International Journal of Systematic and Evolutionary Microbiology (2001), **51**, 737–749 Printed in Great Britain

Phylogenetic relationships among algae based on complete large-subunit rRNA sequences

Abdelghani Ben Ali,[1] Raymond De Baere,[1] Gert Van der Auwera,[2] Rupert De Wachter[1] and Yves Van de Peer[3]

[1] Department of Biochemistry, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Wilrijk, Belgium

[2] Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium

[3] Department of Biology, University of Konstanz, D-78457 Konstanz, Germany

Author for correspondence: Yves Van de Peer. Tel: +49 7531 88 2763. Fax: +49 7531 88 3018. e-mail: Yves.Vandepeer@uni-konstanz.de

The complete or nearly complete large-subunit rRNA (LSU rRNA) sequences were determined for representatives of several algal groups such as the chlorarachniophytes, cryptomonads, haptophytes, bacillariophytes, dictyochophytes and pelagophytes. Our aim was to study the phylogenetic position and relationships of the different groups of algae, and in particular to study the relationships among the different classes of heterokont algae. In LSU rRNA phylogenies, the chlorarachniophytes, cryptomonads and haptophytes seem to form independent evolutionary lineages, for which a specific relationship with any of the other eukaryotic taxa cannot be demonstrated. This is in accordance with phylogenies inferred on the basis of the small-subunit rRNA (SSU rRNA). Regarding the heterokont algae, which form a well-supported monophyletic lineage on the basis of LSU rRNA, resolution between the different classes could be improved by combining the SSU and LSU rRNA data. Based on a concatenated alignment of both molecules, the phaeophytes and the xanthophytes are sister taxa, as well as the pelagophytes and the dictyochophytes, and the chrysophytes and the eustigmatophytes. All these sister group relationships are highly supported by bootstrap analysis and by different methods of tree construction.

Keywords: heterokont algae, cryptophytes, chlorarachniophytes, haptophytes, large-subunit rRNA (LSU rRNA)

INTRODUCTION

Generally, on the basis of pigmentation, three main eukaryotic algal groups have been discerned, namely the chlorophytes or green algae (characterized by the presence of chlorophyll *a* and *b*), the rhodophytes or red algae (chlorophyll *a* and phycobilins), and the chromophytes or yellow-brown algae (chlorophyll *a* and *c*, and absence of chlorophyll *b*). The latter group, which is polyphyletic, is further subdivided into four taxa, namely the cryptophytes, the haptophytes, the dinoflagellates and the heterokont algae, on the basis of pigmentation and plastid ultrastructure, flagellar apparatus and small-subunit rRNA (SSU rRNA) phylogenies (Whatley, 1989; Bhattacharya *et al*., 1992; Leipe *et al*., 1994; Cavalier-Smith *et al*., 1994a; Medlin *et al*., 1995).

The cryptophytes or cryptomonads (Gillot, 1990) are unicellular biflagellated organisms for which the evolutionary history is unclear and controversial. In previous studies, they have been phylogenetically positioned with chlorobionts (Eschbach *et al*., 1991), other chromophytes (Cavalier-Smith *et al*., 1994a), glaucocystophytes (Bhattacharya *et al*., 1995a; Ragan & Gutell, 1995; Van de Peer *et al*., 1996a) and *Acanthamoeba* (McFadden, 1993). The haptophytes or prymnesiophytes (Green *et al*., 1990) are unicellular flagellate cells, characterized by a filiform organelle associated with the flagella, called the haptonema. Also for this group of organisms, the phylogenetic status is rather unclear (Daugbjerg & Andersen, 1997a; and references therein). The heterokont algae, also referred to as 'stramenochromes' (Leipe *et al*., 1994) and 'Ochrista' (Cavalier-Smith *et al*., 1994b), consist

This paper was presented at the XIIIth meeting of the International Society for Evolutionary Protistology in České Budějovice, Czech Republic, 31 July–4 August 2000.

Abbreviations: LSU RNA, large-subunit rRNA; NM, nucleomorph; SSU RNA, small-subunit rRNA.

Table 1. The different algal taxa, classified on the basis of their main pigments

Numbers between brackets indicate the number of membranes surrounding the plastids.

Algae with:		
Chlorophyll *a* and *b*	**Chlorophyll *a* and phycobilins**	**Chlorophyll *a* and *c***
Chlorophytes (2)	Rhodophytes (2)	Cryptophytes* (4)
Chlorarachniophytes (4)	Glaucophytes (2)	Haptophytes (4)
Euglenophytes† (3)		Dinoflagellates (3)
		Heterokont algae: (4)
		Bacillariophytes
		Chrysophytes
		Dictyochophytes
		Eustigmatophytes
		Pelagophytes
		Phaeophytes
		Phaeothamniophytes‡
		Raphidophytes‡
		Synurophytes‡
		Xanthophytes

* Although the cryptophytes are classified as algae that contain chlorophyll *a* and *c*, they also contain phycobilins as accessory pigments. Furthermore, they obtained their plastids by engulfing a eukaryotic alga that most probably was related to the red algae (see text).

† Euglenophytes are included in the table, although their phylogenetic position is not discussed here. The phylogenetic position of the euglenophytes is controversial and on the basis of rRNA data, *Euglena* and relatives, which share a common origin with the kinetoplastids, usually branch off well before the other 'crown' taxa. On the contrary, on the basis of protein data, *Euglena* and the kinetoplastids diverge much later (Baldauf *et al*., 2000; Van de Peer *et al*., 2000a).

‡ No LSU rRNA data are available yet for these organisms.

of different subgroups that share morphological features such as the possession of tripartite tubular flagellar hairs, similar flagellar anchorage systems (based on four microtubular roots) and electron-dense mitochondria with short tubular cristae (Patterson, 1989). The major classes in this group comprised the phaeophytes, chrysophytes, synurophytes, xanthophytes, eustigmatophytes, raphidophytes, bacillariophytes, dictyochophytes, pelagophytes and phaeothamniophytes (Hibberd & Leedale, 1970, 1971, 1972; Ettl, 1978; Silva, 1980; Andersen, 1987; Heywood, 1990; Andersen *et al*., 1993, 1998, 1999; Potter *et al*., 1997; Bailey *et al*., 1998). The heterokont algae are thus a diverse assemblage of many different smaller groups of algae. The evolutionary relationships between these different groups have been widely studied on the basis of SSU rRNA sequences (Ariztia *et al*., 1991; Bhattacharya *et al*., 1992; Andersen *et al*., 1993; Bhattacharya & Medlin, 1995; Saunders *et al*., 1995, 1997; Cavalier-Smith *et al*., 1995; Cavalier-Smith & Chao, 1996; Van de Peer *et al*., 1996b), although classifications have been based upon ultrastructural and biochemical observations (Andersen, 1991; Williams, 1991a, b). Furthemore, it seems that the heterokont algae, as a group, are most closely related to certain heterotrophic phyla such as oomycetes and hyphochytriomycetes (Forster *et al*., 1990; Bhattacharya *et al*., 1991, 1992; Cavalier-Smith *et al*., 1994b; Leipe *et al*., 1994, 1996; Van der Auwera *et al*., 1995) and form together the Heterokonta (Cavalier-Smith *et al*., 1994b) or the stramenopiles (Patterson, 1989). Finally, the dinoflagellates are unicellular biflagellated organisms that are a sister group of the apicomplexans, while both of them form, together with the ciliates, a monophyletic group called the alveolates (Gajadhar *et al*., 1991; Patterson & Sogin, 1993; Van de Peer & De Wachter, 1997a).

