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Evolution

Mitochondrial DNA sequences in single hairs from a southern African population

(!Kung/polymerase chain reaction/geographic structuring/population genetics/molecular anthropology)

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ABSTRACT **Hypervariable parts of mitochondrial DNA (mtDNA) were amplified enzymatically and sequenced directly by using genomic DNA from single plucked human hairs. This method has been applied to study mtDNA sequence variation among 15 members of the !Kung population. A genealogical tree relating these aboriginal, Khoisan-speaking southern Africans to 68 other humans and to one chimpanzee has the deepest branches occurring amongst the !Kung, a result consistent with an African origin of human mtDNA. Fifteen cases of unrelated individuals having identical sequences in the most variable parts of the mtDNA control region were found *within* populations of !Kung, Western Pygmies, and Eastern Pygmies, but no cases of identity were evident *among* these populations. This and other evidence of geographic structuring of the mitochondrial diversity in Africa, together with knowledge of the rate of accumulation of base changes in human mtDNA, implies that the average rate at which female lineages have moved their home bases during hunter–gatherer times could be as low as 13 meters per year. The technique of enzymatic amplification and direct sequencing applied to readily collected, highly stable biological materials such as hairs makes it possible to examine with high resolution many representatives of virtually any population.**

Nucleotide sequences provide the highest possible resolution for examining molecular evolution in populations. The study of genealogical relationships among individuals within a species requires rapidly evolving, non-recombining sequences such as those found in maternally inherited mtDNA (1). mtDNA accumulates base changes at a rate 5–10 times that of nuclear DNA, and the 1.1-kilobase (kb) control region is its fastest-changing segment (2, 3). It is therefore appropriate for the study of the origin of modern humans and the history of modern populations of our species.

Among those aboriginal peoples of southern Africa that speak click languages, the !Kung have attracted the interest of many researchers due to their isolation and retention until recently of a hunting and gathering life-style (4). Their continued genetic isolation makes them an interesting population for evolutionary studies using mtDNA. This study presents sequences from 14 !Kung individuals from a population of less than 500 living in the Xangua valley in northwestern Botswana along the Namibian border. These samples were collected in a novel way, as single plucked scalp hairs from which DNA was extracted and enzymatically amplified by using the polymerase chain reaction (PCR) (5).

Use of the PCR also allowed us to rapidly acquire sequence information from a further 69 humans, of which 47 are native Africans. With a total of 83 humans surveyed, this study represents the largest collection of mitochondrial sequences examined within a single species.¶ A published sequence for the common chimpanzee (6) provides an outgroup for rooting a genealogical tree relating the mtDNA types. This tree is consistent with that obtained from restriction-site data in the placement of the common human mtDNA ancestor in Africa and in the overall time scale. However, the use of a much larger and more geographically and ethnically diverse African sample coupled with sequence information allows us to see a distinct regionality in the mtDNA tree and make inferences about the movement of female hunter-gatherers.

MATERIALS AND METHODS

Geographic Origins of DNA. Fourteen !Kung and two Herero samples were collected in Dobe, Botswana. One !Kung sample (no. 15) was collected in Chumxoi, Namibia (T. Jenkins, personal communication). Seventeen Western Pygmy samples (Biaka Pygmies) originated in the Central African Republic, and 20 Eastern Pygmy samples (Mbuti Pygmies) were from the Ituri Forest of Zaire. Seven of the African samples were Nigerian. The eight Asian samples included three Chinese, one Taiwanese, one Japanese, one Philippino, one Tongan, and one H'mong Vietnamese. The aboriginal Australian sample was from Perth. The four European and eight African American samples were from individuals living in the United States, and the human reference sequence (7) was assumed to be of northern European origin.

