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Accurate sex identification of ancient human remains using DNA shotgun sequencing

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ABSTRACT

Accurate identification of the biological sex of ancient remains is vital for critically testing hypotheses about social structure in prehistoric societies. However, morphological methods are imprecise for juvenile individuals and fragmentary remains, and molecular methods that rely on particular sex-specific marker loci such as the amelogenin gene suffer from allelic dropout and sensitivity to modern contamination. Analyzing shotgun sequencing data from 14 present-day humans of known biological sex and 16 ancient individuals from a time span of 100 to ~70,000 years ago, we show that even relatively sparse shotgun sequencing (about 100,000 human sequences) can be used to reliably identify chromosomal sex simply by considering the ratio of sequences aligning to the X and Y chromosomes, and highlight two examples where the genetic assignments indicate morphological misassignment. Furthermore, we show that accurate sex identification of highly degraded remains can be performed in the presence of substantial amounts of present-day contamination by utilizing the signature of cytosine deamination, a characteristic feature of ancient DNA.

1. Introduction

Without information on the biological sex of human remains, archaeological and anthropological hypotheses about division of labor, social stratification and religion in prehistoric human societies cannot be accurately tested. While morphological methods have a relatively high success rate on near-complete and adult skeletons, they lack power for identification of juvenile and fragmentary remains (Black, 1978a; Brown et al., 2007). While many methods have been developed for application on juvenile remains (Ditch and Rose, 1972; Black, 1978a,b; Weaver, 1980; Rosing, 1983; Schutkowski, 1993; Molleson et al., 1998; Loth and Henneberg, 2001; Cardoso, 2008; (Wilson et al., 2008; Veroni et al., 2010; Adler and Donlon, 2010) they are generally less reliable for females than males (Black, 1978b; Weaver, 1980; Schutkowski, 1993; Scheuer, 2002; Wilson et al., 2008; Galdames et al., 2008) as well as for populations other than the one directly used for morphological reference (Hunt, 1990; Scheuer, 2002; Sutter, 2003; Vlak et al., 2008; Cardoso and Saunders, 2008; Galdames et al., 2008; Wilson et al., 2010).

Molecular sex identification has hitherto been focused on differentiating between the X-linked amelogenin gene and the amelogenin pseudogene on the Y-chromosome, and has been applied to a variety of ancient remains (e.g. Gibbon et al., 2009; Sullivan et al., 1993; Götherström et al., 1997; Faerman et al., 1995; Hummel and Herrmann, 1991; Stone et al., 1996; Lassen et al., 2000; Morill et al. 2008; Tschentscher et al. 2008; Daskalaki et al., 2011; Quincey et al., 2013). However, these molecular methods have been hampered by concerns about authenticity, arising both from high risk of allelic dropout (Kim et al., 2013), and the danger of contamination from exogenous present-day sources in archaeological material (Malmström et al., 2005). Even small amounts of such contamination could bias the sex identification, and methods to deal with contaminated specimens require the sequencing of a large number of clones (Helgason et al., 2007). Moreover, approaches targeting specific genome-regions (using primer based PCR) require longer template fragments than direct sequencing and may thus increase the rate of contamination (Krause et al., 2010), and the patterns of post-mortem damage that can be used to authenticate putatively ancient DNA are strongest at the ends of molecules (Briggs et al., 2007) which are preferentially lost in such primer-based approaches. Since both Y-and X-linked markers are common in the population (approximate allele frequencies of 1/4 and 3/4 respectively) and contains few polymorphic

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sites to generate genetic fingerprints, support for authenticity cannot be based on differences between genotypes determined from the remains and the genotypes of personnel that have been in vicinity of the remains, unless more informative markers such as Y-chromosome microsatellites are analyzed simultaneously, which is unlikely to be widely applicable for heavily degraded DNA.

