0014-4754/94/060592-10$1.50 + 0.20

Genetic variation in the New World: ancient teeth, bone, and tissue as sources of DNA

D. A. Merriwether*, F. Rothhammer[a] and R. E. Ferrell

Department of Human Genetics, University of Pittsburgh, 130 DeSoto St., (Pennsylvania 15108, USA) and [a]Department of Cell Biology and Genetics, University of Chile, Santiago (Chile)

Abstract. Examination of ancient and contemporary Native American mtDNA variation via diagnostic restriction sites and the 9-bp Region V deletion suggests a single wave of migration into the New World. This is in contrast to data from Torroni et al.[34] which suggested two waves of migration into the New World (the NaDene and Amerind). All four founding lineage types are present in populations in North, Central, and South America suggesting that all four lineages came over together and spread throughout the New World. Ancient Native American DNA shows that all four lineages were present before European contact in North America, and at least two were present in South America. The presence of all four lineages in the NaDene and the Amerinds argues against separate migrations founding these two groups, although admixture between the groups is still a viable explanation for the presence of all four types in the NaDene.
Key words. Mitochondrial DNA; ancient DNA; Native Americans.

Introduction

With the advent of the Polymerase Chain Reaction (PCR)[24] it has become possible to recover sufficient DNA from small amounts of biological material to perform fairly complex genetic analyses of both ancient and contemporary populations. This has enabled anthropologists, archaeologists, and geneticists to collaborate in the collection and analysis of ancient human remains and of contemporary populations. One of the most exciting applications of these collaborations has been the investigations into the peopling of the New World through the study of ancient and contemporary genetic variation[5,14,28]. A growing number of laboratories are investigating ancient and/or contemporary mtDNA variation[2,6,10–13,15–23,25–27,33–39]. The first mtDNA paper to investigate Amerindian mtDNA variation was Wallace et al.[36] (1985), using 6 restriction enzymes to generate restriction fragment length polymorphism (RFLP) haplotypes of each individual's entire mitochondrial genome. This analysis showed that Amerindian mtDNAs were a subset of Asian mtDNA types, but at different frequencies than the Asian types, providing good indications of a founder effect for the New World. The Wallace laboratory later expanded this to 14 and 18 enzyme RFLP haploytypes of both Amerindian and Asian populations[2,25,33–37] and revealed that all Native American mtDNA variation in the New World today was descended from just four maternal mtDNA lineages. These lineages (later labeled lineages A, B, C, and D) could be defined by a HaeIII 663 site gain, the presence of the Region V 9-bp deletion, a HincII 13259 site loss, and an AluI 5176 site loss respectively (numbers refer to the Anderson et al. reference sequence[1] base positions). All subsequent Native Americans possessing pure (non-admixed) maternal Amerindian ancestry can be traced back to one of these founding lineages. This work was later confirmed by sequence analysis of the mitochondrial D-Loop[6,12,16,27,34,35,38,39] by a number of different labs. The four founding lineages can now also be defined by a number of nucleotide substitutions in the D-Loop[6,12,16,17,27,34,35,38,39]. In addition, we can now be sure that we are dealing with Native American (or at least Asian) mtDNA types, since the HaeIII 663 site gain and the Region V 9-bp deletion (with some rare exceptions) are Asian-specific (or specific to populations of Asian descent such as the American Indians and Pacific Islanders). While the HincII 13259 and AluI 5176 site losses are not Asian-specific, they are Asian-specific when accompanied by AluI 10394 and DdeI 10397 site gains. In addition, some combinations of D-Loop sequence substitutions and the diagnostic restriction sites are also Asian or Amerindian-specific, enabling us to determine the 'Amerindian-ness' of a particular mtDNA sample. This is critical in establishing that the DNA under investigation is Amerindian, and not a modern laboratory contaminant.

Most of the initial research on the peopling of the New World has involved contemporary populations, although Hauswirth and colleagues[3,21] published ancient mtDNA data on the 8,000–9,000-year-old Windover site in 1986 and 1988. The large scale RFLP and D-Loop sequence population studies of Native Americans now provides an excellent data set with which we can probe ancient mtDNA variation. Population and Region-specific markers have been identified for a number of populations and geographic regions which will enable us to directly assess the relationsips between ancient and contemporary populations in the New World (as well as the relationships between different

ancient populations and between different contemporary populations). Examples of these types of studies will be presented later in this paper.

In addition to direct comparisons of mtDNA haplotypes, it is also possible to incorporate other types of data into an analysis. For example, on our Copan Mayan samples from the Maya Classic Period, we have stable isotope data for each individual to indicate diet, and burial status information indicating social status. The different mtDNA patterns observed can be compared with their status and diet to see if particular mtDNA lineages are associated with social status. Some skeletal remains are known to belong to sacrificial victims who were captured in wars with neighboring groups, and their mtDNA patterns can help elucidate what neighboring populations were like. There are remains at the Copan site spanning many hundreds of years, many associated with specific cultural phases. The mtDNA patterns from different cultural phases can be contrasted to see if these phases represent an importation of culture of the existing population, or a replacement of the existing population by a new culture. There are many possible applications for genetic data.

Materials and methods

Description of the populations and the contemporary samples appear in greater detail in Merriwether et al.[20]; however, table 1 contains the list of ancient samples used in this study and table 2 lists contemporary samples from our research and those from the literature. Figure 1 displays the map locations of many of the populations discussed in this paper.