Types of Template DNA. Enzymatic amplification of control regions from 82 individuals proceeded from template mtDNA of various states of purity. Forty-four of these mtDNAs were present in purified genomic DNA from 37 cell lines and 7 blood samples. An additional 16 were purified mtDNAs from placentas (8). !Kung sample 15 was purified mtDNA derived from cell line GM3043 (Human Genetic Mutant Cell Repository, Camden, NJ) (8). A total of 21 individuals were studied using genomic DNA isolated from hair. Single plucked scalp hairs were collected in Dobe, Botswana, from 15 unrelated !Kung and 2 unrelated Herero. These hairs were stored in 100% ethanol for 3–4 months prior to DNA extraction, while the other hairs (4 European and 1 African American) were freshly plucked. The hairs were digested with proteinase K as described (9) in a total volume of 0.5 ml. A negative control with no hair or DNA was also taken through the entire procedure in order to detect contamination. Hair DNA was extracted with an equal volume of phenol/chloroform (1:1, wt/vol) and then back-extracted

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Abbreviation: PCR, polymerase chain reaction.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M28893–M28912).

with TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA). The aqueous fractions were extracted once more with phenol/chloroform and finally with 1-butanol. The resulting 1-ml volume was reduced to 40 μl following centrifugation three times with TE in a Centricon-30 microconcentrator (Amicon) as per the manufacturer's instructions.

Amplification and Sequencing of Single-Stranded DNA. One microliter (i.e., 2.5%) of the recovered hair DNA was subjected to 35 cycles of amplification in a 50-μl reaction volume with 2 units of *Thermus aquaticus* (*Taq*) DNA polymerase (United States Biochemical). Each cycle consisted of denaturation at 93°C for 45 sec, annealing at 55°C for 1 min, and extension at 74°C for 3 min. The primers for each reaction were present in unequal amounts (50:1 molar ratio) in order to generate single-stranded DNA for direct sequencing (10). Two segments of the control region, each ≈400 base pairs (bp) long, were amplified with the following primers: segment I, L15996 (5′-CTCCACCATTAGCACCCAAAGC-3′) and H16401 (5′-TGATTTCACGGAGGATGGTG-3′); segment II, L29 (5′-GGTCTATCACCCTATTAACCAC-3′) and H408 (5′-CTGTTAAAAGTGCATACCGCCA-3′). The L or H in the primer name refers to the light or heavy strand, respectively, and the number identifies the base at the 3′ end according to the numbering of the reference sequence (7). Asymmetric priming was done in both directions so as to make it possible to check a polymorphism or ambiguous base detected with one primer by sequencing the complementary strand. PCR products were prepared for sequencing by filtration in a Centricon-30 unit as described above. Seven microliters of the 40 μl retentate was used for sequencing with the Sequenase kit (United States Biochemical) and 2-deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate, following the manufacturer's recommendations. Electrophoresis of reaction products through 6% polyacrylamide/7 M urea gels with wedge spacers (BRL) was carried out for 2.5 hr at 40 mA. Gels were fixed in 5% glacial acetic acid/5% methanol for 45 min, dried, and exposed to film for 12–72 hr. In 4 of the 16 African hair DNAs, but none of the other samples, faint sequencing ladders made it necessary to first perform a double-stranded amplification of the regions of interest followed by the asymmetric priming PCR amplifications. In this procedure the double-stranded product was purified by electrophoresis in 0.04 M Tris acetate through a 2% SeaPlaque (FMC) agarose gel. Approximately 10 μl of the band was excised and melted in 500 μl of water, and 1 μl of this mixture was used in the asymmetric amplification.

RESULTS AND DISCUSSION

DNA from Hair. Only 1 of 22 hair samples failed to yield a product upon amplification, a success rate of >95%. The unsuccessful hair did not appear to contain any of the root material, which is much more amenable to amplification than the hair shaft material (9). No contaminating DNA was detected in the negative controls for extraction and PCR procedures. In addition, the sequence of mtDNA from hair is clear, and there are no background signals that might indicate contamination (Fig. 1).

Sequence Polymorphism. An initial study of complete control-region sequences from 21 humans of varied origins allowed us to identify the most variable segments (L.V., unpublished data; T.D.K., unpublished data). Nearly all (92%) of the polymorphisms were found to be contained in two ≈400-bp segments, which were then sequenced in a further 65 individuals. Fig. 2 shows the 36 positions at which the !Kung sequences differ from the reference sequence in these two hypervariable segments. These changes are almost all transitions, with the exception of a single transversion and two nonunique length changes. The two length mutations each involve the gain of a cytosine on either side of the thymine at control-region position 856 (base 310 of reference sequence). This thymine residue therefore interrupts a region that contains from 12 to 14 consecutive cytosines, suggesting that a possible mechanism for generating length mutations in this region may be slipped mispairing involving this long series of cytosines (11).

