
Freezer anthropology: new uses for old blood

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Archived blood fractions (plasma, settled red cells, white cells) have proved to be a rich and valuable source of DNA for human genetic studies. Large numbers of such samples were collected between 1960 and the present for protein and blood group studies, many of which are languishing in freezers or have already been discarded. More are discarded each year because the usefulness of these samples is not widely understood. Data from DNA derived from 10–35-year-old blood samples have been used to address the peopling of the New World and of the Pacific. Mitochondrial DNA haplotypes from studies using this source DNA support a single wave of migration into the New World (or a single source population for the New World), and that Mongolia was the likely source of the founding population. Data from Melanesia have shown that Polynesians are recent immigrants into the Pacific and did not arise from Melanesia.

Keywords: mitochondrial DNA; New World; Polynesia; Oceania; 9-bp deletion; Melanesia

1. INTRODUCTION

In the heyday of large-scale anthropological fieldwork in the 1960s and 1970s, many tens of thousands of blood samples were drawn from worldwide populations to study everything from blood group polymorphisms, protein polymorphisms and isozymes (Neel 1973, 1978*a,b*; Neel *et al.* 1974; Neel & Ward 1970; Arends *et al.* 1967, 1970; Friedlaender 1975, 1987; Friedlaender & Steinberg 1970; Salzano *et al.* 1977; Spielman *et al.* 1972, 1974, 1979; Tanis *et al.* 1974; Hill *et al.* 1989), to malaria (Kelly 1990; Clark & Kelly 1993), to hepatitis (Mazzur *et al.* 1973). When blood is allowed to sediment for 1–3 days in a vacutainer containing an anti-coagulant, it separates into three distinct layers: (i) the red cells settle to the bottom and are a deep, dark red colour; (ii) the white cells form a thin milky layer on top of the red cells, known as the buffy coat; and (iii) the serum plasma forms the top half of the sample and is usually a milky to clear yellow colour. In the era prior to the routine collection of molecular sequence data, it was the plasma portion or the red cell portion that were commonly of interest, while the DNA white cells in the buffy coat were often discarded and were considered a contaminant of the other two fractions. Often these fractions were separated right in the field with a portable centrifuge, or by normal gravity, retaining the plasma and the red cells in separate vials and often discarding the buffy coats on the spot. Luckily, under these less than optimal conditions, there are usually plenty of white cells ‘contaminating’ both of the other blood fractions, and it is this that is the target of molecular geneticists today. The red cells do not contain nuclei, but can contain mitochondria, so they can be a source of mitochondrial DNA. White cells will often stick to clumps of red cells, and so co-sediment with the red cells. Many researchers stored the samples as whole blood, clotted blood or whole blood stains on Guthrie

cards or filter paper. All these are potential sources of DNA.

These early blood samples can be of tremendous value. At the very least they represent a specific snapshot in time of the genetic variation present in the populations being sampled. Some may represent populations that have since gone extinct or been admixed into a larger neighbouring population, or have undergone some kind of severe population bottleneck or expansion since the original sampling occurred. Since these samples were used for some specific purpose, there are often many other interesting data points associated with each sample (blood type, pedigrees, morphological data, language, geographical location, isozyme data, etc.) which can be compared to the molecular genetic data typically being collected today. Perhaps most importantly, few studies since the beginning of the 1980s have collected on the large scales of many of the early studies. Just a few of the many examples included:

- (i) The Harvard Solomon Islands Project (over 6000 individuals sampled between the late 1950s and the mid-1980s by Jonathan Friedlaender and colleagues);
- (ii) James V. Neel and colleagues Amazonian Indian studies (9800 individuals sampled from South America between 1967 and 1977);
- (iii) Moses Schanfield’s worldwide isozyme studies, combining plasmas from many studies from the 1960s and 1970s;
- (iv) Baruch Blumberg’s studies of viral evolution and spread in the Pacific in the 1960s and 1970s includes many thousands of plasma samples.

While researchers still work in all these areas, it is rare to find a project that collects such large sample sizes, both in numbers of populations and numbers of individuals per population, making these early (mostly plasma) collections a unique and extremely useful resource.

