Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8750–8753, September 1992
Evolution

Class-level relationships in the phylum Cnidaria: Evidence from mitochondrial genome structure

(Anthozoa/Hydrozoa/phylogeny/Scyphozoa)

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Communicated by Frank H. Ruddle, June 12, 1992

ABSTRACT **The phylogenetic relationships of the Recent cnidarian classes remain one of the classic problems in invertebrate zoology. We survey the structure of the mitochondrial genome in representatives of the four extant cnidarian classes and in the phylum Ctenophora. We find that all anthozoan species tested possess mtDNA in the form of circular molecules, whereas all scyphozoan, cubozoan, and hydrozoan species tested display mtDNA in the form of linear molecules. Because ctenophore and all other known metazoan mtDNA is circular, the shared occurrence of linear mtDNA in three of the four cnidarian classes suggests a basal position for the Anthozoa within the phylum.**

The sister-group relationships of the cnidarian classes have been debated for over a century (1, 2) and are of particular significance for two reasons. (*i*) The Cnidaria lie close to the base of the metazoan lineage. Recognition of the most primitive class bears directly on theories regarding the radiation of the Eumetazoa and the origin of the coelom, and on the assessment of the primitive axis of symmetry in the Metazoa (3–5). (*ii*) The life cycle of three of the cnidarian classes includes an adult stage, the pelagic medusa, which is radically different from the benthic adult of the fourth class. Recognition of the basal class thus bears on whether the medusa is a morphological and ecological innovation or the original body plan of the phylum (6, 7).

The debate over cnidarian phylogeny has been limited by a paucity of characters that are independent of life-cycle variation. Indeed, the degree of disagreement is such that scientists in the United States are routinely trained in the tradition that the class Hydrozoa is primitive (8), whereas European scientists are trained to regard the class Anthozoa or Scyphozoa as primitive (9).

Molecular characters have proved especially useful in other cases where classical approaches to phylogenetic reconstruction have failed to distinguish clearly between competing hypotheses. Large-scale structural alternations, such as inversions documented in chloroplast DNA (10), have proven particularly useful characters for phylogenetic reconstruction. A structural modification of the cnidarian mitochondrial genome has been documented by Warrior and Gall (11), who demonstrated that some cnidarians possess a linear mitochondrial genome. We have undertaken a survey of 42 cnidarian species to determine the phyletic distribution of the two conformations of the molecule. A ctenophore species was also examined, as the Ctenophora have been argued to be a sister group to the Cnidaria (3, 8, 12). We suggest that presence of linear mtDNA is a synapomorphy that supports a basal position for the Anthozoa.

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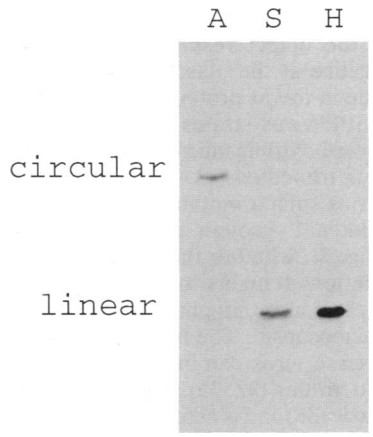

FIG. 1. Autoradiogram showing mtDNA from the anthozoan *Condylactis gigantea* (lane A), the scyphozoan *Cassiopea* sp. (lane S), and the hydrozoan *Hydra oligactis* (lane H). DNA in lane A is circular, whereas DNA in lanes S and H is linear.

MATERIALS AND METHODS

mtDNA structure was determined by its electrophoretic migration relative to linear and circular standards on 0.6%, 0.9%, and 1.2% agarose gels containing ethidium bromide at 0.5 μg/ml and 0.9% agarose gels without ethidium bromide. Whole genomic DNA was extracted as described in Shure *et al.* (13), without banding on a CsCl gradient. DNA electrophoresis and Southern blotting conditions were according to published procedures (14). Restriction-digested λ DNA served as a linear control, and purified manatee (*Trichechus menatus*) mtDNA served as a circular control.

