Oral Presentation (AQ-9)

Identification of Sumateran Wild Boar Meat (*Sus scrofa vittatus*) by Restriction Fragment Length Polymorphism (RFLP) Analysis of *Cytochrome b* Gene

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INTRODUCTION

Sumateran wild boars have been super abundant in Sumateran forest. In Indonesia, this wildlife condition has led to the exploitation for commercial purpose. The high number of Sumateran wild boars population increases wild boar hunting resulting in an abundant availability of wild boar meat in the food market with extremely cheap price. The macroscopic similarity of wild boar meat and beef has prompted the local people to abuse this situation by selling wild boar meat in traditional market as beef. Based on annual record from Cilegon Class II Quarantine Office in 2014, there were nine smuggling cases or a total of 21.556 kg of wild boar meat smuggling effort that were prevented by Cilegon Quarantine officers. The number of food safety concerns related to smuggling of wild boar or counterfeiting beef with wild boar is a very detrimental condition for consumers, especially consumers in traditional markets.

The checking of genuineness or validity of food products is an important effort to protect people from consuming unhealthy food and to indicate whether the food is halal or not. Studies of meat detection should be continuously developed as an effort to protect consumers. Genetic method is the most specific and sensitive method to check food ingredients authenticity by detecting the presence of genetic material or deoxyribonucleic acid (DNA). It results from the specific character of the structure of DNA particles and the possibility of using the information included in them. The most frequent loci used for species identificationin phylogenetics and biodiversity studies are mitochondrial cytochrome b (cyt *b*).

Genetic method is the most specific and sensitive tool for analyzing the authenticity of food ingredients in a molecular level by means of detecting the presence of genetic material or deoxyribonucleic acid (DNA). One of the various methods could be used to detect genetic material is polymerase chain reaction (PCR). Specifically, one of such method frequently used in food industry to observe animal derived product fabrication is PCR restriction fragment length polymorphism (RFLP). PCR-RFLP is based on the comparison of the bands profile generated after certain enzymes digest the DNA target. PCR-RFLP is appropriate for meat testing due its ability in exploiting sequence variation in designated DNA region that allows species differentiation even from closely related species through DNA fragment restrictions selected by suitable restriction enzyme. PCR-RFLP is advantageous since it is simple, cheaper, and easier to be adjusted for routine big-scale studies such as surveillance program.

MATERIALS AND METHODS

Fresh pork (*Sus scrofa domestica*) (n=7) and frozen beef (*Bos taurus*) (n=7) were obtained from wet market in Bogor city. Fresh Sumateran wild boar meat (*Sus scrofa vittatus*) (n=7) was obtained from Way Kanan Regency, Lampung Province. The DNA of fresh raw beef, pork, and wild boar meat were extracted using DNA extraction kit (Qiamp® DNA Mini Kit, Qiagen, Germany), according to the protocol from manufactur.

A total of 3 pair primers were utilized in each PCR reaction. A pair of mitochondria cyt *b* primers used in this work was described by Kocher et al (1989). This study also used Porcine-specific primer and bovine-specific primers were described by Cheng et al (2003).

PCR amplicons of the *cyt b* gene were subjected to restriction enzyme digestion with restriction enzyme *Alu*I, *Bsa*JI, *Hind*III, *Rsa*I, *Hae*III, and *Taq*$^{\alpha}$I according to the suppliers' instructions (Biolabs).

RESULT AND DISCUSSION

The PCR amplification analysis was conducted using cyt *b* and species-specific primers on pork (*Sus scrofa domestica*), Sumateran wild boar meat (*Sus scrofa vittatus*) and beef (*Bos taurus*). Figure 1 shows the PCR amplification products of pork, Sumateran wild boar meat and beef by cyt *b* primers that produced a single band with molecular size of 359 bp, respectively. Meanwhile, using the species-specific for porcine and bovine species primers, pork and Sumateran wild boar meat also produced a single band with molecular size of 212 bp and 271 bp for beef (Figure 2).

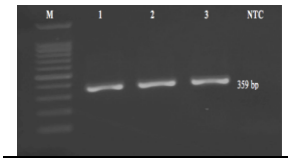

Fig 1. Agarose gel electrophoresis of amplification product of cyt *b* gene. (M:1 kb marker, 1: pork, 2: Sumateran wild boar , 3: beef, 4: NTC).

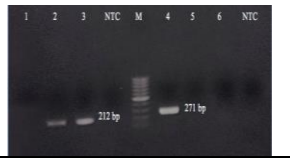

Fig 2 Polymerase chain reaction (PCR)-amplification of porcine specific and bovine specific gene. (M:1 kb marker, 1: pork, 2: Sumateran wild boar meat, 3: beef, 4: pork, 5: Sumateran wild boar, 6: beef, 7:NTC).

The method for species identification of meat products has increasingly turned towards DNA-based techniques in overcoming the limitations of existing methods (Rahmati et al 2016). Genetic traceability is based on the identification of both animals and their products through the DNA analysis.

