobacterial homolog of Toc75 (Slr1227 or SynToc75) was shown to be essential for the viability of the organism (Reumann *et al.*, 1999). Based on its apparent similarity in its sequence to prokaryotic channel secretion virulence factors such as ShIB from *Serratia marcescens*, Slr1227 was postulated to play a role in peptide secretion (Reumann and Keegstra, 1999; Reumann *et al.*, 1999); however, its biological function remains unknown.

Eckart *et al.* (2002) reported the presence of a 66 kDa protein similar to, but distinct from, psToc75 in the outer envelope membrane of pea chloroplasts. Based on the internal peptide sequences of the pea 66 kDa protein, they identified an *Arabidopsis* cDNA clone that encodes an ortholog to the 66 kDa protein, and named the protein atToc75-V, which shows 22% sequence identity to psToc75 (Eckart *et al.*, 2002). More recently, the presence of atToc75-V in the *Arabidopsis* chloroplastic envelope membrane was confirmed by a proteomic approach (Froehlich *et al.*, 2003). Based on an analysis using the web-based prediction program ChloroP (Emanuelsson *et al.*, 1999), Eckart *et al.* (2002) postulated that atToc75-V was synthesized as a larger precursor of 80 kDa and processed to be a mature form of 69 kDa. This hypothesis, which has not been tested experimentally prior to this report, stands in contrast to the case of nearly all proteins identified so far in the chloroplastic outer envelope membrane, which do not have cleavable transit peptides. The only known exceptions are psToc75 and its orthologs, such as atToc75-III, whose biogenesis is mediated by unique bipartite transit peptides that show no sequence similarity to the predicted transit peptide of atToc75-V (Inoue and Keegstra, 2003; Tranel and Keegstra, 1996).

Recently, the relationships between putative protein-translocating outer membrane porins and other related

proteins from various organisms were established based on their amino acid sequences and on their predicted secondary structure: several Toc75 homologs, including Slr1227, atToc75-III, and psToc75, were grouped into the two-partner secretion (TPS) family (Yen *et al.*, 2002). Furthermore, Gentle *et al.* (2004) showed that based on conserved amino acid sequences, the plant Toc75 clustered with the cyanobacterial proteins but did not group with eukaryotic and proteobacterial Omp85 homologs. Finally, Eckart *et al.* (2002) postulated that atToc75-V represented the most ancestral form of the Toc75-like channel in chloroplasts. However, there has been no detailed analysis of phylogenetic relationships between Toc75 homologs from plants and cyanobacteria.

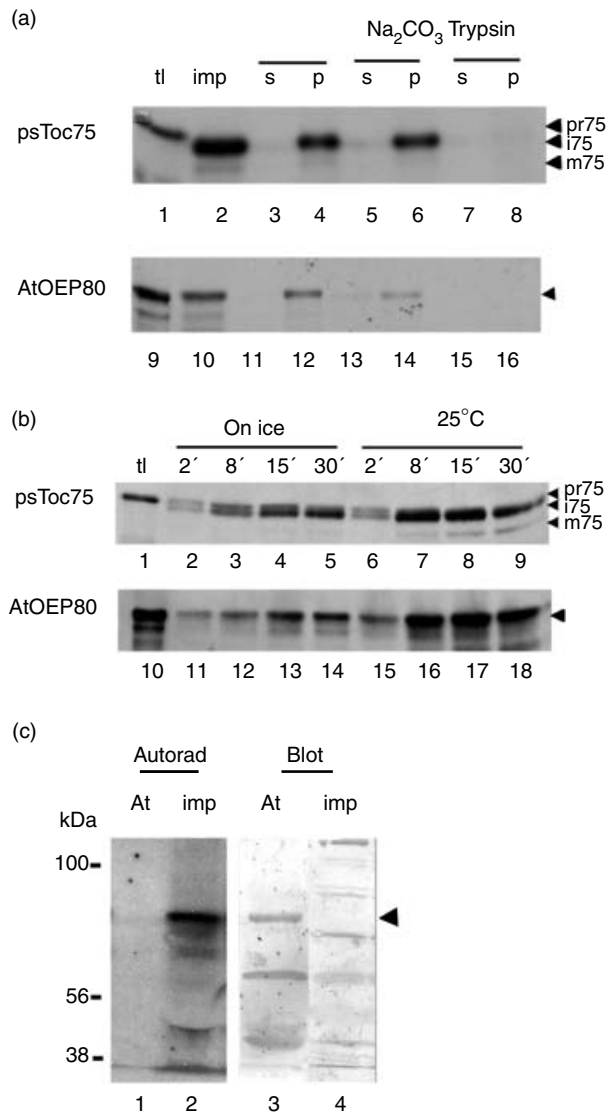
In the present report, we extended the study on the protein previously known as atToc75-V in order to gain insights into the evolutionary origin of the chloroplastic protein translocation machinery. First, we found by immunoblot experiments that the apparent molecular weight of the endogenous atToc75-V is 80 kDa, and renamed the protein (AtOEP80) *Arabidopsis thaliana* outer envelope protein of 80 kDa. Next, we found that Toc75 and AtOEP80 are targeted to the chloroplastic outer envelope by different mechanisms. Finally, we conducted detailed phylogenetic analyses of Toc75 homologs including Toc75, AtOEP80, and related proteins from various organisms, and found that Toc75 and OEP80 diverged early in the evolution of plastids from their common ancestor with modern cyanobacteria.

