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HSP90, Tubulin and Actin are Retained in the Tertiary Endosymbiont Genome of *Kryptoperidinium foliaceum*

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ABSTRACT. The dinoflagellate *Kryptoperidinium foliaceum* has replaced its ancestral peridinin-containing plastid with a fucoxanthin-containing diatom plastid via tertiary endosymbiosis. The diatom endosymbiont of *K. foliaceum* is much less reduced than well-studied endosymbiotic intermediates, such as cryptophytes and chlorarachniophytes, where relict nuclear genomes are retained in secondary endosymbionts. The *K. foliaceum* endosymbiont retains a prominent nucleus, multiple four-membrane plastids, and mitochondria, all within a relatively large volume of cytoplasm that is separated from the host cytoplasm by a single membrane. Here we report the first protein-coding gene sequences from the *K. foliaceum* endosymbiont and host nuclear genomes. We have characterised genes for nucleus-encoded cytosolic proteins, actin (from endosymbiont), alpha-tubulin (from both), beta-tubulin (from host), and HSP90 (from both), in addition to homologues from pennate diatoms *Nitzschia thermalis* and *Phaeodactylum tricornutum*. Phylogenetic reconstruction shows that the actin is diatom-derived, the beta-tubulin dinoflagellate-derived, while both diatom- and dinoflagellate-derived alpha-tubulin and HSP90 genes were found. The base composition biases of these genes co-varied with their phylogenetic position, suggesting that the genes still reside in their respective genomes. The presence of these genes implies they are still functional and more generally indicates that the endosymbiont is less genetically reduced than those of cryptophytes or chlorarachniophytes, raising the interesting question of whether any genes have transferred between the two nuclear genomes.

Key Words. Alpha-tubulin, beta-tubulin, dinoflagellate, fucoxanthin, gene transfer, genome reduction, nucleomorph, pennate diatom, plastid replacement, tertiary.

PLASTIDS and mitochondria are now known to have originated by endosymbioses involving a cyanobacterium and an alpha-proteobacterium respectively, but the history of plastids has many extra layers of complexity because this event only explains a fraction of plastid diversity. The original endosymbiosis with a cyanobacterium is now referred to as the primary endosymbiosis, and it led to the plastids in glaucophytes, red algae, green algae, and plants. In contrast, all other algal groups acquired their plastids by secondary endosymbiosis with either a red or green alga (Archibald and Keeling 2002). In practically all of these cases the algal endosymbiont is reduced to its plastids and an extra membrane, but in two cases, the cryptophytes and chlorarachniophytes, the endosymbionts have retained functioning, vestigial nuclei. These relict nuclei, called nucleomorphs, retain well-characterised, highly reduced genomes that largely encode housekeeping genes as well as a small number of genes for proteins targeted to the plastid (Gilson, Maier, and McFadden 1997; Gilson and McFadden 2002). Whether these genomes are now in the process of being eliminated or have been frozen for some reason is unknown, but in either case they represent important intermediates in the process of endosymbiont reduction.

Virtually all photosynthetic dinoflagellates have a secondary plastid derived from a red alga that is known as the peridinin-containing plastid. However, this secondary peridinin plastid has been supplanted in a handful of dinoflagellate lineages. *Lepididinium* has undergone what has been termed a serial secondary endosymbiosis by acquiring a green algal plastid (Watanabe et al. 1987; Watanabe et al. 1990). At least three (Schnepf and Elbrachter 1999) groups have acquired tertiary plastids by forming an endosymbiotic partnership with other secondary algae: 1) Photosynthetic *Dinophysis* species have a cryptophyte-derived plastid; 2) *Karenia* and *Karlodinium* have haptophyte-derived plastids; 3) *Kryptoperidinium* and *Durinskia* have a diatom-derived plastid (Chesnick, Morden, and Schmieg 1996; Chesnick et al. 1997; Hackett et al. 2003; Hallegraeff and Lucas 1988; Hewes et al. 1998; Schnepf and Elbrachter 1988; Tangen and Bjørnland 1981; Tengs et al. 2000). *Kryptoperidinium foliaceum* (previously placed in the genera *Glenodinium* and *Peridinium*) was originally noted for its dual nuclei, and then for containing the typical chrysophyte pigment fucoxanthin (Dodge 1971; Withers and Haxo 1975). Morphological observations and phylogenetic analyses of the large and small subunits (*rbcL* and *rbcS*) of plastid ribulose-1, 5-bisphosphate carboxylase demonstrated the existence of a membrane-bound endosymbiont and suggested it was of diatom origin (Chesnick, Morden, and Schmieg 1996; Jeffrey and Vesk 1976; Kite and Dodge 1985). Subsequently, small subunit ribosomal RNA (SSU rRNA) phylogenies placed the endosymbiont among the pennate diatoms (Chesnick et al. 1997). A closely related species, *Durinskia baltica*, has also been shown to contain such a plastid (as *Peridinium balticum* in Tomas and Cox 1973). The endosymbiosis appears to be permanent and obligatory, and these two species are now thought to be the product of a single endosymbiosis (Inagaki et al. 2000). Interestingly, *K. foliaceum* has retained a tri-membrane carotene-containing eyespot organelle that has been interpreted as a relict of the original, tri-membrane peridinin-containing plastid (Dodge 1983; Dodge and Crawford 1969).