Beside these four main groups of algae, a few smaller groups of algae exist. One of these is the chlorarachniophytes, an intriguing group of marine, unicellular amoeboflagellate algae showing a possible common ancestry with filose amoebae (Bhattacharya *et al*., 1995b; Van de Peer *et al*., 1996a). Together with the cryptophytes, haptophytes and heterokont algae, the chlorarachniophytes contain plastids surrounded by four membranes, and are believed to have acquired these by engulfing another eukaryotic plastid-containing algae (secondary endosymbiosis; Gibbs, 1981; Douglas *et al*., 1991; Maier *et al*., 1991; Cavalier-Smith, 1993, 1995; Whatley, 1993; Martin *et al*., 1998; Palmer & Delwiche, 1998; Delwiche, 1999). Chlorarachniophytes and cryptophytes still contain a small nucleus-like structure called the nucleomorph (NM)

(Ludwig & Gibbs, 1989; Gibbs, 1993; McFadden, 1993), situated between the two inner and the two outer membranes surrounding the plastid and which is a remnant of the endosymbiont's nucleus. Since the NM of cryptomonads and chlorarachniophytes still encode 18S rRNA, it could be demonstrated that the endosymbionts of chlorarachniophytes are probably related to green algae, while those of cryptomonads are most probably related to red algae (Van de Peer *et al*., 1996a). Although the latter relationship is usually not supported by bootstrap analysis, it has been suggested previously on the basis of SSU rRNA (Douglas *et al*., 1991; Maier *et al*., 1991; Maerz *et al*., 1992). In haptophytes and heterokont algae, a remnant of the endosymbionts nucleus does not exist anymore and therefore the true nature of the endosymbiont that gave rise to their plastids can only be inferred through sequence analysis of plastid genes. Dinoflagellates have plastids that are surrounded by three membranes. As recently demonstrated, the DNA organization of these plastids differs radically from all other plastid genomes. Dinoflagellate plastids have a unique genome organization in which each gene is located on its own minicircular chromosome (Zhang *et al*., 1999). A brief summary of the different algal taxa, classified on the basis of their main pigments and the number of membranes surrounding the plastids, is shown in Table 1.

In this paper, we have focused on (1) the phylogenetic position and relationships of the different groups of algae discussed above and on (2) the phylogenetic relationships between different taxa that belong to the heterokont algae. Regarding the latter, Saunders *et al*. (1995, 1997) and Potter *et al*. (1997) suggested, by combining ultrastructural, biochemical and SSU rRNA sequence data, that all heterokont algae with a highly reduced flagellar apparatus such as bacillariophytes (diatoms), pelagophytes, and dictyochophytes, form a monophyletic group. However, in other analyses on the basis of the SSU rRNA (Van de Peer *et al*., 1996b, 2000a; Leipe *et al*., 1996) and on the basis of *rbcL* data (Daugbjerg & Andersen, 1997b) such a monophyly is not supported. Overall, despite several studies, the deeper branching relationships within the heterokont algae remain unclear and need further investigation.

In order to address the questions raised above, we determined the complete, or nearly complete, large-subunit rRNA (LSU rRNA) sequence of the bacillariophytes (diatoms) *Cylindrotheca closterium* and *Rhizosolenia setigera*; the dictyochophyceae *Apedinella radians*, *Dictyocha speculum* and *Rhizochromulina* cf. *marina*; the pelagophyceae *Aureococcus anophagefferens* and *Pelagomonas calceolata*; the haptophyte *Prymnesium patelliferum* and *Phaeocystis antarctica*; the cryptophyte *Guillardia theta* (nuclear gene); and the chlorarachniophyte *Chlorarachnion* 'strain CCMP621' (nuclear gene). Phylogenetic trees were constructed with different methods and on the basis of LSU rRNA as well as on concatenated sequence alignments of LSU and SSU rRNA.

METHODS

Sequence determination. The LSU rDNA of all species was amplified by PCR, either in one or several overlapping fragments. The primer combinations that were used for these PCRs are listed in Table 2, and their location is indicated relative to the 18S and 28S rDNA gene sequences from *Saccharomyces cerevisiae* (Van der Auwera *et al*., 1994, 1995). All reactions were performed in 50 µl containing 200 µM of each dNTP, 0·5 µM of each primer and 0·025 U/µl *Taq* DNA polymerase in the appropriate buffer (Roche Diagnostics). Thirty cycles were performed, each consisting of a denaturation step of 1 min at 94 °C, an annealing step of 1 min at various temperatures, and an annealing step at 72 °C for different amounts of time. The exact annealing temperature and extension time of each reaction is listed in Table 2. The cycling was always preceded by a denaturation step of 2 min at 94 °C and followed by an extension step of 10 min at 72 °C.

The LSU rDNA of the chlorarachniophyte strain CCMP 621 was amplified in two overlapping fragments (Table 2). Because the template contained nuclear as well as NM DNA, each PCR product was probably a mixture of two amplicons. As only one amplification product was observed in each reaction, they could not be separated on the basis of their length. However, since the NM sequence had already been determined (Gilson & McFadden, 1996), it was possible to digest each amplicon with a panel of restriction enzymes that were known to cut the NM fragment. Enzymes that only cut the NM fragment could be readily identified since they generate the restriction fragments characteristic of the NM, while leaving the nuclear PCR product intact. It was observed that digestion of the second PCR fragment did not result in the expected NM fragments. Since it is our experience that the antisense primer used to generate this fragment is unsuccessful in many amplifications, it was assumed that this primer worked well on the nuclear DNA, while the NM DNA was selected against because too many mismatches were present. As a result no further steps were needed to obtain the pure second nuclear DNA fragment. In order to obtain the first pure nuclear amplification product, total genomic DNA was digested with the restriction enzyme *Pst*I, which was shown to cut only the NM DNA between both primer annealing sites used in the PCR. When the digested DNA was used as template, only the nuclear genome was amplified, while the NM DNA was not intact and therefore could not be amplified.

The LSU rDNA of *Guillardia theta* was amplified in one fragment covering the 28S rDNA (Table 2). The amplification of the fragment covering 5.8S rDNA ($\pm$162 nucleotides) and the 5′ end of the 28S rDNA ($\pm$40 nucleotides) was unsuccessful and as a result these sequences were not determined. As in the case of the chlorarachniophyte CCMP621, the PCR product was probably again a mixture of nuclear and NM DNA. In order to obtain pure nuclear DNA, the same approach was followed, using *Bam*HI to cut the NM DNA between both primer annealing sites used in the PCR.

Table 2. Amplified PCR fragments

Organism*	PCR conditions†			
	Sense primer	Antisense primer	Extension (min)	Annealing (°C)
CCMP621	1624–1643 (18S)	1940–1917 (28S)	3	55
	1252–1274 (28S)	3378–3357 (28S)	3	55
Guillardia theta	26–46 (28S)	3378–3357 (28S)	4	50
Prymnesium patelliferum				
Phaeocystis antarctica	1624–1643 (18S)	660–636 (28S)	2	55
Aureococcus anophagefferens	26–46 (28S)	2210–2185 (28S)	2	55
Pelagomonas calceolata	1917–1940 (28S)	3378–3357 (28S)	2	55
Apedinella radians				
Dictyocha speculum				
	1624–1643 (18S)	660–636 (28S)	2	55
Rhizochromullna cf. *marina*	26–46 (28S)	2210–2185 (28S)	2	55
	1252–1274 (28S)	3126–3106 (28S)	2	55
	1624–1643 (18S)	660–636 (28S)	2	59
Cylindrotheca closterium	26–46 (28S)	1858–1841 (28S)	2	54
Rhizosolenia setigera	1252–1274 (28S)	2413–2393 (28S)	2	59
	1917–1940 (28S)	3378–3357 (28S)	2	57

* The LSU rDNA of all species listed in the same row (separated by the horizontal lines) were amplified using the same fragments and conditions.

† Each line represents a different PCR reaction, indicating the annealing temperature, extension time and primers used. The position of the primers is given relative to the 18S or 28S rDNA genes of *S. cerevisiae* (in parentheses).

The LSU rDNAs of *Aureococcus anophagefferens*, *Pelagomonas calceolata*, *Apedinella radians*, *Dictyocha speculum*, *Rhizochromulina* cf. *marina* and both haptophytes, viz. *Prymnesium patelliferum* and *Phaeocystis antarctica*, were amplified in three overlapping PCR fragments, while four amplicons were needed to cover the *Cylindrotheca closterium* and *Rhizosolenia setigera* genes (Table 2).

The PCR amplified fragments were purified with the QIAquick PCR purification kit (Qiagen) and then used as template for an asymmetric PCR, using the same conditions as for the PCR but omitting one of the primers. The asymmetric PCR products were again purified with the QIAquick PCR purification kit and used as template for cycle sequencing reactions. This procedure is a modification of the one of Allard *et al*. (1991).

