J Mol Evol (1998) 47:21–31

JOURNAL OF MOLECULAR EVOLUTION

The Effects of Social Structure, Geographical Structure, and Population Size on the Evolution of Mitochondrial DNA: II. Molecular Clocks and the Lineage Sorting Period

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Abstract. Evolutionary geneticists have increasingly used sequence variation in mitochondrial DNA (mtDNA) as a source of historical information. However, conclusions based on these data remain tentative because a sufficiently clear understanding of the evolutionary dynamics of mtDNA has yet to be developed. In this paper we present the results of computer simulations designed to illustrate the effects of social structure, geographical structure, and population size on the rate of nucleotide substitution and lineage sorting of mtDNA. The model is based in part on the social structure of macaque monkeys. Simulated populations of females were divided into 25 social groups; the animals in each were distributed in a hierarchy of four dominance rank categories. The probabilities for offspring survivorship were varied among dominance ranks to reflect the fitness consequences of social structure. Population size was varied across runs from 100 to 300 females. The pattern of female migration was also varied to mimic either the island model or the stepping-stone model. All these variables are shown to affect the lineage sorting period (LSP), and certain combinations of parameter values can cause the retention of mtDNA polymorphisms for a very long time. In addition, the simulations exhibited a negative relationship between the LSP and substitution rate over a modest and realistic range of LSP values. An important implication of these results is that estimates of time since isolation based on the assumption of a constant molecular clock may be biased and unreliable.

Key words: Mitochondrial DNA — Molecular evolution — Population genetics — Molecular clock — Lineage sorting period

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Introduction

Examination of mitochondrial DNA (mtDNA) is yielding an impressive amount of information for geneticists interested in reconstructing organismal phylogenies, charting historical biogeography, and estimating the amount of time that has passed since cladogenic events (e.g., Avise 1986; Avise et al. 1987; Cann et al. 1987). The popularity of mtDNA for such studies has developed in part because the genetics of this molecule differs from nuclear DNA in several ways that offer practical advantages in the collection and analysis of data (Brown 1983; Honeycutt and Wheeler 1989; Melnick and Hoelzer 1993): (1) It is relatively small (about 16,000 bp in most vertebrates); (2) it is maternally inherited (but see Neale et al. 1989 for an exception in plants); (3) the genome is haploid; (4) it does not undergo recombination; (5) it exhibits relatively rapid sequence evolution (Brown et al.

1979); and (6) the genetic organization of the entire mitochondrial genome is known for a diverse array of species. These features have proven particularly valuable for phylogenetic reconstruction of recently diverged taxa because matrilineal relationships can be traced directly without the complications resulting from the recombination of genomes. However, while several decades of theoretical and empirical attention have revealed many of the conditions that affect allele frequencies in the nuclear genome, the factors that influence the frequencies of mitochondrial haplotypes and the rate of divergence in mtDNA variation between populations are less clear. Some authors have begun to develop population genetic models for extranuclear genomes (e.g., Chapman et al. 1982; Birky et al. 1983, 1989; Neigel and Avise 1993; Takahata and Maruyama 1981; Takahata 1985; Takahata and Palumbi 1985), but these models generally do not consider the potentially confounding effects of social or geographic structure on mtDNA evolution.

Given the maternal, clonal inheritance of this molecule, social relationships among females might be expected to influence mtDNA evolution. While female social behavior varies widely across species, the model presented here focuses on the effects of social behavior as exhibited by female macaque monkeys because we have an abundance of data on the extent and distribution of variation in mtDNA for numerous macaque species that show surprisingly high levels of intraspecific variation. By including aspects of macaque population biology that might affect mtDNA evolutionary dynamics in the model, we might gain a better understanding of the pattern of mtDNA variation observed in macaques. In addition, although the social behaviors implemented in this model are designed with macaques in mind, many other species exhibit aspects of these behaviors (Greenwood 1980); thus, results produced from the analysis of this model are generally relevant to the evaluation of mtDNA variation in a wide variety of taxa.

The principal advantage of this approach to modeling is that some of the characteristics of natural systems, which cannot be easily incorporated into analytical models, can be explored. Of course, this simulation model also makes simplifying assumptions and explores the effects of some factors, such as migration and social structure, in rather narrowly defined ways. Nevertheless, models like this can be instructive by illustrating the potential effects of these factors under conditions outside the scope of existing analytical models. The data generated from such a simulation model can be used to assess the robustness of existing analytical models with regard to some of their simplifying assumptions.

Macaque Monkeys

Extremely high levels of intraspecific mtDNA sequence variability have recently been found in monkeys of the genus *Macaca*. Sequence differences of 3% or more occur within most species examined (e.g., *M. tonkeana,* Williams 1990; *M. mulatta,* Melnick et al. 1993; *M. sinica* and *M. assamensis,* Hoelzer et al. 1993; *M. nemestrina,* Melnick et al. unpublished data). These levels of divergence within species are similar to those found between many macaque species (Hayasaka et al. 1988; Zhang and Shi 1989; Hoelzer et al. 1993; Melnick et al. 1993), as well as between other congeneric mammalian species (Honeycutt and Wheeler 1989). A conspicuous feature of the behavior of macaques that may account for such exceptional intraspecific diversity is that they live in highly complex, socially structured populations (Melnick and Pearl 1987). Therefore, to explore the implications of social structure for mtDNA evolution, many of the specific features of macaque social structure, as well as more general cases, were examined through a computer simulation.

Among macaques, female dominance interactions within groups often determine access to critical resources and thus can be correlated with reproductive success (e.g., Dittus 1977, 1979; Mori 1979). As a consequence, the sorting of mtDNA haplotypes will be directly affected by these interactions in each generation. To the extent that a female's dominance rank is inherited, whether by cultural ''convention,'' as in macaques, or as a correlate of some genetic trait, the increases and decreases in frequencies of particular haplotypes will be reinforced each generation. Furthermore, the evolutionary fate of new mtDNA mutations is tied to the reproductive success of the female lineage that descends from the matriarch in which the mutation occurred.

