arrived in Melanesia are thought to have had a matrilocal and matrilineal society.[9] Thus, both factors are suggested to have played a role in the admixture procedure that occurred in Melanesia between early Austronesians and local non-Austronesians and gave rise to the people currently living on the many Pacific islands known as "Polynesia."

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The Crucial Role of Calibration in Molecular Date Estimates for the Peopling of the Americas

To the editor: In a recent study of Native American mitochondrial genomes, Fagundes et al.[1] claimed to have found molecular evidence that the colonization of the New World occurred well before the appearance of the Clovis cultural horizon (c. 12.6–13.2 thousand years [kyr] ago[2]). To support this claim, the authors performed a variety of phylogenetic analyses, including Bayesian date estimation and skyline-plot inference, using the software BEAST.[3] A very similar conclusion was reached in a recent study by Achilli et al.,[4] who estimated that each of the major Native American haplogroups coalesced around 19 kyr ago. A key failing of these studies, however, was an underappreciation of the importance of calibration choice. In fact, upon closer examination of the calibration techniques involved in the two studies, there appears to be little support for an American colonization event significantly antedating the earliest physical evidence of human occupation.[5,6]

Fagundes et al.[1] employed two approaches to calibrating their date estimates. The first, which was also used by Achilli et al.[4] in their study, assumed a global substitution rate of 1.26×10^{-8} subs/site/year, originally obtained by Mishmar et al.[7] with the use of a human-chimpanzee calibration at 6.5 Myr. The second method was to include a chimpanzee sequence in the phylogenetic analysis, again fixing the age of the human-chimpanzee split to 6.5 Myr. The date estimates produced under the two calibration methods were very similar, which is not surprising given that they were effectively based on the same calibration. However, using only a single calibration point makes date estimates sensitive to calibration choice, particularly when fossil information has been condensed to a point estimate in spite of uncertainty over the timing for the human-chimpanzee split.

Fagundes et al.[1] do consider one alternative rate, estimated exclusively from synonymous substitutions within the human phylogeny by Kivisild et al.[8] They acknowledge that using this rate would have shifted their own coalescence time estimates about 5 kyr closer to the present, thereby invalidating their claim of a significantly pre-Clovis occupation of the New World. They dismiss this synonymous substitution rate, however, on the grounds that it has been questioned elsewhere[9] and because it is not as widely cited as the Mishmar rate. Achilli et al.[4] raised similar doubts concerning the Kivisild rate.

This uncritical dismissal of alternative rates reflects an unsettling trivialization of the effect of calibration choice. Recent observations have indicated that substitution rates estimated within species are considerably higher than those estimated on phylogenetic (interspecific) scales.[10–12] The consequence of this pattern is that it is inadvisable to assume an interspecific rate in an intraspecific analysis;[13] this also applies to studies of human evolution, with mounting evidence that the use of a human-chimpanzee calibration is generally inappropriate.[14–17]

We reanalyzed the data of Fagundes et al.[1] and Achilli et al.,[4] using the Bayesian phylogenetic software BEAST, in order to estimate the coalescence times of five Native American haplogroups (A2, B2, C, D1, and X2a). Rather than calibrating our analysis with either the Mishmar rate or the age of the human-chimpanzee split, we used a set of three biogeographic calibrations within the human tree to obtain a rate estimate from a data set used in our recent study of human mitochondrial substitution rates.[15] Our methodology consisted of two steps, which are outlined below.

Step 1: Estimating the Coding-Region Rate

An alignment of 177 mitochondrial genomes, sampled primarily from macrohaplogroups M and N, was obtained from our previous study on human mitochondrial substitution rates.[15] Details of data selection and sequence alignment were described previously.[15] The aligned genomes were truncated to leave the coding region (sites 577–16023 of the Cambridge Reference Sequence[18]) for analysis, following both Fagundes et al.[1] and Achilli et al.[4]

In order to estimate substitution rates and divergence times from the coding-region alignment, Bayesian phylogenetic analysis was performed with the use of BEAST 1.4.7.[3] To match the settings used by Fagundes et al.,[1] we used the HKY+G model of nucleotide substitution without partitioning the alignment and used a Bayesian-skyline-plot approach in order to integrate over different coalescent histories.[19]

Posterior distributions of parameters, including divergence times and substitution rates, were estimated by Markov chain Monte Carlo (MCMC) sampling in BEAST. In each analysis, samples were drawn every 10,000 MCMC steps from a total of 20,000,000 steps, following a discarded burn-in of 2,000,000 steps. Convergence to the stationary distribution and sufficient sampling were checked by inspection of posterior samples.

