ELSEVIER

FEBS Letters 338 (1994) 133-136

FEBS 13578

FEBS LETTERS

Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes

Gert Van der Auwera, Sabine Chapelle, Rupert De Wachter*

Universiteit Antwerpen, Departement Biochemie, Universiteitsplein 1, B-2610 Antwerpen, Belgium

Received 6 December 1993

Abstract

The 5.8S and 28S rRNA sequences of the oomycete *Phytophthora megasperma* were determined in order to study the secondary structure of these molecules and to assess the phylogenetic position of the oomycetes among the eukaryotes. Preliminary results point to an affiliation between the oomycetes, dinoflagellates and ciliates, a cluster which seems related to the fungi. In the course of this work, we developed a set of primers which allow sequencing and PCR amplification of eukaryotic large ribosomal subunit RNA genes of a wide range of phylogenetically distant organisms.

Key words: Oomycete; *Phytophthora megasperma*; Large ribosomal subunit rRNA; Evolution; Secondary structure; Primer

1. Introduction

Whittaker [1] divided the eukaryotes into four kingdoms: Protista, Fungi, Animalia and Plantae. While the latter three kingdoms are monophyletic evolutionary lineages, the Protista form an artificial grouping, its members being defined as all the eukaryotes not belonging to one of the other three kingdoms [2]. The phylogenetic relationship between the different protist groups and the other eukaryotes has partially been elucidated on the basis of evolutionary trees derived from SSU rRNA sequence alignments [3]. These trees show a number of deeply diverging lines comprising, in order of divergence, the protist groups Diplomonada, Microsporidia, Euglenozoa, and the slime moulds. On the other hand, the branching order between a number of later-diverging taxa, viz. the Metazoa, Chlorobionta (plants and green algae), Fungi, and a number of protist taxa like the Rhodophyta, Oomycota, Chromophyta, Apicomplexa, Dinoflagellata and Ciliata, is less well resolved. Using nuclear LSU rRNA (28S and 5.8S rRNA) instead of SSU rRNA as a molecular clock, we are attempting to clarify this phylogeny. The first LSU rRNA sequence we determined is that of the oomycete *Phytophthora megasperma* (ATCC 12316, MUCL 11644), which is presented in this paper. Because LSU rRNA sequences are already known for representatives of the Dinoflagellata, Ciliata, Fungi, Chlorobionta and Metazoa [4], the relationship between these taxa and *Phytophthora megasperma* can be assessed preliminarily.

Since we want to determine the LSU rRNA sequence of phylogenetically distant organisms in the future, we developed a set of primers for sequencing and PCR which can be used for a wide range of eukaryotic nuclear LSU rRNA genes.

* Corresponding author. Fax: (32) (3) 820 2248.

Abbreviations: LSU rRNA, large subunit rRNA (28S + 5.8S); SSU rRNA, small subunit rRNA (18S); ATCC, American Type Culture Collection; MUCL, Mycothèque de l' Université Catholique de Louvain; PCR, polymerase chain reaction.

2. Materials and methods

2.1. DNA isolation

DNA was isolated from biomass supplied by the MUCL culture collection (Louvain-la-Neuve, Belgium). About 2.5 g of frozen cell paste (−70°C) was crushed in the presence of liquid nitrogen and alumina. Then, 6 ml were added of a buffer containing 10 mM $MgCl_2$, 50 mM NaCl, 1% SDS and 50 mM Tris adjusted with HCl to pH 7.4. Subsequently, the mixture was thawed, extracted once with phenol/chloroform/isoamylalcohol (25:24:1) and once with chloroform/isoamylalcohol (24:1). The DNA was precipitated in the presence of 0.3 M NaAc pH 5.6 by the addition of one volume isopropanol. The pellet was washed once with 70% ethanol and redissolved in a buffer containing 0.1 mM EDTA and 10 mM Tris adjusted to pH 7.4 with HCl.

2.2. PCR amplification and cloning of the LSU rRNA genes

The primers used for PCR and sequencing are listed in Table 1. The 5.8S and most of the 28S rRNA gene were amplified in one PCR on 10 ng of genomic DNA using primers 1 and 29 which are situated near the 3′ end of the 18S and 28S rDNA, respectively.

