Identification of Polynesian mtDNA haplogroups in remains of Botocudo Amerindians from Brazil

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There is a consensus that modern humans arrived in the Americas 15,000–20,000 y ago during the Late Pleistocene, most probably from northeast Asia through Beringia. However, there is still debate about the time of entry and number of migratory waves, including apparent inconsistencies between genetic and morphological data on Paleoamericans. Here we report the identification of mitochondrial sequences belonging to haplogroups characteristic of Polynesians in DNA extracted from ancient skulls of the now extinct Botocudo Indians from Brazil. The identification of these two Polynesian haplogroups was confirmed in independent replications in Brazil and Denmark, ensuring reliability of the data. Parallel analysis of 12 other Botocudo individuals yielded only the well-known Amerindian mtDNA haplogroup C1. Potential scenarios to try to help understand these results are presented and discussed. The findings of this study may be relevant for the understanding of the pre-Columbian and/or post-Columbian peopling of the Americas.

ancient DNA | human migration | mitochondrial DNA | South America | Paleoindians

The origin of the first Americans has been a controversial topic since the beginning of the 19th century (1, 2). Three main hypotheses for the pre-Columbian peopling of America are presently being entertained. The first hypothesis, called "Two Components," is based on morphological studies of crania from ancient and modern Native Americans. Two apparently polar morphologies have been identified: first, a generalized (or Paleoamerican) one with dolichocephalic features, and second, a specialized Mongoloid morphology with brachycephalic features (3). The foremost representatives of the Paleoamerican type in Brazil are the skeletons from Lagoa Santa, Brazil (4), and the specialized morphology is seen in the vast majority of extant Amerindians. Proponents of this model argue that two distinct populations entered the Americas by the end of the Pleistocene, and that the transition between the cranial morphology of the Paleoamericans and the morphology of later Native Americans, which occurred around 8,000–9,000 y before present, was abrupt because of total or partial population replacement (5–9). Of interest is the observation that multivariate analyses of skull measurements of the Paleoamerican group have shown similarity to some African groups, Australians, Melanesians, and Easter Islanders (5).

In apparent contrast, molecular data, mostly obtained through the study of extant populations, have proposed a single migration wave into South America (Single Wave model), although North America may have received additional migrations on a minor scale from Beringia in the Holocene (10). Analyses of complete mitochondrial genomes have identified at least 15 founding lineages in the extant Amerindian gene pool, including the Pan-American mtDNA haplogroups A2, B2, C1b, C1c, C1d, C1d1, D1, and D4h3a, as well as some haplogroups restricted to northern North America (sub-Arctic and Arctic regions), A2a, A2b, D2a, D3, and X2a (11–13), and others that have been exclusively found in populations located in the southernmost part of South America, including B2l, D1g, and C1b13 (14, 15). It has been proposed that most of these founding lineages were generated during a period of refuge of about 5,000 years in Beringia (13, 16). In contrast, genetic analyses of the nonrecombining portion of Y chromosome haplotypes have shown a significant founder effect (17). More than 80% of Amerindian Y chromosomes belong to the single haplogroup Q1a3a-M3, whose precursors could be traced back to central Siberia (18–20). Although this genetic evidence is suggestive of a single founding population for all Native South Americans, we have to realize that these studies have been largely limited to extant Amerindian groups or to relatively recent skeletal remains. Thus, molecular studies have not been capable of testing appropriately the Two Components model yet.

Based on geometric morphometric studies, González-José et al. (21) have proposed that the Paleoamerican and Mongoloid cranial morphologies are not separate "types," but simply extremes in a continuous gradient of variation. The authors postulate that the morphological pattern defining the Paleoamerican remains can be found in the range of extant Amerindian populations and that the previous separation of samples into discrete categories represented subjective assignments. González-José et al. presented a third model, called "Recurrent Gene Flow," which is capable of integrating morphological and molecular data (21). Using 2D geometric morphometric data they propose that this model has better explanatory performance than either the Single Wave or the Two Components models.

