ARTICLE

Molecular Characterization of *SRY* Gene in the Philippine Carabao (*Bubalus bubalis*) swamp buffalo populations

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ABSTRACT

The Philippine carabao (*Bubalus bubalis*) swamp buffalo is considered the national animal and locally known as "carabao" or "kalabaw." The resilience of Philippine carabao (PC) in working under stressful conditions and the interest to improve its potentials in meat and milk gave rise to the national upgrading program of the species through crossbreeding of swamp and riverine buffaloes. Hence, there is a need to sustain and strengthen the conservation management of the PC. The Y-linked DNA polymorphism is valuable for studying phylogeny, phylogeography, and domestication of the buffaloes from a male perspective. The study aimed to characterize the partial sequence of *SRY* gene for the paternal identification of PC in application toward selection, breeding, and conservation of the species in the Philippines. 150 blood samples from PC were randomly selected from 26 collection sites in the Philippines, and 11 reference *SRY* sequences were retrieved from NCBI Genbank. Results revealed the absence of nucleotide and amino acid sequence differences within swamp buffaloes. However, the *SRY* gene coding region sequences showed a single nucleotide polymorphism (SNP) in which cytosine (C) in riverine and guanine (G) in swamp type were observed. This resulted in missense replacement of glycine in swamp instead of arginine in riverine. A point mutation for nucleotide and amino acid was informative to delineate *B. bubalis* subspecies for swamp and riverine buffaloes. Few animals with riverine paternal identity based on the partial sequence of *SRY* gene reflected the male introgression of riverine into the modern PC population.

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INTRODUCTION

Water buffaloes (*Bubalus bubalis*) are of two types, the river and swamp. These vary in karyotypes, morphology, purposes, and domestication. The river-type has 50 chromosomes, a large body size ranging from 450 to 1,000 kg with curly horns, and is primarily used for milk and meat production. In contrast, swamp-type has 48 chromosomes, a small body size ranging from 325 to 450 kg with crescent horns, and is mainly used as draught animal (Cockrill 1981; FAO 2005; Nanda and Nakao 2003; Castillo, 1971). The riverine was domesticated in the western part of South Asia while the swamp was in China and through Southeastern Asia (Cockrill 1981; Kumar et al. 2007a; Kumar et al. 2007b; Lei et al. 2007; Chen and Li 1989; Lau et al. 1998).

KEYWORDS

B. bubalis, Philippine carabao, *SRY* gene, swamp-type

The Philippine carabao (PC) is a swamp-type that is considered as the national animal and is locally known as "carabao" or "kalabaw." The continuing propagation of the species is attributed to its economic importance in agriculture, especially in the lives of the smallhold farmers in rice cultivation. Swamp buffalo is an excellent source of power to cultivate agricultural land and pull heavy loads (Villamor 2015). The government upholds the conservation and propagation of buffaloes reported to have a total population of approximately 2.90 million animals (Philippine Statistics Authority 2020). The resiliency of the PC working under stressful conditions and the interest to improve its potentials in meat and milk gave rise to the national upgrading program of the species through crossbreeding between the female swamp and male riverine buffaloes, which resulted in the crossbred buffalo that had 49 chromosomes. However, a drawback of crossbreeding program was the difficulty of monitoring the species identification of crossbred buffaloes when moving from one place to another without pedigree records, particularly those raised in the backyard. Hence, there is a need to sustain and strengthen the conservation and management of PC.

The common practice of selecting swamp buffaloes for conservation and management in open nucleus herd, village-based, or institutional herd was based primarily on size, growth rate, and reproduction ability (Cruz 2012). Thus, confirming *B. bubalis* sub-species identification implies using DNA-based molecular characterization for a specific and efficient way to resolve the species identification. Various genetic markers were used to determine the genetic variation between the swamp and riverine buffaloes. These included restriction fragment length polymorphisms and restriction endonucleases digestion in mitochondrial DNA (mtDNA), cytochrome *c* oxidase subunit I (COI), cytochrome *b* (cyt *b*), and microsatellites markers that are informative to determine the maternal identity and genetic diversity of water buffaloes (Amano et al. 1994; Tanaka et al. 1995; Paraguas et al. 2017; Kikkawa et al. 1997; Zhang et al. 2007). However, the paternal identity and genetic variations using the Y-linked *SRY* gene need to be established in various water buffalo populations.

