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Polymorphism and Association of the Novel KCTD2 Gene with Flavor and Odor in Indonesian Local Sheeps

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ABSTRACT

A candidate marker that influences the flavor and odor of Indonesian sheep that is the Potassium Channel Tetramerization Domain Containing 2 (KCTD2) gene. This study aims to examine the polymorphism and association between the KCTD2 gene and lamb flavor and odor in Indonesian local sheeps. This study used DNA taken from the longissimus dorsi (LD) muscle of 100 rams including 75 Javanese Thin-Tail sheep (JTTS), 10 Javanese Fat-Tail Sheep (JFTS), and 15 Jonggol Sheep (JS), with ranged in weight from 20 to 35 kg and were 10 to 12 months old. The PCR-RFLP technique and GLM test analysis were used to identify polymorphisms and association of KCTD2 gene. The restriction enzyme BafI digests the PCR-RFLP product of the KCTD2 gene. The results showed that the KCTD2 gene was polymorphic (CC and CT). KCTD2 gene analysis showed a significant ($P<0.05$) association with 3-Methylphenol (MP). This research provide information regarding the role of the KCTD2 gene in lamb flavor and odor, especially in 3-Methylphenol (MP) compounds, and explain KCTD2 as a functional gene in the selection of premium sheep with low flavor and odor in lamb meat.

Keywords: Flavor, KCTD2, Odor, PCR-RFLP, Sheep

Introduction

Sheep is a source of animal protein that has economic value and high adaptability to various environmental conditions. The prospect of sheep farming in Indonesia has the potential to be developed because in addition to meeting domestic demand it also has the opportunity to become an export commodity (Falahudin and Imanudin, 2018). Popular Indonesian breeds in the country and notable breeds include Javanese Fat-Tail sheep (JFTS), Javanese Thin-Tail sheep (JTTS) and Jonggol Sheep (JS) (Listyarini *et al*., 2022). Javanese Fat-Tail sheep (JFTS) are white, hornless, and is a predominant breed in East Java, whereas JTTS are small and generally white breed predominant in West Java. Jonggol Sheep (JS) are crossbred with thin tailed sheep (50%) with GS (50%) and have no or less fat, white and black stripes fleece, and rams have horns while ewes don't have horn (Sodiq and Tawfik 2004; Edey, 1983; Jarmuji, 2014).The level of lamb meat production in Indonesia in 2020 was 54,188.48 tons and in 2021 it was 55,863.16 tons lower than production in 2019 which was 70,072.93 tons (BPS, 2020). These data indicate the level of consumption of lamb meat has decreased. One of the reasons for the low consumption of lamb meat, among others, is the perception in society that lamb meat has a distinctive smell and has a negative effect on health. Therefore, efforts to improve genetic quality are needed to produce premium lamb meat. Genetic quality improvement using a molecular approach has been carried out through several studies related to candidate genes controlling meat quality traits (Harahap *et al*., 2021).

The results of transcriptomic studies through RNA Sequencing (RNA-Seq) have produced the KCTD2 (Potassium Channel Tetramerization Domain Containing 2) gene as a candidate gene for controlling lamb meat quality, and it is located on chromosome 11 in sheep. Information regarding the KCTD2 gene in sheep is still limited, especially in terms of meat quality. The KCTD2 gene has been widely reported regarding its role in the human nervous system. The KCTD2 gene is a protein found in glial cells (Teng *et al*., 2019). According to the study by Angrisani *et al*. (2021), it was reported that KCTD2 promotes the ubiquitination and degradation of the c-Myc oncogene and reduces the levels of KCTD2 mRNA present in glioma cells. This KCTD2 locus has an important function in mitochondrial energy

production and hyperpolarization of neurons during conditions of cellular stress, such as hypoxia or glucose deprivation (Boada *et al*., 2014). The KCTD2 gene is predicted to have an important role in controlling protein metabolism in sheep, protein is closely related to flavor and odor. The chemical reaction process triggers the formation of flavor and odor in lamb meat, according to Wasserman (1972), the protein and fat components in meat undergo physical and chemical changes when heated and depending on the temperature and air level. Myofibrillar proteins will shrink and produce meat juices. The meat liquid will mix with melted lipids, allowing the mixing of water- and fat-soluble components. Specifically, the main reaction of flavor formation meat is the Maillard reaction, Stecker and degradation lipid and thiamine degradation reactions (Brunton *et al*., 2002; Jayasena *et al*., 2013). This study aims to analyze the polymorphism and the association between the KCTD2 gene with flavor and odor in Indonesian local sheeps.