## Results

### *AtOEP80 is paralog to the chloroplastic protein translocation channel Toc75*

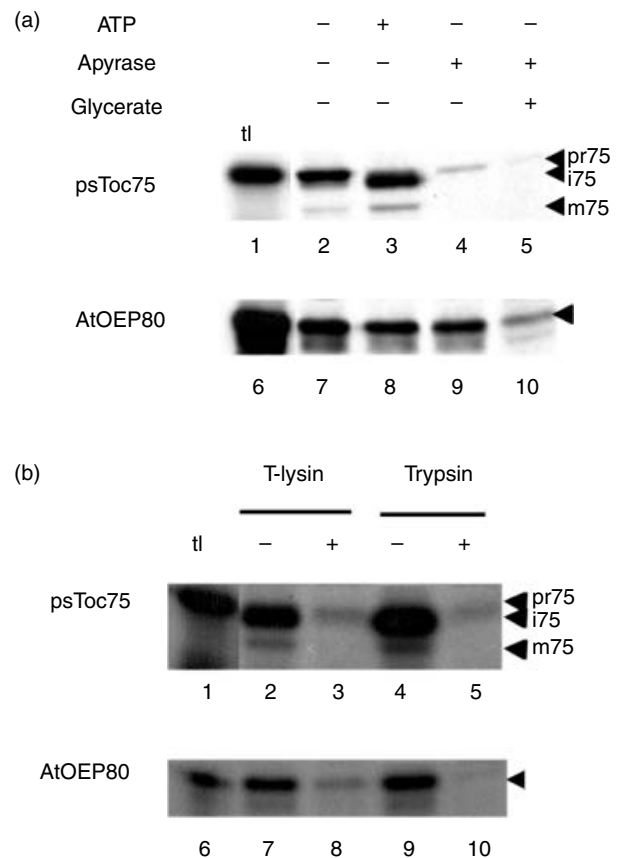
The cyanobacterial protein Slr1227, or SynToc75, was identified by searching for genes encoding psToc75 homologs in the genome of *Synechocystis* sp. PCC 6803 (Bolter *et al.*, 1998; Reumann *et al.*, 1999). The recent completion of *Arabidopsis* genomic sequencing (*Arabidopsis* Genome Initiative, 2000) prompted us to search for genes encoding any plant proteins similar to Slr1227, other than *atTOC75-III*. As a result, we identified a gene in chromosome V of the *Arabidopsis* genome (At5g19620) that encodes a protein with a sequence identity of 31% to Slr1227, which is higher than that to psToc75 (22%). Eckart *et al.* (2002) previously identified the same gene, named its product atToc75-V, and predicted that it was synthesized as a larger precursor of 80 kDa, and processed to be a mature form of 69 kDa. This prediction would make it similar to psToc75, but different from other outer membrane proteins, which do not have N-terminal cleavable targeting sequences (Schleiff and Klösgen, 2001). As a first step to characterize the newly identified Toc75 paralog from *Arabidopsis*, we sought to determine its native molecular weight. To this end, we





**Figure 2.** AtOEP80 is targeted to the chloroplastic outer envelope without a cleavable transit peptide.

(a) The radiolabeled precursor proteins (tl, 10% of the translation product subjected to the assay; lanes 1 and 9) were incubated with intact chloroplasts under the import condition. The chloroplasts were re-isolated, lysed hypotonically (lanes 3, 4, 11, and 12), or resuspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub> (lanes 5, 6, 13, and 14), or treated with trypsin and lysed hypotonically (lanes 7, 8, 15, and 16), before fractionated into the supernatant (s) and the pellet (p) by centrifugation, then proteins in each fraction were separated by SDS-PAGE and radiolabeled proteins were visualized by fluorography. The precursor form of Toc75 (pr75) was processed to the intermediate form (i75), then to the mature form (m75), as indicated with arrowheads. AtOEP80 did not change its molecular weight before and after the import as indicated with an arrowhead. (b) The radiolabeled precursor protein (tl, 10% of the translation product subjected to the assay; lanes 1 and 10) was incubated with intact chloroplasts on ice (lanes 2–5; 11–14) or at 25°C (lanes 6–9; 15–18) for the time indicated, and then chloroplasts were reisolated and analyzed as described above. (c) *Arabidopsis* chloroplasts (At, lanes 1 and 3) and pea chloroplasts containing imported radiolabeled AtOEP80 (imp, lanes 2 and 4) were run side by side, blotted on the same PVDF membrane, and analyzed by autoradiograph (Autorad, lanes 1 and 2) or immunoblots against AtOEP80 antisera (blot, lanes 3 and 4). AtOEP80 is indicated with an arrowhead.

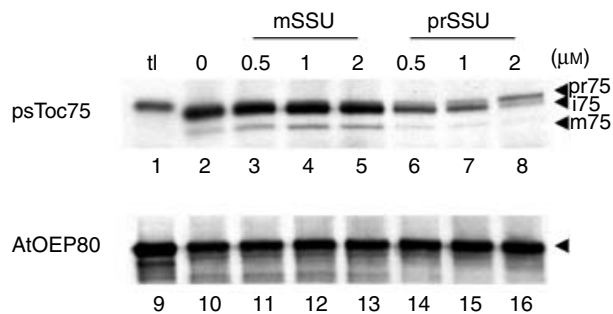


**Figure 3.** Both Toc75 and AtOEP80 require ATP and protease-sensitive components for their targeting.

(a) ATP in the translation products and chloroplasts used for the protein import assay was depleted by apyrase alone (lanes 4 and 9) or apyrase and glycerate (lanes 5 and 10). tl, 10% of the translation product subjected to the assay (lanes 1 and 6).

(b) Chloroplasts were treated with thermolysin (T-lysin; lanes 3 and 8) or trypsin (lanes 5 and 10) before subjected to the protein import assay. Mock-treated chloroplasts were also used (lanes 2, 4, 7, and 9). tl, 10% of the translation product subjected to the assay (lanes 1 and 6).

