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# Microsatellite Evolution in the Mitochondrial Genome of Bechstein's Bat (*Myotis bechsteinii*)

**Frieder Mayer,[1] Gerald Kerth[2]**

[1] Institute of Zoology II, University of Erlangen–Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany
[2] Institute of Zoology, University of Zürich, CH-8057 Zürich, Switzerland

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**Abstract.** Being highly polymorphic, microsatellites are widely used genetic markers. They are abundant throughout the nuclear genomes of eukaryotes but rare in the mitochondrial genomes (mtDNA) of animals. We describe a short but highly polymorphic AT microsatellite in the mtDNA control region of Bechstein's bat and discuss the role of mutation, genetic drift, and selection in maintaining its variability. As heteroplasmy and hence mutation rate were positively correlated with repeat number, a simple mutation model cannot explain the observed frequency distribution of AT copy numbers. Because of the unimodal distribution of repeat numbers found in heteroplasmic individuals, single step mutations are likely to be the predominant mechanism of copy number alternations. Above a certain copy number (seven repeats), deletions of single dinucleotide repeats seem to be more common than additions, which results in a decrease in frequency of long alleles. Heteroplasmy was inherited from mothers to their offspring and no evidence of paternal inheritance of mitochondria was found. Genetic differences accumulated with more distant ancestry, which suggests that microsatellites can be useful genetic markers in population genetics.

**Key words:** Mitochondrial DNA — Control region — Microsatellite — Chiroptera — *Myotis bechsteinii*

*Correspondence to:* Frieder Mayer; *email:* fmayer@biologie.uni-erlangen.de

## Introduction

Microsatellites by definition are the shortest repeated DNA sequences, ranging from 1 to 6 bp (base pairs) in length. During the last decade, they have become widely used genetic markers in genome mapping, forensics, and molecular ecology for three major reasons (for reviews see Goldstein and Schlötterer 1999). (1) A high mutation rate alternating the copy number of repeats causes substantial allelic diversity. Heterozygosity of autosomal loci is usually above 0.7 in outbred populations. (2) The polymerase chain reaction (PCR) allows fast typing of genotypes and length polymorphisms can be easily detected using high-resolution polyacrylamide gel electrophoresis. (3) Microsatellites are common and widely distributed in the nuclear genome of eukaryotes and also occur, although at a much lower frequency, in the genomes of mitochondria and chloroplasts.

Because of their importance in modern molecular biology, the mutation mechanisms underlying the evolution of nuclear microsatellites have been studied intensively (reviewed by Ellegren 2000). A stepwise mutation mechanism is caused by slippage during DNA replication, if repetitive sequences re-anneal out-of-frame after displacement of the nascent strand (Levinson and Gutman 1987; Tautz and Schlötterer 1994). Germline analyses revealed that single-step mutations are more frequent than multistep changes (Primmer et al. 1998; Weber and Wong 1993). Furthermore, gains in repeat numbers seem to be more common than losses and mutation rate may be pos-

itively correlated with allele size (Amos et al. 1996; Primmer et al. 1998). Nevertheless, constraints on an upper limit of allele size apparently exist (Garza et al. 1995).

In contrast to nuclear microsatellites, little is known about microsatellites in the genomes of organelles. Microsatellites were reported from chloroplasts and mitochondria in plants (Doyle et al. 1998; Echt et al. 1998; Gugerli et al. 2001; Powell et al. 1995; Vendramin et al. 1999). In the yeast *Saccaromyces cerevisiae* mitochondrial microsatellite mutations were studied by inserting a reporter gene containing out-of-frame insertions of poly(AT) or poly(GT) tracts into the mitochondrial genome (Sia et al. 2000). Poly(GT) tracts were considerably less stable than poly(AT) tracts, and in contrast to nuclear microsatellites, alternations usually involved single repeat deletions rather than single repeat additions. In animals, due to the condensed length of their mitochondrial genome, repetitive elements are rare and usually are found only in the nontranscribed control region (Fumagalli et al. 1996; Lunt et al. 1998; Wilkinson et al. 1997). Microsatellites are known from the 3′ end of the control region, i.e., between the conserved sequence block one (CSB1) and the $tRNA^{Phe}$ gene. In several vespertilionid bat species including Bechstein's bat (*Myotis bechsteinii*), a hexanucleotide unit is repeated at least 20 times: $(CGCATA)_{22}$ in *Myotis bechsteinii* (GenBank accession number AY030078), $(GTATAC)_{31}$ in *Myotis myotis* (Petri et al. 1996), $(GCATAC)_{36}$ in *Plecotus auritus* (GenBank AY030077), and $(GCATAC)_{27}$ in *Pipistrellus abramus* (GenBank AB061528). In the turtle *Pelomedusa subrufa* a $(TA)_n$ microsatellite is part of a larger repeat unit (Zardoya and Meyer 1998). In seals and carnivores the repeat motifs $(AC)_{1–4}GT$ are repeated many times (Arnason et al. 1993; Arnason and Johnsson 1992; Hoelzel et al. 1993, 1994).

