Volume 269, number 2, 445-449 FEBS 08818 September 1990

The 18S ribosomal RNA sequence of the sea anemone *Anemonia sulcata* and its evolutionary position among other eukaryotes

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Received 19 June 1990

Evolutionary trees based on partial small ribosomal subunit RNA sequences of 22 metazoa species have been published [(1988) Science 239, 748–753]. In these trees, cnidarians (Radiata) seemed to have evolved independently from the Bilateria, which is in contradiction with the general evolutionary view. In order to further investigate this problem, the complete srRNA sequence of the sea anemone *Anemonia sulcata* was determined and evolutionary trees were constructed using a matrix optimization method. In the tree thus obtained the sea anemone and Bilateria together form a monophyletic cluster, with the sea anemone forming the first line of descent of the metazoan group.

Small ribosomal subunit RNA sequence; Metazoan evolution; *Anemonia sulcata*

1. INTRODUCTION

In the past, the study of the evolution of metazoa mainly has been accomplished on the basis of morphological and embryological criteria. However, this classical approach is not adequate to unravel all metazoan evolutionary events. Therefore, in addition to the traditional approach, a molecular approach has been applied to investigate patterns in metazoan evolution. In this way, protein sequences such as those from cytochrome *c* [1], and 5S ribosomal RNA sequence data [2–5] have been used as a tool to study metazoan evolution. Recently, due to the enormous progress in cloning and sequencing techniques, the sequences of larger molecules such as small ribosomal subunit RNA (srRNA) have become available for studying the evolution of organisms in general and more specific those of metazoa (e.g. [6–8]).

Up to now 62 complete srRNA sequences of eukaryotes, among which 6 vertebrates, 4 arthropods and 1 nematode, have been published and compiled under the form of an alignment [9]. In addition to these complete sequences, Field and coworkers [6] have determined the partial srRNA sequences of 22 metazoa species belonging to 10 different invertebrate phyla. The phylogenetic trees constructed on the basis of the latter sequences using a matrix distance method, showed that within the Eumetazoa, two main groups are distinguishable, viz. Radiata (Cnidaria) and Bilateria, which originate from a different ancestor. The cnidarians seemed to be more affiliated with plants, fungi and ciliates than with other animals. This result is in contradiction with the view of most zoologists, who consider the Radiata as the most primitive Eumetazoa, which gave rise to the Bilateria [10]. Also on the basis of 5S rRNA sequence data, Cnidaria and Bilateria together form a monophyletic group [2–5]. Consequently, several authors [11–13] have proposed to treat the molecular results of Field et al. [6] with caution. The suggested polyphyletic origin of metazoa can be due to the use of partial srRNA molecules and/or to shortcomings of the algorithm used for tree construction. Field et al. [14] reported a somewhat more careful appraisal of the relationship of Radiata and Bilateria when using the bootstrapping method [15] for the random choice of positions. In most cases (54%) the matrix algorithm generated the same topology as obtained earlier [6], but in all other cases, Radiata seemed to form a sister group of the Bilateria. Recently, the evolutionary parsimony method [16] has been applied [17] to the metazoan partial srRNA sequence data set [6]. In the tree thus constructed, Cnidaria and Bilateria seem to have originated from a common ancestor [17]. In order to further investigate these contradictory results, the complete sequence of the sea anemone *Anemonia sulcata* was determined, aligned with other srRNA sequences and phylogenetic trees were inferred using a matrix optimization method [18].

2. MATERIALS AND METHODS

2.1. *DNA extraction of nucleic acids*

The sea anemone *Anemonia sulcata* (phylum Coelenterata or

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Abbreviations: srRNA, small ribosomal subunit RNA; srDNA, gene coding for the srRNA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Cnidaria, classis Anthozoa) grows on rocks in the Mediterranean Sea and was bought in an aquarium shop. In order to obtain genomic DNA of a length of about 50 kb, 5 g of fresh tissue was homogenized in the presence of 2 g alumina and liquid nitrogen in a mortar. The residue was suspended in 50 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 1% sodium dodecyl sulphate, adjusted with HCl to pH 7.4. Phenol extractions and precipitations of nucleic acids were as described elsewhere [19].

