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Supplementary Materials for

Insights into the genetic histories and lifeways of Machu Picchu's occupants

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Supplementary Text

Abstract in Spanish

Resumen: Machu Picchu, la hacienda real del Emperador Inca Pachacuti (1420- 1532 CE.), tenía una población permanente de *yanacona*s, individuos de diferentes grupos étnicos traídos para servir a la familia real y la élite Inca por vida, que residían en el sitio. Poco se sabía de estos individuos que al morir eran sepultados en los alrededores del sitio y como se relacionaban a los habitantes de la capital Inca, Cusco. Hemos generado datos de todo el genoma de 34 individuos que fueron enterrados en Machu Picchu, y 34 individuos de varios sitios en el Cusco. Nosotros hemos contextualizado estos datos con estudios arqueológicos y fuentes históricas. El resultado del análisis muestra que la población de los yanacona de Machu Picchu es altamente heterogénea, con individuos que exhiben ancestros genéticos asociados con grupos de muchas regiones del imperio Inca y la Amazonía. Estos resultados sugieren una comunidad diversa en la cual los miembros con diferentes historia y genéticas vivían, reproducían y eran enterrados juntos.

Ethics Statement

The human remains discussed in this paper are considered the cultural heritage of Peru. This project has been part of the research effort envisioned by the MOU between Yale and the Universidad Nacional de San Antonio Abad del Cusco (UNSAAC) associated with the return of all Machu Picchu collections from the 1912 Yale Peruvian Scientific Expedition as described by Salazar and Burger (*95*). This agreement included a commitment to continued research on Machu Picchu and Inca culture in general, as well as community education via displays at the Museo Machu Picchu, an institution administered by UNSAAC. The remains of all individuals from Machu Picchu are curated at this museum. Samples for analysis were exported in accordance with the Ley General del Patrimonio Cultural de la Nación (Law No. 28296) in collaboration and with the permission of the local and national Peruvian Ministry of Culture (Oficio N260-2017-SR-UNSAAC; RD000016-2017-DGM-VMPCIC-MC; RV026-2018-VMPCIC-MC). Over the 11 years since the signing of the MOU, the results and insights produced by investigations of the collections have been continuously communicated by LS, RB, and LFS to, and discussed with the community, general public and university community in Cusco (including the final results before publication), and elsewhere in Peru through conference talks, publications, workshops, and exhibits. These efforts have fostered international collaboration among academics and university students from the United States and Peru, and local community members, as well as producing a series of academic publications (*25*, *27*, *95*–*100*). We plan to continue our work with local leaders and collaborators and hope to generate further educational outreach resources to support our work with local public and private schools in and around Cusco. Our plans also include multilingual additions to the permanent exhibitions at the Museo Machu Picchu with a new exposition focusing on the DNA research discussed in this article. This will allow the efficient and accessible dissemination of the new insights from the ancient genetic research to the descendants of the community that lived at this site as well as for the people of Peru outside of Cusco and broader audiences from many Latin American and other nations. Salazar, Fehren-Schmitz and Burger also plan to organize a workshop in Cusco in August, 2023 for the students and faculty at UNSAAC presenting the principles, accomplishments, and potential of DNA research in the Andes.

Radiocarbon Dating

This study presents 41 AMS radiocarbon measurements obtained from human skeletal remains from 10 archaeological sites in the Inca heartland (Table S1b). These measurements add to a growing corpus of absolute dates from the Inca occupation in the Cuzco region (*101*). In recent years, scholars have questioned the historicist frameworks (*102*) of Inca chronology presented by John Howland Rowe that wase based on often contradictory, or unreliable, colonial period documents (*101*, *103–105*) . As an alternative, archaeologists are increasingly relying on radiocarbon dating to develop chronological models that are independent of the biases of colonial sources (*106–108*).

A challenge for interpreting radiocarbon dates in South America centers on determining whether the Northern Hemisphere (IntCal20; (*109*)) or Southern Hemisphere (SHCal20; (*110*)) curve is the most appropriate for calibration (*47*, *108*, *111*). This is because there is uncertainty regarding the degree of atmospheric mixing from the Northern Hemisphere in different parts of the continent due to the South American Southern Monsoon (*112*). As a result, air and moisture from the Northern Hemisphere reaches the eastern Andes, which includes the Cuzco region.

To address this ambiguity, some scholars have employed a mixed calibration curve that takes inputs from both IntCal and SHCal (*110*, *112*). This type of calibration has been previously utilized at Machu Picchu, as well as Inca sites in the northern Andes (*25*, *47*, *113*). All dates discussed in the main text of our paper are based on calibrations using this mixed curve. However, because there is still uncertainty regarding calibration, we also present dates calibrated with SHCal20, IntCal20, and the mixed curve in Table S1B and Figure S1A for comparative purposes (*108*). Comparison of the three types of calibration shows subtle differences, with IntCal20 *generally* producing slightly earlier dates than SHCal20 and the mixed curve at the 68.3% and 95.4% confidence intervals. However, these differences are significant given the relatively short duration of the Inca Empire.

Radiocarbon Dating Machu Picchu

There are 26 radiocarbon dates from 26 different individuals from Machu Picchu. Radiocarbon measurements range between 550±20 BP and 345±20 BP and when calibrated span much of the 15th through early 16th century CE (*25*). For the present study, we reanalyzed the 26 Machu Picchu dates using a single -phase model (*47*) to produce a more robust absolute chronology. The command input for OxCal v. 4.4 was as follows:

```
Plot()
 {
  Curve("IntCal20","IntCal20.14c");
  Curve("SHCal20","SHCal20.14c");
  Mix_Curve("Mixed","IntCal20","SHCal20",U(0,100));
  Outlier_Model("General",T(5),U(0,4),"t");
  Sequence()
   {
    Boundary("S");
    Phase("Inca")
```

```
   {
   R_Date("UCIAMS-222473", 550,20){Outlier(0.05);};
   R_Date("UCIAMS-222472", 540,20){Outlier(0.05);};
   R_Date("UCIAMS-222474", 540,15){Outlier(0.05);};
   R_Date("UCIAMS-226319", 525,20){Outlier(0.05);};
   R_Date("UCIAMS-226330", 515,20){Outlier(0.05);};
   R_Date("UCIAMS-226318", 510,20){Outlier(0.05);};
   R_Date("UCIAMS-222465", 485,15){Outlier(0.05);};
   R_Date("UCIAMS-226321", 475,20){Outlier(0.05);};
   R_Date("UCIAMS-226332", 475,20){Outlier(0.05);};
   R_Date("UCIAMS-226329", 470,20){Outlier(0.05);};
   R_Date("UCIAMS-222475", 455,20){Outlier(0.05);};
   R_Date("UCIAMS-222471", 450,20){Outlier(0.05);};
   R_Date("UCIAMS-226316", 440,20){Outlier(0.05);};
   R_Date("UCIAMS-222467", 425,20){Outlier(0.05);};
   R_Date("UCIAMS-226331", 425,20){Outlier(0.05);};
   R_Date("UCIAMS-226317", 420,20){Outlier(0.05);};
   R_Date("UCIAMS-226322", 415,25){Outlier(0.05);};
   R_Date("UCIAMS-226325", 415,20){Outlier(0.05);};
   R_Date("UCIAMS-226328", 415,20){Outlier(0.05);};
   R_Date("UCIAMS-222466", 405,15){Outlier(0.05);};
   R_Date("UCIAMS-226324", 400,20){Outlier(0.05);};
   R_Date("UCIAMS-222469", 395,20){Outlier(0.05);};
   R_Date("UCIAMS-222470", 390,15){Outlier(0.05);};
   R_Date("UCIAMS-222468", 385,20){Outlier(0.05);};
   R_Date("UCIAMS-226323", 375,20){Outlier(0.05);};
   R_Date("UCIAMS-226320", 345,20){Outlier(0.05);};
};
  Boundary("E");
  };
  };
```

The results of this analysis (Table S1C and Figure S1B) suggest a start date of 1400-1435 CE and an end date of 1470-1520 CE at the 95.4% confidence interval (or 1410-1426 CE [start] to 1480-1507 CE [end] at the 68.3% confidence interval). It should be noted that the latest date in our model (UCIAMS-22632) may be an outlier and had a low agreement index (29.9). Removal of this outlier changed the chronological model only slightly (1401-1436 CE [start] to 1468-1511 [end] 95.4% CI). These results reinforce the conclusions originally reached by Burger et al. (*25*) that suggest that people began to be buried at Machu Picchu by c. 1420 CE. The revised chronology of Machu Picchu is also supported by recently published radiocarbon dates on charcoal from Machu Picchu (n=3) and the nearby satellite sites of Chachabamba (n=5) and Choquesuysuy (n=3) (*113*).

The earliest Machu Picchu dates indicate that the site was occupied at least 20-30 years earlier than textual sources, which place Pachacuti's ascension to power at 1438 CE (*25*). These early dates fit within recent radiocarbon dates from different parts of Tawantinsuyu that suggest that Inca expansion occurred earlier than historicist models would imply (*2*, *101*, *47*, *108*, *114*).

Significantly, the chronological model also indicates that the latest burials at Machu Picchu date to c. 1520 CE and predate the Spanish conquest (1532/1533 CE). The radiocarbon data is supported by the near absence of Colonial Period material culture at the site. There are no examples of artifacts of Spanish origin such as nails, metal hinges, and ceramic tiles, objects that are common in the early Colonial Period occupation at nearby Vilcabamba (c. 1536-1572 CE) (*115*). Furthermore, a study of the animal bones from Machu Picchu did not find any European introduced fauna (*28*).

Site Description & Individuals Studied

The human remains discussed in this article come from burial contexts from several sites distributed throughout the Inca heartland of the Cusco region. This region consists of the imperial capital of Cusco, an urban center located at approximately 3450 m asl. While there is a long history of settlement at Cusco, it was apparently transformed into an imperial capital in the early 15th century. Because the site was heavily impacted by the Spanish conquest, as well as subsequent damage, our understanding of the layout of Cuzco is based on a combination of Colonial Period documents and archaeological data. During the Late Horizon, Inca Cuzco was characterized by palaces, temples, plazas, and elite residences, often built from cut stone ashlar masonry. In this study human remains were sampled from burials at San Sebastian, Kusicancha, Casa Concha, Sacsahuaman, and Qotakalli. The immediate region surrounding Cuzco consisted of a wide variety of different types of settlement including royal estates located along the Urubamba River (Machu Picchu, Torontoy, and Ollantaytambo). Recent synthesis of existing data demonstrates that there was a great deal of heterogeneity in terms of Inca mortuary practices in the Cuzco region (*116*).

Machu Picchu

During the 1912 archaeological investigations of the Yale Peruvian Scientific Expedition, George Eaton and Ellwood Erdis identified and excavated 107 burial caves on the ridges of Machu Picchu concentrating in four clusters referred to as Cemeteries 1, 2, 3, and 4 (Fig. 1b) (*20*, *26*, *117*). Most of the burials were located along the margins of the palace complex. Cemetery 1 is situated among the boulders to the northeast of the architectural core. Cemetery 2 is found on the slopes to the east and Cemetery 3 was located along the northern slopes of Machu Picchu mountain to the south (*117*). Additional burials have been recovered on the western terraces and are referred to here as Cemetery 4 (*21*, *23*). The burial caves and niches mostly contained commingled human skeletal remains of several individuals in varying states of preservation and few grave goods. Morphological assessments allowed us to assign the human remains to 177 distinct individuals (*20*, *26*). The individuals have been studied for intentional cranial deformation types (*20*), cranial morphology (*20*), strontium ^{87}Sr/^{86}Sr (*12*), and other stable isotopes (*18*, *19*). We were able to obtain tooth and bone samples suitable for ancient DNA analyses from 82 of these individuals, of which 34 yielded DNA sufficient for genome wide DNA analysis. For more details on the site refer to the main text. The skeletons studied here were part of the Bingham collection at the Peabody Museum of Natural History and all have been returned by Yale University to the Museo Machu Picchu in Cusco, Peru under the administration of UNSAAC.

Ollantaytambo

Ollantaytambo was a royal estate established in the Urubamba Valley by Pachacuti approximately 60 km to the north of Cusco. This large Inca site includes palaces, religious and defensive structures, storehouses, roads, bridges, fountains, and elaborate terracing. The residential area consists of enclosed compounds each of which have several one-room buildings surrounding an open patio (*118*). The burials investigated in this study, however, date much earlier than Pachacuti's reign. There was no available information on the exact contexts and location of the individual's burials. Two radiocarbon measurements yielded calibrated dates of 1034-1160 CE and 1280-1388 CE (Table S1b). There is a poorly understood Late Intermediate Period occupation at Ollantaytambo as demonstrated by the presence of the Killke pottery style (*119*) and the extant radiocarbon dates suggest that the individuals might have been associated with this Late Intermediate Period occupation of Ollantaytambo. The individuals sampled for this study are all curated at the Museo Inka, Cusco.

Torontoy

Torontoy is located at 2400 masl on the right bank of the Urubamba drainage approximately 83 km northwest of the city of Cusco. It is now situated within the Machu Picchu Historic Sanctuary (Figure 1). It is characterized by classic Inca stone architecture including trapezoidal doors and niches, tightly fitted masonry, patios, multi-room compounds, and a stone bath chamber. It has been investigated by archaeologists of the Peruvian Ministry of Culture from 2007-2020. The burials were found during the excavation of these structures. The genomic data of the three individuals reported here was first published in Nakatsuka et al. (*34*). Two of these burials were dated to 1442-1498 CE and 1427-1480 CE respectively and are congruent with the presence of imperial Inca architecture (Table S1). The samples of the individuals were suggested for sampling and provided by the Peruvian Ministry of Culture, Cusco, without further details on the specific burial context.

