Restriction-Site Heteroplasmy in Anchovy (Engraulis encrasicolus) Indicates Incidental Biparental Inheritance of Mitochondrial DNA

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Presence in the same individual of two highly diverged types of mitochondrial DNA (mtDNA) implies either that the two types accumulated mutational differences while coexisting in the same female lineage or that two independently diverged lineages anastomosed through biparental transmission. Cleavage-site mtDNA analysis in anchovies revealed 3 heteroplasmic individuals among 435 examined. All three contained the same two types of molecules that differed at 7 cleavage sites in a total of 26 surveyed by seven restriction endonucleases. Estimates of the time of heteroplasmy persistence in other species are much shorter than the time needed for this level of divergence. No heteroplasmic individuals were found for mtDNA molecules differing by fewer cleavage sites. This also argues against the hypothesis of gradual divergence in a single lineage, as it would imply selective removal of much more frequently occurring lower-level heteroplasmies. The two types of molecules found in heteroplasmy were the most common in the population, as expected from the hypothesis of paternal leakage. We conclude that the heteroplasmy that we have observed resulted from biparental mtDNA inheritance in anchovies.

Introduction

For many years the prevailing view about animal mitochondrial DNA (mtDNA) was that it is strictly maternally inherited (e.g., see Avise and Lansman 1983). Experiments designed to detect low levels of “paternal leakage” failed to produce positive evidence (Hayashi et al. 1978; Lansman et al. 1983; Gyllensten et al. 1985). However, recent observations of cases of heteroplasmy (co-occurrence of more than one type of mtDNA molecule in the same individual) for highly diverged molecules have suggested the possibility of paternal contribution (Satta et al. 1988; Hoeh et al. 1991). Direct experimental evidence for this came from Drosophila (Kondo et al. 1990) and mice (Gyllensten et al. 1991) hybrids. Here we present evidence for biparental mtDNA inheritance in a fish, the anchovy Engraulis encrasicolus. Our observations are similar to those of Hoeh et al. (1991), who suggested this type of inheritance in mussels.

Material and Methods

In the context of a study of population differentiation (Magoulas 1990), anchovies were collected from the Aegean and Ionian seas (on the eastern and western sides of the Greek peninsula). The fish were obtained from local fishing boats on landing.
were kept on ice until arrival in the laboratory, and then were frozen at -30°C until processed. Total DNA was extracted from the liver, according to the method of Harrison et al. (1985), and was digested with restriction endonucleases for 5–8 h, according to the manufacturer's instructions. The fragments were separated by electrophoresis on 0.7% agarose gels at 1.0 V/cm for 10–15 h and then were transferred to nylon membranes according to a method described by Maniatis et al. (1982, pp. 383–386). For routine scoring we employed three informative enzymes (i.e., endonucleases whose cleavage profiles were polymorphic in the sample), BglI, BglII, and HindIII. Cleavage profiles for these endonucleases were assayed using, as a probe, linearized sardine mtDNA, extracted according to the method of Lansman et al. (1981) and labeled with 32P by the random-priming technique of Feinberg and Vogelstein (1983). A selected sample of individuals (including the heteroplasmic individuals; see below) were examined for two more enzymes—BamHI and EcoRI—by using, as a probe, either cloned fragments of the mtDNA molecule of the American shad Alosa sapidissima or homologous (anchovy) mtDNA extracted according to the method of Lansman et al. (1981), and the probe was labeled with digoxigenin according to the instructions of the supplier (Boehringer and Mannheim catalog no. 1093657).