In the context of the genetic reconstruction of the Native American history, the study of the Botocudo Indians from Brazil might provide new information. Also known as Aimores, these Amerindians inhabited adjacent areas in the present-day states of Minas Gerais, Espírito Santo and Bahia, in Southeastern Brazil (22). They were a large community, divided into many separate groups, some of them in conflict with each other. In common, they were Macro-Je speakers, had a nomadic hunter-gatherer lifestyle, and used typical ornaments in the lips and ears, called "botoques"

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by the Portuguese colonizers. In 1808 the Portuguese Crown declared "Just War" (*Bellum iustum*) against all Indian tribes that did not accept European laws (23). The fierce Botocudo were targeted in such wars and, in consequence, became virtually extinct by the end of the 19th century (24). Their importance for the history of the peopling of the Americas was revealed by studies reporting that the Botocudo had cranial features that consistently were described as intermediate between the polar Paleoamerican and Mongoloid morphologies (25, 26). Multivariate analyses of the cranial measures of different Amerindian and Paleoamerican groups from Brazil indeed concluded that the Botocudo Indians presented sufficient similarities with the Lagoa Santa Paleoamericans to be considered candidates to be their possible descendants (27).

The Museu Nacional/Universidade Federal do Rio de Janeiro (UFRJ) in Rio de Janeiro has a collection of Botocudo skulls dating to the 19th century and we extracted DNA and partially sequenced the mtDNA from teeth obtained from 14 of them. Usually, when human groups colonize the territory of another population there is introgression of the mtDNA of the autochthonous population into the "invading" one because of sexually asymmetric reproduction (28). Thus, we reasoned that if the Botocudo Indians were indeed a product of genetic admixture between the original Paleoamericans and a second wave of Amerindians with specialized cranial morphologies, the chance of finding any DNA evidence of such event would be greater using the matrilineal mtDNA.

We describe herein the discovery of unexpected mitochondrial lineages considered as typically Polynesian in teeth obtained from two Botocudo skulls. We shall first present our molecular results, which have been reproduced independently in two different laboratories. We then present possible scenarios to explain how this Polynesian-Botocudo contact might have occurred. The first scenario, prehistoric, is related to the possibility of genetic continuity between the Paleoamericans from Lagoa Santa and Botocudo Indians. We follow with an imaginable historic pre-Columbian scenario, involving opportunities for more recent direct contact between Polynesia and South America before the European arrival. We then present two possible modern post-Columbian scenarios, both related to the slave trade. One scenario is connected with the practice of the "blackbirding" trade, which occurred in 1860s and brought ~2,000 Polynesians to Peru. The second scenario is the possible arrival of Polynesian haplogroups to Brazil in modern times through the African slave trade from Madagascar, where 20% of the mtDNA belong to the B4a1a1a haplogroup (29).

Results

We sequenced the control region (first and second hypervariable segments: HVSI-HVSII) and typed specific mutations of the coding region of mtDNA extracted from teeth of 14 different Botocudo skulls. As reported previously (30), 12 of these skulls clearly belonged to the Amerindian haplogroup C1 and will not be further discussed here. The extracts from the remaining two skulls, MN00015 and MN00017, yielded mitochondrial sequences belonging to haplogroup B, with unexpected ancestries. Their description and analysis constitute the core of the present article.

Phylogenetic Analysis of Polynesian Sequences Found in Brazil. The skulls identified as MN00015 and MN00017 in the Museu Nacional/UFRJ in Rio de Janeiro were both from adult male Botocudo individuals from the Rio Doce valley in the state of Minas Gerais, Brazil, as registered by annotations written directly on the outer aspect of their respective parietal bones. According to information of the log book of the museum, it is most likely that these crania arrived there on August 25, 1890. The date of death is not known with certainty, but it is almost certainly the second half of the 19th century.

We first extracted DNA from an intact premolar tooth of skull MN00015. Sequencing of mtDNA identified the following mutations: 73G, 146C, 151T, 6719C, 12239T, 15746G, 16189C, 16217C, 16247G, 16261T. The 9-bp deletion between the *COII* and *tRNA* (Lys) genes characteristic of haplogroup B (31) was also present. This set of mutations classified the haplotype as B4a1a, found mainly in Taiwan, Island Southeast Asia and populations on the Pacific Islands (32, 33). Further analysis identified the mutation 14022G, which classified the sample in haplogroup B4a1a1 (32–34). The presence of the mutation 16247G (HVSI) and 6905A (ancestral allele), further characterized the sequence as belonging to haplogroup B4a1a1a (32–34). This haplogroup is found at high frequency in Polynesia, Micronesia, parts of Near Oceania, and Easter Island (33–36).

