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DIFFERING LEVELS OF AMONG-POPULATION DIVERGENCE IN THE MITOCHONDRIAL DNA OF PERIODICAL CICADAS RELATED TO HISTORICAL BIOGEOGRAPHY

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Abstract.—Mitochondrial DNA (mtDNA) haplotypes were determined for 118 individuals of 13- and 17-year periodical cicadas (genus *Magicicada*) collected from 16 localities throughout the Midwest and eastern United States. Two distinct mtDNA lineages, identified as A and B, differ by 2.5% based on analysis of fragment patterns and restriction maps. Observed levels of mtDNA diversity within each lineage are low compared to estimates for other taxa. The two lineages are regionally segregated, with the boundary line occurring at a latitude of approximately 33° North. The levels of mtDNA diversity and population genetic structure differ within the two lineages. There is a remarkably low level of mean mtDNA divergence and no genetic structure in lineage A, whereas lineage B exhibits an order of magnitude higher level of mtDNA diversity and significant genetic structure among sampled populations. The low level of mtDNA diversity in cicadas may be attributed to (1) a population bottleneck that most likely occurred during the Pleistocene, (2) recent colonization following the retreat of the glaciers and the expansion of deciduous forests, and/or (3) high among-family reproductive variance (as a consequence of large population size, high fecundity, aggregative behavior of adults, and clumping of eggs). The difference in mtDNA diversity and population genetic structure between the lineages suggests that they experienced different biogeographic histories; we relate this to Pleistocene changes.

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The determination of genetic relatedness among individuals and the extent of the differences between them provide the basis for inferring evolutionary history. Patterns of relatedness are the result of processes occurring over two time scales: evolutionary time that encompasses broad-scale changes in prevailing environmental conditions, and ecological time over which population processes (e.g., migration, local extinction, and colonization) occur. A primary goal of evolutionary biology is to unravel the interaction and assess the relative importance of these short- and long-term processes. Perhaps the greatest understanding of these processes of evolution emerges from studies involving a number of closely related taxa that represent a spectrum of levels of diversification.

As a consequence of emergence periodicity and differences among populations in the timing of reproduction, periodical cicadas manifest both geographic and temporal population structure. In addition, because *Magicicada* is widely distributed throughout deciduous forests east of the Great Plains (Simon, 1988), it is possible to sample genetic diversity across a large geographic area, providing the means to assess the level of genetic differentiation over varying degrees of reproductive isolation.

The systematics of the genus is complicated owing to the unique life history characteristics. There are three morphologically, behaviorally, and ecologically distinct periodical cicada taxa, abbreviated as Decim, Cassini, and Decula (Simon, 1988). In turn, each taxon contains a pair of sibling species that differs in life cycle length, for a total of six named species: *Magicicada septendecim, M. tredecim, M. cassini, M. tredecassini, M. septendecula, and M. tredecula* (Alexander and Moore, 1962). Furthermore, the species of *Magicicada* are divided into year classes. A year class is defined as all individuals emerging in a given year. There are a total of 15 year classes, of which 12 comprise 17-year cicadas and the remaining 3 are 13-year cicadas (see Simon, 1988). Each year class has been designated as a distinct brood and is identified by a unique Roman numeral (Marlatt, 1907). Because these cicadas have only one breeding season and are periodical, the different year classes are reproductively isolated from one another in time (except see Martin and Simon, 1988). On this basis they have been recognized as incipient species (White, 1978).

The natural history of periodical cicadas is well known (see Marlatt, 1907; Lloyd and Dybas, 1966). The life cycle consists of five

nymphal stages that live underground, feeding on the xylem fluid of a variety of tree roots (White and Strehl, 1978). After either 13 or 17 years underground, the 5th instar nymphs emerge from the soil, metamorphose into adults, and reproduce. Emergence events are periodic (sensu Bulmer, 1977), highly synchronized (Hoppensteadt and Keller, 1976), and involve large numbers of individuals (Dybas and Davis, 1962). Indeed, the immense number of cicadas that emerges in a given year inspires the common name, locusts.

Previous research on the evolutionary relationships among year classes of 13- and 17-year Decim has shown that (1) there are no fixed protein allelic differences among populations either within or among year classes, and (2) within a given year class there is little among-population genetic heterogeneity (Simon, 1979; Archie et al., 1985). Simon (1983) was able to distinguish cicadas from different year classes based on analysis of the wing venation, which showed significant among-brood and within-population variability but little among-population variability; however, these morphometric differences contain no phylogenetically useful information (Simon, 1990). The inability of these methods to resolve the extent that populations are genetically differentiated, either geographically or temporally, led us to use mitochondrial DNA (mtDNA) restriction site analysis (Wilson et al., 1985; Avise et al., 1987; Moritz et al., 1987).

This paper focuses on two different year classes, one each of the Decim sibling-species pair: *Magicicada septendecim* (17-year, Brood X) and *M. tredecim* (13-year, Brood XIX). We report here a survey of mtDNA diversity for populations of these two widely distributed year classes. The estimates of mtDNA relatedness are interpreted in the light of historical biogeography, natural selection, and the life history characteristics of periodical cicadas to assess the relative roles that these factors play in the generation and distribution of genetic variation.

