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**KEY WORDS** ancient DNA; ABO blood group; entrance of humans into the Americas; ancestry informative markers

**ABSTRACT** In this study, we explore the geographic and temporal distribution of a unique variant of the O blood group allele called \(O1^V{G542A}\) which has been shown to be shared among Native Americans but is rare in other populations. \(O1^V{G542A}\) was previously reported in Native American populations in Mesoamerica and South America, and has been proposed as an ancestry informative marker. We investigated whether this allele is also found in the Tlingit and Haida, two contemporary indigenous populations from Alaska, and a pre-Columbian population from California. If \(O1^V{G542A}\) is present in Na-Dene speakers (i.e., Tlingits), it would indicate that Na-Dene speaking groups share ancestry with other Native American groups and support a Beringian origin of the allele, consistent with the Beringian Incubation Model.

Native American branches of the human mitochondrial tree are differentiated from their Asian sister branches by a number of unique mutations (Tamm et al., 2007; Achilli et al., 2008; Fagundes et al., 2008b; Peregó et al., 2009, 2010). This pattern is consistent with the “Beringian Incubation Model” (or “BIM”), which posits a period of isolation of the proto-Native American population prior to entering the Americas, during which this differentiation accumulated via mutation and genetic drift (Tamm et al., 2007; Fagundes et al., 2008a; Kitchen et al., 2008; Mulligan et al., 2008). While most Native Americans are the direct maternal descendants of a single Asian source population that migrated into the Americas approximately 15,000–20,000 years before present (YBP) (see recent review by Kemp and Schurr (2010)), mitochondrial DNA (mtDNA) evidence also supports more recent migration of humans into North America carrying subhaplogroups D2a and D3, which is largely associated with Aleut and Eskimo prehistory (Tamm et al., 2007; Crawford et al., 2010). The BIM does not specify the number of migrations into the American continents, as multiple waves could have originated from the same Beringian population.

If the BIM is correct, it predicts that a number of Native American ancestry informative markers (AIMs) exist across the genome that evolved prior to the initial entry of humans into the Americas and, thus, should be widely distributed in both North and South American populations. The 9 repeat allele (9RA) at the D9S1120 locus represents one such marker, as it has been observed in all Native American populations genotyped at the locus, yet is absent from all other world-wide populations that have been similarly screened, except for the Chukchi and Koryaks across the Bering Strait (Schroeder et al., 2007, 2009). The distribution of the Y-chromosome M3 single nucleotide polymorphism (SNP) (Underhill et al., 1996), which defines the Q1a3a1a branch of the human Y-chromosome tree,

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largely mirrors the geographic pattern of the 9RA (Underhill et al., 1996; Karafet et al., 1997, 1999, 2001; Lell et al., 1997, 2002; Bortolini et al., 2003; Zegura et al., 2004; Dulik et al., 2012a, 2012b). The presence of the 9RA and M3 SNP in Na-Dene and Aleut-Eskimos speakers is also important because it supports a shared origin of these two groups with all other Native American populations. Because multiple waves of migration could have originated from the same ancestral population at different points in time, and because of the random nature of genetic drift, it is possible for populations that have diverged from a common ancestor to exhibit different mitochondrial markers and yet share a vast part of their nuclear genome. The strength of genetic drift is four times greater on the mitochondrial genome than it is on any autosomal DNA locus. Additional Native American AIs have been identified, such as the ABCA1 (ATP-binding cassette transporter A1), C230 variant (Acuna-Alonzo et al., 2010; Hunemeier et al., 2012), or Albumin variants Mexico and Naskapi (Smith et al., 2000), which are very widespread, but these probably evolved after the last entry into the Americas and, thus are not informative to the BIM.

