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Additional primer sets for an amelogenin gene PCR-based DNA-sex test

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Abstract When aligning the human amelogenin X (AmelX)-gene with the human amelogenin Y (AmelY)-gene, 19 regions of absolute homology ranging in size from 22 to 80 bp, 5 deletions located on AmelX from 1 to 6 bp and 5 deletions 1–183 bp long located on AmelY can be observed. The regions of absolute homology are used to design primer sets which span deletions of the X- and/or Y-chromosome. The PCR products generated on the X- and Y-homologues differ in size. New regions of deletions and newly designed primers are described to facilitate the integration of the amelogenin sex test into multiplex PCR reactions.

Key words Amelogenin · Sex test · Deletions · PCR · Alignment · STRs

Introduction

In forensic DNA typing investigations the determination of sex by PCR can give important additional information (Kreike and Lehner 1995; Levinson et al. 1992; Nakagome et al. 1991; Sasaki et al. 1995; Suzumori et al. 1992; Tanoue et al. 1992). Sex determination can also be useful in the identification of archeological remains (Faerman et al. 1995) and in characterizing certain sex chromosome abnormalities such as Klinefelter's syndrome (Sullivan et al. 1993)

The human amelogenin gene sequenced by Nakahori et al. (1991 a, b) (GenBank Accession number M55418 for AmelX and M55419 for AmelY) can be used to determine the sex of the donor of a sample because it resides on both the X- and Y-chromosome in humans (Nakahori et al. 1991 b). The human AmelX-gene has a size of 2872 bp and is located on the p22 region of the X-chromosome, while the human AmelY-gene has a size of 3272 bp and is located on the 11p12.2 region of the Y-chromosome (Bailey et al. 1992; Nakahori et al. 1991 a, b). The amelogenin sex test is based on the identification of single copy X- and Y-homologous regions and was first described for PCR by Nakahori et al. (1991 a, b). Several PCR primer sets have been developed to use this gene as a sex test (Akane et al. 1991, 1992; Bailey et al. 1992; Faerman et al. 1995; Sullivan et al. 1993). The most commonly used amelogenin PCR-based sex test is the one described by Sullivan et al. (1993), in which primers flank a 6 bp deletion within intron 1 of the homologues resulting in 106 and 112 bp PCR products from the X- and Y-chromosome respectively. Although this sex test is robust, sensitive, rapid and reliable the integration of this test into multiplex amplification with short tandem repeats (STRs) e.g. HumCD4 (Edwards et al. 1991; Kimpton et al. 1993; Urquhart et al. 1995) sometimes results in overlapping of fragments. To overcome this drawback we investigated possibilities to design adapted primers such that the size of the amelogenin gene amplification products can be altered to suit multiplexing with STR loci. We determined regions of homology between the AmelX and AmelY genes, where primer sequences could be designed. In this paper we will show that there is an upper and lower size limit in changing the primer sequences and therefore to integrate this system into coamplification reactions with STR loci.

Material and methods

DNA was extracted from human female and male blood samples using an organic extraction procedure (Sambrook et al. 1989). DNA was quantitated using the TKO100 fluorimeter (Hoefer Scientific instruments). To determine regions of absolute homology between AmelX (GenBank Accession No. M55418) and AmelY (GenBank Accession No. M55419) genes, the software GeneRunner Version 3.04 from Hastings Software N.Y. 1994 was used. Table 1 lists the regions of absolute homology observed after aligning the sequences of AmelX and AmelY for PCR primers and Fig. 1 shows a schematic illustration of these regions. The size of the deletions and their locations are listed for the AmelY-gene in

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Table 1 Regions of absolute homology between AmelX and AmelY obtained after alignment of the human amelogenin AmelX and AmelY-gene and analysis for PCR primers

No.	Location on AmelX [bp]	Size of region of absolute homology [bp]	Location on AmelY [bp]
1	186– 265	80	186– 265
2	287– 310	24	287– 310
3	368– 412	44	375– 418
4	899– 927	29	721– 749
6	1005–1026	22	822– 843
7	1341–1364	24	1157–1181
8	1375–1399	24	1192–1215
9	1400–1423	24	1217–1240
10	1425–1462	38	1242–1280
11	1512–1533	33	1330–1362
12	1599–1624	26	1417–1442
13	1655–1680	26	1473–1498
14	1711–1739	29	1532–1560
15	1820–1853	34	1642–1674
16	1965–2020	56	1785–1840
17	2058–2095	38	1878–1915
18	2197–2256	60	2017–2076
19	2398–2441	44	2218–2261

Table 2 Size and location of deletions on the 3272 bp AmelY-gene and on the 2872 bp AmelX-gene. Data obtained after aligning the human amelogenin AmelX- and AmelY-gene sequences

