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Comparison of morphological and molecular genetic sex-typing on mediaeval human skeletal remains☆

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ABSTRACT

Archaeological excavations conducted at an early mediaeval cemetery in Volders (Tyrol, Austria) produced 141 complete skeletal remains dated between the 5th/6th and 12th/13th centuries. These skeletons represent one of the largest historical series of human remains ever discovered in the East Alpine region. Little historical information is available for this region and time period. The good state of preservation of these bioarchaeological finds offered the opportunity of performing molecular genetic investigations. Adequate DNA extraction methods were tested in the attempt to obtain as high DNA yields as possible for further analyses. Molecular genetic sex-typing using a dedicated PCR multiplex ("Genderplex") gave interpretable results in 88 remains, 78 of which had previously been sexed based on morphological features. We observed a discrepancy in sex determination between the two methods in 21 cases. An unbiased follow-up morphological examination of these finds showed congruence with the DNA results in all but five samples.

1. Introduction

The small Austrian village of Volders is located in the lower Tyrolean Inn Valley. This region had already been settled between the late Neolithic to Early Bronze Age and the Roman era, and was later colonized by Rhaetian and German speaking tribes, including the Bavarians. During the Roman era Volders acted as an important station along the thoroughfare connecting the Italian peninsula with the North. In the end of the 6th century Bavarians settled in the region and lived side by side in admixture with the local inhabitants and the Romans [1].

The reconstruction of early mediaeval rural populations has usually been limited to archaeological, anthropological, and historical research. In Volders, however, excavations carried out by municipal archaeologists revealed the presence of an early mediaeval cemetery (Fig. S1, [1], Alexander Zanesco, Institute of Archaeologies, Innsbruck) that represents one of the largest series of historical human remains found in Tyrol. In an area covering approximately 140 m^2 and two main allocation layers, a total of 153 graves were documented containing a total of 141 nearly complete skeletons. These were subsequently examined and dated between the 5th/6th and 12th/13th centuries [1].

This skeletal assemblage is exceptional for the Alpine region both with respect to the number of individuals as well as to the state of skeletal preservation (Fig. S2a, b, [2], Alexander Zanesco, Institute of Archaeologies, Innsbruck). The cemetery is located close to the upper rim of the ancient bank of the Inn River, which may be one reason for the good state of preservation of some of the burials. The geological layers underneath the burials, which for the most part were interred in soil, are comprised of loose riverbed gravel and stones. This allowed for rapid drainage of rainwater and subsequent better bone preservation. Skeletons that were buried deeper and covered in this gravel were actually preserved less well, since the stones exerted a grinding action on the bones. The archaeological survey brought interesting findings such as different directional orientations of the burials (mainly east–west with a few north–south, Fig. S1, Alexander Zanesco, Institute of Archaeologies, Innsbruck), the presence of stone encirclements around some of the graves and clothing accessories (iron belt

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buckles with silver inlay, knives, metal belt strap ends and combs) which are typical of the late Roman and the early mediaeval periods [1]. The recovered remains appeared to be suitable for molecular genetic analyses through which – by interdisciplinary collaboration – more light may be shed on the make-up of this past population. Prior to DNA extraction initial experiments were conducted to determine the most suitable DNA extraction method. After extraction DNA was quantified using a real-time PCR approach and sex-typed with a previously described, home-made PCR multiplex ("Genderplex") [3]. The results were compared to morphological sex typing and the findings are discussed highlighting the advantages and limitations of the applied methods.

2. Materials and methods

2.1. Samples

Following completion of the archaeological and anthropological investigations the skeletal remains were stored at room temperature in carton boxes at the Museum of Industry and Prehistory in the neighbouring town of Wattens for about 10 years. A total of 305 samples including femora and humeri as well as teeth (preferentially molars) were chosen for molecular genetic investigations, as those were the most promising of the available tissues according to our experience. Small pieces (ca. 2 cm × 1 cm × 1 cm) of each bone specimen were excised with a bone saw and molars were extracted using forceps. Buccal swabs were collected under written consent from a total of 81 individuals who handled the remains during the excavation process and the anthropological work (n = 22). The associated DNA profiles were added to those of the entire laboratory staff (n = 59) to build a contamination elimination dataset.

2.2. DNA extraction

2.2.1. Physical and chemical sample pre-treatment

The mechanical and chemical processing of the samples was performed with the necessary care required for challenging samples [4,5].

A total of 194 samples were taken from the 141 skeletons (Table 1) and subjected to mechanical surface cleaning with sterile scalpel blades. Samples were then bathed in sodium hypochlorite (≥4% active chlorine, Sigma Aldrich, St. Louis, MO, USA) at room temperature for 15 min, washed in purified water (DNA/RNA free), rinsed in absolute ethanol for 5 min and UV irradiated for 10 min (λ = 254 nm). Samples were dried in a closed laminar flow cabinet over night and then powdered using a vibrating ball mill (Laarmann Group BV, Roermond, The Netherlands).

