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The kinship of two 12th Dynasty mummies revealed by ancient DNA sequencing

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ABSTRACT

We resolve a longstanding question regarding the kinship of two high-status Egyptians from the 12th Dynasty, Nakht-Ankh and Khnum-Nakht, whose mummies were discovered in 1907 by Egyptian workmen directed by Flinders Petrie and Ernest Mackay. Although their coffin inscriptions indicate that Nakht-Ankh and Khnum-Nakht were brothers, when the mummies were unwrapped in 1908 the skeletal morphologies were found to be quite different, suggesting an absence of family relationship. We extracted ancient DNA from the teeth of the two mummies and, following hybridization capture of the mitochondrial and Y chromosome fractions, sequenced the DNA by a next generation method. Analysis of single nucleotide polymorphisms showed that both Nakht-Ankh and Khnum-Nakht belonged to mitochondrial haplotype M1a1, suggesting a maternal relationship. The Y chromosome sequences were less complete but showed variations between the two mummies, indicating that Nakht-Ankh and Khnum-Nakht had different fathers. Our study emphasizes the importance of kinship in ancient Egypt, and represents the first successful typing of both mitochondrial and Y chromosomal DNA in Egyptian mummies.

1. Introduction

The elaborate funerary practices of the ancient Egyptians have bequeathed to modern scholars a rich collection of human, animal, plant and material remains dating from the 4th to 1st millennia BC. Central among these remains are the mummified bodies of the Egyptians themselves which, together with the inscriptions in their tombs, provide a record of social organization from the pre-Dynastic Period onwards. However, evidence regarding family relationships, obtained from morphological examination of mummies and interpretations of inscriptions, can be ambiguous. An example is provided by two high-status Egyptians, Nakht-Ankh and Khnum-Nakht, whose relationship has been debated since the discovery of their tomb by Sir William Flinders Petrie and Ernest Mackay in 1907.

The tomb of Nakht-Ankh and Khnum-Nakht (Fig. 1) was unusual in being completely undisturbed prior to its excavation (David, 2007). Nakht-Ankh and Khnum-Nakht lived during the 12th Dynasty (1985–1773 BCE) in Middle Egypt and are thought to have been of noble lineage, as indicated by the elite location of their tomb in the Deir Rifeh cemetery, by their grave goods, and by the inscriptional evidence on the coffins. Although Nakht-Ankh was the elder by 20 years, Khnum-Nakht was the one to die first. For Nakht-Ankh, who according to inked dates on the bandages appears to have died six months later, the mummification and burial were carefully undertaken, compared with a lower standard of care for Khnum-Nakht, possibly indicating that the latter died unexpectedly. Flinders Petrie and Mackay concluded that Nakht-Ankh and Khnum-Nakht were brothers, partly because the two bodies were buried adjacent to one other, but also because the inscriptions on the coffins mention the female name Khnum-Aa, who is described as 'lady of the house' and referred to as the mother of both Nakht-Ankh and Khnum-Nakht. However, the inscriptions were less informative regarding the paternal filiation, the father of both men indicated only by a generic title and not by name (Murray, 1910).

The two mummies were transferred to the Manchester Museum in 1908, and subjected to additional tests which revealed substantial differences in the cranial and postcranial morphologies of the skeletons, suggesting that Nakht-Ankh and Khnum-Nakht were not related. This hypothesis gained further support during the 1970s, when analysis of the residual skin tissue indicated differences in pigmentation, arguing against a shared parentage, and three-dimensional reconstruction of the skulls emphasized the remarkable difference in cranial morphology (David, 2007). More recently, amplification by the polymerase chain reaction (PCR) and direct sequencing of part of the mitochondrial DNA hypervariable region (mtDNA HVR) from the two mummies found