Unfortunately, there are few molecular data from either the host or symbiont of *K. foliaceum*. Yet, it is a remarkable system to study the physical and genetic reduction characteristic of endosymbionts because it appears to be in the early stages of this process. While the endosymbiont appears to be obligately intracellular, it is far less reduced than the endosymbionts of either cryptophytes or chlorarachniophytes (Fine and Loeblich 1976; Jeffrey and Vesk 1976; Morrill and Loeblich 1977). The *K. foliaceum* endosymbiont has lost motility and its distinctive diatom wall, and the endosymbiont nuclear genome of its sister species, *D. baltica*, appears to divide amitotically (Tippit and Pickett-Heaps 1976). Nevertheless, the symbiont has retained a comparatively large cytoplasmic space and nucleus. It is unclear whether the nucleus should be referred to as a nucleomorph, so for the present it will be called the endosymbiont nucleus. Mitochondria, which have been lost in all other plastid-endosymbionts, are retained (Kite, Rothschild, and Dodge 1988; Rizzo and Cox 1976, 1977). *Kryptoperidinium foliaceum* is accordingly quite complex at the genome level: it contains two nuclear genomes, two mitochondrial genomes, and at least one plastid genome. Moreover, the diatom endosymbiont apparently resides in the host cytoplasm (since there is only one membrane separating the host and endosymbiont nuclei), in contrast to the cryptophyte and chlorarachniophyte endosymbionts, which re-

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side in the host endomembrane system (Eschbach et al. 1990; Gilson 2001).

These differences and the relative rarity of such endosymbiotic events make *K. foliaceum* an interesting point of comparison with the much better studied cryptophyte and chlorarachniophytes. Here we report the first protein-coding genes from the nucleus of the endosymbiont, together with homologues from the host genome and from other diatoms. We have examined four genes with interesting functional implications and varying presence or absence in cryptophyte and chlorarachniophyte nucleomorph genomes: heat-shock protein 90 (HSP90, which is present in both nucleomorphs), alpha- and beta-tubulin (which are present in cryptophytes but apparently not in chlorarachniophytes), and actin (which is present in neither nucleomorph).

MATERIALS AND METHODS

DNA isolation, amplification and sequencing. Cultures of *Kryptoperidinium foliaceum* (Center for Culture of Marine Phytoplankton, CCMP 1326) and *Nitzschia thermalis* (Canadian Centre for the Culture of Microorganisms, CCCM 608) were maintained in f/2-Si medium on a 13/11 light/dark cycle. *Kryptoperidinium foliaceum* was kept at room temperature (23–25 °C); *N. thermalis* at 16 °C. Cells were harvested by centrifugation and DNA purified using the DNeasy Plant DNA isolation kit (Qiagen, Mississauga, ON). DNA from *Phaeodactylum tricornutum* (CCMP 630) was kindly provided by J. T. Harper. Alpha-tubulin, beta-tubulin, HSP90, and actin genes were amplified using the following primers: 5′-TCCGAATTCARGTNG GNAAYGCNGGYTGGGA-3′ and 5′-CGCGCCATNCCYTCN CCNACRTACCA-3′ (alpha-tubulin), 5′-GCCTGCAGGNCAR TGYGGNAAYCA-3′ or 5′-TCCTCGAGTRAAYTCCATYTC RTCCAT-3′ and 5′-CAGGTCGGTCARTGYGGNAA-3′ (beta-tubulin), plus diatom specific beta-tubulin primers 5′-ATBGCK GCNGCMGTNTGYGGNCATA-3′ and 5′-CCACGTCTCCTG SACRGCVGTGGT-3′. 5′-GTCAAGCAYTTYWSNGTNGAR GGNCA-3′, 5′-GGAGCCTGATHAAYACNTTYTA-3′, and 5′-GTCCCGCAGNGCYTGNGCYTTCATDAT-3′ (HSP90), and 5′-GAGAAGATGACNCARATHATGTTYGA-3′, and 5′-GGC CTGGAARCAYTTNCGRTGNAC-3′ (actin). PCR products were cloned using pCR2.1 TOPO cloning kit (Invitrogen, Burlington, ON), and both strands sequenced using Big Dye terminator chemistry. New sequences have been deposited into GenBank, accession numbers AY713387-AY713398.

Phylogenetic analysis. Conceptual translations were added to existing amino acid alignments (Keeling and Leander 2003; Saldarriaga et al. 2003). *Thalassiosira pseudonana* (CCMP 1335) alpha- and beta-tubulin, actin, and HSP90 sequences were assembled from genome sequence data, produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/).