Our next step was the replication of the results through the analysis of a second tooth from the same MN00015 skull, using identical methodology. All mutations were confirmed. A third tooth from MN00015 was then sent to the Centre for GeoGenetics in Copenhagen for independent validation. The 9-bp deletion in the intergenic *COII*/*tRNA* (Lys) region was confirmed by PCR and blind analysis of the control region confirmed the presence of 73G, 146C, 151T, 16189C, 16217C, 16247G, 16261T. Most importantly, the presence of 6719C, 12239T, 14022G, 15746G, and the ancestral allele at 6905 were also authenticated. Thus, we obtained confirmation in two laboratories that the mtDNA of the MN00015 skull belonged to haplogroup B4a1a1a, which is characteristic of Polynesians.

Mitochondrial DNA analysis from a tooth extracted from the second skull, MN00017, also revealed the presence of the 9-bp deletion of haplogroup B and the presence of the following mutations: 73G, 146C, 151T, 16126C, 16189C, 16217C, 16261T, and 14022G, thus classifying it as haplogroup B4a1a1 (32). These results were independently confirmed through the extraction of DNA from a second tooth of the same skull.

Discussion

This study is unique in reporting the presence of mtDNA haplotypes considered to be typically Polynesian in the gene pool of an extinct Brazilian Amerindian group. These results have bona fide scientific status as indicated by the fact that: (*i*) they were found in two different Botocudo skulls; (*ii*) the initial discovery made with the MN00015 skull was independently replicated in DNA from other teeth from the same cranium in two different laboratories; and (*iii*) these mtDNA sequences were retrieved from crania collected in the 19th century from an inland Native American population in Brazil.

Our findings raise an important question: How did these Polynesian sequences show up in an Amerindian population living in a region in the interior of Brazil? We cannot claim to have an answer, but we would like to discuss possible scenarios, presented here in the chronological order of the possible contact.

The first scenario, prehistoric, is related to the possibility of genetic continuity between the Paleoamericans from Lagoa Santa and Botocudo Indians (26, 27, 37), which indeed originally had motivated this study. It is conceivable that the Lagoa Santa Paleoamericans carried ancient mtDNA sequences related to those of modern Polynesians, possibly because of a contact with their ancestors, and passed them on to early Amerindians, along with genes associated with Paleoamerican skull morphology. Indeed, there have been several previous proposals that the survival of Paleoamerican morphologies in modern Amerindian groups might be related to gene flow between these groups (37, 38). This theory has been proposed for Fuegians (38–40), for a modern Amerindian group that lived in the isolated area located in Baja California (38), and also for skeletal remains from Sabana de Bogotá in Colombia (37).

However, for this scenario to be acceptable it has to be chronologically compatible with what is known about the evolutionary

history of the mtDNA "Polynesian motif" (14022G, 16217C, 16247G, 16261T), which has been associated with the Austronesian expansion and settlement of Polynesia and Micronesia (34) at a time scale more recent than peopling of the Americas. Notably, the Polynesian motif has additionally been found in Indonesian populations at low frequencies (41) and also in Madagascar (29, 42).

Polynesian islands were apparently populated around 3,000 y ago (33, 36). Nevertheless, the time to the most recent common ancestor (TMRCA) for the Polynesian motif in Papuans and Polynesians was calculated as 9,300 ± 2,000 y before present (34). Razafindrazaka et al. (29) obtained a similar TMRCA of 8,300 ± 3,100 y before present. These TMRCA dates for the Polynesian motif appear too recent for having been introduced into the Americas by the Paleoamerindian migration. Still, we cannot rule out this possibility—however improbable—because, as pointed out by Friedlander et al. (34), the variances of these coalescence estimates may be greater than generally acknowledged.

An additional problem with this scenario is the fact that in all of the positions that were examined by us, the sequences of Botocudos had exact matches to the common haplotypes observed in Polynesians, particularly in the control region. Prima facie, considering an ancient Paleoamerican origin of the Botocudo haplotypes, we should expect new "private" mutations to have appeared. On the other hand, because we did not sequence the whole mtDNA, we cannot rule out the existence of such variations in the coding region.

Another imaginable pre-Columbian scenario involves opportunities for more recent direct contact between Polynesia and South America before the European arrival. Such possibility of a direct movement from Oceania across the Pacific Ocean to the Americas was raised by Cann (43) on a discussion of the origin of the Amerindian B haplogroup. This finding prompted Bonatto et al. (44) to evaluate the likelihood of a Polynesian-Amerindian contact having occurred and conclude against it, although they could rule out neither minor contact events nor nonmaternal genetic exchange. New evidence from human and nonhuman material has become available since then. For example, there were archeological findings of Polynesian chicken bones in the Arauco Peninsula, in Chile (45) and evidence has been found in Easter Island of pre-Columbian presence of sweet potato and bottle gourd, both typical of South America (46, 47). Independent of the plausibility or implausibility of the pre-Columbian arrival of Polynesians to the South American Pacific coast, there still would remain the need to explain how these migrants crossed the Andes and ended up in Minas Gerais, Brazil. We feel that such a scenario is too unlikely to be seriously entertained.

