ARTICLE

Genotyping and assessment of microsatellite DNA markers for genetic diversity and potential forensic efficacy of Philippine Carabao (*Bubalus bubalis*) swamp buffalo

Aivhie Jhoy DS. Escuadro* and Lilian P. Villamor

Animal Genetic Resources Section, Cryobank Unit, Philippine Carabao Center, National Headquarters and Genepool, Science City of Munoz, Nueva Ecija, 3120, Philippines

ABSTRACT

The genetic characterization of the Philippine carabao (PC) (*Bubalus bubalis*) swamp buffalo population contributes a significant role in determining the genetic variation and population differentiation for strategic conservation management. This study was undertaken to evaluate the 30 FAO microsatellite loci for the allele frequencies, polymorphism, and potential forensic efficacy for recommending a set of marker panel for genetic diversity and parentage testing of Philippine Carabao. A total of 277 alleles were observed in 488 unrelated individuals from the PC population. The mean number of alleles per locus was 9.2, ranged from 19 (DRB3) to 1 (HMHIR, ILSTS008, and RM099), while the PIC generated a mean value of 0.547 ranging from 0.083 (CSSM045) to 0.811 (DRB3). Furthermore, the exact test conducted for HWE revealed that most markers had significantly deviated from the equilibrium *(P<0.05)*. Twenty-seven STR markers are highly and reasonably informative loci, which are suitable DNA markers in determining the genetic variation of the Philippines swamp populations. Moreover, three loci showed potential use in forensic studies with 73.8% combined power of exclusion and 99.5% combined discriminatory power to distinguish samples from different individuals. Thus, this study provided baseline information on the applicability of FAO microsatellite markers to determine the genetic diversity and potential forensic efficacy in the Philippine swamp buffalo populations.

*Corresponding author
Email Address: aivhiejhoyescuadro@gmail.com
Date received: May 25, 2021
Date revised: October 22, 2021
Date accepted: October 26, 2021

KEYWORDS

polymorphism, heterozygosity, microsatellite DNA markers, forensic efficacy, Philippine Carabao

INTRODUCTION

The Philippine carabao (*Bubalus bubalis*) swamp buffalo is one of the economically important livestock species, specifically in many agricultural provinces in the country. Swamp buffaloes play a vital role in the sustainability of farmlands for it is primarily considered as a draft animal. However, a conclusive study on its genetic diversity is still uncertain.

Molecular markers are powerful tools for understanding the evolution and genetic diversity based on DNA sequence polymorphism (Navani et al. 2002). The mitochondrial DNA (mtDNA) has been extensively used in phylogenetic and genetic diversity analyses. Since the haploid mtDNA carried by the mitochondria has a maternal mode of inheritance and a high mutation rate, it has become an excellent source of information in evolutionary studies. During the past years, few samples of the Philippine carabao sub-population had been part of genetic diversity studies of Asian water buffaloes using the mitochondrial DNA D-loop and cytochrome *b* molecular markers (Barker et al. 1997; Lau et al. 1998). Other studies proved that the mtDNA known cytochrome c oxidase subunit I (COI) is one of the most conserved mitochondrial protein-coding genes in animals that displayed significant phylogenetic signal (Lunt et al. 1996; Mueller, 2006; Wilson-Wide et al. 2010; Paraguas et al. 2018). In the Philippines, the COI gene marker revealed its usefulness in discriminating breeds of *B. bubalis* swamp-type with the riverine-type and confirming the identity of Calayan swamp buffaloes into its subspecies level (Paraguas et al. 2018). However, information extracted from mtDNA could be limited to the maternal lineage. In support of the mitochondrial information, another genetic marker from a nuclear gene would be highly recommended.

The microsatellite markers, known as the short tandem repeats (STRs), are informative markers used to understand population genetics, genetic divergence estimation, parentage testing, and individual identification (Hoffmann et al. 2004). The Food and Agriculture Organization (FAO) recommended 30 microsatellite loci that can be used to assess the genetic diversity and population structure among buffaloes. However, in developing the genetic maps, the characterization and optimization of polymorphic microsatellite markers were suggested (Nagarahan et al. 2009). These microsatellite markers were previously used to determine the genetic diversity of Southeast Asian buffalo breeds (Barker et al. 1997). With the advent of molecular genetic markers, microsatellite loci were now widely used to evaluate the genetic diversity of water buffaloes from various countries in Asia, including China (Zhang et al. 2008), Guilan, Iran (Aminafshar et al. 2008), India (Tantia et al. 2006), and Vietnam (Berthouly et al. 2010). The previous report of Cacho et al. (2013) utilized 110 cattle microsatellite markers for four water buffalo breeds from the institutional herds in the Philippines. However, only 10 STR loci indicated polymorphism in 26 swamp buffaloes. The limited number of loci and animals could be insufficient to capture the overall genetic diversity of PC.

