Lack of founding Amerindian mitochondrial DNA lineages in extinct Aborigines from Tierra del Fuego–Patagonia

Carles Lalueva*, Alejandro Pérez-Pérez*, Eva Prats1,+Lluís Cornudella1,* and Daniel Turbón

INTRODUCTION

The peopling of the New World has engendered much discussion and heated controversy (1). Multiple migrations across the Beringia landbridge have been proposed to explain population diversity in America on the basis of diverse evidence (2) inclusive of differing dental characters (3). These migratory waves have been correlated with the distribution of native American languages into three main linguistic stocks: Amerind, NaDene and Eskimo-Aleut (4). The two latter groups are thought to have been the last to enter the continent while Paleoindians are currently considered the initial immigrants (5). Despite some acceptance, the three-wave theory has also received considerable criticism (6–8) even on linguistic grounds (9,10). Because of its original genetic diversity of the asiatic ancestors, which possibly occurred during the passage through Beringia (12). In opposition, the existence of variants of the major founding mtDNA lineages (14,16) has been considered evidence in support of a single wave of migration into the New World (8).

Herein we report the first screening for Amerindian mtDNA lineages of skeletal remains from four extinct aboriginal populations of South America who inhabited Tierra del Fuego (Selknam, Yamana, Kaweskar and Aonikenk). These aborigines from Fuego–Patagonia occupied the southernmost edge of the continent (17) and their ancestors might well have been some of the founding colonists of America. The ancestry of the Selknam began to split off the aboriginal cluster in Patagonia~12 000–10 000 years BP and became confined to Isla Grande in Tierra del Fuego at least 6000 years ago (17), when the last landbridges in the Magellan Straits were flooded (18). Both were terrestrial populations and shared similar skull morphologies. The Yamana and Kaweskar lived along the coast of Tierra del Fuego. They constituted marine, hunter-gatherer groups, morphologically related and unambiguously distinguishable from the Selknam and the Aonikenk. The ancestors of these canoeists have also been traced to Tierra del Fuego from 6000 years BP. Archaeological evidence has documented ecological adaptations to the Beagle channel environment as well as a highly developed maritime way of life (19). All these indigenous peoples were decimated upon contact with Europeans, leading to their virtual extinction between the turn of the XIX century and the early part of the XX. The Aonikenk had already vanished during the XVII and XIX centuries and there are no contemporary survivors descended from the Selknam.

The extinction of aboriginal populations in America that followed cultural displacement upon European contact has hindered genetic analyses, especially in the far South and the Northeast where this contact was most damaging. Recently, the feasibility of retrieving DNA from ancient remains has made possible the examination of extinct populations, overcoming the extinction gap and providing valuable genetic information which...
might otherwise be lost. Ancient DNA has been most often recovered from mummified soft-tissues (20,21) but it has also been successfully extracted from bones (22,23), thus making DNA from archaeological samples of solid-tissues and museum specimens amenable to analysis.

RESULTS

Analysis of mtDNA restriction site polymorphisms

Ancient mtDNA was successfully recovered from 60 skeletal samples of a total of 75 individuals (71 teeth and four rib fragments) mainly belonging to the extinction period (XIX–XX centuries), yet including two samples from skeletal finds 4030 and 5000 years of age, respectively, uncovered in Cueva Lago Sofia and the Marazzi sites. Samples were removed from skeletons held at the Museo Nacional de Historia Natural in Santiago de Chile, the University of Magallanes in Punta Arenas (Chile), and at the Museum of Ushuaia and the CENPAT-CONICET (Argentina). Some specimens from Laguna del Juncal (Río Negro, Argentina) were included in the Aonikenk sample. Group attributions of the specimens were retrieved from museum records. Samples were exhaustively cleansed before processing, the DNA extracted by an established procedure and subjected to polymerase chain reaction (PCR) amplification as described under Materials and Methods.

