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Case report

NOVEL MUTATION SITE IN *HOXD1* AND *RXFP2* GENES DETECTED IN A POLYCERATED GARUT SHEEP (*Ovis aries*)

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Garut sheep (*Ovis aries*) is one of Indonesian native sheep breeds from West Java which is bred for meat production purposes. Despite this, Garut rams with good horn characteristic are also used for the fighting contest festival which is the famous cultural tradition in West Java. This study was aimed to detect the mutation site in the two functional genes controlling horn development *i.e. HOXD1* and *RXFP2*. In this study, a rare polycerated (4 horns) Garut sheep was found at Cipacing Village, Sumedang Regency of West Java and used for investigation and compared with nine wildtype (2 horns) Garut sheep. Therefore, the partial sequences of *HOXD1* (788 bp) and *RXFP2* (505 bp) genes were amplified for the forward sequencing analysis. Results showed a presence of two novel mutations in the tested Garut sheep, namely g.742_743indel. TAAG in the *HOXD1* gene and g.2218A>T in the *RXFP2* gene. Specifically, a polycerated Garut sheep had the deletion 4 bp allele in *HOXD1* gene and T allele of *RXFP2* gene. In conclusion, horn characteristics of Garut sheep were controlled by novel mutation site in *HOXD1* and *RXFP2* genes.

Keywords: Garut sheep, mutation, polyceraty, *HOXD1, RXFP2*

INTRODUCTION

Garut sheep (*Ovis aries*) is one of the genetic resources of native Indonesian livestock which has high economic and cultural value. The Garut sheep have been registered as one of the Indonesian native sheep breeds since 2011 through the decision of Indonesian Ministry of Agriculture No: 2914/Kpts/OT.140/6/2011 [1]. Apart from being used as meat-producing breed, Garut sheep has also an important role in the tradition of West Javanese society, especially in fighting contests. In Garut, the naming and breeding of sheep are deeply rooted in cultural practices, with historical

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significance tied to agility competitions. These cultural aspects influence the perception and selection of sheep traits, including horn characteristics [2], as for fighting sheep, the horn characteristics are important to be selected by the farmers. Generally, the horns of fighting Garut sheep are massive, curling and diverging [3].

Policeraty, the presence of more than two horns, is a very rare phenotype variation found in the Garut sheep population. Although this trait has been observed in several sheep breeds in the world [4-6], the genetic basis that controls polyceraty in Garut sheep is still unknown. Polyceraty in Garut sheep, has never been reported before in the scientific literature. The inheritance of horn traits in sheep is complex and varies across breeds. Recent genomic studies have identified significant SNPs on chromosome 10 associated with horn phenotypes, with the *RXFP2* gene region being particularly important [7]. In Icelandic sheep, the *RXFP2* insertion on chromosome 10 shows some association with polledness, but not perfect segregation, especially for scurs and oval horns [8]. Similarly, in breeds with variable horn status, this insertion does not consistently segregate with horn status [9]. Horn inheritance in Jacob sheep appears to have sex-limiting factors, with 4-horned dams more likely to produce 4-horned offspring than 4-horned sires [10]. Otherwise, the early research indicated that large horns are dominant in males but recessive in females [11].

Besides of the *RXFP2* gene, the *HOXD1* gene is also associated with polyceraty, with a 4 bp deletion present in all multi-horned sheep and some polled animals from multi-horned families (17). The ovine *HOXD1* gene (GenBank: NC_030809.1) located on chromosome 2 along 2,239 bp with two exons. While, the ovine *RXFP2* gene (NC_056063.1) located on chromosome 10 along 61,966 bp with 18 exons. Previously, an indel mutation along 4-bp (AGTA) in the intron 1 of the *HOXD1* gene that associated with polyceraty trait in Manx Loaghtan sheep (2). Subsequently, an indel mutation along 1,781 bp was occurred in the 3'UTR of the *RXFP2* gene and affecting the polledness in Bundner Oberlander sheep [12].

