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PCR-based sexing of caprine embryos using *Sry*/*Aml-X* duplex PCR and *Sry*/DVEPC041 duplex PCR

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ABSTRACT

This study presents the development of a PCR-based method for sex determination of caprine embryos by *Sry* and *Aml-X* gene detection. DNA sequencing of the PCR products amplified from the target loci confirmed that these amplicons were indeed amplified from portions of the caprine *Sry* and *Aml-X* genes. Furthermore, the primer sets were found to be specific for sexing of caprine of various breeds. An alternative PCR-sexing protocol was also developed to verify embryo sexing results and it was a pit-stop using *Sry* and DVEPC041 primer sets. For the pit-stop PCR protocol, the *Sry* primer was best added after the first 6 cycles to obtain bands of equal intensity at 116 bp and 160 bp when identifying the male sex. However, application of the same pit-stop PCR protocol using genomic DNA of various caprine breeds also produced acceptable and accurate sex identification despite showing slight diversion of the expected result.

Key words: Caprine, Polymerase chain reaction, Sex determination

Sex determination is important in livestock industry to identify foetal gender for breeding, culling and eliminating expenses of the progeny test programmes. Consequently, sex predictability could be compromised for artificial insemination using sex-sorted sperm. Polymerase chain reaction (PCR) is the method of choice due to acceptable reliability, high sensitivity, inexpensiveness and rapidity (Dervishi *et al*. 2011, Saberivand and Ahsan 2016). In previous study, PCR duplex protocol using DNA extracted from blood sample of caprine was reliable, accurate, reproducible and efficient for caprine sexing (Phua *et al*. 2003), and then the present study was continued to refine this protocol for sexing of caprine embryos.

There has been little information on sex determination of caprine embryos using a PCR method (Chen *et al*. 2007). Therefore, the objectives of this study were to standardise a caprine blood sexing duplex PCR protocol (Phua *et al*. 2003) for application in caprine embryo sexing, and to develop and optimise an alternative PCR duplex protocol for sexing caprine embryos.

MATERIALS AND METHODS

Blood samples were obtained from both sexes of caprine, ovine, bovine, murine and human. DNA was extracted according to Phua *et al*. (2003). The DNA samples obtained were then quantified for concentration and purity using a spectrophotometer. The primer sets were designed to amplify a portion of the caprine *Sry* (GenBank accession no. Z30646) and *Aml-X* (GenBank accession no. AF215887) genes, respectively. The primer sequences were as follows:
Sry.5: 5´ ATGAATAGAACGGTGCAATCG 3´
Sry.3: 5´ GAAGAGGTTTTCCCAAAGGC 3´
Aml-X.5: 5´ CAGTAGCTCCAGCTCCAGCT 3´
Aml-X.3: 5´ GTGCATCCCTTCATTGGC 3´

The master mix was vortexed, centrifuged and each portion aliquoted into a reaction tube. Finally, the reaction tubes were given a short spin and transferred to the thermal cycler for PCR. The PCR programme consisted of denaturation (94°C, 5 min), followed by 34 cycles of denaturation (94°C, 45 sec), annealing (58°C, 45 sec) and primer extension (72°C, 60 sec). The final cycle was followed by a final extension (72°C, 7 min) and indefinite hold time at 25°C. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml). The products were viewed using UV illuminator for their desired bands. Caprine oocytes were retrieved from donor does as described by Nor Farizah *et al*. (2015). The embryos were produced by *in vitro* maturation, fertilisation and culture of oocytes following the method of Phua *et al*. (2015).

Standardisation of amplified caprine Sry and Aml-X sequences (Experiment 1): The *Sry* and *Aml-X* primer sets were tested for inter-species specificity. Blood samples were

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collected, and the DNA was extracted and quantified, and the DNA dilution prepared. PCR products were electrophoresed in agarose gel (2%), stained with ethidium bromide (0.5 µg/ml), viewed using UV illumination and documented. The primer sets were also tested for intra-species specificity. Blood samples from both female and male of the following breeds were collected, viz. Saanen, Anglo-Nubian, Jamnapari (Indonesian variant), Jamnapari (Malaysian variant), Boer, Jermasia and Katjang.