As described previously by us,[15] internal calibration was conducted by specifying priors on the ages of three nodes in the tree. The time to the most-recent common ancestor (TMRCA) of haplogroup P was assumed to follow a lognormal distribution, with a minimum of 40,000 years, with a mean of 45,000 years, and with 95% of the distribution lying between 40,000 and 55,000 years. The TMRCAs of haplogroups H1 and H3 were each assumed to follow a normal distribution, with a mean of 18,000 years and an SD of 3500 years;[20,21] approximately 95% of the distribution lies between 11,000 and 25,000 years. Justifications for these calibrations are described elsewhere.[15]

Step 2: Reanalysis of the Data of Fagundes et al. and Achilli et al

The sequences used in the studies by Fagundes et al.[1] and Achilli et al.[4] were collected from GenBank. These represented all 86 of the genomes analyzed by the former but only 148 of the 185 genomes analyzed by the latter. The remaining genomes, which were obtained from published studies and subsequently corrected for errors, were unavailable from Achilli et al.[4] The absence of 37 genomes, all from haplogroup A2, is unlikely to have a noticeable effect on estimates of coalescence times for the total haplogroup.

The two alignments were analyzed with the use of BEAST, with the same settings as in Step 1. Instead of internal calibration, we used the posterior rate estimate in Step 1 to specify a prior distribution for the substitution rate in the present analyses. The prior rate was assumed to be normally distributed, with a mean of 2.038×10^{-8} subs/site/year and an SD of 2.064×10^{-9} subs/site/year.

The coalescence times of haplogroups A2, B2, C, D1, and X2a were estimated from the data of Fagundes et al.,[1] and the coalescence times of haplogroups A2, B2, C1, and D1 were estimated from the data of Achilli et al.[4]

Our coalescence-time estimates are closer to the present than are those obtained by either of the original studies[1,4] (Table 1) but are very similar to those estimated by Tamm et al.,[22] who obtained a mean estimate of 13.9 kyr by using the Kivisild rate with a median-joining network. In contrast with the interpretations of Fagundes et al.[1] and Achilli et al.,[4] our date estimates are unable to exclude the hypothesis of a colonization event coincident with the archaeological dates for the Americas. There is a similar contraction in the time scale of our Bayesian skyline plot, suggesting that rapid population expansion occurred around 10–12 kyr ago (Figure 1). These results present a considerably different scenario from that visualized by Fagundes et al.[1] and Achilli

Table 1. Coalescence-Time Estimates for Native American Haplogroups

	Coalescence-Time Estimate (Years)							
	Fagundes et al.				Achilli et al.			
	Original Estimate		Present Study[a]		Original Estimate		Present Study[a]	
Haplogroup	Mean	95% HPD[b]	Mean	95% HPD[b]	Mean	95% CI	Mean	95% HPD[b]
A2	21,290	(16,550 – 28,130)	13,840	(9,380 – 18,700)	17,200	(13,870 – 20,530)	14,970	(10,030 – 20,600)
B2	22,140	(17,570 – 28,730)	14,070	(9,670 – 18,680)	21,200	(16,500 – 25,900)	14,440	(10,190 – 19,120)
C	20,680	(16,830 – 26,260)	13,260	(9,360 – 17,630)	23,800	(15,370 – 32,230)	15,600	(10,870 – 20,830)
D1	21,430	(16,850 – 28,730)	13,930	(9,550 – 19,200)	18,600	(14,090 – 23,110)	13,670	(9,570 – 18,400)
X2a	20,730	(16,100 – 29,000)	13,340	(9,140 – 18,920)	–		–	
Average	20,730		13,690		20,200		14,670	

[a] For calibration of these coalescence-time estimates, a normally distributed prior (mean 2.038×10^{-8} subs/site/year, SD 2.064×10^{-9}) was placed on the substitution rate.
[b] HPD: highest posterior density.

et al.,[4] in which this population expansion commenced toward the end of the last glacial maximum in Beringia.