Sequencing reactions were performed with Cy5-labelled primers and the Thermo sequenase fluorescent-labelled primer cycle sequencing kit from Amersham Pharmacia Biotech according to the manufacturer's instructions. Reactions were analysed on an ALF express automatic DNA sequencer (Amersham Pharmacia Biotech). For sequencing, the primers published in Van der Auwera *et al*. (1994, 1995, 1997) were used, and one new primer was synthesized which anneals at position 46–26 in the *Saccharomyces cerevisiae* 28S rRNA gene and has the sequence ATATGCTTAARTTCAGCGGGT. The 5.8S and 28S rDNA were sequenced completely, except for 5.8S rDNA and about 40 nucleotides at the 5′ end of the 28S rDNA of *Guillardia theta*. Also about 40 nucleotides at the 3′ end of the 28S rDNA of all the species were not determined because they could not be amplified by PCR due to the lack of a suitable primer beyond the 3′ end of the gene, as was also the case for about 80 nucleotides at the 5′ end of 5.8S rDNA and about 250 nucleotides at the 3′ end of the28S rDNA from *Rhizochromulina* cf. *marina*. Sequencing was done on both strands, except for about 50 nucleotides at both ends of the 5.8S rDNA and the 5′ end of the 28S rDNA for CCMP621, *Phaeocystis antarctica*, *Rhizochromulina* cf. *marina* and *Aureococcus anophagefferens*, which were sequenced on one strand only. When several PCR products were used to cover the entire gene, the overlapping regions were sequenced to make sure that all products are from the same template.

Sequence alignment and tree construction. The LSU rRNAs determined in this study (accession numbers can be found in Table 3) were added to the European LSU rRNA sequence database (De Rijk *et al*., 2000). Sequences were aligned with the DCSE sequence editor (De Rijk & De Wachter, 1993), taking into account both primary and secondary structure information. Neighbour-joining (Saitou & Nei, 1987) trees based on Kimura (1980) and substitution rate calibrated (SRC) distances taking into account among-site rate variation (Van de Peer *et al*., 1996b) were constructed and drawn with TREECON for Windows (Van de Peer & De Wachter, 1997b). Bootstrapped maximum-parsimony trees were constructed with PAUP* (Swofford, 1998). Maximum-likelihood trees were inferred by the PUZZLE program (Strimmer & von Haeseler, 1996). Secondary structure models were drawn with the software RNAViz (De Rijk & De Wachter, 1997), a versatile program developed to draw secondary structures of molecules in a fast and user-friendly way.

Table 3. Lengths and accession numbers of the 5.8S and 28S rDNA sequences (only the length of the determined part is given)

Organism	Molecule	Length (bp)	Accession number
Chlorarachnion sp. CCMP621	5.8S	163	AF289035
	28S	3296	AF289036
Guillardia theta	5.8S	–	–
	28S	3324	AF289037
Prymnesium patelliferum	5.8S	162	AF289038
	28S	3204	AF289038
Phaeocystis antarctica	5.8S	162	AF289039
	28S	3267	AF289040
Aureococcus anophagefferens	5.8S	162	AF289041
	28S	3272	AF289042
Rhizochromulina cf. *marina*	5.8S	84	AF289043
	28S	3083	AF289044
Apedinella radians	5.8S	162	AF289045
	28S	3226	AF289045
Dictyocha speculum	5.8S	162	AF289046
	28S	3251	AF289046
Pelagomonas calceolata	5.8S	162	AF289047
	28S	3269	AF289047
Rhizosolenia setigera	5.8S	162	AF289048
	28S	3331	AF289048
Cylindrotheca closterium	5.8S	162	AF289049
	28S	3195	AF289049

RESULTS AND DISCUSSION

For all species, approximately 40 bases at the 3′ end of the 28S rDNA were not determined, because these were not amplified in the PCR reactions due to the lack of a primer downstream of the 3′ end. Apart from that, complete 5.8S and 28S rDNA sequences from the chlorarachniophyte *Chlorarachnion* strain CCMP621 (host), the haptophytes *Prymnesium patelliferum* and *Phaeocystis antarctica*, the bacillariophytes *Cylindrotheca closterium* and *Rhizosolenia setigera*, the dictyochophytes *Apedinella radians*, *Dictyocha speculum* and *Rhizochromulina* cf. *marina*, and the pelagophytes *Aureococcus anophagefferens* and *Pelagomonas calceolata* were determined. For the cryptophyte *Guillardia theta* (nuclear gene), the complete LSU rDNA was determined except for the 5.8S rDNA and about 40 nucleotides at the 5′ end of the 28S rDNA. The LSU rRNA sequence from *Rhizochromulina* cf. *marina* was determined except for about 80 nucleotides at the 5′ end of the 5.8S rDNA and about 250 nucleotides at the 3′ end of the 28S rDNA. The lengths of the sequences and their accession numbers are given in Table 3.

Phylogenetic position of the algal taxa

Fig. 1 shows a neighbour-joining tree considering among-site rate variation and based on 85 LSU rRNA sequences of different eukaryotes belonging to the so-called crown taxa (Knoll, 1992; Van de Peer *et al*., 2000a). Estimation of nucleotide substitution rates and estimation of evolutionary distances taking into account the specific distribution of substitution rates (Van de Peer *et al*., 1996b) was based on the complete LSU rRNA, except for the variable regions of the molecule for which alignment was problematic indicated in Fig. 5 (see also De Rijk *et al*., 2000). As can be seen in Fig. 1, several well-supported monophyletic groups can be discerned: viz. the animals, red algae, land plants plus green algae, fungi, stramenopiles and alveolates. Stramenopiles and alveolates appear as sister groups, albeit not supported by bootstrap analysis (bootstrap support, BS, 56 %). Nevertheless, a sister relationship of alveolates and stramenopiles or heterokonts has been suggested before on the basis of SSU rRNA, although also never statistically supported (Saunders *et al*., 1995; Van de Peer & De Wachter, 1997a). The chlorarachniophytes, cryptophytes and haptophytes seem to form independent lineages, not specifically related to any of the other eukaryotic taxa. Also this finding is in good agreement with SSU rRNA data published previously (Van de Peer & De Wachter, 1997a; Van de Peer *et al*., 2000a). Interesting to note is that a close relationship between fungi and animals is hardly ever supported by LSU rRNA data (Fig. 1; Van der Auwera *et al*., 1998; Van de Peer *et al*., 2000b), although this clade is firmly established by SSU rRNA analyses (Wainright *et al*., 1993; Van de Peer & De Wachter, 1997a; Van de Peer *et al*., 2000a), by their unique possession of a large insertion in protein synthesis elongation factor-1α (EF-1α; Baldauf & Palmer, 1993), and by analyses of the conservative,

