Divergent mtDNA lineages of goats in an Early Neolithic site, far from the initial domestication areas

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Goats were among the first farm animals domesticated, ≈10,500 years ago, contributing to the rise of the ''Neolithic revolution.'' Previous genetic studies have revealed that contemporary domestic goats (*Capra hircus*) show far weaker intercontinental population structuring than other livestock species, suggesting that goats have been transported more extensively. However, the timing of these extensive movements in goats remains unknown. To address this question, we analyzed mtDNA sequences from 19 ancient goat bones (7,300–6,900 years old) from one of the earliest Neolithic sites in southwestern Europe. Phylogenetic analysis revealed that two highly divergent goat lineages coexisted in each of the two Early Neolithic layers of this site. This finding indicates that high mtDNA diversity was already present >7,000 years ago in European goats, far from their areas of initial domestication in the Near East. These results argue for substantial gene flow among goat populations dating back to the early neolithisation of Europe and for a dual domestication scenario in the Near East, with two independent but essentially contemporary origins (of both A and C domestic lineages) and several more remote and/or later origins.

archaeology | ancient DNA | livestock origins | Neolithic expansion | *Capra*

After the initial plant and animal domestications in the Near East, *ca.* 11,500 and 10,500, respectively, years ago (ya) (1, 2), Neolithic culture diffused into Europe along two main routes (3, 4) (Fig. 1). From their initial domestication areas (5–7), goats were introduced into Europe by following these routes. Archaeological data and radiocarbon dates on seeds or bones provide support for an earlier arrival in western Europe (namely France) via the Mediterranean route rather than the "Danubian" route (4, 8, 9).

Genetic studies of present-day domestic goats have revealed multiple highly divergent maternal lineages (A, B, C, D, and E) (10–12). The time since divergence among the main lineages A, B, and C vastly predates the time of domestication suggested from the zooarchaeological records, indicating that these three lineages arose from genetically discrete populations rather than from a single wild population (10). In addition, genetic data have revealed that the degree of phylogeographic structuring is far weaker in domestic goats than in other livestock species (13–17), which probably results from high gene flow at the intercontinental level, suggesting that goats have been extensively transported (10).

It is intriguing to consider at what time period the movements responsible for high gene flow among domestic goat populations might have taken place. These movements might go back as far as the first wave(s) of expansion of farming that originated from the Near East *ca.* 9,500 ya (6). Alternatively, the extensive mixing might have started much later, when people improved new domestic animal types and spread them throughout the Old World at different periods, e.g., Late Neolithic (wool sheep) (18, 19), Roman times (introduction of large cattle) (20, 21), or during the rise and spread of the modern breeds of ungulates at the end of the 19th and beginning of the 20th centuries.

To investigate whether extensive mixing had taken place at the beginning of the Neolithic or during subsequent historical times, we analyzed 24 ancient goat bone samples originating from Southern France (Ardèche) at the Early Neolithic site of Baume d'Oullen (22). This site is very well suited to test for ancient mixing among goat populations because the two earliest Neolithic layers (C6 and C5) have yielded >5,000 identified animal bone specimens associated with a large number of human artifacts dating to the Cardial and Epicardial periods, respectively (22–24). These Early Neolithic cultures are dated from the middle of the 8th millennium to the very beginning of the 7th millennium before present (B.P.) (8, 9) and represent the second step of the Neolithic in this area, just after the Impressa wave, dated in this area to 7,700–7,600 cal. B.P. (4, 9, 23) (Fig. 1).

Results

Our zooarchaeological analyses at Baume d'Oullen and at a range of Early Neolithic sites from Southern Europe reveal that the number of goats was low with reference to sheep and even to cattle and pigs (see Table 2, which is published as supporting information on the PNAS web site). These data indicate that Early Neolithic farmers were breeding and transporting relatively small flocks of goats. These small local flocks were, however, probably more or less interbred at the regional scale with other Early Neolithic flocks, because the contacts between the small human communities were strong enough to generate large and rather homogeneous cultural areas such as the one of

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Abbreviations: AMS, accelerator mass spectrometry; cal. B.P., calibrated radiocarbon date B.P.; ya, years ago.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ847506–DQ847511).