The *SRY* is a non-recombining region located in Y-chromosome (Kikkawa et al. 2003). Moreover, its functional region had been initially used to determine the testis in mammals, and it became a practical approach for sex determination by amplifying male-specific *SRY* fragments (Fu et al. 2007). *SRY* gene was reported to be informative in animals' genetic diversity and evolution studies, including the domesticated buffaloes (Kikkawa et al. 2003). Another related study reported the complete river-type *SRY* sequence, confirming the evolutionary divergence between cattle and river-type 10 million years ago (Parma et al. 2004). Furthermore, the *SRY* gene had been a powerful marker used in bovine phylogeography in Y-chromosome and speciation (Mohamad et al. 2009; Nijman et al. 2008). Also, the paternal origin or Y-linked DNA polymorphism had been important in the study of evolutionary relationships, domestication, phylogeography, and phylogeny of the buffaloes from a male perspective (Bradley et al. 1994; Hanotte et al. 2000; Kikkawa et al. 2003; Zhang et al. 2006). The polymorphic Y-linked marker effectively determined the degree of male introgression of riverine into the swamp buffalo populations (Edwards et al. 2000). Thus, the objective of the study was to characterize the *SRY* gene for the paternal identification of PC.

MATERIALS AND METHODS

Site Selection and Sample Collection

The collection sites were coordinated to various regional managers of the Philippine Carabao Center (PCC), local government units, and local swamp buffalo raisers. The selected sites were mountainous and coastal parts of the country to ensure that no introduction of artificial insemination program of the riverine buffaloes was done. All PC was initially identified based on morphological characteristics, including body color, which ranged from light gray to slate gray, presence of chevron and white socks, and sickle-curved horns following the recommendation of Castillo 1998.

In this study, 150 blood samples from PC were randomly selected from 26 collection sites in the Philippines (Table 1). These farm animals lacked pedigree records due to the absence of a breeding program. Thus, measuring the relatedness of individual animals was done before the collection of blood samples. In addition, the animal owner or farmer was interviewed to determine the absence of full-sibs and half-sibs among the animals.

Table 1: Sample size of Philippine carabao from major islands sub-groups in the Philippines that were included in the study.

Population	Collection Site	Code	Sample Size
Luzon			**91**
	Aurora	AU	8
	Batanes	BTS	5
	Benguet	BGT	6
	Calayan Island	CAL	9
	Camarines Sur	CAM	5
	Ilocos Norte	ILO	3
	Kalinga	KA	6
	La Union	LA	2
	Occidental Mindoro	OCM	15
	Pangasinan	PA	5
	Romblon	CAR	16
	Zambales	ZAM	7
	Nueva Ecija	CLD	1
	Sorsogon	SOR	3
Visayas			**45**
	Bohol	BHL	12
	Capiz	CPZ	12
	Guimaras	GMR	7
	Iloilo	G-ILL	1
	Leyte	LYT	5
	Samar	SMR	4
	Talibon	TAL	4
Mindanao			**14**
	Bukidnon	BKD	5
	North Cotabato	NCOT	1
	Sarangani	SRN	1
	South Cotabato	SCOT	1
	Sultan Kudarat	SK	6

DNA Extraction and PCR Amplification

The genomic DNA was extracted from the whole blood samples using the commercially available DNA extraction kit (Promega ReliaPrep™), following the manufacturer's recommended procedure with minor modifications. These modifications included the additional step of centrifugation (14,000rpm) for 1 min to remove traces of Column Wash Solution after the third washing. Also, the incubation time of DNA in Nuclease-Free water was extended to 5 min prior to elution step in order to obtain high yield and good quality of DNA (Villamor et al. 2021).