Location of deletions on AmelX [bp]	Size of deletion [bp]	Location of deletions with intron or exon on AmelY	Corresponding location of the deletions on AmelY
585– 769	185	intron 2	495
865– 868	4	intron 2	690
951– 955	5	intron 2	772
1761	1	intron 3	1581
2720–2721	2	exon 4	2539
	not present		2691–3272

Table 2. The primer sequences used were 5′ CCC TTT GAA GTG GTA CCA GAG CA 3′ (position 1657–1679 on AmelX and position 1475–1497 on AmelY; AMELU1) for the upstream strand and sequence 5′ GCA TGC CTA ATA TTT TCA GGG AAT A 3′ (position 1712–1736 on AmelX and position 1533–1557 on AmelY; AMELD1) for the complementary strand flanking the 3 bp deletion within exon 3 of the X-homologue and resulting in 80 bp and 83 bp PCR-products from the X- and Y-chromosome, respectively. The primer sequences 5′ TGG ATG TAA ACA CAG TGC CTG 3′ (position 1446–1466 on AmelX and position 1263–1283 on AmelY; AMELU2) for the upstream and 5′ CAT CTT GAG TGT AAG AGT CCG ATA G 3′ (position 1512–1536 on AmelX and position 1330–1354 on AmelY; AMELD2) for the downstream strand flank the 1 bp deletion within intron 2 of the X-homologue resulting in 90 bp and 91 bp PCR products from the X- and Y-chromosome, respectively. Primers were synthesized by Roth, Karlsruhe, Germany. Amplification of X- and Y-specific DNA fragments was performed with one primer set (AMELU1 and AMELD1 or AMELU2 and AMELD2) in a 50 µl reaction volume using a Perkin Elmer Thermocycler 9600 with 1.25 U Taq-polymerase (Gibco BRL), 0.1 µM of a primer set and 0.2 µM of each dNTP using 0.1 up to 10 ng of template DNA. Samples were amplified through 30 cycles comprising of 1 min. 95° C, 30 s 55° C, 30 s 72° C. Of the amplified products 9 µl was separated on a 9%T, 3%C_{PDA} (PDA: piperazine diacrylamide) and 60 mM formate polyacrylamide gel by horizontal electrophoresis (PAGE) for 3 h at maximum settings of 600 V and 25 mA and visualized by silver staining (Budowle et al. 1991).

Results and discussion

When aligning AmelX and AmelY sequences, regions of absolute homology and regions of deletions can be determined (Fig. 1). A total of 19 regions of absolute homology can be displayed varying in size from 22 to 80 bp (black boxes in Fig. 1, exact sequence positions for AmelX and AmelY are listed in Table 1) and representing conceivable regions to design primers.

Primers selected from the regions of absolute homology including more than one deletion were used by several authors (Akane et al. 1991, 1992; Bailey et al. 1992; Eng et al. 1994; Faerman et al. 1995; Nakahori et al. 1991 a, b; Sullivan et al. 1993). A PCR-based amelogenin sex test using only the 6 bp X-chromosome specific deletion in intron 2 was described by Sullivan et al. (1993). Using the flanking regions of this deletion to design primers results in 112 bp Y- and 106 bp X-specific fragments. The

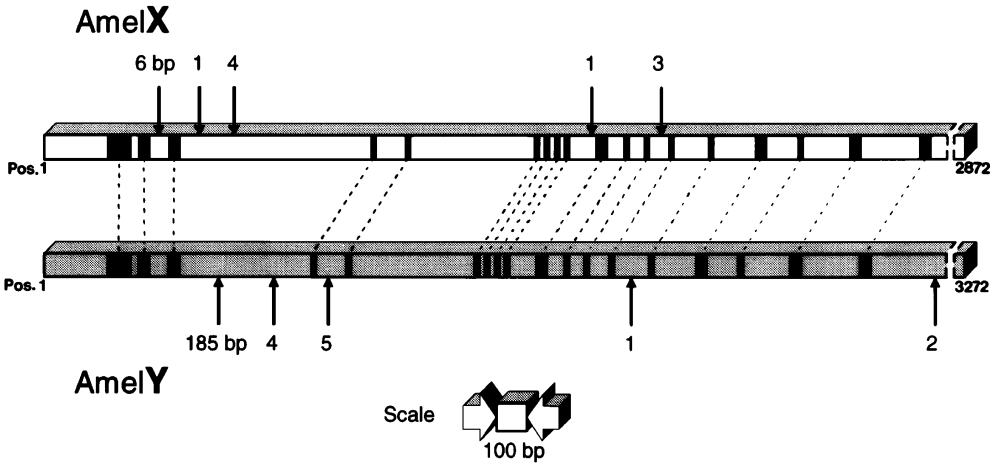

Fig. 1 Schematic of the location of the regions of absolute homology and regions of deletions between the AmelX- and AmelY-gene. The black boxes depict the regions of absolute homology analysed for PCR primers, the regions of deletions are depicted as arrows. The arrows above the AmelX-gene show the regions of deletions on the AmelX-gene. The arrows below the AmelY-gene show the localization of the deletions on the AmelY-gene. The numbers indicate the size of the deletions

