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Identification of a Differentiation Factor of Indonesian Ongole Cattle Breeds Based on Microsatellite Markers and Mitochondrial DNA

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ABSTRACT

This study was conducted to identify the differentiation factor of Indonesian Ongole cattle breeds (Sumba Ongole and Ongole Grade) based on the 12 microsatellite markers and Cyt b gene polymorphism. A total of 50 blood samples (25 samples for each cattle breed) were used in this study. The multiplex DNA fragment analysis was conducted for allele identification based on the microsatellite markers. The haplotype identification (based on the mitochondrial DNA) was conducted using restriction fragment length polymorphism (RFLP) analysis with three restriction enzymes i.e. HinfI, HaeIII, and XbaI. Twelve microsatellite loci in this study revealed high polymorphism. A total of 82 alleles were detected in the SO cattle and 117 alleles were detected in the PO cattle. The TGLA227 and ETH225 were specific locus candidates which are different in the size and the number of alleles in the SO and PO cattle breeds. The B (HinfI), D (HaeIII), and Y (XbaI) haplotypes were found only in the PO cattle breed samples. The X haplotype was found in all samples of the SO cattle breed but was not found in all samples of the PO cattle breed. The Y haplotype was found in all samples of the PO cattle breed but was not found in all samples of the SO cattle breed. It can be concluded that the TGLA227 and ETH225 (based on microsatellite markers) and the B, D, X, and Y haplotypes (based on the mitochondrial DNA) can be considered as the differentiation factors between the SO and PO cattle breeds.

Keywords: Differentiation, Identification, Microsatellite, Mitochondria, Ongole

Introduction

Sumba Ongole (SO) are one of the local Indonesian cattle breeds. The existence of the SO cattle in Indonesia began since the Indian Ongole breed was imported from India in 1914 and centralized in Sumba Island (East Nusa Tenggara Province) for breeding programs (Ministry of Agriculture of the Republic of Indonesia, 2014). Since then, the Ongole generations resulted from breeding programs in the Sumba Island have been known as Sumba Ongole (SO) cattle (Hardjosubroto, 2004). The SO cattle have excellent potential to gain higher dressing percentage (>50%) compared with other local cattle breeds in Indonesia (Agung *et al*., 2015).

The phenotype characteristics of the SO cattle are closely similar with the Ongole Grade's cattle (known as PO cattle and spread out across in the Java Island), and it is difficult to identify these two Ongole breeds based on the phenotypic parameters because the PO cattle is a crossbred of uncontrolled mating of the SO cattle breed and Java cattle breed (Suyadi *et al*., 2014) or other Indonesian local breeds (Sudrajad and Subiharta, 2012). In order to resolve the difficulties in the SO and PO identification, a scientific investigation is needed to find the differences between the SO and PO cattle breeds based on their genetic information.

The development of molecular genetic analysis has made it possible to study the potency of certain cattle breeds at the deoxyribonucleic acid (DNA) level. Microsatellites are defined as sequential repeats of a 1–6 nucleotide motif and found throughout the genomes of prokaryotes and eukaryotes (Haasl and Payseur, 2012). The microsatellite markers can be used for parentage verification (Radko, 2010), paternity testing (Stevanovic *et al*., 2010), assessing the genetic diversity (Seo *et al*., 2017), and also can be used for estimating the genetic differentiation (Rutledge *et al*., 2010).

Mitochondria have been characterized as the powerhouses of the cell, because their most basic function is oxidative phosphorylation (Ladoukakis and Zouros, 2017). Mammalian mitochondrial DNA is a gene-dense, double-stranded DNA (dsDNA) molecule of 16.6 kb, which encodes 11 messenger RNAs (mRNAs) (translated to 13 proteins), 2 ribosomal RNAs (rRNAs) (12S and 16S rRNA), and 22 tRNAs

(Gustafsson *et al*., 2016). The mitochondrial DNA can be used for investigation the genetic diversity and genetic structure in certain animal breeds (Sharma *et al*., 2015). The cytochrome b (Cyt b) gene is one of genes that are located in the mitochondrial DNA (Stewart and Chinnery, 2015) and can be used to investigate the origin of certain animal species (Satish *et al*., 2009; Zarringhabaie *et al*., 2011; Farag *et al*., 2015). The differentiation factor of Indonesian Ongole cattle breeds (SO and PO) might be found based on the microsatellite markers or the Cyt b gene due to the microsatellite markers can be used to identifying the relationship among livestock breeds (Maretto *et al*., 2012) and the variation in the Cyt b gene can be used for the comparison study of different animal species (Munira *et al*., 2016). This research was conducted to identify the differentiation factor of Indonesian Ongole cattle breeds (SO and PO) based on 12 microsatellite markers and Cyt b gene polymorphisms.

