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**New Evidence of Ancient Mitochondrial DNA of the Southern Andes (Calchaquí Valleys, Northwest Argentina, 3600–1900 Years BPA)**

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## **Abstract**

Genetic studies on pre-Hispanic populations of the Southern Andes have been increasing steadily in the last decade. Nevertheless, ancient DNA characterization of Formative Period archaeological human remains is particularly scant, especially for Northwest Argentina. In order to enlarge current information on genetic characterization of the first agricultural communities of the Southern Calchaquí Valleys, we present and discuss the first mitochondrial aDNA information obtained on samples dated to ca. 3600-1900 years BP from the Cajón Valley, Catamarca Province. Reproducible mtDNA HVR-1 sequences were obtained in 7 individuals. Mitochondrial HVR-1 haplotypes were assigned to three of the four founding haplogroups D1 (57.1%), C1 (28.5%) and B2 (14.2%), with absence of A2. Our results show that the Cajón Valley sample -with predominance of D1 and C1- differ from that commonly observed in ancient and modern Andean populations, which usually show a high prevalence of haplogroup B2. The fact that the Cajón Valley and Pampa Grande (Salta Province, Argentina) share a prevalence of haplogroup D1 could provide additional evidence to support possible genetic affinities between the Valleys and the eastern sub-Andean region during the Formative Period in Northwest Argentina, expanding the archaeological evidence of contact between both populations. Future complete mitogenomes analysis will provide substantial information to formulate new hypotheses about the origins and phylogenetic relationships between the individuals of the Cajón Valley and other groups from the Andes, Gran Chaco and the Amazon.

Paleogenetic analysis of human remains have proved to be an invaluable source of information about the biological history, population dynamics and continuity and/or replacement of ancient populations (Pääbo et al. 2004; Fehren-Schmitz et al. 2010; Llamas et al. 2016). Ancient DNA (aDNA) combined with archaeological and anthropological evidence provides unique insights to key problems within the study of past events and lifestyles such as the peopling of the Americas, migrations, social structures, kinship relations, and social organization (Baca et al. 2012, 2014; Llamas et al. 2016; Cocilovo et al. 2017; Posth et al. 2018).

Although studies of genetic diversity on pre-Hispanic populations of the Southern Andes (SA) have been increasing steadily in the last decade, ancient DNA characterization of Northwest Argentina (NWA) archaeological human remains is still scant. This lack of information is specially pronounced for the Formative Period (FP) (ca. 1500BC-1000AD), time of the first agricultural sedentary communities which constitutes the main focus of this paper. To date, only three studies are available for this chronological moment in NWA: one for Pampa Grande site in the eastern slope of the Andes (Carnese et al. 2010), another for Salvatierra site in the northern Calchaquí Valleys (Russo et al. 2014), and the third one for Flores 1 site in Quebrada de Humahuaca (Russo et al. 2016).

For the following Late or Regional Developments Period (RDP) (ca. AD 1000-1450), seven studies were carried out on samples from different regions of NWA: Quebrada de Humahuaca (Mendisco et al. 2011, 2014, 2018; Russo et al. 2016, 2018a, 2018b), the Northern Calchaquí Valleys (Mendisco et al. 2014), and the Puna highlands of Jujuy Province (Mendisco et al. 2014; Postillone et al. 2017). For the subsequent Inca Period (ca. AD 1450 until the Spanish conquest) aDNA analyses for NWA include samples from Quebrada de Humahuaca (Russo et al. 2017), and the three Llullaillaco mummies of Salta Province (Wilson et al. 2007; Llamas et al. 2016) as well as the Aconcagua mummy from

Mendoza Province (Cuyo region) (Gómez-Carballeda et al. 2015). To date, no successful aDNA results have been reported for the Archaic Period (ARC) in NWA (ca. 8000-4000 BP).

Concerning the FP, main interest of the present study, Carnese et al. (2010) were pioneers in aDNA research in NWA. They analyzed the hypervariable region 1 of the mitochondrial DNA (mtDNA HVR-1), 9 autosomal STRs, and 11 Y chromosomal STR on 21 individuals from Pampa Grande site (Salta Province, eastern flank of the Andes) dated to 1310±40 years BP (YBP). This collection, excavated in the 1970s, is unique for the period and area for the great number of recovered individuals as well as for the exceptional preservation of the human remains and associated objects (Aparicio 1941; González 1972; Baffi et al. 1996). Genetic results showed that the frequencies of mtDNA haplogroups -A2 (11%), B2 (47%) and D1 (42%)- were in similar proportion to the ancient Andean populations of Peru and San Pedro de Atacama, Chile. By contrast, Y chromosome haplotype diversity turned out to be specific to Pampa Grande, that is, not comparable to any other studied South American population.

Salvatierra is another large and well-preserved collection of human remains of the FP from NWA. Also located in Salta Province, but in the Northern Calchaquí Valleys, Salvatierra (SsalCac91) is a funerary site dated to 2205±140 YBP (Tarragó 1996). A total of 66 individuals were excavated in the 1970s. Russo et al. (2014) studied mtDNA SNPs on 19 individuals from this collection obtaining positive results in only five samples, four belonging to haplogroup B2 and one to haplogroup D1.

Flores 1 site, located in Quebrada de Humahuaca (Jujuy Province) dated to the final stages of the FP (1450-1050 YBP), is the third sample with aDNA evidence for NWA. Russo et al. (2016) analyzed autosomal and uniparental genetic markers in two adult individuals recovered from the same grave. Results showed that both samples belonged to mitochondrial haplogroup B2 with a private mutation in the nucleotide position 16111G, not observed so far

in other pre-Hispanic or contemporary populations. Additionally an incomplete STR autosomal profile was obtained.

For other regions of the Central and Southern Andes (CA and SA) paleogenetic studies on FP samples are also scarce. To date, published results include populations of the coast and highlands of Peru (Kemp et al. 2009; Fehren-Schmitz et al. 2015; Llamas et al. 2016; Posth et al. 2018), the Bolivian Altiplano (Rothhammer et al. 2003; Llamas et al. 2016), and the upper valleys of northern Chile (Moraga et al. 2005) (Table 1).

The meager available paleogenetic evidence for FP populations highlights the need of increasing research on aDNA characterization on samples from NWA as well as other regions of Central and Southern Andes. In this study we present and discuss the first mitochondrial aDNA analyses on samples from the Cajón Valley (CV) dated to ca. 6000-1900 YBP. Results obtained expand current information on genetic characterization of the first agricultural communities of the FP of the Southern Calchaquí Valleys in NWA, and contribute to the understanding of local historical processes, their relationship with other South American pre-Hispanic and contemporary populations, and to understand the microevolutionary mechanisms (i.e. gene drift, gene flow) that could have acted in local/regional differentiation processes.

### ***Archeological Background of the Cajón Valley***

The earliest evidence for the peopling of NWA goes back to 11000 YBP indicating that the first hunter-gatherer groups already inhabited this area during the Late Pleistocene and Early Holocene (Martínez 2018). The most ancient proof of occupation –between ca. 10800 and 8000 YBP- is restricted to caves and rock shelters of the dry Puna environments (López et al. 2009). At high altitude valley areas, the record of human occupation dates back to ca. 7820-

7420 YBP (Martínez et al. 2013). The CV provided evidence of human presence as early as ca. 6000 YBP, corresponding to sample C640 included in this paper.

In NWA, the second millennium BC witnessed the transition from the specialized hunter-gatherers of the Archaic Period to the early farming villages of the FP (Aschero and Hocksman 2011). By the Middle Holocene (ca. 8400-5200 YBP) a major environmental change encouraged a modification in hunter-gatherer mobility patterns, pre-dating both the appearance of the domesticated llama (*Lama glama*) and the construction of corral structures that suggest the keeping of camelids in captivity (Yacobaccio 2003). In the Puna -the high plateau between northern Chile and Argentina- this stage of incipient pastoralism was characterized by a low density sparsely distributed population, reduced residential mobility, craft specialization, weak social hierarchies and long distance exchange networks (Yacobaccio 2004; Aschero 2007; Aschero and Hocsman 2011).

The pastoralist and agricultural lifestyle, lead by subsistence diversification through the introduction of plant cultivation and animal domestication, consolidated during the Formative Period (ca. 1500 BC-1000 AD) (Núñez Regueiro 1974; Tarragó 1980, 1992; Olivera 2001; Scattolin 2006, 2007). Although mostly self-sufficient, these communities were also involved in dynamic social interactions oriented towards the distribution and exchange of local products on a regional scale possibly enhanced by the introduction of llama caravans (Nuñez and Dillehay 1979; Browman 1980; Nielsen 2013).