In post-Columbian times, a potential source for the presence of Polynesian haplogroups in Brazil might be the blackbirding trade that occurred in 1860s and brought ~2,000 Polynesians to Peru (48). However, to the best of our knowledge there is no evidence of transportation of any of these Peruvian slaves to the Brazilian territory in this period. The less than 300 individuals who survived were repatriated after the abolition of slavery in Peru in 1896 (48). It is also significant that mtDNA analyses in Peru and Chile have not found Polynesian sequences in the gene pool of extant populations (49–51). A possible exception was the finding of the motif seen in the HVSI of Polynesians (16189C, 16217C, 16247G, 16261T) in two individuals from a Peruvian archeological site from the 14th and 15th centuries (52). However, the coding mutation 14022G that is critical for definition of the Polynesian motif was not investigated in these remains. Although the transition at 16247 has been used to identify a Polynesian motif in earlier studies, it appears to be hypermutable, possibly because of numerous back-mutations, and thus not ideal for ancestry assignment (34). In fact, Baca et al. (52) themselves assigned these samples to haplogroup B2.

The last scenario that we wish to assess is the possible arrival of Polynesian haplogroups to Brazil in modern times through the African slave trade from Madagascar, where 20% of the mtDNA lineages belong to the B4a1a1a haplogroup (29). In 1807 Britain outlawed the Atlantic slave trade, making it illegal for British ships to transport slaves. The Royal Navy then began to patrol the waters off West Africa to enforce the so-called "Blockade of Africa." British cruisers actually succeeded in capturing 169 Brazilian ships in that region in the period 1815–1850 (53). One form of evading the blockade was to switch the trade to the East Coast of Africa, from where ships could take a South Atlantic route to Brazil. Thus, Mozambique, a previously minor source of trade, became in the 19th century a very important port of origin of slaves to Brazil, drawing captives from an ample area that included neighboring Madagascar (53, 54). It has been estimated that between 1817 and 1843 ~120,000 slaves were brought from that region to Brazil (53), some of them probably going to Minas Gerais, Espirito Santo, and Bahia, to work close to areas occupied by Botocudos. It is known that in these regions, some Botocudo Indians had been pacified during the 19th century and were drafted to work side-by-side with African slaves in plantations (55), an environment potentially conducible to gene flow. Another possibility would be that female slaves from Madagascar living in these regions might have been kidnapped by Botocudo Indians or had run away and find refuge among them, thus creating conditions for introgression of their mtDNA in the Amerindian population. In fact, the kidnapping of a female by Botocudo Indians (Aimores) is a central theme of an 1870 Brazilian opera ("Il Guarany," composed by Carlos Gomes). Although fanciful, this is perhaps the most likely scenario among those that can be entertained.

In conclusion, we found evidence of Polynesian mtDNA haplogroups in 2 of 14 Botocudo skulls that were studied (30), with independent confirmation of the findings in two separate laboratories. As indicated previously, these findings have bona fide scientific status. We have entertained several possible models to try to explain how these Polynesian sequences were found in individuals from an Amerindian population living in a region in the interior of Brazil. At present, our results do not allow us to accept or definitely reject any of these scenarios. We hope that further molecular studies will settle the question and will clarify the relevance of our findings for a more complete understanding of pre-Columbian migratory routes of people into the Americas.

GENETICS

Materials and Methods

Samples. Three teeth extracted from Botocudo skull MN000015 were provided by the Museu Nacional/UFRJ (Rio de Janeiro, Brazil): a premolar was initially analyzed in Belo Horizonte, Brazil. Subsequently, another premolar tooth from the same skull was analyzed for confirmation in Belo Horizonte and a third one was sent to Copenhagen for independent replication. Two teeth were extracted from Botocudo MN000017: first, a molar tooth was analyzed and, subsequently, a premolar tooth from the same skull was analyzed for confirmation, both in Belo Horizonte.