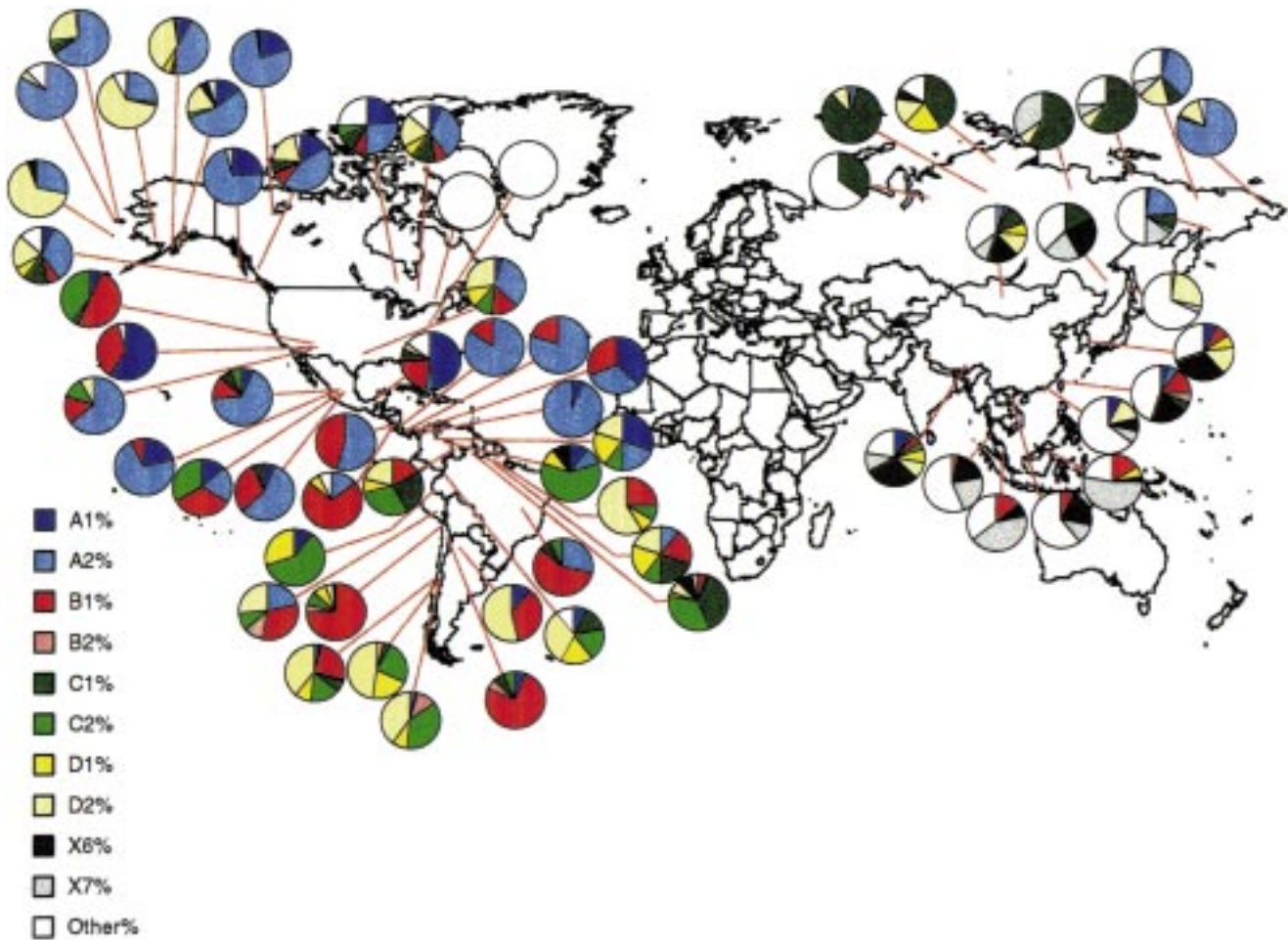


Figure 1. World map showing pie-chart frequencies of the nine primary New World founding haplotypes. Data from Merriwether & Ferrell (1996).

Until Ryk Ward demonstrated the use of old plasma samples in his study of the Pacific Canadian Nootka Native Americans (Ward *et al.* 1991), almost all molecular genetic studies of humans had used cell-line DNA, placental DNA (which can be retrieved in large quantities), and buffy coat DNA. This is largely due to the legacy of Southern blotting techniques (Southern 1975) which required copious amounts of high-quality DNA to accurately type polymorphisms. With the advent of PCR (Saiki *et al.* 1988) and its eventual spread to all areas of molecular biology—genetic data collection, large amounts of template DNA were no longer required to retrieve even high resolution data from blood samples. Even after Ward showed that old plasma samples can be excellent sources of DNA for sequencing and RFLP studies, few others made use of these resources. Exceptions include the large-scale studies of the peopling of the New World using mostly plasma samples by Merriwether and colleagues (Merriwether *et al.* 1994, 1995, 1996; Merriwether & Ferrell 1996) and by the Douglas Wallace laboratory (Torroni *et al.* 1993*a,b*) which used cell-line DNA, buffy coat DNA and some plasmas from the Neel collection. Kenneth Weiss and colleagues at Penn State (Weiss *et al.* 1994; Buchanan *et al.* 1993) performed a number of tests on the use of old archival samples (using the Neel collection), including optimizing the Lone Linker genomic amplification technique, as well as testing numerous extraction protocols for efficiency versus these old

samples. The Weiss laboratory has been at the forefront of developing and testing curating techniques for archival blood and DNA samples.