San Sebastian

San Sebastian is an area that has been engulfed by the urban expansion of Cusco. It is located 1.3 km north of the Plaza Mayor. The Inca royal highway (known to the Incas as the *capac ñan*) and a major stone- lined Inca canal traverses the archaeological zone, which was heavily occupied in Inca times. Recent excavations have confirmed the presence of important Killke, Inca and Colonial occupations as well as possible evidence for the production of Inca ceramics (*120*). Inca burials have been uncovered in association with residential compounds there. Radiocarbon dating of burials (n=3) from these contexts range from 1300-1400 CE to c. 1440-1500 CE (Table S1?). The former dates correspond with current estimates for the age of Killke style pottery, while the latter corresponds with the dates associated with Imperial Inca. The remains of these individuals were part of the Yale Peabody Museum Bingham – Machu Picchu collections, and there was no context information besides the site name provided. The individuals have been repatriated to Peru.

Kusicancha

Kusicancha is situated in the historic center of Cusco across from the Temple of the Sun (Coricancha). Protected from development for over half a century by army barracks, archaeological investigations by the National Institute of Culture unearthed a complex of Inca residences organized along a grid and built using the classic elements of Inca masonry.

Numerous burials were found in and around the houses, as well as evidence of cooking and other secular activities (*121*). The two radiocarbon dates from individuals from Kusicancha studied here suggest that the site was occupied in the 15th and perhaps early 16th century. The samples of the individuals were suggested and provided by the Peruvian Ministry of Culture, Cusco, without further details on the specific burial context in Kusicancha.

Qotakalli

Qotakalli is a small site on a bluff overlooking the banks of the Watanay River. It is located near the Cusco airport in the SE sector of the expanding urban center, roughly 4.5 km from the Plaza Mayor. The dominant occupation of the site was Inca but remains dating to pre-Inca times were leveled to permit the construction of the Inca center. Investigations by Peruvian government archaeologists unearthed 84 rectangular structures organized in two rows flanking a central road. The dead were buried in and around their houses and 37 burials were documented. One of the burials in this study was radiocarbon dated to 1414-1450 CE and a second to 1470-1630. This latter date has a 61.1% probability of falling 1540-1630, which corresponds to the early Colonial Period. If this is the case it would support other archaeological evidence that suggest that Qotakalli was occupied during the Colonial period but at a reduced scale (*122*). The samples of the individuals were suggested and provided by the Peruvian Ministry of Culture, Cusco, without further details on the specific burial context in Kusicancha.

Sacsahuaman

Sacsahuaman is a large site on a steep hill overlooking the city of Cuzco. The site had a long history of use during the Early Intermediate Period, Middle Horizon and Late Intermediate Period, but the major constructions date to Inca times. Famous for its massive megalithic retaining walls, Sacsahuaman has sometimes been assumed to be a fortress, but there is evidence for its use as a venue for Inca religious and civic ceremonies, as well as large-scale storage of military and other items. Excavations by Luis Valcárcel in 1933-34 (*123*, *124*) and more recent excavations by representatives of the Peruvian government in 1999 and 2001 unearthed numerous commoner and high status Inca burials, with a high proportion of probable elite females (*2*, *54*, *124–126*). Our study produced a single radiocarbon measurement from one of these burials that calibrates to 1433-1480 CE. The individuals sampled for this study are all curated at the Museo Inka, Cusco. There was no available information on the exact contexts and location of the individual's burials.

Casa Concha

Casa Concha is a colonial residence built above an Inca royal palace. Located close to Cusco's Plaza Mayor, Casa Concha was restored by the INC in collaboration with UNSAAC. Today it is the site of the Museo Machu Picchu where the artifacts from Machu Picchu recovered by the 1912 Yale Peruvian Scientific Expedition are housed. During the restoration of the complex, fine Inca masonry was uncovered along with Inca burials, the most important of which featured a large Inca vessel holding the deceased and associated with a set of Inca miniature vessels. This individual (CCA-7-2) was radiocarbon dated to 1417-1450 CE. The other individual studied here was found adjacent to the aforementioned individual.

Kanamarka

Kanamarka is a small site located in the province of Espinar, District of Alto Pichigua roughly 145 km south of Cusco. At 3950 masl, Kanamarka is in the high *puna* grasslands above the agricultural zone. It has Inca style masonry and buildings that are rectangular and circular in form. An elite female Inca burial was uncovered at Kanamarka in 2004, together with 37 other burials (*127*). A second burial sector was found surrounding an early colonial church, estimated to have only been in use for approximately 50 years (1532 to 1580 CE) (*128*), which is supported by two radiocarbon dates of 1460-1625 CE and 1497-1636 CE. The individuals investigated in this study were suggested and provided by the Peruvian Ministry of Culture, Cusco and come from both burial sectors, however without further details on the specific burial context.

Genetic relationships between newly sampled individuals and various Prehispanic and modern-day groups in- and around the Andes

Y chromosome and mitochondrial DNA analyses

We were able to determine the Y-chromosomal haplogroup to at least some extend for 22 of the 27 biologically male individuals. For 17 of these the Y-chromosomal coverage allows us to further determine that their haplogroup falls into the Q1b1a1a (M3) haplogroup cluster which is dominant in South America (*129*), while for the remaining 5 we can confirm that they fall into the broader Q1b clade (Table S1). Due to the generally limited and heterogenous y-chromosomal coverage we obtained we urge not to over emphasize on the determined terminal haplogroup here.

To identify the mitochondrial haplotypes of the individuals, we manually analyzed each variant as described in Llamas et al. (*73*). All mitochondrial reads mapped to the rCRS using BWA were visualized in Geneious v7.1.3 (Biomatters; available from https://www.geneious.com/) for each sample. Initially, SNPs were called in Geneious for all polymorphisms with minimum coverage 5 and a minimum variant frequency 0.8. The assembly and the resulting list of SNPs were verified manually and compared to SNPs reported at phylotree.org (mtDNA tree Build 17 [18 Feb 2016] (*130*). We excluded common indels and mutation hotspots at nucleotide positions 309.1C(C), 315.1C,AC indels at 515–522, 16182C, 16183C, 16193.1C(C), and C16519T (*131*) and embedded the consensus mitochondrial genomes in the existing mitochondrial tree (mtDNA tree Build 17 [18 Feb 2016]) using the online tool HaploGrep2 (*132*) to determine the haplotypes. Most mitochondrial linages observed in this study are found throughout the Central Andes and in adjacent Amazonian populations. MP3a, MP4d, MP55 exhibit haplotypes (C1b2, B2b+152, C1b23) that so far have been only reported from groups living in what is today Peru's North Western Amazonia region, or Ecuador (Wayku, Loja)(*133*). MP31a exhibits a haplotype that is considered to be restricted to modern day Ecuador in its distribution (*134*). MP27 and MP71 exhibit B2ai haplotypes that haven been reported for pre-colonial and modern day groups from Bolivia (*135*).

Genomic Diversity Estimates

The basal working hypothesis of this project is that Machu Picchu was a royal estate, and that the individuals buried there were retainers of diverse geographic origin and/or genetic ancestry. Several Inca relocation policies are known but those affecting individuals rather than larger groups are believed to be more relevant to the situation at Machu Picchu (*17*, *136*):

- **Acllacona**, chosen women, removed from their family unit at young age and trained to weave and perform religious and ceremonial duties, and to be given as wives to the families in Cusco and those subjects being rewarded for their service.
- **Yanacona**, males that were similarly separated from their homes at a young age and permanently assigned to state or aristocratic service.
- **Camayoq,** skilled specialists designated by the Inca state to carry out specific activities such as metallurgy and the reading of quipus. According to Rowe (*11*), it was possible for a Camayoq to also be a yanacona**.**
- **Mitmakuna**, groups of people permanently resettled from one province to another for purposes of political control, economic development and cultural homogenization.

If the people buried at Machu Picchu proved to be of diverse origins and ancestries, and not from a local group or groups, , we would hypothesize that they were yanacona and acllacona and expect the genomic diversity observed to exceed the average degree of genetic diversity observed for other groups. To test this hypothesis we performed conditional heterozygosity analyses for the Machu Picchu and Cusco groups reported here, and previously reported genomic data from several Prehispanic and modern-day Andean and South American groups from rural and urban contexts (*32*, *34*, *46*, *82*, *86*), using POPSTATS (*86*). Conditional heterozygosity is an estimate of genetic diversity in a group obtained by sampling a random allele from each of two randomly chosen individuals at a known panel of polymorphisms. To further test if the conditional heterozygosity observed for the Machu Picchu burial population increases over time, we grouped the directly dated individuals based on estimated date from AMS measurements, with each group consisting of individuals with similar median ages, to some extent reflecting the assumed expansion phases of the Inca Empire in Rowe's historical chronology (*102*). The results of this analysis are shown in Figure 2.

We found that no matter if we group all individuals together or separate them by median age based on the AMS dates, Machu Picchu always exhibits rates of variation at polymorphic sites higher than observed for any other pre-Hispanic or modern-day group from the Andes, except for the populations from the contemporary Peruvian capital in Lima. We also observe no significant differences in the diversity estimates for the different expansion phases of the Inca, thus in our sample it appears that diversity at Machu Picchu did not increase over time, as it might have been expected from Rowe's chronology of expansion. This observation is relevant for understanding the history and timing of expansion of the Inca Empire. Traditionally, scholars have relied on a historical model developed by John Rowe (*102*) that hypothesizes that imperial conquests outside of the Cuzco heartland began in 1438 CE under Pachacuti Inca Yupanqui. The Rowe model was based primarily on the Spanish chronicle written by Cabello de Balboa (c. CE 1586). However, in recent years this orthodoxy has been questioned by archaeologists based on a growing corpus of radiocarbon dates in the Inca heartland (*25*, *113*) and in the provinces (*106–108*, *137*) that suggest that Inca expansion was underway earlier than previously thought. The presence of individuals from the north coast of Peru, as well as different parts of the western Amazon at Machu Picchu suggest that these regions were incorporated into Tahuantinsuyu at least two decades earlier, based on radiocarbon dates from Machu Picchu (*25*, *113*). Our findings suggest the need to reassess the traditional history of Inca imperial expansion using archaeological evidence.

In accord with observation made in previous studies (*34*, *138*) we further observe that pre-Hispanic groups from urban contexts, such as the ones from the Inca capital Cusco investigated in this study, and from the Middle Horizon center of Tiahuanaco , exhibit higher rates of variation compared to groups from more rural archaeological contexts, although still less than Machu Picchu. The degree of diversity in Cusco discussed here may not fully reflect the complexity of the urban population of the capital and will have to be reevaluated in the future with a larger sample of specimens from other sections of the city.

To further explore if there might be a gendered process underlying the observed genetic diversity, we also calculated mitochondrial nucleotide diversity (π)(*139*) for Machu Picchu, Cusco, and several pre-Hispanic and modern day South American populations (*50*, *138*, *140–149*) using Arlequin 3.5 (*150*), restricting the analysis to the Hypervariable Region 1 (HVR1) for data availability reasons. The analysis shows that again Machu Picchu as well as the Late Horizon population of Cusco exhibit higher degrees of nucleotide (genetic) diversity than all other groups (Figure S5).

Establishing ancestry and population structure in the Urubamba Valley and Cusco preceding the Imperial Inca period

With the Urubamba Valley being home to Machu Picchu, and nearby Cusco being the capital of the Inca Empire it is necessary to establish the population structure and ancestry of people inhabiting the areas before the Late Horizon in order to investigate change due to Inca policies. The radiocarbon dates obtained from the individuals buried at Ollantaytambo in the Urubamba Valley indicate that some of the burials pre-date Inca expansion (see Table S1). Thus, they represent the ancestral groups inhabiting the area. For Cusco itself we found that two burials from the San Sebastian that date to the Late Intermediate Period (LIP, ~1300-1400 CE), and one to the Late Horizon (LH). Chronologically the San Sebastian individuals seem to precede the Inca expansion phase (*151*, *152*). and since they are the earliest individuals analyzed for Cusco, we use them as reference ancestral population for Cusco in subsequent analyses. However, considering the impossibility to determine if the individuals from San Sebastian are strictly “pre-imperial”, and in general the low number of individuals, we caution that indeed the individuals from San Sebastian might not be representative for the pre-imperial Inca population of Cusco. To investigate the population of Cusco, it would be necessary to obtain genomes from earlier dating individuals. However, since the emphasis of this study is to characterize the genomic diversity of the individuals of Machu Picchu, not Cusco, it is appropriate to compare the Machu Picchu individuals to the San Bastien individuals, to highlight potential differential ancestries.

We first tested if the LIP and LH individuals from San Sebastian are genetically homogenous using f4-statistics of the type f4(Mbuti, X; SanSebastian_LIP, SanSebastian_LH), were X is any population in the 1240k datasets, with the ancient Andean populations reported in Nakatsuka et al (*34*) grouped as suggested by the authors (NorthPeruCoast, NorthPeruHighland, CentralPeruCoast, SouthPeruCoast, SouthPeruHighland, TiticacaBasin, NorthChile) to increase statistical power (Table S2). The test confirms that both groups from a clade, and we combined the San Sebastian individuals into one population (Peru_SanSebastian_combined) for all subsequent analyses.

Next, we tested if Late Intermediate Period populations from the Urubamba Valley and Cusco were genetically homogenous using f4-statistics of the type f4(Mbuti, X; Ollantaytambo_LIP, SanSebastian_combined), with X again being the same populations / groupings as in the test before. The test reveals that both groups do not form a clade, and that the Ollantaytambo individuals share significantly more alleles with ancient individuals from what Nakatsuka et al. defined as SouthPeruHighland. This group includes individuals from the Departments of Ayacucho and Huancavelica, and also the Inca period group Kanamarka1 which is south of Cusco (Table S2).

We then tested if either Ollantaytambo_LIP or SanSebastian_combined form a clade with the geographically close SouthPeruHighlands ancestry group defined by Nakatsuka et al. (*34*) using f4-statistics of the type f4(Mbuti, X; SanSebastian or Ollantaytambo, SouthPeruHighlands). Ollantaytambo forms a clade with SouthPeruHighlands, sharing significantly more alleles with Ollantaytambo, modern Quechua speakers from Peru, and Kanamarka1 than SanSebastian combined.