Large-scale high-throughput shotgun sequencing has emerged as a revolutionary technology in the field of ancient DNA (e.g. Poinar et al., 2006; Green et al., 2006; Rasmussen et al., 2010; Skoglund et al., 2012; Keller et al., 2012; Meyer et al., 2012). While high-throughput sequencing has proven to be efficient for aneuploidy detection in present-day high-quality DNA sources (e.g. Bianchi et al., 2012; Liang et al., 2013), it has not been widely applied to sex identification using ancient DNA. Furthermore, determining whether small degraded DNA fragments derive from an X-chromosome or a Y-chromosome is complicated by the homology shared between the two. For example, a Neandertal individual from Vindija cave (Vi33.16) was identified as male based on the presence of sequences matching the Y-chromosome by two early studies (Green et al., 2006; Noonan et al., 2006) but a later study changed that assignment to female by carefully excluding regions where the X and Y chromosomes share homology (Green et al., 2010). Here, we suggest a simpler strategy that makes use of all the relevant information in alignments to both the sex chromosomes, leveraging the power of shotgun sequencing without the need for detailed filtering of the Y-chromosomal reference sequence.

2. Materials and methods

We collected shotgun sequencing data from the literature for 16 ancient human remains sequenced using Illumina (unless otherwise noted) and SOLID technology. These included a 100 year old hair sample from an aboriginal Australian (Rasmussen et al., 2011), a 4000 year old hair sample from a Paleo-eskimo (Saqqaq) (Rasmussen et al., 2010), four ~5000 year old Neolithic Scandinavian individuals (Skoglund et al., 2012), one 5300 year old individual from the Italian Alps ('Ötzi' the Tyrolean Iceman) sequenced using SOLID technology (Keller et al., 2012), two ~7000 year old Mesolithic Iberian individuals (Sánchez-Quinto et al., 2012), one Denisovan individual from Denisova cave in Russia which is at least 50,000 years old (Krause et al., 2010; Reich et al., 2010; Meyer et al., 2012), and finally six Neandertal individuals from Vindija cave in Croatia, El Sidron cave in Spain, Mezmaiskaya cave in Russia, and Feldhofer cave in Germany, all dated to between ~38,000 and ~70,000 years ago (Green et al., 2010). We validated the approach using a calibration panel based on seven present-day individuals sequenced to low coverage using Illumina and SOLID technology by the 1000 genomes project (1000 genomes project consortium, 2012), as well as 7 present-day males sequenced by the Neandertal and Denisova genome consortia (Green et al., 2010; Reich et al., 2010). The data from Ötzi (Keller et al., 2012) and the Neolithic individuals from Skoglund et al. (2012) were remapped to the human reference genome using `BWA 0.5.9` (Li and Durbin 2009 with the seed disabled (parameters -l 16500 -n 0.01 -o 2).

Sex assignment (where samples with no evidence for the presence of a Y chromosome are denoted 'female' and samples with evidence of a Y chromosome are denoted 'male') was performed by computing the number of alignments to the Y chromosome (n_Y) as a fraction of the total number of alignments to both sex chromosomes ($n_X + n_Y$) which we denote R_Y where $R_Y = n_Y/(n_X + n_Y)$. We discarded alignments with a mapping quality of less than 30 (90 in the case of the six Neandertals, which were mapped using the ANFO software (Green et al., 2010)). Assuming that each sequence read from the sex chromosomes is an independent draw from two possible outcomes (Y- or X-chromosome), a 95% confidence interval (CI) was computed by a normal approximation as $R_Y \pm 1.96 \times R_Y \times (1 - R_Y)/(n_X + n_Y)$. We provide a python script (Additional File 1) to compute this statistic that also performs sex identification according to the guidelines suggested below.

