Natural radioactivity and human mitochondrial DNA mutations

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Edited by Henry C. Harpending, University of Utah, Salt Lake City, UT, and approved August 9, 2002 (received for review July 5, 2002)

Radioactivity is known to induce tumors, chromosome lesions, and minisatellite length mutations, but its effects on the DNA sequence have not previously been studied. A coastal peninsula in Kerala (India) contains the world's highest level of natural radioactivity in a densely populated area, offering an opportunity to characterize radiation-associated DNA mutations. We sampled 248 pedigrees (988 individuals) in the high-radiation peninsula and in nearby low-radiation islands as a control population. We sequenced their mtDNA, and found that the pedigrees living in the high-radiation area have significantly ($P < 0.01$) increased germ-line point mutations between mothers and their offspring. In each mutation case, we confirmed maternity by autosomal profiling. Strikingly, the radioactive conditions accelerate mutations at nucleotide positions that have been evolutionary hot spots for at least 60,000 years.

Natural radiation varies geographically, but it is never absent and has been irradiating all forms of life since the beginning of evolution. Chromosome lesions and cancer are well known macroscopic results of ionizing radiation (1). Current research is now focusing on the effects of radiation on the DNA sequence itself and has recently revealed an intriguing multigenerational destabilization in repetitive DNA loci in the germ line of irradiated mice (2). Such findings are of particular significance to present and future descendants of professional radiation workers (3), and of people exposed to radiation in Hiroshima, Nagasaki (4), and Chernobyl (5), but also to evolutionary geneticists who rely on a constant molecular mutation rate for reconstructing prehistory from extant alleles. In humans, long-term experimental irradiation and monitoring for point mutations across generations would be impractical and unethical, so instead we have taken advantage of the natural setting in the south Indian state of Kerala, the coast of which contains the world's highest levels of natural radioactivity in a densely populated area. The increased radioactivity is due to the local abundance of monazite, a mineral containing ≈10% thorium phosphate. The radioactive strip measures an area of only 10 km by 1 km, but supports a population of several thousand, whose traditional occupation is fishing (6). The biologically effective radiation dose received by the coastal population is 10,000–12,000 μSv per year, approximately 10 times greater than the worldwide average. Studies in Kerala on rat morphology (6), Down's syndrome (7, 8), chromosomal aberrations (9), and congenital malformations (10) could not conclusively reveal significant abnormalities in the local residents, and direct DNA sequencing has never been performed in intergenerational radiation studies in Kerala, or indeed elsewhere in the world.

In this study, we sequenced the noncoding mtDNA control region of 988 Keralese individuals from 248 families to directly determine whether lifelong exposure to high levels of natural radiation increases the mutation rate, and if so, whether its effects differ from long-term evolutionary mutational change. MtDNA is inherited maternally and is therefore simple to trace within pedigrees; another technical advantage is the high copy number of several thousand per cell. In addition to the intrinsic medical, forensic, and evolutionary interest in mtDNA (11–16), the mtDNA control region is ideal for radiological studies for two reasons: first, the normal mtDNA control region mutation rate is high enough (17) for us to expect mutations even in the control pedigrees, and second, mutational hot spots are known in detail from evolutionary mtDNA studies (18), allowing a comparison between prehistoric DNA sequence evolution and current radiation-associated mtDNA evolution.

Subjects and Methods

Saliva samples were obtained with informed consent from 730 healthy individuals from 180 families (spanning 595 mtDNA transmissions) living in the high-radiation seashores of Puthenthura (lat 8°57.2′N, long 76°31.8′E), Neendakara (lat 8°56.8′N, long 76°32.1′E), and Chavara (lat 8°57.8′N, long 76°31.8′E). The samples were taken mainly from families living between the highway and sea, where the radioactivity is highest (6). Furthermore, we sampled 258 control individuals (from 68 families spanning 200 mtDNA transmissions) who lived 3 km to the southeast on low-radiation islands off Mukkad (lat 8°55.4′N, long 76°33.4′E), namely the Fatima, Kanakkan, Puthen, and Arulappan islands. Nine of the families were sampled from Mukkad itself and nine from Saktikulamkara (lat 8°55.4′N, long 76°32.5′E). The sampling locations are indicated in Fig. 1. Individuals were selected for sampling if they and their sampled maternal ancestors had spent their lives at the sampling locations. These criteria excluded migrants between high- and low-radiation areas in our study. In practice, few such recent or ancestral migrants were encountered. The inhabitants of the high- and low-radiation areas are phenotypically, culturally, and linguistically indistinguishable, and we found the same major mtDNA clades in the radiation and control areas (Fig. 2 and Table 1). The residents in both areas are mainly Hindu (non-Brahmin) who nevertheless eat fish. The sampling time-depth is 2.9 generations in both areas. In the high-radiation area, 20 families were sampled in 2 generations, 155 families in 3 generations, and 5 families in 4 generations. In the low-radiation area, 14 families were sampled in 2 generations, 49 families in 3 generations, and 5 families in 4 generations. The average mtDNA generation times in the high- and low-radiation areas are also very similar. The sample generation time (based on all sampled mothers) is 25.0 (SD 6.1) and 26.5 (SD 7.2) years, respectively, and the long-term generation time (based on dead mothers and postmenopausal mothers >55 years old) is 29.7 (SD 7.4) and 31.6 (SD 7.8) years, respectively. The Keralese mtDNA generation time of ≈30 years may appear high, but is identical to the preindustrial European and Canadian average (19–21).