The drawbacks to plasma lie primarily in the degraded nature of most of the DNA that can be retrieved from it. Unless it is stored in liquid nitrogen, the DNA in the plasma will eventually, and rather quickly (in just a few years at -20°C or over five years at -80°C), degrade into small pieces, most of which are under 400 bp in length. This small size obviates the use of a number of different strategies such as the Wallace laboratory method of amplifying the entire mitochondrial DNA genome in nine or ten overlapping amplicons 1000–2500 bp in length (Torroni *et al.* 1993*a,b*). It also disallows the sequencing of long stretches in a single reaction, requiring many smaller PCRs and sequencing reactions to cover the same length of DNA. A further problem is that the nuclear genes are far more difficult to recover from old plasma samples and are often impossible to recover without additional genomic amplification procedures to increase the concentration of the DNA (Cheung & Nelson's (1996) DOP method, Sun *et al.*'s (1995) PEP method, Weiss *et al.*'s (1994) Lone Linker method). Mitochondrial DNA, however, is usually quite easy to recover from old blood samples, presumably due to the much larger copy number (there are on average 750 mitochondria per cell and 2.5 mtDNAs per mitochondrion) versus nuclear DNA (a fraction over two copies per cell on average, and only one copy for parts of the Y and X

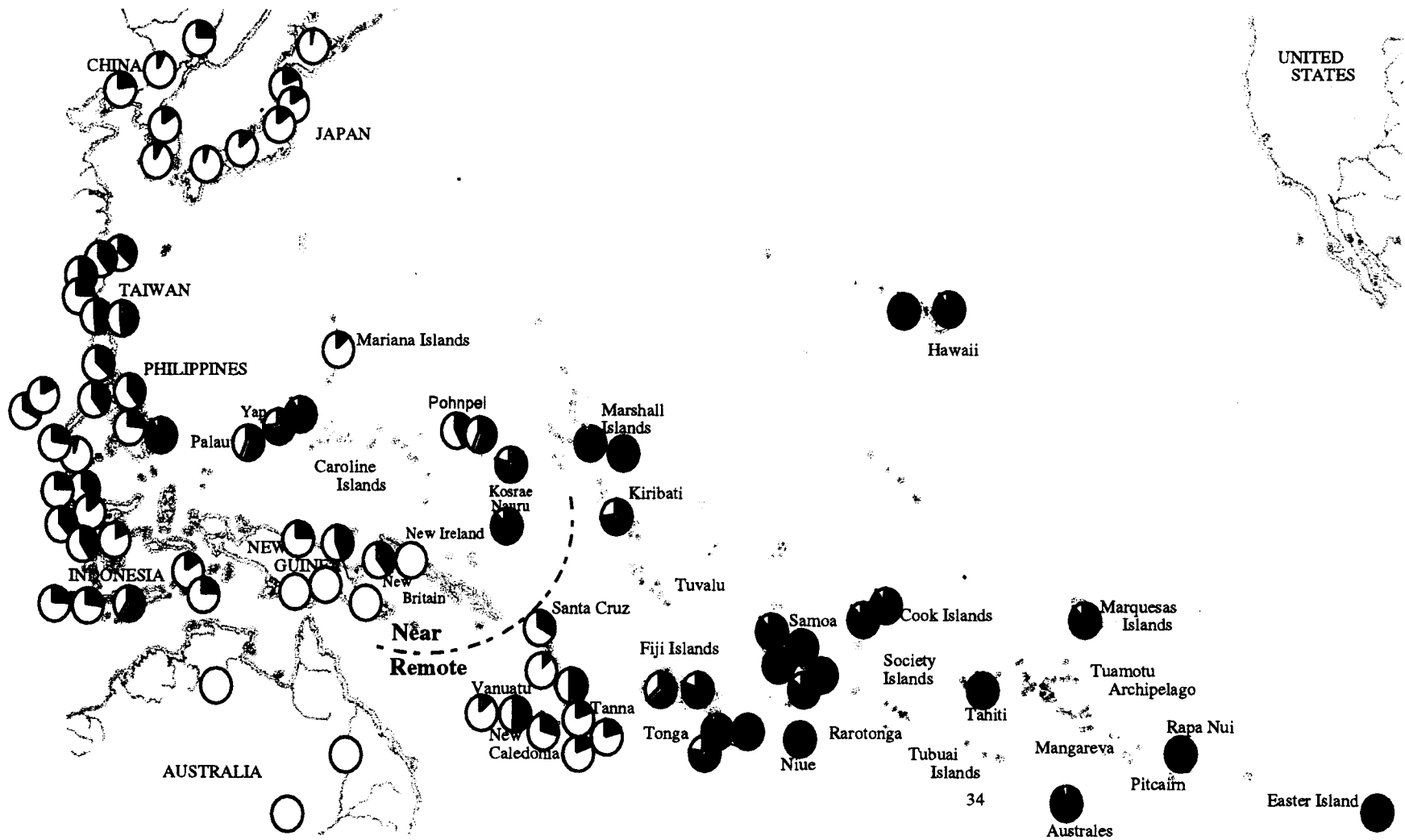


Figure 2. Map of the Pacific showing pie-chart frequencies of the 9-bp region V deletion. Data from Merriwether *et al.* (1999).

Green Island

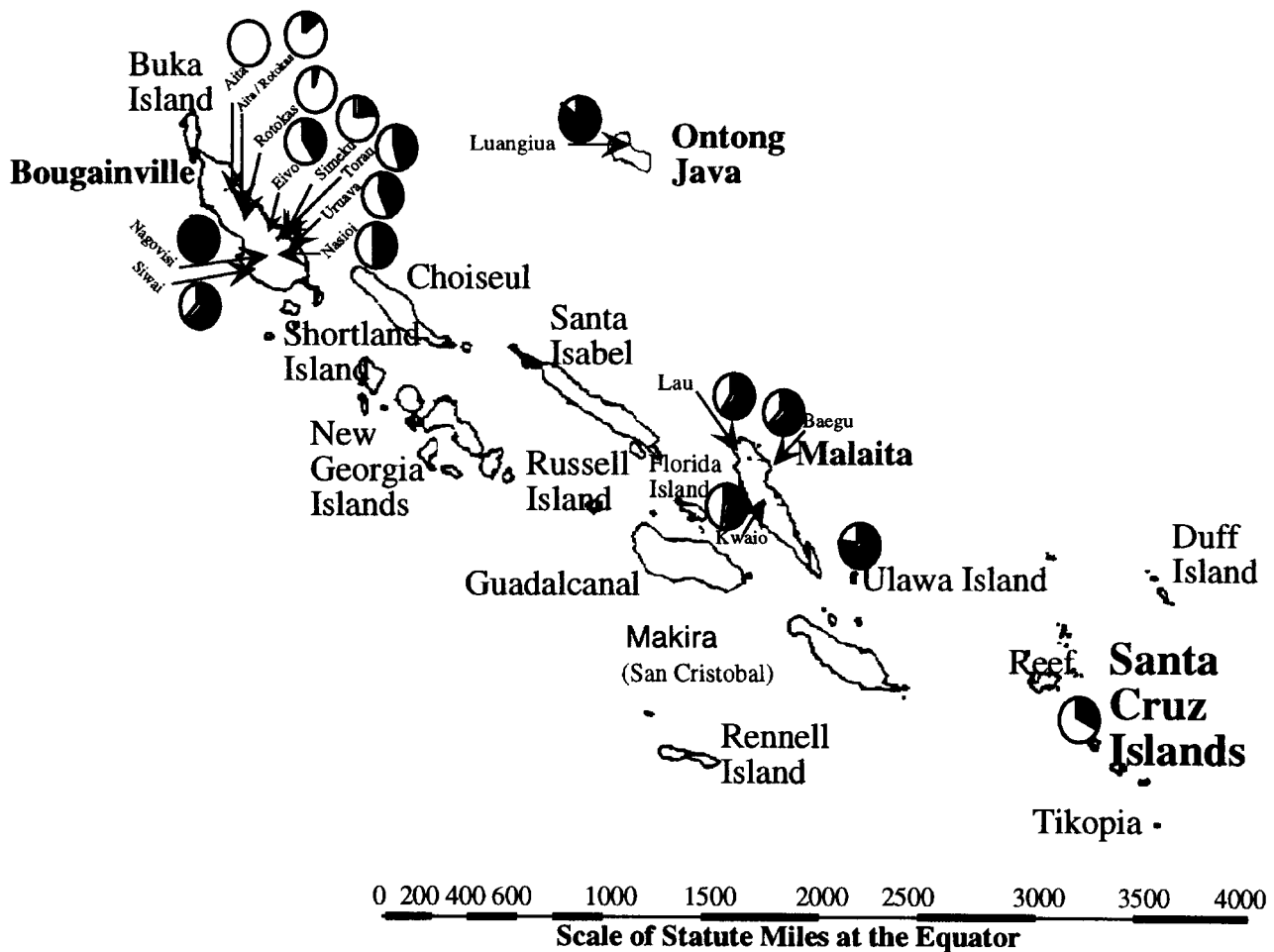


Figure 3. Map of Island Melanesia showing pie-chart frequencies of the 9-bp region V deletion. Data from Merriwether *et al.* (1999).