Materials and Methods

Blood sample and DNA collection

A total of 50 heads of cattle including the SO cattle (n=25; all individual cattle samples belonged to several private farmers in the Sumba Island) and the PO cattle (n=25; all individual cattle samples belonged to Research Center for Biotechnology farm in West Java) were used for the blood sampling purpose. Blood samples (3-5 mL) were taken from the *coccygeal* veins using *Venoject* and collected in *Vaccutainer* tubes containing an anticoagulant. The blood samples were used in the DNA extraction process using the Genomic DNA Mini kit (Geneaid Biotech Ltd., Taiwan) following the manufacturer's protocol. A total of 12 microsatellite-labeled primers (part of the 30 primers recommended by Food and Agriculture Organization of the United Nations (FAO)) were used in the polymerase chain reaction (PCR) process (primers sequence, annealing temperature, the range of PCR product size, and label used were based on Agung *et al*. (2015)). Amplification of the Cyt b gene was performed using primers based on Hartatik *et al*. (2015) i.e. forward (5'-aaaaaccaccgttgttattcaacta-3') and reverse (5'-gcccctcagaatgatatttgtcctca-3').

DNA amplification

The PCR reagents are composed of: KAPA2G Robust Hot Start Ready Mix PCR Kit (Kapa Biosystems, Cape Town, South Africa), forward and reverse primers (200 ng/µL), DNA samples (5-50 ng/µL), and H_2O up to 25 µL final volume. The PCR program was set as follows: denaturation at 94°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing 58°C to 64°C (depending on the primers) for 45 seconds, extension at 72°C for 45 seconds; and a final extension at 72°C for 5 minutes on Mastercycler® Gradient (Eppendorf, Hamburg, Germany). The PCR products were then visualised by electrophoresis process (1% agarose gel, SyBr® staining, and captured in GBOX documentation System (Syngene, UK)).

Allele and haplotype identification

Multiplex DNA fragment analysis was conducted afterwards for allele identification. The multiplex DNA fragment analysis was conducted in the 1st BASE Laboratory, Malaysia. The haplotype identification was conducted using restriction fragment length polymorphism (RFLP) analysis with three restriction enzymes i.e. HinfI, HaeIII, and XbaI. The use of HinfI, HaeIII, and XbaI enzymes in this study were based on Hartatik *et al*. (2015), Farag *et al*. (2015), and Mohamad *et al*. (2009) respectively. The reagents for RFLP analysis were composed of: 3 µl PCR products, 1.4 µl H_2O, and 1 unit (± 0,6 µl) of restriction enzyme HinfI, HaeIII, or XbaI including its buffer (New England Biolabs, USA). The reagents were incubated at 37°C for 1 hour, and it was followed by electrophoresis process. The restriction sites were as follows: G|ANTC, GG|CC, and T|CTAGA for HinfI, HaeIII, and XbaI enzymes respectively.

Data analysis

Data of allele's size (unit in base pairs) were generated using the multiplex DNA fragment analysis. The data was processed using CONVERT version 1.3.1 (Glaubitz, 2004) to convert the size of alleles observed for each individual sample to assure suitability for further data analysis. The converted data was processed using POPGEN version 1:32 program (Yeh and Boyle, 1997) to generate observed number of alleles (n_A), effective number of alleles (n_e), observed heterozygosity value (H_o), expected heterozygosity value (H_e), and allele frequency. The converted data was also processed using CERVUS version 3.0.7 program (Kalinowski *et al*., 2007) to obtain the polymorphism information content (PIC) value. The haplotypes data were generated using PCR-RFLP analysis with three restriction enzymes. The PCR-RFLP products were visualised by electrophoresis process. Individual cattle haplotype was determined based on the differences in the number and size of the visualised bands. The frequency of the haplotype was calculated using MS Excel 2007 program based on Nei and Kumar (2000): $\chi_{ii} = (n_{ii}/N)$ for haplotype frequency, where: χ_{ii} = frequency of ii^{th} haplotype; n_{ii} = number of individuals with ii haplotype; N = number of samples.