Considering the potential of microsatellite markers, it is necessary to first evaluate the utility of these markers in genotyping the Philippine carabao. This study was undertaken to evaluate the 30 FAO microsatellite marker for the information content, heterozygosity, and its potential efficacy for recommending a set of marker panels for genetic diversity and parentage testing of Philippine carabao.

MATERIALS AND METHODS

Sites and sample selection

Selection of these areas was properly coordinated with the PCC regional centers and respective LGU's to ensure that there was no introduction of riverine buffaloes through artificial insemination programs. Sampling sites were identified as isolated areas such as mountainous parts and coastal islands of the country. In this study, 488 fresh blood samples of Philippine swamp buffalo were randomly selected from 27 Philippine different populations of Luzon, Visayas, and Mindanao islands (Table 1). The distinct morphological features, including one to two white chevrons on the neck, white stocking on the forelegs and hindlegs, and sickle-curve horn shape, were considered preliminary evaluation of animals as swamp buffaloes.

Table 1: Sample size of Philippine Carabao from Luzon, Visayas, and Mindanao that were included in the analysis.

Major Island	Administrative Region	Population	No. of Samples
LUZON	Ilocos Region	Ilocos	16
		Pangasinan	14
		La Union	15
	Cagayan Valley	Calayan Island	35
		Cagayan	21
		Batanes	27
	Cordillera Administrative Region	Kalinga	18
		Benguet	12
	Central Luzon	Aurora	15
		Zambales	14
	Southern Tagalog	Carabao Island, Romblon	33
		Occ. Mindoro	13
	Bicol Region	Camarines Sur	14
		Sorsogon	14
VISAYAS	Western Visayas	Capiz	22
		Guimaras	20
		Gigantes Island, Ilo Ilo	9
	Central Visayas	Pitogo Island, Bohol	35
		Talibon, Bohol	11
	Eastern Visayas	Samar	7
		Leyte	6
MINDANAO	Northern Mindanao	Bukidnon	15
	Southern Mindanao Soccsksargen	Davao	20
		South Cotabato	20
		North Cotabato	11
		Sultan Kudarat	25
		Sarangani	26
TOTAL	12	27	488

DNA Isolation and PCR Optimization

Genomic DNA was extracted from the whole blood samples using the commercially available DNA extraction kit from RealiPrep™ Blood gDNA with some modifications (Villamor et al. 2021). The polymerase chain reaction (PCR) was composed with the final volume of 15µl and concentration of the following; 1X colorless GoTaq® reaction buffer, 1.33mM $MgCl_2$, 0.2mM dNTPs, 0.5µM of each forward and reverse primer, 1 Unit GoTaq® Polymerase, and at least 3.33ng of genomic DNA (gDNA).The PCR thermal cycling conditions were optimized for all 30 STR markers used in the study with initial denaturation at 95˚C for 2 minutes, followed by 35 cycles of 95˚C for 30 seconds, 48-67.1˚C for 30 seconds, 72˚C for 30 seconds, and final extension of 72˚C for 5 minutes. The PCR products with various allele sizes were visualized using 2% agarose in gel electrophoresis by GelRed™ staining. Amplified PCR products were sent to 1stBASE Sequencing Malaysia for fragment analysis. All loci with at least a 95% success rate of amplified PCR products were included in the analysis.

