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Genetics

Atypical mitochondrial DNA from the deep-sea scallop *Placopecten magellanicus*

(size polymorphism/heteroplasmy/restriction mapping)

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ABSTRACT **The mitochondrial DNA of most metazoan animals is highly conserved in size, averaging about 17 kilobase pairs (kbp). The mitochondrial DNA from the deep-sea scallop *Placopecten magellanicus*, in contrast, has been found to be approximately 34 kbp long. It is also highly variable in size from individual to individual and is unusual in the extent of its size variation. Mitochondrial DNAs from individuals collected at the same site differ by as much as 7 kbp. The size variation is due largely to differences in the number of copies of a tandemly repeated 1.2-kbp element.**

Measurements of the size of mitochondrial DNA (mtDNA) from many phylogenetically diverse sources have led to the view that the molecule is remarkably conserved in size within the metazoa, pared down to near the limit required to encode the proteins, transfer RNAs, and ribosomal RNAs in its genome (1). For example, restriction mapping and sequencing studies yield size ranges of 15.7 to 19.5 kilobase pairs (kbp) for the Drosophilids (2), 15.7 kbp for sea urchins (3), 16.5 kbp for humans (4), 17.4 kbp for the blue mussel (5), and 17.5 kbp for 11 species of *Cnemidophorus* lizards (6). The molecule has been called "an extreme example of genetic economy" (7), in part because sequencing studies show a lack of significant noncoding regions, both 5′ and 3′, flanking mitochondrial genes. Many of the genes are butt-joined and a few overlap by a small number of base pairs (8). The economy of size observed has been maintained through more than 600 million years of evolutionary divergence and is therefore regarded as universally stable (7).

We report here on an atypically large and variable mtDNA, that of the commercial sea scallop *Placopecten magellanicus*. In a survey of more than 250 animals from several populations, we found that the scallop's mtDNA is more than 30 kbp long (approximately twice the expected length) and is strikingly variable in size from individual to individual. Lengths observed to date range from a minimum of approximately 32.1 kbp to a maximum of approximately 39.3 kbp.

MATERIALS AND METHODS

Scallop mtDNA. Live scallops were obtained from the Department of Fisheries and Oceans, Canada. Mitochondria were isolated from individual adductor muscles essentially as described (9), except that homogenization was carried out in 500 mM sucrose/150 mM KCl/2 mM EDTA (pH 7.4) and the clarified homogenate was treated with protease type XXIII (Sigma) at 10 mg/ml for 1–2 hr at 37°C to reduce viscosity. After collection of mitochondria from a sucrose step gradient (1 M and 1.5 M), DNA was purified and digestions were carried out as described (10). Yields have been on the order of 1 μg of mtDNA per 3 g (wet weight) of tissue. CsCl/ethidium bromide gradients for isolation of covalently closed circular DNA were as described (10).

Cloned *Drosophila yakuba* mtDNA for Hybridization. Fragments of *D. yakuba* mtDNA cloned in plasmid pBR322 were kindly provided by D. R. Wolstenholme (University of Utah). Plasmid pdyHB has been sequenced and found to contain genes encoding a subunit of the respiratory-chain NADH dehydrogenase (ND 2, formerly URF 2) (partial), cytochrome oxidase subunit I, cytochrome oxidase subunit II, ATPase 8, ATPase 6, and cytochrome oxidase subunit III (partial) (11, 12). Plasmid pdyHC has been sequenced and contains the ND 2 gene (partial), the small rRNA gene, and three tRNA genes (13, 14).

Hybridizations to Cloned *D. yakuba* mtDNA. After electrophoresis, *Eco*RI-digested mtDNA was transferred from a 0.8% agarose gel to nitrocellulose membrane (Schleicher & Schuell). ^{32}P-labeled probes were obtained by nick-translation (10) of whole plasmids containing inserts of *D. yakuba* mtDNA. Hybridization and washing were done under low-stringency conditions: prehybridization was in 5× standard saline citrate/0.1% $NaDodSO_4$/5× Denhardt's solution containing DNA carrier at 250 μg/ml, with incubation at 50°C for 3 hr; hybridization was as above except that the membranes were incubated overnight; washing was done twice in 0.2× standard saline citrate/0.5% $NaDodSO_4$ at 50°C for 1 hr. (Standard saline citrate is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.)