To assess the robustness of this approach to contamination from present-day humans, we artificially added sequences from a present-day male from Sardinia to the sequence data from the Mezmaiskaya 1 Neandertal which is identified as a female (see Green et al., 2010 and Fig. 1). We compared the estimated R_Y using unfiltered data to the estimated R_Y using only sequences that displayed a C→T mismatch in the first 15 bp of the sequence read

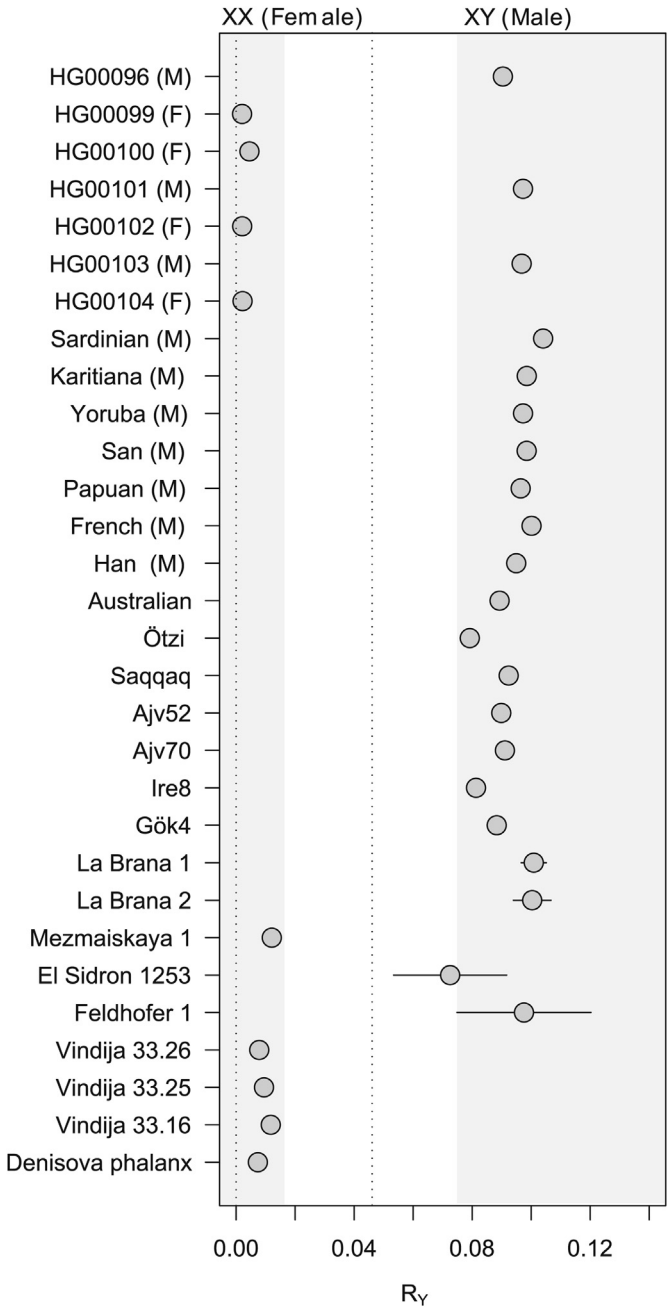

Fig. 1. Observed fraction of Y chromosome alignments compared to the total number of alignments to the X and Y chromosome in 14 present-day humans of known sex (F: Female; M: Male) and 16 historical/ancient humans. The fraction sequences aligned to the Y-chromosome is expressed as a ratio of the total number of sequences aligned to either sex chromosome (R_Y). Error bars represent 95% confidence intervals.

(Skoglund et al., 2012). To test the power of the approach as a function of the number of sequences, we down-sampled data from five individuals to various sizes ranging between 1000 and 1,000,000 sequences.

3. Results

Based on the calibration panel comprising 4 females and 10 males, we find that the highest fraction of alignments to the Y-chromosome R_Y were ~0.0022 in females and ~0.09 in males (Fig. 1), with no apparent differences between SOLID and Illumina platforms. We found that ancient samples were also separated into two clear categories for degraded samples from different tissues where the sex is known, such as the ~100 year old male aboriginal Australian hair sample, the pelvis sample from the 5300 year old male Tyrolean Iceman, and the more than 50,000 year old archaic female individual represented by the Denisova phalanx (Fig. 1). This suggests that our approach can provide a general framework for sex identification. We decided to proceed with limits based on the most extreme R_Y in each category adjusted for the variance observed in each samples. Specifically, we assigned a sample as female if its CI upper bound for R_Y was lower than 0.016 (obtained by adding the maximum value observed in all ancient and modern female samples to 3 standard errors of the mean). We assigned a sample as male if its R_Y CI lower bound was higher than 0.075 (obtained by the minimum value R_Y observed in all male samples [excluding Mezmaiskaya 1 and El Sidron 1253 due to low coverage] subtracted by 3 standard errors of the mean).