Methods

Periodical cicadas were collected from localities across the entire range of the 1985 emergence of 13-year cicadas and the 1987 emergence of 17-year cicadas, corresponding to Broods XIX and X, respectively (Table 1). The cicadas were frozen in liquid nitrogen in the field and then stored at −70°C until analysis. Individual female abdomens with eggs and thorax muscle were homogenized in 250 m*M* sucrose, 75 m*M* EDTA, 50 m*M* Tris, pH 7.5 buffer, and purified mitochondrial DNA was obtained by differential centrifugation, membrane lysis, and ultracentrifugation in a cesium chloride–ethidium bromide gradient (Lansman et al., 1981; Palumbi and Wilson, 1990). Due to the small amount of tissue available and subsequently the low concentration of mtDNA in solution, supercoiled mtDNA was not visible in the gradient. We used the relative location of the mtDNA band visible in pooled samples from 10 individuals to gauge the general region of the gradient to save in single cicada preparations during fraction recovery. Enough pure mtDNA was obtained from single individuals in this way to perform greater than 30 digestions. For the analysis of single individuals, mtDNA was digested using 10 restriction enzymes that cut the DNA at two or more sites: *Ava*II, *Bcl*I, *Bgl*II, *Bst*NI, *Cla*I, *Eco*RI, *Eco*RV, *Mbo*I, *Msp*I, and *Xba*I. Fragments were visualized by end-labeling and separated in 1.0–2.0% agarose gels (Lansman et al., 1981). The haplotype of each individual was determined by analysis of fragment patterns. In addition to the analysis of single individuals of 13- and 17-year cicadas from Broods XIX and X, respectively, pooled samples of 15 individuals from the Broods I, V, VII, and IX were used to evaluate the relatedness among different year classes of 17-year Decim.

Endonuclease Restriction Site Mapping

The low level of mtDNA diversity found at a given sampling site allowed the pooling of 8–10 individuals to obtain more concentrated DNA samples for restriction site mapping. All the restriction sites for the enzymes *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Bsp*HI, *Bst*EII, *Cla*I, *Hin*dIII, *Msp*I, *Pst*I, and *Xba*I were mapped by single and double digestions of whole mtDNA molecules. For the enzymes *Bcl*I and *Eco*RI only 4 of the 14 and 9 of the 12 sites were mapped, respectively. For any given individual, approxi-

TABLE 1. Composite haplotypes for all individuals surveyed in this study.

Pattern[a]	Haplotype	Lineage	Life cycle	Location[b] (sample size)
AAAAAAA	A	A	17	Knox Co., TN (7) Vanderburg Co., IN (11) Vermillion Co., IL (4) Hillsdale Co., MI (4) Suffolk Co., NY (12)
AAAAAAA	A	A	13	Lee Co., IA (6) Piatt Co., IL (4) Pike Co., IL (4) Washington Co., IL (8) Columbia Co., MO (6) Fulton Co., AK (10)
AAAAAJA	A1	A	13	Columbia Co., MO (1)
CAAAAAA	A2	A	13	Fulton Co., AK (1)
ABBBBBB	B	B	13	McCurtain Co., OK (7) Searcy Co., AK (4) Trigg Co., KY (6) Wilkes Co., GA (4) Greenwood Co., SC (7)
ABDBBBB	B1	B	13	McCurtain Co., OK (1) Searcy Co., AK (1)
ABEBBBB	B2	B	13	Trigg Co., KY (2) Greenwood Co., SC (2)
ABFBBBB	B3	B	13	Greenwood Co., SC (1)
ABBBGBB	B4	B	13	Searcy Co., AK (3)
ABBBHBB	B5	B	13	Greenwood Co., SC (1)
ABBBHBK	B6	B	13	Greenwood Co., SC (1)

[a] The most common pattern is listed as A. Seven enyzmes are polymorphic. The patterns for these enzymes are listed in this order: *Bcl*I, *Bgl*II, *Bst*NI, *Cla*I, *Eco*RI, *Mbo*I, *Msp*I. The enzymes *Ava*II, *Eco*RV, and *Xba*I are monomorphic. The letter codes correspond with the fragment pattern profiles given in the Appendix.
[b] See Figure 3 for the county locations.

mately 50% of the fragments visualized by end-labeling were physically mapped in the genome. Cicada mtDNA was also digested with *Hin*fI, *Xmn*I, and *Dra*I and the fragment sizes estimated. The enzymes *Afl*II, *Kpn*I, *Sac*II, *Sal*I, and *Xho*I did not cleave the mtDNA molecule. The percentage nucleotide divergence between the two most common haplotypes was estimated by the maximum likelihood method of Nei and Tajima (1983) using site data and averaging the methods of Engels (1981) and Nei and Li (1979) using fragment data.

Estimation of Genetic Differentiation

To facilitate estimation of sequence divergence and population structure, for each polymorphic enzyme, the minimum number of restriction site changes that distinguish haplotypes was estimated from analysis of fragment patterns. Individuals were characterized by the presence or absence of a site at all polymorphic sites. The resultant matrix allowed the calculation of mean maximum likelihood within- and between-group genetic differentiation estimates using the algorithm of Nei and Tajima (1983). Genotypic diversity was calculated as in Ball et al. (1988). Within- and among-population probabilities of genetic identity were calculated as in Takahata and Palumbi (1985) and the level of among-population genetic structure estimated using the Gst statistic (Nei, 1973; Takahata and Palumbi, 1985). Gst is a measure of the amount of genetic variation that is due to among-population heterogeneity (Nei, 1973). The significance of the Gst statistic was evaluated by the bootstrap method (Efron, 1979) as adapted for Gst by Palumbi (Palumbi and Wilson, 1990): individuals were assigned to populations randomly, keeping the sample size per population the same, and then recalculating the Gst statistic for the shuffled haplotypes. This procedure was replicated 1,000 times and the observed Gst was compared to the distribution of random Gst's. The existence of significant among-popu-