Determining the Size of Scallop mtDNA. Digested DNA fragments were separated in 1% agarose gels, stained with ethidium bromide, and photographed as described (10). Electron microscopy of purified mtDNA molecules spread under standard conditions was done as described (15). Intact phage λ DNA was used for calibration of magnification. Mapping of restriction endonuclease sites was accomplished by analysis of complete single and double restriction enzyme digestions.

DNase Treatment of Intact Mitochondria. The mitochondrial fraction collected after banding in a sucrose step gradient was resuspended in 1 ml of 500 mM sucrose/150 mM KCl/2 mM EDTA, pH 7.4. *Eco*RI-cut phage λ size markers were added to the intact mitochondrial fraction and the sample was split into two equal aliquots. $MgCl_2$ (20 mM) and DNase (Worthington, 50 μg/ml) were added to one of the aliquots. Both were incubated 60 min on ice and swirled every 15 min. EDTA (50 mM) was added to both samples at the end of the incubation to inhibit further digestion. Mitochondria were lysed, and DNA was extracted, prepared

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Abbreviation: mtDNA, mitochondrial DNA.

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for digestion, and electrophoresed in agarose gels as described above. A Joyce–Loebl scanning microdensitometer was used to compare bands on a gel quantitatively by determining the relative intensity of ethidium bromide staining.

RESULTS

Unusually Large Size of the mtDNA. The evidence we present to document the size of the molecule is of two types: (*i*) multiple restriction digests from which an internally consistent circular map can be derived and (*ii*) contour measurement from electron micrographs of individual DNA molecules. Determinations of length by both methods agree well. Fig. 1*A* shows the patterns obtained by single digestions with *Eco*RI (lane 1), *Bam*HI (lane 2), and *Pst* I (lane 3) and by double digestion with *Eco*RI/*Bam*HI (lane 4), *Eco*RI/*Pst* I (lane 5), and *Bam*HI/*Pst* I (lane 6) of the mtDNA from a single individual. The sum of the fragment sizes from each digestion is consistently approximately 34 kbp. The data from fragments produced by enzyme cleavage were used to construct the map presented in Fig. 1*B*. Summation of small fragments to surmise the size of fragments expected from single digestions is consistent in all cases within experimental error. Electron micrographs were prepared as described in *Materials and Methods*, using purified mtDNA. The average size of the DNA was determined to be 34.8 ± 1.5 kbp (n = 12) by measurement of the contour length, a figure in good agreement with the estimated size of 33.6 kbp obtained for the mtDNA from the individual used for Fig. 1*A*.

Verification of the Mitochondrial Origin of the Purified DNA. The unexpectedly large size of the DNA purified from scallops prompted three experiments designed to verify its mitochondrial origin. First, the mitochondria from eight individuals were combined after isolation from sucrose gradients, and the DNA was extracted and centrifuged through a CsCl/ethidium bromide gradient. The band of higher density, corresponding to covalently closed circular DNA, was removed, digested, and run in an agarose gel. The four-band *Eco*RI pattern obtained after digestion was identical to that we have observed with other animals (as in Fig. 1*A*), demonstrating that the DNA we extract from scallops exists in the cell in a covalently closed circular form, as does mtDNA from other animals (16).

Hybridization using cloned DNA of known mitochondrial origin as probes of scallop DNA provided a second means of verifying the mitochondrial identity of the 34-kbp DNA. Fig. 2*A* shows the results of low-stringency hybridization of ^{32}P-labeled pdyHB from *D. yakuba* (lane 2) and pdyHC from *D. yakuba* (lane 3) to scallop DNA. Both clones contain several mtDNA genes. In lane 2, hybridization to the 2-kbp fragment is apparent (this band does not contain repeated DNA; see below), whereas in lane 3, there is hybridization to the two larger *Eco*RI fragments. The hybridization in lane 4 was done under identical conditions, using ^{32}P-labeled pBR322, the vehicle into which the *D. yakuba* fragments were cloned. No hybridization to scallop DNA is detectable, demonstrating that the homology detected in lanes 2 and 3 is due to the mtDNA inserts.