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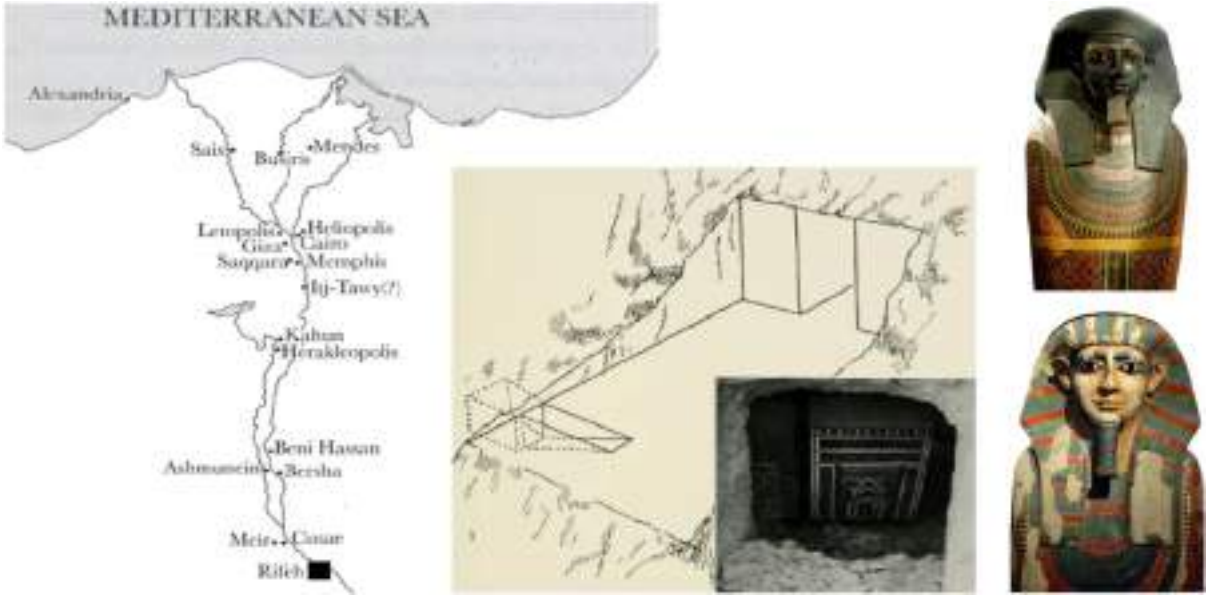

Fig. 1. The tomb of the 'mummy brothers'. Site map showing the burial location at Rifeh, with a diagram of the tomb, and the burial of the two coffined mummies *in situ*. On the right are the inner coffins of Nakht-Ankh (above) and Khnum-Nakht (below).

sequence differences, from which it was concluded that Nakht-Ankh and Khnum-Nakht were maternally unrelated but had been adopted by Khnum-Aa (Matheson et al., 2014).

Direct DNA sequencing of amplified segments of mtDNA is now viewed as unreliable with archaeological material in general (Brown and Brown, 2011) and Egyptian mummies in particular (Marchant, 2011). We therefore revisited the question of kinship between Nakht-Ankh and Khnum-Nakht by carrying out next generation sequencing of extracts of dental samples, with sequencing libraries prepared from bulk extracts (shotgun sequencing) and from extracts that had been enriched for mtDNA or Y chromosomal DNA by hybridization capture.

2. Methods

2.1. Teeth samples

Two teeth from each mummy were extracted from the maxillae in one sampling stage. The second right molar and the second left molar were extracted from Nakht-Ankh (samples NAI, NAII), while the third right molar and the third left molar were extracted from Khnum-Nakht (KNI, KNII). The teeth that were taken were ones attached relatively firmly to their sockets to ensure that they belonged to the correct individual, and no damage was caused to the skull during the extraction. At a later stage, a third sample was obtained from Khnum-Nakht, a second left mandibular molar (KNIII), due to poor DNA preservation in the previous two teeth. Teeth were extracted under clean conditions by personnel wearing protective clothing including forensic suits, face masks, hair nets, goggles and two pairs of sterile gloves.

2.2. Ancient DNA authentication regime

Ancient DNA (aDNA) analyses were performed in a suite of independent, physically isolated laboratories, each with an ultrafiltered air supply maintaining positive displacement pressure and a managed access system. All surfaces within the laboratories were periodically sterilized by UV irradiation and cleaned with 5% bleach and 70% ethanol, and all utensils and equipment were treated with DNA-Away (Molecular Bioproducts) before and after use. Items such as test tubes were UV irradiated (254 nm, 120,000 μJ cm^{-2} for 2 × 5 min, with 180° rotation between the two exposures) before use. Aqueous solutions were similarly irradiated for 15 min. Personnel wore protective clothing including forensic suits, face masks, hair nets, goggles and two pairs of sterile gloves at all times. DNA extractions were carried out in a Class II biological safety cabinet in one laboratory within the facility, and sequencing libraries were prepared in a laminar flow cabinet in a second, physically-isolated laboratory. All DNA extractions were accompanied by two extraction blanks (normal extraction but without skeletal material) per five samples, and these tested for contamination by the PCRs described below. Every set of 5–7 PCRs was also accompanied by at least two PCR blanks (set up with water rather than DNA extract).

