AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY 132:000–000 (2007)

A Preliminary Analysis of the DNA and Diet of the Extinct Beothuk: A Systematic Approach to Ancient Human DNA

Melanie Kuch,[1,2] Darren R. Gröcke,[3] Martin C. Knyf,[3] M. Thomas P. Gilbert,[4] Ban Younghusband,[5] Terry Young,[5] Ingeborg Marshall,[6] Eske Willerslev,[4] Mark Stoneking,[7] and Hendrik Poinar[1,2,8]*

[1]*McMaster Ancient DNA Center, McMaster University, Hamilton, ON L8S 4L9, Canada*
[2]*Department of Anthropology, McMaster University, Hamilton, ON L8S 4L9, Canada*
[3]*Stable-Isotope Biogeochemistry Laboratory, School of Geography and Earth Sciences, McMaster University, Hamilton, ON, Canada L8S 4L9*
[4]*Ancient DNA and Evolution Niels Bohr Institute and Institute of Biology, University of Copenhagen, Copenhagen, Denmark*
[5]*Discipline of Genetics, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3V6*
[6]*Institute of Social and Economic Research, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3V6*
[7]*Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany*
[8]*Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada L8S 4L9*

KEY WORDS ancient human DNA; Beothuk peoples; native American Y SNP

ABSTRACT We have used a systematic protocol for extracting, quantitating, sexing and validating ancient human mitochondrial and nuclear DNA of one male and one female Beothuk, a Native American population from Newfoundland, which became extinct ~180 years ago. They carried mtDNA haplotypes, which fall within haplogroups X and C, consistent with Northeastern Native populations today. In addition we have sexed the male using a novel-sexing assay and confirmed the authenticity of his Y chromosome with the presence of the Native American specific Y-QM3 single nucleotide polymorphism (SNP). This is the first ancient nuclear SNP typed from a Native population in the Americas. In addition, using the same teeth we conducted a stable isotopes analysis of collagen and dentine to show that both individuals relied on marine sources (fresh and salt water fish, seals) with no hierarchy seen between them, and that their water sources were pooled or stored water. Both mtDNA sequence data and Y SNP data hint at possible gene flow or a common ancestral population for both the Beothuk and the current day Mikmaq, but more importantly the data do not lend credence to the proposed idea that the Beothuk (specifically, Nonosabasut) were of admixed (European-Native American) descent. We also analyzed patterns of DNA damage in the clones of authentic mtDNA sequences; there is no tendency for DNA damage to occur preferentially at previously defined mutational hotspots, suggesting that such mutational hotspots are not hypervariable because they are more prone to damage. Am J Phys Anthropol 132:000–000, 2007.

Newfoundland, a sub-Arctic island of about 112,000 sq km off the coast of Canada, was home to the Beothuk at the time of John Cabot's arrival in 1497. This native group is believed to have numbered between 500 and 700 individuals (Marshall, 1996) at that time. These hunters and fishers moved with the seasons between the coast and the interior. From early contact onward the Beothuk avoided Europeans, retreating to less accessible harbors and coves on the coast and to inland areas away from European fishing premises and settlements. They also did not develop trade relations, and rejected European firearms, foodstuff, and clothing (Marshall, 1996). As the settler population expanded and Montagnais (Innu) from Labrador and Mi'kmaq from Cape Breton Island came to hunt and fish in Newfoundland more extensively, the Beothuk were unable to procure sufficient subsistence within the areas left to them. Competition for resources and resulting animosities led to a cycle of violence between Beothuk and some of the newcomers (Howley, 1915; Marshall, 1996). This conflict, combined with disease and possibly other factors, brought about their demise in the late 1820s (Marshall, 1981). In 1819 members of the small group of remaining Beothuk, including Demasduit and her husband, chief Nonosabasut, encountered a party of 10 armed English settlers at Red Indian Lake. In the ensuing confrontation on the frozen lake, chief Nonosabasut was killed, as was his brother, and his wife Demasduit was taken captive. It was hoped that she could mediate peace with her people but she died from tuberculosis before she could rejoin

Grant sponsor: Social Sciences and Humanities Research Council; Grant numbers: 410-2004-0579 (HP) and 410-2006-1191 (DG).

*Correspondence to: Hendrik Poinar, Department of Anthropology, McMaster University, 1280 Main St. West, Hamilton, ON, Canada L8S 4L9. E-mail: poinarh@mcmaster.ca

Received 26 April 2006; accepted 1 November 2006.

DOI 10.1002/ajpa.20536
Published online in Wiley InterScience (www.interscience.wiley.com).

her kin. Although Demasduit provided a list of Beothuk words and a few years later her niece, Shanawdithit, the last known Beothuk, gave some additional cultural information (Howley, 1915; Hewson, 1978; Marshall, 1996), we know little about many aspects of Beothuk culture, diet, history and origin, and about their relationship with neighboring native groups. While the Beothuk's prehistoric ancestors in Newfoundland have been traced by archaeological methods to 1900 BP (Renouf, 2004; personal communication), links with the earlier Maritime Archaic Indian population (5500 to 3000 BP in Newfoundland) have been suggested but not confirmed.

To investigate the origin and diet of these two Beothuk and their relation to current day native peoples of the Northeast, we analyzed mtDNA, nuclear DNA, and stable isotopes from a tooth each from their skulls, using a quantitatively focused approach for ancient human DNA.

MATERIALS AND METHODS

DNA analyses

One tooth each was removed from the skulls of the two Beothuk individuals, Demasduit and Nonosabasut, which are currently housed in the National Museums of Scotland in Edinburgh. They have been dated between 1819 and 1820 respectively. In an attempt to cause as little further damage to the external phenotype of the teeth as possible, we embedded the tooth from Demasduit, which was already broken into two pieces, in RTV silicone rubber (RTV11, GE Silicones) (Gilbert, 2004) {No. 1698}. Excess silicone covering the tip of the root was removed, the root was cut open, and the inside of the root cavity, including the dentine, was removed using a drill. The powder was then collected. We used 40 mg of this powder for the DNA extraction. From the male sample, a small broken-off piece from the root of the tooth, which was in poor shape due to an abscess, was broken into smaller pieces, and 60 mg were used for the DNA extraction.

We decalcified the tooth powder and the pieces of tooth using 1 ml of 0.5 M EDTA (pH 8.0) at room temperature overnight in the dark on a rotating wheel. Post demineralization, we kept the EDTA solutions for future processing. We digested the remaining pellets in 1 ml of digestion buffer (10 mM Tris (pH 8.0), 10 mM NaCl, 5 mM CaCl, 2 mM EDTA, 1% SDS, 0.5 mg/ml PK, 10 mg/ml DTT, 10 mM PTB) at 55°C overnight in the dark on a rotating wheel. This resulted in almost complete digestion of the remaining powder or pieces. We extracted these solutions using Phenol (1x) and Phenol/Chloroform/Isoamylalcohol (25:24:1) (2x). The supernatant from the digest and the remaining EDTA-buffer from the initial demineralization step (stored in the interim at 4°C) were separately filtered through pre-moistened microcon YM-10 (Millipore, USA). After concentrating to about 100 μl, both the supernatant from the digest as well as the EDTA-buffer were mixed twice with 100 μl of H_2O and further centrifuged to concentrate. Approximately 100 μl of final extract was recovered from the digest and ~150 μl from the EDTA-buffer. Following failed attempts to quantitate DNA from either extracts, due to obvious inhibition, we purified the extracts using the silica based QIAquick PCR Purification Kit (Qiagen Inc., Canada) as per manufacturers instructions. We concentrated both extracts, EDTA and digest, as they will be referred to from this point forward, down to 50 μl total volume.

