

MORPHOLOGY AND PHYLOGENY OF SWAMP BUFFALOES (*Bubalus bubalis*) IN CALAYAN ISLAND, CAGAYAN

Alexander M. Paraguas¹, Therese Patrickka C. Cailipan¹,
Ester B. Flores² and Lilian P. Villamor¹

ABSTRACT

Swamp buffalo is considered as a single and homogenous breed in the Philippines. The study aimed to establish the morphological and molecular characteristics of swamp buffaloes in Calayan Island, Cagayan. Morphology and morphometrics of body, horn, face and neck were recorded. A total of 64 sequences from the mtDNA *cytochrome c oxidase I (COI)* and 21 *SRY* sequences were included for phylogenetic analysis for maternal and paternal lineages, respectively. On average, males have significantly greater values ($P<0.05$) than females for traits measured except for height and distance and width of horns which indicated sexual dimorphism. The coat color for gray is predominant over the black and brown. The inferred phylogenetic tree based on *COI* sequences, with high statistical support (BP=100%), showed one clade and separated by two clusters of swamp and riverine buffaloes. This showed the usefulness of *COI* and *SRY* genetic markers to discriminate breeds of *B. bubalis* swamp-type with riverine-type and revealed the high affinity of swamp buffaloes in the Calayan Island with those in the PCC Institutional Herds and Asian countries. Therefore, the combined data on the morphological and molecular characteristics showed that the buffaloes in Calayan Island are of swamp-type buffaloes and suitable for the establishment of the Philippine carabao conservation.

Key words: *Bubalus bubalis*, Calayan Island, *COI*, conservation, *SRY* gene

INTRODUCTION

In Asian countries mostly in the Philippines, *Bubalus bubalis* or water buffalo is an effective source in the agricultural economy and food security. They are also known for their role in draft power in farming system mainly in production of main agricultural crops (Cruz, 2012). With significant variations, water buffalo was categorized in two major types, the riverine-type and swamp-type.

The Philippines has always considered the swamp buffalo as a single and homogenous breed. Characterization of buffalo breeds are usually just based on the phenotypic

¹Cryobank Unit, Philippine Carabao Center National Headquarters and Genepool, Science City of Munoz, Nueva Ecija,
²Genomics and Bioinformatics Section, Philippine Carabao Center National Headquarters and Genepool, Science City of
Munoz, Nueva Ecija (e-mail: lpv.2012@gmail.com).

information. According to Hebert *et al.* (2003), few taxonomists can critically identify more than 0.01% of the estimated 10–15 million species and a community of 15000 taxonomists will be required to identify species on morphological diagnosis is to be sustained. Unfortunately, the limitations inherent in morphological-based identification systems and the reduced group of taxonomists elicited the need for a new approach to taxon recognition or for species identification. The worldwide status of breeds raised a need to assess the between-breed diversity using the molecular approach. This encompassed the use of more informative genetic markers that can be used to understand the genetic diversity in various populations, including the water buffalo.

Currently, Calayan Island was declared as another swamp buffalo conservation site in the Luzon Island. This was a joint effort by the Department of Agriculture-Philippine Carabao Center (DA-PCC) together with the DA-Region II, Cagayan State University (CSU), and the Local Government Unit in Calayan Island, Cagayan. The site is located about 24 miles (39 km) west-south-west of Babuyan Island, off the north coast of the Philippines. It is the largest of the Babuyan Islands group in the West Philippine Sea and consists mainly of mountainous and uneven land, with the highest land in the center and with low gaps in places. The DA-Region II reported that artificial insemination of exotic germplasm from riverine buffaloes and shipments of live bulls were not introduced in the Island for several years (personal communication with Dr. Franklin Rellin, Regional Director at PCC-CSU).

