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Highlights

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Relic excavated in western India is probably of Georgian Queen Ketevan

Niraj Rai [a], Nizamuddin Taher [b], Manvendra Singh [a], Gyaneshwer Chaubey [d], Aditya Nath Jha [a], Lalji Singh [a,c], Kumarasamy Thangaraj [a,*]

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- Discovery of relic of Queen Ketevan of Georgia
- This discovery has international importance, as it is associated with historical events enacted in Iran, Georgia and India.
- This is also the first ancient DNA report from India.
- Our genetic study corroborating the archaeological and literary evidences
- The results are authenticated with cloning and genotyping.

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Table S1 Genotyping results of 108 mtDNA diagnostic markers of QKT1, QKT2 and QKT3.

Table S2 mtDNA HVSI haplotype of contemporary Georgian individuals.

Table S3 Haplogroup affiliations of contemporary Gowli population of Goa, India.

Fig. S1 Electropherogram of 12 cloned sequences of QKT1 having C16327T mutation.

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Relic excavated in western India is probably of Georgian Queen Ketevan

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ABSTRACT

History has well documented the execution of Queen Ketevan of Georgia by the Persian Emperor of modern day Iran. Based on historical records, in 1624 two Augustinian friars unearthed the queen's remains and one of them brought the relic to the St. Augustine convent in Goa, India. We carried out ancient DNA analysis on the human bone remains excavated from the St. Augustine convent by sequencing and genotyping of the mitochondrial DNA. The investigations of the remains revealed a unique mtDNA haplogroup U1b, which is absent in India, but present in Georgia and surrounding regions. Since our genetic analysis corroborates archaeological and literary evidence, it is likely that the excavated bone belongs to Queen Ketevan of Georgia.

1. Introduction

It has been documented that in the year 1613, the emperor of Persia, Shah Abbas I led an army to conquer the Georgian kingdom and took Queen Ketevan as prisoner (Gulbenkian, 1985). For about a decade (1614 to 1624) the queen remained in Shiraz, Iran as a prisoner of Shah Abbas I (Gulbenkian, 1985). In the year 1624, the Persian emperor attempted to convert the queen to the Islamic faith and make her join his harem. He sent soldiers to Shiraz to carry out these instructions, under threat of torture and death. Resisting his wishes, Queen Ketevan was tortured and finally strangled to death on 22 September 1624 (Gulbenkian, 1985). A year (1623) before this event, two Augustinian friars had arrived in Shiraz to start a mission and gained the queen's trust, becoming her confessors (Gulbenkian, 1985). These friars unearthed the remains of the queen and hid them from 1624 to 1627. In 1627, some of these remains (the right arm) were brought to Goa and kept in a black box or stone sarcophagus on the second window along the Epistle side of the chapter chapel in the St. Augustine convent (Fig. 1) (Gulbenkian, 1985; Rêgo, 1958).

Considering the importance of Queen Ketevan for the Georgian people, Soviet Union–Russian–Georgian officials requested the Government of India to help in tracing the relics of Queen (Saint) Ketevan in Goa. Since 1989, various delegations from Georgia have worked together with the Archaeological Survey of India (ASI) to locate Ketevan's relics within the St. Augustinian convent in Old Goa (India). This historical convent was founded by a group of Augustinian friars, who have arrived in Goa in 1572 (Da-Fonseca, 1994). Over time it was enlarged and rebuilt, becoming one of the most noteworthy buildings of old Goa (Kloguen, 1988). In 1835, the church underwent partial demolition, and in 1842 the main vault of the church collapsed; the convent rapidly transformed into ruins. By this time, the valuable articles belonging to the religious complex had been either sold or lost (Da-Fonseca, 1994).

