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Short Communication: Genetic diversity of the *IGF2* gene as a source of genetic marker for halal authentication

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Abstract. *Hamdani AD, Madihah, Suryohastari RB, Virgianti DP, Putrie RFW. 2018. Genetic diversity of the* IGF2 *gene as a source of genetic marker for halal authentication. Nusantara Bioscience 10: 203-209.* The main issue of halal authenticity is the availability of reliable and rapid analytical methods to identify animal species in raw and processed food. The two most popular procedures for identifying the source of the meat are protein-based and DNA-based methods, including mass spectroscopy (MS) using a peptide marker or PCR based DNA marker, respectively. This study aims to investigate the genetic diversity of insulin-like growth factor 2 (*IGF2*) gene, mainly from porcine (*Sus scrofa*) and bovine (*Bos taurus*) as a source of genetic marker for halal authentication by in-silico analysis using bioinformatic tools. Multiple sequence alignment and phylogenetic tree construction of IGF2 protein sequences from representative species of mammals, birds, and fishes showed the paraphyletic relationship between the IGF2 protein of *S. scrofa* and *B. taurus.* Protein structure analysis revealed differences in helical structures near the carboxyl end of the protein, while gene analysis showed different number of exon and motifs. By in-silico analysis, we have designed a peptide marker from amino acids at position of 93-107 (*S. scrofa*) and 93-112 (*B. taurus*) from peptide preptin region that resulted in different pattern of mass spectrum between the two species. We have also identified two DNA markers that can be detected by PCR using two primers sets designed from the *IGF2* transcript sequences, to examine the presence of porcine in the sample. Thus, this study provides new genetic predictive markers, derived from the *IGF2* gene, to identify the source of meat for halal authentication.

Keywords: *Bos taurus*, DNA marker, IGF2, insulin-like growth factor 2, peptide marker, *Sus scrofa*

Abbreviations: DCS: differential scanning calorimetry, ELISA: enzyme-linked immunological assay, HPLC: high-performance liquid chromatography, IGF2: insulin-like growth factor 2, IMF: intramuscular fat, PCR: polymerase chain reaction, MS/MS: tandem mass spectrometry

INTRODUCTION

The halal authenticity of food is an issue that is growing in awareness and concern worldwide, especially in Muslim countries. The major authenticity concerns for Muslim consumer in meat and meat products include pork substitution, the use of prohibited ingredients, pork intestine casings and non-halal method of slaughter (Nakyinsige et al. 2012). A reliable, sensitive and exact procedure for the authentication is crucial for law enforcement, quality assurance, and consumer protection. The testing procedures commonly employed for halal authenticity is either chemical or biological approaches, using-lipid based, protein-based, or DNA-based methods. (Ballin, 2010; Premanandh and Bin Salem. 2017; He and Yang 2018).

Lipid-based method for halal authentication is differential scanning calorimetry (DCS) to analyze oils and fats (Guntarti et al. 2017). Protein-based method has been used to detect pork in highly processed food, by high-performance liquid chromatography (HPLC), tandem mass spectrometry (MS/MS) (Von Bargen et al. 2014) for hemoglobin myoglobin (Montowska and Pospiech, 2011), and enzyme-linked immunological assay (ELISA) for troponin I (Zvereva et al. 2015). For the DNA-based assay, different methods on PCR has been applied to meat specification by amplifying species-specific sequence, such as PCR-RFLP alone or coupled with slab gel electrophoresis (Erwanto et al. 2014; Hsieh et al. 2016). DNA usually can be detected to analyze contaminants in raw material as well as in processed food. Both nuclear and mitochondrial genes have been targetted for identification of meat species (Fajardo et al. 2010). Among mitochondrial genes, the Cytochrome b and DNA D-loop region fragments are the most commonly used markers for halal authentication (Erwanto et al. 2014; Hamzah et al. 2014;

Kumar et al. 2015; Hsieh et al. 2016). Some examples of meat authentication from nuclear markers are growth hormone, actin, or the melanocortin receptor 1 (MC1R) genes (Fajardo et al. 2010). It should be noted that no method has gained supremacy. The application will depend on the practical scenario to determine which method could be applied in the food industry.

