Molecular Identification of Lice from Pre-Columbian Mummies

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Background. Three distinctly different lineages of head and body lice are known to parasitize humans. One lineage includes head and body lice and is currently worldwide in distribution (type A). The other 2 (types B and C) include only head lice and are geographically restricted. It was hypothesized that head louse phylotypes were exchanged only recently, after European exploration and colonization (after Columbus).

Methods. To determine which louse type or types were found in the Americas before European colonization, we used polymerase chain reaction in 2 laboratories to amplify DNA from 2 genes (*Cytb* and *Cox1*) belonging to 1000-year-old lice collected from Peruvian mummies.

Results. Only the worldwide type (type A) was found. Therefore, this phylotype was worldwide before European colonization, as type A lice were common in Europe, Africa, and Asia.

Conclusions. The findings of this study show that several phylotypes of head lice have coexisted for centuries in humans and support the claim that type A lice were present in the Americas before the time of Columbus.

Human lice are strictly restricted to human beings and differ from the lice of apes. Until recently, lice were categorized into 3 species: the pubic louse *(Pthirus pubis),* the body louse *(Pediculus corporis),* and the head louse *(Pediculus capitis)* [1]. Although many synonyms exist for these louse taxa, such as *Pediculus humanus capitis* for head lice and *Pediculus humanus humanus* and *Pediculus vestimenti* for body lice, the International Commission on Zoological Nomenclature currently recognizes human head and body lice as subspecies (*P. humanus capitis* and *P. humanus humanus,* respectively). Recent genetic studies have found that, besides *P. pubis,* 3 phylotypes of human lice *(P. humanus)* are currently prevalent on earth, with only 1 phylotype including body lice. Their estimated diverging time is dated 0.7–1.2 million years ago [2], long before the coalescence to a single lineage of their human host [3].

To date, 6 studies [2, 4–8] have addressed the phylogenetic relationships of human head and body lice on the basis of modern lice. Taken together, the findings of these 6 studies agree on the basic structure of the phylogenetic tree for *P. humanus*. Mitochondrial DNA (mtDNA) studies have shown that there are 3 distinctly different clade phylotypes of *P. humanus* found among modern humans (figure 1, redrawn from Reed et al. [2] and Kittler et al. [4]). The most common mtDNA phylotype is found among both head and body lice (type A) (figure 1) and is worldwide in distribution. The second mtDNA group (type B) (figure 1) occurs only in head lice and has been found in the New World, Europe, and Australia. The third type (type C) (figure 1)has been found only among head lice from Nepal and Ethiopia.

The geographic distribution of these 3 louse clades is interesting and warrants further investigation. Lice from Asia and Africa fall almost exclusively into the clade of type A lice, with the exception of a few lice that make up clade C (figure 1). These type C lice appear to be quite uncommon, in light of the DNA sequences deposited in GenBank. In contrast, the type A louse can be found worldwide, and type A is by far the most common phy-

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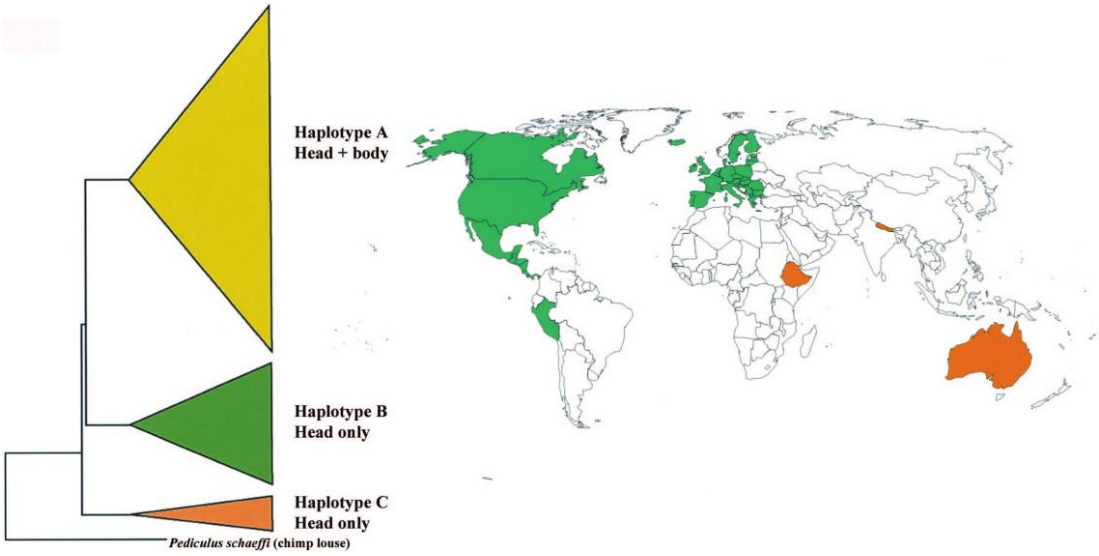

Figure 1. Phylogenetic tree summarizing the results of Kittler et al. [4] and Reed et al. [2]. Both studies examined *Cox1* sequence data to find 3 clades of human lice. One clade contained both head and body lice, whereas the other 2 clades each contained only head lice. The current repartition of phylotypes are indicated in yellow (type A), green (type B), and orange (type C).