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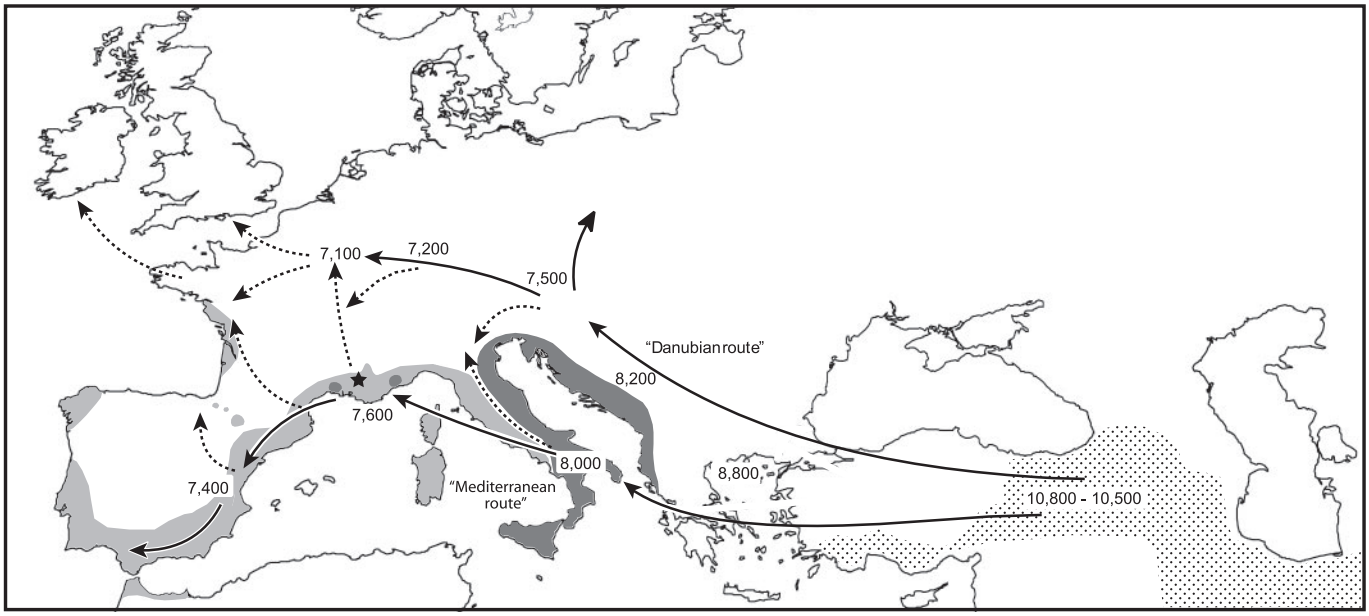

Fig. 1. Map shows occidental part of the current geographic distribution of the wild goat, *Capra aegagrus* (dotted area), as well as the two main waves for the initial advancement of the Neolithic culture into Europe: the Mediterranean route and the Danubian route (4, 8, 9). The location of Baume d'Oullen is indicated by a star. The dates on the map are calibrated radiocarbon date-derived B.P. (cal. B.P.). Solid-line arrows indicate main flow; broken-line arrows indicate possible secondary flows. Dark gray zones indicate the area of the Impressa culture (8,000–7,500 cal. B.P.); light gray zones indicate the area of the Cardial and cultures (between 7,500 and 6,800 cal. B.P.) (4).

the Cardial pottery, which spread from the Tyrrhenian area to the French Midi, Spain, and Northern Morocco (4, 8) (Fig. 1).

In this context, we can argue that if no extensive gene flow had taken place during the Neolithic expansion between the Eastern and the Western Mediterranean Basin, we would expect to find low genetic diversity in goats from the Cardial area as expressed in the Baume d'Oullen site, because of successive founder effects (from the Near East to the Western Mediterranean Basin) that would have led to rapid loss of mtDNA types because of the small population size at the regional scale (25). On the contrary, if an extensive mixing had already started during the Impressa or the Cardial waves (7,700–7,000 ya) of expansion into Europe, we would expect the diversity of ancient goats to be high in Baume d'Oullen.