The 3′-terminal fragment of the 28S rRNA gene lying beyond primer 29 was amplified with the inverse PCR technique [5]. To this end, genomic DNA was digested with *Hin*dIII (BRL, Gaithersburg MD, USA), which had been shown to create a fragment of about 4.5 kb extending from nucleotide 113 of the 28S rDNA into the intergenic spacer downstream of it. After extraction and precipitation, the resulting fragments were ligated in the presence of 0.02 U/μl T4 DNA ligase

0014-5793/94/$7.00 © 1994 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(93)E1495-8

(BRL, Gaithersburg, MD, USA) at the very low concentration of ~1 ng/μl, favouring intramolecular reactions. The inverse PCR was performed on 50 ng of the ligated DNA fragments using primers 13 and 20 (Table 1) in order to yield an amplification product overlapping with the one obtained by means of primers 1 and 29 as described above.

Both amplifications were performed using the hot-start PCR technique in 100 μl of 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatine and 10 mM Tris adjusted to pH 8.3 with HCl. We used 0.5 μM of each primer together with 200 μM of each dNTP (Pharmacia, Uppsala, Sweden) and 0.025 U/μl Taq DNA polymerase (Boehringer Mannheim, Germany). Thirty cycles were performed, each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 3.5 min primer extension at 72°C. After completion of all the cycles, an additional polymerisation step was added at 72°C for 10 min. Both PCR products were purified on agarose gel [6] and ligated into a dT-tailed vector according to [7]. The recombinant plasmids were introduced into *E. coli* DH5α cells by electroporation. Screening [8] was performed with a PCR product obtained from genomic DNA by amplification with primers 4 and 29 (Table 1), which was labelled with ^{32}P using the nick translation kit from Amersham (Amersham, UK).

2.3. Sequencing

In order to minimize sequencing errors introduced by Taq DNA polymerase during PCR, 12 clones of the PCR product were pooled as well as 7 clones of the inverse PCR product. Sequencing was performed using primers 2–32 (Table 1) with the Sequenase Kit from USB (Cleveland, Ohio, USA), the T7 Sequencing kit from Pharmacia (Uppsala, Sweden) and the Bst DNA Sequencing kit from Bio-Rad (Richmond, California, USA), all according to the manufacturer's instructions. Sequences were determined on both strands except for position 1–59 and 92–163 of the 5.8S rDNA and position 1–54 of the 28S rDNA.

2.4. Alignment and tree construction

The sequence of *Phytophthora megasperma* was included in an alignment comprising 42 known eukaryotic nuclear LSU rRNA sequences (some of which are partial) maintained in our research group. Positions which could not be unambiguously aligned were omitted from the phylogenetic analysis. Evolutionary trees were constructed on the remaining 3038 alignment positions which include 2723 out of the 3860 nucleotides of *Phytophthora megasperma* LSU rRNA. Trees were constructed with the transformed-distance [9] and neighbour-joining [10] method using the software package Treecon [11]. Evolutionary distances were calculated according to an equation [12] which takes into account insertions and deletions and corrects for multiple substitutions according to Jukes and Cantor [13]. Bootstrap analysis [14] was performed on 100 samples unless mentioned otherwise.

