The Use of Amelogenin Gene in Sex Determination from Human Skeletal Fragments and Teeth Specimens

İnsan İskeleti Parçalarından ve Diş Örneklerinden Amelogenin Genini Kullanarak Cinsiyet Belirleme

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ABSTRACT

Alternative approaches to sex determination of DNA samples involve investigation of regions of the amelogenin gene. This is the gene that encodes tooth enamel and is present on both the X and Y chromosomes. A review composed via Medline Internet search of literature and contributions from our experiences as well as experiences from colleagues. The rareness of failures in sex determination provides confidence in current techniques, but amelogenin gene method (singly) of sex determination is not without failures. Amelogenin PCR method/system of sex determination should not, at the moment, completely replace traditional methods of sex identification. Hence, sex identification with amelogenin gene, of subjects for forensic purposes should be conducted as much as possible through a multiple morphological-molecular combined methods to avoid fallibility of *amelogenin* gene.
Key words: Amelogenin, bone, tooth, sexing.

ÖZET

DNA örneklerindeki cinsiyet tayini için alternatif yaklaşımlardan biri de Amelogenin gen bölgeleridir. X ve Y kromozomlarının üzerinde bulunan bu gen diş minesini de kodlar. Medline araştırma sitesinde makale ve deneyimler taranarak kendi deneyimlerimizin dışında farklı araştırmalarda gözden geçirilmektedir. Mevcut tekniklerle cinsiyet tayini başarısızlıkları seyreklik gösterirken, Amelogenin

gen yöntemi tek başına başarılı değildir. Amelogenin PCR yötemi/sisteminde cinsiyet tayini şu anda geleneksel yöntemin yerini almamalıdır. Bu nedenle özellikle adli amaçlı konularda Amelogenin geniyle cinsiyet tayininin yanılınabilirliğini önlemek için bir çok morfolojik-moleküler yöntemle birleştirilerek yapılmalıdır.

Anahtar Kelimeler: Amelogenin geni, kemik, diş, cinsiyet

Amelogenin

Amelogenin is a low-molecular-weight protein found in developing tooth enamel, and it belongs to a family of extracellular matrix (ECM) proteins. Developing enamel contains about 30% protein, and 90% of this is comprised of *amelogenins*[1].

In the early stages of tooth development, internal enamel epithelial cells differentiate into ameloblasts which synthesize and secrete specific proteins as enamel matrix. Protein concentrations as high as 25-30% can be found in newly-secreted enamel. The matrix proteins then decrease during the maturation of the enamel and are replaced by apatite crystals. These proteins are thought to play important roles in the process of enamel mineralization[1].

There are two classes of proteins in the early enamel, *amelogenin* and enamelin. *Amelogenin,* which was termed by Eastoe[2] is a discrete and major constituent unique to the developing enamel. This protein contains high concentrations of proline, glutamine, leucine and histidine. *Amelogenin* is now well characterized from amino acid sequencing data and the gene structure[1]. Another protein, enamelin, termed by Termine *et al.*[3] is a minor protein component present in a form associated with the mineral phase in the developing enamel.

Identification of the Amelogenin Gene

The *amelogenin* genes were identified on sex chromosomes in both mouse and man first by Lau *et al.*[4]. They constructed a recombinant cDNA for mouse *amelogenin* and used it as a specific hybridization probe. They found that there is only one *amelogenin* gene in the mouse genome, which is located on the X-chromosome. The analysis established the locus of th*e amelogenin* gene to the distal portion of the mouse X-chromosome. On the other hand, two copies of the *amelogenin* genes were detected in the human male genome by Southern blot analysis of human-rodent cell hybrids. One copy of the *amelogenin* genes was located on the

distal short arm of the X-chromosome in the p22.1-p22.3 region. The second copy of the *amelogenin* gene was localized near the centromere of the Y-chromosome. However, they did not determine DNA sequences for human and mouse *amelogenin* genes. At about the same time, human *amelogenin* gene, assumed to be derived from X-chromosome, was sequenced by Shimokawa *et al*.[5].

Thereafter, it has been confirmed that there are two *amelogenin* genes, one on the X-chromosome and the other on the Y-chromosome in some animals and a single gene resides exclusively on the X-chromosome in other animals[1].

