Mitochondrial DNA Differentiation during the Speciation Process in *Peromyscus*[1]

John C. Avise, John F. Shapira, Susan W. Daniel, Charles F. Aquadro, and Robert A. Lansman
University of Georgia

We address the problem of the possible significance of biological speciation to the magnitude and pattern of divergence of asexually transmitted characters in bisexual species. The empirical data for this report consist of restriction endonuclease site variability in maternally transmitted mitochondrial DNA (mtDNA) isolated from 82 samples of *Peromyscus polionotus* and *P. leucopus* collected from major portions of the respective species' ranges. Data are analyzed together with previously published information on *P. maniculatus*, a sibling species to *polionotus*. Maps of restriction sites indicate that all of the variation observed can be reasonably attributed to base substitutions leading to loss or gain of particular recognition sites. Magnitude of mtDNA sequence divergence within *polionotus* (maximum $\simeq$ 2%) is roughly comparable to that observed within any of five previously identified mtDNA assemblages in *maniculatus*. Sequence divergence within *leucopus* (maximum $\simeq$ 4%) is somewhat greater than that within *polionotus*. Consideration of probable evolutionary links among mtDNA restriction site maps allowed estimation of matriarchal phylogenies within *polionotus* and *leucopus*. Clustering algorithms and qualitative Wagner procedures were used to generate phenograms and parsimony networks, respectively, for the between-species comparisons. Three simple graphical models are presented to illustrate some conceivable relationships of mtDNA differentiation to speciation. In theoretical case I, each of two reproductively defined species (A and B) is monophyletic in matriarchal genealogy; the common female ancestor of either species can either predate or postdate the speciation. In case II, neither species is monophyletic in matriarchal genotype. In case III, species B is monophyletic but forms a subclade within A which is thus paraphyletic with respect to B. The empirical results for mtDNA in *maniculatus* and *polionotus* appear to conform closely to case III. These theoretical and empirical considerations raise a number of questions about the general relationship of the speciation process to the evolution of uniparentally transmitted traits. Some of these considerations are presented, and it is suggested that the distribution patterns of mtDNA sequence variation within and among extant species should be of considerable relevance to the particular demographies of speciation.

1. Number 5 in the series "The Use of Restriction Endonucleases to Measure Mitochondrial DNA Sequence Relatedness in Natural Populations." Key words: mitochondrial DNA, mammalian speciation, molecular evolution, restriction digests.

Address for correspondence and reprints: John C. Avise, Department of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602.

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Introduction

In recent years there has been tremendous interest in the molecular changes associated with speciation. One major motivation for this interest is that any genetic changes (such as chromosome rearrangements or iteration patterns of repetitive DNA) found to be regularly correlated with speciation *might* also be causally responsible for morphological divergence and/or reproductive isolation. Thus for many groups of organisms, information has been gathered about the genetic differences between closely related species. For example, in the multilocus protein-electrophoretic literature, more than 3,800 genetic distances between pairs of species have been recorded for vertebrates alone (Avise and Aquadro 1982); and the relevance of allozyme data to the speciation process has been extensively discussed (Lewontin 1974; Ayala 1975, 1976; Nei 1975). Early suggestions that changes in genome regulation (rather than replacement substitutions in structural genes) might be responsible for most organismal evolution have stimulated searches for other classes of genetic characters associated with species formation (Wallace 1963; Ohno 1969; Stebbins 1969; Britten and Davidson 1971; Wilson 1976). White (1978) makes a strong case that structural chromosomal rearrangements have commonly played major roles in initiating divergence and speciation. Recent discoveries of previously unsuspected classes of DNA (e.g., repetitive DNA and transposable elements) and mechanisms of DNA change (e.g., gene conversion and molecular drive) have led to speculation about how these processes may be related to evolution and speciation (Schopf 1981; Bonner 1982; Dover and Flavell 1982; Milkman 1982).

In this paper we address the problem of mitochondrial DNA (mtDNA) differentiation during speciation. Our concern with this issue might seem surprising. To the best of current knowledge, mtDNA appears to be strictly maternally inherited in higher animals (e.g., Dawid and Blackler 1972; Francisco et al. 1979; Lansman et al. 1983*b*). The apparent asexual transmission of mitochondria means that female lineages are genetically and evolutionarily isolated from one another in mtDNA composition no matter whether they belong to the same or to different species. Thus, from the point of view of mtDNA, sexual reproduction and speciation might seem to be unimportant processes (except through possible selection effects involving interactions between mtDNA and different nuclear genotypes). Furthermore, it remains a viable hypothesis that much of the extensive mtDNA polymorphism commonly assayed within and among closely related vertebrate species is selectively neutral. The majority of within-species polymorphism is attributable to base substitutions rather than to additions, deletions, or rearrangements (Aquadro and Greenberg 1983; Avise and Lansman 1983; Greenberg et al. 1983); in base sequences encoding polypeptides, silent third-position changes greatly outnumber substitutions which replace amino acids (Anderson et al. 1982; Brown et al. 1982). Why then should it be of interest to examine the differentiation of mtDNA during speciation?

It is precisely because mtDNA's are asexually transmitted, and because many of their polymorphisms may not be under strong selection, that they may provide excellent genetic markers for inferring the evolutionary histories and population dynamics of female lineages ancestral to extant species. In an example of this approach, Brown (1980) interpreted the exceptionally low level of mtDNA poly-

morphism within living humans as evidence for a severe population bottleneck in our female ancestors about 180,000–360,000 yr ago. Thus even if most assayable mtDNA divergence does not directly underlie reproductive isolation and speciation, it remains of interest because of the inferences it may allow about the genealogical and demographic histories of female populations undergoing speciation. This is the thesis we will address here.

More specifically, the purposes of this report are: (1) to estimate empirically levels and patterns of mtDNA divergence between the very closely related rodent species *Peromyscus maniculatus* and *P. polionotus* (samples of *P. leucopus* were also included as an "outgroup"), (2) to provide general conceptual scenarios of how observable mtDNA differentiation among extant species might be related to the process of species formation, and (3) to interpret the empirical data on *Peromyscus* in the context of these conceptual possibilities.