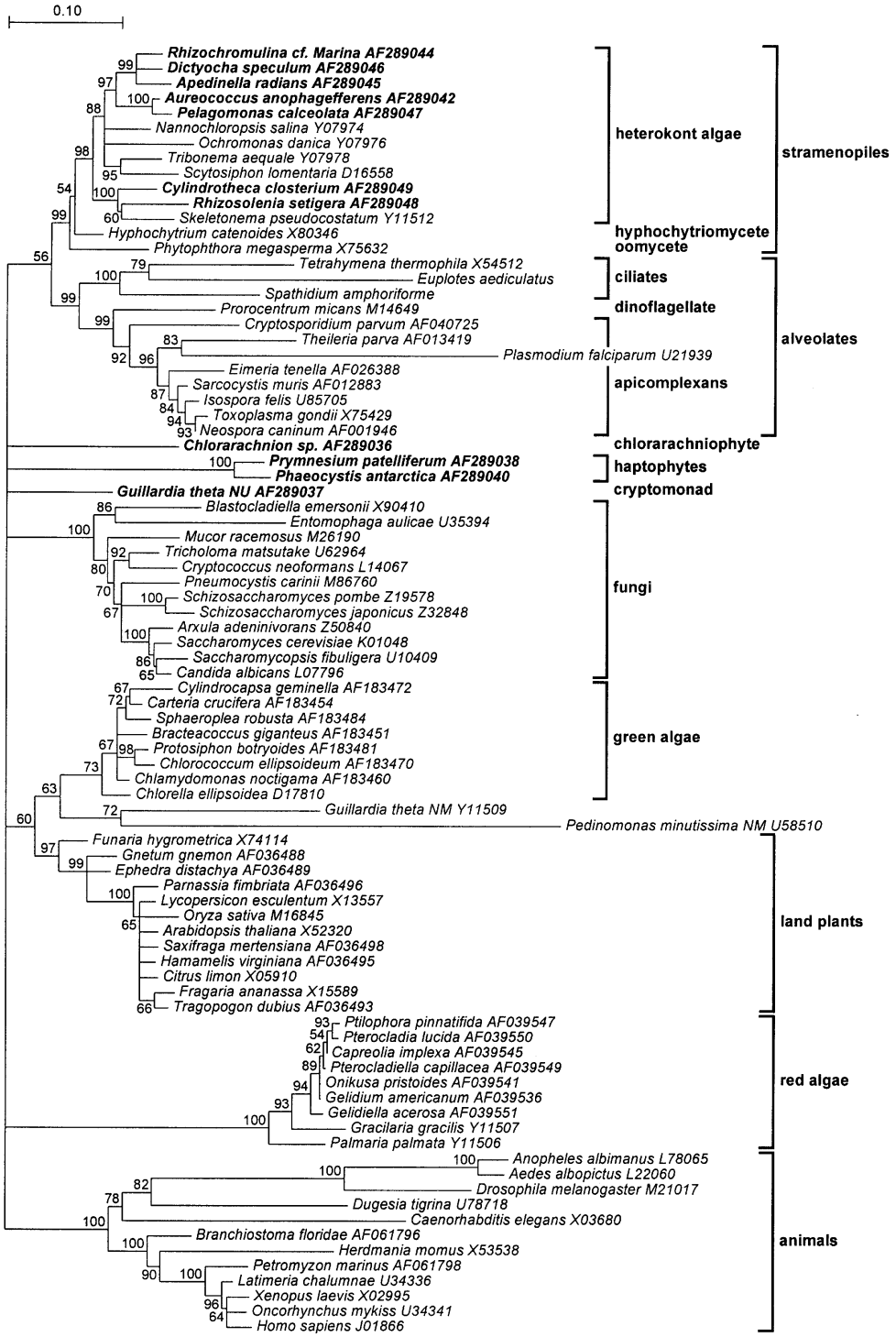

Fig. 1. Unrooted evolutionary tree of 85 eukaryotic LSU rRNA sequences constructed by neighbour-joining from a distance matrix based on substitution rate calibration (Van de Peer *et al.*, 1996b). Algae for which the sequence was determined in this study are in bold. The evolutionary distance between two organisms is obtained by summing the lengths of the connecting branches along the horizontal axis, using the scale on top. Bootstrap values (Felsenstein, 1985) above 50% (out of 500 resamplings) are shown at the internodes. Branches supported by less than 50% are drawn as unresolved. Taxon designations are placed to the right of the corresponding clusters.

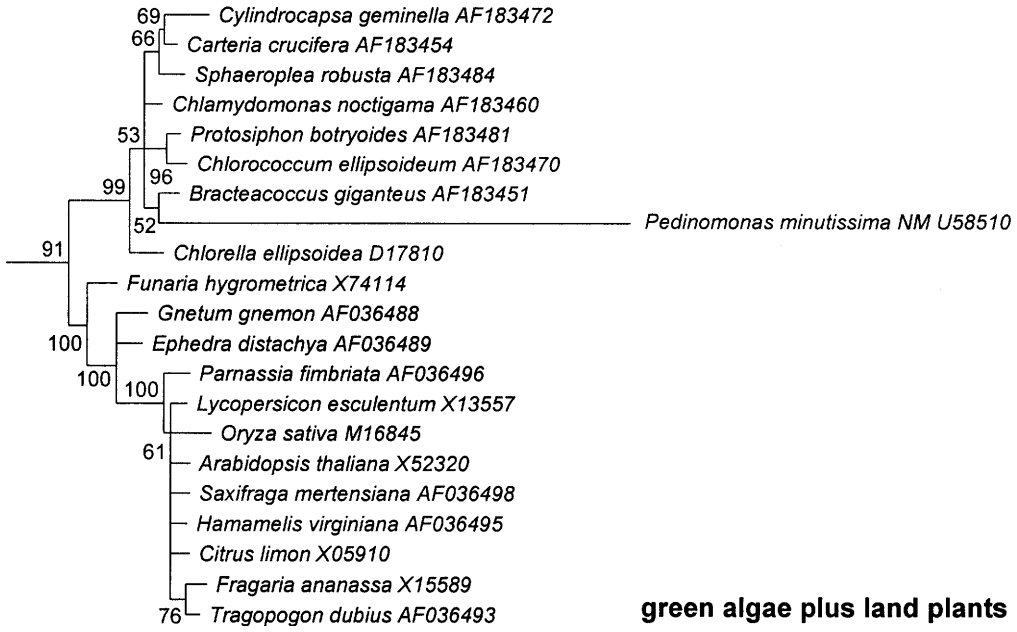

Fig. 2. Detail of the tree topology and bootstrap support for the green algae plus land plants but with removal of the NM sequence of the cryptomonad *Guillardia theta* (Fig. 1). See text for details.

taxonomically well-sampled proteins α-tubulin, β-tubulin, EF-1α and actin (Baldauf, 1999).

In the tree of Fig. 1, the NM LSU rRNA of the chlorarachniophyte *Pedinomonas* is clustered with the LSU rRNA of the cryptomonad NM *Guillardia*, although their genomes are most probably unrelated (Van de Peer *et al.*, 1996b). Although this clustering is thus unexpected it has been noticed before on the basis of SSU rRNA (Cavalier-Smith *et al.*, 1994a; Van de Peer *et al.*, 1996b; Van de Peer & De Wachter, 1997a). However, this attraction appears to be artifactual and caused by the increased evolutionary rate of the NM rRNAs, although SRC is applied to reduce long-branch attraction artifacts (Van de Peer *et al.*, 1996a). The strong attraction of the cryptomonad NM sequence by the chlorarachniophyte NM can be demonstrated by omitting the latter from the analysis. When the chlorarachniophyte NM is omitted from the analysis and only the cryptomonad NM is included (not shown), it forms an independent lineage, clearly separated from the green algae and land plants, which still form a very well-supported clade. However, on the basis of LSU rRNA, the red algal ancestry of the cryptomonad endosymbiont, as suggested previously (Douglas *et al.*, 1991; Maier *et al.*, 1991; Maerz *et al.*, 1992; Van de Peer *et al.*, 1996a) cannot be demonstrated. When only the chlorarachniophyte NM sequence is included, its phylogenetic position remains basically unchanged, but the bootstrap support for the clade formed by green algae plus land plants increases spectacularly (Fig. 2). The strong artificial attraction between the NM rRNAs remains embarrassing but possibly might be explained by co-variation (Van de Peer, unpublished) or convergence in their sequences due to similar evolutionary pressure after becoming endosymbionts (Ishida *et al.*, 1999).

Maximum-parsimony analysis (not shown) shows the same overall tree topology as that found on the basis of distance analysis and the same major clusters are recognized. The divergence order between the different major clusters is largely unresolved, except that stramenopiles and alveolates are found as sister groups, but again not supported by bootstrap analysis (bootstrap proportion, BP, 51%). Contrary to the distance tree shown in Fig. 1, the NM sequences form a monophyletic (BS 84%) but independent lineage and thus do not group with the green algae. Furthermore, even when the NM sequence of the cryptomonad *Guillardia* is omitted from the analysis, the NM sequence of *Pedinomonas* does not share a common ancestry with the green algae, although this is highly supported in distance analysis (BS 99%), at least when among-site rate variation is taken into account. If this is not the case, also in distance trees the NM sequence of *Pedinomonas* forms an independent evolutionary lineage (not shown).

As can be seen in Fig. 1, many of the deeper divergences in the eukaryotic crown are unresolved. Actually, the resolution for deeper divergences within the LSU rRNA tree seems to be less robust than in SSU rRNA trees, where one usually finds a close and statistically well-supported relationship between animals and fungi, and between stramenopiles and alveolates (e.g.

