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# Comparative molecular characterization of Forkhead box protein 3 (FoxP3) gene of swamp-type (*Bubalus carabanensis*) and riverine-type (*Bubalus bubalis*) water buffaloes

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ABSTRACT

FoxP3 is a forkhead family member that plays an important role in the development and function of a type of CD4 + T cell called T regulatory cells. Molecular characterization of FoxP3 gene in swamp- and riverine-type water buffaloes was conducted to determine its homology and compare it to the FoxP3 gene of other animal species (cattle, goat, sheep, horse, pig, cat, and dog), determine its unique characteristics in water buffaloes, and provide a reference for future studies to analyze its immunological function. FoxP3 nucleotide sequence of swamp- and riverine-type water buffaloes was 99% identical, whereas its protein translation revealed 97% homology. FoxP3 of swamp- and riverine-type water buffaloes were compared to FoxP3 of other animal species and revealed a high degree of homology which suggests that they may have the same biological properties. This study is the first report that describes the genetic characteristic of FoxP3 gene in water buffalo.

## 1. Introduction

Forkhead box protein 3 (FoxP3), an X-linked gene (located in Xp11.23), is a forkhead family member that plays an important role in the development and function of a type of CD4+ T cell called T regulatory cells [1], thus called the "master regulator" for the Treg lineage [2], which is essential for maintaining immune tolerance to self.[1] Induction of the Foxp3 gene in normal naïve T cells converts them to Treg-like cells with *in vivo* and *in vitro* suppressive function. This indicates that Foxp3 functions in controlling expression of critical suppression-mediating molecules [2]. Ziegler [3] stated that lack of Foxp3 leads to development of fatal autoimmune lymphoproliferative disease. Furthermore, retroviral-mediated introduction of Foxp3 can cause conventional CD4 + T cells to acquire a regulatory-like phenotype and be capable of suppressing immune responses both *in vitro* and *in vivo*.

The swamp-type (*Bubalus carabanensis*) and riverine-type (*Bubalus bubalis*) buffaloes are the known types of domesticated water buffalo. Swamp-type is usually gray, but there are also significant numbers which are pink, albino which are white, or piebald which are gray with white patches, whereas riverine-type is usually much darker but often have white markings on the head, legs or tail [4].

For the advancement of buffalo management and health, studies about its genetic immunity may be helpful. Genotype of Foxp3 in water buffaloes, was not yet characterized, is not well understood. Since Foxp3 is known to be responsible for the development of the regulatory T cells [1] which act to suppress immune responses, hence maintaining homeostasis and self-tolerance [5], it may also have specific significance regarding the immune responses of water buffaloes as compared to other species.

Foxp3 gene of water buffaloes has been molecularly characterized and compared to the Foxp3 gene sequence of other species. This is one of the primary steps in understanding the specific role of FoxP3 in water buffalo's immune system. Thus, this study can be a foundation to know its involvement in disease susceptibility or resistance and probably to improve the gene selection for breeding.

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## 2. Materials and methods

### 2.1. Blood sample

A total of 23 blood samples were collected from the jugular and/or ear vein of randomly selected animals using syringes with heparin as anticoagulant. Blood samples from riverine-type buffaloes were collected from the Philippine Carabao Center (PCC) herd. Meanwhile, swamp-type buffaloes were collected from the municipalities of Nueva Ecija, Philippines. The buffy coat was then collected and transferred in a 1.5 ml centrifuge tube and washed with 1000 μL of $NH_4Cl$ through mixing in the vortex and centrifugation for 5 min at 3000 rpm. The supernatant was discarded and the white pellet remained in the tube. The DNA yield from buffy coat extraction is higher compared to whole blood extraction method, hence used in this study.