In this study, we investigated the evolution of an $(AT)_{5–14}$ microsatellite, which was found at the 5′ end of the control region of the Bechstein's bat's mitochondrial genome (Kerth et al. 2000). To the best of our knowledge, this study is the first approach to investigate the role of microsatellite stability and genetic drift in the evolution of an animal's mitochondrial microsatellite. Genetic polymorphism, i.e., the microsatellite's length variability, was studied within individuals (degree of heteroplasmy), among maternally related bats, and among maternally unrelated bats coming from different colonies. Distinguishing matrilineages is possible in Bechstein's bats because females live in closed societies (maternity colonies) that are demographically independent from each other due to absolute female natal philopatry (Kerth et al. 2000).

## Materials and Methods

### *Samples*

A total of 635 adult female Bechstein's bats were sampled in 33 maternity colonies occurring in Germany and Switzerland. We sampled between 1 and 90 females per colony. Bechstein's bat form maternity colonies comprising on average about 20 adult females, while males live solitarily (Kerth 1998). Members of a given colony are maternally closely related, belonging to one or two matrilineages only (Kerth et al. 2000). Therefore, colony members often resemble each other in their mitochondrial genome.

### *Genetic Analysis*

Total genomic DNA was isolated from a small wing tissue sample of all individuals (Kerth et al. 2000). To amplify the AT microsatellite a PCR was performed with primers P and B (Kerth et al. 2000) in a total volume of 15 µl, using approximately 20 ng total genomic DNA, 0.1 µl Pharmacia Taq polymerase, 1.5 µl 10 × PCR buffer, and final concentrations of 0.24 µM primers, 1.5 mM $MgCl_2$, and 0.2 mM dNTPs. An initial heating period of 4 min at 95°C was followed by 30 cycles each consisting of 94°C for 30 s, 55°C for 45 s, and 72°C for 50 s. The PCR was terminated by a 20-min incubation at 72°C. The length of the amplification products was determined by capillary gel electrophoresis with an ABI 310 automatic gene analyzer (Applied Biosystems). Primer B was labeled with the fluorescent dye FAM, TET, or HEX in order to size amplification products from three PCRs in a single run. Haplotypes were distinguished by their amplification length.

Sequencing of PCR products of 80 individuals with primer P confirmed that only a variable copy number of AT repeats caused the observed length polymorphisms in the fragment analysis. In all cases the number of AT repeats (5 to 11) was identical to the repeat number predicted by the length of the dominant amplified fragment. We did not find a single deletion or insertion outside the AT microsatellite.

To quantify the reproducibility of the PCR we sampled eight individuals twice and analyzed both samples. In addition, we evaluated the possibility of PCR artifacts by cloning PCR products from two individuals. One individual was homoplasmic for five AT repeats. The other bat was heteroplasmic for seven and eight repeats with an intensity of 67% of the $(AT)_8$ fragment and 33% of the $(AT)_7$ fragment. We analyzed five clones from each individual as described above. PCR amplification of five clones of the homoplasmic individual always resulted in a single band with the expected length of 5 AT repeats (100% band intensity in all cases). Amplification of five clones of the heteroplasmic individual revealed a fragment of eight AT repeats with a band intensity ranging from 98.2 to 99.0%. The shift from 67% from the bat DNA to at least 98% from the cloned PCR product indicates that PCR artifacts have only a minor effect on our measurement of heteroplasmy, if they occur at all.

To investigate the inheritance of heteroplasmy from mother to offspring we compared the individual distribution of AT copy numbers in 15 mother–offspring pairs, sampled in one colony where all members were heteroplasmic. Mother–offspring assignment was based on behavioral observations and the analysis of up to 11 nuclear microsatellites (Kerth et al. 2002b).

