Ancient Marine Hunter-Gatherers From Patagonia and Tierra Del Fuego: Diversity and Differentiation Using Uniparentally Inherited Genetic Markers

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ABSTRACT

Objectives: The human population history from Patagonia and Tierra del Fuego has been of great interest in the context of the American peopling. Different sources of evidence have contributed to the characterization of the local populations, but some main questions about their history remain unsolved. Among the native populations, two marine hunter-gatherers groups inhabited the Patagonian channels below the 478S: Kawéskar and Yámana. Regardless of their geographical proximity and cultural resemblance, their languages were mutually unintelligible. In this study we aim to evaluate the genetic diversity of uniparental genetic markers in both groups and to test if there is a high genetic differentiation between them, mirroring their linguistic differences.

Material and Methods: Ancient DNA was extracted from 37 samples from both populations. We compared their genetic variability of their mitochondrial lineages and Y-STR as well as with other modern native populations from the area and further north.

Results and Discussion: We observed an important differentiation in their maternal lineages: while Kawéskar shows a high frequency of D (80%), Yámana shows a high frequency of C (90%). The analysis of paternal lineages reveals the presence of only Q1a2a1a1 and little variation was found between individuals. Both groups show very low levels of genetic diversity compared with modern populations. We also notice shared and unique mitochondrial DNA variants between modern and ancient samples of Kawéskar and Yámana. Am J Phys Anthropol 158:719–729, 2015.   © 2015 Wiley Periodicals, Inc.

The evolutionary history of populations from Patagonia and Tierra del Fuego has been of great interest in the context of the entrance of humans into the Americas, particularly because of evidence for early human presence in the area (Dillehay, 2009). Evidence of human occupation of Patagonia [42°S latitude to Cape Horn (56°S)], dates to the Pleistocene-Holocene border [13,000–8,000 years before present (YBP)] (Miotti and Salemme, 2004; Borrero, 2008). The first inhabitants faced changing climatic conditions in the context of the Last Glacial Maximum, where the increase of temperature and the ice recession affected sea levels, vegetation and fauna (Coronato et al., 1999; Heusser, 1994)

The first inhabitants of the area were terrestrial hunter-gatherers, having subsisted on guanaco, rheas, small mammals, and other now extinct fauna and flora. The earliest evidence does not support adaptation to littoral zones, although a non-specialized exploitation of marine resources is possible (Dillehay et al., 2008; Piana and Orquera, 2009). By 6,000 YBP littoral zone adaptations are observed to have been concentrated in three areas: (1) Chiloé, (2) Strait of Magellan—Otway Sound, and (3) the Beagle Channel (Piana et al., 2012; San Román, 2014). The oldest dates have been found in the last two areas in the sites Punta Santa Ana (6,350 YBP) and Túnel I sites (6,400 YBP), respectively. The archaeological evidence and dates are not clear enough to identity the origin of this lifeway and, thus, it is still a subject of debate (Orquera et al., 2011).

From the combination of archaeological and ethnohistoric evidence, two groups are recognized according to their different subsistence activities: marine versus terrestrial hunter-gatherers (Gusinde, 1986). At the time of European contact, members of the marine group included, from north to south, the Chono, Kawéskar (or Alakalufe), and Yámana (or Yaghan). The terrestrial group included the Tehuelche, in the Argentinian pampa and Selk'nam and Haush in Tierra del Fuego (Fig. 1). The Chono people became extinct during the 18th century (Cooper, 1946) and, along with Haush, they are the least well-known group. In contrast, Kawéskar, Yámana, Tehuelche, and Selk'nam have been frequently

Fig. 1. Location of native populations of Patagonia and Tierra del Fuego at the time of European contact.

material culture (e.g., harpoons with detachable points and canoes) and exhibited similar social characteristics, their languages were very different and mutually unintelligible (Emperaire, 1963; Gusinde, 1986 [1937], 1991 [1974]; Barceló et al., 2011; Orquera et al., 2011; Chapman, 2012).

From a global perspective, craniometric variation measures from hunter-gatherers of Patagonia and Tierra del Fuego (particularly Selk'nam, Kawéskar, and Yámana) cause them to cluster together. One possible explanation for this observation is that they all share relatively recent common ancestry (González-José et al., 2002). Locally, however, it is possible to differentiate the marine from the terrestrial groups based on craniometric measures (Hernández et al., 1997; González-José et al., 2004; Pérez et al., 2007). Mitochondrial DNA (mtDNA) studies of modern descendants (only Kawéskar and Yámana) and ancient remains (Selk'nam, Kawéskar, and Yámana) demonstrate that these populations exhibit only lineages belonging to haplogroups C and D (Lalueza et al., 1997; García-Bour et al., 2004). Some of the sub-haplogroups identified among these populations include C1, D1g and D4h3, the last of which is highly frequent, particularly in marine hunter-gatherers (García-Bour et al., 2004; Moraga et al., 2000, 2010). Y-chromosomes among these populations include lineages belonging to Q-M3 (García-Bour et al., 2004), now Q1a2a1a1 according to the International Society of Genetic Genealogy (ISOGG) 2015 Y-DNA Haplogroup Tree (Version 10.01; January 1, 2015).

The study of uniparentally inherited genetic markers has been particularly useful for the reconstruction of the evolutionary histories of human populations, especially when it is possible to measure temporal variability through the study of ancient skeletal remains. Today, Kawéskar and Yámana descendants are scarce and much of their history has been lost, making the study of their origin(s) and differentiation difficult. Previous studies using ancient DNA (aDNA) only identified major haplogroups using RFLP or sequences of a small fragment of the first hypervariable region (HVR1) of the mitochondrial genome (Lalueza et al., 1997; García-Bour et al., 2004). Our goal is to test the degree of genetic differentiation between these groups using mitochondrial and Y-chromosome lineages characterized from ancient human remains. Regardless of the close geographical proximity of these groups and their cultural resemblance, we hypothesize a high genetic differentiation between them, mirroring their linguistic differences. In order to better contextualize their population histories, we also compare their genetic variation with modern indigenous groups around the area and further to the north.