In this study, we investigated a nuclear gene, namely insulin-like growth factor 2 (*IGF2*) gene, as an alternative source of markers for halal authentication in-silico using bioinformatics tools. *IGF2* gene has been used as markers associated with meat and carcass quality in porcine (Davoli and Braglia, 2008). *IGF2* is a fetal growth and differentiation factor that plays an important role in muscle growth and myoblast proliferation and differentiation. The bovine *IGF2* gene maps to chromosome 29. Polymorphisms in the *IGF2* gene are associated with growth traits and may be used for marker-assisted selection in a beef cattle breeding program (Huang et al. 2013). Genetic variation in the promoter region of the *IGF2* gene is associated with intramuscular fat (IMF) content in porcine skeletal muscle, and that greater expression of the *IGF2* gene is associated with higher IMF content (Aslan et al. 2012; Wu et al. 2013).

MATERIALS AND METHODS

Phylogenetic analysis

Phylogenetic analysis was performed using protein sequences of IGF2 from representative species of mammals, birds, and fishes. The protein sequences were downloaded from UniProt (www.uniprot.org). The IGF2 protein sequences from *Oryctolagus cuniculus* (B7NZU3), *Bos taurus* (P07456), *Ovis aries* (P10764), *Rattus norvegicus* (P01346), *Mus musculus* (P09535), *Homo sapiens* (P01344), *Sus scrofa* (P23695), *Canis lupus familiaris* (J9P961), *Danio rerio* (Q9PUD0) and *Gallus gallus* (P33717) were aligned using MEGA7 software to construct a phylogenetic tree by Maximum Likelihood method with 1000 bootstrap replicates and to conduct evolutionary analysis. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

Protein analysis

Analysis of protein structure using PredictProtein (http://ppopen.informatik.tu-muenchen.de/) and the prediction of tertiary protein structure was modeled using Swiss-Model (https://swissmodel.expasy.org/interactive). The physico-chemical properties of IGF2 protein included hydrophobicity, solubility, isoelectric point, and pI. Hydrophobicity was analyzed using Kyte-Dollylte Hydropathy plot (https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=misc1), solubility was analyzed by Protein-Sol (https://protein-sol.manchester.ac.uk/), while molecular weight and isoelectric point (Pi) were estimated by Compute pI/Mw (https://web.expasy.org/compute_pi/).

Further analysis was to design peptide markers that involved analysis of IGF2 peptides, cleavage with trypsin enzymes to search for unique sequences of peptides, as well as in-silico mass spectrum analysis. The properties of peptide regions were analyzed using Protparam online analysis (http://expasy.org/protparam/). The masses of IGF2 peptides and their post-translational modifications was calculated by PeptideMass (https://web.expasy.org/peptide_mass/), using trypsin to cleave the protein with zero missed cleavage. Unique peptide for both species was selected, and the y-ion intensities of the theoretical spectrum for those peptides were predicted by OpenMS-Simulator (http://bioinfo.ict.ac.cn/OpenMS-Simulator/). The list of peak masses and intensities of the selected peptide sequence resulted from OpenMS-Simulator were used to generate the mass spectrum using Mass Spectrum Generator (http://www.sisweb.com/mstools/spectrum.htm).

Gene analysis

The *IGF2* gene sequences from *Sus scrofa* (GeneID: 396916) and *Bos taurus* (GeneID: 281240) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/gene/). The structure of mRNA transcript was created with SnapGene program. The gene motifs were identified by the Nsite program (http://linux1.softberry.com/berry.phtml). To determine the polymorphic coding sequence in the *IGF2* gene, the mRNA transcripts were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The polymorphic region was subjected to design primers using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) and the in silico PCR was performed at UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgPcr) using pig genome (assembly: Feb.2017(Sscrofa11.1/susScr11)) and cow genome (assembly: Jun. 2014 (Bos_taurus_UMD_3.1.1/bosTau8)) as a template. The identity of the amplicon was also confirmed by Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast), using the designed primer against *Sus scrofa* (taxid: 9823) and *Bos taurus* (taxid 9913) genome as the template.