Alignments consisted of 50, 47, 54, and 44 sequences, and 375, 363, 505, and 206 unambiguously aligned sites for alpha-tubulin, beta-tubulin, HSP 90, and actin, respectively (available upon request). Phylogenetic trees were inferred for all four individual gene data sets using distance and maximum likelihood. TREE-PUZZLE 5.0 (Schmidt et al. 2002) was used to calculate a distance matrix under the Whelan and Goldman (WAG) model of substitution frequencies, and each was corrected for site-to-site rate variation using a discrete gamma distribution with 8 variable rate categories plus one invariable category. The amino acid frequencies, proportion of invariable sites, and shape parameter alpha were estimated from the data with TREE-PUZZLE 5.0. Alpha parameters (α) and proportion of invariable sites (i) for alpha-tubulin, beta-tubulin, HSP90, and actin were α = 0.63, 0.44, 0.85, 0.47 and I = 0.25, 0.00, 0.09, 0.11, respectively.

Distance trees were inferred from each distance matrix by weighted neighbour-joining (WNJ, Weighbor 1.0.1) (Bruno, Socci, and Halpern 2000) and Fitch-Margoliash (FITCH, Fitch 3.572) (Felsenstein 1997). For all four data sets 100 (Fitch Margoliash) or 500 (weighted neighbor-joining) bootstrap replicates were analysed using PUZZLEBOOT (A. Roger, M. Holder, http://www.tree-puzzle.de) with the alpha shape parameter and the proportion of invariable sites from the original data.

Protein maximum-likelihood (ML) trees were inferred for all four data sets using ProML 3.6 (Felsenstein 1997) under the Jones, Taylor, and Thornton (JTT) substitution frequency matrix with global rearrangements and two input order jumbles. Site-to-site rate variation was modeled on a gamma distribution with four variable rate categories and one invariable category. Rates and frequencies were estimated using TREE-PUZZLE 5.0. Maximum-likelihood bootstrapping was performed with 100 replicates, only one category of sites, global rearrangements, and one jumble.

RESULTS AND DISCUSSION

Host and endosymbiont nuclear-encoded protein coding genes from *Kryptoperidinium foliaceum*. Altogether, eight new homologues of alpha-tubulin, beta-tubulin, actin, and HSP90 were characterised from *K. foliaceum*, as well as alpha-tubulin, actin, and HSP90 from the pennate diatom *N. thermalis* and actin from *P. tricornutum*. The phylogenies of these four genes were constructed to distinguish homologues from the host and endosymbiont nuclear genomes of *K. foliaceum*. The overall characteristics of these phylogenies resembled those seen in previous analyses (Edgcomb et al. 2001; Keeling and Leander 2003; Saldarriaga et al. 2003; Stechmann and Cavalier-Smith 2003), and most importantly the dinoflagellates and diatoms consistently formed strongly supported clades.

Three distinct alpha-tubulin genes were amplified from *K. foliaceum*, two of which were highly similar at the amino acid level. In alpha-tubulin phylogeny (Fig. 1), the two similar *K. foliaceum* sequences branched strongly within the dinoflagellate clade (97–100%), while the third gene branched with moderate to strong support in the diatom clade (63–96%), specifically with the pennate diatom *N. thermalis* (97–100%). This pattern is precisely that predicted for a host genome origin for the two similar genes and an endosymbiont genome origin for the third.

The beta-tubulin phylogeny included two similar *K. foliaceum* genes (Fig. 2) and showed both sequences branching with strong support in the dinoflagellate clade (90–98%), to the exclusion of *Oxyrrhis* and all other species. In this case, both of these sequences are predicted to have originated in the host genome. Given the presence of an endosymbiont-derived alpha-tubulin, however, it is likely that an endosymbiont derived beta-tubulin does exist, but may be too divergent to amplify, despite much effort and multiple primer-sets. This notion is supported by the slightly divergent nature of the pennate diatom alpha-tubulins (since the two often co-evolve), the divergent nature of the centric diatom beta-tubulins (which, together with brown algae, do not branch with oomycete heterokonts), and the failure to amplify the beta-tubulin from *N. thermalis* or the *K. foliaceum* endosymbiont with universal or heterokont-specific primers, nor any combination of the two.

A single *K. foliaceum* actin sequence was characterised. In actin phylogenies (Fig. 3) this gene grouped within a moderately supported heterokont clade (70–83%), specifically within the strongly supported diatom subgroup (97–100%). Within the diatoms, the centric and pennate types do not form discrete clades, but the *K. foliaceum* sequence does branch weakly with