The Philippine carabao gene pools were continuously established in the open nucleus herds from the major islands of Luzon, Visayas, and Mindanao. The animal selections of better stocks from the surrounding communities were limited to the size, growth rate, and reproduction ability (Cruz, 2012). To strengthen the strategy for the selection of swamp buffaloes, molecular characterization is essential. This involves the use of informative genetic markers in order to confirm the species identification of the maternal and paternal lineages. The *cytochrome c oxidase I (COI)* or *COX1* gene is suitable for this role since the mutation rate is often fast enough to discriminate closely related species, and it is conserved among same species. According to Lunt *et al.* (1996), the size and structure of *COI* gene have been well conserved in the animal groups, a feature which makes it especially suitable for evolutionary studies. Based on the studies of Mueller (2006) and Wilson-Wilde *et al.* (2010), it was also one of the most conserved mitochondrial protein-coding genes in animals and thus displayed a better phylogeny signal.

Paternal origin or Y-linked DNA polymorphism was of great value for the study of evolutionary relationships (Bradley *et al.*, 1994; Hanotte *et al.*, 2000; Kikkawa *et al.*, 2003). Y-specific SNP marker was expected to be a useful marker for investigating domestication of buffalo and its phylogeny from a male perspective. Hence, the *SRY* gene, a non-recombining region located in Y-chromosome and a male specific gene was fit for the study of animal evolution and genetic diversity.

Analysis of datasets of variable markers of *B. bubalis* offers a promising approach to address some of major concerns in phylogeny of the species and its population. The study aimed to establish the morphological and molecular characteristics of swamp buffaloes in Calayan Island.

MATERIALS AND METHODS

Physical characteristics such as color of the body coat, forehead, iris, muzzle, forelegs and hind legs were recorded for each selected animal. Additionally, coat pattern, ear shape, ear and horn orientation were also noted. Age and sex were determined for each animal presented for sampling. Measurements were taken for the heart girth (body circumference immediately posterior to the front leg), body length (distance from shoulder point to pin bone), height at withers (the vertical distance from the ground level to the highest point of withers), width of head (between horns), face length (the distance between head and muzzle), horn length (the distance from the tip to the base of the horn both along greater and smaller curvatures), horn circumference at base, neck circumference (at the midway from the junction with shoulder and junction with head) according to Khan *et al.* (2013).

Univariate analysis was performed on morphometric traits using the linear model having effect of sex and age:

$$Y_{jkl} = \mu + S_j + A_k + e_{jkl}$$

Wherein:

Y_{jkl}	= Individual observation
μ	= Overall Mean
S_j	= Effect of the j^{th} sex
A_k	= Effect of the k^{th} age category of the buffalo
e_{jkl}	= Random error

Least squares ANOVA were computed separately for each variable in the model using the Fit Model platform of JMP13 statistical software (SAS, 2016). Tukey HSD test was used to compare variables on each factor identified. Sex was classified into male and female and age was classified into age categories, 0-2.99 years old, 3-5.99 years old, 6-8.99 years old, 9-11.99 years old, 12-14.99 years old and >15 years old. Age of an animal was determined based on interview of the owner and verified by dentition.

The genomic DNA was extracted from whole blood samples using the commercially available DNA extraction kit from Promega following the manufacturers' recommended procedure (ReliaPrep™ Blood gDNA Miniprep System Technical Manual) with few modifications (Villamor *et al.*, 2016).

Two primer sets, *COI* and *SRY*, were used for this study. The *COI* gene was amplified using published primer sequences (Hebert *et al.*, 2003). The PCR reaction was carried out in a total volume of 30 μ L containing the DNA template (about 3-7 ng), 1X Colorless GoTaq® Flexi Reaction Buffer, 2mM MgCl₂, 0.2mM dNTP, 0.5mM of each primer, and 0.125 U GoTaq® DNA polymerase. The PCR amplifications were performed using a programmable thermal cycler conditions: initial denaturation of 2 min at 95°C followed by 35 cycles of denaturation of 30s at 95°C, annealing of 30s at 51°C, and extension of 30s at 72°C, and final extension of 5 min at 72°C. The *SRY* gene was amplified using published primer sequences (Zhang *et al.*, 2006) and following the optimized PCR reactions and thermal cycler conditions by Cailipan and Villamor (2017). The expected PCR products of 650-800 bp for *COI* and 200-300 bp were visualized using 2% agarose in gel electrophoreses by GelRed™ staining. PCR products were sent to First Base Laboratories, Sdn Bhn, Malaysia for

both forward and reverse primers on ABI PRISM 3730xl Genetic Analyzer (AB, USA) and BigDye®Terminator v3.1 Cycle Sequencing Kit (AB, USA).