Statistical data analysis

The genotyping for each locus was scored using Geneious Prime® (v2021.1 Biomatters Ltd.). Microsatellite plugin was downloaded in order to remove excess peaks following the

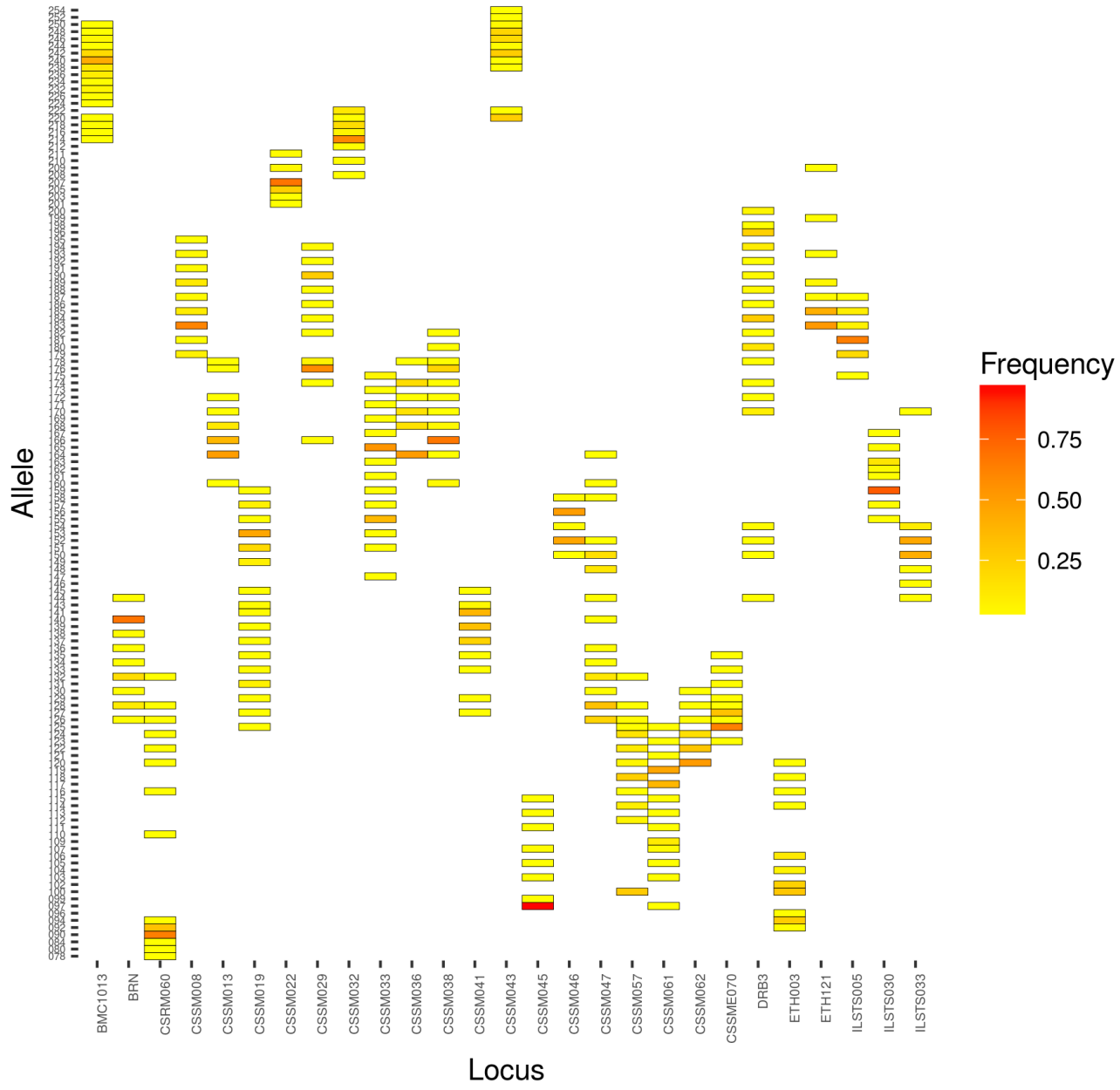

Figure 1: Heatmap of the allele frequencies in the Philippine Carabao populations across 27 microsatellite loci.

internal size standard of GeneScan™ 500LIZ™. Allele scores and bins were manually checked and exported through the .csv file. The genetic diversity parameters for the number of alleles per locus (N_a), observed (H_o), and expected heterozygosity (H_e) were computed using GenAlEx 6.503 software (Peakall and Smouse, 2012). The polymorphism information content (PIC) was calculated using Cervus 3.0 (Marshall et al. 1998). The criterion for PIC by Botstein et al. (1980) was used to define the varying degrees of PIC, highly informative for PIC $\geq$ 0.5, reasonably informative for 0.5 > PIC > 0.25, and slightly informative for PIC < 0.25. The exact test for the deviation from Hardy-Weinberg equilibrium (HWE) was calculated using Cervus 3.0 (Marshall et al. 1998). The null allele frequency (r) was estimated using the Micro-checker (Van Oosterhout et al. 2004) and interpreted following the criteria by Chapuis and Estoup (2006) wherein frequency of ($r < 0.05$) was negligible, moderate ($0.05 \leq r < 0.20$), or large ($r \geq 0.20$). The microsatellite markers were further evaluated for potential parentage capability and forensic efficacy based on the recommendation of Fernando, (2013 unpublished). The probability of exclusion (PE) and the combined power of exclusion (CPE), discriminant capability (PD), and the combined discriminant capability (CPD) were calculated using FORSTAT v1.0 software (https://fdl-uwc.shinyapps.io/forstat/).