In mammals, *amelogenin* is said to possess seven exons, although exon 4 is lacking in most species studied, and two extra exons (8 and 9) are found in human and rodents[6]. The five first exons (exon1—exon5) are small (42—56 bp) and exon 6 (426 bp) and 7 (160 bp) are large. Exon 1 is uncoding, most exon 2 sequence codes the signal peptide, and the only first three nucleotides of exon 7 are coding for the protein[6]. The C- and N-terminal regions are hydrophilic, while the central region (most of exon 6) is hydrophobic. A*melogenin* is subjected to extensive alternative splicing[7]. The N-terminal region contains amino acids that are suspected to be involved in various functions: a phosphorylation site[8], alpha helices[9], a binding site to *N*-acetylglucosamine and keratins[10]. The hydrophilic C- and N-terminal regions interact with the surrounding environment (cell processes and hydroxyapatite crystals) and the hydrophobic region (exon 6) forms the core of the nanospheres, which are responsible for the enamel microstructure[11]. The C-terminal region plays an important role in contributing to stabilize these nanospheres[12].

Nucleotide Sequences of Human Amelogenin Genes

Human *amelogenin* gene was first isolated and sequenced by Shimokawa *et al*.[5]. A human genomic DNA library in the phage-vector was screened by plaque hybridization using a bovine *amelogenin* cDNA probe. One clone containing a 13-kb insert, probably from X-chromosome, was obtained. Restriction enzyme fragments were subcloned and sequenced by the dideoxy chain termination method. Some segments of the isolated human genomic DNA corresponded to the bovine *amelogenin* sequence. At that time, the presence of at least four exons in the human gene structure was suggested. First coding exon was not identified, although nucleotides of three putative exons (corresponding to exons 3, 5 and 6 by Salido *et al*.[13]) were sequenced and the deduced amino acid sequence was reported[1].

Nakahori *et al.*[14] reported two genomic sequences for human *amelogenin* from the X- and Y-chromosomes. They isolated a Y-specific clone encoding *amelogenin* which was used as a probe. Southern hybridization analysis of male and female genomic DNAs detected a male-specific band on the Y-chromosome and a band common to male and female on the X-chromosome. By digestion analysis with restriction enzymes, the difference between the X- and the Y-chromosome sequences was confirmed. They sequenced the nucleotides in the homologous regions from the X- and Y-chromosomes and aligned them with the best possible match. There was 88.9% homology between the X and Y nucleotide sequences. First exon was not identified and three presumed exons (exons 3, 5 and 6 by Salido *et al.*[13]) both on the X and Y (93% homology) were recognized and amino acid sequences were deduced[1].

Fincham *et al.*[15] reported the differences in human *amelogenin* components according to the sex of the individual. They extracted enamel protein samples from fetal and postnatal human deciduous dentitions and the proteins were analyzed by electrophoresis. *Amelogenin* bands specific for male-derived specimens were detected and a sex-linked dimorphism of *amelogenin* was indicated. Later, in 1992, Salido *et al.*[13] reported that human *amelogenin* genes are expressed from both the X- and Y-chromosomes. A human genomic library was screened with a probe of mouse *amelogenin* cDNA, and positive clones from the X- and Y chromosomes were isolated. Both genes spanned more than 9 kb and 7 exons were identified in the sequences of both the X- and Y derived clones of *amelogenin* genes. Complete sequences of human *amelogenin* protein including signal peptide of 16 residues on both X- and Y-chromosomes were identified[1].

The mature human *amelogenin* from the X-chromosome skipping exon 4 is a protein of 19.8 kilo Daltons with 175 amino acid residues and which from Y is a 20 kDa protein with 176 amino acids. The methionine at residue 29 of the Y -chromosome *amelogenin* is absent from the X *amelogenin*, due to a 3-base deletion in the human X *amelogenin* gene. The overall sequence similarity between the X- and Y derived cDNAs is 91%. The protein coding regions (exons 2-6) are highly conserved, with a similarity index between 93% and 100%, whereas the 5' and 3' untranslated regions (exons 1 and 7) are much less conserved[1].