A particularly salient aspect of macaque social structure is female philopatry. Social groups are organized according to matrilineal relationships (Sade 1972) and, while groups occasionally split when they become too large, females seldom transfer between groups (Sade 1972; Dittus 1975; see Moore 1984 for exceptions). The effects of group fission will be explored elsewhere (Hoelzer et al. in preparation), while the effect of migration rate on the maintenance and distribution of mtDNA variation is addressed in this paper. Dominance relationships among females within a group are quite stable. Status is inherited directly from mother to daughter by convention (Sade 1972; Schulman and Chapais 1980) and dominance rank is very rarely changed by aggressively challenging a female of higher rank (Walters and Seyfarth 1986).

Methods

The Simulation. One hundred, 200, or 300 females are initially distributed evenly among each of the four dominance ranks in each of the 25 social groups. Each female in the founding population is assigned a single identifying mutation, but no two founders share any mutations. Every adult female then gives birth to two daughters and dies prior to

the next round of reproduction; thus the generations are nonoverlapping. Daughters acquire new mtDNA mutations at the rate of one in 100 births without any provision for reverse, convergent, or parallel mutations. Estimates of natural mutation rates for mammalian mtDNA range from 5×10^{-9} to 5×10^{-8} mutations/site/year after selection (Wallace et al. 1987). The mtDNA molecule of macaques contains about 16,800 bp (Hayasaka et al. 1988; Melnick et al. 1992) and their generation time is about 10 years (Dittus, personal communication), so the mutation rate of 0.01 mutations/genome/generation is at the high end of a reasonable range. Daughters are then subjected to the survival probabilities of their inherited rank to determine which ones will make up the reproductive adult population.

Density-dependent mortality is built into the simulated population in two ways. First, the whole population is subject to a variable governor, which maintains the overall population close to the original size. Second, the size of local social groups is limited to 2.5 times the average group size to simulate local resource limitations. Because this model only deals with the females, actual group and population sizes would be larger. Group sizes vary stochastically below the maximum, which allows for the occasional extinction of local groups, as is known to occur in macaques (Dittus 1988). Indeed, the simulated population is appropriately described as a metapopulation (Murphy et al. 1990), as the model allows for both the extinction and recolonization of discrete sites.

The number of females that migrate between groups prior to reproduction is determined by the operator-specified migration rate. Migrants are either constrained to neighboring groups, as in a two-dimensional stepping-stone model (Kimura and Weiss 1964), or are free to choose among any of the 25 sites, as in the island model (Wright 1931, 1943). These procedures correspond to the presence or absence of geographical structure, respectively. In both cases, females from the lowest dominance rank of large groups move into the highest available rank of smaller groups in such a way that they enter a rank inferior to existing residents, or equal to residents of rank 4. In this way, migrating individuals from the bottom rank could potentially enter another group at rank 2 or 3 if there were only one or two residents present, thus improving their fitness and that of their daughters. A migrant could also potentially move into a vacant site and acquire the fitness of the top dominance rank.

When a dominance rank within a particular group becomes empty due to the mortality of all daughters born into that rank the remaining females are redistributed among the ranks to maintain a reasonable distribution of fitness differences among group members. The details of this procedure and all other aspects of the computer program are described elsewhere (Wallman et al. 1996).

Analyses. The following migration rates were used for both the island and two-dimensional stepping-stone models: 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 0.99. The rank-related survival probabilities were also varied systematically among the following sets (listed from highest to lowest dominance rank): 0.5, 0.5, 0.5, 0.5; 0.8, 0.6, 0.4, 0.2; and 0.8, 0.4, 0.3, 0.2. The equivalence of survival probabilities removes the effects of dominance, whereas the other two sets distribute survival probabilities in different ways and reduce the effective number of females (N_e) in the population.

The number of mutations shared by all members of the population (i.e., substitutions) was calculated when all of the founding matrilines but one had gone extinct and at generation multiples of 500 thereafter. The period prior to this point, called the lineage sorting period (LSP), measures time in a forward direction. The LSP is synonymous with fixation time (sensu Tajima 1990) and related to coalescence time (Hudson 1990) in that both tie existing genetic variation to an ancestral source; however, coalescence follows the confluence of matrilines as they are traced back in time, whereas the LSP traces the spread of a new mutation forward in time (Fig. 1). After the initial LSP, the single foundress to which all remaining matrilines can be traced may not be the same as the *most recent* shared matrilineal ancestor for the population because additional lineage sorting may have occurred within the lineage descended from the successful foundress prior to the loss of all other founding lineages. Therefore, the LSP is $\geq$ the time since the *most recent* shared matrilineal ancestor existed (i.e., the coalescence time).