We further used qpWave to tests if any pairing of Ollantaytambo_LIP, SanSebastian_combined, or PeruSouthHighland indicates genetic homogeneity (Table S2). The results confirm the F4 statistics showing that that Ollantaytambo_LIP and SouthPeruHighland are consistent with 1 wave of ancestry ($p>0.01$ for rank 0), though the support of the model is relatively low ($p= 0.0745$). Neither the combination of SouthPeruHighland and SanSebastian_combined nor Ollantaytambo_LIP and SanSebastian are consistent with 1 wave of ancestry, however the latter model is close to significance ($p = 0.0456$), indicating that the differences in ancestry are subtle.

To further explore what additional ancestry SanSebastian might harbor compared to SouthPeruHighlands we used qpADM to test 2-way admixture models employing the rotating model approach suggested by Harney et al. (*153*), as described in Methods. The 2-way admixture model with the most support ($p= 0.2058$) fits San Sebastian as a mixture of 80±8% ancient PeruSouthHighland ancestry and 20±8% ancestry related to ancient TiticacaBasin populations. However, other significant models indicate a mixture of Andean ancestry sources with TitcacaBasin. Considering the high amount of genetic ancestry shared between all Andean groups, and the relatively recent divergence, it is to be expected that several qpADM models produce significant support, and it is possible that the true sources of the population are missing (*34*, *153*).

Since we do not have any older populations from the Cusco Region we cannot directly test if the introduction of the TitcacaBasin related ancestry was due to a relatively recent migration event or if the genetic differentiation between Cusco and the adjacent SouthHighland and Urubamba regions has deeper chronological roots. We used the software DATES (*154*) which models allele covariance over genetic distance to measure admixture dates to get a better understanding when the admixture event could have occurred. We selected PeruSouthHighland and TiticacaBasin as sources to model SanSebastian's ancestry and ran the software with standard parameters. The analysis indicates that the admixture event happened 295.29 generations (~8,260 years ago) however with an enormous error of 186.102 generations (std. err. = ± ~5211 years, Z= 3.135). The error indicates that the results are highly unreliable, which could also be driven by the fact that each of the groups has less than 10 individuals (number recommended by the authors).

Genomic Ancestry of the Urubamba Valley and Cusco Populations

To explore if the individuals analyzed in this study coming from sites in the Urubamba Valley or the Cusco region are of local ancestry, we computed f4-statistics of the type f4(Mbuti, X; Ollantaytambo_LIP, Urubamba/Cusco) and f4(Mbuti, X; SanSebastian_combined, Urubamba/Cusco), where X is any population in the 1240k or HumOrg dataset, and Cusco/Ollantaytambo are the respective individuals grouped following the qpWave test of genetic homogeneity described above (e.g. Peru_Kanamarka1_LH). Figure S7 summarize the F4 statistics emphasizing on the regional Andean ancestry groups determined by Nakatsuka et al. (*34*) and several modern-day and ancient groups representing other geographic regions in South- and Central America. For the complete overview of computed F4-statisctics refer to Table S6.

We further used qpWave to test if any of the Cusco / Urubamba groups is consistent with 1 wave of ancestry (p>0.05 for rank 0 when paired with either SanSebastian_combined, Ollantaytambo_LIP, any of the regional Andean ancestry clusters determined by Nakatsuka et al., or other non-Andean South Americans. The compiled results using both the full 1240k and the HumOrg dataset can be found in Table S4. In the following we discuss all observation by site, and determined subgroup:

CasaConcha1 (n=1): The F4-statistics (Table S6) show that this individual exhibits an increased affinity for the SouthPeruCoast ancestry group when compared to SanSebastian_combined, however only when using the full 1240k panel, while f4(Mbuti, X; Ollantaytambo, CasaConcha1) indicates that the individual shares more alleles with individuals from the pre-colonial TiticacaBasin. The qpWave test (Table S4), indicates that CasaConcha1 and SanSebastian can be modeled as consistent with 1 wave of ancestry though the significance is relatively low (p=0.0745). Since SanSebastian can be modeled as a mixture of SouthPeruHighand and TiticacaBasin ancestry we explored several 2-way mixture models using qpADM employing the rotating strategy as described above (see Table S5). There is a total of four admixture models that produce significant values (p>0.5), with the two highest p-values supporting a model suggesting admixture between TiticacaBasin (60±14%) and PeruSouthCoast (40±14%) ancestry (p=0.808), or TitcacaBasin (42±15%) and PeruSouthHighand (58±15%) ancestry (p=0.355). The positioning in the F3-outgroup Tree (Figure S7) is closer to other Titicaca Basin groups, while in the PCA (Figure 2) the individual is drifted towards individuals form the SouthPeruCoast. Considering the generally low genetic differentiation between the Andean ancestry groups and the close relatedness between individuals from the Southern Coast and the Southern Highlands, both mixture models could be plausible, and fit with the genetic similarity to San Sebastian as indicated by the qpWave test.

CasaConcha2 (n=1): While both f4-tests (testing cladednes with either Ollantaytambo or SanSebastian) do not produce Z-scores > 3, f4(Mbuti, SouthPeruHighland; SanSebastian, CasaConcha2) is close to significant with a Z=2.943, indicating that this individual shares more alleles with individuals from the SouthHighland ancestry group. The qpWave tests confirm that CasaConcha2 is consistent with 1-wave of ancestry when tested with PeruSouthHighland or Ollantaytambo (p=0.6, p=0.2; Table S4), however not when paired with SanSebastian (p=0.0002). No 2-way qpADM model produced significant results, indicating that the best fit for CasaConcha2 is SouthHighland/Ollantaytambo ancestry.

Kanamarka1 (n=2) & 2 (n=1): F4 statistics for both groups show that they share significantly more alleles with SouthPeruHighland and Ollantaytambo, than SanSebastian does (Figure S8). qpWave indicates that both can be modeled as being genetically homogenous with SouthPeruHighland or Ollantaytambo ancestry (Table S4).

Kanamarka3 (n=1): The f4-tests show that SanSebastian and Ollantaytambo do not from a clade with this biologically female individual labeled Kanamarka3 (Figure S8). When using the geographically more diverse HumOrg dataset, f4(Mbuti, Ecuador; SanSebastian or Ollantaytambo, Kanamarka3) shows that Kanamarka3 shares significantly more alleles with Kichwa speaking individuals from the Ecuadorian lowlands reported by Barbieri et al (*33*). The latter study indicated genetic differences between the populations of the Ecuadorian Amazon and Southern Colombia (NorthAmazon in Barbieri et al), and groups inhabiting the North Western Peruvian Amazonian regions (e.g. Wayku, San Martin). Both the f3-outgroup analysis (Figure S4) and the F4 statistics (Table S6) indicate that the affinity of Kanamarka3 is for NorthAmazon (Kichwa) ancestry and not for Peruvian Amazon ancestry. Using qpADM (Table S5) we model Kanamarka3 as a mixture of 36±8% Ecuadorian (Kichwa) ancestry, and 64±8% ancient PeruNorthCoast ancestry (p= 0.57), however a rivaling model suggesting PeruNorthHighland instead of NorthCoast ancestry also reaches significance (p=0.15). That is not surprising considering that both the NorthCoast and NorthHighland clusters share significant amounts of ancestry.

Kusicancha1 (n=3): F4 statistics indicate that the individuals grouped as Kusicanca1 form a clade with SanSebastian but not with Ollantaytambo (Table S6; Figure S8). This is confirmed by qpWave indicating that Kusicancha1 and SanSebastian can be modeled as consistent with 1-wave of ancestry (Table S4). Like SanSebastian, Kusicancha1 can be modeled as a 2-way mixture between SouthHighland (83±10%) and TiticacaBasin (17±10%) ancestry using qpADM (Table S5).

Kusicancha2 (n=1): The f4-statistics indicate that this biologically female individual shares significantly more alleles with individuals from the ancient NorthPeruHighlands ancestry cluster than individuals from Ollantaytambo or SanSebastian. When computing f4(Mbuti, X; Kusicancha2, NorthPeruHighland) the results indicate that Kusicancha2 does not form a clade with the NorthHighland cluster and is most likely admixed. This is confirmed by all tested qpWave models (Table S4) which show that the individual's ancestry cannot be modeled as consistent with 1-wave of ancestry in any of the tested pairings. To explore potential 2-way admixture models we used qpADM employing the rotating source strategy described before.

While a number of models come up as significant, the highest p-values are observed for 2-way models of admixture between PeruSouthHighland and PeruNorthHighland (51%-49%; ±15%, p=0.572) or PeruSouthCoast and PeruNorthHighland (37%-63%; ± 19%, p=0.509). The errors observed for both models are high, and due to the significant amount of ancestry shared between populations from the SouthernCoast and Highlands it is no possible to determine which is more likely.

Qotakalli1 (n=3): The f4 statistics show that Qotakalli1 is not consistent with Ollantaytambo associated ancestry (Table S8), but with SanSebastian, which is further confirmed by the qpWave (p=0.137, Tab. 2). Like SanSebastian, Qotakali1 can be modeled as a mixture of PeruSouthHighland (83±10%) and TiticacaBasin (17±10%) associated ancestry (p=0.39) using qpADM (Table S5).

Qotakalli2 (n=1): This biologically female individual shares significantly more alleles with NorthPeruHighland than SanSebastian or Ollantaytambo as indicated by F4-statisctics (Figure S7, Table S6). Further, the test f4(Mbuti, X; Qotakali2, NorthPeruHighland) produces no significant statistics, indicating that Qotakalli2 is of NorthHighland ancestry (Table S7). Latter is supported by qpWave modeling Qotakalli2 and NorthHighland as being consistent with one wave of ancestry (p=0.165, Table S4).

Sacsahuaman1 (n=1): This male individual does not form a clade with Ollantaytambo or SanSebastian in the F4 tests (Figure S7, Table S6), and cannot be modeled as consistent with 1-wave of ancestry with any of the populations tested (Table S4). F3outgroup statistics described before indicate that this individual shares most genetic drift with ancient and modern-day individuals from the Titicaca Basin and the Bolivian Andes (Figure S3, Figure S4), however the PCA (Figure 2) places the individual closer to SouthHighland individuals, indicating that it could be of admixed ancestry. Using qpADM we tested several two-way admixture models using the rotating strategy described above. We find support for a model assuming a mixture between SouthPeruHighland (34± 23%) and Titicaca Basin (66±23%) associated ancestry (p=0.561, Table S5), however the error is very high. The significantly higher proportion of Titicaca Basin ancestry could explain why this individual does not form a clade with SanSebastian or other groups that we were able to model as mixtures of SouthHighland and TiticacaBasin ancestry (all having a higher proportion of SouthHighland ancestry, Table S5).

Sacsahuaman2 (n=3): F4 statistics, testing symmetry between Sacsahuman2 and Ollantaytambo or SanSebastian show that the group has a significant attraction to NorthPeruHighland ancestry (Figure S7), however f4(Mbuti, X; Sacsahuaman2, PeruNorthHighland) also shows that Sacsahuaman2 does not form a clade with latter (Table S7). None of the 120 2-way admixture models tested with qpADM using the rotating strategy are a good fit. However, we were however able to model Sacsahuaman2 as a 3-way mixture (p=0.0642) between PeruNorthHighland (31±9%), PeruSouthHighland (44±10%) and TiticacaBasin (25±7%) ancestry (Table S5).

Sacsahuaman3 (n=1): This female shares significantly more alleles with individuals from the Titicaca Basin (Figure S7) when computing f4(Mbuti, X; Ollantaytambo, Sacsahuaman3). When replacing Ollantaytambo with SanSebastian combined in the test, there are no significant results, but the highest f4-scores show increased affection to Titicaca Basin populations. The test f4(Mbuti, X; TiticacaBasin, Sacsahuman3) shows that Sacsahuman3 forms a clade with TiticacaBasin (Table S7), which is confirmed by qpWave modeling both groups to be consistent with 1-wave of ancestry (Table S4), and also consistent with the placement of the individual in the PCA (Figure 2).

Torontoy1 (n=2) and Torontoy2 (n=1): These three individuals from the Urubamba Valley palace site of Torontoy have been reported in Nakatsuka et al. (*34*). The individuals from Torontoy1 are of Peruvian North Coast ancestry, while Torontoy2 is of Titicaca Basin ancestry. Our analysis here confirmed the observations made in the aforementioned paper (Table S6, Figure S7).

In summary, the Late Horizon groups from Cusco studied here exhibit mixtures of diverse Andean ancestries. The two main sources of non-SouthHighland (the geographic region including Cusco) ancestry are TiticacaBasin and NorthHighland associated genetic ancestry. About 82% (14 of 17) of the individuals from urban Cusco exhibit some degree of Titicaca Basin ancestry. While none of the four individuals from Kanamarka located to the south of Cusco exhibit TiticacaBasin ancestry, they can be fitted as consistent with SouthHighland ancestry, or in the case of the female individual Kanamarka3, as an admixture of PeruNorthCoast and Ecuadorian Amazon (Kichwa) ancestry.

Genomic Ancestry of the individuals buried at Machu Picchu

As for the Cusco / Urubamba groups we computed f4-statistics of the type f4(Mbuti, X; Ollantaytambo, MPindividual) and f4(Mbuti, X; Ollantaytambo, MPindividual) to test if any of the individuals buried at Machu Picchu exhibits local ancestry associated with the key regions of this study: the Urubamba Valley (home to Machu Picchu), or Cusco (the nearby capital of the Inca Empire). To not lose any information on the life history of the individuals, and to ensure we can compare the genetic results to isotopic, morphological, and archaeological data we did not group any of the Machu Picchu individuals other than for selected analyses. This limits the statistical power for some analyses presented here, especially for individuals with low-coverage and when using only the SNPs covered by the HumOrg reference panel. Figure S8 summarizes the F4 statistics emphasizing the regional Andean ancestry groups determined by Nakatsuka et al. (*34*) and several modern-day and ancient groups representing other geographic regions in South- and Central America. For the complete overview of computed F4-statisctics refer to Table S6. We also used qpWave to test if any of the individuals can be modeled as genetically homogenous with any of the regional Andean ancestry clusters determined by Nakatsuka et al. 2020, or other non-Andean South Americans. The compiled results using both the full 1240k and the HumOrg dataset can be found in Table S4.