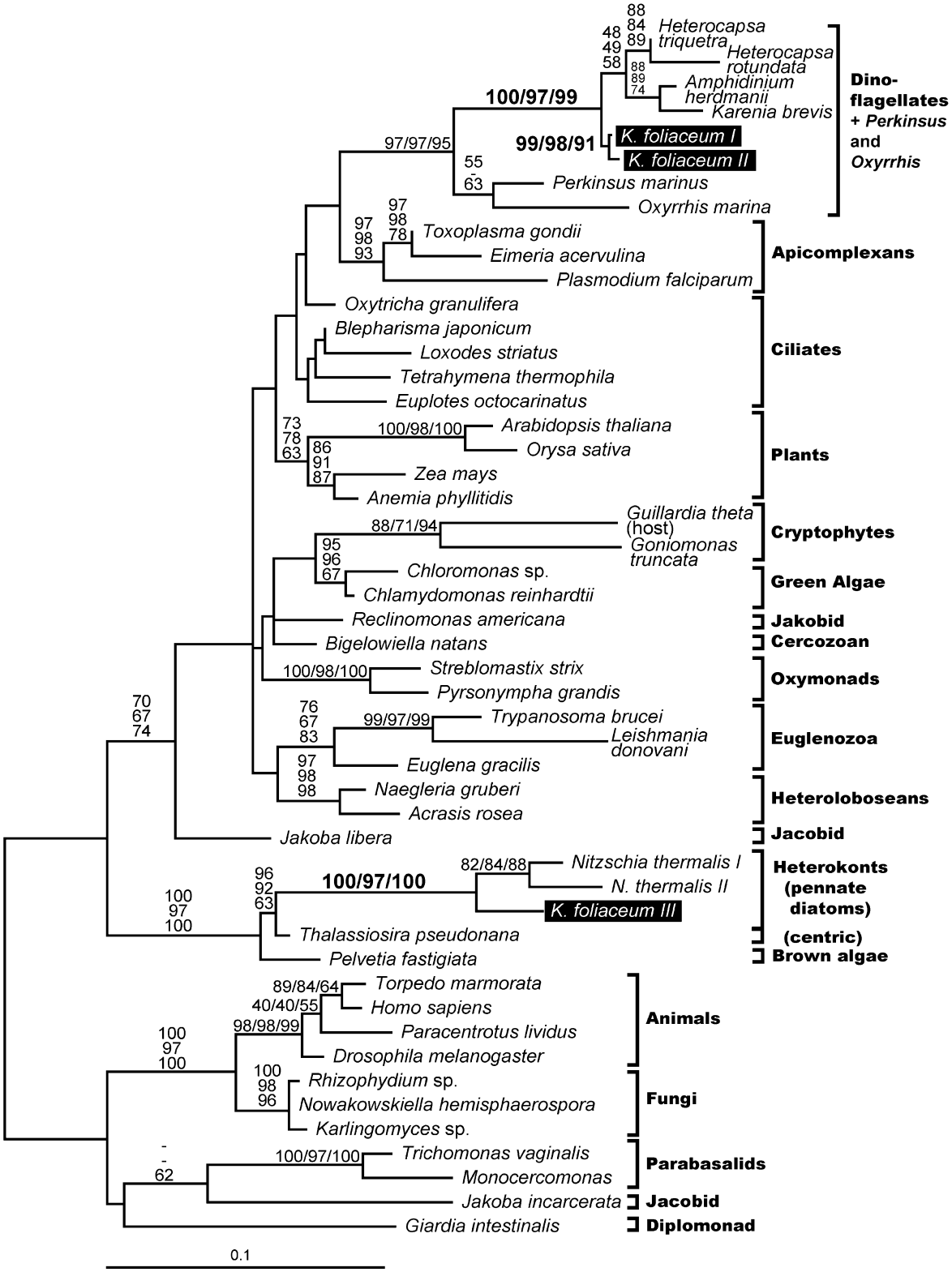

Fig. **1**. Alpha-tubulin maximum likelihood (ML) phylogeny. Bootstrap values shown for weighted neighbor-joining (left, top), Fitch-Margoliash (centre), and maximum likelihood (right, bottom). Major lineages are boxed and labeled to the right. Three *Kryptoperidinium foliaceum* genes are shown in black boxes. Two of these group strongly within the dinoflagellates, and the third groups strongly with pennate diatoms.