Two separate mtDNA segments, 130 bp of the control region and 110 bp of cytochrome *b*, were successfully amplified and sequenced from 19 of the 24 ancient samples tested. The results were confirmed by independent analyses in separate laboratories and sequences were validated by cloning (26) (see Figs. 4 and 5 and Table 3, which are published as supporting information on the PNAS web site). The high rate of success (83%) indicates good preservation of ancient DNA in the samples, which is exceptional although not surprising because of their origin from a cave-like deposit (27–30).

The ancient sequences cluster in two divergent groups that correspond to the A and C lineages previously identified in an extensive contemporary data set of >400 domestic goats (10) (Fig. 2; see also Fig. 6, which is published as supporting information on the PNAS web site). Both lineages A and C currently coexist in Europe, although lineage C was found in <0.5% of the modern samples (10 of 207) and only in Switzerland and Slovenia. In the ancient samples, however, two mtDNA types (or

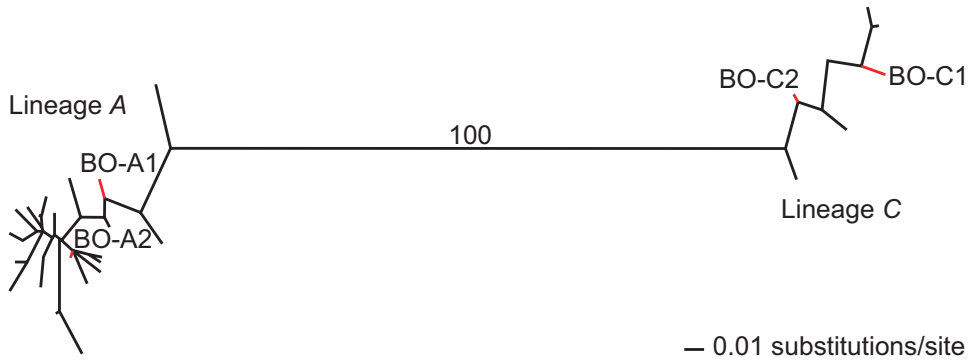

Fig. 2. Neighbor-joining tree shows the ancient haplotypes that cluster in the two divergent lineages A and C. The number on the branch is the percentage of 2,000 bootstrap trees with the same branch structure. The four ancient goat haplotypes from southern France (red branches BO-A1, BO-A2, BO-C1, and BO-C2) are compared with 43 previously published modern goat sequences from local autochthonous breeds. Lineage A sequences are from contemporary goats from France ($n = 14$), Slovenia ($n = 2$), and Switzerland ($n = 18$). Lineage C sequences are from contemporary goats from Slovenia ($n = 6$) and Switzerland ($n = 3$). Lineage C has been identified only in the Slovenian goats and the Swiss Toggenburg goats; it has never been found in contemporary goats from France. Trees constructed by using Bayesian analyses give similar results (see Fig. 6).

Table 1. Control region haplotypes and lineages identified in 19 goat bone samples from the archaeological site of Baume d'Oullen

Layer	Lineage	Haplotype	Samples
C6	A	BO-A1	BO-06, BO-07,* BO-17, BO-22, BO-23†
		BO-A2	BO-28
	C	BO-C1	BO-01,* BO-04, BO-12, BO-14,† BO-15,*† BO-20, BO-24, BO-27
		BO-C2	BO-19†
C5	A	BO-A1	BO-02,*† BO-26
	C	BO-C1	BO-03,* BO-11†

*Radiocarbon dated.
†DNA analysis reproduced in two separate laboratories.

haplotypes) belong to lineage A, and two to lineage C (Fig. 2). The lineage identity (A or C) of both modern and ancient sequences was verified by using both cytochrome *b* and control region markers. These results demonstrate that both lineages A and C were represented among the first populations of domestic goats moving into western Europe.

Both archaeological levels C5 and C6 of the Baume d'Oullen deposit contained the two divergent lineages (A and C), and level C6 contained all four haplotypes (Table 1). Moreover, accelerator mass spectrometer (AMS) radiocarbon dates obtained from five bone samples from the two Early Neolithic levels do not statistically differ from one another and are comprised within the range of *ca.* 7,300–6,900 ya (Fig. 3). This range is well before the first Danubian wave coming from western Germany reached the Mediterranean area (Fig. 1). Therefore, our results clearly indicate that goats from lineages A and C, originating from the Mediterranean wave, lived at the same site within a short time period >7,000 ya.