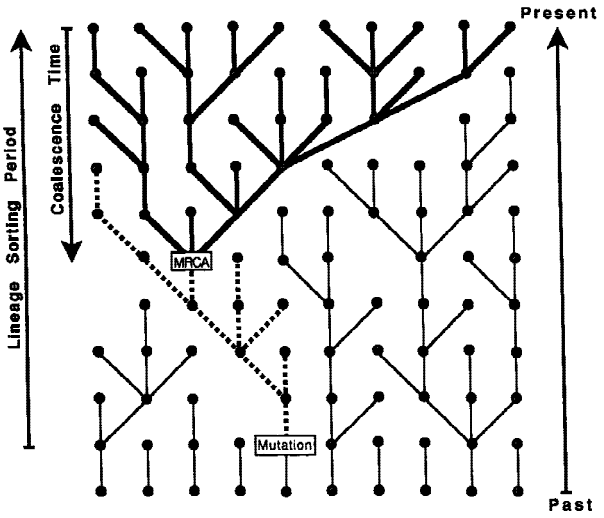

Fig. 1. A schematic representation of lineage sorting and coalescence among haplotypes. Because this figure illustrates the spread and extinction of mtDNA haplotypes in a population initially containing ten females (*hatched circles*), the furcation of lineages moving up (i.e., forward in time) represents the production of multiple daughters by a single mother. The termination of a lineage represents the failure of a mother to produce any daughters. Coalescence time in a population is determined by looking back in time from the present to the existence of the most recent common ancestor (*MRCA*) of all extant members of the population. In this case, the coalescence time is five generations. In contrast, the LSP is determined by starting at the time when a mutation occurred, defining the existence of a new haplotype, and looking forward to the time when all haplotypes in the population are derived from the female with the original mutation. *Bold lines* represent lineages that descend from the MRCA of all extant females. *Dashed lines* represent lineages descending from the female that defined the new haplotype, which eventually became fixed in the population but did not descend from the MRCA.

To further illustrate the distinction between coalescence time and the LSP we can compare the expected values of each under a simple model of population structure, the Wright-Fisher model. For nuclear alleles, coalescence is expected to occur in $4N$ generations (where $N = N_m + N_f$; Nei 1987), and the coalescence of mtDNA haplotypes is expected to occur in one-quarter the time of nuclear alleles (Moore 1995) because mtDNA is a haploid genome that is transmitted only through females. Therefore, mtDNA haplotypes are expected to coalesce in $2N_f$ generations. In contrast, the expected value of the LSP for mtDNA haplotypes is $4N_f$ generations (Tajima 1990).

Substitution rates were calculated by dividing the number of substitutions at the end of the LSP by the length of the LSP. This procedure gave the best estimate of short-term substitution rate relative to the expected value because it avoided the chance that the measurement would be taken after a long interval without substitutions, which would bias the estimate in a downward direction (see below). Because mutations have no direct impact on fitness in this simulation, neutral theory predicts that the substitution rate will be equal to the mutation rate ($\mu = 0.01$; Kimura 1983).

Each simulation was terminated after 10,000 generations or after the LSP, whichever was longer. Ten simulations were run for each combination of parameter values. Means and standard deviations were calculated for the length of the LSP and the substitution rate for each set of simulations. The significance of differences between the means

was determined by examining the distribution of 5,000 permutations in a randomized test (Manly 1992).

Results

The Null Model

A ''null'' model, in which neither social structure nor geographic structure was present, was created by setting all survival probabilities equal to 0.5 and allowing 99% of the individuals to migrate according to the island model each generation. Under these conditions, the simulated population mimicked a single random-mating or panmictic population with no group structure, roughly analogous to that assumed under most analytical models.

The null model yielded the following results: (1) The LSP lasted 292.7 ± 79.8, 640.7 ± 383.2, and 1,248.1 ± 537.8 ($\mu \pm$ sd) generations for population sizes of $N = 100$, 200, and 300, respectively. These means are all significantly different from one another (one-tailed tests, $P < 0.005$). Analytical theory predicts that the LSP for neutral mutants should be $4N_e$ (Kimura and Crow 1963); however, the expected distribution of LSPs is skewed (Kimura 1970), such that a relatively small sample of simulations will underestimate the mean of the theoretical distribution (e.g., Avise et al. 1984). Tajima (1990) overcame this sample size effect by examining the results of 10,000 simulations of evolution in a population with $N = 100$ and found a mean LSP of $3.99N$, but the most frequently observed range of LSPs was $2.5 - 3N$. Thus, the results from this simulation are consistent with the estimates of Avise et al. (1984) and Tajima (1990). (2) The substitution rates, measured at the end of the LSP, were 0.0052 ± 0.0022, 0.0060 ± 0.0033, and 0.0053 ± 0.0031 substitutions/generation for population sizes of $N = 100$, 200, and 300, respectively. As predicted by neutral theory, these rates were not significantly different from one another; however, they were only about half the base mutation rate (0.01 mutations/individual/generation). This discrepancy may be in part due to the arbitrary starting conditions of the simulation, which did not reflect an equilibrium distribution of genetic variation within the population. Nevertheless, the effects of varying parameter values on observed substitution rates cannot be attributed to initial conditions, and those results show that the depression of substitution rate is also caused by the retention of polymorphism (see below). Given a generation time of 10 years and a mtDNA molecule with 16,800 bp in macaques, the observed substitution rates translate into an evolutionary rate of about 3% per 1,000,000 years within a population, or 6% divergence between independent populations per 1,000,000 years. This is about double the empirically derived rate for primate populations (Brown et al. 1979). However, this discrepancy is not unanticipated given that we used a mutation rate that is at the high end of the range of empirically derived values, which span an order of magnitude.