2.2. *Cloning and sequencing*

A *Bam*HI restriction enzyme fragment containing the srRNA gene from base 550 to the 3'-end and an EcoRI restriction fragment which contains the srRNA gene from 5'-end to base 1550 were each cloned in pUC18 [20]. These two overlapping clones were amplified and plasmid DNA was recovered using the boiling method [21]. Sequencing was performed by the dideoxy sequencing method [22], using 16 primers, complementary to evolutionary conserved regions of the coding and non-coding strand of the srDNA [23]. The polymerase used was Sequenase 2.0 (USB, Cleveland, OH) and sequencing conditions were as described by the manufacturer.

2.3. *Alignment and evolutionary tree construction*

The srRNA sequence thus obtained was aligned with other srRNA sequences, compiled under the form of an alignment [9]. On the basis of this alignment, evolutionary trees were constructed. For this purpose a dissimilarity matrix was calculated by pairwise comparison of sequences as described previously [7]. Gaps and insertions, irrespective of their length, are counted as a single evolutionary event and given the same weight as a substitution. The algorithm used to obtain the tree topology was a matrix optimization method [18].

3. RESULTS AND DISCUSSION

3.1. *Primary and secondary structure*

The sequence of the srRNA gene of *Anemonia sulcata* is 1799 nucleotides long. The 5'- and 3'-termini were derived by comparison with other published sequences. The sequence is fitted in the secondary structure model proposed by Neefs et al. [9] and is shown in Fig. 1.

Helices are numbered as described previously [9] i.e. universal helices bear a single number, whereas helices specific for eukaryotes bear a number of the form of Ea-b, where a is the number of the preceding universal helix and b is a serial number. Helices in the E21 area are not numbered continuously because in certain species, extra helices are present. The region comprising helices E21-7 and E21-8 is folded in a pseudoknotted structure [24]. Evidence for the existence of such a pseudoknot will be published elsewhere.

3.2. *Evolution*

In Fig. 2 an evolutionary tree, based on all alignment positions and constructed with a matrix optimization method [18], is depicted. Compared to a previously published tree [23] the early diverging eukaryotic lineages such as a diplomonad, a microsporidian, kinetoplastids etc., have been omitted and only the green plants, the amoeba *Acanthamoeba castellanii*, the ascomycetous fungi, the metazoa, the chromophytes *Ochromonas danica*, *Skeletonema costatum*, the oomycete *Achlya bisexualis*, the dinoflagellate *Prorocentrum micans*, ciliates and sporozoa are included. The slime mold *Dictyostelium discoideum* was taken as an outgroup in order to root the tree. As stated in the introduction Field et al. [6] have published an evolutionary tree based on partial srRNA sequences of 22 metazoa species, in which the Radiata and Bilateria seem to have originated from a different ancestor [6]. In contrast, Lake [17] has analyzed the same partial srRNA data by his evolutionary parsimony criterion and found the two groups to share a common origin. In the tree of Fig. 2 too, the sea anemone and the Bilateria form together a monophyletic group, as expected on the basis of conventional data. The sea anemone diverges first among the metazoa. The dissimilarity between the sea anemone and Bilateria is appreciably higher than the dissimilarities within the Bilateria. The reason why Field et al. [6] have found the Radiata to be closer related to the fungi and plants than to the other metazoa, is probably a consequence of artifacts of the treeing method, as already suggested by other authors [11–13].

Depending on the organisms included in the tree, we also obtained different results with respect to the evolutionary status of the metazoa. Trees comprising all hitherto published complete eukaryotic srRNA sequences, i.e. all organisms included in the tree of Fig. 2 plus those of early diverging species such as a diplomonad, a microsporidian, kinetoplastids, an euglenoid, the amoeba *Naegleria gruberi* and the slime mold *Physarum polycephalum* were constructed. In this case, there was a rearrangement of the clusters (green plants, ascomycetes, ciliates plus sporozoa, metazoa, etc.), discernible in Fig. 2. This is not very surprising in view of the fact that dissimilarity values separating the branching points leading to these clusters are small. In these more complete trees, the sea anemone appeared to be more related to the plants and fungi than to other multicellular animals. When in the tree of Fig. 2 the outgroup species *Dictyostelium discoideum* is replaced by an earlier diverging organism such as the kinetoplastid *Trypanosoma brucei*, the branching order is altered again, with the metazoa forming the earliest diverging cluster, but not comprising the sea anemone which forms the next diverging branch. When both *Dictyostelium discoideum* and *Trypanosoma brucei* are included in the tree construction, the same topology as shown in Fig. 2 is obtained.