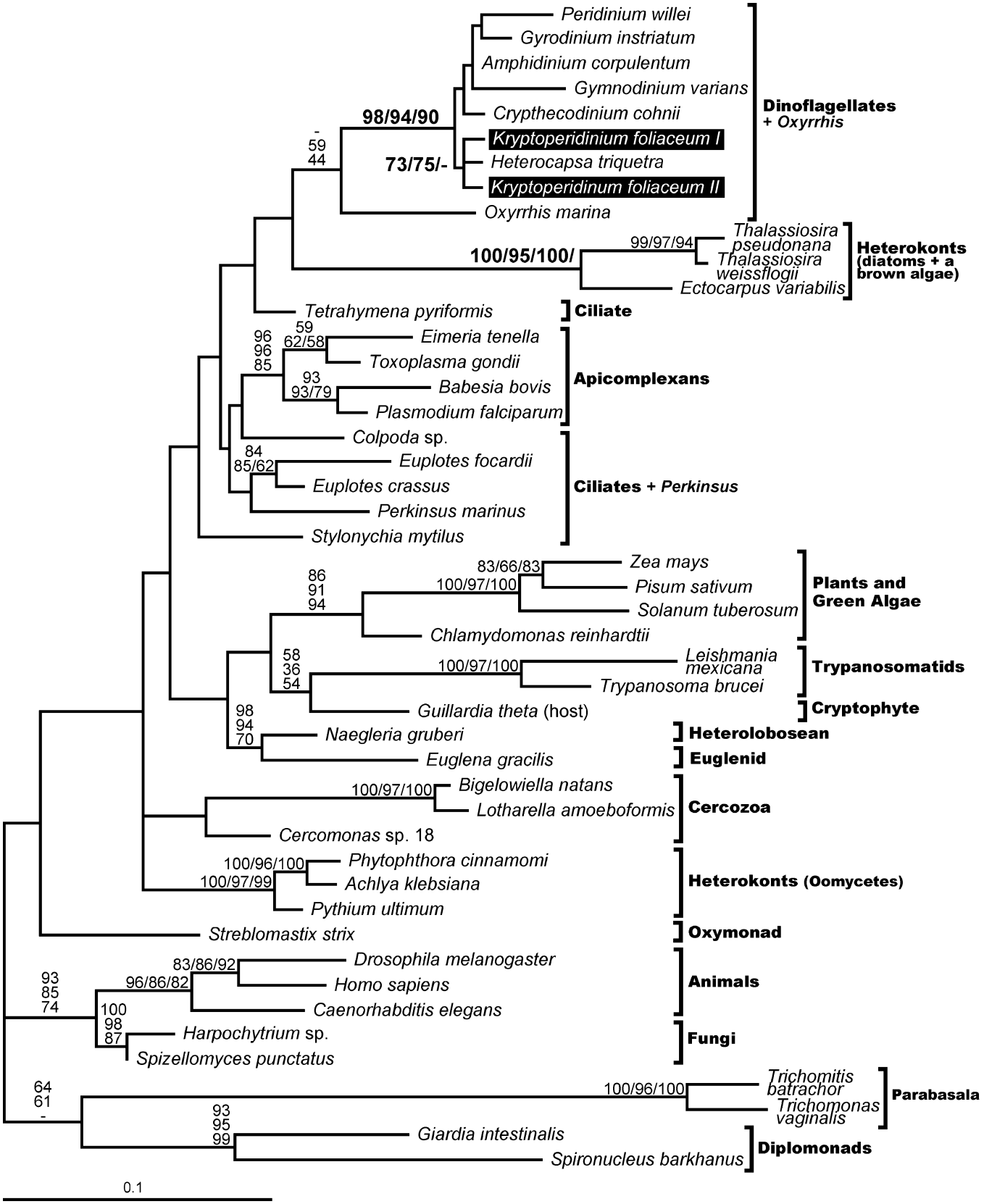

Fig. 2. Beta-tubulin ML phylogeny. Bootstrap values shown for weighted neighbor-joining (left, top), Fitch-Margoliash (centre), and maximum likelihood (right, bottom). Major lineages are boxed and labeled to the right. Both *Kryptoperidinium foliaceum* sequences appear to be host-derived, grouping strongly within the dinoflagellates.