3. Results and discussion

3.1. Primary and secondary structure of the LSU rRNA

The 5.8S and 28S rRNA are 163 and 3697 bases long,

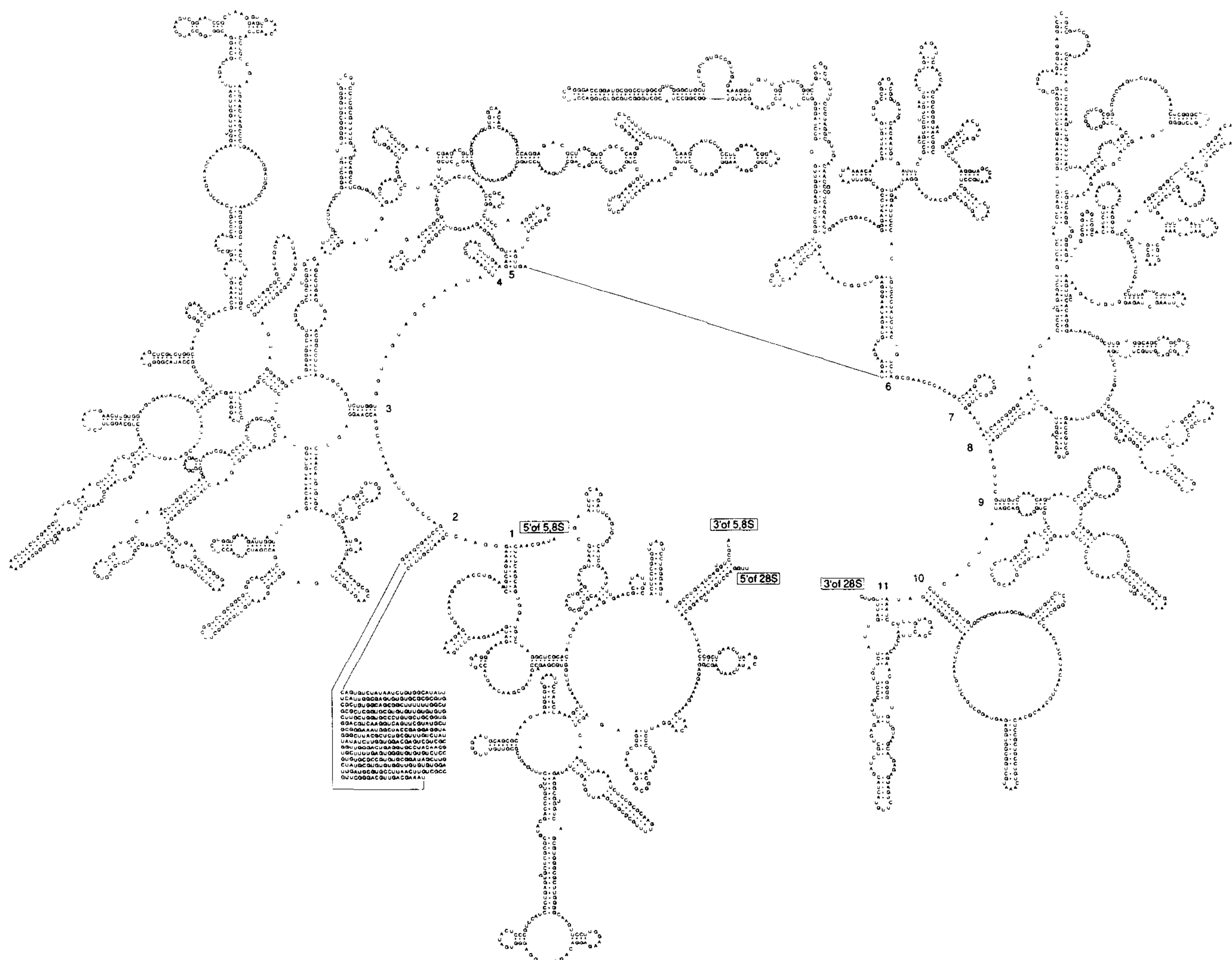

Fig. 1. Secondary structure model of the LSU rRNA of the oomycete *Phytophthora megasperma*. Only helices departing from the single-stranded structure drawn as an inner circle are numbered.

respectively. The 5′ and 3′ termini of both molecules were deduced by comparison with other LSU rRNAs. The sequences are available from the EMBL data bank and have been assigned the accession numbers X75632 (5.8S rRNA) and X75631 (28S rRNA). Fig. 1 shows the secondary structure model of the LSU rRNA as used in our database. This model is basically the same as the one published by Gutell for *Saccharomyces cerevisiae* LSU rRNA [4]. No secondary structure has yet been found for an area situated at the apex of helix 2 (Fig. 1) which has a chain length of 370 nucleotides in *Phytophthora megasperma* but which is variable in length among different species.

Table 1
Primers used for PCR and sequencing

No.	Sequence[a,b]	Strand[c]	Position[d]
	Primer in eukaryotic 18S rDNA		
1	TTTGYACACACCGCCCGTCG	F	1624–1643
	Primers in eukaryotic 5.8S rDNA		
2	RCATCGATGAAGAACGYWG	F	31–49
3	GCGTTCRAAGWBTCGATG	R	108–91
	Primers in eukaryotic 28S rDNA		
4	ACCCGCTGAAYTTAAGCATAT	F	26–46
5	ATATGCTTAARTTCAGCGGGT	R	46–26
6	CGATAGYVRACAAGTA	F	339–354
7	TACTTGTYBRCTATCG	R	354–339
8	ATCTTGGTGGACGAGT	F	643–658*
9	ACTCGTCCACCAAGAT	R	658–643*
10	CCGTCTTGAAACACGGACCAAGGAG	F	636–660
11	CTCCTTGGTCCGTGTTTCAAGACGG	R	660–636
12	CCGAAGTTTCCCTCAGGATAGC	F	947–968
13	GCTATCCTGAGGGAAACTTCGG	R	968–947
14	TCCGCTAAGGAGTGTGTAACAAC	F	1252–1274
15	GTTGTTACACACTCCTTAGCGGA	R	1274–1252
16	GGTYAGTCGRTCCTRAG	F	1519–1535
17	TCTYAGGAYCGACTNAC	R	1536–1520
18	CCGCAKCAGGTCTCCAA	F	1841–1857
19	CTTGGAGACCTGMTGCGG	R	1858–1841
20	GTAACTTCGGGAWAAGGATTGGCT	F	1917–1940
21	AGCCAATCCTTWTCCCGAAGTTAC	R	1940–1917
22	TGATTTCTGCCCAGTGCTCTGAATGT	F	2185–2210
23	ACATTCAGAGCACTGGGCAGAAATCA	R	2210–2185
24	GGGAAAGAAGACCCTGTTGAG	F	2393–2413
25	CTCAACAGGGTCTTCTTTCC	R	2413–2393
26	GGGAGTTTGRCTGGGGCGG	F	2605–2623
27	CCGCCCCAGYCAAACTCCC	R	2623–2605
28	AGGGAACGTGAGCTGGGTTTAGAC	F	2932–2955
29	GTCTAAACCCAGCTCACGTTCCCT	R	2955–2932
30	CTGAACGCCTCTAAGTCAGAA	F	3106–3126
31	TTCTGACTTAGAGGCGTTCAG	R	3126–3106
	Primer in intergenic spacer (IGS)		
32	CGGTTGCAATTGCTTGGG	R	20–37*