CASE PRESENTATION

In the present study, a 4 horned (polyceraty) Garut ram was investigated from Cipacing Village, Sumedang Regency, West Java of Indonesia. In addition, nine wildtype (2 horned) Garut rams were used as control animals. The DNA extraction was done from all the collected blood samples using Genomic DNA Extraction Kit (Geneaid, Taiwan) following the manufacturer's protocols. The amplification of *HOXD1* and *RXFP2* genes was performed in a total volume of 30 µL consisting of 9 µL of DNA template; 0.6 µL of each primer; 15 µL of PCR mastermix (MyTaqTM HS Red Mix, Bioline) and 4.8 µL of nuclease-free water. A primer pair was designed using Primer3Plus package (https://www.primer3plus.com) to amplify ovine *HOXD1* gene along 788 bp (Figure 1).

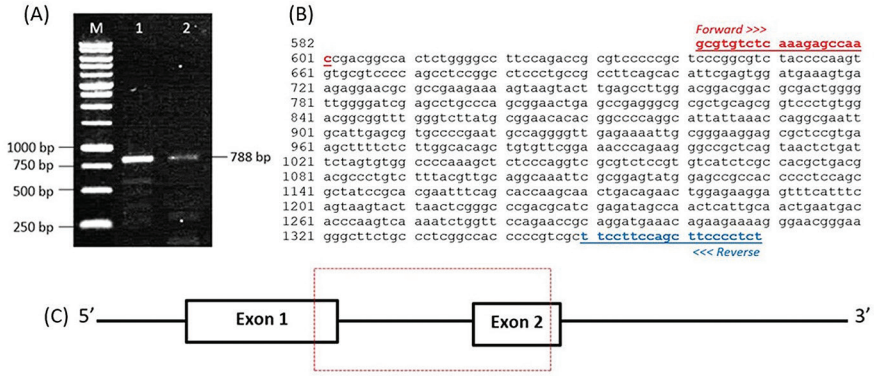

Figure 1. The target sequence of *ovine HOXD1* gene. **A:** the amplification of *HOXD1* gene (GenBank: NC_030809.1) along 788 bp. **B:** primer position in the target sequence of *HOXD1* gene. C: location of observed sequence from exon 1 to exon 2 (red box) of *HOXD1* gene

While, for amplification of the ovine *RXFP2* gene along 505 bp, a previously primer pair [9] was used as shown in Figure 2.

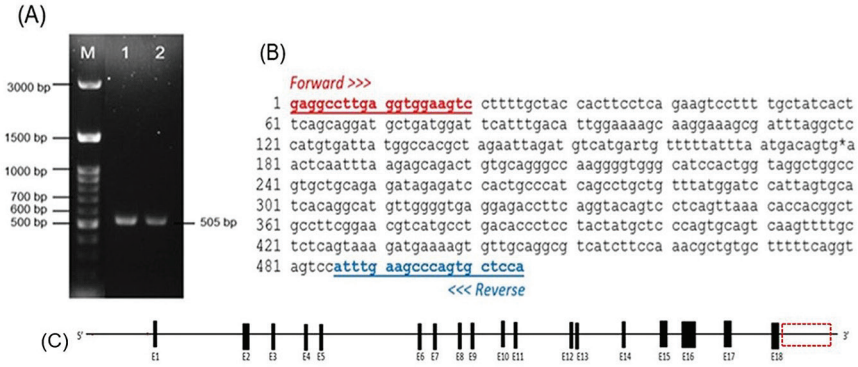

Figure 2. The target sequence of *ovine RXFP2* gene. **A:** the amplification of *RXFP2* gene (GenBank: KX084523.1) along 505 bp. **B:** primer position in the target sequence of *HOXD1* gene. C: location of observed sequence at 3'UTR (red box) of *RXFP2* gene. An indel mutation of 1,781 bp (*) was detected at the position between 179th and 180th nucleotide.

The PCR program for amplification of the *HOXD1* gene consisted of 1 cycle of pre-denaturation at 95 °C for 4 min, followed by 37 cycles of denaturation at 95 °C for 30 s; annealing at 48.8 °C for 30 s; initial extension at 72 °C for 30 s and final extension at 72 °C for 30 s. While, the *RXFP2* gene was amplified with 1 cycle of pre-denaturation at 94 °C for 4 min, followed by 37 cycles of denaturation at 94 °C for 30 s; annealing at 58.5 °C for 30 s; initial extension at 72 °C for 31 s and final extension at 72 °C for 4 min. The electrophoresis analysis of DNA samples was performed at 100 V for 30

min with 1% agarose gel and visualized using Gel Documentation System (UVITEC, UK). The forward sequencing analysis was performed by Apical Scientific laboratory service (Malaysia).