The partial sequence of *SRY* gene was amplified using the primers B*SRY*S, 5′- ATGTGAAAGGGGAGAAAATG-3′ and 5′- AGGTCGATA TTTATAGCCCG -3′of Zhang et al. 2006. Amplification was carried out using Veriti® 96-well thermal cycler with an initial denaturation at 10 min at 95°C, followed by 30 cycles of the 30s at 95°C, 30s at 48°C, 30sat 72°C and final extension of 5min at 72°C. The PCR products ranged from

200-265 bp and were visualized on 2% agarose gel and stained using GelRed™. PCR products were purified using the commercially available kit following the manufacturer's recommended protocol (QIAquick PCR Purification Kit Protocol). Sequencing of purified PCR products was outsourced from the Apical Scientific Sequencing Laboratory in Malaysia.

Sequence Analysis

All partial sequences of the *SRY* gene were aligned using ClustalW and analyzed using Molecular Evolutionary Genetics Analysis (MEGA X) (Thompson et al. 1994; Kumar et al. 2018). The analysis included 150 water buffaloes from this study, and 11 reference *SRY* sequences were retrieved from NCBI Genbank. These were represented by one swamp and seven riverine buffaloes from the Philippines (MG461046; MG461048-MG461054, 209bp), two swamp buffaloes from China (DQ119747.1 and FJ546414.1, 1070bp), and one riverine buffalo (AY341337.1, 848bp). For further analysis, the nucleotide sequences were translated into protein sequences to determine the amino acid polymorphism using MEGA X (Kumar et al. 2018).

RESULTS

DNA sequences (MG46046-MG461175; MZO14907-MZO14936) generated from this study were submitted to NCBI Genbank. All genomic DNA (gDNA) isolates were successfully amplified, except from a female buffalo used as a control sample (Fig. 1). The coding region starts at base pair 1 and ends at base pair 219. With a focus only on the Philippine carabao from 26 collection sites, a point mutation was not detected on the *SRY* gene sequences. A similar pattern of absence of polymorphism in *SRY* gene sequences was observed between PC and two Chinese swamp buffaloes (FJ546414.1 and DQ119747.1) (Fig. 2). Likewise, absence of polymorphism in *SRY* nucleotide and amino-acid sequences was also observed between Philippine carabao and two Chinese swamp buffaloes (Fig. 3). The coding region yielded a 73 amino-acid sequences. The open reading frame (ORF) of the partial *SRY* included the high mobility group (HMG) box, which started at amino-acid 7 and ended at amino-acid 73.

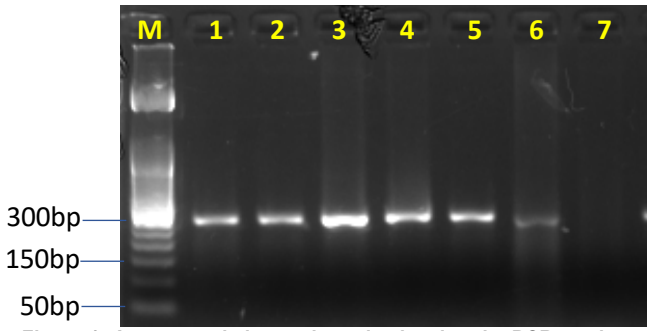

Figure 1: Agarose gel electrophoresis showing the PCR product of *SRY* gene. (Lane M: 50bp DNA Ladder; Lanes 1-6: Male buffalo; and Lane 7: Female buffalo (control))

Single nucleotide polymorphism (SNP) and an amino-acid difference were detected between *B. bubalis* subspecies swamp and riverine. When comparing the *SRY* gene coding region sequences of water buffaloes, a SNP was detected at base pair 70, in which guanine (G) for swamp buffalo while cytosine (C) for river buffalo were observed (Fig. 2). The *SRY* amino-acid sequence revealed a missense replacement at amino-acid 24, which encoded glycine (C) in swamp and arginine (R) in riverine type (Fig. 3).