A major transcript was shown to be derived from the X-chromosome by the technique of Real Time (RT)-PCR. However, faint Y-derived transcripts which hybridized only with the Y-specific probe were detected. The result indicated that the *amelogenin* sequence on the Y-

chromosome is also transcribed, although at a level much lower (about 10% of the total) than that of the X-homolog[1].

Mapping of Human Amelogenin Gene Loci on the X- and Y-Chromosomes

Lau *et al.*[4] mapped human *amelogenin* genes on the short arm of the X- chromosome in the p22.1-p22.3 region and in the pericentric region of the Y-chromosome. The *amelogenin* sequence on the human Y-chromosome was tentatively mapped onto the proximal long arm in the Yq11 region, on the basis of retention of the Y locus in a fibroblast cell line GM-1709 which was derived from a phenotypic female with an intact X-chromosome and an isochromosome for the long arm of the Y-chromosome[4]. However, a controversy arose over the localization of human *amelogenin* gene on the Y-chromosome. Nakahori *et al.*[16] mapped the *amelogenin* sequence on the short arm at Y-chromosome using newly cloned Y-specific fragment probes. Bailey *et al.*[17] also reported that the location of the Y *amelogenin* sequence was found in the short arm, Yp.

According to Salido *et al.*[13] aberrant Y-chromosome in GM-1709 was redefined as dicentric with a breakpoint in the p11.2 region, thus raising the possibility that the Y locus may be on the short arm of the Y-chromosome. They mapped the Y locus by fluorescent *in situ* hybridization using a Y-genomic clone as a probe. Metaphase Y-chromosomes with hybridization signals were examined and the *amelogenin* locus was found clearly on the short arm of the Y-chromosome in the p11.2 region, and this is generally agreed upon[13, 17]. Precise mapping of human *amelogenin* gene (AMG) in the p22 region on the X-chromosome[18] and the homolog (AMGL) on the Y-chromosome [19] were reported.

Phylogeny and Evolution of Amelogenin Gene

As described above, it is well established that there are two *amelogenin* genes, one on the X- and the other on the Y-chromosome in some mammals, with a single gene residing exclusively on the X-chromosome in other vertebrates. Male and female genomic DNA samples from a variety of animals were investigated using the "Noah's ark blot" by Nakahori *et al.*[14] and also using the PCR method by Bailey *et al*[17].

It seems to be reasonable that the *amelogenin* genes reside on both the X- and Y-chromosomes in anthropoids (gorilla, chimpanzee and orangutan) as well as in man. Some old-world monkeys (Japanese monkey, rhesus monkey and crab-eating macaque) have *amelogenin* genes on the X- and Y-chromosomes, while other old-world monkeys (baboon, patas monkey, green monkey and talapoin) have no Y-homolog. A new-world monkey, tamarin, has only one *amelogenin* gene on the X-chromosome, while the capuchin monkey, another new-world variety, has both the X and Y genes. Only one *amelogenin* gene locus resides on the X-chromosome in the mouse and rat, but bovine genes are on the X and Y-chromosomes. The data of chromosomal distribution of *amelogenin* gene(s) cannot simply be attributed to single gene duplication or translocation via phylogenetic lineage. Multiple duplication or deletion is required to conform to the phylogenetic distribution of the species[1].

It is of particular interest that the *amelogenin* gene homologs are located on the autosomes but not on sex chromosomes in the primeval forms of mammals, monotreme (platypus) and marsupial (wallaby), according to the study of Watson *et al.*[20]. These authors determined that *amelogenin* gene homologs were located on chromosomes 5q and 1q in wallaby, and on chromosomes 1 and 2 in platypus (duckbill) by Southern blot analysis. The adult duckbill has no true teeth, although the hatchling has an egg tooth and very young duckbills transiently possess teeth with typically mammalian characteristics. And this primitive egg-laying mammal has *amelogenin* gene-like loci on autosomes. However, the authors were not able to determine whether these homologous sequences are active *amelogenin* genes or pseudogenes. They surmised that the *amelogenin* gene region must have been translocated to X- and Y-chromosomes before the divergence of primates and other mammals[1].

