

# Polled Intersex Syndrome and Polledness in Goats: Molecular Aspects

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## ABSTRACT

The aim of the present study was to confirm the presence of the polled intersex syndrome (PIS) deletion in hermaphroditic individuals and polled bucks, transmitting at least one of its copies to PIS individuals. Two sex-reversed animals (Czech White Improved and Polish White Improved), two polled and two horned bucks were examined. Three primer pairs were designed for the identification of the PIS deletion in the sex-reversed individuals and polled bucks. Additional 24 primer pairs were used for the identification of the deletion region in sex-reversed individuals. Selected PCR products were sequenced. The identification of the PIS deletion in hermaphroditic individuals and polled bucks based on the primers designed on the reference template was impossible. Therefore, an attempt was made to determine the reasons for the failure of deletion identification in these individuals. For the polled bucks, the deletion region was amplified similarly to the horned ones, except for an initial sequence. In the two sex-reversed individuals, four inserts were identified in the deletion region. Despite the fact that PIS in goats has been known to breeders and scientists for several decades, many of its aspects, including the molecular reason, are still controversial and require further detailed research.

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## Authors' Contribution

IS conception of the work, drafting the work. JU acquisition and analysis of data, drafting the work. DZ interpretation of data, drafting the work. ECP interpretation of data, drafting the work. WG design of the work and revising it critically for important intellectual content. MW, acquisition of data, revising the work critically for important intellectual content. JW, acquisition of data, revising the work critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

## Key words

Hermaphrodites, Polled bucks, Deletion, Sex reversal, Amplification, Sequencing

## INTRODUCTION

Polled intersex syndrome (PIS) has been known to goat breeders for decades (Soller *et al.*, 1969). It occurs in homozygous 60,XX (SRY-) polled females and is accompanied by significant masculinization of the reproductive system (Szatkowska *et al.*, 2014a). This phenomenon is inherited in an autosomal recessive

way, whereas polledness, occurring in both sexes, is an autosomal dominant trait. In 1996, Vaiman *et al.* (1999) located the PIS locus on the q43 region of chromosome 1. In 2001, Pailhoux *et al.* (2001) found that the 11.7-kb deletion in this region is responsible for PIS. Repetitive DNA sequences are mainly located in the deletion region, constituting a palindrome system. The most important ones are the two LINE, placed in an inverted orientation and forming the main palindromic structure, as well as the two repetitive elements located in the central part of the deletion and capable of loop formation (Pailhoux *et al.*, 2001). The whole described palindromic structure may form the hairpin regulatory element acting as a male gonad-specific transcription factor. It is likely involved in chromatin structure changes together with the SRY protein binding to the central part of the loop, the two LINE and two conservative regions 1 and 3 (Pailhoux *et al.*, 2001). Such a construct acts as a transcriptional repressor of the

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four target genes (*PISRT1*, *PISRT2*, *PFOXic*, *FOXL2*), from which two are the most significant ones: *PISRT1* and *FOXL2*, located 20 and 200 kb from the PIS deletion site, respectively (Pannetier *et al.*, 2012). An alternative smaller loop is formed in females by LINE2 together with conservative regions 2 and 3. It acts as an enhancer in the absence of the SRY protein (Pailhoux *et al.*, 2001).

*PISRT1* (PIS-regulated transcript 1) contains the TATA box and the polyadenylation signal sequence but does not include an open reading frame or intron sequences. It encodes the 1.5-kb transcript (Boulanger *et al.*, 2008). It is known that its expression in the gonads of PIS-/- sex-reversed goats is extinguished between 36 and 40 days postcoitum (dpc), which coincides with the expression of the *SOX9* gene, i.e., the main factor promoting testis development, besides the *SRY* gene. On the other hand, its high-level expression in horned PIS+/+ female goats and polled PIS+/- heterozygotes is already noticeable at 34 dpc and continues in the gonads of adult individuals, whereas the transcription level of *PISRT1* in the developing male gonads shows sexual dimorphism from 34 to 70 dpc, i.e. lower in males than in females, and then its expression starts to increase, reaching a maximum value during the perinatal period and remaining at a high level until sexual maturity (Pailhoux *et al.*, 2001).