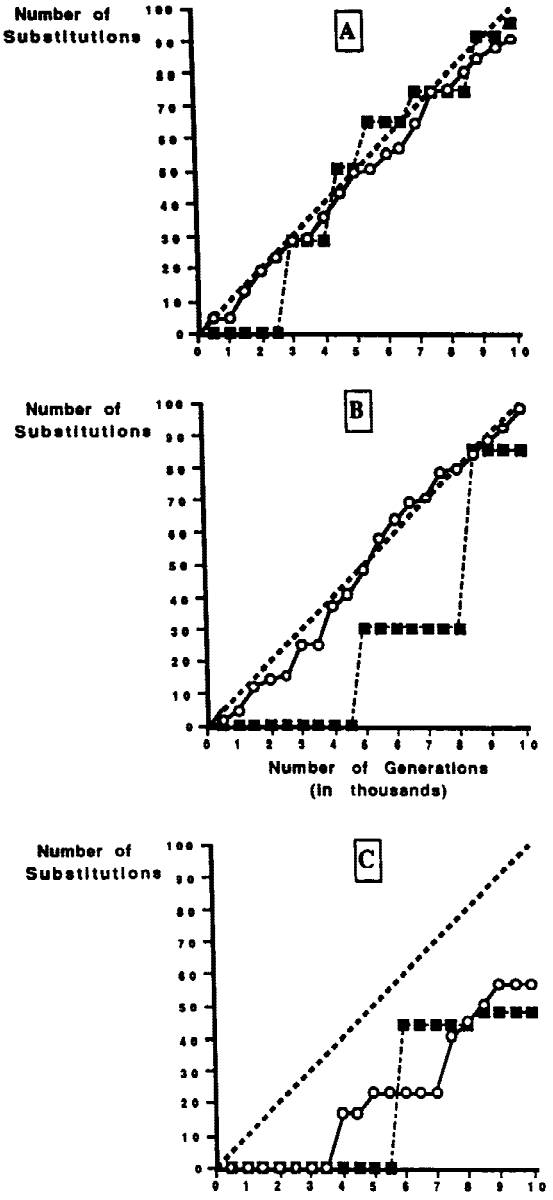

Fig. 2. The patterns of mutation substitution with time. Each set of data points represents a single simulated population. *Filled squares connected by a thin broken line* represent the two-dimensional stepping-stone model of migration, while *open circles connected by a solid line* represent the island model of migration. In all cases $N = 100$ and $m = 0.005$. The *dashed, diagonal line* represents the expectation of neutral theory, in which substitution rate equals the mutation rate. The different graphs illustrate the patterns seen under different social structures: **A** no social structure (i.e., fitnesses are equal across dominance ranks), **B** dominance ranks exhibit relative fitnesses of [0.8, 0.6, 0.4, 0.2], **C** dominance ranks exhibit relative fitnesses of [0.8, 0.4, 0.3, 0.2].

The Effects of Social and Geographic Structure

Patterns of Substitution and the LSP

Figure 2 illustrates the accumulation of substitutions over time in the simulated population ($N = 100$) with a

migration rate of $m = 0.005$ under both the island model and the stepping-stone model of migration. Under the island model the population accrues substitutions regularly and at a rate roughly equal to that predicted by neutral theory, unless the dominance-fitness relationship is skewed to the advantage of the most dominant matriline. When the fitnesses are skewed in this way the substitution rate is generally reduced and the pattern of substitutions is more irregular. The *near* concordance of populations without skewed fitnesses with the substitution rate predicted by neutral theory appears to contradict the results from the null models reported above. However, the apparent discrepancy can be explained by the procedure used to calculate substitution rates. The measurement is taken immediately following the LSP, which is a relatively brief period under the island model. The number of substitutions at that point is commonly about half the number predicted by neutral theory, but the continued gradual accumulation of substitutions closely parallels the predicted line. In contrast, the pattern of substitutions under the stepping-stone model follows a step function: periods without the occurrence of substitutions followed by the fixation of many mutations at once. The size of the steps is increased by the association of fitness with dominance status and even further by skewing that relationship to favor the success of the single most dominant matriline. The punctuated pattern of substitution under the stepping-stone model is caused by the retention of ''ancient'' polymorphisms that prevent the fixation of mutations. The coexistence of two matrilines that share a most recent common ancestor thousands of generations back ends when one of those matrilines goes extinct. At this time all of the mutations that have accumulated and become fixed within the surviving lineage also become fixed in the overall population. This produces the punctuated burst of substitution apparent as the step-up in Fig. 2.

Changes in the Lineage Sorting Period

The duration of the LSP was strongly affected by several factors (Fig. 3, Table 1). The most easily interpreted patterns emerged in the absence of a dominance-fitness association (Fig. 3A,B). For most rates of migration, the LSP varies in the neighborhood of $4N$. However, the LSP increases dramatically as m falls below 0.05, and this increase is more extreme for the two-dimensional stepping-stone model than for the island model. Indeed, analytical results show that the LSP *must* increase to infinity as m approaches zero, because N_e approaches infinity under these conditions (Nei and Takahata 1993).

When different fitnesses were assigned to different dominance ranks the relationship between the LSP and migration rate changed dramatically (Fig. 3C–F). Only at very high rates of migration was the LSP close to $4N$. LSPs increased substantially above $4N$ with decreasing m; first in the largest population ($N = 300$; $0.8 < m < 0.9$), then in the intermediate population ($N = 200$; $m \approx 0.8$), then in the smallest ($N = 100$; $m \approx 0.7$). In the smallest population, the LSP varied between $20N$ and $30N$ for $m \leqslant 0.5$. The extent of increase in the LSP at lower rates of migration was positively associated with population size, the two-dimensional stepping-stone model of migration, and the skewed fitness structure.

The increase in LSP caused by an association between dominance ranks and fitness in this model can be understood in terms of N_e. Skewing the fitnesses of females decreases N_e within groups, because the mtDNA lineages represented by low-ranking females are likely to go extinct or have reduced representation among the daughters in the next generation; however, the same factors increase N_e for the total population, because mtDNA lineages from the higher dominance ranks are unlikely to migrate or go extinct for a long time. The positive relationship between population subdivision and total N_e and a concomitant increase in the coalescence time are known from analytical models (Hoelzer 1997); thus, the effect of dominance structure on LSP observed in this model is not unexpected.

More surprisingly, the functional relationship between migration rate and the LSP changed when dominance-fitness associations were combined with larger population sizes. With a fitness distribution of (0.8, 0.6, 0.4, 0.2), the largest LSPs occurred at intermediate migration rates, although the LSPs were still shorter for the highest migration rates than for the lowest. Unfortunately, a quantitative assessment of LSP values for intermediate rates of migration was impossible due to a memory constraint inherent in the computer program. However, the inverted U-shaped function is evident from the ends of the distribution. Data points are only available for the high end of the range of migration rates with the fitness distribution of (0.8, 0.4, 0.3, 0.2), where the LSP increased dramatically with decreasing migration.

