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Genetic Analysis of Early Holocene Skeletal Remains From Alaska and its Implications for the Settlement of the Americas

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ABSTRACT Mitochondrial and Y-chromosome DNA were analyzed from 10,300-year-old human remains excavated from On Your Knees Cave on Prince of Wales Island, Alaska (Site 49-PET-408). This individual's mitochondrial DNA (mtDNA) represents the founder haplotype of an additional subhaplogroup of haplogroup D that was brought to the Americas, demonstrating that widely held assumptions about the genetic composition of the earliest Americans are incorrect. The amount of diversity that has accumulated in the subhaplogroup over the past 10,300 years suggests that previous calibrations of the mtDNA clock may have underestimated the rate of molecular evolution. If substantiated, the dates of events based on these previous estimates are too old, which may explain the discordance between inferences based on genetic and archaeological evidence regarding the timing of the settlement of the Americas. In addition, this individual's Y-chromosome belongs to haplogroup Q-M3*, placing a minimum date of 10,300 years ago for the emergence of this haplogroup. Am J Phys Anthropol 132:000–000, 2007.

Studies of variation in the first hypervariable region (HVRI) of the mitochondrial genome have concluded that humans first entered the Americas between 15,000 and 40,000 years ago (YBP) (Forster et al., 1996; Bonatto and Salzano, 1997b; Stone and Stoneking, 1998; Schurr, 2004b). The earliest of these dates are inconsistent with accepted evidence from the archaeological record and have significant implications for the route by which humans could have entered the Americas (i.e. coastal versus inland, ice-free corridor route) (Fiedel, 2001, 2004; Schurr, 2004b). However, conclusions drawn from the molecular studies rest on the assumption that diversity of Native American mitochondrial DNA (mtDNA) ultimately derives from only five haplotypes, those representing the American founders of mitochondrial haplogroups A, B, C, D, and X (Schurr, 2004b). If additional, unrecognized haplotypes were brought to the Americas, the previously estimated dates for the peopling of the Americas are incorrect. Moreover, the accuracy in dating events with molecular data ultimately depends on the accuracy of the calibration of the molecular clock. Phylogenetic estimates (i.e. long-term) of rates of HVRI evolution ranging from ~10 to 20%/site per million years (myr) have been used to date the peopling of the Americas (Bonatto and Salzano, 1997a; Stone and Stoneking, 1998). In contrast, pedigree-derived estimates (i.e. short-term) for the rate of HVRI evolution range from 34 to 47.5%/site per myr (Siguroardottir et al., 2000; Howell et al., 2003), but have not been

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considered in dating the settlement of the Americas. Recently, Ho et al. (2005) and Ho and Larson (2006) have argued that the measurable rate of molecular evolution declines systematically with time, explaining why the short-term and long-term evolutionary rates differ so greatly. Their argument also suggests that the phylogenetic estimates of HVRI evolution may not be appropriately applied to date relatively recent events (such as the settlement of the Americas or even the emergence of *Homo sapiens*). In this case, if an inaccurate calibration of the molecular clock has been employed, previous molecular studies have also miscalculated the timing of the settlement of the Americas.

The investigation of ancient DNA (aDNA) provides a unique opportunity to test hypotheses generated by modern DNA studies. While modern DNA studies make predictions about the past based on lineage coalescence, aDNA studies can place lineages at specific temporal and geographic axes. While very few human remains from the Americas predating 7,000 YBP have yielded genetic information (Smith et al., 2005), previous studies of mtDNA extracted from Native American remains predating 8,000 YBP provide evidence of the antiquity of haplogroups B (Stone and Stoneking, 1996) and C (Kaestle, 1998) in the Americas. In both cases, the individuals were determined to belong to the accepted founding haplotypes of their respective haplogroups and, therefore, these data are consistent with the five-founder-lineage paradigm (i.e. one representing each haplogroup). Calibration of the human mtDNA clock has not been previously attempted using aDNA, as it has for other animal species (Lambert et al., 2002; Shapiro et al., 2004).

To test assumptions about the number of founding lineages and calibration of the mtDNA clock, we used aDNA techniques to extract and analyze DNA from 10,300-year-old skeletal remains discovered in On Your Knees Cave (OYKC) on the northern tip of Prince of Wales Island, Alaska (Dixon, 1999). This study demonstrates that assumptions about the genetic composition of the Native American founder population are incorrect and that previous calibrations of the mtDNA clock may be flawed.

MATERIALS AND METHODS

Skeletal remains

The human remains found in OYKC, representing a man who died in his mid-twenties, are comprised of: 1) a mandible recovered in two pieces, 2) the partial remains of a right pelvis and several small articulating and nonarticulating fragments, 3) one cervical vertebrae, 4) one lumbar vertebrae, 5) three thoracic vertebrae, 6) three ribs (one recovered in two fragments), 7) three incisors, and 8) one canine. The mandible contains all of its molars, premolars, and canines, but is missing all four incisors. The mandible was dated to 9730 ± 60 ^{14}C YBP (CAMS-29873) and the pelvis to 9880 ± 50 ^{14}C YBP (CAMS-32038) (Dixon et al., 1997; Dixon, 1999). These two dates overlap at two sigma and suggest the individual dates to circa 9,800 ^{14}C YBP. Delta ^{13}C values of −12.5% (CAMS-29873) from the mandible and −12.1% (CAMS-32038) from the pelvis (Dixon et al., 1997; Dixon, 1999) indicate that this individual subsisted on a marine-based diet and, therefore, the age of the skeletal remains should be reduced to circa 9,200 ^{14}C YBP (Josenhans et al., 1995; Southon and Fedje, 2003). The accuracy of this correction was demonstrated when the lower left second incisor that fit the mandible was recovered from the microblade horizon dated to 9,200 ^{14}C YBP. The corrected date is further consistent with the cultural occupation of the cave based on the radiocarbon age of three charcoal samples: 1) 8,760 ± 50 ^{14}C YBP (CAMS-43991), 2) 9,210 ± 50 ^{14}C YBP (CAMS-43990), and 3) 9,150 ± 50 ^{14}C YBP (CAMS-43989) (Dixon et al., 1997; Dixon, 1999). The radiocarbon age of 9,200 YBP corresponds to a calendrical date of 10,300 YBP, which was used for all analyses in this study.

DNA extraction

Initial attempts to extract DNA from a rib and a vertebra failed (data not shown). Later, a molar was sent to the Molecular Anthropology Laboratory at UC Davis for analysis. Following successful analysis of the tooth, DNA from a second molar was independently extracted and analyzed at Trace Genetics (Richmond, CA) to confirm the results obtained at UC Davis. At both of these facilities, DNA extraction and PCR set-up were performed in laboratories that are separated from those wherein modern DNA is extracted and from the post-PCR laboratory. Negative extraction and PCR controls served as monitors for potential contamination generated during the extraction and analyses of the DNA at both UC Davis and Trace Genetics.

Approximately 0.12 g of molar were removed by carefully separating a root from the remainder of the tooth. Because aDNA occurs in low copy number and is highly degraded (Pääbo, 1990; Lindahl, 1993), aDNA extractions and PCR amplifications are highly susceptible to contamination from modern sources. The tooth root was submerged in 6% sodium hypochlorite (full strength Clorox Bleach) for 15 min to remove any surface contamination (Kemp and Smith, 2005). The bleach was poured off and the sample rinsed with DNA-free ddH_2O (Gibco) to remove any remaining bleach. The possibility that contamination directly arose from the authors who performed the molecular analyses could be precluded because their mtDNA and Y-chromosome haplogroups differ from those to which the OYKC individual was assigned. The root was transferred to a 15 mL conical tube and demineralized by gentle rocking in 2 mL molecular grade 0.5 M EDTA, pH 8.0 (Gibco), for 6 days at room temperature. Three milligrams of Proteinase K were added to the sample, followed by incubation at 65°C for 4.5 h. DNA was first extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the EDTA, which was then vortexed briefly and centrifuged at 3,100 rpm for 5 min. The aqueous phase was removed and subsequently extracted using phenol:chloroform:isoamyl alcohol (25:24:1), as just described. A third extraction was performed using an equal volume of chloroform:isoamyl alcohol (24:1), which was then vortexed briefly and centrifuged at 3,100 rpm for 3 min. DNA was precipitated from solution by adding one half volume of room temperature 5 M ammonium acetate and, to this combined volume, one volume of room temperature absolute isopropanol (Hänni et al., 1995), then storing the solution overnight at room temperature. The DNA was pelleted by centrifuging the tube for 30 min at 3,100 rpm The isopropanol was discarded and the sample air-dried for 15 min. The DNA was washed with 1 mL of 80% ethanol by vortexing, pelleted again by centrifuging for 30 min at 3,100 rpm, and air-dried for 15 min after decanting the ethanol. The DNA was resuspended in 300 μL of DNA-free ddH_2O and silica extracted (Höss and Pääbo, 1993) using the Wizard PCR Preps DNA Purification System (Promega), following the manufacturer's

instructions except that: 1) the "Direct Purification Buffer" was not added and 2) DNA was finally eluted with 100 μL DNA-free ddH_2O.