*FOXL2* (forkhead box L2) contains one exon. The protein encoded by this gene belongs to a large family of transcription factors, including 37 members, involved in different biological processes such as the normal development of the tissues originating from all three germ layers, the control of metabolic processes and the cell cycle (Uhlenhaut and Treier, 2006). In the gonads of female goats, the expression of *FOXL2* is initiated between 34 and 36 dpc and remains at a high level both in the pre- and postnatal period. It is maintained in adult individuals (Pailhoux *et al.*, 2002). In males, however, *FOXL2* transcripts are undetectable during the so-called gonadic-switch time period (34–36 dpc). What is interesting is the fact that *FOXL2* expression in polled XX PIS-/- female goats drastically decreased at early stages of gonadogenesis (36 dpc), which resulted in the appearance of *SOX9* transcripts, controlling *AMH* expression (40 dpc). This cascade of transcriptional events, leading to the initiation of the male developmental pathway, is responsible for the masculinization of XX PIS-/- fetuses at early stages of sex determination and differentiation (Pailhoux *et al.*, 2005).

So, why are detailed molecular studies on the background of the PIS phenomenon still interesting? There are several reasons for this. The first one is associated with the molecular diagnosis of sex-reversed individuals, which constitute an interesting model for the understanding

of the molecular mechanisms of sex determination, differentiation and reversal, not only in goats but also other mammalian species. The second reason refers to the studies on homozygous polled bucks. Reports on decreased reproductive potential in such males appeared already in the 1960s (Soller *et al.*, 1963, 1969). This problem has not been studied so far, mainly due to the lack of tools for the molecular identification of the dominant polled gene. However, if polled males transmit one polled allele to at least half of their offspring (heterozygous inheritance), they also propagate one sex-reversal allele, since these traits are linked. Therefore, polled males must be heterozygous (PIS+/-) or homozygous (PIS-/-) for the 11.7-kb deletion linked to hermaphroditism. Consequently, it can be assumed that the issue of homo- or heterozygosity of such polled males should be determinable based on its identification.

Taking into account the above-mentioned reasons and the doubts about the occurrence or precise localization of the discussed deletion, the aim of the present study was to confirm its presence in hermaphroditic individuals and polled bucks, transmitting at least one of its copies to PIS individuals.

## MATERIALS AND METHODS

Peripheral blood was collected from the external jugular vein of the two sex-reversed individuals (Czech White improved with the proportion of the Saanen breed and Polish White improved with the proportion of the Alpine breed). The diagnosis of sex-reversal was based on the masculinization changes of the external and internal genitalia, histological examination of the gonads, the *AMGLX* and *AMGLY* gene analysis allowing the determination of genetic sex (F 5'-CAGCCAAACCTCCCTCTGC-3', R 5'-CCCGCTTGGTCTTGTCTGTTGC-3') and the absence of *SRY* sequence in the genomic DNA (F 5'-CTGGTGAACGAAGACGAAAGG-3', R 5'-GCCTTCCGACGAGGTCGATACTTA-3') (Fig. 1). The analysis also included two polled and two horned bucks (for comparison).

DNA was isolated with the MasterPure™ Genomic DNA Purification Kit (Epicentre Biotechnologies). The DNA extractions were stored at -20°C for further analysis. The PCR reaction contained approximately ~50 ng of DNA template, 0.3 units of DreamTaq DNA Polymerase, 1×PCR Buffer, 1.5 mM of MgCl<sub>2</sub>, 200 μM of each dNTP, 15 pmol of each primer and filled up to 15 μL with deionized water.

The first three primer pairs (Del- 1F: 5'-TGCTTCTCCACTGGCAGAGT-3', Del- 1R: 5'-TGCAATGCCAATAATAGAGAAAA-3'; Del -2F: 5'-GCATTGAGGCCAGATCCTTA-3',

Del -2R: 5'- AAAAAGGGGAGTTGAAGAAGAAA -3';  
 Del -3F: 5'- GGGCTTTGCATGTGGTAGAT-3',  
 Del -3R: 5'- CCCAGAAGAAAAGATTTCAAACA -3')  
 were designed for the identification of the PIS deletion in sex-reversed individuals and polled bucks, which, theoretically, should be the carriers of the 11.7-kb PIS deletion, at least in the heterozygous form. The reference template contained only the flanking regions of the 11.7-kb PIS deletion, which is presented in Figure 2.