The inferred phylogeny was independently constructed from the combined sequences of *COI* and *SRY* from this study and GenBank at National Center for Biotechnology Information (NCBI). The multiple sequence alignments were performed using ClustalW in MEGA 6.06 (Thompson *et al.*, 1994) and BioEdit (Hall, 1999). The calculation of the over-all mean diversity and pairwise sequence divergence was run using the Tamura 3-parameter model (T92) (Tamura *et al.*, 2011). Phylogenetic tree was done using Neighbor Joining method (NJ) in MEGA 6.06 with the following parameters: test and number of phylogeny bootstrap and 1000 replicates; model of T92; transitions and transversions as substitutions to include; uniform rates among sites; and use of complete deletion for data subset (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

The morphology and morphometric characteristics of swamp buffaloes in Calayan Island served as the baseline information for conservation in the Island (Table 1). On average, males have significantly greater values ($P < 0.05$) than females for all traits except for the height and the set of the horns (distance between horns and width between horns) indicating sexual dimorphism. The coat color for gray is predominant over the black and brown. Albinoids were not observed in the population although a spotted coat pattern in one individual may indicate a carrier of albinism gene (Table 2). This remains to be validated with other swamp buffaloes across the country. Horns of swamp buffaloes are generally described as semi-circle or crescent horns (Castillo, 1971), while buffaloes in Calayan Island have horn tips pointing laterally rather than curved or pointing inward. This observation will have to be compared with other local populations in other provinces. Generally, the values obtained are typical of description of the swamp buffaloes in the Philippines (Castillo, 1971).

A total of 64 *COI* sequences were completely submitted to GenBank at NCBI and were included in the phylogenetic analysis (Table 3). The low mean diversity of 0.9% (0.009 units) and with lack of sequence divergence implied closer relationship within the swamp-types. The same pattern was observed from previous study on domestic buffaloes swamp-type breeds in the Philippines (Bondoc, 2013). The inferred phylogenetic tree based on *COI* sequences, with high statistical support (BP=100%), showed one clade and separated by two clusters of swamp and riverine buffaloes. This implies the usefulness of *COI* markers to discriminate breeds of *B. bubalis* swamp-type with riverine-type in the Philippines and in other countries. Also, results of the phylogenetic analysis revealed the high affinity of swamp buffaloes in the Calayan Island with those in the PCC Institutional Herd and Asian countries particularly from China, India, and Vietnam (Figure 1). The utility of the *COI* sequences confirmed the identity of the buffaloes in the conservation site to the subspecies level as *B. bubalis* swamp-type.

Based on the 219 base pairs of the *SRY* gene, 21 nucleotides sequences were used to infer phylogenetic tree using NJ showed high level of support to the two sub-groups riverine and swamp buffaloes (Figure 2). The over-all mean diversity was 0.3% (0.003 units). Comparing the *SRY* gene coding region sequences of swamp with the riverine, it showed that one nucleotide aligned locating at the site of 82 base position was different in swamp

Table 1. Average measurements of 10 morphological traits of swamp buffaloes in Calayan Island.

Morphology	No. of samples	Overall		Male		Female	
		Mean, cm	±SE	Mean, cm	±SE	Mean, cm	±SE
Body							
Height	74	129.6	1.3	130.5	2.1	128.6	1.5
Heart Girth	73	193.4	2.1	197.0 ^a	3.6	189.7 ^b	2.4
Body Length	20	143.4	2.3	153.6 ^a	4.1	133.1 ^b	2.3
Horn							
Horn greater curvature	20	62.4	2.5	70.2 ^a	4.3	54.7 ^b	2.5
Horn lesser curvature	20	48.4	2.0	53.6 ^a	3.5	43.1 ^b	2.0
Horn circumference base	20	30.5	0.4	36.0 ^a	0.6	25.1 ^b	0.4
Distance between horns	20	51.6	2.4	51.8	4.2	51.5	2.4
Width between horns	20	17.0	0.4	17.8	0.7	16.3	0.4
Face and Neck							
Face length	20	54.1	1.0	57.4 ^a	1.6	50.7 ^b	1.0
Neck circumference	20	102.6	1.9	115.6 ^a	3.3	89.5 ^b	1.9

