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Mitochondrial DNA Size Variation Within Individual Crickets

Abstract. *The mitochondrial DNA's of two closely related cricket species (genus* Gryllus*) share a size polymorphism as evidenced by analysis of restriction fragment patterns. Moreover, 12 of 100 field-collected crickets are heteroplasmic, that is these individuals have more than one size class of mitochondrial DNA. No heteroplasmy for restriction site variation is observed. Intraindividual variation in cricket mitochondrial DNA provides a useful marker for studying the transmission genetics of mitochondrial DNA. Available data on patterns of variation in mothers and offspring suggest that random segregation of mitochondrial DNA variants does not occur rapidly in cricket germ-cell lineages.*

Studies of mitochondrial DNA (mtDNA) in animals have revealed high levels of intraspecific variation and substantial differentiation between closely related species (*1–3*). It has been suggested that mtDNA evolves at a rate significantly faster than that of single-copy nuclear DNA (*3, 4*). Despite the rapid accumulation of differences between lineages, there is generally little or no variation in populations of mtDNA molecules within individuals. Virtually all individuals of animal species examined thus far (mostly vertebrates and *Drosophila*) are homoplasmic; that is, they carry only one type of mtDNA (*2*). Heteroplasmy has been observed or inferred in only a very few species, and in most cases has been analyzed only in a single individual or maternal lineage (*2, 5, 6*).

Homoplasmy is, in part, a consequence of the maternal inheritance of mtDNA. However, the relation between degrees of variation within individuals and degrees of differentiation between individuals also depends on (i) rates of mutation (which generate diversity) and (ii) rates of fixation and loss within germ-cell lineages (which reduce diversity within but not between such lineages) (*5, 7, 8*). A high mutation rate coupled with rapid fixation or loss can lead to the rapid evolution of mtDNA and the absence of individual heteroplasmy (*5, 7, 8*). Under these conditions, the heteroplasmic state (which must intervene if maternal lineages are to become differentiated) exists only briefly.

Here we report the existence of heteroplasmy for mtDNA size variants in natural populations of field crickets. We show that two closely related cricket species share a mtDNA size polymorphism and that in both species there are a substantial number of heteroplasmic individuals. We suggest that the addition or deletion (or both) events responsible for the size variation occur with high frequency and that the size polymorphism has arisen independently in the two lineages. Direct observations of patterns of mtDNA variation in a heteroplasmic female and her offspring indicate that the heteroplasmic state is not transient but is transmitted from mother to offspring. Segregation of mtDNA variants in cricket germ-cell lineages does not occur rapidly.

Our initial reason for examining mtDNA variation in field crickets was to identify suitable genetic markers for characterizing patterns of genetic exchange across a hybrid zone between the closely related species *Gryllus pennsylvanicus* and *Gryllus firmus* (*9*). To do this, we collected crickets from a series of populations across the hybrid zone in Connecticut (*10*). Total DNA was isolated from field-collected individuals and digested with restriction endonucleases (*11*). The resulting digests were processed on 0.7 or 1.2 percent agarose gels and transferred to nitrocellulose filters (*12*). Pure mtDNA was isolated from a laboratory isofemale line of *Gryllus assimilis* (*13*). The purified mtDNA was nick-translated (*14*) to yield a ^{32}P-labeled probe that could be hybridized with the nitrocellulose filters. The mtDNA fragment patterns were then visualized by autoradiography.

In an initial survey of fragment patterns produced by 16 restriction endonucleases, four of the enzymes (Apa I, Hinc II, Hind III, Xba I) showed variation in fragment patterns that could be attributed to gain or loss of a recognition site. Using these variable sites as markers, we determined the composite mtDNA genotypes of all field-collected individuals. Two composite genotypes, labeled AAAA and BBBB (*15*), accounted for 96 percent of our sample. These differed from each other by gain or loss of a single recognition site for each of the four enzymes. In addition, we detected two rare mtDNA genotypes, which in each case could be derived from the common ones by one further single site