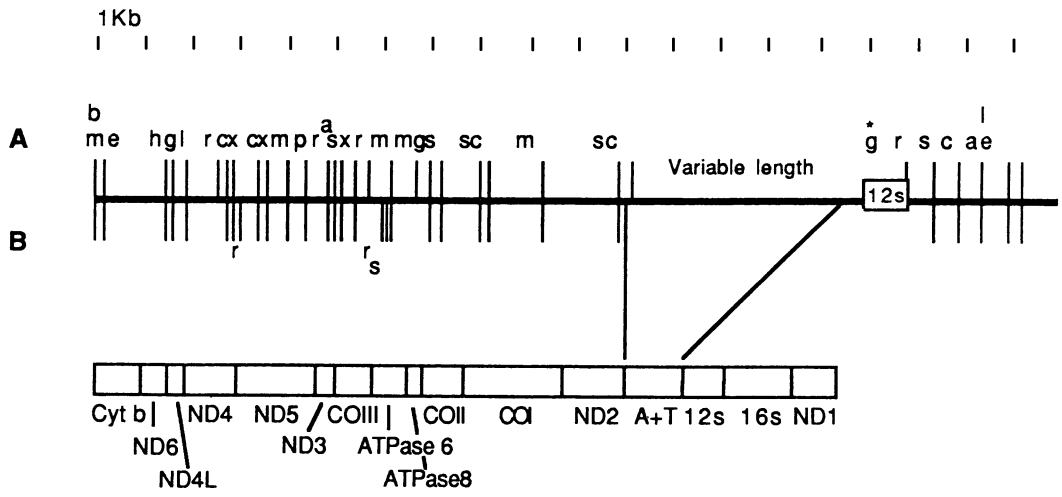

FIG. 1. Restriction enzyme maps for the two most common mtDNA genomes, A and B. Letter codes are as follows: a = *Ava*II, b = *Bam*HI, c = *Cla*I, e = *Bst*EII, g = *Bgl*II, h = *Hin*dIII, l = *Bcl*I, m = *Msp*I, p = *Pst*I, r = *Eco*RI, s = *Bsp*HI, x = *Xba*I. The gene order map for *Drosophila yakuba* is given for comparison. The asterisks above the polymorphic *Bgl*II restriction site indicate that this site was used in addition to the variable length region to orient the restriction map, assuming that the variable length region in *Magicicada* is homologous with the A + T region in *Drosophila*. The orientation of the map explicitly assumes that the gene order in cicadas is similar to *Drosophila* (although this may not be the case). With the exception of *BCl*I and *Eco*RI, all restriction sites for the enzymes listed above are on the map. The extent of the variable length region is uncertain and was estimated by subtracting the mean length of coding sequences of the mtDNA molecule derived from a variety of organisms from the total size of the cicada genome. Furthermore, there is a small (≈400 ± 100 bp) difference in the size of the two genomes (A and B) that occurs in the variable length region (see Fig. 2) that is not indicated on the map.

lation genetic structure was indicated if the calculated Gst statistic was greater than 95% of the randomly generated Gst values.

RESULTS

The Decim Mitochondrial Genome

Restriction site analysis consistently identifies two distinct mitochondrial lineages in Decim (Martin and Simon, 1988), labeled type A and type B (Fig. 1). (The fragment sizes for all restriction endonuclease digestions are presented in the Appendix.) The estimate of percentage sequence divergence between lineages A and B is 2.4 ± 0.5% based on fragment data (Engels, 1981; Nei and Li, 1979) and 2.6 ± 1.0% based on cleavage map comparisons (Nei and Tajima, 1983, Eqs. 23 and 25). There is also a size difference between the two lineages, with type A being 20 kb ± 500 bp and type B being approximately 400 ± 100 bp smaller, or 19.6 kb ± 500 bp (Fig. 2). In addition, genome size variability was often exhibited among individuals possessing the same lineage type (data not shown). Based on this, and observations from other taxa with similar hypervariable regions of the mtDNA genome (Solignac et al., 1983; Densmore et al., 1985; Harrison et al., 1985), we assume that the variable-length region in cicadas is likely homologous to the AT-rich region of *Drosophila* (Clary and Wolstenholme, 1985). This assumption is further supported by the fact that the small 12 S ribosomal subunit, which is adjacent to the AT-rich region in *Drosophila,* was found to be adjacent to the variable length region in cicadas. By knowing the relative locations of the variable length region and the 12 S gene, we oriented the restriction map (Fig. 1) using sequence data for the 12 S rRNA gene that contained the polymorphic restriction site for *Bgl*II (Simon and Pääbo, unpubl. data).

Geography, Life Cycle Length, and Mitochondrial Genotype

The two mitochondrial lineages are regionally segregated: lineage A is found in northern populations whereas lineage B oc-

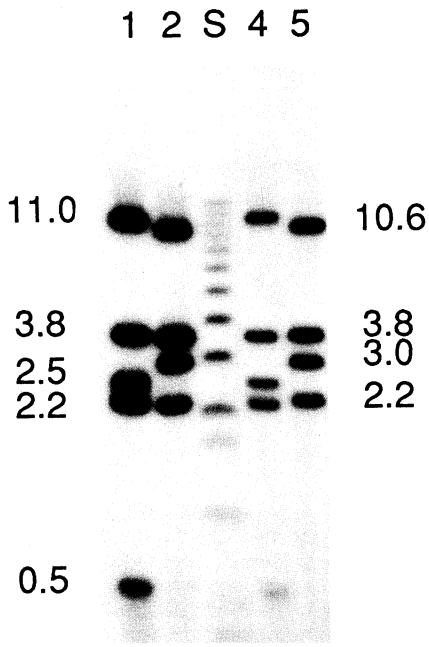

FIG. 2. Digestion profile of *Msp*I illustrating the difference in size between the two distinct genotypes A and B. Lanes 1 and 4 (lineage A) are two individuals from Suffolk Co., NY and lanes 2 and 5 (lineage B) are from Greenwood Co., SC. A 1 kb ladder was used as the standard (lane S). The fragment sizes in kilobases (kb) are given. The largest fragment for both genotypes exhibits the length difference between the two mtDNA genomes. We failed to detect any fragments smaller than 500 base pairs for *Msp*I digestions. *Msp*I recognizes a diagnostic site between the two lineages: the 3 kb fragment in genotype B has been cleaved into two fragments of 2.5 and 0.5 kb.