MATERIALS AND METHODS

Samples

Seventeen ancient Kawéskar and 20 ancient Yámana samples (represented by bone and teeth) were analyzed (Supporting Information Table S1). The samples were provided by the "Instituto de la Patagonia" (Punta Arenas, region of Magallanes and Antártica Chilena) and "Museo Martín Gusinde" (Puerto Williams, Navarino Island, region of Magallanes and Antártica Chilena). Three samples assigned to Kawéskar were obtained from Moraga et al. (2010). The samples originated from archaeological rescue or were incidental finds (Legoupil, 1987, 2000; Aspillaga et al., 1999; San Román and

mentioned since the European contact. Most of the ethnographic observations of these groups originate from the work of Martin Gusinde (1918–1924) who focused on Selk'nam, Kawéskar, and Yámana. While the traditional way of life of these groups had been severely modified, Gusinde (1986 [1937], 1991 [1974]) contributed importantly to the knowledge of these groups by recording the memories of their descendants.

Selk'nams were characterized by their tall stature and bow hunting abilities, as were the other terrestrial hunter-gatherer groups. Their most important prey was the guanaco, supplemented with small mammals, birds and marine resources. They formed highly territorial groups comprised of a few families, totaling about 30 individuals. Kawéskar and Yámana were characterized by shorter stature and the use of highly specialized tool assemblages to depend almost entirely on littoral resources, particularly pinnipeds, marine birds and mussels (Orquera and Piana, 2005; Orquera et al., 2011). Their social organization was focused around nuclear families. Although both Kawéskar and Yámana shared much

TABLE 1. mtDNA primers

Name	Primer (5′–3′)	Coordinates	Annealing temperature (°C)	Size	Reference
HgA_f	GTAGCTTACCTCCTCAAAGCAA	584–606	55	145	Moraga et al., 2000
HgA_r	AGGGTGAACTCACTGGAACG	709–729			Moraga et al., 2000
HgB_f	CACAGTTTCATGCCCATCGT	8,195–8,214	55	113/122	Moraga et al., 2000
HgB_r	ATGCTAAGTTAGCTTTACAGTGG	8,294–8,316			Moraga et al., 2000
HgC_f	AATCGTAGCCTTCTCCACTTCA	13,236–13,257	55	130	Modified from Kemp et al., 2007
HgC_r	GGAGCACATAAATAGTATGGC	13,345–13,365			Moraga et al., 2000
HgD_f	CCTAACTACTACCGCATTCCTA	5,099–5,120	55	113	Moraga et al., 2000
HgD_r	GGGTGGATGGAATTAAGGGTGT	5,190–5,211			Kemp et al., 2007.
HV1_1f	AGTCTTTAACTCCACCATTAGC	15,966–15,988	51	149	This work
HV1_1r	GTGGCTGGCAGTAATGTACG	16,095–16,115			This work
HV1_2f	GGAAGCAGATTTGGGTACCA	16,035–16,054	51	203	Modified from Kemp et al., 2007
HV1_2r	TGTGTGATAGTTGAGGGTTG	16,218–16,237			Handt et al., 1996
HV1_3f	CACCATGAATATTGTACGGT	16,112–16,131	51	211	Kemp et al., 2007
HV1_3r	TGGCTTTATGTACTATGTACT	16,302–16,322			Modified from Handt et al., 1996
HV1_4f	CCCCATGCTTACAAGCAAGT	16,190–16,209	51	213	Kemp et al., 2007
HV1_4r	TGGTCAAGGGACCCCTATCT	16,383–16,402			Modified from Kemp et al., 2007
HV2_1f	GGGAGCTCTCCATGCATTTGGTA	34–56	51	207	Modified from Kemp et al., 2007
HV2_1r	TATTATTATGTCCTACAAGCA	220–240			Modified from Gabriel et al., 2001
HV2_2f	GCACCCTATGTCGCAGTATCTGTC	109–132	51	177	Kemp et al., 2007
HV2_2r	GTTATGATGTCTGTGTGGAA	266–285			Modified from Gabriel et al., 2001
HV2_3f	TAT TTATCGCACCTACGTTC	155–174	51	200	Modified from Gabriel et al., 2001
HV2_3r	GTTTGGCAGAGATGTGTTTAAGT	331–353			Modified from Kemp et al., 2007

Morello, 2001). All correspond to marine hunter-gatherers from the late Holocene (less than 2,000 BP) and from insular or coastal contexts without connection with other terrestrial groups like Selk'nam and Tehuelche (Lalueza et al., 1995; Guichón et al., 2001; Schinder and Guichón, 2003). The remains were found buried in shell middens or caves, most of them only accessible by sea. The approximate location of the sites from which the samples originated and further information about them are depicted in Supporting Information Table S1 and Figure S1.

DNA extraction

At the University of Chile, all extractions and polymerase chain reaction (PCR) setups were carried out in a laboratory dedicated exclusively to aDNA analysis, one separated from the modern lab. This laboratory has positive pressure (filtered air HEPA quality treated with ultraviolet light) and UV lamps on all working surfaces. Only disposable sterile plastic materials and DNA-free reagents were used. Reagents and samples were manipulated only within a laminar flow cabinet. One blank extraction was included in every extraction set of five samples. All steps of DNA extraction and amplification were carried out at the Faculty of Medicine, University of Chile (UChile). Additionally, 12 samples (11 bones and one tooth) were replicated in the Washington State University (WSU) laboratory of Molecular Anthropology and Ancient DNA.