PCR and sequencing

The extract was screened for the polymorphisms that define Native American mitochondrial haplogroups A, B, C, and D (Schurr et al., 1990; Forster et al., 1996). It was also subsequently screened for markers definitive of macrohaplogroup M (of which haplogroups C and D are members) and subhaplogroups D3 and D4, which are common variants of haplogroup D found in Siberia (Starikovskaya et al., 2005). Molecular sex determination was performed by screening a length dimorphism in the amelogenin gene (Sullivan et al., 1993). After molecular confirmation that the OYKC individual was male, the extract was screened for the polymorphisms that define Y-chromosome haplogroups Q-M242* (Seielstad et al., 2003) and Q-M3* (Underhill et al., 1996). PCR conditions and primer coordinates (or sequence) for each marker tested are provided in Table 1.

Approximately 5 μL of each amplicon were separated on 6% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light to confirm successful PCR amplification for later restriction enzyme digestion or to identify the length polymorphisms. The remainder of each amplicon was digested for ~3 h at 37°C with 1 unit of the appropriate restriction enzyme. The digested products were separated and visualized as described earlier to identify the presence or absence of the restriction sites that characterize mtDNA haplogroups A, C, D, M, and Y-chromosome haplogroups Q-M3* and Q-M242*. Defining polymorphisms of subhaplogroups D3 and D4 were screened by direct sequencing of the amplicons (Table 1).

Nucleotide positions (nps) 16011-00537 of the mitochondrial genome, nearly representing the entire displacement loop (D-Loop), were sequenced in eleven small (<200 bp) overlapping fragments. Primers D-Loop 1-11 were used, and their coordinates, along with PCR conditions, are provided in Table 2. To confirm amplification, 3–4 μL PCR product were visualized on a polyacrylamide gel as described above. Single stranded DNA (e.g. excess primers) was destroyed by adding the remaining PCR product to a 60 μL *Exo*I digestion cocktail that contained 40 U *Exo*I (New England Bio Labs) and 0.33× *Exo*I buffer. This reaction was incubated at 37°C for 90 min, followed by an 80°C hold for 20 min to denature the *Exo*I. The *Exo*I digested DNA was filtered through a Millipore 96-well Montage PCR Microfine Plate and resuspended in 25 μL ddH_2O. This product was submitted for direct sequencing to the Division of Biological Sciences (DBS) Automated DNA Sequencing Facility at the University of California, Davis. Sequencing was performed in both directions and sequences were read off both strands.

Authentication of results

DNA was independently extracted at Trace Genetics, using the protocol described above that was employed at UC Davis, from 0.23 g of tooth root removed from a second molar. The extract was screened, as described above, for the mitochondrial haplogroup D marker, the amelogenin alleles, and the Y-chromosome haplogroup Q-M3* marker. The Y-chromosome Q-M242 polymorphism was screened with a real-time "taqman" assay for SNP detection (Applied Biosystems).

Nucleotides 15996-00537 were sequenced from the extract in eleven small (<200 bp), overlapping fragments, as described earlier. The primers used to sequence this extract included some of those cited above (D-Loop 5, 6, 10, and 11), in addition to others cited in Table 2: D-Loop 1.1, 2.1, 3.1, 4.1, 7.1, 8.1, 9.1. PCR conditions are found in Table 2. Reactions for both forward and reverse sequencing were prepared as described earlier.

Comparison to published sequences

Nucleotide positions 16011-16362 of the OYKC sequence were compared to those of 3286 Native American sequences from published works, two unpublished studies (Johnson and Lorenz, unpublished data; Kemp et al., unpublished data), and other sequences available from Genbank (Table 3). The sequence was also compared to 3824 Asian mtDNA sequences from published works and others available from Genbank (Table 4). The comparative sequences were screened for fourteen pairs of mutations exhibited by the first HVRI of the OYKC individual: 1) 16092(C) and 16223, 2) 16092(C) and 16241(G), 3) 16092(C) and 16301(T), 4) 16092(C) and 16342(C), 5) 16092(C) and 16362(C), 6) 16223(T) and 16241(G), 7) 16223(T) and 16301(T), 8) 16223(T) and 16342(C), 9) 16241(G) and 16301(T), 10) 16241(G) and 16342(C), 11) 16241(G) and 16362(C), 12) 16301(T) and 16342(C), 13) 16301(T) and 16362(C), and 14) 16342(C) and 16362(C). Comparisons were limited to polymorphisms found in nps 16011-16362 because few Native American and Asian mtDNA sequences extended beyond this region. Because many authors of the comparative literature did not make haplogroup assignments, our screening was not limited to individuals belonging to haplogroup D. A search for 16223(T) and 16362(C) was not performed because this combination of polymorphisms is known to be associated with haplogroups other than haplogroup D (e.g. Native American haplogroups A and C).

Phylogenetic analyses and likelihood ratio tests

To determine if nucleotide substitution rates have remained constant over time in the subhaplogroup of haplogroup D containing the OYKC haplotype, two sets of phylogenetic analyses were performed. The first set included sequences spanning nps 16001-00684 (including much missing data) and the second included only nps 16185-16362 of these sequences. In each case, hierarchical LRTs were performed using Modeltest 3.6 (Posada and Crandall, 1998) to select the maximum likelihood model of sequence evolution. The maximum likelihood haplotype tree was constructed in PAUP* 4.0 (Swofford, 2003) using a heuristic search with TBR branch-swapping and the selected model of evolution. A LRT was then performed to compare the rate constant (molecular clock) and rate variable models of sequence evolution. The statistical significance of the LRT statistic (Λ) at the 0.05 level of probability was determined using the χ^2 distribution with $n - 2$ degrees of freedom (df), where n is the number of haplotypes in the phylogenetic tree (Huelsenbeck and Rannala, 1997).

Calibration of the molecular clock

After determining that sequence evolution in the OYKC subhaplogroup of D is consistent with the rate-constant model, the molecular clock was calibrated by using the age of the OYKC remains as a *minimum* date for the

TABLE 1. Mitochondrial and Y-chromosome haplogrouping primers and amelogenin primers, with annealing temperatures

Target region	Defining marker	Primer	Primer coordinates[a]/ sequences (5′–3′)	Annealing temperature	Primer citation
Mitochondrial haplogroup A	*Hae*III 663 (+)	611F	00591-00611	55°C	Stone and Stoneking, 1993
		743R	00743-00765		
Mitochondrial haplogroup B	9 base pair deletion	8215F	08195-08215	55°C	Wrischnik et al., 1987
		8297R	08297-08316		
Mitochondrial haplogroup C	*Alu*I 13262 (+)	13256F	13237-13256	55°C	Parr et al., 1996
		13397R	13397-13419		
Mitochondrial haplogroup D	*Alu*I 5176 (–)	5120F	05099-05120	55°C	Parr et al., 1996
		5190F	05190-05211		
Mitochondrial subhaplogroup D3[c]	*Taq*I 10180 (–)	10120F	10120-10143	66°C[b]	This study
		10287R	10266-10287		
Mitochondrial subhaplogroup D4[c]	RsaI 10646 (+)	10555F	10555-10576	68°C[b]	This study
		10728R	10705-10728		
Mitochondrial haplogroup M	AluI 10397 (+)	10353F	10353-10370	49°C	This study
		10478R	10459-10478		
Sex determination: amelogenin	male = 106/112, female = 106/106	Amel-A	CCCTGGGCTCTGTAAAGAATAGTG	55°C	Sullivan et al., 1993
		Amel-B	ATCAGAGCTTAAACTGGGAAGCTG		
Y-Chromosome M242 region	*Dde*I (+), IVS-866 of DBY gene	M242F	AACTCTTGATAAACCGTGCTG	56°C	Bolnick, 2005
		M242R	AACACGTTAAGACCAATGCTAA		
Y-Chromosome M3 region	*Mfe*I (–), DYS199 C→T	M3F	CGCGGGATAAATGTGGCCAAGTTTT	50°C	Bolnick, 2005
		M3R	AGGTACCAGCTCTTCCCAATT		

Fifteen microliter PCR amplification reactions contained: 0.32 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 2.4 mM primers, 0.3 U of platinum *Taq* (Invitrogen), and 1.5 μL of template DNA. Mitochondrial DNA was amplified with 40 cycles of PCR and the amelogenin and Y-chromosome markers with 60 cycles. PCR conditions were as follows: denaturation at 94°C for 3 min, followed by 15-s holds at 94°C, the annealing temperature specified below, and 72°C, followed by a final 3 min extension period at 72°C.