Material and Methods

Taxa Examined

Many of the approximately 60 named species of the rodent genus *Peromyscus* have been placed within taxonomic "species groups" which reflect probable evolutionary affinities. *Peromyscus leucopus* belongs to the *Leucopus* group. The *maniculatus* species group consists of about six recognized species—the widespread *maniculatus*, which occurs throughout most of North America, and geographically peripheral species in the highlands of Mexico (*melanotis*), Gulf of California (*sejugis, sleveni*), Pacific Northwest (*sitkensis* and possibly *oreas*), and southeastern United States (*polionotus*). *Polionotus* is generally thought to have evolved from *maniculatus*-like stock originally isolated on Floridian islands as a result of changing sea levels during the Pleistocene (Blair 1950).

The geographic ranges of *polionotus* and *maniculatus* do not overlap, so the magnitude of reproductive isolation in natural sympatry is unknown. In the laboratory, reproductive isolation between *polionotus* and tested *maniculatus* exists primarily in the form of prezygotic mating barriers (Blair and Howard 1944), coupled with difficulties of implantation and embryo survival following those successful matings which do occur (Maddock and Chang 1979). (Reproductive relationships among geographic samples of *maniculatus* are poorly known, but at least some populations have been reported to exhibit mild reproductive barriers as well [Dice 1968].) Glazier (1980) suggests that *polionotus* and *maniculatus* might best be considered semispecies within a larger superspecies complex.

Two earlier reports in this series also dealt with mtDNA evolution in *Peromyscus*. The first paper (Avise et al. 1979) introduced the approach of comparing mtDNA restriction digest patterns in samples of *P. maniculatus, P. polionotus,* and *P. leucopus* collected from a few localities. In a later study (Lansman et al. 1983*a*) we greatly extended the data base for *maniculatus* by employing additional enzymes and by mapping restriction sites ("site analysis") in mtDNA from mice collected from about 40 localities across the North American continent. Site analyses provide more refined estimates of sequence divergence than do fragment comparisons alone and in addition provide more direct information about the nature of evolutionary change in the mtDNA molecule itself. The new data for this current report consist of "site analyses" of mtDNA from a total of 82 newly collected individuals of *polionotus* and *leucopus* taken from major portions of their respective geographic ranges in eastern North America. These new data will

be analyzed together with the restriction site data previously published for *maniculatus*. Collection sites for *polionotus* and *leucopus* are listed in tables 1 and 2, respectively.

Laboratory Procedures and Data Analyses

Mice were returned live to the laboratory, where crude "cytoplasmic nucleic acid" fractions were prepared from livers plus kidneys and hearts of individual animals, according to published procedure (Lansman et al. 1981). These were digested with the following eight restriction enzymes: (1) *Hin*cII, (2) *Bgl*II, (3) *Hin*dIII, (4) *Bst*EII, (5) *Eco*RI, (6) *Bam*HI, (7) *Xba*, and (8) *Hpa*II. Digested fragments were "end labeled" with ^{32}P-dATP using DNA polymerase as described by Brown (1980). The labeled fragments were separated by molecular weight on agarose gels and detected by autoradiographic procedures (Lansman et al. 1981).

One sample each of *polionotus* and *leucopus* was also used to construct recombinant plasmids by inserting all of the *Bam*HI fragments of these genomes into pBR322. Much of the site mapping was accomplished by analyzing results of double-enzyme digests of the cloned sequences. The construction of clones and the mapping experiments were conducted as in Lansman et al. (1983*a*) (Shapira and Lansman, unpublished). The approximately 16,000 base-pair mtDNA genome of *Peromyscus* was arbitrarily assigned a total map length of 100 units, so each

Table 1
mtDNA Clones Observed in Geographic Samples of *Peromyscus polionotus*

Clone Number	Composite mtDNA Genotype[a]	Counties and States of Collection	Number of Individuals
1	AAPBAEUX	Clay, Houston, Marion, Wayne, Ga.; Pike, Ala.	20
2	AAPMBEUY	Taylor, Toombs, Ga.	2
3	AAPBAEUZ	Taylor, Ga.	1
4	AAPLAEUY	Twiggs, Ga.	1
5	AAPLADUY	Twiggs, Ga.	1
6	AAPHAEUX	Pike, Ala.	1
7	CAPBAEUX	Okaloosa, Fla.	5
8	EAPBAEUX	Jackson, Fla.	1
9	HAPBAEUX	Jackson, Fla.	1
10	AAPMAEUY	Bullock, Toombs, Wilcox, Ga.	5
11	AAPMPEUY	Bullock, Ga.	3
12	AAMBAEUX	Okaloosa, Fla.	2
13	σAPHBEUX	Okaloosa, Fla.	3
14	σAPHAEUX	Okaloosa, Fla.	1
15	BAMMADUY	Ben Hill, Wayne, Ga.	2
16	AAPMADUY	Toombs, Ga.	1
17	AAPIAEUX	Indian River, Marion, Fla.	7
18	AAPIAEWX	Indian River, Fla.	1
19	AAPIADUX	Indian River, Fla.	1
20	AANIAEUX	Marion, Fla.	4
21	AAPKAEUX	Marion, Fla.	4
22	BAPKAEVZ	Wayne, Ga.	1

[a] Letters, from left to right, refer to restriction morphs for *Hin*cII, *Bgl*II, *Hin*dIII, *Bst*EII, *Eco*RI, *Bam*HI, *Xba*, and *Hpa*II, respectively (see fig. 2).

Table 2
mtDNA Clones Observed in Geographic Samples of *Peromyscus leucopus*

Clone Number	Composite mtDNA Genotype*	Counties and States of Collection	Number of Individuals
1.....	αBAPEMAK	Cheshire, N.H.	1
2.....	αBAOEMAK	Cheshire, N.H.	1
3.....	αBBOEMAJ	Union, Ga.	1
4.....	αBAOEMAE	Clarke, Ga.; Macon, N.C.	2
5.....	αBAPEMAJ	Macon, N.C.	1
6.....	αBAOENAS	Chester, Pa.	1
7.....	αBBOEMAE	Pickaway, Ohio	1
8.....	αBAODMAE	Giles, Va.	1
9.....	αBAOEMAJ	Giles, Va.	1
10.....	αBCOEMBS	Giles, Va.	1
11.....	δBAPFPAJ	Reeves, Tex.	2
12.....	δBAQCOAG	Jefferson, Kans.	1

[a] Letters, from left to right, refer to restriction morphs for *Hin*cII, *Bgl*II, *Hin*dIII, *Bst*EII, *Eco*RI, *Bam*HI, *Xba*, and *Hpa*II, respectively (see fig. 3).

map unit corresponds to about 160 base pairs. Map position zero indicates the unique site at which the endonuclease *Pst*I cleaves most *maniculatus* mtDNA.