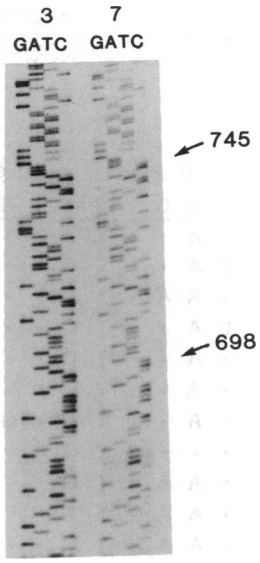


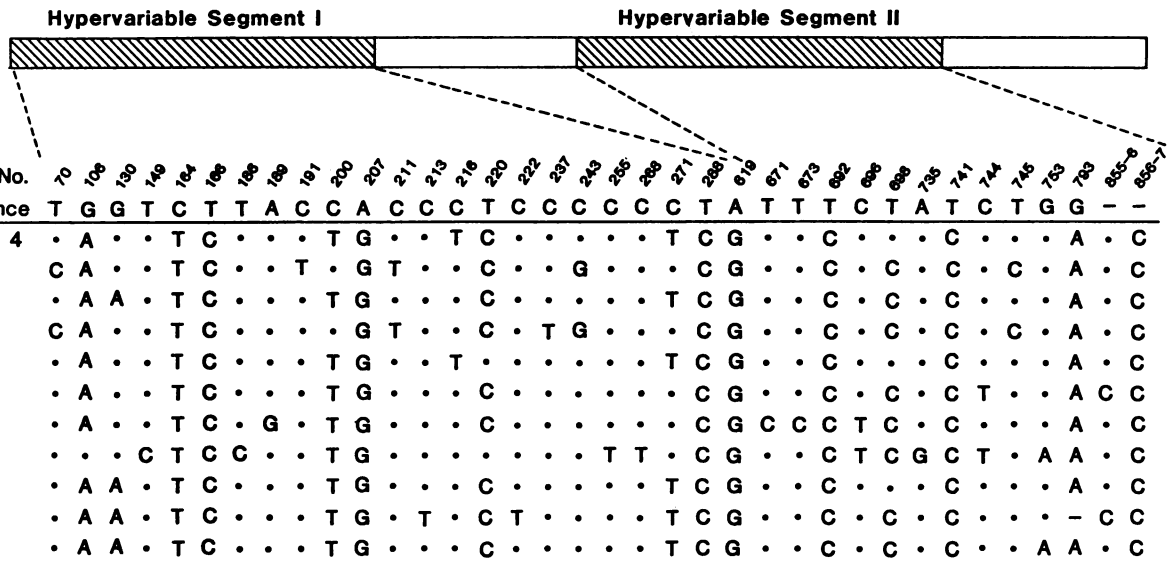
FIG. 1. Direct sequencing of single-stranded DNA enzymatically amplified from hair of !Kung individuals 3 and 7. Sequencing was done with the L29 primer. Arrows point to T ⇌ C polymorphisms at positions 698 and 745 of the control region.

Eight of the 36 positions are monomorphic in the !Kung, with a further 14 varying in only one individual. Five of the 36 positions have not been observed to vary in any other human population. However, many more individuals must be examined before any mutations can be considered markers for particular populations. Fig. 1 is a photograph of a sequencing gel showing variation at two of these five positions.