The distortion of the local tree topology as a consequence of the incorporation of early diverging lineages has also been described by Olsen [25]. Since the incorporation of such lineages can severely influence the branching order among later diverging lineages, care should be exerted in the selection of species for the construction of trees by a matrix method and the effect of changing the composition of the sets should be evaluated.

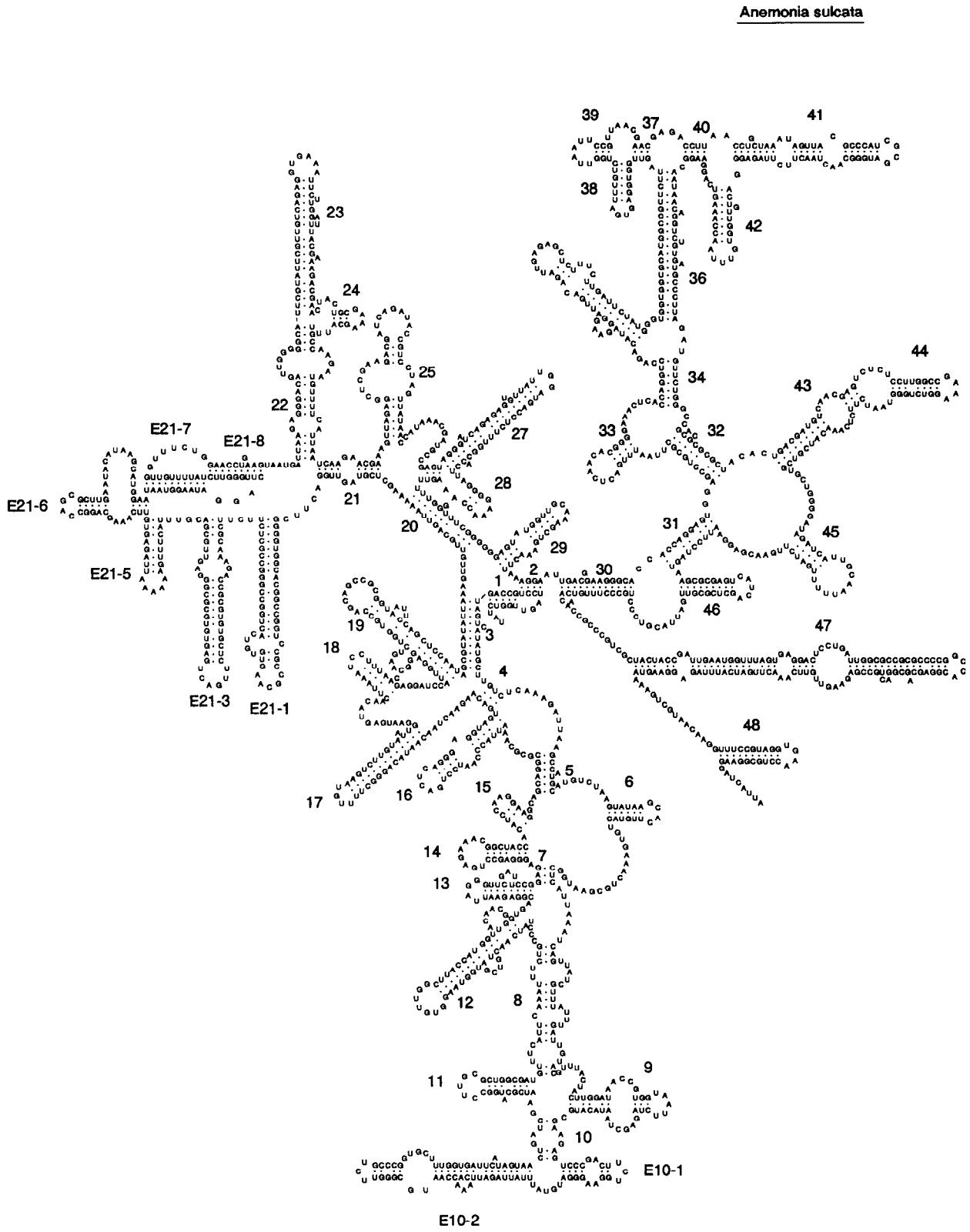

Fig. 1. Secondary structure model of the srRNA of *Anemonia sulcata*. The helix numbering is as described in the text. The numbering in the E21-n region is not continuous because helices E21-2 and E21-4, which appear only in a limited number of species, are lacking in *Anemonia sulcata*.