### 2.2. RNA extraction

RNA extraction, based on the procedure of Mingala et al. [6] was preceded by dissolving the isolated cells with 1000 μL of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The tube was then incubated at room temperature for 10 min to permit the complete dissociation of the nucleoprotein complex. After incubation, 200 μL of chloroform was added to cause proteins to become denatured and become soluble in the organic phase while nucleic acids remain in the aqueous phase. Then, it was mixed in the vortex to have a homogenous mixture. The mixture was centrifuged at 14,000 rpm for 15 min at 4 °C and the aqueous layer was separated by careful pipetting and transferred into a new 1.5 ml centrifuge tube with 500 μL of 100% isopropanol for RNA isolation. The solution was mixed by inverting the tubes repeatedly, and was incubated at room temperature for 10 min to precipitate the RNA. Then, it was centrifuged for 10 min at 14,000 rpm at 4 °C. The supernatant was removed carefully in order to avoid discarding the pellet. The tube was washed and shook gently with 1000 μL of 75% ethanol, and centrifuged for 5 min at 8000 rpm at 4 °C. After centrifugation, the supernatant was discarded and the RNA pellet was air dried. After drying, the RNA pellet was suspended with 30 μL of RNase free distilled water stored at −20 °C.

### 2.3. Complementary deoxyribonucleic acid synthesis

The cDNA synthesis was carried out following the procedure described in cDNA Synthesis Kit Manual by Takara (Takara Bio Inc., Otsu, Shiga, Japan) wherein the primer mixture of RNA was prepared in a PCR tubes containing 1 μL of random 6 primers, 1 μL of deoxynucleotide (dNTP), 3 μL of template RNA and 5 μL of RNase free distilled water. The mixture was incubated in a PCR machine at 65 °C for 5 min and was cooled immediately on ice. In a new PCR tube, a reaction mixture was prepared containing 4 μL of 5X primeScript Buffer, 0.5 μL of RNase Inhibitor (40 U/μL), 1.0 μL of PrimeScript RTase (200 U/μL) and 10 μL of RNase free distilled water. Then, the 10 μL of template RNA primer mixture was mixed to the reaction mixture gently and was immediately incubated at 30 °C for 10 min, increased to 50 °C for 45 min, and increased further to 95 °C for 4 min. The total reaction mixture was then stored at 4 °C until use.

### 2.4. PCR amplification of β-actin gene

β-actin gene was used as housekeeping gene which is involved in the regulation of basic and ubiquitous cellular functions required for the survival of most cell types. Hence, PCR amplification of β-actin gene served as quality check of isolated cDNA samples. The β-actin sequences have the forward 5′-CGC ACC ACC GGC ATC GTG AT-3′ primers and reverse 5′-TCC AGG GCC ACG TAG CAG AG-3′ primers [7]. A total volume of 10 μL of master mix composed of 4.2 μL DDW, 2 μL 5X buffer, 1.2 μL Magnesium Chloride ($MgCl_2$), 0.5 μL dNTPs, 0.5 μL of 10 pmol forward and reverse primers, 0.1 μL Taq enzyme and 1.0 μL of DNA template was prepared.

The β-actin primer set (F/R) PCR profile cycle was: initial denaturation for 5 min at 94 °C, 35 cycles of denaturing step for 30 s at 94 °C, annealing step for 30 s at 55 °C, and extension step for 30 s at 72 °C, followed by additional extension step for 5 min at 72 °C to complete the reaction [7]. The β-actin primer set (F/R) was used for PCR amplification of β-actin encoding gene and the amplified sequence weight was 200 base pairs (bp). Only samples positive for β-actin gene were subjected to polymerase chain reaction amplification of FoxP3 gene.

### 2.5. Primer designing

The primers used in the PCR amplification of FoxP3 gene were designed using the Primer-Blast of the National Center for Biotechnology Information, an all-purpose public tool that helps users design target-specific primers [8].