[a] Coordinates, numbered according to the Cambridge Reference Sequence (Andrews et al., 1999).

[b] Starting annealing temperature for touch-down PCR, as described in Table 2.

[c] Definitive markers of subhaplogroups D3 and D4 were screened by direct sequencing. PCR amplification reactions followed conditions described in Table 2.

TABLE 2. Sequencing primers used in this study, with annealing temperatures

Target region	Primer	Coordinates[a]/sequence (5′–3′)	Annealing temperature	Citation
D-Loop 1	15986F	15986-16010 GCACCCAAAGCTAAGATTCTAATTT	62°C[b]	This study
	16153R	16132-16153 CAGGTGGTCAAGTATTTATGGT		
D-Loop 1.1	15995F	15976-15995 TCCACCATTAGCACCCAAAG	55°C	This study
	16139R	16132-16153 CAGGTGGTCAAGTATTTATGGT		
D-Loop 2	16106F	16106-16126 GCCAGCCACCATGAATATTGT	62°C[b]	This study
	16251R	16230-16251 GGAGTTGCAGTTGATGTGTGAT		
D-Loop 2.1	16131F	16112-16131 CACCATGAATATTGTACGGT	55°C	This study
	16218R	16218-16237 TGTGTGATAGTTGAGGGTTG		
D-Loop 3	16190F	16190-16209 CCCCATGCTTACAAGCAAGT	58°C[b]	This study
	16355R	16331-16355 GGGATTTGACTGTAATGTGCTATGT		
D-Loop 3.1	16209F	16192-16209 CCATGCTTACAAGCAAGT	50°C	This study
	16368R	16368-16385 TCTGAGGGGGGTCATCCA		
D-Loop 4	16232F	16232-16249 CACACATCAACTGCAACT	58°C[b]	This study
	16404R	16383-16404 GGTGGTCAAGGGACCCCTATCT		
D-Loop 4.1	16287F	16268-16287 CACTAGGATACCAACAAACC	55°C	Eshleman, 2002;
	16420R	16420-16439 GCACTCTTGTGCGGGATATT		Kaestle, 1998
D-Loop 5	16353F	16353-16372 CCCTTCTCGTCCCCATGGAT	62°C[b]	This study
	16549R	16530-16549 GGGGAACGTGTGGGCTATTT		
D-Loop 6	16470F	16470-16493 GGGGGTAGCTAAAGTGAACTGTAT	62°C[b]	This study
	00106R	00082-00106 CGGCTCCAGCGTCTCGCAATGCTAT		
D-Loop 7	00034F	00034-00058 GGGAGCTCTCCATGCATTTGGTATT	62°C[b]	This study
	00185R	00160-00185 CCTGTAATATTGAACGTAGGTGCGAT		
D-Loop 8	00112F	00112-00135 CCCTATGTCGCAGTATCTGTCTTT	62°C[b]	This study
	00275R	00249-00275 CTGTGTGGAAAGTGGCTGTGCAGACAT		
D-Loop 8.1	00133F	00109-00132 GCACCCTATGTCGCAGTATCTGTC	55°C	This study
	00256R	00256-00275 CTGTGTGGAAAGTGGCTGTG		
D-Loop 9	00184F	00184-00208 GGCGAACATACTTACTAAAGTGTGT	62°C[b]	This study
	00356R	00331-00356 GGGGTTTGGCAGAGATGTGTTTAAGT		
D-Loop 9.1	00185F	00162-00184 CGCACCTACGTTCAATATTACAG	55°C	This study
	00336R	00336-00355 GGGTTTGGCAGAGATGTGTT		
D-Loop 10	00255F	00255-00279 GCACAGCCACTTTCCACACAGACAT	62°C[b]	This study
	00436R	00415-00436 GGGGTGACTGTTAAAAGTGCAT		
D-Loop 11	00369F	00369-00393 CCCTAACACCAGCCTAACCAGATTT	62°C[b]	This study
	00560R	00538-00560 GGGGTTTGGTTGGTTCGGGGTAT		

Thirty microliter PCR amplification reactions contained: 0.32 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 2.4 mM primers, 0.3 U of platinum *Taq* (Invitrogen), and 3.0 μL of DNA template. Each portion of the hypervariable region was subjected to 60 cycles of PCR, as follows: 3 min denaturing at 94°C, followed by 15-s holds at 94°C, at the annealing temperature, and at 72°C, followed by a final 3 min extension period at 72°C.

[a] Coordinates, numbered according to the Cambridge Reference Sequence (Andrews et al., 1999).

[b] Touch-down PCR used, decreasing the annealing temperature 0.1°C after each cycle.

emergence of the clade. Our calibration assumes that a single founder type of the OYKC subhaplogroup of D was carried to the Americas. Nucleotide diversity (π) was estimated in Mega 3.0 (Kumar et al., 2004) for the following four sets of the data: 1) complete sequences ranging from nps 16185-16362 (Chumash 1 and 2 were excluded from the analysis due to missing data), an analysis representing the best compromise between sample size and length of the first HVRI sequence, 2) all sequences ranging from nps 16024-16383 (including missing data), 3) only those sequences ranging from nps 16024-16383 that are missing $\leq$33% of the data (i.e. sequences that include at least 240 bp), and 4) only those sequences ranging from nps 16024-16383 that are missing $\leq$25% of the data (i.e. sequences that include at least 270 bp). In each case, the standard error of nucleotide diversity was estimated by performing 100,000 bootstrap replicates of the data in Mega 3.0 (Kumar et al., 2004). The molecular clock was calibrated by dividing π by twice the age of the OYKC remains, producing an estimate of percent evolution per site per year. We have found that rates of evolution are inconsistently reported and compared in the literature. While divergence rate and substitution rate (sometimes incorrectly called "mutation rate") are sometimes directly compared, the divergence rate is actually twice the substitution rate because divergence can result from a substitution in either of two lineages that share a common ancestor with equal probability. Here, we calculate the rate of evolution by dividing π by twice the age of the OYKC remains, producing an estimate of percent evolution per site per year, which is equivalent to the substitution rate or one half the divergence rate.