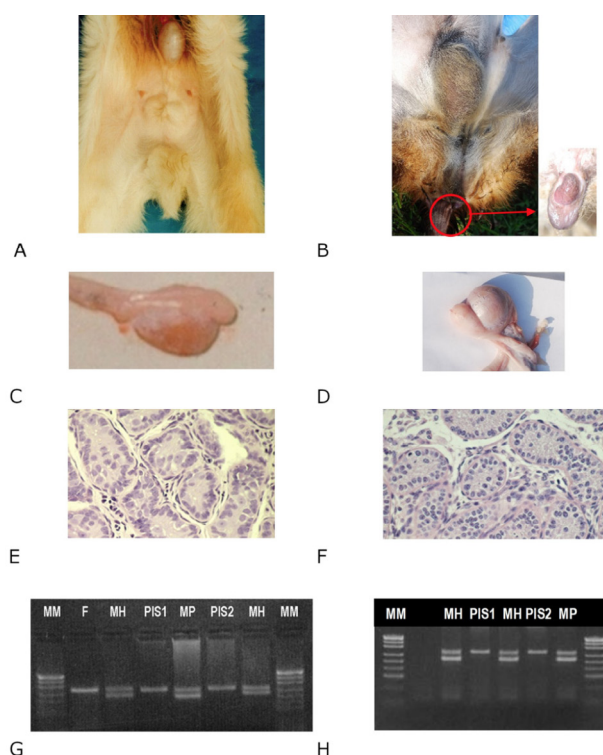


Fig. 1. Phenotypic description of the PIS individuals (masculinization of external and internal genitalia, histological examination of the gonads) and a sex identification test (based on the *AMGLX*, *AMGLY* and *SRY* genes). **A**, Male-specific external genitalia; caudally displaced hypoplastic penis; absence of the urethral process; problems with urination. **B**, Male-specific external genitalia; one testis in the scrotum, the second one in the groin area; enlarged clitoris. **C**, Testes with epididymides. **D**, Testes with epididymides. **E**, In the testis of the animal, the seminiferous tubules lacked lumen and contained Sertoli cells and primordial cells located on the basal lamina and occasionally in the center of the tubules. **F**, the seminiferous tubules lacked lumen and contained spermatogonia and Sertoli cells; the interstitial tissue with a small group of Leydig cells. **G**, *AMGLX*, *AMGLY*, MM, DNA ladder; F, female; MH, horned male; MP, polled male; PIS1 and PIS2, sex-reversed individuals; **H** – *SRY*, MM, DNA ladder; F, female; MH, horned male; MP, polled male; PIS1 and PIS2, sex-reversed individuals.

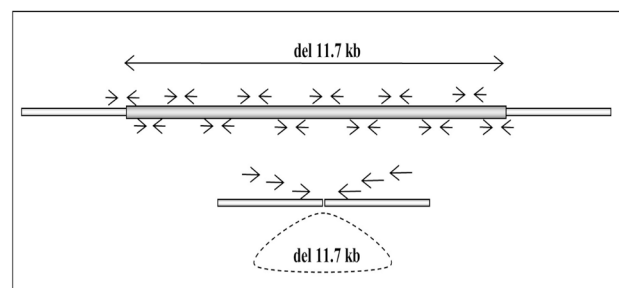


Fig. 2. Experimental layout (the reference template containing only the flanking regions of the 11.7-kb PIS deletion).

The next 24 primer pairs (Primer 3 Input version 0.4.0), including the whole deletion region with the flanking sequences (GenBank, AF404302), were used for the identification of the deletion region in the sex-reversed individuals, in which it should occur.

Selected PCR products were visualized, purified (Gel-out) and randomly sequenced at the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics in order to confirm their homology to the reference sequence (Accession Number AF404302).

## RESULTS

The identification of the PIS deletion in the hermaphroditic individuals and the polled bucks based on the primers designed on the reference template, taking into account only flanking regions, turned out to be impossible, despite using three pairs of primers covering different sites. Although, after the tedious standardization of reaction conditions (primer annealing temperature, the number of cycles), it was possible to obtain the product of expected length without unspecific bands (Fig. 3),

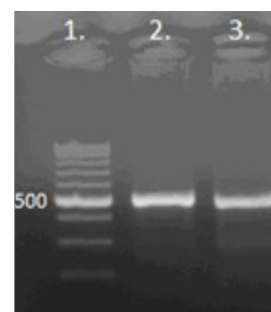


Fig. 3. PCR results for the identification of the PIS deletion: Lane 1: 100–1000-bp DNA ladder, lane 2: hermaphrodite, lane 3: hermaphrodite.