RESULTS

A total of 277 alleles across 30 loci ranged from 1 allele (RM099, ILSTS008, and HMHIR) to 19 alleles (DRB3) (Figure 1). Results revealed 17 highly informative markers (PIC $\geq$ 0.5), 10 reasonably informative (0.5 > PIC > 0.25), one slightly informative marker (PIC < 0.25), and three monomorphic markers. The mean PIC value of 0.547, ranged from 0.811 (DRB3) to 0.083 (CSSM045). However, 27 microsatellite DNA markers detected a reduced number of observed than the expected heterozygosity. The locus DRB3 presented the highest expected heterozygosity of 0.831, while CSSM045 had the lowest of 0.084 (Table 2).

Three out of 27 loci, including CSSM047, CSSM013, and CSSM033, have been noted as suitable markers for forensic

Table 2: Genetic diversity measures and null allele frequencies of 30 microsatellite loci in Philippine Carabao populations.

LOCI	N_a	H_o	H_e	PIC	*r*
DRB3	19	0.505	0.831	0.811[a]	0.2329***
CSSM057	12	0.725	0.818	0.793[a]	0.0663**
CSSM047	14	0.763	0.791	0.760[a]	0.0190*
CSSM043	11	0.603	0.779	0.742[a]	0.1472**
ETH003	11	0.702	0.767	0.728[a]	0.0463*
BMC1013	16	0.679	0.747	0.717[a]	0.0389*
CSSM019	17	0.603	0.734	0.707[a]	0.1047**
CSSM041	9	0.610	0.691	0.631[a]	0.0601**
CSSM036	6	0.577	0.663	0.617[a]	0.0801**
CSSM061	13	0.568	0.640	0.573[a]	0.0464*
CSSM008	9	0.477	0.590	0.565[a]	0.0956**
CSSM062	6	0.528	0.621	0.554[a]	0.0839**
CSSM032	8	0.541	0.592	0.553[a]	0.0437*
ILSTS033	7	0.534	0.612	0.532[a]	0.0645**
CSSM029	11	0.500	0.584	0.532[a]	0.0787**
CSSM013	8	0.566	0.601	0.523[a]	0.0287*
CSSM033	14	0.567	0.580	0.503[a]	0.0107*
ILSTS005	6	0.482	0.537	0.492[b]	0.0561**
ETH121	7	0.480	0.563	0.472[b]	0.0566**
BRN	9	0.476	0.490	0.450[b]	0.0121*
CSSM038	11	0.423	0.494	0.446[b]	0.0825**
CSSM046	5	0.322	0.541	0.435[b]	0.2685***
CSSME070	9	0.466	0.498	0.417[b]	0.0098*
CSSM022	6	0.371	0.465	0.403[b]	0.1149**
CSRM060	14	0.435	0.487	0.400[b]	0.0328*
ILSTS030	8	0.290	0.369	0.343[b]	0.0987**
CSSM045	8	0.052	0.084	0.083[c]	0.2593***
RM099	1				
ILSTS008	1				
HMHIR	1				
Total no. of alleles	**277**				
Mean	**9.2**	**0.512**	**0.598**	**0.547**	

N_a = Number of alleles; **H_o** = Observed heterozygosity; **H_e** = Expected heterozygosity; **PIC** = Polymorphism information content; [a]Highly informative [b]Reasonably informative [c]Slightly informative marker; *r* = Null alleles frequencies (Chakraborty), *Negligible **Moderate ***Large

efficacy, with an expected heterozygosity greater than 0.6, PIC value of above 0.5, a locus with more than five alleles, and absence of null alleles. Forensic assessment of these loci resulted in PE with the highest value of 0.531 in CSSM047, the lowest value of 0.252 in CSSM013, and with CPE of 0.738. These markers also revealed PD, ranging from 0.926 in CSSM047 to 0.734 in CSSM013 and CPD of 0.995 (Table 3 and Figure 2).