Within this framework, we confidently assign the sex of several individuals which are in agreement with previous assignments, such as the Saqqaq Paleo-Eskimo assigned as male (Rasmussen et al., 2010) two Iberian Mesolithic skeletons that were relatively complete (both assigned as male), 4 out of 6 Neandertal specimens of which Feldhofer 1 is assigned as male and the remaining 3 female (Green et al., 2010) and the Denisovan phalanx originating from a female individual (Reich et al., 2010). Notably, we find that the 4 individuals sequenced by Skoglund et al. (2012) are all male, despite that two of them (Ire8 and Gök4) had previously been assigned as female based on morphological criteria (Gejvall, 1974; K-G Sjögren, pers. comm). For the two remaining individuals, Ajv70 is assigned as male in agreement with morphological evidence (Molnar, 2008) whereas the fourth individual had not been previously assigned due to its young age (~7 years) (Molnar, 2008). Mezmaiskaya 1 and El Sidron 1253 could not be confidently assigned due to the R_Y CI lower bound overlapping the assignment threshold for males, but since they are both consistent with a male sex and inconsistent with being female, it is likely that more sequencing will show unambiguously that they are indeed male, as suggested by Green et al. (2010). Alternative less stringent criteria would be to set a threshold intermediate to the values observed in males and females, which in our case would be $R_y = 0.044$. A sample would then be assigned if the confidence interval does not overlap this central threshold, in which case we would assign the Mezmaiskaya 1 and El Sidron 1253 Neandertals as males.

To investigate the minimum amount of reads required for sex identification, we down-sampled data from five individuals, and found that for all these individuals, their sex was confidently identified down to approx. 100,000 sequences, with less confident (but still accurate) assignment down to 10,000 sequences in many cases (Fig. 2).