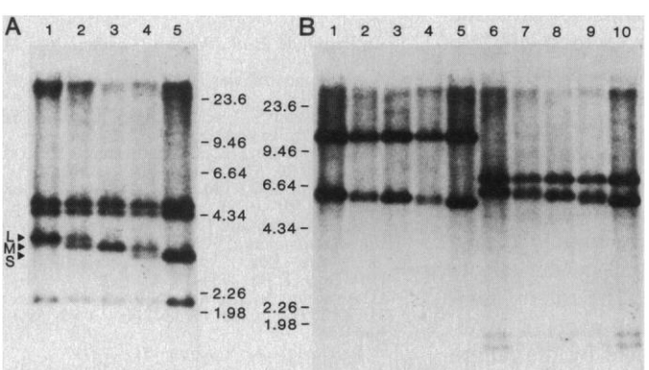

Fig. 1. (A) Eco RI digests of DNA from five individuals of *G. firmus* from Guilford, Connecticut. The fragment patterns for each individual include fragments of approximately 4.9, 4.6, 2.1, and 1.2 kb (the smallest fragment cannot be seen in this autoradiograph). In addition, there is a fifth fragment which appears to come in three sizes (labeled L, M, S). Individuals in lanes 2 and 4 appear to have two size classes of this variable sized fragment. (B) DNA from the same individuals digested with Ava I (lanes 1 to 5) and Hind III (lanes 6 to 10). In both cases gels were 0.7 percent agarose.

change. The two common composite genotypes correspond to the two different species (AAAA, *G. pennsylvanicus*; BBBB, *G. firmus*), but in the hybrid zone there is evidence of introgression of AAAA mtDNA into *G. firmus*.

In addition to the expected variation in fragment patterns due to gain or loss of restriction sites, we also observed differences that reflect variation in the size of the mitochondrial genome. For example, Eco RI digests of DNA from most individuals (of both species) showed five mtDNA fragments (Fig. 1A). Four of these fragments were identical in all individuals, whereas the fifth fragment varied in size. Three size classes were evident (referred to as S, M, and L), corresponding to total genome sizes of approximately 15.8, 16.1, and 16.4 kb (Fig. 1A, lanes 1, 3, and 5). In 12 of the 100 crickets sampled, Eco RI digests showed six bands (Fig. 1A, lanes 2 and 4). These individuals had the four "constant" fragments plus two of the three size classes of the fifth ("variable") fragment. Variation in Eco RI fragment pattern was not restricted to one of the composite mtDNA genotypes defined by restriction site variation. In fact, each of the five Eco RI restriction fragment patterns could be found among individuals having the AAAA and the BBBB mtDNA genotypes.

We interpret these results as follows. Both the AAAA and the BBBB composite genotypes exist in three discrete size classes that differ from each other by about 300 bp. Moreover, a substantial number of crickets of both species are heteroplasmic for these size variants and therefore exhibit two size classes of the variable fragment.

Clearly, if variation in Eco RI fragment patterns reflects differences in the overall size of the mtDNA, such variation should show up in digests with other enzymes. It is evident from Fig. 1B, which shows Ava I and Hind III digests of total DNA from the same individuals represented in Fig. 1A, that the size relations are the same for each enzyme and that individuals identified as heteroplasmic from Eco RI digests appear heteroplasmic in the Ava I and Hind III digests as well. Other enzymes (Cla I, Hinc II, Sac I, and Xba I) also give results consistent with this interpretation (*16*).

Size variation in mtDNA has been observed in a number of animal species and appears to occur most frequently in the D-loop region in vertebrates and in the A+T-rich (A, adenine; T, thymine) region in *Drosophila* (*17*). In *Drosophila* interspecific variation in the size of the

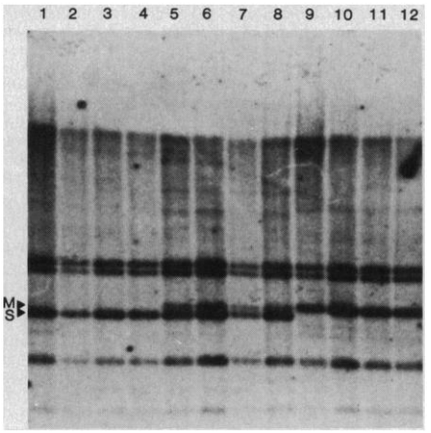

Fig. 2. Eco RI digests of DNA from three field-collected females (lanes 1, 5, 9). These three females contained mtDNA that is marked S (lane 1), M/S (lane 5), or M (lane 9). Lanes 2 to 4, 6 to 8, and 10 to 12 contained DNA from laboratory-reared offspring of each of these females. We have examined the mtDNA's from ten offspring of the heteroplasmic female in lane 5 (three of which are shown here), and all show patterns similar to those seen in lanes 6 to 8.

A+T-rich region has been well documented (*18*) and accounts for the large range of mtDNA sizes in that genus. Intraspecific size polymorphisms within the A+T-rich region have been observed in species in the *D. melanogaster* species group (*19*). In one of these species (*D. mauritiana*), mtDNA from a single isofemale line has been shown to be heteroplasmic for two size variants (*6*).