Individuals with NWAmazonian ancestry (n=8)

The f4-tests for 6 biologically female individuals **(MP3a, MP4b, MP4d, MP4e, MP4f, MP61)**, four from the same burial cave, reveal that they share significantly more alleles with North Western Amazonian populations (1240k: Piapocco; HumOrg: San Martin) when compared to either Ollantaytambo or SanSebastian (Figure S8, Table S6), which is consistent with their placement in the PCA (Figure 2), as well as the F3-outgroup analysis (Figure S4). When using the HumOrg dataset that includes both the Piapocco group form South Venezuela, and groups from the North Western Peruvian Amazon (e.g. San Martin, Wayku) the f4-values for the latter are higher in all 6 cases, indicating that this is the best fitting (geographic) ancestry source (Figure 3). The qpWave tests (Table S4) show that all five individuals can be modeled as consistent with 1-wave of ancestry when pairing them with Piapocco (using 1240k), and MP4d also shows a weak fit with Karitiana (p=0.084). When using the HumOrg panel for qpWave, all individuals fit with San Martin (Peruvian northwest Amazonia), however only MP4e also fits with Piapocco. All tests indicate that the individuals are of unadmixed Amazonian ancestry.

We computed f4-statisctics of the type f4(Mbuti, X; MPa, MPb), where MPa and MPb are each one of the six individuals with Amazonian ancestry to test if the individuals share the exact same ancestry (Table S8 - MPvsMP). There are no significant statistics for any pairing with a SNP overlap > 25k SNPs, confirming genetic homogeneity for all five (to the extent of our statistical resolution), which is consistent with the degree of shared genetic drift, leading them to cluster in the F3 heat matrix (Figure S5). To further evaluate which Amazonian population could be the best source for the ancestry of all 6 individuals we computed f4-statistics of the type f4(Mbuti, Amazonia; MPind, Amazonia) where Amazonia are groups representing broader geographic regions of North and Western South America: NorthWestern (Peruvian) Amazon (Wayku, SanMartin, Cocama), NorthAmazon-Kichwa (Ecuador, Inga), NorthEasternAmazon (Arara, Urubu Kapoor), SouthWesternAmazon & Chaco (Karitiana, Xavante, Guarani). The compiled statistics in Table S8 show that in all tests NorthWesternAmazonia seems to be best fit (if SNP overlap > 100k SNPs).

To get a better understanding with which Peruvian Amazonian group the Machu Picchu individuals share most ancestry we computed further statistics using the HO_Omni dataset. While the SNP overlap for this dataset is relatively low (125,320 SNPs), it includes several groups living in the Peruvian Amazon along the Eastern Slopes of the Andes, like the Cashibo, Shipibo, and Ashaninka, with the latter group known to the Inca as Anti or Campa, which also gave the name to the eastern Antisuyu province of the Tahuantinsuyu (*155*). Due to the low amount of SNPs we only included the individuals with the highest coverage: MP3a, MP4b, MP4f, MP61. We computed outgroup-f3 statistics, and plotted heatmaps and constructed a NJ-tree as described under “F3 Statistics”. The tree shows all four individuals grouping in a clade with Ashaninka, Cashibo, and Shipibo, which form a broader clade with NW-Peruvian groups, diverged from other Amazonian groups (Figure S5). The f3-heatmaps also show that the four individuals tested share most genetic drift with these CentralPeruvianAmazon groups (Figure S4). We computed f4-statistics of the type f4(Mbuti, MachuPicchu_Amazon; NW_PeruvianAmazon, Amazon), where MachuPicchu_Amazon is MP3a, MP4f, and MP61 grouped, NW_PeruvianAmazon is either Wayku or Smartin, and Amazon is any other Amazonian group. The tests show that the grouped MP individuals share more alleles with Smartin than with non-Peruvian Amazonian groups like Karitiana, Xavante, or Ecuadorian_Kichwa speakers (f4=-0.00230 to -0.00130; Z= -5.9 to -3.8); however, share less alleles with Smartin than with the Central_PeruAmazon groups: Cashibo (f4 = 0.002, std. err. = 0.0005; Z=4.224), Shipibo (f4 = 0.00047, std. err. = 0.0003; Z=4.825), and Ashaninka (f4 = 0.003, std. err. = 0.0004; Z=4.912). The combined analyses show that the modern-day NW_PeruvianAmazon and Central_PeruvianAmazon groups share most of their ancestry, and that the ancient Machu Picchu individuals share more ancestry with the latter group, especially modern-day Ashaninka people. The Ashaninka communities are geographically closets to Machu Picchu, living in the lowlands of the Departments Junín and Ucayali, north of Machu Picchu and Cusco (Figure 1).

Another individual, **MP51** (adult biologically male), also forms no clade with Ollantaytambo or SanSebastian, and while not significant, all f4-tests indicate a strong affinity for Amazonian, and ancient Caribbean groups, and several of the Machu Picchu individuals discussed before. In the PCA the individual is positioned with individuals from the Central Peruvian Coast. It also is placed together with **MP50** an young adult female individual in the PCA. The f4-tests show no significant break of clade, however, non-significant affinity for the same populations as MP51. Since both the NorthPeruCoast and CentralPeruCoast ancestry clusters (*30*, *33*, *34*) exhibit admixture with Amazonian ancestry we computed f4-statistics of the type f4(Mbuti, X; AnyCoast, MP50 or MP51), were "AnyCoast" is either NorthPeruCoast, CentralPeruCoast, SouthPeruCoast (Table S7 – MP50-51vsCoasts). However, both individuals do not form a clade with any of the three coastal groups, which is also confirmed by the qpWave tests (Table S4). We further computed f4(Mbuti, X; MP50, MP51), which indicates that both form a clade / share the same ancestry (Table S7 – MP50-51vsCoasts). We tested several 2-way mixture models in qpADM using the rotating strategy described before. For MP51, three models find significant support, each modeling the individual as a mixture between SouthPeruCoast and a second Amazonian source (SanMartin, Piapocco, Karitiana). The models considering a NorthWest Amazonian source (SanMartin, Piapocco) produce the highest p-values (Table S5). The model with the highest p-value (p=0.93) suggests that MP51's genome is a mixture of 71±5% SouthCoast and 29±5% SanMartin associated ancestry. There are also several supported models for MP50a. Again, all of them model the ancestry of the individual as a mixture between PeruSouthCoast and a NorthWest or South Amazonian source. The model with the highest p-value (0.246) suggests a mixture of 63±8% SouthCoast ancestry, and 37±8% ancestry associated with Piapocco.

Individuals with SouthAmazonian ancestry (n=2)

The f4-tests (Figure S8) indicate that the biologically female individuals labeled **MP5a** and **MP107b** also exhibit excess allele sharing with Amazonian individuals when compared to Ollantaytambo or SanSebastian (Table S4). However, in contrast to the previously discussed individuals, these individuals seem to have a significant affinity for ancestry observed in individuals from the Southern Amazon (e.g. Karitiana, Xavante) and Gran Chaco (Guarani, Chane)(Table S7).

The qpWave tests (Table S4) show that **MP5a**'s ancestry cannot be modeled as consistent with 1-wave of ancestry with any of the reference populations used, and f4-statistics of the type f4(Mbuti, X; MP5a, Chane or Karitiana) confirm that the individual is not of strict SouthAmazon/Chaco ancestry but shares alleles with Andean populations (Table S7). Using the rotating qpADM approach we find several supported 2-way mixture models indicating SouthPeruHighland, but also NorthChile or TiticacaBasin as one source of ancestry and an Amazonian population as the second source (Table S5). Highest support is found for models assuming a mixture of ~30% SouthPeruHighland ancestry and ~70% either Chane or Karitiana associated ancestry using both the 1240k or HumOrg panel (e.g.: 70±9% Chane, 30±9% SouthPeruHighland, p=0.5). The relatively low genetic differentiation between Andean and also non-Andean groups is the most likely explanation for the multiple possible models observed, and there could be also previously unobserved lineages that influence our observation.

For MP107 the situation is less clear. While the f4-tests reveal that there is an increased affinity for Amazonian populations, especially Karitiana, none of these have Z-scores >3. None of the performed qpWave tests are consistent with 1-wave of ancestry (Table S4). Since some of the observations made for MP5a and MP107b seem to overlap we tested f4(Mbuti, X; MP107, MP5a) which reveals that both individuals form a clade, however the SNP overlap is <100k SNPs, meaning that we could lack statistical power to detect genetic distinctness (Table S7). However, to increase our statistical power we combined both individuals in a group: MachuPicchu_SouthAmazon_Admixed. Using this newly combined grouped we performed additional f4-tests of the type f4(Mbuti, X; Andes, MachuPicchu_SouthAmazon_Admixed) where Andes is either PeruSouthHighlands, TiticacaBasin, or Ollantaytambo. The tests show that MachuPicchu_SouthAmazon_Admixed shares significantly more alleles with several Amazonian, Gran Chaco, or Eastern South American Groups than the Andean groups, with highest positive f4-scores being observed for groups like Chane, Guarani, Karitiana (Table S7). Since all our analyses indicate that the source of Amazonian ancestry in MP5 and MP107 seems to differ from the source that contributed to the ancestry observed for the other Amazonian individuals (e.g. MP4b, MP61), we grouped the latter, and computed f4-statisctics of the type f4(Mbuti, X; MachuPicchu_NW-Amazon, MachuPicchu_SouthAmazon_Admixed) to verify that observation. X in these tests is any of the Amazonian, Gran Chaco, or Eastern South American groups in our dataset (HumOrg). We observe negative f4-values for Peruvian and North West Amazonian groups, and positive f4-values for South Amazonian / Gran Chaco groups like Guarani, Chane, Karitiana, indicating that the grouped individuals MP5 and MP107 share more alleles with latter groups (Table S7). When the individuals are grouped, qpADM shows that there are numerous 2-way mixture models that could explain their ancestry. The models with the highest p-values suggest mixtures between SouthPeruHighland (~40-50%) as Andean source (followed by either NorthChile or TiticacaBasin), and SouthAmazonian/Chaco populations (e.g. Chane, Guarani) as other source (~ 60-50%; Table S6).

All analysis performed here indicate that there is a high certainty that both MP5a and MP107 are of mixed ancestry, with some ancestry deriving from a Southern Andean group, and some from groups inhabiting Amazonia, or the Gran Chaco today. The genetic landscape and population history of Amazonia is complex, and it is at this point impossible to directly identify the potential source group that contributed to the ancestry of the two individuals, however a source ancestral to groups today inhabiting the Southern Amazonian regions, and the Gran Chaco seems to be most likely. Archaeological and ethnohistoric sources both confirm interactions between the Inca and the ancestors of the Chane in the foothills of the Bolivian Andes, which makes our conclusion plausible (*43*).

Individuals with Ecuador / Kichwa associated ancestry (n=4)

The f4-tests indicate that 4 individuals **(MP9b, MP31a, MP63, MP78)** buried at Machu Picchu (3 female, 1 male, not counting the individual Kanamarka3) share significantly more alleles with Ecuadorian (label: Ecuador) and Southern Colombian (Inga) Kichwa speakers than the individuals from Ollantaytambo or SanSebastian (Figure S8). Using qpWave we also determine that none of the individuals are of strict Kichwa-speaker associated ancestry (Table S4). We used the rotating qpADM approach to test several 2-way mixture models for each of the individuals. For MP9b, MP31a, MP63, and MP78 the feasible models with the highest p-values all suggest that the individuals can be modeled as having between 45-57% Ecuadorian Kichwa and Peruvian North Coast associated ancestry comparable to the Kanamarka3 female discussed earlier (Table S5). However, we also observe competing models for each of the individuals that suggest admixture between Ecuadorian Kichwa associated ancestry with ancestry observed for other coastal groups (Central, South) or individuals from the NorthPeruHighlands. We observe that the higher the coverage for the tested individual is the lower is the number of 2-wave mixture models that are supported, which is to be expected, especially when considering the relatively low amount of genetic differentiation between the Andean ancestry clusters. To get a better estimate of admixture proportions we first did f4-statistcis testing pairwise cladality for each of the individuals: f4(Mbuti, X; MPa, MPb), were MPa and MPb are one four of the MP individuals or Kanamarka 3. The tests show that none of the individuals exhibits a significantly increased affinity for another population. However, the SNP overlap for some of the F4-statistics is below 50k SNP (Table S7). We then grouped all individuals and repeated the qpADM analysis using the grouped individuals as target. The resulting supported models confirm the induvial based tests. A model considering a mixture of 46±6% Ecuadorian-Kichwa associated ancestry and 54±6% NorthCoast ancestry produces the highest p-value (p=0.41), however other models considering other Andean sources (NorthHighland, CentralCoast, SouthHighland) are supported as well (Table S5).

There are no published ancient or modern-day genomes from individuals living in the Ecuadorian highlands or along the coast which makes it hard to assess if the observed admixture between PeruNorthCoast ancestry and Kichwa ancestry actually indicates a recent admixture event, or if it reflects the genetic ancestry of an unsampled group somewhere in Ecuador.