Despite the exact location of the queen's relics mentioned in Portuguese document (Rêgo, 1958), all efforts to find them were unsuccessful due to difficulties in interpretation of the convent. The ground map of the St. Augustinian convent was reconstructed on the basis of the literary source (Rêgo, 1958) and with the help of the local historians. After several attempts and a topographical survey within the Augustinian convent, the chapter chapel and window mentioned in literary sources were located by archeologists of the ASI, Goa-circle, assisted by a Portuguese researcher in May 2004 (Singh, 1996; Taher et al., 2004). As per the literary sources, the relic box of QKT1 was expected to be at the second window of the chapter chapel towards the Epistle side. Therefore, this area was systematically explored and searching for the black box (stone sarcophagus), either intact or broken, was the primary interest of the excavation team. Further, while clearing the collapsed wall in which the second window was present, a long arm bone was found in the sediments and was registered as QKT1. Since the relic box was made up of stone, it was broken into pieces due to the collapse of the wall. Fortunately, the team had found the intact lid (Fig. 2), which provides a clue that the relic box might have placed at

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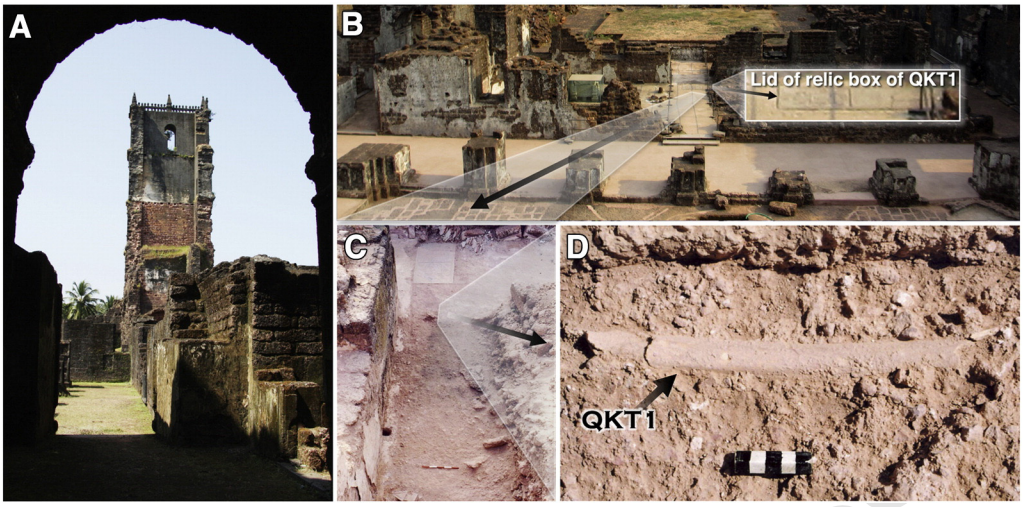
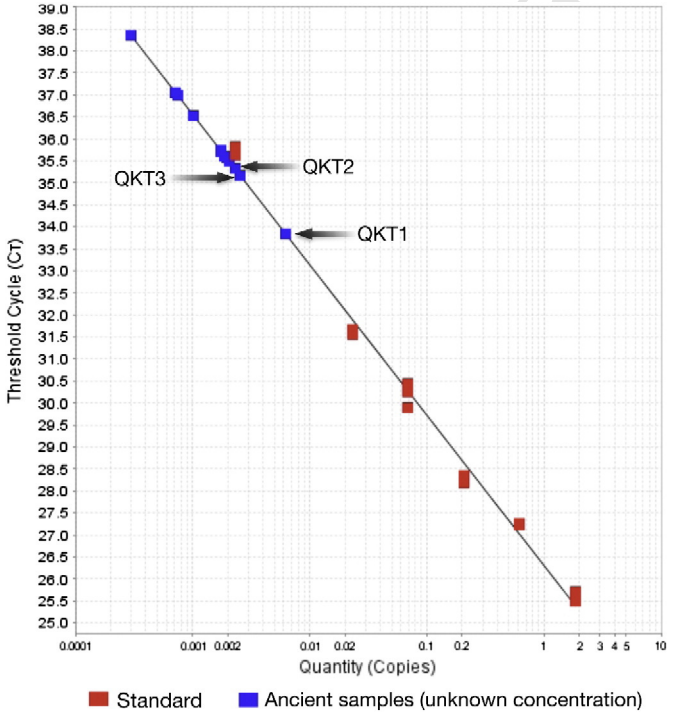