chromosomes). Thus, to date, most studies using plasma have involved mtDNA. It is a similar problem to that faced by researchers working with ancient DNA, where there is a relatively small number of mostly degraded DNA molecules from which to amplify. As with any suboptimal source of DNA (like ancient DNA, for example), extra care must be taken to ensure the authenticity of the DNA that is extracted. Contamination by modern, largely intact, DNAs may be preferentially amplified over the fragmented and damaged plasma-derived DNA. This is especially true when large fragments are being amplified, and there is little or no plasma-derived DNA of that size to act as a template for PCR. Negative PCR and extraction controls should be run for every extraction, and every PCR, to test for contamination of the extract or PCR.

2. APPLICATIONS OF FREEZER ANTHROPOLOGY

There have been a number of applications of 'freezer anthropology' in the past eight years, largely by Merriwether and collaborators, most of which have centred on the peopling of the New World (Merriwether *et al.* 1994, 1995, 1996; Merriwether & Ferrell 1996) and the peopling of the Pacific (Merriwether *et al.* 1999; Green *et al.* 1999). Both of these sets of studies relied primarily on DNA from plasma collected in the late 1960s to the mid-1980s.

The volume of the plasma in these studies ranged from 10 ml down to 20 μ l. MtDNA was recovered and typed by PCR and RFLP, PCR detectable insertion-deletion events and/or directly sequenced. So far, more than 98% of over 4200 samples have successfully amplified for at least one mtDNA marker using old plasma as the source of DNA (although many of these were for primer pairs less than 250 bp apart).

3. THE PEOPLING OF THE NEW WORLD

For many years there have been two primary competing hypotheses involving the peopling of the New World. Both involve migrations across the Bering Strait, presumably via a land bridge and possibly later by boat. The two hypotheses differ in the number and timing of the wave(s) of migration into the New World. The single-wave hypothesis posits a single source population and one (possibly quite prolonged) wave of migration. The second hypothesis posits at least three waves of migration, from one or more sources, corresponding to Greenberg's (1960, 1987; Greenberg *et al.* 1986) three linguistic groups (Amerind, NaDene and Eskaleut). The precise timing of the three-wave hypothesis migrations is not widely agreed upon. Torroni *et al.* (1992, 1993a,b) propose divergence times of (i) 20–41 000 BP for haplogroups A, C and