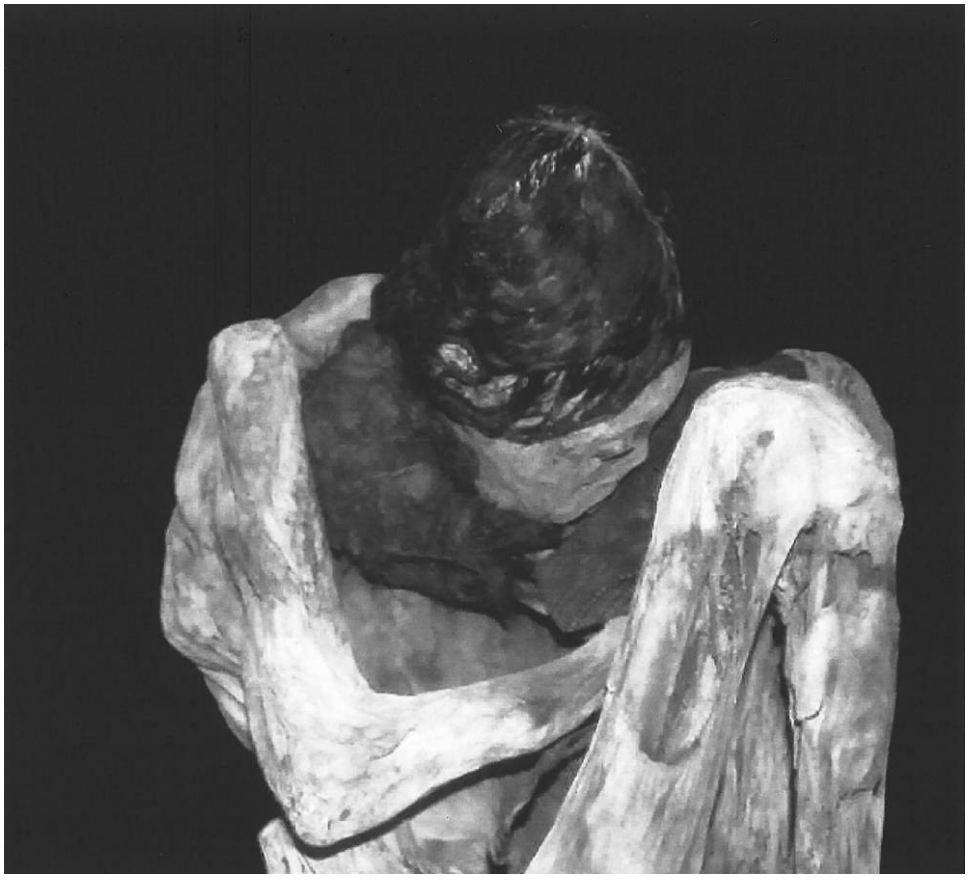

Figure 2. Image of Chiribaya mummy from Peru showing intact hair that is still braided. The 2 heads from which our lice were collected (not shown) for work on ancient DNA were disembodied, presumably the result of looters.