^{ab}Different letters in a row are significantly different ($P < 0.05$)

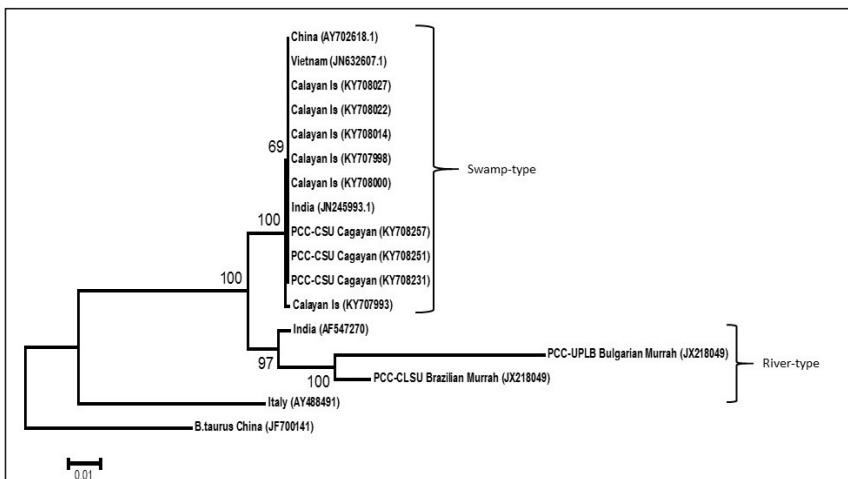


Figure 1. Inferred phylogeny of the cytochrome c oxidase subunit I sequences from Calayan Island, Cagayan PCC-CSU, and retrieved NCBI sequences.

Table 2. Frequencies of categorical variables for descriptive traits recorded on swamp buffaloes of Calayan Island.

Phenotype	Categories			
Color	Black	Brown	Gray	Whitish
Coat	0.40	0.12	0.48	
Forehead	0.19	0.65	0.15	
Foreleg		0.01	0.14	0.85
Hindleg		0.01	0.14	0.85
Muzzle	1.00			
Iris	0.99	0.01		
Coat Pattern	Plain	Spotted		
	0.99	0.01		
Horn Orientation	Curved	Tips pointing laterally		
	0.15	0.85		
Ear Shape	Rounded	Straight edged		
	0.01	0.99		
Ear Orientation	Lateral			
	1.00			