Contamination precautions

Stringent precautions were employed to eliminate contamination of ancient samples by modern DNA and laboratory-DNA sources. Lab coats, gloves, and masks were worn during all extraction steps. Extractions took place under a laminar-flow hood which is under continuous ultraviolet light when it is not in use. Gloves were changed regularly to avoid cross-sample contamination. All reagents for extraction and amplification (except enzymes, Taq polymerase, and primers) were also UV irradiated in a Statagene UV crosslinker. All tubes were similarly irradiated. Pipettes were washed in 10% Chlorox bleach and then UV irradiated. Final PCR cocktails (containing everything but the target DNA and Taq polymerase) were then filtered through a 30,000 or 100,000 MW spin column to filter out any contaminating DNAs. Aerosol-free pipette tips were used exclusively to help prevent carry-over contamination. All reagents were kept separate in a separate location from where DNA was stored and amplified. Extractions took place in a separate lab under a laminar-flow hood, as did PCR set-up. Dedicated pipettes were used which had never been in contact with amplified DNA. Additional precautions relating directly to the materials being extracted are discussed in the following section.

Mummified tissue, bone and teeth

Preparation of mummified tissue, bone and teeth was carried out according to Pääbo[22].

Mummified tissue preparation. 0.1 to 1.0+ grams of mummified tissue were removed from an interior portion of each sample, placed in a disposable sterile Petri dish, and minced with a disposable scalpel blade. Some samples were pre-frozen in liquid nitrogen prior to mincing to aid the breakup of the tissue. The interior portions only were used for extraction to avoid contamination with modern DNA due to handling. The minced tissue was then placed in a 15 ml conical tube for further processing.

Bone preparation. The outside surfaces of bone pieces were washed in sterile distilled water, followed by 10% bleach, to remove previous contamination due to handling, and were allowed to dry. 0.1 to 1.0+ grams of bone (in this case all bones were ribs) were sealed in a Seal-A-Meal bag and suspended in liquid nitrogen until frozen, then crushed (inside the plastic bag) with a hammer or other heavy blunt object. Keeping the bone sealed in a bag helps prevent contamination from the liquid nitrogen, the hammer and containers. The bone fragments and dust were placed in a 15 ml conical tube for further processing.

Teeth preparation. Individual teeth were chosen for extraction based upon lack of caries and cracks and an intact closed root system. Molars and premolars were chosen over other teeth when a choice was possible because their size and root structure make them easier to crack open without destroying the tooth (this is important when the teeth must be returned to a collection). Teeth were then soaked in hydrogen peroxide

Table 1. Samples from this study (ancient)

Tribe (Location)	Sample size and site name	DNA source	Collected by:
Chinchorro (N. Chile)	12 Azapa	Mummy	Rothhammer
Inca (N. Chile)	4 Camarones	Mummy	Rothhammer
Fort Ancient (West Virginia)	12 Mann Site	Rib Bone	Reed
Maya (Honduras)	30 Copan	Rib Bone	Reed

Table 2. New World and Siberian samples from the literature (modern)

Tribe (location)	Sample size and village name	Number deleted	DNA source collected by
Aymara (N. Chile)	89 Visviri	53	
Aymara (N. Chile)	25 Caquena (Poll 1)	14	
Aymara (N. Chile)	13 Parinacota	7	
Aymara (N. Chile)	11 Codpa	7	
Aymara (N. Chile)	9 Guallatiri	5	
Aymara (N. Chile)	9 Putre	4	
Aymara (N. Chile)	13 Illapata	12	
Aymara (N. Chile)	16 Esquina	9	
Aymara (N. Chile)	15 Lluana Valley	8	
Aymara (N. Chile)	15 Guanacagua	15	
Pehuenche (S. Chile)	76 Trapa Trapa	7	
Pehuenche (S. Chile)	28 Butalelbum	1	
Huilliche (S. Chile)	91 Huilliche	23	Merriwether et al.[20]
Atacameno (C. Chile)	126 San Pedro de Atacama	93	
Quechua (S. Peru)	23 Quechua	7	
Mohawks (Canada)	172 Mohawks	17	
Dogrib (N. W. Canada)	134 Dogrib	0	
Muskoke (Oklahama)	71 Muskoke	11	
Eskimos (Alaska)	156 Old Harbor	4	
Eskimos (Alaska)	56 Ouzinkie	0	
Eskimos (Alaska)	72 Gambell	0	
Eskimos (Alaska)	68 Savoonga	0	
Eskimos (SW Alaska)	174 Yupiks	0	
Aleuts (Alaska)	78 St. Paul	0	
Nigerians	191 Nigerians	3	
Maya (Mexico)	37 Yucatec Maya	8	
Ticuna (Venezuela)	31 Ticuna	0	Torroni et al.[34]
Pima (Arizona)	31 Pima	14	
Navajo (Arizona)	48 Navajo	18	
Nuu-Chah-Nulth (SW Canada)	62	2	Ward et al.[38]
Athabaskan (Alaska)	22 Athabaskan	0	
Inupiaq Eskimo (Alaska)	16 Inupiaq	0	
Yup'ik (Alaska)	9 Yupik	0	
Yup'ik (Siberia)	25 Yup'ik	0	Shields et al.[26]
Chukchi	46 Chukchi	0	
Altai	25 Ust-Kan,	3	
(South Central Asia)	7 Chibit,	0	
	13 Ust-Ulagan	0	
Mapuche (Chile)	45 (9 villages)	9	
Colombian	20 (4 villages)	4	Horai et al.[12]
Bella Coola	25	2	Torroni et al.[33]
Inuit	35	0	
Dogrib	12	0	
Navajo	10	6	
Chipewa	18	2	
Cheyenne/Araphoe	5	1	
Kickapoo	7	2	
Bella Coola	11	0	
Cherokee	18	5	
Sioux	17	3	
Yokuls	8	3	
Choctaw	20	3	
Chickasaw	8	3	
Creek	18	2	
Zuni	20	11	Lorenz et al.[15]
Washo	17	5	
Hualapai	5	2	
Havasupai	5	2	
Yavapai	5	3	
Paipai	8	7	
Quechan	14	7	
Kumial	16	10	
Cochimi	13	6	
Chumash	8	0	
Jemez	14	9	
Nahua	16	3	
Pima	7	6	
Paiute	7	1	
Shoshone	7	1	

