The Identification of Variation Sequences from Ovulation Rate Gene as Genetic Candidate for Twin Birth Markers in East Java Local Cows

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Abstract. The level of public consumption of beef continues to increase along with public awareness of the importance of animal protein needs. This increase continues every year. However, this is not balanced with an adequate increase in the amount of production, so the right solution is needed to overcome it. The purpose of this study was to identify variations and expressions of gene ovulation rates as genetic candidates for multiple birth markers in East Java-local cows. The identification was carried out in several stages including DNA isolation, PCR amplification, electrophoresis, sequencing, and MEGA 7 analysis. The results obtained from the isolation stage were then analyzed using MEGA 7. This analysis is a computer application program designed to compare and analyze homologous gene sequences. The program is also equipped with the results in the form of phylogenetic trees and evolutionary distance matrices. The results gained showed the analysis of sequencing results using MEGA7 software; there were variations in the sequence of nucleotide bases in the four samples. A number of these differences indicated that the four samples had differences, but there was still kinship. The analysis results showed that the primary ovulation rate gene was one of the marker genes that could be used in the detection of multiple births in local cattle in East Java.

Keywords: Ovulation rate gene, local cattle genetic variation, expression of protein function

1. Introduction

Cows are one of the ruminants that contribute significantly to fulfilling the needs of animal protein [1] primarily for poultry [2]. The data on beef consumption in the community continues to increase every year; this high demand is related to national economic growth, increased public awareness, population growth, and public purchasing power [3]. The high demand is expected to continue to rise until 2019, which is equal to 661.8 thousand tons/year [4]. The facts indicate that the increase in demand for community meat consumption has not been balanced with the addition of adequate production quantities [5].
Cows are generally produced from areas that have a high potential for livestock commodities. East Java Province is one of the suppliers of beef at 4.3 million or around 29% of the national cattle population [6]. However, this production is still relatively slow and has not been able to meet the demand for meat, so an alternative solution is necessary in the form of optimal management, especially for the management of livestock genetic resources [7] through natural reproductive efficiency in cows that have the potential to produce twins. The genetic analysis of marker genes for reproductive features can provide quality early detection of animals for twin births as a form of reproductive efficiency [8]. One of the marker genes, in this case, is the twin birth control gene, ovulation rate gene.

Ovulation rate gene is a determinant of ovulation level which affects production efficiency and is closely related to twin births [9] and genetic relations between twin births and ovulation rates range from 0.75 to 1.0 [10]. The ovulation rate gene is detected in chromosomes 7 and 23 [11]. In chromosome 7 was a UWCA20 marker correlated with one phenotypic standard deviation and this accounts for 10% of the phenotypic variation in the form of ovulation rate. Kappes study [12] showed that the choice of genome scanning strategies from cattle populations for increased twinning was conducted by identifying genes in certain chromosomes that play a role in the level of ovulation rate. The pregnancy process of cattle (Bos taurus) starts from the stage of ovum formation (ovulation), fertilization, and birth itself. The physiological pregnancy process of cattle is influenced by several factors, one of which is factor related to the quality and level of ovulation. Genes that affect the level of ovulation play a role and are directly involved in follicular development or ovulation. Two genes that directly express their expression have been influenced by the gene ovulation rate, both of which play an important role in the ovulation process, namely the Booroola fecundity gene (FecB) [13] and the Inverdale (FecX) fecundity gene [14] that function to inhibit steroidogenesis granulosa cells that cause advanced follicular maturation [15].

The use of genetic markers for gene identification and variations in sequences is relatively more advantageous, because it can be done easily, quickly and cheaply but has high accuracy [16]. Besides, being beneficial in terms of technique, the use of DNA markers can be used to calculate genetic variations related to quantitative characteristics. Thus, in the long term, it can be used as a tool to help the genetic selection of animals that are qualified (superior) [17]. The identification of gene ovulation rate in twin cows can be used in determining genetic variation by looking at differences in base sequence in several local cows in East Java. The more varied the genes in the population, the genes can increase the value of heterozygosity. Therefore, the value of heterozygosity influences the diversity of the population itself. Hence, this gene is very potential for breeding due to the high heterozygosity value. The results of this analysis can be used to see the expression of proteins produced from these genes in their role in regulating twin births in cattle.

