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Extreme Sequence Heteroplasmy in Bat Mitochondrial DNA

Barbara Petri, Arndt von Haeseler and Svante Pääbo*

Institute of Zoology, University of Munich,
P.O. Box 202136, D-80021 München, Germany

* Corresponding author

Mitochondrial heteroplasmy is shown to be extensive when amplification products from the mitochondrial control region are cloned and sequenced from a European bat species. In contrast, a mitochondrial ribosomal RNA gene does not exhibit substantial levels of heteroplasmy when analyzed in an identical way. In the bat, heteroplasmy with respect to length as well as sequence seems to be transmitted from mother to offspring. Thus, the intra-individual sequence diversity seems to accumulate within the female germ line and its extent to be controlled primarily by purifying selection. Similar experiments in humans and a marsupial suggest that heteroplasmy may not be as uncommon among mammals as hitherto thought.

Key words: Bats / DNA sequence variation / Heteroplasmy / Humans / Mammals / mtDNA.

Introduction

Mitochondrial heteroplasmy, i.e. the coexistence within one individual of two or more mitochondrial genomes (mtDNA) that differ in primary structure, has been observed in a number of species, including eutherian mammals such as rabbit (Mignotte *et al.*, 1990; Biju-Duval *et al.*, 1991), monkey (Hayasaka *et al.*, 1991), American evening bat (Wilkinson and Chapman, 1991), pig (Ghivizzani *et al.*, 1993), elephant seal (Hoelzel *et al.*, 1993), and shrew (Stewart and Baker, 1994) as well as marsupials (Janke *et al.*, 1994) and monotremes (Janke *et al.*, 1996). In all these cases, the mitochondrial genomes within an individual differ in size due to variable copy number of repeated sequences located in the control region, which encodes no structural genes but contains sequences necessary for the replication and transcription of the mitochondrial genome. In contrast, heteroplasmy involving genomes that differ by base substitutions has been reported to be virtually absent in humans (Monnat and Loeb, 1985) except in diseases caused by mitochondrial mutations (see Wallace, 1993, for a review). However, recently, a number of cases have been reported where heteroplasmy occurs in healthy humans (Gill *et al.*, 1994; Comas *et al.*, 1995; Ivanov *et al.*, 1996; Howell *et al.*, 1996). Similarly, substitutional differences between genomes within a lineage of cows have been shown to occur (Olivo *et al.*, 1983; Laipis *et al.*, 1988; Ashley *et al.*, 1989). Furthermore, in some non-mammalian species, e.g. mussel (Hoeh *et al.*, 1991) and anchovy (Magoulas and Zouros, 1993), heteroplasmy with high levels of within-individual sequence divergence exists, and in those cases biparental inheritance of mitochondrial DNA has been inferred (Magoulas and Zouros, 1993) or shown (Skibinski *et al.*, 1994; Zouros *et al.*, 1994) to be the functional origin of the heteroplasmy. Here, we show that the coexistence within an individual of several different mitochondrial genomes, carrying many substitutional differences, is not the exception but the rule in bats. In other mammals, including humans, a similar situation, albeit to a lesser extent, may exist.

Results

The Mitochondrial Control Region of *Myotis myotis*

The nucleotide sequence of the mitochondrial control region from one individual of the European Vespertilonid bat *Myotis myotis* was determined and found to be 1548 bp long (Figure 1). Three conserved sequence blocks (CSBs), which have been observed in other mammals (Walberg and Clayton, 1981) are also found in *M. myotis*. A repeated element of 82 bp with the consensus sequence 5′-AAATTATTTACCACATGAATATTAAACAAGTACATCAGGAATATTAATATTACATAATACATACTATGTATAATTGTACATT-3′ was identified between the gene for the tRNA for proline and the CSBs. Furthermore, a motif of six nucleotides (GTATAC) repeated 31 times in the sequenced sample was found between CSB I and CSB II. In order to study the variation in length, as well as primary sequence, within and between individuals in the region containing the 82-bp motifs, primers flanking the repeat array were designed (Figure 1) and used for enzymatic amplifications.

Length Variation and Heteroplasmy

Enzymatic amplifications were performed from more than 200 bats, and amplification products were found to vary in length between approximately 400 bp and 800 bp among individuals. Direct sequencing showed that the variation in size was due to the 82-bp-repeat unit which varied in copy number between three and eight. Furthermore, approximately 40% of the animals displayed amplification products of two sizes.

