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=== REVIEW ===

THE EVOLUTION OF GENDER DETECTION PROTOCOLS IN BIOARCHAEOLOGICAL STUDIES

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SUMMARY. Accurate gender attribution has always been a priority in forensic casework and has always concerned the archeologists. Characteristics of forensic DNA samples analysis are similar to that of ancient DNA, small quantities of workable molecules being the main resemblance. The most frequently used markers for molecular sex attribution are the single copy gene for amelogenin located on X (AMELX) and Y (AMELY) chromosomes. Worldwide used sex determination kits were designed based on amelogenin genes amplification, especially useful in forensic casework. Sometimes these sex tests fail due to allelic dropout. New molecular markers for sex identification are constantly developed to overcome this problem. Another issue characteristic for ancient DNA studies is the contamination of samples with modern molecules of DNA. The accuracy of sex tests for ancient DNA studies depends on the possibility to discriminate between authentic ancient DNA and modern contaminant DNA.

Keywords: AMELX, AMELY, ancient DNA, molecular sex attribution, physical anthropology, sex attribution.

Introduction

Planktonic organisms, living suspended in the water, form one of the most important pelagic communities, next to the nekton

Correct sex identification has always preoccupied archeologists or anthropologists and has major importance in forensic casework for nowadays population. In past populations, the status of man and woman significantly varied and the dynamics of relations between sexes changed during time. Physical anthropology may work out identifying with a higher degree of certainty the gender of skeletal remains by analyzing a few markers that indicate the sex.

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The most common physical markers are the skull and the pelvis. Skull features tend to be more robust in the man than the female and the sub pubic region presents important differences due to the fact that females are capable of giving birth (Buikstra, 1994). The pelvic region markers are more reliable in sex attribution compared to those on the skull. These protocols, however, show no results when infant remains are analyzed. Alternative methods regarding tooth crown diameter in deciduous (Black, 1978) or permanent teeth (Cardoso, 2008) were developed for sex identification of immature individuals.

All anthropological data tend to be influenced by observer subjectivity and cannot be obtained when fragmentary remains are analyzed. Considering these limitations, a molecular sex test can provide additional information. Of course molecular methods have their own limitations regarding DNA's status of preservation or its contamination with modern molecules, but, in connection with traditional anthropological methods can offer a bigger picture, more complete picture.

Sex tests based on the amelogenin gene

The interest in ancient human DNA extraction and molecular analysis begins at the end of the 1980's, when ancient Egyptian mummy DNA was cloned (Pääbo, 1985). Since then, new extraction techniques were developed in order to overcome the degradation process that DNA suffers over time and the contaminants present in each sample. In order to obtain molecular information about an individual's sex, X and Y chromosome investigation is required.

Forensic casework needed methods to determine the sex of individuals from forensic samples. Before the PCR technique was developed other methods for correct sex identification were applied, beginning with the visualization of sex chromatin (Zech, 1969). Alternative methods for detection of the Y chromosome based on the analysis of Southern hybridization patterns were developed (Vergnaud *et al*., 1984). Another technique to discriminate between males and females based on digestion with restriction enzyme (*Hae*III) revealed a specific pattern of bands for the Y chromosome (Ludes *et al*., 1990). The development of the PCR technique (Mullins and Faloona, 1987) offered new avenues of investigation and determination of sex chromosomes in humans.

The most frequently on both X and Y chromosomes is the gene encoding amelogenin. The amelogenin is a fetal tooth matrix protein (Termine *et al*, 1980). Being single copy genes, they are suitable for rapid sex identification. AMELX is located on the X chromosome Xp22.1-22.3, while AMELY on Yp11.2. AMELX gene is expressed at ten times higher levels than AMELY (Haas-Rochholz and Weiler, 1997). Analyzing their sequences, 19 regions of absolute homology can be identified, consisting of 20-80bp, 5 deletions of 1-6bp on the X chromosome and 5 deletions of 1-183bp on the Y chromosome (Haas-Rochholz and Weiler, 1997).

Generally, homologous regions are used for the design of amplification primers. The length of the obtained fragments is different due to the presence of different deletions on both X and Y chromosome.

First amplification of certain X and Y chromosomal regions was carried out using a modern DNA sample, for forensic application (Akane *et al*, 1991). The designed primers amplified fragments of 788bp on Y chromosome, respectively 977bp on X chromosome.