An alternative hypothesis, originally posited by Greenberg et al. (1986), details separate source populations for “Amerinds,” Na-Denes, and Aleut-Eskimos, groups that are argued to have entered the Americas in that order. A recent genome-wide study by Reich et al. (2012) has found support for at least three streams of gene flow from Asia into the Americas. Most Native Americans were found to descend from a single source population, which Reich et al. (2012) termed “First America.” Reich et al. (2012) found that Na-Dene and Eskimo-Aleut populations derive most of their genomes from the First American stream, but each also traces some ancestry to additional streams of gene flow into the Americas (where the Na-Dene Chipewyan derive ~10% of their ancestry from a second stream and Eskimo-Aleuts derive ~43% of their ancestry from a third stream). The First American population may, at first glance, appear nearly synonymous with Greenberg et al.’s (1986) Amerind, a controversial linguistic grouping (Nichols, 1990, Campbell, 1997). However, because the majority of Na-Dene and Eskimo-Aleut ancestry originates from the First American population (Greenberg et al., hypothesis), First American is not equivalent to or distinct from Na-Dene and Eskimo-Aleut (as Amerind is). However, because all Native American populations are largely descended from the same ancestral population, and because they diverged recently from Asian populations, it is expected they carry a majority of shared polymorphisms. This is the case for genome-wide analyses, in which the majority of polymorphisms have little informative power for resolving the specific histories of Native American populations. Focusing on polymorphisms that are unique to the Americas and absent in Asian populations may be more informative for resolving the specific relationships between Native American populations.

Early evidence that Native Americans are genetically distinct from other human populations was found in the ABO blood group system. Many Native American populations are fixed for O alleles, a pattern that is not found in any other human populations (Swerdlow et al., 1994; Molnar, 2002; Llop et al., 2006). While the O allele is not the only allele present in Native American populations, there is a very strong North-South decline in the frequencies of the A and B alleles, which are found predominantly in populations belonging to the Na-Dene and Aleut-Eskimos linguistic groups (Szathmary, 1979). Indigenous populations of South America are fixed for the O allele (Mourant et al., 1976).

Most O alleles evolved from A alleles by a deletion in position 261 of exon 6 of the ABO gene. This mutation “A261” creates a premature stop codon, resulting in a truncated protein lacking glycosyltransferase function (Gagneux and Varki, 1999). Although, O alleles are not deleterious, the shortened protein is no longer functional, and thus the gene accumulates mutations in a neutral fashion. Among all human populations there are at least 40 neutral variants of the O allele (Yip, 2002).

Sequence level data has provided additional resolution of O allele diversity exhibited by Native Americans (Estrada-Mena et al., 2010). Some alleles may be restricted to a single population, such as O05 and Ov7 that have, thus far, been exclusively described in the Cayapa of Ecuador and O32 and O33 in the Aymara of the Bolivian Andes (Estrada-Mena et al., 2010). Other less derived O alleles, for example, O1 and O1v, are shared between all prehistoric and contemporary Native American populations sampled to date (Halverson and Bolnick, 2008; Estrada-Mena et al., 2010; Georges et al., 2012), as well as with Old World populations (Chester and Olsson, 2001). One allele of particular interest, dubbed “O1vG542A,” has been found in various Mesoamerican and South American populations (Table 1), and may very well be unique to the Americas because it was not observed in Japanese, Chinese, and Koreans (Estrada-Mena et al., 2010). If O1vG542A is truly a unique American allele and is found in all Native American populations, it would support a single ancestral population for those that carry the allele, consistent with the BIM, as argued by Estrada-Mena et al. (2010).

However, two questions must be answered before the O1vG542A allele can be considered a Native American AIM or evidence for the BIM. First, how widespread is the O1vG542A allele in the Americas? Is it found throughout the Americas, or is it restricted to Mesoamerica and South America? Because indigenous populations north of central Mexico have not yet been screened for this allele, it is possible that the defining mutation is found only in Mesoamerica and South America. If that is the case, then the continued ancestry of the Native Americans to the Americas, and its frequency in Mesoamerica and South America would reflect the population history of those regions rather than the initial migration of people into the Americas.

Second, was the O1vG542A allele present in the Americas in prehistoric times, or was it introduced by admixture after European contact in AD 1492? No pre-contact populations have yet been screened for this allele. The O1vG542A mutation has been reported in at least one individual in an European population (Yip, 2000) and one individual in a Middle-Eastern population (Yip et al., 2006). Therefore, it is possible the allele may have been introduced from the Old World after AD 1492. Alternatively, the presence of O1vG542A in Europe and the Middle East might be due to mutational homoplasy at the ABO locus or even postcontact movement from the Americas to the Old World.