Result and Discussion

Based on allele identification using multiplex DNA fragment analysis, twelve microsatellite loci in this study revealed high polymorphism, and 199 alleles were detected with 82 alleles in the SO cattle and 117 alleles in the PO cattle. Based on the allele distribution (Table 1 and Table 2), the TGLA122 locus has the highest n_A value in the SO cattle while the TGLA122 and

Table 1. Alleles distribution and its frequency based on 12 microsatellite loci in the SO cattle breed

Loci	Allele	Allele frequency	Loci	Allele	Allele frequency	Loci	Allele	Allele frequency
INRA023	197	0,080	SPS113	131	0,160	TGLA126	111	0,020
	199	0,040		133	0,220		117	0,140
	201	0,100		135	0,040		119	0,040
	203	0,100		137	0,340		123	0,320
	205	0,020		139	0,160		125	0,280
	209	0,060		141	0,040		127	0,200
	211	0,020		147	0,020	TGLA227	**78**	0,780
	215	0,580		**157**	0,020		**80**	0,040
CSSM66	178	0,180	SPS115	242	0,180		**84**	0,040
	180	0,040		244	0,440		**86**	0,020
	182	0,040		246	0,060		**88**	0,020
	196	0,020		**250**	0,060		**92**	0,020
	198	0,020		252	0,080		**94**	0,020
	220	0,700		254	0,060		**96**	0,020
ILSTS006	292	0,060		**258**	0,020		**100**	0,040
	294	0,500		**262**	0,020	ETH225	**135**	0,396
	296	0,240		**264**	0,080		**139**	0,042
	298	0,160	TGLA122	136	0,160		**143**	0,062
	300	0,020		140	0,040		**145**	0,021
	302	0,020		142	0,060		**149**	0,021
BM1824	181	0,200		144	0,120		**155**	0,458
	183	0,560		152	0,280	TGLA53	135	0,805
	185	0,160		154	0,100		137	0,083
	189	0,020		158	0,020		**139**	0,028
	195	0,020		160	0,020		141	0,028
	197	0,020		162	0,180		161	0,056
	199	0,020		**164**	0,020	BM1818	262	0,240
							264	0,760

bold=the alleles that was not found in the PO cattle breed.

Table 2. Alleles distribution and its frequency based on 12 microsatellite loci in the PO cattle breed

Loci	Allele	Allele frequency	Loci	Allele	Allele frequency	Loci	Allele	Allele frequency
INRA023	**195**	0,040	SPS113	131	0,060	TGLA227	**71**	0,062
	197	0,200		133	0,100		**77**	0,542
	199	0,080		135	0,060		**79**	0,084
	201	0,040		137	0,220		**81**	0,062
	203	0,100		139	0,120		**83**	0,021
	207	0,100		141	0,060		**87**	0,021
	209	0,080		**143**	0,060		**91**	0,062
	211	0,020		**145**	0,080		**93**	0,042
	215	0,320		147	0,040		**99**	0,104
	219	0,020		**149**	0,180	ETH225	**128**	0,020
CSSM66	178	0,160		**151**	0,020		**134**	0,100
	180	0,180	TGLA53	**133**	0,033		**136**	0,060
	182	0,020		135	0,534		**138**	0,060
	184	0,020		137	0,067		**140**	0,020
	188	0,020		141	0,033		**142**	0,060
	192	0,040		**143**	0,067		**144**	0,040
	196	0,020		**149**	0,067		**146**	0,040
	198	0,020		**153**	0,033		**150**	0,100
	220	0,520		**159**	0,033		**154**	0,400
ILSTS006	**276**	0,022		161	0,033		**156**	0,060
	284	0,022		**163**	0,067		**158**	0,020
	286	0,022		**165**	0,033		**164**	0,020
	288	0,022	SPS115	242	0,180	BM1824	**177**	0,020
	290	0,108		244	0,420		181	0,360
	292	0,022		246	0,200		183	0,340
	294	0,326		**248**	0,040		185	0,120
	296	0,174		252	0,100		**191**	0,140
	298	0,282		254	0,060		195	0,020
TGLA122	**134**	0,020	TGLA126	**103**	0,042	BM1818	**248**	0,040
	136	0,120		**107**	0,021		**256**	0,040
	140	0,020		111	0,042		**258**	0,020
	142	0,120		**115**	0,063		**260**	0,100
	144	0,040		117	0,208		262	0,280
	146	0,040		119	0,146		264	0,240
	148	0,040		**121**	0,146		**266**	0,080
	150	0,060		123	0,062		**268**	0,020
	152	0,160		125	0,083		**270**	0,180
	154	0,180		127	0,125			
	158	0,040		**129**	0,062			
	160	0,020						
	162	0,149						