These primers cannot be used when working with ancient DNA due to its degradation. Fragments that long are rarely amplifiable on such samples (Pääbo, 1989). New sets of primers were designed, many of them being suitable to amplify smaller fragments not only important when working with ancient DNA but in forensics too, because analyzed samples may have degraded DNA as well.

Two sets of primers were designed to amplify the same segment on X and Y chromosome obtaining a fragment of 106bp on the X chromosome and a fragment of 112bp on Y, respectively a 212bp fragment on the X chromosome and a 218bp fragment on the Y chromosome (Sullivan *et al*, 1993). The difference in length is due to a deletion of 6bp in the intron 1 of the X chromosome, difference that makes the segments separable by electrophoresis. This method is fast and fragments on both X and Y chromosomes are amplified in one reaction, X chromosome being used as a positive control.

Primers designed by Sullivan were included in commercial kits like AmpFlSTR® SGM Plus® (Holt *et al*, 2000) and used in forensic casework all over the world. Usually when they are contained in commercial kits, the sexual markers are associated with other markers for example Y-STR (Short Tandem Repeats) markers. At least 13 STR loci are used in forensic investigation (Holt *et al*., 2000). This kind of multiplex amplifications may conduct to overlapping fragments. Primers amplifying 80bp on the X chromosome and 83bp on the Y chromosome were designed to better suit multiplex reactions (Haas-Rochholz and Weiler, 1997).

Alternative primers for sex identification were designed targeting other regions of amelogenine genes on both X and Y chromosomes in order to amplify sort segments suitable for ancient DNA studies. Targeting a deletion in the first intron in the Y chromosome, a system of three primers was used to amplify both fragments on X and Y chromosomes in one reaction obtaining a 330bp segment on the X chromosome respectively a 218bp on the Y chromosome (Faerman *et al*, 1995). Forward primers were the same on both chromosomes while the reverse primer on the X chromosome matches an X specific sequence, absent on the Y chromosome. The primer specific for the Y chromosome matches the joint of the deletion break point (Faerman *et al*, 1995). In order to obtain improved results while working with ancient highly degraded DNA, Faerman proposed a new primer for X specific amplification in order to obtain a smaller fragment consisting of 270bp, suitable when working with ancient DNA (Faerman and Bar-Gal, 1998).

On the same principle of using three primers to obtain both segments for X and Y chromosome in one reaction, new primers were designed in order to obtain a 196bp amplification product on the X chromosome and a 136bp amplification product on the Y chromosome (Götherström *et al*., 1997). The downstream primer specific for the Y chromosome matches to a related region on the X chromosome, a 304-312 additional amplification product being expected. This additional product was found just when modern DNA samples were analyzed. While working with ancient DNA, due to its degradation, this undesired additional product couldn't be amplified.

Another method involving amelogenin gene implies using a set of primers amplifying the same segment of 112bp on exon 6 of both the X and Y chromosome (Stone, 1996). Using a dot blot procedure amplification products were assigned to X and Y chromosomes due to the fact that the Y chromosome presented 8 SNPs in the amplified region when compared to the X chromosome.

In order to obtain smaller fragments suitable when working with ancient DNA or forensic samples, new primers were designed that lead to the amplification of even shorter segments. Targeting a deletion of 3bp on the Y chromosome, a 48bp segment was amplified on the X chromosome and a 45bp one on the Y chromosome (Tschentscher *et al*., 2008).

Alternatives to amelogenin gene sex tests

Sometimes sex identification using amelogenin may go wrong. If the fragment specific to the Y chromosome is missing, an individual can be catalogued as female. Using commercial kits based on primers designed by Sullivan, 4.5% of Nepalese males were identified as females because no Y specific amplification took place (Jha *et al*, 2010). The amplification of the Y specific fragment failed because AMELY was missing. Man lacking AMELY - called 'deleted-amelogenin males' (Thangaraj, 2002) - were also identified in India and Malaysia (Chang *et al*., 2003), Israel (Michael and Brauner, 2004), Spain (Bosch *et al*., 2002), Austria (Steinlechner *et al*., 2002). The absence of AMELY is explained by the complete deletion of the Y's chromosome short arm (Michael and Brauner, 2004, Lattanzi *et al*., 2005). In some cases the X chromosome's amplification fails. A rare (C→G) mutation was identified in the binding region of the X specific primer which lead to the failure of the amplification (Maciejewska and Pawłowski, 2009).