### 2.6. Nested PCR amplification of FoxP3 gene

For the primary standard PCR reaction, a 10 μL total volume composed of 3.6 μL DDW, 2 μL 5X Buffer, 1 μL $MgCl_2$, 0.8 μL dNTP, 0.5 μL each for forward and reverse primer of the target gene, 0.1 μL Taq enzyme, and 1.5 μL cDNA, were mixed in a PCR tube on ice. The first primer sequence had a forward 5′-GTT TCC CAC AAG CCA AGC TG-3′ and reverse 5′-CTC TGC ACC AAG GAT GCT CA-3′. The FoxP3 PCR profile cycle used were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturing step for 30 s at 94 °C. The annealing step assigned for FoxP3 was 55 °C for 45 s. The extension, on the other hand, was 72 °C for 1 min and 30 s and final extension was 72 °C for 10 min.

The nested PCR reaction was performed using the same 10 μL reaction with the cDNA replaced by the primary PCR product. The second primer had a forward 5′−CCA CAA GCC AAG CTG ATC CT-3′ and reverse 5′−CCG CAG GGA CCA AGA AGT AA-3′. PCR was done under the same profile cycle condition except that the annealing temperature was 59 °C.

All PCR products were analyzed using 2% agarose gel and viewed under short UV illumination.

### 2.7. Purification of FoxP3 and DNA sequencing

PCR products were prepared and sent to First Base (Malaysia) for the purification of FoxP3 and DNA sequencing. Sequence data analysis was done using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information.

### 2.8. Homology analysis

Homology analysis was performed using the Basic Local Alignment Search Tool (BLAST) and CLUSTAL W Multiple Alignment program in BioEdit version 7.2.5. SignalP4.1 server was also used to predict the cleavage site in swamp and riverine buffaloes while TMHMM server v. 2.0, as well as NetNGlyc 1.0 server, were used in predicting transmembrane helices and possible N-linked glycosylation sites in the protein sequence of FoxP3 gene, respectively.

Phylogenetic analysis was performed, as well, to determine evolutionary relationship between FoxP3 of different animal species. Phylogenetic tree was constructed using the neighbour-joining method by the MEGA5 software.

## 3. Results

### 3.1. Sequence analysis of FoxP3

The amplicon size of FoxP3 for both types of water buffaloes was 1296 bp encoding for 432 amino acids is shown in Table 1.The

**Table 1**
Nuclotide and amino acid percentage homology of FoxP3 in different species with their references to swamp-type and riverine-type water buffalo.

| Species | Nucleotide (N) and Amino Acid (AA) Homology Percentages (%) | | | | Accession No. | |
|---|---|---|---|---|---|---|
| | Swamp | | Riverine | | | |
| | N[+] | AA[++] | N[*] | AA[**] | N | AA |
| Cattle | 98 | 97 | 99 | 99 | NM_001045933.1 | NP_001039398.1 |
| Goat | 97 | 97 | 98 | 99 | XM_005700741.2 | XP_005700798.1 |
| Sheep | 97 | 97 | 98 | 99 | NM_001144947.1 | NP_001138419.1 |
| Pig | 91 | 93 | 92 | 95 | NM_001128438.1 | NP_001121910.1 |
| Horse | 91 | 91 | 92 | 93 | NM_001163272.1 | NP_001156744.1 |
| Cat | 90 | 91 | 91 | 93 | NM_001083952.1 | NP_001077421.1 |
| Dog | 89 | 90 | 90 | 92 | NM_001168461.1 | NP_001161933.1 |

[+] Accession no. LC122555.1.
[++] Accession no. BAU50489.1.
[*] Accession no. LC122554.1.
[**] Accession no. BAU50488.