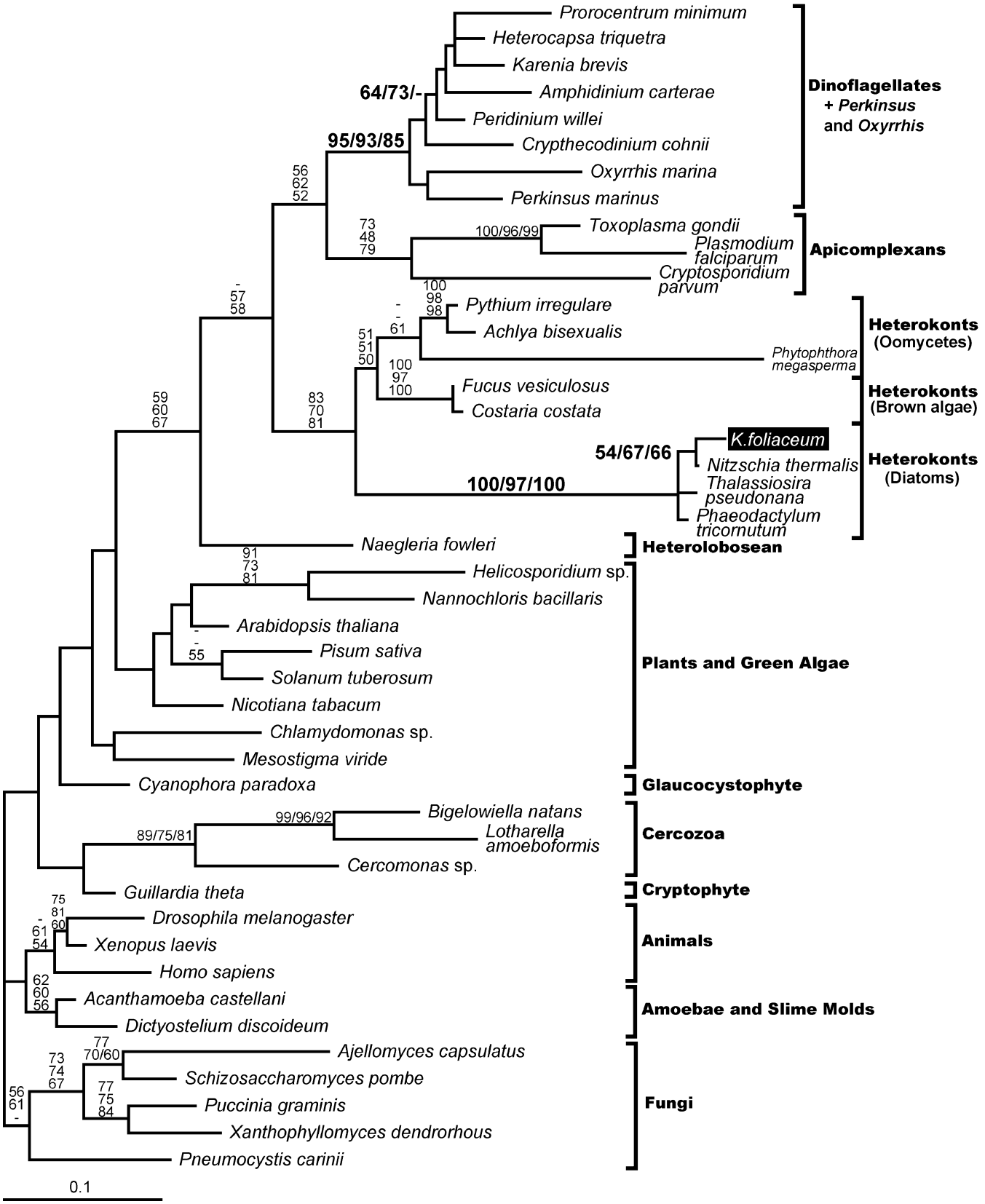

Fig. **3**. Actin ML phylogeny. Bootstrap values shown for weighted neighbor-joining (left, top), Fitch-Margoliash (centre), and maximum likelihood (right, bottom). Major lineages are boxed and labeled to the right. Apicomplexans, dinoflagellates, and heterokonts are strongly supported in this analysis, and their positions are in accordance with well-established relationships. Ciliates were excluded from this analysis as they are highly divergent and polyphyletic in actin (Keeling 2001). The *Kryptoperidinium foliaceum* sequence groups strongly within the heterokonts.

that of the pennate *N. thermalis*, altogether strongly supporting an endosymbiont origin for this gene.

Lastly, two HSP90 genes were characterised from *K. foliaceum*. In the HSP90 phylogeny (Fig. 4), these branched with strong support within the dinoflagellate (94–100%) and diatom (94–100%) clades, respectively. Once again, this is indicative of a host and endosymbiont origin for these genes. Overall, the HSP90 phylogeny is the most well supported of the four analysed and the most consistent with well-established relationships: dinoflagellates were sisters to apicomplexans, and the alveolate clade including ciliates was resolved with high bootstrap support. Similarly, the second HSP90 copy showed a strong affinity to the diatoms (although to neither centric nor pennate forms), which grouped with other heterokonts.

In summary, the phylogenies support the conclusion that *K. foliaceum* possesses alpha-tubulin and HSP90 genes of both host and endosymbiont origin, as well as at least host beta-tubulin and endosymbiont actin. In analyses including both centric (*Thalassiosira*) and pennate (*Nitzschia* and *Phaeodactylum*) diatoms, endosymbiont-derived *K. foliaceum* genes showed an affinity to the pennate diatoms over the centric forms in alpha-tubulin and actin, and no affinity in HSP90. Overall, this is in agreement with previous molecular studies based on endosymbiont nuclear SSU rRNA, which suggested a pennate diatom ancestry for the *K. foliaceum* endosymbiont (Chesnick et al. 1997).

Structural features of host- and endosymbiont-derived genes in *Kryptoperidinium foliaceum*. The phylogenetic history of a gene provides a strong inference for its location in the cell, but it remains possible that endosymbiont-derived genes are encoded in the host nuclear genome, and vice versa. Without physically localizing a gene, the genome in which it is encoded will always be in some doubt, but there are certain characteristics that have been used successfully, together with phylogenetic history, to provide a very accurate prediction.

Cryptophyte and chlorarachniophyte nucleomorph genes exhibit several physical characteristics distinct from host nuclear genes that allow these genes to be distinguished. Chlorarachniophyte nucleomorph genes have minute, 18- to 20-bp introns that readily distinguish them from those of the host nuclear genes (an estimated 168-bp on average) (Gilson and McFadden 2002). The difference between cryptophyte nuclear (50- to 74-bp) and nucleomorph (42- to 52-bp) introns is not as pronounced, but still somewhat useful (Douglas et al. 2001). Chlorarachniophyte and cryptophyte nucleomorph genes also exhibit a strong AT-bias, so that nucleomorph and nuclear genes can be as much as 25% different in overall AT-content (Douglas et al. 2001; Gilson and McFadden 1996).

Unfortunately, none of the *K. foliaceum* genes characterised here contained introns, but the AT-content of each *K. foliaceum* sequence was calculated to determine if there was a bias between dinoflagellate-derived genes vs. diatom-derived genes (Table 1). The mean AT content of the five dinoflagellate-identified genes is 42.5%, ranging from 39.2–46.6%. The three diatom-identified sequences have a mean AT content of 48.8% (47.8–51.6%). Accordingly, there is an AT-bias of approximately 6% in the diatom-derived genes, and no overlap in the ranges of AT-content between the two sets of genes. This bias supports the conclusion that the two phylogenetic classes of genes in *K. foliaceum* likely reside in different genomes: the dinoflagellate genes in the host nucleus and the diatom genes in the endosymbiont nucleus. While the 'total-nucleotides' AT bias (7%) may be less than that observed in cryptophyte and chlorarachniophyte genomes, AT bias in 'third-position-only' shows a larger separation between host and endosymbiont genes (18%). The diatom-identified genes from *K. foliaceum* have a mean third position AT content of 36.7%, ranging from 35.2–38.8%. In contrast, mean AT content at third position of the dinoflagellate-identified genes is 18.9%, ranging from 11.5—23.6% (for a difference of approximately 18% between host and endosymbiont genes). A codon bias analysis was also done, but no clear trends were observed. Codon biases and AT biases are complimentary and potentially useful tools for sorting sequence data; a 6% or 18% AT bias allows for preliminary predictions about the location of genes encoded in *K. foliaceum*.