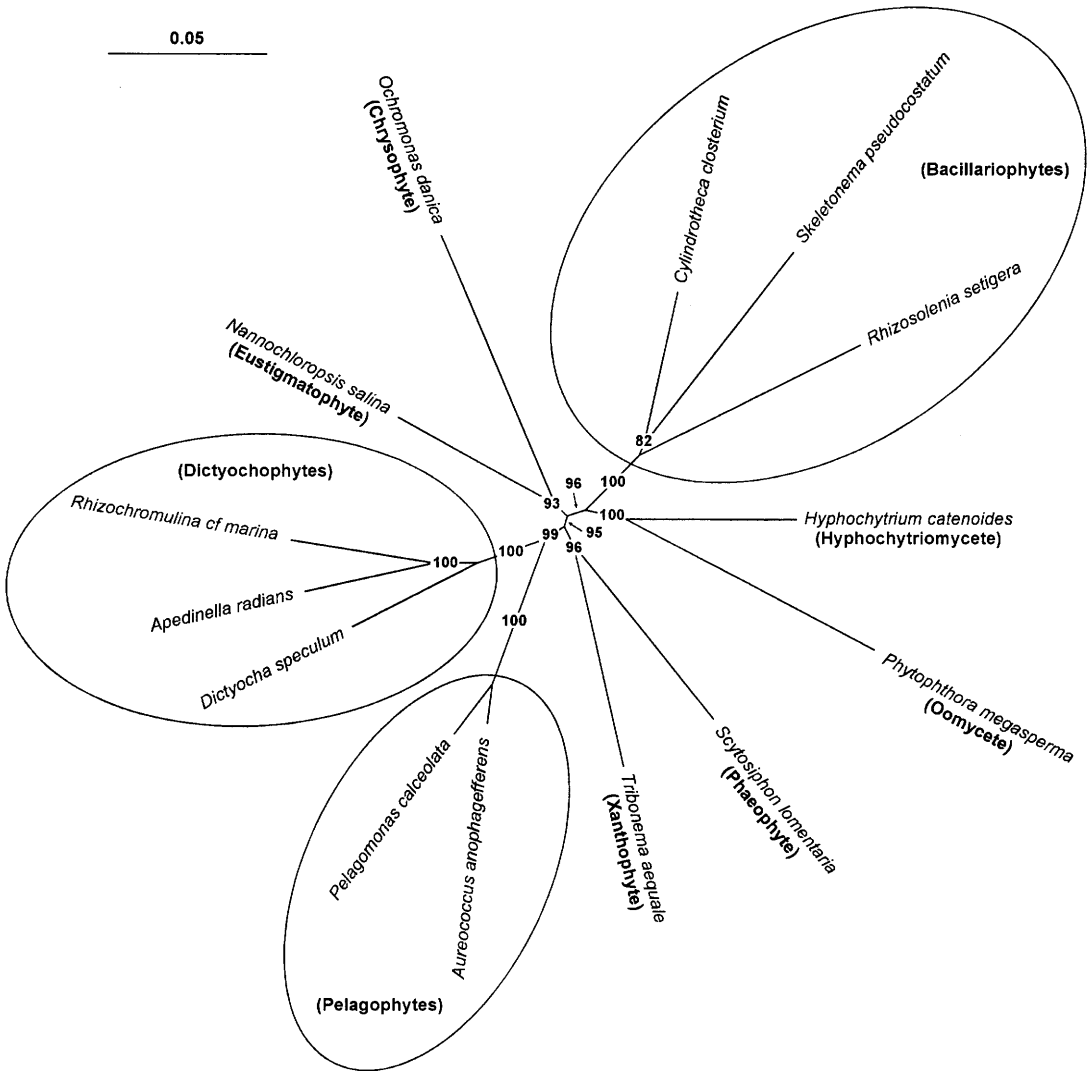

Fig. 3. Unrooted phylogenetic tree for the Heterokonta based on a concatenated alignment of SSU and LSU rRNA sequences.

Van de Peer & De Wachter, 1997a). On the other hand, in some cases the LSU rRNA seems to be more informative than the SSU rRNA. On the basis of LSU rRNA the common ancestry of Fungi and Microsporidia, the latter of which are amitochondrial parasites previously thought to be the descendants of a primitive, ancient eukaryotic lineage (Vossbrinck *et al.*, 1987), could be confirmed (Van de Peer *et al.*, 2000b). Although this relationship has been supported by protein-coding genes such as tubulin, RNA polymerase, and others (Keeling & Doolittle, 1996; Hirt *et al.*, 1999), such a common origin cannot be confirmed on the basis of SSU rRNA (Philippe & Germot, 2000; Philippe *et al.*, 2000; unpublished results). This again demonstrates that, despite considerable debate on the vices and virtues of various molecular phylogenetic markers, all have their strengths and weaknesses.

Nevertheless, it has been recently demonstrated that the branching order between many deep divergences can possibly be resolved by combining several molecular markers (Moreira *et al.*, 2000; Baldauf *et al.*, 2000). Therefore, in order to find out whether the small or scarcely sampled algal taxa such as the chlorarachniophytes, cryptomonads and haptophytes are important missing links among major clades, it would be highly interesting to determine the sequences of some protein-coding genes for these organisms.

Phylogenetic relationships within the heterokont algae

On the basis of the SSU rRNA, the phylogenetic relationships between the different taxa that belong to the so-called heterokont algae cannot be deduced unequivocally (Ariztia *et al.*, 1991; Andersen *et al.*, 1993, 1998, 1999; Potter *et al.*, 1997; Lavau *et al.*,

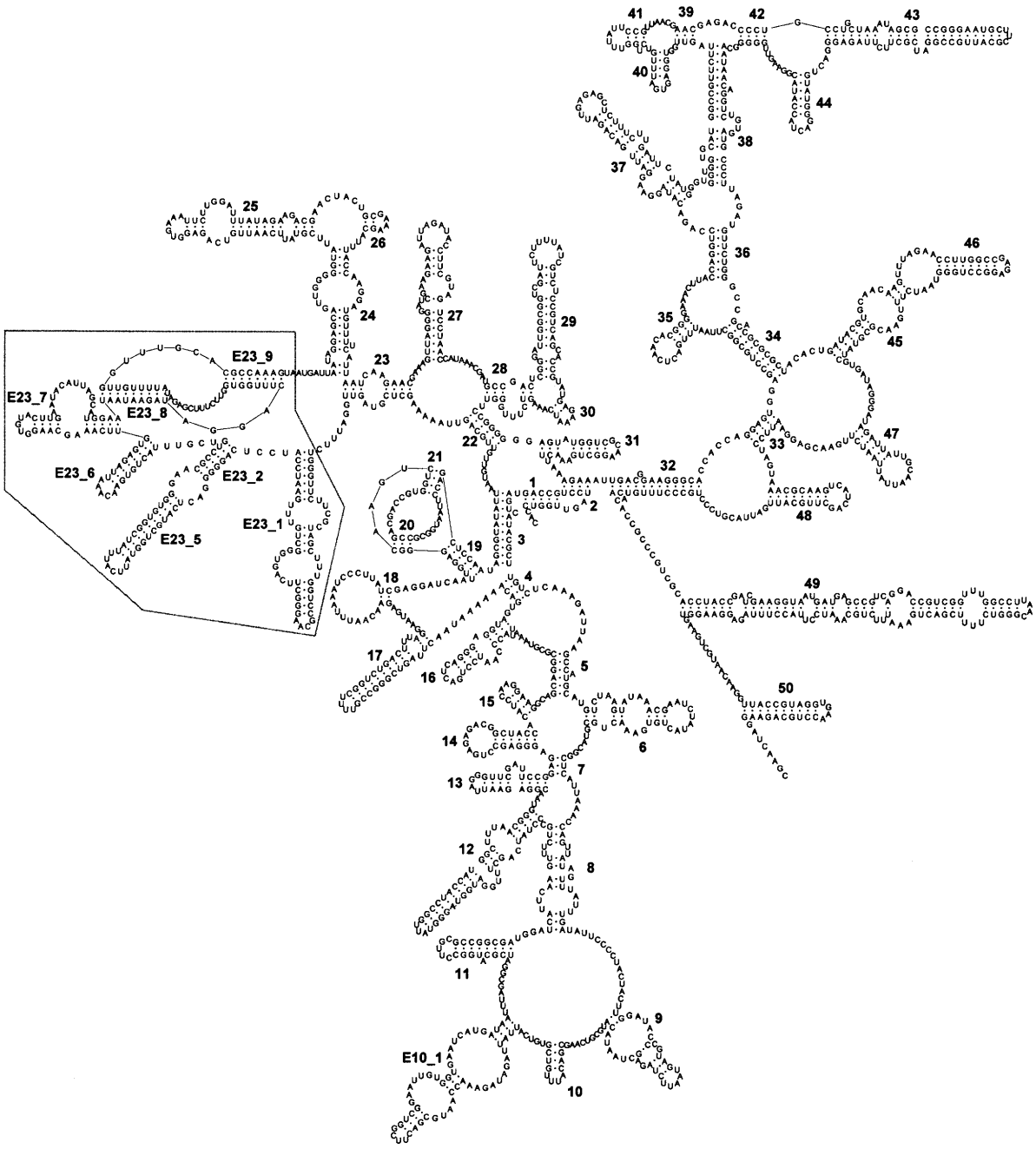

Fig. 4. Secondary structure model of the SSU rRNA of the dictyochophyte *Apedinella radians*. The hypervariable region E23, indicated by a box, was omitted from the alignment.