2. Materials and Method

2.1. Materials
The sources of DNA in this study were blood samples of local cattle from Pujon, Batu, Situbondo, and Pacitan regions, the primer of gene ovulation rate: Forward 5’–TTTGCATGTCTA CCCTCTGC-3’ and reverse primer Reverse 5’–GGCTGTGAGATGAACCTTGC- 3’, the chemicals used in the extraction of DNA isolation, PCR and electrophoresis.

2.2. Method

2.2.1. Blood Sampling
Bovine blood sampling from each sample was carried out through the jugular vein in the neck using venoject and then inserted into a 5 mL of vacuum/airtight tube (vacutainer). Vacutainer used was equipped with EDTA which functions as an anticoagulant.
2.2.2. DNA Isolation
DNA extraction uses a modification of the Sambrook method [18]. About 500 μL of blood samples was then lysed with lysis buffer consisted of 350 μL TE, 40 μL SDS 10%, and 20 μL Proteinase K (10 mg/mL). DNA purification was carried out using the phenol-chloroform method added with 40 μL of isoamyl chloroform alcohol (24: 1) and NaCl; it was then slowly shaken. The DNA phase and phenol phase were separated by sniffing at a speed of 13000 rpm for three minutes. DNA was deposited using 2x absolute ethanol volume. Then, the resulted DNA deposits were washed using 70% absolute ethanol as much as 100 μL. After that, it was centrifuged again and washed again using 70% absolute ethanol as much as 100 μL; then, the supernatant was discarded and the pellet was dried and then dissolved again with 25 μL of buffer TE.

2.3. PCR Amplification and Electrophoresis Technique
PCR amplification used a volume of 10 μL consisting of 1 μL of DNA, 0.5 μL of forward primer, 0.5 μL of reverse primer, 5 μL of mixed-PCR (dNTP, buffer, Taq polymerase, H2O), 2.75 μL of ddH2O and 0.25 μL of BSA. Incubation was carried out on a thermocycler machine (Thermal PC Cycler MP4 PCR). The PCR program arrangement consisted of 95 °C hot start for 2 minutes, and then the initial denaturation at 92 °C for 1 minute, the annealing stage at 45-60 °C for 1 minute, the extension stage at 72 °C for 1 minute, and the post extension at 72 °C for 5 minutes.

The visualization of DNA fragments from PCR amplification was carried out by electrophoresis technique. The preparation of gel was by mixing 1.5 grams of agarose powder, 30 mL of TBE 1X buffer solution of 30 mL, and 2 μL of EtBr dye. Electrophoresis was undertaken at a voltage of 50 V with a time of 60 minutes. When the tape was known, the sample was ready to be sequenced.

2.4. Genetic Analysis of Sequencing Results with MEGA 7
The sequencing results were then furtherly analyzed which aimed to determine the sequence changes from the sample using MEGA 7 software. Then, the results of the analysis would be translated using bioinformatics software namely ExPASy and swiss models to see the reading of DNA sequences into amino acids [19].

3. Results and Discussion
3.1. Sequencing and Genetic Variation Analysis
The results of the isolation obtained showed that the DNA concentration of each sample was good enough at 58.77, 59.19, 48.97, and 66.77 ng/μL, and DNA purity of 1.8- 2.0. The results of PCR amplification carried out indicated that the twin marker genes namely ovulation rate gene could be identified at a temperature of 60 °C with a base length of 100-200 bp (Figure 1).

The PCR results were then sequenced to determine the sequence of nucleotide bases encoded from this amplified DNA molecule. To find out and see the existence of genetic variation, the application of sequence scanner and MEGA 7 was used. Based on the analysis using sequence scanner and MEGA 7, there were variations in the nucleotide base sequence in the four samples. The changes in the nitrogen base sequence were all shown in the form of substitution mutations that produced differences in amino acids. This change, in amino acids, produced two possibilities that are explained in the following Figure 2.
**Figure 1.** PCR product of ovulation rate gene (optimization of the temperature of annealing, 1= 50 °C, 2= 52 °C, 3= 54 °C, 4= 56 °C, 5= 58 °C, and 6= 60 °C). The optimum annealing temperature was 60 °C and band in range of 100-200 bp.

