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Mitochondrial DNA analysis of ancient human bones excavated from Nukdo island, S.Korea

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We have performed analyses using ancient DNA extracted from 25 excavated human bones, estimating around the 1st century B.C. Ancient human bones were obtained from Nukdo Island, which is located off of the Korean peninsula of East Asia. We made concerted efforts to extract ancient DNA of high quality and to obtain reproducible PCR products, as this was a primary consideration for this extensive kind of undertaking. We performed PCR amplifications for several regions of the mitochondrial DNA, and could determine mitochondrial haplogroups for 21 ancient DNA samples. Genetic information from mitochondrial DNA belonged to super-haplogroup M, haplogroup D or its sub-haplogroups (D4 or D4b), which are distinctively found in East Asians, including Koreans or Japanese. The dendrogram and principal component analysis based on haplogroup frequencies revealed that the Nukdo population was close to those of the East Asians and clearly distinguished from populations shown in the other regions. Considering that Nukdo is geologically isolated in the southern part of the Korean peninsula and is a site of commercial importance with neighboring countries, these results may reflect genetic continuity for the habitation and migration of ethnic groups who had lived in a particular area in the past. Therefore, we suggest that phylogenetic analyses of ancient DNA have significant advantages for clarifying the origins and migrations of ethnic groups, or human races. [BMB reports 2010; 43(2): 133-139]

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INTRODUCTION

Forensic identification of individuals is based on the analysis of genetic markers, such as SNPs (single nucleotide polymorphisms) or STRs (short tandem repeats), present in chromosomal or mitochondrial DNA (1-3). This approach has been extended to ethnic analysis in human population genetics. In particular, ancient DNA analysis has been increasingly used to obtain genetic and heredity information for human beings from the past (4, 5). However, several obstacles exist for ancient DNA analysis (6-9). Because ancient DNA is physiologically degraded, as well as contaminated by inhibitors due to burial in the earth for long periods, its analysis is difficult by PCR amplification. In addition, it may be spuriously contaminated by foreign DNA during processing of the ancient DNA. Therefore, ancient DNA analysis requires more systematic approaches, including improved DNA purification or strategic PCR amplifications.

Mitochondrial DNA is particularly suited to searching the genetic information of ancient DNA due to the presence of high copy numbers of mitochondria in cells. Furthermore, 16,769 bp of mitochondrial DNA have been fully sequenced, making SNP information easily accessible (10, 11). As mitochondria are transmitted to the next generation only by the maternal gamete during fertilization, we can assume the maternal heritage of analyzed samples. To date, previous studies using ancient DNA have determined mitochondrial haplogroups arising from distinct polymorphisms in non-coding control regions of mitochondrial DNA (12, 13). However, non-coding control regions are not only prone to copying errors during biological replication, but are also prone to ambiguous PCR amplification. Thus, the confidence of phylogenetic results based only on sequences from control regions has been questioned (14). In addition, as insufficient criteria or different clades

may be used among researchers, several problems have arisen regarding the determinations of mitochondrial haplogroups.

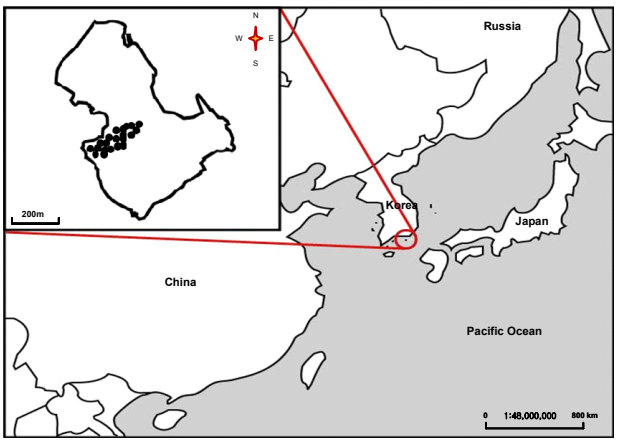

Fig. 1. Geographical map of Nukdo and excavation sites for the ancient bones. Nukdo is at the southern end of the Korean peninsula, administered by Sacheon City, Kyungsang Namdo, Republic of Korea. The Korean peninsula is located in far eastern Asia, which includes the countries of Mongolia, China, Korea and Japan. The small closed circles in the inset show the excavation sites for the ancient bones.