7 and 10). When chloroplasts were pre-treated with proteases, namely, thermolysin or trypsin, before the import assay, the targeting of both psToc75 and AtOEP80 to the chloroplastic envelope was significantly reduced, with a larger reduction by trypsin over thermolysin (Figure 3b, compare lanes 2 and 3, 4 and 5, 7 and 8, and 9 and 10, respectively). These results indicate that proteinaceous components are required for the targeting of these proteins. We performed immunoblot analysis of the protease-treated chloroplasts used in this study, and found that (i) the amount of one of the receptor components of the protein import machinery, Toc159, was reduced below the detection limit by both proteases; (ii) the level of endogenous Toc75 was partially reduced by trypsin but not changed by thermolysin; and (iii) the amount of a stromal chaperon, Hsp93, appeared to



**Figure 4.** A substrate for general import pathway inhibits targeting of Toc75, but not that of AtOEP80 to chloroplasts.

The chloroplast protein import assay of Toc75 (lanes 1–8) and AtOEP80 (lanes 9–16) was performed with the presence of 0.5, 1, or 2  $\mu\text{M}$  of the precursor of Rubisco small subunit (prSSU, lanes 6–8; 14–16), or its mature portion (mSSU, lanes 3–5, 11–13) that were produced in bacteria, or without any of these proteins (0, lanes 2 and 10). tl, 10% of the translation product subjected to the assay (lanes 1 and 5).

be unchanged (data not shown). Therefore, proteins in the outer envelope should be responsible for the targeting of AtOEP80.

As the presence of a transit peptide in its N-terminus was predicted, and its targeting required both ATP and protease-sensitive components at the chloroplast envelope, one might assume that, although there is no cleavable transit peptide, the targeting of AtOEP80 is partly mediated by the general import pathway. In order to test this hypothesis, we examined effects of a substrate of the general pathway, namely, the precursor of the small subunit of Rubisco, on the targeting of AtOEP80. As expected, the targeting of psToc75 was inhibited by the addition of the precursor (Figure 4, lanes 2, 6–8), but not by that of the mature form of the small subunit of Rubisco (Figure 4, lanes 2–5). On the contrary, neither the precursor nor the mature form of the Rubisco small subunit affected the targeting of AtOEP80 (Figure 4, lanes 10–16). Therefore, we concluded that the targeting of AtOEP80 is independent of the general import pathway.

#### *Phylogenetic relationships among AtOEP80, Toc75, and their cyanobacterial homologs are weakly resolved*

In order to get better insights into their evolutionary relationships, we extended the phylogenetic analyses of Toc75 homologs by incorporating all sequences available from cyanobacterial genomic and plant EST and genomic databases. We found that a green alga, *Chlamydomonas reinhardtii*, contained at least one gene each orthologous to Toc75 and AtOEP80, respectively, although we could not incorporate them into our analysis as these sequences were not long enough (data not shown). No genes encoding proteins homologous to Toc75 were identified from red algae such as *Porphyra yezoensis* (Asamizu *et al.*, 2003), which might be the result of the lack of their complete

sequence information. For the analyses, we also included sequences of the Omp85 family, one of which was shown to be present in the outer membrane of a Gram-negative bacterium, *Neisseria meningitides*, and to be responsible for biogenesis of outer membrane proteins (Voulhoux *et al.*, 2003), and sequences of the ShIB family, some of which were shown to function as virulence factor transporters (Sanchez-Pulido *et al.*, 2003), as outgroups to root the trees and to examine the possibility that the plant and cyanobacterial proteins do not form a monophyletic group. In order to minimize potential phylogenetic reconstruction artifacts derived from the use of distant outgroups, we sought to use only conserved and reliably aligned sequence regions. As a result, we found that (i) the N-terminus was extremely variable and could not be reliably aligned as previously described (Reumann *et al.*, 1999); (ii) a region corresponding to the 485 C-terminal amino acids of atToc75-III could reliably be aligned for 19 sequences (results not shown). As a large number of partial sequences were available in databases, we also constructed an alignment corresponding to the stretch of 107 amino acids in the C-terminus of atToc75-III, which included 75 sequences (Table 1).

We explored the effects of analyzing alignments of different lengths (longer and shorter), and both amino acid and nucleotide sequences, on the inferred relationships among the sequences. We also conducted both maximum parsimony and maximum likelihood analysis, the latter implemented in a Bayesian framework. Individual data are available upon request to the authors. As exemplified in Figure 5, the trees resulting from the analysis of 75 sequences of shorter lengths provided strong support for a clade including all the sequences of the Toc75 family and for one including all but two of the putative OEP80 homologs. OsOEP80B and ZmOEP80B, from rice and maize, respectively, were not consistently united with the other OEP80 sequences, and appear at the bottom of the tree in Figure 5. The sequences of the ShIB family were not resolved as monophyletic in any of the analyses. The relationships among Toc75, OEP80, and the cyanobacterial sequences were weakly resolved in all of the analyses: that is, none of the following three possible topologies could be rejected as significantly worse than any other under either optimality criterion for either alignment: (i) Toc75 and the cyanobacterial sequences form a monophyletic group; (ii) OEP80 and the cyanobacterial sequences form a monophyletic group; (iii) Toc75 and OEP80 form a monophyletic group.