The third line of evidence showing the mitochondrial origin of the 34-kbp DNA is a demonstration that the 34-kbp DNA is sequestered from DNase digestion prior to extraction from the mitochondrial membrane. Isolated mitochondria were treated with DNase, and the DNA was extracted and run in a 1% agarose gel (Fig. 2*B*). *Eco*RI-cut DNA from phage λ was added to the mitochondria prior to DNase digestion, to document the activity of the DNase. The mitochondria giving rise to the DNA in lanes 1 and 2 were DNase-treated before DNA extraction; the mitochondria giving rise to the DNA in lanes 3 and 4 were not DNase-treated before DNA extraction. The DNA in lanes 2 and 4 was *Eco*RI-digested after extraction from mitochondria. Densitometric tracings of the bands in lanes 2 and 4 were used to quantify the effect of DNase on the scallop-derived versus the phage λ-derived DNA. Approximately 80% of the scallop-derived DNA was present in each of the four bands after DNase digestion,

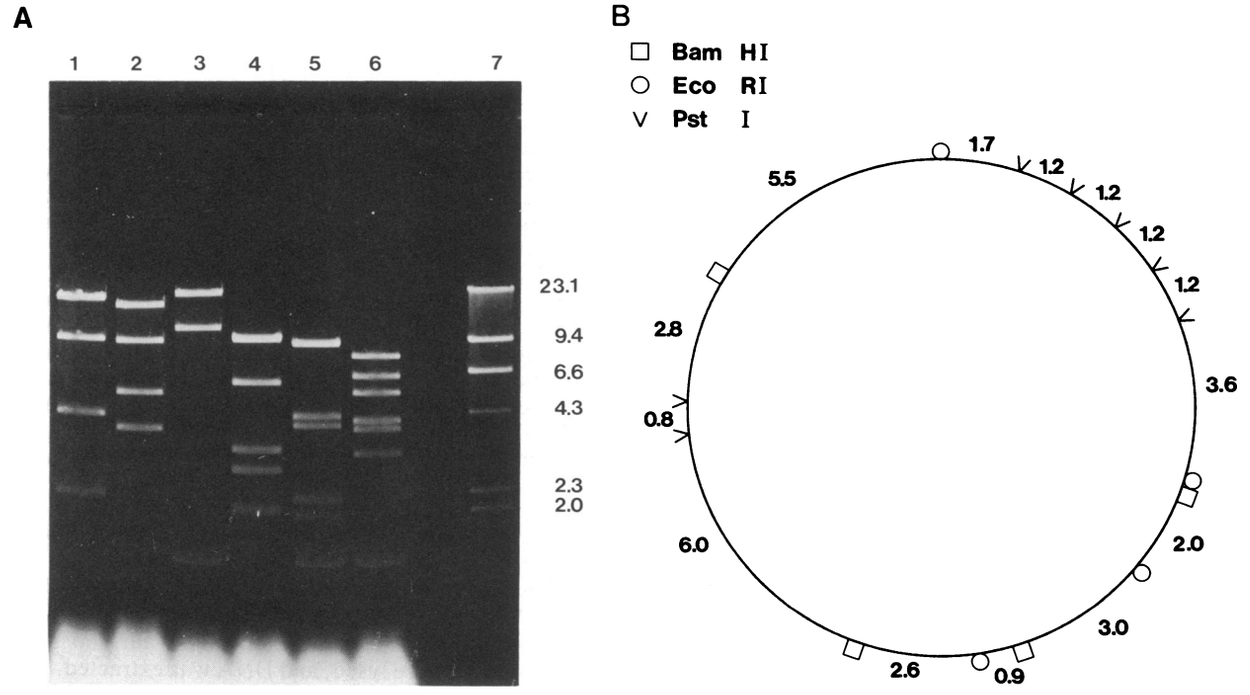

FIG. 1. Size of mtDNA of *P. magellanicus*. (*A*) Single and double restriction enzyme digestion patterns. Lanes: 1, *Eco*RI; 2, *Bam*HI; 3, *Pst* I; 4, *Eco*RI/*Bam*HI; 5, *Eco*RI/*Pst* I; 6, *Bam*HI/*Pst* I; 7, *Hin*dIII-digested phage λ DNA for size standards (markers in kbp at right). mtDNA recovered from a single animal was used for all digestions. (*B*) Restriction map derived from size data in *A*. Fragment sizes (kbp) are indicated.