DISCUSSION

Allele frequency, polymorphism, and heterozygosity

The evaluation of 27 STR loci showed a substantial amount of alleles and polymorphism, which detected abundant genetic diversity in the PC population. This indicated the applicability of the microsatellites in determining the PC genetic diversity. The allele frequency and an average PIC value obtained from this study were significantly higher than the 96 alleles and average PIC value of 0.492 was observed in the previous study of Philippine swamp buffalo (Cacho et al. 2013). The previous study reported the limited number of samples collected from the institutional herd of the Philippine Carabao Center (PCC). It could be insufficient to capture the overall genetic diversity of PC. In this present study, several factors, including the increase in samples, collection sites, and loci, could explain the remarkable increase in the number of alleles and polymorphism content obtained in the PC population. Thus, information based on the high allele frequency and higher polymorphism (PIC > 0.5) are distinctly informative in population genetic studies (Reinosa et al. 2015) following the standard category as described by Botstein et al. (1980). However, three STR markers that exhibited a single allele per locus were considered non-informative genetic markers. A similar study of Zhang et al. (2008) in Chinese buffaloes reported that RM099, HMH1R, and ILSTS008 generated only one allele both in the swamp and riverine buffaloes except for RM099 that generated two alleles for the riverine. On the other hand, the reduced number of

Table 3: Forensic efficacy parameters for the three STR loci.

Statistical Parameters	STR Loci		
	CSSM033	CSSM013	CSSM047
PD	0.734	0.765	0.926
PM	0.265	0.234	0.073
PE	0.253	0.252	0.531
PI	1.154	1.152	2.105

PD = Discriminatory Power, **PM** = Match Probability, **PE** = Power of Exclusion, **PI** = Paternity Index

observed than the expected heterozygosities could contribute to the general excess of homozygotes. This pattern agreed with the report of Cacho et al. (2013) that described the observed excess homozygosity in four breeds of Philippine water buffalo populations could be explained by the breeding scheme in the country. Similar with the previous reports, the observed homozygote excess in the present study could also be linked to the farm animals included in the analysis that has no structured pedigree records and breeding programs. However, average heterozygosity between 0.3 to 0.8 indicated the capacity of loci to be used in the evaluation of populations' genetic variation (Takezaki and Nei, 1996).

HWE and null alleles

The significant deviation of the PC population from HWE of all STR markers could be attributed to genetic evolutionary forces such as the absence of random mating, migration, and genetic drift as described by Selkoe and Toonen, (2006). Relative to this result, loci that departed from the equilibrium also showed a high frequency of null alleles. This pattern was also detected in the study of Nagarahan et al. (2009), wherein 62% of the loci that deviated from HWE showed the presence of null alleles in water buffaloes in India. The validation of genotyping errors using MICRO-CHECKER software asserted that the presence of null alleles was caused by general excess in homozygosity and no indication of scoring errors attributed to stuttering or large allele dropout. Thus, identification of loci with significant deviation to HWE and presence of null alleles could not be grounds to discard genetic markers for population diversity analysis (Selkoe and Toonen, 2006). On the other hand, analysis involving the loci with significant deviation to HWE had less effect when allelic diversity was high and sample sizes were moderate (Guo and Thompson, 1992). If these loci will be used in the future for parentage testing, these markers, however, would require high accuracy of genotyping and exclusion of loci with null alleles (Selkoe and Toonen, 2006).

Potential forensic efficacy

The evaluation of three loci for their potential forensic efficacy indicated that single-locus PE and CPE of these markers indicated a low degree of exclusion power in comparison with the 99.9% CPE, which is sufficient to achieve the correct parentage in buffaloes (Kathiravan et al. 2012; Borghese, 2005), cattle (Heyen et al. 1997), and goats (Luikart et al. 1999).

The present study suggested that an increase of markers should be considered to obtain a higher power of exclusion. Alternatively, high CPD detected higher power in discriminating breeds between members of the population that was comparable to the 99.9% CPD obtained in Serbia cattle (Stevanovic et al. 2010) and in Spanish cattle (Jimenez-Moreno et al. 2015) that was considered as the required level of discrimination power in a parentage analysis (Vankan and Faddy, 1999; Perez-Miranda et al. 2005). Therefore, the CPD obtained in the present study also implied the capability of loci to distinguish different individuals with a probability of 99.5%.