Coding regions in mitochondrial DNA also contain distinct nucleotide changes that depend on ethnicity or race, and are useful for determining mitochondrial haplogroups (15). Recent work suggests that it is indispensable to correctly determine mitochondrial haplogroups by analyzing both coding and control regions of mitochondrial DNA.

Our interdisciplinary approach has overcome several obstacles for genetic analysis of human beings from the past (16). We have improved a method for the extraction of high-quality ancient human DNA and strategically amplified several coding and control regions in order to analyze SNPs. Mitochondrial DNA sequences were systematically trimmed by the DNASTAR program and were determined to be suitable for haplogroups. In this study, we analyzed the excavated bones of ancient human beings who had lived around the 1st B.C. on Nukdo, which is a small island administered off of the Korean peninsula. To determine mitochondrial haplogroups, we attempted to analyze several coding regions as well as control regions of the mitochondrial DNA. Based on these results, we suggest that mitochondrial DNA analysis of ancient human traces excavated from an island might reflect typical ethnic haplogroups.

Table 1. Primer pairs for amplification of coding and control regions for mitochondrial DNA

Numb.	Localization	Sequences	size	SNP and haplogroups
Primer 1	MT663-584F	5′-AGC TTA CCT CCT CAA AGC-3′	155	(A) 663 A-G
	MT663-739R	5′-GTG GTG ATT TAG AGG GTG-3′		
Primer 2	MT3010-2932F	5′-GGG ATA ACA GCG CAA TCC-3′	145	(D4) 3010 G-C
	MT3010-3077R	5′-GTC TGA ACT CAG ATC ACG-3′		
Primer 3	MT3394-3279F	5′-CAG AGG TTC AAT TCC TCT TC-3′	286	(M9a) 3394 T-C
	MT3394-3565R	5′-TTC ATA GTA GAA GAG CGA TG-3′		
Primer 4	MT4715-4621F	5′-GTT CCA CAG AAG CTG CCA TC-3′	355	(CZ) 4715 A-T
	MT4715-4976R	5′-TCC ACC TCA ACT GCC TGC TA-3′		(G) 4833 G-C
Primer 5	MT5178-5041F	5′-TAG CAG TTC TAC CGT ACA AC-3′	240	(D) 5178 C-A
	MT5178-5281R	5′-GTG AAT TCT TCG ATA ATG GC-3′		
Primer 6	MT5417-5336F	5′-CCT CTA CTT CTA CCT ACG-3′	158	(N9) 517 G-A
	MT5417-5494R	5′-AAA GGG GAG ATA GGT AGG-3′		
Primer 7	MT8281-8190F	5′-CAA ACC ACA GTT TCA TGC-3′	198	(B) 8291-8299 9 bp deletion
	MT8281-8388R	5′-ACG GTA GTA TTT AGT TGG G-3′		
Primer 8	MT9824-9743F	5′-AGA GTA CTT CGA GTC TCC-3′	253	(M7) 9824 T-A
	MT9824-9996R	5′-AAG AGT AAG ACC CTC ATC AA-3′		
Primer 9	MT10398-10085F	5′-CAA CAC CCT CCT AGC CTT AC-3′	398	(M) 10398 A-G
	MT10398-10483R	5′-TAA ATG AGG GGCATT TGG-3′		
Primer 10	MT12705-12616F	5′-TTG TTC GTT ACA TGG TCC-3′	180	(R) 12705 C-C
	MT12705-12796R	5′-GCA AGA AGG ATA TAA TTC C-3′		
*HV1	MT-HV1-F15974	5′-ACT CCA CCA TTA GCA CCC AA-3′	447	
	MT-HV1-R16421	5′-TTG ATT TCA CGA GGA TGG T-3′		
*HV2	MT-HV2-F15	5′-CAC CCT ATT AAC CAC TCA CG-3′	414	
	MT-HV2-R429	5′-CTG TTA AAA GTG CAT ACC GCC A-3′		

*HV: hypervariable region

Table 2. Information on ancient human bones excavated from Nukdo and their mitochondrial haplogroups