In two more recent studies using Bayesian-skyline-plot analysis of the mitochondrial coding region, Kitchen et al.[23] and Atkinson et al.[14] estimated that population expansion in the Americas began 15 kyr and 18 kyr ago, respectively. However, the time scale of Kitchen et al.[23] was based on a substitution rate obtained by Ingman et al.[24] with a human-chimpanzee calibration. The Ingman rate is slightly faster than the Mishmar rate because the former assumed a date of 5 Myr for the divergence whereas the latter used a value of 6.5 Myr; nevertheless, both rate estimates are interspecific in nature, meaning that the estimate of Kitchen et al.[23] can be grouped with those of Fagundes et al.[1] and Achilli et al.[4] The contrast between our estimated chronology and that of Atkinson et al.,[14] who used a similar methodology involving biogeographic calibration, is most likely due to the effect of rate variation among lineages. The internal diversity of mtDNA haplogroup Q, which was employed as the sole calibration in the study by Atkinson et al.,[14] is substantially less than that of the similarly aged haplogroup P,[25] which we used as one of three biogeographic calibrations. Therefore, a rate estimate calibrated with the use of haplogroup Q is likely to yield a comparatively slower substitution rate than one calibrated with the use of haplogroup P. In turn, this appears to have led to an overestimate of the antiquity of the population expansion in the Americas in the study by Atkinson et al.[14]

The differences among all of the various analyses predominantly derive from different approaches to calibration. In our recent study of human mitochondrial substitution rates,[15] we found evidence to support the validity of the calibration technique used by Kivisild et al.[8] to infer a synonymous substitution rate. This, in turn, lends support to the date estimates obtained by Tamm et al.,[22] which are very similar to the results of the present study. We also note that our estimated time for population expansion overlaps with both the end of the Younger Dryas, ~11.3 kyr ago, and the chronology for the rapid spread of the Clovis culture, ~13 kyr ago.[2] If we accept the alternative hypothesis that the genetic signal for demographic expansion is coincident with the last glacial maximum, then

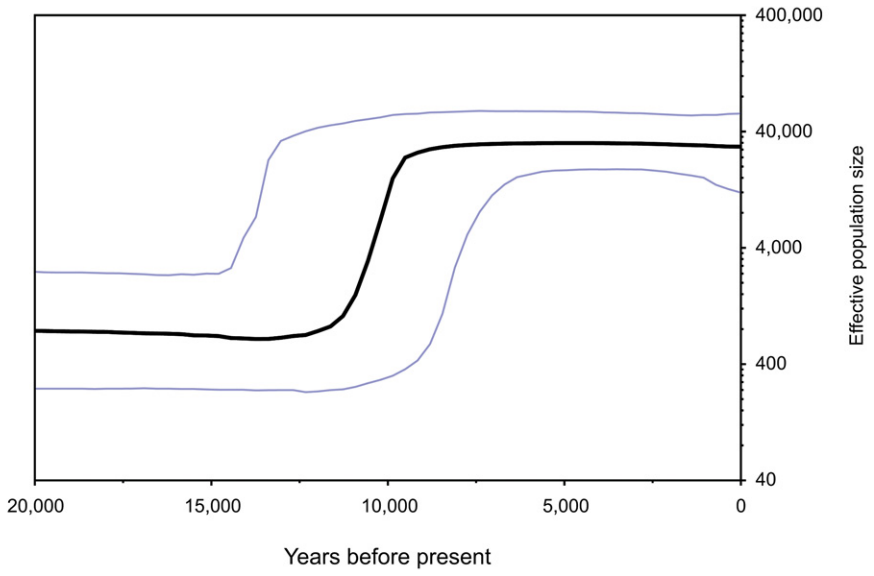

Figure 1. Bayesian Skyline Plot of Native American Population-History
Bayesian skyline plot, obtained with the use of BEAST, showing population history estimated from the coding regions of 86 Native American mitochondrial genomes. The vertical scale measures the effective population size, assuming a generation time of 25 years. There is evidence of population expansion commencing around 12–13 kyr before the present.

it is difficult to explain why there is not another population increase associated with the Clovis archaeological horizon. The older dates also require additional explanation for the absence of archaeological evidence in the Americas during this phase and for why populations should be showing significant signals of expansion under such unfavorable climatic conditions.