Development of an alternative caprine sex-determination protocol (Experiment 2): Two X-specific microsatellites were selected, viz. DVEPC041 and DVEPC065. For convenience, the forward and reverse primers of DVEPC041 were designated 'X041f' and 'X041r', respectively, and the forward and reverse primers of DVEPC065 were designated 'X065f' and 'X065r', respectively. The primer sequences are listed below:

X041f : 5′ ATTTGTATTAGTCAGAGTCCTC 3′
X041r : 5′ TTCCTATAATCAATCTCTCTTTCTG 3′
X065f : 5′ CTCTAATATAAGTTATTCAGGAG 3′
X065r : 5′ GACCACCAGGGAAGTCTTG 3′

After conducting initial trials of single microsatellite primer etc PCR reactions, two sets of duplex reactions involving *Sry* primer set with each microsatellite primer set were optimised for annealing temperature, $MgCl_2$ concentration, DNA concentration and a modified PCR approach known as 'pit-stop PCR'. The optimised reaction conditions obtained for pit-stop *Sry*/DVEPC041 and *Sry*/DVEPC065 duplex PCRs were tested for their ability to accurately sex various caprine breeds. For this experiment, the DNA solution (100 ng/µl) was diluted to 10 ng/µl, in which only 10 ng DNA template was used per reaction tube.

Sexing of caprine embryos (Experiment 3): Reliability and efficiency of *Sry*/*Aml-X* duplex PCR and pit-stop *Sry*/DVEPC041 duplex PCR in sexing caprine embryos was tested. First, the embryos were removed of their zona pellucida and the blastomeres dissociated and transferred into PCR reaction tubes. Every two PCR reaction tubes containing blastomeres originating from the same embryo was noted. Before performing PCR, the DNA contained in the blastomere(s) was extracted directly in the PCR reaction tube. PCR was done using the thermal cycler GeneAmp^{Ò}PCR system 9600 (Perkin-Elmer, USA). Sexing results from both *Sry*/*Aml-X* and *Sry*/DVEPC041 duplex PCRs were compared and verified with each other.

RESULTS AND DISCUSSION

Standardisation of amplified caprine Sry and Aml-X sequences (Experiment 1): The amplicon was found to be most identical (96%) to the caprine *Sry* gene. The submitted sequence matched nucleotides 33 to 116 of the published *Sry* sequence, with only three 1-nucleotide gaps in between. Submission of the DNA sequence of *Aml-X* PCR product (amplified from male caprine DNA) to the BLAST database search revealed the highest match (93% identical) with the caprine *Aml-X* gene. The submitted sequence matched nucleotides 130 to 325 of the published *Aml-X* sequence, with two 1-nucleotide gaps in between. Submission of the DNA sequence of *Aml-X* PCR product (amplified from female caprine DNA) to the BLAST database search also revealed the highest match (98% identical) with the caprine *Aml-X* gene. The submitted sequence matched nucleotides 141 to 399 of the published *Aml-X* sequence, with two 1-nucleotide gaps in between.

Female caprine and ovine were identified by presence of one band at 300 bp, and male caprine and ovine by presence of two bands at 300 bp and 116 bp, respectively (Fig. 1). No band was observed in both sexes of bovine, human and murine. PCR-sexing done on genomic DNA of female Alpine and Toggenburg caprine, and female and male Saanen, Anglo-Nubian, Jamnapari (Indonesian variant), Jamnapari (Malaysian variant), Boer, Jermasia and Katjang caprine revealed 100% (16/16) accuracy in sex identification. All female caprine of the breeds tested were identified by presence of one band at 300 bp and male caprine by presence of two bands at 300 bp and 116 bp, respectively (Fig. 2).

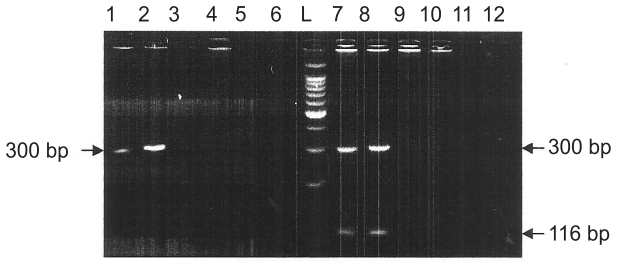

Fig. 1. Inter-species specificity of *Sry* and *Aml-X* primer sets in various species. Lanes 1 to 5 contain female caprine, ovine, bovine, human and murine DNA, respectively. Lanes 7 to 11 contain male caprine, ovine, bovine, human and murine DNA, respectively. Lanes 6 and 12 are negative control. The primer sets were indicated by presence of one band at 300 bp for female caprine and ovine, and presence of two bands at 300 bp and 116 bp, respectively, for male caprine and ovine. L, 100 bp DNA ladder.