[a] The sequence of primer 1 was taken from [15], all other primers are newly designed. Primers 2–7 and 10–31 can be used for most eukaryotic nuclear LSU rDNAs. Primers 8, 9 and 32 are specific for *Phytophthora megasperma*.
[b] R = A and G; Y = C and T; M = A and C; K = G and T; W = A and T; B = G, C and T; V = G, A and C; N = G, A, T and C.
[c] F: forward primers, these have an rRNA-like sequence. R: reverse primers, these have a sequence complementary to that of the rRNA.
[d] The numbering corresponds to the rRNA sequence of *Saccharomyces cerevisiae* as published in [16], [17] and [18], except for primers 8, 9 and 32 (marked with *) for which the numbering and sequence are that of the rRNA of *Phytophthora megasperma* (this paper).

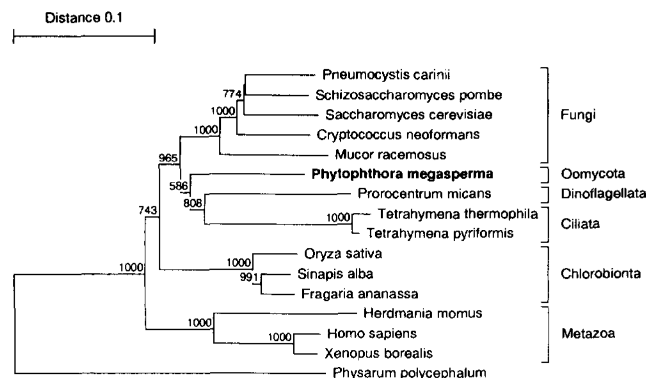

Fig. 2. Evolutionary tree constructed by the neighbour-joining method with 1000 bootstrap samples. Bootstrap values above 50% are indicated at each node. Distances are measured in substitutions per nucleotide and calculated as described in section 2. The sequences were obtained from the EMBL data bank.

3.2. Phylogenetic analysis

An evolutionary tree was constructed from an alignment of 35 available complete eukaryotic nuclear LSU rRNA sequences with the neighbour-joining method using *Escherichia coli* as outgroup. This tree showed a cluster comprising the Metazoa, Chlorobionta, Fungi and species belonging to the protist taxa Dinoflagellata, Oomycota and Ciliata. Eleven other protist species were situated outside this cluster, on more deeply diverging branches, and can thus be used as outgroups for examining this cluster. These species were the diplomonads *Giardia muris*, *G. intestinalis* and *G. ardeae*, the euglenophyte *Euglena gracilis*, the flagellates *Trypanosoma brucei*, *T. cruzi* and *Crithidia fasciculata*, the slime moulds *Didymium iridis*, *Physarum polycephalum* and *Dictyostelium discoideum*, and the amoeba *Entamoeba histolytica*. This tree topology is consistent with that of trees obtained from eukaryotic SSU rRNA alignments [3].