Calibration of the phylogenetic dispersion (ρ)

Phylogenetic dispersion (ρ) and its standard error were estimated as described by Forster et al. (1996) and Saillard et al. (2000) in two manners from median joining networks constructed in Network v. 4.1.0.9 (Bandelt et al., 1999). All of our calibrations of ρ assume that a single founder type of the OYKC subhaplogroup of D was carried to the Americas. The first considered complete sequences spanning nps 16185-16362, so the Chumash 1 and 2 sequences were again omitted from the analysis due to missing data. In the second estimate of ρ, only complete sequences spanning nps 16090-16365 were considered, resulting in an estimate based on 15 lineages descended from the OYKC founding haplotype. In both cases, the age of the OYKC remains was divided by ρ to produce a rate of phylogenetic dispersion (i.e. one mutation per unit

TABLE 3. Native American populations to which the On Your Knees Cave sequence was compared

Population	*N*	Number of haplogroup D	Number of Ds related to OYKC	Reference
Acomawi	1	0	0	Kaestle, 1998
Akimel O'odham	80	1	0	Kemp, 2006
Akimel O'odham	3	0	0	Torroni et al., 1993
Aleut	163	107	0	Rubicz et al., 2003
Apache	6	0	0	Malhi et al., 2003
Apache	1	0	0	Horai et al., 1993
Apache	146	0	0	Budowle et al., 2002
Apache	1	1	0	Torroni et al., 1993
Athapaskan (Alaska)[a]	18	0	0	Shields et al., 1993
Athapaskan (Alaska)[a]	20	0	0	Simonson et al., 1999; F184627-AF184638, AF184640-AF184647
Bella Coola[a]	40	10	0	Ward et al., 1993
Bella Coola	5	4	0	Malhi et al., 2004
Bella Coola	4	1	0	Torroni et al., 1993
Bella Coola	2	0	0	Lorenz and Smith, 1997
Boruca	2	1	0	Torroni et al., 1993
Brazilian (Amazon Region)	92	11	0	Santos et al., 1996
Brazilian (Ancient)	18	1	0	Ribeiro-dos-Santos et al., 1996
Brazilian (Mixed)	82	13	1	Alves-Silva et al., 2000
Cayapa	120	26	26	Rickards et al., 1999
Cherokee (OK Red Cross)	15	0	0	Malhi et al., 2001
Cherokee (Stillwell)	15	0	0	Malhi et al., 2001
Cheyenne/Arapaho	4	0	0	Malhi et al., 2001
Chickasaw	8	0	0	Bolnick and Smith, 2003
Chickasaw/Choctaw	2	0	0	Bolnick and Smith, 2003
Chilean (Indigenous)[a]	45	15	0	Horai et al., 1993
Chippewa	8	0	0	Malhi et al., 2001
Chippewa (Turtle Mountain)	2	0	0	Malhi et al., 2001
Chippewa (Wisconsin)	19	0	0	Malhi et al., 2001
Choctaw	14	0	0	Bolnick and Smith, 2003
Choctaw	1	0	0	Lorenz and Smith, 1997
Chumash	20	4	4	Johnson and Lorenz, 2006
Chumash	5	0	0	Lorenz and Smith, 1997
Ciboney[b]	14	5	0	Lalueza-Fox et al., 2003
Cochimi	1	0	0	Malhi et al., 2003
Columbia Plateau[b]	2	0	0	Malhi et al., 2004
Columbian (Indigenous)	20	1	0	Horai et al., 1993
Columbian Mummy[b]	6	0	0	Monsalve et al., 1996
Cora	72	3	0	Kemp, 2006
Creek	21	8	0	Bolnick and Smith, 2003
Deleware	1	0	0	Malhi et al., 2001
Dogrib	2	0	0	Torroni et al., 1993
Dogrib	1	0	0	Lorenz and Smith, 1997
Douglas Site[b]	1	0	0	Malhi et al., 2004
Embera	42	0	0	Kolman and Bermingham, 1997
Gaviao[a]	27	19	0	Ward et al., 1996
Guaymi	1	0	0	Torroni et al., 1993
Haida	1	0	0	Torroni et al., 1993
Haida[a]	40	2	0	Ward et al., 1993
Hopewell[b]	34	7	0	Mills, 2003
Hopi	1	1	0	Lorenz and Smith, 1997
Hualapai	54	0	0	Kemp, 2006
Huetar	27	7	0	Santos et al., 1994
Huichol	56	0	0	Kemp, 2006
Inuit (Alaska)	2	0	0	Shields et al., 1993
Inuit (Canadian)	46	0	0	Simonson et al., 1999; AF186706-AF186751
Inuit (Greenland)	82	0	0	Saillard et al., 2000
Inuit (Western Greenland)[a]	11	0	0	Shields et al., 1993
Inuit (Western Greenland)[a]	16	0	0	Simonson and Shields, 1999, AF184648-AF184663
Inupiaq	6	0	0	Shields et al., 1993
Inupiaq	10	0	0	Simonson et al., 1998; AF082222-AF0822231
Iowa	3	0	0	Malhi et al., 2001
Jemez	67	0	0	Kemp, 2006
John Day Site[b]	1	0	0	Malhi et al., 2004
Karok	1	0	0	Kaestle, 1998

(*continued*)

TABLE 3. (Continued)

Population	*N*	Number of haplogroup D	Number of Ds related to OYKC	Reference
Kickapoo	5	0	0	Malhi et al., 2001
Kiliwa	1	0	0	Malhi et al., 2003
Klunk Mound[b]	39	5	1	Bolnick, 2005
Kraho	3	0	0	Torroni et al., 1993
Kumeyaay	1	0	0	Malhi et al., 2003
Makiratare	1	0	0	Torroni et al., 1993
Mapuche	40	10	0	Ginther et al., 1993
Mapuche	34	14	2	Moraga et al., 2000
Mataco	3	1	0	Torroni et al., 1993
Maya	3	0	0	Horai et al., 1993
Maya	4	1	0	Torroni et al., 1993
Mexican-American	4	4	4	Johnson and Lorenz, unpublished
Mexico (North-Central)	63	1	1	Green et al., 2000
Micmac	4	0	0	Malhi et al., 2001
Mixe	49	6	0	Kemp, 2006
Mixtec	65	3	0	Kemp, 2006
Nahua	5	0	0	Lorenz and Smith, 1997
Nahua-Atocpan	44	1	1	Kemp, 2006
Nahua-Cuetzalan	29	1	0	Kemp, 2006
Navajo	180	2	0	Budowle et al., 2002
Navajo	4	0	0	Malhi et al., 2003
Navajo	2	0	0	Torroni et al., 1993
Navajo[a]	14	0	0	Simonson and Shields, 1997; AF011670-AF011683
Nespelem Site[b]	1	0	0	Malhi et al., 2004
Ngobe	45	0	0	Kolman et al., 1995
Norris Farm[b]	51	5	0	Stone and Stoneking, 1998
Northern Paiute	2	0	0	Malhi et al., 2004
Northern Paiute	30	12	0	Kaestle, 1998
Nuu-Chah-Nulth	1	0	0	Malhi et al., 2004
Nuu-Chah-Nulth[a]	65	27	0	Ward et al., 1991
Ojibwa	3	0	0	Torroni et al., 1993
Ojibwa	2	0	0	Lorenz and Smith, 1997
Pawnee	3	0	0	Malhi et al., 2001
Pehuence	24	6	0	Moraga et al., 2000
Pomo	4	1	0	Kaestle, 1998
Ponca	3	0	0	Malhi et al., 2001
Potowatomi	1	0	0	Malhi et al., 2001
Pyramid Lake[b]	17	8	0	Kaestle, 1998
Quapaw	3	0	0	Malhi et al., 2001
Quechua (Peruvian)[a]	105	27	1	Fuselli et al., 2003
Salinan	1	0	0	Lorenz and Smith, 1997
SanPoil[b]	1	1	0	Malhi et al., 2004
Seminole	3	0	0	Bolnick and Smith, 2003
Seri	27	0	0	Kemp et al., unpublished
Shawnee	2	0	0	Malhi et al., 2001
Shoshone	3	1	0	Kaestle, 1998
Sioux/Caddoan	2	0	0	Malhi et al., 2001
Sisseton/Wapheton	16	0	0	Malhi et al., 2001
Snake River[b]	1	0	0	Malhi et al., 2004
Tainos[b]	19	4	0	Lalueza-Fox et al., 2001
Takic	4	0	0	Lorenz and Smith, 1997
Tarahumara	55	4	1	Kemp, 2006
Ticuna	3	1	0	Torroni et al., 1993
Tierra del Fuego[b]	24	10	2	García-Bour et al., 2004
Tlingit	6	0	0	Simonson and Shields, 1997; AF012827-AF012832
Tlingit	2	0	0	Torroni et al., 1993
Tohono O'odham	38	0	0	Kemp, 2006
Vantage Site[b]	7	3	0	Malhi et al., 2004
Washo	20	3	0	Kaestle, 1998
Washo	2	0	0	Lorenz and Smith, 1997
Wintun	1	0	0	Kaestle, 1998
Wishram	16	7	0	Malhi et al., 2004
Wounan	33	0	0	Kolman and Bermingham, 1997
Xavante	26	0	0	Ward et al., 1996
Yaghan	15	9	2	Moraga et al., 2000
Yakima[a]	41	6	0	Shields et al., 1993
Yanomama	155	19	0	Williams et al., 2002