To address these two questions, we screened a large number of Tingits from southeastern Alaska, a more limited sample of Haida and Inuit from Alaska, and one ancient pre-Colombian population from California for the O1vG542A allele. The analysis of these populations...
allowed us to determine if O1vG542A is found in more widespread Native American populations than previously described and if the allele was present in the Americas prior to European contact.

If the O1vG542A allele is found in these populations, it would suggest that the allele originated before their divergence, with all North and South American populations inheriting this allele from a common ancestral population. It is possible, however, that this allele is common to all of these populations due to gene flow between the separate “waves” of migrants that introduced genetic variation to the American continents, following the observations of Reich et al. (2012). In this case, a simple migration model can be used to estimate the level of gene flow between populations that is required to explain the modern frequencies of O1vG542A and whether this level of migration is feasible based on our current understanding of Native American prehistory.

**METHODS**

**Indigenous Alaskan samples**

All Tlingit, Haida, and Inuit samples were collected by Brian M. Kemp and Kari B. Schroeder during Celebration 2008 in Juneau, Alaska. This study was approved by the Institutional Review Board (IRB # 10379) at Washington State University. After a thorough review of the purposes and use of the DNA study and samples, Sealaska Heritage Institute agreed to support the study in its Celebration.

The Tlingit language is probably related to Eyak and Athapaskan languages (Campbell, 1997; Vajda, 2010), which together represent a linguistic macrogroup that is nearly synonymous with “Na-Dene” as envisioned by Greenberg et al. (1986). However, the inclusion of Haida in Na-Dene is a controversial addition (Campbell, 1997). Mitochondrial and Y-chromosomal DNA variation exhibited by the Tlingit and Haida provide no compelling evidence for very recent common ancestry between these two populations (Schurr et al., 2012). In the study of Reich et al. (2012), the Chipewyan, a population belonging to the Athapaskan language family, was used as a representative of Na-Dene. For comparison, the Tlingit are considered Na-Dene in this study, but with the above caveat about the validity of this linguistic grouping in mind. The Haida are not considered to be a Na-Dene population here, but they are informative about how widespread the O1vG542A allele is in southeast Alaska. The Inuit belong to the Aleut-Eskimo linguistic group (Campbell, 1997).

All DNA was extracted from saliva using the Norgen Saliva DNA Isolation Kit (Cat# RU45400). Due to polymerase chain reaction (PCR) inhibitors coextracted with these samples, DNA extracts were diluted 1:10. Seventy-two Tlingit samples, four Haida, and three Inuit were typed for ABO alleles using a modified PCR-RFLP method following Hummel et al. (2002). These samples represent individuals for which Tlingit, Haida, and Inuit ancestry was self-reported (Supporting Information Table S1). Although participants were asked for their tribal affiliation for up to two generations, in some cases this information was incomplete. To minimize the inclusion of individuals with non-Native American ancestry, individual who explicitly reported non-Native American ancestry on either parental line were excluded from analysis.

**TABLE 1. Sample size and counts of total chromosomes, O alleles, O1 alleles, and O1vG542A alleles; frequencies of O1vG542A alleles relative to O alleles, total O alleles, and total chromosomes of all O alleles, in all American populations sampled at the time of this study.**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Count of O1vG542A alleles</th>
<th>Count of O alleles</th>
<th>Count of total chromosomes</th>
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<tr>
<td>Acuna</td>
<td>85</td>
<td>100</td>
<td>128</td>
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<tr>
<td>Aymars</td>
<td>83</td>
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To amplify exons 6 and 7 of the ABO locus, we performed 15 µL PCRs containing 0.32 mM dNTPs, 1× PCR Buffer, 1.5 mM MgCl₂, 0.24 µM primers, 0.3 U of platinum Taq (Invitrogen), and 3 µL template DNA. We used the primers described by Hummel et al. (2002). Negative controls (PCR reactions to which no DNA template was added) accompanied every set of PCRs to monitor the presence of contaminating DNA. PCR conditions were as follows: 94°C for 3 min, 60 cycles of 15 s holds at 94°C, 56°C (for Exon 6) and 53°C (for Exon 3), and 72°C, followed by a final 3 min extension period at 72°C. Amplification success was determined by visualizing 5–6 µL of the amplicons on a 6% polyacrylamide gel stained with ethidium bromide under UV light.