For accurate sex identification, especially important in forensic casework, alternative regions to amelogenin are necessary to be investigate since amelogenin tests are not totally reliable. The halphoid satellite family consists of repetitive DNA and is found on the pericentromeric region of each human chromosome (Willard, 1985). Repetitive sequences offer greater amount of template for PCR than single copy gene sequences (Pascal *et al.*, 1991). Specific primers for the Y chromosome,

flank a 170bp fragment (Wolfe *et al*,. 1985), while on the X chromosome a 130bp fragment is expected after amplification (Willard, 1985). Using these primers separately, PCR was performed. Other primers amplifying an X specific segment of 157bp and a Y specific segment of 200bp were designed to be used in the same reaction (Neeser and Liechti-Gallati, 1995).

ZFY is a gene located on the Y chromosome and encodes a zinc finger protein known as testis determining factor (TDF) (Page *et al*., 1987). ZFX is located on the X chromosome. Two primers were designed for amplifying a 209bp fragment on both chromosomes. The X fragment contained a restriction enzyme site for *Hae*III determining two smaller fragments consisting of 172bp and 37bp while the fragment corresponding to the Y chromosome contained a supplementary restriction site for the same enzyme which can split the 172bp segment in two fragments: one of 88bp and the other of 84bp (Stacks and Witte, 1996).

Sex-determination region Y (SRY) is located on the Y chromosome (Santos, 1998) and can be used in addition to the amelogenin test in order to verify the presence of the Y chromosome. Two primers flanking a fragment of 96bp were designed in order to suit the amplification conditions of commercially STR and amelogenin kits (Drobnič, 2006).

Authenticating ancient DNA results

One of the major issues when working with ancient DNA is represented by contamination with modern DNA (Richards *et al*., 1995). Firstly, the remains found come in contact with the archaeologists and they are the major source of contamination (Sampietro *et al*., 2006) compared with the input of modern DNA molecules coming from anthropologists and genetic researchers. Both teeth and bones are equally exposed to contamination especially prior to DNA extraction (Malmström *et al*., 2007, Sampietro *et al*., 2006). To avoid contamination, standard protocols for organizing a laboratory where ancient DNA is analyzed were elaborated (Shapiro, 2012). In order to eliminate the laboratory specific contamination the same probe can be analyzed in other ancient laboratory physically separated from the first one (Richards *et al*., 1995). An efficient method to decrease the quantity of modern DNA present in bone samples is the incubation of the bone powder in bleach (Malmström *et al*., 2007).

To authenticate DNA samples analyzed by PCR techniques a few features of ancient DNA might be taken into account. Molecular size of ancient DNA fragments is low (Pääbo, 1989), more purines are present before strand breaks (Briggs *et al*., 2007), a high number of post mortem C→T substitutions at the ends of fragments are present (Briggs *et al*., 2007) as well as miscoding lesions (Pääbo, 1989). Dealing with contamination requires a large number of cloned amplification products (Kolman and Tuross, 2000).

For sex determination, in order to overcome difficulties characteristic to ancient DNA like the presence of small fragments, small quantities of DNA and high number of cloned amplification products for authenticating the probes, new sequencing techniques can be applied. Using shotgun sequencing reliable sex determination was possible on ancient remains from 100 to 70.000 years ago (Skoglund *et al*., 2013). Discriminating ancient DNA molecules from modern ones was based on the presence of C→T substitutions present at the ends of the fragments. High throughput sequencing starts to be applied to analyze ancient DNA molecules (Bianchi *et al*., 2012).

Conclusions

Molecular sex attribution complements physical anthropological studies applied on ancient human remains and is essential in forensic casework. Amelogenin single-copy genes are widely used for sex determination. Diverse fragments of these genes are targets for PCR reactions. The length of targeted segments gets smaller and smaller to suit ancient DNA work. Failures in amplifying amelogenin genes, especially in man, have been reported. Complementing methods for the X and Y chromosomes are developed. Other molecular methods are applied in order to facilitate the authenticating process of ancient molecules in the context of exogenous DNA contamination.

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