At the University of Chile, two protocols were employed for DNA extraction: a phenol-chloroform based method and a silica based one. First, each sample was immersed in 6% w/v sodium hypochlorite for 10 min and rinsed five times in DNA-free water to remove any trace of bleach. Then, a total of 15 ml of EDTA 0.5M (pH 8.0) per gram of material was added to each sample (between 400 and 600 mg of sample), followed by gentle rocking at room temperature for 48 h. Following incubation, proteinase K was added to a final concentration of 0.2 mg/ml and the samples incubated at 53°C for 16 h. In the

phenol-chloroform extraction method, one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. Tubes were vortexed and centrifuged at 4,500 rpm for 1 min. The supernatant (aqueous phase) was transferred to a new tube and one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added again. After vortexing and centrifugation at 4,500 rpm for 1 min, the supernatant was again removed and transferred to a new tube. Each sample was mixed with one volume of phenol:chloroform (25:24), vortexed and centrifuged for 1 min at 4,500 rpm. The supernatant was transferred to a new tube, to which one volume of 5M ammonium acetate and two volumes of isopropanol 100% were added. Following overnight incubation at room temperature, each sample was centrifuged at 6,000 rpm for 30 min. The supernatant was discarded and the pellet washed in 1 ml of 70% ethanol. After centrifugation and complete elimination of the ethanol, the samples were eluted in 100 μl of double distilled water.

The silica extraction was performed using the QIAGEN kit (MinElute PCR Purification Kit) following the instruction of the manufacturer, with minor modifications recommended elsewhere (Raghavan et al., 2014). Briefly, after digestion the samples were concentrated using a 30 kDA centrifugal filter units (Millipore) down to 250 μl. A total of 13 volumes, instead of 5, of binding buffer were added to each sample. In the elution step, spin columns were incubated with 100 μl of double distilled water at 37°C for 10 min.

At WSU, DNA was extracted in a dedicated aDNA laboratory, using between 100 and 200 mg of material and following the described for WSU by Cui et al. (2013) or by a modified Kemp et al. (2007) method described by Moss et al. (2014).

Mitochondrial DNA

Defining markers of haplogroups A, B, C and D (Torroni et al., 1993) were screened. The primers used at the University of Chile are described in Table 1 and the PCR conditions were as follows, considering a final

TABLE 2. Y Chromosome primers

Name	Polymorphism	Primer (5′–3′)	T_m (°C)	Size	References
rs3894 Q-M3	SNP: C - T	CCTGACAATGGGTCACCTCT	55	126	This work
		CCAGATATTACATGGGACATGC			
DYS391	STR: (TCTA)6-14	TTCATCATACACCCATATCTGTC	55	89-121	Gusmao et al., 2000
		GATAGAGGGATAGGTAGGCAGGC			
DYS392	STR: (TAT)6-17	AAAAGCCAAGAAGGAAAACAAA	57	93-125	Ruitberg y Butler, 2000
		AAACCTACCAATCCCATTCCTT			
DYS393	STR: (AGAT)10-17	GTGGTCTTCTACTTGTGTCAATAC	59	108-140	Myhre et al., 2000
		GAACTCAAGTCCAAAAAATGAGG			
DYS439	STR: (AGAT)9-14	ACATAGGTGGAGACAGATAGATGAT	59	116-136	Ruitberg y Butler, 2000
		GCCTGGCTTGGAATTCTTTT			

TABLE 3. Modern samples

Sample	N	References
Aymara	39	De Saint Pierre et al., 2012
Atacameño	28	De Saint Pierre et al., 2012
Pehuenche	86	De Saint Pierre et al., 2012
Huilliche	57	De Saint Pierre et al., 2012
MapucheCHL (Chile)	19	De Saint Pierre et al., 2012
MapucheARG (Argentina)	51	De Saint Pierre et al., 2012
Tehuelche	29	De Saint Pierre et al., 2012
Kawéskar	13	Moraga et al., 2010
Yámana	21	Moraga et al., 2000

Samples of native groups from Chile and Argentina.

volume of 25 μl: 3 μl of template DNA, 2 units of Go Taq Hot Start polymerase (Promega), 2.5 μl of the appropriated buffer, 200 μM of each dNTPs, 25 pmol of each primer and 100 μg of BSA. The cycling conditions were: one cycle at 94°C for 5 min followed by 45 cycles of 40 s at 94°, 40 s at the annealing temperature (see Table 1) and 40 s at 72°, and one cycle at 72°C for 3 min. Successful amplification was visualized by electrophoresis on 3% agarose gels. The 9 bp deletion, characteristic of haplogroup B, was screened in this manner by directly observing separation of the amplicons. The remaining amplicons were digested with the appropriate enzyme (*Hae*III, *Hinc*II, and *Alu*I for the haplogroups A, C, and D, respectively) and results again visualized by electrophoresis on 3% agarose gels. At WSU, defining markers of haplogroups A-D were screened following Kemp et al. (2007).

Hypervariable regions 1 (HVR1) and 2 (HVR2) were amplified in three fragments each, using the primers shown in Table 1. The PCR reactions were the same as described before. The PCR products were visualized by eletrophoresis in a 3% agarose gel and then sequenced in both directions at Macrogen.Inc, Korea. Sequencing HVR1 and HVR2 at WSU was conducted following Kemp et al. (2007). In the samples (three) previously published by Moraga et al. (2010), only the fragment HV1_1 (Table 1) was amplified and sequenced in order to match the lecture range.

Y chromosomal DNA

Molecular sex determination was performed by screening the dimorphism in the amelogenin gene using the primers describe by Sullivan et al. (1993). Further analysis of the Y-chromosome included screening the samples for the M3 marker (SNP rs3894) characteristic of haplogroup Q1a2a1a1 according to Underhill et al. (1996),

and the following short tandem repeats (STRs): DYS391, DYS392, DYS393, DYS439. All primers and annealing temperatures are described in Table 2. The PCR conditions were as follows: in a final volume of 25 μl, the following reagents were added 3 μl of DNA, 2 units of Go Taq Hot Start polymerase (Promega), 2.5 μl of buffer 5×, 200 μM of each dNTPs, 25 pmol of each primer and 100 μg of BSA. The cycling conditions were: an initial denaturation step at 95°C for 2 min followed by 60 cycles of 40 s at 94°C, 40 s at the annealing temperature (Table 2) and 40 s at 72°C; and a final step at 72°C for 5 min. The extension temperature of the STR markers DYS391 and DYS393 was set at 60°C. PCR products were visualized by electrophoresis in a 3% agarose gel and sequenced in both directions at Macrogen Inc., Korea. The sequences were aligned to the Y chromosome reference available in NCBI using the software Geneious v6.0 (www.geneious.com). The polymorphism characteristic of M3 was directly confirmed, while the STRs were identified using the "search motif" tool available in Geneious and directly confirmed.