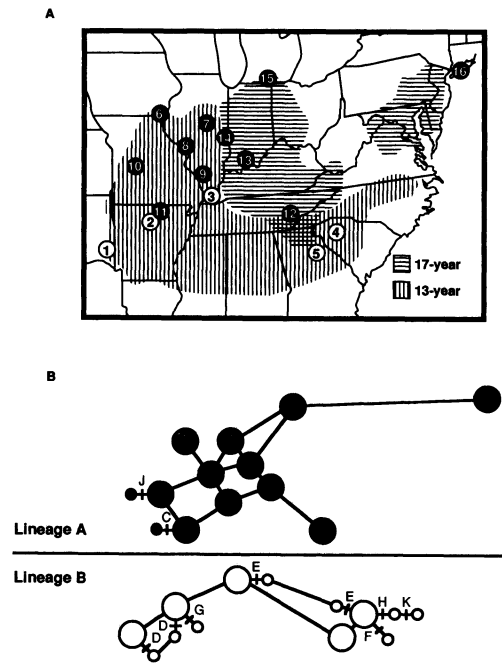

FIG. 3. (A) Map of the distribution of the two year classes of 13- and 17-year periodical cicadas. Closed circles indicate lineage A whereas open circles represent lineage B. Numbers refer to the following collecting sites: 1, McCurtain Co., OK; 2, Searcy Co., AK; 3, Trigg Co., KY; 4, Greenwood Co., SC; 5, Wilkes Co., GA; 6, Lee Co., IA; 7, Piatt Co., IL; 8, Pike Co., IL; 9, Washington Co., IL; 10, Columbia Co., MO; 11, Fulton Co., AK; 12, Knox Co., TN; 13, Vanderburg Co., IN; 14, Vermillion Co., IL; 15, Hillsdale Co., MI; 16, Suffolk Co., NY. (B) Haplotype network summarizing the relationships of cicadas within lineages A and B. Circles designate haplotypes. The relative locations of the haplotypes correspond with the collecting localities shown in A. The solid lines connecting nearest-neighbor haplotypes show the minimum number of mutational steps. Each unique change is indicated by a tick mark. Haplotypes connected by a line without a tick mark are identical. The letters at each tick mark correspond with the variant haplotypes listed in Table 1 and the Appendix.

curs in the southern populations (Fig. 3). The transition from one lineage type to the other occurs over a relatively short distance at a latitude of approximately 33° (parallel with the northern border of Tennessee). We did not detect areas in the range of either 13- or 17-year cicadas where both lineage types were present.

Only lineage A is found in the 1987 year class of 17-year cicadas whereas the 1985 year class of 13-year cicadas has both type A and B genomes. All populations of 13-year Decim sampled from southeastern Iowa to northern Arkansas and east to the Illinois–Indiana border (hereafter designated the midwest) contain the type A genome (which is characteristic of 17-year Decim), whereas 13-year Decim populations sampled elsewhere in their range have the type B genome. This mtDNA genotype identity of midwestern 13- to 17-year cicadas is paralleled by similarities in allozyme frequencies and abdominal sternite coloration. Based on these data, Martin and Simon (1988) concluded that the 13-year Decim in the midwest are descendents of 17-year Decim that permanently switched their life cycle from 17 to 13 years. The indication of life cycle length plasticity suggests that the current taxonomy that assigns species status to 13- and 17-year Decim is mis-

TABLE 2. Maximum likelihood estimates of mean within-taxon sequence divergence, π (Nei and Tajima 1983) and genotypic diversity [$1 - \Sigma f_i^2$, where f_i is the frequency of the ith genotype (Ball et al. 1988)] for periodical cicadas.

	Number of individuals	Mean number of restriction sites	π	$1 - \Sigma f_i^2$
Lineage A	78	55	0.0001	0.052
Lineage B	40	56	0.0012	0.472
Total	118	67	0.0133	0.460

leading. Thus, in the remainder of the paper we will refer to A and B lineages rather than to *M. septendecim* and *M. tredecim* because the mitochondrial lineages more accurately represent the true, well-differentiated monophyletic groups.

Levels of Mitochondrial DNA Divergence

The haplotypes for all individuals sampled are presented in Table 1. We detected a total of 10 mitochondrial haplotypes. Of a sample size of 118 individuals, 88% possess either the A or B haplotype. (For all four individuals from collection site 14 and all four individuals from site 15 there are no data for *Eco*RV because of insufficient DNA samples. All eight of these individuals were assumed to have fragment pattern X, which is reasonable considering that all 110 specimens surveyed possessed the X fragment pattern for *Eco*RV. Note that all monomorphic enzymes are designated as pattern type X in the Appendix.) Of the 14 individuals possessing variant haplotypes, 13 differ from one of the two most common haplotypes (A and B) by a single site (or length) difference, and the remaining variant haplotype (B6) differs from haplotype B by two site differences. One *Bst*NI variant (pattern D, Appendix) sampled in lineage B is missing a fragment. This digestion profile appeared in two individuals from separate localities, corresponding to genotype B1 in Table 1. The absence of the 3,400 bp band can perhaps be explained by the existence of an approximately 400 bp length mutation, resulting in comigration of this fragment with the 3,800 bp fragment. For purposes of calculating an mtDNA diversity index, this haplotype was assumed to differ from haplotype B by a single site change. None of the variant haplotypes was shared between the A and B lineages.

For each individual we sampled 55–56 restriction sites, corresponding to approximately 300 bp or 1.5% of the genome. There is little mtDNA differentiation (Table 2). Based on a sample size of 78 individuals for lineage A, the level of mean mtDNA sequence variation is less than 0.05% ($P = 0.0001$). Correspondingly there is very little mtDNA diversity (Table 2). Only two variant haplotypes were observed in lineage A and these were sampled from two different populations in the midwest (Table 1). These two haplotypes differ from the most common type by a single site gain and site loss for the enzymes *BCl*I and *Mbo*I, respectively (Table 1 and Appendix).