2.3. DNA extraction

Each tooth was cleaned externally by placing it, with the roots pointing upwards, in a small beaker containing sufficient 3% sodium hypochlorite solution to reach a level just below the root holes. After 5 min the tooth was removed, dried with a paper towel, placed in a second beaker and rinsed in Millipore water, again without inundating the root holes. After drying, a 37% phosphoric acid gel (Schwartz et al., 1991) was applied to the tooth surface, left for 1 min, then wiped off. The tooth was rinsed in Millipore water, dried for 10 min, and 50–70 mg dentine powder collected using a dental pick (Alakoc and Aka, 2009). DNA was then extracted from the powder by two standard methods, as described in Rohland and Hofreiter (2007) for NAI and KNI, and Dabney et al. (2013) for NAII, KNII and KNIII.

2.4. PCR and Sanger sequencing

We initially performed three overlapping PCRs (Bouwman et al., 2008) directed at the mtDNA HVRI locus (revised Cambridge Reference Sequence [rCRS] coordinates 16,028–16,195, 16,147–16,294, 16,210–16,340) to assess DNA preservation and endogenous DNA content. PCRs were set up using the Qiagen Multiplex PCR kit in a final volume of 25 μl per sample consisting of 3 μl DNA extract, 10 μM each primer, 1 × Qiagen master mix. Thermal cycling conditions were: 95 °C for 15 min; 44 cycles of 0.5 min at 94 °C, 1.5 min at the annealing temperature, 1 min at 72 °C; 15 min at 72 °C. PCR products were visualized in 1.5% agarose gels, purified (Qiagen MinElute Purification kit), cloned (Thermo Scientific CloneJET PCR Cloning kit) into *Escherichia coli* XL1-Blue competent cells (Agilent), and inserts amplified by colony PCR in 20 μl reactions comprising 10 μM each primer, 2.5 mM

Table 1
Mitochondrial DNA SNPs.

Position	SNP	Coverage		Phylogenetic value[a]
		Nakht-Ankh (capture/shotgun)	Khnum-Nakht (capture only)	
73	A → G	12/–	15	Informative
195	T → C	8/3	14	Informative
263	A → G	23/4	19	Informative
489	T → C	9/–	9	Informative
750	A → G	20/5	18	Informative
813	A → G	8/–	8	Informative
1438	A → G	21/4	9	Informative
2706	A → G	14/12	9	Informative
3705	G → A	31/–	17	Informative
4,769	A → G	7/1	4	Informative
6446	G → A	25/2	13	Informative
6671	T → C	15/5	9	Informative
6680	T → C	13/5	7	Informative
7028	C → T	37/5	15	Informative
8251	G → A	62/5	10	Private
8701	A → G	16/3	15	Informative
8860	A → G	19/3	16	Informative
9540	T → C	10/2	5	Informative
10,398	A → G	19/9	13	Informative
10,400	C → T	21/10	13	Informative
10,873	T → C	13/2	5	Informative
11,719	G → A	67/7	17	Informative
12,346	C → T	44/2	5	Informative
12,403	C → T	67/3	5	Informative
12,705	C → T	28/2	13	Informative
12,950	A → C	22/7	14	Informative
14,110	T → C	23/5	15	Informative
14,766	C → T	24/3	11	Informative
14,783	T → C	12/2	9	Informative
15,043	G → A	21/2	20	Informative
15,301	G → A	50/3	16	Informative
15,326	A → G	51/4	28	Informative
16,129	G → A	50/2	11	Informative
16,145	G → A	38/–	7	Private
16,189	T → zC	23/–	5	Informative
16,223	C → T	53/4	10	Informative
16,249	T → C	30/4	10	Informative
16,311	T → C	13/–	9	Informative
16,359	T → C	7/–	8	Informative
16,519	T → C	4/4	14	Hotspot

