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Genetic differentiation between wildtype and spotted swamp buffaloes (*Bubalus bubalis*) of Indonesia based on ISSR and RAPD markers

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Abstract

The swamp buffaloes (*Bubalus bubalis*) are the important livestock that used for draught, meat production and cultural tradition ceremony in Indonesia. This study was aimed to characterize the wildtype and spotted buffaloes based on inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. Total of twenty-two buffaloes (13 wildtype and nine spotted) were used for the experimental animals. Two ISSR primers (P01, P02) and three RAPD primers (SRILLS-2, SRILLS-5, SRILLS-6) were assessed to discriminate two types of buffalo. Results showed that the ISSR primers had a higher of polymorphic informative content (PIC) value than RAPD primers. Therefore, P01 had the highest of polymorphic informative content (PIC) value (0.412) and SRILLS-5 as the lowest (0.063). The Shannon's diversity index (I) value in both technics were >1.00 and indicating a various fragments size resulted in each primer. However, the RAPD technique can discriminate two types of buffalo better than ISSR technique. In the pool animals, haplotype one of SRILLS-6 (16 heads) was more frequent than other haplotypes and followed by and haplotype two of P02 (14 heads). Despite this, two types of swamp buffalo in the present study also can be discriminated with combination of ISSR and RAPD technics accurately. In conclusion, the ISSR and RAPD primers in the present study can be used as the genetic markers to differentiate swamp buffaloes of Indonesia.

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Introduction

Swamp buffaloes (*Bubalus bubalis*) are the important livestock in Indonesia that kept for many purposes such as meat production and draught resource. Otherwise, the buffaloes are used in the cultural tradition ceremony in many places of Indonesia. However, the population of buffalo in Indonesia was decreased from 1,154,226 heads in year 2020 to 556,794 heads in year 2024 (1). Actually, a spotted coat colour patterns of buffalo was found in Indonesia at Toraja region of South Sulawesi (2) and Mamasa district of West Sulawesi (3). In year 2012, the buffaloes at Toraja region including of spotted buffalo have been designated as the Indonesian buffalo through the Indonesian Ministry of Agriculture Decree No: 2845/Kpts/LB430/8/2012 (4). According to the mitochondrial D-loop region, the spotted

buffalo (Toraya buffalo) are classified in the separated cluster with Indonesian buffaloes from Sumatera, Java, and South Sulawesi (5). Actually, a spotted colour pattern in the Toraya buffalo was caused by a nonsense mutation of c.328C>T (p. Arg110) and c.840+2T>A (p. Glu281_Leu282Ins8) in the *Microphthalmia-associated transcription factor* (*MITF*) gene (6). Presently, a genetic diversity in the animals can be evaluate with inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) methods (7). Previously, an ISSR technique has been used for genetic characterization in sheep (8-10), goat (11-13), cattle (14-16), horse (17) and buffalo (18). While evaluation of the genetic diversity with a RAPD technique has been performed in many livestock animals such as sheep (19), goat (20,21), cattle (22,23), horse (24) and buffalo (25-27). Unfortunately, a study to evaluate the genetic diversity in the livestock based on ISSR and RAPD technics were not reported. However, both technics have been used for genetic characterization in the silkworm / *Bombyx mori* (28).

This study was aimed to characterize wildtype and spotted swamp buffaloes in Indonesia using ISSR and RAPD technics. The results in the present study are essential for the breeding and genetic conservation programs for Indonesian swamp buffalo in the future.

Materials and methods

Ethical approval

This study obtained approval from the scientific broad, National Research and Innovation Agency, Indonesia. (Approval issue 050/KE.02/SK/03/2023).