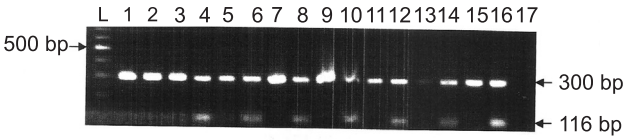

Fig. 2. Intra-species specificity of *Sry* and *Aml-X* primer sets in various caprine breeds. One band (300 bp) denotes female and two bands (300 bp, 116 bp) denote male. Lane number: 1, Alpine; 2, Toggenburg; 3, 4, Saanen; 5, 6, Anglo-Nubian; 7, 8, Jamnapari (Indonesian); 9, 10, Jamnapari (Malaysian); 11, 12, Boer; 13, 14 Jermasia; 15, 16, Katjang. Lane 17 is control. L, 100 bp DNA ladder.

Submission of the sequenced caprine *Sry* and *Aml-X* products obtained from PCR-sexing as described by Phua *et al.* (2003) to the BLAST database search revealed the highest match to the caprine *Sry* and *Aml-X* genes, respectively. The sequenced caprine *Sry* PCR product was 96% identical, *Aml-X* (from male caprine DNA) 93%

identical and *Aml-X* (from female caprine DNA) 98% identical to that of the corresponding published sequences. These results provided strong evidence that the primers were correctly amplified from portions of the caprine *Sry* and *Aml-X* genes as intended. In testing for inter-species specificity of the *Sry* and *Aml-X* primer sets, the result indicated these primer sets were specific only for sexing caprine and ovine. This result implies that the *Sry* and *Aml-X* primer sets originally intended for caprine sexing can also be applied in ovine sexing. In testing for intra-species specificity of the *Sry* and *Aml-X* primer sets, sex identification was accurate for sexing caprine of various breeds.

Development of an alternative caprine PCR-sexing protocol (Experiment 2): Using standard PCR conditions and female and male caprine DNA, primer set for DVEPC041 yielded a product at 160 bp for both sexes, and primer set for DVEPC065, a product at 200 bp for both sexes. The annealing temperature that worked best for *Sry*/DVEPC041 duplex PCR ranged from 48.0– 48.8ºC. The annealing temperature that worked best for *Sry*/DVEPC065 duplex PCR ranged from 54.0– 55.4ºC.

In the *Sry*/DVEPC041 duplex PCR, the most suitable $MgCl_2$ concentration was 1.50 mM. In the *Sry*/DVEPC065 duplex PCR, the most suitable $MgCl_2$ concentration was 1.25 mM. For the *Sry*/DVEPC041 duplex PCR, the desired bands at 116 bp (*Sry* product) and 160 bp (DVEPC041 product) were present up to 2 ng of DNA template. For the *Sry*/DVEPC065 duplex PCR, the desired bands at 116 bp (*Sry* product) and 200 bp (DVEPC065 product) were present up to 1 ng of DNA template. For pit-stop *Sry*/DVEPC041 duplex PCR, equal banding intensity (116 bp and 160 bp) was obtained when the *Sry* primer set was added after the first 6 cycles. Addition of the *Sry* primer set after the first 8 and 10 cycles resulted in *Sry* bands of lesser intensity than the DVEPC041 bands, and after 12 cycles, the *Sry* band was no longer present. For pit-stop *Sry*/DVEPC065 duplex PCR, equal banding intensity (116 bp and 200 bp) was obtained when the *Sry* primer set was added after the first 6 cycles. Addition of the *Sry* primer set after the first 8 and 10 cycles resulted in *Sry* bands of lesser intensity than the DVEPC065 bands, and after 12 cycles, the *Sry* band was no longer present. For pit-stop *Sry*/DVEPC041 duplex PCR, successful amplification was observed in reactions containing as low as 10 ng DNA template. For pit-stop *Sry*/DVEPC065 duplex PCR, successful amplification was observed in reactions containing as low as 10 ng DNA template.