Table 2. (Continued)

Tribe (location)	Sample size and village name	Number deleted	DNA source collected by
Argentine Mapuche	39	15	Ginther et al.[6]
Ojibwa	43	3	Torroni et al.[33]
Boruca	14	10	
Kuna	16	0	
Guyami	16	5	
Bribri/Cabecar	24	11	
Yanomama	24	4	
Piaroa	10	0	
Makiritare	10	0	
Macushi	10	2	
Wapishana	12	3	
Ticuna	31	0	Torroni et al.[34]
Kraho	14	8	Torroni et al.[33]
Marubo	10	0	
Mataco	28	10	
Eskimos (Siberia)	50	0	Torroni et al.[35]
Chuckchi (Siberia)	24	0	
Koryaks (Siberia)	46	0	
Evens (Siberia)	43	0	
Yukagirs (Siberia)	27	0	
Nganasans (Siberia)	49	0	
Sel'Kups (Siberia)	20	0	
Nivkhs (Siberia)	57	0	
Udegeys (Siberia)	46	0	
Evenks (Siberia)	51	0	

The following list consists of populations from the literature with sample sizes of less than five. The names of each population is followed by the sample size, the number of deleted individuals in parthenteses, and the references is in superscript: Yaghan 2 (0)[20]; Tohono-O'odham Pomo 1 (0)[34]; Hopi 1 (1)[34]; Tlingit 2(0)[34]; Yukagir 4(0)[26]; Nanaika 1 (0)[26]; Lamut 1 (0)[26]; Chuvanka 2 (0)[26]; Koryak 1 (0)[26]; Brazilian 3 (1)[12]; Haida 4 (0)[15]; Hupa 2 (2)[15]; Apache 4 (1)[15]; Shawnee 2 (0)[15]; Narragansett, Acoma, Ponca, Hidatsa, Iowa, Winnebago, Otoe, Minnok, Winlun, Coos, Achomawai, Gabrielero, Luiseno, Kawaiisu, Cora, Cahuvilla, Juaneno 1 each (0)[15]; Yurok 1 (1)[15]; Pawnee 3 (2)[15]; Crow 1 (1)[15]; Ohlone 4 (3)[15]; Semoinole 3 (0)[15]; Karok 2 (1)[15]; Pomo 2 (1)[15]; Mojave 3 (2)[15]; Cocopa 3 (3)[15]; Kanya 3 (3)[15]; Salinan 4 (1)[15]; Kiowa 3 (0)[15]; Taos 2 (1)[15]; San Idellonso 1 (1); Hope 3 (3)[15]; Comanche 2 (2)[15]; Kilanernuk 4 (1)[15]; Tubatulabal 4 (2)[15]; Femandeno 1(1)[15].

(3–30%) for 10–30 min, rinsed with distilled water, and then rinsed thoroughly with 10% bleach to destroy any contaminating DNA on the surface. The tooth was then rinsed again with distilled water to remove any traces of the bleach, and was UV irradiated for 10 min. A sharp metal probe (fire and bleach sterilized) was gently hammered into the dentine between the roots of the tooth just below the crown to crack the tooth open and expose the pulp. The two halves of the tooth were then placed in a 15 ml conical tube for further processing.

Digestion

To each prepared sample (still in a 15 ml conical tube) was added ~4 ml of extraction buffer (10 mM Tris-HCl pH 8.01, 2 mM EDTA, 10 mg/ml DTT, 0.5 mg/ml Proteinase K, 0.1% SDS). Each tube was then incubated overnight at 37 °C with slow agitation (teeth were incubated for 48 to 72 h at 37 °C).

Phenol extraction. An equal volume of phenol was added to each tube and the tubes were rotated at 37 °C for 10 min. The tubes were spun down to 1500–3000 rpm to separate the phases, and the aqueous layers were removed and placed in new 15 ml tubes. This extraction was then repeated once with phenol and finally with 24:1 chloroform:isoamyl alcohol.

Purification. The aqueous layer from the chloroform:isoamyl alcohol extraction was placed in a 30,000 MW cutoff microconcentrator (Millipore Ultra-Free MC; or Amicon Centricon or Microcon 30). If the volume exceeded the 0.4 ml volume of the microcentrators, then the extract fluid was added after each spin until it had all been added. The samples were spun down to the hold-up volume of the concentrator, until all of the aqueous layer of each extraction had been concentrated. Then the samples were washed 2–3 times in distilled water, and finally once in 1M Tris-EOTA pH 8.0. The samples were then used directly as template for PCR reactions.

Basic PCR protocol

The basic PCR protocol was carried out according to Saiki et al.[24]. 30 µl reactions were carried out using a Perkin-Elmer Cetus 9600 thermocycler using 3 µl of 10 × PCR buffer, 0.7 µl of each primer (20 µM), 1.2 µl of dNTP mix (10 mM), 20.5 µl dH_2O, and 0.2 µl of Taq or Polymerase (5 units/µl). 5 µl of extract DNA and

Figure 1. Map of the distribution of some of the populations described in this study, and from the published literature. Underlined populations indicate 'ancient' population locations.