(*continued*)

TABLE 3. (Continued)

Population	*N*	Number of haplogroup D	Number of Ds related to OYKC	Reference
Yanomama	3	1	0	Torroni et al., 1993
Yavapai	1	0	0	Malhi et al., 2003
Yuman (Baja)	10	0	0	Lorenz and Smith, 1997
Yupik	25	1	0	Simonson and Shields, 1997; AF011645-AF011669
Yurok	2	0	0	Malhi et al., 2001
Zapotec	74	3	1	Kemp, 2006
Zoro	30	18	0	Ward et al., 1996
Zuni	30	0	0	Kemp, 2006
TOTAL	*3286*	*474*	*47*	

Shown are the number of individuals sampled from each population, the number of individuals that belong to mitochondrial haplogroup D (if known), and the number of these individuals that belong to the same subhaplogroup to which the On Your Knees Cave individual belongs.
[a] Haplogroups determined from the sequence, as haplogroup defining polymorphisms were not screened by the authors.
[b] Ancient DNA/Pre-Columbian population.

TABLE 4. Asian sequences to which the On Your Knees Cave sequence was compared

Population	Location	*N*	Number of haplogroup D[a]	Number of Ds related to OYKC	Reference
Alor	Indonesia	4	0	0	Redd et al., 1995
Altaian		17	?	0	Shields et al., 1993
Altaian		7	0	0	Derenko et al., 2001
Ami	Taiwan	10	0	0	Sykes et al., 1995
Asian		15	?	0	Jorde et al., 1995
Asian		37	?	0	Handt et al., 1998
Ata	Taiwan	4	0	0	Sykes et al., 1995
Ataval	Taiwan	1	0	0	Sykes et al., 1995
Borneo		6	?	0	Lum et al., 1998
Bun	Taiwan	8	?	0	Sykes et al., 1995
Cambodia		12	?	0	Jorde et al., 1995
Cantonese		20	?	0	Betty et al., 1996
Chinese		1	0	0	Lum et al., 1994
Chinese	Beijing	1	?	0	Arnason et al., 1996
Chinese	Southern	10	?	0	Vigilant et al., 1991
Chinese		300	?	0	Yao et al., 2002b
Chinese		14	?	0	Jorde et al., 1995
Chinese		8	?	0	Yao et al., 2002a
Chinese		6	?	0	Horai and Hayasaka, 1990
Chinese		19	?	0	Lum et al., 1998
Chuckchi	Chukchee Peninsula	60	?	0	Voevoda et al., 1994; AF212373-212432
Chuckchi		3	?	0	Shields et al., 1993
Chukchi	Russia–Chukotka	66	8	0	Starikovskaya et al., 1998
H'mong		1	?	0	Lum et al., 1994
H'mong		1	?	0	Vigilant et al., 1991
Han	Fengcheng	51	10	0	Yao et al., 2002a
Han	Kunming, Yunnan	43	6	0	Yao et al., 2002a
Han	Qingdao, Shandong	50	19	1	Yao et al., 2002a
Han	Taiwan	7	1	0	Torroni et al., 1993
Han	Wuhan, Hubei	42	3	0	Yao et al., 2002a
Han	Yili, Xinjiang	47	10	0	Yao et al., 2002a
Han		167	?	0	Oota et al., 2002
Han		30	4	0	Yao et al., 2002a
Han		69	10	0	Kivisild et al., 2002
Havik	India	48	?	0	Mountain et al., 1995
Hiri	Indonesia	6	?	0	Redd et al., 1995
India		298	?	0	Kivisild et al., 1999
India		1	?	0	Horai and Hayasaka, 1990
Indonesian	Flores	6	?	0	Redd et al., 1995
Indonesian		1	?	0	Vigilant et al., 1991
Indonesian		1	?	0	Horai and Hayasaka, 1990
Indonesian		6	?	0	Lum et al., 1994
Inuit	Siberia	3	?	0	Shields et al., 1993
Inuit	Russia - Chukotka	79	16	0	Starikovskaya et al., 1998
Itel'men	Siberia	47	0	0	Schurr et al., 1999
Japanese		19	?	0	Jorde et al., 1995

(*continued*)

TABLE 4. (Continued)

Population	Location	*N*	Number of haplogroup D[a]	Number of Ds related to OYKC	Reference
Japanese		1	?	0	Vigilant et al., 1991
Japanese		61	?	0	Horai and Hayasaka, 1990
Japanese		1	?	0	Lum et al., 1994
Japanese		27	?	0	Lum et al., 1998
Japanese		89	?	0	Oota et al., 2002
Javan	Java	17	?	0	Lum et al., 1998
Jety-Asar[b]	Syr-Darya River, Eastern Aral	3	?	0	Ovchinnikov et al., 1999
Kadar	India	7	?	0	Mountain et al., 1995
Kazak	China-Xinjiang	28	?	0	Yao et al., 2000
Kazak+Kirg	China	149	?	0	Comas et al., 1998
Korean		3	?	0	Horai and Hayasaka, 1990
Korean		306	?	0	Lee et al., 1997
Korean		7	?	0	Lum et al., 1998
Korean		4	2	0	Torroni et al., 1993
Korean		66	?	0	Pfeiffer et al., 1998
Koryak	Siberia	155	2	0	Schurr et al., 1999
Lahu		3	?	0	Yao et al., 2002a
Malaysian		7	?	0	Jorde et al., 1995
Moken	Indian Ocean	8	?	0	Lum et al., 1998
Mongolian		103	?	0	Kolman et al., 1996
Mongolian[b]	Egyin Gol Valley	47	17	0	Keyser-Tracqui et al., 2003
Mukri	Indian	43	?	0	Mountain et al., 1995
Pai	Taiwan	9	?	0	Sykes et al., 1995
Pai	Taiwan	1	?	0	Horai and Hayasaka, 1990
Phillippino		23	?	0	Lum et al., 1998
Phillippino		36	?	0	Sykes et al., 1995
Roti	Indonesia	5	?	0	Redd et al., 1995
Sabah/Kota Kinabalu	Indonesia/Borneo	37	?	0	Sykes et al., 1995
Siberian	Southern	533	89	0	Starikovskaya et al., 2005
Siberian	Western	38	?	0	Voevoda and Shkapenko, 1999; AF214068-AF214105
Siberian[b]	Lokomotiv Site	10	2	0	Mooder et al., 2005
Siberian[b]		1	0	0	Ricaut et al., 2004
Siddis		8	?	0	Thangaraj et al., 1999
Taiwan		1	?	0	Vigilant et al., 1991
Ternate	Indonesia	1	?	0	Redd et al., 1995
Thai	Northern	32	?	0	Yao et al., 2002b
Thai		9	?	0	Lum et al., 1998
Tibetan		40	?	0	Yao et al., 2002b
Timor	Indonesia	2	?	0	Redd et al., 1995
Uighurs		55	?	0	Comas et al., 1998
Urak Lawoi	Indian Ocean	8	?	0	Lum et al., 1998
Uygurs	Xinjiang	45	?	0	Yao et al., 2000
Vietnamese		9	?	0	Jorde et al., 1995
Vietnamese		22	?	0	Lum et al., 1998
Vietnamese		35	?	0	Oota et al., 2002
Yupik	Siberia	77	?	0	Simonson and Shields, 1997; AF013633-AF013709
TOTAL		*3824*	*≥199*	*1*	

Shown are the number of individuals sampled from each population, the number of individuals that belong to mitochondrial haplogroup D (if known), and the number of these individuals that belong to the same subhaplogroup to which the On Your Knees Cave individual belongs.

[a] An entry of a question mark in this field indicates that the study did not determine the haplogroup affiliation of the sequences.

[b] Ancient sample(s).

of time). Like other estimates of the rate of evolution based on dating the age of a founding lineage of a clade (Forster et al., 1996), ours is a *maximum* estimate because the origin of the founding lineage might predate the event used to calibrate the molecular clock.