DNA Extraction and Analysis in Belo Horizonte. The surfaces of the teeth were cleaned by soaking them in a 6% (vol/vol) NaClO solution for 15 min, followed by rinsing in double-distilled UV-irradiated water. Each tooth was ground using mortar and pestle. The powder (0.5 g) was transferred into sterile 15-mL tubes and DNA was extracted according to the protocol published by Rohland and Hofreiter (56). Briefly, the powder was incubated at room temperature in 10 mL of solution containing 0.45 M EDTA and 0.25 mg/mL proteinase K (pH 8.0) for 24 h. After centrifugation (1,118 × *g*), the supernatant was transferred into 40 mL binding buffer (5 M GuSCN, 25 mM NaCl, 50 mM Tris). A volume of 100 μL silica suspension was added and the pH was adjusted to 4.0 with concentrated HCl. The tubes were rotated at room temperature for 3 h. The silica pellet was collected by centrifugation and washed once with 1 mL binding buffer and twice with washing buffer. DNA was eluted in 50 μL TE buffer (pH 8.0). A negative extraction control to which no tooth powder was added accompanied each sample extraction.

PCR amplifications of mtDNA were conducted in 20-μL volumes, containing 3 μL of DNA extract (not quantified) using two units Taq DNA polymerase (Taq Platinum; Invitrogen) and standard conditions. Thermal cycling used the following parameters: 94 °C for 4 min, followed by 44 cycles at: 94 °C for 30 s, 60 °C for 30 s and 68 °C for 30 s, with final extension at 72 °C. PCR products were visualized by electrophoresis and afterward precipitated with PEG8000. The primer sequences are described in Table S1.

For sequencing, PCR products were mixed with 5 μM of primer (Table S1) and 4 μL of the DYEnamic ET dye terminator kit (GE Healthcare), followed by injection on a MegaBace 1000 sequencer (GE Healthcare). Analysis used the Bioedit v7.0.9 software.

Additionally, 3 μL of PCR products were directly digested at 37 °C with 1 unit of the appropriate restriction enzyme for 2 h (Table S2). The products were separated by electrophoresis in polyacrylamide gels and stained with silver salts for identification of the presence or absence of the restriction sites that characterize Amerindian haplogroups A, C, or D, and also to test for the 9-bp deletion characteristic of haplogroup B.

Independent Replication of the Results in Copenhagen. For DNA extraction and analysis, the tooth was first soaked in 10 mL of 0.1 M HCl solution (UV 254 nm 20 min) and incubated on a shaker for 5 min. The HCl solution was discarded and the teeth were washed with 10 mL Millipore water (UV 254 nm 20 min) and incubated on a shaker for 2 min. A 10 mL 95% ethanol solution was added and incubated for 2 min. The tooth was left to dry overnight. The roots of the tooth were cut using sterile cutting disks (UV 254 nm 20 min) to expose the pulp (root chamber). Fine-grained powder (0.5 g) was obtained using sterile round-headed drill bits. Before starting the DNA extraction protocol, the powder was soaked in 1 mL 0.5% bleach solution, incubated for 15 min and washed twice with HPLC water. The powder was incubated overnight at 55 °C in 1.6 mL Yang buffer (5 mL 10 M urea and 45 mL 0.5 M EDTA) and 8.7 μL (20 mg/mL) Proteinase K was added. After centrifugation (600 × *g* for 5 min), the supernatant was transferred to Amicon filters (wiped with 0.5% bleach) and spun down at 4,000 rpm to concentrate the sample (final volume 250 μL). The volume recovered was transferred to 15-mL tubes containing 1,250 μL of Qiagen PB buffer and aliquots of 720 μL were spun down in QIAquick PCR Purification Kit (Qiagen) filters at 14,000 rpm (1 min). Next, 720 μL of Qiagen PE buffer was added to the QiaQuick filter and spun down at 14,000 rpm (1 min). DNA was eluted by adding 45 μL EB buffer, incubated for 2 min, and spun down at 14,000 rpm for 1 min (repeated twice). The 90 μL DNA extract was transferred to new nonirradiated tubes and stored at −20 °C.

PCR reactions were set up in 25-μL volumes containing 3 μL of DNA extract (not quantified), 0.2 mM of each dNTP, 0.3 μM of each primer, 2.5 mM Mg_2SO_4, 1.75 μL 99.5% DMSO, and two units of Taq DNA polymerase (Taq Platinum; Invitrogen) in a buffer made up of 20 mM Tris•HCl pH 8.4 and 50 mM KCl. Cycling conditions were: initial denaturing at 94 °C for 5 min, 40 cycles involving 94 °C for 15 s, 46 °C for 20 s, and 68 °C for 30 s, and final extension at 72 °C for 7 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Primer sequences are described in Table S1.