Individuals of North and Central Peruvian Coast associated ancestry (n=4)

The F4 tests indicate that three biologically female individuals (**MP33, MP42b, MP53a**) and one biologically male individual **(MP84c)** buried at Machu Picchu share significantly more alleles with individuals from the PeruNorthCoast, and PeruCentralCoast ancestry regions defined by Nakatsuka et al. (*34*). In the case of MP53a we actually observe no significant statistics, just a trend, which can most likely be explained by the low coverage obtained for that individual (~33k SNPs). In the PCA all individuals group with ancient and modern-day individuals from the North- and Central coast. Again, MP53a drifts away, probably due to low coverage. To test if the individuals can be modeled as being of strict coastal ancestry, we computed f4(Mbuti, X; MP, North- or Central- or SouthCoast) and used qpWave to test if any of the individuals can be modeled as homogenous with any of the ancestry clusters. Both F4 (Table S6) and qpWave (Table S4) confirm that MP42b and MP84c are of NorthPeruCoast associated ancestry. For MP33 we observe cladality when compared to both North- and CentralCoast populations, and the qpWave analysis shows that the individual ancestry can be modeled as consistent with 1-wave of ancestry with both coasts, CentralCoast producing the highest p-value (p=0.6672, Table S4). The coverage for MP53a is so low that qpWave actually finds support for a number of Andean populations, with CentralCoast producing the highest p-value (p=0.6981). It is not possible to differentiate between North- and Central coast ancestry for these two individuals. The genetic differentiation between the two regions is in general very low, and the two sampling points defining each ancestry cluster are relatively geographically distant from each other (Figure 1), which could mean that the ancestry observed for MP33, and maybe MP53a, reflects the ancestry of populations that existed between the sampling points. Due to the low coverage, there is no certainty that our suggestions for MP53a's ancestry are correct, we infer North-Central-Coast ancestry from our combined observations made with PCA, F3-outgroup, and non-significant F4 statistics.

Individuals with NorthPeruHighland ancestry (n=1)

The 40–50-year-old biologically male individual labeled MP77a shares significantly more alleles with individuals from the North Peru Highlands than individuals from Ollantaytambo or San Sebastian (Figure S8). We computed f4-statistics of the type f4(Mbuti, X; MP77a, PeruNorthHighland) which indicates that the individual's ancestry is not consistent with being completely of NorthHighland ancestry (Table S7). Using qpADM we can model the individual's ancestry as a mix of 52±15% PeruSouthHighland and 48±15% PeruNorthHighland ancestry (Table S5), comparable to what we observed for the Cusco region individuals grouped in Kusicancha2 and Sacsahuaman2. Again, we have to mention that this does not really allow us to precisely determine the geographic origin of the individual's ancestry, they could either be of recent admixed ancestry, or from a region between the populations used to defined the two geographical ancestry clusters that has not been sampled so far. It is to be expected that there will be a gradient of ancestry between regions and not a clear-cut boarder.

Potential admixture between SouthCoast and NorthChile ancestry (n=1)

The juvenile male individual **MP48** does not produce any significant statistics in the f4-tests, however in both tests using SanSebastian or Ollantaytambo there is a number of populations that the individual seems to have affinity for close to statistical significance (Table S6). qpWave indicates that the individual's ancestry is neither consistent with 1-wave of ancestry when paired with Ollantaytambo nor SanSebastian. Thus, we used qpADM as described above finding highest support both when using the full 1240k or the HumOrg set for a model considering the individual's ancestry to be a mixture of 33% SouthPeruCoast and 63% NorthChile ancestry, however with a large error of ±20% (Table S5).

Individuals with TiticacaBasin and/ or SanSebastian associated Ancestry (n=4)

Individual MP80 clusters with ancient- and modern-day individuals from the Titicaca Basin in the PCA (Fig. 2). The f4-tests reveal that the individual shares significantly more alleles with individuals of TiticacaBasin associated ancestry than individuals from Ollantaytambo and Cusco (San Sebastian). In qpWave the individual can be modeled as consistent with one wave of ancestry when paired with Titicaca (Table S4; Fig. 3), suggesting that the individual is of unadmixed Titicaca Basin associated ancestry.

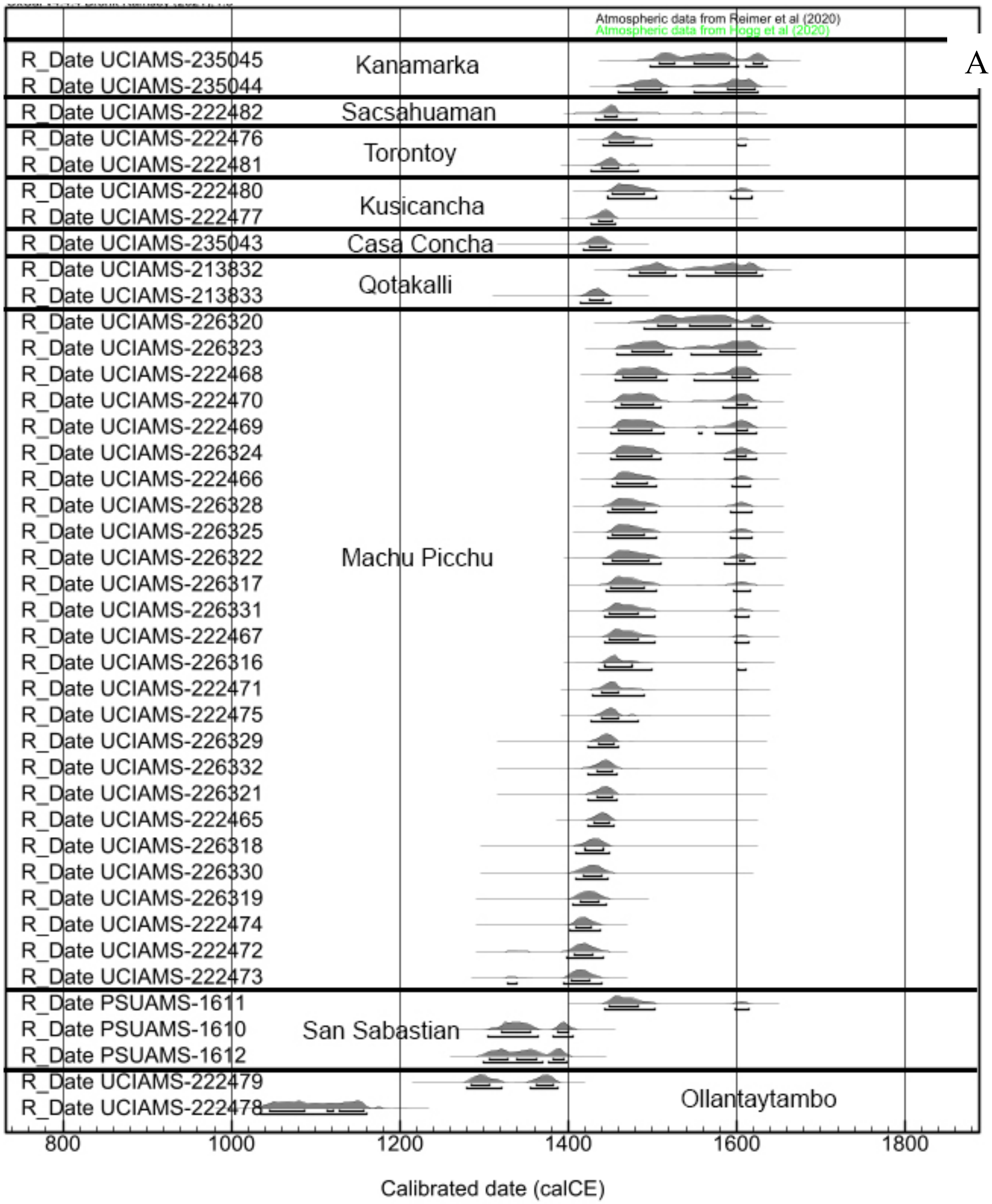
The f4-tests for the biologically male individual **MP4i** indicate that it shares significantly more alleles with Ollantaytambo than SanSebastian, but also more alleles with TiticacaBasin individuals when compared to Ollantaytambo (Figure S8). So, while SanSebastian ancestry seems to be defined by an admixture of SouthHighland and TiticacaBasin ancestry, it does not seem to be a good fit for this individual, which is also supported by the qpWave test showing that MP4i and SanSebastian are not consistent with 1-wave of ancestry (Table S4). Using the rotating qpADM approach we find support for a model assuming MP4i's ancestry to be a mixture between 65%±10 SouthPeruHighland ancestry and 35±10% TiticacaBasin ancestry (Table S5).

MP27 (biologically male) forms a clade with SanSebastian, but not Ollantaytambo (Table S6). The qpWave test indicates that this male individual is consistent with 1-wave of ancestry when paired with SanSebastian. Using qpADM we can model the individual's ancestry to be a mix of 65±19% SouthPeruHighland and 35±19% TiticacaBasin associated ancestry ($p=0.669$). The error is of course very high, which indicates a weakness in the fit of this model, and there are other supported models, however with much lower p-values, that point to admixture of PeruSouthHighland and a number of other (mostly Amazonian) sources.

MP71 is another biologically male individual for which the F4 tests indicate an increased affinity for TiticacaBasin when compared to Ollantaytambo (Figure S8). When tested with SanSebastian, MP71 shares increased affinity with the individual Torontoy2 (Table S6), which we previously determined to be of TiticacaBasin ancestry, indicating that the individual might harbor more TiticacaBasin ancestry than SanSebastian. The PCA places the individual also close with Torontoy2 and other modern-day Titicaca Basin individuals (Figure 2). When computing f4(Mbuti, X; MP71, TiticacaBasin) the test however reveals that MP71 doesn't form a clade with TiticacaBasin (Table S7). Using qpADM we can model the ancestry of the individual as a mix of 71±15% TiticacaBasin and 29±15% SouthPeruHighland associated ancestry.

Individuals of Ollantaytambo-SouthHighland Ancestry (n=6)

The f4-statisctics (Figure S8) indicate that five individuals buried at Machu Picchu, three biological males **MP32, 45a, 55**, and two biological females **MP23** and **42a**, form a clade with Ollantaytambo, but not with SanSebastian. The qpWave tests confirm that the individuals are of SouthHighland ancestry, also represented in Ollantaytambo (Table S4).


A
Atmospheric data from Reimer et al (2020)
Atmospheric data from Hogg et al (2020)
R_Date UCIAMS-235045
R_Date UCIAMS-235044
Kanamarka
R_Date UCIAMS-222482
Sacsahuaman
R_Date UCIAMS-222476
R_Date UCIAMS-222481
Torontoy
R_Date UCIAMS-222480
R_Date UCIAMS-222477
Kusicancha
R_Date UCIAMS-235043
Casa Concha
R_Date UCIAMS-213832
R_Date UCIAMS-213833
Qotakalli
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R_Date UCIAMS-226330
R_Date UCIAMS-226319
R_Date UCIAMS-222474
R_Date UCIAMS-222472
R_Date UCIAMS-222473
Machu Picchu
R_Date PSUAMS-1611
R_Date PSUAMS-1610
R_Date PSUAMS-1612
San Sabastian
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R_Date UCIAMS-222478
Ollantaytambo
800
1000
1200
1400
1600
1800
Calibrated date (calCE)