Sequence Heteroplasmy

From six bats exhibiting size heteroplasmy, the two length variants were isolated from agarose gels and cloned. In

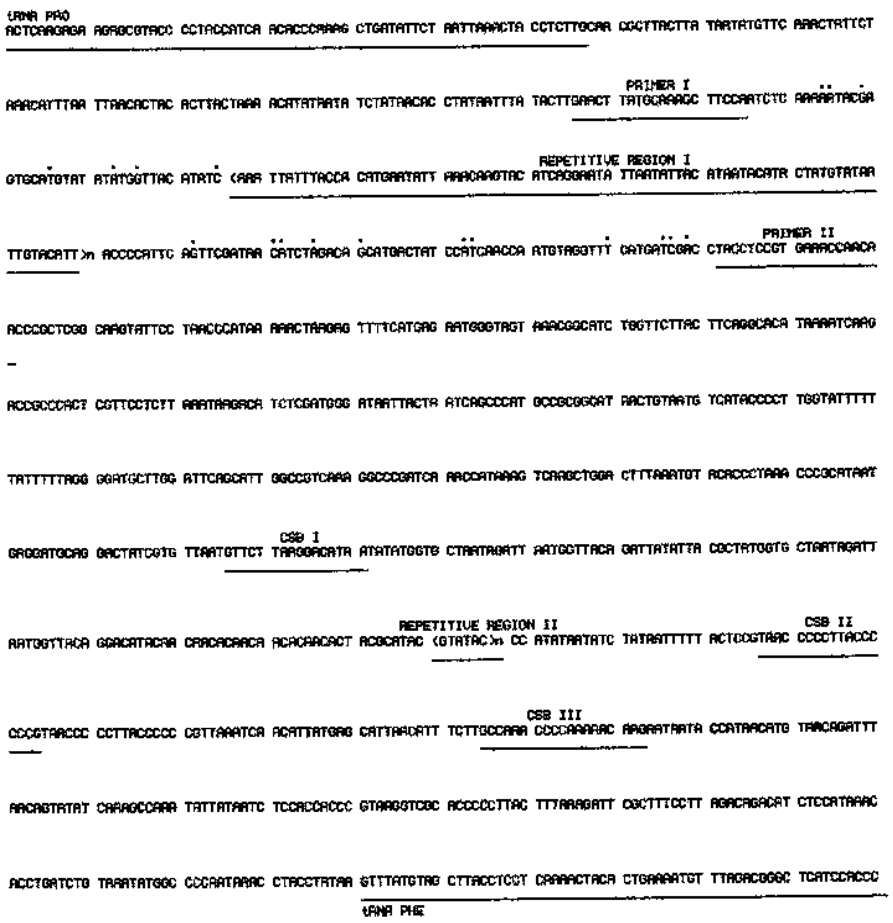


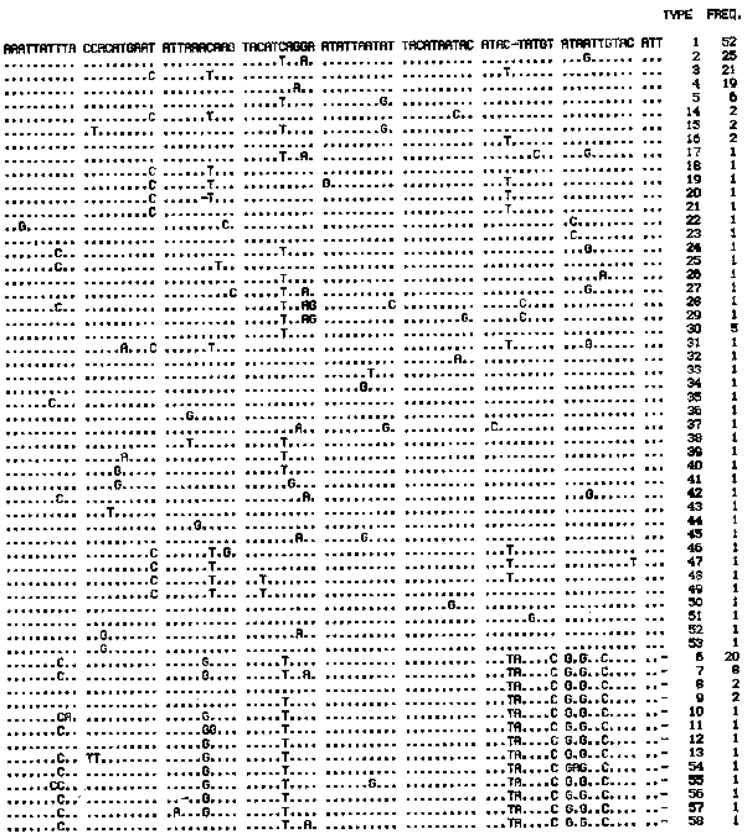
Fig. 1 Nucleotide Sequence of the Mitochondrial Control Region of *Myotis myotis*.
The tRNA genes for prolin and phenylalanine, which flank the control region, are underlined as well as the conserved sequence blocks (CSBs) (Walberg and Clayton, 1981). The arrays of repeated sequences and the primers used in this study are indicated. The 82-bp-repeat has been shown to occur in 1 – 8 copies per molecule. Dots indicate positions that are affected by substitutions in the region flanking the repeat (c.f. Figure 2, below).