In general, the bulk of natural radioactive dose received by soft tissues such as the gonads is derived from three sources: terrestrial, dietary, and cosmic radiation; altogether they amount to an

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: np, nucleotide position.

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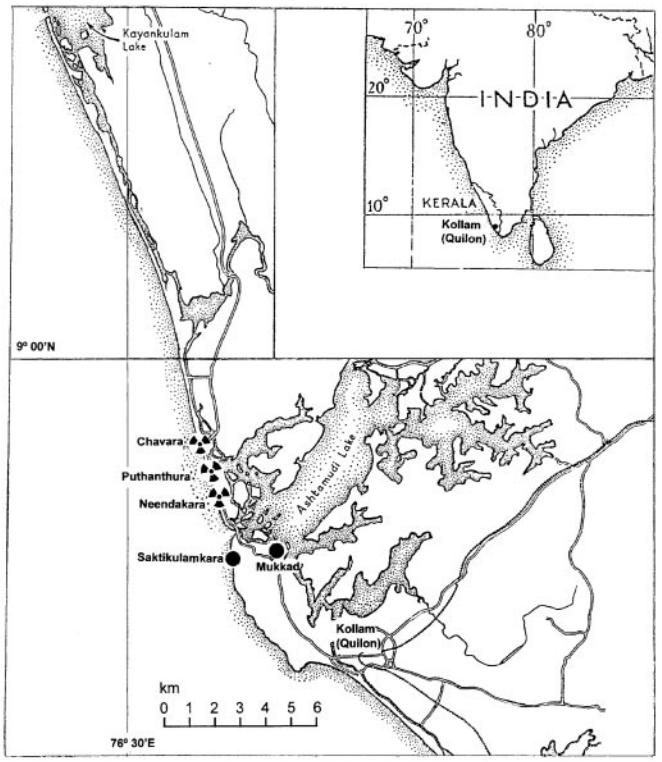

Fig. 1. High-radiation and low-radiation localities sampled in this study. The radioactivity in the peninsula increases from Kayankulam Lake to Ashtamudi Lake, with a peak radioactivity around Chavara. Radioactive areas are easily identifiable by the presence of black monazite sand, rather than the white nonradioactive sand elsewhere. The control samples were taken mainly from four lake islands off Mukkad, and partly from the white sand seashore of Saktikulamkara. The bridge across the mouth of Ashtamudi Lake was built in the 1920s. The District capital of Kollam (formerly Quilon) is shown for orientation.