RESULTS AND DISCUSSION

Phylogenetic analysis

The IGF2 proteins from mammals were clustered in one group, which further divided into two subclusters (Figure 1). Meanwhile, IGF2 protein from fish (*Danio rerio*) and Aves (*Gallus gallus*), each separated in a different cluster. The phylogenetic tree showed that the IGF2 protein of *Sus scrofa* and *Bos taurus* were clustered together and formed paraphyletic groups.

Protein analysis

Both IGF2 proteins from *S. scrofa* and *B. taurus* showed similar physicochemical properties, with slightly higher solubility, Pi, and molecular weight of IGF2 protein from *S. scrofa* (Table 1).

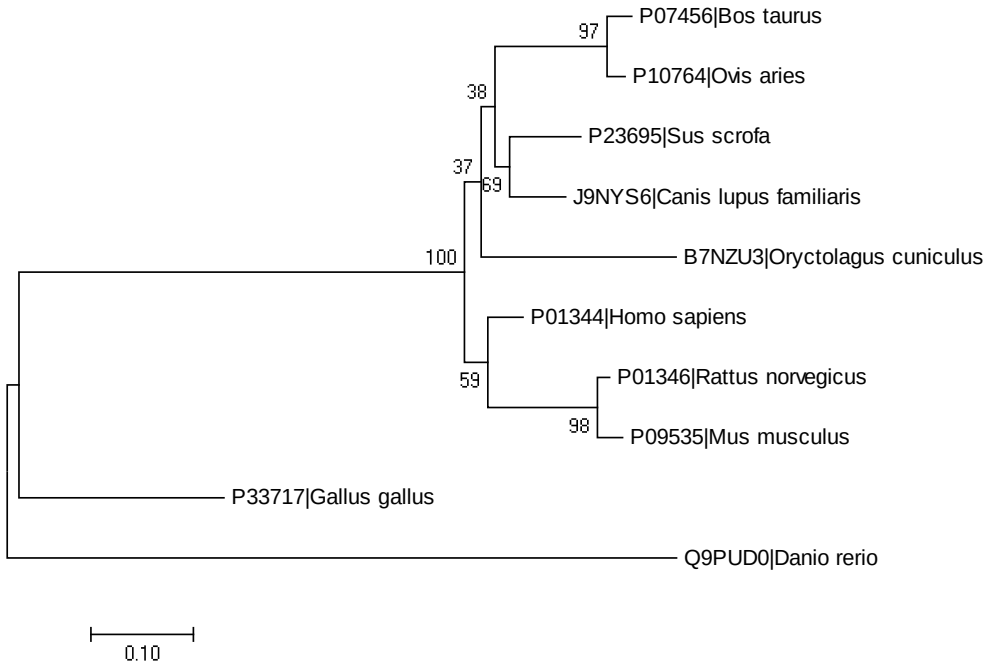

Figure 1. Maximum Likelihood tree based on the JTT matrix-based model. The analysis involved ten amino acid sequences. There was a total of 174 positions in the final dataset. The tree with the highest log likelihood (-1796.3218) is shown. The percentage of trees in which the associated taxa clustered together is displayed next to the branches.