No.	Sample	Excavated region	Part of the bone	Haplo-group	Coding region												Other polymor-phism	Control region	
					M/N 10398	M/N 10400	D 5178	M9 3394	M7 9824	CZ 4715	D4 3010	R 12705	A 663	N9 5417	B 9 bp DEL	G 4833		*HV1	*HV2
					A	C	C	T	T	A	G	C	A	G		A			
1	KR0004	Nuk-do 7	Femur	D	G	T	A				G							16223T 16362C/T	73G 152C 263G
2	KR0005	Nuk-do 23	Left Tibia	D	G		A				G/A							16223T 16362C/T	73G 152C 263G 309C IN 310C/T 315C IN
3	KR0007	Nuk-do 18	Left Tibia	D	G	T	A				G							16223T 16362C	73G 152C 263G 309C IN 310C
4	KR0009	Nuk-do 4	Left Femur	D	G	T	A				G							16189C	
5	KR0017	Nuk-do 6	Right Tibia	D	G	C/T	A				G							16129A 16223T 16362C	
6	KR0002	Nuk-do 2	Tibia	D4	G	T	A				A							16223T	73G 152C/T 263G 309C IN 310C 315C IN
7	KR0011	Nuk-do 2	Right Tibia	D4	G		A				A							16223T 16362C	
8	KR0014	Nuk-do 26	Left Humerus	D4	G		A				A							16223T 16362C/T	73G 152C/T 263G
9	KR0015	Nuk-do 4	Left Tibia	D4	G		A				A							16223T 16362C/T	73G 98C/T 152C 263G 315C IN
10	KR0018	Nuk-do 6	Left Humerus	D4	G		A				A							16223T 16362C/T	73G 152C 263G 315C IN
11	KR0019	Nuk-do 7-1	Right Humerus	D4	G	T	A				A							16223T 16362C	73G 152C/T 263G 315C IN
12	KR0022	Nuk-do 7-4	Right Humerus	D4	G	T	A				A							16223T	
13	KR0029	Nuk-do 8	Right Tibia	D4	G	T	A				A							16223T 16362C/T	73G 263G 315C IN
14	KR0030	Nuk-do 10	Right Tibia	D4	G	T	A				A							16223T 16362C/T	73G 263G 315C IN
15	KR0016	Nuk-do 9	Right Tibia	D4b	G	T	A				A							16223T 16319A	73G 189G 194T 195C 204C 207A 263G 309C IN 315C IN
16	KR0027	Nuk-do 3	Left Huckle Bone	D4b	G		A				A							16187T 16223T 16290T 16319A	
17	KR0006	Nuk-do 24	Right Femur	M	G	C/T	C											16223T 16362C/T	73G 152C 263G 309C IN 310C/T 315C IN
18	KR0008	Nuk-do 26	Left Tibia	M	G	T	A/C											16223T 16362C/T	73G 152C 263G 309C IN 310C/T
19	KR0013	Nuk-do 11	Left Tibia	M	G	T	C		T	A						A	4769G	16223T 16362C/T	
20	KR0032	Nuk-do 13	Left Femur	M	G	T	C		T	A						A	4769G 4883T	16223T 16362C	73G 152C 263G 315C IN
21	KR0035	Nuk-do 21	Ribs	M	G	C/T	C			A						A	4769G	16161A 16223T 16234C/T 16250C/T 16362C/T	
22	KR0010	Nuk-do 15	Right Tibia															16223T 16362C/T	73G 152C 263G 315C IN
23	KR0012	Nuk-do 7 F-8 IV	Right Femur															16126T/C 16176T 16223T	73G 189G 194T 195C 204C 207A 263G 309C IN 315C IN
24	KR0020	Nuk-do 7-2	Right Humerus															16223T	73G 152C/T 194C/T 204T/C 263G 309C IN 315C IN
25	KR0021	Nuk-do 7-3	Right Humerus															16223T 16362C	195C 204C 215G 263G 315C IN

*HV: hypervariable region

RESULTS

Geographical and archaeological background of Nukdo island

Nukdo is a small island of 0.56 km^2 at the southern end of the Korean peninsula administered by Sacheon City, Kyungsang Namdo, Republic of Korea (Fig. 1). Human habitats and tombs have been excavated on Nukdo since 1979. Large amounts of non-decorated pottery (Mumon pottery) were excavated from several sites (17, 18). It also included numerous human bones, jars, coffins, shell mounds, and so on. Based on the chronological context of the discovered artifacts and grave types, these artifacts were estimated to be ancient relics of human beings who had lived during the 1st century B.C., from the late bronze to the early iron ages (17). In addition, foreign artifacts, such as Chinese coins and Yayoi-style pottery (ancient Japanese origin), were also excavated from Nukdo, suggesting that ancient human beings on Nukdo might have inhabited this area and interacted with neighboring countries.