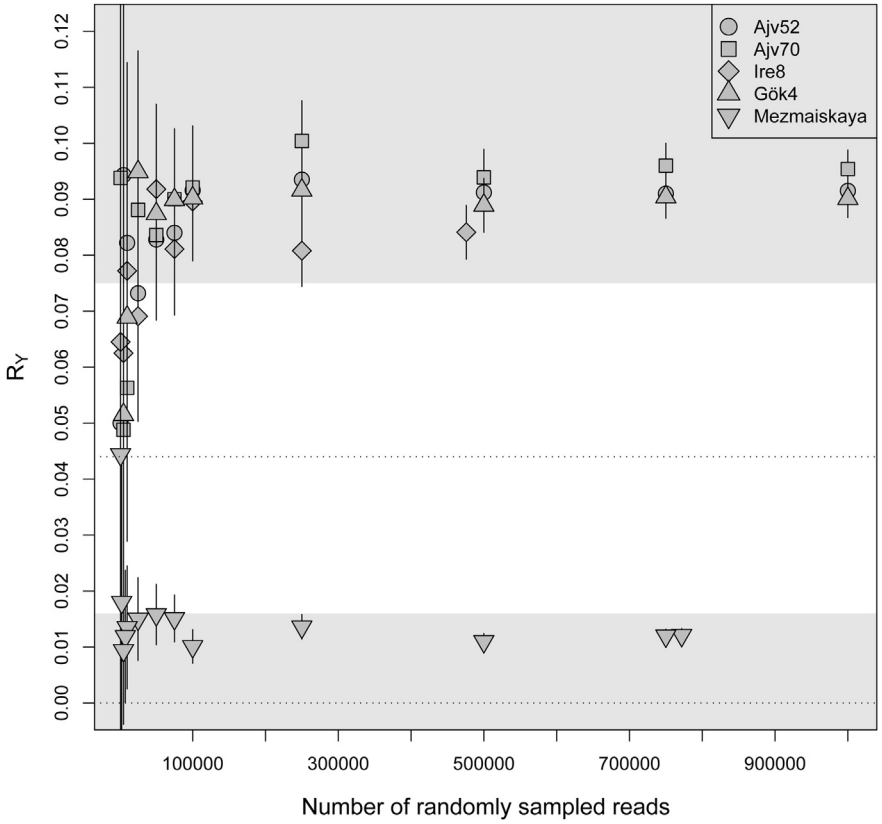

Fig. 2. Sex identification is robust for as little as 100,000 endogenous human sequences (corresponding to 3000–5000 sequences from the sex chromosomes). We randomly sampled between 1 million and 1000 sequences from five individuals in our reference data set. The fraction sequences from aligned to the Y-chromosome is expressed as a ratio of the total number of sequences aligned to either sex chromosome (R_Y). Error bars represent 95% confidence intervals.

Analyzing data from a Neandertal female to which we had artificially added various amounts of contamination from a present-day human male, we found that sex assignment was unambiguous even for contamination of a few percent, and that contamination levels >60% resulted in the sex being assigned to the sex of the contaminating individual (male) ($R_Y > 0.077$) (Fig. 3). If the sex-identification was restricted to sequences displaying post-mortem degradation (PMD, in the form of a C→T mismatch to the reference in the first 15 bp of the sequence read) according to the approach of Skoglund et al. (2012), the sex was correctly and unambiguously inferred as female for contamination levels up to 40%, and undetermined for levels >40%.

To investigate if contamination could influence the discrepancy between morphological and molecular assignments, we re-analyzed the 4 ancient Scandinavians sequenced by Skoglund et al. (2012), restricting our analysis to the subset of sequences that were obtained using HiFi polymerase (the Phusion polymerase results in depletion of PMD nucleotide misincorporations), and found that the sequences that displayed evidence of post-mortem degradation had an R_Y of 0.088–0.097 in the four individuals (including the near-complete and unambiguously male Ajv70 skeleton), suggesting that contamination by present day-males cannot explain the results for Ire8 and Gök4.

4. Discussion

We have shown that shotgun sequencing data can be used to accurately identify the biological sex of the individual in human remains (even from relatively sparse sequence data) by considering the fraction of sequences aligned to the Y-chromosome compared to the total fraction of sequences aligned to both sex chromosomes (R_Y). We demonstrate that sex can be assigned as male if, for instance, the CI lower bound for R_Y is greater than 0.077, and female if the CI upper bound is lower than 0.016. There are possibly some factors that could cause deviations from these empirical criteria that result in assignment being left undefined despite a small confidence interval (which was not the case for any of the individuals investigated in this study). For example, if an individual had an XXY, XXXY, or XYY karyotype, if there is contamination from an exogenous source (of opposite sex), or if the data production and/or alignment strongly deviates from the standard procedures used for the data analyzed here.

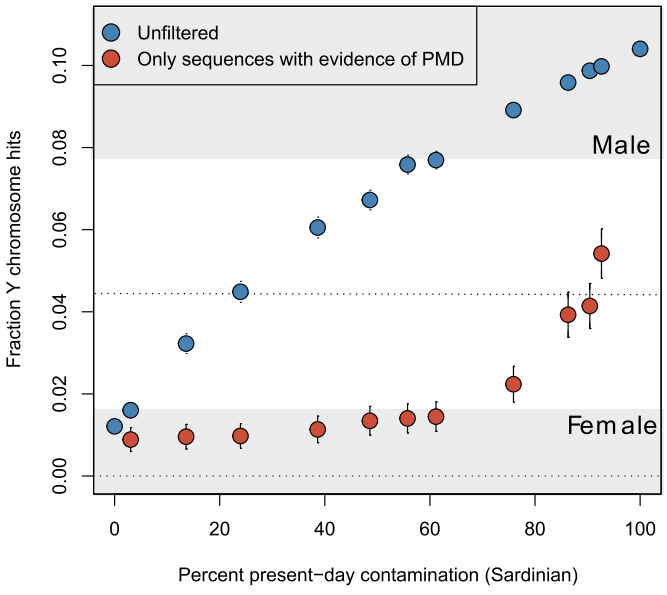

Fig. 3. Observed fraction of sequences aligning to the Y chromosome compared to the total number of sequences aligned to the X and Y chromosome (R_Y) when artificially contaminating data from the Mezmaiskaya 1 Neandertal (female) with data from a present-day Sardinian male. PMD: post-mortem damage.