bold=the alleles that was not found in the SO cattle breed.

ETH225 were the loci with highest n_A value in the PO cattle.

The TGLA227 locus in the SO cattle breed has an even-numbered allele size characteristic (e.g. alleles 78, 80, etc.), whereas in the PO cattle breed it was odd-numbered (e.g. 71, 77, etc.). In contrast, the ETH225 locus has an odd-numbered allele size (e.g. alleles 135, 139, etc.) in the SO cattle breed, but it has even-numbered allele size (e.g. alleles 128, 134, etc.) in the PO cattle breed. These results were in agreement with Agung *et al*. (2015), who reported the odd-numbered alleles in the ETH225 locus and the even-numbered alleles in the TGLA227 locus. However, due to the limited number of samples in this study, a further investigation using a great number of samples for each cattle breed that represents the population of the SO and PO cattle breeds in Indonesia needs to be conducted.

Based on the alleles variation found in the SO and PO cattle, there were several specific locus or allele candidates. The TGLA227 and ETH225 were specific locus candidates which are different in the size and the number of alleles in the SO and PO cattle breeds. This is an indication that the TGLA227 and ETH225 loci might be used to separate the SO and PO cattle breeds. The specific locus in certain cattle breeds was also reported in the Simmental cattle breed. The TGLA53 allele 168 was a specific allele candidate for the Simmental purebred cattle, and the TGLA122 allele 181 was a specific allele candidate for the Simmental crossbred (Agung *et al*., 2016).

The highest H_o value in the SO cattle breed population was 1.00 (SPS113) and the lowest was 0.16 (BM1818). Meanwhile, the highest H_o value in the PO cattle breed population was 0.92 (SPS113) and the lowest was 0.44 (ILSTS006) (Table 3).

The H_o value can be used for detecting the level of genetic diversity and inbreeding process within a population (Cervini *et al*., 2006). Unfortunately, the 12 microsatellite markers in the SO cattle and PO cattle in this study mostly were have low H_o value and can be interpreted that the level of genetic diversity was low. However, the high level of genetic diversity in the SO and PO cattle population were represented by TGLA122, ETH225, and SPS113 loci that have high H_o value.

The TGLA122 locus has the highest PIC value in SO cattle (PIC=0.81) and PO cattle (PIC=0.87). Meanwhile, the lowest PIC value in the SO cattle was 0.30 (BM1818) and in the PO cattle was 0.63 (CSSM66). The PIC value at 12 microsatellite loci in the PO cattle breed population in this study was more than 0.5 (PIC>0.5). Hence, every locus in this study was highly informative for detecting the level of genetic diversity in the PO cattle population. Meanwhile, there were four microsatellite loci in the SO cattle breed that have the PIC value less than 0.5 (PIC<0.5). As the result, not every locus in this study can be used to detect the level of genetic diversity in the SO cattle population. In addition, Czerneková *et al*. (2006) reported that low PIC value can be interpreted that certain conservation process has been carried out in a particular population.