RESULTS

The mtDNA of the OYKC individual exhibited the *Alu*I site loss at np 5176 and the *Alu*I site gain at np 10397 and, therefore, belongs to haplogroup D. The sample did not exhibit the *Taq*I site loss at np 10180 or the RsaI site gain at np 10646 and, therefore, does not belong to subhaplogroups D3 or D4 (according to the nomenclature of Starikovskaya et al., 2005). The D-loop sequence of this individual, determined from nps 16011-00537, exhibits the following mutations, relative to the Cambridge Reference Sequence (CRS) (Andrews et al., 1999): 16092(C), 16223(T), 16241(G), 16301(T), 16342(C), 16362(C), 0073(G), 00152(C), 00263(G), 00309.1(+C), 00315.1(+C), and 00489(C). The assignment of the mtDNA this individual to haplogroup D and its HVRI sequence (nps 16011-16382) were replicated at UC Davis through a second

TABLE 5. Hypervariable region polymorphisms[a] exhibited by the On Your Knees Cave sample and related samples

Sample	Hypervariable region polymorphisms	Sequence Read	Reference
OYKC	16092, 16223, 16241, 16301, 16342, 16362, 00073, 00152, 00263, 00309.1(+C), 00315(+C), 00489	16011-00537	This study.
KlunkMound[b]	16223, 16241, 16301, 16318, 16342	16016-16367	Bolnick, 2005
Chumash 1[d]	16223, 16241, 16301, 16342	16051-16343	Johnson and Lorenz, 2006
Chumash 2[d]	16223, 16241, 16301, 16342	16051-16360	Johnson and Lorenz, 2006
Chumash 3	16223, 16241, 16301, 16342, 16362	16021-16395	Johnson and Lorenz, 2006
Chumash 4	16223, 16241, 16301, 16342, 16362	16021-16408	Johnson and Lorenz, 2006
Mexican-Am 1	16223, 16241, 16250, 16301, 16330, 16342, 16360, 16362	16086-16395	Johnson and Lorenz, unpublished
Mexican-Am 2	16223, 16241, 16301, 16342, 16362	16060-16395	Johnson and Lorenz, unpublished
Mexican-Am 3	16142, 16223, 16241, 16270, 16301, 16342, 16362	16051-16382	Johnson and Lorenz, unpublished
Mexican-Am 4	16223, 16241, 16342, 16362	16021-16390	Johnson and Lorenz, unpublished
Mexican	16223, 16234(G, 16241, 16301, 16342, 16362	16001-16404	Green et al., 2000
Tarahumara	16223, 16241, 16301, 16342, 16362, 16519, 00071(-G), 00073, 00152, 00263, 00309.1(+C), 00309.2(+C), 00315(+C), 00489, 00523.1(+C), 00523.2(+A), 00533	16001-00686	Kemp, 2006
Nahua	16092, 16223, 16241, 16301, 16342, 16362, 16519, 00071(-G), 00073, 00152, 00263, 00315(+C), 00489, 00533	16001-00686	Kemp, 2006
Zapotec	16129, 16223, 16241, 16301, 16342, 16362, 00073, 00152, 00263, 00309.1(+C), 00309.2(+C), 00315(+C), 00489, 00523.1(+C), 00523.2(+A)	16001-00686	Kemp, 2006
Cayapa 1	16223, 16241, 16291, 16301, 16342, 16362, 00073, 00152, 00263,	16111-16362, 00063-00291	Rickards et al., 1999
Cayapa 2	16223, 16241, 16291, 16342, 16362	16185-16370	Rickards et al., 1999
Cayapa 3	16223, 16241, 16291, 16301, 16342, 16362	16185-16370	Rickards et al., 1999
Cayapa 4	16223, 16241, 16291, 16342, 16359, 16362	16185-16370	Rickards et al., 1999
Brazilian	16114, 16179, 16223, 16241, 16288, 16301, 16342, 16362, 00073, 00152, 00263, 00309.1(+C), 00309.2(+C), 00315(+C),	16060-16362, 00072-00337	Alves-Silva et al., 2000
Quechua	16223, 16241, 16255, 16301, 16342, 16362	16024-16383	Fuselli et al., 2003
Mapuche	16223, 16241, 16301, 16342, 16362, 00073, 00152, 00263	16001-16400, 00030-00407	Moraga et al., 2000
Yaghan	16223, 16234, 16241, 16311, 16342, 16362, 00073, 00152, 00263	16001-16400, 00030-00407	Moraga et al., 2000
Tierra del Fuego 1[c]	16223, 16241, 16342	16156-16393	García-Bour et al., 2004
Tierra del Fuego 2[c]	16223, 16311, 16342, 16362	16156-16393	García-Bour et al., 2004
Han Chinese	16148, 16223, 16249, 16301, 16342, 16362, 00073, 00152, 00263, 00309.1(+C), 00309.2(+C), 00315(+C)	16001-16497, 00031-00407	Yao et al., 2002a

[a] All polymorphisms are transitions, unless otherwise noted (plus sign, nucleotide insertion(s); dash, nucleotide deletion).
[b] Klunk Mound DNA was extracted from skeletal remains dating to 1,825 ± 75 BP (Bolnick, 2005).
[c] Tierra del Fuego samples are represented by a skeletal population dating 100–400 YBP (García-Bour et al., 2004).
[d] At the time of these analyses the state of np 16362 was unknown in these samples. With additional sequencing to 16362, Johnson and Lorenz (2006) determined both to exhibit a cytosine (C) at the position.

extraction of the first tooth, as described in the Materials and Methods section. The sex of this individual was determined to be male, confirming the morphological assessment (Dixon, 1999). The Y-chromosome of this individual exhibits the M242 and M3 markers and, therefore, belongs to haplogroup Q-M3*. The assignment of this individual's mtDNA to haplogroup D, the presence of the D-loop mutations cited above, and the sex determination and Y-chromosome assignment to haplogroup Q-M3* were confirmed with DNA extracted from a second tooth in an independent laboratory at Trace Genetics, in Richmond, CA. In the case of the D-loop sequence, matching, overlapping fragments from multiple amplifications of samples processed in independent laboratories strongly suggest that the results are authentic and unbiased by DNA damage or contamination.

Forty-seven (~1.4%) of the 3286 Native American comparative sequences (or ~10% of Native Americans belonging to haplogroup D) belong to the OYKC subhaplogroup of haplogroup D. All 47 exhibit a thymine (T) at np 16223 and a cytosine (C) at np 16342 and match the CRS (Andrews et al., 1999) at np 16325 (Table 5 and Fig. 1), setting them apart from all other members of haplogroup D in the Americas. Furthermore, 46 of these samples exhibit a guanine (G) at np16241, with an apparent reverse mutation at that position in the Tierra del Fuego 2 lineage (Table 5 and Fig. 2). Forty-four of these individuals were sampled from contemporary populations, whereas the remaining three were sampled from ancient human remains. The Klunk Mound individual is from a burial population at the Peter Klunk Mound Group in Illinois, which is affiliated with the Illinois Hopewell cultural phenomenon and dates to 1,825 ± 75 YBP (Bolnick, 2005). The two individuals from Tierra del Fuego are represented by skeletal remains that date to 100–400 YBP (García-Bour et al., 2004).

Of the 3824 comparative Asian sequences, only one, that of a Han Chinese from Qingdao, Shangdon (Yao

Fig. 1. Map of the Americas that indicates the approximate locations of the On Your Knees Cave individual and related samples. Sample size (*N*) of one at each location unless otherwise noted.

et al., 2002a), is known to belong to the subhaplogroup of haplogroup D containing the OYKC haplotype. This individual's mtDNA exhibits mutations at nps 16223(T) and 16342(C) and matches the CRS (Andrews et al., 1999) at np 16325 (Table 5), mutations common to all of the Native American haplotypes in the OYKC subhaplogroup.

LRTs on the related sequences spanning nps 16001-00684 and the sequences restricted to nps 16185-16362 yielded similar results, with both supporting the null hypothesis of rate constancy (nps 16001-00684: $-2 \log \Lambda = 28.418$, $df = 19$, $P = 0.078$; nps 16185-16362: $-2 \log \Lambda = 11.605$, $df = 13$, $P = 0.560$). Consequently, the mtDNA clock was calibrated using the age of the OYKC skeletal remains as a minimum date for the emergence of the founding haplotype of the clade in the Americas. Dividing our estimate of π by twice the age of the OYKC sample indicates that nps 16185-16362 of HVRI evolve at a rate of 44%/site per myr (95% CI 4.8–82%/site per myr) (Table 6a). HVRI sequences spanning 16024-16383 were estimated to evolve at a rate of 34–44%/site per myr (95% CI 15–82%/site per myr) (Table 6a).