Changes in the Substitution Rate

Substitution rate, measured at the end of the lineage sorting period, also varied in response to some of the model parameters (Fig. 4, Table 2), although the high level of stochastic variation in substitution rate tended to obscure the patterns. Again, the simplest patterns emerged in the absence of fitness-dominance relationships. The substitution rate decreased with increasing population size. No clear effect on mean substitution rate was detected from the rate or pattern of migration, although the variation in substitution rate appears somewhat reduced under the two-dimensional stepping-stone model.

The dependence of substitution rate on population size and the similarity of results from the two models of migration remained consistent when fitness-dominance relationships were varied. However, this form of social structure seems to cause the apparent substitution rate to rise with increasing migration rate, at least for high rates of migration (> about 0.7). It is unlikely that this obser-

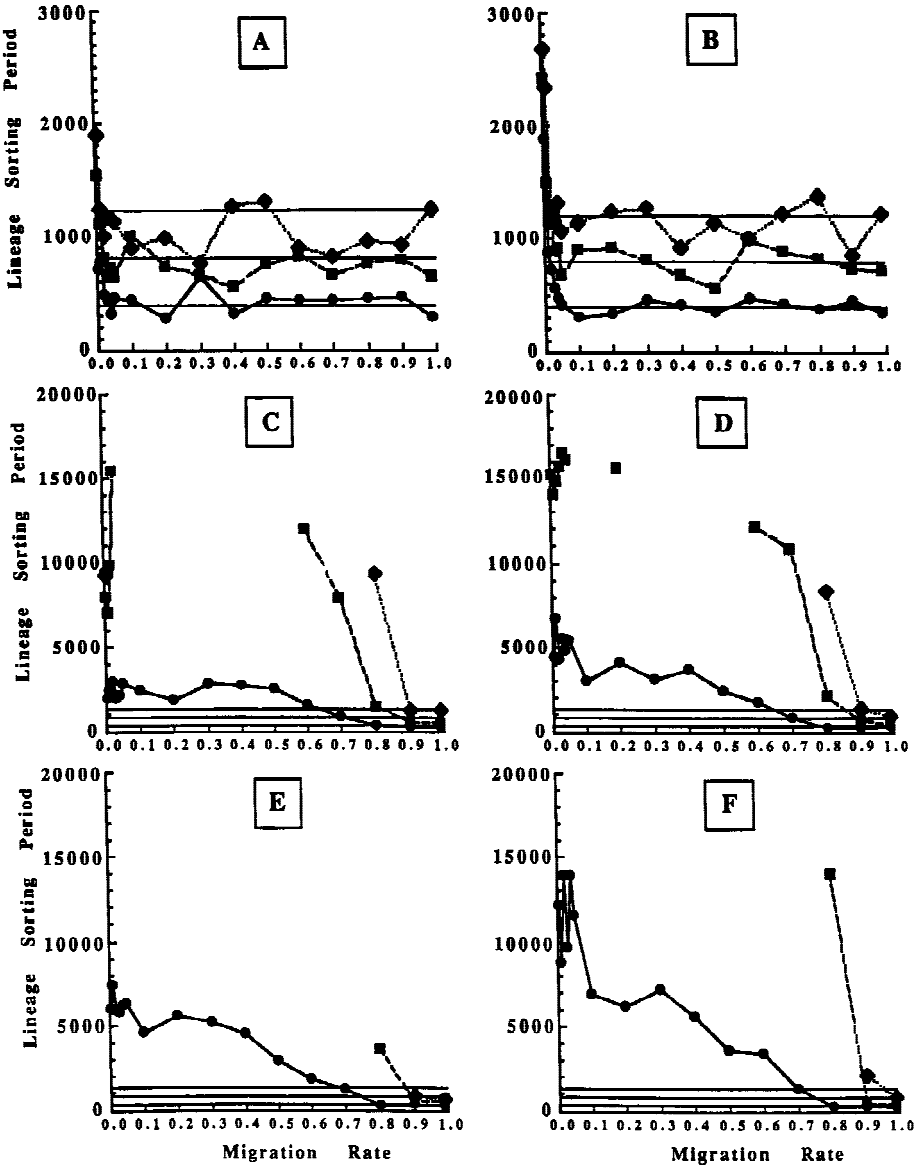

Fig. 3. The relationship between the LSP and migration rate. Each point represents the mean of ten simulations. Points are omitted where one or more simulations ended unsuccessfully by exceeding the memory capacity of the program, because the length of the LSP was positively correlated with the likelihood that a simulation would be aborted; therefore, an estimate of these points would be biased. *Circles connected by solid lines* represent $N = 100$. *Squares connected by dashed lines* represent $N = 200$. *Diamonds connected by dotted lines* represent $N = 300$. *Solid horizontal lines* indicate the predicted values of LSP for different population sizes (i.e., LSP $= 4N$). In **A, C,** and **E** migration occurs according to the island model; in **B, D,** and **F** the two-dimensional stepping-stone model applies. Relative fitnesses across dominance ranks are [0.5, 0.5, 0.5, 0.5] in **A** and **B,** [0.8, 0.6, 0.4, 0.2] in **C** and **D,** and [0.8, 0.4, 0.3, 0.2] in **E** and **F**.