addition, the single amplification product from one bat homoplasmic with respect to length was similarly isolated and cloned. Finally, the longer product from the offspring of one of the heteroplasmic individuals was similarly analyzed. One to ten clones of each size variant were sequenced. In total, 211 82-bp-repeats were compared. Fourthyeight sequence positions were variable, which together defined 58 repeat types (Figure 2). Thirteen repeats were observed more than once, the most common one 52 times.

In nine cases, two to ten clones were sequenced from the molecules of one and the same size and, surprisingly, clones differed by up to 8 substitutions in the repeat arrays. From the individuals B and H, which were maternally related, ten clones of the same size were sequenced from each animal and three and five, respectively, were found to be identical within and between the two animals in the repeat arrays whereas the others differed from each other.

The 150-bp-long single copy sequences that flank the repeat units also varied among clones representing molecules of the same as well as different size classes within and between individuals (Figure 2). Up to three substitutions and two insertion/deletions were found between clones of one and the same size class within an individual. Differences among molecules of different size classes within one individual ranged up to 4 substitutions and two insertion/deletions. Thus, the primary sequence variation is extensive among molecules of the same as well as different sizes. This is the case in individuals that are heteroplasmic as well as homoplasmic in terms of numbers of repeats. The variation in the repeats is apparently not generated by recombinational shuffling of repeats within the arrays since the variation also occurs in the regions flanking the repeats.

Fig. 2 MtDNA Repeat Types and Arrays in *Myotis myotis*.
Above, the sequences of the 82-bp-repeats determined from eight bats are shown as well as the frequencies with which they were found in the bats. Below, the sequence of repeats in clones from the animals are shown, numbered as above, as well as substitutions in the flanking sequences at positions indicated by dots in Figure 1.



A_1	A A G T A G C	21	22	1	2	6			A G A A G A T T T C A
A_2	· · · · · · A	3	23	25	2	13			G · · · · · · · · · ·
A_3	· · · · · · ·	18	2	24	11				G · · · · · · · · · ·
A_4	· · · · · · ·	3	1	2	6				G · · T · · · · · · ·
A_5	· G · · · · ·	20	2	9					G · · · · · · · · · ·
B_1	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
B_2	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
B_3	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
B_4	· · · · · · ·	3	1	4	27	12			G · · · · · · · · · ·
B_5	· · · · · · ·	3	1	4	17	6			G · · · · · · · · · ·
B_6	· · · · G · ·	3	1	4	2	56			G · · · · · · · · · ·
B_7	· · T · · · ·	3	1	4	2	57			G · · · · · · · · · ·
B_8	· · · · · · ·	3	1	45	2	55			G · · · · · · · · · ·
B_9	· · · · · · ·	3	16	4	2	6			G · · · · · · · · · ·
B_{10}	· · · · · · ·	3	44	4	2	54			G · · · · · · · · · ·
B_{11}	· · · · · · ·	19	16	8					B · · · · · · · · · ·
C_1	· · · · · · ·	14	15	5	5	5	28	6	G · · · A · · · · · ·
C_2	· · · · · · ·	14	15	5	5	5	29	6	G · · A · · A · T G
C_3	· · · · · · ·	3	3	1	26	2	10		G · · · · · · · · · ·
D_1	· · · C · · ·	31	33	1	4	2	6		G · · · · · · · · · ·
D_2	· · · · · · ·	3	34	1	37	42	6		G · · · · · · · · · ·
D_3	- T · · · · ·	1	1	1	41	7			G A · · · · C · · · ·
D_4	· · · · · · -	1	1	1	40	7			G A · · · · C · G · ·
E_1	· · · · · · ·	1	1	1	1	30	7		G A · · · · G · · · ·
E_2	· · · · · · ·	32	1	1	39	7			G A · · · · C · · · ·
F_1	· · · · · · ·	1	1	1	38	43	8		G A · · · · C · · · ·
F_2	· · · · · · ·	1	35	36	30	7			G A · · · · C · · · ·
F_3	· · · · · · ·	1	1	1	30	7			G A · · · · C · · · ·
G_1	· · · · · · ·	1	1	1	1	1	9		G A · · · G C · · · ·
G_2	· · · · · · ·	1	1	1	1	30	7		G A · · · · G · · · ·
G_3	· · · · · · ·	1	53	1	1	30	7		G A · · · · C · C · ·
H_1	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
H_2	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
H_3	· · · · · · ·	3	1	4	2	6			G · · · · · · · · · ·
H_4	· · · · · · ·	3	1	4	2	6			· · · · · · · · · · ·
H_5	· · · · · · ·	3	1	4	2	6			· · · · · · · · · · ·
H_6	· · · · · · ·	3	50	4	2	6			· · · · · · · · · · ·
H_7	· · · · · · ·	47	1	4	2	6			· · · · · · · · · · ·
H_8	· · · · · · ·	46	1	4	2	6			G · · · · · · · · · ·
H_9	· · · · · · ·	48	1	52	2	6			· · · · · · · · · · ·
H_{10}	· · · · · · ·	49	51	4	2	58			G · · · · · · · · · ·