Enzyme digestion for Rsal and HpyCH4IV (Exon 6) and NfI/III and MnlI (Exon 7) was performed at approximately 36°C for >3 h. Digested amplicons were visualized by separating 4–5 µL on a 6% polyacrylamide gel stained with ethidium bromide under UV light. For individuals exhibiting the O1v allele, an additional PCR fragment was amplified and sequenced using forward primer O1v542R (GGGCCAGCTCTCCACTA) and reverse primer O1v542R (GAACACGGGATCACGATCT), under the above described PCR conditions with an annealing temperature of 58°C. Amplicons were prepared for sequencing following Kemp et al. (2007) and sequenced in both directions at Eimin BioPharm (Hayward, CA).

Ancient Ohlone samples

Ancient DNA (aDNA) was analyzed from burials excavated from the Yukisma site (CA SCL-38), an ancestral Muwekma Ohlone burial site located in northern Santa Clara County, California. The site is located approximately 6 miles southeast of the southern portion of San Francisco Bay (Leventhal et al., 1993; Morley, 1997; Jurmain, 2001; Gardner et al., 2011). This site falls under the Tamyen-speaking territory, part of the Ohlone languages in the Utian family (Bellifemine, 1997; Jurmain, 2001). One or more skeletal elements from each of the 252 burials were reserved for genetic analysis prior to remaining skeletal elements being reinterted. With approval from the Museum of California and the samples were transferred to the Kemp Lab of Molecular Anthropology and aDNA at the University of Texas at Austin for radiocarbon dating. Radiocarbon dating places deposition from 250 B.P to at least 2,200 B.P, but the burials are predominately from the beginning to middle of the Late Period (740–230 B.P) (Leventhal et al., 1993; Bellifemine, 1997).

All extractions and PCR preparations were conducted in a dedicated aDNA facility, with amplification and post-PCR processing conducted in a laboratory located in a separate building. Approximately 0.5 g of material was carefully removed from each of 68 ribs. Each sample was submerged in 6% sodium hypochlorite (full strength household bleach) for 15 min (Kemp and Smith, 2005). Each sample was rinsed 1–2 times with DNA free water (Gibco) to remove the bleach. DNA was extracted following the method described by Kemp et al. (2007) with the following modifications. Following isopropanol precipitation, the pelleted DNA was resuspended in 300 µL of 60–65°C DNA-free ddH₂O and incubated at 60–65°C for 20 min and vortexed three times before silica extraction using the Wizard PCR Preps DNA Purification System (Promega).

Each sample was first screened for the markers diagnostic of Native American mtDNA haplogroups A, B, C, and D following Kemp et al. (2007). To confirm that the screened individuals conform to haplogroup frequencies expected in Native Americans from the California and Great Basin areas, a Fisher’s exact test for haplogroup frequencies was performed between the ancient Ohlone population and the Cahuilla, Costanoan, Modern Great Basin (multiple groups), Sahaptain (200 YBP, protohistoric/historic) (Malhi et al., 2004), Serano/Vanyume, Sierra Miwok, Tubat, and Yokuts modern populations (Johnson and Lorenz, 2006). Also compared were two prehistoric populations: CA-SCL-287/SMA-263 (1176–1534 YBP), which is considered to be an ancestral Muwekma Ohlone Site and is located on the border of Santa Clara and San Mateo Counties, CA, within the vicinity of CA-SCL-38 (Monroe et al., 2009); and CA-SOL-270 (ca. 2000 YBP), which is located in Solano, County, CA east of the San Francisco Bay and also thought to be part of the historic Ohlone tribal area (Eshleman, 2002).