### *Data Analysis*

Individuals were classified according to their most common AT repeat number (dominant band). To analyze the frequency distribution of copy numbers within the population we calculated the

**Table 1.** Frequencies of AT copy number and diversity indices in 18 nursing colonies and in the total sample of 303 individuals

| | | Mean frequency of | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Colony | $N$ | $(AT)_5$ | $(AT)_6$ | $(AT)_7$ | $(AT)_8$ | $(AT)_9$ | $(AT)_{10}$ | $(AT)_{11}$ | $(AT)_{12}$ | $(AT)_{13}$ | $(AT)_{14}$ | $K_c$ | Mean $K_b$ | $G_{IP}$ |
| IB1 | 20 | 0.726 | 0.000 | 0.060 | 0.213 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.423 | 0.108 | 0.746 |
| UH | 19 | 0.947 | 0.053 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.100 | 0.000 | 1.000 |
| SB | 15 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | |
| RH | 2 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | |
| DU | 2 | 0.000 | 0.725 | 0.147 | 0.128 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.437 | 0.435 | 0.003 |
| BA | 14 | 0.006 | 0.964 | 0.030 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.071 | 0.060 | 0.144 |
| HB | 9 | 0.000 | 0.027 | 0.418 | 0.000 | 0.014 | 0.098 | 0.038 | 0.186 | 0.147 | 0.074 | 0.758 | 0.332 | 0.561 |
| GS1 | 19 | 0.000 | 0.089 | 0.901 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.180 | 0.161 | 0.106 |
| BW | 2 | 0.000 | 0.077 | 0.922 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.143 | 0.143 | 0.003 |
| GS2 | 5 | 0.000 | 0.020 | 0.980 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.038 | 0.035 | 0.080 |
| KL | 3 | 0.667 | 0.000 | 0.038 | 0.296 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.467 | 0.067 | 0.857 |
| RB | 15 | 0.000 | 0.000 | 0.043 | 0.506 | 0.123 | 0.325 | 0.000 | 0.000 | 0.000 | 0.000 | 0.622 | 0.266 | 0.572 |
| El | 10 | 0.000 | 0.000 | 0.004 | 0.730 | 0.266 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.397 | 0.066 | 0.834 |
| SD | 40 | 0.000 | 0.002 | 0.227 | 0.757 | 0.013 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.375 | 0.295 | 0.214 |
| GB1 | 18 | 0.000 | 0.000 | 0.125 | 0.808 | 0.068 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.327 | 0.135 | 0.587 |
| GB2 | 18 | 0.000 | 0.017 | 0.205 | 0.061 | 0.701 | 0.015 | 0.000 | 0.000 | 0.000 | 0.000 | 0.462 | 0.156 | 0.663 |
| BG | 2 | 0.000 | 0.000 | 0.500 | 0.000 | 0.161 | 0.000 | 0.306 | 0.033 | 0.000 | 0.000 | 0.629 | 0.259 | 0.589 |
| BS | 90 | 0.000 | 0.000 | 0.000 | 0.001 | 0.043 | 0.284 | 0.499 | 0.155 | 0.018 | 0.000 | 0.644 | 0.437 | 0.322 |
| Total | 303 | 0.170 | 0.061 | 0.153 | 0.220 | 0.076 | 0.104 | 0.152 | 0.052 | 0.010 | 0.002 | 0.853 = $K_t$ | 0.238 | 0.721 |

*Note*. $K$ indices were calculated from AT copy number frequencies within an individual ($K_b$) and mean values (columns 3–12) for each colony ($K_c$) using the formula $K = 1 - \sum p_i^2$. Total gene diversity ($K_t$) was calculated by using mean frequencies of the total sample. The $G_{IP}$ statistic describes the proportion of diversity within colonies, which is attributed to differences between individuals (Rand and Harrison 1989).

frequency of dominant AT repeat numbers per colony for all 33 colonies sampled. In addition, we quantified individual heteroplasmy within 304 bats coming from 18 colonies. Each individual had a dominant band, although relative band intensities varied substantially among individuals (e.g., Fig. 4). Bats were treated as heteroplasmic if, besides the dominant AT repeat, we detected one or more additional copy numbers, each having a peak area (determined with Genescan Version 3.1; Perkin Elmer) of at least 10% of the dominant band's peak area. This minimum threshold might lead to an underestimation of the proportion of heteroplasmic individuals, but it accounts for the possibility of slippage during PCR amplification. Using a 10% threshold is conservative because we observed a substantial number of bats which had no additional copy number beside the dominant AT repeat number or in which the additional band had less than 10% of the dominant band's peak area. Furthermore, additional bands were often one repeat longer than the dominant band, whereas slippage products are usually one repeat unit shorter than the template. Finally, cloning a heteroplasmic individual led to homoplasmic clones, suggesting that PCR artifacts contribute very little to the observed distribution of band intensities in individuals.

To quantify the degree of heteroplasmy per individual we calculated the gene diversity within an individual based on the frequencies ($p_i$) of all AT copy numbers ($i$), with $i$ ranging from 5 to 14 in Bechstein's bat: $1 - \sum p_i^2$. Hence homoplasmic individuals have a gene diversity of zero. Gene diversity within an individual is equivalent to the $K_b$ value of Birky et al. (1983). In a similar way gene diversity can be calculated for other levels of organization by using mean frequencies of AT copy number classes (Table 1; for technical details see Birky et al. [1983] and Rand and Harrison [1989]): $K_c$ = diversity within a colony and $K_t$ = diversity within the total sample. $K_c$ and $K_t$ were estimated from the mean frequencies of AT repeat numbers of each colony or of the total sample, respectively (columns 3 to 12 in Table 1). The total mtDNA diversity ($K_t$) was apportioned to within-individual, among-individual, and among-colony components as described by Rand and Harrison (1989).