No details of the structure of cricket mtDNA are yet available, but from restriction site mapping we have localized the site of addition or deletion to a 800-bp region of the mtDNA where the variable length fragments produced by digestion with Ava I, Cla I, Eco RI, Hinc II, Hind III, and Xba I all overlap. We suggest that this region may correspond to the A+T-rich region in *Drosophila*. Given that the size difference between adjacent size classes is in each case 300 bp, it is possible that the addition/deletion events involve changes in copy number of a 300-bp repetitive sequence. A similar type of change (duplication of a 500-bp repeat unit) may explain the size variation in the A+T-rich region of *D. mauritiana* (*6*).

The occurrence of apparently identical size classes in what are presumably independent maternal lineages (the AAAA and BBBB composite genotypes) are most likely the result of parallel changes in the two lineages. The same size classes also appear in crickets from Virginia and Florida that have composite mtDNA genotypes different from those found in Connecticut. A high mutation rate (addition or deletion of a particular 300-bp sequence) would explain both the multiple origins for size variation and the high level of heteroplasmy in natural populations (nearly 20 percent in the BBBB composite genotype). It should be emphasized that there is no evidence for heteroplasmy of mtDNA variants differing in the presence or absence of particular restriction sites. If rates of fixation and loss are independent of genotype, then differences in extent of heteroplasmy between the two types of mtDNA variants (size variants and restriction site variants) must reflect differences in mutation rates.

The discovery of significant levels of heteroplasmy in an organism that can be easily reared in the laboratory with a short generation time promises to make this an excellent animal system for studying the "intracellular population genetics" of mtDNA (*7*). Given the increasing use of mtDNA in evolutionary studies, it is particularly important that we come to understand the dynamics of mtDNA molecules in individual animals so that we can correctly interpret patterns of variation in animal populations.

As a first step, we have examined the mtDNA fragment patterns of field-collected females and their laboratory-reared progeny. As expected, homoplasmic females give rise to homoplasmic offspring which have mtDNA of the same size class as their mother (Fig. 2). Of ten offspring of a heteroplasmic female, all were heteroplasmic and appeared to have proportions of the two size variants similar to that of their mother (Fig. 2). This suggests that the effective population size (n_e) of mtDNA molecules in cricket germ-cell lineages is sufficiently large that random drift or random segregation do not have a profound influence on mtDNA variant frequencies in the cell generations that intervene between zygote and unfertilized egg. Quantitative analysis of the variance in proportions of mtDNA size variants among offspring of individual heteroplasmic females will allow estimates of n_e.

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10. We have sampled 100 crickets from nine populations, ranging from pure *G. pennsylvanicus* populations in the northwest hills of Connecticut to pure *G. firmus* along the coast.
11. Total DNA was isolated from individual crickets by a method developed for *Drosophila* in the laboratory of David Hogness (S. Artavanis-Tsakonas, personal communication). Crickets were ground in liquid nitrogen and then homogenized in 5 ml of buffer in the presence of DEPC. The homogenate was incubated at 70°C for 30 minutes, 0.75 ml of 8*M* potassium acetate was added and the mixture was put on ice for an hour. The resulting precipitate was spun down and two volumes of ethanol were added to the supernatant to precipitate DNA. The DNA was pelleted and the pellet was resuspended in TE buffer and treated with ribonuclease. The DNA was then extracted with phenol, phenol:chloroform, and chloroform and precipitated with ethanol.
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13. *Grylus assimilis* is a close relative of *G. pennsylvanicus* and *G. firmus* and was used as a source of pure mtDNA for two reasons: (i) *G. assimilis* has continuous generations in the laboratory (no diapause) and single isofemale lines can be reared conveniently; (ii) in contrast to *G. pennsylvanicus* and *G. firmus*, it is a long-winged species with substantial flight muscles that are a rich source of mitochondria. To obtain pure mtDNA, we homogenized thoracic muscle from 25 to 50 adult crickets and separated mitochondria by differential centrifugation. The mitochondria were lysed with SDS, and mtDNA was purified away from residual nuclear DNA contamination by equilibrium centrifugation in CsCl–propidium diiodide.
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15. The label designates the fragment patterns for the enzymes Apa I, Hinc II, Hind III, and Xba I. The AAAA genotype has the A fragment pattern for each enzyme.
16. One enzyme, Bgl II, did not show any fragment pattern differences among individuals with mtDNA's of different sizes. Moreover, in all digests the sum of the Bgl II fragments was about 1.0 kb less than the sum of the fragments produced by other enzymes. Mapping studies indicate a "missing" 1.2-kb Bgl II fragment which does not show up on our autoradiographs, presumably because no similar sequence occurs in *G. assimilis* mtDNA. Only in Bgl II digests is a fragment produced that falls entirely within the nonhomologous region. We suggest that this rapidly evolving sequence also contains the region of addition or deletion in *G. pennsylvanicus* and *G. firmus*.
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Selective Inhibition of Fibronectin-Mediated Cell Adhesion by Monoclonal Antibodies to a Cell-Surface Glycoprotein