To characterize the Amerindian lineages, specific pairs of primers encompassing the distinctive mtDNA polymorphic sites were designed bearing in mind the reported inverse relationship between size of amplified fragments and PCR efficiency (24). Primers enclosing the cytochrome oxidase II (COII)-tRNA Lys intergenic domain (25) were also used. After amplification, the mitochondrial PCR products were restricted with the specific endonucleases defining each Amerindian haplogroup and then electrophoresed on agarose gels (Fig. 1). All amplified samples were found to fit within the Amerindian gene pool, although restricted to the major mitochondrial haplotype clusters C and D. Haplogroups A and B were not recognized in any of the samples typed and only one sample did not conform to any of the Amerindian mtDNA lineages (see Table 1). The amplifications were reproducible since all DNA samples that allowed for independent duplication (40%) yielded identical results. Replicate amplifications were from single extracts since only small skeletal pieces were made available owing to the uniqueness of the museum specimens examined and they were performed by different workers in physically separated laboratories. Therefore, the absence of Amerindian mtDNA patterns in a single sample might be attributable to present-day European-born contamination, although its sole occurrence among the whole set of samples makes the latter rather unlikely. In this respect, it is worth noting that residual lineages differing from the major Amerindian mtDNA clusters have recently been reported (8,14,16). The probability of European genetic admixture in the analyzed samples is remote, however, since no mixed crossings during the XIX century are documented for Fuego–Patagonia and all samples dated to the XIX century or earlier. In addition, samples with unreliable records were rejected. Multiple DNA extraction controls (with no ancient material added) and PCR blanks were systematically performed in parallel. Blanks were consistently negative, indicating absence of detectable sample contamination.

Table 1. Distribution of mitochondrial haplotypes in extinct Paleoindian populations of Fuego–Patagonia

<table>
<thead>
<tr>
<th>Populations</th>
<th>mtDNA haplotype (%)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>N&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fueguian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaweskar</td>
<td>19</td>
<td>–</td>
<td>–</td>
<td>3 (15.8)</td>
<td>16 (84.2)</td>
<td>–</td>
</tr>
<tr>
<td>Yamana</td>
<td>11</td>
<td>–</td>
<td>–</td>
<td>10 (90.9)</td>
<td>1 (9.1)</td>
<td>–</td>
</tr>
<tr>
<td>Selknam</td>
<td>13</td>
<td>–</td>
<td>–</td>
<td>6 (46.1)</td>
<td>6 (46.1)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>unaffiliated</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2 (100.0)</td>
<td>–</td>
</tr>
<tr>
<td>Subtotal</td>
<td>45</td>
<td>–</td>
<td>–</td>
<td>19 (42.2)</td>
<td>25 (55.6)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Patagonian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aonikenk</td>
<td>15</td>
<td>–</td>
<td>–</td>
<td>4 (26.7)</td>
<td>11 (73.3)</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>–</td>
<td>–</td>
<td>23 (38.3)</td>
<td>36 (60.0)</td>
<td>1 (1.7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Figures indicate number of positive samples followed by relative frequency percentages in parentheses.

<sup>b</sup>Number of subjects.

<sup>c</sup>Haplogroup N includes the single haplotype found not segregating into any of the four major Amerindian haplogroups (13,14,16).
Figure 2. Mitochondrial D-loop sequences from cloned PCR products of representative template extracts of the haplogroups observed upon restriction analysis of the Fuego–Patagonia sample surveyed. DNA from samples F14 (C-positive) and F18 (D-positive) was amplified using primers D18 and D2 (underlined) (27), corresponding to positions 16,192–16,420 of the human mtDNA reference sequence (28), cloned and sequenced. Sequences of D-loop groups C and D reproduce the reported polymorphic nucleotides distinctive of each D-loop group (12,13) with variant positions indicated below the sequences. Dots denote identity to reference sequence.

The restriction site screening in the 60 individuals typed, inclusive of those from Cueva Lago Sofía and Marazzi sites dated respectively to 4030 and 5000 years BP, yielded 23 carrying haplogroup C (38%) and 36 belonging to haplogroup D (60%) (Table 1). It is worth mentioning that the two oldest specimens typed, a Selknam tooth from Cueva Lago Sofía (1-43367) and a tooth from a Fueguian, unaffiliated owing to his ancientness, uncovered at Marazzi (M21-5902) carried haplotypes C and D, respectively. None of the retrieved haplotypes could be fitted into either A or B haplogroups. Although undetected, these haplogroups might still have been present in the populations studied at a frequency higher than 5% (26). However, given the size of the sample analyzed (n = 60), the probability of this is quite low (P = 0.047). This estimation supports the assumption that haplogroups A and B may have been absent after all from the Fuego–Patagonian populations. The haplotype analysis yielded unambiguous haplogroup assignments and no sample showed signs of haplotype combinations (16).