For HVSII fragments, the PCR set up was as follows: 3 μL of DNA extract (not quantified), 0.2 mM of each dNTP, 0.3 μM of each primer, 2.5 mM $MgCl_2$, and two units Smart Taq DNA polymerase (Naxo) in a Smart Taq10× buffer, totaling a final volume of 25 μL. Cycling conditions were: initial denaturing at 95 °C for 15 min, 45 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 30 s, and final extension at 72 °C for 15 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide. The primer sequences are described in Table S1.

For cloning, an aliquot of the PCR product was diluted (1:15) and 1 μL of the mix was added to a 0.5 μL of a solution containing 0.25 μL salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and 0.25 μL TOPO cloning vector (Invitrogen, Life Technology). The mix was incubated for 20 min at room temperature. In the following step, 10 μL of competent cells were added to the mix and the tube was incubated for 20 min on ice. After incubation, the cells were subjected to heat shock for exactly 30 s at 42 °C and immediately transferred to ice for 2 min. A 100 μL volume of SOC medium was added and the tube was incubated with shaking for 1 h at 37 °C (250 rpm). The mix was spread on agar plates with ampicillin and incubated overnight at 37 °C. Between 24 and 32 colonies from each plate were used as template and the PCR set up for the colony PCR was: 1 μL DNA extract (colony immersed in water) was mixed in a solution containing 0.2 mM of each dNTP, 0.3 μM of each primer (M13), 2.5 mM $MgCl_2$, and two units Taq DNA polymerase (Taq Platinum; Invitrogen) in a buffer made up of 20 mM Tris•HCl pH 8.4 and 50 mM KCl, in a final volume of 25 μL. Cycling conditions were: initial denaturing at 95 °C for 1 min, 39 cycles at: 94 °C for 20 s, 54 °C for 25 s, and 72 °C for 30 s, and final extension at 72 °C for 10 min. The primers used for cloning amplification are described in Table S1. The PCR products were shipped for DNA sequencing at Macrogen.

Prevention of Contamination in Both Belo Horizonte and Copenhagen. In both laboratories, all ancient DNA extractions and PCR setups were performed in a physically separated laboratory where no work with amplified DNA had ever been done. The bench was irradiated with UV lamps (254 nm) for 30 min before all experiments and cleaned with highly concentrated sodium hypochlorite solution (2%). Gloves, face masks, hats, laboratory coats, and laboratory equipment (including pipettes, tubes, filter tips, and centrifuges) were sterilized by exposure to UV lamps (254 nm). All metallic material and glassware were sterilized in an oven at 200 °C for at least 6 h (Belo Horizonte). To detect possible contamination by exogenous modern DNA, extraction and amplification blanks were used as negative controls. Finally, all persons involved in the ancient DNA work were genetically typed (HVSI sequencing) and compared with the results obtained from ancient teeth samples.