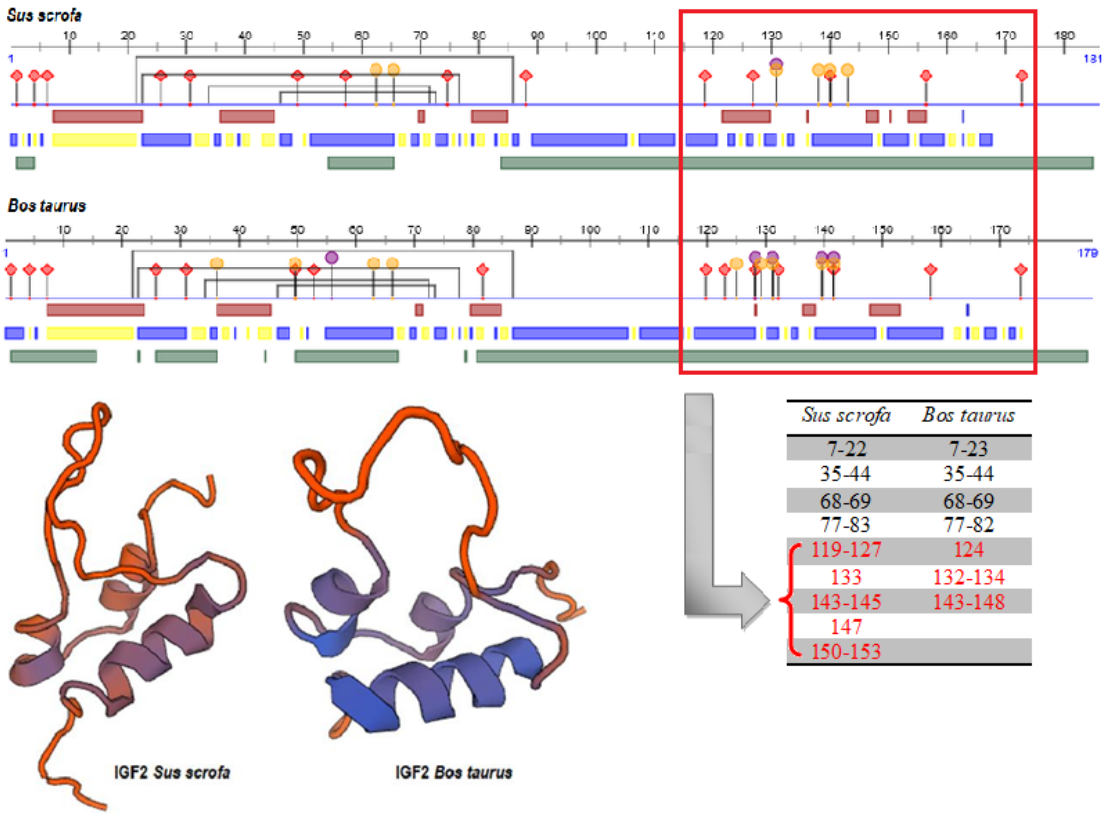

Figure 2. The comparison of IGF2 protein structure from *Sus scrofa* and *Bos taurus*. The above diagram showed the variation in helical structures at the later part of the protein sequence (the box and the table). Similarly, the differences in the protein structure model built for the IGF2 protein were also shown.

Figure 2 showed a similar model for both proteins, with a variation on helical structures, especially toward the carboxyl end. Five helical structures were detected at the end of carboxyl region of the IGF2 protein from *S. scrofa*, as opposed to only three helical structures found in the same region of *B. taurus* IGF2 protein. However, all peptide fragments obtained after cleavage with trypsin were originated from non-helical structures. The protein cleavage by trypsin enzyme resulted in peptide fragments of Insulin-like growth factor II precursor (IGF-II), which consisted of Chain Insulin-like growth factor II (positions 25-91) and Peptide Preptin (positions 93-126) (Table 2).

Greater variation was found in peptide preptin region. The cleavage by trypsin resulted in four peptide fragments in *S. scrofa*, while only three fragments were identified in *B. taurus*, lack of the additional cleavage at the 108-112 region. Also, all comparable fragments had different sequence and various length, indicated potential source for developing markers. To develop MS-based peptide markers, the longest peptide fragment in the preptin region, a position of 93-107 (*S. scrofa*) and 93-112 (*B. taurus*), was subjected to in-silico MS analysis.