2.2.2. Demineralization and DNA extraction

About 100–150 mg bone powder were fully demineralized and lysed in 7 ml lysis buffer (500 mM Na_2EDTA (pH 8.0), 0.5% *N*-lauroylsarcosine sodium salt (both Sigma Aldrich), 250 μl 20 mg/ml proteinase K (Roche, Basel, CH)) in a rotary shaker at 56 °C overnight as detailed in [6]. For the DNA extraction from the demineralization/lysis supernatants three published protocols either directly using the silica columns and buffer set included in the QIAamp DNA Blood Maxi kit (Qiagen, Hilden, Germany; SC method) [7], phenol/chloroform/isoamyl-alcohol (PCI) [8], or a spin filter method (SF) [9] were compared in a series of initial experiments on three specimens. Both the PCI and the SF protocol featured a final purification step on a silica matrix using the MinElute PCR Purification kit (Qiagen) according to the manufacturer's recommendations with two additional washing steps.

In the modified SF protocol the demineralization/lysis supernatants (7 ml) were diluted with an equal volume of water (DNA/RNA free) before concentrating them in centrifugal filter units (Amicon Ultra 15, 30K MWCO, Millipore, Billerica, MA, USA). This additional step in the protocol appeared to attenuate the risk of filter-clogging and reduced the concentrations of the lysis buffer constituents in the retenate recovered from the spin filters. All further steps of the SF protocol were performed as described in [9]. The final volume of DNA extracts was 50–75 μl for all approaches. Reference samples on buccal swabs were DNA extracted using a Chelex (Bio-Rad, Hercules, CA, USA) method according to [10].

Table 1
Number of extractions performed per individual skeleton (including pre-studies and repeat extractions).

Number of DNA extractions (n = 194) performed on individuals (n = 141)	
Single	96
Double	38
Triple	6
Quadruple	1

2.3. Real-time PCR DNA quantification

The quantity of total genomic (g)DNA was determined using a real-time PCR approach targeting human specific AluYb8 sequences according to Walker and colleagues [11] with modifications. To monitor potential inhibition we co-amplified a spiked in vitro mutagenized and cloned part of the human retinoblastoma susceptibility protein 1 (RB1) gene as internal PCR control ($pRB1_{IPC}$) [12]. Amplifications were performed in 20 μl reactions containing 1× TaqMan Universal PCR Master Mix (Life Technologies (LT), Carlsbad, CA, USA), 5 μg non-acetylated bovine serum albumin (Sigma Aldrich), 400 nM each of the two AluYb8 amplification primers (F: CTTGCAGTGAGCCGAGATT, R: GAGACGGAGTCTCGCTCTGTC, [11]), 200 nM AluYb8 probe (FAM-ACTGCAGTCCGCAGTCCGGCCT-NFQ/MGB, [11]), 900 nM RB1-157-F (CCAGAAAATAAATCAGATGGTATGTAACA, [12,13]), 900 nM RB1-IPC-R (TCGTTTCGGAGCGTTGGTTAG, [12]), 200 nM RB1 hybridization probe (VIC-CAGCACTTCTTTTGAGCAC-NFQ/MGB, [12]), and 20,000 $pRB1_{IPC}$ plasmids [12]. Kinetic PCR was conducted in 96-well polypropylene PCR plates on a 7500 Fast Real-Time PCR System (LT) using the "7500 standard setting" for the speed of all temperature transitions. The thermal cycling protocol comprised of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. The 7500 Fast System Sequence Detection Software (v1.4, Applied Biosystems/LT) was used for signal read-out during the annealing/extension step and data analysis. Calibration curves covered a gDNA input range of 15 ng–254 fg per 20 μl reaction. The human genome accommodates multiple AluYb8 repeats, which explains the high sensitivity of the quantification assay used here. For instance, by searching the publicly available databases Gibbons and colleagues [14] constructed a library of 2201 AluYb8 repeats found in the human genome, and by using this information we identified 1072 templates with perfectly matching primer and probe binding sites. The deduced minimum and maximum amplicon lengths were 71 bp (n = 1068) and 94 bp. Based on the sequence information in the AluYb8 repeat library we also designed an alternative TaqMan probe (FAM-CGGACTGCGGACTGCA-NFQ/MGB) and an alternative forward primer (AluYb8-F_2: GGGTGGATCATGAGGTCAGGA). For the AluYb8-F_2 ↔ AluYb8-R plus alternative probe trio 1075 perfectly matching template sequences were obtained (amplicon size range: 196–261 bp, 921 × 235 bp). This "AluYb8 long amplicon" approach (using the 71 bp amplicon PCR conditions but 250 nM each primer, 200 nM alternative probe, and extra AmpliTaq Gold DNA polymerase (2 units, LT) was successfully tested in initial experiments. The linear dynamic range was comparable to that obtained