We compared genetic diversity from 130-bp control region sequences in ancient and modern goats, including the two most polymorphic locations found in Europe today (Switzerland and Slovenia) (see Table 4, which is published as supporting information on the PNAS web site). Nucleotide diversity is high within the ancient samples, mostly because of the presence of both lineages A and C, which are highly divergent. Haplotype diversity in ancient samples (4 mtDNA types for 19 samples) might be underestimated considering that some of the samples sharing the same haplotype might belong to a single individual.

Discussion

Population genetics theory actually predicts it is unlikely that two or more lineages will persist in a population beyond $4N_{ef}$ generations (N_{ef} is the effective number of females) because genetic drift leads to monomorphism (31). In small goat populations (e.g., where $N_{ef} = 10–20$), genetic diversity is lost rapidly (e.g., in 40–80 generations) because of genetic drift, unless gene flow through immigration occurs. Thus it is likely that only one lineage is present after a short time (e.g., only 160–320 years, counting 4 years per goat generation) in a goat population where no exchanges occur. Accordingly, the relatively high diversity found within goats from Baume d'Oullen could have been maintained only if the effective size of the population (N_e) was very large, which could have resulted in either of two ways: (*i*) if the effective size of the goat population in the Cardial area of the Western Mediterranean Basin was very large, an assumption that is not supported by zooarchaeological data, or (*ii*) if substantial gene flow occurred, making the local effective size approach the global N_e (e.g., for the entire goat species). Therefore, such an early diversity seems likely to be explained by a diverse founding pool and a large effective size (i.e., global N_e) resulting from extensive exchanges of goats between the eastern fully Neolithic areas and the western pioneer front(s) of neolithisation, all along the diffusion route from the Middle East into Europe.

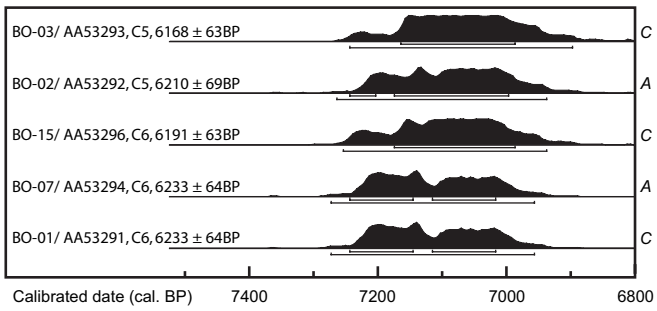

Fig. 3. Calibration histograms in cal. B.P. calendar dates for five AMS radiocarbon-dated goat bones from Baume d'Oullen. Each sample label is followed by its AMS lab number, stratigraphic layer, and uncalibrated date (B.P.) with standard deviation. The calibration histograms for each sample show the probability distribution of possible true calendar ages, and the brackets under each histogram delineate the 1- and 2 σ calendar age ranges. All five samples are statistically the same radiocarbon and calendar age. Mitochondrial lineage (A or C) is indicated on the right.

The presence of the two lineages in southwestern Europe since as early as the beginning of the Neolithic may result from either the succession of different waves of goats bearing different haplotypes between the first Impressa (7,700–7,500 B.P.) and Cardial (7,500–7,000 B.P.) time periods, or from one wave bearing all of the diversity as early as the first Impressa steps. In any case, however, our results reveal that the diversity of present-day goats does not result mainly from any Late Neolithic, Roman, or Modern episode. Instead, these data suggest that extensive gene flow occurred around the time of the first waves of arrival of Neolithic farmers into Europe through the Mediterranean route, *ca.* 7,500 ya. This is evidence of a continuing high degree of interactions (through regional contacts and commerce) along the Mediterranean basin during the Early Neolithic.

This hypothesis of substantial early gene flow is consistent with biological and behavioral characteristics of goats, which are the hardiest of all livestock species and will thrive and breed on minimal food and under extremes of temperature and humidity. Goats can provide clothing, meat, and milk, which was the case at Baume d'Oullen (32), as well as bone, sinew, and dung (33, 34). Goats were also easy to transport over long distances in boats as well as by land because they followed humans easily.