Data analysis

The hypervariable region sequences were edited and aligned with Geneious v6.0 (www.geneious.com). The characteristic mutations of Native American haplogroups were identified by comparing the samples with the revised Cambridge reference sequence (Andrews et al., 1999). Sequence analysis included evaluation of variability within each group, differentiation between groups, and the construction of a haplotype network separately for lineages belonging to different haplogroups. To place the data collected in this study in a regional comparative framework, comparable data from nine modern native populations from Chile and Argentina were included in the analysis (Table 3). The standard diversity index (k: mean number of nucleotide differences between pair of sequences), nucleotide diversity (π), number of haplotypes (h), and haplotype diversity (Hd) were calculated in the software DnaSP 5.1 (Librado and Rozas, 2009) clustering by ethnic group. To evaluate intergroup differentiation we performed an exact test of population differentiation (number of Markov chains = 100,000) and calculated F_{ST} values (with 10,000 permutations) with Arlequin v3.5 (Excoffier et al., 2005; Excoffier and Lischer, 2010). An analysis of molecular variance (AMOVA) was also conducted with Arlequin v3.5 (with 10,000 permutations), dividing the samples into geographic groups: (1) North: Aymara and Atacameño, (2) South: Huilliche, Pehuenche, Mapuche Chile (CHL), Mapuche Argentina (ARG), and Tehuelche, (3) Southernmost: Kawéskar, Yámana, and
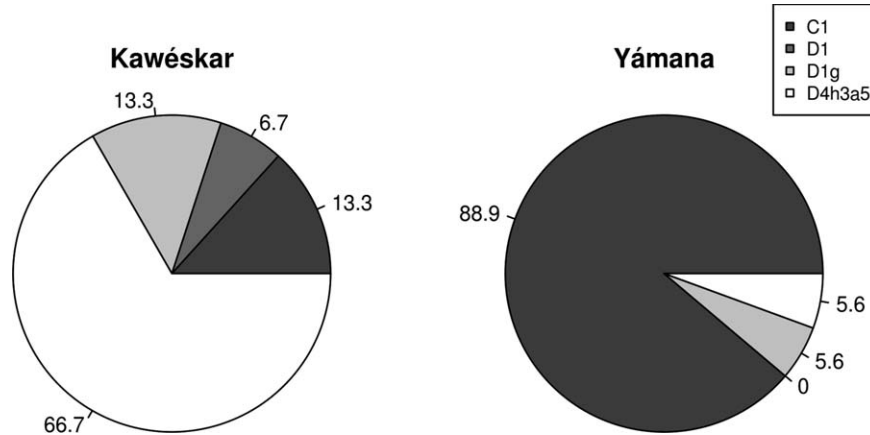
Fig. 2. Percentages of mitochondrial sub-haplogroups in Kawéskar and Yámana samples analyzed in this study.

TABLE 4. Genetic diversity

Sample	N	S	h	Hd	k	π	References
Ancient Kawéskar	15	15	8	0.886	4.59	0.01039	This work
Ancient Yámana	17	17	10	0.838	3.824	0.0075	This work
Aymara	39	66	34	0.993	10.343	0.01048	De Saint Pierre et al., 2012
Atacameño	28	58	22	0.976	12.677	0.01284	De Saint Pierre et al., 2012
Pehuenche	86	37	21	0.9	6.581	0.01075	De Saint Pierre et al., 2012
Huilliche	57	60	31	0.968	11.311	0.01146	De Saint Pierre et al., 2012
MapucheCHL (Chile)	19	41	16	0.982	9.977	0.01011	De Saint Pierre et al., 2012
MapucheARG (Argentina)	51	66	32	0.973	12.063	0.01222	De Saint Pierre et al., 2012
Tehuelche	29	41	18	0.961	10.163	0.0103	De Saint Pierre et al., 2012
Kawéskar	13	33	8	0.897	9.462	0.00959	Moraga et al., 2010
Yámana	21	21	7	0.814	6.819	0.00691	Moraga et al., 2000

The samples analyzed in this study are shown in the first two rows as ancient Kawéskar and ancient Yámana
N: number of individuals; S: number of polymorphic sites; h: number of haplotypes; Hd: haplotype diversity; k: mean number of nucleotide difference between pair of sequences; π: nucleotide diversity.

the ancient samples analyzed in this study. In order to assess alternative clustering, we performed a Spatial Analysis of Molecular Variance (SAMOVA) in the software SAMOVA 1.0. This analysis allows the identification of geographically homogeneous groups, maximizing the differentiation between them (Dupanloup et al., 2002). The analysis was performed with a k (or number of groups) from 2 to 10 and an AMOVA was executed for each clustering in Arlequin v3.5. A Neighbor-joining dendrogram was created in the software MEGA v6 (Tamura et al., 2013) using the F_{ST} matrix.

Median-joining networks for each haplogroup separately were constructed with Network 4.6.1.2 (Bandelt et al., 1999). To minimize reticulation in the networks sites that are prone to homoplasy were eliminated (sites 152) or a lower weight (sites 146, 195, 16,189, and 16,311) was given to them in the matrix according to De Saint Pierre et al. (2012).

RESULTS

Thirty-five of 37 (94.59%) of the samples were characterized by PCR-RFLP and assigned to a mtDNA haplogroup. The Kawéskar are characterized by 18.75% (3/16) of haplogroup C and 81.25% (13/16) of haplogroup D. The Yámana exhibit quite the opposite pattern with haplogroup C at 89.47% (17/19) and haplogroup D at 10.53% (2/19). The absence of haplogroups A and B in both populations is in agreement with prior studies in

the area, where ancient and modern samples had been included (Lalueza et al., 1997; Moraga et al., 2000, 2010; García-Bour et al., 2004). Seven of the samples were successfully replicated at WSU, exhibiting the same haplogroups as identified at UChile. From the remaining five samples, two fail as well at UChile, leaving only three samples that failed at WSU but were successfully analyzed at UChile.