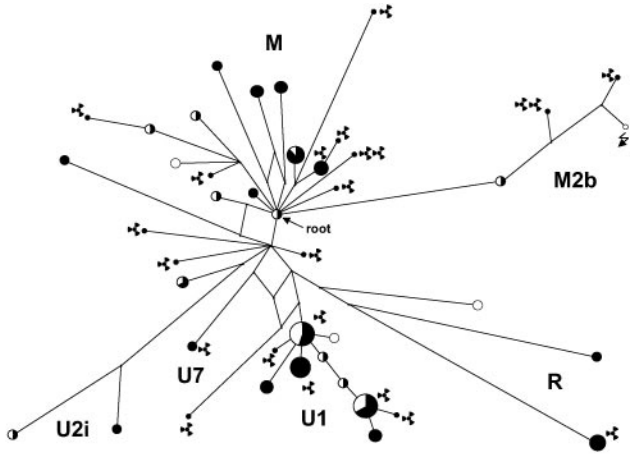

Fig. 2. Skeleton network of Keralese mtDNA. Black shading in the circles indicates samples from the high-radiation area, and white indicates those from the low-radiation area. Links represent mutations and circles represent mtDNA types, the circle size corresponding to the number of families with that type. Mutant family mtDNA types in the high- and low-radiation areas are marked by radiation symbols and a flash symbol, respectively. The root and the mtDNA groups M, U, etc. are indicated in accordance with ref. 35. The network encompasses 203 of the total of 248 families, by selecting mtDNA types occurring more than once and adding the mutant family mtDNA types. Calculations were made with NETWORK 3.111 (www.fluxus-engineering.com) by sequentially using the RM option with $r = 2$ and the MJ option with $\varepsilon = 1$, followed by postprocessing to remove nonparsimonious reticulations in M2b. Length polymorphisms around nucleotide position (np) 16189 and np 309 were disregarded, as was variation at nps 152 and 195. Other hypervariable positions were assigned half weights: nps 16129, 16189, 16311, 16362, and 146, and a transversion at np 16318 was assigned triple weight.

average of 1,100 μSv per year in Germany (22) for example. In Chavara, Puthanthura, and Neendakara the terrestrial radioactive dose (γ-radiation emitted in the ^{232}Th series) has been measured by personal dosimetry and averages 9,000, 8,000, and 6,500 μSv, respectively (coastal sections 6-1, 6-2, and 6-3 in refs. 23 and 24). Gross dietary radiation caused by ^{232}Th (and measured by its daughter nuclide ^{228}Ra) is 162 pCi (1 Ci = 37 GBq) per day in the high-radiation peninsula (25, 26), but the corresponding biological dietary dose is not available in the literature. We therefore calculated the conversion by using information from Eisenbud and Gesell (see ref. 27), who give the conversion factor for ^{226}Ra from μCi per time unit to rem (1 rem = 10^{-2} Sv) per time unit as 25, which we take as an approximation for ^{228}Ra after multiplying by a factor of 5/2.2 to include the α decays of the daughter nuclides of the short-lived ^{220}Rn, whose half-life of 56 s is not long enough to allow diffusion out of the body. However, this conversion factor would refer only to bone dose (radium as an alkaline earth is preferentially deposited in bone). The soft tissue receives ≈10% of whole body radium (28); thus we divided the bone dose by 10 and obtain a soft tissue biological dose of ≈3,000 μSv per year. Dietary potassium-40 intake (25) contributes another 250 μSv according to the potassium-40 conversion factor (27). Cosmic radiation at equatorial latitudes contributes ≈350 μSv at sea level (29). In summary, the gonadal dose in the three high-radiation localities would amount to 10,000–12,000 μSv per year, approximately 10 times greater than in monazite-free areas.

Within each family, only samples covering the maximum number of transmissions were sequenced (for example in the family shown in Fig. 3, only individuals I.1, II.3, III.1, and III.2 were initially screened). When mutations were observed, all family members were sequenced. DNA was extracted from the dried saliva by the Chelex method. Nucleotide positions (nps) 15971 to 00484 (numbering as in ref. 30) were amplified by PCR using primers LF1 (5′-TTA ACT CCA CCA TTA GCA CC-3′) and LF4 (5′-TGA GAT TAG TAG TAT GGG AG-3′) and the amplicon was purified by excision from agarose gel after electrophoresis and then by silica columns (Qiagen, Hilden). This

Table 1. mtDNA clades and radiation-associated mutations in Kerala

	Control		Radioactive		
Clade*	Absolute	%	Absolute	%	Mutations
M	4	6	24	13	5
M1	0	0	10	6	
M2b	4	6	4	2	4†
M3	1	1	1	1	
M4	2	3	10	6	2
M5	1	1	3	2	
R	2	3	15	8	2
R1	0	0	1	1	
U1	46	68	79	44	5
U2i	3	4	13	7	
U7	0	0	5	3	2
Other	5	7	15	8	3
Total	68	100	180	100	23

*Clades as defined in ref. 35.
†Includes the single mutation in the control group.