Compared to lineage A, there is considerably more mtDNA diversity and a 10-fold higher level of mtDNA sequence divergence among cicadas of lineage B (Table 2). Figure 1B provides a graphic representation of relationships among the 13-year cicada B lineage genotypes. Note, there are no fixed differences among populations. In addition, two of the six variant genotypes are shared among localities, leading to high probabilities of gene identity among populations (the mean within- and among-deme probabilities of gene identity are 0.98 and 0.95, respectively).

The amount of the total diversity for lineage B cicadas due to among-population variation is 41% (Gst = 0.413). From the bootstrap analysis for all populations of lineage B, all the randomly generated Gst values were less than that calculated from the observed distribution of haplotypes, indicating that there is significant population genetic structure. The magnitude of the Gst calculated for lineage B cicadas results from an excess of variant haplotypes, mainly among individuals from population 4, and from a unique haplotype (B4, see Table 1) that occurs at high frequency in population 2 (see Fig. 3). The relative contribution of each population to the observed genetic structure was assessed by omitting populations with the greatest within-population diversity until Gst was not significant. This analysis indicated that the observed genetic structure is largely due to the high frequency of the unique B4 haplotype in population 2.

Using the techniques employed here, there is no evidence for differentiation among the year classes of 17-year Decim sampled. The fragment patterns resulting from all restriction endonuclease digestions were identical among Broods I, V, VII, IX, and X.

Discussion

Evolution of the Periodical Cicada mtDNA Genome

There are two distinct mtDNA lineages present in the Decim species that differ in nucleotide sequence by 2.6%. If we assume that divergence occurs at a rate of approximately 2% per million years (Brown, 1985; Wilson et al., 1985; Shields and Wilson, 1987; DeSalle et al., 1987), these two lineages diverged an estimated 1–2 million years ago. However, the average rate of mtDNA evolution in insects and other invertebrates may differ from that observed for higher vertebrates (Powell et al., 1986; Vawter and Brown, 1986; DeSalle and Hunt, 1987). Even if the rate of divergence over short periods of time is similar, the high AT bias observed for insects imposes compositional constraints (Bernardi and Bernardi, 1986), which result in a more rapid saturation effect than is observed for vertebrates (DeSalle et al., 1987), and thereby limits the degree by which two sequences differ as divergence time increases. This is seen to a dramatic extent for *Drosophila* (Clary and Wolstenholme, 1985; DeSalle et al., 1987) with approximately 77% A + T nucleotides. Simon et al. (1989) found 78% A + T for a 326 bp segment of the mitochondrial 12 S gene in 13-year cicadas.

mtDNA Divergence within Lineages

There is a low level of within-lineage mtDNA sequence divergence in Decim relative to other taxa (Table 3). For lineage A, mtDNA diversity is less than 0.05%. Cicadas that are isolated by over 1,500 km are genetically indistinguishable using the methods employed here. Furthermore, the different year classes of 17-year Decim all have identical fragment patterns for every enzyme employed in this analysis. Even in lineage B cicadas with a substantially higher level of mean sequence diversity than is observed in lineage A, there are high probabilities of genetic identity among widely separated populations. This widespread similarity is also apparent from analyses of allozyme data (Archie et al., 1985; Martin and Simon, 1988) and for a morphological character (Martin and Simon, 1988).

The low level of within-lineage mtDNA diversity may be the consequence of (1) a reduced mutation rate, (2) long generation time, (3) strong purifying selection, (4) high among-family reproductive variance, and (5) recent population bottlenecks. The first three explanations involve how variation is generated and eliminated as a function of molecular processes and phenotypic effects of base substitutions. The fourth explanation focuses on aspects of the life history of periodical cicadas and their demographic consequences, while the remaining explanation attempts to relate changes in the environment and the distribution of suitable habitat over hundreds or thousands of generations to current conditions.

If we assume that the ancestral population size of the lineage B year class examined was 100,000,000 [a conservative estimate based on the population census of Dybas and Davis (1962)], the rate of evolution required to generate the level of nucleotide diversity of 0.0012 observed in lineage B cicadas would be 0.00001 substitutions/bp/Myr (see Avise et al., 1988), well below the estimate of 0.01 observed for *Drosophila* (DeSalle et al., 1987) or any other taxa that have been examined (Avise et al., 1987, 1988). Therefore, although the substitution rate in cicadas may differ from *Drosophila,* it is unlikely that a depressed mutation rate explains the low level of mtDNA diversity in cicadas relative to other taxa. Furthermore, the extremely reduced rate calculated for periodical cicadas argues against any significant influence of long generation time. Therefore, although long generation may reduce the rate of evolution in periodical cicadas as predicted based on theoretical (Kimura, 1979; Ohta, 1987) and empirical analyses (Wu and Li, 1985), its effect remains to be evaluated using DNA sequence data and appropriate time calibrations.

Given that the rate of molecular evolution is a function of both the mutation and fixation rates (Brown et al., 1979), it may be that the reduced level of within-lineage

TABLE 3. Comparison of levels of mean within-taxon mtDNA sequence divergence, π for various species of invertebrates and vertebrates.