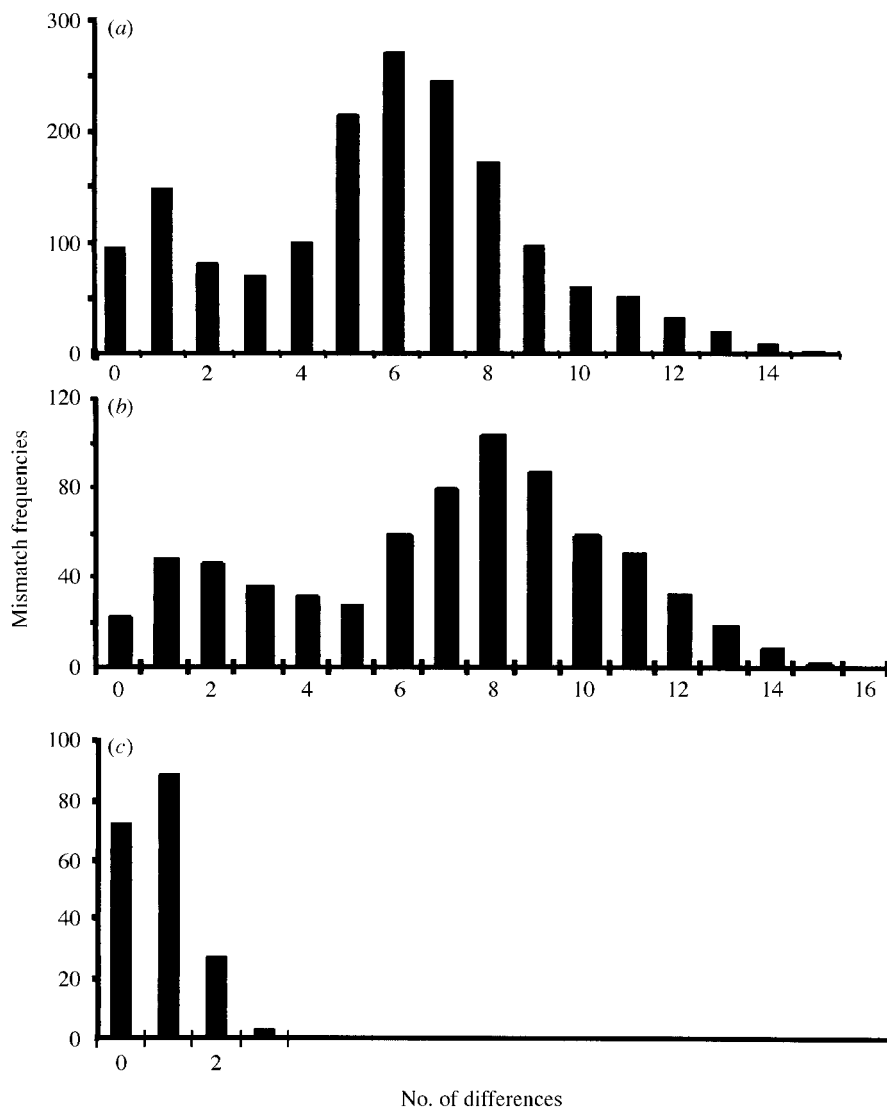


Figure 4. Histogram of pairwise mtDNA sequence differences among Santa Cruz Islanders between nt 16258 and nt 00283 (numbers based upon the Anderson *et al.* (1981) reference sequence). (a) Mismatch distribution for all individuals; (b) mismatch distribution for non-9-bp-deleted individuals, and (c) for 9-bp-deleted individuals. Data and more detailed figures are from Green *et al.* (1999).

D, and 612 000 for haplogroup B, for the Amerinds, (ii) 510 000 BP for haplogroup A in the NaDene, and (iii) <5000 BP for the Eskaleuts.

Torroni *et al.* (1994b) later fine-tuned the date, using Chibchan-specific mutations and a 9000 entry time of the Chibchans into Central America from fossil evidence, to 22 000–29 000 BP for all the Amerind groups. The molecular dates are dependent on the accuracy of the estimate of the rate of mutation for the regions of the mtDNA being studied (and the constancy of that rate over a relatively short period of time evolutionarily). The molecular dates also depend on knowing which mitochondrial mutations arose in the New World and which arose on the other side of the Bering Strait and migrated over. While improved estimates of the mutation rates should eventually be generated, it is impossible to know if you have tested all the relevant (source) populations on the other side of the Bering Strait. Dating the migrations notwithstanding, the question of the number of waves of migration can be evaluated with mtDNA data.

In 1985, Wallace *et al.* demonstrated that New World natives contained mtDNA RFLP mutations that were a subset of those mutations found in Asia, some of which were at drastically altered frequencies in the New World (indicating a strong founder effect). Schurr *et al.* (1990)

were the first to show that almost all Native American variation fell within four major haplogroups (labelled A, B, C and D). Numerous papers by Antonio Torroni, Douglas Wallace and co-authors (Torroni *et al.* 1993a,b, 1994b,c; Wallace & Torroni 1992; Torroni & Wallace 1995) bore out the 'four founding lineage' hypothesis for North, Central and South America. It was the contention of the Wallace laboratory that those four lineages were the only types that entered the New World, and that only one version (or haplotype) of each haplogroup entered the New World during each migration. They further argued that there were three to four waves of migration, the first being the Amerinds (containing haplogroups A, C and D in one wave, and haplogroup B migrated over later), the second being the NaDene (containing only haplogroup A), and the third being the Eskaleuts (containing haplogroups A and D). They argued that any other haplogroups than these found in these groups were due to admixture with the surrounding Amerind populations.

Several groups (Bailliet *et al.* 1994; Merriwether *et al.* 1994, 1995; Merriwether & Ferrell 1996) later proposed that there were more than just the 'four founding lineages' that entered the New World. Bailliet *et al.* (1994) argued that groups A, C and D could be broken up into A1, A2, C1, C2, D1 and D2 by the presence or absence of the