Extraction of ancient DNA and determination of mitochondrial haplogroups

We collected 25 human bones from 21 excavation sites and analyzed the genetic information for ancient human beings on Nukdo. We made concerted efforts to extract ancient DNA of high quality and to obtain reproducible PCR products, as this was a primary consideration for this extensive kind of undertaking. These were heavily discolored during early DNA extraction processing from bone samples, which is evidence for poor preservation of human bones (data not shown). We used an ion-exchange column to purify high quality ancient DNA as previously reported (16). This procedure could also remove unknown PCR inhibitors and microbial contamination, which are hurdles to be overcome while handling ancient DNA (19). In order to effectively perform PCR amplifications on ancient DNA, we designed 12 specific primer pairs for coding and control regions, which can uniquely determine mitochondrial haplogroups for East Asians (Table 1). To enhance the credibility of the sequencing data, 2 different technicians performed the PCR amplifications for the same ancient DNA samples. Non-reproduced sequence information was excluded from the data. At the beginning of the experiments, we checked for correct band amplifications of HV1 (447 bp) and HV2 (414 bp) in the control regions, and compared with rCRS and SNP information obtained for HV1 and HV2. Although SNPs for the control regions depend on the quality of the ancient DNA samples, we found common high mutation rates at several positions: 16223 or 16362 for HV1, and 73, 153 or 263 for HV2 (Table 2). Minor mutations were also found for other positions, depending on the samples.

After confirming the sequencing data for the control regions, we performed PCR amplifications for 10 coding regions. Like the nucleotide substitutions shown in the control regions, we also found common mutations at high frequencies in the coding regions: A→G transition at position 10398, C→A transversion at position 5178, and G→A transition at position 3010. These positions are important for determining haplogroup D, or D4. However, there were no nucleotide substitutions at position 5178 for 5 samples (KR0006, KR0008, KR0013, KR0032 and KR0035). In addition, as we also failed to obtain PCR bands for determining the M7 or M9 haplogroups for these 5 samples, we assumed that these 5 ancient DNA samples belonged to the M super-haplogroup. Finally, we determined 5 mitochondrial haplogroups from 21 ancient DNA samples. These belonged to the M super-haplogroup (5), D haplogroup (5) and the sub-haplogroups D4 (9) or D4b (2) (Table 3).

Table 3. Distribution of mitochondrial haplogroups of the analyzed ancient DNA

Haplogroup	Number	Percentage
M	5	23.8%
D	5	23.8%
D4	9	42.9%
D4b	2	9.5%
Total	21	100%

Dendrogram and PC map analysis for mtDNA haplogroup

Dendrogram and principal component (PC) analysis of mitochondrial haplogroup frequencies were compared with data that were previously published by the other groups including East Asians (Mongolian, Japanese, Chinese, and Korean), South Asian (Indian), Southwest Asia (Yemen), European (Swedish) and Central American (Cuban) populations (15, 20-27). Dendrogram analysis suggests that the Nukdo population was closely related to East Asian populations, whereas they greatly deviated from Yemen, Swedish and Cuban (Fig. 2). Interestingly,

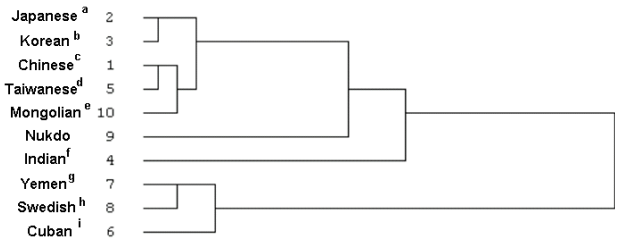

Fig. 2. Phylogenetic analysis of the Nukdo population. The Nukdo haplogroup was compared with 9 populations that were previously published by other groups. In this analysis, the Nukdo population is closely related to the modern East Asian population that includes the Chinese, Japanese, Taiwanese, Mongolians and Koreans. [a]Data from ref. (25), [b]Data from ref. (24), [c]Data from ref. (15), [d]Data from ref. (20), [e]Data from ref. (22), [f]Data from ref. (27), [g]Data from ref. (21), [h]Data from ref. (23), [i]Data from ref. (26).