The theoretical mass spectrum of selected IGF2 peptide generated from in-silico MS analysis showed a different number of peaks, as well as the position and intensity of each peak due to differences in amino acids sequence (Figure 3). The distinctive pattern between *S. scrofa* and *B. taurus* peptide was notably observed in the m/e region of 1000-1600, particularly in their ion intensities.

Gene analysis

The properties of the *IGF2* gene from *S. scrofa* and *B. taurus* were shown in Table 3. There were 12 exons identified in *S. scrofa* while only 10 found in *B. taurus* (Table 3). The protein length differed from each other only by two amino acids, with nucleotide frequencies slightly varied in G and T frequencies. Both genes had different number of motifs. The *IGF2* gene from *S. scrofa* contained 24 motifs of 18 different regulatory elements (REs), while 18 motifs of 17 different REs had been found in the gene from *B. taurus*. The same motifs had also been identified in chicken, rat, cattle, mouse, human, and rabbit (data not shown).

For developing PCR-based DNA marker, the *IGF2* mRNA transcripts (Figure 4) were subsequently used to design the primer (Table 4). The targeted amplicon was the polymorphic regions identified in the last fragment of coding sequence (Figure 4), following the alignment of both sequences. The primer that resulted in different-size amplicon products could not be designed since the Tm of

Table 1. The comparison of physicochemical properties of IGF2 protein from *Sus scrofa* and *Bos Taurus*

Properties	*Sus scrofa*	*Bos taurus*
Hydrophobicity	Hydrophilic	Hydrophilic
Solubility value	0.592	0.467
Estimated Pi	9.94	8.8
The molecular weight (msa)	20315.51	19681.51

Table 2. The comparison of peptide fragments properties after protein cleavage by trypsin.

Precursor	Properties	Mass	Position	Peptide sequence
Chain Insulin-like growth factor II at positions 25-91	*Sus scrofa*			
	Theoretical pI: 6.41	2629.238	25-48	AYRPSETLCGGELVDTLQFVCGDR
	Mw (average mass): 7502.49	1698.808	74-89	SCDLALLETYCATPAK
	Mw (monoisotopic mass): 7497.57	1187.596	49-58	GFYFSRPASR
	Formula: $C_{322}H_{506}N_{94}O_{101}S_6$	1055.465	65-73	GIVEECCFR
	Sequence covered by trypsin cleavage: 88.1%			
	Bos taurus			
	Theoretical pI: 6.41	2629.238	25-48	AYRPSETLCGGELVDTLQFVCGDR
	Mw (average mass): 7532.52	1698.808	74-89	SCDLALLETYCATPAK
	Mw (monoisotopic mass): 7527.58	1203.591	49-58	GFYFSRPSSR
	Formula: $C_{323}H_{508}N_{94}O_{102}S_6$	1055.465	65-73	GIVEECCFR
	Sequence covered by trypsin cleavage: 88.1%			
Peptide Preptin at positions 93-126	*Sus scrofa*			
	Theoretical pI: 9.52	1654.844	93-107	DVSTPPTVLPDNFPR
	Mw (average mass): 4027.55	712.33	116-120	YDTWK
	Mw (monoisotopic mass): 4025.06	589.3052	121-125	QSAQR
	Formula: $C_{187}H_{277}N_{49}O_{51}$	563.3187	108-112	YPVGK
	Sequence covered by trypsin cleavage: 88.2%			
	Bos taurus			
	Theoretical pI: 4.58	2035.0229	93-112	DVSASTTVLPDDVTAYPVGK
	Mw (average mass): 3877.32	1146.5618	113-120	FFQYDIWK
	Mw (monoisotopic mass): 3874.94	619.3158	121-125	QSTQR
	Formula: $C_{177}H_{267}N_{43}O_{55}$			
	Sequence covered by trypsin cleavage: 97.1%			

Notes: Signal in positions 1-24 and Propep in positions 92-179 have been removed. Peptides displayed >500 Da. No post-translational modifications occur in all peptides