1997; Saunders *et al*., 1995, 1997; Bailey *et al*., 1998; Van de Peer & De Wachter, 1997a; Van de Peer *et al*., 2000a). Therefore, we determined the complete or nearly complete LSU rRNA sequences of several species for which the SSU rRNA sequence was already known. Fig. 3 shows an unrooted phylogenetic tree of the Heterokonta (heterokont algae plus oomycetes, and hyphochytriomycetes) based on the concatenation of SSU rRNA and LSU rRNA sequences. The complete SSU rRNA sequence was used, except for the hypervariable region E23 (see Fig. 4), while three hypervariable regions of the LSU rRNA were omitted (see Fig. 5). Since sequence dissimilarities between heterokonts are rather small, evolutionary distances were estimated according to Kimura (1980). As expected, other distance estimates gave the same tree topology.

In the combined analysis, the clustering of the dictyochophytes and the pelagophytes, as already suggested

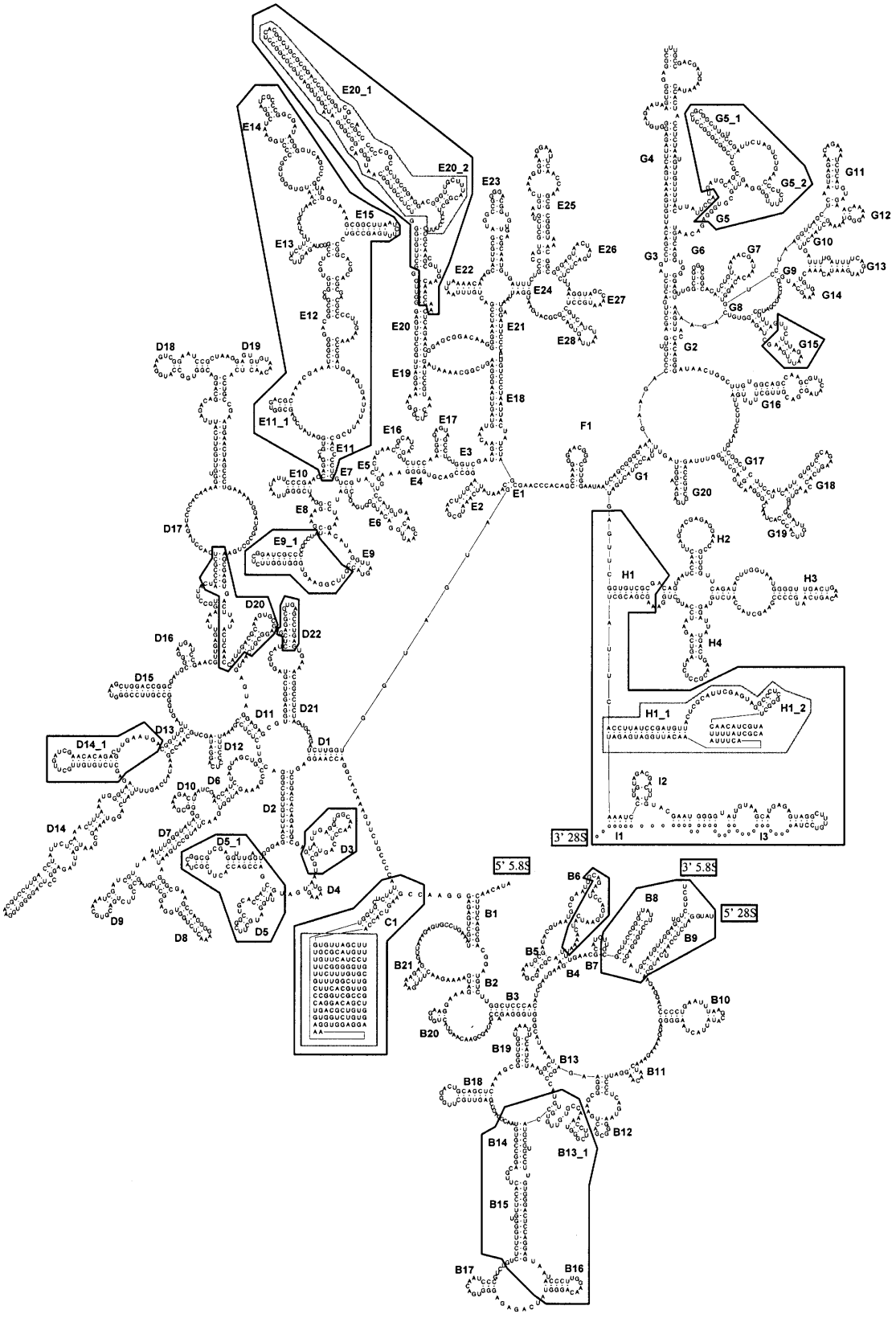

Fig. 5. Secondary structure model of the LSU rRNA of the dictyochophyte *Apedinella radians*. For the construction of the LSU tree of Fig. 1 all the regions indicated in black boxes were omitted. For the construction of the tree based on the concatenated alignment of SSU rRNA and LSU rRNA sequences of Heterokonta (Fig. 2), only the hypervariable regions indicated in grey boxes were omitted.

on the basis of SSU rRNA (Saunders *et al*., 1995; Van de Peer & De Wachter, 1997a) and LSU rRNA alone (Fig. 1), is confirmed: both groups share a common ancestry highly supported by bootstrap analysis (BP 99%). The close relationship between xanthophytes and phaeophytes, as previously suggested on the basis of rRNA data (Ariztia *et al*., 1991; Bhattacharya *et al*., 1992; Leipe *et al*., 1994; Saunders *et al*., 1995; Potter *et al*., 1997; Van de Peer & De Wachter, 1997a; Van der Auwera *et al*., 1997) is also confirmed (BP 96%). On the basis of the concatenated sequence alignment, also the chysophytes (represented here solely by *Ochromonas danica*) and the eustigmatophytes (here represented by *Nannochloropsis salina*) seem to be sister taxa, highly supported by bootstrap analysis. This sister group relationship could not be demonstrated when SSU rRNA or LSU rRNA sequences were analysed separately (Van de Peer & De Wachter, 1997a; Fig. 1). Furthermore, it should be pointed out that this sister-group relationship only holds when neither the chrysophytes nor the eustigmatophytes form the first diverging lineage within the Heterokonta. Although on the basis of SSU rRNA this cannot be ruled out (Van de Peer & De Wachter, 1997a; Van de Peer *et al*., 2000a), this seems to be unlikely on the basis of LSU rRNA (see Fig. 1), where the heterokont fungi form the first diverging lineages. If this is indeed true, the heterokont algae form a well-supported monophyletic lineage (BP 100%), of which the bacillariophytes form the first diverging taxon. Contrary to what has been previously suggested, the bacillariophytes do not form a monophyletic cluster with the pelagophytes and dictyochophytes, although they do share the possession of a reduced flagellar apparatus (Saunders *et al*., 1995). Maximum-likelihood analysis (not shown) shows the same topology and similar support as the tree in Fig. 3. Maximum-parsimony supports all the sister-relationships discussed above, but support is lacking for any of the relationships between classes of heterokont algae at a deeper level.

ACKNOWLEDGEMENTS

Y.V.d.P. acknowledges the support of the University of Antwerp (Belgium) and the University of Konstanz (Germany, Professor Axel Meyer). The authors thank Geoff McFadden for DNA from the chlorarachniophyte strain CCMP621, Uwe G. Maier for DNA from *Guillardia theta*, Linda Medlin for DNA from *Prymnesium patelliferum* and *Phaeocystis antarctica* and Robert A. Andersen for DNA from the different heterokont algae discussed in this paper. Y.V.d.P. and G.V.d.A. are Research Fellows of the National Fund for Scientific Research – Flanders (Belgium).

REFERENCES

Allard, M. W., Ellsworth, D. L. & Honeycutt, R. L. (1991). The production of single-stranded DNA suitable for sequencing using the polymerase chain reaction. *Biotechniques* **10**, 24–26.

Andersen, R. A. (1987). The Synurophyceae classis nova: a new class of algae. *Am J Bot* **74**, 337–353.

Andersen, R. A. (1991). The cytoskeleton of chromophyte algae. *Protoplasma* **164**, 143–159.

Andersen, R. A., Saunders, G. W., Paskind, M. P. & Sexton, J. P. (1993). Ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. *J Phycol* **29**, 701–715.

Andersen, R. A., Brett, R. W., Potter, D. & Sexton, J. P. (1998). Phylogeny of the Eustigmatophyceae based upon the 18S rRNA gene, with emphasis on Nannochloropsis. *Protist* **149**, 61–74.