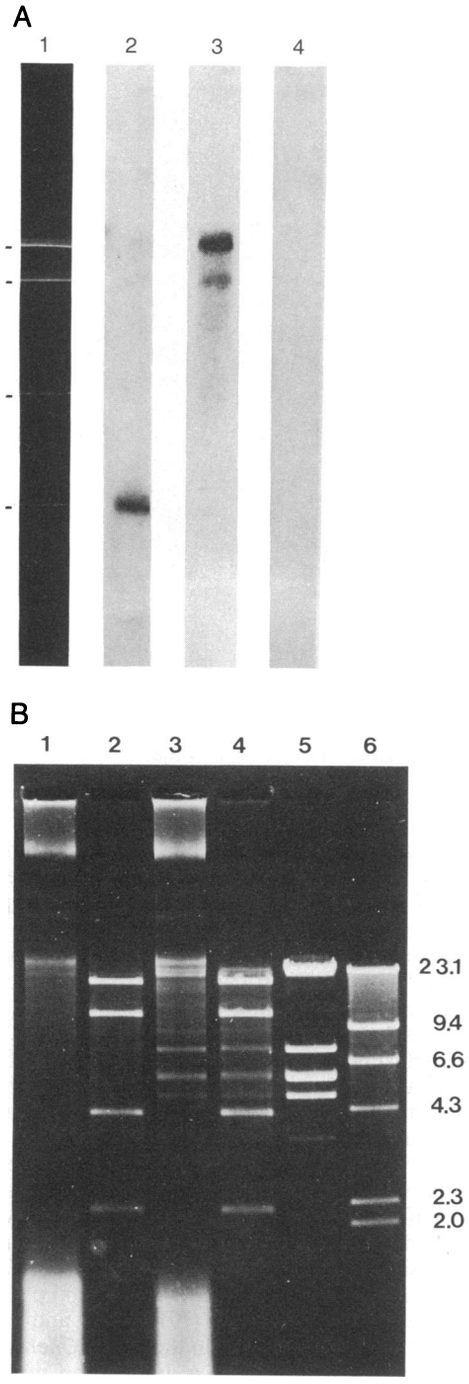

FIG. 2. (*A*) Hybridization of cloned mtDNA from *D. yakuba* to *Eco*RI-cut scallop DNA. Lane 1: ethidium bromide staining of the 0.8% agarose gel of *Eco*RI-cut scallop mtDNA prior to transfer to nitrocellulose. Lane 2: hybridization of *D. yakuba* 4.8-kbp insert encoding cytochrome oxidase subunits I–III, ATPase subunits 6 and 8, and several tRNA genes. Lane 3: hybridization of *D. yakuba* 2.3-kbp insert containing the small rRNA gene, the A+T-rich region, and several tRNA genes. Lane 4: hybridization of nick-translated pBR322 to mtDNA. Low-stringency hybridization conditions are described in *Materials and Methods*. (*B*) mtDNA-derived fragments are resistant to exogenous DNase before lysis of the mitochondrial membrane. Isolated mitochondria were mixed with *Eco*RI-cut phage λ DNA and treated as described in *Materials and Methods*. Lane 1: digestion with DNase only. Lane 2: digestion with both DNase and *Eco*RI. Lane 3: no digestion. Lane 4: digestion with *Eco*RI but not DNase; Lane 5: *Eco*RI-digested λ DNA. Lane 6: *Hin*dIII-digested phage λ DNA.

whereas less than 10% of the phage λ-derived DNA remained in each band. The majority of the scallop DNA is, therefore, present in a sequestered form within the cell.

High Frequency of mtDNA Length Variation from Individual to Individual. *P. magellanicus* mtDNA displays a remarkable frequency of length variation. Lanes 1–6 in Fig. 3*A* show *Eco*RI-restricted mtDNA from six different individuals collected from the same site. The largest DNA fragment and the two smallest fragments are constant in size from individual to individual. In contrast, five different sizes are apparent for the second largest fragment in each lane. The mtDNA of one individual (lane 1) has a total size of approximately 38.1 kbp, with a minimum of seven copies of the 1.2-kbp repeated element (see below). Another individual (lane 2) is heteroplasmic, containing two size classes of mtDNA: one is approximately 39.3 kbp, with at least eight copies of the repeated element, and the other is approximately 34.5 kbp, with at least four copies of the repeated element. In lanes 3 and 5, the mtDNA is approximately 34.5 kbp, with a minimum of four copies of the repeat. In lane 4, the mtDNA is approximately 33.3 kbp, with a minimum of three repeats; in lane 6, the mtDNA is approximately 36.9 kbp, with a minimum of six copies of the repeat.