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1. Rivet P (1908) La race de Lagoa-Santa chez le population precolombiennes de L'Equateur. *Bull Mem Soc Anthropol Paris* 9:209–268.
2. Hrdlicka A (1912) *Early Man in South America* (Smithsonian Institution, Washington).
3. Lahr MM (1996) *The Evolution of Modern Human Diversity: A Study of Cranial Variatio* (Cambridge Univ Press, Cambridge).
4. Neves WA, Prous A, González-José R, Kipnis R, Powell J (2003) Early Holocene human skeletal remains from Santana do Riacho, Brazil: Implications for the settlement of the New World. *J Hum Evol* 45(1):19–42.
5. Neves WA, Pucciarelli HM (1991) Morphological affinities of the first Americans: An exploratory analysis based on Early South American human remains. *J Hum Evol* 21(4): 261–273.
6. Hubbe M, Neves WA, Atui JPV, Cartelle C, Silva MP (2004) A new early human skeleton from Brazil: Further support to the "two main biological components model" for the settlement of the Americas. *Current Research in the Pleistocene* 21:77–81.
7. Neves WA, Hubbe M (2005) Cranial morphology of early Americans from Lagoa Santa, Brazil: Implications for the settlement of the New World. *Proc Natl Acad Sci USA* 102 (51):18309–18314.
8. Hubbe M, Neves WA, Harvati K (2010) Testing evolutionary and dispersion scenarios for the settlement of the new world. *PLoS ONE* 5(6):e11105.
9. Hubbe M, Harvati K, Neves W (2011) Paleoamerican morphology in the context of European and East Asian late Pleistocene variation: Implications for human dispersion into the New World. *Am J Phys Anthropol* 144(3):442–453.
10. Reich D, et al. (2012) Reconstructing Native American population history. *Nature* 488 (7411):370–374.
11. Tamm E, et al. (2007) Beringian standstill and spread of Native American founders. *PLoS ONE* 2(9):e829.
12. Perego UA, et al. (2009) Distinctive Paleo-Indian migration routes from Beringia marked by two rare mtDNA haplogroups. *Curr Biol* 19(1):1–8.
13. Perego UA, et al. (2010) The initial peopling of the Americas: A growing number of founding mitochondrial genomes from Beringia. *Genome Res* 20(9):1174–1179.
14. Bodner M, et al. (2012) Rapid coastal spread of First Americans: Novel insights from South America's Southern Cone mitochondrial genomes. *Genome Res* 22(5):811–820.
15. de Saint Pierre M, et al. (2012) An alternative model for the early peopling of southern South America revealed by analyses of three mitochondrial DNA haplogroups. *PLoS ONE* 7(9):e43486.
16. Fagundes NJ, Kanitz R, Bonatto SL (2008) A reevaluation of the Native American mtDNA genome diversity and its bearing on the models of early colonization of Beringia. *PLoS ONE* 3(9):e3157.
17. Pena SD, et al. (1995) A major founder Y-chromosome haplotype in Amerindians. *Nat Genet* 11(1):15–16.
18. Santos FR, et al. (1999) The central Siberian origin for native American Y chromosomes. *Am J Hum Genet* 64(2):619–628.
19. Zegura SL, Karafet TM, Zhivotovsky LA, Hammer MF (2004) High-resolution SNPs and microsatellite haplotypes point to a single, recent entry of Native American Y chromosomes into the Americas. *Mol Biol Evol* 21(1):164–175.
20. Karafet TM, et al. (2008) New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. *Genome Res* 18(5):830–838.
21. González-José R, Bortolini MC, Santos FR, Bonatto SL (2008) The peopling of America: Craniofacial shape variation on a continental scale and its interpretation from an interdisciplinary view. *Am J Phys Anthropol* 137(2):175–187.
22. Paraiso MHB (1992) *História dos Índios no Brasil [History of Indians in Brazil]* (FAPESP/SMC/Companhia das Letras, São Paulo), pp 413–430.