Hypervariable region sequences, generated for 33 of 37 samples (89.19%), allowed for the identification of sub-haplogroups C1, D1, D1g, and D4h3a5 in the Kawéskar and Yámana. The basic informative sites of each sub-haplogroup were: (1) for C1: 16,223, 16,298, 16,325, 16,327, 73, 249d, 263m and 290-291d, (2) for D1: 16,223, 16,325, 16,362, 73, and 263; D1g, same as the previous plus the site 16,187, and (3) for D4h3a5: 16,051, 16,223, 16,241, 16,342, 16,362, 73, 263 (Tamm et al., 2007; Perego et al., 2009, 2010). The frequencies of these haplogroups are depicted in the Figure 2. Most of the D lineages identified in Kawéskar are assigned to D4h3a5, while in Yámana no further characterization than C1 was possible with the lecture range obtained with the sequences.

The results of the genetic diversity analyses are presented in Table 4. Considering haplotype diversity and nucleotide diversity, both the ancient and modern samples of Kawéskar and Yámana exhibit the lowest values compared with the other groups considered here. This
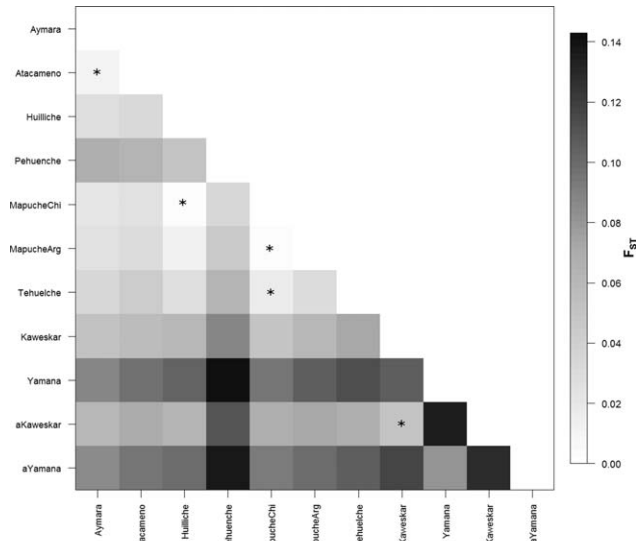
Fig. 3. F_{ST} values between modern and ancient samples. The ancient samples analyzed in this study are shows as aKawéskar and aYámana. The values with asterisk have a P value >0.05.

result is concordant with the presence of only C and D lineages in those samples, while the comparative samples also have members of haplogroups A and B.

The results of the exact test of population differentiation (Supporting Information Table S2) reveals a significant difference between ancient Kawéskar and ancient Yámana (P value = 0.00053). All comparisons between ancient and modern samples were significantly different, except that between ancient Kawéskar and modern Kawéskar (P = 0.05908). The F_{ST} values between pair of samples were plotted in Figure 3 and their exact values are found in Supporting Information Table S3. The F_{ST} value obtained between ancient Kawéskar and ancient Yámana was one of the highest in the analysis (0.12876, P value = 0.00089) consistent with them exhibiting a moderate level of genetic differentiation (Hartl and Clark, 1997). The comparison of the ancient and modern sample of Kawéskar reveals a low F_{ST} value (0.05097) although not significantly different from zero (P = 0.08415). The range of F_{ST} values with the other samples was 0.06169 to 0.10998 (with all P values < 0.05). In the case of comparison between ancient and modern Yámana, we observed an F_{ST} value of 0.0876 (P value = 0.01960), with a range of 0.08637 to 0.13826 (with all P values <0.05) between ancient Yámana and the other modern samples.

The neighbor-joining dendrogram show in Figure 4 reveals a northern branch composed by Aymara and Atacameño and a southern branch composed by Pehuenche, MapucheCHL, MapucheARG, Huilliche, and Tehuelche. The modern and ancient samples from the southernmost area, which included Kawéskar and Yámana, do not cluster together in agreement with the genetic differentiation described above.

Dividing populations by geography in the AMOVA [North (Aymara, Atacameño), South (Pehuenche, MapucheCHL, MapucheARG, Huilliche, Tehuelche) and Southernmost (Kawéskar, ancient Kawéskar, Yámana, ancient Yámana)] shows a low genetic differentiation between groups with a F_{CT} of 0.02643 (P value = 0.00188) and most of the variance is explained by
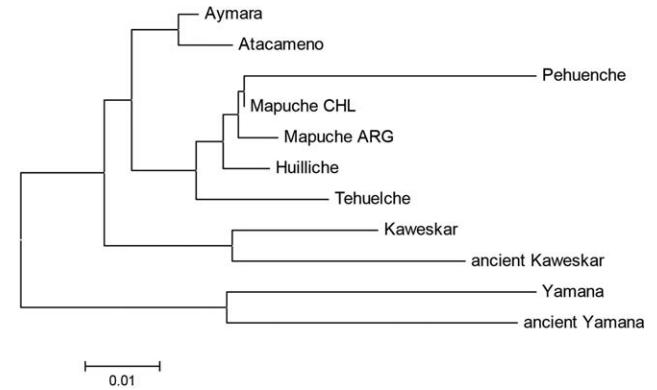

Fig. 4. Dendrogram neighbor-joining. The ancient samples analyzed in this study are shown as ancient Kawéskar and ancient Yámana.

intra-group differences (93.08%). According to SAMOVA (Supporting Information Table S4), the highest F_{CT} value was obtained for $k = 8$ [(1) Aymara-Atacameño; (2) Pehuenche; (3) Huilliche-MapucheCHL-MapucheARG; (4) Tehuelche; (5) Kawéskar; (6) Yámana; (7) ancient Kawéskar; (8) ancient Yámana] showing a moderate differentiation between groups ($F_{CT} = 0.05374$, P value <0.001) and with most of the variation explained within samples level (93.59%).