Within individuals, $C_I = \overline{K_b}/K_t$
Among individuals within colonies, $C_{IP} = (\overline{K_c} - \overline{K_b})/K_t$
Among colonies within the total sample, $C_{PT} = (K_t - \overline{K_c})/K_t$

## Results

### *Overall Variation of Copy Number*

The AT microsatellite locus is located downstream of the origin of replication of the H strand (oriH) in a 195-bp nonrepetitive region between the R1 repeat array and the tRNA for proline. The AT repeats apparently lie outside of the displacement loop region, as several copies of D-loop termination-associated sequences (TAS) are located only in the R1 repeat region (Fig. 1). The dominant AT copy number ranged from 5 to 14 in 635 individuals analyzed. Comparing the frequencies of AT copy numbers among the 33 colonies studied revealed that the haplotype carrying seven repeat units was most frequent (Fig. 2A). From seven to higher repeat copy numbers the frequency of haplotypes decreased significantly (Sperman correlation coefficient $r_s = 1.0$, $p < 0.001$). Fewer than 5 or more than 14 repeat units were not observed.

### *Copy Number Variation Within Individuals (Heteroplasmy)*

The proportion of individuals that were heteroplasmic increased with increasing AT repeat numbers. It

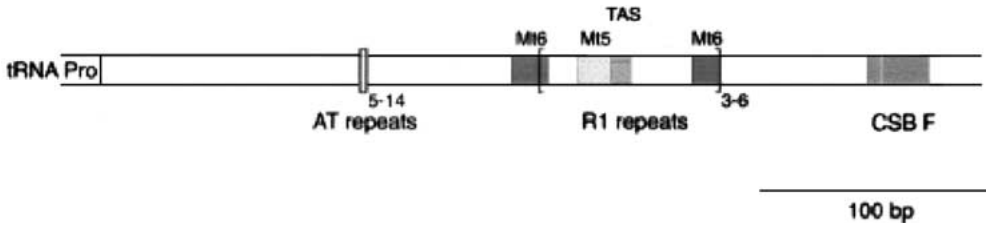


**Fig. 1.** Schematic sequence of the left control region domain of the mitochondrial genome of Bechstein's bat. The location of the *tRNA-Pro* gene, the AT microsatellite, and R1 repeats are given. TAS, termination-associated sequences. mt5 and mt6 are sequences that are widely conserved among bats and mammals and are located within R1 repeats (Wilkinson et al. 1997).

ranged from close to 0 to 100% (Fig. 2B). For example, all but 1 of 52 individuals with five AT repeats were homoplasmic. In contrast, all individuals having haplotypes of 10 or more AT repeats were heteroplasmic. The individual degree of heteroplasmy, i.e., gene diversity within an individual, increased with higher AT copy numbers (Fig. 2C). The average proportion of the most common AT copy number per individual decreased with increasing numbers of AT repeats, hence less frequent copy numbers gained in intensity (Fig. 3). Within a heteroplasmic individual maximally four different AT repeat numbers were found and the distribution of the AT copy numbers was usually unimodal. In other words, AT copy numbers were mostly consecutive within individuals (examples for mothers and their offspring are given in Fig. 4). However, 2 of 635 individuals (0.3%) showed a bimodal distribution of AT repeats. In one individual 7 and 9 AT copies and in the other individual 8 and 10 AT copies were more intense than the respective intermediate AT copy number.

### *Inheritance from Mother to Offspring*

The amplification pattern of the AT microsatellite in a mother closely resembled that of her offspring (Fig. 4). In all 15 mother–offspring pairs both individuals carried the same dominant copy number, with their intensity differing between 0.5 and 14.8% (mean ± SD: 6.1 ± 4.9%). This deviation did not differ from that of eight bats, which were sampled and analyzed twice (range, 0.7 to 15.5%; mean ± SD, 4.3 ± 4.9%).