Species		Reference
Insects		
Cicadas		
Magicicada		This study
Lineage A	0.0001	
Lineage B	0.001	
Diceroprocter apache	0.012[a]	Martin (unpub. data)
Flies		
Drosophila silvestris	0.048	DeSalle et al. (1986)
D. heteroneura	0.033	
Other invertebrates		
Sea urchins		
Strongylocentrotus spp.	0.001–0.005	Palumbi and Wilson (1990)
S. purpuratus	0.01	Vawter and Brown (1986)
Molluscs		
Mytilus spp.	0.007–0.013	Skibinski (1985)
Vertebrates		
Rodents		
Geomys pinetis		Avise et al. (1979)
Within region	0.003	
Between regions	0.034	
Peromyscus maniculatus		Lansman et al. (1983)
Eastern USA	0.015	
Continent wide	0.031	
Birds		
Parus atricapillus	0.004	Mack et al. (1986)
Agelaius phoeniceus	0.002	Ball et al. (1988)
Fish		
Freshwater		
Lepomis spp.	0.007–0.087	Bermingham and Avise (1986)
Marine		
Merluccius spp.	0.005–0.006	Becker et al. (1988)

[a] Based on sampling a mean number of 32 restriction sites for a total of 12 individuals collected from a single location (Tucson, Arizona).

mtDNA diversity is the result of strong purifying selection. Although selection has been documented to operate on mitochondrial variants, estimates of its intensity are low and its effects are sporadic (MacRae and Anderson, 1988) and therefore insufficient to account for the orders of magnitude difference in the rates of evolution between flies and cicadas (as calculated above).

A factor that may have contributed to the low level of mtDNA diversity is the fact that some females leave more offspring than others [deviation from a Poisson distribution of offspring (Avise et al., 1988)]. High among-family reproductive variance has been invoked to explain the low level of genetic diversity observed in sea urchins (Palumbi and Wilson, 1990) and *Anguilla* eels (Avise et al., 1988), species with large population sizes and high fecundities: Decim have large population sizes and high fecundities (300–600 eggs/female) and the distribution of adults and eggs in nature is clumped (White, 1980; Simon et al., 1981), thereby enhancing among-female reproductive variance.

The paucity of mtDNA diversity in Decim suggests that there may be a substantial discrepancy between present day population size—in which N can be up to 1,500,000 per acre (Dybas and Davis, 1962)—and evolutionary effective population size. We can assess the magnitude of the difference between N and N_e by estimating N_e using neutral theory (Nei, 1987). If we assume Decim is in genetic equilibrium, that there is no selection operating on the restriction sites examined, and the rate of substitution per year is similar to that observed for *Drosophila* (DeSalle et al., 1987), the substitu-

tion rate per generation, ϵ, is 2.6×10^{-7} for 13-year cicadas and 3.4×10^{-7} for 17-year cicadas. Based on these values, the estimate of N_e for the cicadas possessing lineage A is $\pi/\epsilon \approx 0.0001/3.4 \times 10^{-7} \approx 300$. Similarly, for cicadas with lineage B, $N_e \approx \pi/\epsilon \approx 0.0012/2.6 \times 10^{-7} \approx 4{,}600$. Because the assumptions of the model may not hold, the estimated values of N_e should be viewed as heuristic approximations. On the basis of these approximations, the estimated N_e for lineage A and B are at least five orders of magnitude below current population sizes.

This pattern appears to be common among a variety of taxa. Avise et al. (1988) show that for three vertebrates, effective population sizes (N_e) can be two to three orders of magnitude lower than present day census sizes (N) of breeding populations. This has also been reported for one group of marine invertebrates (Palumbi and Wilson, 1990). The disparity between N and N_e is derived from the lack of agreement between observed genetic heterogeneity and predicted heterogeneity based on census estimates of N. A general conclusion from these studies is that present day ecological and demographic conditions may not necessarily represent long-term evolutionary conditions.

The large difference between N_e and N could be explained by historical population bottlenecks (Nei and Graur, 1984; Nei, 1987; Avise et al., 1988). Approximately 30,000 years BP the interglacial climate began deteriorating, culminating in a glacial maximum at 18,000 years BP (Watts, 1983; Anderson et al., 1988). During the climatic deterioration, the distributional range for periodical cicadas must have contracted, driving the extinction of more northern populations of cicadas, or perhaps forcing northern cicadas to migrate south [similar phenomena have been hypothesized for a variety of North American temperate insects (Ross, 1966; Howden, 1969; Morgan and Morgan, 1980), plants (Adams, 1977; Schwargerle and Schaal, 1979; Webb, 1981; Bennett, 1985; Cwynar and MacDonald, 1987; Delcourt and Delcourt, 1987; and references therein), and vertebrates (Highton and Webster, 1976; Sattler and Guttman, 1976; Bellemin et al., 1978; Larson et al., 1984; Bermingham and Avise, 1986; Sage and Wolff, 1986; MacNeil and Strobeck, 1987; Ball et al., 1988)]. Relatively small pockets of deciduous forests are thought to have provided refugia (Delcourt and Delcourt, 1987, and references therein) that would have allowed cicadas to have survived the glacial maximum.

For nuclear-encoded traits we might not expect the same reduction in genetic diversity, despite the presumed restricted range of cicada populations during the most recent Pleistocene glaciation event. This is because periodical cicadas require large population sizes to successfully reproduce (Karban, 1982). Therefore, we predict that there should be a substantial level of nuclear gene diversity [as is observed for sea urchins (Britten et al., 1978)]. Furthermore, the level of heterozygosity should not differ dramatically between lineage A and lineage B cicadas. Simon (1979) examined variation at 20 allozyme loci and found that heterozygosity in these cicadas is not significantly reduced in comparison to other taxa (Nei, 1987), and that there was no difference between 13- and 17-year cicadas for the loci examined.

Based on the available data, we believe that the hypotheses with the most explanatory power regarding the low level of mtDNA diversity in periodical cicadas are (1) the reduction in population sizes due to the Pleistocene glacial maximum and (2) recent population expansion coupled with high among-family reproductive variance.

Distribution of Genetic Diversity in Lineage A versus B

In contrast to the complete lack of any observable mtDNA differences among populations of lineage A cicadas, there is among-population genetic differentiation in lineage B cicadas. Theoretical studies have demonstrated the dependence of population differentiation on time (Tajima, 1983). This suggests that the difference in population genetic differentiation may reflect differences in the relative magnitude of divergence times for populations with lineage A compared to lineage B.