To determine the number of mtDNA molecules in our extracts, we designed a quantitative PCR (qPCR) assay using the L16209 and H16303 (according to Anderson) primer pair for the mtDNA hypervariable region I (HVRI). This section of HVI contains 5 of the 10 single nucleotide polymorphisms, which help define the five major Native American haplogroups known from present day indigenous populations (A, B, C, D, and X) (Schurr et al., 1990, Forster et al., 1996, Malhi et al., 2002). We generated a standard curve by amplifying a serial dilution with the following copy numbers: 31,250, 6,250, 1,250, 250, 50, and 10 generated from a purified PCR product of known DNA concentration (measured by UV spectrophotometry). For each quantitation we ran two standards in parallel. This assay had a reproducible sensitivity at ~10 DNA copies. The efficiency of the assay was between 95 and 100% as calculated from the slope of the standard curve (Fig. 4a). We quantified nuclear DNA using the cMyc proto-oncogene on chromosome 8q24 using a previously described assay (Morin et al., 2001), except that we replaced the probe with SYBR green (Fig. 4b). To ensure that the PCR was not inhibited due to coeluates in the DNA extract, we quantitated undiluted extracts as well as 1:10 dilutions of the extracts. Each 20 μl qPCR reaction contained the following; 1X PCR Buffer II, 2.5 mM $MgCl_2$, 400 μM dNTPs (each), 300 nM each primer (L16209, H16303; cMyc_E3_F1, cMyc_E3_R1), ref dye (1:500 dil., Stratagene), 0.167X SYBRgreen (Sigma), 1 unit AmpliTaq Gold, and 2.5 or 5 μl of DNA template. The temperature profile for the reaction included an initial activation of the enzyme at 95°C for 7 min, followed by 50 cycles of the following: 95°C for 30 sec, 58°C (HVRI)/59°C (cMyc) for 30 sec and 72°C for 30 sec followed by a final cycle of 95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec (to generate a dissociation curve) and a final extension of 72°C for 15 min.

All PCR reactions were carried out in 20 μl reaction volumes at 1X PCR Buffer II, 2.5 mM $MgCl_2$, 400 μM dNTPs (each), 300 nM each primer, 1 unit AmpliTaq Gold, and 2.5 μl of template. Cycling parameters were 95°C for 7 min, followed by 40–50 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 45–60 sec with a final extension of 72°C for 15 min. We used the following primer combinations: L16287/H16379, L16209/H16379, L16131/H16379, and L16055/16218 for HVI regions (Stone and Stoneking, 1998); and L13257/H13377 for the Hinc II site at position 13259 that helps define haplogroup C. Each PCR product was amplified twice, once from the extract and once from the EDTA solution used for demineralization. In addition, only the amplification products for the Hinc II site were gel-purified and re-amplified, and 5 μl of the re-amplification products were incubated with 1X PCR Buffer II, 2.5 mM $MgCl_2$, 500 μM dATP, and 0.6 units AmpliTaq Gold in a 10 μl reaction prior to cloning. As all Y SNP amplifications for contemporary populations are 200bp or larger, and it is unlikely that this will be attainable from fossil extracts, we designed new primers around the Y-SNP QM3, present in >60% of all Native American Y chromosomes (Zegura et al., 2004), to amplify a 50bp product. The primers used were the following Y-SNP_M3(DYS199)_F: 5′-AATGGGTCACCTCTGGGACT, Y-SNP_M3(DYS199)_R: 5′-CATTTTAGGTACCAGCTCTTCCTAA. Amplifications for the Y SNP involved the following parameters: 95°C for 7 min, followed by 60 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec with a final extension of 72°C for 15 min. Five microliters of template DNA was used for each amplification.

TABLE 1. The number of amplifiable molecules of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) as determined via qPCR[1]

	mtDNA				nDNA		Totals	
Sample	DNA	DNA 1:10	EDTA	EDTA 1:10	DNA	DNA 1:2	mtDNA (per gram tooth)	nDNA (per gram tooth)	Ratio (mt/n)
Nonosabasut	407 ± 50%	44	99	9	137	50	136,000; 85,000–250,000	20,833 (11,670–33,330)	6.5 (2.5–22)
Desmaduit	210 ± 50%	19	190	18	3	1	84,000; 100,000–300,000	600 (250–1,250)	140 (80–1,200)

[1] MtDNA qPCR contained 2.5μl of extract as did the 1:2 dilution of the nDNA. The straight nDNA contained 5μl of extract.

We cloned all PCR products using the TOPO TA cloning kit and TOP 10 competent cells as per manufacturers instructions, except that we scaled down to one-quarter reactions (Invitrogen, Canada). Clones were screened using blue/white selection and amplified using the M13 F/R primer pairs. These PCR products were purified using AcroPrep 96 Filter Plates (Pall) and Multiscreen $PCR_{\mu 96}$ Plates (Millipore). The purified products were quantitated using a UV spectrophotometer and each product sequenced in one direction. Using BigDye version 1.1 on an ABI 3130 (ABI) as per manufacturers suggestions.

We applied a novel (Kuch and Poinar, in preparation) amelogenin sexing assay, designed for highly degraded human DNA, to both extracts. The reaction volume was 20 μl and the conditions were the following: 1X PCR Buffer II, 4.5 mM $MgCl_2$, 400 μM dNTPs (each), 300 nM each primer (Amelo_F, Amelo_R), 200 nM each probe (Amelo-X (Vic), Amelo-Y (Fam)), 1 unit AmpliTaq Gold, and 5 μl of DNA template. The temperature profile for the reaction included an initial activation of the enzyme at 95°C for 7 min, followed by 50 cycles of the following: 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec followed by a final extension of 72°C for 15 min.

Stable-isotope analysis

Collagen is a useful material for understanding the diet of ancient humans, since it remodels itself throughout the life of an individual and thus isotopically records a lifetime-average of diet (Gröcke, 1998). In order to obtain carbon- and nitrogen-isotope ratios from the small amount of dentine made available, we modified the standard collagen extraction method (Katzenberg and Weber, 1999) by only de-carbonating the dentine sample and conducting bulk organic isotope analysis on the remaining material. Carbon- and oxygen-isotope analysis of the enamel carbonate was performed on <2 mg of tooth enamel. All stable-isotope measurements were performed in the Stable-Isotope Biogeochemistry Laboratory at McMaster University. Organic carbon- and nitrogen-isotope analysis was obtained using a COSTECH elemental analyzer connected to a ThermoFinnigan Delta-Plus XP. Enamel carbonate carbon- and oxygen-isotope analysis was obtained using a VG OPTIMA with an Isocarb system. Isotopic measurements were checked using internal standards calibrated against international standards (NBS 19 and 21). Isotopic results are reported in the standard delta (δ) notation in parts per thousand (‰) relative to VPDB (carbon and oxygen) and atmospheric N_2 (nitrogen): i.e., for carbon, $\delta^{13}C‰ = (^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{standard}) - 1 \times 1000)$.

RESULTS

DNA results

We quantitated the number of 133bp mtDNA fragments in 2.5 μl and 5 μl from the male and female DNA digests, as well as the EDTA fraction, via qPCR (Table 1). To test for inhibition in the extracts, we quantitated 1:10 fold dilutions of all extracts. Quantitative PCR of 5 μl and the 1:10 fold dilutions from both the male and female extract indicated slight inhibition in the female extract, thus we attempted quantitation using 2.5 μl. The DNA digest from the male tooth contained ~400 (200–600) copies in 2.5 μl, and thus 8,000 (4,000–12,000) copies in the total extract (50 μl), The EDTA extract contained four fold (1.3–12-fold) less DNA (~100 copies) (50–150) or