Fig. 1. A. A view of the ruined bell tower of the church of Our Lady of Grace within the St. Augustine convent. B. A general view of the excavated area within the chapter chapel. C. View of the lid of relic box of QKT1 where the long bone was located. D. Closer view of the right arm bone, along with sediments. Photographs presented in Fig. 1 were taken by one of the co-authors, Nizamuddin Taher.

the second window. During the excavation, two other bone relics (QKT2 and QKT3) were also recovered from outside the second window, with two intact black boxes with their lids. According to the descriptions of Friar Purificacao in 1723 (interpreted and presented in Portuguese in 1958), the archeologist believed that these two bone relics (QKT2 and QKT3) might belong to Friar Guilherme de Sto. Agostinho and Friar Hyeronimo da Cruz, which were placed at this location (Rêgo, 1958) (for the video visit, see http://www.ccmb.res.in/sct_videos/). While the archeological and historical data are consistent with the relic being the remains of Queen Ketevan, the evidence cannot be considered conclusive without direct confirmation of her identity. Keeping in mind the importance of mtDNA analysis for historical samples, with proven application in history, archaeology, and human evolution (Cooper and Poinar, 2000; Haak et al., 2010a; Rasmussen et al., 2010), we successfully analyzed the hyper-variable segment I (HVSI), as well as selective diagnostic substitutions in the mitochondrial DNA (mtDNA) coding region, to establish the authentic identity of the bone samples.



Q8 **Fig. 2.** QPCR /plot of QKT1, QKT2 and QKT3 along with reference control samples.

2. Methods

2.1. Samples

The three bone samples (specimen reference: QKT1, QKT2 and QKT3) were recovered from the Augustinian complex, Goa, India by the excavation team of the Archeological Survey of India (ASI), Ministry of Culture, Government of India (ASI, Goa Circle, Goa, India) in 2004. They have been treated with care and stored in separate boxes. Details of the three samples are in Table 1. The samples have been kept at the repository of ASI, Goa Circle under ASI regulations.

A part of sample from each bone was brought to the ancient DNA laboratory of the CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India in order to establish their genetic identity. All necessary consents were obtained for the described study from the Archeological Survey of India, Government of India, which complied with all relevant regulations.

2.2. Ethics statement

The hunt for the relic of Queen Ketevan of Georgia was the combined effort of the Georgian Government and Ministry of External Affairs, Government of India. We received the buccal swab of native Georgian individuals with their informed written consent through the Director of the Church of Queen Ketevan at Tibilisi, Georgia.

Table 1
Archeological details of all the three bone samples excavated from the St. Augustine complex.

Sl. no.	Index	Location	Skeletal part	Dimension
1	QKT1	At 10.12 ft from the 2nd window of chapter chapel	Diaphysis of a long bone	18 cm * 2.5 cm * 0.5 cm
2	QKT2	Near the coping stone of the second window of chapter chapel	Unidentified bone fragment	10 cm * 3.0 cm * 0.2 cm
3	QKT3	Outside the second window at the lower level of the same deposit	Unidentified bone fragment	7.0 cm * 1.0 cm * 0.7 cm

2.3. DNA extraction from the bones

We applied very stringent standards for the authentication of ancient DNA (Cooper and Poinar, 2000; Haak et al., 2010b; Paabo et al., 2004) for all the samples. DNA was extracted from all the three bones (QKT1, QKT2 and QKT3) independently in a dedicated ancient DNA laboratory. At least three independent DNA extractions by two independent researchers were performed to demonstrate repeatability of the results. A single sample per day was extracted with extraction controls. Throughout the extraction procedure, extreme care was taken to avoid contamination with modern DNA.