The median-joining network depicting members of haplogroup C clearly segregates C1b13 lineages, which are in high frequency in the south of Chile and Argentina, but absent in the ancient Kawéskar and Yámana samples (Fig. 5). Only three modern samples (one Kawéskar and two Yámana) were identified as C1b13, while the ancient samples are concentrated in the main haplogroup or sub-haplogroups derived from it, particularly in Yámana. Ancient Yámana samples show unique sub-haplogroups, standing out those shared with modern Yámana samples.

Figure 6 shows the median-joining network of haplogroup D lineages. In this case, there are three main sub-haplogroups: D1, D1g, and D4h3a5. Two different D1g lineages were identified in ancient Kawéskar: (1) one with a transition at np 16,245, and (2) one with transitions at nps 16,189, 16,209, and 195. The other D lineage identified was D4h3a5, mostly observed in the Kawéskar. Moreover, a D4h3a5 variant with transitions at nps 16,213 and 16,271 was identified in four ancient Kawéskar from three sites. Additionally, some modern and ancient samples of Kawéskar and Yámana have transitions at np 16,311, while Tehuelche carry a transition at np 16,301.

The screening of the length dimorphism of the amelogenin gene was possible for 11 of 17 Kawéskar samples (64.7%) and 18 of 20 Yámana samples (90%). This allowed the identification of six and eight males, respectively, data that agrees with the morphological estimation with the exception of three individuals. For these three individuals it is possible that their sexes were misidentified because of the absence of clear morphological indicators. The Q-M3 marker (locus r3894) was successfully screened in 64% of the males (five Kawéskar and four Yámana), with all of them exhibiting the mutation and thus belonging to subhaplogroup Q1a3a1a. Y-STRs analysis was successful for three markers and the results are shown in Table 5. It was not possible to get information on DYS439 due to difficulties in generating
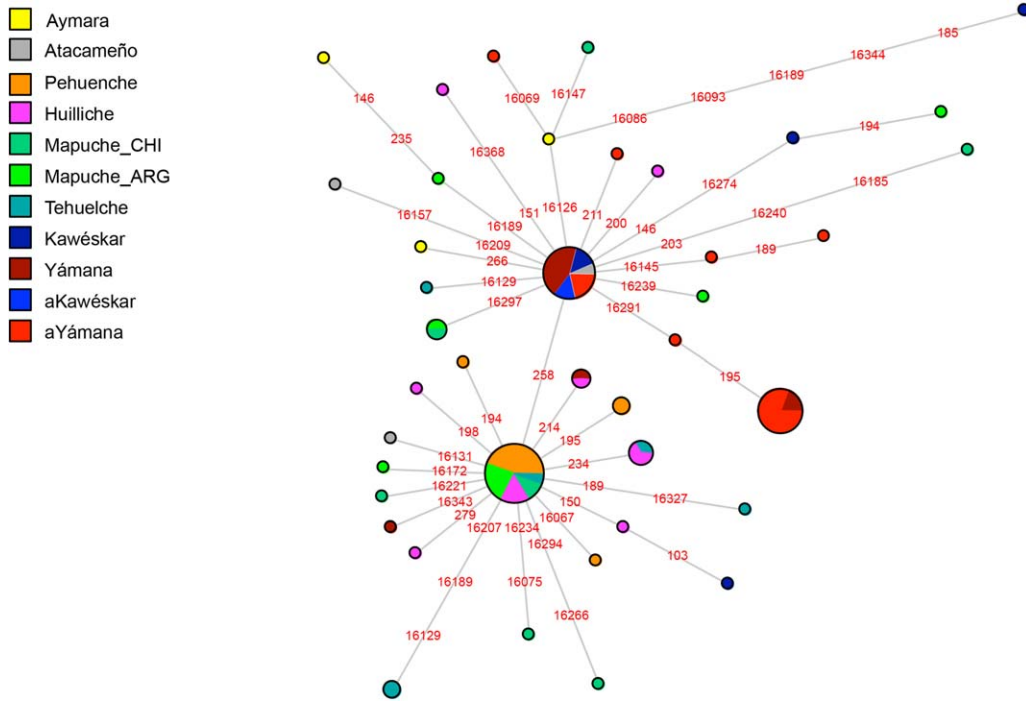
Fig. 5. Median-joining network of haplogroup C1. The ancient samples analyzed in this study are shown as aKawéskar and aYámana. The D-loop fragments analyzed were: HV1: 16055-16382; HV2: 57-330. The analysis was performed in the software Network v4.612 using the median joining criterion.

Fig. 6. Median-joining network of haplogroup D. The ancient samples analyzed in this study are shown as aKawéskar and aYámana. The D-loop fragments analyzed were: HV1:16050-16382; HV2: 57-330. The analysis was performed in the software Network v4.612 using the median joining criterion.
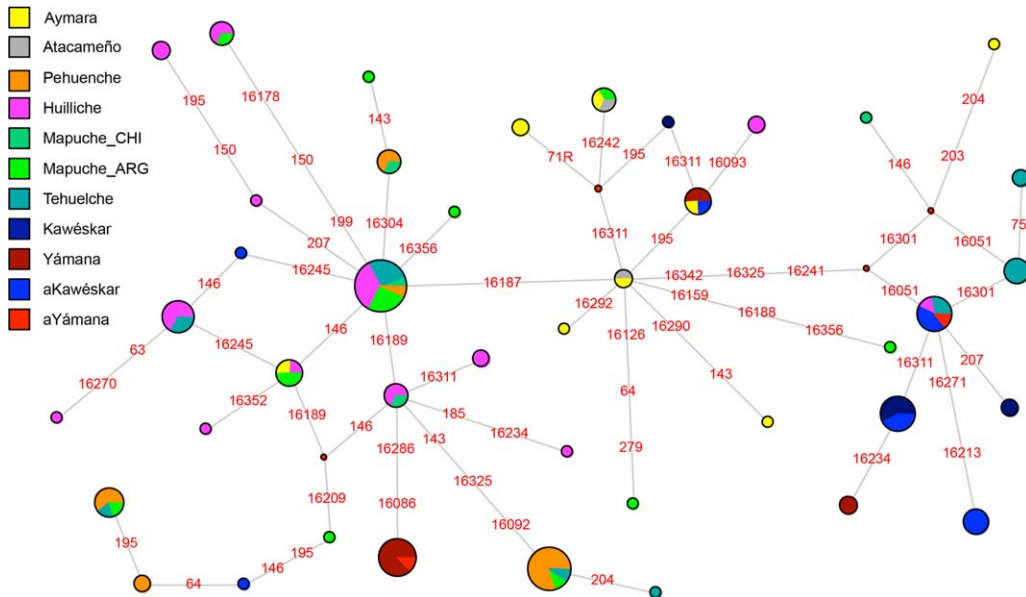
readable sequences. Only DYS392 had some variability in the sample with two alleles (14 and 16). This result allow for the characterize of two haplotypes in the sample: (1) DYS391(10 RA)-DYS392(14 RA)-DYS393(13 RA) and (2) DYS391(10 RA)-DYS392(16 RA)-DYS393(13 RA).