The outcomes of our reanalysis illustrate the crucial role of calibrations in obtaining robust date estimates and highlight the wide range of rate estimates currently used for calibration despite evidence to suggest that some of these might be misleading. Although our own estimates are unable to exclude the hypotheses presented by Fagundes et al.[1] and Achilli et al.,[4] they also demonstrate that it is not possible to rule out a scenario in which the timing of the colonization of the Americas closely matches that suggested by the current archaeological evidence. Improvements in the precision of the coalescence-time estimates with the use of our approach will be possible with increased availability of sequence data, especially from ancient DNA, which is able to offer precise calibrations within the human tree.[16] Methods that are currently in development will be able to utilize multi-locus data in order to recover complex population histories.[14] Finally, we hope that the identification of well-supported calibrations within the human tree will encourage a movement away from uncritical usage of the human-chimpanzee calibration.

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Web Resources

The URL for data presented herein are as follows:

GenBank, www.ncbi.nlm.nih.gov/Genbank/

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Reply to Ho and Endicott

To the editor: Ho and Endicott (H&E) propose an alternative interpretation for our findings,[1] stating that Native American mtDNA demography is better associated to a more recent Clovis population expansion than to a pre-Clovis expansion. They base their scenario exclusively on results obtained with the use of substitution rates derived from internal calibrations for mtDNA evolution.[2]

We agree with H&E that improvements in mtDNA-evolutionary-rate estimation are needed to better clarify details of human prehistory, including the peopling of the New World. We also agree that perhaps a better method to achieve this could be the use of intraspecific calibration. However, there are a number of issues regarding the specific internal calibrations that they proposed for human mtDNA evolution that render their rate estimate questionable.

H&E's internal calibration is based on haplogroup diversification associated to two biogeographical events. Their oldest calibration, associated to the peopling of Sahul, uses a single haplogroup (P), even though there is at least one more haplogroup (Q) that could be associated to this event. Previously, when Haplogroup Q was used for a similar internal calibration, the mtDNA rate estimated was much slower than that of H&E, and expansion dates were closer to those of our study.[3] However, H&E prefer to disregard Haplogroup Q rather than use information from both haplogroups, with the sole justification that it would result in a slower substitution rate and consequently older population expansion. Their other calibration event is also problematic. They assumed that haplogroups H1 and H3 expanded 18 thousand years (kyr) ago (95% HPD 24–11 kyr ago) in Europe around the end of the LGM (last glacial maximum). If we accept this calibration and our estimate that the Native American (NA) haplogroups expanded ~18 kyr ago, the basic diversity statistics (e.g., rho and TMRCA [time to the most recent common ancestor]) should be similar for both sets of haplogroups because such statistics are independent of any absolute rate. Moreover, if we accept the H&E estimates that the NA haplogroup expansion occurred ~12–10 kyr ago, NA haplogroup statistics should be ~40% *lower* than H1 and H3. Actually, NA haplogroup values for most of these statistics are ~70% *higher* than H1 and H3, thus almost three times higher than expected under H&E calibration assumptions and results. The results can be explained by accepting our estimates of ~18 kyr ago for the expansion of the NA haplogroups and ~11 kyr ago for the expansion of H1 and H3, as originally estimated[4] with a phylogenetic mutation rate.

Another illustration of the notion that H&E's rates seem to be exaggeratedly fast can be found by the application of the substitution rate that they proposed for the noncoding region (Table 3, D-loop, in [2]) to this same region of our NA mtDNA sequences (Figure 1). This results in an average coalescence time for the NA haplogroups of ~11 kyr ago and a population expansion of ~9–7 kyr ago. These dates are clearly irreconcilable with even the most radical supporters of a later entry for the peopling of the Americas (see below). Interestingly, another substitution rate based on pedigree studies[5] indicates an expansion around 15 kyr ago, much closer to our original estimate.

The assumption that diversification of a sample (usually a single haplogroup) does not predate the biogeographical event it represents may also be an important source of error. In a previous study on mtDNA calibration,[6] Ho et al. used the peopling of the Americas by humans as a calibration point, assuming that this event is represented by the coalescence of all sequences from all haplogroups found in a single North American tribe. This is completely mistaken, given that this coalescence can be traced back to the coalescence of macrohaplogroups M and N in Asia $>$ 50 kyr ago.[2] This example illustrates very well how an uncritical use of knowledge about human evolutionary history can undermine internal calibrations.

Contrary to H&E's claims, their scenario for the peopling of the Americas is harder to reconcile with archeological data. The coalescence of each Native American haplogroup, estimated by them as occurring ~13.9 kyr ago, must of course predate the expansion event. However, this date is too recent, given that there is now convincing evidence that humans were already in the southern tip of South America at least 14.5 kyr ago.[7] Similarly,