Ceramic style distributions, shared technological traditions, raw material provenance and the occurrence of foreign objects at very large distances from their sources have led many authors to propose the existence of extensive routes of exchange and networks between different ecological regions of NWA, Northern Chile and Southern Bolivia (Núñez and Dillehay 1979; Núñez 1994; Nielsen 2006; Lazzari et al. 2009, 2017). These regular long distance movements of people and things constitute a key issue in archaeology and has

fostered hypothesis on social structures in the past. For example, there is wide consensus among researchers that reciprocity and redistribution among kin-based or ethnic groups was the main driving force for the circulation of goods and people. These regular movements might have also favored interethnic marital exchange and the renewal of friendship and political alliances (Nielsen 2013). Bioarchaeology has also given independent support to this hypothesis. The evaluation of cranial phenotypic differences by means of quantitative traits lead Cocilovo et al. (2017) to propose the existence of possible links and relationships between San Pedro de Atacama (Northern Chile) and the Puna de Jujuy (NWA) during the Middle and Late Periods.

The end of the FP is characterized by significant social changes. The following Late or Regional Developments Period (RDP) (ca. AD 1000–1450) witnesses the consolidation of highly hierarchical societies with clear-cut territorial boundaries maintained through the political control of other ecological areas, agglomerated settlements and warfare. These changes were in probable response to a cycle of severe droughts that triggered conflicts over the control of more stable water sources for irrigation (Nielsen 1996, 2001). Towards the middle of the 15th century AD, with the annexation of NWA to the Inca Empire, local groups were displaced and reorganized, Inca craft styles and architectural patterns were transferred to the conquered areas as one form of control and domination (Cremonte and Williams 2007). Soon after, the Europeans colonized the area, leading to massive changes in local culture.

Located in the Southern Calchaquí Valleys (Catamarca Province), the Cajón Valley extends north-south along 90 km between W66°00' 66°30' and S26°10' 27°00' (Figure 1). This valley occupies a strategic point in the landscape that could have been of fundamental importance in the transit and exchange networks in the past, connecting people and resources of the Puna, the valleys and the eastern lowlands (Scattolin et al. 2009a, 2009b).



Within this valley, archaeological research in the locality of La Quebrada (Santa María Department, Catamarca Province) has been growing steadily for the last decades (Scattolin and Gero 1999; Gero and Scattolin 2002; Scattolin et al. 2009a, 2015) filling a longstanding gap of information of first agricultural communities of the FP in this region.

Throughout the last 15 years, the chance finding of human remains has been an important part of the archaeological research in La Quebrada. A total of 12 burial contexts of diverse characteristics and chronologies -including at least 20 individuals- have been excavated within a 10 km<sup>2</sup> radius from Cardonal, Bordo Marcial and Yuopian dwelling settlements (Cortés 2011, 2013; Figure 1). Cardonal and Bordo Marcial were assigned by various radiocarbon dates to the first centuries AD (ca. 1800-1900 YBP) (Scattolin et al. 2015, 2009a, 2009b) while Yutopian presents components dated to both FP and RDP (Scattolin and Gero 1999). Accelerator Mass Spectrometry dates (AMS) obtained from human remains evinced a previously unexpected deep chronological occupation of this valley. While some funerary contexts were contemporaneous to the occupation of the dwelling sites (around 2000 YBP), others predated these settlements. In fact, burial contexts have provided unquestionable proof that human occupation of the southern CV goes back to the Archaic Period, proving at least 5000 years of occupation of this area, from ca. 6000 to 1300 YBP. Of this sample of twelve individuals, eleven were selected for aDNA analyses.

## **Materials and Methods**

### ***Samples and Contexts***

Eleven individuals from eight different funerary contexts were analyzed (Table 2; Figure 2). The individual from burial C640, a 40-50 year old woman, is the most ancient sample dated to the Archaic period, ca. 6000 YBP (Table 2, Figure 2). The human remains were found on the surface in a very poor state of preservation. Samples from contexts C1225 and C1242

were both dated to ca. 3600 YBP. Individual C1225, an adult male, was placed in a pit dug directly in the ground in a genuflected position. Sample C1242 was a young child 5-6 years of age. This individual was found incomplete and in a very poor state of preservation.

Individuals C440-1 to 4 belong to a collective burial where the disarticulated, commingled and fragmented bones of at least other 10 individuals (including both female and male adults, and children of various ages) were found. The context was dated to ca. 3000 YBP (Table 2).

This tomb stands out as the human remains were associated to an anthropomorphic mask which is, so far, the most ancient intentionally shaped copper object of the Andean region (Scattolin et al. 2010; Cortés and Scattolin 2017). The four individuals considered for the analysis include two subadults, a 4-5 year old child (C440-1) and another of approximately 10-12 years of age (C440-2), and two adults of undetermined sex (C440-3 and C440-4).

The remaining four individuals in the sample are dated between ca. 2100 and 1900 YBP (Table 2), that is, probably contemporaneous to the occupation of the nearby villages of Cardonal and Bordo Marcial (Figure 1). Individual C1222, an adult male of 30-40 years of age, was buried in a hyperflexed position in a clearly delimited pit dug in the ground. A few meters away, the disarticulated and scattered remains of a woman of 25-35 years of age (C1223) were recovered. Individual C639, a woman of 20-25 years of age, was found in a partially destroyed burial context. Bones of fetus or newborn around her abdominal area indicated she was probably pregnant at the time of death or buried with her child. The last individual in the sample (C641) is an adult male whose body had also been disposed in a hyperflexed position and buried in a site known as Duna Cemetery. This large dune of fine white sand has been used as a cemetery during the pre-Hispanic past, and has also been subject of systematic looting in modern times. The radiocarbon date of this individual of ca. 1900 YBP (Table 2) indicates that this cemetery was in use contemporary to the occupation of the dwelling sites of Cardonal and Bordo Marcial. In sum, the analyzed sample comprises

11 individuals dated between 6000 and 1900 YBP including 8 adults (3 males, 3 females and 2 of indeterminate sex), and 3 subadults between 4 and 12 years old (Table 2) (for further details on the excavated contexts see Cortés 2011, 2013).

### ***Mitochondrial DNA Analysis***

**Ethics Statement.** The aDNA study was approved by the Ethics Committees of Puerto Madryn Zonal Hospital (Resol.004/2017). The excavation of archeological human remains has been endorsed by permission of the Anthropological Directorate of Catamarca Province (Dirección de Antropología de Catamarca, Argentina). The Ingamana Indigenous Community gave its consent to the archaeological investigations in the study area. The extraction and transportation of samples of the human remains were approved by the National Institute of Anthropology (INAPL, Argentina), and the National Registry of Archaeological Sites, Collections and Objects (RENYCOA, Argentina).

**Prevention of Contamination.** Sample processing followed strict decontamination and monitoring procedures to minimize the risk of contamination and to ensure the reliability of the results (Pääbo et al. 2004; Gilbert et al. 2005; Carnese et al. 2010). All analyses were carried out at the Instituto de Diversidad y Evolución Austral (IDEAus-CONICET-CENPAT, Argentina) in a molecular laboratory exclusively destined for processing samples of low DNA content with pre- and post-PCR separated areas. Before manipulation all laboratory equipment was cleaned with 10% sodium hypochlorite, rinsed with ultrapure water and irradiated with UV light. Extraction blanks and PCR positive and negative amplification controls were included in each analysis. To test the reproducibility of results, PCRs were performed at least twice per sample. All personnel involved in the manipulation of the samples were genotyped for mtDNA.

**DNA Extraction.** Bone and tooth samples were first washed with 10% sodium hypochlorite, rinsed with DNase- and RNase-free water and irradiated with UV light during 30 minutes on each side in order to remove surface exogenous DNA. Powder sample was obtained using a grinder mill (IKA- A11 Basic) and a Dremel drill. For each sample, DNA was extracted from 80-100 mg of the pulverized material. The decalcification was performed through 24 hours incubation with 1.5 ml of 0.5 mM EDTA (pH 8) in an orbital shaker. The pellet sample was digested with a solution containing 0.5 mM EDTA (pH 8), 10 mM Tris HCl (pH 8), 2% SDS, 0.3 mM sodium acetate and 60ul proteinase K (20µg/ml), and incubated overnight at 56°C in a digital dry bath. Extractions were carried out with two different protocols: a commercial kit QIAamp DNA Investigator (Qiagen) and the standard phenol:chloroform:isoamyl alcohol (25:24:1) plus purification with Wizard SV Gel and PCR Clean Up System kit (Promega).