Low Diversity Within Hunter–Gatherers. As is apparent in Fig. 2, a group of 4 and a group of 2 of the !Kung individuals have identical sequences over ≈740 bp of the control region. Individuals with identical sequences have also been found in other African populations of small size (summarized in Table 1). Eastern Pygmies of the Central African Republic have a discontinuous population of ≈33,000, but the group sampled contained <6000 individuals (13). Similarly, Western Pygmies in Zaire have a total population size of 35,000, which is further divided into three language subgroups, only one of which was sampled (13, 14). Total !Kung population size has been estimated at 5000–10,000 (15). It is unlikely that more sequence from the control region will find distinguishing polymorphisms in the identical individuals, since the ≈740 bases sequenced here contain >90% of the polymorphism in the control region.

A further estimate of variation within populations is given by the diversity value, h, as shown in Table 1. This index does not reach its maximal value of 1 in the two Pygmy groups and the !Kung but does so in the group of 31 other people. The probability of identity, calculated as the sum of the squares of the frequencies of types, again shows that it is more likely to find identical individuals within the !Kung or Pygmies.

The statistical significance of the differences in diversity values can be evaluated in the following way. The ratio of the number of individuals with identical mtDNAs to individuals with unique mtDNAs can be compared across populations and tested for homogeneity by a χ^2 analysis. By this test, diversity values do not differ among the three African populations but the combined African group of !Kung and



Base No.	70	106	130	149	164	166	186	189	191	200	207	211	213	216	220	222	237	243	256	268	271	288	519	671	673	692	698	698	735	741	744	746	753	769	855-6	855-7
Reference Sequence	T	G	G	T	C	T	T	A	C	C	A	C	C	C	T	C	C	C	C	C	C	T	A	T	T	T	C	T	A	T	C	T	G	G	–	–
!Kung 1, 2, 3, 4	•	A	•	•	T	C	•	•	•	T	G	•	•	T	C	•	•	•	•	•	T	C	G	•	•	C	•	•	C	•	•	•	A	•	C
5	C	A	•	•	T	C	•	•	T	•	G	T	•	•	C	•	•	G	•	•	•	C	G	•	•	C	•	C	•	C	•	C	•	A	•	C
6	•	A	A	•	T	C	•	•	•	T	G	•	•	•	C	•	•	•	•	•	T	C	G	•	•	C	•	C	•	C	•	•	•	A	•	C
7	C	A	•	•	T	C	•	•	•	•	G	T	•	•	C	•	T	G	•	•	•	C	G	•	•	C	•	C	•	C	•	C	•	A	•	C
8	•	A	•	•	T	C	•	•	•	T	G	•	•	T	•	•	•	•	•	•	T	C	G	•	•	C	•	•	C	•	•	•	A	•	C
9,10	•	A	•	•	T	C	•	•	•	T	G	•	•	•	C	•	•	•	•	•	•	C	G	•	•	C	•	C	•	C	T	•	•	A	C	C
11	•	A	•	•	T	C	•	G	•	T	G	•	•	•	C	•	•	•	•	•	•	C	G	C	C	C	T	C	•	C	•	•	•	A	•	C
12	•	•	•	C	T	C	C	•	•	T	G	•	•	•	•	•	•	•	•	•	T	T	•	C	G	•	•	C	T	C	G	C	T	•	A	A	•	C
13	•	A	A	•	T	C	•	•	•	T	G	•	•	•	C	•	•	•	•	•	•	T	C	G	•	•	C	•	•	C	•	•	•	A	•	C
14	•	A	A	•	T	C	•	•	•	T	G	•	T	•	C	T	•	•	•	•	•	T	C	G	•	•	C	•	C	•	C	•	•	•	–	C	C
15	•	A	A	•	T	C	•	•	•	T	G	•	•	•	C	•	•	•	•	•	•	T	C	G	•	•	C	•	C	•	C	•	•	A	A	•	C

FIG. 2. Variable sites in control regions of !Kung mtDNA. Base No. refers to the position of a base in the control region, with base 1 in the control region corresponding to base 16024 by the standard numbering (7). Dots indicate identity with the published reference sequence (7), while dashes represent deletions. Individuals listed in the same row have identical sequences. Positions 216, 220, 243, 745, and 753 are not observed to vary in any other human population.

Pygmies is less diverse than the group of 31 other people ($P < 0.001$). Populations can be further compared by the percent sequence differences calculated from pairwise comparisons (Table 1). The !Kung are less diverse than the Pygmy groups by all of these measures, but larger sample sizes are needed to see whether the difference is statistically significant.