**Figure 2.** The comparison of alignment results of the nucleotide sequence of the ovulation rate gene in the four samples using MEGA7 software

| Domain | ii | si | sv | R | TT | TC | TA | TG | CT | CA | CG | AT | AC | AA | AG | GT | GC | GA | GG | Total | Domain Info |
|--------|----|----|----|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|      |             |
| 1. Av  | 106| 6  | 11 | 0.5| 19 | 2  | 0  | 2  | 1  | 39 | 2  | 1  | 2  | 2  | 33 | 1  | 1  | 1  | 2  | 2  | 15  | 123.0 Data |
| 1st   | 35 | 3  | 4  | 0.8 | 5  | 1  | 0  | 0  | 0  | 14 | 1  | 0  | 0  | 0  | 12 | 0  | 0  | 1  | 1  | 4  | 41.0 1st Pos Data |
| 2nd   | 35 | 2  | 5  | 0.3 | 7  | 0  | 0  | 1  | 0  | 12 | 1  | 0  | 0  | 0  | 11 | 0  | 0  | 0  | 0  | 1  | 41.0 2nd Pos Data |
| 3rd   | 37 | 1  | 2  | 0.5 | 7  | 0  | 0  | 0  | 0  | 12 | 0  | 0  | 1  | 0  | 10 | 0  | 0  | 0  | 0  | 0  | 7  | 41.0 3rd Pos Data |

**Note**

ii = identical pairs of nucleotide  
si = transitional pairs of nucleotide  
sv = transversional pairs of nucleotide  
R = si/sv (ratio between transitional and transversional mutation)

**Figure 3.** The results of the number of identical base pair frequencies, base pairs undergoing transitions, and base pairs undergoing transversion in all three samples

The number of changing nucleotides (mutations) and base positions of the codon is shown in figure 3. The results of the analysis of four samples showed that the number of transversions was 11 nucleotides consisting of four events in first base, five events in second base and two events in the third base. The position of each event is shown in figure 1. The transitions were only six events with the distribution in first base position were 3 nucleotides, the second position with two nucleotides and the position of third base was one nucleotide. A number of differences were shown in the four samples causing differences in amino acids formed. The difference in the sequence of nucleotide bases to the four samples showed that there were variations between the four cows. Related to this gene, the rate of ovulation is an essential phenotypic property as an important component of litter size in porcine [20]. Compared to previous research, a mutation that occurred in BMP15 (morphogenetic bone protein 15)
could increase ovulation rates in heterozygous organisms and decrease follicular development in homozygous organisms [21]. To compare with this study, the information about mutations in genes, that missense mutations in GDF9 can produce a variety of ovulation levels in Finnsheep [22] and this study, there were no sequences that caused missense. Related to the explanation of Graur and Li [23], substitution mutations that occurred in protein-coding regions could be classified according to their effect on translation products. Synonymous mutations are mutations that do not cause changes in the amino acids encoded, whereas non-synonymous mutations are mutations that cause changes in amino acids due to codon changes. This mutation is said to be a missense mutation because of an amino acid change. This variation can also be used to determine the phylogeny of the four cows through phylogenetic analysis. This analysis also used MEGA7 software [19] which is presented in the form of phylogeny tree construction as shown in Figure 4. To further clarify the existence of sequential variations, an analysis of the genetic relationship to the four research samples was carried out using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) method with 1000x bootstrap presented in Figure 3.

Figure 4. Phylogeny tree (Neighbour-Joining tree) of local cows (Pujon, Batu, Pacitan, and Situbondo)

The results of the analysis using the UPGMA method showed that there were three different groups or cluster for those cow samples (Figure 4). In the first group, the bootstrap value was 85%, which means that 85% of the 1000x replication of cattle had the proximity shown by the cows in the Pujon region with the cattle in Batu region [19]. However, both of these cows are closely related to Pacitan and Situbondo cattle. This case is because the cows in the Situbondo area are the cows derived from cross-breeding between the Ongole Breeds (PO) and Simmental cattle. Brahman cattle are widely found in the Pacitan region while the Pujon and Batu regions are mostly Simmental cows. Individual variations, especially based on the diversity of genotypic variations play a significant role in breeding livestock if there is no variation in genotypic livestock populations [24, 25]. The higher genotype variation in the population suggested has impacted the greater expected improvement in animal quality. For this reason, further analysis is needed regarding the function of genes that experience this variation. If gene functions do not change, this gene is a gene conserved with variation.