The level of phylogenetic dispersion (ρ) measured within the OYKC clade between nps 16185-16362 ($\rho = 1.222$, SE $= 0.618$) results in a calibration of one mutation per 8,429 years for this stretch of HVRI (Table 6b). Figure 2 exhibits the network from which this measure of phylogenetic dispersion was calculated. Phylogenetic dispersion estimated from nps 16090-16365 ($\rho = 1.118$, SE $= 0.328$, network not

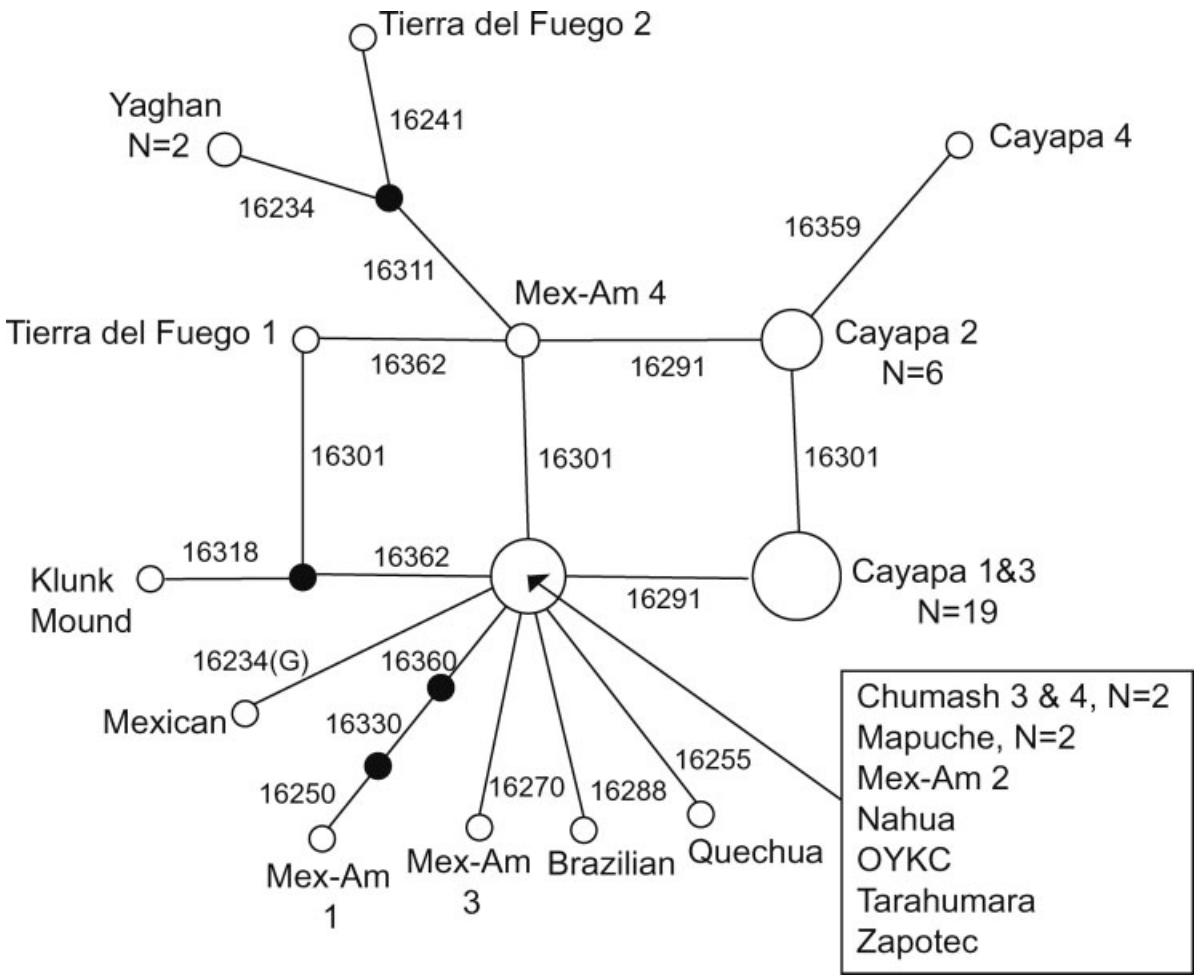

Fig. 2. Network displaying the relationship between the On Your Knees Cave individual and related samples. Black nodes represent hypothetical haplotypes that must have existed (or do exist), but have not been sampled. Sample size (*N*) of one at each node unless otherwise noted.

depicted) results in a calibration of one mutation per 9,213 years (95% CI 5,806–22,294 years) (Table 6b).

DISCUSSION

The OYKC teeth contain well-preserved mitochondrial and sex-chromosomal DNA. DNA was not preserved in the bones of the same individual, which is consistent with previous evidence that DNA preserves better in teeth than in bones (Shook, 2005). The age of the skeletal remains establishes a minimum date of 10,300 YBP for the origin of the OYKC mtDNA haplotype and the Y-chromosome haplogroup Q-M3*.

By the criteria of Torroni et al. (1993), the OYKC haplotype qualifies as an additional founding Native American haplotype, representing a distinct subhaplogroup of haplogroup D that was brought to the Americas. First, descendants of the OYKC haplotype are found in a number of linguistically and geographically diverse populations distributed from Alaska to the southern tip of South America (Fig. 1). Second, the OYKC haplotype is centrally positioned in a phylogeny (Fig. 2). Third, although an exact match of this haplotype was not detected in Asia, the haplotype of one Han Chinese is clearly related to the OYKC haplotype as it exhibits 16223(T) and 16342(C), and matches the CRS at 16325, three polymorphisms common to all of the Native American haplotypes belonging to the OYKC subhaplogroup of haplogroup D (Table 5). Moreover, the antiquity of the OYKC skeletal remains is consistent with the hypothesis that it represents a founder haplotype, as in the case of the oldest known representatives of haplogroups B (Stone and Stoneking, 1996) and C (Kaestle, 1998) in the Americas. The older a lineage, the higher its probability of representing, or closely resembling, the founding haplotype of the haplogroup of which it is a member. This observation may serve as an addition criterion to the standards of Torroni et al. (1993). Rickards et al. (1999) first proposed that derivatives of this haplotype, common among the Cayapa, represented additional founders, but were criticized because they failed to determine the haplogroup to which the hapolotypes belong (Schurr, 2004b). Here we report that the Cayapa samples related to the OYKC sample all exhibit the *Alu*I site loss at np 5176 and the *Alu*I site gain at np 10397 and, therefore, do indeed belong to haplogroup D (Rickards et al., unpublished data). The confirmation that the OYKC subhaplogroup of haplogroup D represents an additional founder type, combined with the recent discovery of two ~5,000-year-old burials from the China Lake site in British Columbia that belong to haplogroup M

TABLE 6. Rates of HVRI evolution measured in this study: (a) molecular clock and (b) phylogenetic dispersion

Sequence read	Samples excluded	Nucleotide diversity and standard error	Percent change/site/Myr[a]	Rho and standard error	One mutation occurs every[a]
a. Molecular clock					
16185-16362	Chumash 1 and 2	$\pi = 0.009$, SE = 0.004	44% (5–82%)		
16024-16383	None	$\pi = 0.009$, SE = 0.004	44% (5–82%)		
16024-16383	Those missing >33% data	$\pi = 0.008$, SE = 0.002	39% (19–58%)		
16024-16383	Those missing >25% data	$\pi = 0.007$, SE = 0.002	34% (15–53%)		
b. Phylogenetic dispersion					
16185-16362	Chumash 1 and 2			$\rho = 1.222$, SE = 0.618	8,429 years (upper bound 4,190 years[b])
16090-16365	Brazilian, Chumash 1 and 2, all Cayapa and Tierra del Fuego			$\rho = 1.118$, SE = 0.328	9,213 years (5,806–22,294 years)

[a] Numbers rounded to nearest whole.
[b] Lower bound could not be calculated as $\rho = -0.14$.