Assuming that periodical cicadas did experience a severe population bottleneck during the most recent Pleistocene glacial maximum, population size has expanded

rapidly. The pattern of colonization in periodical cicadas was probably concordant with that for deciduous forests following the retreat of the glaciers (Howden, 1969); therefore, expansion of periodical cicadas to their present day range can be approximated by the distribution maps for deciduous forest trees at different times before the present based on pollen records (Bennett, 1985; Delcourt and Delcourt, 1987). On the basis of this, because of their northern distribution, all populations of lineage A Decim sampled are likely to have been recently derived (<12,000 years BP). This time frame is shorter than the resolution capability of standard mtDNA restriction site analysis and may explain our inability to detect mtDNA divergence among populations of lineage A.

In contrast to the northern distribution of lineage A, lineage B is found in the proximity of known Pleistocene refugia (Delcourt and Delcourt, 1977; Watts, 1983; Delcourt and Delcourt, 1980, 1987). The southern distribution of lineage B and the indication of significant genetic structure suggest that these populations are older. Furthermore, if we assume that the rate of evolution of mtDNA for periodical cicadas is constant and similar to *Drosophila,* then generating the level of mtDNA divergence observed (for lineage B, $\pi = 0.12\%$) would require at least 60,000 years. This suggests that 60–70% of the genetic diversity originated prior to the glacial maximum.

We suggest that lineage B periodical cicadas maintained mtDNA diversity through the Pleistocene bottlenecks because they occupied more than one glacial refuge. Vegetation maps for the glacial maximum reconstructed from pollen profiles provide evidence for multiple deciduous forest refugia that were patchily distributed during the glacial maximum (Delcourt and Delcourt, 1987). These patches of forest varied in area, species richness, and proximity to other patches. Therefore, the present day distribution of mtDNA haplotypes may be in large part determined by habitat heterogeneity that existed during the glacial maximum.

For cicadas with lineage B, there is a high proportion of shared rare haplotypes between distant populations. This may be an artifact of recently formed populations, providing information more relevant to historical patterns of genetic structure and gene exchange than to present day conditions (Larson et al., 1984). Alternatively, shared rare haplotypes may indicate secondary contact between previously isolated populations. Based on a maximum population dispersal rate of 6 km per generation (White et al., 1983), it is conceivable that the patterns of shared haplotypes among populations possessing lineage B are due to gene flow.

The Pleistocene Glacial Maximum and the Existence of Two Distinct Lineages

There are three hypotheses to explain the existence and regional segregation of the two, distinct lineages. The first two maintain that the two lineages refuged sympatrically in a single refugium, while the alternative view is that the lineages A and B persisted through the glacial maximum in geographically isolated refugia.

If both maternal lineages refuged sympatrically, then cicadas possessing lineage A would have expanded their range in concert with the expansion of deciduous forests, and as a result of stochastic lineage extinction or natural selection, lineage A could have been lost in southern populations. A number of features of periodical cicadas argue against this hypothesis. Presently, there are very few cases in which 13- and 17-year cicada populations exist sympatrically. This is presumably due to competition between the nymphs of different ages (Lloyd and Dybas, 1966). In cases in which 13- and 17-year cicadas have been suspected of being sympatric, 13-year cicadas have been inferred to be competitively dominant (Lloyd et al., 1983). Assuming that periodical cicadas were restricted to refugia for at least 100 generations (Watts, 1983), we believe that competition would have led to the extinction of 17-year cicadas (see Martin and Simon, 1988). Furthermore, if 13- and 17-year cicadas had been sympatric, there would have probably been gene flow between the two life cycle types as a consequence of simultaneous emergences every 221 years so that we would expect to see a mixture of lineages. The abrupt regional transition between lineages and the lack of

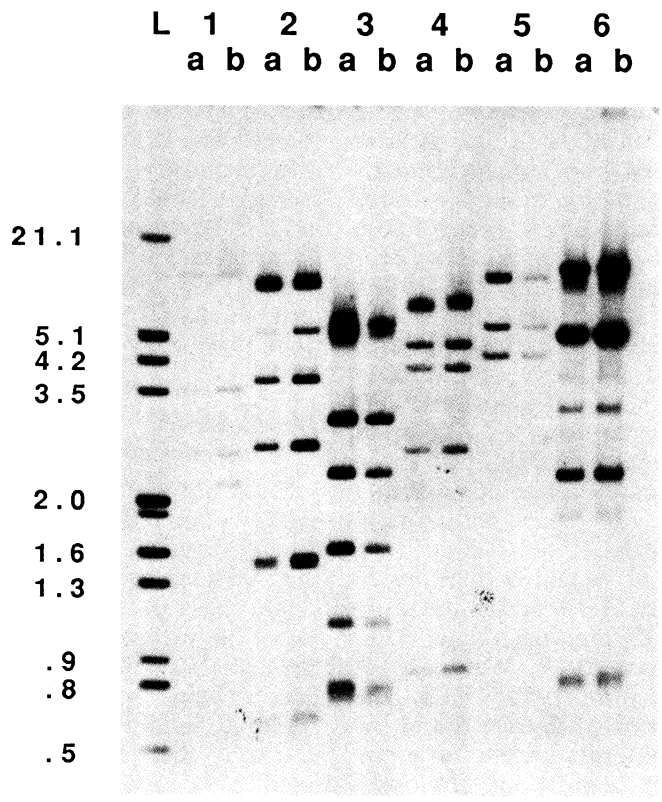

FIG. 4. Restriction fragment profile for pooled samples of 15 individuals from Broods I(a) and VII(b). Lanes 1 = *Msp*I, 2 = *Bcl*I, 3 = *Hin*fI, 4 = *Cla*I, 5 = *Bgl*II, and 6 = *Eco*RI. Lane L is a size standard (*Eco*RI–*Hin*dIII double digest of λ DNA). Fragment sizes for the restriction digestion patterns are given in the Appendix.

intermixing of the two lineages suggest that gene flow did not occur; however, the level of gene flow may have been too small to detect using the methods employed here.