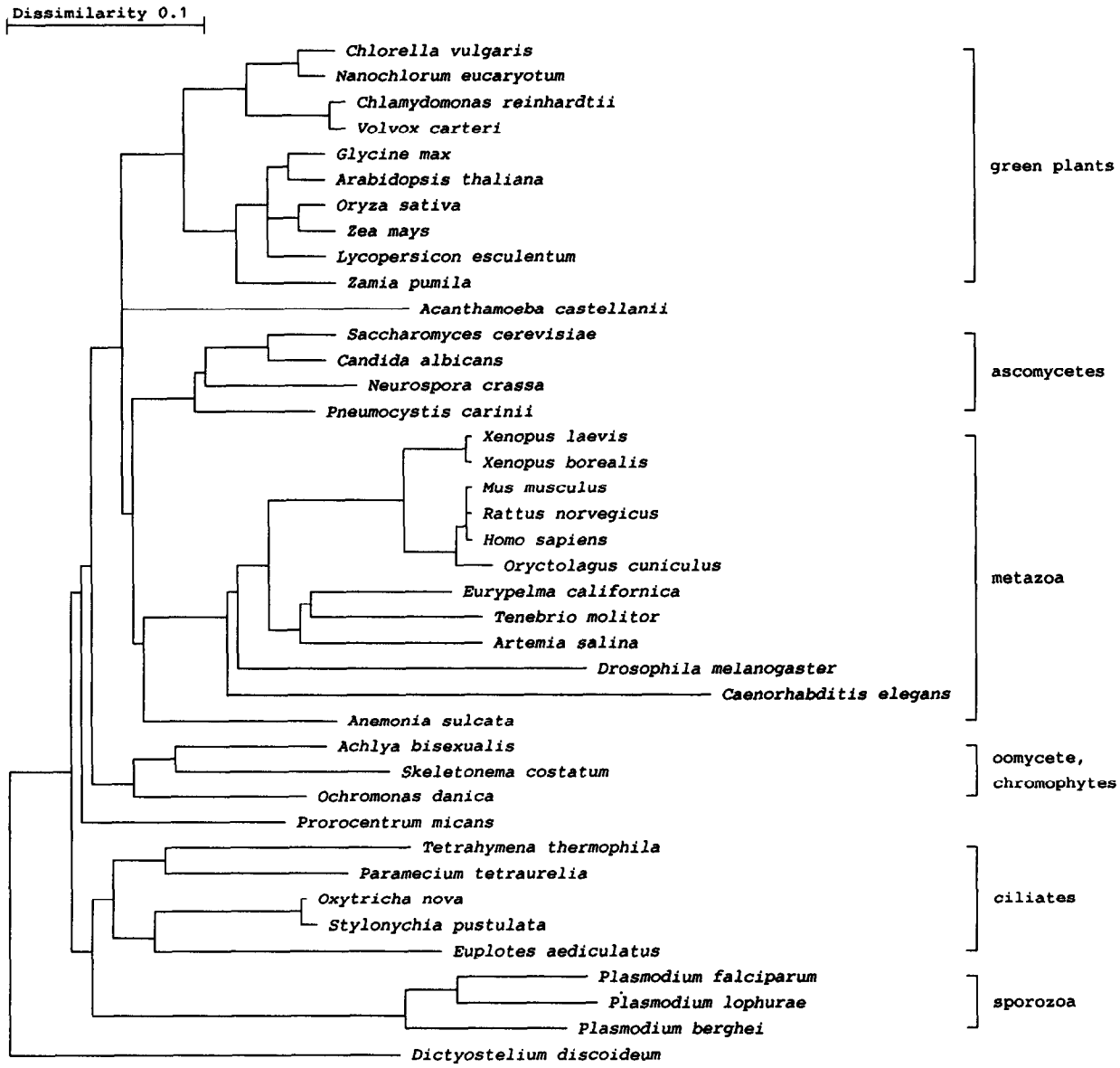

Fig. 2. Evolutionary tree based on 40 complete eukaryotic srRNA sequences. Taxon designations are placed to the right of the corresponding cluster. The dissimilarity between two species is obtained by summing the lengths of the connecting branches, measured along the horizontal axis and using the scale at the top. Branches originating at levels differing less than 0.0025 in dissimilarity are drawn as diverging simultaneously.

Acknowledgements: Our research was supported by Grant Bio/03 of the Belgian National Incentive Programme on Fundamental Research in Life Sciences, and by the N.F.W.O. Yves Van de Peer and Jean-Marc Neefs hold an I.W.O.N.L. scholarship.

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