Amelogenesis Imperfecta

Amelogenesis imperfecta is an inherited disease affecting the formation of tooth enamel in primary and permanent dentition. In general, there are two different phenotypes in clinical manifestations, the hypoplastic and the hypomineralization. The former is characterized by enamel that does not attain normal thickness during development, whereas in the latter the enamel is softer than normal. Genetically, amelogenesis imperfecta contains heterogeneous and autosomal dominant, autosomal recessive and X-linked forms which have been included in the different classification schemes with subdivision of these categories according to the perceived phenotype[21]. Affected (hemizygous) males and (heterozygous) females in the same

family have different clinical manifestations, with the teeth of heterozygous females exhibiting vertical markings of the enamel. Therefore, it was suggested that this type of amelogenesis imperfecta is caused by a structural alteration in the *amelogenin* gene[1].

The review is aimed at bringing out latest works and challenges experienced in using *amelogenin gene* primers in sex determination from human skeletal fragments and teeth specimens. This review is composed via Medline Internet search of literature and contributions from our experiences as well as experiences from colleagues.

The Application of Amelogenin Gene in Sex Determination from Human Skeletal Fragments and Teeth Specimens

Sex determination is not only a useful tool but of extreme necessity in forensic investigations specifically in cases of sexual assault, aged blood stains and human skeletal remains whereby rapid, sensitive, accurate and reliable methods of investigation are of the utmost importance[22]. The genetic difference between males and females is defined by the presence or absence of the Y-chromosome. The majority of the DNA of the sex chromosomes is specific to either the X or Y form. Closer inspection of *amelogenin* gene on each of the sex chromosomes identified regions of true homology, but also identified a number of sequence differences between the two forms[14]. Because of these homologous regions, PCR systems could now be designed to amplify both X- and Y-chromosomes in a single reaction by placing primers in homologous positions on the *amelogenin* gene that flank areas of difference between the X and Y forms. This duel amplification from a single set of primers provides an internal PCR control, meaning reaction failure will affect both X- and Y-chromosomes, removing the possibility of false-positive results that could be encountered by separate amplification strategies. Early systems designed to amplify regions of the *amelogenin* gene focused around an 189bp deletion present in the first intron of the X homolog of the gene[14] and required large amounts of template DNA to be entered into the amplification reaction[23]. The starting amount of template DNA was quickly reduced by optimization of the reaction leading to reported success rates from quantities equivalent to a single diploid cell[24].

The biggest breakthrough in DNA sex typing came with the publication of an alternative target sequence within *amelogenin* gene[25]. This system was again designed to co-amplify X- and Y-chromosomes in a single reaction, but was based around a shorter, 6bp X-chromosome

deletion, allowing for amplification of shorter target sequences. Instead of long-length products (977bp and 788bp), amplified fragments were a mere 106bp and 112bp for the X- and Y-chromosomes respectively, and these targets could also be amplified from minute amounts of template DNA. This system was compatible with both low copy number and degraded DNA, and as such quickly became the system of choice within both forensic and archaeological disciplines. The short product lengths of these redesigned amplicons also had the advantage of falling within the size range of forensically important STR loci, and as such were perfectly suited to multiplexing within DNA profiling kits. Today, forensic DNA profiling is primarily carried out using commercially produced kits, designed to amplify up to 16 STR loci simultaneously. All kit primers for amplification of the *amelogenin* loci are designed around the 6bp deletion of intron 1 of this gene[26].

An additional bonus of this particular system comes with the detection method of choice. Because the *amelogenin* DNA fragments can be co-amplified along with autosomal STR loci within commercial DNA profiling kits, the fluorescent labeling and visualization method used for this process provides a degree of quantitative information along with fragment-length measurement. The quantitative data can then be used to determine the ratio of X- to Y- chromosomes present in the analyzed sample. Male/female DNA mixtures are often encountered during forensic investigation, especially in sexual assault investigations. The ability to calculate the relative ratio of male to female DNA present in a given mixture can be extremely useful for downstream analysis. This quantification will also detect chromosome duplication events of the sex chromosomes, for example, to detect the presence of an extra X- chromosome in XXY males. This system is not useful for correct sex determination in sex- reversal syndromes such as XY females and XX males where a mutational event or translocation results in the loss or gain of the *amelogenin* gene[27].