Finally, we sought to establish the evolutionary relationships between OEP80 and Toc75 by comparing their gene structures. In the *Arabidopsis* genome, three genes have been identified to encode proteins orthologous to psToc75: *atTOC75-I*, *atTOC75-III*, and *atTOC75-IV*, among which only *atTOC75-III* appears to encode a full length coding sequence, and was shown to be expressed (Jackson-Constan and Keegstra, 2001); but, no gene sequence was found to show a

**Table 1** Sequences analyzed in this study

Group <sup>a</sup>	Name	Organism	i.d. <sup>b</sup>
Toc75	atTOC75-III	<i>Arabidopsis thaliana</i>	30692756
Toc75	atTOC75-IV	<i>Arabidopsis thaliana</i>	18413189
Toc75	bvTOC75	<i>Beta vulgaris</i>	26113883
Toc75	caTOC75	<i>Capsicum annuum</i>	22781791
Toc75	crTOC75A	<i>Citrus reticulata</i>	28616985
Toc75	crTOC75B	<i>Citrus reticulata</i>	28616984
Toc75	gmTOC75	<i>Glycine max</i>	TIGR_TC180820
Toc75	haTOC75	<i>Helianthus annuus</i>	TIGR_TC12671
Toc75	hvTOC75	<i>Hordeum vulgare</i>	TIGR_TC90082
Toc75	leTOC75	<i>Lycopersicon esculentum</i>	TIGR_TC117445
Toc75	lsTOC75	<i>Lactuca sativa</i>	TIGR_TC330
Toc75	mtTOC75	<i>Medicago truncatula</i>	20291249
Toc75	osTOC75A	<i>Oryza sativa</i>	32972018
Toc75	osTOC75B	<i>Oryza sativa</i>	32980034
Toc75	osTOC75C	<i>Oryza sativa</i>	32995594
Toc75	ppTOC75	<i>Physcomitrella patens</i>	37827066
Toc75	psTOC75	<i>Pisum sativum</i>	633606
Toc75	potTOC75	<i>Populus trichocarpa</i>	JGI
Toc75	soTOC75	<i>Saccharum officinarum</i>	35118629
Toc75	spTOC75	<i>Sorghum propinquum</i>	11409262
Toc75	stTOC75	<i>Solanum tuberosum</i>	TIGR_TC66558
Toc75	taTOC75	<i>Triticum aestivum</i>	TIGR_TC133426
Toc75	vvTOC75	<i>Vitis vinifera</i>	TIGR_TC16362
Toc75	zmTOC75A	<i>Zea mays</i>	TIGR_TC192715
Toc75	zmTOC75B	<i>Zea mays</i>	TIGR_TC192716
OEP80	AtOEP80	<i>Arabidopsis thaliana</i>	30687407
OEP80	BnOEP80	<i>Brassica napus</i>	32498116
OEP80	CrOEP80	<i>Citrus reticulata</i>	38025963
OEP80	GmOEP80	<i>Glycine max</i>	TIGR_TC201900
OEP80	HvOEP80	<i>Hordeum vulgare</i>	TIGR_TC108413
OEP80	LeOEP80	<i>Lycopersicon esculentum</i>	TIGR_TC124893
OEP80	OsOEP80A	<i>Oryza sativa</i>	32976046
OEP80	OsOEP80B	<i>Oryza sativa</i>	32974320
OEP80	PpOEP80	<i>Physcomitrella patens</i>	37837067
OEP80	PitOEP80	<i>Pinus taeda</i>	37564120
OEP80	StOEP80	<i>Solanum tuberosum</i>	21922105
OEP80	VvOEP80	<i>Vitis vinifera</i>	TIGR_TC16457
OEP80	ZmOEP80A	<i>Zea mays</i>	TIGR_TC198407
OEP80	ZmOEP80B	<i>Zea mays</i>	TIGR_TC202002
Cyano	NosAlr0075	<i>Nostoc</i> sp. PCC 7120	17135053
Cyano	NosAlr2269	<i>Nostoc</i> sp. PCC 7120	17131360
Cyano	NosAlr4893	<i>Nostoc</i> sp. PCC 7120	17134030
Cyano	NosATC29133A	<i>Nostoc punctiforme</i> strain ATCC 29133	JGI
Cyano	NosATC29133B	<i>Nostoc punctiforme</i> strain ATCC 29133	JGI
Cyano	NosATC29133C	<i>Nostoc punctiforme</i> strain ATCC 29133	JGI
Cyano	NosATC29133D	<i>Nostoc punctiforme</i> strain ATCC 29133	JGI
Cyano	SynSlr1227	<i>Synechococcus</i> sp. PCC 6803	1652591
Cyano	TheBP1	<i>Thermosynechococcus elongatus</i> BP-1	22295515
Cyano	Trichodesmium	<i>Trichodesmium erythraeum</i>	JGI
Cyano	PmaCCMP1375	<i>Prochlorococcus marinus</i> CCMP1375	33238397
Cyano	PmaMED4	<i>Prochlorococcus marinus</i> MED4	33640262
Cyano	PmaMIT9313	<i>Prochlorococcus marinus</i> MIT9313	33635263
Cyano	SynWH8102	<i>Synechococcus</i> sp. strain WH8102	33638775
Omp85	BabOmp1	<i>Brucella abortus</i>	1262286
Omp85	CcrOmp	<i>Caulobacter crescentus</i> CB15	13423361
Omp85	CelC34E10	<i>Caenorhabditis elegans</i>	14550347
Omp85	DmeCG7639	<i>Drosophila melanogaster</i>	7303109
Omp85	EcoEcfK	<i>Escherichia coli</i>	15529631
Omp85	EcoYtfM	<i>Escherichia coli</i>	1790666

Table 1 Continued

Group <sup>a</sup>	Name	Organism	i.d. <sup>b</sup>
Omp85	HinD15	<i>Haemophilus influenzae</i>	1573932
Omp85	HsaCGI51	<i>Homo sapiens</i>	15929538
Omp85	NcrTob55	<i>Neurospora crassa</i>	32409934
Omp85	NmeOmp85	<i>Neisseria meningitides</i>	7378858
Omp85	PaePA3648	<i>Pseudomonas aeruginosa</i>	9949799
Omp85	RprOmp1	<i>Rickettsia prowazekii</i>	3860726
Omp85	SceSam50	<i>Saccharomyces cerevisiae</i>	6324302
Omp85	SmeSMc03097	<i>Sinorhizobium meliloti</i>	15075850
Omp85	Spoc17C9.06	<i>Schizosaccharomyces pombe</i>	5869957
Omp85	VchVC2252	<i>Vibrio cholerae</i> O1 biovar eltor	9656810
Omp85	XfaXf1231	<i>Xylella fastidiosa</i>	9106207
ShIB	BpeFhaC	<i>Bordetella pertussis</i>	313840
ShIB	EchHecB	<i>Erwinia chrysanthemi</i>	1772622
ShIB	EtaAsp	<i>Edwardsiella tarda</i>	2244626
ShIB	HduHhdB	<i>Haemophilus ducreyi</i>	1151071
ShIB	SmaShIB	<i>Serratia marcescens</i>	556419

Sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>), DOE Joint Genome Institute (<http://www.jgi.doe.gov>), and The Institute of Genomic Research (<http://www.tigr.org/tdb/tgi/plant.shtm>).