Forkhead or the “winged-helix” superfamily domain, identified using the BLAST, was located from positon 337 up to position 409 (Fig. 1). The transcription factor of the Forkhead was identified as well, which was located in positions 335 to 429. All cysteine residues were all intact in the conserved region of the sequences and 3 N-linked glycosylation sites were observed in positions 318 (NST), 388 (NLS), and 426 (NPT). Moreover, differences in amino acid of swamp- and riverine-type water buffaloes were observed in position 80, wherein alanine (A) was substituted by threonine (T), in position 156, wherein alanine (A) was substituted by aspartic acid (D),in position 163, wherein arginine (R) was substituted by lysine (K), in position 172, wherein proline (P) was substituted by glutamine (Q), in position 177, wherein proline (P) was substituted by histidine (H), in position 178, wherein arginine (R) was substituted by lysine (K), in position 196, wherein asparagine (N) was substituted by histidine (H), in position 201, wherein tryptophan (W) was substituted by arginine (R), in position 206, wherein lysine (K) was substituted by asparagine (N), and position 215, wherein leucine (L) was substituted by isoleucine (I). Additionally, there was only one amino acid substitution observed between riverine-type water buffalo and cattle, in which alanine (A) in riverine buffalo was substituted by valine (V) in cattle located in position 404.

### 3.2. Phylogenetic analysis

The phylogenetic analysis of FoxP3 (Fig. 2) showed a close relationship between buffalo and cattle, showing 90% identity. Generally, it revealed a 100% identity between buffalo, cattle, sheep, goat, and pig which emphasized the close relationship between species of order Artiodactyla. Human FoxP3 was included as outgroup in the phylogenetic tree.

## 4. Discussion

The FoxP3 gene, as part of the Forkhead transcription factor complex or the Forkhead box genes, play an important role in the immune system including the correct performance of cellular and humoral immune response [9]. In this study, FoxP3 gene of swamp-type and riverine-type water buffaloes were amplified and characterized through DNA sequencing and phylogenetic analysis. These are the primary steps in understanding the specific role of FoxP3 in water buffalo’s immune system. FoxP3 of water buffaloes were also compared to existing FoxP3 sequences in the Genbank, particularly from cattle, goat, sheep, pig, horse, dog, cat and human.

The FoxP3 gene of riverine-type water buffalo had the highest homology to cattle, with only 1 amino acid substitution present. This supports the earlier claims of Abdel-Rahman [10] that there is an evidence of high genetic similarity between cattle and buffalo linked to their moleculer markers. On the other hand, riverine and swamp buffaloes contained a number of amino acid substitutions suggest that there are unique characteristics within both swamp and riverine buffaloes in molecular level despite of their close similarities. This can be attributed to the observation of Mingala et al. [11] that swamp-type water buffalo is more disease tolerant than riverine-type water buffaloes. However, it is not yet known if the substitutions have something to do with the claims of disease resistance. The substitutions observed may also be a result of selective pressure. Based on the study of Padiernos and Mingala [12], selective pressure for substitution exists when a disease has a significant effect on morbidity and mortality before the age of reproduction, and may occur when polymorphisms are protective in some diseases. Also, recent evidences indicated that among the diverse environmental factors that acted as selective pressures during evolution, pathogen load had the strongest influence [12].

The homology analysis revealed a high degree of homology between the FoxP3 of swamp-type and riverine-type water buffalo and the FoxP3 of other animal species from Genbank, which indicates highly conserved biological activities, as the conserved regions in the sequences were intact for all the animal species. Homology is further determined by the potential N-linked glycosylation sites which were conserved in all animal species in the study. Additionally, the cysteine residues, considered the most conservative residue in primary structure of homologous proteins [13], also contributed to the homologous properties of the FoxP3 of different species. These observations may suggest that FoxP3 gene of swamp-type and riverine-type water buffaloes may have the same biological properties with the FoxP3 of other animal species in the study.