DISCUSSION

Earlier genetic investigations of hunter-gatherers groups from Patagonia and Tierra del Fuego highlighted an interesting characteristic of these populations

TABLE 5. Results of Y-STR analysis

Sample	Population	DYS391	DYS392	DYS393
IPK05	Kawéskar	10	16	13
IPK08	Kawéskar	10	16	13
IPK09	Kawéskar	10	16	13
IPK10	Kawéskar	-	-	13
IPK13	Kawéskar	10	16	13
IPY04	Yámana	10	14	13
IPY10	Yámana	10	16	13
MG30a	Yámana	10	16	13

Negative sign (-) indicates failure to amplify.

regarding to their mitochondrial DNA variation particularly the lack of some major Native American lineages (A and B) and a low genetic diversity (Lalueza et al., 1997; García-Bour et al., 2004). In agreement with those studies, the data presented in this study reveals similar patterns. In addition, we found an uneven distribution of C and D lineages, with the Kawéskar exhibiting a high proportion of haplogroup D and Yámana a high proportion of haplogroup C. Genetic diversity among Kawéskar and Yámana is consistently low, in agreement with the presence of only C and D lineages. This low genetic diversity has been previously attributed to the founder effect during the peopling of the continent and the area (Moraga et al., 2010). No human remains related to marine hunter-gatherers groups have been previously identified as belonging to either haplogroup A or B, but some early dated skeletal remains (pre 4,500 YBP) from terrestrial hunter-gatherers in the archaeological site "Baño Nuevo-1" had been identified as members of haplogroup B (Mena et al., 2003; Moraga et al., 2010: Reyes et al., 2012). Considering the distribution of haplogroups along Chile, haplogroup B shows a latitudinal cline with higher frequencies in the North (Aymara and Atacameño) and minor frequencies southward, while haplogroups C and D increase their frequencies to the South. This pattern may suggest that the absence or low frequency of B in Southern Patagonia is due to genetic drift (García et al., 2006; Moraga et al., 2010; De Saint Pierre et al., 2012).

The lower genetic diversity found in Kawéskar and Yámana could also be attribute to the population dynamics of these groups and their social structure. Demographic reconstructions using mitochondrial DNA sequences of the D-loop of modern Kawéskar and Yámana have shown evidence of little population growth, which is in agreement with a hunter-gatherer lifestyle (De Saint Pierre et al., 2012). Our results also show higher values of F_{ST} between the Patagonian samples (Kawéskar and Yámana) and the rest of the populations, which may have resulted from isolation of these populations in the southernmost area, with limited genetic exchange with populations from north Patagonia. The maintenance of a small population sizes together with some degree of isolation could have both contribute to the observed low level of genetic diversity. Considering the residence and reproductive patterns, Gusinde (1986 [1937], 1991 [1974]) described that the main social unit in both Kawéskar and Yámana, was the nuclear family. A higher level of organization was a kinship group, composed by closely related nuclear families that shared a geographical area. The pattern of residence was patrilocal and followed a lineage exogamy, avoiding the unions inside kinship groups and between close relatives. The union between ethnic groups was not banned, but it could have had limited impact in the genetic structure of each group since it was not frequently observed (Gusinde, 1991 [1974]). Considerer together, those patterns may contribute to the differentiation between Kawéskar and Yámana, but have little impact in the levels of diversity found.

A decrease in genetic diversity could also be associated to a reduction in the population size. Population estimations during the first years of European contact totaled around 5,000 Kawéskar individuals and 3,000 Yámana (Martinic, 1989), while by the mid-19th century, populations sizes were estimated to have been reduced to 250 and 50 individuals, respectively (Gusinde, 1986 [1937], 1991 [1974]). Although there are just a few radiocarbon dates of the samples, most of them have been associated to historical times and the severe reduction in population sizes could be reflected in these individuals. Raff et al. (2011) suggested that regional differentiation and haplogroup patterns in mtDNA have not changed in the last few thousand years and that recent historical events do not seem to have a great impact in those patterns. However, we cannot discard the impact of this reduction since only few individuals older than 2,000 YPB have been analyzed in the area.

Regardless their low genetic diversity, our sample suggests a higher differentiation between Kawéskar and Yámana, as revealed in the exact test of population differentiation and F_{ST} values. Moreover, the AMOVA test is in agreement with a very low level of genetic structure, mainly in the southernmost group, reinforcing the previous idea of a limited genetic exchange between those groups. The highest F_{CT} value obtained by SAMOVA was for a $k = 8$, showing only two groups with more than one population composed by the Northern and Southern samples respectively. Considering the comparison between samples, the F_{ST} values and the exact test of population differentiation, there is no difference between the ancient and modern samples of Kawéskar, both showing a high proportion of the lineage D4h3a5, together with Tehuelche (27.6%). Meanwhile, the exact test of population differentiation in ancient Yámana shows significant difference with all the samples, including the modern Yámana. Despite the difference, it is important to notice that the F_{ST} value between the ancient and modern samples of Yámana is one of the lowest in the matrix and both samples share some unique sub-haplogroups, suggesting some degree of continuity between them. One variant is derived from C1 and characterized by the transitions at nps 16,291 and 195 and the other is derived from D1g plus the transitions at nps 16,086, 16,189, and 16,286.