Size Variation of Scallop mtDNA Is Due Largely to the Number of Copies of a Tandemly Repeated 1.2-kbp Element. Fig. 3*B* shows the mtDNA from two individuals [one heteroplasmic (lanes 2 and 4), the other homoplasmic (lanes 1 and 3)] digested with *Eco*RI (lanes 1 and 2) or *Pst* I (lanes 3 and 4). *Pst* I cuts within the repeated element, so that the variation in size of the mtDNA evident in lanes 1 and 2 disappears and is replaced by a variation in the intensity of staining (and therefore in the number of copies) of the fragment at the 1.2-kbp position after digestion with *Pst* I (arrow). Seven size classes differing by 1.2-kbp increments have been observed thus far in a survey of approximately 300 animals. The smallest contains at least two copies of the repeat; the largest contains at least eight copies of the repeat. (When the smallest observed mtDNA is digested with *Pst* I, a 1.2-kbp fragment is generated; since digestion of n repeats generates $n - 1$ fragments, this molecule must contain at least 2 copies of the repeated sequence.)

DISCUSSION

The question of genetic interaction of commercial scallop populations is of vital concern for management of the scallop harvest. Allozyme frequencies have been determined for the main populations around Nova Scotia (17), but the results did not permit discrimination between two possibilities: (*i*) that gene flow is large enough to replenish an overfished local stock or (*ii*) that gene flow is large enough to produce a genetically homogenizing effect but not large enough to compensate for overfishing. An analysis of scallop mtDNA was undertaken in the hope that it would prove to be a more sensitive tool for stock discrimination than enzyme polymorphism has been. Preliminary results suggest that stock differentiation may be difficult based on restriction analysis of mtDNA, but the unusual character of the mtDNA has resulted in an unexpected array of interesting biological problems.

Large Size of Scallop mtDNA. Scallop mtDNA departs from the generally accepted model of metazoan mtDNA (e.g., see ref. 18) in several respects. The major departure is in its large size, which is due in part to the presence of repetitive sequences. Even in the absence of the identified repetitive sequences, the molecule is nearly 2-fold larger than the majority of metozoan mtDNAs described to date. A 2-fold increase suggests the possibility of a duplication event as the origin of the size difference. Our data do not support duplication as the origin, for two reasons. First, there is no