Highly informative microsatellite markers confirmed their usefulness in determining the genetic diversity of Philippine carabao sub-populations in Luzon, Visayas, and Mindanao. Therefore, these markers should be useful for genetic admixture analysis, identification of possible population structure that

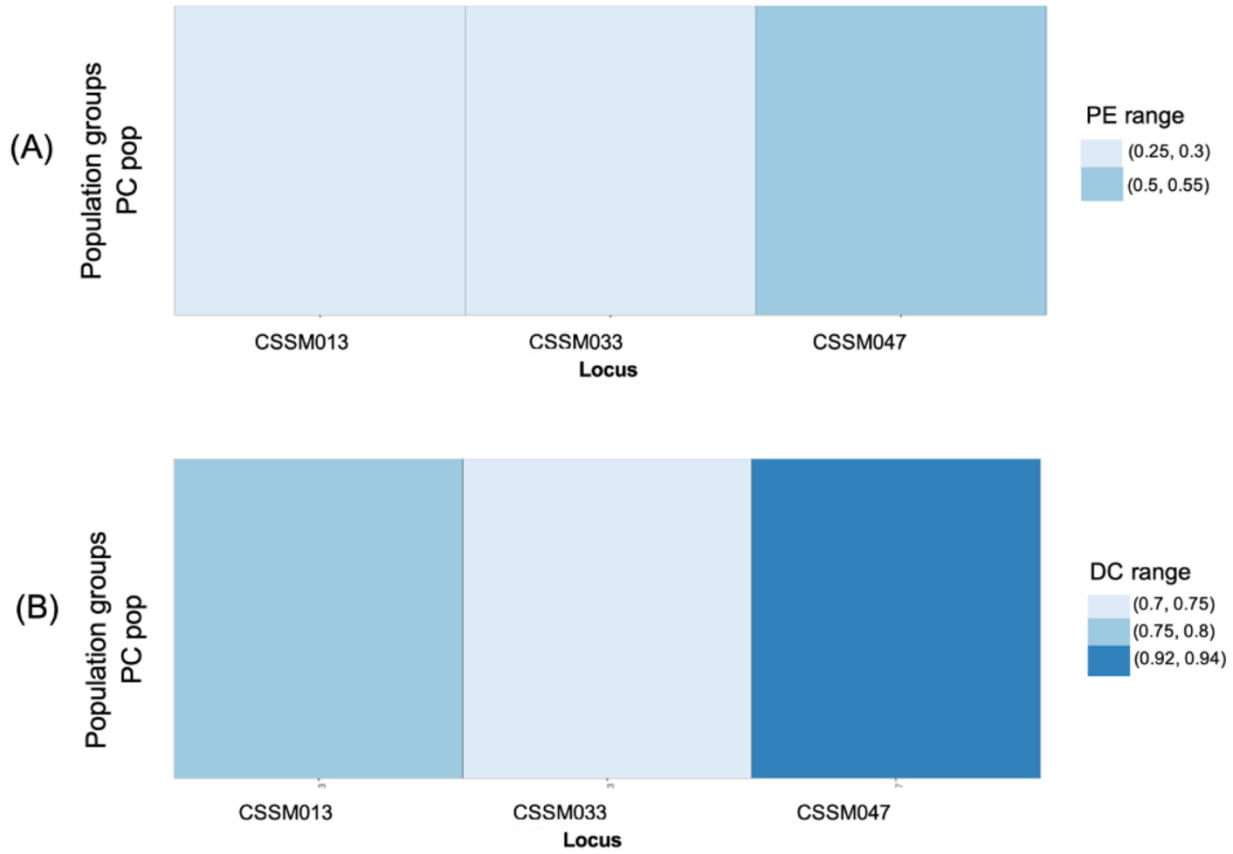

Figure 2: Heatmap of power of exclusion (A) and discrimination capacity (B) of three loci in the Philippine Carabao population.

could be a basis to strengthen PC conservation management. Moreover, the potential efficacy of three loci could be considered for future forensic studies. However, increasing the number of loci is recommended to achieve higher statistical power suitable for forensic applications such as paternity testing in the PC population.