Many of the data analyses follow procedures utilized in earlier papers of this series (Lansman et al. 1983*a*). Thus for each restriction enzyme and for each animal, the "raw" data consist of a restriction site map given an arbitrary letter designation for purposes of bookkeeping. The letter designations have been standardized to those of *maniculatus* presented earlier (in other words, a *Bgl*II map "B" for *leucopus* and/or *polionotus* would carry the identical restriction sites as a *Bgl*II map "B" in *maniculatus*). Considering data from all eight endonucleases, we summarize the "composite mtDNA genotype" of each animal in an eight-letter code (see note to table 1). For convenience, we say that individuals sharing a given composite genotype belong to the same mtDNA clone (with respect to the observed restriction sites). For comparisons *within* the species *leucopus* and *polionotus* (where mutation distances proved to be small), the eight-letter codes facilitate the linking of mtDNA clones into unrooted phylogenetic networks by a process which involves adding new clones to positions in a growing network requiring the smallest number of mutation steps (see Lansman et al. 1983*a*). Such networks among conspecifics were subsequently superimposed over the geographic sources of collections.

In comparisons between species and more distantly related genotypes, a useful alternative representation of raw data is a matrix of presence-absence information for each restriction site in each mtDNA clone. Our final matrix for the three species of *Peromyscus* had 10,070 elements (106 restriction sites × 95 mtDNA clones). Minimum mutation distances (the minimum number of mutation steps separating pairs of clones) were counted directly from the matrix. Estimates of nucleotide sequence divergence (p) between mtDNA clones were calculated by the cleavage site method of Nei and Li (1979, eq. 8). The final value of p was weighted according to relative numbers of cleavage sites produced by the endonucleases recognizing four- and six-base cleavage sites.

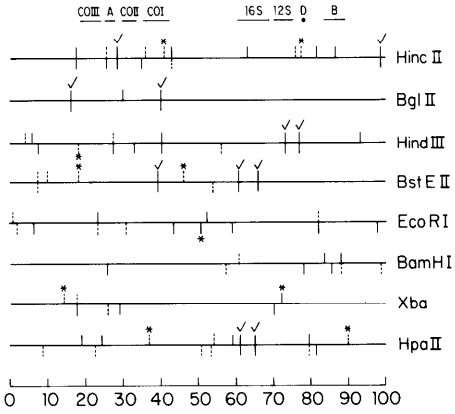

FIG. 1.—Map positions of all restriction sites observed in *Peromyscus polionotus* and *P. leucopus*. The mtDNA molecule is shown in linear form with the scale beginning at the zero *Pst*I site. Sites in *polionotus* are shown above each line, those in *leucopus* below. Conserved sites (those present in all samples from a species) are shown by solid lines; variable sites (those absent in one or more samples) are shown by dashed lines. Stars indicate sites mapped to only one of two possible alternative positions. Checks indicate conserved sites that were also shared by all assayed members of *P. maniculatus*. The approximate positions of coding sequences at the top are for cytochrome oxidase (CO) subunits, ATP-ase (A), cytochromc B (B), thc D-loop origin of H-strand replication (D), and 16S and 12S rRNA, all determined by comparison with *Mus musculus* mtDNA (Bibb et al. 1981; see Lansman et al. 1983*a*).

For a preliminary identification of phenetic groupings in the data, a matrix of minimum mutation distances between representative mtDNA genotypes was subjected to a clustering analysis by the unweighted pair-group method with arithmetic means (UPGMA [Sneath and Sokal 1973]). The original presence-absence matrix of restriction sites was also used to generate an initially unrooted phylogeny by the Wagner parsimony method (Eck and Dayhoff 1966; Kluge and Farris 1969) implemented in computer programs kindly supplied by Joe Felsenstein. This parsimony method allows character-state changes in both directions ($0 \longrightarrow 1$ and $1 \longrightarrow 0$), assumes no knowledge of ancestral states, and seeks to minimize total changes along the network.

Results

mtDNA Variability within *polionotus* and *leucopus*

The eight restriction endonucleases used to assay the 82 samples of *Peromyscus polionotus* and *leucopus* revealed a grand total of 74 restriction sites (48 in each species), distributed along the mtDNA molecule as shown in figure 1. As was also true for the earlier data on *maniculatus*, all of the observed genetic changes can reasonably be attributed to base substitutions leading to loss or gain of particular restriction sites; additions, deletions, or rearrangements affecting more than about 50–150 base pairs (0.3–1.0 map unit or less) would usually have been detectable but were not observed. Thus variable restriction sites behave independently and are gained or lost without detectable alterations in the sizes of the restriction fragments in which they occur.

Considering restriction enzymes one at a time, we were able to link mtDNA restriction maps for conspecifics of *polionotus* and *leucopus* into relatively unambiguous minimum path networks which represent the probable pathways of genotypic interconversions during evolution (figs. 2, 3). For example, *polionotus*

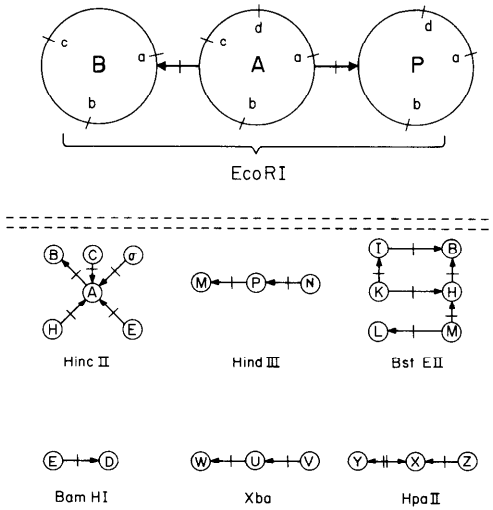

FIG. 2.—*Top,* Locations of restriction sites in the three *Eco*RI maps (P, A, and B) observed in *Peromyscus polionotus*. Lines connecting maps indicate probable phylogenetic links; arrows indicate direction of loss of a restriction site (but do not necessarily imply direction of evolution). *Bottom,* Probable phylogenetic links among the maps produced by each of six other restriction endonucleases. For *Bgl*II, all *polionotus* samples exhibited an identical three-site map (fig. 1).