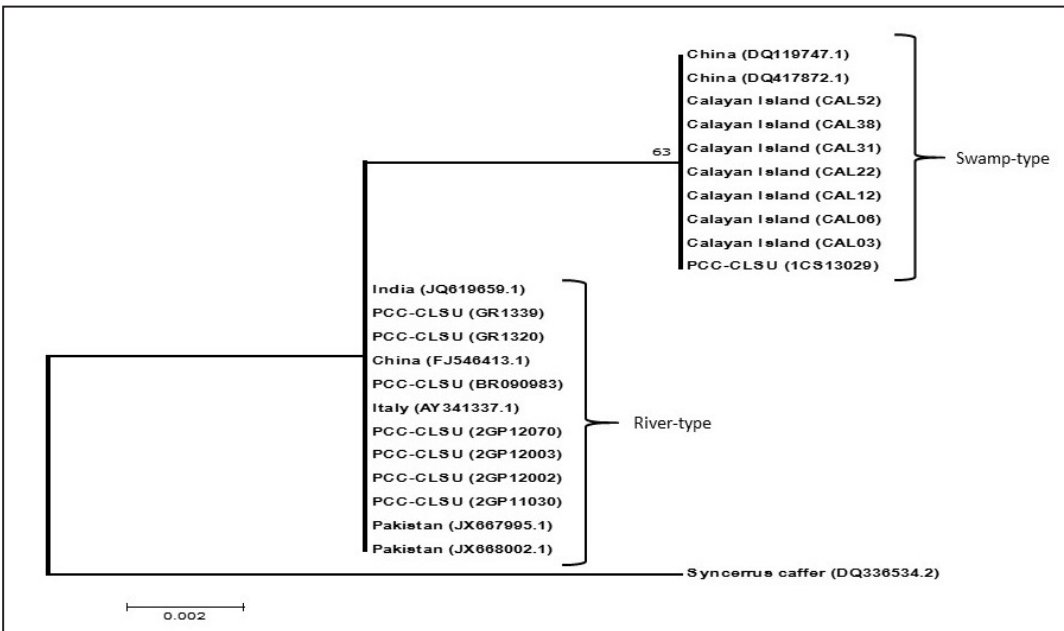


Figure 2. Inferred phylogeny of the *SRY* sequences from Calayan Island, Cagayan, PCC-CSU, and retrieved NCBI sequences.

Table 3. Population, buffaloes breeds, and number of sequences in *COI* and *SRY* from the Philippines and retrieved NCBI.

Population	Breed	No. of <i>COI</i> sequences	GenBank Accession No.	No. of <i>SRY</i> sequences	GenBank Accession No.
Calayan Island, Cagayan	Swamp	39	KY707993-29, KY708242, KY708237	5	MG461090-94
PCC-CSU, Cagayan	Swamp	17	KY708227-32, KY708235-36, KY708243-44, KY708251-57	0	
PCC-CLSU, Nueva Ecija	Swamp	0		1	MG461046
	River	0		7	MG461048-54
	Swamp	3	AY702618, JN632607, JN245993	2	DQ119747, DQ417872
Retrieved from NCBI	River	4	AF547270, AY488491, JX218048-49	5	FJ546413, Q619659, JX667995, JX668002, AY341337
	<i>Bos taurus</i> (Out group)	1	JF700141	0	
	<i>Syncerus caffer</i> (Out group)	0		1	DQ336534
Total		64		21	

and riverine, with cytosine (C) for riverine and guanine (G) for swamp. Similarly, the study of Zhang *et al.* (2006) exhibited the same result, in which in site 202 bp of *SRY* gene coding region had cytosine (C) for riverine and guanine (G) for swamp-type which led to a mis-sense replacement of glycine instead of arginine. The inferred phylogeny of both genetic markers, *COI* and *SRY*, showed discrimination between swamp-type and river-type. The results served as baseline information in establishing the species identification of the swamp-type buffaloes in support to the conservation management of the swamp-type buffaloes in Calayan Island.

CONCLUSION

Morphological and molecular characterizations of swamp buffaloes in the Calayan Island were important tools to establish the species identification of the animals toward conservation management. Body measurements in the Calayan swamp buffaloes indicated sexual dimorphism. Phenotypic characteristics of the swamp buffaloes were consistent with the description of swamp buffaloes of the previous studies. The *COI* and *SRY* genetic markers were powerful enough to discriminate subspecies of *B. bubalis* to swamp and river types. The use of *COI* and *SRY* genes confirmed the identification of the maternal and paternal lineages, respectively, of the Calayan buffaloes to the sub-species level as swamp-type. Therefore, the combined data on the morphological and molecular characteristics revealed that the buffaloes in Calayan Island are of swamp-type buffaloes and suitable for the establishment of the Philippine carabao conservation.

ACKNOWLEDGEMENT

Funding for this initiative was provided by the Department of Agriculture Biotechnology Program and Department of Agriculture Bureau of Agricultural Research (DA-BAR) for the project [DABIOTECHR1506]: Genetic Diversity of the Philippine Carabao using mtDNA (*COI*) and microsatellite markers (FAO STRs) and the Philippine Carabao Center-National Headquarters and Genepool. The authors were grateful for the leadership of Dr. Franklin Rellin, Center Director of PCC-Cagayan State University and the assistance of Mr. Lino Llopis, Municipal Agriculturist Officer (MAO) officials of the Local Government Unit of the Calayan, Island, Cagayan.