This study successfully identified two novel mutations associated with polyceraty in Garut sheep, namely g.742_743indel.TAAG in the *HOXD1* gene and g.2218A>T in the *RXFP2* gene. The *HOXD1* mutation was identified as a 4-bp deletion, while the *RXFP2* mutation involved a single nucleotide substitution, resulting in genotypic differences between the polycerated and wildtype individuals. These findings are consistent with previous studies highlighting the crucial role of *HOXD1* and *RXFP2* in horn development in sheep [6,12]. However, this is the first scientific report of these specific mutations in Garut sheep, suggesting that the mentioned genotypes may be unique to this population or exhibit a different inheritance mechanism compared to other sheep breeds.

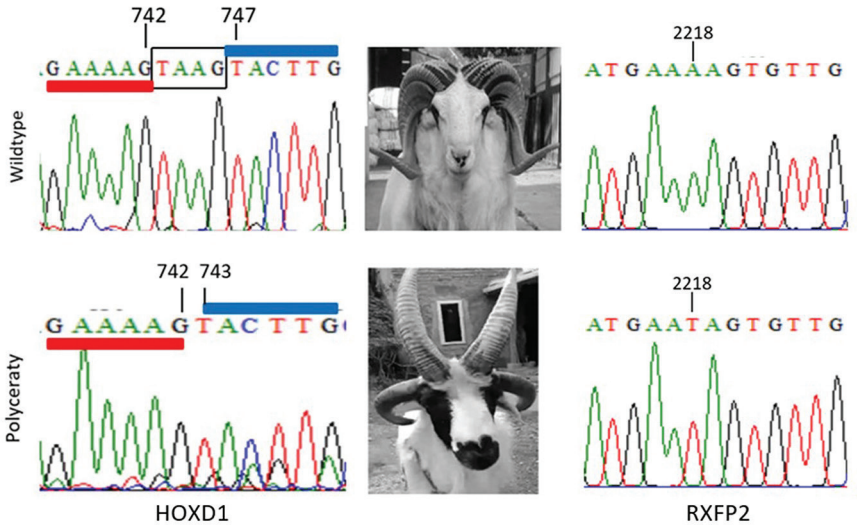

Figure 3. Detection of novel mutation site in *HOXD1* (g.742_747indel.TAAG) and *RXFP2* (g.2218A>T) genes of polycerated Garut ram

The *RXFP2* gene has been widely recognized as a key candidate in the inheritance of horn characteristics in sheep [7,13]. In several breeds, mutations in the *RXFP2* have been linked to horned or polled phenotypes. In this study, the g.2218A>T mutation found in polycerated Garut sheep suggests that the T allele may contribute to the expression of more than two horns. Interestingly, the absence of heterozygous AT individuals in the wildtype population indicates that this mutation may be closely associated with the expression of polyceraty or may have a recessive effect that only manifests in TT homozygous individuals. Notably, the 1.78 kb indel previously reported in the *RXFP2* and associated with polledness in Soay and Merino sheep [12] was not detected in the Garut sheep population examined in this study. This

suggests that the g.2218A>T variant in the *RXFP2* gene may be more specifically linked to polyceraty in Garut sheep rather than the polledness mechanism found in other breeds. Furthermore, mutations c.1957C>T (Val653Met) and c.1999C>T (Glu667Lys) previously identified in Tibetan sheep [14] were also absent in the tested Garut sheep population, reinforcing the genetic uniqueness of this breed.