**HVR-1 Sequencing.** The analysis of the mtDNA hypervariable region 1 (HVR-1) was performed by the amplification of three overlapping portions, using three sets of primers (F15998-R16142; F16120-R16239; F16208-R16410) described by Adachi et al. (2009) and Gabriel et al. (2001) (Supplementary material: Supplementary Table S1). When haplogroup B was detected, an alternative primer R16167 (5'-GGGTTTGATGTGGATTGGG-3') (Ricaud et al. 2004) was used for sequencing the second segment. PCR was performed on a final volume of 25ul, using a HotStarTaq Master Mix Kit (Qiagen). The use of a high quality commercial master mix, including all PCR components, minimizes the steps of handling reagents and therefore the risk of potential contamination of the samples. The cycling conditions were: predenaturation 10 min at 95°C followed by 45 cycles of denaturation, 45 s at 94°C, annealing 45 s at 54°C and extension 45 s at 72°, final extension 5 min at 72°C, and

holding at 15°C. PCR products were visualized under UV in a 2% agarose gel stained with GelRed™ and purified with Illustra ExoProStar (General Electrics) enzyme following manufacturer's recommendation. The HVR-1 amplified fragments were sequenced in forward and reverse direction, in an ABI Prism 3130 capillary genetic analyzer (Applied Biosystems) in the IDEAus sequencing service.

Consensus sequences were mapped to the revised Cambridge Reference Sequence (rCRS) (Andrews et al. 1999) using Sequencher v4.9 Demo (GeneCodes) and edited with Bioedit v7.0.5 (Hall 1999) program. The mtDNA haplogroups were assigned using Phylotree build 17 (van Oven 2015) and Haplogrep 2 (Weissensteiner et al. 2016). In order to analyze the distribution of the haplotypes obtained and their continuity or replacement across time and space, our HVR-1 haplotypes were compared with those obtained by other authors for 56 archeological and modern populations from NWA and others from CA and SA (Table 2). Genetic distances (*Fst*) estimation between pairwise populations and its representation in a multi-dimensional scaling (MDS) plot were performed respectively with Arlequin v.3.5.2 (Excoffier and Lischer 2010) and the FactoMineR (Lê et al. 2008) package for R 3.1.1. Median-joining networks were constructed using NETWORK v. 4.6.1.3 software program (Bandelt et al. 1999).

## **Results**

In spite of the variable preservation conditions of the human remains, reproducible HVR-1 sequences were obtained in 7 out of 11 individuals. Best results were obtained with the organic extraction instead the commercial kit QIAamp DNA Investigator (Qiagen), in particular regarding the DNA amplification quality. As shown in Table 3, five different HVR-1 haplotypes were assigned to haplogroup D1 (57.1%), C1 (28.5%) and B2 (14.2%). A partial haplotype obtained for sample C1223 could not be assigned to a specific haplogroup.

For lineage D1, four individuals shared the diagnostic nodal mutations, and two subadult individuals from the copper mask burial (C440-1 and C440-2) also shared the 16327T private mutation. For haplogroup C1, two individuals (C1222 and C641) carried the nodal mutations, and one of them (C641) also showed the diagnostic A>G substitution at nucleotide position (np) 16051 characteristic for the variant C1d (Achilli et al. 2008). Haplogroup B2 was only assigned to the most ancient individual (C1242) and haplogroup A2 was not detected in the sample.

Median-joining networks were constructed only for haplogroup C1 and D1 due the low representation of B2 in the studied sample. Additionally, two phylogenetic trees of lineages B2, C1 and D1, were built comparing the CV HVR-1 haplotypes with other CA and SA ancient and modern populations (Supplementary material: Supplementary Figure S1 and Supplementary Figure S2, respectively).

Fifty-two out of 111 ancient and modern samples analyzed for the haplogroup C1, including the individual C1222 (2164±47 YBP), carried the nodal haplotype (Figure 3). Moreover, the individual C641 (1915±47 YBP) shared the same haplotype with the transition 16051 with other eight samples: an Inca Period individual (600-500 YBP) from San Sebastián, Peru (Llamas et al. 2016), seven modern samples from Catamarca (n=3) and Salta (n=3) both provinces of NWA (Tamm et al. 2007; Perego et al. 2010), and one contemporary Aymara sample from Bolivia (Gayà-Vidal et al. 2011). For the lineage D1, 47 out of 95 ancient and modern individuals analyzed, including samples C639 (2056±48 YBP) and C1225 (3678±39 YBP), carried the nodal haplotype (Figure 4). The two subadults from the copper-mask burial (C440-1 and C440-2; 3001±49 YBP) carried the same transition 16327, not observed in any of the ancient or contemporary comparative samples analyzed (Figure 4).

Using mtDNA haplogroup frequencies (Table 1), pairwise  $F_{ST}$  genetic distances were estimated between the CV and other 36 ancient and modern samples from CA and SA, each

one composed of at least 5 individuals (Supplementary material: Supplementary Table S2).

The CV sample showed lower genetic distances ( $F_{st}$  0.0742-0.2908) and non-significant differences ( $p$ -value  $> 0.05$ ) with four FP populations: one from of the eastern flank of the Andes in NWA -Pampa Grande (PG) ( $F_{st} = 0.0742$ )-, another from the northern Calchaquí Valleys -Salvatierra (SA) ( $F_{st} = 0.2908$ )-, and two from the coast of Peru -Río Grande de Nasca Drainage (RC1) ( $F_{st} = 0.0791$ ) and Huaca Pucllana Lima (HPL) ( $F_{st} = 0.0869$ ). Likewise, CV showed low genetic distances ( $F_{st}$  0.0225-0.2811) with RDP and Inca samples from Jujuy Province, NWA (MU, TIL, DON, EHU) and the coast of Peru (PA, HPY, PV). The comparison between our results and the mtDNA haplogroup frequencies of modern samples shows low genetic distances between the CV and contemporary samples of the provinces of Catamarca (CAT) ( $F_{st} = 0.0791$ ) and Salta (SLT) ( $F_{st} = 0.0959$ ) of NWA. Nonetheless, it is important to emphasize that the genetic distances and significances ( $p$ -values) could be affected by the different sample sizes. Inter-population genetic distances ( $F_{ST}$ ) (Supplementary material: Supplementary Table S2) were represented in a multidimensional scaling plot (Figure 5).

## Discussion

Although archaeological evidence indicate that NWA has been inhabited at least since 11000 YBP (Martínez 2018), our knowledge of the genetic constitution of pre-Hispanic populations still remains incipient. Genetic studies carried out in ancient Andean human remains show high frequency of mitochondrial haplogroup B2 (Moraga et al. 2005; Kemp et al. 2009; Rothhammer et al. 2009; Baca et al. 2012, 2014; Mendisco et al. 2014; Russo et al. 2014, 2016; Fehren-Schmitz et al. 2015; Llamas et al. 2016), in accordance with the frequencies observed in contemporary Andean populations (Barbieri et al. 2011; Gayà-Vidal et al. 2011; de Saint Pierre et al. 2012).

In the present study, mtDNA HVR-1 haplotypes obtained in seven samples of the Cajón Valley (ca. 3600-1900 YBP) showed highest frequencies of haplogroup D1 (57.1%) followed by C1 (28.5%), while the characteristic Andean haplogroup B2 was registered in the lowest frequency (14.2%) and A2 was absent. Considering that the small sample size could be affecting the estimation of haplogroup frequencies, the following discussion is focused in the most representative lineages observed in the Cajón Valley: Haplogroups C1 and D1.

Four out of seven HVR-1 haplotypes obtained were assigned to the haplogroup D1, which is one of the pan-American mtDNA haplogroups (together with A2, B2, C1b, C1c, C1d, D4h3a and X2a), with an estimated entry time to America from Beringia between 15-18 thousand years ago (kya) (Tamm et al. 2007; Achilli et al. 2008; Perego et al. 2009). Individuals C639 (2056±48 YBP) and C1225 (3678±39 YBP) carried the D1 nodal haplotype, while samples C440-1 and C440-2 (3001±49 YBP) additionally shared the same private mutation in the position 16327, not observed among any of the analyzed haplotypes (Figure 4). As mentioned before, these last two samples correspond to subadult individuals found in a collective burial along with other 12 individuals buried with an anthropomorphic copper-mask. The fact that the both individuals share the mtDNA HVR-1 haplotype with a same private mutation -and the possibility of both children being related by maternal lineage- could provide additional evidence to the proposed hypothesis about the mask being the material referent of the Andean figure of “ancestor”, acting as a synthesis for the community buried in the sepulcher (Cortés 2013). However, genomic analysis of higher resolution power must be carried out to confirm the possibility of consanguinity between these two individuals.