*Eco*RI patterns "B" and "P" differ from each other by two restriction site changes but are both only one site loss from "A" (fig. 2). Similarly, by parsimony criteria, the four *Eco*RI maps of *leucopus* can be linked in a unique fashion (fig. 3).

The composite mtDNA genotypes of *polionotus* and *leucopus,* which summarize the combined data for all restriction enzymes, are listed in tables 1 and 2, respectively. Estimated phylogenetic networks (Lansman et al. 1983*a*) were generated from these composite genotypes and superimposed over the geographic sources of collections, with results shown in figures 4 and 5. For *polionotus,* a predominant mtDNA clone (no. 1) was observed in 20 mice collected from five counties ranging from extreme eastern Georgia, through central and southwest Georgia, and into southeastern Alabama. This genotype was one mutation step removed from each of seven other mtDNA clones observed in adjacent geographic regions (nos. 3, 6, 7, 8, 9, 12, 17). MtDNA clone "17," the most common genotype in central Florida, was in turn one mutation step removed from each of four additional mtDNA clones (nos. 18–21). In figure 4, for purposes of graphical clarity, the network positions of a few mtDNA clones are not pictured. Clone 15 is linked by two mutation steps to clone 16, clone 22 is three steps from clone 21, and clone 10 is three steps from clone 6.

Especially for these closely related mtDNA clones, the particular links in the network should not be interpreted as definitive statements of matriarchal relationships for two reasons. First, we have utilized only eight restriction enzymes to sample an average of about 38 sites per mtDNA genome. Because of problems of sampling error, it is very likely that particular branching patterns would be altered with additional data; thus the given network might better be thought of as an estimation of observed mtDNA character-state relationships rather than of whole organismal relationships (Avise 1983). Second, even when interpreted as observed character-state relationships, several ambiguities due to homoplasy (the

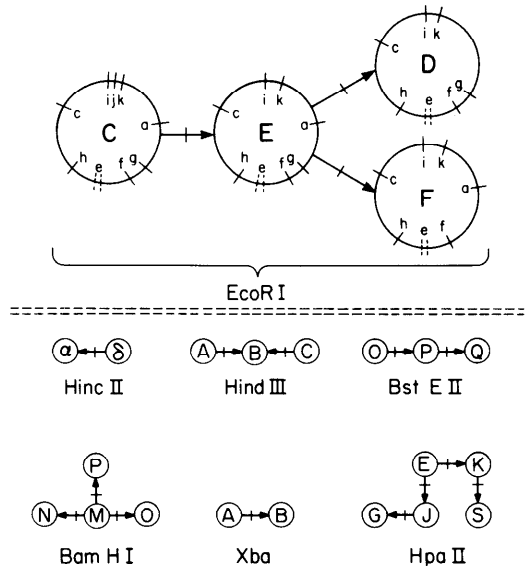

FIG. 3.—*Top*, Locations of restriction sites in the four *Eco*RI maps (C, E, D, and F) observed in *Peromyscus leucopus*. Site "e" was mapped only to one of the two alternative positions indicated by the dashed lines. Lines connecting maps indicate probable phylogenetic links; arrows indicate direction of loss of a restriction site (but do not necessarily imply direction of evolution). *Bottom*, Probable phylogenetic links among the maps produced by each of six other restriction endonucleases. For *Bgl*II, all *leucopus* samples exhibited an identical two-site map (fig. 1).

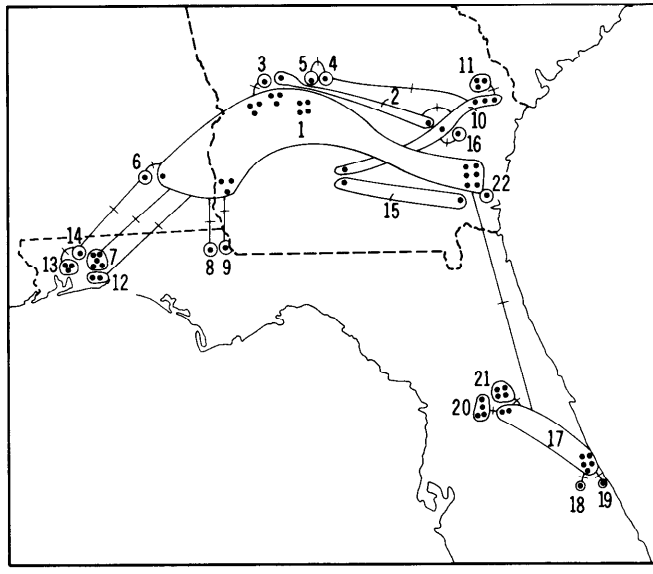

FIG. 4.—Geographic distribution and estimated phylogenetic network of mtDNA clones in *Peromyscus polionotus*. Numbers refer to composite genotypes as in table 1. Branches connect clones that are related by a single mutation step; in aggregate they form a network based on minimum mutation distance. The placements of clones 10, 15, and 22 (which are not shown fully in the figure) are discussed in the text. Black dots indicate individual mice sampled.