25 µl of reaction cocktail was then added to each tube. The tubes were sealed and placed in the thermocycler. Cycling parameters varied with the primer sets utilized and the template. Cycling on the 9600 typically consisted of a 30 µl reaction running for 37 cycles of 94 °C for 0.5 min, 55 °C for 0.25 min, and 72 °C for 0.25 min, followed by a 4 °C soak.

It was necessary to dilute the original DNA extract 50:1 or 100:1 to achieve amplification with many samples. This was due to some form of inhibitor present in the extract. This was demonstrated by placing modern DNA extracts in a tube with ancient DNA extracts and observing a dramatic decrease in amplified product. In some cases, no product was observed at all. Not all samples required this dilution, but most did. The extracts often contained a brown, red, yellow, or black color, even after all of the extraction and purification steps.

Results

Extractions were carried out on many different tissue types, including liver, kidney, muscle, rib bone, and teeth. In one mummified individual where multiple tissues were available for extraction, kidney and muscle extracts yielded more DNA than liver, heart or lung tissue when equal volumes of tissue were extracted from different parts of the same individual. All individuals were tested for Schurr et al.'s[25] four founding lineage types, as well as the Asian-specific markers described above. Few individuals were amplifiable for all five primer sets necessary to specify the four lineages and the two Asian-markers. This may be remedied by purifying the samples further using silica to bind the DNA, a process we are now trying in the lab. In any case, the results are only of use in direct comparison to modern mtDNA data on Native Americans. Figure 2 shows the frequencies of the 9-bp deletion in a large number of worldwide populations. Figure 3 shows the frequencies of each of the four founding lineage types (A, B, C and D). Ancient DNA frequencies in figures 2 and 3 are indicated by the underlined populations. Note the low frequency or absence of the Region V deletion in any of the New World ancient populations. It is especially striking in Northern Chile, where the deletion is absent in the Azapa Valley mummies of the Chinchorro and Alto Ramirez cultures studied in this paper and by

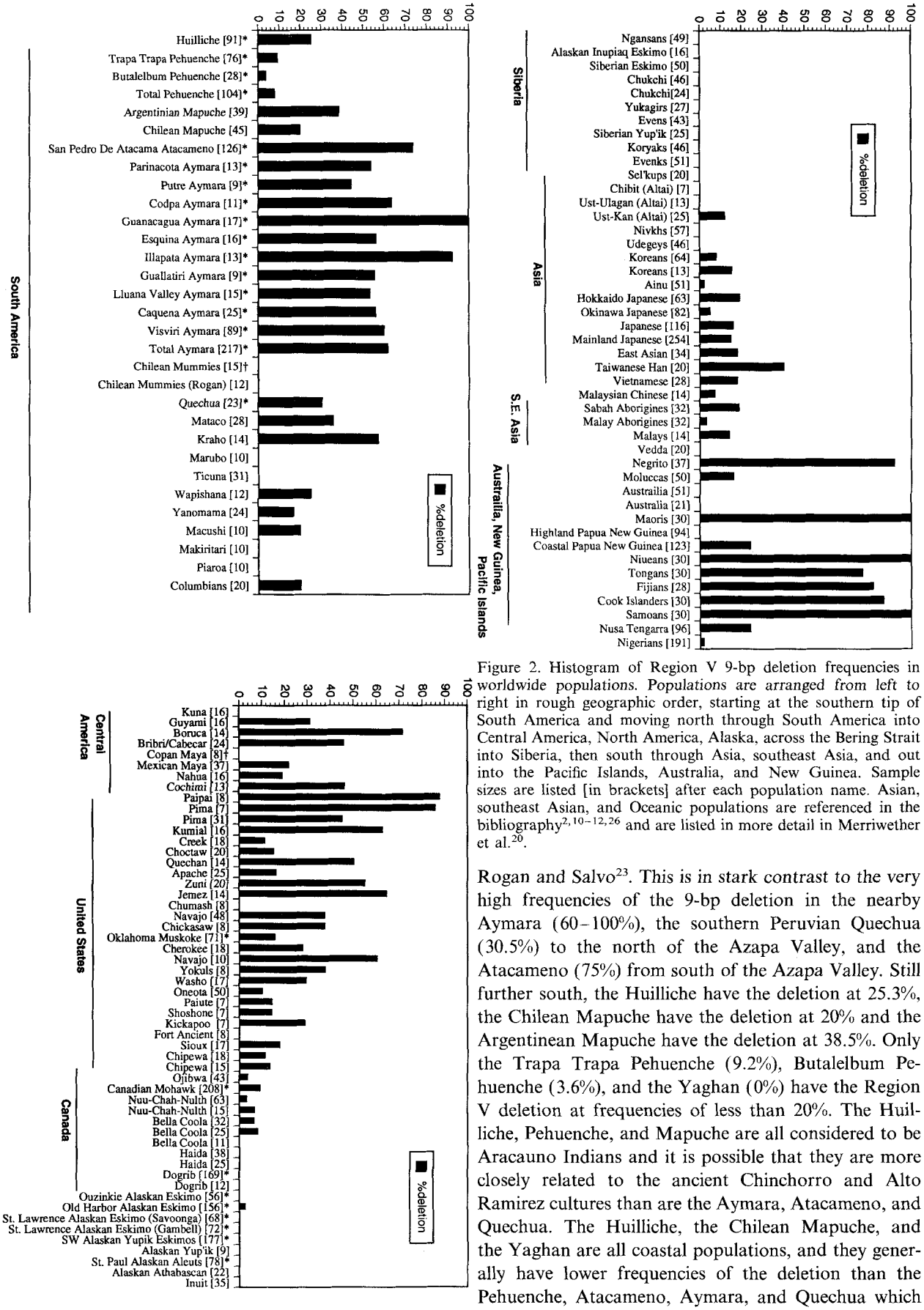

Figure 2. Histogram of Region V 9-bp deletion frequencies in worldwide populations. Populations are arranged from left to right in rough geographic order, starting at the southern tip of South America and moving north through South America into Central America, North America, Alaska, across the Bering Strait into Siberia, then south through Asia, southeast Asia, and out into the Pacific Islands, Australia, and New Guinea. Sample sizes are listed [in brackets] after each population name. Asian, southeast Asian, and Oceanic populations are referenced in the bibliography[2,10–12,26] and are listed in more detail in Merriwether et al.[20].