Comparing the Cajón Valley HVR-1 haplotypes with other Central and Southern Andes ancient and modern populations, the resulting median-joining networks (Figures 3 and 4) showed a majority of sequences within the ancestral C1 and D1 haplotypes, with derived haplotypes diverging only for one or two single mutations, and some shared haplotypes. This



result could support a scenario of extensive networks and multiple contacts within the NWA and Andean region as a whole.

In Formative Period populations, haplogroup D1 exhibits in general relative low frequencies, with the exception of the Pampa Grande samples of the eastern flank of the Andes in NWA (Carnese et al. 2010) and RGND (Coast) samples from Peru (Fehren-Schmitz et al. 2014) (Table 1). According to Carnese et al. (2010), the high frequency of haplogroup D1 could indicate a genetic affinity between Pampa Grande and eastern lowlands populations (Toba and Wichi). However, the high frequency of a specific maternal lineage in a sample is also suggestive of possible matrilinearity/matrilocality, associated to cultural practices, which not only cause gene flow but also introduces biases in its sense and direction.

Haplogroup C1 was registered in two individuals of the CV, C1222 (2164±47 YBP) and C641 (1915±47 YBP). The first one carried the C1 nodal haplotype while the individual C641 was assigned to the ancestral lineage C1d due the presence of the diagnostic A>G substitution at np 16051 (Achilli et al. 2008). Lineage C1 is one of the pan-American haplogroups, and has been subdivided into three branches originated in Beringia: C1b, C1c, and C1d (Tamm et al. 2007). Among these, haplogroup C1d has been defined as the latest lineage with an estimated entrance to the American Continent between 7.6 and 10.9 kya (Tamm et al. 2007; Achilli et al. 2008; Perego et al. 2009). The analysis of 63 mitochondrial genomes from different populations of America (Perego et al. 2010) and 271 HVR-I and HVR-II sequences -including NWA and Uruguay- (Figueiro et al. 2011) have led to conclude that the geographic distribution of the C1d ancestral mutations is wide enough to assume that this haplogroup entered the continent as a part of the initial peopling of the Americas, about 15-17 kya. The analysis of more than 300 mtDNA haplotypes from different American populations (Perego et al. 2010; Figueiro et al. 2011) suggests that C1d entered the continent as a part of the initial peopling.

Both in Central and Southern Andes pre-Hispanic populations (Table 1), haplogroup C1 is frequent in the FP samples of RGND coast and highlands (Fehren-Schmitz et al. 2014), Huaca Pucllana Wari, Huaca Pucllana Lima, all from Peru, as well as in Tiwanaku samples from the Bolivian Altiplano (Llamas et al. 2016). In NWA haplogroup C1 is less frequent, probably due the low number of samples analyzed in each site, nevertheless it is present in sample LA18 from Los Amarillos in Quebrada de Humahuaca (Mendisco et al. 2011), as well as in two individuals from the Inca Period, one of the three Lullailaco mummies of Salta Province (Wilson et al. 2007; Llamas et al. 2016) and the Aconcagua mummy from Mendoza Province (Gómez-Carballa et al. 2015) (Supplementary material: Supplementary Figure S1). Haplogroup C1d was only registered in a sample of the Inca Period (ca. 550 YBP) from San Sebastián, Peru (Lamas et al. 2016).

Although lineage C1 was not detected in PG the fact that Pampa Grande and Cajón Valley samples share a prevalence of D1 could give additional support the longstanding argument of possible connections between communities settled in different ecological regions of NWA (Núñez and Dillehay 1979; Browman 1980; Nielsen 2013). However, this hypothesis -based on HVR-1 results- needs to be subject of further testing by higher resolution power genomic analysis. As mentioned before, archeological evidence has strongly endorsed the existence of extensive exchange networks throughout NWA, as well as northern Chile since the Archaic Period. For example, analyses of ceramic styles of the Formative Period -in particular the first millennium AD- from the Cajón Valley have shown great similitude with other sites of the eastern flank of the Andes. The ceramic style known as “Candelaria” (Heredia 1968, 1974) is found in Pampa Grande (Baldini et al. 1998, 2003) and in other sites of the eastern flank of the Andes as well as in the Cajón Valley (Scattolin et al. 2009a, b). From an archaeological perspective, this sharing of ceramic styles have led to argue that people from the Cajón Valley and the eastern flank of the Andes shared common

ways of doing things (Scattolin 2007; Scattolin et al. 2009a, b), suggesting possible frequent relationships between people inhabiting these two environments. Also, the presence of foreign vegetable resources in the sites of the Cajón Valley (Bugliani et al. 2009) -such as *Anadenanthera* sp., an alucinogenous plant that grows under 2000 masl- gives additional support to this hypothesis. As mentioned, reciprocity and redistribution among ethnic groups (as observed in modern populations) is thought to have been the main driving force for the circulation of goods and people (Nielsen 2013). These regular movements might have also favored interethnic marital exchange and gene flow. Although it is still difficult to assess the partial coincidence in aDNA frequencies to gene flow between both regions, the relationships between the Valleys and the eastern slope of the Andes is valid for further consideration through future higher resolution DNA analyses and a more widespread sampling of populations of both regions.

## **Conclusion**

Paleogenetic studies in NWA Formative Period populations are particularly scant. This research provided the first aDNA data for individuals from the Southern Calchaquí Valleys dated to ca. 3600-1900 YBP. In spite of the small sample size and its limitations, this is so far the only genetic information available for the region and period studied.

Results show that the maternal lineages retrieved from our sample -with predominance of haplogroup D1 and C1- differ from that commonly observed in ancient and modern Andean populations, usually characterized by a strong dominance of haplogroup B2. Moreover, if we consider samples with high frequencies of D1 or C1 independently results show relative low frequencies of these lineages during the Formative Period both in Central and Southern Andes.

Although similar frequencies of lineages C1 and D1 were found in RGND coast region from Peru, the marked differences on the samples sizes undermines any solid hypothesis regarding a common population origin of both populations.

The present study has provided preliminary information on possible genetic affinities between the Southern Calchaquí Valleys and the eastern sub-Andean region during the Formative Period in NWA, in particular, Pampa Grande. Notwithstanding, as mentioned, the small sample size as well as the low resolution of HVR-1 technique hampers the chance of giving strong sustenance to the longstanding hypotheses on relationships between different ecological regions in NWA during the Formative Period. In this sense, future agenda is set up to obtaining complete mitogenomes that will improve the power of resolution necessary to define mitochondrial variants characteristic of certain geographic regions and/or population groups. Additionally, by incorporating samples from other regions not included in this paper, we expect to formulate new hypotheses about the origins and phylogenetic relationships between the individuals of the Southern Calchaquí Valleys and other population groups from the eastern slope of the Andes, Gran Chaco and Amazonas lowlands as well as the southernmost extreme of the Patagonian Andes.

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**Table 1. Ancient and Modern Populations Analyzed and Respective mtDNA Haplogroup (Hg) Frequencies**