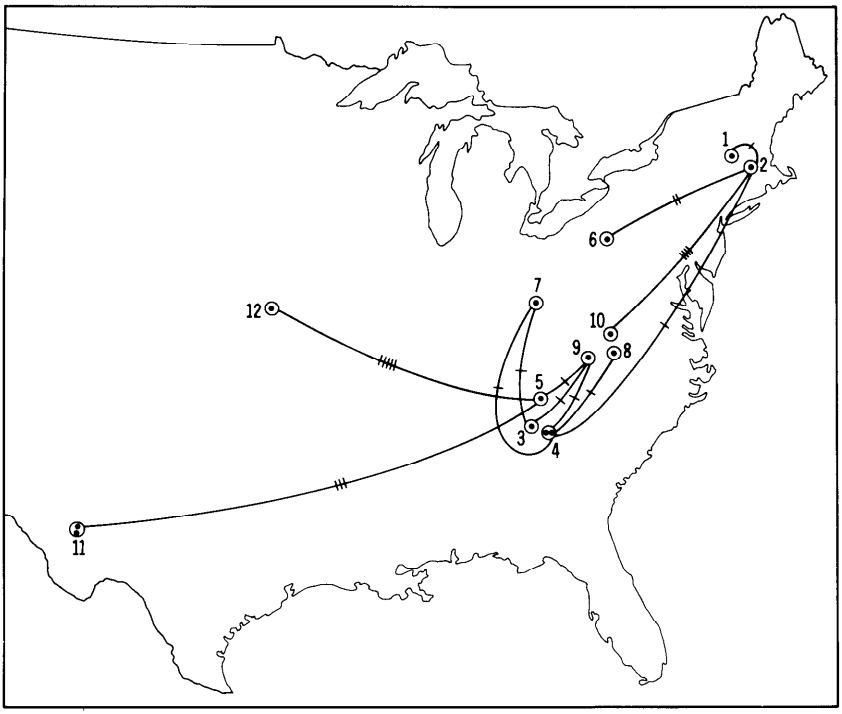

FIG. 5.—Geographic distribution and estimated phylogenetic network of mtDNA clones in *Peromyscus leucopus*. Numbers refer to the composite genotypes listed in table 2. Branches of the network interconnect pairs of clones separated by minimum mutation distance. The observed numbers of mutation steps separating clones are indicated by lines crossing branches of the network. Black dots indicate individual mice sampled.

convergent loss or gain of particular restriction sites) are apparent. For example, *Bam*HI map "D," which differs from "E" by absence of a site at position 60.5, was shared by clones 19 and 5, which were otherwise only distantly related in the network (fig. 4). When genotypes from pairs of enzymes are considered jointly, it can be shown that no network alteration can eliminate this kind of homoplasy. Thus the *Bam*HI genotypes "D" and "E," and the *Hpa*II genotypes "X" and "Y," were observed in all possible combinations (DX, DY, EX, EY) in *polionotus*. No matter which of these clonal patterns is considered ancestral, and no matter what additional data from other enzymes might reveal, it is inevitable (barring recombination) that for the character conflict exemplified by *Bam*HI and *Hpa*II there has been evolutionary convergence to a common restriction morph at least once (LeQuesne 1969). At least five similar instances of convergence are apparent in our samples of *polionotus*. This finding of homoplasy in the mtDNA restriction site data for *polionotus* parallels our earlier findings of extensive restriction site convergence in *maniculatus* (Lansman et al. 1983*a*).

For our samples of *leucopus*, no single mtDNA genotype predominated in abundance or in geographic distribution (fig. 5). Most mtDNA clones interconnect by single mutation steps in the network, but three genotypes (nos. 10, 11, and 12) were several mutation steps from all others. Two of these were from western portions of the range of *leucopus* (Kansas and Texas), and the third was from Virginia. Baker et al. (1983) have recently described two well-defined chromo-

Table 3
Estimates of Nucleotide Sequence Divergence between Representative mtDNA Clones in *Peromyscus polionotus*, *P. leucopus*, and *P. maniculatus*

Clone Number	*P. polionotus*					*P. maniculatus*					*P. leucopus*				
	1	7	17	15	10	35	1	32	23	30	2	4	6	11	12
1.....		.002	.002	.019	.011	.037	.026	.043	.046	.060	.156	.142	.150	.126	.133
7.....	1		.004	.022	.013	.039	.022	.040	.043	.056	.158	.136	.151	.128	.126
17.....	1	2		.021	.013	.039	.027	.044	.048	.056	.158	.145	.151	.128	.134
15.....	7	8	8		.007	.052	.033	.058	.054	.072	.155	.141	.147	.124	.140
10.....	4	5	5	3		.044	.029	.047	.047	.060	.151	.138	.144	.122	.137
35.....	14	15	15	19	16		.029	.033	.050	.049	.138	.133	.132	.118	.116
1.....	10	10	11	13	12	12		.038	.039	.046	.156	.121	.151	.130	.120
32.....	16	15	17	20	18	14	16		.059	.047	.163	.141	.156	.142	.140
23.....	17	16	18	19	19	19	15	21		.023	.166	.144	.159	.151	.149
30.....	19	18	18	24	21	21	19	19	10		.167	.143	.159	.151	.150
2.....	45	44	46	44	44	45	42	47	46	49		.003	.005	.021	.020
4.....	44	43	45	42	43	44	41	46	45	48	1		.008	.010	.016
6.....	43	42	44	42	43	43	41	45	44	46	2	3		.020	.026
11.....	39	38	40	38	39	41	40	43	42	44	6	5	8		.013
12.....	41	40	42	42	43	41	39	43	45	46	8	7	10	6	

NOTE.—Entry above diagonal: mean sequence divergence estimated from analyses of proportions of shared restriction sites; below diagonal: minimum mutation distances between clones. Margin numbers label the mtDNA clones as in tables 1 and 2 of the present report, and (for *maniculatus*) table 1 of Lansman et al. (1983*a*). For this matrix, the product-moment correlation between the distance measures is >0.99.

somal forms of *P. leucopus* distinguishable by three euchromatic pericentric inversions. The "southern race" was found in southern Kansas, Oklahoma, and Texas, while the "northern race" was found in remaining portions of the species range. Among our samples, judging from geographic location, the Kansas sample (no. 12) is near the apparent boundary of the northern and southern chromosome forms, and only our Texas sample (no. 11) is clearly referable by geography to the southern race.

In an earlier study of *maniculatus*, five major genetic assemblages within the species could be identified on the basis of mtDNA genotype: central states, southern California, Texas-Mexico, eastern states, and northern Michigan (Lansman et al. 1983*a*). Within any given assemblage, most estimates of mtDNA nucleotide sequence divergence were less than 2%, and clones usually interconnected along branches that were only one- to three-mutation steps in length. Levels of mtDNA sequence divergence within *polionotus* (table 3; figs. 4, 5, 6) appear roughly comparable to those within a given assemblage of *maniculatus*. Thus the maximum p among *polionotus* mtDNA clones is about 2% (table 3). The maximum mtDNA sequence divergence within *leucopus* is somewhat greater ($\simeq$4%; table 3, fig. 6).

mtDNA Divergence among *maniculatus*, *polionotus*, and *leucopus*

Estimates of nucleotide sequence divergence between representative mtDNA genotypes of *maniculatus*, *polionotus*, and *leucopus* are presented in table 3. Distances derived from site analyses and minimum mutation steps are highly correlated for these data (product-moment correlation > 0.99). In site comparisons between *maniculatus* and *polionotus*, $p \simeq 0.045$ (range 0.02–0.07). In comparisons of *leucopus* versus *maniculatus* and *polionotus*, the respective estimates of sequence divergence are as follows: $\bar{p} \simeq 0.14$ (range 0.12–0.17), and $\bar{p} \simeq 0.14$ (range