Samples that yielded Native American mtDNA types were screened for ABO variation as described for modern samples. This was replicated three times by F.A.V. (Table 2), and once more by B.M.K., following recommendations by Halverson and Bolnick (2008). Results were screened against the mitochondrial and blood types of the researchers to eliminate possible contamination originating from them. None of the researchers belong to any of the mitochondrial haplogroups found in the samples. While one researcher (F.A.V.) has the genotype O1vO1vᵀᴹ, this genotype was not found in any of the ancient samples. To settle differences in typing between replicates, this study assumes that allelic drop-out is the most likely explanation. Note that each allele is determined by a combination of four restriction enzymes, and so allele drop-out in one PCR translates to two genotypes (Table 2, Hummel et al., 2002). For example, in sample B171, allelic drop-out explains the shift from the genotype AO1v (+/-, +/-) into O11vO1v (+, +) as these two genotypes are differentiated by heterozygosity at only one locus.

Ancient samples that tested positive for both mtDNA and the ABO locus were sent to the aDNA laboratory at the University of Texas at Austin for independent confirmation (Table 2). Samples were extracted following the protocol described by Bolnick and Smith (2007). The mtDNA haplogroup of each sample was determined, and ABO genotyping was performed 1–5 times per sample.

Gene flow

To estimate the degree of gene flow between Na-Dene and First American groups sufficient to explain the presence of O1vᵀᴹ in both groups, we performed a simple estimation of gene flow following Schroeder et al. (2007). Specifically, if the Tingit descend from a separate founding population in which O1vᵀᴹ was not present, the amount of gene flow (m) required to bring O1vᵀᴹ to the frequencies observed today is calculated as $m = \frac{p_1}{p_1 + p_2}$, where $p_1$ is the observed average frequency of O1vᵀᴹ in the Na-Dene, $p_2$ is the observed average frequency (with a minimum and maximum range) of O1vᵀᴹ in the other (non-Na-Dene) Native American groups; and $p_2$ is the frequency of O1vᵀᴹ in the Na-Dene prior to gene flow, which was zero.
RESULTS

Modern Alaskan samples

Of 144 chromosomes representing 72 Tlingit samples typed for ABO using PCR-RFLPs, 129 exhibited O alleles and 77 of these were typed as O1v alleles (Table 1). Sequencing revealed 16 of the 144 alleles to be O1v(G542A) (11%). The frequency of the O1v(G542A) allele among the Tlingit falls within the range of frequencies reported for other Native American populations, ranging from 4 to 69% (Estrada-Mena et al., 2010). The relative frequency of the O1v(G542A) allele of all O1v alleles among the Tlingit is 21%, which also falls in the range of Native American frequencies (9–74%, Table 1, Fig. 1). One of the four Haida exhibited one O1v(G542A) allele and none of the three Inuit exhibited this allele.

Ancient Ohlone samples

Forty-one of the sixty-eight (60%) ancient samples were typed as belonging to one of the four major Native American mtDNA haplogroups A-D. A Fisher’s exact test shows that the ancient Ohlone population is not significantly distinguishable at the 0.05 level of probability from Cahuilla, Costanoan, Modern Great Basin (multiple groups), Sahaptain (200YPB, protohistoric/historic) (Malhi et al., 2004), Serano/Vanyume, Sierra Miwok, Tubat, and Yokuts populations (Johnson and Lorenz, 2006) as well as the CA-SCL-287/SMA-263 (1176–1954 YBP) (Monroe et al., 2009) and CA-SOL-270 (ca. 2,000 YBP) (Eshleman, 2002) prehistoric populations. These results suggest that the mtDNA results from these samples are authentic, as the data make phylogenetic sense given where the population was sampled.

Out of these 41 samples, 15 produced data for the ABO locus (Table 2). Ten of the 30 chromosomes exhibited O1v alleles, of which two were typed as O1v(G542A). Six other samples carrying the O1v allele failed to yield readable sequence information. The frequency of the O1v(G542A) mutation in O1v chromosomes is, therefore, 0.20 or higher. In addition, we identified three individuals carrying the A allele, which is rare in North American Native Americans. The importance of precontact presence of A alleles has been discussed elsewhere (see Halverson and Bolnick, 2008).

Gene flow

If the presence of O1v(G542A) in the Na-Dene (i.e., Tlingit) is the result of admixture with other Native American groups, the frequency of O1v(G542A) observed today in Na-Dene would require gene exchange of 48% (17% minimum and 100% maximum, using the lowest and highest frequencies, respectively, of O1v(G542A) observed among a non-Na-Dene population) with First Americans.