Andersen, R. A., Van de Peer, Y., Potter, D., Sexton, J. P., Kawachi, M. & LaJeunesse, T. (1999). Phylogenetic analysis of the SSU rDNA from members of the Chrysophyceae. *Protists* **150**, 71–84.

Ariztia, E. V., Andersen, R. A. & Sogin, M. L. (1991). A new phylogeny for chromophyte algae using 16S-like rRNA sequences from *Mallomonas papillosa* (Synurophyceae) and *Tribonema aequale* (Xanthophyceae). *J Phycol* **27**, 428–436.

Bailey, J. C., Bidigare, R. R., Christensen, S. J. & Andersen, R. A. (1998). Phaeothamniophyceae classis nova: a new lineage of chromophytes based upon photosynthetic pigments, rbcL sequence analysis and ultrastructure. *Protist* **149**, 245–263.

Baldauf, S. L. (1999). A search for the origins of animals and fungi, comparing and combining molecular data. *Am Nat* **154**, S178–S188.

Baldauf, S. L. & Palmer, J. D. (1993). Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. *Proc Natl Acad Sci U S A* **90**, 11558–11562.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. (2000). A kingdom level phylogeny of eukaryotes based on combined protein data. *Science* **290**, 972–977.

Bhattacharya, D. & Medlin, L. (1995). The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions. *J Phycol* **31**, 489–498.

Bhattacharya, D., Stickel, S. K. & Sogin, M. L. (1991). Molecular phylogenetic analysis of actin genic regions from *Achlya bisexualis* (Oomycota) and *Costaria costata* (Chromophyta). *J Mol Evol* **33**, 525–536.

Bhattacharya, D., Medlin, L., Wainright, P. O., Ariztia, E. V., Bibeau, C., Stickel, S. K. & Sogin, M. L. (1992). Algae containing chlorophyll $a+c$ are polyphyletic: molecular evolutionary analysis of the Chromophyta. *Evolution* **46**, 1801–1817; erratum **47** (1993), 986.

Bhattacharya, D., Helmchen, T., Bibeau, C. & Melkonian, M. (1995a). Comparisons of nuclear-encoded small-subunit ribosomal RNAs reveal the evolutionary position of the Glaucocystophyta. *Mol Biol Evol* **12**, 415–420.

Bhattacharya, D., Helmchen, T. & Melkonian, M. (1995b). Molecular evolutionary analyses of nuclear-encoded small subunit ribosomal RNA identify an independent rhizopod lineage containing the Euglyphina and the Chlorarachniophyta. *J Eukaryot Microbiol* **42**, 65–69.

Cavalier-Smith, T. (1993). The origin, losses and gains of chloroplasts. In *Origins of Plastids*, pp. 291–348. Edited by R. A. Lewin. London: Chapman & Hall.

Cavalier-Smith, T. (1995). Membrane heredity, symbiogenesis, and the multiple origins of algae. In *Biodiversity and Evolution*, pp. 75–114. Edited by R. Arai, M. Kata & Y. Doi. Tokyo: The National Science Museum Foundation.

Cavalier-Smith, T. & Chao, E. E. (1996). 18S rRNA sequence of *Heterosigma carterae* (Raphidophyceae), and the phylogeny of heterokont algae (Ochrophyta). *Phycologia* **35**, 500–510.

Cavalier-Smith, T., Allsopp, M. T. E. P. & Chao, E. E. (1994a). Chimeric conundra: are nucleomorphs and chromists monophyletic or polyphyletic? *Proc Natl Acad Sci U S A* **91**, 11368–11372.

Cavalier-Smith, T., Allsopp, M. T. E. P. & Chao, E. E. (1994b). Thraustochytrids are chromists, not Fungi: 18S rRNA signatures of Heterokonta. *Philos Trans R Soc Lond Biol* **346**, 387–397.

Cavalier-Smith, T., Chao, E. E. & Allsopp, M. T. E. P. (1995). Ribosomal RNA evidence for chloroplast loss within Heterokonta: pedinellid relationships and a revised classification of Ochristan algae. *Arch Protistenkd* **145**, 209–220.

Daugbjerg, N. & Andersen, R. A. (1997a). Phylogenetic analyses of the rbcL sequences from haptophytes and heterokont algae suggest their chloroplasts are unrelated. *Mol Biol Evol* **14**, 1242–1251.

Daugbjerg, N. & Andersen, R. A. (1997b). A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded rbcL sequence data. *J Phycol* **33**, 1031–1041.

Delwiche, C. F. (1999). Tracing the thread of plastid diversity through the tapestry of life. *Am Nat* **154**, 164–177.

De Rijk, P. & De Wachter, R. (1993). DCSE, an interactive tool for sequence alignment and secondary structure research. *Comput Appl Biosci* **9**, 735–740.

De Rijk, P. & De Wachter, R. (1997). RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Res* **25**, 4679–4684.

De Rijk, P., Wuyts, J., Van de Peer, Y., Winkelmans, T. & De Wachter, R. (2000). The European large subunit ribosomal RNA database. *Nucleic Acids Res* **28**, 177–178.

Douglas, S. E., Murphy, C. A., Spencer, D. F. & Gray, M. W. (1991). Cryptomonad algae are evolutionary chimaeras of two phylogenetically distinct unicellular eukaryotes. *Nature* **350**, 148–151.

Eschbach, S., Wolters, J. & Sitte, P. (1991). Primary and secondary structure of the nuclear small subunit ribosomal RNA of the cryptomonad *Pyrenomonas salina* as inferred from the gene sequence: evolutionary implications. *J Mol Evol* **32**, 247–252.

Ettl, H. (1978). Xanthophyceae. In *Susswasserflora von Mitteleuropa*, Bd. 3, 1. Edited by H. Ettl, H. J. Gerloff & H. Heynig. Stuttgart: Teil, Gustav Fisher.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**, 783–791.

Forster, H., Coffey, M. D., Elwood, H. & Sogin, M. L. (1990). Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. *Mycologia* **82**, 306–312.

Gajadhar, A. A., Marquardt, W. C., Hall, R., Gunderson, J., Ariztia-Carmona, E. V. & Sogin, M. L. (1991). Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata*, and *Crythecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. *Mol Biochem Parasitol* **45**, 147–154.

Gibbs, S. P. (1981). The chloroplasts of some algal groups may have evolved from endosymbiotic eukaryotic algae. *Ann N Y Acad Sci* **361**, 193–208.

Gibbs, S. P. (1993). The evolution of algal chloroplasts. In *Origins of Plastids*, pp. 107–121. Edited by R. A. Lewin. London: Chapman and Hall.

Gillott, M. (1990). Phylum Cryptophyta. In *Handbook of Protoctista*, pp. 139–151. Edited by L. Margulis, J. O. Corliss, M. Melkonian & D. J. Chapman. Boston, MA: Jones and Bartlett.

Gilson, P. R. & McFadden, G. I. (1996). The miniaturized nuclear genome of eukaryotic endosymbiont contains genes that overlap, genes that are cotranscribed, and the smallest known spliceosomal introns. *Proc Natl Acad Sci U S A* **93**, 7737–7742.

Green, J. C., Perch-Nielsen, K. & Westbroek, P. (1990). Phylum Prymnesiophyta. In *Handbook of Protoctista*, pp. 293–317. Edited by L. Margulis, J. O. Corliss, M. Melkonian & D. J. Chapman. Boston, MA: Jones and Bartlett.

Heywood, P. (1990). Phylum Raphidophyta. In *Handbook of Protoctista*, pp. 318–325. Edited by L. Margulis, J. O. Corliss, M. Melkonian & D. J. Chapman. Boston, MA: Jones and Bartlett.

Hibberd, D. J. & Leedale, G. F. (1970). Eustigmatophyceae: a new algal class with unique organization of the motile cell. *Nature* **225**, 758–760.

Hibberd, D. J. & Leedale, G. F. (1971). A new algal class: the Eustigmatophyceae. *Taxon* **20**, 523–525.

Hibberd, D. J. & Leedale, G. F. (1972). Observations on the cytology and ultrastructure of the new algal class, Eustigmatophyceae. *Ann Bot* **36**, 49–71.

Hirt, R. P., Logsdon, J. M., Jr., Healey, B., Dorey, M. W., Doolittle, W. F. & Embley, T. M. (1999). Microsporidia are related to Fungi: evidence from the largest subunit of RNA polymerase II and other proteins. *Proc Natl Acad Sci U S A* **96**, 580–585.