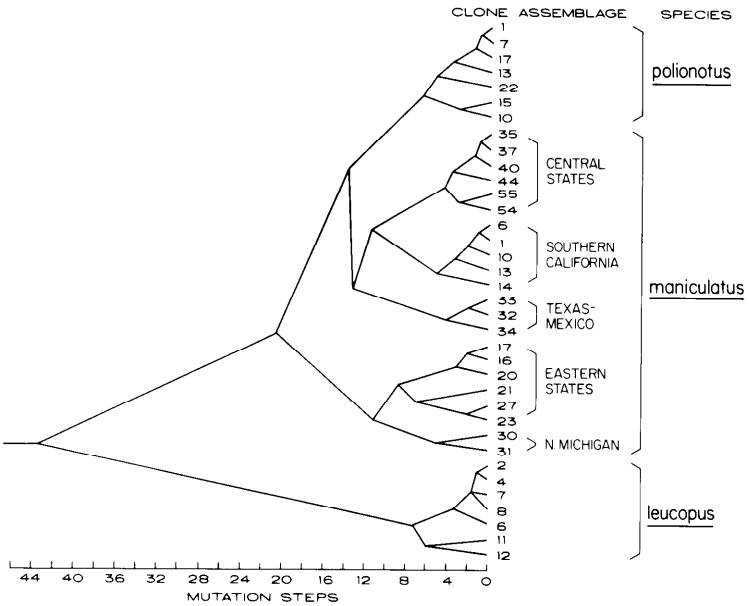

FIG. 6.—UPGMA dendrogram for representative mtDNA clones of *Peromyscus polionotus*, *P. leucopus*, and *P. maniculatus* generated by cluster analysis of a matrix of minimum mutation distances between mtDNA genotypes. The five "assemblages" of *maniculatus* mtDNA clones are the same as those described in our earlier study (Lansman et al. 1983*a*). The mtDNA clones are numbered as in tables 1 and 2 of the present study and table 1 of Lansman et al. (1983*a*). In this and in fig. 7, possible trifurcations in the data (e.g., clones 1, 7, 17) are shown as bifurcations to indicate one of several equally parsimonious trees that could have been drawn.

0.12–0.16). All of these between-species estimates of sequence divergence are considerably lower (roughly one-third to one-half the magnitude) than our previously published estimates for these same species based on fewer samples (Avise et al. 1979). Presumably the differences are attributable to the larger cohort of enzymes included in the present study (eight vs. six), to the fact that only three of eight endonucleases were shared by the two studies, and to the greater reliability of current estimates due to precise site mapping. In any event, these results underline the need for caution in interpreting absolute levels of mtDNA sequence divergence based on information from small numbers of restriction enzymes (Li 1981; Nei and Tajima 1981).

A UPGMA phenogram for 36 representative mtDNA clones of *maniculatus*, *polionotus*, and *leucopus* is shown in figure 6. This particular phenogram was generated from a matrix of minimum-mutation distances (the matrix of p values would cluster similarly since the distance measures were highly correlated). Five *maniculatus* clusters, representing the previously identified genotypic assemblages (Lansman et al. 1983*a*), are again apparent. All *polionotus* clones form a tight cluster, but this cluster falls well within a larger grouping formed by all *polionotus* and *maniculatus*. *Leucopus* samples also form a well-defined cluster which is phenetically distant from the *polionotus-maniculatus* grouping. On the average, in our survey about 43 mutation steps distinguish any *leucopus* sample from any *maniculatus* or *polionotus*.

A Wagner network generated from the presence-absence restriction site matrix of representative *Peromyscus* clones is shown in figure 7. Results agree closely with those of the phenetic analysis: all five assemblages of *maniculatus* can be identified, *polionotus* genotypes form a branch of the network, the *polionotus* branch stems from a point within the larger network linking the *maniculatus*