DISCUSSION

Finding the O1v(G542A) variant in the Tlingit and Haida, as well as in a pre-Colombian North American population, allows several conclusions to be drawn. First, the distribution of O1v(G542A) has now been widened by over 3,000 kilometers north, across all Native American populations sampled at the time of this study, including a Na-Dene population. This finding indicates close ancestry among all groups sampled for the ABO locus. The distribution of O1v(G542A) is now more consistent with the previously reported distribution of the 9RA
at the D9S1120 microsatellite, which also includes Aleut-Eskimos. It is possible that O1v<sup>GS42A</sup> will be detected in these populations as well, although this will require further work. Here, the allele was not detected among the Inuit, but only three samples were included in this study.

Second, finding O1v<sup>GS42A</sup> in a pre-Colombian Ohlone population demonstrates that the allele was present in the Native American population prior to European contact. While this observation may be unsurprising given the distribution of the O1v<sup>GS42A</sup> allele among contemporary Native Americans, it is an important direct observation. Taken together with the observation of O1v<sup>GS42A</sup> in all Native American populations tested for this allele, and the rarity of the allele in non-Native American populations, the most likely explanation is that O1v<sup>GS42A</sup> originated in the population ancestral to all Native Americans and became widespread as this population migrated and spread into the continent, consistent with the BIM proposed by Tamm et al. (2007). In this case, the few alleles previously reported outside of America are most likely either the consequence of gene flow into Europe and the Middle East from the Americas or mutational homoplasy.

An alternative hypothesis accounts for the presence of alleles such as O1v<sup>GS42A</sup> and 9RA as the result of secondary gene flow between Native American groups descended from different source populations, more closely following the three wave migration model originally posited by Greenberg et al. (1986). While it is possible that O1v<sup>GS42A</sup> originated in the Amerinds and was absent in a secondary wave that contributed to Na-Dene ancestry, this scenario requires a high level of gene flow (48%) to explain the frequency of this allele in the Tlingit. This result is consistent with the estimated level of gene flow necessary to explain the shared presence of 9RA (91.9–92.8%, Schroeder et al., 2007). Based on a nuclear genome-wide survey of variation, Reich et al. (2012) argued for at least three streams of population movement into the Americas, followed by extensive gene flow between them. For example, in their study, they found that the Chipewyan, a Na-Dene population, derives 90% of its ancestry from the First Americans population, with the remaining 10% of their ancestry from a genetically distinct source population. Yet, the most parsimonious explanation for the presence of O1v<sup>GS42A</sup> and 9RA in such high frequencies among both First American and Na-Dene populations is shared ancestry. According to the study of Reich et al. (2012), the Na-Dene are indeed unique among Native American populations, but the fact remains that they are still 90% First Americans. While these genetic data cannot inform us if the remaining 10% of their ancestry was associated with the introduction of Na-Dene languages into the Americas, if that were true, it would provide very weak support for the model originally proposed by Greenberg et al. (1986), which argued for biologically distinct origins of Amerind, Na-Dene, and Eskimo populations. In a strict sense, Greenberg et al.’s model assumed discrete biological groups, which are not consistent with the high degree of gene flow inferred from Reich et al.’s (2012) data.

Finally, taken together, this new evidence from a locus unlinked with D9S1120, the Y-chromosome M3 marker, or the mitochondrial genome further supports the shared ancestry of all modern Native Americans, which are descended from a population that evolved in isolation for enough time to allow for the rise of unique variants. In
this case, it will be particularly informative to screen additional Na-Dene populations, as well as Aleut-Eskimos and northeastern Siberian groups, to take advantage of expanding studies of Native American ancestry into relatively unexplored nuclear markers.

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LITERATURE CITED


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AQ5: Please spell out RFLP during the first instance.

AQ6: Please note that the references “Olsson et al., 1998, 1999, 2000; Estrada-Mena et al., 2008, 2009, 2011, 2012; Roubinet et al., 2001, 2002; Barjas-Castro et al., 2003” are provided in the table but not detailed in the list. Please provide the details in the list.