The accumulation of interindividual differences was studied by an analysis of copy number variation within and between nursing colonies. A hierarchical analysis was possible, because female Bechstein's bats form nursing colonies of maternally related females (Kerth et al. 2000). Analyzing gene diversity within colonies ($K_c$) and within the total sample ($K_t$) (Table 1) revealed that in several colonies within-colony gene diversity ($K_c$) exceeded average individual gene diversity ($\overline{K_b}$). This is expressed by the *G*-statistic (Rand and Harrison 1989), which quantifies

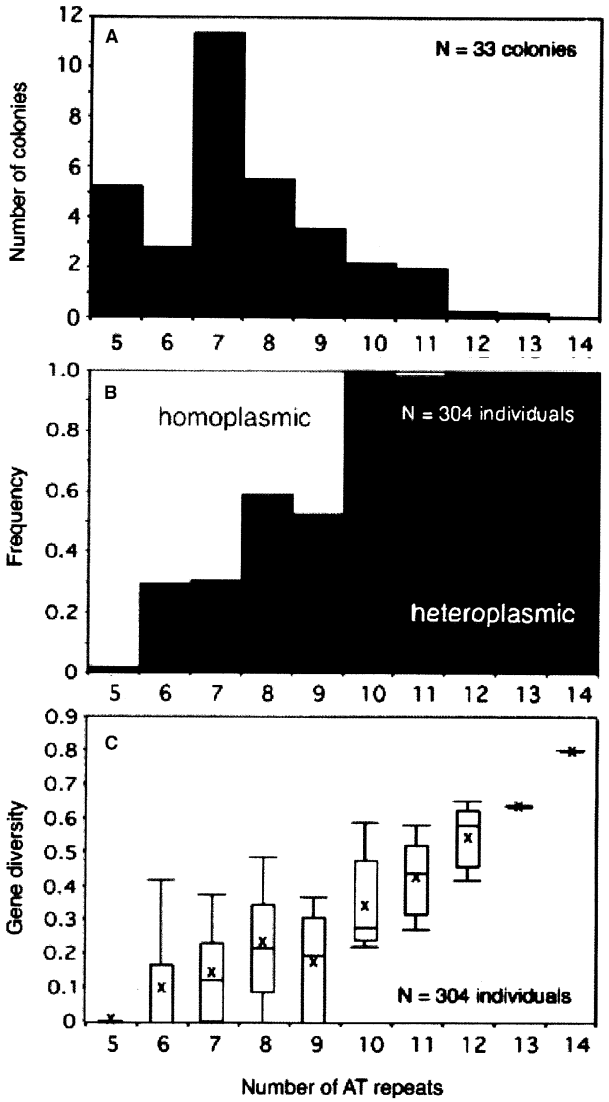


**Fig. 2.** (**A**) Number of AT repeats among colonies, (**B**) proportion of homoplasmic (white) and heteroplasmic (black) individuals, and (**C**) gene diversity within individuals, i.e., degree of heteroplasmy. Individuals were classified according to the dominant AT copy number. In A each colony was counted once considering the frequency of different AT copy numbers within a colony. One to 90 individuals were sampled per colony.

the excess of colonies' gene diversity in comparison to mean individual gene diversity (last column in Table 1): $G_{IP} = (K_c - \overline{K_b})/K_c$. In two colonies (SB and