mtDNA was visualized by hybridization with probes from the gene encoding mitochondrial large ribosomal subunit rRNA (rDNA). Hydrozoan mtDNA was visualized by using a 3.15-kilobase (kb) fragment cloned from *Hydra vulgaris* known to contain the entire large ribosomal subunit gene (11). rDNA probes to visualize mtDNA for the remaining classes were obtained as PCR amplification products. Primers were chosen based on regions of mitochondrial rDNA sequence similarity between the hydrozoan *Hydra vulgaris* and the anthozoan *Metridium senile*. Anthozoan and ctenophore probes were amplified by using primer 1, 5′-TCGACTGTTTACCAAAAACATAGC, and primer 2, 5′-ACGGAATGAACTCAAATCATGTAAG, which flank 634-base-pair (bp) *Hydra vulgaris* and 758-bp in *Metridium senile*. Scypho-

Abbreviations: TEM, transmission electron microscopy; rDNA, rRNA-encoding DNA.

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zoan and cubozoan probes were obtained with primer 1 and primer 3, 5′ GTCGCCCCAACTAAACTACCAAACTT, which flank 342 bp in *Hydra vulgaris* and 444 bp in *Metridium senile*. Probes were amplified from the following species: anthozoans *Renilla mulleri* and *Astrangia asteriaformes*, scyphozoan *Chrysaora quinquecirrha*, cubozoan *Carybdea marsupialis*, and ctenophore *Mnemiopsis mccradyi*. Sequencing of anthozoan, scyphozoan, and cubozoan PCR products verified that amplified products were mitochondrial rDNA fragments. Probes were labeled with [^{32}P]dATP and random oligonucleotide primers according to published procedures (15).

Table 1. mtDNA conformation in the phyla Ctenophora and Cnidaria

Taxon	Species	mtDNA structure	
		Shape	Size, kb
Phylum Ctenophora			
Class Tentaculata			
Lobata	*Mnemiopsis mccradyi*	Circular	ND
Phylum Cnidaria			
Class Anthozoa			
Stolonifera	*Clavularia* sp.	Circular	ND
Gorgonacea	*Leptogorgia virgulata*	Circular	ND
	Lophogorgia sp.	Circular	ND
	Eugorgia sp.	Circular	ND
Pennatulacea	*Renilla mulleri*	Circular	ND
Zoanthidea	*Zoanthus solanderi*	Circular	ND
Actiniaria	*Calliactis tricolor*	Circular	ND
	Condylactis gigantea	Circular	ND
	Haliplanella luciae	Circular	ND
	Haloclava producta	Circular	ND
	Metridium senile	Circular	ND
	Nematostella vectensis	Circular	ND
Scleractinia	*Astrangia asteriaformes*	Circular	ND
	Phyllangia sp.	Circular	ND
	Siderastrea sp.	Circular	ND
Corallimorpharia	*Corynactis californica*	Circular	ND
Ceriantharia	*Ceriantheopsis americana*	Circular	ND
Class Cubozoa			
Carybdeida	*Carybdea marsupialis*	Linear	16
Class Scyphozoa			
Stauromedusae	*Craterolophus convolvulus*	Linear	18
	Manania auricula	Linear	18
Semaeostomeae	*Aurelia aurita*	Linear	18
	Cyanea capillata	Linear	17
Rhizostomeae	*Cassiopea* sp.	Linear	17
Class Hydrozoa			
Trachymedusae	*Liriope* sp.	Linear	18
Leptomedusae	*Eirene viridula*	Linear	16
	Obelia dichotoma	Linear	16
	Sertularia sp.	Linear	17
Anthomedusae	*Cordylophora lacustris*	Linear	15
	Eleutheria dichotoma	Linear	16
	Eudendrium carneum	Linear	16
	*Hydra vulgaris**	Linear doublet	8
	*Hydra littoralis**	Linear doublet	8
	Hydra magnipapillata	Linear doublet	8
	Hydra japonica	Linear doublet	8
	Hydra attenuata	Linear doublet	8
	Hydra fusca	Linear	16
	Hydra robusta	Linear	16
	Hydra oligactis	Linear	16
	Hydra viridissima	Linear	16
	Hydra circumcincta	Linear	16
	Hydra utahensis	Linear	16
	Hydra hymanae	Linear	14
	Hydractinia symbiolongicarpus	Linear	16
	Millepora conplanata	Linear	17
	Podocoryne carnea	Linear	16
	Tubularia sp.	Linear	16
Limnomedusae	*Gonionemus vertens*	Linear	18
Siphonophora	*Physalia physalis*	Linear	16

ND, no data.
*These data are reported in ref. 11.