Since also the sex of individuals with very low coverage, such as the La Brana 2 and Feldhofer 1 Neandertal, can be reliably identified, our results show that it may be efficient to screen many individuals simultaneously using pooled sequencing with indexed reads. Specifically, we find that only about 100,000 human sequences are necessary for accurate sex identification (with clear indications already at about 10,000 sequences), corresponding to approximately 0.002X coverage of the genome (assuming an average read length of 55). Assuming 1% endogenous human DNA of the skeleton (and 25% of all sequences being clonal), 0.002X genome coverage corresponds to about 13 million shotgun sequences. For example, 13 individuals could be pooled on an Illumina HiSeq 2000 lane given a total yield of 187 million paired-end reads. This example would thus correspond to less than 300 U.S. dollars (USD) per sample in a sex identification study (assuming 135 USD for library building and 2000 USD for a lane of Illumina HiSeq 2000 sequencing), which is less than typical costs for radiocarbon dating. The fraction retrieved endogenous DNA can range from ~0.1% to ~80% even at the same excavation site (Reich et al., 2010), which would change the necessary sequencing effort accordingly. However, using less stringent (but still accurate) criteria such as a central threshold at R_y 0.04, the estimated cost here could potentially be reduced ten-fold.

Our analyses also show that genetic identification can provide new resolution to sex identification previously performed using morphology. The identified sex of two individuals (male) differ from that reported from the morphological analysis, and the high statistical support as well as analysis of sequences with evidence of post-mortem degradation demonstrate that the morphological sex assessment of these individuals is unreliable. The Ire8 individual has been assessed as a female individual (Gejvall, in Janzon, 1974). The cranium of this individual is preserved while most of the post-cranial skeleton is damaged. Thus, no assessment of sex on the pelvic bone was possible. The cranium of Ire8 displays a female morphology compared to other Pitted Ware culture individuals, but the assessment was not conclusive (Petra Molnar, personal communication). The other individual with a different molecular sex assessment was a morphologically identified female from a megalithic burial structure in Gökhem parish (Frälsegården) (Karl-Göran Sjögren, personal communication). This individual is only represented by a mandible, which exhibits a female character, but also here the assessment was not fully diagnostic. Furthermore, the individual was a young adult and the sex-indicating morphology was possibly not yet fully developed. Finally, it is possible that the general criteria used for morphological sexing is not fully applicable to the Stone Age population in question (see e.g. Murail et al., 1999; Kjellström, 2004.)

The approach of interrogating both Y and X chromosomal DNA sequence data utilizes the expectation that males have half the amount of X-chromosomal genetic material compared to females in addition to their Y-chromosomal genetic material, whereas comparing the X-chromosomal genetic material to a similarly sized autosome (Green et al., 2010) only utilizes the former source of information. Carefully excluding homologous regions between the X and Y has also been suggested for sex identification (Green et al., 2010), but our approach is more easily reproducible for different genome assemblies while retaining high accuracy. We also show that our approach is robust to contamination in the sense that intermediate levels of contamination result in sex remaining undetermined. This is in contrast to PCR-based analysis of the amelogenin locus in which case a handful of contaminating Y-

chromosomal molecules may result in the sex being erroneously assigned as male. We recommend that for ancient remains, authenticity should also be supported by observation of post-mortem degradation (Briggs et al., 2007; Sawyer et al., 2012), and that the inferred sex is consistent if the analysis is restricted to sequences with evidence of degradation (Skoglund et al., 2012). Finally, if possible, it should also be established that the vast majority of sequences stem from a single individual (Rasmussen et al., 2010; Krause et al., 2010).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2013.07.004.

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