<sup>a</sup>Toc75, plant Toc75 orthologs; OEP80, plant OEP80 orthologs; Cyano, sequences from cyanobacteria; Omp85, the Omp85 family; ShIB, the ShIB family.

<sup>b</sup>NCBI gene index number, TIGR Tentative Consensus (TIGR\_TC), or JGI.

significant similarity ( $E < 0.01$ ) to that of *AtOEP80* in the *Arabidopsis* genome other than itself. Therefore, we compared predicted structures of the four genes: *atTOC75-I*, *atTOC75-III*, *atTOC75-IV*, and *AtOEP80* (Figure 6). *atTOC75-I* and *atTOC75-IV* appear to be truncated forms of *atTOC75-III*, while *AtOEP80* is significantly different from the other three genes in the number and the size of the exons and introns. These observations support the hypothesis that the gene duplication for four Toc75 homologs would have taken place in the following order: divergence of *AtOEP80* in the very early stage of evolution, followed by more recent gene duplication to *atTOC75-I*, *atTOC75-III*, and *atTOC75-IV*.

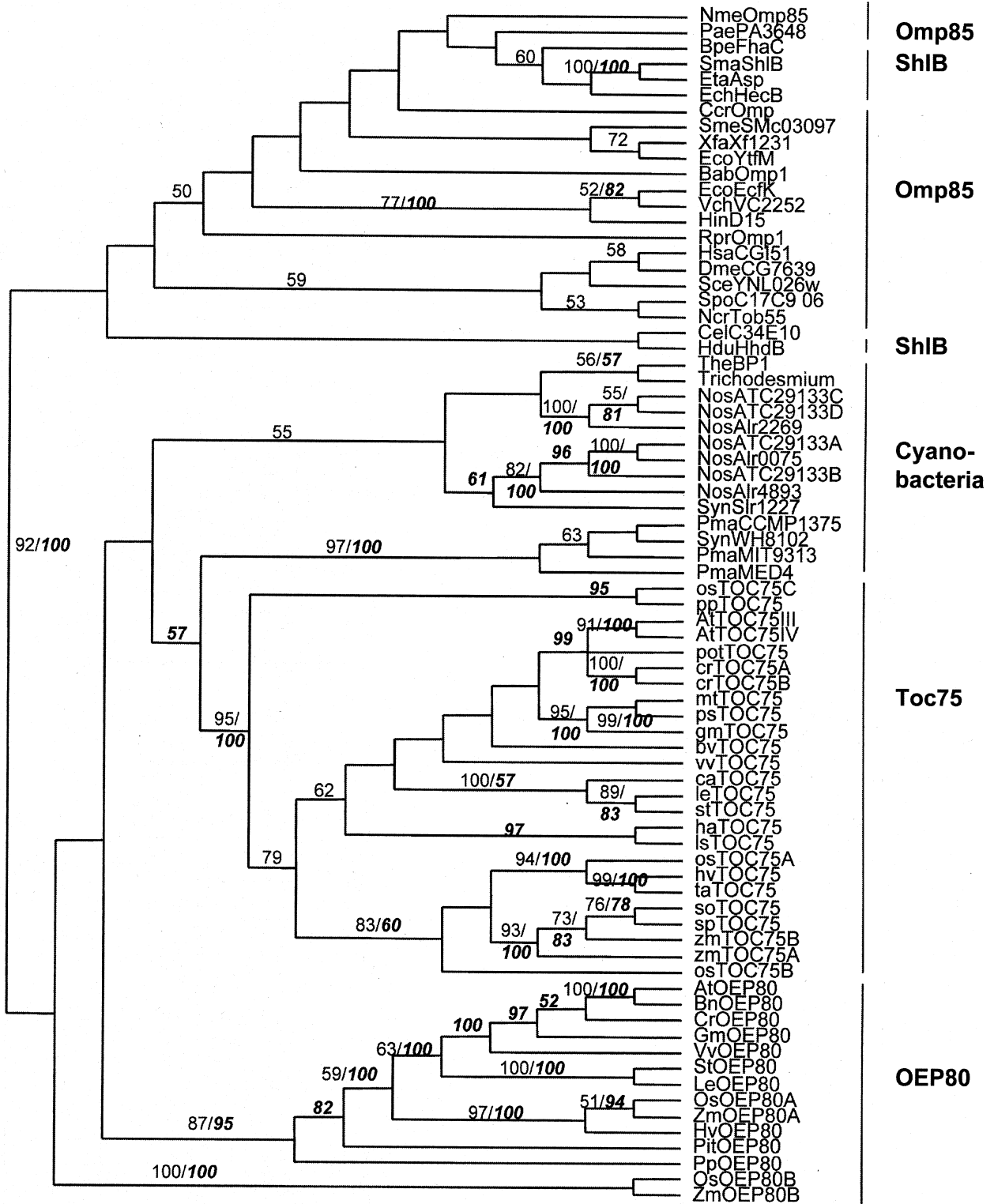
## Discussion

During the course of the evolution of organelles such as mitochondria and plastids, genes from the endosymbionts are believed to have been transferred to the host nucleus and relinquished from the bacterial genome (Martin, 2003). Therefore, the establishment of protein translocation machinery at their envelope membranes was a prerequisite for the successful transformation of the prokaryotic endosymbionts into semiautonomous organelles. Currently, however, it remains unclear how these events took place. The main objective of the present study was to shed light on this problem by studying the newly identified paralog to the chloroplastic protein translocation channel Toc75.