Ishida, K., Green, B. R. & Cavalier-Smith, T. (1999). Diversification of a chimeric algal group, the chlorarachniophytes: phylogeny of nuclear and nucleomorph small-subunit rRNA genes. *Mol Biol Evol* **16**, 321–331.

Keeling, P. J. & Doolittle, W. F. (1996). Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. *Mol Biol Evol* **13**, 1297–1305.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Knoll, A. H. (1992). The early evolution of eukaryotes: a geological perspective. *Science* **256**, 622–627.

Lavau, S., Saunders, G. W. & Wetherbee, R. (1997). A phylogenetic analysis of the synurophyceae using molecular data and scale case morphology. *J Phycol* **33**, 135–151.

Leipe, D. D., Wainright, P. O., Gunderson, J. H., Porter, D., Patterson, D. J., Valois, F., Himmerich, S. & Sogin, M. L. (1994). The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. *Phycologia* **33**, 369–377.

Leipe, D. D., Tong, S. M., Goggin, C. L., Slemenda, S. B., Pieniazek, N. J. & Sogin, M. L. (1996). 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. *Eur J Protistol* **32**, 449–458.

Ludwig, M. & Gibbs, S. P. (1989). Evidence that nucleomorphs of *Chlorarachnion reptans* (Chlorarachniophyceae) are vestigial nuclei: morphology, division and DNA-DAPI fluorescence. *J Phycol* **25**, 385–404.

McFadden, G. I. (1993). Second-hand chloroplasts: evolution of cryptomonad algae. *Adv Bot Res* **19**, 189–230.

Maerz, M., Wolters, J., Hofmann, C. J., Sitte, P. & Maier, U. G. (1992). Plastid DNA from Pyrenomonas salina (Cryptophyceae): physical map, genes, and evolutionary implications. *Curr Genet* **21**, 73–81.

Maier, U. G., Hofmann, C. J., Eschbach, S., Wolters, J. & Igloi, G. L. (1991). Demonstration of nucleomorph-encoded eukaryotic

small subunit ribosomal RNA in cryptomonads. *Mol Gen Genet* **230**, 155–160.

Martin, W., Stoebe, B., Goremykin, V., Hansmann, S., Hasegawa, M. & Kowallik, K. V. (1998). Gene transfer to the nucleus and the evolution of chloroplasts. *Nature* **393**, 162–165.

Medlin, L. K., Cooper, A., Hill, C., Wrieden, S. & Wellbrock, U. (1995). Phylogenetic position of the Chromista plastids based on small subunit rRNA coding regions. *Curr Genet* **28**, 560–565.

Moreira, D., Le Guyader, H. & Philippe, H. (2000). The origin of red algae: implications for the evolution of chloroplasts. *Nature* **405**, 69–72.

Palmer, J. D. & Delwiche, C. F. (1998). The origin of plastids and their genomes. In *Molecular Systematics of Plants* 2, pp. 375–409. Edited by D. E. Soltis, P. S. Soltis & J. J. Doyle. New York: Chapman and Hall.

Patterson, D. J. (1989). Stramenopiles: chromophyte from a protistan perspective. In *The Chromophyte Algae: Problems and Perspectives*, pp. 357–379. Edited by J. C. Green, B. S. C. Leadbeater & W. L. Diver. Oxford: Clarendon Press.

Patterson, D. J. & Sogin, M. L. (1993). Eukaryote origins and protistan diversity. In *The Origin and Evolution of Prokaryotic and Eukaryotic Cells*, pp. 13–46. Edited by H. Hartman & K. Matsuno. River Edge, NJ: World Scientific Publishing.

Philippe, H. & Germot, A. (2000). Phylogeny of eukaryotes based on ribosomal RNA: long branch attraction and models of sequence evolution. *Mol Biol Evol* **17**, 830–834.

Philippe, H., Lopez, P., Brinkmann, H., Budin, K., Germot, A., Laurent, J., Moreira, D., Müller, M. & Le Guyader, H. (2000). Early branching or fast evolving eukaryotes? An answer based on slowly evolving positions. *Proc Roy Soc Series B* **267**, 1213–1222.

Potter, D., Saunders, G. W. & Andersen, R. A. (1997). Phylogenetic relationships of the Raphidophyceae and Xanthophyceae as inferred from nucleotide sequences of the 18S ribosomal RNA gene. *Am J Bot* **84**, 966–972.

Ragan, M. A. & Gutell, R. R. (1995). Are red algae plants? *Bot J Linn Soc* **118**, 81–105.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Saunders, G. W., Potter, D., Paskind, M. P. & Andersen, R. A. (1995). Cladistic analyses of combined traditional and molecular data sets reveal an algal lineage. *Proc Natl Acad Sci U S A* **92**, 244–248.

Saunders, G. W., Potter, D. & Andersen, R. A. (1997). Phylogenetic affinities of the Sarcinochrysidales and Chrysomeridales (Heterokonta) based on analyses of molecular and combined data. *J Phycol* **33**, 310–318.

Silva, P. C. (1980). Names of classes and families of living algae. *Regnum Veg* **103**, 1–156.

Strimmer, K. & von Haeseler, A. (1996). Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol Biol Evol* **13**, 964–969.

Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, MA: Sinauer Associates.

Van de Peer, Y. & De Wachter, R. (1997a). Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18S rRNA. *J Mol Evol* **45**, 619–630.

Van de Peer, Y. & De Wachter, R. (1997b). Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. *Comput Appl Biosci* **13**, 227–230.

Van de Peer, Y., Rensing, S. A., Maier, U. G. & De Wachter, R. (1996a). Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. *Proc Natl Acad Sci U S A* **93**, 7732–7736.

Van de Peer, Y., Van der Auwera, G. & De Wachter, R. (1996b). The evolution of stramenopiles and alveolates as derived by "substitution rate calibration" of small ribosomal subunit RNA. *J Mol Evol* **42**, 201–210.

Van de Peer, Y., Baldauf, S. L., Doolittle, W. F. & Meyer, A. (2000a). An updated and comprehensive rRNA phylogeny of (crown) eukaryotes based on rate calibrated evolutionary distances. *J Mol Evol* **51**, 565–576.

Van de Peer, Y., Ben Ali, A. & Meyer, A. (2000b). Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. *Gene* **246**, 1–8.

Van der Auwera, G. & De Wachter, R. (1997). Complete large subunit ribosomal RNA sequences from the heterokont algae *Ochromonas danica*, *Nannochloropsis salina*, and *Tribonema aequale*, and phylogenetic analysis. *J Mol Evol* **45**, 84–90.

Van der Auwera, G., Chapelle, S. & De Wachter, R. (1994). Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. *FEBS Lett* **338**, 133–136.

Van der Auwera, G., De Baere, R., Van de Peer, Y., De Rijk, P., Van den Broeck, I. & De Wachter, R. (1995). The phylogeny of the Hyphochytriomycota as deduced from ribosomal RNA sequences of *Hyphochytrium catenoides*. *Mol Biol Evol* **12**, 671–678.

Van der Auwera, G., Hofmann, C. J., De Rijk, P. & De Wachter, R. (1998). The origin of red algae and cryptomonad nucleomorphs: a comparative phylogeny based on small and large subunit rRNA sequences of Palmaria palmata, Gracilaria verrucosa, and the Guillardia theta nucleomorph. *Mol Phylogenet Evol* **10**, 333–342.

Vossbrinck, C. R., Maddox, J. V., Friedman, S., Debrunner-Vossbrinck, B. A. & Woese, C. R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature* **326**, 411–414.

Wainright, P. O., Hinkle, G., Sogin, M. L. & Stickel, S. K. (1993). Monophyletic origins of the metazoa: an evolutionary link with fungi. *Science* **260**, 340–342.

Whatley, J. M. (1989). Chromophyte chloroplasts: a polyphyletic origin? In *The Chromophyte Algae: Problems and Perspectives*, pp. 125–144. Edited by J. C. Green, B. S. C. Leadbeater & W. L. Diver. Oxford: Clarendon Press.

Whatley, J. M. (1993). Membranes and plastid origins. In *Origins of Plastids*, pp. 77–106. Edited by R. A. Lewin. London: Chapman and Hall.

Williams, D. M. (1991a). Cladistic methods and chromophyte phylogeny. *Biosystems* **25**, 101–112.

Williams, D. M. (1991b). Phylogenetic relationships among the Chromista: a review and preliminary analysis. *Cladistics* **7**, 141–156.

Zhang, Z., Green, B. R. & Cavalier-Smith, T. (1999). Single gene circles in dinoflagellate chloroplast genomes. *Nature* **400**, 155–159.