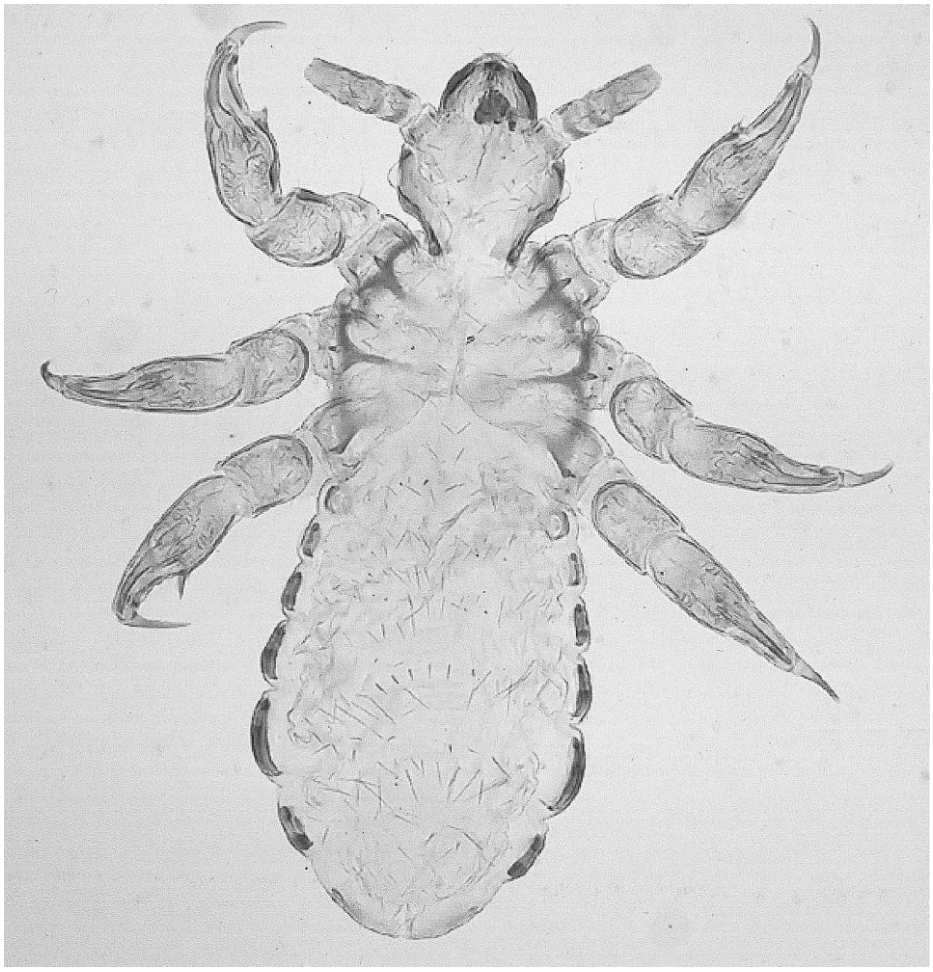

Figure 3. Image of a louse sampled in a Peruvian mummy. The louse was close to hatching when it died.

lotype. Lice from Europe, Australia, and the Americas contain a mixture of types A and B lice (figure 1).

Lice are well known for their long coevolutionary histories shared with avian and mammalian hosts (e.g., see Reed et al. [2] and Hafner et al. [9]). Studies of DNA sequence data from human head and body lice have confirmed events in human evolution, such as the time since divergence with chimpanzees (~5.6 million years in chimp and human lice [2]) and a population expansion in human lice coincident with the out-of-Africa expansion of humans ~100,000 years ago [2]. However, these lice have also elucidated events in human evolution that are uncertain from host fossil and genetic data. For example, Kittler et al. [4] estimated the date that modern humans began wearing clothing by estimating the age of the human body louse, which lays its eggs and lives within clothing. It is likely that further study of lice and other host-specific parasites of humans will clarify additional events in human history that are unknown or unclear, such as the timing and route of the peopling of the Americas.

One of the theories explaining the coexistence of 3 phylotypes of head lice is that European and American lice were mixed after the arrival of Columbus, with phylotype A originating from Europe and phylotype B from America. The recent collection of lice from pre-Columbian mummies in Peru afforded us the rare opportunity to type these ancient American lice. Because head lice have been recovered from New World mummies with radiocarbon dates as old as 10,000 years BP, we know that lice arrived in the New World with the first peoples near the end of the Pleistocene [10]. Because the first lice found in the Americas were head lice and not body lice, it is conceivable that ancient lice in the Americas could have been from any of the 3 phylotypes (A, B, or C). Knowing that phylotype A was present in pre-Columbian America may help us to understand whether louse-transmitted diseases (transmitted by body lice only—i.e., phylotype A [11]) were prevalent before the arrival of Columbus [12].

METHODS

Samples of archaic lice. Archaeologists led by one of us (S.G.) excavated naturally preserved mummies in the extremely arid southern Peruvian coastal desert from 1999 to 2002. The mummies belonged to the post-Tiwanaku Chiribaya culture [13],

Table 1. Specimens examined for the combined cytochrome oxidase 1 *(Cox1)* and cytochrome *b (Cytb)* data set (figure 5), including taxonomic name, country, voucher identification no. (ID) listed in GenBank, and accession nos. for the *Cox1* and *Cytb* genes in GenBank.