Rogan and Salvo[23]. This is in stark contrast to the very high frequencies of the 9-bp deletion in the nearby Aymara (60–100%), the southern Peruvian Quechua (30.5%) to the north of the Azapa Valley, and the Atacameno (75%) from south of the Azapa Valley. Still further south, the Huilliche have the deletion at 25.3%, the Chilean Mapuche have the deletion at 20% and the Argentinean Mapuche have the deletion at 38.5%. Only the Trapa Trapa Pehuenche (9.2%), Butalelbum Pehuenche (3.6%), and the Yaghan (0%) have the Region V deletion at frequencies of less than 20%. The Huilliche, Pehuenche, and Mapuche are all considered to be Aracauno Indians and it is possible that they are more closely related to the ancient Chinchorro and Alto Ramirez cultures than are the Aymara, Atacameno, and Quechua. The Huilliche, the Chilean Mapuche, and the Yaghan are all coastal populations, and they generally have lower frequencies of the deletion than the Pehuenche, Atacameno, Aymara, and Quechua which

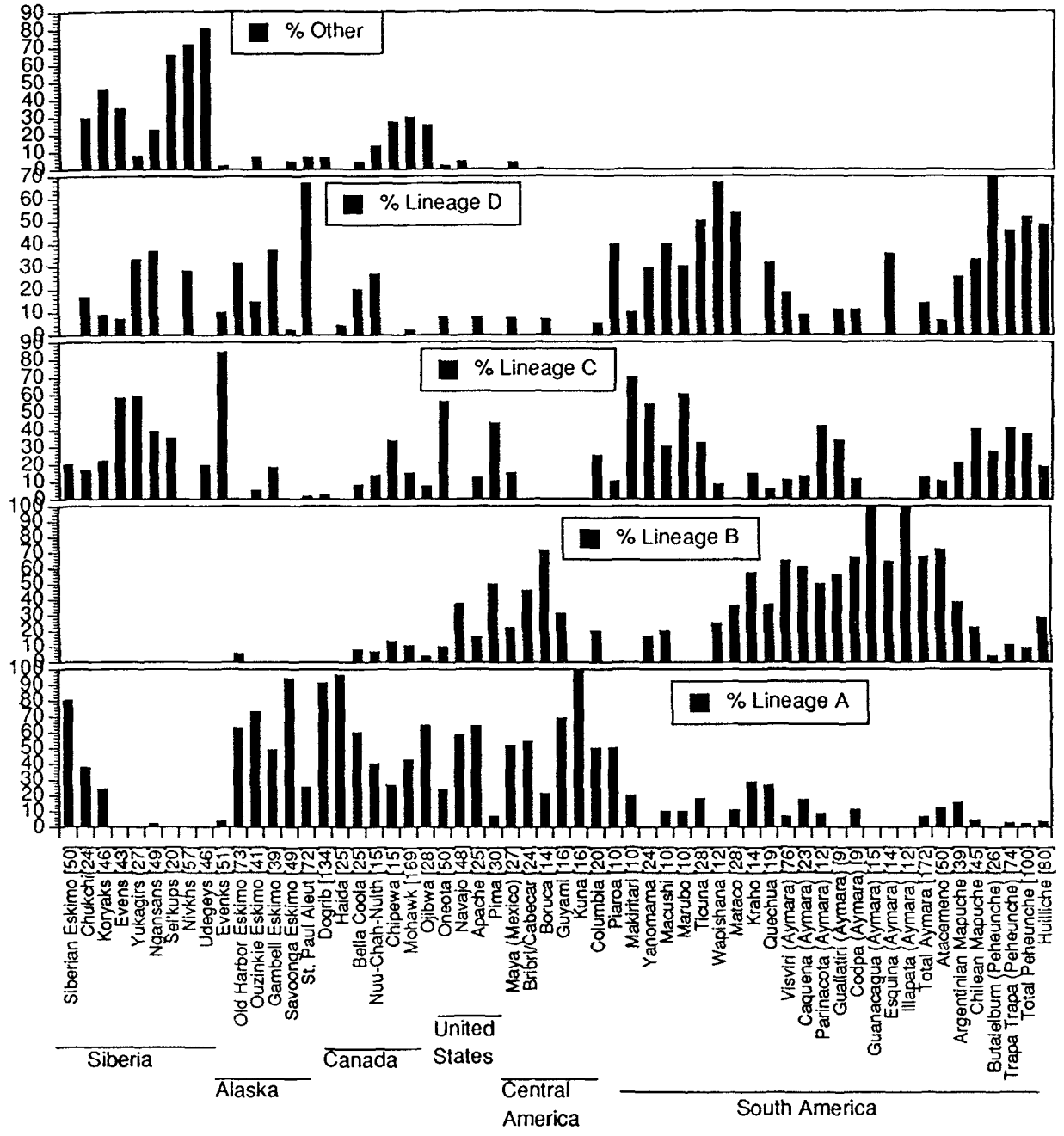

Figure 3. Histogram of the frequencies of the four founding lineage types in Siberia and the New World. All non-founding lineage types are classified as 'other'. Populations are arrayed in rough geographic order starting in Siberia and moving across the Bering Strait into Alaska, then southward through North, Central, and South America, down to the southern tip of South America. Sample sizes are listed [in brackets] after each population name.