Since the paper by Hochmeister *et al*.[28] reporting the use of DNA extracted from human femoral bone of a corpse submerged 18 months under water and a 11-year-old mummified- corpse, using restriction fragment length polymorphisms (RFLP) and variable tandem repeats (VNTR) loci amplified by PCR, as well HLA DQ A1, other studies in the literature report using restriction fragment length polymorphisms (RFLP), short tandem repeat (STR) and *amelogenin* typing kits, PCR amplified sequences of the HLA DRB1 gene and PM Amplitype kits with bones using different methods of extraction[29].

From Genova in Italy, Berks in UK and Moscow in Russia, Manucci et al.[30] reported that gender identification of forensic samples was determined by amplifying a segment of the X-Y homologous gene *amelogenin.* Using a single pair of primers spanning part of the first intron, 106 and 112 by PCR products were generated from the X and Y homologous respectively, which were then resolved by agarose gel electrophoresis.

Faerman et al.[31] reported from Hadassah, Jerusalem, on the establishment of a sensitive and reliable method of sex determination in skeletal remains based on amplification of single-copy *amelogenin*-encoding gene (*AMG*). The Y allele carries a small deletion in the first intron, facilitating the design of distinct X- and Y-specific polymerase chain reactions. Using this method, the sex of 18 individuals, including young children, out of 22 examined from periods ranging from 200 to around 8000 years ago. In no case was there a discrepancy between the DNA and the morphometric studies[31].

A study by Alvaraz Garcia et al.[32] used the homologous gene *amelogenin* to determine sex in dental pulp subjected to various environmental factors.

The applicability of the amplification of 9 STR loci and *amelogenin* was shown by typing bones and teeth, from few months to up to 3,000 years of age, using the Ampf/STR Profiler Plus. This method proved to be efficient in the analysis of ancient degraded DNA, in addition to the positive aspect of the utilization of minimal amounts of the sample[33].

From the Pennsylvania State University, Pennsylvania, and the University of Munich, Germany, Stone et al.[34] reported on the accurate genetic sex determination of 19 out of 20 ancient human skeletons dating to A. D. 1300. For sex determination using DNA, primers for PCR were designed to amplify a small fragment (112 bp) in exon 6 of the *amelogenin* gene[34].

In 1997, Cattaneo et al.[35] analyzed the genetic sex of 32 human skeletons using PCR amplification for HLA DPB1 (327 pb), *amelogenin* (106/112 pb), and mtDNA loci. The best amplification was that of the mtDNA, followed by HLA DPB1 that amplified at the same frequency of the *amelogenin* loci; and amplification was possible in 11 of a total of 32 skeletons[35].

Evison et al.[36] analyzed human bone samples from 1986 to 1994 in forensic cases or exhumed material that had been buried from 1904 to 1984, as well as from human teeth and blood stains that were 3 months to 91 years old. The amplification and analysis of *amelogenin,* HLA-

DPB1 gene and mtDNA sequences were performed. There was no correlation between the ages of specimen and the degree of DNA preservation.

Faerman *et al.*[37] from the Hebrew University, Hadassah, Jerusalem, Israel, determined the sex of infanticide victims from the late Roman era through ancient DNA analysis. They applied a highly sensitive method based on PCR amplification of the X and Y *amelogenin* alleles. The reaction yielded distinguishable X- and Y-chromosome products by the simultaneous use of three primers (M4, M5 and M6). Amplification was successful for 19 out of the 43 ancient specimen tested. Fourteen specimens were found to be males and five females giving a significantly higher frequency of boys than girls. There were no inconsistencies or conflicting data for any of the specimen.

To determine the effect of environmental factors on the preservation of DNA, 38 archaeological teeth from 18 individuals of approximately similar age (900 to 2000 years BC), but greatly differing in terms of site milieu, were analyzed for aDNA content. The STR loci HUMvWA31A, HUMTH01, HUMFES/FPS and *amelogenin* were analyzed. Differences in quantity and quality of the results are attributed to the respective prevailing environmental factors or to respective storage conditions[38].