Period	Region/Country/Area	Site/Population	n	mtDNA Segment	Hg A2	Hg B2	Hg C1	Hg D1	Reference	
Archaic (ARC)	CA/Peru/ Highlands	Cuncaicha	2	mitogenome	2 (100%)	0	0	0	Posth et al. (2018)	
	CA/Peru/ Highlands	Lauricocha	4	HVR-1/mitogenome	3 (75%)	1 (25%)	0	0	Fehren-Schmitz et al. (2015), Llamas et al. (2016)	
	CA/Peru/ Highlands	La Galgada (Kotosh)	4	mitogenome	1(25%)	1(25%)	1(25%)	1(25%)	Llamas et al. (2016), Posth et al. (2018)	
	SA/Chile/Northern coast	Camarones (Chinchorro) [CH]	14	RFLP /HVR-1	7 (50%)	5 (35.7%)	1 (7.1%)	1 (7.1%)	Moraga et al. (2005)	
	SA/Chile/Northern coast	Camarones (Chinchorro)	1	mitogenome	1 (100%)	0	0	0	Llamas et al. (2016)	
Formative Period (FP)	SA/NWA/Eastern Andean slope/Salta	Pampa Grande [PG]	19	HVR-1	2 (10.5%)	9 (47.4%)	0	8 (42.1%)	Carnese et al. (2010)	
	SA/NWA/Northern Calchaquí Valley/Salta	Salvatierra [SA]	5	RFLP	0	4 (80%)	0	1(20%)	Russo et al. (2014)	
	SA/NWA/Quebrada de Humahuaca/Jujuy	Flores 1	2	HVR-1	0	2 (100%)	0	0	Russo et al. (2016)	
	CA/Coast/Southern Peru/Coast/RGND	Río Grande de Nasca drainage [RC1]	10	HVR-1	4 (3.7%)	11 (10.1%)	32 (29.6%)	61 (56.4%)	Fehren-Schmitz et al. (2014)	
	CA/Southern Peru/Highlands/RGND	Río Grande de Nasca drainage [RH1]	8	HVR-1	2 (5.1%)	22 (56.1%)	11 (28.2%)	4 (10.2%)	Fehren-Schmitz et al. (2014)	
	CA/Peru/Highlands	Lauricocha	39	HVR-1	2 (5.1%)	22 (56.1%)	11 (28.2%)	4 (10.2%)	Fehren-Schmitz et al. (2014), Fehren-Schmitz et al. (2015), Llamas et al. (2016)	
	CA/Peru/Highlands	Cuncaicha	1	mitogenome	1 (100%)	0	0	0	Posth et al. (2018)	
	CA/Peru/Coast	Huaca Pucllana (Wari) [HPW]	1	mitogenome	1 (100%)	0	0	0	Llamas et al. (2016)	
	CA/Peru/Coast	Huaca Pucllana (Lima) [HPL]	10	mitogenome	2 (20%)	3 (30%)	5 (50%)	0	Llamas et al. (2016)	
	CA/Peru/Coast	Huaca Pucllana (Lima) [HPL]	9	mitogenome	1 (11.1%)	4 (44.4%)	3 (33.3%)	1 (11.1%)	Llamas et al. (2016)	
	CA/Peru/ Highlands	Conchopata [CON]	14	HVR-1	4 (28.6%)	7 (50%)	2 (14.3%)	1 (7.1%)	Kemp et al. (2009)	
	SA/Bolivia/Altiplano	Tiwanaku [TIW]	9	mitogenome	1 (11.1%)	5 (55.5%)	3 (33.3%)	0	Llamas et al. (2016)	
	SA/Chile/Upper Northern Valleys	Azapa, Alto Ramírez, Late Alto Ramírez, Maitas-Chiribaya, Tiwanaku, Cabuza [UNV]	19	RFLP /HVR-1	6 (31.6%)	8 (42.1%)	5 (26.3%)	0	Moraga et al. (2005)	
	Regional Developments Period (RDP)	SA/NWA/Quebrada de Humahuaca/Jujuy	Muyuna [MU]	6	HVR-1	0	0	0	6 (100%)	Russo et al. (2016)
		SA/NWA/Quebrada de Humahuaca/Jujuy	Los Amarillos [LAM]	16	mtSNP/HVR-1	12 (75%)	1 (6.2%)	1 (6.2%)	2 (12.5%)	Mendisco et al. (2011, 2014, 2018)
SA/NWA/Quebrada de Humahuaca/Jujuy		Banda de Perchel	2	mtSNP/HVR-1	1 (50%)	0	1 (50%)	0	Mendisco et al. (2014)	



	SA/NWA/Quebrada de Humahuaca/Jujuy	Huichairas	1	mtSNP/HVR-1	1 (100%)	0	0	0	Mendisco et al. (2014)
	SA/NWA/Quebrada de Humahuaca/Jujuy	Tilcara [TIL]	6	mtSNP/HVR-1	1 (16.6%)	4 (66.6%)	0	1 (16.6%)	Mendisco et al. (2014)
	SA/NWA/Quebrada de Humahuaca/Jujuy	Juella	3	mtSNP/HVR-1	0	1 (33.3%)	1 (33.3%)	1 (33.3%)	Mendisco et al. (2014)
	SA/NWA/Quebrada de Humahuaca/Jujuy	Sarahuaico	2	mtSNP/HVR-1	0	2 (100%)	0	0	Mendisco et al. (2014)
	SA/NWA/Quebrada de Humahuaca/Jujuy	San José	1	mtSNP/HVR-1	0	0	1 (100%)	0	Mendisco et al. (2014)
	SA/NWA/Quebrada de Humahuaca/Jujuy	Huacalera	1	mtSNP/HVR-1	0	0	0	1 (100%)	Mendisco et al. (2014)
	SA/NWA/Northern Calchaquí Valley/Salta	Tero	4	mtSNP/HVR-1	1 (25%)	2 (50%)	1 (25%)	0	Mendisco et al. (2014)
	SA/NWA/Northern Calchaquí Valley/Salta	Fuerte Alto	2	mtSNP/HVR-1	2 (100%)	0	0	0	Mendisco et al. (2014)
	SA/NWA/Puna de Jujuy /Jujuy	Doncellas	3	mtSNP/HVR-1	0	2 (66.6%)	1 (33.3%)	0	Mendisco et al. (2014)
	SA/NWA/Puna de Jujuy /Jujuy	Doncellas [DON]	10	HVR-1	5 (50%)	1 (10%)	2 (20%)	2 (20%)	Postillone et al. (2017)
	SA/NWA/Puna de Jujuy /Jujuy	Agua Caliente	4	HVR-1	1 (25%)	1 (25%)	1 (25%)	1 (25%)	Postillone et al. (2017)
	SA/NWA/Puna de Jujuy /Jujuy	Casabindo	1	HVR-1	1 (100%)	0	0	0	Postillone et al. (2017)
	SA/NWA/Puna de Jujuy /Jujuy	Río Negro	1	HVR-1	0	0	1 (100%)	0	Postillone et al. (2017)
	CA/Peru/Highlands/Ayacucho	Huari [HUA]	18	HVR-1	3 (16.7%)	4 (22.2%)	10 (55.5%)	1 (5.6%)	Kemp et al. (2009)
	CA Coast/Southern Peru/Coast/RGND	Río Grande de Nasca drainage [RC2]	22	HVR-1	1 (4.5%)	13 (59%)	6 (27%)	2 (9%)	Fehren-Schmitz et al. (2014)
	CA /Southern Peru/Highlands/RGND	Río Grande de Nasca drainage [RH2]	38	HVR-1	2 (5.2%)	20 (52.6%)	12 (31.5%)	4 (10.5%)	Fehren-Schmitz et al. (2014)
	CA/Peru/Coast	Pasamayo (Chancay) [PA]	7	mitogenome	1 (14.2%)	2 (28.5%)	3 (42.8%)	1 (14.2%)	Llamas et al. (2016)
	CA/Peru/Coast	Huaca Pucllana (Ychsma) [HPY]	15	mitogenome	0	8 (53.4%)	4 (26.6%)	3 (20%)	Llamas et al. (2016)
	SA/NWA/Quebrada de Humahuaca/Jujuy	Pukará de la Cueva	1	HVR-1	1 (100%)	0	0	0	Russo et al. (2018a)
	SA/Chile/Northern Desert Valleys/Atacama	Pica Ocho	1	mitogenome	1 (100%)	0	0	0	Posth et al. (2018)
	SA/Chile/Northern Coast/Desert Valleys	Gentilar [GEN]	15	RFLP/HVR-1	3 (20%)	8 (53.3%)	3 (20%)	1 (6.7%)	Moraga et al. 2005
Inca (INC)	SA/NWA/Quebrada de Humahuaca/Jujuy	Esquina de Huajra [EHU]	5	HVR-1	3 (60%)	0	2 (40%)	0	Russo et al. (2017)
	SA/NWA/Highlands/Salta	Llullaillaco	3	HVR-1/mitogenome	0	0	1 (33.3%)	2 (66.6%)	Wilson et al (2007), Llamas et al. (2016)
	SA/NWA/Highlands/Mendoza	Cerro Aconcagua	1	mitogenome	0	0	1 (100%)	0	Gómez-Carballa et al. (2015)
	SA/Chile/Tarapacá	Pica (Pica-Tarapacá) [PIC]	5	mitogenome	2 (40%)	3 (60%)	0	0	Llamas et al. (2016)