Sequence Diversity within *vs.* among Individuals

To test whether the different molecules found within one individual are more similar to each other than to those of randomly chosen individuals from the population, pairwise alignments were computed for all pairs of the cloned repeat arrays, allowing for as many insertion/deletions of repeat units as necessary to maximize the similarity between molecules. For molecules of the same length the mean pairwise sequence differences of the aligned molecules ranged from 0.5 to 2.4 percent within one individual, from 0.6 to 2.3, and 0.3 to 3.4 percent among individuals in the German and Portugese nursery colonies, respectively, and from 0.3 to 3.4 percent among individuals from the pooled sample of the colonies. For pairwise comparisons between molecules of different size classes, the mean sequence differences (ignoring repeats that were aligned with gaps) varied between 0.5 and 3.0 percent within an individual, and 0.6 and 2.9 percent among all the animals analyzed. Thus, divergences between molecules within one individual were of the same magnitude as the divergences between molecules from any two animals in different European populations.

Maternal Inheritance of Heteroplasmy

In order to investigate whether the length variation is inherited from mother to offspring, 17 homoplasmic and 6 heteroplasmic mother/pup pairs were analyzed. The results showed that all pups had inherited homoplasmy or heteroplasmy from their mothers (Figure 3). Furthermore, in four of the heteroplasmic pairs, the approximate proportions of the two sizes in the heteroplasmic pups were similar to those of their mothers whereas in two cases offspring appeared to differ in the proportions of the molecules compared to their mothers. Further quantitative studies are necessary to clarify how much the heteroplasmy varies between different tissues within an individual and between generations. However, it is clear from these data that female animals generally transmit the state of being either homoplasmic or heteroplasmic with respect to repeat length to their offspring.

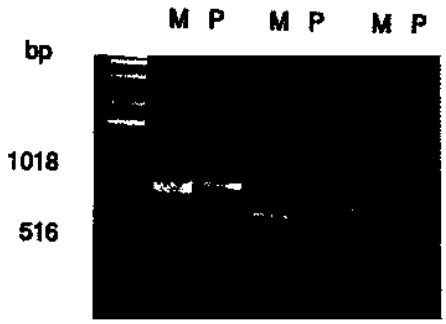


Fig. 3 Amplifications of the Repeat Array from Three Representative Mother (M)-Pup (P) Pairs, the First Two Heteroplasmic and the Last Homoplasmic.
In the first pair, the relative amounts of the two length variants differ between mother and offspring.

To investigate whether sequence heteroplasmy with respect to substitutions may be transmitted from mother to offspring, ten clones were isolated and sequenced from the offspring of individual B in which three out of ten clones were identical to each other in the repeat array (Figure 2) as well as the flanking region. In the offspring (individual H), three clones were identical to the three clones that were identical to each other in the mother whereas two differed from these by one substitution in the flanking sequence. The remaining five clones in the offspring differed by one to six substitutions from each other as well as from the clones derived from the mother. Thus, heteroplasmy with respect to as from the clones derived from the mother. Thus, heteroplasmy with respect to numbers of repeat units as well as sequence types seems to be transmitted from mother to offspring.