From the United States of America, Seo *et al.*[39] reported that they analyzed the maternity of two newborns who were murdered and abandoned >5 and 10 years, by amplification and direct sequencing of mitochondrial DNA control regions. Sexes of the remains were determined to be female and male by amplifying a segment of the X-Y homologous gene, *amelogenin.*

Hummel *et al.*[40] conducted a study on 33 human skeletons to evaluate the morphological sex determination based on traits of the ossa coxae and the cranium. The evaluation criterion was genetic analysis of the *amelogenin* gene, which represents part of X-and Y-chromosome. They found that 88% of the morphological determination matched the genetic sex[40].

Mays and Faerman[41] reported from English Heritage Centre for Archaeology, Fort Cumberland, Eastney, Portsmouth, UK and Laboratory of Biological Anthropology and Ancient DNA, Hadassah School of Dental Medicine, Hebrew University, Jerusalem Israel, on sex identification in some putative infanticide victims from Roman Britain using ancient DNA. Sex identification was performed using a method based on PCR amplification of the X and Y *amelogenin* alleles. The reaction yielded distinguishable PCR products of the X and Y *amelogenin* alleles by the

simultaneous use of three primers. The primers M4, M5 and M6 amplify PCR products of 329-bp (X allele)   and 235-bp (Y allele). Altogether nine specimen were identified as male, four as female. Again, the sex of 11 infants could be identified: nine males and two females. Importantly, only the Y *amelogenin* allele could be amplified in all nine male samples, thus reflecting a relative poor state of DNA preservation in the bone specimen[41].

Matheson and Loy[42] reported from the University of Queensland, Brisbane, Australia, on the use of *amelogenin* gene in the genetic sex identification of 9400-year-old ten human skull samples from Çayönü Tepesi, Turkey. The X chromosome amplification with the M4 and M5 primers produced a 331bp fragment while the Y chromosome amplified with M4 and M6 to produce a 237bp produc. However, the *amelogenin* X-specific detection system only amplified three of the samples. Generally, the *amelogenin* amplifications did not amplify efficiently and reliably, this could be due to degree of fragmentation of the DNA in these same samples[42].

From the University of Göttingen, Germany, Diane *et al.*[43] introduced a polymerase chain reaction-based multiplex approach to improve the certainty of molecular sex identification on archaeological skeletal material. They coamplified *amelogenin*, two X-chromosomal short tandem repeats (STRs) (DXS6789 and DXS9898) and two Y-chromosome specific STRs (DYS391 and DYS392). The amplification results of this multiplex approach back each other up, and enable reliable sex identification.

From the Masaryk University, Czech Republic, Vaňharová and Drozdová[44] reported on the sex determination of 53 skeletal remains of 4000 year old children and juveniles from Hoštice 1 za Hanou by ancient DNA analysis. They amplified the SRY locus and the *amelogenin*. Concordance between the genetic and archeological determinations occurred in 13 cases out of 21 (~62%)[44].

From Johannesburg, South Africa, Gibbon *et al.*[45] designed two novel methods of molecular sex identification from 30 skeletal tissues, using *amelogenin* gene. The classification of sex using the first system was determined by analysing the sequence for 10 sex-specific single nucleotide polymorphisms (SNPs) to determine whether both the X and Y (male) or only the X (female) was represented. System two spans an indel (6 bp) in addition to 10 sex-specific SNP[45]. Using both systems of sex identification 14 (46.66%) specimens produced results, and of these 12 (85.71%) were males and 2 (14.28%) were females. Based on this data, 4 (28.57%) of the specimens in the sample possess the same result using both methods.

Method one produced 2 (14.28%) specimens and 8 (57.14%) specimens were achieved using system two[45].

From the Aristotle University of Thessaloniki, Greece, Kovatsi et al.[46] reported on the successful genetic sex identification from 10 human teeth using *amelogenin* gene locus, through DNA repair. The amplification of a single 106 bp segment of the *amelogenin* gene corresponding to the X chromosome indicates the female origin of those samples. The double PCR bands (106 bp and 112 bp), corresponding to the X and Y chromosomes, indicate the male origin of the samples[46]. The *amelogenin* gene was amplified by PCR in 8 out of 10 DNA extracts (amplification success rate 80%) following DNA repair, while prior to this repair treatment no DNA extract yielded PCR products for this gene. Four out of the ten teeth examined were found to belong to females, another four were found to belong to males and two could not be typed[46].