In the present study, most of the PC (97% or 146/150) revealed the paternal identity of swamp buffalo, while few of the animals (3% or 5/150) belonged to riverine buffalo. Farm animals, including ILO13 from Ilocos Norte, LYT11 from Leyte, CLD02 from Nueva Ecija, OCM54 from Occidental Mindoro, and BKD15 from Bukidnon, exhibited more distinct features of a swamp-type rather than a river-type (Fig. 4). However, all these five samples were confirmed to have a riverine paternal sequence using the *SRY* gene sequence.

DISCUSSIONS

A lack of mutation on the nucleotide and amino-acid sequences within swamp or riverine samples indicated that the coding region of the *SRY* gene was highly conserved within the *B. bubalis* subspecies. However, a type-specific SNP of the *SRY* gene detected in water buffalo subspecies enabled the usefulness of the genetic marker to confirm the paternal identity of water buffaloes. The procurement and dispersal of the animals depend on the intended application of the local and national buffalo programs toward milk and meat production to riverine and resulting crossbreeds, while as source of draught power for swamp buffalo. Therefore, the common practice of animal selection based on morphological characteristics could be unreliable and could lead to misidentification.

The *SRY* genetic marker has a potential application in species identification of the male perspective in water buffaloes. *SRY* DNA-based identification elucidated that the five PC included in this study (ILO13, LYT11, CLD02, OCM54, and BKD15) had a riverine paternal identity and most probably were putative crossbreds from the female swamp and male riverine. With the initial assignment of buffaloes to swamp-type based on physical features, the study revealed a low level of discordance between morphological and molecular identifications. In another related study, the discordance occurred when native cattle of Bangladesh and Nepal were identified as zebu cattle instead of taurine based on their morphology. However, the *SRY* gene confirmed that these samples were taurine cattle. These findings generally suggested that the cattle from Asian countries were hybrids (Kikkawa et al. 2003).

Few animals identified with riverine paternal parent indicated that male introgression of riverine occurred into the Philippines swamp populations. This was expected in populations in areas wherein Artificial Insemination (AI) for upgrading swamp buffalo were introduced. The Philippines is one of the two countries in Asia which implemented programs on a large scale of crossbreeding and backcrossing to produce genetic potentials for milk and meat. Crossbreeding and backcrossing in the Philippines were limited before 1974. However, in 1982, the upgrading of swamp buffaloes was initiated after the approval and implementation of the project by the United Nations Development Programme-Food and Agriculture Organization (UNDP-FAO) (Cruz 2009). Species identification could still be unresolved when identifying the crossbred offspring of swamp x riverine crossbreeding rely solely through morphological characterization.

The SNP position and non-synonymous substitution in a DNA sequence from this study resulted in a missense mutation. The *SRY* SNP position and amino acid polymorphism showed a similar pattern between indigenous swamp buffaloes and introduced river buffalo from China (Zhang et al. 2006). In another previous study, the selection of pure swamp-type breed, as part of the conservation and management of species, used the *SRY* gene to establish paternal identification of male buffaloes in the establishment of the Carabao Sanctuary in