The low diversity observed among the !Kung in this study is consistent with the low average heterozygosity observed in a set of nuclear markers (16). The !Kung population is small, and the gene flow that does occur is from the !Kung into their neighbors, not the reverse (16). There are other Khoisan-speaking groups in the region, but they share languages related to Nama Hottentot (17). The !Kung have moved into the region from southern Angola and are linguistically and culturally distinct (17).

An estimate of long-term effective population size, N, of the !Kung can be calculated using the mean pairwise sequence divergence, 0.88% (2). Assuming a rate of control-region sequence evolution of 8.4% per million years (see below) and a generation time of 20 years, N for the !Kung is ≈5000 females, in good agreement with census estimates. This value does not support a postulate of extreme long-term bottlenecks.

Genealogical Tree. A genealogical tree relating the 84 individuals was constructed by the PAUP computer program (18) using the parsimony method and 105 phylogenetically informative sites. Outgroup rooting with the published common chimpanzee sequence eliminates the necessity of assuming constant rates of evolution in all lineages. Branch swapping using MacClade 2.1 (19) was performed in order to examine alternate topologies of identical or similar length. A resulting minimal-length tree has 72 types and a length of 363 mutations (Fig. 3). The consistency index is a measure of homoplasy and the value of 0.35 indicates that each position changed an average of three times along the tree. Each of the five deepest nodes (denoted by arrows in Fig. 3) in the human part of the tree gives rise to one or more lineages that are exclusively African and specifically !Kung. Only nodes that are more recent than these give rise to lineages that are exclusively non-African. This implies an African origin of human mtDNA, a result consistent with that obtained from restriction analysis using midpoint rooting (8). An alternative tree in which the deepest node leads first to the clade of four Asians requires eight additional mutations and so is much less parsimonious.

The chimpanzee sequence allows us to calibrate the rate of control-region sequence divergence. The apparent chimpanzee/human sequence difference of 13.6% is adjusted to 42% by means of a multiple-hit correction that accounts for the loss of the record of transitions over evolutionary time. This method counts each transversion event as equal to 10 transitions (21). Since the chimpanzee/human split is estimated to have occurred 5 ± 2 million years ago, 1% sequence divergence occurs in 71,000–167,000 years, for an average rate of control-region sequence divergence of about 8.4% per million years.

The time at which the common ancestor of modern human mtDNAs existed in Africa can be calculated from this rate. The ancestral type links types that have diverged by an average of 2.00%, and therefore existed about 238,000 years ago. This value is within the range of 140,000–280,000 years estimated by restriction mapping (8).

Restriction and Sequence Analyses Are Concordant. Sequence analysis of 740 bp of the control region yields an amount of information comparable to that from high-resolution restriction analysis of the entire mtDNA genome.

Table 1. Variability in the control region of three human populations

Population	Sets of identical mtDNAs	Diversity	Probability of identity	% sequence difference
15 !Kung	One pair, one quartet	0.93	0.13	0.88
17 Western Pygmies	One pair, one trio	0.97	0.09	1.96
20 Eastern Pygmies	Three pairs, one trio	0.97	0.08	1.48
31 other people	None	1.00	0.03	1.93

Diversity is $h = (1 - \Sigma x_i^2)n/(n - 1)$, where x_i is the frequency of a type and n is the number of individuals (12).