are not coastal (and are generally high altitude populations). The Azapa Valley archaeological sites are not high altitude sites, and the low deletion frequency in both the ancient and contemporary coastal populations may indicate shared ancestry. This may also indicate that the coastal populations have a different origin from the altiplano cultures. Much higher resolution data will be necessary to address this question, and mtDNA D-loop sequence data has already been collected on representatives of many of the contemporary populations. D-loop sequencing on the ancient samples is the next step. The lack of the deletion in ancient Azapa Valley individuals may well be due to inadequate sampling, and we are now extracting DNA, and typing 50 additional Chinchorro and 30 additional Alto Ramirez individuals.

Mayans from the Classic Period at Copan (a lowland Maya city) were all lineage C or lineage D, in direct

contrast to the contemporary Mexican Maya (Yucatec speakers) from the Yucatan Peninsula which had all four lineages, but were primarily lineage A and B. Once again, small sample sizes may be the reason for this finding, and more individuals are being typed. Additional Late Classic Maya individuals from the city of Iximche in Guatemala (a highland Maya capital) are being typed. This may shed some light on the differences between highland and lowland Maya, and on the present day descendants of the great Maya empire.

Lastly we noted that all the Fort Ancient individuals typed from West Virginia and from Ohio were devoid of the deletion, and the West Virginia Fort Ancient individuals were all lineage C. We are expanding our study to include over 150 additional Fort Ancient, Monongehela, and Adena individuals from Pennsylvania, Ohio, and West Virginia in order to gain a better picture of these extinct Ohio River Valley cultures, and to gain clues as to their linguistic affiliations through comparison to contemporary Northeastern Amerindian groups.

Discussion

A number of interesting features of the peopling of the New World have come from the growing body of mtDNA literature on both ancient and contemporary populations. The finding by Schurr et al.[25] that all Native Americans could trace their maternal heritage back to just one of four founding lineages has had a profound effect on how one now examines the existing variation in the New World. Plotting the distribution of these lineages against putative patterns of migration into and throughout the New World has yielded some interesting (and sometimes conflicting) conclusions[12,20,26,27,31,33–39]. Merriwether et al.[20] note that the four lineages are spread throughout North, Central, and South America and all four lineages are present in many populations. In fact, the vast majority of populations in which 10 or more individuals were surveyed report at least three of the four lineages being present. Merriwether et al.[20] argue that this distribution can best be explained by a single migration into the New World which contains all four of these lineages. It is inconceivable that a smaller number of lineages entered (A, C, and D), and that a later lineage (B) migrated into the same areas throughout the entire New World. The data from Torrino et al.[33–35] demonstrate that within lineage B divergence is much less than the divergence within any of the other three lineages, indicating a more recent origin (or entry) for the lineage B individuals. Merriwether et al.[20] suggest that this may be due to multiple members of lineages A, C, and D entering the New World which carried within-lineage variants for each lineage, and that lineage B brought over only one type (or far fewer lineage B variants). Variation which pre-dates the expansion into the New World would seem to be the best explanation for the apparently large difference in coalescence times observed between lineage B and any of the other three lineages. It would be useful to see larger sample sizes examined, from both Amerind and NaDene populations, and then to look at within-population within-lineage variation for multiple populations. This is needed to give a more accurate picture of the age of each lineage, and whether or not there are multiple arrivals of some lineages. The lack of divergence of the lineage B individuals needs to be explained, if it is a real and pervasive phenomenon in New World populations. It is here that ancient DNA may contribute to the discussion. To date, there have been no reported appearances of the 9-bp deletion in (the relatively small number of) samples that have been typed from South America which are over 500 years old. Indeed, no 'ancient' population has displayed high frequencies of the deletion in the New World. Once again, sample sizes have been very small for most studies. Stone et al.[29] did report the presence of the 9-bp deletion in a pre-historic Oneota cemetry in about 10% of the 50 individuals typed, so it is certainly present in the New World, and unpublished work has shown that it was present in North America at least 8,000 years ago. Sequencing of these deleted individuals should shed more light on the question of the amount of variation contained in the lineage B individuals who originally populated the New World.

Torroni et al.[33] proposed that there was evidence for two separate waves of migration (Amerind first, and NaDene later) into the New World, once again based upon the difference in the amounts of divergence within the Amerinds and within the NaDene. Once again, larger scale within-population within-linage comparisons are necessary to better evaluate this hypothesis. The larger number of Amerind populations may be influencing the fact that there is greater within-Amerind divergence than within-NaDene divergence. Torrini et al.[33–35] note that the NaDene are primarily lineage A, and this is confirmed by the data in figure 2. However, figure 2 demonstrates that all North American populations (except the St. Paul Aleuts, Pima, and the ancient Oneota) are predominately lineage A, and lineage A is the next most common type in the St. Paul Aleuts and the ancient Oneota. Furthermore, Merriwether et al.[20] studied over 100 additional individuals from the same Dogrib population as Torroni et al.[33] and found that the Dogrib also possess lineage C at low frequency as well as some (presumably) Caucasian haplotypes. In fact, when all of the other NaDene populations sampled to date are examined, they all possess lineages other than A. The Haida have lineages A and D, the Navajo have lineages A and B, and the Apache have all four lineages. Torroni et al.[33–35] explain this as admixture with Amerind groups, and this may well be the best explanation, but we have no basis for evaluating this