REFERENCES

- Bondoc OL. 2013. Genetic diversity and relationship of domestic buffalo and cattle breeds and crossbreeds (Subfamily Bovinae) in the Philippines based on the *cytochrome c oxidase I (COI)* gene sequence. *Philipp Agric Sc* 96(1): 93-102.
- Bradley DG, MacHugh DE, Loftus RT, Snow RS, Hoste CH and Cunningham EP. 1994. Zebu-aurine variation in Y chromosomal DNA: a sensitive assay for genetic introgression in West African trypano tolerant cattle populations. *Anim Gen* 25: 7-12.
- Cailipan TP and Villamor LP. 2017. Paternal lineage of swamp buffaloes from 14 Luzon populations using *SRY*- gene. Poster presented at 54th PSAS Scientific Seminar and Annual Convention, Cebu City, Philippines.
- Castillo L.S. 1971. Proposal for a new scientific name for the carabao-*Bubalus carabanensis* (Linn.). *Philipp J Anim Sci* 8(2): 155-156.
- Cruz LC. 2012. Transforming swamp buffaloes to producers of milk and meat through crossbreeding and backcrossing. *J Anim and Plants Sci* 22 (3 Suppl.): 157-168.
- Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95-98.

- Hanotte O, Tawah CI, Bradley DG, Okomo M, Verjee Y, Ochieng J and Rege JEO. 2000. Geographic distribution and frequency of a taurine *Bos taurus* and an indicine *Bos indicus* Y specific allele amongst sub-Saharan African cattle breeds. *Mol Ecol* 9: 387-396.
- Hebert PDN, Cywinska A, Ball SL and deWaard JR. 2003. Biological identification through DNA barcodes. *Proc Biol Sci* 270(1512): 313-321.
- Khan M, Rahim I, Rueff H, Jalali S, Saleem M, Maselli D, Muhammad S and Wiesmann U. 2013. Morphological characterization of the Azikheli buffalo in Pakistan. *Anim Genet Resour* 52: 65–70.
- Kikkawa Y, Takada T, Sutopo, Nomura K, Namikawa T, Yonekawa H and Amano T. 2003. Phylogenies using mtDNA and *SRY* provide evidence for male-mediated introgression in Asian domestic cattle. *Anim Gen* 34: 96–101.
- Lunt DH, Zhang DX, Szymura JM and Hewitt GM. 1996. The insect *cytochrome oxidase I* gene: Evolutionary patterns and conserved primers for phylogenetic studies. *Insect Mol Biol* 5(3): 153-165.
- Mueller RL. 2006. Evolutionary rates, divergence dates, and the performance of mitochondrial genes in bayesian phylogenetic analysis. *Syst Biol* 55(2): 289-300.
- Saitou N and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4): 406-425.
- SAS Institute Inc. 2016. Cary, North Carolina 27513-2414, USA.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10): 2731-2739.
- Thompson JD, Higgins DG and Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22(22): 4673–4680.
- Villamor LP, Paraguas AM, Escuadro AJD, Cailipan TP and Flores EB. 2016. Genetic diversity of the Philippine carabao using mtDNA (*COI*) and microsatellite markers (FAO STRs). Annual Report- Year 1. Presented at the DA Biotech Program Annual Project Review and Assessment of the DA Livestock Biotech, Manila, Philippines.
- Wilson-Wilde L, Norman J, Robertson J, Sarre S and Georges A. 2010. Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (*COI*) gene. *Forensic Sci Med Pathol* 6: 233-241.
- Zhang Y, Dongxiao S, Ying Y and Zhang Y. 2006. A Y-linked SNP in *SRY* gene differentiates Chinese indigenous swamp buffalo and introduced river buffalo. *Asian-Australas J Anim Sci* 19(9): 1240-1244.