A section of each bone was first polished with sandpaper and then drilled to provide roughly 1 g of powdered bone sample. Bone powder was dissolved in 4.0 ml extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS, and 500 μg/ml proteinase K) and incubated in a shaking incubator at 55 °C overnight, followed by incubation at 37 °C for 12 h. After this, we used the QIAquick PCR Purification Kit (Qiagen), to trap the DNA fragments that are larger than 100 bp and smaller than 10 kb while excluding nucleotides, proteins, and salts. These columns are suitable for ancient DNA as the templates are highly degraded and the target regions for amplification are generally quite small (100 to 250 bp). DNA molecules that are larger than 10 kb and associated with ancient remains are more likely to be postmortem and/or modern bacterial or fungal DNA instead of authentic ancient DNA.

The extraction solution was centrifuged at 10,000 *g* for 10 min and 4.0 ml aliquots of the supernatant were transferred to 50 ml tubes and mixed with 5 volumes of QIAquick PB buffer. From this, 750 μl was loaded directly onto QIAquick columns and centrifuged at 12,800 *g* for 1 min. The flow-through was discarded and the process repeated until all of the extract had passed through the column. The DNA was washed with 750 μl of QIAquick PE buffer and centrifuged for 1 min. The flow-through was discarded and the DNA was then eluted from the column by loading 40 μl of elution buffer followed by centrifugation for 1 min.

2.4. Quality control for ancient DNA extraction and amplification:

The ancient human bones used in this study were treated as previously suggested by other groups (Champlot et al., 2010; Cooper and Poinar, 2000; Haak et al., 2010b; Paabo et al., 2004). Our major concern throughout the entire work was to avoid contamination by exogenous DNA. All steps (bone cleaning, cutting of bone samples, drilling, DNA extraction and PCR preparation) were carried out in two separate laminar airflow rooms. The ancient DNA rooms are designed with positive pressure created by high filtered air (HEPA 0.5 μm filters), an exclusive water supply and ultraviolet (UV) irradiation excluding contamination. Pipettes and other instruments used for DNA extraction were cleaned using commercial bleach and 70% alcohol followed by UV irradiation for at least 45 min. DNA extraction and PCR setup were carried out in two different dedicated chambers. Disposable full body suits and gloves were used throughout the processing of samples. Aerosol barrier tips (Fisher Scientific) and ultrapure DEPC treated water (Invitrogen, Life Technologies) were used for all the experiments carried out in the ancient DNA laboratory.

2.5. DNA extraction from buccal swab of contemporary individuals

To determine the degree of association of our ancient DNA sequence data with the Georgian people, we collected buccal swabs of 30 individuals with informed consent from different parts of Eastern Georgia, with the help of the Archeological Survey of India. DNA from the 30 buccal samples was extracted in normal laboratory using QIAamp DNA Swab BioRobot Kit (Qiagen).

2.6. Quantification of ancient DNA

Prior to amplification, qPCR was used to determine the amount of DNA in QKT1 (presumed to be the bone of Queen Ketevan of Georgia), using a human DNA Quantification Kit (Applied Biosystems, Foster City, USA) (Alonso et al., 2003, 2004). Two microliters of DNA was used in a final reaction volume of 10 μl according to the manufacturer's instructions. We used the ABI Prism 7500 Sequence Detection System (7500 SDS) and SDS software (V 1.0) for real-time data collection and analysis (Applied Biosystems, Foster City, USA). Care was taken to avoid contamination with modern DNA. We quantified the ancient DNA content of both QKT1 and known ancient samples of East Asian origin to rule out any irregularities in the experiment (Fig. 2).