[a] Informative SNPs are polymorphisms that are substantially different between populations and can be associated with a specific haplogroup. Hotspot SNPs exist in many different populations and are thought to be subject to positive selection. Private SNPs are polymorphisms that have been observed once or a few times, often in members of the same family or within a limited population. Neither hotspot nor private SNPs were used for haplogroup characterization.

dNTPs, 1 × *Taq* buffer (New England Biolabs), 1.25 units *Taq* DNA polymerase (New England Biolabs), with cycling at: 95 °C for 3 min; 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C; 15 min at 72 °C. Colony PCR products were sequenced (GATC Biotech, Cologne) and aligned with the rCRS using Geneious v8.1.8 (created by Biomatters, available from http://www.geneious.com/).

2.5. Library preparation, in-solution hybridization capture and DNA sequencing

Seven dual-indexed Illumina libraries were prepared in total (Meyer and Kircher, 2010) including blanks. Libraries were enriched by in-solution hybridization capture (MycroArray Mybait target enrichment kits, according to the manufacturer's instructions for degraded samples) with baits for the entire mtDNA, and for approximately 500 kb of the NRY comprising 655 non-repetitive loci, most of these in the ampliconic and X-degenerate regions and excluding most of the X-transposed and heterochromatic regions (Lippold et al., 2014). For mtDNA, a single enrichment was performed with samples NAII, KNI and KNIII, and a double enrichment with KNII; for the NRY, double enrichments were performed for NAII and KNIII, with sequences for NAII obtained after both the first and second rounds of enrichment. After enrichment, samples were quantified by qPCR (Roche LightCycler 480) and fluorimetry (Qubit 2.0), amplified for twelve PCR cycles, requantified and the length distributions assessed (Agilent 2100 Bioanalyzer). Samples were then pooled in equimolar ratios and sequenced from both ends in a single flow cell (Illumina 2500 HiSeq). Shotgun sequencing was also performed with samples NAII and KNII. Sequence data are curated at the European Nucleotide Archive under project accession number PRJEB20294 (secondary accession ERP022437).

2.6. Data handling and analysis

Raw data were processed using AdapterRemoval v2.1 (Schubert et al., 2016) with the following parameters: *trimns*, *minlength 25*, *trimqualities*, *minquality 30*, *collapse*. We retained those fragments whose forward and reverse pairs overlapped by at least 11 bp and which could therefore be collapsed into single sequences. Reads which did not overlap were examined in a separate round of analysis using bedtools v2.26.0 and only those found to belong to the same sequence were retained. Mapping was performed with BWA v.0.7.5a–r405 (Li and Durbin, 2009) using default settings of the *aln*, *samse* and *sampe* commands with the exception of the *t32* and *l1000* parameters which are suggested for aDNA. The enriched mtDNA datasets were mapped against the rCRS from the UCSC database (GRch38 assembly). Y chromosome enriched reads were mapped against the human genome assembly hg19 (GRch37), again from the UCSC database. We chose this build for the Y chromosome data because it has no masked regions. Picard v1.95 was used to clean and sort the reads by their 5′–3′ coordinates using the default *CleanSam* and *SortSam* commands. The resulting BAM files containing the mapping information were processed with SAMtools v.1.2 (Li et al., 2009) to extract the mapped reads, using the *view* command with the *b*, *q20* and *F4* parameters. Duplicate sequences were removed using Picard *MarkDuplicates*. We also performed local indel realignment and recalibration with GATK v3.5 (McKenna et al., 2010) because of the damaged nature of aDNA. Single nucleotide polymorphisms (SNPs) were identified using HaplotypeCaller from the GATK package, applying both default parameters, and considering only the depth of coverage (DP > 3). SNPs were validated by manual inspection using Geneious v8.1.8.

Mitochondrial DNA haplogroup assignment was performed using Haplogrep (Kloss-Brandstätter et al., 2011). For the NRY data, the presence of modern contamination in the sequence datasets was assessed with PMDtools (Skoglund et al., 2014) using a PMD threshold score < 0. For all datasets, the DNA fragmentation and misincorporation patterns were assessed with MapDamage v.0.3.3 (Ginolhac et al., 2011). Additional tests for authenticity of the Y chromosome SNPs are described in the Supplementary text.