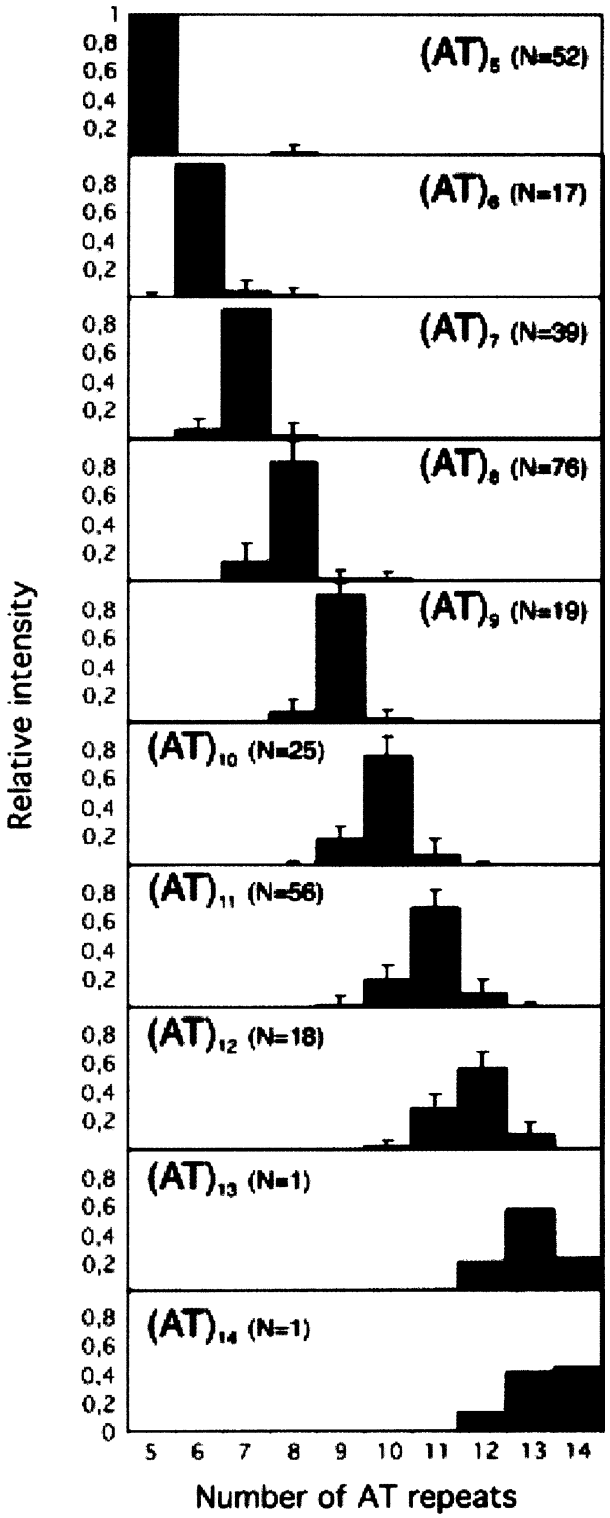


**Fig. 3.** Mean and standard deviation of band intensities of individuals, which were categorized according to the dominant AT copy number (5 to 14 AT repeats).

RH) all sampled individuals were monomorphic and homoplasmic for five AT repeats, thus $G_{IP}$ could not be calculated. Lower values of $G_{IP}$ and hence little genetic variation among members of a colony were found in five colonies (DU, BA, GS1, BW, and GS2), whose members had low repeat numbers (six or seven

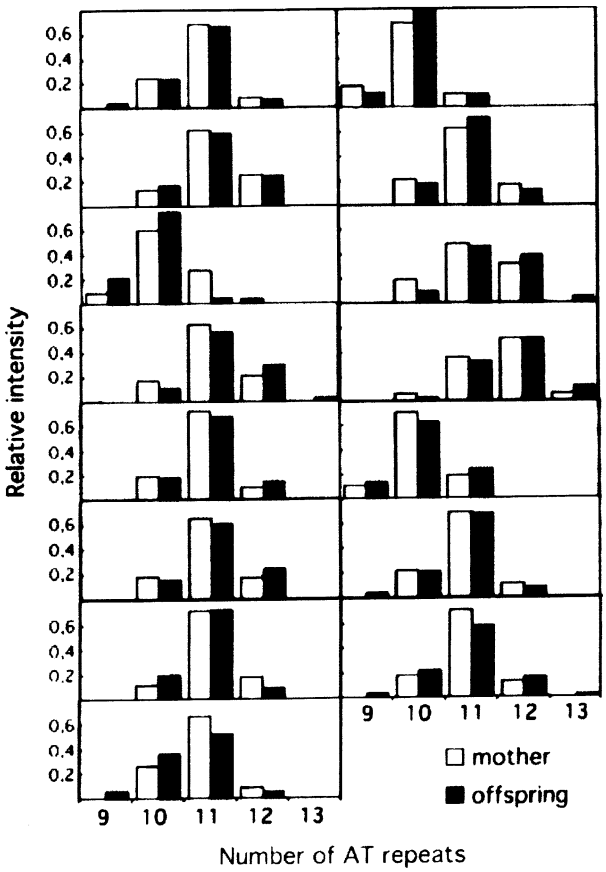


**Fig. 4.** Intensity of different AT copy numbers in 15 mother–offspring pairs.

AT repeats), and hence many individuals were homoplasmic. In contrast, $G_{IP}$ values were higher in the remaining 11 colonies, whose members had high AT copy numbers and/or showed a bimodal distribution of $(AT)_n$ frequencies. Bimodal distribution of AT repeats is caused by colony members belonging to different matrilineages (Kerth et al. 2000).

Mitochondrial gene diversity within the total sample of 303 individuals was hierarchically partitioned into diversity within an individual ($C_I$), among individuals within colonies ($C_{IP}$), and among colonies within the total sample ($C_{PT}$). As expected, the three diversity components summed up to 1. Most of the total diversity can be attributed to variation among colonies ($C_{PT}$ = 0.605). The remaining 39.5% of mtDNA variation partitions about equally into within-individual ($C_I$ = 0.192) and among-individual ($C_{IP}$ = 0.203) variation, if the mean $K_b$ value for the total sample is calculated as the average of colonies' mean $K_b$ values, i.e., each colony contributes equally to the mean $K_b$ value for the total sample.