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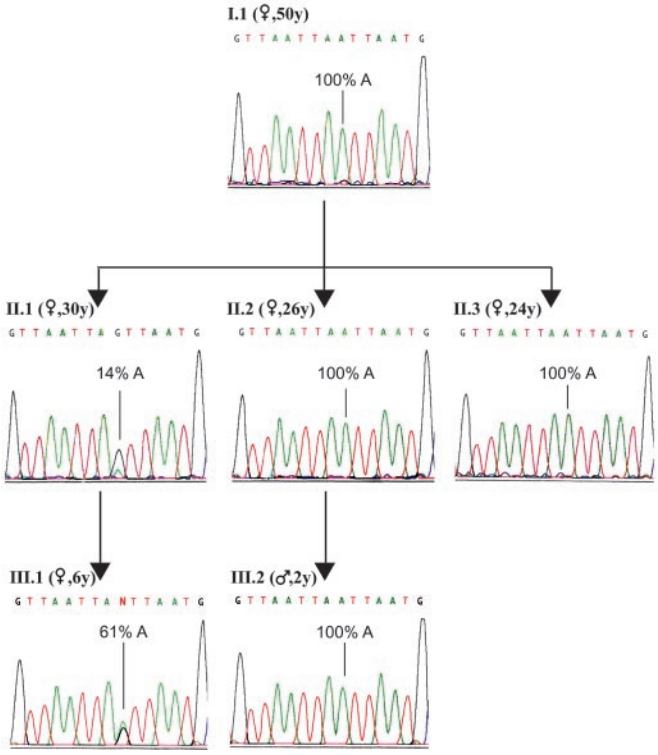

Fig. 3. Inheritance of a new mtDNA mutation. Each member of this family was born and lived exclusively in the high-radiation peninsula of coastal Kerala, India. The percentages of the new variant were determined by gravimetrically comparing the reduction of the A peak area relative to a neighboring reference peak in this forward sequencing reaction. Analogous evaluation of the reverse sequencing reactions (not shown) yielded similar values, both agreeing with our counts of bacterially cloned mtDNA amplicons.

purification step ensured a low baseline in the following sequencing reaction, performed with the Perkin–Elmer Big Dye Terminator kit by using primers LF1 and LF4 as well as primers LF2 (5′-GAG GAT GGT GGT CAA GGG AC-3′) and LF3 (5′-CAC CCT ATT AAC CAC TCA CG-3′). The sequences were analyzed on an Applied Biosystems Prism 310 Genetic Analyzer, and each was determined at least from nps 15990–16390 and from nps 35–465. The data are presented in Table 3, which is published as supporting information on the PNAS web site, www.pnas.org. Each sample was sequenced twice; except when a mutation was discovered, in which case the sample was reextracted, reamplified, and sequenced for a third and fourth time. Mutations were scored with the Sequence Navigator (ABI, Weiterstadt, Germany) and were always rechecked manually. When heteroplasmy was observed, the proportions of both the mutant and the original nucleotide were determined gravimetrically by using an internal reference peak area (31). Mutant and original nucleotide peak areas were always determined from forward and reverse sequencing reactions from two independent PCR amplifications; the error was always found to be less than 10%; nevertheless we considered only peak area changes of more than 20% as real changes to further minimize any risk of false positives. The gravimetric method relies on the low baseline of Big Dye Terminator chemistry on the ABI 310 Genetic Analyzer, and was confirmed in the Freiburg laboratory by conventional bacterial cloning (32) of 100 PCR amplicons for a sample containing two heteroplasmic mutations at nps 144 and 152. In this sample, the two independent gravimetric measurements yielded 39% and 40% for 144C (cloning yielded 49% 144C), and 56% and 58% for 152C (cloning yielded 52% 152C).

When putative mtDNA mutations were observed within families, maternity testing was performed by using the nonaplex STR kit AmpFlSTR Profiler (PE Applied Biosystems, Weiterstadt). Maternity was accepted if the probability exceeded 99.15%.

Results and Discussion

Three children were identified as undisclosed adoptions on the basis of the maternity test, leaving 988 individuals in the study. Without maternity testing, erroneous results would have been obtained, wrongly suggesting 25 full "mutations" instead of none. In the genetically related family members, we observed 22 partial (heteroplasmic) mutations in 595 high-radiation transmissions, and only 1 mutation in 200 low-radiation transmissions, significantly more at $P < 0.01$ (χ^2 test). An example of an mtDNA mutation passed down in a pedigree is shown in Fig. 3. In the 22 high-radiation mutations, 18 families had 1 heteroplasmic position, and 2 families had 2 heteroplasmies (one family at nps 144 and 152 and another at nps 16189 and 16272). It is striking that we observed only heteroplasmic mutations, and that, furthermore, no mutation was observed to attain fixation in any individual even after sequencing up to two descendent generations. Previous pedigree studies yielding implausibly fast mtDNA mutation rates lack either maternity tests or an adequate differentiation between full and heteroplasmic mutations (see ref. 17, p. 364), and may therefore tend toward overestimation.