			Accession no.	
Louse	Country	Voucher ID	*Cox1*	*Cytb*
Pediculus humanus capitis	United States	11.01.2000.2	AY695979	AY696047
P. humanus capitis	United States	11.01.2000.3	AY695980	AY696048
P. humanus capitis	United States	11.01.2000.4	AY695981	AY696049
Pediculus humanus humanus	United States	11.29.2000.1	AY695970	AY696038
P. humanus humanus	United States	11.29.2000.2	AY695971	AY696039
P. humanus capitis	Honduras	01.31.2001.1	AY695976	AY696044
P. humanus capitis	Honduras	01.31.2001.2	AY695947	AY696015
P. humanus capitis	Philippines	01.31.2001.3	AY695977	AY696045
P. humanus capitis	Philippines	01.31.2001.4	AY695978	AY696046
P. humanus capitis	Philippines	01.14.2002.4	AY695951	AY696019
P. humanus capitis	Philippines	01.14.2002.5	AY695952	AY696020
P. humanus capitis	Philippines	01.14.2002.6	AY695975	AY696043
P. humanus capitis	Philippines	01.14.2002.7	AY695953	AY696021
P. humanus humanus	United States	01.21.2002.1	AY695958	AY696026
P. humanus humanus	United States	01.21.2002.2	AY695959	AY696027
P. humanus humanus	United States	01.21.2002.3	AY695960	AY696028
P. humanus humanus	United States	01.21.2002.4	AY695961	AY696029
P. humanus humanus	United States	01.21.2002.5	AY695962	AY696030
P. humanus humanus	United States	05.29.2002.1	AY695972	AY696040
P. humanus humanus	United States	05.29.2002.2	AY695973	AY696041
P. humanus capitis	Honduras	06.26.2002.1	AY695948	AY696016
P. humanus capitis	Honduras	06.26.2002.3	AY695949	AY696017
P. humanus capitis	Philippines	06.26.2002.7	AY695954	AY696022
P. humanus capitis	Philippines	06.26.2002.8	AY695989	AY696057
P. humanus capitis	Philippines	06.26.2002.9	AY695957	AY696025
P. humanus capitis	Honduras	06.27.2002.2	AY695991	AY696059
P. humanus capitis	Honduras	06.27.2002.3	AY695992	AY696060
P. humanus capitis	Honduras	06.27.2002.4	AY695944	AY696012
P. humanus capitis	Honduras	06.27.2002.5	AY695950	AY696018
P. humanus capitis	Philippines	06.27.2002.6	AY695993	AY696061
P. humanus capitis	Philippines	06.27.2002.7	AY695955	AY696023
P. humanus capitis	Philippines	06.27.2002.8	AY695956	AY696024
P. humanus capitis	Philippines	06.27.2002.9	AY695994	AY696062
P. humanus capitis	Philippines	06.27.2002.10	AY695990	AY696058
P. humanus humanus	Socotra Island, Yemen	08.14.2002.7	AY695974	AY696042
P. humanus capitis	Papua New Guinea	08.14.2002.3	AY695995	AY696063
P. humanus capitis	Papua New Guinea	08.14.2002.4	AY695996	AY696064
P. humanus capitis	Papua New Guinea	08.14.2002.5	AY695997	AY696065
P. humanus capitis	Papua New Guinea	08.14.2002.6	AY695998	AY696066
P. humanus humanus	Canada	11.19.2002.1	AY695963	AY696031
P. humanus humanus	Canada	11.19.2002.2	AY695964	AY696032
P. humanus humanus	Canada	11.19.2002.3	AY695965	AY696033
P. humanus humanus	Canada	11.19.2002.4	AY695966	AY696034
P. humanus humanus	Canada	11.19.2002.6	AY695967	AY696035
P. humanus humanus	Canada	11.19.2002.7	AY695968	AY696036
P. humanus humanus	Canada	11.19.2002.8	AY695969	AY696037
P. humanus capitis	Papua New Guinea	11.19.2002.11	AY695982	AY696050
P. humanus capitis	Papua New Guinea	11.19.2002.12	AY695983	AY696051
P. humanus capitis	Papua New Guinea	11.19.2002.14	AY695984	AY696052
P. humanus capitis	United States	12.30.02.1	AY695985	AY696053

(continued)