Materials and Methods

Animals and samples

The DNA was extracted from the longissimus dorsi (LD) muscle of 100 Indonesian rams, including 75 JTTS, 10 JFTS, and 15 JS. The sheep were 10 to 12 months old and had weigh ranging from 20 to 35 kg. Sheep are slaughtered by halal butchers who are supported by the Institutional Animal Care and Use Committee (IACUC).

Flavor and odor analyses

On samples of meat rams, flavor and odor components were analyzed. Using the Gas Chromatography-Mass Spectophotometry (GC-MS) instrument, the volatile flavor and odor components were extracted using the Likens-Nickerson method, a mix of distillation and solvent extraction (Reinecceius, 1997). MNA, MP, MI or skatole, MOA, and EOA were the phenotypic measurements for lamb flavor and odor. Low and high lamb odors scents were characterized as lamb with a fat BCFA (MNA and MP) of greater than 215 µg/g and less than 229 µg/g, respectively (Watkins *et al*., 2014). Low and high skatole samples of sheep were those with a fat skatole content of fewer than 0.25 µg/g and more than 0.25 g/g, respectively, for the flavor (Gunawan *et al*., 2013; Strathe *et al*., 2013).

PCR-RFLP amplification and DNA extraction

Using the Geneaid gSYNC DNA Extraction Kit (Catalog Number: GS050/100/300), genomic DNA was extracted from the longissimus dorsi muscle samples in accordance with the 's protocol. KCTD2 gene fragments using the GeneAmp ESCO PCR system, DNA amplification was initiated by denaturation at 95°C for 1 minute. The second stage consisted of 35 cycles at 95°C for 15 seconds, primary annealing at 59°C for 15 seconds, 72°C for 15 seconds, and primary extension at 72°C for 1 minute, the final stage cooling at a temperature of 15ºC for 5 minutes. The PCR product was electrophoresed using 1.5% agarose gel media and then visualized using a thermocycler machine with the help of a UV transilluminator to see DNA bands. The PCR products were cut using the PCR-RFLP technique with BafI enzyme and incubated at 37°C for 4 hours. The results were then electrophoresed again using 2% agarose gel, then visualized to see the resulting genotype (TT= 441 bp; CC= 272, 169 bp; CT= 441, 272, and 169 bp) (Table 1).

Statistical analysis

The level calculated were allele frequency, genotype frequencies, and Hardy-Weinberg equilibrium values (Nei and Kumar, 2000). In addition, the associations between KCTD2 gene polymorphism (g. 55318263 T>C) with phenotype were computed using PROC GLM procedures to analyze the effects of genotype (Minitab 19 Software).

$$Y_{ijk} = \mu + G_i + Bj + E_{ijk}$$

Where:
Y_{ijk} = the flavor and odor compound (MNA, MP, MI or skatole, MOA, and EOA)
μ = the population mean;
G_i = the fixed effect of i-th genotype (i = CC and CT);
B_j = the fixed effect of j-th breed (j = JTTS, JFTS, and JS);
E_{ij} = the residual error.

Results and Discussion

Novel polymorphism of the KCTD2 gene

The study of polymorphism novel KCTD2 gene was successfully amplified using PCR-RFLP with enzyme restriction BafI. For SNP g. 55318263 T>C KCTD2, DNA restriction fragments were found to be CC = (272, 169 bp); CT = (441, 272, and 169 bp) (detailed in Figure 1). The KCTD2 gene displays two genotypes, CC and CT, which are combinations of the T and C alleles. Nitrogen base alterations alter the size of restriction fragments that are metabolized by particular restriction enzymes (Bardakci, 2001).

The genotype and allele frequencies of the KCTD2 gene in Javanese thin-tailed sheep (JTTS), Java fat-tailed sheep (JFTS), and Jonggol sheep (JS) were not in Hardi-Weinberg Equilibrium. In this case, the KCTD2 gene in our populations, homozygote TT was absent, and homozygote CC and heterozygote CT were more common (Table 2). According to Gunawan *et al*. (2018a), if the obtained allele frequency is less than 99%, the allele frequency is polymorphic. The imbalance could be caused by intense selection, non-random mating, and mutations when examined in light of the requirements for the validity of Hardy-Weinberg (Khasanah *et al*., 2016).