PCR and Cloning Artifacts

To rule out the possibility that variation would be generated by mutations during PCR and cloning, 360 bp of the 12S rRNA gene from two bats (individuals A and D) were amplified via PCR and cloned under conditions identical to those used for the control region. Four and two clones were sequenced from each of the two individuals, respectively, and no within- or between-individual sequence differences were observed among these six clones (data not shown).

In other experiments, a cloned copy of the human control region was amplified from approximately 10 000 copies and the amplification product was cloned in a fashion identical to the one used above. Eight clones were sequenced and only one substitution was found (C. Kilger, S. Germer, and H. Zischler, unpublished). Thus, neither the amplification process itself nor any particular property of the control region sequences *per se* are responsible for the high level of variation observed. Furthermore, when a mother and her pup were studied, sequence variants that differed from all other individuals studied, but were identical between the two related animals, were retrieved (Figure 2). Thus, although the mtDNA from the bats have not been cloned directly in order to exclude that the PCR generates the sequence variation observed, we feel that the results can be interpreted as largely reflecting the variation among molecules *in vivo*.

Discussion

The finding of extreme sequence heteroplasmy in bats is in apparent contradiction to the observation that upon direct sequencing of mitochondrial control regions from mammals, where a consensus sequence of the entire amplification product is determined, one sequence is generally observed. However, the extent of sequence variation is so great that only rarely will any particular sequence variant reach frequencies in an individual (approx. 10 – 20%) that allow it to be clearly seen in direct sequencing reactions. In the rare cases when this happens, it may often be overloo-

ked or regarded as a technical problem (Comas *et al.*, 1995). Furthermore, the level of heteroplasmy observed is in apparent contradiction to the observation of Monnat and Loeb (1985), who examined 248 clones of mitochondrial DNA from five humans and found only one intra-individual sequence difference in 49 000 base pairs. Similarly, in human leukemic cells, these authors failed to find evidence of sequence variation (Monnat *et al.*, 1985). Since the majority of the data presented in those studies refer to protein- and tRNA-encoding genes and the clones from the 12S rDNA sequences did not vary within individual bats, it may be that the sequence variation within an individual is subject to purifying selection. In that case, primarily positions that are under little functional constraint might vary in an individual and regions of the mitochondrial genome which evolve rapidly within a species, as well as between species, would be expected to show the most variation within individuals. However, since silent positions in protein-coding genes would still be expected to show variation under such a scenario, it is possible that the discrepancy between these results could reflect a difference in the inherent mutation rate of the control region as opposed to other parts of the mitochondrial genome. Furthermore, the data could point to a substantial difference between bats and other mammals, including humans, in the level of sequence heteroplasmy.

In order to investigate whether sequence heteroplasmy is limited to bats or if it is a general feature of mammalian mtDNA, and if so, whether it is related to the presence of variable numbers of repeated sequences in the control region, amplifications were peformed from an opossum *(Didelphis virginiana)*, a marsupial mammal, and a human. While the mtDNA of the former animal exhibits length heteroplasmy due to repeated sequences in the control region (Janke *et al.*, 1994), neither obviously repeated sequences (Anderson *et al.*, 1981) nor length heteroplasmy have been observed in humans except in association with rare neuromuscular diseases (see *e.g.* Wallace, 1993) and in the case of a homopolymeric tract of cytosine residues (Bendall and Sykes, 1995). From the opossum, 528 bp of the mitochondrial control region were sequenced from seven clones. All clones differed from each other by one to four substitutions (data not shown). From the hypervariable region I of the human control region, 21 clones, each containing 286 bp, were sequenced. Of these, 13 clones were identical while eight differed by one to three substitutions (data not shown). Thus, these observations support the recent observations of heteroplasmy in humans (Ivanov *et al.*, 1996; Howell *et al.*, 1996) and indicate that substantial levels of mitochondrial heteroplasmy can exists also in other mammals, albeit at lower levels than in bats. Further work is needed to clarify to what extent heteroplasmy exist in other species, including humans.