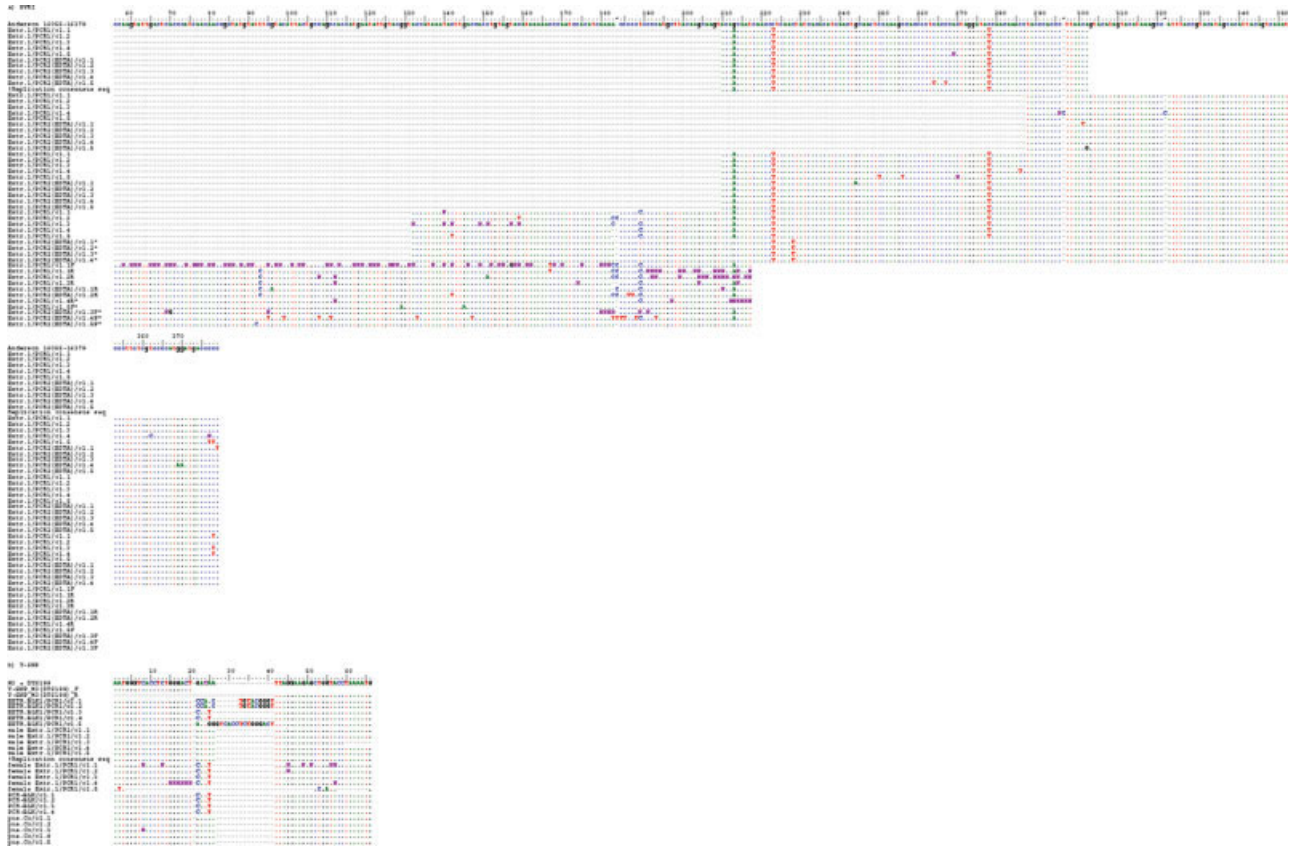

Fig. 1. (**a**) Sequences of clones from Nonosabasut's hypervariable region 1 mtDNA sequence, and (**b**) Y SNP QM-3. Dots indicate identity to the Cambridge Reference Sequence at top (Anderson et al., 1981). Ambiguous bases are indicated by standard abbreviations. * denotes contaminants and † indicates consensus replication sequence. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

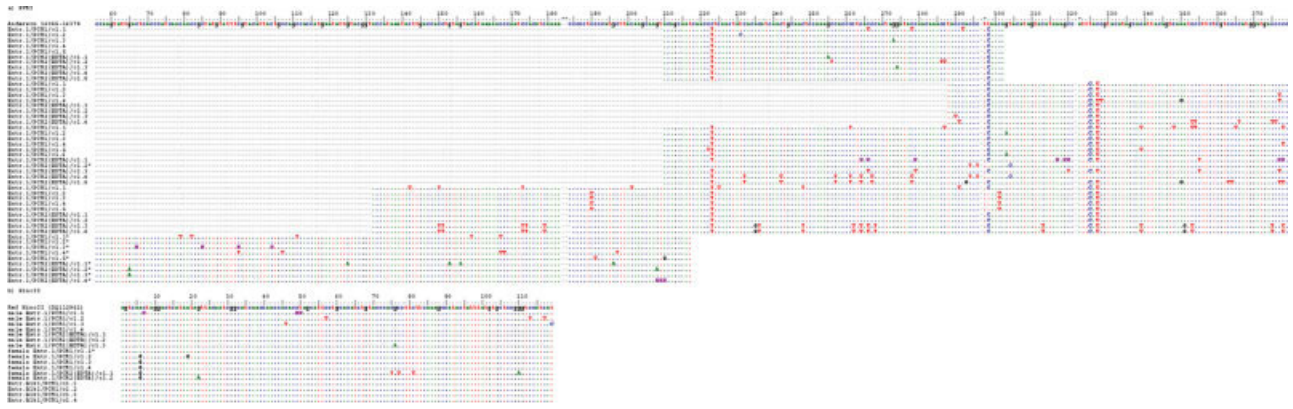

Fig. 2. (**a**) Sequences of clones from Desmaduit's hypervariable region 1 mtDNA sequence, and (**b**) Hinc III region. Dots indicate identity to the Cambridge Reference Sequence at top (Anderson et al., 1981). Ambiguous bases are indicated by standard abbreviations. * denotes possible contaminants. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

2,000 (1,000–3,000) copies per extract. Thus, the male tooth yielded ~125,000 (85,000–250,000) copies per gram. The female tooth digest contained ~200 (100–300) copies per 2.5 μl, 4,000 (2,000–6000) copies per extract (50 μl) and, surprisingly, the same number of copies in the EDTA solution as in the digest (190 vs. 210 copies/2.5 μl). Thus, there were a total of ~200,000 (100,000–300,000) copies per gram of tooth and therefore little difference in the number of amplifiable mtDNA copies per gram tooth, between the (EDTA + digest) male and female extracts. As all quantitative values could be the result of a mixture of endogenous and exogenous DNA contamination we cloned and sequenced the qPCR product.

The qPCR product (L16209/H16303) was cloned for the male (m) and the female (f) extracts, and five clones from each PCR product were sequenced (Figs. 1a and 2a).

TABLE 2. Substitutions found in consensus sequences derived from clones from both the male and female DNA extracts[1]

HVR position	13259	16093	16189	16213	16223	16278	16298	16325	16327	QM3 (YSNP)
European	A	T	T	G	C	C	T	T	C	C
Nonosabasut	•	**C**	**C**	**A**	**T**	**T**	•	•	•	T
Desmaduit	G	•	•	•	**T**	•	**C**	**C**	**T**	na

[1] Numbers refer to position in the mitochondrial hypervariable region. Dots indicate identity to the "European" Cambridge Reference Sequence (CRS) (Anderson et al., 1981) as well as Ysnp data (Underhill et al., 2000); na, not applicable.