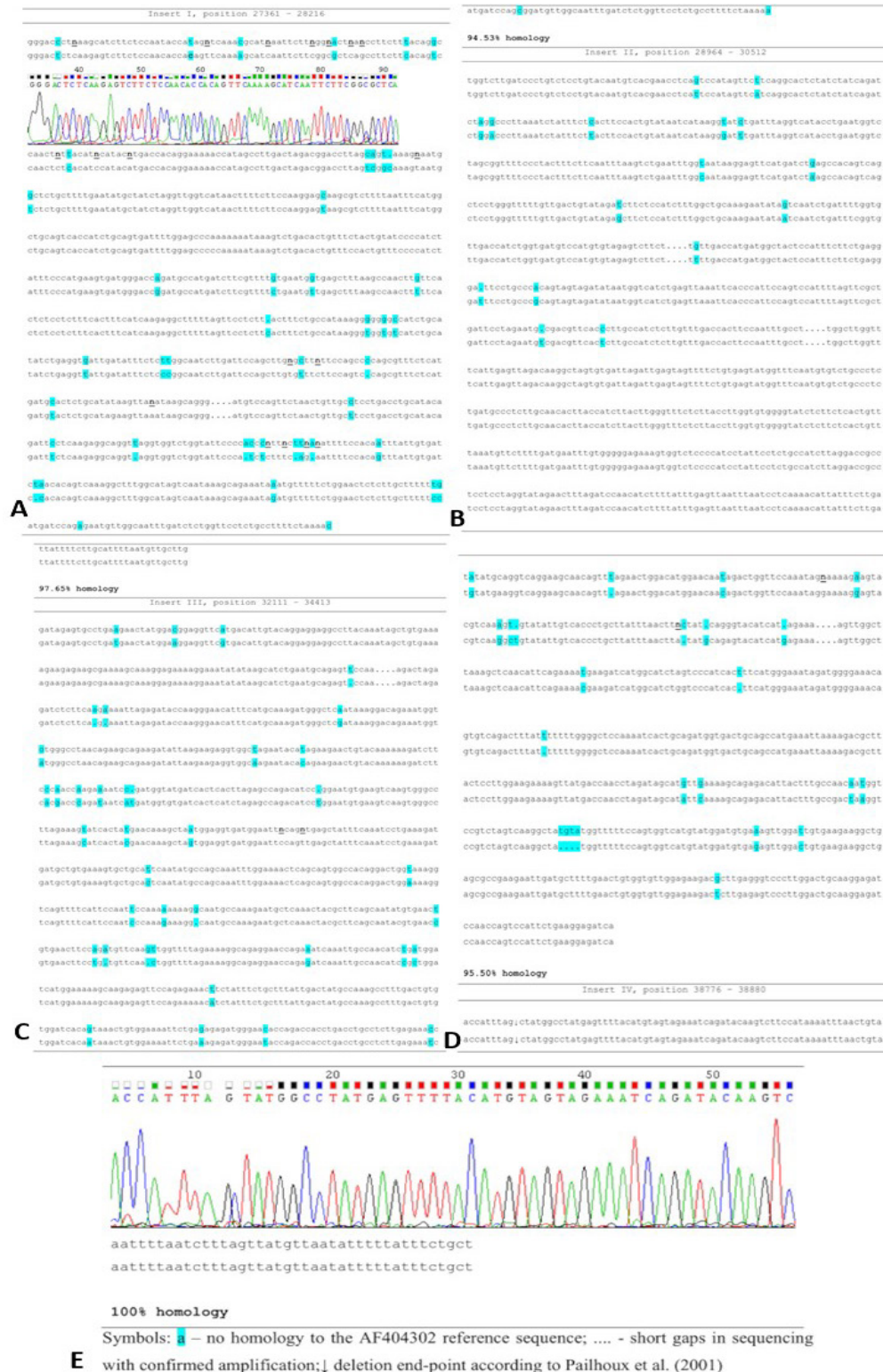


Fig. 4. Insert sequencing results in hermaphrodites.

A, part 1 (symbols explained in part E); B, part 2 (symbols explained in part E); C, part 3 (symbols explained in part E); D, part 4 (symbols explained in part E); E, part 5.

the sequencing results (or lack of results) did not confirm this. It could have been caused by the weak sequence homology of the designed primers to the reference template.

At the next stage of the present study, an attempt was made to determine the reasons for the failure of deletion identification in the individuals that should be its carriers. For this purpose, an amplification was carried out using the primers designed on the 11.7-kb deletion template. The reactions were run for the two sex-reversed individuals and two polled bucks, however, it was unknown whether the latter two were homo- or heterozygous for polledness. Two horned bucks were included in the study for comparison.