hypothesis. The NaDene look remarkably similar to other North American Native American populations. Many of the NaDene do possess a unique (NaDene-specific) RsaI 16329 restriction site gain[33] indicating shared heritage best explained by descent from a common NaDene founding population. Here again, ancient DNA is the best suited approach to evaluate between competing hypotheses about the origin of the NaDene. Ancient NaDene must be sampled and typed to see which of the four founding lineages they possess, and to see if additional variation was lost. Bottlenecking of the NaDene could explain the reduced within-NaDene divergence time in comparison to the within-Amerind divergence time. The large number and variety of Athabaskan-speaking NaDene found today may argue against such a bottleneck. Ewens-Watterson[5,40,41] and Tajima [32] tests would be useful to see if NaDene populations are in equilibrium. If they had passed through a bottleneck severe enough to halve the within-population variation (as compared to the Amerinds) then we would expect this to show up in these tests. Indeed, we would expect to see an excess of the most common haplotypes and of singleton (or rare, newly mutated) haplotypes[18]. The data exist to test some of these hypotheses right now for modern populations.

In summary, there are still exciting and important questions to be resolved about the timing, size, and routes of migration into the New World, and ancient DNA is emerging as the best candidate to choose between the different scenarios.

Acknowledgments. Many thanks to D. Reed for providing the Copan and Fort Ancient samples and to S. Barton and C. Orrego for help forwarding the Azapa Valley samples. We thank E. Szathmary for providing the Dogrib samples and for useful discussions of the problem of the peopling of the Americas. Thanks to the Wenner Gren Foundation for providing financial support to D.A.M. and R.E.F., and to the W. M. Keck Center for Advanced Training in Computational Biology for providing support for D.A.M.

* Corresponding author

1 Anderson, S., Bankier, A. T., Barrel, B. G., DeBulin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G., Sequence and organization of the human mitochondrial genome. Nature *290* (1981) 457–465.

2 Ballinger, S. W., Schurr, T. G., Torroni, A., Gan, Y. Y., Hodge, J. A., Hassan, K., Chen, K.-H., and Wallace, D. C., Southeast Asian mitochondrial DNA analysis reveals genetic continuity of ancient Mongoloid migrations. Genetics *130* (1992) 139–152.

3 Doran, G. H., Dickel, D. N., Jr, W. E. B., Agee, O. F., Laipis, P. J., and Hauswirth, W. W., Anatomical, cellular and molecular analysis of 8,000-year-old human brain tissue from the Windover archaeological site. Nature *323* (1986) 803–806.

4 Ewens, W. J., The sampling theory of selectively neutral alleles. Theor. Popul. Biol. *3* (1972) 87–112.

5 Ferrell, R. E., Bertin, T., Barton, S. A., Rothhammer, F., and Schull, W. T., The multinational Andean genetic and health program. IX. Gene frequencies and rare variants of 20 serum proteins and erythrocyte enzymes in the Aymara of Chile. Am. J. hum. Genet. *32* (1980) 92–102.

6 Ginther, C., Corach, D., Penacino, G. A., Rey, J. A., Carnese, F. R., Hutz, M. H., Anderson, A., Just, J., Salzano, F. M., and King, M.-C., Genetic Variation among the Mapuche Indians from the Patagonian region of Argentina: Mitochondrial DNA sequence variation and allele frequencies of several nuclear genes, in: DNA Fingerprinting: State of the Science, pp. 211–219. Eds S. D. J. Pena, R. Chakraborty, J. T. Epplen and A. J. Jeffreys. Birkhäuser Verlag, Basel 1993.

7 Greenberg, J. H., The General Classification of Central and South American Indian Languages, in: Selected Papers of the Fifth International Congress of Anthropological and Ethnological Sciences. A. F. C. Wallace. University of Pennsylvania Press, Philadelphia 1960.

8 Greenberg, J. H., Language in the Americas. California Stanford University Press, Palo Alto 1987.

9 Greenberg, J. H., Turner, C. G., II, and Zegura, S. L., The settlement of the Americas: A comparison of linguistic dental and genetic evidence. Curr. Anthrop. *27* (1986) 477–497.

10 Harihara, S., Momoki, H., Suutou, Y., Shimizu, K., and Omoto, K., Frequency of the 9-bp deletion of mitochondrial DNA among Asian populations. Hum. Biol. *64* (1992) 161–166.

11 Hertzberg, M., Mickleson, K. N. P., Serjeanston, S. W., Prior, J. F., and Trent, R. J., An Asian specific 9-bp deletion of mitochondrial DNA is frequently found in Polynesians. Am. J. hum. Genet. *44* (1989) 504–510.

12 Horai, S., Kondo, R., Nakagawa-Hattori, Y., Hayasaki, S., Sonoda, S., and Tajima, K., Peopling of the Americas, founded by four major lineages of mitochondrial DNA. Molec. Biol. Evol. *10(1)* (1993) 23–47.

13 Johnson, M. J., Wallace, D. C., Ferris, S. D., Rattazzi, M. C., and Cavalli-Svorza, L. L., Radiation of human mitochondrial DNA types analyzed by restriction endonuclease cleavage patterns. J. molec. Evol. *19* (1983) 255–271.

14 Laughlin, W. S., and Harper, A. B., (Eds) The First Americans: Origins, Affinities, and Adaptations. Gustav Fischer, New York 1979.