Sample collection and research site

Twenty-two heads of swamp buffaloes (mixed sex) with wildtype (13 heads) and spotted (9 heads) coat colour patterns of swamp buffalo (*Bubalus bubalis*) were used for the investigation. The wildtype buffaloes (black colour) were kept with semi-extensive system at Sijunjung Regency of West Sumatera, Indonesia. While the spotted buffaloes were kept with intensive system at Toraja Regency of South Sulawesi, Indonesia (Figure 1). Amount 3 ml of blood samples from each animal were taken from jugular vein using venoject vacutainer tube containing EDTA. The DNA extraction was performed with Genomic DNA extraction kit (Geneaid, Taiwan) following the manufacturer's instructions and stored on freezer (-20°C) until the further analysis.

PCR amplification

The PCR reaction for ISSR and RAPD technics were performed in a total volume of 10 µl consisted of 3 µl of DNA template (21.59-49.15 ng/µl of DNA concentration); 5 µl of PCR master mix; 0.4 µl of primer and 1.6 µl of nuclease-free water. Two ISSR primers (P01 and P02) and three RAPD primers (SRILLS-2, SRILLS-5 & SRILLS-6) were used for genetic characterization of investigated animals (Table 1). The amplification of ISSR and RAPD markers were performed in 1 cycle of pre-denaturation at 94 °C for 4 min and following 35-45 cycles of denaturation at 94 °C for 1 min; annealing at 50.4-60 °C for 1 min; initial extension at 72 °C for 2 min and final extension at 72 °C for 7 min. Therefore, DNA visualization was performed in 2% stained agarose gel and captured with G-box documentation system (UVITEC, UK).

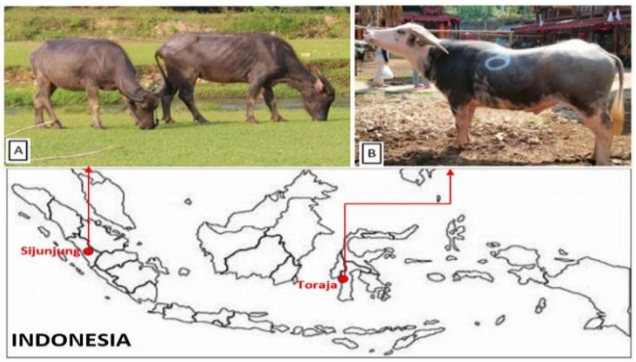

Figure 1: The phenotype characteristic of wildtype swamp buffalo (personal picture) from Sijunjung Regency (A) and spotted swamp buffalo from Toraja Regency (B) of Indonesia.

Table 1: Primer list for ISSR and RAPD analyses

Marker	Primer ID	Sequence	Temperature (°C)	Reference
ISSR	P01	5'- AGA GAG AGA GAG AGA GAG C -3	50.4	(9)
	P02	5'- GAG AGA GAG AGA GAG AGA C -3'	50.4	
RAPD	SRILLS-2	5'- CCC AGG AAC TGA TCG CAC AC -3'	59.1	(27-29)
	SRILLS-5	5'- GGC AAG CTG GTG GGA GGT AC -3'	60.0	
	SRILLS-6	5'- ATG TGT GCG ATC AGT TGC TG -3'	56.0	

Five genetic diversity parameters were evaluated in this study belonging to the total of DNA fragments, number of polymorphic loci, number of effective alleles / fragments (n_e), Shannon's diversity index (I) and polymorphic informative content (PIC). While the n_e, I and PIC values were calculated using mathematical formula (30-32) as follow: $n\_e=1/(\sum P_i^2)$, $I=-\sum p_i \, Ln(P_i)$, $PIC=1-\sum P_i^2$. where, P_i is the frequency of the i^{th} allele. Therefore, a Heatmapper

package (33) was used to obtain the dendogram of wildtype and spotted swamp buffaloes based on ISSR, RAPD and both combination analyses.