Table 3. The properties of the IGF2 gene from *Sus scrofa* and *Bos taurus*

Gene ID (NCBI)	Organism	Gene size (NCBI)	Exon count (NCBI)	Product size	Nucleotide frequencies (Nsite)	Motif [c] (Nsite)
396916	*Sus scrofa*	27235 bp	12	181 aa	A- 0.17 G- 0.31 T- 0.19 C- 0.33	24 motifs of 18 different REs
281240	*Bos taurus*	27317 bp	10	179 aa	A- 0.17 G- 0.30 T- 0.20 C- 0.33	18 motifs of 17 different REs

Note: [a]real or consensus sites, with 80% level of homology between known RE and motif

Table 4. The in-silico PCR-based DNA marker design from a polymorphic region in the *IGF2* coding sequence

Gene ID (NCBI)	Primer design (Primer3)	Amplicon size (UCSC in-silico PCR)		Amplicon identity (Primer-Blast)	
		S. scrofa	***B. taurus***	***S. scrofa***	***B. taurus***
396916 (*S. scrofa*)	Forward: AAGTCCGAGAGGGACGTGT Reverse: AATTTGGCTCACTTCCGATG	547 bp	None	IGF2	None
281240 (*B. taurus*)	Forward: CACAGCAGCCCGACTAGC Reverse: AAGGTGACACTCGGTGGTCT	498 bp	None	IGF2	None