We can exclude the possibility that we are mostly observing somatic mutations rather than germ-line mutations by analyzing saliva, because in 10 of 10 mutation cases where a sample from a descendant of the mutant individual is available, the mutant nucleotide was passed down to the descendant in clearly detectable amounts (>20%). We can furthermore exclude the possibility that we are observing mtDNA lineage-specific events, as the mtDNA profiles of the high- and low-radiation areas are similar, and, in any case, the observed mutations are not restricted to any particular mtDNA clade (Table 1 and Fig. 2).

The radiation-associated mtDNA mutations in this study share both hallmarks of mutations reconstructed in the evolutionary tree of Eurasian mtDNA (Table 2), which has a coalescent age of about 60,000 years (33, 34). First, both evolutionary and radiation-associated mutations are strongly biased toward transitions. According to Table 2, within the mtDNA region considered here, evolutionary mutations consist of 95.5% transitions, 3.0% transversions, and 1.5% insertions/deletions. The radiation-associated mutations consist of 95.5% transitions and 4.5% transversions. Second, the evolutionary and radiation-associated mutations occur predominantly at the same nucleotide positions (Fig. 4). This visual impression can be quantified by comparing the nucleotide mutation rates. The average evolutionary mutation rate is 0.46 mutation per nucleotide position (267 mutations in 579 nucleotides of HV1 and HV2). If radiation-associated mutations were distributed randomly across the sequence, their mutation rate would be the same, namely 0.46. However, the radiation-associated mutations have hit nucleotide positions that mutate, on average, 12 times faster during evolution (a mutation rate of 5.55, based on 122 evolutionary mutations at the 22 radiation-associated nucleotide positions). The observation that radiation accelerates point mutations at all is unexpected, at first glance, because radiation was, until recently, thought to generate primarily DNA lesions (1). A potential explanation is provided by our additional observation that these radiation-associated point mutations are also evolutionary hot spots, indicating that the radiation indirectly increases the cell's normal (evolutionary) mutation mechanism (5).

There are no previously published comparisons of evolutionary and radiation-associated heritable mtDNA point mutations, and thus our results are difficult to compare with other studies on radiation-associated mutational change. Perhaps the most

Table 2. Ancient mutations in the Eurasian mtDNA tree and new mutations in Kerala families

Nucleotide positions (nps) (transitions unless specified)	No. of mutations*		
	Evolutionary	High radiation	Low radiation
152	16	3 (C/T)	
16189	12	3 (C/T)	
16362	12		
195 (10C & 1A)	11	1 (C/T)	
146	10		
16129, 16311	6		
16093	5	2 (C/T)	
150, 200, 16304	5		
228	4	1 (A/G)	
16274, 16278, 16126	4		
189	3	1 (A/G)	
194	3	1 (C/T)	
93, 151, 199, 204, 16179, 16234, 16256, 16266, 16357, 16390	3		
16223	2	1 (C/T)	
207	2	1 (A/G)	
72, 73, 143, 153, 185, 188, 198, 225, 234, 239, 246, 247, 263, 16134,	2		
16163, 16193, 16218, 16239, 16249, 16255, 16289, 16292, 16294,	2		
16318 (G & T), 16319, 16325, 16356	2		
214	1	1 (A/G)	
291	1	1 (A/T)	
16291	1	1 (C/T)	
16320	1		1 (C/T)
55, 56T, 65.1T, 66T, 95C, 114del, 240, 249del, 250, 257, 285, 295,	1		
16095, 16104, 16111, 16162, 16166, 16172, 16174, 16176, 16181,	1		
16183, 16186, 16187, 16188, 16192, 16207, 16209, 16213, 16221,	1		
16224, 16227, 16233, 16245, 16248, 16261, 16263, 16265C, 16270,	1		
16270.1C, 16280, 16286, 16288, 16290, 16295, 16296, 16298,	1		
16302, 16316, 16324, 16327A, 16335, 16336, 16344, 16346C, 16348,	1		
16352, 16353, 16354, 16366	1		
144	0	1 (C/T)	
215	0	3 (A/G)	
16272	0	1 (A/G)	
All other nucleotides within nps 16093–16390 and within nps 35–315	0		
Total mutations	267	22	1

*Evolutionary mutations are counted from mtDNA trees of Macaulay *et al.* (36) and Fig. 2. Length changes at nps 16182/3 and 309 are disregarded.