Table 1. ***(Continued)***

Louse	Country	Voucher ID	Accession no.	
			Cox1	*Cytb*
P. humanus capitis	United States	12.30.02.4	AY695943	NA
P. humanus capitis	United States	12.30.02.13	AY695940	AY696009
P. humanus capitis	United States	12.30.02.14	AY695941	AY696010
P. humanus capitis	United States	12.30.02.15	AY695987	AY696055
P. humanus capitis	United States	12.30.02.16	AY695988	AY696056
P. humanus capitis	United States	12.30.02.17	AY695945	AY696013
P. humanus capitis	United States	12.30.02.18	AY695946	AY696014
P. humanus capitis	Peru (ancient)	NA	EF653431	EF653430
Pediculus schaeffi	Uganda	05.23.2003.1	AY696599	AY696067
Pedicinus hamadryas	United States (captive)	02.04.2001.3	AY696007	AY696069
P. hamadryas	United States (captive)	01.14.2002.2	AY696006	AY696068
Pthirus pubis	United States	02.04.2001.1	AY696003	NA
P. pubis	United States	12.06.2001.2	AY696002	NA
P. pubis	United Kingdom	01.14.2002.1	AY696000	NA
P. pubis	United States	1.21.02.2	AY696001	NA
P. pubis	United States	08.14.2002.1	AY696004	NA
P. pubis	United States	08.14.2002.2	AY696005	NA
Fahrenholzia pinnata	United States	12.27.02.2	AY696008	DQ104217

NOTE. NA, not available.

which was located in this area (Osmore drainage) from sea level to an altitude of ~3000 m [14–16]. Two mummified heads with their hair (long and still braided) and scalp intact were collected and stored at Centro Mallqui (figure 2). Because the corresponding bodies were destroyed by looting, it was impossible to determine the sex of the mummies. The heads were found at the Chiribaya Baja site (south of the Moquegua River, 8 km from the coast), which has a mean calibrated age of 1025 AD. Lice were collected ($n = 407$ and 545) using forceps and were preserved in 96% ethanol (figure 3). Subsamples of the lice were deposited in the Insect Genomics Collection of the Whiting Lab at Brigham Young University (IGC PH52 and PH53) and were sent to 2 independent laboratories for ancient-DNA work, in accordance with established authenticity criteria [17].

Work on ancient DNA in Florida and Marseilles. Extractions were done in the dedicated ancient-DNA laboratory at the Florida Museum of Natural History, where no previous work on lice had been performed. We used a modified silica-based extraction method, following the method of Boom et al. [18] and Höss and Pääbo [19]. Two to 5 lice were ground together in liquid nitrogen and incubated for 48 h with agitation at 55°C in 600 μL of extraction buffer (7.5 mol/L guanidinium thiocyanate, 0.1 mol/L Tris hydrochloride [pH 6.4], 0.02 mol/L EDTA [pH 8.0], and 1.3% Triton X-100). After centrifugation, 500 μL of supernatant was removed to a second tube containing an additional 500 μL of extraction buffer and 40 μL of saturated silica suspension (SiO_2 in water). DNA was bound to the silica for 10 min at 27°C and was then pelleted by centrifugation, washed twice with extraction buffer and twice with 70% ethanol supplemented with 10 mmol/L sodium chloride, and eluted in two 75-μL volumes of Tris-EDTA buffer at 60°C. Mock DNA extractions (containing no lice) and negative polymerase chain reaction (PCR) controls were used to detect contamination. PCR primers for the *Cox1* (cytochrome oxidase subunit 1) gene were L6625 and H7005 [9]. PCR primers for the *Cytb* (cytochrome b) gene were CytbF1 (5'-GAG CGA CTG TAA TTA CTA ATC-3'), CytbR1 (5'-CAA CAA AAT TAT CCG GGT CC-3'), CytbF2 (5'-GAG GAG GGT TTT CAG TTA-3'), and CytbR2 (5'-ACT TTA TCA CTA TCC AAA TC-3'). PCR products were purified using Eppendorf PerfectPreps and were sequenced on an ABI automated sequencer. Cloning was performed on a subset of PCR products to test for polymorphisms resulting from the pooling of louse individuals during DNA extraction. Cloning methods followed those of Reed and Hafner [20]. The resulting sequences were deposited in GenBank.

At the Marseille laboratory, 2 vials of lice were prepared, each with 3 lice. Lice were imaged (figure 3), rinsed with distilled water, dried on sterile filter paper, and then crushed individually in sterile Eppendorf tubes. DNA was extracted using the QIAamp Tissue Kit in a room dedicated for DNA extraction. PCR and sequencing for *Cytb* was performed as described elsewhere [5, 21].

GenBank sequence data. To examine the phylogenetic relationships of the 3 previously described clades of *P. humanus* (figure 1), we downloaded the largest single-gene data set possible from GenBank that contains all 3 clades. This data set com-

prises 483 bp of the *Cox1* mtDNA gene from 167 specimens. The data were aligned unambiguously by eye. A second data matrix was constructed that consisted of louse individuals from GenBank with both *Cox1* gene and *Cytb* gene sequences available (table 1). This data set maximized the number of characters in common with our ancient DNA but lacked members from the type C clade. Our ancient-DNA sequences were unambiguously aligned (742 nt from 2 genes) with sequences from GenBank for the following taxa: *P. humanus, Pediculus schaeffi* (from chimpanzees), *P. pubis* (from humans), *Pedicinus hamadryas* (from baboons), and *Fahrenholzia pinnata* (from rodents) (see table 1 for GenBank accession numbers).