Results and Discussion

Four hundred thirty-five individuals were examined for the enzymes BglI and BglII, and a subset of 174 were examined for the enzyme HindIII (Magoulas 1990). Fourteen composite (three-enzyme) genotypes were observed, of which three—AAA, BBC, and BBB (where the first letter refers to the BglI cleavage profile, the second to BglII, and the third to HindIII)—were most common, accounting for 58%, 21%, and 16% of the total sample, respectively. In the whole sample of 435, 3 heteroplasmic individuals were found, all of which were A/B for BglI, A/B for BglII, and A/C for HindIII. In all three individuals (two of which are shown in fig. 1), the BglI A, BglII A, and HindIII A bands were stronger than the BglI B, BglII B, and HindIII C bands. From these observations we concluded that the three heteroplasmic individuals were of the type AAA/BBC, with the first genotype representing the majority of mtDNA molecules in each individual. The observation that in all three heteroplasmic individuals the two mtDNA molecules differed for all three enzymes that we employed suggested that these two molecules might be highly diverged and that they could produce different cleavage profiles if digested with additional enzymes. This turned out to be the case for two new enzymes—BamHI and EcoRI—that were found to be informative, among four examined for this purpose (AccI and EcoRV were found to be monomorphic for all animals tested). The results from the scoring of a randomly selected sample of 58 type AAA individuals and 31 type BBC individuals are shown in table 1. It follows from the table that the great majority of AAA animals have the composite five-enzyme pattern AAAAA and that the great majority of BBC animals have the pattern BBCBB. By the time the BamHI and EcoRI polymorphisms were observed, the DNA of one of the three heteroplasmic individuals was exhausted. The other two individuals were indeed found heteroplasmic for these two enzymes. Moreover, the bands of the EcoRI A and BamHI A profiles were stronger than those of the B profiles, in agreement with the hypothesis that the two mtDNA molecules contained by these individuals were of the type AAAAA and BBCBB (fig. 1).

The approximate relative amounts of the two genotypes in the heteroplasmic animals were estimated from the intensity of the discriminating bands by using the image-analysis program Optical Pattern Recognition System, version 1.08 (BioSonics).
The less frequent genotype (BBCBB) varied between 30% and 40%. This high percentage of the minor type argues against the possibility of accidental contamination of DNAs from different animals, as it would necessitate a substantial mixing of preparations during DNA extraction on three independent occasions, an event that can be excluded under our experimental conditions.

Genotypes AAAAA and BBCBB differ in 7 cleavage sites of a total of 23 revealed by these five enzymes (fig. 2). The sequence divergence between these two genotypes (when account also is taken of the two AccI and one EcoRV sites, for which we have seen no polymorphism) is estimated at 2.82% (Nei and Li 1979). Although this estimation is subject to a large stochastic error, because of the small number of cleavage sites and because these sites might not be representative of the whole molecule, it seems that this level of within-species divergence is among the highest values observed in marine fishes (see Gonzalez-Villaseñor and Powers 1990, table 3). Theoretically, there can be two explanations for the presence of two highly diverged molecules in the same individual. One explanation is that the molecules have accumulated nucleotide differences while in a state of uninterrupted heteroplasmy along a single lineage. The other explanation is that the two molecules diverged as separate lineages and were brought together in the same individual through an event of paternal leakage.

In our study, the evidence favors the second explanation. If the rate for mtDNA divergence is assumed to be 2%–4%/nucleotide site/Myr (Cann et al. 1987), the hypothesis that the AAAAA/BBCBB condition represents a stage in the evolutionary divergence of an uninterrupted heteroplasmic line requires that the heteroplasmacy originated 0.7–1.4 Mya and that it resisted stochastic assortment for an equal number of generations [anchovies have roughly one generation per year (e.g., see Blaxter and
Table 1
Five-Enzyme Genotypes of Two Sets of Individuals, One of Type AAA and the Other of Type BBC, for BglII, BglII, and HindIII

<table>
<thead>
<tr>
<th>BglI, BglII, and HindIII</th>
<th>BamHI</th>
<th>EcoRI</th>
<th>Composite Genotype</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>A</td>
<td>A</td>
<td>AAAAAA</td>
<td>55</td>
</tr>
<tr>
<td>AAA</td>
<td>C</td>
<td>A</td>
<td>AAACA</td>
<td>2</td>
</tr>
<tr>
<td>AAA</td>
<td>A</td>
<td>D</td>
<td>AAAAD</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>BBC</td>
<td>B</td>
<td>B</td>
<td>BBCBB</td>
<td>30</td>
</tr>
<tr>
<td>BBC</td>
<td>B</td>
<td>C</td>
<td>BBCBC</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>31</td>
</tr>
</tbody>
</table>

Hunter 1982, pp. 11 and 20; Ivanov and Beverton 1985, p. 37). Lower rates of mtDNA divergence will produce a longer estimate for the age of heteroplasmy. Even after allowance is made for a large standard error for the estimates of divergence, this appears to be too high a number of generations for the survival of heteroplasmy. In cows fixation of alternative genotypes occurred, apparently, in only five generations (from data in Hauswirth and Laipis 1982; Olivo et al. 1983), and in *Drosophila* (Solignac et al. 1984), as well as in crickets (Rand and Harrison 1986), the genotype-frequency variance among progeny of heteroplasmic females suggested that fixation is complete within a few hundred generations.