hypervariable *Hae* III restriction site at nucleotide (nt) 16517. This was verified by Merriwether & Ferrell (1996) and Easton *et al.* (1996). Bailliet *et al.* (1994) also argued that there were some other types found in the New World that were not a part of A, B, C or D (calling them E or X). Merriwether & Ferrell (1996) and Easton *et al.* (1996) defined two new, Asian-specific haplotypes called X6 and X7 that were found in North and South America as well as all over Asia (figure 1). X6 and X7 differ at the *Hae* III 16517 site, but both have site gains at *Alu* I 10397 and *Dde* I 10394. The *Alu* I 10397 site is Asian-specific (Ballinger *et al.* 1992) and is not seen in African or European populations. X6 and X7 were found in the remote Brazilian Amazonian Yanomami Indians from Surucucu. This area had not been contacted by missionaries until the 1960s, and Easton *et al.* (1996) found X6 and X7 in individuals born before known contact. This, coupled with the fact that it involves an Asian-specific mutation and the fact that women are required to move to introduce mitochondrial lineage, makes it seem very unlikely that X6 and X7 were due to recent, post-contact admixture with Western populations. This was further borne out when X6 and X7 were found in the ancient Windover site in Florida (8000–9000 years old) by William Hauswirth and collaborators. Therefore, Merriwether proposed that X6 and X7 should be labelled as additional founding lineages (or founding haplogroups). The fact that multiple variants of each of the other founding haplogroups are present in North, Central and South America, and throughout Asia and Siberia indicates that they arose on the other side of the Bering Strait and entered the New World with the initial migrants. In addition, the presence of all the major haplogroups and most of the major haplotypes in all three putative waves of migration (Amerind, NaDene and Eskaleut) is difficult to reconcile with three separate waves of migration. It is much more parsimonious with a single wave of migration containing all these types, followed by linguistic and cultural diversification after or during entry. Torroni *et al.* (1993b) argued that Siberia is the likely origin of the founding population, but they lack haplogroup B (Shields *et al.* 1992) and several of the subtypes of the other haplogroups. Merriwether *et al.* (1996) and Kolman *et al.* (1996) demonstrated that Mongolia shares more variation with the New World than any other population. The one exception to this was Tibet (Torroni *et al.* 1994a; D. A. Merriwether and Beall, unpublished data), but the Tibetans sampled are believed to be migrants from Mongolia in historic times. The data on the Nootka, Haida, Aymara, Atacameno, Dogrib, Yamomami, Makiritari, Matacao, Wapishana, Ticuna, Kraho, Cuna, Bribri-Cabecar, Piaroa, Alaskan Eskimos (south-west Alaskan Yupik, St Lawrence Island, Kodiak Island) and Aleuts (St Paul, Pribiloff Island) were derived from archival plasma and settled red cell samples ranging from 10–35 years old (at the time of the studies). Others, such as the Mvskoke and Mohawk were amplified from 5–10-year-old plasmas. Indeed, the conclusion that there was only one wave of migration into the New World, rather than three, was largely derived from these archival blood samples. Besides the RFLP data described above, several labs used D-loop or control region data from the mitochondrial DNA hypervariable major non-coding region

to demonstrate support for a single wave of migration into the New World (Ward *et al.* 1991, 1993; Torroni *et al.* 1993a,b, 1994b,c; Batista *et al.* 1995; Kolman *et al.* 1996; Kolman & Bermingham 1997; Forster *et al.* 1996; Bonatto & Salzano 1997; Ginther *et al.* 1993; Horai *et al.* 1993; Shields *et al.* 1993; Merriwether 1993; Merriwether *et al.*, unpublished data).

4. THE PEOPLING OF THE PACIFIC

The origin(s) of the Pacific Islanders has been of longstanding interest to ethnologists, linguists, archaeologists, and biological anthropologists for many years (Friedlaender 1975, 1987; Kirch 1997; Bellwood *et al.* 1995; Bellwood 1979; Terrell 1986, 1977; Spriggs 1997; Serjeantson & Gao 1995; Serjeantson *et al.* 1992; Spurdle *et al.* 1994; Stoneking *et al.* 1986, 1990). One of the few things about Oceanic populations that has become clear from these different fields of analysis is that the Polynesians seem to represent a distinct group that seems to have arrived in remote Oceania within the last 5000 years (at most). Polynesians speak a group of languages within the Austronesian language family (Pawley & Ross 1993; Kirch 1997). What is less well agreed upon is where the Polynesian culture and people originated from. It is most widely believed that the Polynesians are derived from the Lapita culture which spread throughout Near Oceania and into Remote Oceania as far as Samoa and Tonga (summarized well by Kirsch in his 1997 book, *The Lapita peoples*).

Two competing hypotheses have primarily been argued for the origin(s) of the Lapita peoples. One, by Peter Bellwood (1978; Bellwood *et al.* 1995), dubbed the 'fast train' or 'express train' model by Jared Diamond (1988), postulates that the Polynesians resulted from a recent migration out of South-east Asia (Aboriginal Taiwan populations have been invoked as the source, as well as some Indonesian populations), passing through (island hopping) the already heavily occupied islands in Melanesia, and proceeding straight out into the Pacific with little admixture on the way. The other hypothesis, put forth by John Terrell, called the 'pea soup' or 'voyaging corridor' model, postulates that the Polynesians could have arisen *in situ* in Melanesia, and then spread out, and that continual interaction between South-east Asia, Melanesia, and Polynesia best explains the current variation. The first mtDNA studies of the Pacific (Hertzberg *et al.* 1989) centred on one marker (a 9-bp deletion between the COII gene and the tRNA for lysine in region V of the mitochondrial genome), and it was found that this Asian marker was present in 90–100% of the individuals on most Polynesian and Micronesian Islands (see figure 2). This was borne out in subsequent studies (Lum *et al.* 1994; Lum & Cann 1998; Harihara *et al.* 1992; Passarino *et al.* 1993; Melton *et al.* 1995; Redd *et al.* 1995; Sykes *et al.* 1995; among others), but these studies either skipped or only looked at the periphery of Melanesia (i.e. New Guinea and Vanuatu), rather than the heartland of Island Melanesia in Near Oceania. Merriwether *et al.* (1999) demonstrated that the 9-bp deletion is found at high frequencies throughout the coastal populations of Melanesia (see figure 3) and even many of the inland populations (ranging from 12–100%).