Table 1. The effects of simulation parameters on the duration of the lineage sorting period

	Fitness structure		
Parameter	0.5 0.5 0.5 0.5	0.8 0.6 0.4 0.2	0.8 0.4 0.3 0.2
↑ Population size	↑	↑	↑
↓ Migration rate	↑, especially for $m < 0.05$	↑ with $N = 100$; Highest at intermediate values of m with $N = 200, 300$	↑ with $N = 100$; ↑ at intermediate values of m with $N = 200, 300$
[a]↑ Geographic structure	↑, especially for $m < 0.05$	↑, especially for small m	↑, especially for small and medium m with $N = 100$; ↑ for high m with $N = 200, 300$

[a] Comparison of the two-dimensional stepping-stone model of migration with the island model

vation can be explained by the decrease in N_e that occurs with increasing migration because changes in m most strongly affect N_e for low values of m (Chesser 1991), whereas the effect described here is most evident at high rates of migration. Again, the data suggest that the reverse relationship may apply for low rates of migration in larger populations, creating a U-shaped function, but the scarcity of data points for intermediate and low levels of migration does not permit a thorough analysis of this possibility.

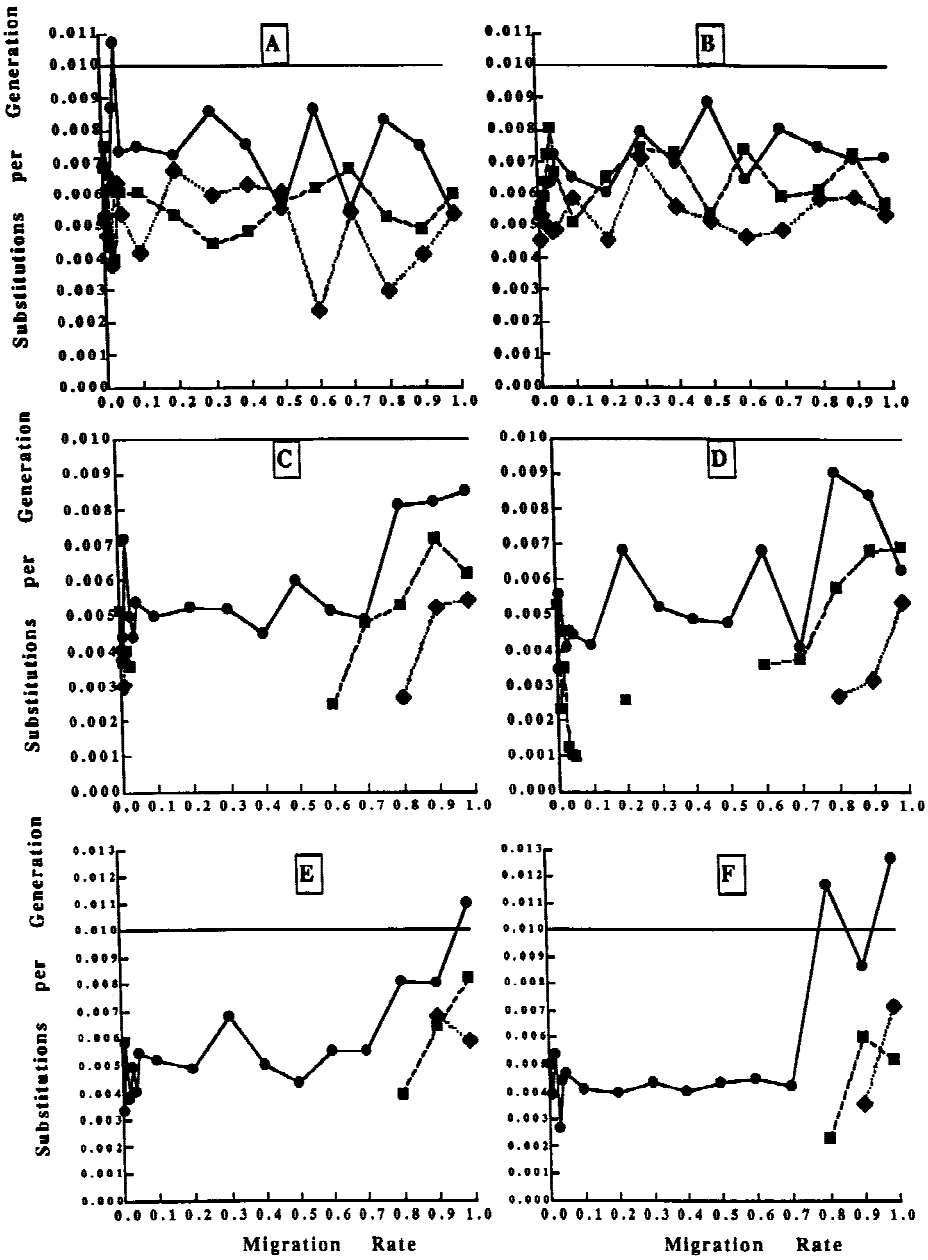

Fig. 4. The relationship between substitution rate and migration rate. This figure is organized in the same way as Fig. 3.

Table 2. The effects of simulation parameters on apparent substitution rate following the lineage sorting period

	Fitness structure		
Parameter	0.5 0.5 0.5 0.5	0.8 0.6 0.4 0.2	0.8 0.4 0.3 0.2
↑ Population size	↓	↓	↓
↓ Migration rate	No effect detected	↓ with $N = 100$; lowest at intermediate values of m with $N = 200$; ↓ at high values of m with $N = 300$	↓ with $N = 100$; ↓ at high values of m with $N = 200, 300$
[a]↑ Geographic structure	No effect	No effect	No effect

[a] Comparison of the two-dimensional stepping-stone model of migration with the island model

Relationship Between the LSP and Substitution Rate

The neutral theory of molecular evolution, which predicts that substitution rate will equal mutation rate, does not consider the potential influence of the LSP or other parameters included in the present model. Combining the results from all simulations run during the course of this study shows that substitution rate decreases monotonically with increasing LSP (Fig. 5). The relationship is