## Discussion

The described short dinucleotide microsatellite, with a variable copy number of 5 to 14 AT repeats, is the

third type of repetitive DNA found in a bat species' control region of the mitochondrial genome. While R1 and R2 repeats occur in several vespertilionid bat species (Wilkinson et al. 1997) and other mammals (Fumagalli et al. 1996), the dinucleotide AT microsatellite is known only from Bechstein's bat (Kerth et al. 2000). In addition, the AT microsatellite is located outside of the displacement loop, while R1 and R2 repeats are located between termination-associated sequences (TASs) and the $tRNA^{Phe}$ gene (Wilkinson et al. 1997). A variable copy number of AT repeats was the only source of length variation of the DNA sequence between the $tRNA^{Pro}$ gene and R1 repeats among the Bechstein's bats investigated. Length of this region is highly variable between bat species (Wilkinson et al. 1997) and two insertions/deletions of a single nucleotide were found in the bat *Myotis myotis* (Petri et al. 1996).

A second AT microsatellite was found in the mitochondrial genome of Bechstein's bat (Kerth et al. 2002a). It is located outside the control region in a noncoding region between the genes 16S rRNA and tRNA-Leu. Dominant repeat numbers ranged from 5 to 10 repeats among 1433 bats and the frequency of repeat numbers decreased from 6 repeats to higher copy numbers (data not shown). Therefore, this second mitochondrial microsatellite showed a range and frequency distribution of the dominant AT copy number similar to those of the described AT microsatellite in the control region. At both loci, four or fewer AT repeats were never observed, although many bats carried five repeats and a large number of animals from a wide geographic range were investigated.

### *Heteroplasmy*

Most individuals analyzed were heteroplasmic, i.e., they carried mitochondrial molecules with different AT copy numbers. The frequency of heteroplasmy increased with higher AT copy number. Generally, the degree of heteroplasmy depends on the mutation rate and the strength of genetic drift in the maternally inherited mitochondrial genome. In addition, biparental inheritance (paternal leakage) could lead to heteroplasmy.

In the case of paternal inheritance of mitochondria, some heteroplasmic individuals should carry greatly different AT copy numbers. However, among the 635 individuals, only 2 bats showed a bimodal frequency distribution of AT repeats ($7+9$ and $8+10$ AT copies were dominant, respectively). All other animals either were homoplasmic or had a unimodal distribution of AT copy numbers. The lack of paternal leakage is best illustrated in the colony BS, with the largest sample size. All 90 BS colony members had between 10 and 12 AT copies, while low copy numbers (5 and 7) were common in neighboring colonies and among solitary males found in the vicinity of the colony BS (Kerth et al. 2000; Kerth and Morf 2004). But although female Bechstein's bats are known to mate with males born outside their colony (Kerth et al. 2002a, b; Kerth and Morf 2004), none of the females of the BS colony was heteroplasmic for a high and a low copy number. Thus, if it exists at all, paternal inheritance must be extremely rare and cannot account for the observed pattern of heteroplasmy in Bechstein's bats.

Heteroplasmy was inherited from mothers to offspring (compare Petri et al. 1996). The frequency distribution of AT repeat copy numbers differed within mother–offspring pairs to a similar degree as in reanalyzed individuals where deviations were due to PCR inaccuracies. A de novo generation of heteroplasmy in each generation is unlikely, because substantial differences between mothers and their offspring (e.g., shifts in the dominant AT copy number) were not found (Fig. 4) but would have been expected under this scenario, especially in mothers with high AT copy numbers. Despite the apparent fidelity in inheritance of heteroplasmy from mothers to their offspring, genetic differences within a matrilineage accumulated over generations because the mean genetic variation among individuals within a colony amounted to 20.3% of the total variation. Genetic drift in the maternal germline and mutations must account for interindividual differences. The hierarchical structure of mtDNA variation revealed the highest genetic differentiation among colonies (60.5% of total variation), which was three times higher than the genetic diversity among and within individuals. This partitioning of variation reflects maternal relatedness among colony members. Individuals of the same colony are more similar to each other than to individuals of other colonies, because females are highly philopatric and hence individuals of the same colony can be maternally closely related (Kerth et al. 2000).

Genetic variation within a colony strongly depended on the repeat copy number. In colonies where individuals carried high AT copy numbers, some colony members even differed in their dominant AT copy number, although they belonged to the same mitochondrial lineage (based on sequencing a part of the mitochondrial control region; cf. Kerth et al. [2000]). In contrast, in colonies where bats carried five AT repeats the bats were monomorphic. High levels of heteroplasmy led to more variation among colony members and hence to higher $G_{IP}$ values (Table 1). This follows the expectation and was also shown for crickets of the genus *Gryllus* which carry one to seven copies of a tandemly repeated sequence 220 bp long in their mitochondrial genome (Rand and Harrison 1989).