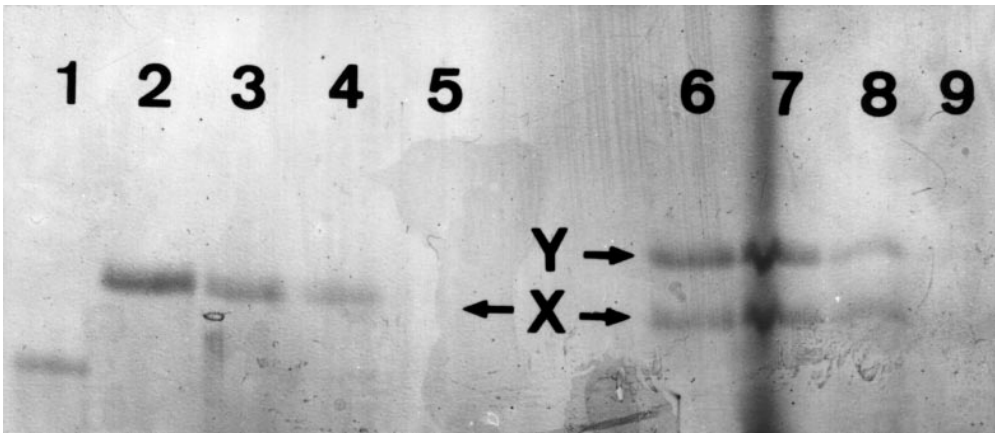

Fig. 2 Detection of 3 bp deletion of the AmelX-gene after PCR for female and male DNA on a silverstained polyacrylamide gel (9%T, 3%C_{PDA}, 60 mM Formate). Lane 1, 75 bp fragment of 1 kb markers (Gibco BRL). PCR-products of female DNA are shown in lane 2 to 5 and of male DNA in lane 6 to 9. 2 ng (lane 2 and 6), 1 ng (lane 3 and 7), 0,1 ng (lane 4 and 8) and 0,05 ng (lane 5 and 9) template DNA was used for PCR with primers AMELU1 and AMELD1. The sizes of the PCR-fragments are 80 bp for the X-chromosome specific and 83 bp for the Y-specific fragment

largest DNA fragment that might be produced using the flanking region of homology is 212 bp for the X-specific and 218 bp for the Y-specific fragment (Sullivan et al. 1993). If the primers are selected from other regions of homology other X- or Y-specific deletions would be included (Akane et al. 1991 and 1992).

We investigated the size of flanking sequences of homologous regions of the AmelX and AmelY-gene for possible primers that produce smaller PCR fragments. These sequences were analyzed with respect to general PCR primer design restrictions (GeneRunner Hastings Software 1994). The 22 bp region of homology (No. 6 in Table 1) was associated with an unfavourable melting temperature (T_m) for one PCR primer (< 43° C). Furthermore when aligning the AmelX- and AmelY-gene, five deletions ranging from 1 to 6 bp were located on the AmelX only (Fig. 1, arrows above the AmelX-gene). The arrows under AmelY in Fig. 1 indicate the location of the deletions ranging in size from 1 to 185 bp detectable only on the AmelY-gene (Table 2). When comparing our data on the size and number of deletions on the AmelY with those of Nakahori et al. (1991 a), deviations could be observed. The authors described seven deletions with sizes of 177, 8, 4, 5, 1, 1 and 3 bp, while we observed only five (Table 2). The large size of one of the deletions on the AmelY causes a shift in the locations of the regions of absolute homology (dashed lines in Fig. 1). One possibility is the design of primers that flank the last 1 bp deletion of intron 2 of AmelX resulting in a 92 bp X-chromosome specific fragment and a 91 bp Y-chromosome specific fragment (for primer sequences see chapter material and methods). The sensitivity achieved using these primers was 0.1 ng of template DNA. Using primer AMELU1 and AMELD1 the 3 bp deletion region in the exon 3 of AmelX (compare Table 1; last row) could be amplified and result in a 80 bp X-chromosome specific and a 83 bp Y-chromosome specific fragment (Fig. 2). Using primerset AMELU1 and AMELD1 0.1 ng template DNA was sufficient for detection.

Our results show that when selecting primers from other regions of homology shorter fragments might be produced to determine the sex of the donor of a sample. Smaller sized fragments might be desirable when the DNA is substantially degraded. Also when developing multiplex PCR where STR loci and the amelogenin sex test could be coamplified, a PCR amelogenin fragment smaller than 100 bp might facilitate multiplex amplification. With suitable equipment the 1 bp deletion on the AmelY can be detected. The largest fragment size that might be produced when using the primers from the flanking regions of homology are 212 and 218 bp (Budowle et al. 1991).

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