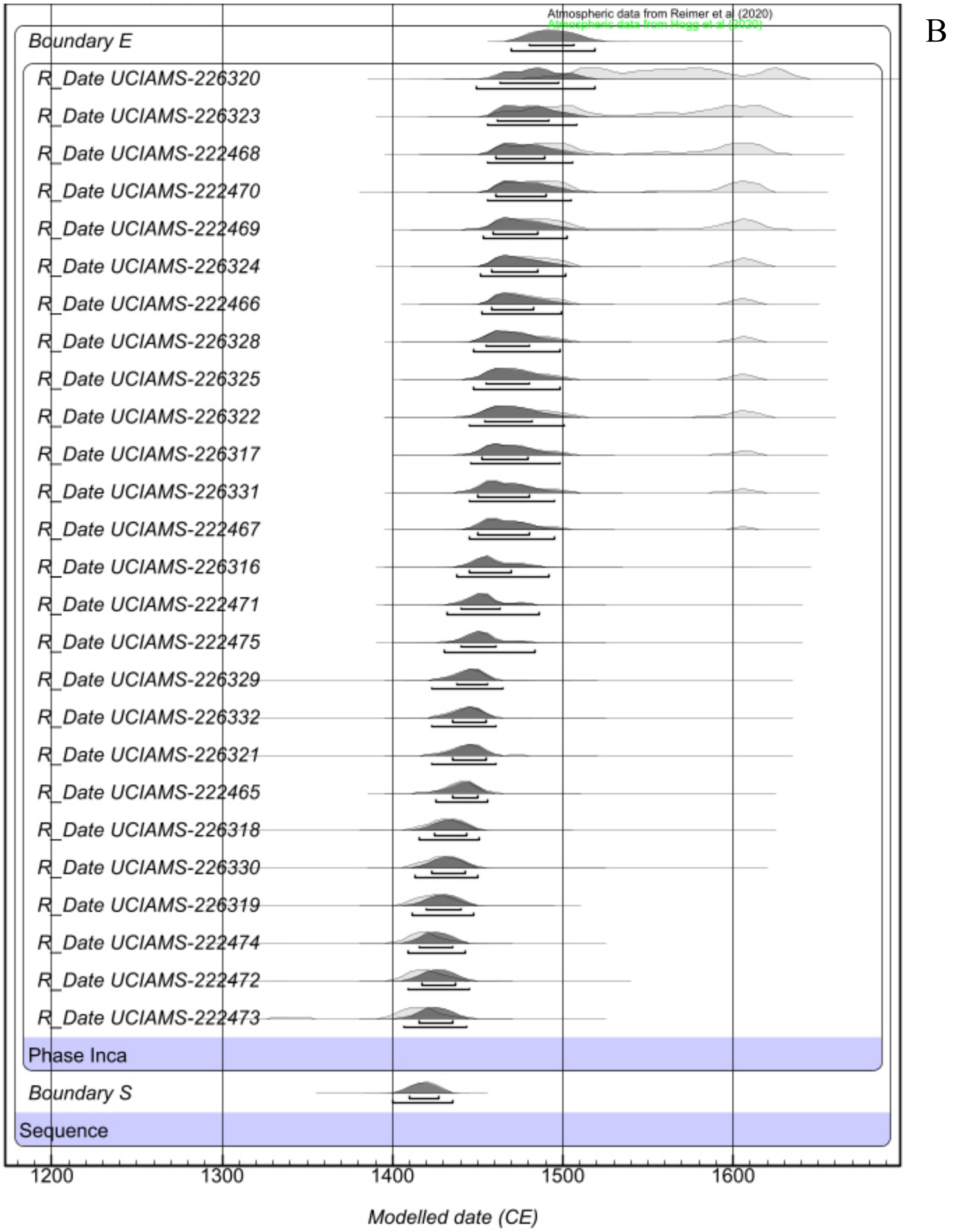
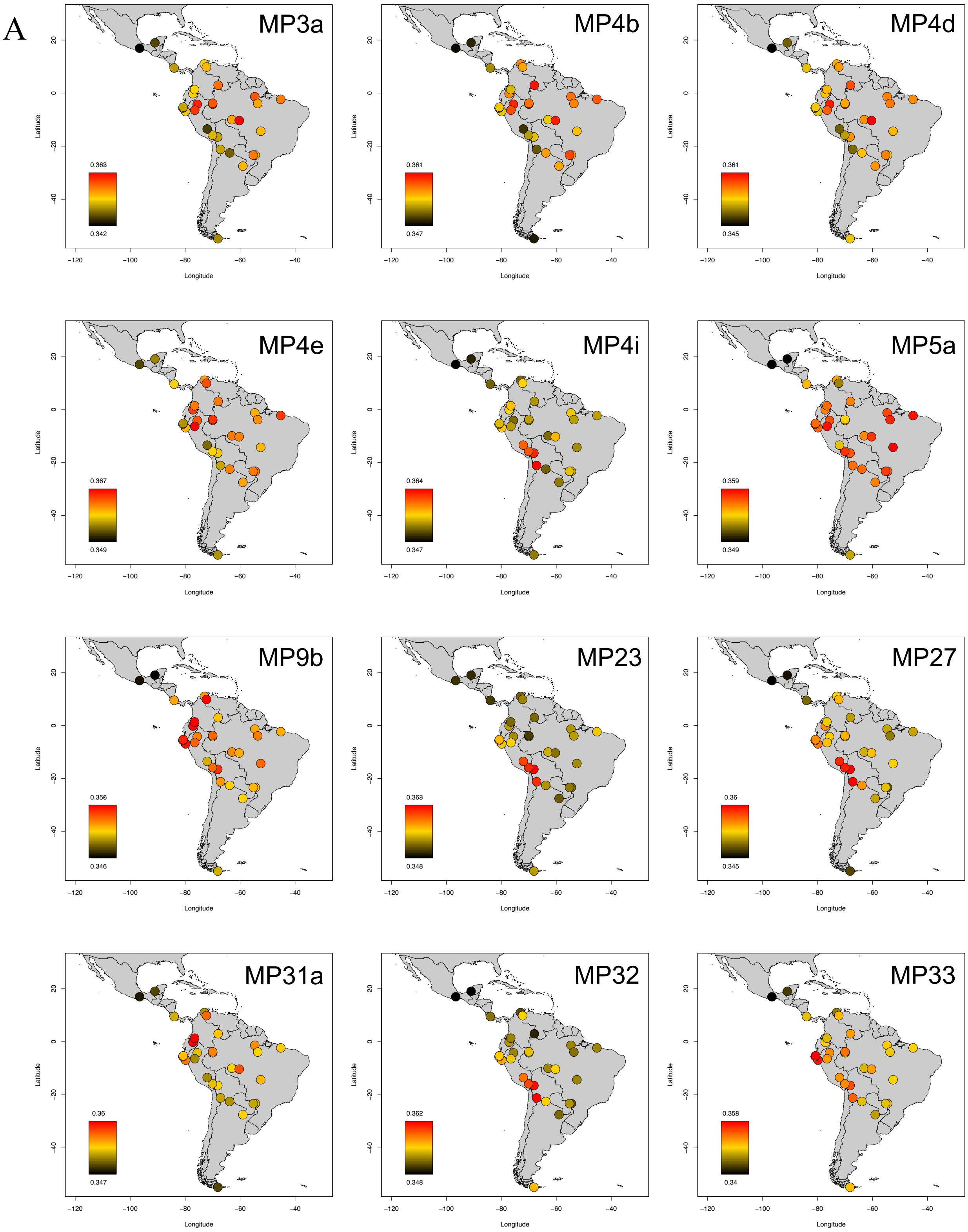


Fig. S1 Calibrated and modelled radiocarbon dates: A. Multiplot of AMS radiocarbon dates discussed in the text (Table S1b). Graphic was produced using OxCal v4.4 with the mixed calibration curve **(112)**. B. Single-phase Bayesian model for only the Machu Picchu dates. The IntCal20 Northern Hemisphere (***109)*** and IntCal20 Southern Hemisphere (***110***) radiocarbon age calibration curves were used in the mixed calibration and model.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MP4i
0.364
0.347
MP5a
0.359
0.349
MP9b
0.356
0.346
MP23
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MP27
0.36
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MP31a
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MP32
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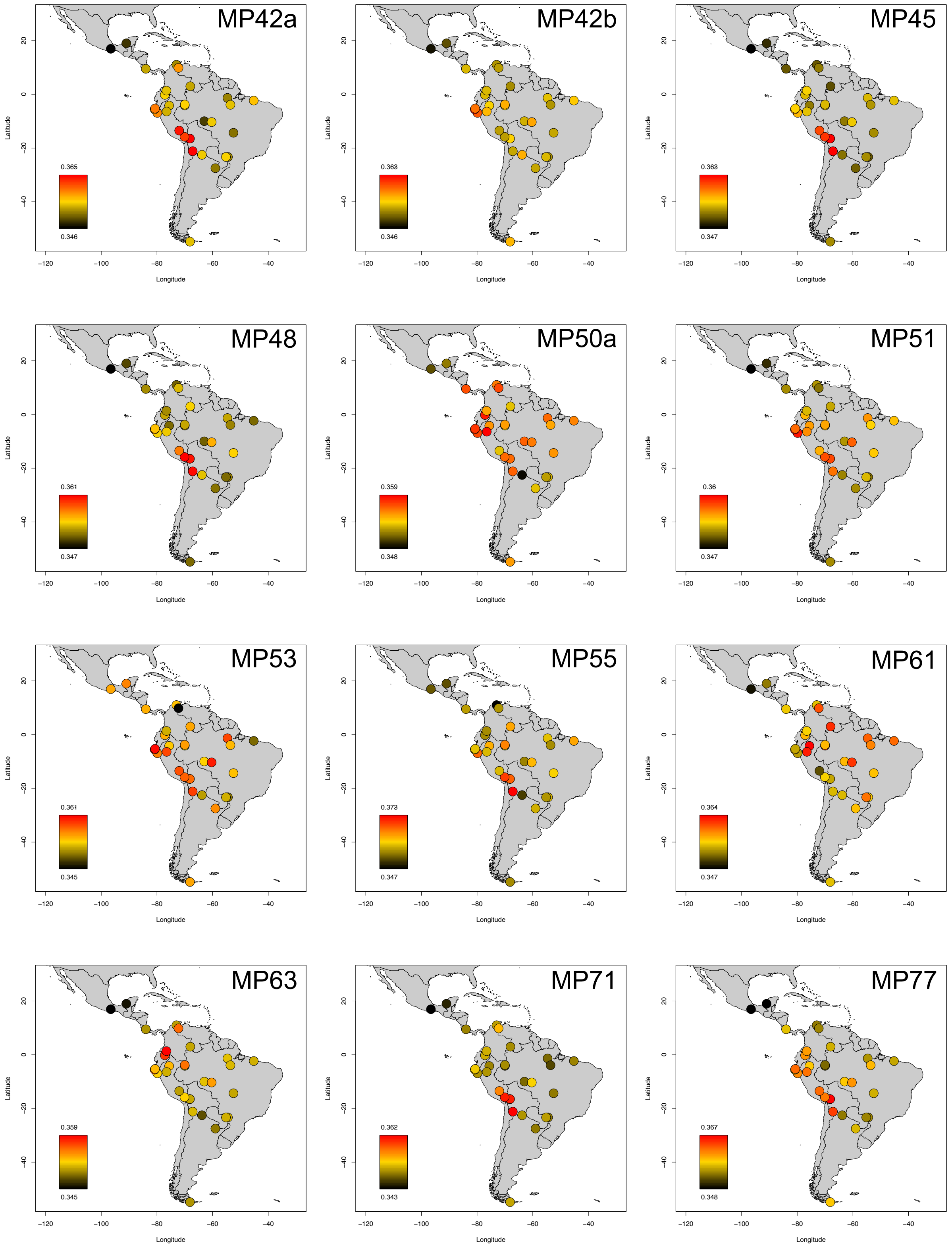
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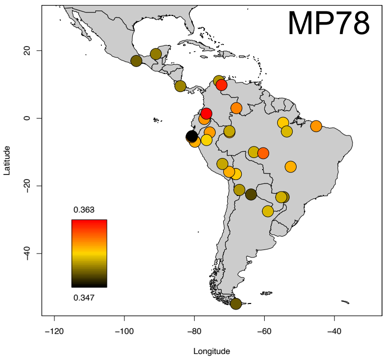
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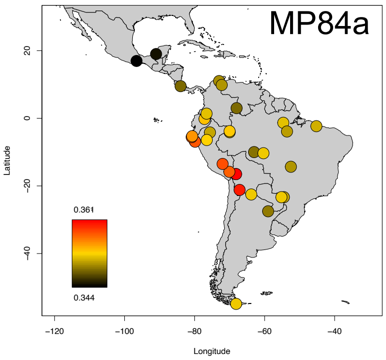
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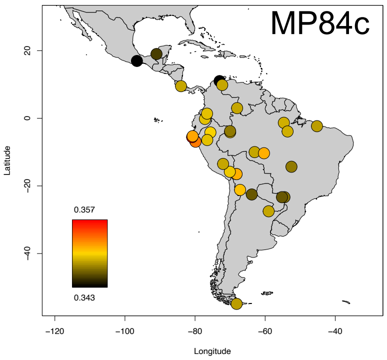
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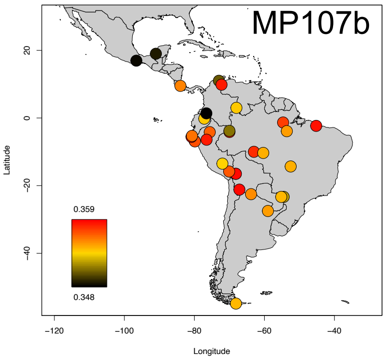
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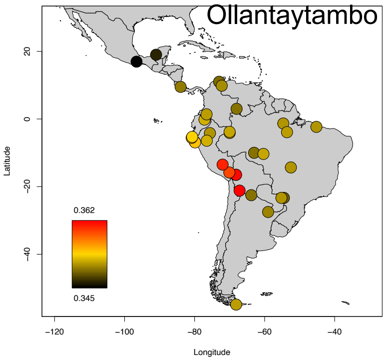
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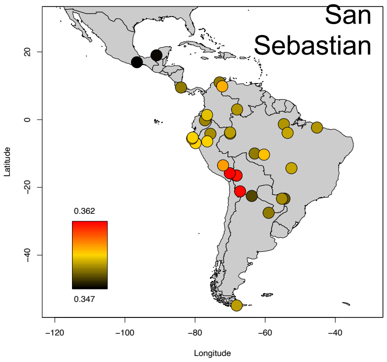
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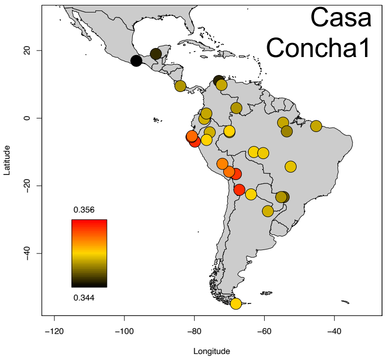
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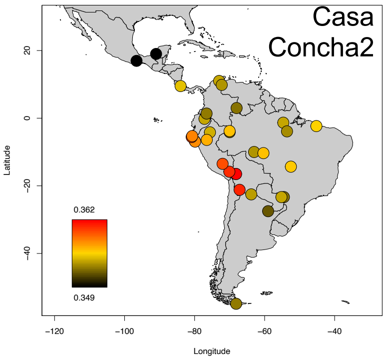
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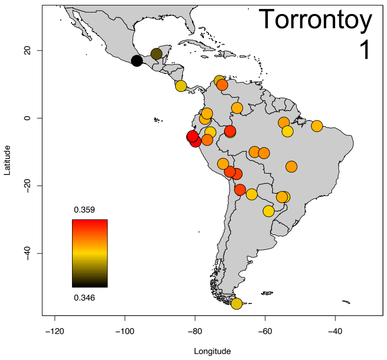
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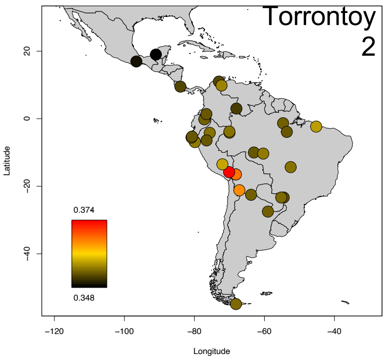
Longitude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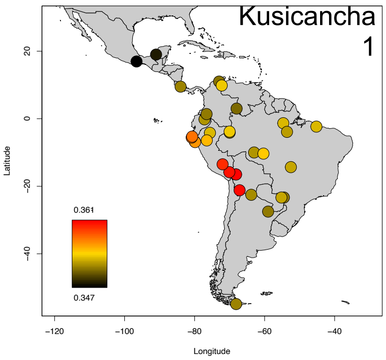
Longitude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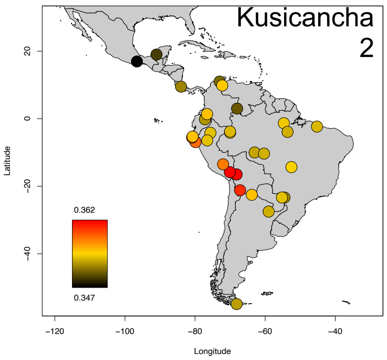
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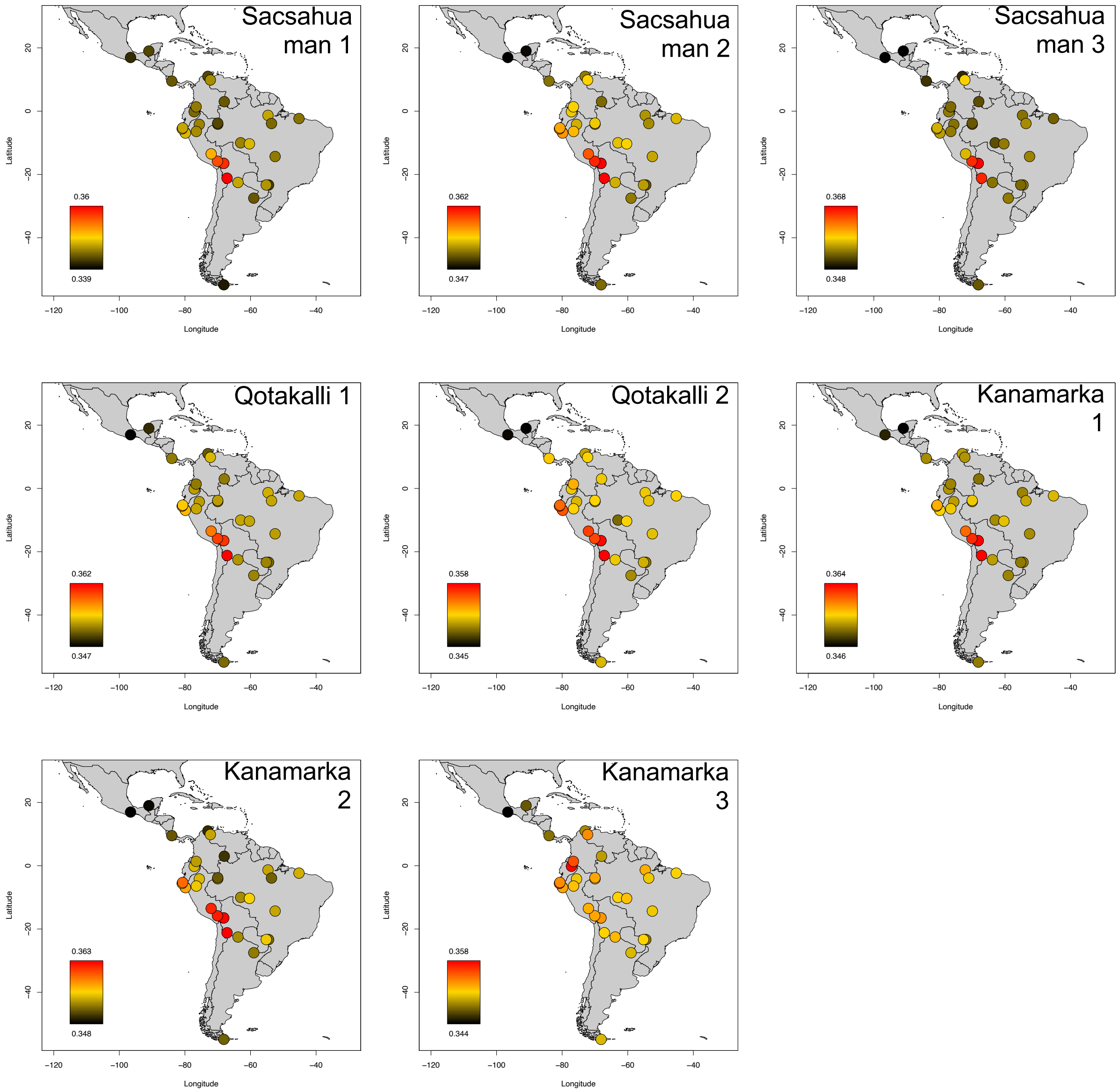
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Qotakalli 2
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0.345
Kanamarka 1
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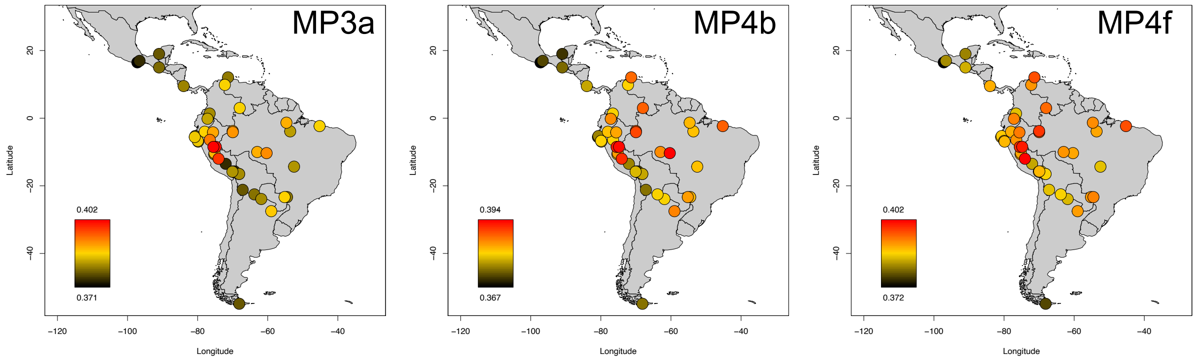
-40