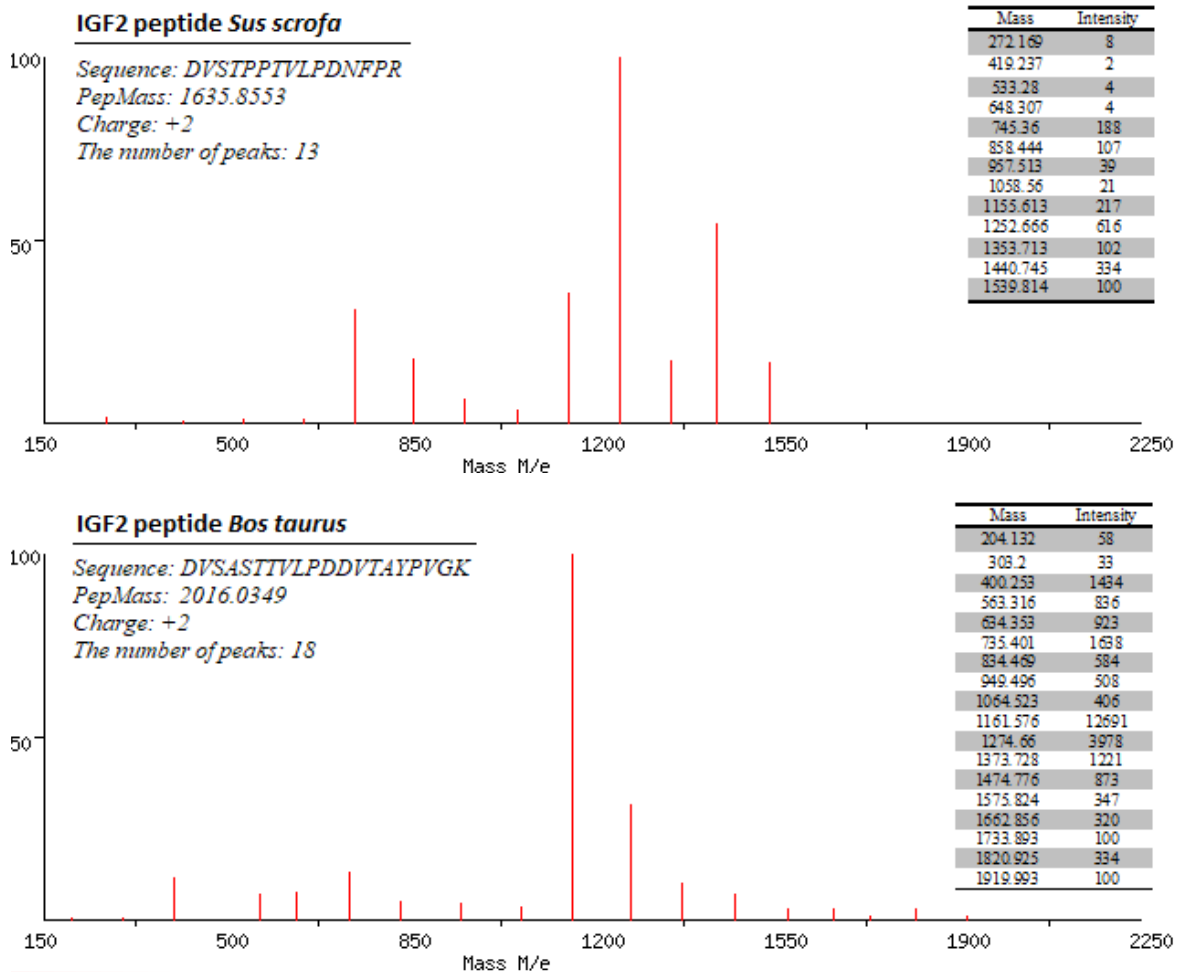

Figure 3. The comparison of theoretical mass spectrum between selected IGF2 peptides from peptide preptin at position 93-107 (*S. scrofa*) and 93-112 (*B. taurus*)

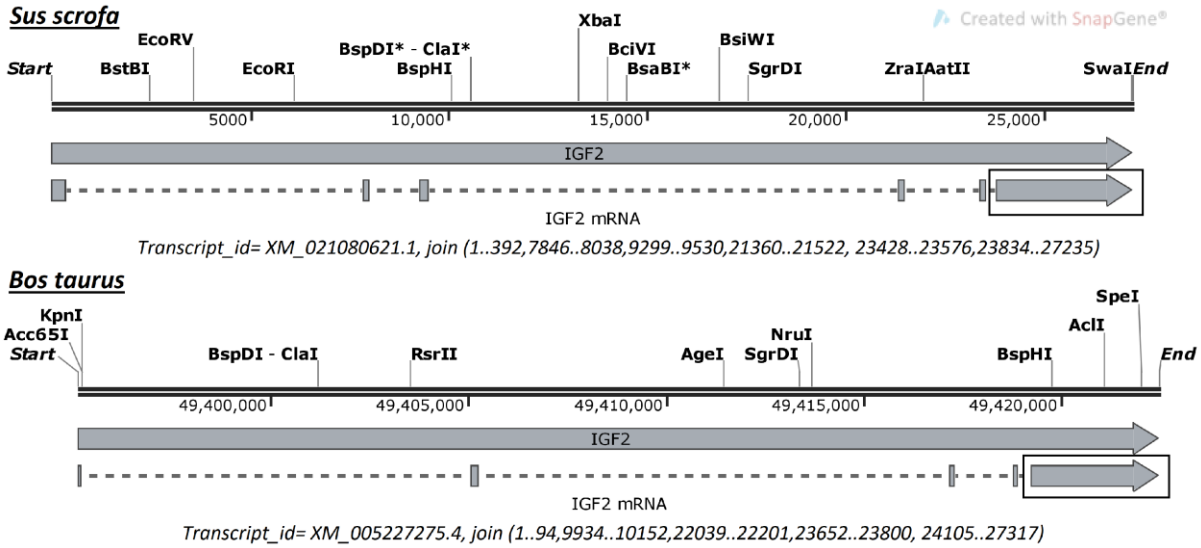

Figure 4. The longest sequences that account for more than 3000 bp (in the box) from the *IGF2* mRNA transcript of *S. scrofa* (transcript_id=XM_021080621.1) and *B. taurus* (transcript_id=XM_005227275.4) were used to design the primer for PCR detection.

designed primers were all above the 70ºC (data not shown), not at all suitable for PCR. Instead, the primers that produced only one specific amplicon in *S. scrofa* genome were obtained, with the amplicon size at around 500 bp. Further confirmation by primer-Blast showed that the primers indeed amplified DNA sequence from the *IGF2* gene in *S. scrofa*, but none was amplified from *B. taurus* genome.