4. Conclusion
The results of sequencing analysis and variation indicated that there were variations in the four samples of cattle from Pujon, Batu, Pacitan, and Situbondo. Further analysis is needed regarding the function of this gene.

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References

[1] Susanti, Y., Priyarsono, S.D., & Mulatsih, S. 2014. Pengembangan Peternakan Sapi Potong Untuk Peningkatan Perekonomian Propinsi Jawa Tengah: Suatu Pendekatan Perencanaan Wilayah. *Jurnal Agribisnis Indonesia*. 2: 177-190.

[2] Rusdianan, S & Maesya, A. 2017. Pertumbuhan Ekonomi Dan Kebutuhan Pangan Di Indonesia. *Jurnal Sosial Ekonomi dan Kebijakan Pertanian*. 6: 12-25.

[3] Olmo, L.; Ashley, K.; Young, J.R; Suon, S.; Thomson, P.C.; Windsor, P.A & Bush, R.D. 2017. Improving smallholder cattle reproductive efficiency in Cambodia to address expanding regional beef demand. *Trop Anim Health Prod*. 49(1):163-172. doi: 10.1007/s11250-016-1175-6.

[4] Badan Pengkajian Dan Pengembangan Kebijakan Perdagangan. Analisis Outlook Pangan 2015-2019. 2014. Jakarta: Kementrian Perdagangan Republik Indonesia.

[5] Dahlanuddin, Henderson, B.; Dizyee, K.; Hermansyah & Ash, A. 2017. Assessing the sustainable development and intensification potential of beef cattle production in Sumbawa, Indonesia, using a system dynamics approach. *PLoS One*. 17;12(8):e0183365. doi: 10.1371/journal.pone.0183365.

[6] Agus A. & Mastuti Widi, T. S. 2018. Current situation and future prospects for beef cattle production in Indonesia - A review. *Asian-Australas J Anim Sci*. Jul;31(7):976-983. doi: 10.5713/ajas.18.0233.

[7] Chamdi, Achmad Nur. 2005. Karakteristik Sumberdaya Genetik Ternak Sapi Bali (Bos d Ovarian Follicular Fluid of). *IOP Conf. Series: Earth and Environmental Science*. 276: 012040 doi:10.1088/1755-1315/276/1/012040.

[8] Weller, J.I., Golik, M., Seroussi, E., Ron, M & Ezrat, E. Detection of Quantitative Trait Loci Affecting Twinning Rate in Israeli Holsteins by The Daughter Design. *American Dairy Science Association*. 2008: 91: 2469-2474

[9] Echternkamp S.E., Spicer L.J., Gregory K.E., Canning S.F & Hammond J.M. 1990. Concentrations of Insulin-Like Growth Factor-I in Blood and Ovarian Follicular Fluid of Cattle Selected for Twins. *Biol. Reprod*, 43: 8-14.

[10] Gregory K.E., Bennett G.L., Van Vleck L.D., Echternkamp S.E & Cundiff L.V. 1997. Genetic And Environmental Parameters for Ovulation Rate, Twinning Rate, And Weight Traits In A Cattle Population Selected for Twinning. *J. Anim. Sci.*, 75: 1213-1222.

[11] Blattman AN, Kirkpatrick BW, Gregory KE. 1996. A search for quantitative trait loci for ovulation rate in cattle. *Anim Genet*. Jun;27(3):157-62.

[12] Kappes SM, Bennett GL, Keele JW, Echternkamp SE, Gregory KE, Thallman RM. 2000. Initial results of genomic scans for ovulation rate in a cattle population selected for increased twinning rate. *J Anim Sci*. 2000 Dec;78(12):3053-9.