A study conducted by Pinheiro et al. [14] has documented FoxP3 expression by subpopulations of both CD4+ and CD8 + T cells in dogs. Characterization of the phenotype and function of canine CD4 + CD25 $^{high}$ FOXP3$^{high}$ T cell provides direct evidence of their suppressive function *in vitro*. In dogs, CD5, a type I transmembrane glycoprotein of the scavcenger receptor cysteine-rich superfamily [15], is expressed by both T cells [16] and, at low levels, natural killer cells [17]. The expression of FOXP3 + T cells lacking CD5 expression or FOXP3 expression occurred in cells other than lymphocytes, such as in neoplastic tissue. It is reported that significant increases in the proportions of FOXP3+ CD4 + T cells is not elicited without the addition of IL-2 and TGFβ [18]. Relatively little is known about regulatory T cells in dogs and goats, though indirect evidence for their existence has been available for several years [14].

Meanwhile, CD4 + CD25+ Tregs might play important role in the persistent infection of porcine reproductive and respiratory syndrome (PRRS), a disease which is characterized by reproductive failure in sows and boars [19]. Based on the results of the study conducted [20], real-time PCR data showed that the level of FoxP3 mRNA in PBMCs of PRRSV-infected pigs was significantly higher than that of control pigs.

The observed depletion of FoxP3+ cells, presumed to be T-regs in fetal sheep, could be mediated by different mechanisms. These reduced numbers could be caused by increased death or decreased proliferation of regulatory T-cells [21]. On the other hand, T-reg cells could lose Foxp3 expression under inflammatory conditions.

In horses, a study conducted by Olofsson et al. [22], findings indicated an increase in IL-17α in horses affected with CASP and a lowered expression of IL-12 in horses with CSP. The cytokine profile was related to the histopathological pattern of inflammation and the numbers of Tregs correlated to cytokine expression.

Regulatory T cells (Treg) are increased and directly infected by feline immunodeficiency virus (FIV) and likely play a role in other feline autoimmune, neoplastic, and infectious diseases. Phenotypically, Tregs are best characterized by surface expression of CD4+ and CD25+ and intranuclear expression of the forkhead transcription factor Foxp3. A real-time PCR assay was developed and used to show Foxp3 mRNA