Data analysis

Five genetic diversity parameters were evaluated in this study belonging to the total of DNA fragments, number of polymorphic loci, number of effective alleles / fragments (n_e), Shannon's diversity index (I) and polymorphic informative content (PIC). While the n_e, I and PIC values were calculated using mathematical formula (30-32) as follow: $n\_e=1/(\sum P_i^2)$, $I=-\sum p_i \text{ Ln}(P_i)$, $PIC=1-\sum P_i^2$. where, P_i is the frequency of the i^{th} allele. Therefore, a Heatmapper package (33) was used to obtain the dendogram of wildtype and spotted swamp buffaloes based on ISSR, RAPD and both combination analyses.

Results

In this study, four haplotype (H) patterns in were observed in each P01, P02 and SRILLS-2 markers. While, SRILLS-5 and SRILLS-6 had five and three haplotype patterns, respectively (Table 2). In the ISSR technique, the highest number of DNA fragments were found in H3 of P01 marker (10 fragments) and H2 of P02 marker (9 fragments). Meanwhile, the highest number of DNA fragments in each RAPD marker were found in H1 of SRILLS-6 (11 fragments) and H4 of SRILLS-5 (7 fragments). In the SRILLS-2 marker, the H1, H3 and H4 had the similar number of fragments (5 fragments) and these value were the highest than other haplotypes (Table 2). Interestingly, many haplotypes in the P01 (H4) and P02 (H1, H3) primers were absence in the spotted buffaloes. While, many haplotypes in the SRILLS-2 (H1, H2), SRILLS-5 (H1, H2, H3) and SRILLS-6 (H2) were absence in the spotted buffaloes. In general, H2 (P02) and H1 (SRILLS-6) were most frequently observed in the pool animals.

Two ISSR primers and three RAPD primers were polymorphic with the PIC value of 0.78 - 0.92 (Table 3). In the pool animals, the n_e value in P01 and P02 markers were about 12 and showed higher than three RAPD primers under study. While, the n_e value in the pool animals with SRILLS-6 was 11.79 that the highest value than SRILLS-2 (8.23) and SRILLS-5 (10.33). The results of amplification in two ISSR primers (P01, P02) and three RAPD primers (SRILLS-2, SRILLS-5, SRILLS-6) were ilustrated in figures 2 and 3, respectively. Otherwise, the RAPD and combination combination ISSR-RAPD technics were more accurate to characterize two different patterns of buffalo under study (Figure 4). According to Figure 4, the two ISSR primers in this study can not discriminate two different color patterns of buffalo accurately. Subsequently, the wildtype buffaloes were classified in two different clusters of wildtype A (9 heads) and wildtype B (4 heads) based on both ISSR primers.

Table 2: Haplotype diversity in each primer for wildtype and spotted swamp buffaloes (*Bubalus bubalis*) of Indonesia

Primer ID	Haplotype	Number of fragments	Length (bp)	Number of observed individuals		
				Wildtype	Spotted	Total
P01	1	5	2000; 1400; 1100; 800; 750	5	1	6
	2	8	2100; 1500; 1400; 1300; 1000; 990; 850; 700	0	6	6
	3	10	2100; 1500; 1400; 1300; 1000; 990; 850; 700; 550; 490	2	2	4
	4	5	1000; 800; 700; 500; 480	3	0	5
P02	1	7	2500; 2000; 1700; 1300; 1100; 800; 600	3	0	3
	2	9	2500; 2000; 1800; 1700; 1300; 950; 800; 600; 550	6	8	14
	3	4	1100; 1000; 800; 650	1	0	1
	4	6	2500; 1600; 1500; 1400; 1100; 600	0	1	1
SRILLS-2	1	5	1100; 800; 700; 550; 350	5	0	5
	2	2	1600; 1100	2	0	2
	3	5	1600; 1100; 800; 700; 350	1	6	7
	4	5	2500; 1600; 1100; 800; 700	0	1	1
SRILLS-5	1	2	2500; 600	5	0	5
	2	2	2500; 2000	3	0	3
	3	3	2500; 2400; 1400	1	0	1
	4	7	1600; 1000; 900; 800; 600; 500; 350	0	4	4
	5	8	2400; 1600; 1000; 900; 800; 600; 500; 350	0	3	3
SRILLS-6	1	11	2500; 1600; 1500; 1400; 1300; 1200; 1000; 700; 600; 500; 400	10	6	16
	2	10	2500; 2300; 1500; 1400; 1300; 1200; 1000; 800; 700; 500	2	0	2
	3	7	1500; 1200; 1000; 800; 700; 600; 500	0	1	1