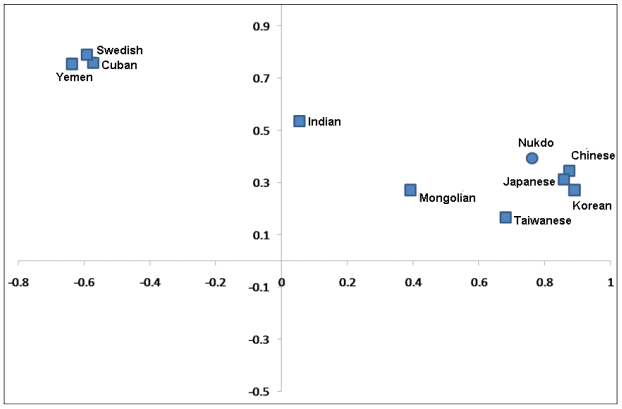

Fig. 3. Principal component analysis of the Nukdo population. In this analysis, the Nukdo population is more closely related to the Chinese, Japanese and Korean populations than the Taiwanese and Mongolian populations. Squares represent modern populations in 9 regions, and a circle represents the ancient Nukdo population.

Nukdo haplogroup covered that of the most East Asian populations including Korean, Japanese, Chinese, Mongolian, and Taiwanese. The PC plot consistently confirmed the previous finding that the Nukdo population was very similar to most East Asian populations and clearly distinguished from the Yemen, Swedish and Cuban populations (Fig. 3). However, the Nukdo population was found to be more closely related to the Han Chinese, Japanese and Korean populations than the Mongolian population, among East Asians.

DISCUSSION

Our interdisciplinary approach to ancient DNA was applied to ancient samples in order to analyze their genetic information. When handling ancient DNA, many criteria for ancient DNA analysis should be adhered to in order to validate their authenticity (19). Among the important criteria, 2 key points are how to extract ancient DNA of high quality and how to obtain reproducible sequence information at the molecular level. Therefore, our strategic approach concentrated on ancient DNA extraction to improve PCR amplification.

Previous studies reported that it is difficult to amplify ancient DNA because of fragmentation (28, 29). However, we could obtain PCR products of more than 400 bp, which provides convincing evidence that improved DNA extraction lead to an increased PCR success rate for this kind of extensive analysis of ancient human populations (16). In addition, we could directly compare the sequencing data of PCR products that were obtained by 2 different technicians, which validated the genetic information from ancient DNA. Therefore, we suggest that this strategic quality control for 2 key steps for ancient DNA analysis allowed successful determinations for genetic information of ancient human beings.

According to previous genetic studies on mitochondrial DNA, it is assumed that the earliest settlement of the East Asian regions occurred about 45 thousand years ago (22, 30). Thereafter, ethnic groups were formed in the same area, evolving into countries by historical accident such as war or conquest. Such events were likely the impetus for genetic diversity between ethnic groups, which then evolved into actual different genetic backgrounds. In general, Northern and Eastern Asian lineages were shown to be wide spread in different branches of the M macro-haplogroup, such as the C, D, G, M, M3, M7-M11, M13 and Z haplogroups, whereas Eastern Eurasian lineages were represented by the haplogroups A, N9a and Y, which belong to the major N haplogroup (22, 31).

Moreover, ethnic groups containg varying mitochondrial haplogroup frequencies, even among ethnic groups that share similar appearances. According to molecular genetic analysis on present-day human populations, the M and D haplogroups constitute about 50 to 60% of the Korean and Japanese populations, but only about 20 to 40% of the Chinese population (12, 25). The Mongolian population, however, was proven to contain a relatively heterogeneous distribution consisting of the C, D, A and B-derived haplogroups (31). Our analyses of ancient bones from Nukdo showed that these could be classified as the M super-haplogroup (23.8%), the D haplogroup (23.8%) and the sub-haplogroups D4 (42.9%) or D4b (9.5%), suggesting the typical ethnic haplogroups for Koreans or Japan, along with the geographical background of these human beings of the past. In other experiments using ancient bones obtained from Mongols or Uzbeks, we also obtained distinct mitochondrial haplogroups for these ethnic groups (unpublished data), again showing that systematic analysis of ancient DNA is very informative for understanding the genetic backgrounds of human beings from the past. Our results also showed that most haplogroups are super-ordinated haplogroups, such as the M or D types. Previous studies also showed less diversity of ancient traces that is probably related to excavations from smaller areas than is the case for the present-day human population (32). In addition, the genetic backgrounds for ancient humans might be mainly distributed in the super-ordinated haplogroups due to a restrictive number of samples. Therefore, it is plausible that ancient humans in a small area progressed to genetic diversity by genetic mixtures and drift.