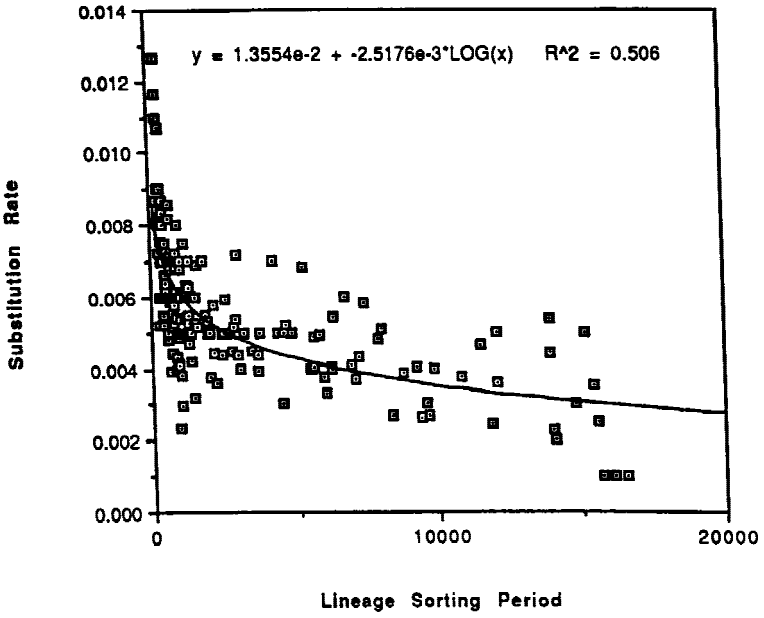

Fig. 5. The relationship between the LSP and the substitution rate. Each point represents the mean of ten simulations for a given set of parameter values and all combinations of parameter values are combined in this figure. The points have been fitted with a logarithmic regression. The regression equation is given along with the *r*-squared value.

best explained with a logarithmic regression. The substitution rate dropped rapidly until the LSP reached about 500 generations, after which it decreased much more gradually.

Discussion

Like any model, the computer simulation presented here contains properties that limit its general applicability. For example, it is possible that some of the effects of varying social structure in this model depend strongly on the near-perfect transmission of dominance from mother to daughter, especially in the higher dominance categories, found in macaques (Sade 1972; Schulman and Chapais 1980). Social structure could, of course, differ in other relevant ways. For instance, status might be determined by individual competitive ability, so that aggressive interactions could continually alter dominance relationships. In such systems, dominance potential might be inherited from each parent but actual status might change with age, vigor, and/or experience. It would be valuable to model such social structure to see if the effects on mtDNA evolution were substantially different from those in the model presented here.

Exploration of different parameter combinations in the simulation model described here confirms some of the conclusions of earlier models regarding the LSP, such as the positive relationship between population size and the LSP (Avise et al. 1984, Tajima 1990); however, this model also reveals other potentially important factors. For example, the length of the LSP can be strongly affected by rates and patterns of migration in a subdivided population. The length of the LSP directly translates into the sizes of the steps in Fig. 2. Figure 5 shows that the length of the LSP is also negatively related to the substitution rate. The depression of substitution rate is reflected by the distance between the peaks of the steps in Fig. 2 and the dashed line indicating the prediction of neutral theory. This distance is related to two factors in this model, one of which is important in natural populations. First, the starting conditions for the simulation do not reflect a natural distribution of genetic variation among individuals in the population, which might reduce the number of substitutions observed at the end of the LSP. More important, the observed number of substitutions is reduced by the retention of polymorphisms during the extinction of a particularly ancient lineage. Consequently, the number of substitutions observed in the population as a whole is less than the number of substitutions that could be counted for any single haplotype.

This perspective on lineage sorting and the process of substitution suggests that a biased underestimation of time since the divergence of mtDNA haplotypes, based on the extent of sequence divergence, can occur for two reasons. (1) The effective substitution rate will be depressed more over shorter divergence times relative to longer ones (Fig. 6A). This means that a molecular clock based on outgroup comparisons will frequently overestimate the substitution rate that should be used in the comparison of more recently diverged populations. (2) A potentially more serious reduction in the effective substitution rate relative to the underlying, long-term substitution rate can be produced by sampling long after the last burst of substitutions (Fig. 6B). This period represents the maintenance of ancient polymorphisms and can affect both the calibration and the application of a molecular clock. In practice the sampling of populations is