Beyond the *RXFP2*, the *HOXD1* gene plays a critical role in morphogenesis and skeletal structure development in animals [6]. The discovery of the g.742_743indel. TAAG mutation in this study further supports the hypothesis that the *HOXD1* is involved in regulation of horn numbers in Garut sheep. A previous study on Manx Loaghtan sheep identified a 4-bp indel in the *HOXD1* gene associated with polyceraty [6], but the mutation found in this study differs in both location and nucleotide composition. The genetic divergence between Garut and Manx Loaghtan sheep may have contributed to the variation in the *HOXD1* indel type observed. Additionally, mitochondrial D-loop sequence analysis classified Garut sheep as genetically closer to Merino and Romney sheep [15], suggesting that the genetic basis of polyceraty in Garut sheep may share similarities with these breeds rather than with Manx Loaghtan sheep.

These findings align with previous Genome–wide Association Studies (GWAS), which identified the *HOXD* gene family as a major candidate for the regulation of polyceraty in various sheep breeds, including Damara, Jacob, Navajo-Churro, Altay, Mongolian, and Sishui Fur sheep [16-18]. In addition to the *HOXD1*, other genes such as the *MTX2*, *EVX2*, *KIAA1715*, and the *ADAMTS12* have been implicated in polyceraty expression in sheep [19,20]. Although the *HOXD1* and the *RXFP2* mutations identified in this study show a strong association with polyceraty in Garut sheep, further investigations using GWAS with a larger population are necessary to determine whether additional genetic factors contribute to polyceraty expression in this breed.

This study highlights the importance of future research to explore the inheritance mechanism of the identified mutations. Since the polycerated Garut sheep analyzed in this study lacked a documented pedigree, a detailed genetic segregation analysis within structured populations is necessary to elucidate the inheritance patterns of the *HOXD1* and the *RXFP2* mutations. Additionally, gene expression analysis using RNA-seq could provide further insights into how these mutations affect molecular pathways governing horn development. Overall, this study provides compelling evidence that polyceraty in Garut sheep is controlled by novel mutations in the *HOXD1* and the *RXFP2* genes, highlighting the unique genetic variation in this breed. These findings have potential applications in marker-assisted breeding programs aimed at maintaining and enhancing the frequency of polyceraty in Garut sheep populations. However, further studies are required to fully understand how these mutations interact in horn development and their broader implications for the genetic diversity of Garut sheep.

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Ethical Statement

This study was approved by the Ethics Committee for the Maintenance and Use of Experimental animals, National Research and Innovation Agency (Approval Number: 129/KE.02/SK/06/2023).

Authors' contributions

JA and WPBP conceived the idea and planned the manuscript. JA contributed to sample preparation. MR contributed in laboratory analysis. MM contributed to manage the ethical approval. WPBP, HH and EH have made significant scientific support and also contributed to the interpretation of the results. All authors provided significant contributions by giving feedback and helped to shape the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NOVO MESTO MUTACIJE U GENIMA *HOXD1* I *RXFP2* OTKRIVENO KOD POLICERATNE OVCE RASE GARUT (*Ovis aries*)

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Garut ovca (*Ovis aries*) je jedna od indonežanskih autohtonih rasa ovaca iz Zapadne Jave koja se uzgaja za proizvodnju mesa. Uprkos tome, Garut ovnovi sa dobrim karakteristikama rogova se takođe koriste za festivale borilačkih takmičenja, što je poznata kulturna tradicija u Zapadnoj Javi. Cilj ove studije je bio da se otkrije mesto mutacije u dva funkcionalna gena koji kontrolišu razvoj rogova, tj. *HOXD1* i *RXFP2*. U ovoj studiji, retka policeratna (4 roga) Garut ovca je pronađena u selu Cipasing, u oblasti Sumedang u Zapadnoj Javi, i korišćena je za istraživanje i upoređivanje sa devet divljih tipova (2 roga) Garut ovaca. Stoga su parcijalne sekvence gena *HOXD1* (788 bp) i *RXFP2* (505 bp) amplifikovane za analizu direktnog sekvenciranja. Rezultati su pokazali prisustvo dve nove mutacije kod testiranih ovaca rase Garut, naime g.742_743indel.TAAG u genu *HOXD1* i g.2218A>T u genu *RXFP2*. Konkretno, policerirana ovca rase Garut imala je alel sa delecijom od 4 bp u genu *HOXD1* i alel T gena *RXFP2*. Zaključno, karakteristike rogova ovaca rase Garut bile su kontrolisane novim mestom mutacije u genima *HOXD1* i *RXFP2*.