The amount of sequence diversity among molecules within an individual bat matches that among the homologous sequences in the population of bats. Two possibilities, which are not necessarily mutually exclusive, are conceivable to explain the generation of the intra-individual diversity. First, the diversity could accumulate in female lines over time; second, the mitochondrial diversity which exists in the population could penetrate the female line by the contribution of male mitochondrial genomes at fertilization. Our results indicate that the former possibility is sufficient to explain the observed heteroplasmy. Size homoplasmy and heteroplasmy were transmitted from mother to pup in all mother/offspring pairs analyzed, and in one case where numerous clones in a mother contained one particular sequence, the same sequence was also found in a similar proportion in the offspring. These observations call into question the existence of a dramatic bottleneck in population size of mitochondrial DNA molecules in the female germ line. Additional support for this comes from studies of human families, where mitochondrial heteroplasmy persists in several maternally related individuals (Howell *et al.*, 1992; Ivanov *et al.*, 1996; Howell *et al.*, 1996). Thus, substitutions can be expected to accumulate in the female germ line over a time span of many organismal generations. The other possible source of heteroplasmy, paternal contribution, has been demonstrated in mussels. However, in mammals paternal mitochondrial contribution is at best very low (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995). In humans, less than one out of 10 000 mitochondrial DNA molecules stems from a putative paternal contribution (C. Kilger, unpublished). Thus, no effective homogenizing mechanism between generations seems to exist in the female germ line. Consequently, mitochondrial sequence diversity can accumulate over generations. Furthermore, the fact that regions of the mtDNA that evolve more slowly than the control region, e.g. ribosomal RNA genes and protein coding genes contain little intra-individual sequence diversity (Monnat and Loeb, 1985), indicates that purifying selection acts on the molecules in the female germ line.

The fact that as much sequence diversity exists within an individual as in the entire population of bats indicates that the effective population size of mitochondrial DNA molecules of the female germ line in *Myotis myotis* is as large as, or larger than, the effective population size of female animals. This allows the coalescent of within-individual sequences to be as deep as that of the consensus sequences determined for individuals in the population. The relationship between the intra-individual and inter-individual sequence diversity obviously depends on the population history of the species under study as well as the mechanisms of mitochondrial DNA replication and transmission in the female germ line. It will be interesting to determine how the relative coalescent of intra- and inter-individual sequences differs in various species and populations.

Materials and Methods

Samples, DNA Isolation, PCR, Cloning and Sequencing

Tissue samples of 3 mm^2 were removed from the tail membranes of 80 *Myotis myotis* individuals from two nursery colonies in

Bavaria, Germany (Au Church and Beyharting Church) and of 21 individuals from one nursery colony in Portugal (Mina Lousal Cave). Mother/pup pairs were sampled when pups were still attached to their mothers' nipples. DNA was either phenol or salt extracted (Maniatis *et al.*, 1982), yields being approximately 15 μg per sample. The mitochondrial control region from *Myotis myotis* was amplified and sequenced using primers L15926, L16007 and H00651 (Kocher *et al.*, 1989). Two primers (5′-GAACTTATGCAAAGCTTCCA-3′ and 5′-GGGTTGGTTTCACGGAGGTA-3′) were designed and used to amplify a region exhibiting length variation. Primers L1091 and H1478 (Kocher *et al.*, 1989) were used to amplify the 12S RNA gene. Opossum control region sequences were amplified using primers L 16652 (5′-CCTTACCCCCTAAACAAGAA-3′) and H17079 (5′-TTAAGCTACATTAACTTTGTG-3′) (Janke *et al.*, 1994), whereas human control region sequences were amplified with primers L16078 (5′-AGTATTGACTCACCCATCAA-3′) and H16410 (5′-GCGGGATATTGATTTCACGG-3′) (Anderson *et al.*, 1981). L and H in the primer designations refer to the light and heavy strands and the numbers to the nucleotide at the 3′-end of the primer in the respective mitochondrial sequence. PCR products were either directly sequenced (Bachmann *et al.*, 1990) with the primers used for amplifications, or cloned. In the latter case, the bands were excised from agarose gels, purified by glassmilk (Geneclean, Bio 101, La Jolla, Ca.), ligated into a plasmid vector (TA Cloning, Invitrogen, San Diego, Ca.) and transformed into the *Escherichia coli* Sure™ strain (Stratagene, La Jolla, Ca.). Alternatively, 5 μl of the total PCR amplification were used for ligation. Clones were sequenced from both directions using primers in the vector and in the amplification product.

Sequence Analysis

Alignment of the repeat region was performed with a modified Needleman-Wunsch procedure (Needleman and Wunsch, 1970) where the similarity between two repeats defined the scoring function with no penalty for insertions and deletions of repeat units. Pairwise comparisons were carried out using the PAUP (Swofford, 1993) program package.

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