In this paper, we have analyzed ancient DNA excavated from Nukdo that is a historically significant site of the past, and estimated that ancient humans being might have an effect on the present-day human population for the same place or for those of neighboring countries. Strategic ancient DNA analysis has proven to provide scientific evidence for archaeological and evolutionary studies of the human population. Therefore, this analysis is very informative for examining the genetic continuity or diversity between ethnic groups. There are significant insights that interdisciplinary chronicler analysis can provide for more progressive genetic information on ethnic origins at a certain area (territory) and genetic migrations into (or out of) neighboring countries.

MATERIALS AND METHODS

Extraction of ancient human DNA

The ancient human bones used in this study were archeologically estimated to be buried around 1st B.C. Ancient DNA of high quality was extracted from the ancient bones according to an improved protocol that we have described (16). Briefly, the bones were fragmented using sterile saw blades, and several millimeters of the bone surface were removed using autoclaved sandpaper. Unknown contaminants were removed using 5% detergents and UV irradiation. Then, samples were ground with a Mixer Mill, MM301 (Retsch, Germany) and decalcified with EDTA for 1-2 days. After incubating ground bones with DNA extraction buffer, an adjusted ion-exchange column was used for DNA purification.

PCR amplification and sequencing

Twelve PCR primer pairs were selected from mitochondrial DNA and synthesized by Genotech Inc., Korea. These primer pairs were used for specific PCR reactions for ancient human DNA (Table 1). Ten coding regions were amplified to determine mitochondrial haplogroups, and 2 control regions were amplified to determine sub-haplogroups. A typical 25 μl PCR reaction contained 1 μl of template, 10 pmol primer sets, 200 μM dNTP mixture, 1.5 U Super-therm Gold Hotstart Taq polymerase (Hoffman-LaRoche, USA), 1X PCR buffer containing 2 mM $MgCl_2$ and 400 μg/ml BSA. PCR products were separated by 1% agarose gel electrophoresis. A desired single band was cut from the gel, and recovered with agarose beads (Takara, Japan). The eluted DNA band was directly sequenced using an ABI Prism 310 sequencer (Macrogen, Korea). The same primers used for amplification were used for sequencing.

Data analysis and haplogrouping

Human Mitochondrial DNA Revised Cambridge Reference Sequences (http://www.mitomap.org/mitoseq.html) were aligned with sequencing data using the DNASTAR program (10, 11). The data were edited in an unbiased manner. We ordered sequencing data for several coding regions, HVS-I and HVS-II, of the mitochondrial DNA using an MS-Excel program and determined the haplogroups according to a classification strategy. Dendrogram analysis was performed using the SPSS 12.0 program. For comparison of the dendrogram and PC map analyses, the published data from modern Mongolian (22), Japanese (25), Chinese (15), Korean (24), Indian (27), Yemen (21), Swedish (23) and Cuban (26) populations were used.

Quality control for ancient DNA handling

All procedures were maintained using the guided regulations for ancient DNA handling previously suggested by other groups (19). The most important consideration was contamination by exogenous DNA. All steps (bone cutting, surface removing, powdering, DNA extraction, PCR preparation and post-PCR work) were carried out using a laminar air flow clean bench in a separate clean room. Appliances for DNA extraction were cleaned using undiluted commercial bleach, UV-irradiation at 254 nm for at least 1 h and followed by autoclaving. PCR work for ancient DNA analysis was isolated from other work, as well as wearing gowns and gloves to prevent potential contamination. For all manipulations, sterile aerosol-barrier (filter tip) pipettes and pipette tips were used. To control contamination during experimental processing, we always performed PCR amplification with blank (no DNA) and technician's DNA as negative and positive controls, respectively. PCR amplifications were repeated twice or more.

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