Functional implications of endosymbiont genes. The *K. foliaceum* plastid endosymbiont presents a unique opportunity to study the early stages of endosymbiotic genome reduction from a functional perspective. Based on the pennate diatom identity of the endosymbiont and its current wall-less, immobile, amitotic state, one would expect that the endosymbiont genome has lost many genes, especially those related to wall structure and deposition, motility, and mitotic nuclear division. However, compared to the nucleomorphs of cryptophytes and chlorarachniophytes, the *K. foliaceum* endosymbiont nucleus appears to be at a relatively early stage of reduction, which is consistent with our observations that it retains genes that have been lost by nucleomorphs.

The presence of HSP90 is the least surprising of the four genes and does not give much insight into endosymbiont reduction, since both cryptophyte (Douglas et al. 2001) and chlorarachniophyte (unpubl. data) nucleomorphs also retain HSP90 genes in their genomes. HSP90 plays a wide variety of roles in eukaryotic cytoplasm, including as a chaperone in protein folding, and because of this, it is probably one of the last proteins lost in a degrading endosymbiont.

Actin and the tubulins, on the other hand, are not always retained, and their presence in *K. folicaeum* is of more interest. Alpha- and beta-tubulin are component parts of microtubules, whose activity is most apparent in the flagella, mitotic spindle, and cytoskeleton. Actin is also a prominent protein in the cytoskeleton and it plays a central role in the gliding motility of diatoms. Alpha-, beta-, and gamma-tubulins are present in the nucleomorph of the cryptophyte *G. theta* (Keeling et al. 1999), but are apparently absent from chorarachniophyte endosymbionts (unpublished data). Actin, on the other hand, appears to be absent from both endosymbionts (although an actin gene of possible endosymbiont origin has been found in the host nucleus of the cryptophyte *Pyrenomonas helgolandii* (Stibitz, Keeling, and Bhattacharya 2000)). In the *K. foliaceum* endosymbiont, while there is no gliding motility, cell wall or frustule, and presumably no mitosis, both alpha-tubulin and actin remain. The presence of actin is especially interesting because it has been lost in both cryptophyte and chlorarachniophyte nucleomorphs, and because it is a major component of diatom motility (Poulsen et al. 1999). Its possible function in the *K. foliaceum* endosymbiont is not clear, but its presence hints that the general cytoskeleton of the *K. foliaceum* endosymbiont is less reduced than those of either cryptophytes or chlorarachniophytes. The endosymbionts of *K. foliaceum* and *D. baltica* and their division have been extensively studied by electron microscopy, and no direct evidence of microtubules has been found during division or growth phases (Tippit and Pickett-Heaps 1976). Microtubules were also notably absent in and around the endosymbiont nuclei during sexual reproduction in *D. baltica* (Chesnick and Cox 1987). In *G. theta*, where tubulins are also present despite a lack of observed microtubules, it has been suggested that they may fulfill some alternate biological role that does not require microtubules (Keeling et al. 1999). Alternatively, both *K. foliaceum* and *G. theta* endosymbionts may contain microtubules that are highly specialized and appear for short periods of the life cycle, or in restricted numbers and size, making them very