N: number of observations

Table 3: Genetic diversity in each primer for wildtype and spotted swamp buffaloes (*Bubalus bubalis*) of Indonesia

Primer ID / Marker	Pattern	n_e	I	PIC
P01 / ISSR	Wildtype	12.93	2.58	0.78
	Spotted	10.95	2.44	0.86
	Pool	12.18	2.56	0.90
P02 / ISSR	Wildtype	9.45	2.31	0.91
	Spotted	10.63	2.40	0.91
	Pool	12.51	2.57	0.92
SRILLS-2 / RAPD	Wildtype	4.84	1.72	0.92
	Spotted	6.37	1.88	0.91
	Pool	8.23	2.19	0.92
SRILLS-5 / RAPD	Wildtype	4.45	1.61	0.89
	Spotted	7.34	2.02	0.91
	Pool	10.33	2.29	0.92
SRILLS-6 / RAPD	Wildtype	11.24	2.47	0.79
	Spotted	10.75	2.41	0.91
	Pool	11.79	2.50	0.88

n_e: number of effective alleles/fragments; I: Shannon's diversity index; PIC: polymorphic informative content.

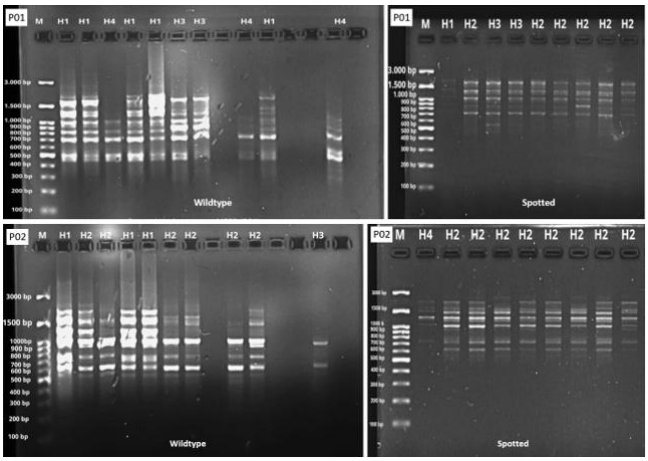

Figure 2: The patterns of haplotype (H) fragment from two ISSR primers (P01 and P02) in wildtype and spotted swamp buffalo (*Bubalus bubalis*) of Indonesia. M: DNA ladder 100 bp.

Discussion

In Bali cattle (*Bos javanicus*), a P01 primer in the ISSR technique was polymorphic with presence of three haplotypes with PIC value of 0.46 (16). Despite this, the P01 primer were polymorphic in Tuvinian sheep (*Ovis aries*) with PIC value of 0.25 to 0.45 (34). Otherwise, P01 and P02 primers in the Saburai goats had the PIC value of 0.87 and 0.67, respectively (13). Contrast, the P01 and P02 primers had the PIC value of 0.06 in Russian cattle and 0.07 in Friesian Holstein cattle (15,35). In the Arabian horses (*Equus caballus*), P01 and P02 primers had the PIC value of 0.28 and 0.44, respectively (17). Subsequently, P01 and P02 primers in the Anatolian water buffaloes had the PIC value of 0.13-0.24 (6-8 fragments) and 0.19-0.59 (4-11 fragments), respectively (36). The PIC value can be described as low (<0.10), moderate (0.11 - 0.30) and high (>0.30) categories (37). In addition, the markers were classified as informative when PIC was > 0.50 (18).