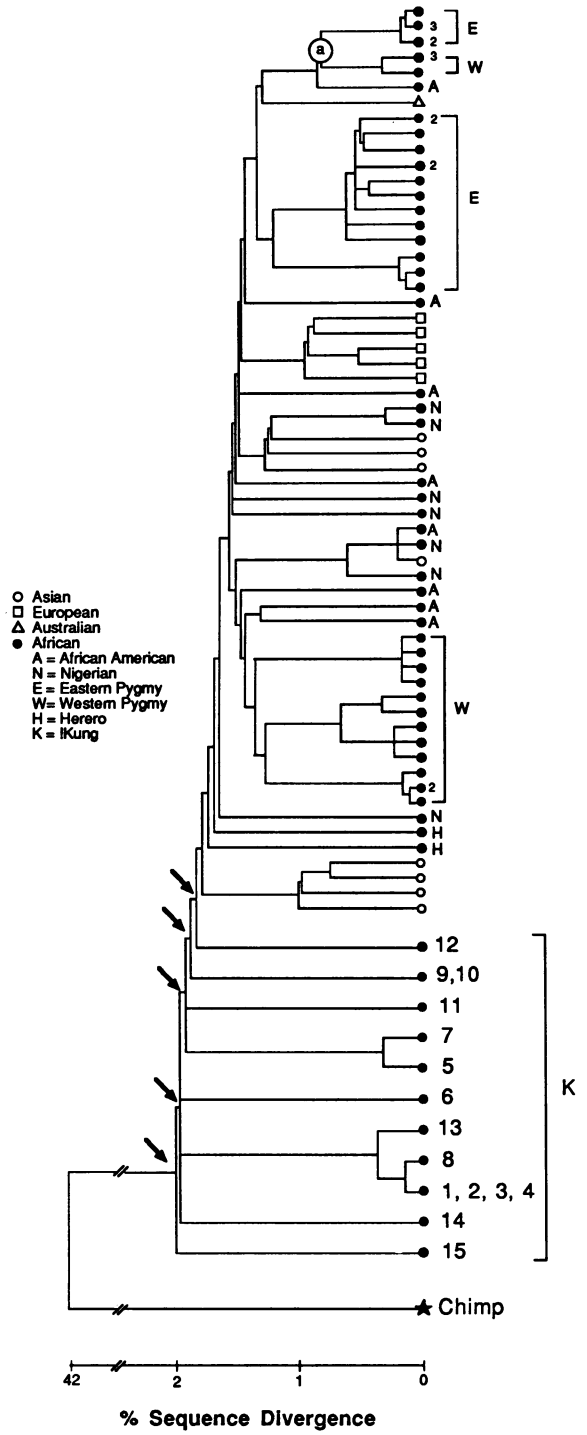


FIG. 3. Genealogical tree for mtDNAs of 84 individuals rooted by using the chimpanzee as an outgroup. This tree is one of several with identical length, but these trees differ only in some rearrangements of terminal branches. Branch points are approximately placed with respect to the scale of percent sequence difference. Arrows indicate the five deep African nodes in the human portion of the tree. The circled letter a denotes the most recent common ancestor of Eastern and Western Pygmy mtDNAs. The !Kung types are individually labeled with large numbers. Multiple Pygmy individuals with identical sequences are indicated with small numbers. Alternative trees were evaluated by comparing the number of steps required for each character on each tree (20). A character is said to favor a tree if it requires fewer changes. A one-tailed binomial test determines whether there is a significant difference in the number of characters favoring one tree over an alternative.

The mean number of differences between any two types on the restriction analysis tree is 9 (ref. 8), while an average of 16 changes separate two types on the tree presented here (Fig. 3). Sequence information is not subject to ambiguities that arise in restriction analysis, where apparent length mutations may in fact be conformational substitutions altering the shape and mobility of the DNA (22). Sequence data have an additional advantage in allowing use of an outgroup, the chimpanzee, to root the tree, thus eliminating the necessity of assuming constant rates of change. The tree obtained from high-resolution restriction analysis of 148 human mtDNAs (8) and the tree presented here are alike in supporting an African origin of human mtDNA. The estimates by the two different methods of the time of existence of the common human ancestor also overlap.

High Geographic Specificity of Types. Restriction analysis has shown that mtDNA types of similar ethnic background tend to be associated in genealogical trees (8, 23). Control-region sequences reveal population and geographic specificity within the broad category of African types. This increased resolution is apparent in the characteristics of individuals with identical types. Identity is limited to individuals within the same population and there is no sharing of mtDNA types between Eastern Pygmies, Western Pygmies, and the !Kung. This is in contrast to low-resolution restriction mapping studies, in which Bantu and !Kung populations share several types and one type is even shared by Bantu, !Kung, Europeans, and Asians (23).