Green *et al.* (1999) sequenced 380 nts (finding 46 polymorphic sites) in 49 unrelated Santa Cruz Islanders

(a non-Austronesian Melanesian population on the boundary of Near and Remote Oceania). The Santa Cruz samples were collected in 1971 by Scott Mazzur and Baruch Blumberg as part of a study on hepatitis and molecular evolution. They demonstrated that the 9-bp-deleted individuals in Melanesia are of very recent origin by generating a pairwise mismatch distribution for the 49 individuals (30% of which were 9-bp deleted). This distribution (see figure 4) is bimodal, with all of the 9-bp deletion lineages falling within the first mode with an average pairwise distance of only 1 (the same as most Polynesian populations with the 9-bp deletion for this fraction of the mtDNA control region, nts 16280–0041). The non-9-bp-deleted lineages were shown to be eight or more differences apart, on average. There were also some non-9-bp-deleted individuals in the first mode and their origins are being investigated. This scenario does not fit with a recent origin in Melanesia and spread to the Pacific. When we look at the broader picture of mtDNA variation across the Pacific and South-east Asia, there is a general increase in frequency as one moves from South-east Asia, through Melanesia, and out into Polynesia and Micronesia. There are large regions of non-deleted individuals throughout South-east Asia, Australia, New Guinea, and Island Melanesia, but they are mostly in the highlands in Melanesia. Australia largely lacks the deletion. This fits with a picture where the original inhabitants of Near Oceania–Melanesia (which arrived at least 40 000 years ago in New Guinea, 35 000 years ago in New Britain and New Ireland, and 29 000 years ago in North Bougainville) did not carry the 9-bp deletion, and a much more recent migration into and through Melanesia by individuals with the 9-bp deletion in the last 3000–5000 years. There is strong evidence for heavy and/or continuous intermixing between the resident Melanesian groups and the migrating Polynesian groups. Thus, the true picture is a mix of both hypotheses. The data are entirely consistent with a migration out of South-east Asia through Indonesia, New Guinea, Melanesia, and out into the Pacific, but with very heavy interchanges of genes along the way. It fits best with those interchanges being due to the ancestral Polynesians settling among the Melanesians, since we see very few non-9-bp-deleted lineages in Polynesia or Micronesia (i.e. there has been less admixture of Melanesians into Polynesians, and considerable admixture of Polynesians into Melanesians).

All of the data on Melanesia reported here were taken from archival plasma samples collected primarily between 1966 and 1972 in the Solomon Islands, Santa Cruz, Vanuatu, and New Caledonia. There was one return collection to the Solomons in 1986 by Friedlaender. Prior to this study, there was virtually no sampling of the core of Island Melanesia (especially not in Near Oceania). Previous studies (Sykes *et al.* 1995) only looked at Vanuatu and Papua New Guinea (the two extremes of the distribution of Melanesians). While the initial Polynesian studies were done primarily from more recently collected bloods, almost all of Melton *et al.*'s (1996) South-east Asia data was from the archival plasma collection of Dr Saha. It is clear that very old plasmas have played a key role in testing theories of the peopling of the Pacific. The large sample sizes, and exquisite wide-ranging data collected through the Harvard Solomon Island Project,

will undoubtedly continue to provide insights into the prehistory of Oceania. So many studies today are little more than 'postage stamp collecting expeditions', picking up 10–25 samples from each location, rather than longitudinal population studies involving entire villages and regions (like the Solomon Islands Project, or the Amazonian Indian studies of James Neel for a similar example in the New World).

Y-chromosome data should also be instrumental in testing these hypotheses, as mtDNA only measures female movements (it is strictly maternally inherited: Giles *et al.* 1980; Case *et al.* 1981; Hutchinson *et al.* 1974), while the non-recombining portions of the Y chromosome are strictly paternally inherited and trace only male gene flow.

5. CONCLUSIONS

It is possible to test many exciting hypotheses of human evolution, variation and dispersal using DNA derived from even very old blood fractions. While it may take more work to analyse these typically degraded DNAs, it is possible, and it allows the investigators to look back in time at a unique snapshot in the history of human populations.

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