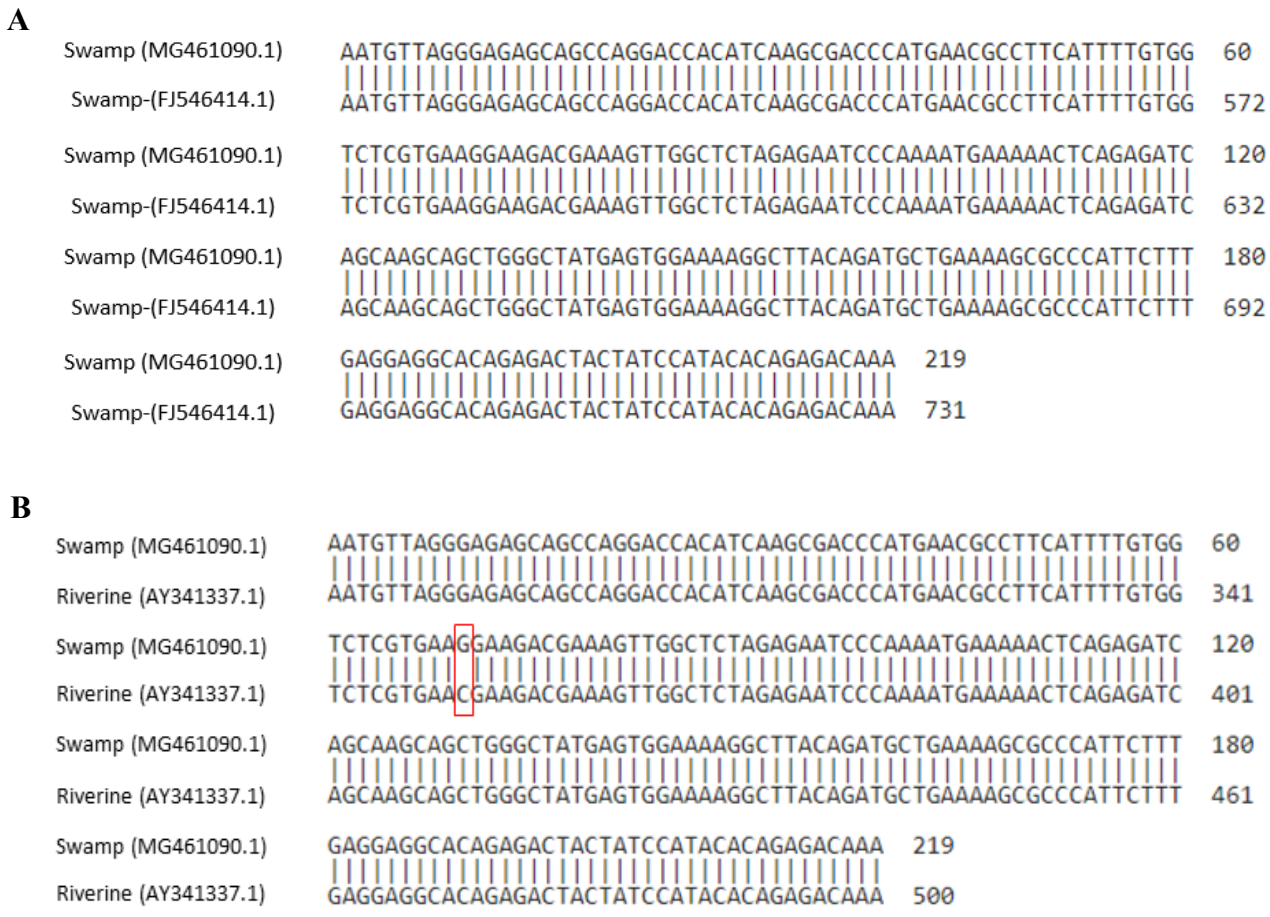

Figure 2: The nucleotide sequences alignment of (A) Philippine carabao (MG461090.1) and China (FJ546414.1) showing no point mutation; and with (B) Riverine (AY341337.1) with a SNP (G70C) appeared in red box.

Figure 3: The amino-acid alignment of (A) Philippine carabao (MG461090.1) and China (FJ546414.1) showing no difference and the (B) predicted *SRY* protein from Philippine carabao (MG461090.1) and riverine (AY341337.1) buffaloes using ORF finder software from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The HMG box region was underlined and difference in amino acid was encircled.

Calayan Island, Cagayan (Paraguas et al. 2017). Thus, the *SRY* gene is an informative genetic marker to determine the paternal identity of the male *B. bubalis* species.