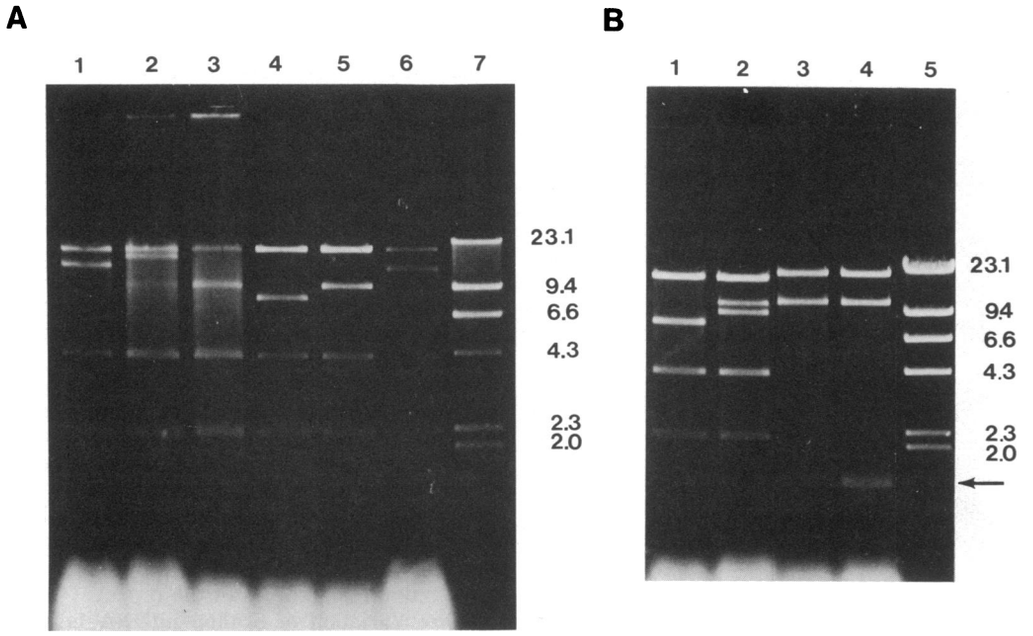

FIG. 3. Size polymorphism and the presence of a 1.2-kbp repeated element within *P. magellanicus* mtDNA. (*A*) Lanes 1–6: *Eco*RI-digested mtDNA from six different animals derived from a single population. Lane 7: *Hin*dIII-cut phage λ DNA for size standards. (*B*) Lanes 1 and 3: mtDNA from the same individual, digested with *Eco*RI or *Pst* I, respectively. Lanes 2 and 4: mtDNA from a second, heteroplasmic individual, digested with *Eco*RI or *Pst* I, respectively. Lane 5: *Hin*dIII-cut phage λ DNA for size standards. The position of the 1.2-kbp band is indicated with an arrow.

symmetry of restriction sites about the molecule. Second, the hybridization of a *D. yakuba* mtDNA fragment encoding cytochrome oxidase subunit I (as well as several other less highly conserved genes) is to a single band of scallop mtDNA. This would be improbable (but not impossible) in a duplicated genome that had undergone little sequence divergence. However, nucleotide divergence would rapidly obscure evidence of a duplication at this level of detection. The cloned *D. yakuba* fragment containing the gene for cytochrome oxidase subunit I is 4.8 kbp in length but hybridizes only to the 2-kbp *Eco*RI fragment from the scallop. It cannot be determined which regions of the two mtDNAs are homologous enough to permit hybridization until sequence data are available from the scallop. However, it is likely that hybridization is due only to a relatively small homologous portion of the 4.8-kbp cloned fragment, probably that which encodes the highly conserved region of cytochrome oxidase subunit I (7). More sensitive methods will be necessary to assess the possibility of a duplication as the origin of the large size. It has been reported (19) that the mtDNA of *Romanomeris culicivorax*, a parasitic nematode, is 26 kbp long. A comparison of the nature of the "extraneous" DNA in these two organisms will be both interesting and informative.

Frequency of Variation. Variation in the number of copies of the 1.2-kbp repeated element generates variability within the population, both in the range of sizes observed and in the frequency with which each size class is observed. Large variation in mtDNA size between individuals of the same species is relatively rare. No variation in length was reported among 432 small mammals of three species surveyed (20–22), or among 193 bluegill sunfish (23). Differences in size have been reported in population surveys of bowfin fish and tree frogs (24), in a nematode (19), in crickets (25), and in several species of lizard (26, 27). The variant animals in these populations appear to be relatively uncommon, the most common type appearing in at least 80% of the population. In the scallop, the frequency of variation is high. Among nearly 300 animals surveyed to date, seven size classes have been observed. The most common type, which contains a minimum of four copies of the 1.2-kbp repeat, is present in just over 50% of the animals (K. Fuller and E.Z., unpublished results).

Heteroplasmy. Heteroplasmy (differences among mtDNA molecules of an individual) has now been documented in a number of species, including domestic cows (28), crickets (25), *Drosophila mauritiana* (29), and lizards (26). Scallops with heteroplasmic mitochondria are present at a frequency of approximately 7%, as judged by the relatively insensitive method of ethidium bromide staining (K. Fuller and E.Z., unpublished results).

Range of Size Variation. Extensive within-species size variation of mtDNA has been documented in trypanosomes and higher plants (29). In contrast, most of the variations observed to date in metazoa have been relatively small. The variation in size reported for two species of *Cnemidophorus* lizard is 0.37 kbp (26); for *D. mauritiana*, 0.5 kbp (30); for *R. culicivorax*, approximately 1 kbp (19). Moritz and Brown (27) reported a size range of 6.2 kbp in the lizard *Cnemidophorus exsanguis*, the result in part of a duplication that includes both regulatory and structural gene sequences. In the scallop, we observed differences of more than 7 kbp between the largest (approximately 39.3 kbp) and smallest (approximately 32.1 kbp) mtDNAs isolated, representing a difference of six copies of the 1.2-kbp element.

As reports of anomalous metazoan mtDNAs continue to accumulate, our view of which features are functionally constrained or which are flexible and subject to evolutionary divergence will be modified. Analysis of the unusual features being catalogued will lead to a better understanding of the mechanisms underlying metazoan mtDNA evolution.

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