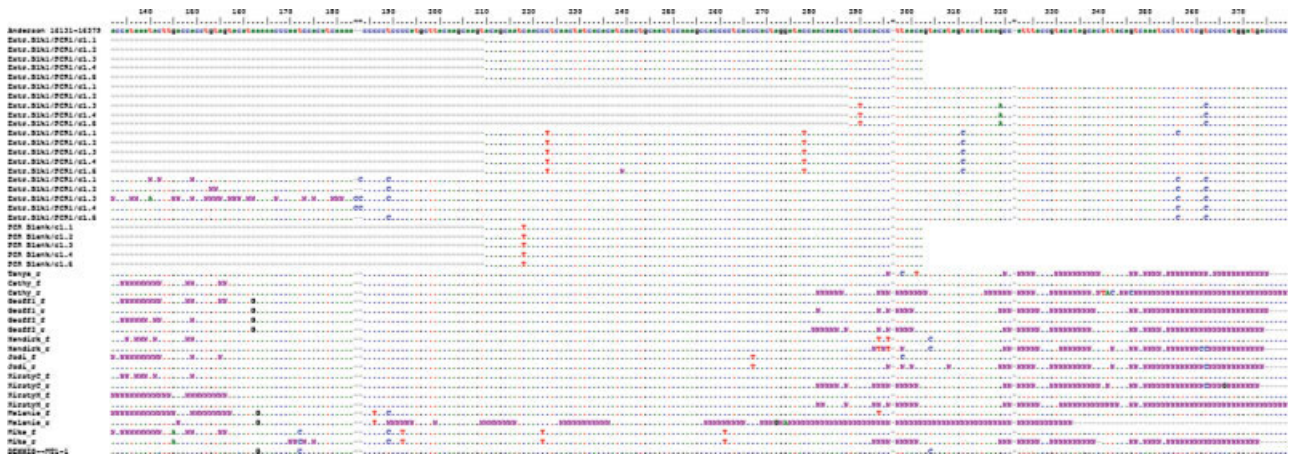

Fig. 3. Sequences of clones from the blank extract of the hypervariable region 1 mtDNA sequence. Dots indicate identity to the Cambridge Reference Sequence at top (Anderson et al., 1981). Ambiguous bases are indicated by standard abbreviations. Sequences of laboratory workers HVRI given at base. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

There were three single nucleotide polymorphism (SNPs) differences to the Cambridge Reference Sequence (CRS) for the male sample, position (+16,000) 213, 223, and 278 and two SNPs for the female sample, positions (+16,000) 223, and 298. Neither of the samples contained more than one consensus sequence. We obtained sequences from 16056 to 16378 of HVRI in five overlapping pieces, as follows. We amplified a 131bp product using the overlapping primer pair 287/379 from both extracts, cloned the products and sequenced 10 (5 digest and 5 EDTA from male) and 8 (4 digest and 4 EDTA from female) clones respectively. These products yielded a single consensus sequence in both extracts again. There were no differences in this stretch from the CRS to the male sample, and for the female the SNP at 16298 was reproduced and two additional SNPs at positions 16325 and 16327 were found; all SNPs are common to haplogroup C.

To ensure that this portion of the sequence was authentic for the male sample we amplified a 209bp product using primer pair 209/379 to incorporate the SNPs in the 209/303 piece, from both extracts, cloned the products and sequenced 10 clones from the male (5 and 5) and 11 (6 and 5) from the female. All clones in the male extract contained the same three SNPs seen in the 209/303 products and lack any additional SNPs between positions 16287/16379. These SNPs indicate that Nonasabut's mtDNA belongs to haplogroup X. The female product contained the same consensus sequence as the two previous products; however, there were many additional singletons, which are likely to reflect damage. We amplified a 287bp product using primer pairs L16131/H16379, cloned the products and sequenced 9 (5 and 4) clones from each extract. Five of the nine clones from the male (all from the extract, not from the EDTA) yield a single consensus sequence which matches the one produced from all 3 previous shorter fragments, but includes an additional SNP at position 16,189. In addition, four of the nine clones (from the EDTA extract) contain a contaminating HVR sequence, which is closest to the CRS when compared to a database of 10,000 or more HVR sequences (HVR base website). The female extract yields a single consensus sequence replicating the original SNPs found at positions 223, 298, 325, and 327, as well as additional SNPs found within the sequences of the clones that appear to be the result of damage to the endogenous DNA and/or polymerase errors. Finally we amplified a 203bp product using primers 16055/16218, cloned the products and sequenced 5 clones from the digest and EDTA extract respectively. In these clones an additional SNP at position 16093 was found in the male, however a possible C at position 16183 could not be unambiguously resolved due to the poly C stretch (16184–16193), thus this position was excluded from the final sequence and further comparative analyses. Clones from the female extract contained no new SNPs.

Finally, to confirm the haplogroup designation of the female as C, we amplified a 167bp piece around the Hinc II site at position 13259 using primers L13257/H13377. Seven (four from digest and three from EDTA) clones were sequenced from the male and six clones (four and two) from the female. Five out of the six clones from the female DNA showed an A-G substitution at position 13263 and therefore lack the Hinc II site, while the male DNA contained the Hinc II site. Thus, these data in conjunction with the HVRI data, suggest assignment of the male to mtDNA haplogroup X and the female to mtDNA haplogroup C (Table 2).

It is interesting to note that only 5 (out of a total 98 sequenced) clones from the demineralized EDTA step

TABLE 3. Mean evolutionary rates for damage sites in clones of amplicons from HV1 from the two ancient samples[1]

	Amplicon			
	131/380	209/303	209/380	287/380
N	31	12	34	19
Mean rate (observed)	0.30	1.40	1.05	0.55
Mean rate (Meyer)	0.74	0.95	0.77	0.71
Mean rate (resamples)	0.75	0.95	0.76	0.72
P	0.99	0.10	0.08	0.72

[1] Damage sites were only counted in clones that also contained substitutions diagnostic for the authentic HV1 sequence inferred for that sample. Amplicons are designated by the amplification primers (+16000); *N* is the total number of damage sites observed per amplicon; the observed mean rate is for the damage sites, based on the per site rates from Meyer et al. (1999); the Meyer mean rate is the overall mean rate from Meyer et al. (1999) for sites within the amplicon; the resample mean rate is the mean rate for 1000 resamples of *N* sites from each amplicon; and *P* is the probability of the observed mean rate (or higher) based on the resamples. The data are combined for the male and female samples as these did not differ significantly from each other (data not shown).

and 2 (out of a total 98 sequenced) clones from the digested DNA extract appear to be the result of exogenous DNA contamination (Figs. 1 and 2). Thus while these skulls had been handled over a period of nearly 200 years, the extracted DNA contained predominantly endogenous and not exogenous DNA, which provides hope for future DNA analysis from teeth.

One extraction blank contained <10 mtDNA copies in 2.5 μl as seen through qPCR. We amplified four HVR1 fragments from this extraction blank, cloned the products and sequenced multiple clones (see Fig. 3). Not surprisingly, the cloned products yielded a minimum of 4 different consensus sequences. None of these sequences were identical to the male or female DNA, and thus they were not cross contaminants, nor do the sequences match any member of our laboratory group (see Fig. 3). Thus, the appearance of a novel sequence in human extracts, which does not match any person from the laboratory, is of little value in assessing the authenticity of ancient human DNA and should not be used to defend the authenticity of a "novel" sequence.

To assess if the sites at which DNA damage was observed ("damage sites") fall preferentially within mutational hotspots in HVRI, for each cloned amplicon we calculated the average evolutionary rate for the damage sites, using previous estimates (Meyer et al., 1999). We then sampled 1,000 times (with replacement), from the distribution of evolutionary rates for the sites covered by each amplicon, a number of sites equal to the number of damage sites in the amplicon, and calculated the average evolutionary rate for each random resample. From the distribution of average evolutionary rates for the 1,000 random resamples, we then obtained the empirical probability of observing the average evolutionary rate for the damage sites in each amplicon (Table 3). For all four amplicons, the average evolutionary rate for damage sites was not significantly greater than that for the random resamples. There is thus no indication that damaged sites in the ancient DNA are occurring preferentially at mutational hotspots in HVRI.