In order to investigate more precisely the relationships between the Metazoa, Chlorobionta, Fungi, Dinoflagellata, Oomycota and Ciliata, eleven neighbour-joining trees were constructed, viz. with each of the aforementioned protist species as outgroup. Fig. 2 shows the tree that was obtained with *Physarum polycephalum* as outgroup. The very same topology was found in 8 out of the 11 trees, except that the Metazoa and the Chlorobionta sometimes formed sister groups. In all but one of these 8 trees, the clustering of the Fungi, the oomycete *Phytophthora megasperma*, the dinoflagellate *Prorocentrum micans* and the ciliates belonging to the genus

Tetrahymena was supported by high bootstrap values (965/1000 in the tree shown in Fig. 2). This result seems to indicate a strong relationship between these groups, which was also found in transformed-distance trees (not shown). The 3/11 remaining trees showed different topologies which were, however, not supported by sufficient bootstrap scores.

Although there is strong evidence that *Phytophthora megasperma* belongs to a cluster that also comprises the dinoflagellates, ciliates and true fungi, there is much less certainty about the position of *Phytophthora megasperma* within this cluster. Indeed, the relationship to the dinoflagellate–ciliate cluster as represented in Fig. 2 is only supported by a bootstrap value of 586/1000, and the dinoflagellate–ciliate cluster itself by a bootstrap value of 808/1000. These results cannot exclude other topologies, where *Phytophthora megasperma* would have either the true fungi, the dinoflagellates or the ciliates as sister group. The latter topology seems the least probable, since it was never encountered in any of the neighbour-joining and transformed-distance trees constructed.

In SSU rRNA trees, the oomycetes and chromophytes are generally clustered with ciliates, apicomplexa and dinoflagellates [3], a topology which is consistent with our results. However, this cluster is not directly associated with the fungi in these trees.

In the future, more LSU rRNA sequences will be determined of different protist groups to assess the eukaryotic phylogenetic relationships more precisely.

Acknowledgements: The authors thank Peter De Rijk for the sequence alignment, and Prof. G. Hennebert for culturing *Phytophthora megasperma* MUCL 11644. This research was supported by an FGWO grant and carried out in the framework of the Institute for the Study of Biological Evolution of the University of Antwerp. Gert Van der Auwera is a research assistant of the NFWO.

References

[1] Whittaker, R.H. (1969) Science 163, 150–160.
[2] Margulis, L. and Schwartz, K.V. (1982) Five Kingdoms - An Illustrated Guide to the Phyla of Life on Earth, Freeman, San Francisco, USA.
[3] Van de Peer, Y., Neefs, J.M., De Rijk, P. and De Wachter, R. (1993) Biochem. Syst. Ecol. 21, 43–55.
[4] Gutell, R.R., Gray, M.W. and Schnare, M.N. (1993) Nucleic Acids Res. 21, 3055–3074.
[5] Ochman, H., Medhora, M.M., Garza, D. and Hartl, D.L. (1990) in: PCR protocols - A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds.) pp. 219–227, Academic Press, London.
[6] Heery, D.M., Gannon, F. and Powell, R. (1990) Trends Genet. 6, 173.
[7] Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1991) Nucleic Acids Res. 19, 1154.
[8] Dale, J.W. and Greenaway, P.J. (1984) in: Methods in Molecular Biology (Walker, J.M., Ed.) vol.2, pp. 277–283, Humana Press, Clifton, New Jersey.
[9] Farris, J.S. (1977) in: Major Patterns in Vertebrate Evolution (Hecht, M.K., Goody, P.C. and Hecht, B.M., Eds.) pp. 823–850, Plenum Press, New York.
[10] Saitou, N. and Nei, M. (1987) Mol. Biol. Evol. 4, 406–425.
[11] Van de Peer, Y. and De Wachter, R. (1993) Comput. Appl. Biosci. 9, 177–182.
[12] Van de Peer, Y., Neefs, J.M. and De Wachter, R. (1990) J. Mol. Evol. 30, 463–476.
[13] Jukes, T.H. and Cantor, C.R. (1969) in: Mammalian Protein Metabolism (Munro, H.N., Ed.) pp. 21–132, Academic Press, New York.
[14] Felsenstein, J. (1985) Evolution 39, 783–791.
[15] Hendriks, L., Goris, A., Neefs, J.M., Van de Peer, Y., Hennebert, G. and De Wachter, R. (1989) System. Appl. Microbiol. 12, 223–229.
[16] Mankin, A.S., Skryabin, K.G. and Rubtsov, P.M. (1986) Gene 44, 143–145.
[17] Rubin, G.M. (1973) J. Biol. Chem. 248, 3860–3875.
[18] Georgiev, O.I., Nikolaev, N., Hadjiolov, A.A., Skryabin, K.G., Zakharyev, V.M. and Bayev, A.A. (1981) Nucleic Acids Res. 9, 6953–6958.