15 Lorenz, J. G., and Smith, D. G., Distribution of the 9-bp mitochondrial DNA Region V deletion among North American Indians. Hum. Biol. (1994) in press.

16 Merriwether, D. A., Mitochondrial DNA D-loop sequence variation in Native South Americans. Am. Soc. hum. Genet. (1993).

17 Merriwether, D. A., Mitochondrial DNA Variation in South American Indians. (1993) University of Pittsburgh, Ph.D. Dissertation.

18 Merriwether, D. A., Clark, A. G., Ballinger, S. W., Schurr, T. G., Soodyall, H., Jenkins, T., Sherry, S. T., and Wallace, D. C., The structure of human mitochondrial DNA variation. J. molec. Evol. *33* (1991) 543–555.

19 Merriwether, D. A., Rothhammer, F., and Ferrell, R. E., Mitochondrial DNA variation in ancient and contemporary Amerindians using the $tRNA^{LYS}$-COII deletion and diagnostic restriction sites. Am. J. hum. Genet. *51(4)* (1992) A13.

20 Merriwether, D. A., Rothhammer, F., and Ferrell, R. E., Distribution of the four-founding lineage haplotypes in Native Americans suggests a single wave of migration for the New World. Hum. molec. Genet. (1994) submitted.

21 Pääbo, S., Gifford, J. A., and Wilson, A. C., Mitochondrial DNA sequences from a 7000-year old brain. Nucl. Acids Res. *16(20)* (1988) 9775–9787.

22 Pääbo, S., Higuchi, R. G., and Wilson, A. C., Ancient DNA and the polymerase chain reaction. J. biol. Chem. *264* (1989) 9709–9712.

23 Rogan, P. K., and Salvo, J. J., Molecular genetics of pre-Columbian South American mummies. Molec. Cell. Biol. *122* (1990) 223–234.

24 Saiki, R. K., Gelfland, D. H. Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A., Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239* (1988) 487–491.

25 Schurr, T. G., Ballinger, S. W., Gan, Y. Y., Hodge, J. A., Merriwether, D. A., Lawrence, D. N., Knowler, W. C., Weiss, K. M., and Wallace, D. C., Amerindian mitochondrial DNAs have rare Asian mutations at high frequencies suggesting a

limited number of founders. Am. J. hum. Genet. *46* (1990) 613–623.
26 Shields, G. F., Hecker, K., Voevoda, M. I., and Reed, J. K., Absence of the Asian-spacific region V mitochondrial marker in Native Beringians. Am. J. hum. Genet. *50* (1992) 758–765.
27 Shields, G. F., Schmiechen, A. M., Frazier, B. L., Redd, A., Voevoda, M. I., Reed, J. K., and Ward, R. H., mtDNA sequences suggest a recent evolutionary divergence for Beringian and Northern North American populations. Am. J. hum. Genet. *53(3)* (1993) 549–562.
28 Shutler, R. Jr, (Ed.) Early Man in the New World. Sage Publications, Inc., Beverly Hills, CA 1983.
29 Stone, A. C., and Stoneking, M., Ancient DNA from a pre-Columbian Amerindian population. Am. J. phys. Anthrop. *92* (1994) 463–471.
30 Szathmary, E. J. E., Genetic markers in Siberian and northern North American populations. Yb. phys. Anthrop. *24* (1981) 37–73.
31 Szathmary, E. J. E., mtDNA and the peopling of the Americas. Am. J. hum. Genet. *53* (1993) 793–799.
32 Tajima, F., Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics *123* (1989) 585–595.
33 Torroni, A., Schurr, T. G., Cabell, M. F., Brown, M. D., Neel, J. V., Larsen, M., Smith, D. G., Vullo, C. M., and Wallace, D. C., Asian affinities and continental radiation of the four founding Native American mitochondrial DNAs. Am. J. hum. Genet. *53* (1993) 563–590.
34 Torroni, A., Schurr, T. G., Yang, C.-C., Szathmary, E. J. E., Williams, R. C., Schanfield, M. S., Troup, G. A., Knowler, W. C., Lawrence, D. N., Weiss, K. M., and Wallace, D. C., Native American mitochondrial DNA analysis indicates that the Amerind and the NaDene populations were founded by two independent migrations. Genetics *130* (1992) 153–162.
35 Torroni, A., Sukernik, R. I., Schurr, T. G., Starikovskaya, Y. B., Cabell, M. F., Crawford, M. H., Comuzzie, A. G., and Wallace, D. C., Mitochondrial DNA variation of aboriginal Siberians reveals distinct affinities with Native Americans. Am. J. hum. Genet. *53* (1993) 591–608.
36 Wallace, D. C., Garrison, K., and Knowler, W. C., Dramatic founder effects in Amerindian mitochondrial DNAs. Am. J. phys. Anthrop. *68* (1985) 149–155.
37 Wallace, D. C., and Torroni, A., American Indian prehistory as written in the mitochondrial DNA: A review. Hum. Biol. *64(3)* (1992) 403–416.
38 Ward, R. H., Frazier, B. L., Dew-Jager, K., and Pääbo, S., Extensive mitochondrial diversity within a single Amerindian tribe. Proc. natl Acad. Sci. USA *88* (1991) 8720–8724.
39 Ward, R. H., Redd, A., Valencia, D., Frazier, B., and Pääbo, S., Genetic and linguistic differentiation in the Americas. Proc. natl Acad. Sci. USA *90* (1993) 10663–10667.
40 Watterson, G. A., The homozygosity test of neutrality. Genetics *88* (1978) 405–417.
41 Watterson, G. A., Allele frequencies after a bottleneck. Theor. Popul. Biol. *26* (1984) 387–407.