Cytochrome b gene (c yt b) is one of the genes that is coded by mitochondrial DNA (mt-DNA) and its gene product plays an important role in electron transfer in the respiration chain. Cyt b gene has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies on the phylogenetic evolution at the intra- and interspecific levels (Kunda et al 2016).

The cyt *b* sequences are good tools for studying a more precise species identification (Foran et al 2015). Mitochondria cyt *b* primer used in this study for differentiation of pork, Sumateran wild boar meet and beef. In this study, the amplification product of pork, Sumateran wild boar meat and beef produced a single band with molecular size of 359 bp, respectively. The molecular size of cyt *b* in this study agreed with previous study (Mutalib et al 2012). Since cyt *b* primer is universal primer, this primer cannot distinct pork, Sumateran wild boar meat and beef origin.

PCR using species-specific primers is one of the most used approaches since it offers simplicity, specificity and high sensitivity for meat authentication studies (Amaral et al 2014). The primers for every species would only amplify the DNA of that particular species (Man et al 2007).

In this study, the species-specific primers used were successfully distinguished porcine meat (pork and Sumateran wild boar) and beef. According to Castello et al (2004), bovine specific primers can be used to detect the presence of beef DNA within pig DNA up to 0.05 % cattle sample concentration within 99.95 % concentration of pig sample. In the other hand, amplification by pig specific primer can be used to directly detect pig-derived materials in a foodstuff (Man et al 2007). Also, this primer can detect pig-derived material up to 0.5 % of concentration within foodstuff and possibly with even lower concentration (Ilhak and Arslan 2006). However, the porcine-specific primer cannot distinguish pork and Sumateran wild boar in this study.

Cyt *b* has been used by various researches to identify meat types (Erwanto et al 2012). *Cyt b* gene has been used in various studies that identifies DNA presence originating from pig or beef in food products or foodstuff and identifying various species, especially in studies utilizing PCR-RFLP (Verkaar et al 2002; Aida et al 2005; Murugiah et al 2009; Mutalib et al 2012; Erwanto et al 2011; Erwanto et al. 2012). Doosti *et al*. (2014) investigated the PCR-RFLP analysis of the mitochondrial *cyt b* gene to differentiate between beef, sheep, pork, chicken, donkey, and horsemeat in meat products (sausages, frankfurters, hamburgers, hams and cold cut meats) and suggested that this method provide a potential technique to rely on for authentication of halal (law or permitted) meat origin.

The PCR amplification product using cyt *b* primers were subsequently examined for PCR-RFLP analysis using six different restriction enzyme of *Alu*I, *Bsa*JI, *Hind*III, *Rsa*I, *Hae*III, and *Taq*$^{\alpha}$I. Table 1 shows the band sizes of cyt *b* PCR amplification products of pork, Sumateran wild boar meat and beef when digested by *Alu*I, *Bsa*JI, *Hind*III, *Rsa*I, *Hae*III, and *Taq*$^{\alpha}$I.

Table 1. Restriction pattern of cyt *b* gene for pig, Sumateran wild boar, and cattle

Species enzymes	Pig	Sumateran wild boar	**Beef**
AluI	244 + 115	244 + 115	190 + 169
BsaJI	228 + 131	228 + 131	320 + 39
HindIII	-	-	-
RsaI	320 + 39	320 + 39	320 + 39
HaeIII	-	200 + 131 + 28	285 + 74
Taq$^{\alpha}$I	228 + 131	228 + 131	-

Two bands were observed in all meats when PCR amplification product was digested with *Alu*I. The PCR-RFLP analysis of pork and Sumateran wild boar meat using *Alu*I produced bands with molecular size of 115 bp and 244 bp, meanwhile, beef amplicon was digested and produced 169 bp and 190 bp (Figure 3).

Similarly, *Bsa*JI produced bands with molecular size of 228 bp and 131 bp for pork and wild boar, meanwhile, beef amplicon was digested and produced 320 bp and 39 bp. *Taq*$^{\alpha}$I restriction

enzyme showed 2 bands with molecular size of 131 bp and 228 bp for pork and Sumateran wild boar meat. However, it failed to digest beef DNA fragments in repeated experiments that produced a single band of 359 bp in size.

Using *Hind*III was undigested all meats DNA. On the other hand, *Rsa*I successfully digested all meats DNA and produced 320 bp. Surprisingly, only *Hae*III was successfully digested Sumateran wild boar amplicons into three fragments of 74 bp, 132 bp and 153 bp (Figure 3).

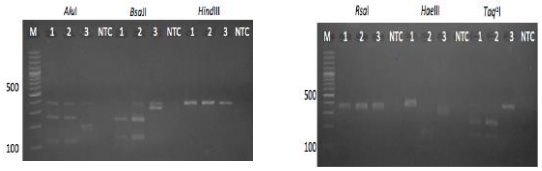

Figure 3. Restriction profiles of the cyt *b* PCR products obtained from pig (1); Sumateran wild boar (2); and cattle (3), after digesting with *Alu*I, *Bsa*JI, *Hind*III, *Rsa*I, *Hae*III, dan *Taq*I. (M): DNA *marker* (Invitrogen), NTC: *Non-template control*.