[13] Galloway S.M., Menatty K.P., Cambridge L.M., Laitinen M.P.E., Juengel J.L., Jokiranta T.S., McLaren R.J., Luiro K., Dodds K.G., Montgomery G.W., Beattie A.E., Davis G.H & Ritvos O. 2000. Mutations in An Oocyte-Derived Growth Factor Gene (Bmp15) Cause Increased Ovulation Rate and Infertility In A Dosage-Sensitive Manner. *Nat. Genet.*, 25: 279-283.

[14] Mulsant P., Lecref F., Fabre S., Schibler L., Monget P., Lanneluc L., Pisselet C., Riquet J., Cribuff E., Thimonier J., Teysnier J., Bodin L., Cognié Y., Chitour N & Olsen J.-M. 2001. Mutation In Bone Morphogenetic Protein Receptor-Ib Is Associated With Increased Ovulation Rate in Booroola Mérino Ewes. *Proc. Natl. Acad. USA* 98 (9): 5104-5109.

[15] Wilson T., Wu X.-Y., Juengel J.L., Ross I.K., Lumsden J.M., Lord E.A., Dodds K.G., Walling G.A., Mcewan J.C., O’connel A.R., Menatty K.P & Montgomery G.W. 2001. Highly Prolific Booroola Sheep Have a Mutation in The Intracellular Kinase Domain of Bone Morphogenetic Protein Ib Receptor (Alk-6) That is Expressed in Both Oocytes and Granulosa Cells. *Biology of Reproduction*, 64 (4): 1225-1235.

[16] Alink. F.M.; Robinson, J.J.; Mylne, M.J.; Kenyon. P.;Watt, R.G.;Wood, M.J. & McEvoy, T.G. 2006. Number of ovulations in Texel x Scottish hill ewes carrying the Inverdale fecundity (FecX(1)) gene. *Vet Rec*. 159(5):154-6.
[17] Allan, M.F., Thallman, R.M., Cushman, R.A., Echternkamp, S.e., White, S.N., Kuehn, L.A., Casaes, E & Smith, T.P. Association of a single nucleotide polymorphism in SPP1 with growth traits and twinning in a cattle population selected for twinning rate. 2007;85: 341-347.

[18] Sambrook, J and Russel, D.W. Molecular Cloning: A Laboratory Manual. 3rd Ed. Vol. 1. New York: Cold Spring Harbour Laboratory Press. 2001.

[19] Raymond, M. and Rousset, F. genepop (Version 1.3d): population genetics, software for exacts test and ecumenicism. Journal of Heredity. 1995; 86: 248-249.

[20] Campbell, E.M; Nonneman, D.J; Kuehn, L.A.; & Rohrer, G. A. 2008. Genetic variation in the mannosidase 2B2 gene and its association with ovulation rate in pigs. Anim Genet. 39(5):515-9. doi: 10.1111/j.1365-2052.2008.01763.x. Epub 2008 Aug 1.

[21] Montgomery, G.W.; Galloway, S.M.; Davis, G.H. & McNatty, KP. 2001. Genes controlling ovulation rate in sheep. Reproduction. 21(6): 843-52.

[22] Mullen, M.P. & Hanrahan, J.P. 2014. Direct evidence on the contribution of a missense mutation in GDF9 to variation in ovulation rate of Finnsheep. PLoS One. 21;9(4):e95251. doi: 10.1371/journal.pone.0095251.

[23] Graur, D. & Li, W-S. 2000. Fundamental of Molecular Evolution. Sunderland-Massachusetts: Sinauer Publishing.

[24] Sukri, A.; Amin, M.; Winaya, A & Gofur, A.. 2014. Substitution and Haplotype Diversity Analysis on The Partial Sequence of The Mitochondrial DNA Cytochrome b of Indonesian Swamp Buffalo (Bubalus bubalis). Biology, Medicine, & Natural Product Chemistry. Volume 3 – Number 2 – 2014: 76 - 81

[25] Amin, M.; Suarsini, E.; Azmi, I. & Gofur. 2016. Phylogenetic Analysis of Local Endemic Buffalo (Bubalus Bubalis) Based on Cytochrome B Gene in Central Indonesia. Jurnal Teknologi. 78:5 (2015) 393–397