For future studies, the study recommends to combine the *SRY* gene and other mtDNA markers such as COI and cyt *b* to confirm the hybrid animals or crossbred offspring. In the Philippines, the routine paternity testing encompasses a panel of

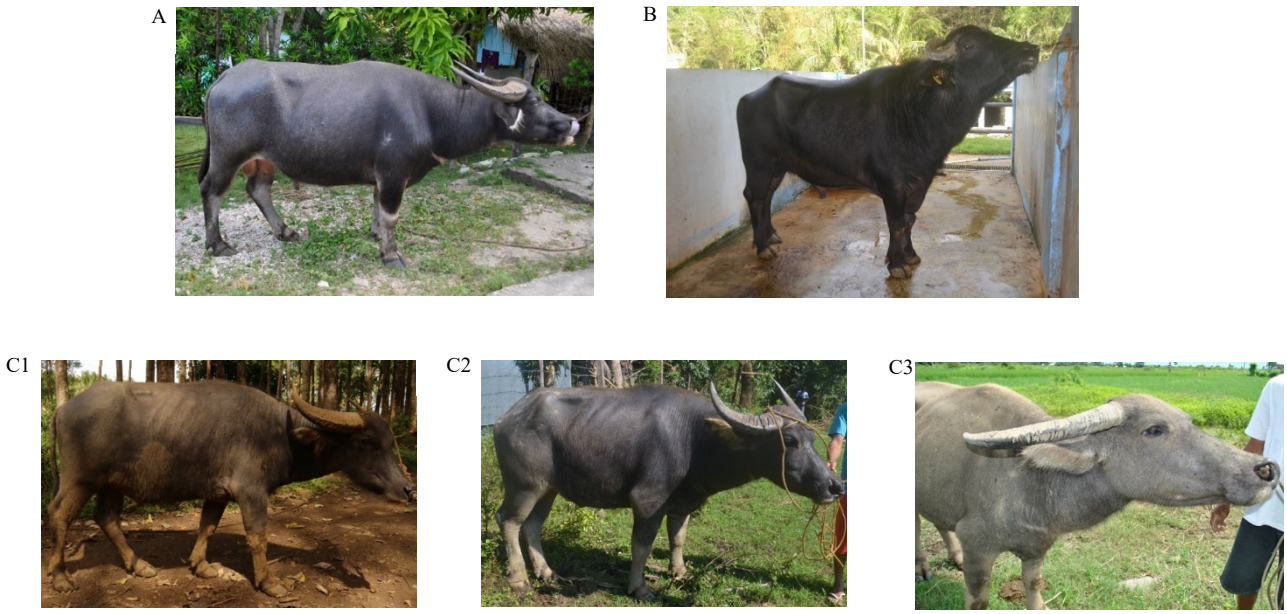

Figure 4: Pure swamp and riverine buffaloes in PCC-CLSU (A and B) and putative crossbreds (C1 to C3) in Bukidnon (BDK15), Occidental Mindoro (OCM54), and Ilocos Norte (ILO13).

15 STR loci to determine the true sire or father of the calf (MN Reyes et al. unpublished observations). The inclusion of the *SRY* gene in the paternity testing panel would be highly recommended since this region is clonally inherited from father to son and identified as a male-specific gene (Lundrigan and Tucker 1994). However, the highly conserved region of the *SRY* gene was uninformative to reveal the genetic diversity within the paternal lineages of swamp buffaloes in the Philippines. Thus, evaluation of additional Y-linked DNA genetic markers such as *ZFY* and *DBY* is recommended to understand the paternal lineages of PC for the reconstruction of population history (Nijman et al. 2008; Zhang et al. 2016; Groeneveld et al. 2010).

CONCLUSION

The research findings revealed a low discordance between PC's morphological and molecular identifications, which confirmed that the former identification system could still be unreliable. Moreover, the study indicated the usefulness of the *SRY* gene marker to delineate swamp from riverine buffaloes and provide evidence on the introgression of Y-chromosomal DNA from riverine buffaloes into the PC populations. Thus, baseline information obtained on *SRY* gene sequences would provide the knowledge of the paternal identity of PC that would be useful in planning effective strategies for selecting PC genetic resources for conservation and management program.

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CONFLICTS OF INTEREST

The authors declared no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

LP Villamor spearheaded the overall concept of the study. TPC Cailipan and LP Villamor performed the fieldwork. TPC Cailipan performed the laboratory experiments and analysis with the supervision of LP Villamor. TPC Cailipan wrote the manuscript with substantial comments, suggestions, and reviews by LP Villamor.

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