	CA/Peru/Coast	Pueblo Viejo [PV]	11	mitogenome	0	5 (45.4%)	4 (36.3%)	2 (18.1%)	Llamas et al. (2016)
	CA/Peru	San Sebastián	2	mitogenome	1 (50%)	0	1 (50%)	0	Llamas et al. (2016)
	CA/Peru/Highlands/Arequipa	Tompullo2 [TP]	24	HVR-1	2 (8.3%)	17 (70.8%)	1 (4.2%)	4 (16.7%)	Baca et al. (2012)
	CA/Peru/Highlands/Arequipa	Acchaymarca [ACC]	11	HVR-1	0	8 (72.7%)	2 (18.2%)	1 (9.1%)	Baca et al. (2014)
	CA/Peru/Highlands/Arequipa	Puca [PUC]	14	HVR-1	6 (42.9%)	6 (42.9%)	1 (7.1%)	1 (7.1)	Baca et al. (2014)
Modern (MDN)	CA/Peru/Altiplano/Titicaca Lake	Quechua [QUP]	37	RFLP/HVR-1	6 (16%)	21 (56%)	5 (14%)	5 (14%)	Barbieri et al. (2010)
	CA/Peru/Altiplano/Titicaca Lake	Aymara [AYP]	20	RFLP/HVR-1	0	18 (90%)	1 (5%)	1 (5%)	Barbieri et al. (2010)
	CA/Peru/Altiplano/Titicaca Lake	Uros [UR]	7	RFLP/HVR-1	1 (14.3%)	5 (71.4%)	0	1 (14.3%)	Barbieri et al. (2010)
	SA/Chile/Puna	Aymara [AYC]	39	Control Region	2 (5.1%)	25 (64.1%)	3 (7.6%)	9 (23%)	de Saint Pierre et al. (2012)
	SA/Chile/San Pedro de Atacama	Atacameño [ATC]	28	Control Region	7 (25%)	16 (57.1%)	3 (10.7%)	2 (7.1%)	de Saint Pierre et al. (2012)
	SA/Bolivia	Aymara [AYB]	96	HVR-1/HVR- 2	7 (7%)	78 (81%)	6 (6%)	5 (5%)	Gayà-Vidal et al. (2011)
	SA/Bolivia	Quechua [QUB]	93	HVR-1/HVR- 2	14 (15%)	57 (61%)	18 (19%)	4 (4%)	Gayà-Vidal et al. (2011)
	SA/NWA/Salta	Salta Capital [SLT]	17	RFLP/HVR-1	4 (23.5%)	6 (35.2%)	5 (29.4%)	2 (11.7%)	Tamm et al. (2007)
	SA/NWA/Catamarca	San Fernando del Valle [CAT]	25	RFLP/HVR-1	6 (24%)	1 (4%)	6 (24%)	12 (48%)	Tamm et al. (2007)

CA: Central Andes; SA: South Andes; NWA: Northwest Argentina. In square brackets, samples selected for *Fst* pairwise analysis.

**Table 2. Samples Analyzed in Present Study**

Sample	Context ID	Procedence	Site	Lab code	<sup>14</sup> C yr BP	Calibrated date (2 sigmas)*	Estimated age (years)	Estimated sex
1	C640	Cajón Valley	El Aumento	AA87287	6133±66	5211-4807 BC	40-50	female
2	C1225	Cajón Valley	El Aumento	AA97850	3678±39	2135-1884 BC	adult	male
3	C1242	Cajón Valley	Pie de Yutopián	AA106689	3679±46	2142-1884 BC	5-6	indet.
4	C440-1	Cajón Valley	Bordo Marcial	AA82256	3001±49	1370-1346 BC	4-5	indet.
5	C440-2	Cajón Valley	Bordo Marcial	AA82256	3001±49	1370-1346 BC	10-12	indet.
6	C440-3	Cajón Valley	Bordo Marcial	AA82256	3001±49	1370-1346 BC	adult	indet.
7	C440-4	Cajón Valley	Bordo Marcial	AA82256	3001±49	1370-1346 BC	adult	indet.
8	C1222	Cajón Valley	Tres Cabezas	AA101317	2164±47	354 BC-AD 2	30-40	male
9	C1223	Cajón Valley	Tres Cabezas	AA101318	2187±45	358 BC-AD 42	25-35	female
10	C639	Cajón Valley	Bordo Marcial	AA87286	2056±48	162 BC-122 AD	20-25	female
11	C641	Cajón Valley	Duna Cemetery	AA87292	1915±47	28-317 AD	20-25	male

\*Calibrations were performed with *Calib-Radiocarbon Calibration Program* (Stuiver and Reimer, 1986-2005), Southern Hemisphere calibration curve. Sex and age estimations were made following standard bioarchaeological methods described in Cortés (2011).

**Table 3. mtDNA HVR-1 Haplotypes (Reading Frame 16024-16365) Obtained from Southern Cajón****Valley Individuals**

Sample ID	<sup>14</sup> C (yr BP)	mtDNA Haplotype	Haplogroup
C1225	3678±39	16223T, 16325C, 16362C	D1
C1242	3679±46	16183C, 16189C, 16217C	B2
C440-1	3001±49	16223T, 16325C, 16327T, 16362C	D1
C440-2	3001±49	16223T, 16325C, 16327T, 16362C	D1
C1222	2164±47	16223T, 16298C, 16325C, 16327T	C1
C1223	2187±45	~, 16223T, ~	nd.
C639	2056±48	16223T, 16325C, 16362C	D1
C641	1915±47	16051G, 16223T, 16298C, 16325C, 16327T	C1/C1d

~: Low quality reading; nd.: No defined haplogroup.

**Supplementary Table S1. Primers Used for HVR-1 Amplification**

Segment	Position	Primer sequence 5' to 3'	Amplicon size	Reference
I	15998- 16142	F15998-CCATTAGCACCCAAAGCTA	144 bp	Adachi et al. 2009
		R16142-ATGTACTACAGGTGGTCAAG		Adachi et al. 2009
II	16120- 16239	F16120-TTACTGCCAGCCACCATGAA	119 pb	Adachi et al. 2009
		R16239-TGGCTTTGGAGTTGCAGTTG		Adachi et al. 2009
III	16208- 16410	F16208-CCCATGCTTACAAGCAAG	202 pb	Adachi et al. 2009
		R16410-GAGGATGGTGGTCAAGGGAC		Gabriel et al. 2001





## Figure Captions

**Figure 1.** Geographic location of the Cajón Valley in the Southern Calchaquí Valleys, NWA.

**Figure 2.** Geographic location of the studied samples in the Southern Cajón Valley (modified from Google Earth, 2017).

**Figure 3.** Median-joining network of haplogroup C1. Network was performed based on 111 ancient and modern individuals and 28 different HVR-1 haplotypes. White circles indicate the proportional number of samples for each node. FP: Formative Period; RDP: Regional Developments Period; INC: Inca Period.

**Figure 4.** Median-joining network of haplogroup D1. Network was performed based on 95 ancient and modern individuals and 20 different HVR-1 haplotypes. White circles indicate the proportional number of samples for each node. FP: Formative Period; RDP: Regional Developments Period; INC: Inca Period.

**Figure 5.** MDS plot of the  $F_{ST}$  pairwise genetic distances between southern CV sample (in bold) and 36 ancient and modern populations (references on Table 1). Stress = 0.1656. ARC: Archaic Period, FP: Formative Period, RDP: Regional Developments Period, INC: Inca Period, MDN: Modern.