Even though damage is not occurring preferentially at mutational hotspots, there still may be a nonrandom distribution of damaged sites across HVRI. To investigate this, we followed the approach of Gilbert et al. (2003) and compared the observed distribution of the number of times a substitution resulting from damage was observed at each site, to the expected Poisson distribution, assuming damage occurs at random. The results (see Fig. 5) indicate that the observed distribution of the number of times each site had a damage substitution does not differ significantly from that expected if such damage is distributed randomly across the 248 positions (16132–16379 inclusive) that were analyzed in HVRI (x^2 test, $P > 0.25$). However, this analysis could be confounded by the fact that the number of amplicons cloned and sequenced for each site varied, ranging from one to four; we therefore repeated the analysis for a subset of 155 positions (16210–16287 and 16303–16379 inclusive) for which clones from three amplicons were sequenced. The observed distribution still did not differ significantly ($P > 0.15$) from the expected distribution obtained by assuming that damage is distributed at random across the HVRI region (data not shown).

We quantitated the number of 81bp nuclear DNA copies using a *c-myc* assay previously described (Morin et al., 2001). The male DNA extract contained ~140 copies (70–200) of an 81bp nuclear fragment in 5 μl of extract, ~1,150 (700–2000) copies per total extract and ~21,000 (11,670–33,330) copies per gram of tooth (Table 1), The female extract contained ~ 3 (1–5) copies per 5 μl of extract, 30 (10–50) per extract and ~600 (250–1,250) per gram of tooth (without the EDTA). Thus there was a ~40-fold (10 to 135-fold) difference in the number of nuclear DNA copies between the male and female teeth. These relative amounts are in stark contrast to the poor morphological preservation of the male tooth, again emphasizing the discordance between morphological and molecular preservation.

The ratio of mtDNA to nuclear DNA is ~7 (2.5–21) for the male and 140 (80–1,200) for the female. Thus the number of amplifiable molecules in the male extract does not follow the often cited 1:1,000 nuclear DNA to mtDNA ratio, however this is confounded by the slightly different sizes of both nuclear and mitochondrial DNA amplicons. To determine if other nuclear DNA loci would be consistent with the *c-myc* results, we attempted to determine the sex for both samples.

In order to confirm the presence of single copy nuclear DNA in our DNA extracts we attempted to sex both the male and female extracts using a novel amelogenin assay (Kuch and Poinar, in preparation) that amplifies an 92bp fragment from both the X and Y chromosome and then discriminates between the X and Y copies of this fragment by two minor groove binding probes. The male DNA extract shows the presence of both the X and Y alleles (Fig. 4c; however, the female extract did not yield any amplification products, consistent with the low nuclear DNA quantity seen for the *c-myc* assay. However as this result could be due to contamination, we looked for a Native American specific SNP on the Y chromosome, to establish the Y chromosome's authenticity in the male's DNA extract, and rule out the possibility of exogenous DNA contamination.

We successfully amplified a single 50bp product from the male extract, from the female and a single amplification blank control, cloned the products and sequenced 5 clones from each (Fig. 1b). Sequences from clones of the

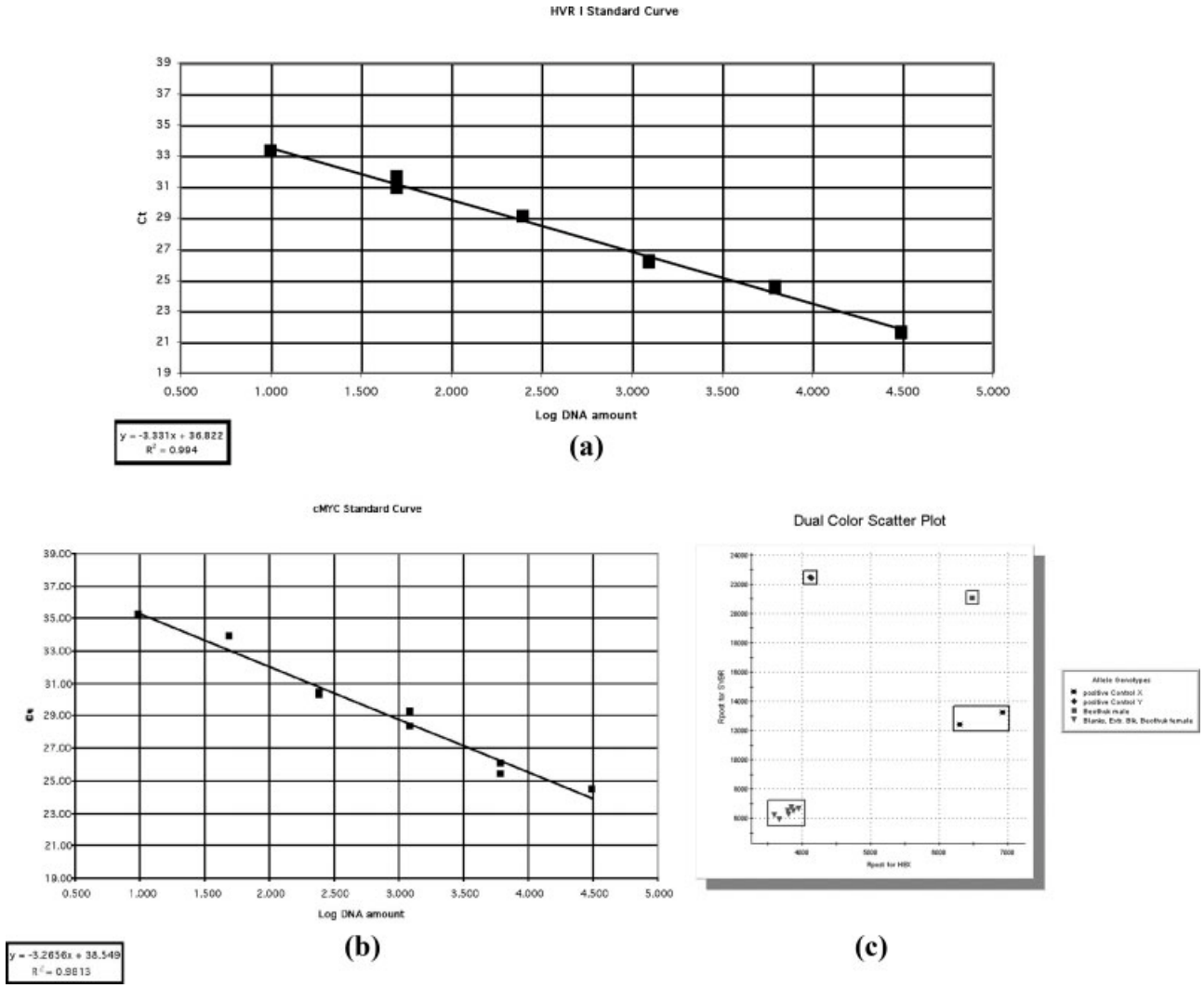

Fig. 4. (**a**) Standard curve of real-time PCR assay for HVRI. Plotted is the initial template quantity (*x*-axis), versus the Ct (threshold cycle). The box shows the slope, *y*-intercept, and R^2 value. (**b**) Standard curve of real-time PCR assay for cMYC. Plotted is the initial template quantity (*x*-axis), versus the Ct (threshold cycle). The box shows the slope, *y*-intercept and R^2 value. (**c**) Allelic discrimination of the Amelogenin gene from Nonosabasut and Desmaduit in comparison to an X and Y standard. Rpost is the final fluorescence reading (in arbitrary units) in a plate-read experiment.

TABLE 4. Isotopic results from the male and female Beothuk samples[1]

	Measured					Predicted		
	$\delta^{13}C_{en}$	$\delta^{18}O_{en}$	C/N	$\delta^{13}C_{org}$	$\delta^{15}N_{org}$	$\delta^{13}C_{diet}$	$\delta^{18}O_{water}$	$\delta^{15}N_{diet}$
Nonosabasut	−12.55	−5.28	+3.18	−15.81	+13.05	−18.81	−7.43	+11.45
Desmaduit	−12.44	−5.15	+3.15	−15.28	+13.20	−18.28	−7.23	+11.60

[1] $\delta^{13}C_{diet}$ and $\delta^{15}N_{diet}$ were determined using a laboratory-based average fractionation factor of –3‰ and –1.6‰ respectively for collagen (Schwarcz and Schoeninger, 1991; Koch, 1998). $\delta^{18}O_{water}$ was determined using Eq. (3) in Dupras and Schwarcz (2001), rearranged to determine a water value. C/N ratios are indicative of pristine collagen (Gröcke, 1997).

blank and the female extract appeared to be the result of primer dimmer formation. Sequences of all clones (Fig. 1b) from the male extract yielded a single consensus sequence. This sequence contains the C to T substitution at position 181 characteristic of the M3 lineage, which is restricted to Native Americans (Underhill et al., 2000); thus the Y allele appears to be an endogenous Native American sequence.