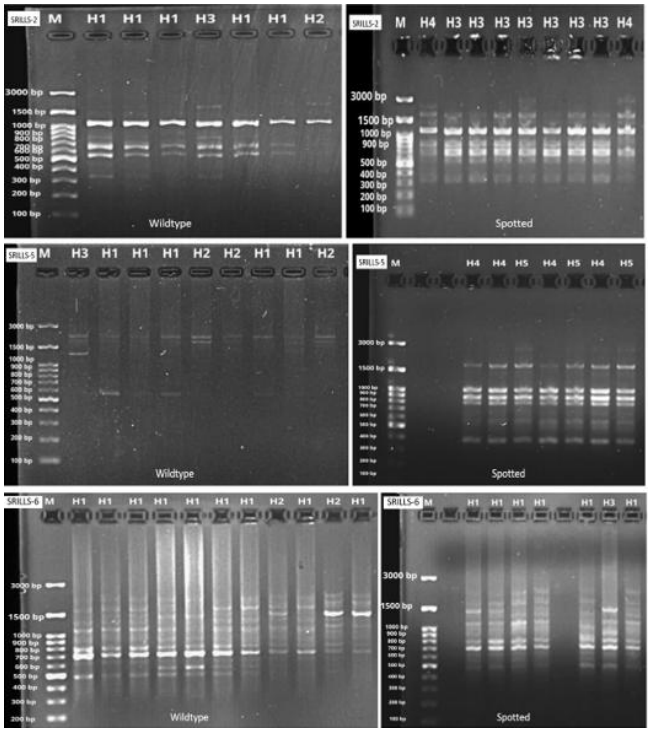

Figure 3: The patterns of haplotype (H) fragment from three RAPD primers (SRILLS-2; SRILLS-5 and SRILLS-6) in wildtype and spotted swamp buffalo (*Bubalus bubalis*) of Indonesia. M: DNA ladder 100 bp.

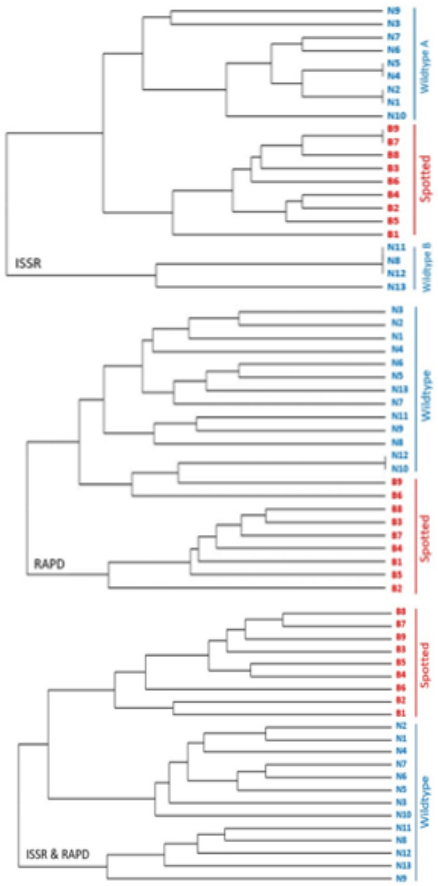

Figure 4: Dendrogram between wildtype (N-) and spotted (B-) swamp buffaloes of Indonesia based on ISSR, RAPD and both combination technics.

In this study, total number of fragments in Indonesian swamp buffaloes were close to the Anatolian water buffaloes with 5-10 fragments for P01 marker and 4-9 fragments for P02 marker. The different results of PIC and number of fragments among buffalo breeds can be caused by the difference of genetic composition in each breed of buffalo. Two ISSR markers in the present study was not accurate for discriminating wildtype buffaloes under study. However, a previous study reported the similar results in Anatolian water buffaloes from Afyon population (36). In this case, many water buffaloes from Afyon region was grouped in separated cluster according to 11 ISSR primers. Moreover, two ISSR primers of $(AC)_9T$ and $(CA)_9T$ can discriminate two coat colour types of Markhoz goats (38).