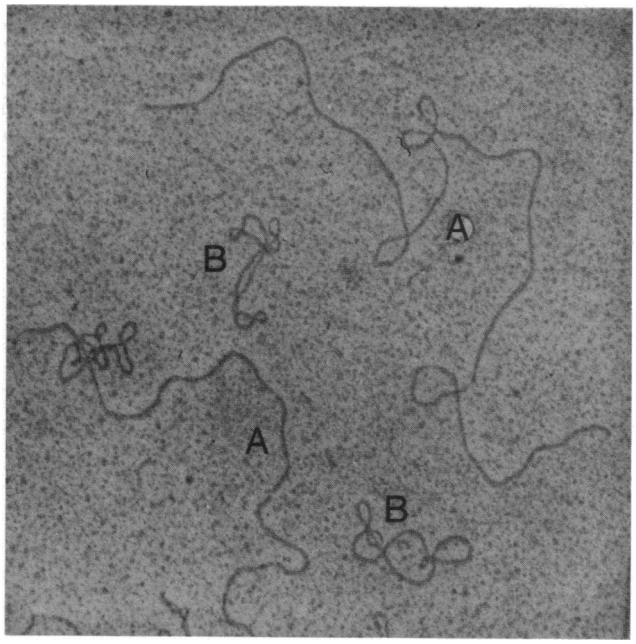

FIG. 2. Transmission electron micrograph of linear mitochondrial genome of the hydrozoan *Physalia physalis* (A) and the circular control (plasmid pBR322) (B).

The results obtained by these procedures were confirmed by electrophoresis of restriction-digested mtDNA molecules and by transmission electron microscopy (TEM). Restriction digestion was done to distinguish linear molecules with specific ends from linear molecules produced by random breakage. DNA of three species showing linear mtDNA (the hydrozoan *Hydractinia symbiolongicarpus* and the scyphozoans *Cassiopea* sp. and *Manania auricula*) was digested. Enzymes recognizing 6-bp sites (*Bam*HI, *Cla* I, *Eco*RI, and *Hin*dIII) were used to produce fragments of several kb in size. As a control, a secondarily linearized mtDNA band present in DNA of the anthozoan *Condylactus gigantea* was excised from a gel and digested. Restriction-digested DNA was hybridized as described above in a Southern analysis.

For TEM, mtDNA-enriched DNA was obtained for two species with linear mtDNA (*Hydractina symbiolongicarpus* and *Physalia physalis*) by using the method for rapid isolation of cytoplasmic nucleic acids described in Lansman *et al.* (16). TEM images were produced according to the spreading procedure of Kleinschmidt (17), with the spreading step using a solution of 0.1 M Tris/0.01 M EDTA/0.01% cytochrome *c*/30% (vol/vol) formamide/DNA at 2.5 μg/ml. The plasmid pBR322 (≈4.4 kb) was used as a circular control. Viewing was done on a Ziess EM-10 TEM.

RESULTS

The electrophoretic results are clear (Fig. 1, Table 1). All Hydrozoa, Cubozoa, and Scyphozoa studied possess linear mtDNA, whereas the anthozoan and the ctenophore species studied possess circular mtDNA. As previously observed by Warrior and Gall (11), we find that the mtDNA of some *Hydra* species are characterized by two ≈8-kb linear molecules.