*Mutation*

The proportion of heteroplasmic individuals increased with higher AT copy number. It is unlikely that genetic drift alone causes this pattern, because the number of mitochondrial molecules within a cell and thus the impact of genetic drift should not vary with AT copy number. Instead, the observed increase in the proportion of heteroplasmic individuals, from close to 0 among bats with 5 AT repeats to 100% for bats that carry 10 or more AT repeats, indicates a strong increase in microsatellite instability. Thus, our data strongly suggest that in the AT microsatellite the mutation rate increases with increasing copy numbers, as has been shown for microsatellites in the nuclear genome (Primmer et al. 1996; Weber 1990; Wierdl et al. 1997).

High levels of heteroplasmy can be maintained only by high mutation rates, counteracting the loss of variability due to genetic drift acting at the time of reduction in mtDNA molecule numbers during oogenesis (Jenuth et al. 1996). All studies we know of suggest that segregation of mtDNA occurs in a population of 10 to 1000 molecules present in oogonia (Krakauer and Mira 1999). If such a mtDNA bottleneck takes place each generation during oogenesis in Bechstein's bats, the mutation rate leading to a deletion or addition of AT repeats must be unusually high to maintain the observed high levels of heteroplasmy. In contrast to our study, analyses of mitochondrial microsatellites in plants revealed little or no heteroplasmy (Doyle et al. 1998; Echt et al. 1998; Gugerli et al. 2001; Powell et al. 1995). This difference could be explained by lower mutation rates and/or more severe bottlenecks of mitochondria in the germ line of plants.

The fact that almost all heteroplasmic bats had a unimodal distribution of AT copy numbers strongly suggests that mutations usually delete or add only a single repeat unit. According to Monte Carlo simulations run with the software package EASYPOP (Balloux 2001), different single step mutation rates (ranging from 0.0001 to 0.41) always had a corresponding number of cellular mtDNA molecules, which resulted in a unimodal distribution at equilibrium (data not shown). Thus, a single step mutation model can explain the unimodal AT repeat pattern observed in heteroplasmic Bechstein's bats. Single step mutations have also been suggested to be the major mutation mechanism for nuclear microsatellites (di Rienzo et al. 1994; Primmer et al. 1998; Weber and Wong 1993).

In the mitochondrial genome of the yeast *Saccharomyces cerevisiae* single repeat deletions were more common than single repeat additions (Sia et al. 2000). This mutation pattern should lead to a deficiency of high copy numbers. Our data support this: the frequency of AT copy numbers among colonies and within heteroplasmic individuals decreased from seven toward higher copy numbers. Thus, and in contrast to studies of nuclear microsatellites, where mutations seem to be biased toward repeat additions (Amos et al. 1996; Primmer et al. 1996; but see also Harr and Schlötterer 2000; Xu et al. 2000), mitochondrial microsatellites may be prone to losing repeat numbers due to mutation. However, a copy number-dependent mutation model, suggested by Xu et al. (2000), could also explain the observed frequency distribution of AT copy numbers. According to this model, a constant mutation rate for expansion combined with an exponentially increasing contraction mutation rate over repeat length can cause a stationary distribution of repeat lengths with rare long alleles. It has recently been shown that a length-dependent mutation pattern agrees with experimental data and can explain the scarcity of long alleles (Calabrese and Durrett 2003; Huang et al. 2002; Lai and Sun 2003).

Alternatively, rarity of high copy numbers can be explained if molecules with high copy numbers have a selective disadvantage and thus cannot attain higher frequencies. Selection against high repeat numbers would be plausible from a mechanistic point of view. Poly(AT) is complementary to itself and thus can form a secondary structure. During replication the newly synthesized AT repeats could displace from the template L-strand and bind to itself, which might delay or even stop replication. Both the formation probability and the stability of such a secondary structure are likely to increase with more AT repeats. Thus, mitochondria carrying too many AT repeats may replicate less frequently.

## Conclusions

Our results show that heteroplasmy increased with higher copy numbers, which could be caused by an increased instability of a longer mitochondrial microsatellite. Genetic variability increased from within individuals (heteroplasmy) over among individuals within colonies to among colonies. Offspring closely resembled their mothers and differences increased with more distant ancestry. Differences accumulate slowly and most mutations involve alternations of a single repeat unit. Therefore, mitochondrial microsatellites can be used as genetic markers to estimate maternal relatedness within matrilineages as well as to distinguish matrilineages. Analyzing mtDNA sequence polymorphism is widely used in phylogeography and in revealing sex-specific dispersal behavior. Hence, mitochondrial microsatellites are highly desired genetic markers because they combine the advantage of high genetic variability to resolve intraspecifc rela-

tionships on the individual and population level with rapid and cheap analysis (Lunt et al. 1998).

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