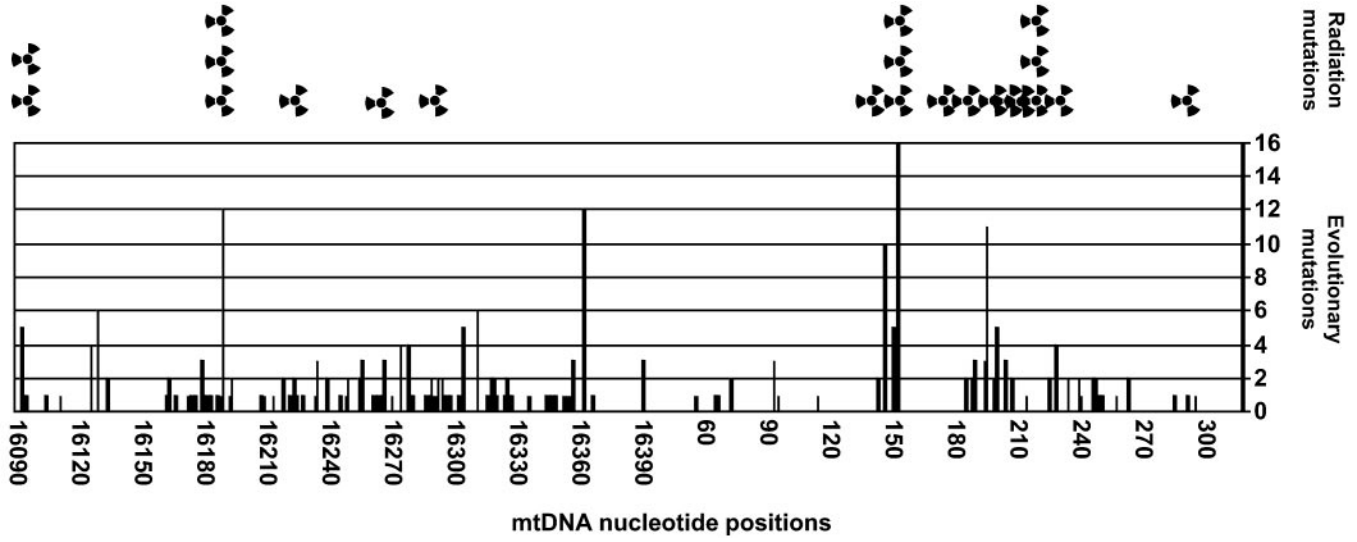

Fig. 4. Ancient mutations in the Eurasian mtDNA tree and new mutations in the Kerala families. The horizontal axis represents the 579 nucleotide positions of the mtDNA control region considered here (nps 16093–16390 and nps 35–315). The column heights are proportional to the absolute number of evolutionary mutations observed at each nucleotide position in a Eurasian mtDNA tree (see ref. 36 and Fig. 2). The radiation symbols mark the nucleotide positions observed to mutate in the families living in the high-radiation area of Kerala. Multiple mutations at a nucleotide position are indicated by a corresponding number of vertically placed radiation symbols. All values for this figure are taken from Table 2. A similar profile of homoplasy at nps 16093–16390 was reported (18).

closely related research in our context is a study (5) on the genetic effects of the Chernobyl fallout, which has exposed parents and their offspring in parts of Byelorussia to increased radiation levels since April 26, 1986. Although those authors investigated autosomal minisatellites rather than maternally inherited mtDNA, and although their initial exposure is much shorter and more intense than our constant natural radiation conditions, the genetic effects may be comparable. The increased minisatellite length mutations in the Byelorussians fell within the normal allelic spectrum, hinting that it is the normal evolutionary mutation mechanism that is accelerated by radiation. mtDNA as a genetic system is well suited to confirm or reject this prediction, as evolutionary mutations can be reconstructed in detail in an mtDNA tree without the complex mutational dynamics that confound the phylogenetic analysis of autosomal minisatellites. As demonstrated, our mtDNA results strongly support an acceleration of the evolutionary DNA mutation mechanism through radiation.

We thank the serology team in Münster (M. Eppink-Walbaum, K. Möller, S. Rand, M. Schürenkamp, U. Sibbing, and K. Waterkamp) for maternity testing, M. Hurles (Cambridge, U.K.) for valuable comments on a previous version of the manuscript, and all donors, helpers, colleagues, and our family in the Kollam District, India. This work was performed by L.F. in partial fulfillment of the requirements for the doctoral degree.

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