A faint circular mtDNA band occurred in a small number of cases ($n = 6$) of species showing an intense linear mtDNA band. Restriction digests showed that mtDNA in these cases consisted of molecules with specific ends rather than broken circular molecules. Digestion of mtDNA from the hydrozoan *Hydractina symbiolongicarpus* and the scyphozoans *Cassiopea* sp. and *Manania auricula* yielded only fragments of discrete sizes, as expected for linear molecules with specific ends (18). Digestion of secondarily linearized mtDNA of the anthozoan *Condylactis gigantea* yielded a continuous range of sizes as expected from a circular molecule subjected to random breakage. Results from electrophoresis were also confirmed by transmission electron micrographs of the mtDNA of the hydrozoans *Hydractina symbiolongicarpus* and *Physalia physalis*. TEM showed linear molecules ≈16 kb in size and no circular molecules larger than the 4.4-kb control (Fig. 2).

DISCUSSION

The distribution of linear mtDNA within the Cnidaria is of phylogenetic significance if the polarity of the character can be determined by comparison with an appropriate outgroup. The metazoan phylum Ctenophora is generally recognized as branching near the base of the metazoa (19) and has been proposed as a sister group to the phylum Cnidaria (3, 8). Our results (Table 1) show that the Ctenophora, like all other Eumetazoa phyla studied (18, 20, 21), possesses circular mtDNA. Although mtDNA genome structure is a single character, the complete uniformity of this character both within each of the cnidarian classes and within the rest of the metazoa suggests that it is a highly conservative trait unlikely to show homoplasy. Such characters are particularly desirable for resolving ancient branching patterns (10).

Three views regarding the relationships of the cnidarian classes have been prominent—namely, that the Hydrozoa (6, 22, 23), Scyphozoa (9), or Anthozoa (24–28) is the most primitive class. The presence of an apparently derived structural alteration in the mtDNA of the Hydrozoa, Scyphozoa, and Cubozoa supports the hypothesis that these three classes form a monophyletic group relative to the Anthozoa. Only one nucleotide-sequence phylogeny has been published that bears on the question of cnidarian relationships. This study (29) presents a phylogeny in which the coelenterates with circular DNA, representing the Ctenophora and the Anthozoa, are sister taxa relative to two hydrozoan species. Although the study lacks any representatives of the Scyphozoa or Cubozoa and is not consistent with most hypotheses of coelenterate evolution, its results are entirely consistent with the observed distribution of circular and linear mtDNA.

The century-old debate over whether the polyp or the medusa came first has traditionally been addressed in the context of the relative placement of the four Recent cnidarian classes (8, 9, 22–25). Our results support the polyp-first hypothesis and bear on a number of classical problems:

(*i*) Evolution of cnidarian life cycles: The medusa-first school holds that the phylum had a holopelagic origin (8, 22, 23). Adherents to this view regard the pelagic trachymedusoid form as primitive and the benthic polyp as secondarily derived. Our results support the alternative hypothesis that the cnidarian ancestor had a benthic stage of the life cycle (9, 25, 27, 28).

(*ii*) Origin of the coelom: Theories involving the enterocoelic origin of the coelom (26, 27) and theories of the radiation of the Eumetazoa require a primitive bilateral polypoid form such as occurs in the Anthozoa [e.g., the bilaterogastrea theory of Jagersten (27) and the turbellian theory of Hadzi (28)]. Our results provide necessary, but certainly not sufficient, support for these claims.

(*iii*) Polarity of developmental diversification: The Anthozoa are far less diverse than the Hydrozoa in their cnidae (30) and are less diverse than all three other classes in their life cycles (8, 9) and modes of cleavage and gastrulation (31, 32). Our findings suggest that the trend in cnidarian evolution has been toward diversification of these features, rather than secondary reduction of developmental diversity.

We thank N. Blackstone, S. Dellaporta, M. Dick, J. Gatesy, W. Hartman, C. Mills, and A. Seilacher for comments on the manuscript; B. Piekos for technical assistance; D. Harvell, F. Henne-

mann, G. Jarms, G. Matsumoto, C. Mills, M. Moore, and T. Sugiyama for assistance in obtaining specimens; D. Wolstenholme and R. Warrior for biological reagents; and the National Science Foundation (BSR-8805961, OCE-9018396, DCB-9018003), Deutsche Forschungsgemeinschaft (III-02-Schi-277/1-2), and Office of Naval Research (N00014-89-J-3046) for financial support.

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