To verify the mtDNA and YSNP results, a sub-sample of the tooth from the male was sent to an independent laboratory in Copenhagen, dedicated to aDNA research. DNA was extracted from the tooth according to the method described above. PCR amplifications of a portion of the mtDNA HV1 region (16209/16303) and the Y Single Nucleotide Polymorphism were independently determined, and the products were cloned and 45 clones were

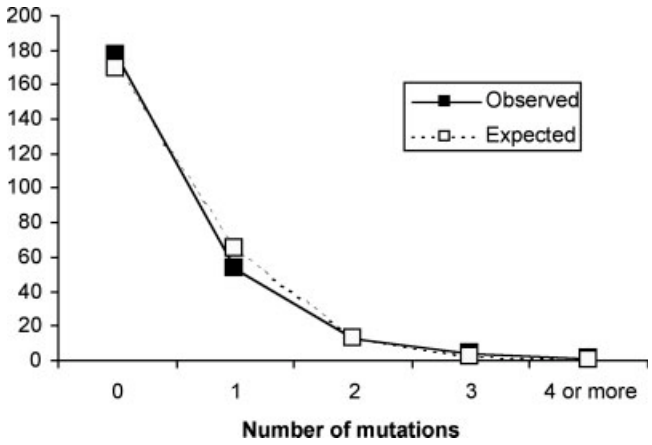

Fig. 5. Distribution of the observed number of mutations per site among cloned sequences from the two ancient specimens (i.e. postmortem damage), compared to the expected distribution assuming that such mutations are occurring at random.

sequenced for the 16209/16303 piece and 34 clones for the Y-SNP. In both cases consensus sequences were identical to the sequences previously determined at McMaster University for those positions (Figs. 1 and 2).

Stable Isotope results

A summary of the stable-isotope results is presented in Table 4. All results are generated from replicate analyses. Analytical reproducibility of replicate analyses of the samples was better than 0.1‰ for carbon, nitrogen and oxygen.

DISCUSSION

DNA preservation and analysis

Our samples contain ~163 (80–240) (male) and 84 (40–120) (female) amplifiable copies of a 133bp mtDNA fragment per microlitre of extract. This is lower than the "1,000" molecule cut off for obtaining authentic endogenous sequences previously suggested by (Handt et al., 1996). Nevertheless, we have obtained reproducible Native American DNA sequences, from amplicons ranging from 133bp up to 287bp in length from both the male and female teeth, despite repeated handling during a time span of nearly 180 years at the National Museums of Scotland. Only four clones from a single amplification (the longest PCR) from the EDTA extract, as well as possibly 5 clones in the 055/218 amplicon from the male (digest and EDTA), appear to be the result of exogenous DNA contamination. For the female in the 055/218 amplicon there are no SNP's to distinguish between endogenous and contaminating DNA, hence the authenticity of this amplicon cannot be determined. Thus, while qPCR is a useful screening method, to set a strict cutoff for the number of amplifiable molecules is not appropriate as tissue types are likely to vary dramatically in preservation capability and contamination resistance. Our qPCR results initially suggested too little mtDNA (<1,000 copies) present in the extract to allow for nuclear DNA analysis (assuming a 1:1,000 nuclear to mtDNA ratio), however the absence of contaminating HVRI DNA sequences in the digests from both samples convinced us to attempt nuclear DNA quantification. The male extract contained 27 (14–40) copies and the female extract 1 (0.2–1) copy per micro liter of an 81bp nuclear DNA fragment. The ratio of nuclear DNA to mtDNA was 1:2–17 for the male and 1:40–600 for the female, both well above the expected ratio. Surprisingly both our samples contain predominantly endogenous DNA and lack heavy exogenous DNA contamination. Teeth therefore represent a relatively less contaminated source of ancient human DNA, than does porous bone.

Sites at which DNA damage occurred were identified as singleton substitutions in clones of the amplicons, and were only counted as damage sites if the clone sequence also contained the relevant substitutions for the authentic ancient HVRI sequence from that sample. The average evolutionary rate (Meyer et al., 1999) of damage sites did not differ significantly from the average evolutionary rate of random resamples (Table 3), indicating that DNA damage in the ancient samples did not occur preferentially at hypervariable positions. Nor is there any significant tendency for damage to occur preferentially at particular sites in the HVRI region (see Fig. 5). These findings contradict a previous study that found that damage sites in ancient human DNA samples were correlated with hypervariable sites, and moreover that damaged sites were distributed nonrandomly (Gilbert et al., 2003). However, the significance of the correlation between damage rates and evolutionary rates was not determined in this study. Moreover, most of the samples came from northern Europeans, and hence it is possible that some of the interclone variation attributed to damage actually reflects contaminating sequences or other artefacts such as jumping PCR. Because our analysis of damage sites was limited to clones, which contained substitutions diagnostic for the authentic Native American mtDNA inferred for each sample, contamination is not a possible explanation for our results. Instead, our results suggest that hypervariable sites in HVRI are not hypervariable because they are more prone to DNA damage (of the type that occurs in ancient DNA). Instead, other mechanisms must be responsible, such as misincorporations during replication (Malyarchuk and Rogozin, 2004).

Nonosabasut's mtDNA contains five SNPs in the HVR1 (16093, 16189, 16213, 16223, 16278) that clearly place it within haplogroup X and the SNP at position 16213 places it within the Native American specific clade X2a (Reidla et al., 2003). Haplogroup X is a common haplotype within Northeastern Native peoples, at frequencies of up to 50% in some populations such as the Mi'kmaq (Malhi et al., 2001), although sample sizes are small ($n = 6$). Nonosabasut's sequence matches 13 of a total of 37 Native American haplogroup X sequences, which represent 12 lineages established in previous work (Smith et al., 1999; Malhi et al., 2001). The matching sequences stem from a single individual of the Mi'kmaq of Nova Scotia, two individuals from the Chippawa (Chipewyan) of North Dakota/Alberta, nine individuals from the Wisconsin Chippawa (Chipewyan) from Hayward/Wisconsin, and a single individual from the Kiowa of Oklahoma. This haplotype is one of the most frequent and represents 35% of all known Native American X haplotypes.

Demasduit's mtDNA contains four SNPs in the HVR1 (16223, 16298, 16325, 16327) and one at position 13259 all of which place it within haplogroup C. Haplogroup C like X, is found at relatively high frequencies up to 35% within Northeastern American populations such as the Wisconsin Chippawa (Malhi et al., 2001). Our sequence

matched six individuals out of 60 Native American haplogroup C sequences, representing 23 lineages, including a single individual from the Mi'kmaq of Nova Scotia, four individuals from the Cherokee of Oklahoma and a single individual from the Stillwell Cherokee of Oklahoma (Malhi et al., 2001). This haplotype is less frequent than the X, being present in ~10% of all known C haplotypes.