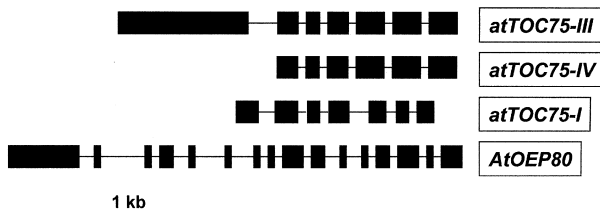
First, we showed that the molecular mass of the new Toc75 paralog in *Arabidopsis* in its native form is 80 kDa; 14 kDa larger than that of the pea ortholog reported by Eckart *et al.* (2002). The reason for this relatively large difference between the two orthologs in their sizes remains

unknown. It also remains unclear whether the sizes of the *AtOEP80* orthologs from different plant species are conserved. We would need to prepare antisera that can recognize orthologs from the various plant species, or to obtain full length cDNAs of various orthologs, and utilize them to address these questions.

Next, we found that the targeting of *AtOEP80* to chloroplasts did not require a cleavable transit peptide although the presence of a 93-amino acid-long transit peptide was predicted (Eckart *et al.*, 2002). It is interesting to note that another chloroplastic outer envelope protein DGD1 was also postulated to have a cleavable transit peptide of 58 amino acids; however, it was targeted to the outer envelope without an apparent change in its molecular weight (Froehlich *et al.*, 2001). Eighty-eight percent of the sequences was correctly classified to be chloroplast-targeted ones by the prediction program ChloroP (Emanuelsson *et al.*, 1999). Therefore, the cases for *AtOEP80* and DGD1 may be examples of false prediction. Alternatively, the predicted transit peptide portion may have some function in the targeting of the protein. A detailed investigation of the effect of deletion of this portion on the targeting should lead us to answer the question. As for the insertion of *AtOEP80*, its temperature dependency could be because of the phase transition of the outer envelope lipids that would be involved in the insertion of the proteins, as was discussed by Schleiff and Klösgen (2001). The targeting of several other outer membrane proteins, such as Toc34 and DGD2, was shown to be enhanced by ATP (Kelly *et al.*, 2003; Li and Chen, 1997). Furthermore, OEP14 and Toc34 were targeted to the envelope in a manner dependent on protease-sensitive components (Tsai *et al.*, 1997; Tu and Li, 2000), although a recent



**Figure 5.** Phylogenetic tree for 75 Toc75-related sequences. Strict consensus tree for two equally most parsimonious trees [ $I = 5,787$ ,  $ci$  (excluding autapomorphies) = 0.22,  $ri = 0.54$ ] from phylogenetic analysis of nucleotide sequences of Toc75, AtOEP80, and homologous proteins from cyanobacteria and various other organisms (Table 1), for an alignment corresponding to the 107 C-terminal amino acids of atToc75-III. Numbers on branches represent bootstrap support values (regular fonts) and Bayesian posterior probabilities (bold italic fonts), both expressed as percentages. Branches lacking one or both values were supported in fewer than 50% of the trees produced by bootstrap parsimony analysis and/or fewer than 50% of the trees from the Bayesian analysis.



**Figure 6.** *atTOC75-I*, *III*, *IV* and *AtOEP80* are different in their gene structures. Closed boxes and lines between them indicate exons and introns, respectively, which are defined by The Institute of Genomic Research (<http://www.tigr.org>; annotation is based on cDNA and gene sequences for *atTOC75-III* and *AtOEP80*, or derived using computer programs only for *atTOC75-I* and *atTOC75-IV*). Scale bar indicates 1 kb.

report using protein-free liposomes argued against the requirement of proteinaceous components for the insertion of Toc34 *in vitro* (Qbadou *et al.*, 2003). It remains unknown whether or not a single energy source and proteinaceous machinery are responsible for the targeting of all chloroplastic outer envelope proteins.

Our extensive phylogenetic analyses of Toc75 homologs resulted in weakly resolved trees. Nonetheless, our analyses supported two conclusions concerning the origins and divergence of the Toc75 and OEP80 families, which, however, did not include the one by Eckart *et al.* (2002) that OEP80 is the more ancestral form. The first conclusion is that Toc75 and OEP80 represent two distinct families of proteins in higher plants. In several species, each of them is present in two or more paralogous copies. Within both families, there appeared to be a lack of correspondence between the gene phylogenies and well-established organismal phylogenies for land plants; for example, sequences from some angiosperms were more closely related to a moss sequence than to other angiosperm sequences. These results suggest that gene duplications occurred within both families early in the evolution of land plants. Analyses of sequences from additional non-angiosperm land plants and those from green algae should help to pinpoint the timing of these ancient duplications. The second conclusion is that both the Toc75 and the OEP80 families may have been derived from cyanobacterial sequences. The strength of this conclusion is, however, restricted by the limited taxon representation of available sequences. Divergence among sequences also severely constrained the length of sequences that we could reliably align. As a result, the exact pattern of relationships among the Toc75, OEP80, and cyanobacterial homologs remains weakly resolved.