Sequence analysis of the mtDNA control region

To verify further the results provided by the analysis of restriction site polymorphisms, cloning of amplified fragments from DNA extracts was attempted. Amplification products corresponding to three individual samples representative of haplogroups C (F3 and F14) and D (F18) were successfully cloned and sequenced. DNA was amplified with primers D18 and D2 enclosing Amerindian haplogroup diagnostic nucleotide transition sites in the displacement (D)-loop hypervariable region of mtDNA (27), corresponding to positions 16,192–16,420 of the human mtDNA reference sequence (28), cloned and sequenced. Sequences of D-loop groups C and D reproduce the reported polymorphic nucleotides distinctive of each D-loop group (12,13) with variant positions indicated below the sequences. Dots denote identity to reference sequence.
haplogroup C, namely, T residues at positions 16 223 and 16 327, plus C variants at positions 16 298 and 16 325. The observed polymorphic positions 16 223 and 16 325 have also been described in other Amerindian groups (13,29). Sample F18 yielded a sequence characteristic of haplogroup D, with two C variants at positions 16 325 (shared with haplogroup C) and 16 362 as well as T residues at positions 16 223 (also present in group C), 16 286, 16 294 and 16 327. The variant positions 16 223 and 16 362 have also been observed in the Kuna populations of Panama (30). The non-diagnostic changes found at positions 16 286 and 16 294 may be possibly attributed either to random mutations due to the hypervariability of the D-loop region or to polymerase error promoted by the intrinsically decayed condition of the ancient DNA templates. This notwithstanding, the variant position 16 294 has also been detected in a Yanomami individual (13). All these sequences were doubly checked by sequencing cloned PCR products from two independent amplifications of DNA extracts yielding identical readings. In summary, most of the substitutions detected in the two samples matched with the mtDNA D-loop sequence polymorphisms previously reported in the Amerinds (13). The D-loop sequencing unambiguously confirmed the assignments deduced from the surveyed restriction site variation. The sequencing of a 121 bp segment cloned from the amplification of sample F3 and spanning the 9 bp long repeat tandemly interspersed between the COII and the tRNA\(^\text{Lys}\) mtDNA genes, confirmed the absence of the 9 bp intergenic deletion associated with Amerindian haplogroup B already noted by gel electrophoresis (Fig. 1).

**DISCUSSION**

Based on D-loop data it has been proposed that the first migrations into America may have occurred between 21 000 and 14 000 years BP (29). D-loop sequence analyses of extant Amerindians (31,32) have pointed to the existence of a genetic diversity wider than previously presumed, although most of the retrieved sequences still cluster into the four basic mtDNA groups. Thus far, near to 1500 living American aborigines have been screened and only 41 (2.7%) fail to segregate into one or other of the primary lineages. Examination of the overall geographic distribution of mtDNA haplogroups in the American continent (Table 2) sustains the existence of opposing clines in the relative frequencies of the mtDNA lineages. Haplogroup A displays a marked decrease in frequency with southerly latitude whereas haplogroup B appears to be essentially confined to the central area of the continent, probably due to having been displaced from the north by successive migrants lacking this lineage. Both are absent from Fuego–Patagonia. In contrast, haplogroups C and D exhibit a marked southward trend towards higher frequencies, attaining the highest values in the Fuego–Patagonian groups. Assuming the absence of selective pressures, such a geographical gradient could be attributed either to random genetic drift or else to distinct evolutionary trends in the Amerindian peoples. These interpretations are not mutually exclusive. The occurrence of early tribalization processes and concomitant tribal isolation events may possibly have contributed to genetic diversification and eventually to lineage extinction. However, the Fueguian canoecists, although inhabiting one of the most extreme world environments, might have thrived in an ecosystem with a plentiful supply of resources, whose abundance and predictability might have allowed for relatively high population densities (19). Since the effective size of human populations (\(N_e\)) may be estimated at about one-third of the total size (33) and based on conservative demographic estimates, an effective population size between 500 and 1300 individuals has been deduced for the Fueguians (17,34,35). However, census surveys conducted by British missionaries late in the XIX century disclosed higher numbers ranging from 2000 to 3000 members (36,37). The incidence of genetic drift under such conditions may be reasonably presumed to have been rather small. Even though computer-simulations make feasible theoretical estimates of random drift, they require that such parameters as generation size, female reproductive population and rate of active reproduction be kept constant over time, no matter the number of generations involved. Since the invariance of these conditions is unlikely in reality, any drawn inference becomes uncertain and fail from conclusive.