2.7. Amplification of mitochondrial DNA (mtDNA)

To amplify HVSI and other informative coding regions of the mtDNA, we have designed 9 sets of primers (Table 2). At least three independent successful amplifications of the same fragment from each sample were performed to validate results. PCR amplifications were carried out in 25 μl reaction mixture containing 5 μl of the ancient DNA extracts, 1 mg/ml bovine serum albumin, 200 mM each dNTP, 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.5 pmol of each primer, and 2 U of AmpliTaq Gold (Applied Biosystems, Foster City, USA). Amplification conditions used were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 46 °C for 30 s, and 72 °C for 2 min; followed by final extension at 72 °C for 5 min. PCR products were visualized on 2% agarose gel and purified with exonuclease and shrimp alkaline phosphatase (GE Health Care, formerly Amersham Biosciences, USA), and subsequently subjected to sequencing PCR using BigDye terminator cycle sequencing kit (Applied Biosystems, USA). After the sequencing reaction, fragments were precipitated with 5 M sodium acetate and ethanol, washed twice with 70% alcohol, dried, dissolved in Hi-Di formamide, and sequenced using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were edited and assembled using the Sequence Analysis and AutoAssembler software (Applied Biosystems, Foster City, USA), respectively. The consensus sequences were compared with our database consisting of about 22,000 individuals from most of the ethnic groups of India and published data sets (Achilli et al., 2005; Behar et al., 2008).

Table 2
Primer sequences used for PCR and sequencing of HVSI and coding regions of mitochondrial DNA.

Primer name	Sequences	Sequence range	Product length
HVSIF	5′GTTCTTTCATGGGGAAGCAG3′	16023–16190	167
HVSIR	5′AGGGGGTTTTGATGTGGATT3′		
HVSIIF	5′CATAAAAACCCAATCCACATCA3′	16160–16390	130
HVSIIR	5′ATGTACTGTTAAGGGTGGGTAGG3′		
HVS3F	5′CACCCCTCACCCACTAGGAT3′	16358–16410	152
HVS3R	5′GCACTCTTGTGCGGGATATT3′		
238F	5′TAACAATTGAATGTCTGCACAGC3′	238–378	150
378R	5′TGGTTAGGCTGGTGTTAGGG3′		
10398F	5′TCATCCCTCTTATTAATCATCATCC3′	10262–10466	204
10398R	5′TGTAAATGAGGGGCATTTGG3′		
12242F	5′TGTCTAACAACATGGCTTTCTCA3′	12243–12408	166
12408R	5′AACGAGGGTGGTAAGGATGG3′		
12705F	5′CCCAAACATTAATCAGTTCTTCAA3′	12668–12835	168
12705R	5′GCTGTGTTGGCATCTGCTC3′		
15043F	5′CGAGACGTAAATTATGGCTGAA3′	14956–15136	181
15043R	5′AGCCTATGAAGGCTGTTGCT3′		
23F	5′TCATTGGACAAGTAGCATCC3′	15811–00005	765
23R	5′GAGTGGTTAATAGGGTGATAG3′		

Primer sequences of 23F and 23R were taken from Anderson et al. (Anderson et al., 1981).

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2.8. Cloning of PCR products

We cloned the PCR products of the QKT1 using the Blunt-ended PCR cloning kit (GE Healthcare, USA), which contains pMossBlue dephosphorylated blunt end vector. Transformation was carried out using DH5α competent cells, as per the manufacturer guidelines. Transformed cells containing insert PCR products were screened using blue white selection of colonies. We picked up 20 white colonies, isolated plasmid DNA using plasmid isolation kit (Qiagen), and then sequenced using forward primer (Fig. S1).

2.9. Validation of sequence data

We validated the sequencing results using MALDI-TOF MS-based SNP genotyping technique (iPLEX Gold from Sequenom, USA). iPlex Gold chemistry requires the input of DNA only in picograms and its reliability and accuracy make it ideal for ancient DNA studies (Lacan et al., 2011; Mendisco et al., 2011). We selected 108 diagnostic markers from the entire mtDNA and designed 5 pools of multiplex primers using the Mass Array Assay design software (version 4.0). The genotyping reaction of QKT1 was performed twice in two different extracts.