Our current data could lead to the hypothesis that Toc75 and OEP80 resulted from duplication of a gene in the prokaryotic lineage that gave rise to the endosymbiont. If this were true, the duplication must have occurred close in time to the endosymbiotic event, as there is only weak support for the sister relationship between Toc75 and

OEP80. If the duplication occurred prior to the endosymbiotic event, we might expect to find extant cyanobacteria bearing both copies. The fact that no such organism has been identified to date suggests either that the duplication occurred after the endosymbiotic event, or that this lineage has gone extinct or is as yet undiscovered. We could also develop an alternative hypothesis that Toc75 and OEP80 are derived from genes present in different ancestral cyanobacteria and arrived in the same genome via lateral gene transfer. In this case, we would expect that only one of these genes was present in the endosymbiont that gave rise to the plastid of modern plants, while the other was transferred, either to the endosymbiont's genome or directly to the host genome, around the time of the endosymbiotic event.

One might speculate that the difference between Toc75 and OEP80 in their targeting mechanism is linked to the scenario that they were derived from different ancestral cyanobacteria. Based on strong similarities in their characteristics at both the structural and functional levels, it has been postulated that the double envelope membrane system of plastids was derived from that of a free-living prokaryotic ancestor (Keegstra *et al.*, 1984). Therefore, one might assume that most outer membrane proteins are conserved in modern cyanobacteria and in plant chloroplasts. Interestingly, however, only beta-barrel proteins have been found in the bacterial outer membrane, whereas both beta-barrel and alpha-helical proteins are present in the chloroplastic outer envelope (Wimley, 2003): the latter seems to have been added to various proteins to fulfill divergent functions of the semi-autonomous organelle. Therefore, it would not be surprising if there were some beta-barrel proteins in the chloroplastic outer envelope which were not derived from the cyanobacterium-like endosymbiont, but rather had arisen from another ancestral cyanobacterium. All of the beta-barrel proteins in the chloroplastic outer envelope identified so far, such as OEP24, OEP16, and AtOEP80, but not Toc75, are targeted to chloroplasts without the aid of N-terminal transit sequence (Schleiff and Klösgen, 2001). One could speculate that (i) the endosymbiont-derived outer membrane proteins have affinity to the outer envelope of the organelle so that they did not need additional sequence for their biogenesis; (ii) the ancestral form of Toc75 may not have existed in the outer membrane of the endosymbiont, thus it needed a cleavable signal to be targeted to the newly acquired organelle to establish its function. In this respect, it would be interesting to test (i) whether or not Toc75 and other related proteins have affinity to the cyanobacterial outer membrane; and (ii) whether or not Toc75 homologs are functionally replaceable.

So, then, what is the biological function of AtOEP80? One can develop several hypotheses based on the current knowledge. A bacterial protein distantly related to Toc75 and OEP80, Omp85 from *N. meningitidis*, was recently

shown to be responsible for directing beta-barrel proteins from the periplasmic space to the outer membrane (Voulhoux *et al.*, 2003). Furthermore, other distant homologs of Toc75, Tob55 from *Neurospora crassa* and Sam50 from yeast, were found to direct beta-barrel proteins that are inserted into the mitochondrial envelope by general protein import apparatus, TOM complex, from intermembrane space to the outer membrane (Gentle *et al.*, 2004; Kozjak *et al.*, 2003; Paschen *et al.*, 2003). Therefore, the function of these homologs, directing beta-barrel proteins from periplasmic space to the outer membrane, appeared to be evolutionary conserved. However, beta-barrel proteins in the chloroplastic envelope appear to utilize mechanisms for their biogenesis distinct from that for the mitochondrial outer membrane proteins: all but one chloroplastic outer envelope membrane proteins do not require the TOC complex-mediated general import pathway for their targeting (Schleiff and Klösgen, 2001). They seem to be targeted to the outer membrane from the outside of the organelle, not from the intermembrane space. Despite this fact and the rather distant relationships with Omp85 homologs, however, AtOEP80 may be responsible for the biogenesis of chloroplastic beta-barrel proteins by assisting their insertions into the outer envelope. Another hypothesis would be that AtOEP80 is part of an alternative protein translocation machinery as suggested by Eckart *et al.* (2002). Currently, we are attempting to test these hypotheses using biochemical and genetic approaches.

## Experimental procedures

### Preparation of anti-AtOEP80 antisera and immunoblot experiments

A peptide of 14 amino acids (Asp-Arg-Lys-Thr-Gly-Glu-Pro-Thr-Lys-Gly-Lys-Thr-Ser-Cys\*) that contains a portion of AtOEP80 (residue 325–337; underlined) and a cysteine residue (asterisk) in its C-terminus was designed as an antigen because it was predicted to be highly antigenic by PeptideStructure (SeqWeb version 2; Accelrys, Inc., San Diego, CA, USA). This peptide sequence was also expected to be highly specific to atToc75-V because there is no cDNA sequence found in the *Arabidopsis* database that is predicted to encode at least five identical continuous amino acids in the peptide. The preparation of the peptide, conjugating it to the carrier protein keyhole limpet hemocyanin via the SH group of the C-terminal cysteine, and raising antisera against the conjugate in a rabbit were carried out by Covance Research Products (Denver, PA, USA). The antisera against psToc75 were kind gifts from Dr Keegstra (Michigan State University). Chloroplasts were prepared from 4-week-old *Arabidopsis* seedlings grown on plates as described (Fitzpatrick and Keegstra, 2001). Chloroplastic fraction containing 10 µg of chlorophyll were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with a blocking buffer, Tris-buffered saline containing 0.05% Tween 20 (TBST) with 5% dry milk, for 30 min, next with the

blocking buffer with sera of 1:2000 dilution for 16 h, rinsed with TBST, incubated with the secondary antibody (alkaline phosphate-conjugated anti-rabbit IgG antibody; KPL Inc., Gaithersburg, MD, USA), then the immunoreaction was visualized using *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt (Promega, Madison, WI, USA) as substrates. The peptide-competition experiments were performed to determine whether the antipeptide antisera recognized the AtOEP80 in chloroplasts. Prior to incubating the antisera with immobilized chloroplastic proteins by the above protocol, the antisera were diluted in 1:2000 with TBST, and were incubated with the antigen peptide (10 µg of peptide ml<sup>-1</sup>) for 30 min at room temperature.