The pit-stop *Sry*/DVEPC041 duplex accurately sexed all caprine although there appeared to be a diversion of the expected result (Fig. 3). All male caprine showed clear 116 bp bands and very faint bands at 350 bp. On the other hand, all female caprine showed clear bands at 350 bp, except for female Jermasia which showed a very faint 350 bp band. The expected DVEPC041 band at 160 bp was very faint and rather indefinite cross all goats. Based on pit-stop *Sry*/DVEPC065 duplex PCR results, male caprine would be expected to show two bands at 116 bp and 200 bp, respectively, and female one band only at 200 bp. The observed result was a total diversion from the expected result (Fig. 4).

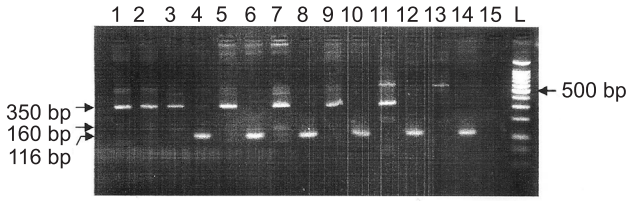

Fig. 3. Pit-stop *Sry*/DVEPC041 duplex PCR using DNA of various caprine breeds. Male caprine denoted by a clear band at 116 bp and female by a clear band at 350 bp. Lane 1, Alpine; 2, Toggenburg; 3, 4, Saanen; 5, 6, Anglo-Nubian; 7, 8, Jamnapari (Indonesian); 9, 10, Jamnapari (Malaysian); 11, 12, Boer; 13, 14, Jermasia. Lane 15 is control. L, 100 bp DNA ladder.

As recommended by Lopes *et al*. (1999), a modified duplex PCR approach known as 'pit-stop PCR' was introduced to circumvent the preferential amplification of *Sry* locus over the microsatellite loci which resulted in equal banding intensity. In pit-stop PCR, the higher yielding primer set (*Sry*) was only added into the reaction mixture after several cycling steps (Lopes *et al*. 1999). From optimisation of pit-stop PCR, it was found that the *Sry* primer set was best added after the 6th cycle for both duplexes.

The pit-stop *Sry*/DVEPC041 duplex results also presented an interesting observation in that all female samples (except for female Jermasia) showed clear 350 bp bands but faint or absent 160 bp bands, and all male samples showed faint or absent 350 bp or 160 bp bands. A possible explanation to this phenomenon is that DVEPC041 microsatellite alleles may be polymorphic and each caprine may be heterozygous or homozygous for the trait, thus influencing the bands' intensity. In a similar work by Bredbacka *et al*. (1995), amplification of the highly repetitive btDYZ-1 region of the bovine Y chromosome which exhibits length polymorphism of TG repeats, produced PCR products of various sizes visualised as a smear in electrophoresis gels. PCR-sexing of caprine embryos using the *Sry*/*Aml-X* protocol is capable of producing accurate sexing results.

Two PCR protocols and result verification (Experiment 3): A summary of results for sexing of caprine embryos,

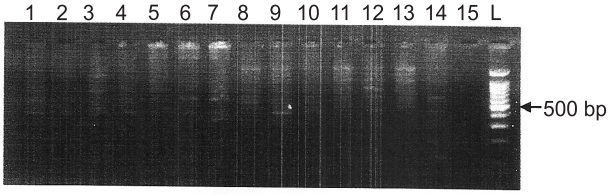

Fig. 4. Pit-stop *Sry*/DVEPC065 duplex PCR using DNA of various caprine breeds. None of the lanes containing caprine DNA showed distinct banding pattern that could be used for identifying the sexes.

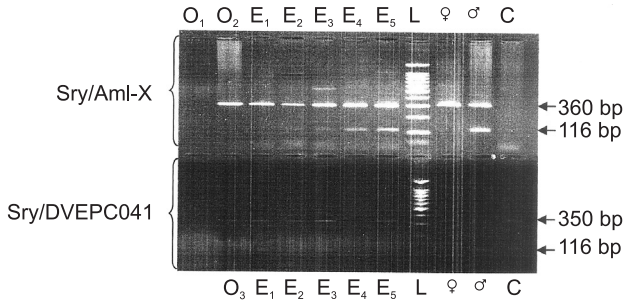

Fig. 5. Representation of caprine embryo sexing results. Shown here are sexing results of 5 embryos (E1 to E5) and 3 oocytes (O1 to O3) using both *Sry*/*Aml-X* and pit-stop *Sry*/DVEPC041 duplex PCRs to verify each other. For both duplexes, 2 bands denote female and one band denotes male. ♀, female positive control; ♂, male positive control; C, negative control; L, 100 bp DNA ladder.