A second argument against the hypothesis that the AAAAA/BBCBB condition evolved along a single lineage is that we have observed no heteroplasmic animal whose molecules differed by fewer cleavage sites. The stochastic decay of heteroplasmy predicts that, in a random sample, heteroplasmic individuals whose two molecules differ by one change should outnumber those whose two molecules differ by two changes, and so on. This argument is independent of (a) assumptions regarding rates of divergence and (b) whether the polymorphic cleavage sites scored are representative of the rate of evolution for the entire molecule. Among the 174 animals scored for BglII, BglII, and HindIII, we found 11 genotypes that were one mutation step removed from one or the other of the three common genotypes (AAA, BBC, and BBB), yet all these rare genotypes were found in homoplasmy. This suggests that in anchovy, as in all known cases in animals, heteroplasmy is short lived, decaying to one or two homoplasmic lines before a second mutational event detectable by our assay could occur.

The hypothesis that highly diverged states of heteroplasmy are generated by events of biparental inheritance predicts that the frequency of heteroplasmic individuals of a certain type will depend not on the number of differences in their component molecules but on the frequencies of these molecules in the population. As noted before, genotypes AAA and BBC were the two most frequent in our sample. The observation that almost all animals of type AAA scored for BamHI and EcoRI were found to be of type AA and that those of type BBC were found to be of type BB (table 1) implies that AAAAA and BBCBB are the two most common genotypes in the population. Biparental inheritance is, therefore, expected to generate heteroplasmy for these genotypes more often than for any other combination.

The presence in the same geographic area of two highly diverged mtDNA types is not a common observation and may suggest a secondary contact of allopatrically evolved populations (Avise et al. 1987). One may speculate that, at the time of contact, anchovy populations had diverged to the point that the presumptive mechanism for
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FIG. 2.—Cleavage maps of the two anchovy mtDNA molecules found in heteroplasmy (types AAAAA and BBCBB). For ease of comparison the circular molecule (~17 kb) is shown linearized at an arbitrary BglI site. Cleavage sites indicated by asterisks are present in one type but not in the other. The maps were constructed using fragment-size data from single and double digests.

paternal mtDNA exclusion became inoperative in “interracial” crosses, so that paternal mtDNA inheritance became possible (or even common) among hybrids. This would agree with the suggestion that biparental inheritance is linked to hybrid crosses (Hoeh et al. 1991). Furthermore, one may assume that the dynamics of heteroplasmy decay were different in these hybrids. For example, the state of heteroplasmy could be maintained under selective pressure to balance out incompatibilities between interracial mtDNA and nuclear genes. Under these assumptions, the few cases of heteroplasmy that we have seen could be remnants of events that ceased to occur after the genetic (nuclear) homogenization of the population. The need to invoke such explanations may become more tempting as the reports of animal biparental mtDNA inheritance become commoner. We note, however, that sympatry of highly diverged mtDNA types in high frequencies provides the most favorable condition for the detection of paternal mtDNA inheritance. In a population containing only minorities of slightly differentiated mtDNA molecules, paternal leakage will only occasionally lead to heteroplasmy, and this will, most likely, be presumed to be due to mutation. Until we have clear evidence that paternal transmission is more frequent in mitochondrially diverse populations, appeals to more involved hypotheses may be premature.

Until very recently, claims of biparental inheritance of animal mtDNA that were based on observations of highly diverged states of heteroplasmy were considered insufficient evidence for the challenge of the established dogma of strictly maternal inheritance. Now that the dogma is overturned, these reports become important for a different reason—they help us form an idea of how widespread incidental paternal mtDNA inheritance is in the animal kingdom. By adding a fish species to the list that already contains a mollusk, an insect, and a mammal, this report adds to the growing suspicion that no animal group may be immune to this phenomenon.

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