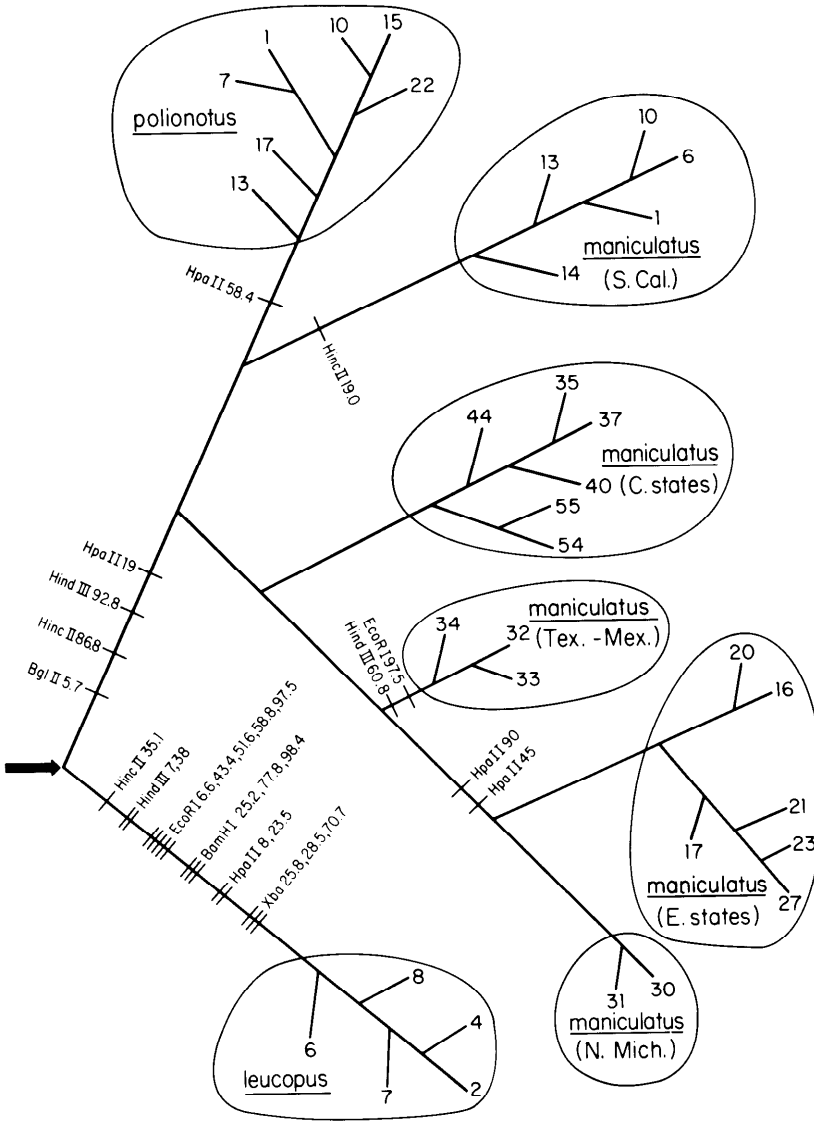

FIG. 7.—Wagner network for representative mtDNA clones of *Peromyscus polionotus*, *P. leucopus*, and *P. maniculatus*. The network was rooted at the arrow to reflect the probable phylogenetic split of *maniculatus-polionotus* versus *leucopus*. Circles encompass assemblages of mtDNA clones of *polionotus* and *leucopus*, and of *maniculatus* defined in our earlier study (Lansman et al. 1983*a*). The structure of the tree is of interest (cf. fig. 6); branch lengths have no meaning. Restriction sites diagnostic for *all* mtDNA clones along various branches of the network are indicated. The mtDNA clones are numbered as in tables 1 and 2 of the present paper and table 1 in Lansman et al. (1983*a*). See legend to fig. 6.

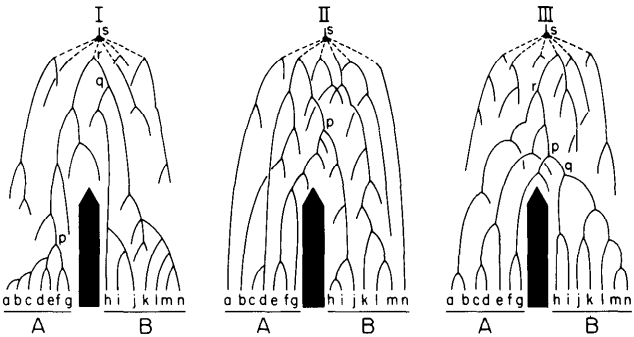

FIG. 8.—Conceptual possibilities for the relationship of mtDNA differentiation to speciation (see text). In each case (I, II, and III) two living species (A and B) are represented by individuals (*a–g* and *h–n*, respectively) whose matriarchal genealogies are indicated in highly schematized form by the branching pattern of the tree. The solid dark arrow indicates the onset of reproductive isolation (speciation) between A and B.

assemblages, and *leucopus* also forms a distinct branch of the tree. The computer output of the Wagner analysis allows identification of those restriction sites which uniquely describe the structure of the tree. Most of these (with the exception of sites present in only one or a few closely related genotypes) are shown in figure 7. Thus 16 sites were present in all assayed mtDNA clones in *leucopus* but absent in all *maniculatus* and *polionotus*. Four sites were shared by all assayed members of *polionotus* and *maniculatus* but were universally absent in *leucopus*. A single site (*Hpa*II 58.4) uniquely defines *polionotus*. In cladistic terminology, since *maniculatus* and the outgroup *leucopus* lack this site, presence of *Hpa*II 58.4 constitutes a synapomorphy (shared-derived state) defining *polionotus* as a clade. There were no restriction sites defining all *maniculatus* assemblages as a clade distinct from *polionotus*.

Nei and Li (1979) have developed a formula to estimate time of divergence between species when data on mtDNA polymorphism within species is also available (see also Ferris et al. 1981, eq. 1). The second term of Nei and Li's equation 25 is meant to correct for mtDNA divergence before species separation (Nei, personal communication). If we assume that *P. polionotus* is most closely related to S. Cal. *P. maniculatus* (fig. 7), and that mtDNA evolves at a rate of 2% base substitutions per million years (Brown et al. 1979), the corrected date for separation of *P. polionotus* from S. Cal. *P. maniculatus* becomes about 1.5 million years. We interpret this as a very provisional estimate.

Discussion

Although some specific models have been developed for the evolution of reproductive isolation through nucleo-cytoplasmic incompatibility (Watson and Caspari 1960; Wright 1969), little attention seems to have been paid to the more general problem of evolution of asexually transmitted characters within otherwise sexually reproducing species (Takahata and Maruyama 1981; Birky et al. 1983). For the evolution of strictly neutral, asexually transmitted characters, of what special significance (if any) is speciation? In particular, how might the distributions of such characters among living populations have been influenced by past speciation events? Some general conceptual possibilities are pictured in figure 8.

In hypothetical case I of figure 8, each reproductively defined species constitutes a monophyletic group (clade) with respect to matriarchal genealogy. All individuals within species A can be traced to a common female parent at point *p*, and all individuals within species B can be traced to a common female parent at *q*. If it is assumed that the female-transmitted traits being assayed (such as mtDNA) diverge at the same constant rate in all lineages, A and B would also appear as coherent clusters in a phenetic analysis. However, the mtDNA sequence diversity within B, much of which would have accumulated from a time vastly predating the onset of reproductive isolation, would be much higher than that within A, all of which would have accumulated from a time well after the speciation event. Also note that sequence divergence *between* A and B was initiated at point *r* and not at the time of species splitting.

In hypothetical case II of figure 8, neither species constitutes a monophyletic group with respect to matriarchal genealogy. Various groupings of individuals (such as *e–j*) do form apparent clades, but these are not consonant with the recognized species boundaries. Depending on the particular comparison, sequence divergence among pairs of conspecifics could range from very low (i.e., individuals *h* vs. *i*) to very high (i.e., individuals *i* vs. *n* which last shared a common female parent at point *s*). In comparisons between representatives of A and B, divergence would have been initiated at points ranging from *p* to *s*, the times at which various pairs of individuals last shared female ancestors. In case III (fig. 8), species B constitutes a monophyletic group with respect to matriarchal genealogy, but species A does not (i.e., species A is paraphyletic [Farris 1974; Wiley 1981]). Expected patterns of divergence within versus between species can again be readily interpreted from the diagram.