CONCLUSION

The overall research findings highlighted the 27 microsatellite markers that are suitable in assessing the genetic diversity of the Philippine carabao sub-population. The study also highlighted the varying polymorphism information of the STR markers that could be utilized to reveal the PC genetic variation and population differentiation. Moreover, three STR markers confirmed their forensic efficacies that could be effectively utilized in forensic studies, such as parentage analysis and individual identification of the Philippine swamp buffaloes.

ACKNOWLEDGMENTS

This research gratefully acknowledged the financial support of the Department of Agriculture (DA) Philippine Carabao Center National Headquarters and Genepool as the implementing agency and the DA Biotechnology Program (DABIOTECHR-1506). The authors would like express gratitude to all the PCC regional centers, LGU's across the 27 provinces, and VBAIT's for their assistance during the sample collection across the country. Moreover, this research would not be possible without the individual efforts of PCC-Cryobank staff: Alexander M. Paraguas, and Therese Patricka C. Cailipan for assistance in the completion of this work.

CONFLICTS OF INTEREST

There is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

The overall concept of the study was led by LP Villamor. AJDS Escuadro and LP Villamor performed the fieldwork. AJDS Escuadro conducted the laboratory experiments and analysis of its results with the supervision of LP Villamor. AJDS Escuadro wrote the manuscript with substantial comment, suggestion and review of LP Villamor.

REFERENCES

Aminafshar M, Amirinia C, Vaez Torshizi R. Genetic diversity in buffalo population of Guilan using microsatellite markers. J Anim Vet Adv 2008; 7(11): 1499-1502.

Barker JSF, Moore SS, Hetzel DJS, Evans D, Tan SG, Bryne K. Genetic diversity of Asian water buffalo (*Bubalus bubalis*): microsatellite variation and a comparison with protein-coding loci. Anim Genet 1997; 28:103-115.

Berthouly C, Rognon X, Nhu Van T, Berthouly A, Thanh Hoang H, Bed'Hom B, Laloë D, Vu Chi C, Verrier E, Maillard JC. Genetic and morphometric characterization of a local Vietnamese swamp buffalo population. J Anim Breed Genet 2010; 127(1): 74-84.

Borghese A. Buffalo production and research. food and agriculture organization of the united nations (FAO). In:

Borghese A, ed. REU Technical Series. 67. Rome, Italy: 2005:49.

Botstein D, White RL, Skolnick M, Davis RW. Construction of genetic linkage map in man using restriction fragment length polymorphism. An J Hum Genet 1980; 32:314-331.

Cacho RGC, Han JM, Herrera JRV, Oh JD, Seong J, Song KD, Cruz LC, Kong HS, Lee HK. Genetic diversity analysis of water buffalo (*Bubalus bubalis* L.) Populations in the Philippines using Bovine microsatellite markers. Philipp J Vet Med 2013: 50(1): 7-15.

Guo SW, Thompson EA. Performing the exact test of hardy-weinberg for multiple alleles. Biometrics 1992; 48(2):361-372.

Heyen D, Beever J, Da Y, Evert R, Green C, Bates SR, Ziegle JS, Lewin HA. 1997. Exclusion probabilities of 22 bovine microsateliite markers in fluorescent multiplexes for semi-automated parentage testing. Anim Genet 1997; 28: 21-27.

Hoffmann I, Marsan PA, Barker JSF, Cothran EG, Hanotte O, Lenstra JA, Milan D, Weigend S, Simianer H. New MoDAD marker sets to be used in diversity studies for the major farm animal species. Recommendations of a Joint ISAG/FAO working group. Proc 29th Int Conf Anim Genet 2004; 107.

Jimenez-Moreno S, David G, Lopez-Oceja A, de Pancorbo M. 2015. Forensic efficacy of twelve STRs in Spanish cattle. Forensic Sci Int Genet Suppl Ser 2015; 5:253-255.

Kathiravan P, Kataria R, Mishra B. Power of exclusion of 19 microsatellite markers for parentage testing in river buffalo (*Bubalus bubalis*). Mol Biol Rep 2012; 39(8): 8217-8223.

Navani N, Jain PK, Gupta S, Sisodia BS, Kumar S. A set of Cattle microsatellite DNA markers for genome analysis of riverine buffalo (*Bubalus bubalis*). Anim Genet 2002; 33(2): 149-154.