A second single-refugium hypothesis is suggested by the direct observations of Kritsky (1988) and the genetic evidence of Martin and Simon (1988) for life cycle length switches. Although all documented life cycle length changes have been from 17 to 13 years (Kritsky, 1988), it is conceivable that cicadas can switch in both directions (e.g., from 13 to 17). During the glacial maximum, there may have been a single year class of 13-year cicadas that was polymorphic for both lineages. The northern 17-year cicadas may be derived from 13-year cicadas that underwent a 4-year deceleration in development and then dispersed northward, perhaps in response to competition with the remaining 13-year cicadas. Again, the abrupt regional transition between lineages and the lack of intermixing of the two lineages argue against this hypothesis.

The existence of two, distinct, regionally segregated lineages suggests that, rather than persisting through the glacial maximum in a single refugium, Decim may have occupied at least two allopatric refugia during the Pleistocene glacial maximum. The palynological data are consistent with this interpretation (Delcourt and Delcourt, 1987).

Segregation of Lineages

There is a north–south regional segregation of the two lineages with the boundary line occurring at a latitude of 33–35° (parallel with the northern border of Tennessee). Interestingly, the location of the boundary line corresponds in latitude with the northern limit of deciduous forest trees during the most recent Pleistocene glacial maxi-

mum (Delcourt and Delcourt, 1987). This clear pattern of mtDNA divergence is also apparent from analyses of one allozyme locus, phosphoglucomutase (Martin and Simon, 1988; Simon, unpubl.), abdominal sternite coloration (Martin and Simon, 1988), and wing morphology (Simon, 1990).

The north–south pattern in cicadas could be explained by deterministic factors. Specific environmental conditions (e.g., soil type, temperature) may select for specific traits [perhaps longer life cycle length (Cox and Carlton, 1988) or migratory ability], in which case the mitochondrial lineage is a marker for a regionally adapted genome. Alternatively, the regional segregation of lineages may be due to stochastic differentiation. Interestingly, the regional segregation pattern that we observed for Decim is also apparent in the horseshoe crab, *Limulus* (Saunders et al., 1986), and is qualitatively similar to a pattern generated by stochastic processes in a species with limited gene flow (Neigel and Avise, 1986). However, similarity between computer-generated distributions and patterns in nature does not constitute strong evidence that the distribution of haplotypes is governed by stochastic factors, only that this is a possibility. As such, we are unable to elect a causal mechanism generating pattern, only identify candidates.

The regional segregation of lineages makes some specific predictions for the distribution of mtDNA haplotypes. All 17-year cicadas are northern (Simon, 1988); therefore, we predict that the different year classes of 17-year Decim should possess lineage A. In fact, this is what is observed: Broods I, IV, V, VII, IX, X and northern populations of 13-year Decim from Brood XIX are all fixed for lineage A (Fig. 4; Martin and Simon, 1988; Martin, Simon and MacIntosh, unpubl. data). Moreover, southernly distributed cicadas should characteristically contain lineage B. Our analysis of southern Decim populations of Brood XIX and preliminary analysis of Brood XXII (which is confined to southern Mississippi and Louisiana) substantiate this prediction. We predict that northern populations of 13-year Brood XXIII, the only other 13-year brood with populations in northern latitudes, will be characterized as belonging to lineage A.

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APPENDIX

TABLE A. Fragment size for restriction enzymes employed in the analysis of 13- and 17-year Decim.[a]

Enzyme	Pattern	Fragment sizes								
*Ava*II	X	13500	6500							
*Bcl*I	A	9600	4000	2500	1400	1350	700	450		
	C	9600	4000	2500	1400	1100	700	450	250	
*Bgl*II	A	9900	5500	4600						
	B	14100	5500							
*Bsp*HI	A	9000	4800	2800	1850	900				
	B	9000	4400	2800	1150	900	700			
*Bst*EII	X	19150	850							
*Bst*NI[b,c]	A	9500	3900	2250	1350	900	600	400	250	
	B	9500	3900	3400	1350	600				
	D	9500	3800	—	1350	600				
	E	9500	3900	3200	1350	600	200			
	F	9500	3900	3400	1350	450	150			
*Cla*I	A	7000	5300	4250	2600	850				
	B	9300	5300	4250	850					
*Dra*I[b]	A	3500	1150	985	925	850	750	700	650	625
	B	3900	2900	1150	985	925	850	750	700	650
*Eco*RI[c]	A	11500	5100	2300	850	350				
	B	9300	6400	1650	1150	900	300			
	G	9300	6400	1650	1450	900				
	H	9500	6400	1650	1200	1150				
*Eco*RV	X	11500	5500	2050	950					
*Hin*fI[b]	A	4900	3000	2300	1600	1100	800			
	B	4400	3000	2300	1600	1300	800			
*Mbo*I[b,c]	A	6300	1300	1200	1100	900	850	800	750	550
	B	5800	1700	1100	900	850	800	750	550	500
	J	6300	1400	1200	1100	900	850	800	750	550
*Msp*I	A	11000	3800	3000	2200	500				
	B	10700	3800	3000	2200					
	K	10700	3000	2950	2200	850				
*Xba*I	X	17100	2100	800						
*Xmn*I	X	12000	5400	2400						

[a] Pattern designations correspond to those in Table 1 and Figure 3B. In cases in which the enzyme is monomorphic, the pattern designation X is used and the fragment size add up to the genome size for lineage type A. For each polymorphic enzyme, the most common pattern is designated as A.

[b] Smaller fragments have migrated off the gel; therefore, fragment sizes do not add up to the total genome size.

[c] Smaller fragments (<400 bp) were not always observed for each individual on the gel. In cases in which there were no apparent restriction site changes, the presence of the fragment in the digestion was inferred.