Phylogenetic analysis. The single-gene *Cox1* gene data set was examined using neighbor joining with a best-fit model of nucleotide evolution (general time reversible model with invariant sites and a gamma-distributed rate parameter) in Modeltest [22]. For the second data matrix, the computer program Modeltest [22] was used as a guide to determine a best-fit [23] maximum-likelihood (ML) model for the molecular data. This model was incorporated into ML heuristic searches in PAUP* [24]. Levels of topological support were calculated from 100 bootstrap replicates.

RESULTS

To maximize the DNA template from mummy head lice, we pooled several lice into single DNA extractions. We performed both direct sequencing on PCR-amplified products as well as sequencing on cloned fragments to test for polymorphisms caused by pooling individual lice. No such variation in the nucleotide sequences was found among sequenced clones. Louse sequences from the Florida laboratory ($n = 3$ extracts from 11 individual lice for the *Cox1* plus *Cytb* genes) were 100% identical to each other for each gene. *Cytb* sequences from the Marseille laboratory were identical to those sequenced at the Florida Museum of Natural History.

Phylogenetic analysis of the *Cox1* data from GenBank confirmed earlier findings [2, 4] showing 3 distinct clades of *P. humanus* (figure 4). One clade contained both head and body lice and was widespread in distribution. The type B clade was geographically restricted to Europe, Australia, North America, and Central America, and the type C clade was restricted to Ethiopia and Nepal. The type A and B clades were sisters to each other, with the type C clade a sister to A plus B.

The ML analysis showed that the mummy louse sequence clustered only with sequences in the type A clade, with 99% bootstrap support (figure 5). The results in the 2 laboratories were congruent and were exchanged at the end, and results for negative controls were negative. We therefore believe that our results are consistent and show that 11th century Americans hosted type A lice.

DISCUSSION

Here, we have reported the genotypes of the oldest tested lice. We believe that our results are consistent, because they were reproduced in 2 independent laboratories, as recommended for archaeology and paleomicrobiology [17, 25]. Given that our ancient lice were collected from the heads of mummies and not from preserved clothing and that head lice (but not body lice) exhibit all 3 mtDNA phylotypes (figure 3), our lice could have exhibited any of the 3 mtDNA lineages. Lice with both the A and B mtDNA phylotypes have been sampled from a single human head in both the United States and Honduras on several occasions, so mixed populations of lice on a single individual are not uncommon (personal observations). However, we can conclude from this study that the pre-Columbian lice sampled in Peru were of the genotype that is currently widespread and common and included both head and body lice (type A) [5].

On the basis of these data, the most parsimonious theory to explain the current dispersal of phylotype A is that it was distributed worldwide before the time of Columbus. Its current presence in all continents is consistent with this explanation. The presence of this phylotype in Africa suggests that it probably emerged in Africa and that the evolution from head to body lice appeared several times [5]. All body lice are derived from phylotype A [5]; for example, the genotype of the 200-year-old body lice found in a mass grave of Napoleon's soldiers was phylotype A [26]. Historical records confirmed that body lice have been found in Europe, Asia, and Africa long before the time of Columbus. Body lice infestation in ancient Egypt was described in the Bible [27]. Moreover, body lice were reported in prehistoric textiles in Austria [28] and in textiles from Masada in Israel 2000 years ago [29]. Body lice were also found in Greenland in a specimen dated AD 990–1350. Communication between Norway, Greenland, and North America may have helped to diffuse lice from Europe to the Americas in the Middle Ages. Evidence of the long-time presence of body lice in Europe, Asia, and Africa, along with our data from the Americas, showed that phylotype A was distributed worldwide before the globalization initiated in the time of Columbus. The existence of body lice, as opposed to head lice, in the Americas before Columbus is controversial [12]. There is a theory suggesting that epidemic typhus resulted from the association between European-borne body lice (imported by Spanish warriors) and Mexican-borne *Rickettsia prowazekii* [30]. This hypothesis has been reinforced recently by the identification of *R. prowazekii* in Mexican ticks [31]. Our findings of phylotype A in South America favor the hypothesis that body lice were present in the Americas before Columbus.