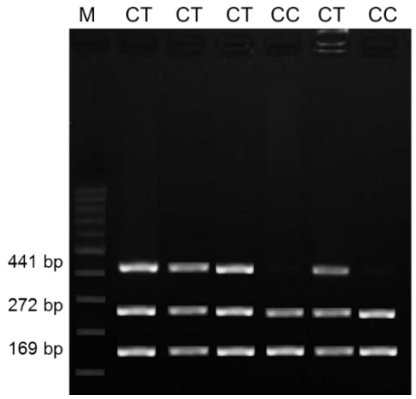

Figure 1. PCR-RFLP result of KCTD2 gene; M= 100 bp ladder size standard; CC (272, 169 bp); CT (441, 272, and 169 bp) genotype; bp= base pair.

Table 1. Primer sequence and accession number of novel gene KCTD2

Gene	Accession Number	Size of PCR	TA ^{O}C	Enzyme	Primer sequence
KCTD2	NC_019468.2	441 bp	59	BafI	F:5' -CAG ATT CCT GGA GCG CTG A3' R:5'-GCA GAA TGG TTG CAG GTG TC3'

Note: Designed using MEGA 7 software.

Table 2. Frequency of genotype and allele of the KCTD2 gene

Sheep Breed	N	Genotype frequency			Allele frequency		χ^2
		TT	CC	CT	T	C	
JTTS	75	0.00 (0)	0.17 (13)	0.82 (62)	0.41	0.59	37.23
JFTS	10	0.00 (0)	0.30 (3)	0.70 (7)	0.35	0.65	39.00
JS	15	0.00 (0)	0.00 (0)	1.00 (15)	0.50	0.50	15.00
Totals	100	0.00 (0)	0.16 (16)	0.84 (84)	0.42	0.58	52.43

N= number of samples; (..)= number of samples with genotypes TT, CC, CT, χ^2 table = 3.84.

Table 3. Association of the KCTD2 gene with flavor and odor

Parameter (µg/g)	Genotype ($\bar{x}$ ± SE Mean)			P Value
	TT (0)	CC (16)	CT (84)	
4-methyloctanoic (MOA)	0.00±0.00	24.6±13.9	124.8±43.2	0.31
4-Ethyloctanoic (EOA)	0.00±0.00	129.0±73.7	127.2±37.7	0.98
4-Methylnonanoic (MNA)	0.00±0.00	28.38±8.71	1149±320	0.13
3-Methylphenol (MP)	0.00±0.00	261±254^{a}	19.29±8.69^{b}	0.02
3-Methylindole (MI)	0.00±0.00	0.51±0.43	1.67±0.56	0.38

$\bar{x}$= means of flavor and odor; SE= standard error; * Mean in the same row with different superscripts differ significantly (P<0.05). The numbers shown in parentheses are the number of individuals with the specified genotype.

Association of KCTD2 gene polymorphism with flavor and odor

SNP g.55318263 T>C The KCTD2 gene was significantly related (P<0.05) to the flavor and odor content of 3-Methylphenol (MP). The sheep with the CT genotype were lower than the CC genotype (Table 3). The levels of 3-Methylphenol (MP) of the KCTD2 gene in rams average 19.29 µg/g. Listyarini *et al.* (2018) reported that the MP compound of the CYP2A6 gene in ram meat was an average of 24.03 µg/g. This shows that the MP compound of the KCTD2 gene is lower than in previous studies. The KCTD2 gene plays an important role in reducing the flavor and odor content in sheep. The low content of flavor and odor in this study can be influenced by the age of slaughter of the sheep which is still relatively young with an age range of 10-12 months and the provision of complete nutritional feed. One of the most practical methods for reducing the levels of rams flavor and odor, according to Gunawan *et al*. (2018b), is genetic selection and breeding. In particular, MNA, MP, MOA, EOA, and MI are the precisely described molecules representing the phenotypic trait (rams flavor and odor) that can be enhanced through genetic selection.

Conclusions

The KCTD2 (g.55318263 T>C) gene was polymorphic (CC and CT). KCTD2 gene analysis showed a significant (P<0.05) association with 3-Methylphenol (MP). This research provide information regarding the role of the KCTD2 gene in lamb flavor and odor, especially in 3-Methylphenol (MP) compounds, and explain KCTD2 as a functional gene in the choice of premium sheep with low flavor and odor in lamb meat.

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