In general, a primer for RAPD technique in the buffalo is short with length about 10 bp and known as OP primers (25,26,39,40). Nonetheless, a different primer has been performed the RAPD technique in buffaloes with the length of 20 bp and known as SRILLS primers (29). According to the SRILLS primers the swamp and water buffaloes of Philippines can be discriminated with SRILLS-2, SRILLS-5 and SRILLS-6. Therefore, these primers also capable to distinguish wildtype and spotted swamp buffalo in the present study accurately. Despite of ISSR and RAPD technics, a microsatellite technique with specific primers can be used to discriminate two different phenotypic traits of livestock including to characterize of polled Bali cattle, horned Bali cattle and Banteng (41). In the present study, the I value in each primer were more than 1.00 and indicating a various fragments size resulted from each primer. The I value usually ranges from 1.50 to 3.50 (42). However, an advanced technology of genome wide association study (GWAS) can be performed to detect the genetic marker for coat colour patterns of buffalo accurately (43).

Conclusion

The primers in the ISSR and RAPD technics were polymorphic with PIC value more than 0.70. However, RAPD technique and combination of ISSR-RAPD technique able to differentiate the wildtype and spotted swamp buffaloes accurately.

Acknowledgment

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Conflict of interest

The authors declare that there is no conflict of interest in the manuscript.

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التمايز الجيني بين جاموس المستنقعات البري والمرقط في إندونيسيا بناءً على علامات *ISSR* و *RAPD*

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الخلاصة

يعد جاموس المستنقعات (*Bubalus bubalis*) من الثروة الحيوانية الهامة التي تستخدم للجر واللحوم وطقوس التقاليد الثقافية في إندونيسيا. هدفت هذه الدراسة إلى توصيف الجاموس البري والجاموس المرقط بناءً على العلامات الجينية *ISSR* و *RAPD*. تم استخدام إجمالي اثنين وعشرين جاموسًا (١٣ جاموسًا بريًا وتسعة جواميس مرقطة) للحيوانات التجريبية. تم تقييم اثنين من بادئات *ISSR (P01*، *(P02* وثلاثة بادئات *RAPD (SRILLS-2*، *SRILLS-5*، *(SRILLS-6* للتمييز بين نوعين من الجاموس. أظهرت النتائج أن بادئات *ISSR* كانت ذات قيمة محتوى معلوماتي متعدد الأشكال (*PIC*) أعلى من بادئات *RAPD*. لذلك، كان لدى *P01* أعلى قيمة محتوى معلوماتي متعدد الأشكال *(PIC)* (٠,٤١٢) و *SRILLS-5* كان الأقل (٠,٠٦٣). كانت قيمة مؤشر التنوع شانون (*I*) في كلتا التقنيتين > ١,٠٠ وتشير إلى حجم شظايا متنوعة ناتجة عن كل بادئ. ومع ذلك، يمكن لتقنية *RAPD* التمييز بين نوعين من الجاموس بشكل أفضل من تقنية *ISSR*. في تجمع الحيوانات، كان النمط الفرداني الأول من *SRILLS-6* (١٦ رأسًا) أكثر شيوعًا من الأنماط الفردانية الأخرى وتبعه النمط الفرداني الثاني من *P02* (١٤ رأسًا). على الرغم من ذلك، يمكن أيضًا تمييز نوعين من جاموس المستنقعات في الدراسة الحالية بدقة باستخدام مزيج من تقنيات *ISSR* و *RAPD*. في الختام، يمكن استخدام بادئات *ISSR* و *RAPD* في الدراسة الحالية كعلامات جينية للتمييز بين جاموس المستنقعات في إندونيسيا.