For the polled bucks, the deletion region was successfully amplified similarly to the horned bucks, except for the initial sequence. In the two sex-reversed individuals, four inserts were identified in the deletion region (Fig. 4) whose homology to the reference sequence (AF404302) was 94.53% (I – 855 bp), 97.65% (II – 1540 bp), 95.5% (III – 2302 bp) and 100% (IV – 9 bp). The sequences of the analogous regions for the polled bucks showed 94.9%, 95.8%, 98.6% and 100% homology, respectively, whereas the sequencing results for selected fragments of the deletion region in the horned males (with regard to the above-mentioned reference sequence) were 99.7% and 100% homologous. Due to partial homology, the inserts identified in the sex-reversed individuals were compared with other sequences deposited in GenBank in order to exclude amplification of other goat genomic regions. BLAST analysis confirmed the strong homology to the reference sequence, on which the presented results were based.

## DISCUSSION

The precise position of the 11.7-kb deletion deposited in GenBank (AF404302) as a causative factor of sex reversal in polled PIS-/- female goats raises doubts in the context of the arguments mentioned in the introduction to the present study. First of all, based on its location, the diagnosis of the PIS cases becomes impossible. It can only be established indirectly on the basis of genetic sex identification, the absence of *SRY* sequence in the genomic DNA and the marked masculinization of the internal and external genitalia in polled female goats (Szatkowska *et al.*, 2014b). Based on its location, it is also impossible to identify the dominant polled allele, linked to the allele responsible for PIS, if both remain a sequence enigma, and, finally (based on its location) it is impossible to identify the homozygous polled bucks, whose breeding condition may be reduced (Szatkowska *et al.*, 2017). But such a conclusion raises doubts if the selection of experimental

bucks was based on the phenotype and not the genotype of polledness.

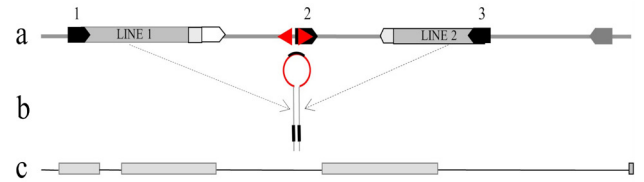


Fig. 5. Deletion region (authors' own work based on Pailhoux *et al.*, 2001); a: the PIS deletion with a marked LINE (in dark grey), repeated elements in the central part of the palindrome (in red), and the elements stabilizing the hairpin and binding to *SRY* (in black); b: the structure of the hairpin regulatory element; c: the PIS deletion with insertion regions identified in the present study in sex-reversed individuals (in grey).

The first two insertion sequences in the deletion region identified in the present study cover, to a large extent, the LINE1 repetitive element but do not contain the most important (in terms of its action on the expression of other genes) palindromic element (Fig. 5). However, this element, located in the central part of the deletion, is activated by the *SRY* protein, absent in the gonads of XX PIS-/- individuals. Similarly, the third insert, partly containing the LINE2 repetitive element, cannot be (in an incomplete form) able to form an alternative loop, acting as an enhancer of the *PISRT1* and *FOXL2* genes. Therefore, it can be presumed that their presence and location do not affect the repression or activation of the target genes indicated by Pailhoux *et al.* (2001), since their potential regulatory function is doubtful. The results obtained in the present study correspond, to some extent, to those reported by Li *et al.* (2011), who also showed that the analysed 11.7-kb fragment is not a complete deletion in polled PIS-/- goats, but a partial deletion in some of its regions. Although, these regions did not cover the insertions obtained in our study, it can ultimately be concluded that the nature of the 11.7-kb deletion is not common in goats, which has been confirmed by recently published studies (Simon *et al.*, 2020), whose authors revealed the presence of a large complex structural variant consisting of a deletion with a total length of 10 159 bp and an inversely inserted approximately 480 kb-sized duplicated segment of a region located approximately 21 Mb further downstream on chromosome 1 containing two genes, *KCNJ15* and *ERG*.

## CONCLUSION

Despite the fact that PIS in goats has been known

to breeders and scientists for several decades, many of its aspects, including the molecular reason, are still controversial and require further detailed research.

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## Ethics statement and IRB approval

According to Polish law, the post-mortem collection of reproductive organs from animals slaughtered on a commercial farm is not classified as experimental work on animals and therefore does not require ethics committee approval (Act of January 15, 2015 on the protection of animals used for scientific or educational purposes).

## Statement of conflict of interest

The authors have declared no conflict of interest.

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