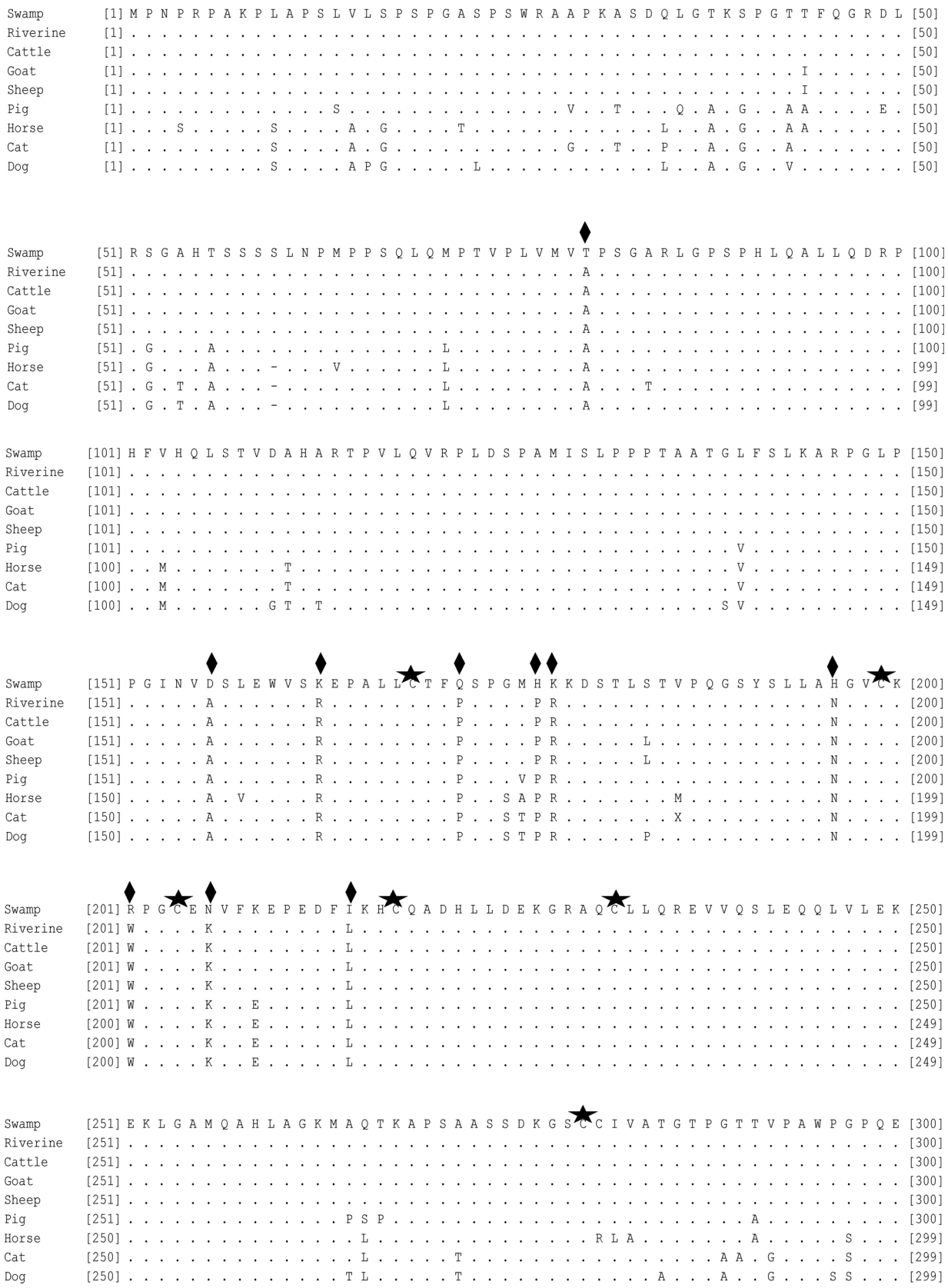


**Fig. 1.** Alignment of the amino acid sequences of FoxP3 genes from swamp-type and riverine-type buffaloes compared with other species. A dot in the sequence signifies the similarity of the amino acids while a dash indicates gap in the alignment. Solid line indicates the Forkhead or the "winged-helix" superfamily domain in the sequence. Enclosed in a box is the transcription factor of the Forkhead family. A solid star indicates the conserved cysteine residue and a solid triangle indicates the potential N-linked glycosylation sites. A solid diamond indicates the amino acid substitutions between swamp and riverine buffaloes and a solid circle indicates the amino acid substitution between water buffalo and cattle.