B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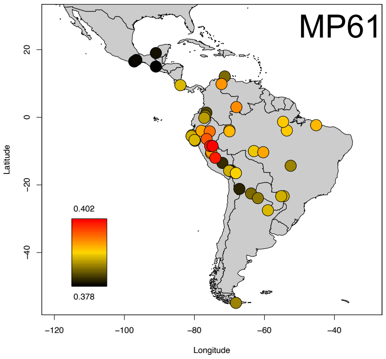

Fig. S2. Heatmap of outgroup-*f_3* statistics for each Machu Picchu individual and the Cusco and Urubamba Valley groups. Color coding is based on statistics of the form *f_3(Mbuti; Ancient, Modern)* – red indicates more shared genetic drift. A. Outgroup-*f3* statistics using the HumanOrigins merged panel; B. Outgroup-*f3* statistics for individuals of Amazonian ancestry using the Omni-HumOrg merged panel to expand group diversity; only individuals with more than 80k SNPs were retained after merge.

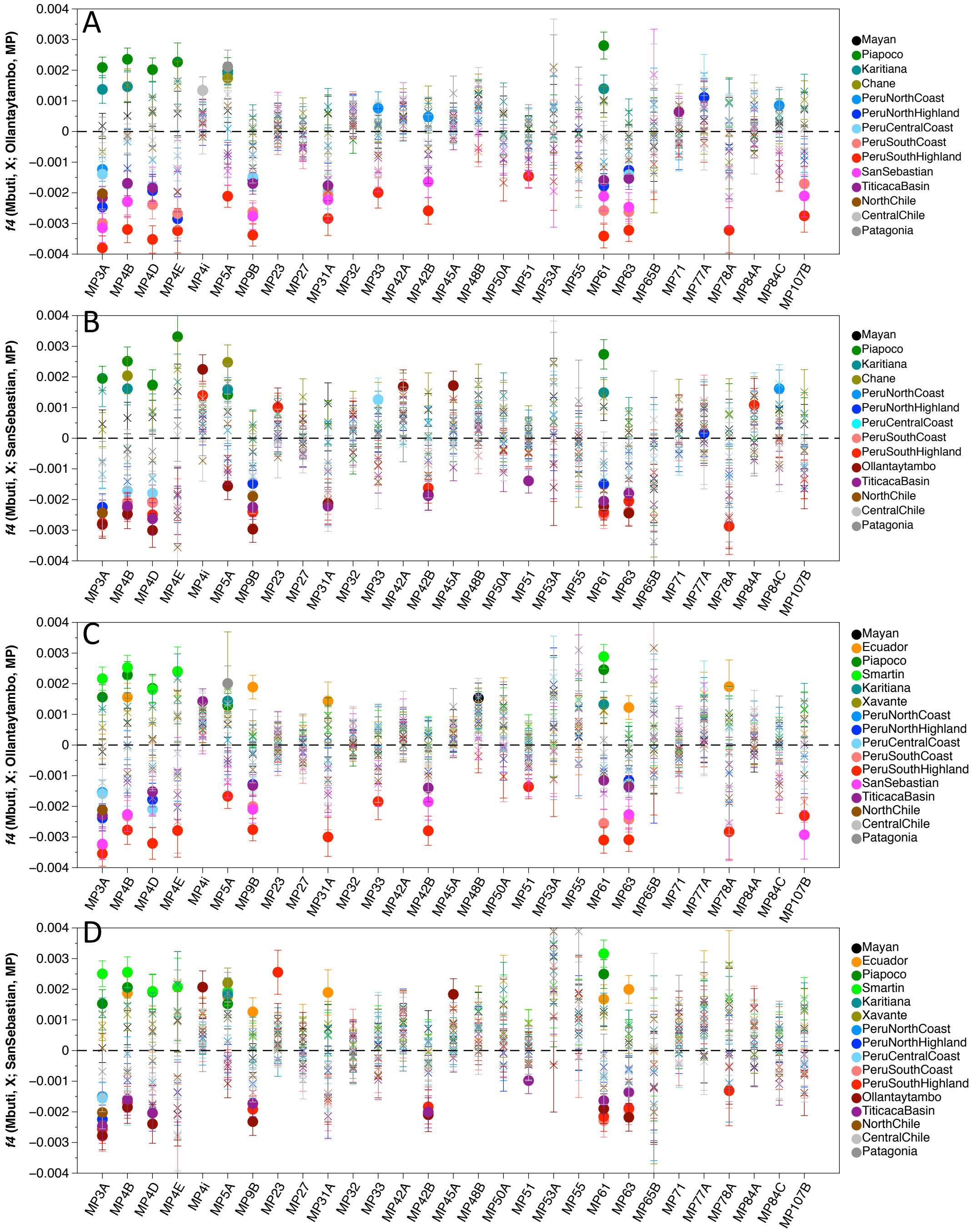

Fig. S3. F4-statisctics of the type f4(Mbuti, X; Ollantaytambo/SanSebastian, MPindividual) using the full 1240k panel (A,B), or the geographically more diverse but lower resolution HumOrg dataset (C, D). For this panel X has been chosen to only represent the Andean ancestry clusters determined by Nakatsuka et al (***34***) and selected populations to represent other South- and Central-American regions, complete statistics can be found Table S6.

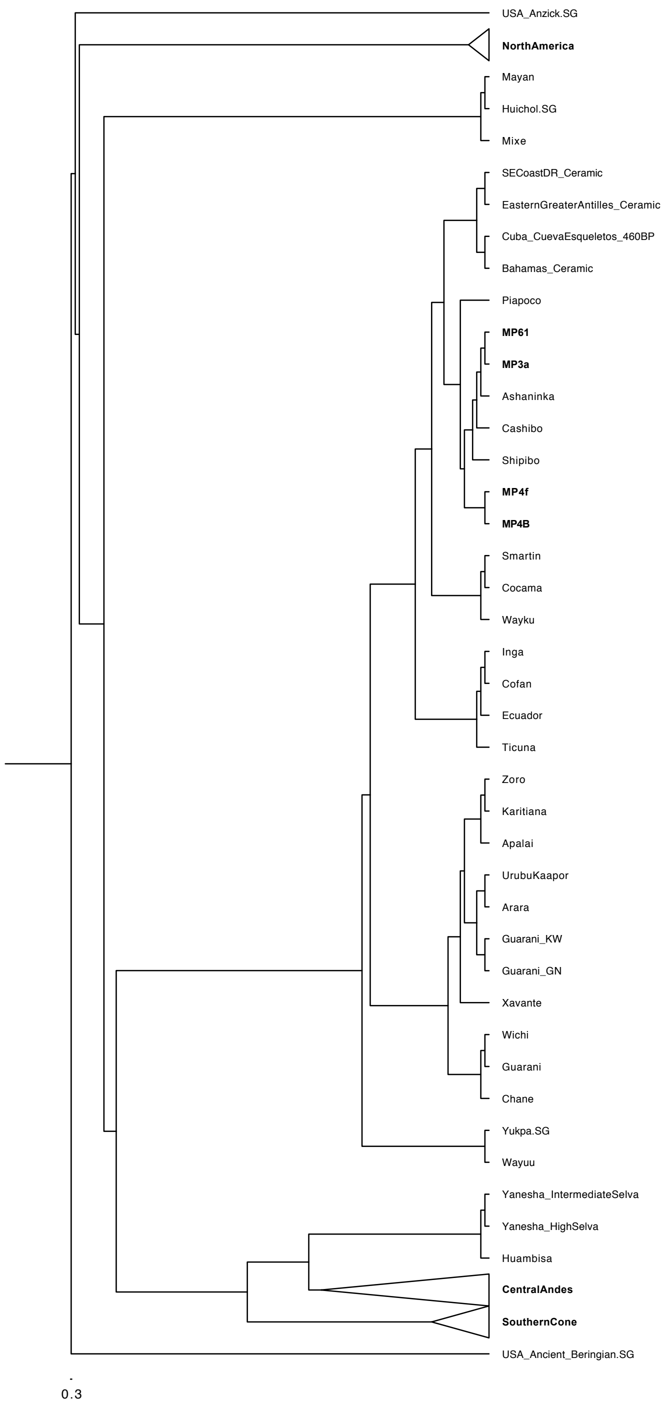

Fig. S4. Neighbor-joining tree based on inverted outgroup-f_3 statistics (1/f_3(Mbuti; Group1, Group2)) using the HO_OMNI SNP set showing the genetic affinity between the Machu Picchu individuals exhibiting Amazonian ancestry and other South American groups. The USA_Ancient_Beringian.SG individual (USA-AK_USR1_Beringian_1140BP.SG) was used as an outgroup for the tree.