### Molecular cloning of AtOEP80 cDNA and chloroplastic protein import assay in vitro

A coding sequence for AtOEP80 was cloned by polymerase chain reaction using primers designed based on the sequence deposited in the database (At5g19620) and cDNA prepared from *Arabidopsis* seedlings as described (Inoue and Keegstra, 2003), and then subcloned into pGEM®-T Easy vector (Promega). The resultant plasmid was named pGEMT-AtOEP80. The identity of the clone was confirmed by sequencing from both ends. For chloroplastic protein import assay, T<sub>N</sub>T® Coupled Reticulocyte Lysate Systems (Promega) was used to prepare radiolabeled precursors. The protein import assay was performed using chloroplasts isolated from pea seedlings as described (Inoue and Keegstra, 2003). For ATP depletion experiments, the radiolabeled proteins were treated with apyrase (Sigma, St Louis, MO, USA), and chloroplasts were treated with glycerate (Sigma) as described (Tranel *et al.*, 1995). The protein import reaction was initiated by adding chloroplasts and maintained in room temperature (25°C) under dark. For protease treatment of chloroplasts prior to the import assay, 0.2 mg of trypsin (2420 BAEE units; Sigma) or thermolysin (Sigma) per chloroplasts containing 0.2 mg chlorophyll was incubated in the import buffer (with 0.2 mM CaCl<sub>2</sub> for thermolysin) of 0.2 ml. After incubation on ice for 30 min, the enzyme reaction was quenched by the addition of 0.4 mg of trypsin inhibitor (for trypsin; Sigma), which had a capacity to inactivate trypsin of 8400 BAEE units, or 10 mM EDTA (for thermolysin). Chloroplasts were then repurified through 40% Percoll cushion, washed with the import buffer once, and the resultant chloroplasts were resuspended in the import buffer and the amount of chlorophyll measured as described (Arnon, 1949). Chloroplasts containing 12.5 µg of chlorophyll were used for the protein import assay with a total volume of 50 µl. Protease treatment of chloroplasts after the import assay was performed as described (Inoue and Keegstra, 2003). The effects of *Escherichia coli*-overproducing proteins on chloroplastic protein import were examined by Inoue *et al.* (2001).

### Phylogenetic analyses

Nucleotide sequences were translated to amino acids in DAMBE (Xia and Xie, 2001); the latter were aligned using ClustalX (Thompson *et al.*, 1997) with default settings and nucleotide sequences were then aligned to the resulting amino acid alignment using DAMBE. Two separate alignments of both the amino acid and the corresponding nucleotide sequences were constructed: one with fewer taxa and a longer portion of the sequence (data not shown) and one with more taxa and a shorter portion of the sequence (e.g. Figure 5).

Phylogenetic analyses of amino acid and nucleotide alignments using the maximum parsimony criterion were implemented in PAUP\* (Swofford, 2002). Heuristic searches were conducted with 100 random taxon addition replicates with TBR branch-swapping and MULPARS in effect and MAXTREES unlimited. All positions were weighted equally; gaps were treated as missing values. Support for branches was evaluated using phylogenetic bootstrapping (Felsenstein, 1985) with 1000 bootstrap pseudoreplicates. For each pseudoreplicate, heuristic searches were conducted with 10 random taxon addition replicates and MAXTREES set at 100.

Bayesian analyses of both nucleotide sequence alignments were conducted using MrBayes v. 3.0B4 (Huelsenbeck and Ronquist, 2001). In each case, the MCMCMC algorithm was run for 1 050 000 generations, four independent chains were run simultaneously, and trees were sampled every 10 generations. The model settings were lset nst=6 rates=invgamma; this corresponds to the GTR + I + G model, which was selected as the best model for both alignments using the hierarchical likelihood ratio test in MODELTEST 3.06 (Posada and Crandall, 1998). The trees from the first 5000 (longer alignment) or 9000 (shorter alignment) generations were eliminated, and a 50% majority rule consensus tree was computed for the remaining trees in PAUP\* (Swofford, 2002); the proportion of trees in which a particular group was recovered was interpreted as the Bayesian posterior probability for that clade (Huelsenbeck and Ronquist, 2001).

In order to test whether or not alternative topologies were significantly worse than the optimal topologies recovered by each analysis, we employed statistical tests of differences in parsimony and likelihood scores, implemented in PAUP\* (Swofford, 2002). We first constructed constraint trees corresponding to the topologies we wished to test. For parsimony analyses, we conducted searches, as described above, on each of the alignments using each of the designated constraint trees. All resulting most parsimonious trees were saved to a single file along with the optimal tree(s) for that alignment; all of these trees were then imported back into the analysis and the significance of the differences in lengths between each tree and the shortest tree(s) was tested using the Kishino and Hasegawa (1989), Templeton (1983), and winning-sites (Prager and Wilson, 1988) tests. To test for significance of differences in log-likelihood scores of the trees resulting from the Bayesian analyses, we filtered the set of trees with each of the constraint topologies. In order to reduce computation time, we saved only two of the trees (if there were any), the first and the last, that conformed to the test topology. All of these trees were saved to a single file and were then imported back into the analysis. The significance of the difference in log-likelihood scores between each tree and the best one was evaluated using the Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) tests, with RELL test distributions estimated using 1000 bootstrap replicates. For these analyses, likelihood scores were calculated using the settings for proportion of invariants, gamma shape, revmat rates, and state frequencies selected by MODELTEST (Posada and Crandall, 1998) for each alignment. For all the tests described above, the differences in parsimony or likelihood score were considered significant at  $P < 0.05$ .

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