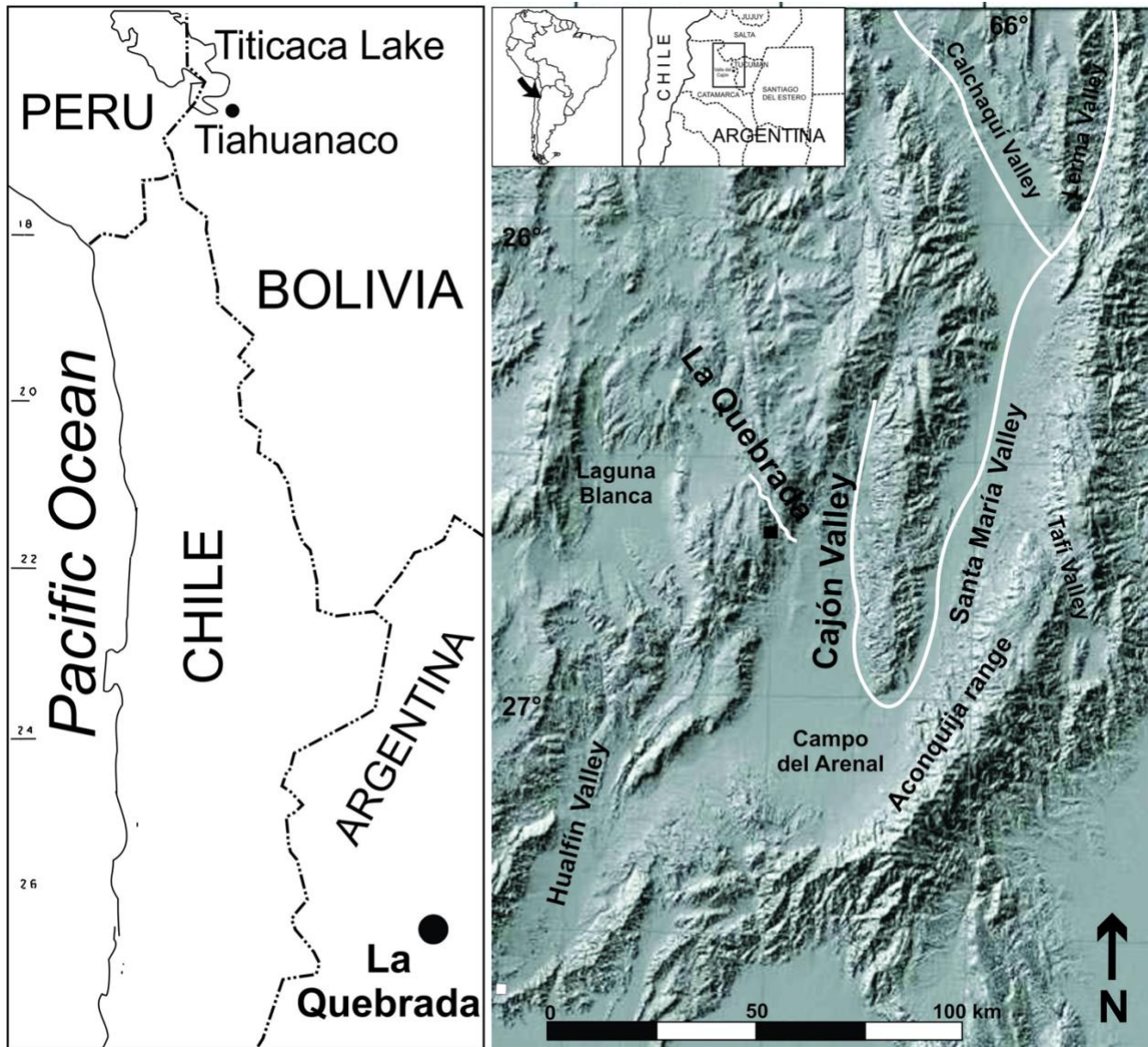
**Supplementary Figure S1.** Phylogenetic tree of Southern Cajón Valley HVR-1 haplotypes and other ancient CA and SA populations. CA: Central Andes, SA: South Andes, NWA:



Northwestern Argentina, ARC: Archaic, FP: Formative Period, RDP: Regional Developments Period, INC: Inca; EH: Early Horizon, EIP: Early Intermediate Period, MH: Middle Horizon, LIP: Late Intermediate Period, LH: Late Horizon, RGDN: Rio Grande de Nasca drainage.

**Supplementary Figure S2.** Phylogenetic tree of Southern Cajón Valley HVR-1 haplotypes and contemporary CA and SA populations. CA: Central Andes, SA: South Andes, NWA: Northwestern Argentina.

Figure 1.



**Figure 2.**

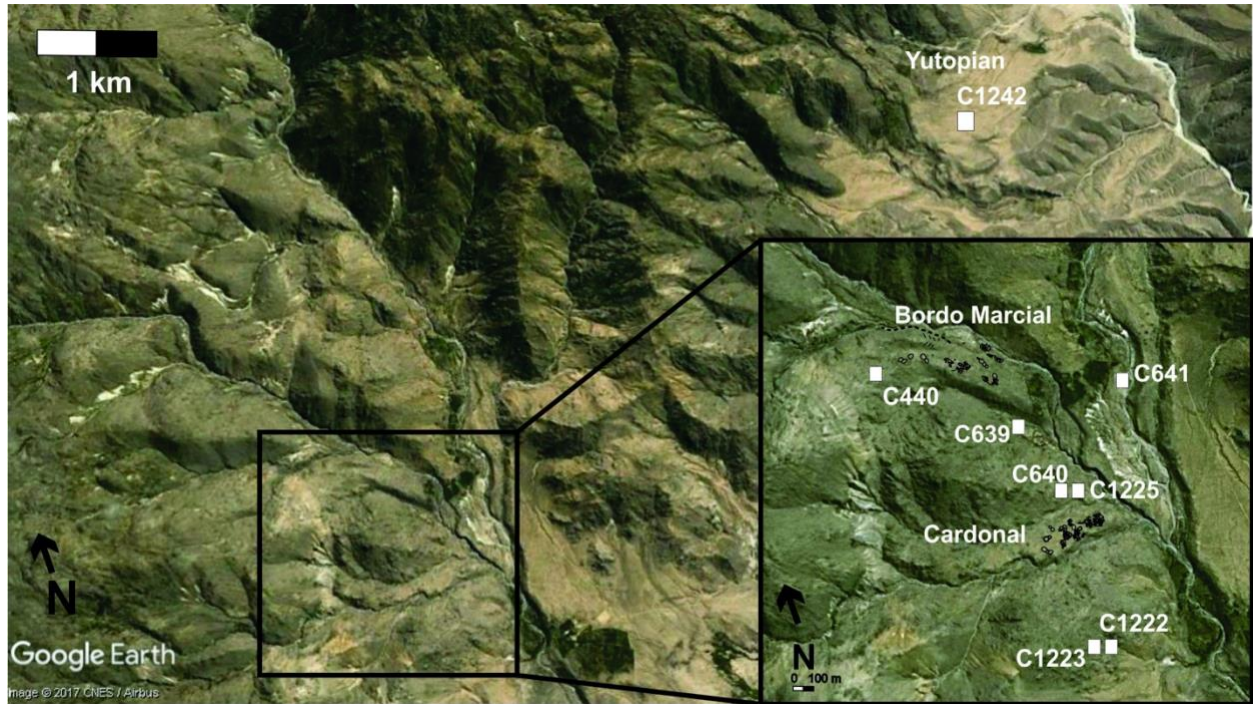


Figure 3.