The early coexistence of both lineage A and C goats in southwestern Europe also implies that both lineages likely arose within similar temporal and geographic parameters. In turn, if it would be confirmed by further analyses in this area, the absence of ancient goats belonging to lineage B, D, or E in the archaeological samples suggests a more removed process, both in timing and geographic center, at the origin of these lineages. Consequently, our results support a dual domestication scenario with two independent but essentially contemporary origins (of both A and C domestic lineages), and several more remote and/or later origins. The two first centers of origins (of A and C lineages) may fit with two of the three main Near Eastern areas where the earliest evidence of domestication has been detected (until now) between 10,500 and 9,000 ya, i.e., the oriental Taurus mountains (6), the Zagros mountains (5, 7), and somewhat less supported, the Jordan valley (35). The more remote and/or later goat domestication origins that would have given birth to lineages B, D, and E may fit the hypothesis of an Indus center of domestication, already accepted as a separate domestication center for cattle (1, 13, 14), or other centers in Central Asia, where so little is known about the domestication of ungulates.

Materials and Methods

Archaeological Data. The archaeological site of Baume d'Oullen is a large cave porch (1,500 m^2) located in the Ardèche low mountains (≈160 m above sea level), that was excavated between 1977 and 1990 by J.-L. Roudil (22). The two 8th/7th millennia B.P. Early Neolithic levels (C6 and C5) extended on only one-tenth (≈160 m^2) of the area of the cave porch. These two layers represent two forms of the typical Western Mediterranean Cardial cultural complex, dated in Southern France between 7,500 and 6,700 cal. B.P., although details are still being debated (23, 36). From a stratigraphical point of view, layers C6 and C5 are not very well differentiated from one another (22), and vertical migrations of items between the two layers is attested. Consequently, it is not surprising that the dates from the two levels do not differ.

Zooarchaeological Data. Animal bones were very well preserved and abundant in the two Early Neolithic layers, C6 (n = 3,639) and C5 (n = 1,118). The zooarchaeological data (Table 2) indicate that in Baume d'Oullen, goat is the least abundant domestic ungulate in the C6 layer (12.0% vs. 22.5% for pig, 23.3% for cattle, and 42.2% for sheep) and in the C5 layer (13.6% vs. 23.1%, 13.9%, and 49.4%, respectively).

AMS Radiocarbon Dating of the Samples. AMS radiocarbon dates were obtained directly from five goat bone samples coming from both C5 and C6 layers and from which DNA was successfully extracted. The radiocarbon dates do not statistically differ from one another and suggest 2σ calibrated ages of 7,272–6,907 cal. B.P., i.e., 5,323–4,958 cal. B.C. (Fig. 3). Uncalibrated ^{14}C dates (B.P.) diverge from actual calendar dates (cal. B.P.) because of cosmic and geophysical phenomena. Calibration using ^{14}C measurements from known-age tree rings transforms ^{14}C measurements and their associated errors into calendar date probability distributions, as shown in Fig. 3. Calibration plots in Fig. 3 were created by using the OxCal 3.10 radiocarbon calibration program (37) and atmospheric data from Reimer *et al.* (38). Good preservation of the five bone samples is indicated by high collagen recovery, C/N ratio values between 3.4 and 3.6 (39), and carbon and nitrogen stable isotope values. These measurements support the reliability of the radiocarbon dates (see Table 5, which is published as supporting information on the PNAS web site).

Authentication of Ancient DNA Sequences. Several lines of evidence support that the sequences described in this paper are authentic ancient DNA sequences, and that no contamination occurred in our samples during the extraction and amplification steps: (*i*) the extraction and amplification of several samples were replicated in two different laboratories (Laboratoire d'Écologie Alpine, Grenoble, and Centre de Génétique Moléculaire et Cellulaire, Lyon) that both contain special extraction and PCR rooms solely devoted to ancient DNA studies; (*ii*) no modern goat material was ever analyzed in Lyons; (*iii*) no contaminating goat DNA was ever detected in the numerous negative controls including, for each set of PCR experiments, an extraction blank without DNA sample, a blank PCR mix, and a third blank (in Lyon) to monitor for aerosol contamination (28); (*iv*) in Lyon, bones of cervids were coextracted with goat bones, in the way of monitoring cross-contamination, and no goat contamination was detected on cervid samples; (*v*) the observed pattern of mutations between clones (Table 3) is consistent with that previously described for ancient DNA because of degradation and chemical modifications of the template (40); and (*vi*) two independent mtDNA markers (cytochrome *b* and control region) identified the same divergent lineages (there are only two haplotypes for cytochrome *b* sequences, one for lineage A and one for lineage C).