The diversification of haplogroup D shows some interesting variants in Kawéskar and Yámana, particularly in the sub-haplogroups D1g and D4h3a5. The D1g lineages are highly frequent in the South of Chile and Argentina and it has been proposed as a founding sub-haplogroup in the area (Bodner et al., 2012). A recent study found a unique variant of this lineage in six modern Yámana (D1g plus transitions at nps 16,086, 16,189, and 16,286), which is also present in one ancient sample analyzed in this work, but no individual belonging to Kawéskar has been found to exhibit this mtDNA variant (De Saint Pierre et al., 2012). Here, we found only one ancient sample of Kawéskar belonging to D1g, one that exhibit the transitions at nps 16,189, 16,209, and 195.

This variant has been previously identified in 5 of 15 terrestrial hunter-gatherer individuals from Lake Salitroso (Santa Cruz, Argentina) dated between 2,750 to 300 cal. BP (Tessone et al., 2005; Moraga, personal communication). A closely related lineage is also present in some modern Pehuenche (5/86), MapucheARG (2/51), and Tehuelche (1/29) (De Saint Pierre et al., 2012) and may also be identified in ancient samples of two Tehuelches, one Kawéskar, and one Yámana analyzed by García-Bour et al. (2004) where only HV1 sequences were collected.

Meanwhile, D4h3a5 shows a high frequency in both Kawéskar samples and Tehuelche. This lineage is a minor founding mitochondrial lineage in the Americas that shows a contemporary frequency of ~1.5% and a distribution principally in the west along the Pacific coast (Kemp et al., 2007; Perego et al., 2009: Cui et al., 2013; Rasmussen et al., 2014). The lineage D4h3a has an estimated age of 13,000 years (Behar et al., 2012) and it has been found only in America in modern and ancient samples (Kemp et al., 2007; Perego et al., 2009; Rasmussen et al., 2014). The general southward increase in frequency of this sub-haplogroup is probably associated with the first migrations that reached the area and followed a Pacific coast route. The high frequency that we report in these samples may be a result of genetic drift affecting these isolated populations. From another perspective, the frequency of D4h3a could have been higher across the continent in early times, but later replaced and lost northward (Cui et al., 2013). Either way, these populations are clear reservoirs for this lineage. As we mentioned before, only the samples of Tehuelche belonging to this lineages have the transition at np 16,301, while the other individuals carry either transitions at np 16,311 or exhibit no derived variations. This may be an indicator of differentiation between terrestrial and marine hunter-gatherer groups, although ancient samples of the terrestrial groups are needed to test this hypothesis. We also found a new variant in this lineage in four individuals, two of them (a female and male) coming from the same site (cave "Estrecho Trinidad") and therefore possibly close relatives. Since the other two individuals come from sites not far from the cave "Estrecho Trinidad" (around 100 km), they may correspond to a kinship group.

The Y-chromosome results show little variation in our sample. Considering each marker separately, a review of uniparental genetic markers in 29 native groups from South America (Bisso-Machado et al., 2012) reveals a frequency close to 80% for DYS391 [10 repeat allele (RA)], 51% for DYS392 (14 RA), 10% for DYS392 (16 RA), and 67% for DYS393 (13 RA). Based on the most frequent alleles per locus, Bisso-Machado et al. (2012) reconstructed the probable ancestral haplotype as DYS391(10 RA)-DYS392(14 RA)-DYS393(13 RA). Later, Bortolini et al. (2003) described this haplotype as the most frequent in several Native American populations (26.5%), while a haplotype with DYS392(16 RA) reachs 0.9%. In the Kawéskar and Yámana individuals, one exhibit the first haplotype and six exhibit the second. Even though only a few individuals and markers were analyzed, the low diversity observed in the Y-chromosomes mirrors that of the mtDNA from these populations.

CONCLUSION

The main goal of this study is to contribute to the local history of Patagonia through the analysis of uniparental genetic markers from ancient remains of two marine hunter-gatherer groups, Kawéskar and Yámana. We were able to successfully analyze the mtDNA and Y-chromosome variation, showing an exceptionally good preservation of aDNA from skeletal elements recovered from this geographical area.

Consistently with prior studies, we observed only mitochondrial lineages belonging to haplogroups C and D. Based on the proportion of these lineages and further characterization of sub-haplogroups, we reported an important differentiation between these groups, with Kawéskar showing a high frequency of D (76.47%), while Yámana shows a higher proportion of C (89.47%). This result agrees with our initial hypothesis, suggesting a high genetic differentiation of these groups that mirror their linguistic difference. Further analysis involving nuclear markers and older samples might be useful to approach the extent and origin of this differentiation. Regarding the Y-chromosome we only identified the Native American lineage Q-M3 (Q1a2a1a1), and the analysis of Y-STR did not reveal differences between the groups.

Compared with modern native indigenous samples from Chile and Argentina, both Kawéskar and Yámana show very low levels of genetic diversity. This result is more likely associated to the population dynamics and social structure of these groups, considering the maintenance of a small population size and their isolation from other groups further north. Interestingly, we also noticed some degree of continuity between the modern and ancient samples of Kawéskar and Yámana. In Kawéskar both samples exhibit a high frequency of D4h3a5 and the exact test of population differentiation shows that the difference between them was not significant. Meanwhile, both samples of Yámana reveal significant differences between them, but they shared some unique mitochondrial DNA variants of haplogroups C and D. This result reflects the affinity between the ancient individuals and the modern native populations from the area, suggesting little influence of other populations in the maternal lineage.

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