oocytes and presumptive zygotes is given in Table 1. A representation of caprine embryo sexing results from the agarose gel is shown in Fig. 5. Out of 20 caprine embryos sexed, only 9 embryos (45%) were successfully sexed, 7 embryos (35%) showed ambiguous results and 4 embryos (20%) showed contradicting results. Out of 62 oocytes sexed using *Sry*/*Aml-X* duplex PCR, 35 oocytes (56.5%) were successfully identified as female, 17 oocytes (27.4%) were wrongly identified as male and 10 oocytes (16.1%) resulted in failed reaction (i.e., no bands). Out of 30 oocytes sexed using pit-stop *Sry*/DVEPC041 duplex PCR, only one oocyte (3.3%) was successfully identified as female, 25 oocytes (83.3%) were wrongly identified as male and 4 oocytes (13.3%) resulted in failed reaction (i.e., no bands). For sexing of presumptive zygotes, out of 11 zygotes sexed using *Sry*/*Aml-X* duplex PCR, 4 zygotes (36.4%) were female, 6 zygotes (54.5%) were male and one zygote (9.1%) resulted in failed reaction (i.e., no bands). Out of 5 zygotes sexed using pit-stop *Sry*/DVEPC041 duplex PCR, none were female, 3 zygotes (60%) were male and 2 zygotes (40%) resulted in failed reaction (i.e., no bands).

Table 1. Results for sexing of caprine embryos, oocytes and presumptive zygotes

Types of samples for PCR-sexing	Quantity	Percentage (%)
Sexing of embryos using *Sry*/*Aml-X* and pit-stop Sry/DVEPC041 duplex PCRs (n = 20 embryos)		
Successful result	9	45
Ambiguous result	7	35
Contradicting result	4	20
Sexing of oocytes using:		
(i)Sry/*Aml-X* duplex PCR (n = 62 oocytes)		
Correctly identified as female	35	56.5
Wrongly identified as male	17	27.4
Failed reaction	10	16.1
(ii)Pit-stop *Sry*/DVEPC041 duplex PCR (n = 30 oocytes)		
Correctly identified as female	1	3.3
Wrongly identified as male	25	83.3
Failed reaction	4	13.3
Sexing of presumptive zygotes using:		
(i)Sry/*Aml-X* duplex PCR (n = 11 zygotes)		
Female	4	36.4
Male	6	54.5
Failed reaction	1	9.1
(ii)Pit-stop *Sry*/DVEPC041 duplex PCR (n = 5 zygotes)		
Female	0	0
Male	3	60
Failed reaction	2	40

The *Sry*/*Aml-X* duplex PCR actually serves as the primary protocol for caprine embryo sexing. The development of an alternative PCR-sexing protocol (pit-stop *Sry*/DVEPC041 duplex) is merely to verify embryo sexing results of the primary protocol. Although embryo sexing results are ideally and ultimately confirmed by live births, however, live birth verification requires a host of other techniques to be refined simultaneously. Therefore, development of an alternative PCR-sexing protocol would provide a more convenient, time-saving and economical approach to verify sexing results of the primary sexing protocol.

Although the use of Y-specific primer set alone is sufficient to determine the sex as had been done successfully in bovine embryo sexing works (Bredbacka *et al*. 1995, Hasler *et al*. 2002), the inclusion of the X-specific primer sets in both *Sry*/*Aml-X* and *Sry*/DVEPC041 duplexes serves a two-fold function – as internal control to discriminate male and female with higher accuracy and as positive control to check for amplification failure should there be absence of Y-specific amplicon.

In conclusion, DNA sequencing of the PCR products amplified from the target loci confirmed that these amplicons were indeed amplified from portions of the caprine *Sry* and *Aml-X* genes. In addition, the *Sry* and *Aml-X* primer sets were found to be specific for sexing caprine of various breeds and sheep. An alternative PCR-sexing protocol was also developed as a means to verify embryo sexing results based on *Sry* and *Aml-X* primer sets. For the pit-stop PCR protocol, the *Sry* primer was best added after the first 6 cycles to obtain bands of equal intensity at 116 bp and 160 bp when identifying the male sex. However, application of the same pit-stop PCR protocol using genomic DNA of various caprine breeds also produced acceptable and accurate sex identification despite showing slight diversion of the expected result.

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