From Malay Peninsular, Shahrul Hisham and his colleagues[47] reported from the University of Kebangsaan Malaysia, Bangi, Selangor and Kuala Lumpur, Malaysia, on the molecular gender determination of 2 ancient human bones, using *amelogenin* gene. They used a cocktail of three primers, consisting of the common forward primer and both the human X-specific reverse primer (*AMELX*) and human Y-specific (*AMELY*) reverse primer. In male samples, the *amelogenin* gene was located on both the X and Y chromosomes and, thus, two bands of PCR products were produced, i.e., 320 bp (*AMELX*) and 235 bp (*AMELY*). On the other hand, as female samples have two X chromosomes, PCR generated only a single band of 235 bp, but in higher concentration than the X chromosome band from males[47].

A study from Czech Republic by Eliásová et al.[48] reported that comparison of genetic methods for sex determination, amplification of *amelogenin* XY and short tandem repeats (STR) analysis, showed 100% accordance in all 11 cases of Knezeves bone samples.

In a recent forensic study for genetic sex determination of burnt powdered skeletal fragments from Sokoto, Northwestern Nigeria, by Zagga et al.[49] a success rate of 33.3% was recorded for correct genetic sex identification using *amelogenin* gene.

In a research study on the polymerase chain reaction (PCR)-based sex determination using unembalmed human cadaveric skeletal fragments from Sokoto, Northwestern Nigeria, Zagga et al.[50] reported that with *amelogenin* gene, genetic sex identification was achieved in four only out of twelve samples.

In a comparative analysis on the efficiency of *amelogenin* gene and *alphoid* repeats in sex determination of embalmed cadaveric skeletal fragments from Sokoto, Nigeria, Zagga *et al.*[51] recorded a success rate of 43% was using *amelogenin* gene.

The Reliability of Amelogenin Gene in Sex Determination of Human Skeletal Fragments and Teeth Specimens

The phenotypic nature of the information provided by investigation of the *amelogenin* locus could potentially present massive problems if an error occurs that is not identified[52]. The failure of a forensic DNA profiling technique to correctly resolve an issue as simple as sex could seriously misdirect the police investigation, as well as cast doubts on the techniques' reliability for criminal prosecution[26].

The reliability of *amelogenin*-based sex testing on hard tissues was first questioned in 1996, by Stone and his colleagues, in a study of ancient human skeletal remains of twenty individuals from Germany, where one case (5%) which was classified as a male based on morphology, but PCR products hybridized only with the X oligonucleotide. This may be the result of insufficient DNA, or mutations in the priming or oligonucleotide binding sites on the Y chromosome copy of the *amelogenin* gene[34].

In a study on sex determination of skeletal remains of 4000 year old children from the Czech Republic, Vanharova and Drozdova[44] reported seven cases in which the results were divergent between archaeological and genetic analyses. About 67% failure rate of genetic sex identification using *amelogenin* gene was reported by Zagga *et al.*[49] in a recent forensic study for genetic sex determination of burnt powdered skeletal fragments from Sokoto, Northwestern Nigeria. A similar failure rate (67%) of *amelogenin* gene sex identification was reported by Zagga *et al*[50] in a different research study on the polymerase chain reaction (PCR)-based sex determination using unembalmed human cadaveric skeletal fragments from Sokoto, Northwestern Nigeria. Zagga *et al.*[51] reported in a comparative analysis on the efficiency of amelogenin gene and alphoid repeats in sex determination of embalmed cadaveric skeletal fragments from Sokoto, Nigeria, that 57% of the samples was wrongly identified as males instead of females with *amelogenin* gene.

Recently, in a single blind study for genetic sex identification using *amelogenin* gene multiplex primers on dry human teeth specimens grouped into embalmed, unembalmed and

deciduous from Sokoto, Northwestern Nigeria, Zagga *et al.*[53] reported that no genetic sex identification was achieved in any of the dry human teeth samples.

Conclusion

Amelogenin PCR method/system of sex determination should not, at the moment, completely replace traditional methods of sex identification. Hence, sex identification with *amelogenin* gene, of subjects for forensic purposes should be achieved as much as possible through a multiple morphological-molecular combined approach.

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