The clustering of the five European mtDNA types in one clade (Fig. 3) appears to contradict the scattered distribution of European types on the tree derived from restriction analysis (8). However, the subset of European types that are of known northern European origin do group more closely together on the restriction analysis tree (M. Stoneking, personal communication). The five individuals sequenced for this study are all of northern European origin and their appearance in one clade is less surprising in view of their limited geographic origin.

The more precise geographical information available for the !Kung confirms the idea that the sharing of types will be limited to individuals in close geographic proximity. Of the four !Kung individuals with identical sequences, three were raised in Dobe and one 20 km away in Kangwa. The other pair of !Kung with identical sequences were both raised in Dobe. One type, no. 15, was collected at Chumxoi, 50 km west of Dobe, and its outermost position in the branching order is consistent with its slightly different geographic origin but is not statistically meaningful.

The Western and Eastern Pygmies each form two clades on the genealogical tree (Fig. 3). A tree in which the Pygmies form a monophyletic group is significantly longer ($P < 0.001$, test described in the legend) than the minimum-length tree presented here. The closest relative of each Pygmy individual is always another Pygmy from the same population, despite the fact that these groups, now separated by ≈1500 km, most likely had a common origin (13). These facts can be used to estimate the rate of movement based on mtDNA. At 1% sequence divergence, an Eastern and a Western Pygmy clade fuse (labeled a in Fig. 3). This corresponds to a time ≈119,000 years ago and gives a rate of successful female hunter–gatherer movement of 0.013 km per year. This is an order of magnitude lower than the migration rate, also estimated from mtDNA analysis, of 0.1 km per year for the colonization of New Guinea and Australia from Southeast Asia (24). An estimate from nuclear gene frequencies of the rate of spread of farmers in Europe is even higher, 1.0 km per year (25). The low hunter–gatherer movement rate may reflect factors such as an absence of conditions conducive to migration or a difference in the patterns of movement by males and females.

CONCLUSION

This study demonstrates the utility of single hairs for molecular anthropology. Hair is superior to other biological materials previously used (e.g., blood, tissue) with respect to ease of sample acquisition, handling, stability, and DNA extraction. In this initial study using hair from 22 individuals, there was only one failure to recover amplifiable DNA, a success rate of >95%. There were no problems with contaminating DNA, which would have been detected in the negative controls included in each step and also would have resulted in sequencing gels of poor quality, in contrast to the results shown in Fig. 1.

The low diversity found in the mtDNA of the !Kung is in accordance with results from nuclear marker studies (15, 26). Moreover, the 11 !Kung types encompass the deepest branches in the human portion of a tree based on sequence analysis of 83 individuals, both confirming and extending the results of mtDNA restriction analysis. The presence of Khoisan outside other populations is seen in a distance tree based on morphological characters (27), but not in a tree based on nuclear genes in which Khoisan appear in an exclusively African branch. Although the Khoisan are linked more closely in the nuclear tree with Ethiopians, Pygmies, and other Africans than with non-African populations, this is not evident in the mtDNA tree (Fig. 3) (28). In addition, a study of nuclear DNA has identified 44 loci that are polymorphic in Pygmies (30). At these loci, 81 of 88 alleles occur in both Pygmy populations, while our results show no sharing of mtDNA types between Pygmy populations. This contrast implies that the low mobility inferred for female hunter–gatherers in Africa may not apply to males.

To our knowledge, this study of 83 individuals is the most extensive published study of mtDNA sequence variation in humans. Control-region sequences offer enough information to study the evolution of discrete populations within Africa. Preliminary indications of a strong correlation between geographical origin and sequence differentiation are apparent and require more samples of known origin to evaluate thoroughly. The implication from mtDNA results of very low rates of female hunter–gatherer movement is a stimulus to gather information from nuclear DNA, such as that encoded by the Y chromosome, in order to compare the dynamics of female- vs. male-mediated movement of DNA. The techniques of enzymatic amplification and direct sequencing of DNA from hair allow surveying of mtDNA variation, and potentially of nuclear DNA markers (29), from many individuals.

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