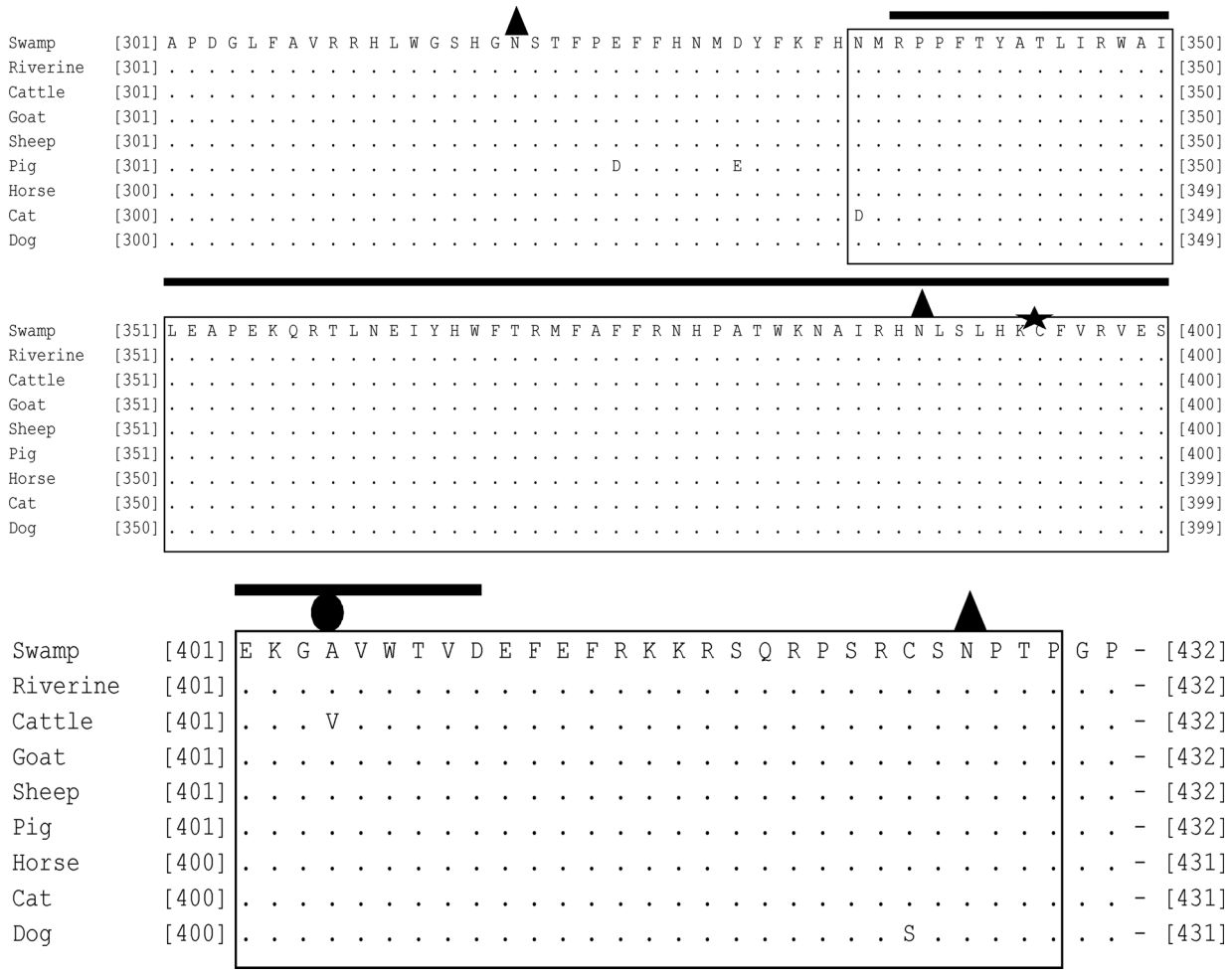


**Fig. 1.** (*continued*)

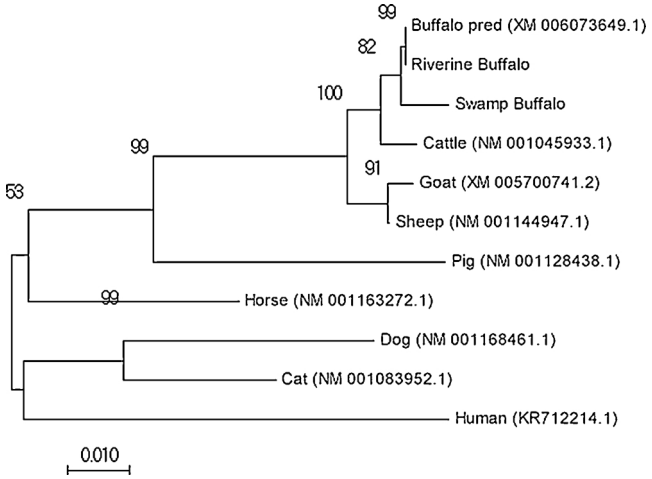


**Fig. 2.** Phylogenetic tree showing the relationship between the FoxP3 sequences of swamp-type and riverine-type buffaloes and the FoxP3 sequences from different species in Genbank.

expression occurs primarily in CD4 + CD25 + T cells [23].