The primary empirical concern of this report is the pattern and magnitude of mtDNA differentiation within and among populations of the closely related species *Peromyscus maniculatus* and *P. polionotus*. From both the Wagner and UPGMA analyses of the restriction site data, the pattern of mtDNA evolution (figs. 6, 7) conforms most closely to case III of figure 8. *Peromyscus polionotus* appears to form a monophyletic matriarchal assemblage (and a phenetic grouping) within the larger clade composed of *polionotus* and *maniculatus*. Thus the various assemblages of *maniculatus* appear to be paraphyletic with respect to *polionotus* in matriarchal ancestry. Which *maniculatus* assemblage is most closely related to *polionotus* is not clearly resolved from our data. However, the magnitude of mtDNA sequence divergence within *polionotus* is roughly comparable to that observed within any of the five geographic assemblages of *maniculatus*.

If our sample of mtDNA restriction sites provides a reliable assessment of overall matriarchal relationships for these species, it appears that populations now classified as *maniculatus* are paraphyletic and can trace ancestries to female parents predating the separation of *polionotus* from *maniculatus*. This scenario is probably not inconsistent with conventional thought. Blair (1950), Hooper (1968), Bowers et al. (1973), and Greenbaum et al. (1978) have all suggested that *maniculatus* represents the ancient, geographically central evolutionary stock from which *polionotus, melanotis, sitkensis, sleveni,* and *sejugis* were budded off via peripheral isolation. If true, the greater total sequence diversity within *maniculatus* is probably expected, as is the potential monophyletic origin of each peripheral isolate.

The primary conceptual concern of this report is how the pattern and magnitude of differentiation in asexually transmitted characters might generally be related to speciation. With the very simple models we have introduced, at this point only a few general observations can be attempted. One prediction from these scenarios is that the initiation of mtDNA sequence divergence *between* living species could predate a speciation, but could not postdate it (fig. 8). This possibility may have important consequences for discussions of rates of molecular evolution. For example, suppose that samples from species A and B show 10% sequence divergence in mtDNA, and from fossil or geographic evidence are known to have speciated 5 million years ago. Even barring potential problems of character-state reversals or convergence, it still does not necessarily follow that rate of divergence is 2% per million years. Depending on the particular (and in practice unknown) matriarchal genealogies involved, rate of sequence divergence could be *lower* than this simply because of the pattern of lineage survivorship.

This potential error is in *some* respects similar to previously recognized sources of error in rate estimates from paralogous versus orthologous protein comparisons (Wilson et al. 1977). In the conventional view of a paralogous comparison of proteins (such as the α and β chains of hemoglobin), any apparent distance between species would have accumulated since the time of gene duplication, which could only be as old as or older than the speciation. (This neglects recently discovered possibilities of "concerted" evolution [Coen et al. 1982].) Similarly, in comparisons of strictly maternally transmitted characters, any apparent distance between species would have accumulated since the time of last common female ancestor, which might also be older than the time of speciation. Recent empirical studies have suggested that mtDNA evolves very rapidly—perhaps five to 10 times more rapidly than single-copy nuclear DNA (Brown et al. 1979; Brown 1983). If the female ancestries of the species used for these comparisons commonly trace to times predating the speciations used in rate calibrations, the *absolute* rates of mtDNA evolution could be inflated for this reason (however, there are also independent mechanistic reasons for supposing that mtDNA has evolved especially rapidly [Brown 1983]).

One way that speciation could have immediate consequences for the pattern of mtDNA evolution is if each speciation is accompanied by a severe population reduction followed by long-term population expansion. With most or all mtDNA sequence diversity lost at the bottleneck, the speciation event would effectively "reset" the molecular clock; subsequently arising sequence diversity *within* a species could be calibrated to the time of speciation. For example, if *polionotus* went through a severe population reduction at time of separation from *maniculatus* perhaps 1–2 million years ago, rate of mtDNA sequence divergence within *polionotus* could be roughly 2% per million years (this is generally comparable to published rates for mtDNA divergence in other mammals [Brown et al. 1979; Ferris et al. 1981]). However, populations certainly may experience bottlenecks at times not associated with speciation. Thus the initiation of sequence divergence within a species could also predate or postdate the speciation process. Furthermore, lineage sorting can be very rapid in stable populations even in the absence of bottlenecks (Avise et al., in preparation).

Another way that past speciations will likely influence patterns of mtDNA relationships among extant species is through the necessary partial correlation between matriarchal heredity and total nuclear genome heredity. The evolutionary

demographies of female lineages have immediate effects on the patterns of evolution of mtDNA, but those females which survive and reproduce contribute to the nuclear as well as the mtDNA gene pools of future generations. The strength of the correlation of mtDNA genotype with nuclear genotype in a given situation will likely depend on mating structure, population size and subdivision, population history, and other related demographic variables. Finally, as already noted, there could be selection pressures causing partial coevolution of nuclear DNA and mtDNA due to functional interactions of their transcribed products.

Clearly, a great deal remains to be learned about the relationship of the speciation process to the evolution of uniparentally inherited traits such as mtDNA. Progress should proceed along two fronts. Empirically, many more examples are needed of the magnitude and pattern of mtDNA differentiation within and among closely related species. Perhaps examples of cases I and II (fig. 8) will be found to complement the example of case III presented here. How often will species boundaries defined by reproductive isolation be concordant with phenetic or cladistic boundaries identified in analyses of mtDNA genotype? Within what limits will conspecific individuals be found to differ in mtDNA genetic composition, and how might these values be related to the evolutionary dynamics of female lineages in particular cases? More empirical work with higher animals is also needed to verify the supposed lack of paternal mtDNA input to progeny; to examine the possibility of recombination or conversion among mtDNA's; to understand how these possibilities, if realized, might affect the conclusions of this and similar studies; and generally to understand the molecular and cellular dynamics of mtDNA variation (Chapman et al. 1982; Lansman et al. 1983*a*).

On the conceptual front, more comprehensive and formal theoretical models relating the dynamics of matriarchal lineages to speciation are required. Under what demographic conditions can concordances between reproductively defined species and matriarchally defined assemblages be anticipated? How often and by how much time might the shared female ancestors of representatives of extant lineages antedate the time of origin of reproductive isolation? How might the evolutionary dynamics of female lineages lead to major genetic gaps in mtDNA sequence among populations (such as those observed in *maniculatus*)? Answers to these and related questions should tell a great deal about the demographies of speciation.

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