It is interesting to note that while some C and X haplotypes are found together at high frequencies in northeastern Native American groups, those of Demasduit and Nonosabasut have (as of yet) not been found together in any current Native American population. However, this may be due to a sampling bias in extant Northeastern peoples, as few groups have been sampled. Neither sequence is itself unique, but their presence in Newfoundland some 200 years ago and as far west as Alberta and south as Oklahoma suggest that both could well have been original founder haplotypes in the New World (Malhi et al., 2001). The presence of both haplotypes among the current Mi'kmaq population suggests either gene flow between them and the Beothuk or a shared ancestral founder population or both. The fact that the languages spoken by both groups are believed to be of Algonquian origin, supports the idea of a common ancestry, though this may date back millennia. It has also been documented that in the early 1800s, when the Beothuk group began to disintegrate, some Beothuk joined the Mi'kmaq, either voluntarily or through kidnapping (Marshall, 1996). However, the numbers are very small and without more sampling of the current Mi'kmaq population in Newfoundland, it is not possible to make a precise statement about common ancestry, possible gene flow, or both. Another population that may have experienced gene flow from the Beothuk and *vice versa* are the Labrador Innu. During the historic period relations between these two groups were friendly and archaeologists and linguists have already proposed a genetic relationship—either due to a common ancestry (both the Innu and Beothuk languages are believed to be part of the Algonquian phylum) or to intermarriage or both (Fitzhugh, 1978; Hewson, 1978; Pastore, 1986).

However, characterization of additional available Beothuk skeletal remains and sampling of Mi'kmaq, Innu and possibly other Northeastern populations should provide a clearer picture.

Due to good DNA preservation in the tooth from Nonosabasut and the low level of DNA contamination we were able to type the Q M3 SNP from the Y chromosome. QM3 along with P-M45 represent the majority of Native American Y chromosomes, with Q haplotypes representing approximateley >75% of all New World Y chromosomes (Zegura et al., 2004). The presence of a Q haplotype in the Beothuk is further proof of the authenticity of the DNA recovered from Nonosabasut's tooth.

The DNA results also shed light on claims of European admixture in the Beothuk, suggested by previous mtDNA analyses (Reed, 2001) and by morphological analyses of the skull, with the latter indicating that "Nonosabasut... has a hooked nose and a mix of Caucasian and Mongoloid features" (S. Black, Anatomy and Forensic Anthropology, Faculty of Life Sciences, University of Dundee; personal communication). Despite these claims both mtDNA from Desmaduit and Nonosabasut, as well as the Y SNP data from Nonosabasut, are clearly of Native American origin, and thus our genetic data do not lend credence to any European admixture in the DNA of Nonosabasut from either of his parents.

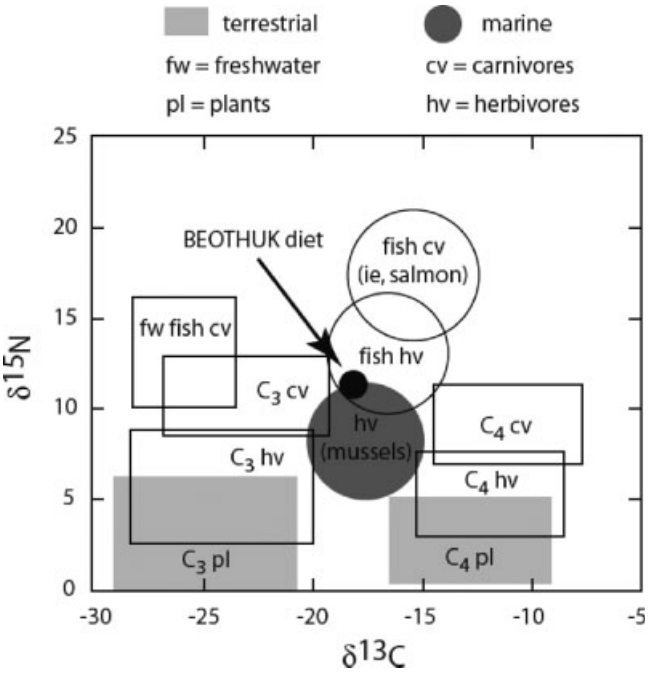

Fig. 6. $\delta^{15}N$ versus $\delta^{13}C$ ranges of various marine and terrestrial dietary sources and trophic level effects. Terrestrial sources are in boxes, while marine sources in circles. Shaded objects represent base level of trophic structure. Note that the estimated value for the Beothuk diet is plotted within the region of marine fish and mussels.

Stable isotope results

As seen in Table 4, the nitrogen-isotope ratios for both Nonosabasut and Desmaduit are quite elevated, suggesting that their diet was not based solely on terrestrial resources. Schoeninger and DeNiro (1984) have shown that $\delta^{15}N$ values less than +9‰ are obtained from individuals who predominantly fed on terrestrial resources, whereas values more than +15‰ are obtained from individuals who predominantly fed on marine resources. However, individuals whose diet consists primarily of freshwater fish also generally exhibit elevated $\delta^{15}N$ values (Schoeninger and DeNiro, 1984). Thus, based on nitrogen isotopes alone, it is difficult to distinguish between a predominantly terrestrial and a predominantly marine diet. Our understanding of Beothuk foraging strategies indicates that their diet was largely composed of caribou and smaller fur bearers, Harp seal, sea birds and their eggs, salmon and other species of fish as well as mollusks and crustaceans (Marshall, 1996). Lakeshore camping was prominent during the winter season and although ice fishing may have taken place, their diet consisted of frozen and stored foods, the latter mostly from caribou killed during their fall caribou drive and some seal. Based on an average fractionation factor between diet and $\delta^{15}N$ of collagen of +1.6‰ from laboratory experiments (Koch, 1998) it is reasonable to suggest that the two Beothuk individuals were consuming a considerable amount of marine-based foods (such as seal, salmon, sea birds) (see Fig. 6). The fact that both individuals have extremely similar $\delta^{15}N$ values suggests that there was no dietary distinction (hierarchy) between them (e.g., the male did not consume the richer, more protein-rich foods).

The carbon-isotope ratios isolated from dentine collagen also suggests a significant marine dietary source ($\sim -18‰$), since most marine organisms, including seals, mollusks, arthropods and fish, give carbon-isotope ratios ranging between $-18‰$ to $-10‰$ (Schoeninger and DeNiro, 1984). Freshwater fish as a dietary source for these individuals is difficult to ascertain since freshwater fish have highly variable $\delta^{13}C$ values, as demonstrated for example from the Lake Baikal region (e.g., from -25 to $-14‰$; Katzenberg and Weber, 1999). As shown in Figure 6, the diet of the two Beothuk individuals plots within the range of marine sources, but we hesitate to suggest it was a dominant source since terrestrial food resources were probably extremely important, especially during the last decades of their existence as it became increasingly difficult for the Beothuk to access the sea shore (Marshall, 1996). Once again, it is important to note that both individuals have very similar $\delta^{13}C$ values, suggesting no significant dietary differences.

Oxygen-isotope ratios from tooth enamel can provide information about the drinking water that an individual consumed at the time of tooth growth, and thus the position of their geographic location relative to this resource (Gat, 1996; Koch, 1998). Modern precipitation $\delta^{18}O$ maps obtainable from the IAEA (2001; http://isohis.iaea.org/) indicate that the annual mean modern $\delta^{18}O$ values of precipitation from Newfoundland range between -10 and $-18‰$, which is more depleted than the water $\delta^{18}O$ values as predicted from the Beothuk teeth values (Table 4). A possible solution to account for this offset would be the use of standing water: accumulated water will evaporate and during this process ^{16}O is preferentially removed, thus the remaining freshwater has more positive $\delta^{18}O$ values (Gat, 1996). Under these circumstances it is reasonable to suggest that the two Beothuk individuals analyzed in this study drank standing (pooled) water either from a lake, a well and/or stored water in vessels that were susceptible to evaporation processes, rather than water from rivers. However, recently Daux et al. (2005) suggests that phosphate oxygen-isotope data from human teeth in France over the Little Ice Age are elevated by $\sim 2‰$ due to a change in the $\delta^{18}O$ value of meteoric water. At present we cannot dismiss such an affect on the isotopic composition of the teeth from these two Beothuk individuals from Newfoundland. Once again, the $\delta^{18}O$ values found in both individuals are very similar, indicating the same water source for both.