2.10. Sex identification using amelogenin marker

Sex of QKT1 sample was assessed using the AmpF*l*STR identifier PCR amplification kit (Applied Biosystems, Foster City, USA). The amelogenin gene is present on both X and Y chromosome. However, there is a 6 bp difference in the region analyzed between these two chromosomes, which enable to differentiate human male DNA from that of female DNA (Fig. 3).

3. Results

Quantification of the DNA extracted from the suspected bone sample of Queen Ketevan (QKT1) revealed a concentration of ≤.01 pg/μl (Fig. 2). Although the copy number of the DNA was very low, we made several attempts to sequence the HVSI and informative diagnostic regions such as substitutions A12308G and G12372A (Table 3). Moreover, we sequenced HVSII position C285T by designing a new primer in order to diagnose the U1b haplogroup (Table 3). The HVS regions incorporate a large amount of variation, which allows detailed definition of the sample's haplotype and subsequent haplogroup assignment (www.phylotree.org).

Sex identification of QKT1 with AmpF*l*STR identifier PCR amplification kit revealed the presence of only the X chromosome-specific fragment of the amelogenin locus, suggesting that the QKT1 is of female origin (Fig. 3). The polymorphisms (16249C–16327T), observed in the QKT1 sample indicate that the putative queen's sample belongs to the West Eurasian specific haplogroup U1b, while QKT2 and QKT3 falls in the South Asian-specific haplogroups R6a (16129A–16266T–161274A–16309G) and U2a (16051G–16206C–16230G), respectively (Table 4). We have also genotyped the 108mtDNA diagnostic sites of all the three samples (QKT1, QKT2 and QKT3) using iPLEX gold technology (Sequenom, USA). This has further helped in reconfirming the haplogroup-defining motif. Although there were a few base modifications (A5301G, C9540T, C13188T, and T16172C) in these samples, the haplogroup defining motifs (A11467G, A12308G, T12705C, T12879C, A13104G and C16327T) were intact (Table S1).

Table 3
Polymorphic motifs at the HVSII and coding region of the QKT1 (presumed the queen's sample) along with other two relics (QKT2 and QKT3), and researchers involved (R1 and R2) in this study.

Samples	Nucleotide position						
	285	10398	10400	12308	12372	12705	15043
[a]rCRS	C	G	C	A	G	T	G
QKT1	T	–	–	G	A	–	–
QKT2	–	–	–	–	–	T	–
QKT3	–	–	–	G	A	–	–
R1	–	A	T	–	T	C	A
R2	–	A	T	–	T	C	A

[a] rCRS: revised Cambridge Reference Sequence.

To further investigate the genetic relationship and ancestry of QKT1, we have sequenced the HVSI region of 30 contemporary Georgian individuals from Eastern Georgia (Table S2). Two Georgian samples matched the defining motif (16249C–16327T) of QKT1, while none of the Indian samples (~22,000) from the published (Sharma et al., 2012; Thangaraj et al., 2010) and our unpublished data were found to carry this motif. To see the geographical distribution of haplogroup U1 haplotypes, we constructed a median-joining network, using available datasets (www.familytreedna.com, www.ianlogan.co.uk). Paraphyletic branch U1a'c mainly carried individuals from South Asia, the Middle East, Caucasia and Europe, whereas the other branch U1b incorporated exclusively European, Caucasian and Near Eastern samples—including two Georgian samples analyzed in the present study (Fig. 4). The presence of the sub-haplogroup U1b among Georgians and people from adjoining regions, coupled with its complete absence among South Asians, strongly links the genetic heritage of QKT1 with Georgia and weakens the possibility of South Asian descent. Samples QKT2 and QKT3 showed a South Asian ancestry, consistent with the historical and archaeological findings (Fig. 4).