3. Results

For Nakht-Ankh, mtDNA enrichment yielded 5,707,546 sequence reads, of which 6138 mapped to the rCRS (Andrews et al., 1999). Forty SNPs were observed with a mean coverage of 18.3 ×, spanning the entire mtDNA (Table 1). Shotgun sequencing gave 1488 reads mapping to the rCRS covering 30 polymorphic sites with 2–14 × coverage, in each case agreeing with the SNP identified from the enriched sample. For Khnum-Nakht, 76,253,577 reads were obtained from the enriched library, 5422 mapping to the rCRS, revealing the same forty SNPs as typed for Nakht-Ankh, with 15.5 × mean coverage (Table 1). No high quality reads were obtained from the shotgun data for Khnum-Nakht. The SNP identities were consistent with mtDNA haplogroup M1a1 with 88.05–91.27% degree of confidence, thus confirming the African origins of the two individuals. The haplotypes included ancestral mutations at positions 489 T → C, 10,398A → G, 10,400C → T and

15,043G → A, which define macrohaplogroup M (Winters, 2010). The M1 branch (Quintana-Muri et al., 1999) was further characterized by a transversion at position 12,950A → C and four transitions in the coding region at positions 6446G → A, 6680 T → C, 12,403C → T, and 14,110 T → C, as well as three transitions 16,129G → A, 16,189 T → C and 16,249 T → C in the control region (Quintana-Muri et al., 1999). Signature motifs of subclade M1a1 are the 3705G → A, 12,346C → T and 16,359 T → C transitions (Gonzalez et al., 2007). To validate our results we considered the complexity of the M haplogroup and examined the SNP data with and without recurring mutations found in several branches of haplogroup M (M4, M5, M34) (Sun et al., 2006), private mutations, hotspots, and mutations that are usually found in haplogroups D, L, N, R and U6 (Olivieri et al., 2006; Sun et al., 2006; Gonzalez et al., 2007; Pennarun et al., 2012), such as position 16,223 (Tanaka et al., 2004), and constructed a median-joining network (Supplementary Fig. 1). The network confirms the close proximity between the mtDNA haplotypes of the two mummies and haplogroup M1a1. The previously published analysis, based on direct sequencing of amplified segments of the mtDNA HVR (Matheson et al., 2014), identified three SNPs (16223C → T, 16261C → T, 16292C → T) in both mummies, only one of which is present in our NGS dataset. We suggest that this previous study was affected by modern contamination, a common problem when PCR is applied to aDNA (Brown and Brown, 2011).

The Y chromosome enrichment system targeted approximately 500 kb of the non-recombining region (NRY). Sequence reads were mapped onto human genome assembly hg19 (GRch37) from the UCSC database. For Nakht-Ankh, we obtained 115,126,472 reads from the first capture round, of which 2776 mapped to the Y chromosome, and 28,053,346 reads from the second capture round, of which 1170 mapped to the Y chromosome. The sequences from the first capture revealed 149 SNPs, with maximum coverage of 22 ×, and the second capture gave 148 SNPs at 50 × maximum coverage (Supplementary Table 1). From the shotgun dataset, 1564 reads mapped to the Y chromosome, including 19 SNPs mostly clustered in the pericentric heterochromatic region, all but one of which was also present in the enriched dataset (Supplementary Table 1). As expected from previous studies of the distribution of SNPs in the Y chromosome (Francalacci et al., 2015), sequence variations were located in the enriched euchromatic region of the NRY, particularly in the X-degenerate region, but were also present in the ampliconic, X-transposed and pericentric heterochromatic regions. Variant authenticity was validated by stringent methods to avoid false assignments and over-interpretation of the results, including comparisons between the enriched and shotgun datasets, comparisons between the two enrichment rounds, and assessment of read depth information (see Supplementary text). Following these tests, 66 SNPs were retained for Nakht-Ankh. For Khnum-Nakht, the double capture yielded 52,405,372 reads of which only 163 mapped to the Y chromosome, indicating a poorer level of DNA preservation. However, 28 SNPs were identified (Supplementary Table 1). The shotgun data for Khnum-Nakht did not yield any reads mapping to the Y chromosome. Of the 28 SNPs, 14 passed the authenticity tests. Comparisons between the datasets showed that eight SNPs were covered in both individuals, three of which failed the authenticity tests (Table 2). Of the remaining five, four have different identities in Nakht-Ankh and Khnum-Nakht, leaving just one SNP that is shared by both individuals. The results therefore indicate that Nakht-Ankh and Khnum-Nakht did not have the same father.