CONCLUSIONS

Using a meticulous and quantitative approach to the investigation of ancient human DNA, we isolated the complete mtDNA hypervariable region 1 sequences from one male and one female individual of the Beothuk, a people who formerly inhabited the Island of Newfoundland but became extinct in the 1820s. We have sexed the male using a novel-sexing assay designed for degraded ancient human DNA and amplified and sequenced, for the first time, an authentic ancient Y SNP. The sequences demonstrate the presence of mtDNA haplogroups X and C, as well as the Y haplogroup Q-M3 in the Beothuk population of Newfoundland some 200 years ago. We also have analyzed dentine collagen and tooth enamel stable-isotope ratios, which indicate that a significant portion of the diet of the two Beothuk individuals consisted of marine foods, that they drank mostly lake water rather than river water, and that both individuals had equal access to all types of food and water.

ACKNOWLEDGMENTS

We thank D. Poinar, Kirsten Bos, Carsten Schwarz, Mike Bunce, J.L Barta, and the McMaster Ancient DNA Centre for constructive criticism of the manuscript as well as three anonymous reviewers. We thank C. Gagosz for support in the project and facilitating access to the teeth for the molecular work and The National Museums of Scotland in Edinburgh for willingly providing teeth.

LITERATURE CITED

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457–465.

Daux V, Lecuyer C, Adam F, Martineau F, Vimeux F. 2005. Oxygen istope composition of human teeth and the record of climate changes in France (Lorraine) during the last 1700 years. Clim Change 70:445–464.

Dupras TL, Schwarcz HP. 2001. Strangers in a strange land: Stable isotope evidence for human migration in the Dakhleh Oasis, Egypt. J Arch Sci 28:1199–1208.

Fitzhugh WW. 1978. Winter cove 4 and the Point Revenge occupation of the Central Labrador coast. Arctic Anthropol 15.

Forster P, Harding R, Torroni A, Bandelt, H-J. 1996. Origin and evolution of Native American mtDNA variation: A reappraisal. Am J Hum Genet 59:935–945.

Gat JR. 1996. Oxygen and hydrogen isotopes in the hydrologic cycle. Annu Rev Earth Planet Sci 24:225–262.

Gilbert MT. 2004. Distribution patterns of postmortem damage in human mitochondrial DNA. Am J Hum Genet 72:32–47.

Gröcke DR. 1997. Stable-isotope studies on the collagenic and hydroxylapatite components of fossils: palaeoecological implications. Lethaia 30:65–78.

Gröcke DR. 1998. Collagenic and carbonate hydroxylapatite spacing in prehistoric humans: possible relationship with dietary protein. Lethaia 31:13–14.

Handt O, Krings M, Ward RH, Paabo S. 1996. The retrieval of ancient human DNA sequences. Am J Hum Genet 59:368–376.

Hewson J. 1978. Beothuk vocabularies, Vol.2. St. Johns: Newfoundland Museum. Technical papers of the Newfoundland Museum.

Higuchi RG, Wrischnik LA, Oakes E, George M, Tong B, Wilson AC. 1987. Mitochondrial DNA of the extinct quagga: Relatedness and extent of postmortem change. J Mol Evol 25:283–287.

Howley JP. 1915. The Beothuks or red indians. Cambridge: Cambridge University Press.

Katzenberg MA Weber A. 1999. Stable isotope ecology and palaeodiet in the Lake Baikal region of Siberia. J Arch Sci 26:651–659.

Koch PL. 1998. Isotopic reconstruction of past continental environments. Annu Rev Earth Planet Sci 26:573–613.

Malhi RS, Eshleman JA, Greenberg JA, Weiss DA, Schultz Shook BA, Kaestle FA, Lorenz JG, Kemp BM, Johnson JR, Smith DG. 2002. The structure of diversity within New World mitochondrial DNA haplogroups: implications for the prehistory of North America. Am J Hum Genet 70:905–919.

Malhi RS, Schultz B, Smith DG. 2001. Distribution of mitochondrial DNA lineages among native American tribes of northeastern North America. Hum Biol 73:17–55.

Malyarchuk BA Rogozin IB. 2004. Mutagenesis by transient misalignment in the human mitochondrial DNA control region. Ann Hum Genet 68:324–339.

Marshall I. 1981. Disease as a factor in the demise of the Beothuk Indians. In: Wilton C, editor. Change and continuity, Vol.1. Toronto: McGraw-Hill Ryerson.

Marshall I. 1996. A history and ethnography of the Beothuk. Montreal: McGill-Queen's University Press.

Meyer S, Weiss G, von Haeseler A. 1999. Pattern of nucleotide substitution and rate heterogeneity in the hypervariable regions I and II of human mtDNA. Genetics 152:1103–1110.

Morin PA, Chambers KE, Boesch C, Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (Pan troglodytes verus). Mol Ecol 10:1835–1844.

Pastore RT. 1986. Excavations at Boyd's cove. The 1985 field season, a preliminary report. In: Thomson JS and Thomson C, editors. Archeology in newfoundland and labrador 1985, Vol. 6. St. Johns: Historic Resources Division, Department of Culture, Recreation and Youth.

Reed AM. 2001. Mitochondrial DNA analysis of Nonosabasut, a Beothuk Indian chief. MA thesis, Ball State University, Muncie, In.

Reidla M, Kivisild T, Metspalu E, Kaldma K, Tambets K, Tolk HV, Parik J, Loogvali EL, Derenko M, Malyarchuk B, Bermisheva M, Zhadanov S, Pennarun E, Gubina M, Golubenko M, Damba L, Fedorova S, Gusar V, Grechanina E, Mikerezi I, Moisan JP, Chaventre A, Khusnutdinova E, Osipova L, Stepanov V, Voevoda M, Achilli A, Rengo C, Rickards O, De Stefano GF, Papiha S, Beckman L, Janicijevic B, Rudan P, Anagnou N, Michalodimitrakis E, Koziel S, Usanga E, Geberhiwot T, Herrnstadt C, Howell N, Torroni A, Villems R. 2003. Origin and diffusion of mtDNA haplogroup X. Am J Hum Genet 73:1178–1190.

Schoeninger MJ, DeNiro MJ. 1984. Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals. Geochim Cosmochim Acta 48:625–639.

Schurr TG, Ballinger SW, Gan Y-Y, Hodge JA, Merriwether DA, Lawrence DN, Knowler WC, Weiss KM, Wallace DC. 1990. Amerindian mitochondrial DNAs have rare Asian mutations at high frequencies, suggesting they derived fro four primary maternal lineages. Am J Hum Genet 46:613–623.

Schwarcz HP, Schoeninger MJ. 1991. Stable isotope analyses in human nutritional ecology. Yearbook Phys Anthropol 34:83–321.

Smith DG, Malhi RS, Eshleman J, Lorenz J, Kaestle F. 1999. Distribution of mtDNA haplogroup X among native North Americans. Am J Phys Anthropol 110:271–284.

Stone AC Stoneking M. 1993. Ancient DNA from a pre-Columbian Amerindian population. Am J Phys Anthropol 92:463–471.

Stone AC, Stoneking M. 1998. mtDNA analysis of a prehistoric Oneota population: Implications for the peopling of the New World. Am J Hum Genet 62:1153–1170.

Underhill PA, Shen P, Lin AA, Jin L, Passarino G, Yang WH, Kauffman E, Bonne-Tamir B, Bertranpetit J, Francalacci P, Ibrahim M, Jenkins T, Kidd JR, Mehdi SQ, Seielstad MT, Wells RS, Piazza A, Davis RW, Feldman MW, Cavalli-Sforza LL, Oefner PJ. 2000. Y chromosome sequence variation and the history of human populations. Nat Genet 26:358–361.

Zegura SL, Karafet T, Zhivotovsky LA, Hammer MF. 2004. High-resolution SNPs and microsatellite haplotypes point to a single recent entry of native American Y chromosomes into the Americas. Mol Biol Evol 21:164–175.