23. Paraiso MHB (1998) O tempo de dor e do trabalho: A conquista dos territórios indígenas nos sertões do leste [The time of pain and labor: The conquest of indigenous territories in eastern inlands]. PhD thesis (Universidade de São Paulo, Brazil).
24. Langfur H (2002) Uncertain refuge: Frontier formation and the origins of the Botocudo war in late colonial Brazil. *Hisp Am Hist Rev* 82(2):215–256.
25. Lacerda F, Peixoto R (1876) Contribuições para o estudo antropológico das raças indígenas do Brazil [Contributions to the anthropological study of indigenous races of Brazil]. *Arquivos do Museu Nacional do Rio de Janeiro* 1:47–79.
26. Peixoto R (1885) Novos estudos craniológicos sobre os Botocudos [New studies on cranial features of the Botocudos]. *Arquivos do Museu Nacional do Rio de Janeiro* 6:5–53.
27. Atui JPV (2005) Morfologia craniana de ameríndios brasileiros recentes e suas implicações para a questão da ocupação do novo mundo: Uma análise exploratória [Cranial morphology of recent Brazilian Amerindians and its implications for the occupation of the New World: An exploratory analysis]. PhD thesis (Universidade de São Paulo, Brazil).
28. Alves-Silva J, et al. (2000) The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet* 67(2):444–461.
29. Razafindrazaka H, et al. (2010) Complete mitochondrial DNA sequences provide new insights into the Polynesian motif and the peopling of Madagascar. *Eur J Hum Genet* 18(5):575–581.
30. Gonçalves VF, et al. (2010) Recovering mitochondrial DNA lineages of extinct Amerindian nations in extant homopatric Brazilian populations. *Investig Genet* 1(1):13.
31. Redd AJ, et al. (1995) Evolutionary history of the *COII/tRNALys* intergenic 9 base pair deletion in human mitochondrial DNAs from the Pacific. *Mol Biol Evol* 12(4):604–615.
32. Trejaut JA, et al. (2005) Traces of archaic mitochondrial lineages persist in Austronesian-speaking Formosan populations. *PLoS Biol* 3(8):e247.
33. Soares P, et al. (2011) Ancient voyaging and Polynesian origins. *Am J Hum Genet* 88(2):239–247.
34. Friedlaender JS, et al. (2007) Melanesian mtDNA complexity. *PLoS ONE* 2(2):e248.
35. Lie BA, et al. (2007) Molecular genetic studies of natives on Easter Island: Evidence of an early European and Amerindian contribution to the Polynesian gene pool. *Tissue Antigens* 69(1):10–18.
36. Thorsby E (2012) The Polynesian gene pool: An early contribution by Amerindians to Easter Island. *Philos Trans R Soc Lond B Biol Sci* 367(1590):812–819.
37. Neves WA, Hubbe M, Correal G (2007) Human skeletal remains from Sabana de Bogotá, Colombia: A case of Paleoamerican morphology late survival in South America? *Am J Phys Anthropol* 133(4):1080–1098.
38. González-José R, et al. (2003) Craniometric evidence for Palaeoamerican survival in Baja California. *Nature* 425(6953):62–65.
39. Lahr MM (1994) The multiregional model of modern human origins: A reassessment of its morphological basis. *J Hum Evol* 26(1):23–56.
40. Lahr MM (1995) Patterns of modern human diversification: Implications for Amerindian origins. *Yearb Phys Anthropol* 38:163–198.
41. Cox MP (2005) Indonesian mitochondrial DNA and its opposition to a Pleistocene era origin of proto-Polynesians in island southeast Asia. *Hum Biol* 77(2):179–188.
42. Soodyall H, Jenkins T, Stoneking M (1995) 'Polynesian' mtDNA in the Malagasy. *Nat Genet* 10(4):377–378.
43. Cann RL (1994) mtDNA and Native Americans: A Southern perspective. *Am J Hum Genet* 55(1):7–11.
44. Bonatto SL, Redd AJ, Salzano FM, Stoneking M (1996) Lack of ancient Polynesian-Amerindian contact. *Am J Hum Genet* 59(1):253–258.
45. Storey AA, et al. (2007) Radiocarbon and DNA evidence for a pre-Columbian introduction of Polynesian chickens to Chile. *Proc Natl Acad Sci USA* 104(25):10335–10339.
46. Yen D (1974) *The Sweet Potato in Oceania: An Essay in Ethnobotany* (Bishop Museum Press, Honolulu, HI).
47. Green RC (2000) A range of disciplines support a dual origin for the bottle gourd in the Pacific. *J Polyn Soc* 109(2):191–197.
48. Maude HE (1981) *Slavers in Paradise: The Peruvian Slave Trade in Polynesia, 1862–1864* (Stanford Univ Press, Stanford, CA).
49. Moraga ML, et al. (2000) Mitochondrial DNA polymorphisms in Chilean aboriginal populations: Implications for the peopling of the southern cone of the continent. *Am J Phys Anthropol* 113(1):19–29.
50. Fuselli S, et al. (2003) Mitochondrial DNA diversity in South America and the genetic history of Andean highlanders. *Mol Biol Evol* 20(10):1682–1691.
51. Lewis CM, Jr., Tito RY, Lizárraga B, Stone AC (2005) Land, language, and loci: mtDNA in Native Americans and the genetic history of Peru. *Am J Phys Anthropol* 127(3): 351–360.
52. Baca M, Doan K, Sobczyk M, Stankovic A, Węgleński P (2012) Ancient DNA reveals kinship burial patterns of a pre-Columbian Andean community. *BMC Genet* 13:30.
53. Curtin PD (1969) *The Atlantic Slave Trade: A Census* (Univ of Wisconsin Press, Madison).
54. Campbell G (1981) Madagascar and the Slave Trade, 1810–1895. *J Afr Hist* 22(2): 203–227.
55. Moreira VML (2010) A guerra contra os índios botocudos e a formação de quilombos no Espírito Santo. [The war against the Botocudo Indians and the formation of African communities in Espirito Santo] *Afro-Asia* 41:57–83.
56. Rohland N, Hofreiter M (2007) Ancient DNA extraction from bones and teeth. *Nat Protoc* 2(7):1756–1762.