Abstract. *Fibroblasts possess several distinct mechanisms that control cellular adhesion to extracellular matrix macromolecules. Monoclonal antibodies to a 140-kilodalton (kD) cell surface glycoprotein inhibited the adhesion of fibroblastic Chinese hamster ovary cells to fibronectin-coated substrata but did not inhibit adhesion to substrata coated with vitronectin, laminin, serum, or other adhesive macromolecules. Thus the 140-kD glycoprotein appears to be involved in the fibronectin-mediated adhesion mechanism but not in other adhesion processes.*

Adhesive interactions between cells and components of the extracellular matrix are important in cell growth and differentiation (*1*), in morphogenetic processes during ontogeny (*2*), and in the metastases of malignant cells (*3*). Progress has been made toward elucidating the structure and function of molecules of the extracellular matrix (such as fibronectin and laminin) that are involved in cell adhesion (*4*). In contrast, there has been relatively little progress in identifying and characterizing plasma membrane components involved in adhesion. A candidate molecule for the cellular receptor for laminin has been identified (*5*); recent identification of a short peptide with adhesion-promoting activity suggests the existence of a specific surface receptor for fibronectin (*6*). However, although gangliosides (*7*), glycosaminoglycans (*8*), and glycoproteins (*9*) have each been suggested as the cellular fibronectin receptor, there is little evidence supporting any of these alternatives. Both large [120 to 160 kilodaltons (kD)] and small (40 to 60 kD) surface glycoproteins have been proposed as the receptor (*10, 11*). We have now developed a monoclonal antibody that inhibits the adhesion of hamster fibroblasts to fibronectin-coated substrata but does not inhibit adhesion to substrata coated with other ligands. The antibody immunoprecipitates a cell surface glycoprotein of approximately 140 kD; this molecule appears to play a central role in fibronectin-mediated adhesion.

Fibroblastic Chinese hamster ovary (CHO) cells were maintained in suspension culture (*12*). These cells have both fibronectin-dependent (type I) and fibronectin-independent (type II) adhesion mechanisms (*12, 13*) and readily adhere to substrata that have been coated with a variety of ligands, including fibronectin and its adhesive fragments, vitronectin, laminin, serum, lectins, and extracellular matrix material (*12–16*).

To isolate monoclonal antibodies to CHO cell surface proteins, we repetitively immunized BALB/c mice with CHO cells that had been trypsinized gently (20 μg/ml, 10 minutes at 25°C) and emulsified in Freund's complete adjuvant. Spleen cells from immunized mice were fused with P3x63-Ag8 myeloma cells, and hybridomas were isolated (*17*). Supernatants of hybridoma cultures were screened either for ability to bind to CHO cell surfaces by means of an enzyme-linked immunosorbent assay (ELISA) (*18*) or for ability to impede CHO cell adhesion to microwells coated with fibronectin. Of 8448 supernatants screened, 193 manifested cell-binding activity while only two displayed adhesion-blocking activity. Selected hybridoma cultures were cloned (*18*); cloned cells were grown in ascites form in pristane-primed BALB/c mice. The serotype of the ascites immunoglobulin was determined with commercial reagents. The antibody was then purified by ammonium sulfate precipitation and ion exchange chromatography (*19*) to approximately 95 percent homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Techniques for the preparation of ligand-coated substrata and for measuring the adhesion of isotopically labeled CHO cells to these substrata have been described (*12, 13*), as are procedures for surface or metabolic labeling of CHO cell membrane proteins (*20*). Immunoprecipitations of nonionic detergent-solubilized membrane proteins were carried out with monoclonal immunoglobulin G (IgG) linked, via a rabbit antibody to mouse globulin, to protein A–Sepharose (Pharmacia) (*21*) or with monoclonal antibody covalently linked to Affigel (Bio-Rad).

Most monoclonal IgG's directed against CHO cell surface components do not affect cell adhesion. However, two clones produced IgG's (PB1 and PB2; subclass IgG_1) capable of effectively blocking adhesion to fibronectin at concentrations of approximately 1 μg/ml (Fig. 1). By contrast, antibodies produced by two other clones (3F9 and 6C10), although directed against abundant surface proteins of 265 kD and 100 kD, respectively, did not block adhesion