Important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments. In addition, the design of PCR-RFLP analyses generally is easy and can be accomplished using public available programs (Rasmussen 2012). The proposed Cyt*b*-PCR-RFLP assay represents a fast and delicate technique appropriate to the detection and authentication of poultry meat species. (Razzaq et al 2017).

PCR-RFLP (PCR restriction fragment length polymorphism) is a stable, rapid, and inexpensive technique that combines PCR and restriction endonuclease digestion and has received much attention in identifying genetic diversity and developing biomarkers for the authentication of adulterants at the species level. Additionally, this technique can semi-quantitatively detect contamination in authentic samples and can be performed quickly and inexpensively Another advantages PCR-RFLP is an accurate and authentic PCR-based authentication method based on (1) using universal primers to amplify a conserved region, (2) digesting the PCR product with specific restriction endonucleases, and (3) separating the digest fragments by agarose gel electrophoresis (Jiang et al 2017).

Dubey et al (2010) on their study said that they have chosen a restriction fragment length polymorphism (RFLP) technique, because it is less time–consuming, it is more cost-effective than DNA sequencing, it requires equipment readily available in most molecular laboratories, and it has proved its utility in species identification.

Another study that conducted by Meganathan et al (2009) stated that a simpler alternative to the DNA sequencing technique is restriction fragment length polymorphism analysis of PCR products (PCRRFLP). This technique proves to be a simple, rapid, and cost-effective method for species identification and has been used in forensic examination to authenticate the confiscated biological materials.

In this study, using the *cyt b* gene, pork, Sumateran wild boar meat and beef produced amplified fragment of 359 bp in size (Figure 1). Furthermore, the PCR-RFLP analysis was conducted the RFLP profiles of all of meats were differentiated by two different restriction site of *Alu*I. However, this enzyme is not able to distinct pork and Sumateran wild boar meat since they have same size of 115 bp and 244 bp for each fragment. Surprisingly, previous study (Mutalib et al 2012) did not find any fragment in wild boar meat using *Alu*I enzyme.

Based on study with other enzymes, *Bsa*JI digestion generated 115 bp and 244 bp for each pork and Sumateran wild boar meat while it produced 320 bp for beef. The fragment size produced by *Bsa*JI in this study is in agreement with previous study (Murugiah et al 2009). It indicates that *Bsa* JI can be used as restriction endonuclease to differentiate porcine meat and beef. However, it is not adequate to distinct pork and Sumateran wild boar meat. In this study, we also compared with other restriction endonuclease, *Taq*$^{\alpha}$I. We found *cyt b* gene digestions originated pork and Sumateran wild boar meat performed with these enzymes resulted in two DNA fragments (228 to 131 bp). However, these enzymes failed to digest beef after repeated experiments. To the best of our knowledge, this is the first study on *Taq*$^{\alpha}$I as restriction enzyme in meat authentication. *Taq*$^{\alpha}$I is not appropriate for differentiation of neither the closely related species pork and Sumateran wild boar nor beef digestion. On the other hand, *Hind*III enzymes did not cleave the PCR products of any of the tested meat, while *Rsa*I restriction enzyme produced 320 bp for all of meats. These results are similar to the previous work (Murugiah et al 2009).

Finally, the *Hae*III cleavage bands visualized in the gel were enough and suitable for the discrimination of all species analyzed. In this study, it is observed at *Hae*III was the only specific enzyme to differentiate between pork, Sumateran wild boar and beef meat.

PCR-RFLP analysis of the *cyt b* gene provides a simple, relatively quick, and accurate identification of Sumateran wild boar species. In this study, PCR RFLP analysis only required 4 hours. Jiang et al (2017) in their study also state that in general, a common PCR RFLP analysis requires 4-5 h, limiting its application in a routine species identification studies

CONCLUSION

The species-specific primer were successfully distinguished porcine meat (pork and

Sumateran wild boar) and beef. Among six tested enzymes, the use of PCR-RFLP analysis of the *cyt b* gene followed by digestion using *HaeIII* provides a simple, relatively quick, and accurate identification of Sumateran wild boar species. This method could be used to identify Sumateran wild boar meat falsified as beef which is typically common practice in Indonesia.

REFERENCES

[1] Doosti A., D. P. Ghasemi, & E. Rahimi. 2014. Molecular assay to fraud identification of meat products. J Food Sci Technol. 51:148–152.

[2] Farag M. R., M. Alagawany, M. E. A. El-Hack, R. Tiwari, & K. Dhama. 2015. Identification of different animal species in meat and meat products: trends and advances. J AAVS. 3:334–346.

[3] Oceja L. A., C. Nunez, M. Baeta, D. Gamarra, & M. M. de Pancorbo. 2017. Species identification in meat products: A new screening method based on high resolution melting analysis of *cyt b* gene. J Food Chem. 237:701–706.

[4] Razzaq A., A. Nawaz, N. Saeed, A. Hayee, & A. Ali. 2017. Modern preventive techniques of meat adulteration for improving public health. J Biosci. 4:127-131.