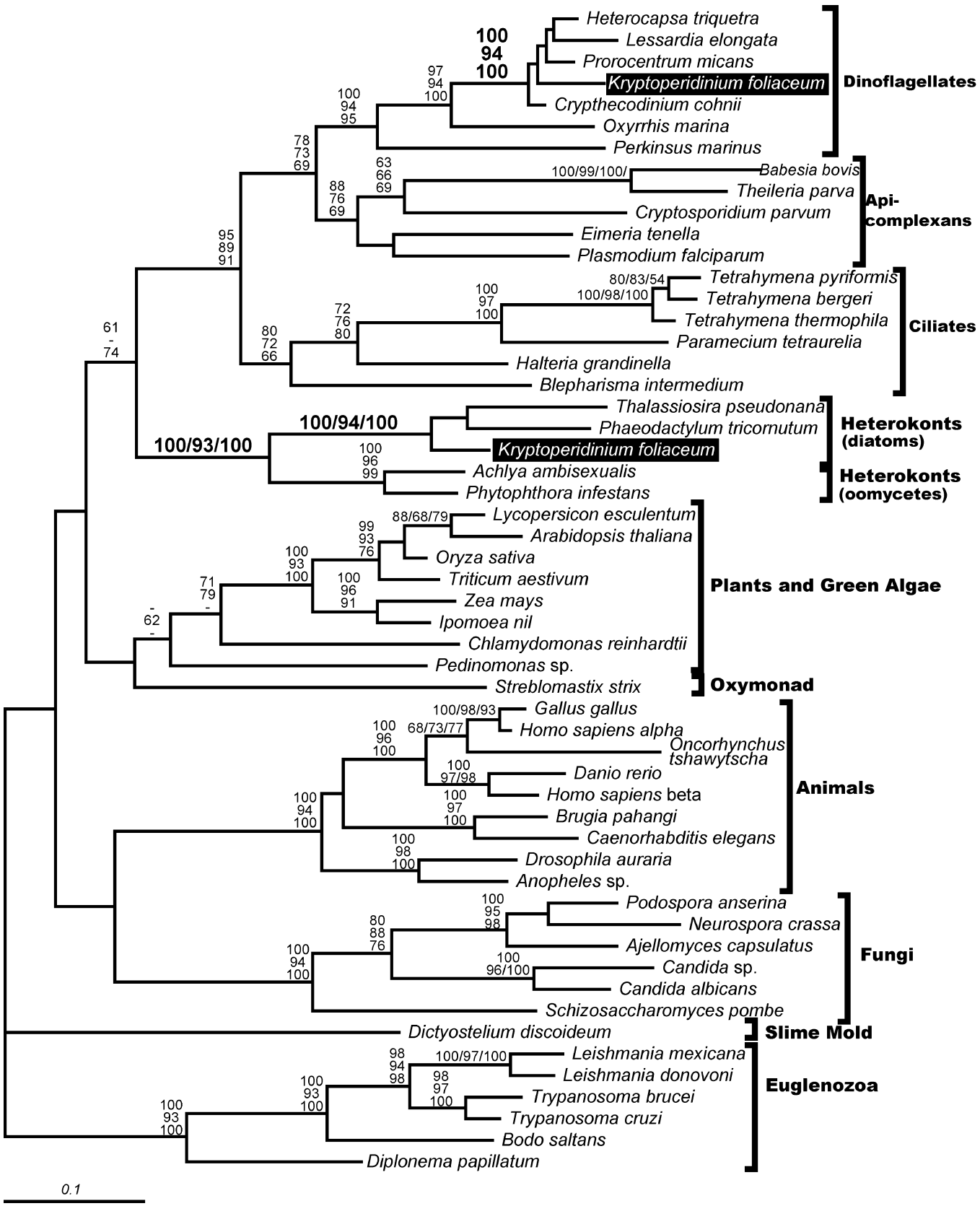

Fig. **4**. HSP90 ML phylogeny. Bootstrap values shown for weighted neighbor-joining (left, top), Fitch-Margoliash (centre), and maximum likelihood (right, bottom). Major lineages are boxed and labeled to the right. One *Kryptoperidinium foliaceum* sequence shows affinity to dinoflagellates, the other to diatoms.

Table **1**. AT content of *Kryptoperidinium* protein-coding genes.

Gene	Inferred Source	AT content (%)	
		Total	3rd Position
HSP90	Host	46.62	17.79
Beta-tubulin	Host	42.93	23.65
	Host	43.10	23.39
Alpha-tubulin	Host	39.15	11.46
	Host	40.88	17.19
Alpha-tubulin	Endosymbiont	46.96	38.80
Actin	Endosymbiont	47.81	35.25
HSP90	Endosymbiont	51.63	36.00

difficult to detect. In *D. baltica*, for example, chromatin condensation and 'crystalline rod' formation in the endosymbiont nucleus were observed after sexual fusion of both host and symbiont cells (as *P. balticum* (Chesnick and Cox 1987). The absence of tubulins from the chlorarachniophyte nucleomorph genome leaves open the possibility that microtubules can be discarded long before the complete reduction of the endosymbiont.

Implications of genetic reduction in *Kryptoperidinium foliaceum*. While the *K. foliaceum* endosymbiont has undergone some reduction (i.e. loss of cell wall and presumed amitosis), the question remains as to whether it has undergone any genetic reduction at all. In other integrated endosymbiotic systems, genetic reduction has partially or completely occurred through some combination of gene loss and transfer, where the products of transferred genes are targeted back to the appropriate compartment. Transfers of plastid-targeted genes and the mechanism by which their products are targeted to the organelle are well known in both primary and secondary plastids (McFadden 1999). In contrast, gene transfer and plastid targeting in tertiary plastids are relatively unknown, and *K. foliaceum* presents a unique case for targeting: instead of being contained in the endomembrane system of the host, freeze-fracture microscopy suggests that the single membrane that separates the symbiont from the host cytoplasm is actually derived from the outer membrane of the symbiont itself (Eschbach et al. 1990). If this is true, the product of any gene that is transferred to the host nucleus must first return to the endosymbiont cytoplasm by an entirely unique method of targeting, perhaps analogous to pinocytosis by the endosymbiont. Determining whether any such transfers to the host nucleus have occurred will potentially provide an important comparison with the better-studied secondary plastids, as it could represent an entirely novel solution to the problem of endosymbiont protein trafficking.

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