Discussion

Phylogenetic analysis showed the paraphyletic relationship between the IGF2 protein of *Sus scrofa* and *Bos taurus*, indicated distinctive protein sequence variation between the two species. According to Huang et al. (2013), IGF2 polymorphisms may be used as a genetic marker, which could be used for the marker-assisted selection in breeding programs for beef cattle. It is expected that the intraspecies polymorphism will be higher between the IGF2 protein of *S. scrofa* and *B. taurus*, hence potential to be developed as genetic markers for halal authentication.

Noteworthy, detection by SDS Page is not suitable for halal detection since the molecular weight of IGF2 protein from both species is not significantly different. However, further protein analysis has confirmed the possibility of utilizing IGF2 as a peptide marker by MS analysis, particularly the preptin region. Preptin is a 34-amino acid peptide that corresponding to Asp^{69}-Leu^{102} of the proinsulin-like growth factor II E-peptide (Buchanan et al. 2001). Following the cleavage by trypsin, a peptide fragment from preptin generates distinctive theoretical mass spectrum between *S. scrofa* and *B. taurus*. Likewise, polymorphism at the preptin sequence, specifically at the fragment region for MS analysis, has also been identified between human and mouse, as well as a rat (Buchanan et al. 2001).

Several proteomic approaches based on the identification of peptide biomarkers have been already reported to reveal food composition, which can detect the addition of processed animal proteins (Sentandreu and Sentandreu, 2011). It is possible because the primary structure of a protein is highly stable (Kumazawa et al. 2016), even in processed food. Therefore, the MS analysis of IGF2 peptide to detect the presence of porcine in meat products is highly applicable.

Another approach for halal authentication using the *IGF2* gene as a source of the marker is by developing a PCR-based DNA marker. The principle of halal detection here is to design primer that will produce a specific amplicon that uniquely belongs to *S. scrofa*, following PCR amplification. As has been shown by the notable differences that exist in both the helical structure toward the carboxyl end as well as in the preptin peptide, the polymorphism in DNA sequence is also expectedly higher in the later part of the coding region. However, instead of different size of amplicon product, the primers that have been designed here specifically amplify the partial *IGF2* coding region from *S. scrofa*, while none obtained from *B. taurus*. Therefore, halal authentication based on PCR can be conducted merely by identifying the presence or absence of amplicon in the samples. This approach is more practical for implementation since the result obtained is more straightforward, as opposed to differentiation by amplicon size. Further laboratory analysis is needed to confirm the reliability and reproducibility of the designed primers for halal authentication.

Sequencing method can also be applied to halal detection, using the polymorphic sequence at a regulatory region, particularly in the *IGF2* promoter region, which associated with intramuscular fat content (Aslan et al. 2012). The motif search has found 17 to 18 different regulatory elements in the gene. However, the motifs have

also found in several animals, such as chicken, rat, cattle, mouse, and rabbit. Further analysis is needed to identify and confirm unique sequence in IGF2 gene that can be subjected for halal detection through sequencing approach.

In conclusion, the *IGF2* gene is a potential source to develop peptide markers for MS analysis and DNA marker for PCR-based analysis in halal authentication, mainly to differentiate bovine and porcine meat in the meat derived products. A peptide fragment from the preptin at position 93-107 (*S. scrofa*) and 93-112 (*B. taurus*) has produced distinctive theoretical mass spectrum pattern and therefore is potentially utilized as peptide marker. Alternatively, two pairs of primers designed from the *IGF2* coding region can also be implemented for halal authentication using a PCR-based method. Further laboratory analysis is needed to validate the in-silico study.

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