Compared with the results from other studies that also used microsatellites which were mostly identical with our study, some differences can be observed. The differences may be in the minimum and maximum sizes of allele, the number of observed alleles, and also the PIC values. The H_o and PIC values in the SO cattle breed in this study for TGLA53, TGLA227, and BM1818 loci were low. This condition was the same with several of *Bos indicus* cattle i.e. the

Table 3. Characterization of the twelve microsatellite markers in the SO and PO cattle breeds

Locus	n_A	n_e	H_o	H_e	PIC	n_A	n_e	H_o	H_e	PIC
	----------SO----------					----------PO----------				
BM1824	7	2,63	0,32	0,63	0,57	6	3,57	0,52	0,74	0,67
ILSTS006	6	2,96	0,32	0,68	0,61	9	4,34	0,44	0,79	0,74
TGLA126	6	4,13	0,52	0,77	0,72	11	8,06	0,83	0,90	0,86
TGLA53	5	1,51	0,28	0,35	0,32	11	3,24	0,73	0,72	0,68
TGLA227	9	1,63	0,36	0,39	0,38	9	3,07	0,58	0,69	0,66
TGLA122	10	5,98	0,80	0,85	0,81	13	8,50	0,88	0,90	0,87
ETH225	6	2,68	0,92	0,64	0,56	13	5,02	0,64	0,82	0,79
INRA023	8	2,71	0,56	0,64	0,61	10	5,58	0,88	0,84	0,80
SPS113	8	4,56	1,00	0,80	0,75	11	7,81	0,92	0,89	0,86
SPS115	9	3,99	0,68	0,76	0,73	6	3,79	0,52	0,75	0,70
BM1818	2	1,57	0,16	0,37	0,30	9	5,30	0,48	0,83	0,79
CSSM66	6	1,90	0,60	0,48	0,44	9	3,01	0,52	0,68	0,63

n_A=observed number of allele; n_e=effective number of allele; H_o=observed heterozygosities; H_e=expected heterozygosities; PIC=polymorphism information content.

Hissar cattle (Rehman and Khan, 2009), Nellore cattle (Cervini *et al*., 2006), and Punganur cattle (Kesvulu *et al*., 2009) but contrast with Brahman cattle (Riojas-Valdes *et al*., 2009). In consequence, the TGLA53, TGLA227, and BM1818 loci were not suitable to investigate the genetic diversity in the SO cattle population. However, the TGLA227 locus was specific locus candidate that might be used to separate the SO and PO cattle breeds.

Based on the mitochondrial DNA analysis results, the size of the PCR product is about 464 base pairs (bp) and the same with the size reported by Hartatik *et al*. (2015). The haplotypes of the mitochondrial DNA were identified based on the differences in size and the number of bands (RFLP product) that appear in the visualisation process. There were two haplotypes for each restriction enzyme (HinfI, HaeIII, or XbaI) in the SO and PO cattle breed population based on the RFLP analysis. The A and B haplotypes were detected using HinfI enzyme (Figure 1), the C and D haplotypes were detected using HaeIII enzyme (Figure 2), and the X and Y haplotypes were detected using XbaI enzyme (Figure 3).

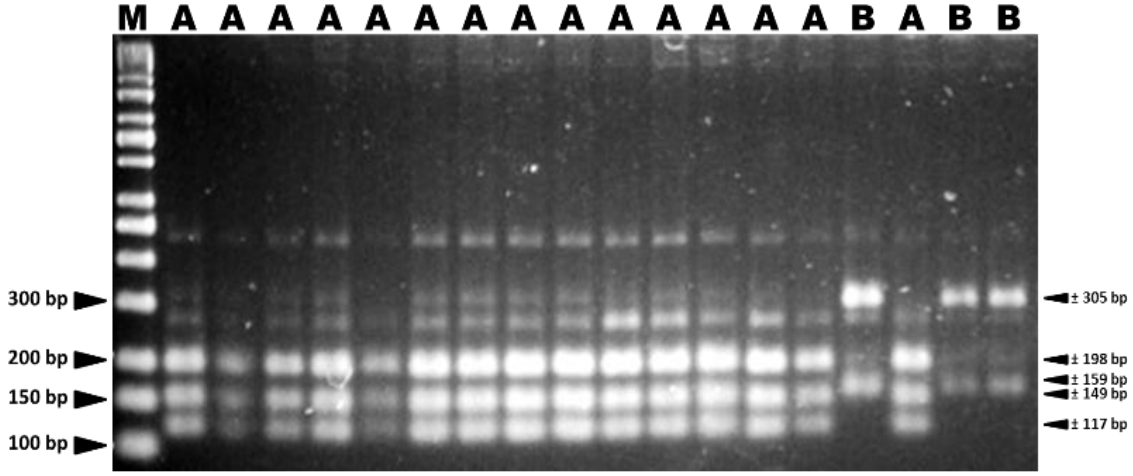

Figure 1. The haplotype visualisation based on the RFLP analysis using the HinfI enzyme (M=100 bp ladder size standard; bp=base pair; A=A haplotype; B=B haplotype).