4. Discussion

The mtDNA, Y chromosome and shotgun sequencing data all indicate a better degree of ancient DNA preservation in the teeth from Nakht-Ankh compared with those from Khnum-Nakht, despite the two mummies being buried adjacent to one another and hence subject to the same diagenetic microenvironment. Both mummies had been bathed in alum, which had previously not been associated with Egyptian practices (Murray, 1910), probably dehydrated with natron (Lucas, 1932), and treated with different preservatives: impure salt for Nakht-Ankh and lime and carbonate for Khnum-Nakht (Murray, 1910). It is unclear if natron was used with Khnum-Nakht, but this is probably irrelevant with regards to DNA preservation. Absence of natron would result in incomplete desiccation, and therefore susceptibility to microbial attack and DNA hydrolysis, but if natron was used it could have interacted with the slaked lime resulting in carbonization which causes further exothermal activity, leading to DNA alkylation damage and strand cleavage (Gilbert et al., 2005). The key factors leading to the differential DNA preservation are probably the lack of evisceration in the case of Khnum-Nakht, evisceration preventing putrefaction and hence being more conducive to DNA preservation (Gilbert et al., 2005), and the generally lower standard of care taken during the mummification process for Khnum-Nakht, which was evident from the state of the mummies when they were unwrapped shortly after their discovery (Murray, 1910).

We followed a strict technical regime designed to avoid contamination of samples with modern DNA (see Methods). We used teeth which were still embedded in the upper or lower jaw, removed these under clean conditions, and following sterilization of the outer surfaces removed dental powder through the root canal and prepared DNA libraries from this powder. Tooth extracts have been shown to be less prone to contamination with extraneous DNA compared with extracts prepared from bone (Evison et al., 1999), and the use of dental powder from within teeth that were *in situ* until sampled further minimizes the risk of contamination. The DNA damage patterns of both the mtDNA and Y chromosome sequence reads were consistent with fragmentation and misincorporation models typical for aDNA (Briggs et al., 2007) (Supplementary Fig. 2). We therefore have confidence in the authenticity of our results. The presence of identical mtDNAs indicates that Nakht-Ankh and Khnum-Nakht had a maternal relationship, consistent with a shared mother or a more distant kinship relationship such as cousins or uncle-nephew. However, based on the common maternal name as documented in the inscriptional evidence, it can be deduced that the two individuals were both children of Khnum-Aa. The differences between the Y chromosome SNPs indicate different paternal lineages, and so we conclude that Nakht-Ankh and Khnum-Nakht were half-brothers.

Our results provide an intriguing insight into one facet of ancient Egyptian kinship, and illustrate the potential use of matrimonial alliance as a means of social reinforcement among the elite and sub-elite. Unfortunately, placing our results in a broader context is difficult because we are unaware of any comparable examples of two men buried together in an intact Pharaonic tomb (*e.g.* Garstang, 1907). There is a separate suggestion of polyandry in the inscriptions on another set of monuments from the same period as the Two Brothers, although these may refer to two women with the same name rather than the same woman having two husbands (Simpson, 1974). The kinship of Nakht-Ankh and Khnum-Nakht also provides an example of the common practice in recorded filiations of this period to give precedence to the maternal rather than paternal line, individual rights being determined by social class rather than gender (Robins, 1993), and can perhaps be looked on as a reflection of the high status accorded to their mother Khnum-Aa in their particular social and family structure.

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Table 2
Y chromosome SNP identities at positions covered for both Khnum-Nakht and Nakht-Ankh. SNPs coloured pink did not pass one or more of the quality filters and were discarded.

Position	Nakht-Ankh		Khnum-Nakht	
	SNP identity	Coverage	SNP identity	Coverage
8,450,740	A	6	G	3
8,450,741	G	6	A	3
8,455,255	A	3	G	2
8,455,257	A	3	G	2
8,656,564	A	9	G	1
21,755,291	C	1	T	3
21,755,314	A	1	A	3
21,755,315	G	1	A	3

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2017.12.025.

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