To improve the management of buffaloes, studies about its immune function are helpful. Forkhead box protein 3 (FOXP3), an X-linked gene (located Xp11.23) is a forkhead family member that plays an important role in the development and function of a type of CD24 + T cell called T regulatory cells [24], thus called the "master regulator" for the Treg lineage, which is essential for maintaining immune tolerance to self [24]. Induction of the FoxP3 gene in normal naïve T cells converts them to Treg-like cells with *in vivo* and *in vitro* suppressive function. This indicates that FoxP3 functions in controlling expression of critical suppression-mediating molecules. According to Ziegler [3], the lack of FoxP3 leads to development of fatal autoimmune lymphoproliferative disease. Furthermore, retroviral-mediated introduction of FoxP3 can cause conventional CD24 + T cells to acquire a regulatory-like phenotype and be capable of suppressing immune responses both *in vitro* and *in vivo*.

For the advancement of buffalo management and health including the Philippine native buffaloes and the Philippines upgraded buffaloes, studies about their genetic immunity might be helpful. Genotype of FoxP3 in water buffaloes, was not yet characterized and not well understood. Since FoxP3 is known to be responsible for the development of the regulatory T cells [24] which act to suppress immune responses, hence maintaining homeostasis and self-tolerance [25], it may also have specific significance regarding the immune responses for water buffaloes as compared to other species.

The Philippine water buffalo not only serves as a farming animal, but can also be a good source of milk and meat for human consumption [26]. In this study, FoxP3 gene of water buffaloes are characterized and compared to FoxP3 gene sequence of other species. The study will serve as a baseline for future researchers to discuss further the immune capacity and disease resistance or susceptibility of water buffaloes in relation to the expression of the FoxP3 gene.

In a study of Mingala et al. [11], the researchers investigated on the moleculo-epidemiological and immunological study through cytokine response assessment to know the dynamics of cytokines in the

initiation, persistence and association to physiological changes of a particular pathogen between swamp-type and riverine water buffaloes.

It was observed that swamp-type buffaloes were more disease tolerant than riverine-type buffaloes based on the gathered infection rate of each examined pathogen, further assessment was done focusing on the two vital cytokines, IFNγ and TNFα [11]. Cytokine expression from ConA-stimulated PBMC revealed that both IFNγ and TNFα were more highly expressed in swamp than in riverine buffalo. To further examine the probable cause of expression differences, the proximal promoter region of these two cytokines were sequenced for the presence of nucleotide polymorphism followed by luciferase assay to analyze the effect of these polymorphisms in gene transcription [11].

Luciferase assay showed that both IFNγ promoter and TNFα promoter in swamp-type water buffalo had higher transcription activity compared to riverine-type water buffalo. These findings confirm that IFNγ and TNFα transcriptions in these animals were highly affected by the disparity in the cytokine promoter region. This suggests that disease tolerance or susceptibility of these buffaloes could be due to the differences in their relative cytokine transcription and may relate to pathogen–host specific pathogenesis [11].

## 5. Conclusions

This study showed that both swamp- and riverine-type water buffaloes had high homology but showed differences in their FoxP3 amino acid sequences which could contribute to the claims of disease resistance in swamp buffalo. Phylogenetic and sequence analyses of different animal species (cattle, goat, sheep, horse, pig, dog and cat) were also included to compare with swamp-type and riverine-type water buffaloes. To further analyze the role of FoxP3 gene, particularly in water buffaloes, it is recommended that a more comprehensive study, such as expression analysis, should be done to evaluate its immune regulatory functions and to determine whether the substitutions observed in the DNA and amino acid sequences are functionally important to the animals.

## Conflict of interest

The authors declare no conflicts of interest.

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