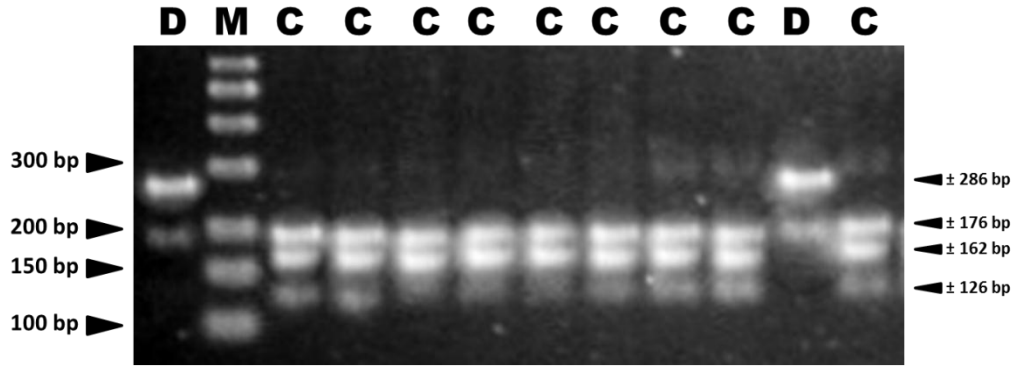

Figure 2. The haplotype visualisation based on the RFLP analysis using the HaeIII enzyme (M=100 bp ladder size standard; bp=base pair; C=C haplotype; D=D haplotype).

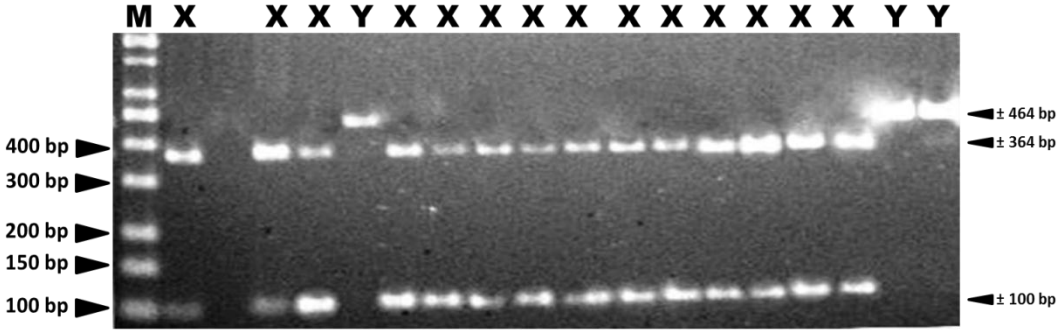

Figure 3. The haplotype visualisation based on the RFLP analysis using the XbaI enzyme (M=100 bp ladder size standard; bp=base pair; X=X haplotype; Y=Y haplotype).

Table 4. The haplotypes frequency in the SO and PO cattle breeds

Breed	n	Haplotype frequency					
		A	B	C	D	X	Y
SO	25	1,00	0,00	1,00	0,00	1,00	0,00
PO	25	0,40	0,60	0,64	0,36	0,00	1,00
Total	50	0,70	0,30	0,82	0,18	0,50	0,50

n=individuals haplotyped.

Based on the haplotype data of the mitochondrial DNA (Table 4), the B (HinfI), D (HaeIII), and Y (XbaI) haplotypes were found only in the PO cattle breed samples. Meanwhile, the X (XbaI) haplotype was found only in the SO cattle breed samples. According to the frequency value of the X and Y haplotypes in the SO and PO cattle breeds, these haplotypes were very potential to become a differentiation factor between the SO and PO cattle breeds.

Conclusions

It can be concluded that the TGLA227 and ETH225 loci (based on microsatellite markers) and the B, D, X, and Y haplotypes (based on the mitochondrial DNA) can be considered as the differentiation factors between the SO and PO cattle breeds.

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