**Table 2.** Average frequency distributions of native mtDNA haplotypes in the American continent

<table>
<thead>
<tr>
<th>Population*</th>
<th>mtDNA haplotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>NaDene-North</td>
<td>209</td>
</tr>
<tr>
<td>NaDene-South</td>
<td>73</td>
</tr>
<tr>
<td>Northern-Amerinds</td>
<td>224</td>
</tr>
<tr>
<td>Central-Amerinds</td>
<td>261</td>
</tr>
<tr>
<td>Southern-Amerinds</td>
<td>676</td>
</tr>
<tr>
<td>Fuego-Patagonians</td>
<td>60</td>
</tr>
</tbody>
</table>


Despite the potential influence of genetic drift, the distinctive distributions in the continent of haplogroup B, which appears to have been absent from Fuego–Patagonians and from aboriginal groups above latitude 55° North in both America and Asia, and A, which is absent as well from the extreme south (Fig. 3), in conjunction with the low probability of having eluded detection in the Fuego–Patagonian sample, suggest that the initial Paleoindian settlers, at least those migrating into South America, possibly lacked haplogroups A and B altogether. Very recently, a Paleoindian culture quite distinct from the early northern Paleoindian cultures of Clovis and Folsom, roughly contemporary with them, has been uncovered in the Brazilian Amazon (5). To explain this finding, it has been proposed that Paleoindian migration patterns might have been more complex than so far presumed and that ancestors to South American Paleoindians might well represent an independent migration event unrelated to the Clovis peoples. Concurrently, in terms of the ancestral source and taking into account that the morphological traits of Paleoindian fossils and Fueguian–Patagonian remains depart from the typical Mongoloid pattern in a number of features (35), it has been suggested that there may have been more than one migratory wave of Asian peoples ancestral to Amerinds. In this regard, the exclusive presence of lineages C and D at the Southern extreme of the continent traces back to a population of more ancient ancestry, distinct from those Amerind populations harbouring all four primary mtDNA
Figure 3. Bar-plot of the overall distribution (%) of the major mtDNA lineages in American aboriginals. Native populations are arranged (left to right) in geographic order North to South through the continent, down to the southernmost edge of South America.

lineages and spread out through North, Central and, partially, South America. Despite claims for considering random drift as the only cause of genetic diversity in the American continent (6), the absence of haplogroups A and B in the Fuego–Patagonians surveyed could be explained by parsimony analysis more easily if their ancestors had not carried them rather than if they had lost both lineages by genetic drift. Undoubtedly, genetic isolation for at least 10,000 years would have contributed to prevent haplogroups A and B from entering the genetic pool of ancient Fuego–Patagonian populations.

MATERIALS AND METHODS

Ancient DNA extraction and purification

Teeth and cortical bone pieces (1–2 g) of well-preserved rib fragments were handled under stringent precautionary measures as described (38) to prevent extraneous contaminations. Whole teeth were sequentially soaked in 15% HCl for 10 min to remove dirt and carbonate deposits, 70% ethanol for 10 min to eliminate acid residues and rinsed in sterile double-distilled water for 30 min. Subsequently, they were irradiated with a 254 nm UV lamp for 15 min. Cleaned fragments were next abraded with a sand-blaster to remove 1 mm of outer layer to minimize contaminations from adventitious mishandling, microbial DNA and soil components, and powdered under liquid nitrogen in a Spex freezer mill fitted with UV-sterilized tubes and impactors.

Powdered tissues were placed in sterile 50 ml conical polypropylene centrifuge tubes and decalcified three times with 0.5 M EDTA, pH 8.0. After centrifugation, pellets were resuspended in 10 ml 0.5 M EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% SDS and proteinase K (50 µg/ml) and incubated overnight at 37°C under gentle gyratory shaking. The digests were centrifuged and the supernatants deproteinized with phenol/chloroform, desalted and concentrated using Centricon-30 microconcentrators (Amicon) to a final volume of 100–300 µl.