Ewing [32] studied large populations of lice in American Indians living at the beginning of the 20th century and found that they included 3 types of head lice, which he named *Pediculus humanus nigritarum* (presumably from Ethiopia; this type may be phylotype C), the common European louse (which may be

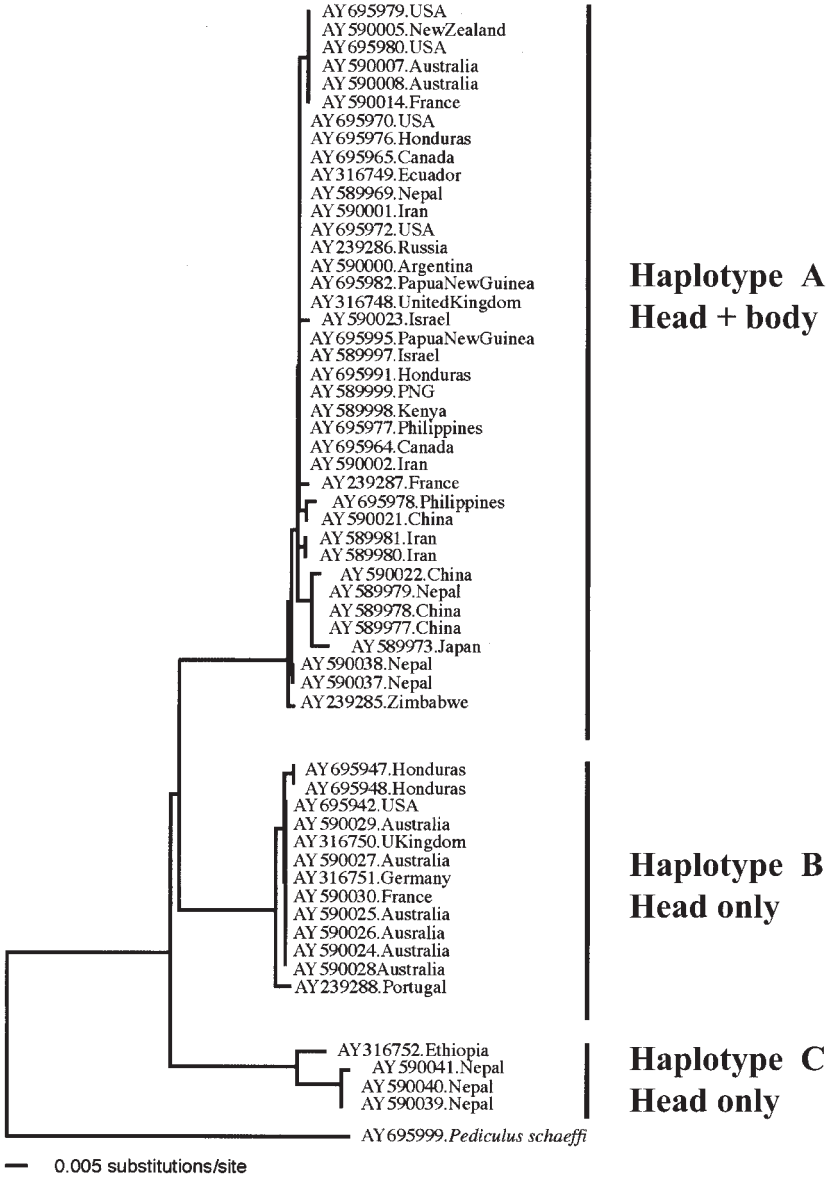

Figure 4. Phylogenetic tree based on the *Cox1* mitochondrial DNA gene showing 3 distinct clades of human head and body lice. As with previous studies, all body lice are confined to a single clade (type A), one that is geographically widespread. Type B lice are confined geographically to North and Central America, Europe, and Australia. Type C lice are restricted to Ethiopia and Nepal. The collection locality is given after the GenBank accession no. for each specimen.

phylotype A), and the American louse (*Pediculus humanus americanus,* which may be phylotype B) [12]. Ewing also reported in 1924 that head lice from American mummies in Peru were phenotypically distinct from head lice from mummies in the southeastern United States [32]. These types may well be phylotype A (currently the only phylotype found in Peruvian mummies) and phylotype B (predominant in the southeastern United States), respectively. However, whether any louse phylotypes other than A were present in the pre-Columbian New World awaits further sampling. Mummies from Arizona have been found to harbor head lice, and it would be interesting to identify their genotype [33].