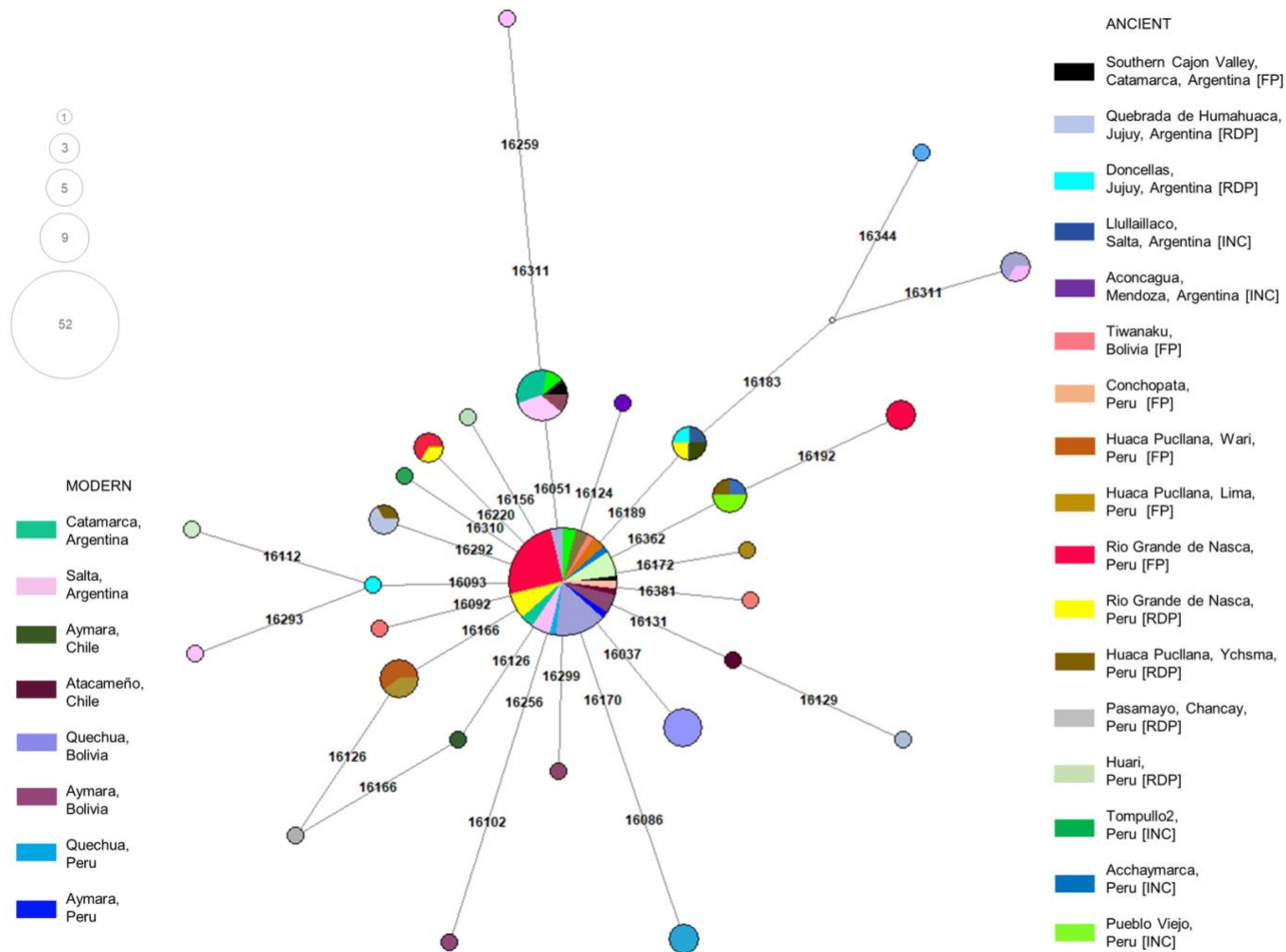
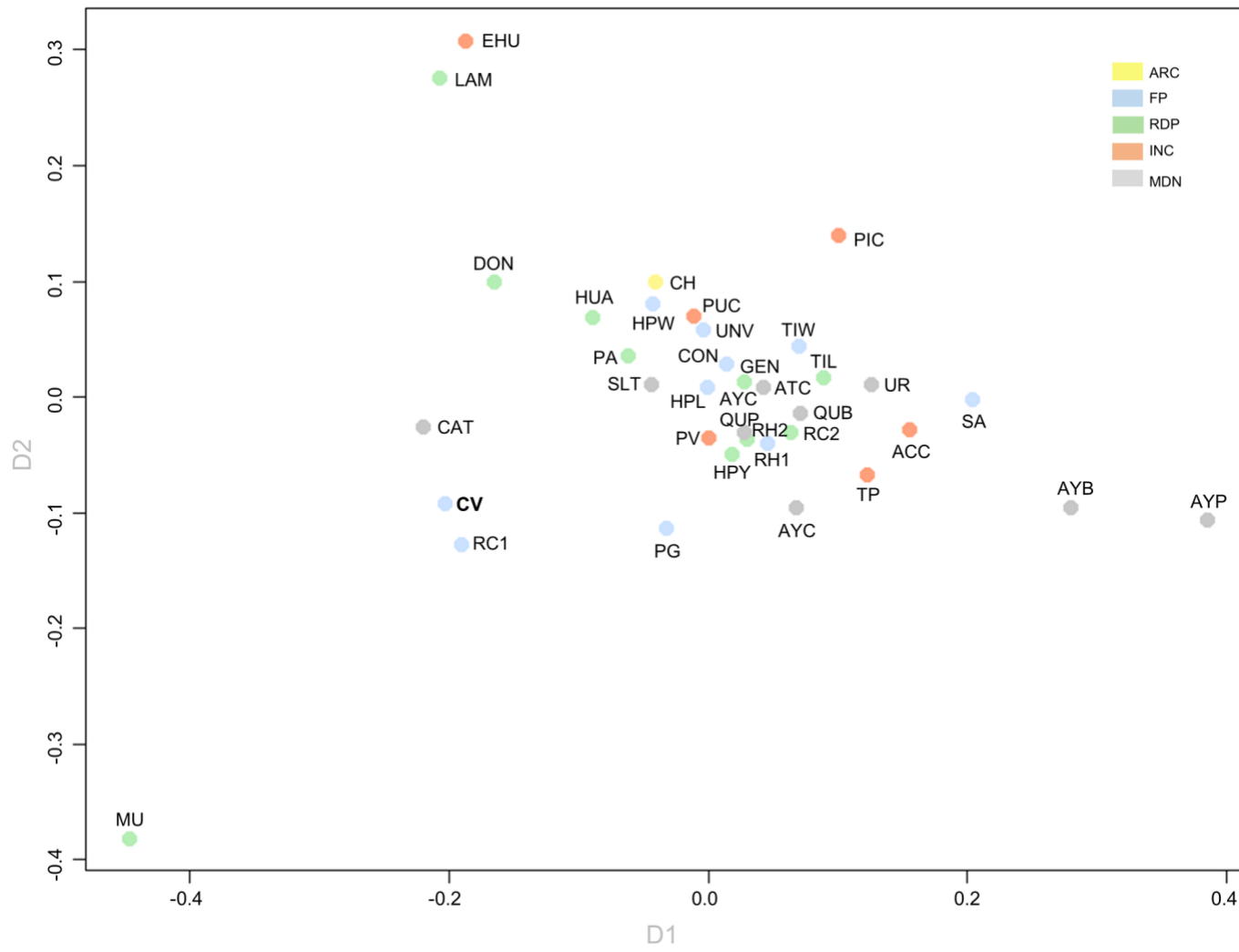
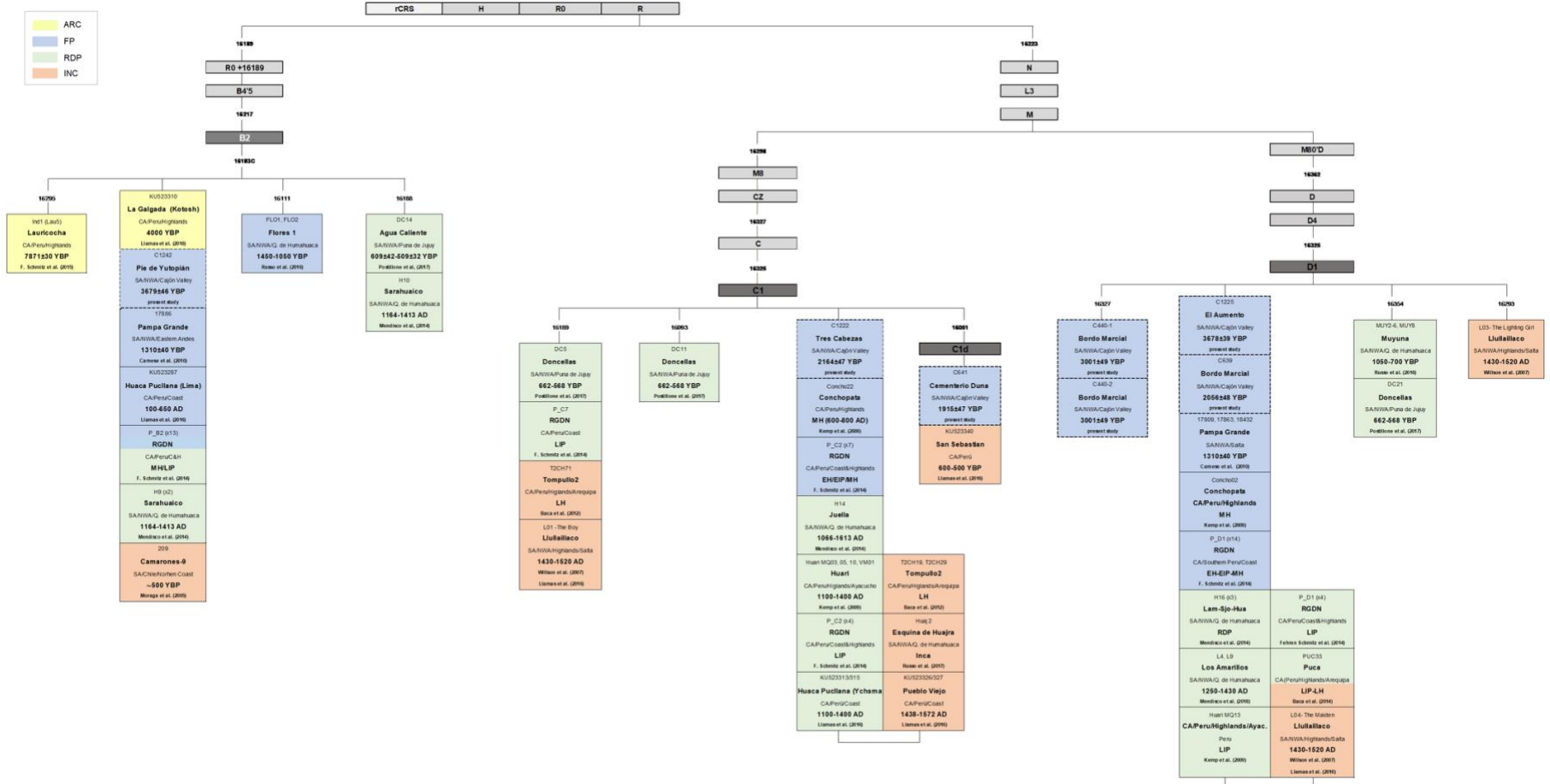




Figure 5.



Supplementary Figure S1.



Supplementary Figure S2.