Enzymatic amplification of ancient mtDNA and restriction analysis

DNA purified from bone and teeth was used to amplify the polymorphic sites defining Amerindian mtDNA lineages. PCR reactions were performed on a Perkin-Elmer thermal cycler with the following profile: 35 cycles for 1 min serial steps at 94°C, 55°C and 72°C in a final volume of 25 µl containing 1 µl of DNA template extract. Reaction mixtures were 20 mM Tris–HCl, pH 8.4, 2.5 mM MgCl₂, 50 mM KCl, 0.2 mM (each) 2′-deoxynucleoside 5′-triphosphate, 0.05% W-1 stabilizer (GibcoBRL), 0.5 µM primers, and 1.0 U Taq DNA Polymerase (GibcoBRL). Negative controls consisting of mock extractions (powdered tissue omitted) and PCR blanks without ancient DNA, were performed in parallel to monitor contamination. Primer sets delineating haplogroups A, C and D respectively, were: L630/H723, 5′-ATGTATAGACGGCTC-3′/5′-TGAACTCACTGGAACG-3′ to produce a 109 bp fragment containing the distinctive HaeIII site at position 663; L13204/H13320, 5′-ACTCTGTTCGCAGCAG-3′/5′-GTGCAGGAA TGCTAGG-3′ yielding a 132 bp segment with the polymorphic HincII and AluI sites at positions 13 259 and 13 262, respectively; L5147/H5252, 5′-AAACTCCAGGACAGCACCAGC-3′/5′-GCCGGTTCG-3′ spanning a 121 bp tract with the AluI site loss at position 5176 of haplogroup D. L and H refer to the light and heavy strand of the mtDNA sequence and the numbers the respective 3′-terminal positions on the light strand (28). For haplogroup B, primers A and B (25) were used.

To screen the restriction site markers defining haplogroups A, C and D, 12.5 µl of the respective unpurified PCR products were digested overnight at 37°C with 5 U of the appropriate endonucleases and electrophoresed on 4% NuSieve-GTG agarose (FMC
BioProducts) gels containing ethidium bromide. For typing the 9 bp deletion defining haplogroup B, PCR products were directly sized by electrophoresis. DNA from a bone sample of a prehistoric Easter Islander known to carry the 9 bp deletion (39) was extracted, amplified and used as a positive control (Fig. 1).

Cloning of amplification products and D-loop sequencing

To screen for variant nucleotides in the hypervariable mtDNA control region, DNA extracts were amplified using primers D18 and D2 (27) and the PCR-amplified fragments purified on low-melt agarose. After purification, DNA fragments were blunt-ended using T4 DNA polymerase and their 5′-ends phosphorylated with T4 DNA kinase. Finally, the latter were ligated with dephosphorylated, Smal-linearized BlueScript SK phagemid (Stratagene) and cloned in competent Escherichia coli XL1-blue cells. Cloned DNA was sequenced by the dideoxy chain-termination method on both DNA strands using the Sequenase kit (Amersham).

ACKNOWLEDGEMENTS

We are greatly indebted to Prof. M. Martinic, from the University of Magallanes in Punta Arenas (Chile) as well as to Dr Silvia Quevedo, from the Museo Nacional de Historia Natural in Santiago de Chile, who committed the Chilean samples to D.T. Quevedo, from the Museo Nacional de Historia, and D. Sheehan for critically reading the manuscript. Supported in part by grants from the Spanish DGICYT to D.T. (PB93-0021) and L.C. (PB94-0042) and funds from the NATO Scientific Affairs Division (CRG93-8024) and the Generalitat de Catalunya to L.C (CRG93-8024) and the Group of Pan-American Congress (GPPN) to L.C. (PB94-0042) by grants from the Spanish DGICYT to D.T. (PB93-0021) and D.T. (PB93-0021) and funds from the NATO Scientific Affairs Division (CRG93-8024) and the Generalitat de Catalunya to L.C (GRQ95-0586) and D.T. (GRQ96-UB2506).

REFERENCES