4. Discussion

The isolation of DNA from ancient biological materials is challenging due to its degradation by oxidative and hydrolytic lesions (Alonso et al., 2004; Haak et al., 2010b; Paabo et al., 2004). Working with ancient materials obtained from tropical countries like India, where humidity causes rapid decomposition is even more difficult. In our study, the excavated skeletal materials were not particularly old and had been

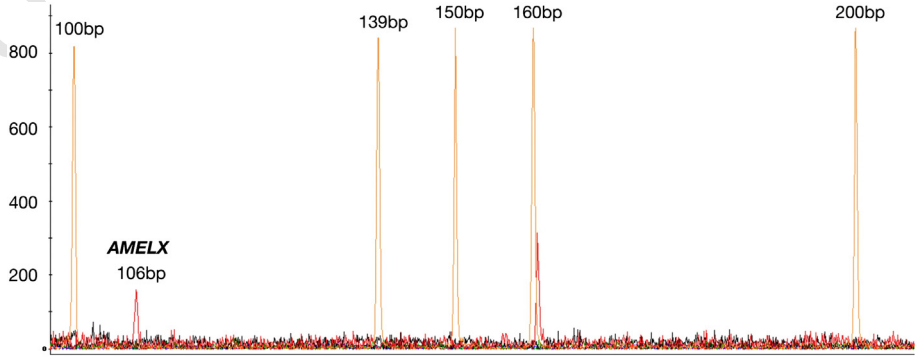

Fig. 3. Sexing of QKT1 using amelogenin locus present in the AmpF*l*STR identifier kit.

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Table 4
Polymorphic motifs at the HVSI of the QKT1 (presumed the queen's sample) along with other two relics (QKT2 and QKT3), and researchers involved (R1 and R2) in this study.

Sample	Nucleotide positions											Haplogroup
	16051	16126	16129	16206	16223	16230	16249	16266	16274	16309	16327	
[a]rCRS	A	T	G	A	C	A	T	C	G	A	C	–
QKT1	–	–	–	–	–	–	C	–	–	–	T	U1b
QKT2	–	–	A	–	–	–	–	T	A	G	–	R6a
QKT3	G	–	–	C	–	G	–	–	–	–	–	U2a
R1	–	C	–	–	T	–	–	–	–	–	–	M
R2	–	–	A	–	T	–	–	–	–	–	–	M

[a] rCRS: revised Cambridge Reference Sequence.

kept in a black box before the collapse of the convent. These conditions aided successful DNA extraction.

Sex identification of QKT1 using the amelogenin marker clearly implies that QKT1 is of female origin (Fig. 3). Considering the fact that all the researchers in our ancient DNA laboratory are male, and that a male team carried out the excavation of the bones, this result argues against modern contamination. Failure to amplify STRs with a larger fragment size (beyond 200 bp) also supports the absence of contamination.

In order to relate the samples to historical and literary sources, we were interested in using genetic variation to identify their maternal lineages. The placement of QTK1 according to HSVI was confirmed by typing further diagnostic mutations in the mtDNA coding regions. To collect information about the phylogeographic distribution of the haplogroup found (U1b), we have surveyed 22,000 HVSI from the Indian subcontinent and also sequenced the HVSI region of 30 contemporary Georgian individuals. Interestingly, none of the Indian sample carried 16249–16327 polymorphisms, which is the characteristic of haplogroup U1b (phylotree.org). However, some samples from South, Central and West Indian populations were found to have its sister haplogroup, U1a (Cordaux et al., 2003; Sharma et al., 2012; Thangaraj et al., 2010). The complete absence of haplogroup U1b in the Indian subcontinent and its presence in high to moderate frequency in the Georgia and adjoining regions (Achilli et al., 2005; Behar et al., 2008) is consistent with QKT1 being a relic of Saint Queen Ketevan of Georgia.