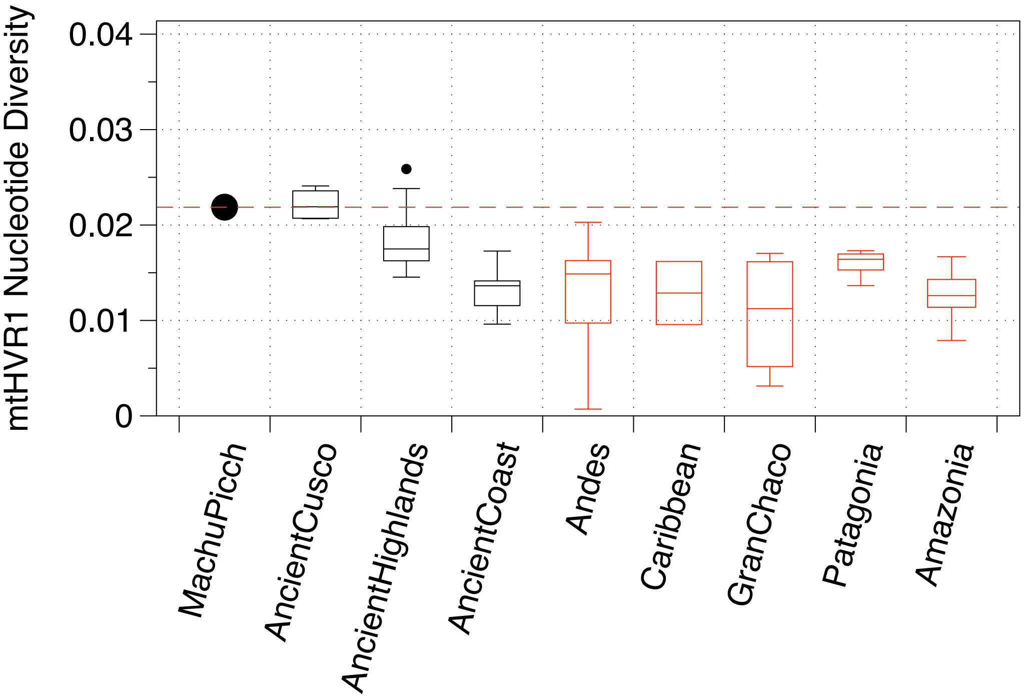

Fig. S5. Mitochondrial nucleotide diversity (π) calculated from HVR1 sequence data from pre-colonial (black) and modern-day (red) South American individuals

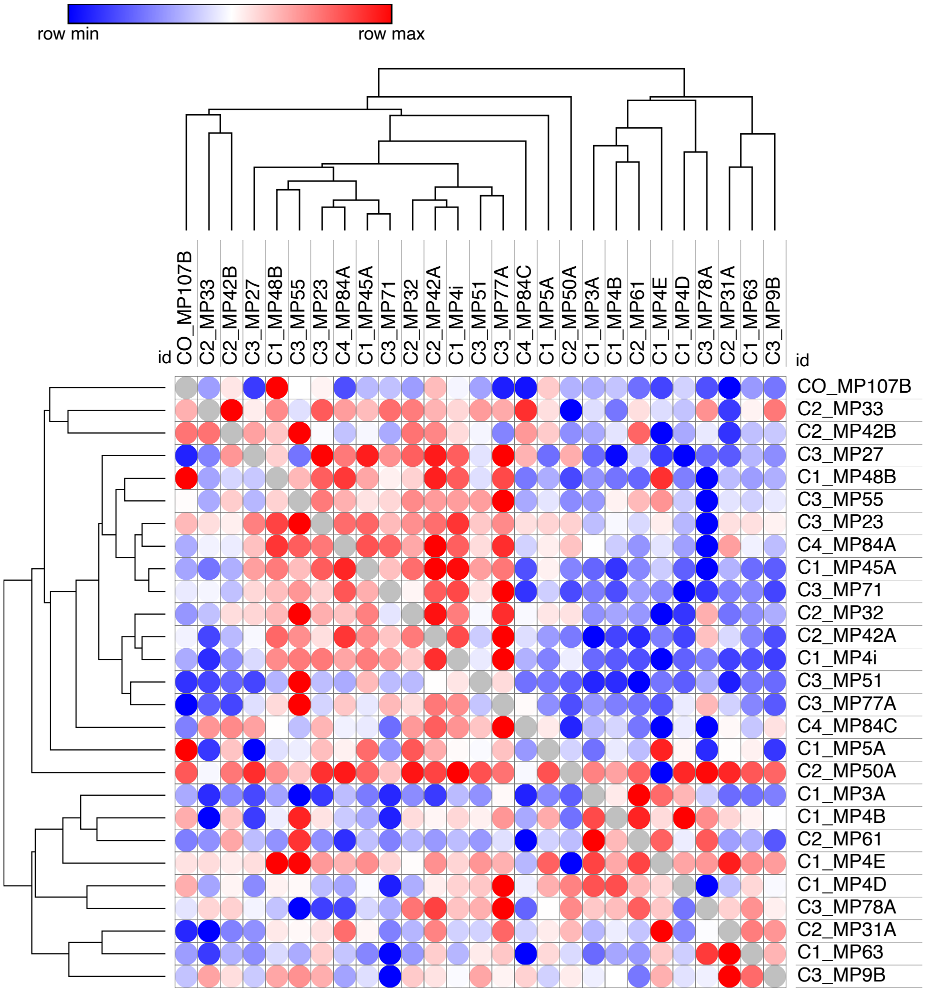

Fig. S6. F3 similarity matrix for the Machu Picchu individuals with generated with Morpheus (https://software.broadinstitute.org/morpheus). Only individuals with >50,000 SNPs are included. The labels C1-4, and CO at the beginning of the individual labels refer to the cemetery the individual was buried in (CO = outlier).

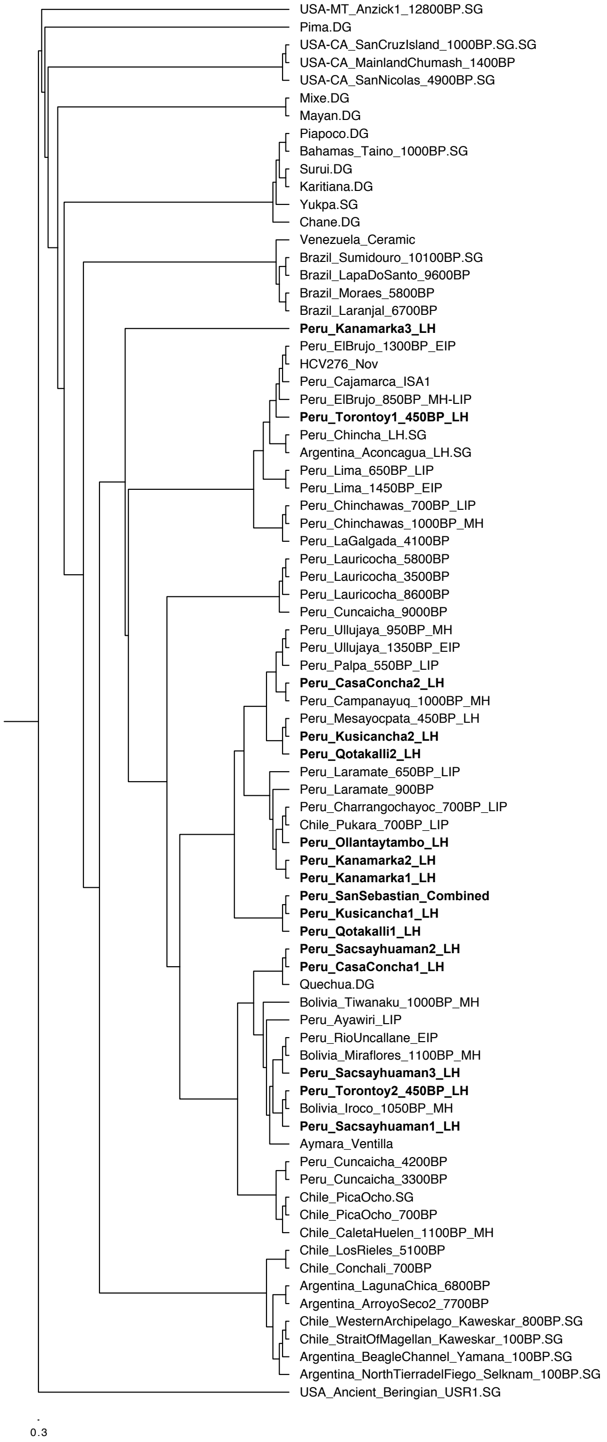

Fig. S7. Neighbor-joining tree based on inverted outgroup-f_3 statistics (1/f_3(Mbuti; Group1, Group2)) using the 1240k SNP set. The USA_Ancient_Beringian.SG individual (USA-AK_USR1_Beringian_1140BP.SG) was used as an outgroup for the tree.

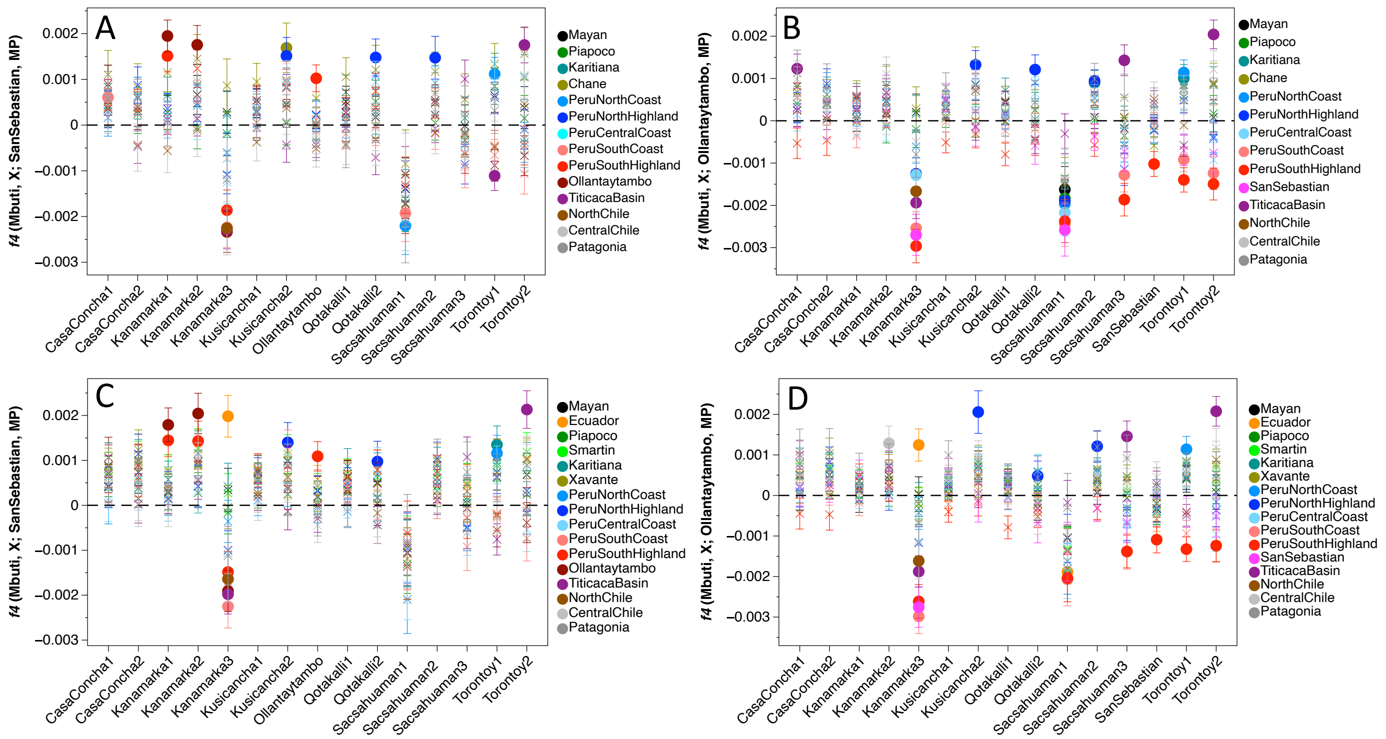

Fig. S8. F4-statisctics of the type f4(Mbuti, X; Ollantaytambo/SanSebastian, Urubamba/Cusco) using the full 1240k panel (A,B), or the geographically more diverse but lower resolution HumOrg dataset (C, D). For this panel X has been chosen to only represent the Andean ancestry clusters determined by Nakatsuka et al (*34*) and selected populations to represent other South- and Central-American regions.

Table S1. (separate file)
Combined table with sequencing statistics, isotopic values, archaeological information, and radiocarbon calibrations / and results for Bayesian model for all individuals investigated in this study.

Table S2. (separate file)
Results of Analyses used to establish ancestry of the individuals form Ollantaytambo, and San Sebastian, and to support grouping of all non-Machu Picchu individuals (F4-statistics, qpADM, qpWave, DATES).

Table S3. (separate file)
F4-statistics of the type f4(Mbuti, X; Ollantaytambo/SanSebastian, MP) for all single Machu Picchu individuals, and Cusco/Urubamba Valley groups.

Table S4. (separate file)
qpWave results

Table S5. (separate file)
F4-statistics to establish source or Amazonian ancestry for several MP individuals

Table S6. (separate file)
qpADM admixture model results

Table S7. (separate file)
Additional f4-statistics to establish individual specific genetic affinities (see Supplementary Text)

Table S8. (separate file)
Relatedness results from READ and lcmlKIN

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