DNA Extraction, Amplification, and Sequencing. In Grenoble, 23 goat bones or teeth were processed by removing the exterior layer with a sterile scalpel blade and grinding the bone to powder in a small mortar, which was bleached and exposed to shortwave UV after each use. DNA was extracted by using the Qiagen tissue kit (Chatsworth, CA.) The primers CAP-FII (5′-GATCTTCCYCATGCATATAAGCA-3′) and CAP-RII (5′-CGGGTTGCTGGTTTCAC-3′) were used to amplify a 130-bp mtDNA fragment of the HVI control region. PCR amplifications were conducted in a 25-μl volume containing 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 200 μg/ml BSA, and 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR mixture underwent an initial step at 95°C for 5 min, followed by 55–60 cycles of 30 s at 95°C, 30 s at 55°C, and 4–10 min at 72°C. A 110-bp stretch of cytochrome *b* was amplified by using primers CapFC1 (5′-CTCTGTAACTCA-CATTTGTC-3′) and CapRB1b (5′-GTTTCATGTTTCTA-GAAAGGT-3′). PCR was identical except that the annealing temperature for cytochrome *b* primers was 50°C. The PCR products were isolated from 1.6% agarose gels and purified by using the Qiaquick gel purification kit (Qiagen). PCR products were sequenced directly, and all sequences were obtained for both DNA strands as previously described (10) except that annealing temperature was 55°C for control region and 50°C for cytochrome *b*. In Lyon, seven samples (Table 1), representative of the different haplotypes identified in Grenoble, and a supplementary one (BO-28, handled only in Lyon), were independently extracted by phenol/chloroform and amplified, according to protocols developed in the laboratory for ancient DNA (28, 29). Only the more variable marker (130 bp of the HVI control region) was amplified by PCR (10 min at 92°C, then 50–60 cycles of 1 min at 92°C, 1 min at 55°C, 45 s at 72°C, and finally 10 min at 72°C), and one to three PCR amplifications per sample were cloned by using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) for sequencing. For each PCR product, three to six clones were analyzed. Clones were amplified by PCR from bacterial colonies by using M13 universal primers (10 min at 94°C, then 30 or 35 cycles of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, and finally 5 min at 72°C) and sequenced with the same primers. The consensus sequence resulting from the individual clones was compared with that of the direct sequence from Grenoble (Figs. 4 and 5). The authentic sequence was always deduced from the consensus between clones from different amplification reactions.

DNA Analysis. Modern goat sequences were obtained from published data (8). Sequences were aligned by eye. Neighbor-joining trees were constructed with Kimura two-parameter corrected distances (alpha shape parameter of the gamma distribution = 0.29; different parameters give very similar results) (10) by using PAUP* software, version 4.0 (Sinauer Associates, Sunderland, MA) (41). Trees constructed by using Bayesian analyses group the sequences into the same two clusters as neighbor-joining trees. Bayesian analyses were performed by using MrBayes 3.1.1 (42) with model parameters selected by the Akaike information criterion (AIC) implemented in MrModeltest2 [a modification by J. A. A. Nylander of Modeltest (43), available at www.csit.fsu.edu/~nylander] (HKY+G). Four runs were done under the following conditions: 1 million generations, four Markov chains using the Metropolis-coupled Markov chain Monte Carlo algorithm, tree sampling every 100 generations, defaults on Bayesian priors, and burn-in value determined after empirical check of stationarity. The mean pairwise sequence differences and nucleotide diversity within populations (44, 45) were calculated by using ARLEQUIN software, version 2.000 (University of Geneva, Geneva, Switzerland) (46), and by using pairwise differences as the genetic distance. To account for

uneven sampling and a possible sampling bias, we used mtDNA haplotypes (instead of sequences) for calculation of the mean number of differences and nucleotide diversity (Table 4).

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