(Malhi et al., in press) indicates that the founding American population was more genetically heterogeneous than previously recognized. Moreover, this newly discovered genetic structure in the Americas indicates that the size of the founding Native America population must have been larger than previously proposed (Hey, 2005).

Presently, the precise nomenclature of the OYKC subhaplogroup of haplogroup D is uncertain. While Yao et al. (2002a) assigned the Han Chinese individual's mtDNA, based on an adenine (A) at np 3010, to subhaplogroup D4, Starikovskaya et al. (2005) regard this mutation as the ancestral state of subhaplogroups D1, D2, D3, and D4. It is likely that whole mitochondrial genome sequences from individuals belonging to the OYKC subhaplogroup of haplogroup D would clarify the relationship of this clade of haplogroup D to others belonging to the same haplogroup.

Our study estimated the average rate of HVRI evolution to be 34–44%/site per myr (Table 6a). Rate heterogeneity across nucleotide positions in HVRI (Meyer et al., 1999) and the exclusion of particular samples, described in the Materials and Methods section, likely contributed to the variation in our average estimates of rates of mtDNA evolution. However, it is interesting that recent aDNA studies of penguins (Lambert et al., 2002) and bison (Shapiro et al., 2004) have also estimated rapid rates of HVRI evolution: 93–96%/site per myr (95% CI 40–144%/site per myr) and 32%/site per myr (95% CI 23–41%/site per myr), respectively. If this rapid rate can be substantiated for humans, its significance is threefold. First, this rate of evolution is faster than previous estimates of ~10–20%/site per myr inferred by phylogenetic methods (Ward et al., 1991; Horai et al., 1995; Bonatto and Salzano, 1997a; Stone and Stoneking, 1998). While the lower bound of our 95% confidence interval overlaps these estimates, the upper bound ranges from 53 to 82%/site per myr. Although our rate represents a maximum, it has not taken saturation into account, which would lead to our rate being underestimated. Similarly, the data presented here also provide evidence that the rate of phylogenetic dispersion (ρ) has been underestimated, as our calibration of ρ, one mutation per 9,213 years for nps 16090-16365, is greater than twice the rate estimated of Forster et al. (1996) of one transition per 20,180 years. The possible underestimate of this rate by Forster et al. (1996) may stem from the incorrect assumption that almost all mtDNA diversity within subhaplogroup A2 was eliminated during the Younger Dryas event. The reason that our rate of phylogenetic dispersion decreased when nucleotides 16090-16184 were added to the analysis (Table 6b) is that, through the addition of these nucleotides, we necessarily had to remove a number of samples (see Materials and Methods). In this case, the removal of samples missing any data from 16090 to 16365 ultimately removed known variation at nps 16249 16288, 16291, and 16359 used to calculate the rate from nps 16185-16362.

Second, our study suggests that the rapid rates of evolution estimated in pedigree studies (Howell et al., 2003 and references therein) extend further back in time than predicted (Gibbons, 1998). A recent synthesis of available pedigree data found the mean estimate of HVR evolution to be 47.5%/site per myr (95% CI 26.5–78.5%) (Howell et al., 2003). Our estimate is intermediate between rates measured in short term (pedigree) and long term (phylogenetic) studies, consistent with the hypothesis that the measurable rate of evolution declines systematically, but not precipitously, with time (Ho et al., 2005; Ho and Larson, 2006). If correct, this evidence lends support to the notion that traditional phylogenetic based estimates of mtDNA evolution are inappropriate for relatively recent prehistoric population events. It is also interesting to note here that while saturation has been argued to explain the lower estimates of molecular evolution inferred by phylogenetic methods (see discussion by Howell et al., 2003), we have demonstrated that the inability to "observe" reversals at hotspots over time has not resulted in a markedly reduced rate of molecular evolution over the past 10,300 years.

Third, if previous estimates of HVRI evolution are too slow, the age of events based on them have been overestimated. This might explain the conflict between the proposed early entrance of humans into the Americas based on molecular evidence (15,000-40,000 YBP, Forster et al., 1996; Bonatto and Salzano, 1997b; Stone and Stoneking, 1998) and evidence from that archaeological record that suggests a later colonization of the Americas (Fiedel, 2001, 2004; Schurr, 2004b). Applying our most conservative rate of 34%/site per myr (95% CI 15–53%/site per myr) to the nucleotide diversity estimate ($\pi = 0.86$) for mtDNA haplogroups A, B, C, and D in Native Americas (Bonatto and Salzano, 1997b), indicates that human entered the Americas ~13,438 YBP (95% CI 8,113–28,667 YBP). While this estimate does not preclude the possibility of an early entry, the estimate is also compatible with an entry more recent than 15,000 YBP. A late human

entry in the Americas (<15,000 YBP) is supported by the fact that no reliably dated human remains have been documented in the Americas that are older than circa 11,000–11,500 ^{14}C YBP (~13,000 YBP) (Dixon, 1999; Johnson et al., 2002). Toth (1991) suggests that if a model is assumed for the colonization of the Americas that uses an ever-increasing human population, the odds of documenting the very earliest evidence of human occupation are very slim. However, the earliest dates on human remains are minimum limiting dates that demonstrate occupation prior to that time, circa 11,000–11,500 ^{14}C YBP. This independently supports our conclusion that initial colonization may have occurred only a few thousand years earlier than the earliest reliable radiocarbon dates on human remains. A late entry is also consistent with Y-chromosome evidence which support an occupation within the past 20,000 years (Bortolini et al., 2003; Seielstad et al., 2003; Zegura et al., 2004). Lastly, The linguistic model of Nettle (1999) can accommodate a recent entry of humans into the Americas.

The OYKC sample also places a minimum date of 10,300 YBP on the emergence of Y-chromosome haplogroup Q-M3*, which is believed to have first evolved in Beringia. This date is older than the estimate of 7,510 ± 681 YBP by Bortolini et al. (2003), falls within the range estimated by Karafet et al. (1999) of 7,900 ± 5,000 YBP, and is younger than Schurr's (2004a) estimate of ~13,800 YBP. Bianchi et al. (1998) and Underhill et al. (1996) have estimated the origin of this haplogroup to predate 22,000 YBP, with a minimum date of 13,500 YBP (Bianchi et al., 1998). The date of OYKC as the minimum age of haplogroup Q-M3* is very near the lower limit (10,100 YBP) of the estimate based on nucleotide diversity within the haplogroup (Zegura et al., 2004). The date of human entrance into the Americas would likely provide a maximum age for this lineage, as it appears to be Native American specific (Underhill et al., 1996). The estimated age for haplogroup Q-M242* (~15,000–18,000 YBP; Bortolini et al., 2003; Seielstad et al., 2003), from which Q-M3* is derived by a single mutation, also places a maximum of age on the Q-M3 mutation. If short tandem repeats (STRs) can eventually be screened in the OYKC individual, and his corresponding Y-chromosome haplotype determined, a rate of Y-chromosome evolution could be estimated as we have done here for mtDNA.

CONCLUSIONS

Our study demonstrates the utility of aDNA evidence for testing hypotheses generated by modern DNA studies. Future genetic analyses of ancient human remains will undoubtedly provide continued insight about human prehistory. The data from this study provide evidence for an additional founding Native American haplotype of haplogroup D, suggesting that other founder haplotypes and/or haplogroups exist, but have yet to be detected or recognized as such (Malhi et al., 2002, in press; Schurr, 2004b). Furthermore, the rate of evolution estimated here suggests that the antiquity of human occupation in the Americas may have been overestimated by previous mtDNA studies. This observation would also hold true for the dating of any other event based on previously underestimated rates of mtDNA evolution. For example, it might also explain why recent molecular dates for human occupation of Southeast Asia, Australia, and the Southwest Pacific exceed archaeological evidence by >15,000 years (Forster and Matsumura, 2005; Macaulay et al., 2005; Merriwether et al., 2005). We are hesitant to place an exact date on the peopling of the Americas based on molecular data, as doing so will require further consideration of the number of founder haplotypes and refinements to the calibration of the molecular clock. In the interim, we believe that the archaeological record will provide the best clues for determining the finer details of when and how humans moved across the globe and first entered the Americas.

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