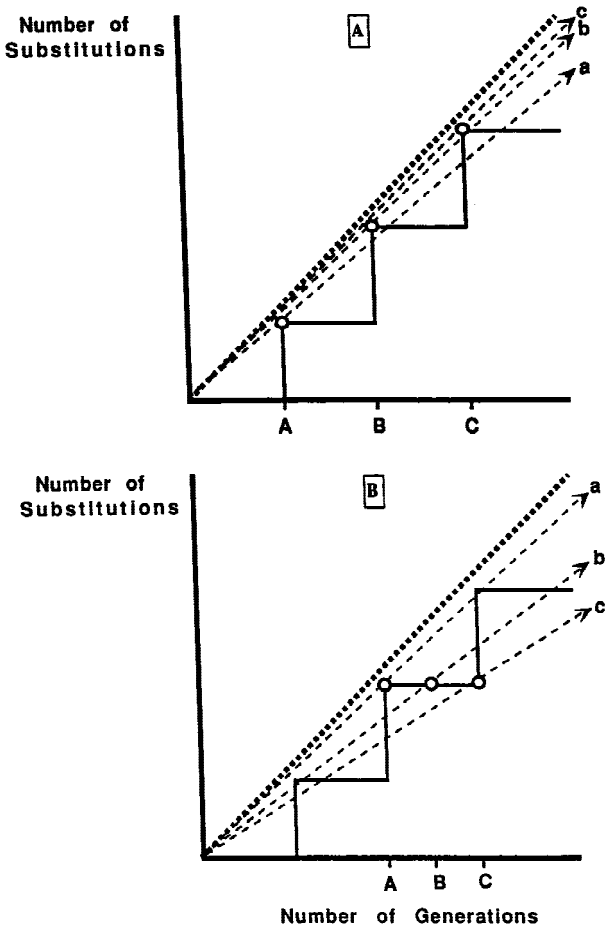

Fig. 6. A schematic illustration of the process of lineage sorting and the pattern of substitutions occurring in this simulation study. The *heavy dashed line* represents the rate of substitution predicted by neutral theory. The *step function* under the heavy dashed line represents the actual pattern of substitutions in a population. **A** *Thin dashed lines a, b,* and *c* represent substitution rates that would be estimated at times *A, B,* and *C,* respectively. The *slopes* of these lines all underestimate the underlying substitution rate despite the fact that they are the best possible times to make such an estimate. **B** This graph illustrates that the underestimation of substitution rate can be even more strongly influenced by the position on the plateau of a step when the estimate is taken.

arbitrary with respect to the timing of fixation events and there is rarely, if ever, data available to estimate the date of the last fixation.

Figure 6 also illustrates our view of the process of neutral evolution in mtDNA. The neutral theory prediction that substitution rate will equal the mutation rate (Kimura 1983) appears to accurately represent the underlying tendency of the simulation data. However, under most conditions, especially when migration follows the two-dimensional stepping-stone model, substitutions occur in a stepwise fashion, with the peaks of the steps usually falling short of the line predicted by neutral theory. Nevertheless, a linear regression through the points representing the number of substitutions at various points in time would parallel the neutral theory line. The same factors that increase the LSP also increase the distance between these two lines. Because these lines are parallel, the relative mean distance between them decreases with time.

Implications for the Molecular Clock

The interrelationships among patterns of migration, social structure, the LSP, and substitution rate suggest several potential problems with the application of the molecular clock to population relationships. First, the high stochastic variability of the substitution rate produces a large inherent error when assuming a constant rate (e.g., Nei 1992). Second, depression of the effective substitution rate by social and geographic structure would cause one to underestimate divergence time when assuming the underlying theoretical rate of molecular evolution or a clock calibrated by outgroup comparison that integrates substitutions over a much longer period of time. Finally, under conditions that promote very long lineage sorting periods, an exceptionally high level of mtDNA diversity could develop within a population such that a newly isolated group could already be quite distinct from most of the parent population as the result of biased sampling (Hoelzer and Melnick 1994). The difference between the two populations, or species, could also be increased by subsequent lineage sorting in the parent population. Consequently, the genetic distance between the two populations would lead to an overestimate of time since isolation.

In practice, the biased and variable depression of apparent substitution rate can be avoided by taking the average divergence between pairs of haplotypes in different populations without correcting for intrapopulational variation. This approach assumes that no mitochondrial gene flow has occurred between populations since isolation, which could result in the comparison of haplotypes that share a common ancestor that lived more recently than the separation of populations. It is also important to note that this procedure does not assure a more accurate time estimate because it does not correct for a biased overestimate caused by ancient polymorphisms or the stochastic errors inherent in both the mutational and sampling processes.

Unfortunately, we are unable to predict when divergence times are more likely to be underestimated or overestimated because measurement points are arbitrary and undefinable relative to the positions of plateaus and substitution events. However, it seems clear that these problems, as well as the stochastic error, decrease with the age of the split under examination. Thus these considerations suggest that applications of the molecular clock to recently isolated taxa should be made with caution. Other authors have pointed out that over long periods of time multiple substitutions at particular sites

erode the relationship between apparent substitution rate and time (Brown et al. 1979; Nei 1987). In effect, following a long period of isolation two lineages can become saturated with substitutional differences so that new mutations are equally likely to be convergent or divergent. Therefore, we suggest that estimates of time since isolation based on mtDNA sequence differences may be most useful within a window of isolation time, beyond the point where the influence of stochastic and biased errors can substantially reduce accuracy of the estimate and before substitutional saturation has occurred. The size and location of this window are likely to vary with the kind of data used (e.g., which gene, transversions only, etc.). The upper bound to this window can be estimated with the appropriate comparisons (Brown et al. 1979), but the lower bound remains elusive. The simulation model explored here points to some of the factors that influence the period of time that must pass before a molecular clock can be applied with confidence, but we are still unable to estimate the duration of this period for particular cases.

Lessons for Macaques and Other Real Populations

Our work on mtDNA variation in macaques provides an example of the maintenance of ancient polymorphism. Mitochondrial DNA sequence differences of 3–5% commonly occur within macaque species (e.g., Williams 1990; Melnick et al. 1992, 1993; Hoelzer et al. 1993), even among conspecifics at the same location (Hoelzer et al. 1994). We find that species with well-dated divergence times based on geological evidence exhibit far greater mtDNA divergence than would be expected based on a straightforward molecular clock rate of 2–4% per 1,000,000 years (Williams 1990; Melnick and Hoelzer 1992), as estimated by Brown et al. (1979, 1982). It is also conceivable that macaques have a dramatically accelerated molecular clock relative to other primates, but there is no direct evidence of this. The results presented here support the hypothesis that the interaction of strong geographical structure caused by female philopatry and the direct inheritance of female dominance has contributed to very long LSPs in this genus. The influence of social structure, geographical structure, and historical population size on mtDNA evolutionary dynamics should be considered in the design of intraspecific sampling and the interpretation of mtDNA data from other taxa, as well.

Acknowledgments. The motivation for this project came from discussions about the unusual features of macaque mtDNA variation within the Melnick lab and with others in the field. Thus, there are many individuals that provided useful contributions to this paper. The manuscript benefited from critical comments by J. Hey and L. Nunney. We would also like to acknowledge access to the computer facilities of the Biological Resources Research Center at the University of Nevada–Reno.

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