The remaining two bone samples (QKT2 and QKT3) exhibit the haplogroups R6a and U2a, respectively. These haplogroups are shared by different ethnic groups in South Asia, and particularly India. We performed a similar search as above for these two haplogroups in order to determine their ethnic and geographic associations. This revealed that the haplogroup R6a is prominent among Western Indian coastal populations (Chaubey et al., 2008). Similarly, haplogroup U2a was seen in several western and northern Indian populations (Palanichamy et al., 2004), particularly in the Gowli populations of Goa with a frequency of 7–10% (Table S3), suggesting that these two bone samples belong to the local populations of Goa. Our findings are consistent with the historical view of Silva Rego's (Rêgo, 1958) description of tombstones and on the basis of which, the location of other two

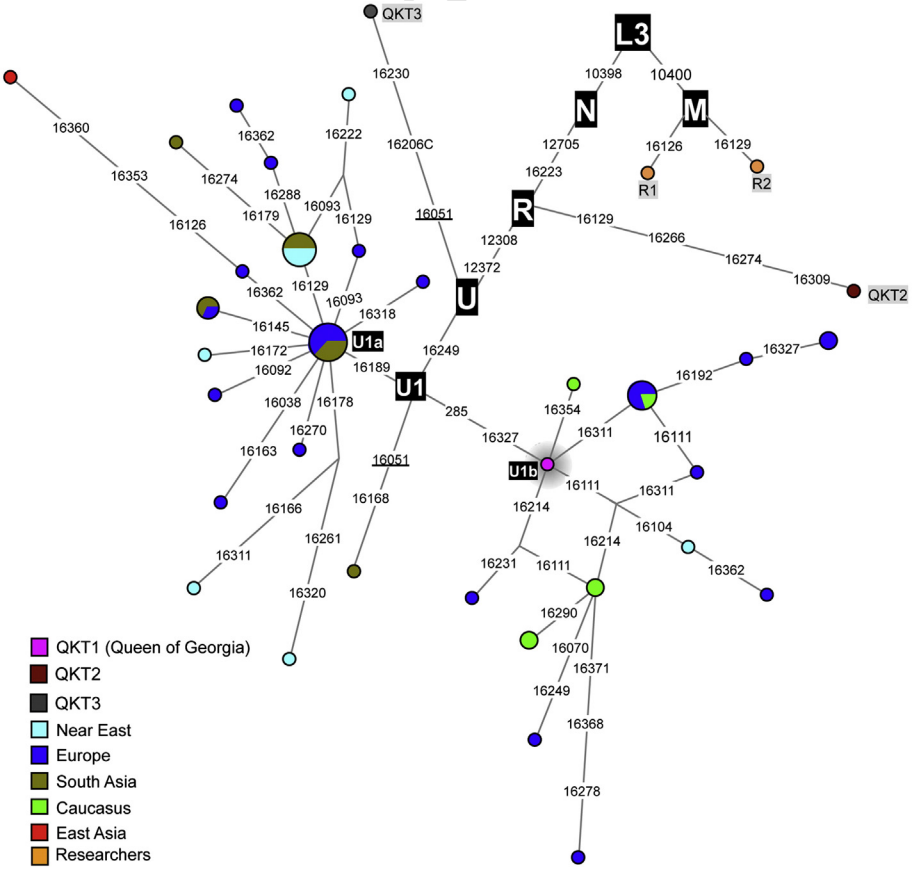

Fig. 4. A phylogenetic median joining network containing different mtDNA haplotypes of haplogroup U1, constructed using Network 4.6. The size of circles is proportional to the number of samples.

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bone remains (QKT2 and QKT3) may have association with the Friar Guilherme de Sto. Agostinho and Friar Hyeronimo da Cruz. Our entire ancient DNA study is corroborating with archaeological and historical records and suggests that the QKT1 is likely to be the remnant of Queen Ketevan of Georgia.

Table S1, Table S2, Table S3, Fig. S1 and a video link http://www.ccmb.res.in/sct_videos/ are provided for background to the project. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mito.2013.12.002.

Author contribution

NR, MS and ANJ performed the experiments. NT and KT have conceived and designed the project. NR, MS and GC analyzed the data. LS contributed the reagents and supervised the entire work. NR, MS, GC, ANJ and KT wrote the manuscript with the contributions from other coauthors.

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