Lau CH, Drinkwater RD, Yusoff K, Tan SG, Hetzel DJS, Barker JSF. 1998. Genetic diversity of Asian water buffalo (*Bubalus bubalis*), mitochondrial DNA D-loop and cytochrome *b* sequence variation. Anim Genet 1998; 29(4): 253–264.

Luikart G, Biju-Duval MP, Ertugrul O, Zagdsuren Y, Maudet C, Taberlet P. Power of 22 microsatellite markers in flourescent multiplexes for parentage testing in Goats (*Capra hircus*). Anim Genet 1999; 30(6): 431-438.

Lunt DH, Zhang DX, Szymura JM, Hewitt GM. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogentic studies. Insect Mol Biol 1996; 5(3): 153-165.

Marshall TC, Slate J, Kruuk LE, Pemberton JM. Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 1998; 7(5): 639-655.

Marques JRF, Martinez AM, Costa MR, Albuquerque M, Quiroz J, Vega-Pla J, Delgado JV. Genetic diversity of Brazilian buffaloes (*Bubalus bubalis*) using DNA microsatellites. Archivos de Zootecnia 2011; 60: 1213-1221.

Mueller RL. Evolutionary rates, divergence dates, and the performance of mitochondrial genes. Syst Biol 2006; 55(2): 289-300.

Nagarajan M, Kumar N, Nishanth G, Haribaskar R, Paranthaman K, Gupta J, Mishra M, Vaidhegi R, Kumar S, Ranjanm AK, Kumar S. Microsatellite markers of water buffalo, *Bubalus bubalis* - development, characterization and linkage disequilibrium studies. BMC Genet 2009; 10: 68

Paraguas A, Cailipan T, Flores E, Villamor L. Morphology and phylogeny of swamp buffaloes (*Bubalus bubalis*) in Calayan Island, Cagayan. Philipp J Vet Anim Sci 2018; 44 (1): 59-67.

Peakall R, Smouse P. GenAlex 6.5: genetic analysis in excel. population genetic software for teaching and research-an update. Bioinformatics 2012; 28(19): 2537-2539.

Pérez-Miranda AM, Alfonso-Sánchez MA, Peña JA, de Pancorbo MM, Herrera RJ. Genetic polymorphism at 13 STR loci in autochthonous Basques from the province of Alava (Spain). Leg Med 2005; 7(1): 58-61.

Promega Corporation Website. 1999. Powerstats Version 1.2. http://www.promega.com/geneticidtools/powerstats/

Reinosa O, Martínez N, da Silva E, Valdés A, Ponce G, da Silva R, Barbosa BS, Filho MG. Genetic relationship between Cuban and Brazilian buffaloes (*Bubalus bubalis*) by microsatellite markers. Revista de Salud Animal 2015; 37(3): 152-163.

Selkoe KA, Toonen RJ. Microsatellites for Ecologist: A practical guide to using and evaluating microsateliite markers. Ecol Lett 2006; 9(5): 615-629.

Stevanovic J, Stanimirovic, Z, Dimitrijevic V, Maletic M. Evaluation of 11 microsatellite loci for their use in paternity testing in Yugoslav Pied cattle (YU Simmental Cattle). Czech J. Anim. Sci 2010; 55: 221-226.

Takezaki N, Nei M. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 1996; 144(1): 389-399.

Tantia M, Vijh R, Mishra B, Bharani Kumar S, Arora R. Multilocus Genotyping to study population structure in three buffalo population of India. Asian-australas J Anim Sci 2006; 19(8): 1071-1078.

Van Oosterhout C, Hutchinson W, Wills D, Shipley P. Micro-checker software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 2004; 4(3): 535-538.

Vankan D, Faddy M. 1999. Estimation of the efficacy and reliability of paternity assignments from DNA microsatellite analysis of multiple-sire matings. Anim Genet 1999; 30(5): 355-361.

Villamor L, Takahashi Y, Nomura K, Amano T. Genetic diversity of Philippine carabao (*Bubalus bubalis*) using mitochondrial d-loop variation: implication to conservation management. Philipp J. Sci 2021; 150(3): 839-848.

Wilson-Wilde L, Norman J, Robertson J, Sarre S, Georges A. Current issues in species identification for forensic science. Forensic Sci Med Pathol 2010; 6(3): 233-241.

Zhang YDS. Genetic variation and divergence among swamp buffalo, river buffalo and cattle: a microsatellite survey on five populations in China. Asian-australas J Anim Sci 2008; 21(9): 1238-1243.