Type B lice are as abundant as type A lice in the New World, although it is not known whether the type B clade came to the New World with the early peoples or more recently with European invaders. The absence of the type B louse from our small sample of ancient lice is insufficient evidence to reject the presence of the type B louse in pre-Columbian America. The acquisition of large numbers of mummy lice suitable for ancient-DNA sequencing seems unlikely, but it will be a priority to test mummies from the southwestern United States. Phylotype B was first found in America [2] but is now found also in Europe and Australia. Its source is unknown, but its current distribution, excluding Africa and Asia, may reflect importation by Europeans returning from America, given

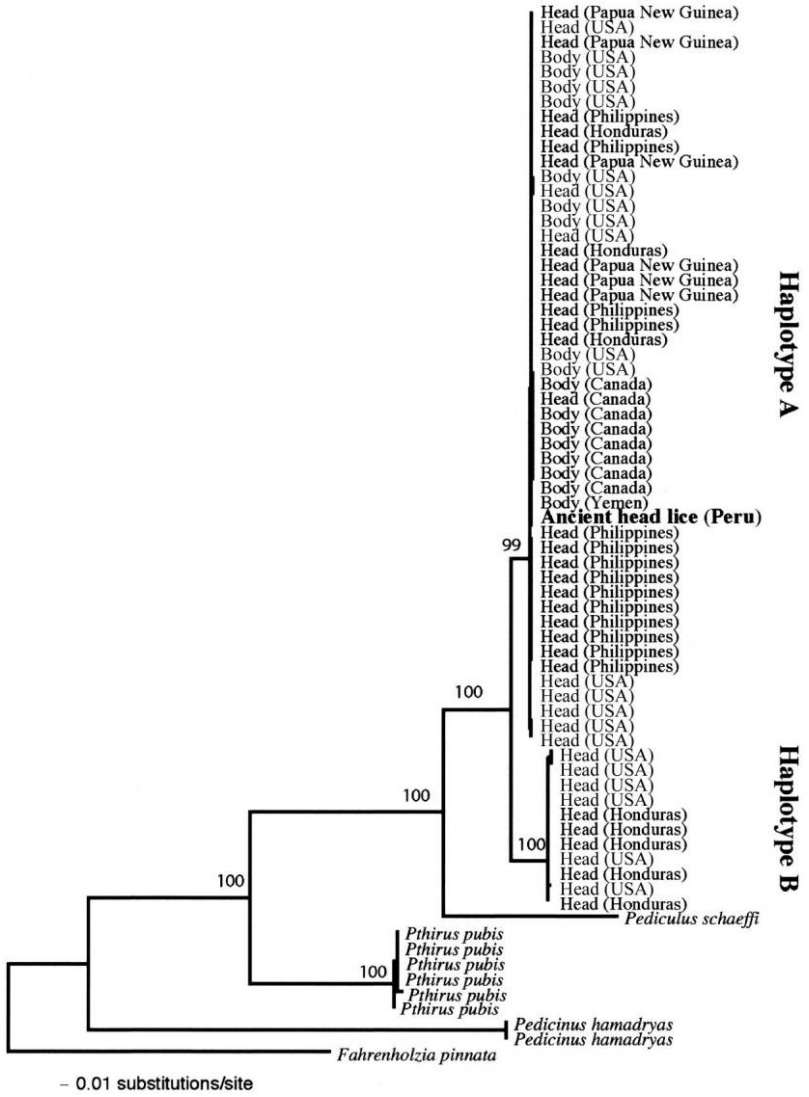

Figure 5. Maximum-likelihood phylogeny of primate lice rooted with a rodent louse, using the *Cox1* and *Cytb* genes. Samples of *Pediculus humanus* form 2 clades (A and B in figures 1 and 4). The ancient-DNA sequences retrieved from mummy lice cluster with sequences exhibiting the A phylotype with 99% bootstrap support (see table 1 for specimen identifiers and GenBank nos.).

that its dispersal follows European colonization where Europeans became the majority of the current population. If true, this contradicts the theory that America was the "melting pot" for the lice [12], favoring rather the hypothesis that America was the source of the louse heterogeneity.

Lice are among the best conserved human parasites. Lice 200 years old were found in a grave of Napoleon's soldiers [26]; 2000-year-old lice were recovered from Masada in Israel [29]; an lice have been recovered from Egyptian mummies. Because of the rapid desiccation of lice that likely occurs during natural human mummification, it is quite possible to amplify and sequence DNA from additional mummy lice. Future collecting efforts should target naturally preserved mummies with hair still intact. Lice from such mummies may provide valuable insight into the pre-Columbian population of body lice and help us understand the distribution of phylotypes A and B in the Americas and the Old World before globalization. Currently, the most likely theory is that phylotype A, issued from Africa, was distributed worldwide. Phylotype B may have survived and developed only in North and Central America, before Columbus, and is now spreading in the world, carried back by Europeans returning from the Americas. Type C is confined to highly mountainous countries of the Old World. In any case, the present work shows that there are several phylotypes of lice with geographical restrictions and that this was true before the arrival of Columbus in the Americas.

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