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PCR amplification, sequencing, and *in silico* analysis of holandric genes *TSPY* and *SRY* in Nili Ravi buffalo

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ABSTRACT

The present work has been designed to study the evolutionary perspectives of two important holandric genes, namely, testis-specific protein, Y-encoded (*TSPY),* and sex determining region, Y-encoded (*SRY*) in Nili Ravi buffalo. These Y-chromosomal genes are responsible for male sex development. The evolutionary aspects of these genes have not been studied in the Nili Ravi buffaloes from India. A study was carried out on the peripheral blood samples of three adult Nili Ravi bulls belonging to university farm. DNA was extracted using the phenol-chloroform isoamyl alcohol (PCI) technique and reported primer sequences were used to amplify the genes (partially) using a thermocycler. Three biological samples were analyzed using Sanger sequencing and were aligned using Clustal Omega. Separate phylogenetic trees for the genes were constructed using MEGA7 software. The selection pressure analysis and evolutionary divergence were individually studied for each of the genes. The current study reports the partial *TSPY* and *SRY* gene sequences of Indian Nili Ravi buffalo. Besides, the molecular evolution analysis indicates that the genes have experienced some purifying selection during the evolutionary process. Molecular characterization of these holandric genes (*TSPY* and *SRY*) in Nili Ravi will find applications in sex determination in Nili Ravi and other buffaloes.

Keywords: Evolution, Nili Ravi, *SRY*, Structure prediction, *TSPY*, Y-Chromosome

Nili Ravi buffaloes are native to the Punjab state of India and are one of the most famous water buffalo breeds. These animals are reared by farmers and dairy entrepreneurs. India is a hub of more than 50% of the world's buffalo population. Nili Ravi is considered one of the best buffalo breeds in India. Globally, Punjab (India) and Pakistan are the hotspots for the black-coloured breed of Nili Ravi. The word 'Nili' is derived from the Sutlej River's blue water. Nili and Ravi were once two separate breeds, but with time and significant crossbreeding, these two breeds got mixed to produce the Nili Ravi breed. The possession of white markings known as *Panch Kalyani* is considered the most desired feature of a female. These buffalo have a lot of phenotypic resemblance with the Murrah buffalo. One significant advantage of this breed over others is in terms of disease resistance. In India, the Nili Ravi can adjust itself to various climatic variations.

Testis Specific Protein Y-encoded (*TSPY*) gene in bovines includes 7 exons that are separated by 6 introns (Vogel *et al.* 1997). Its' length is estimated to be 2.7 kb (Kim *et al.* 1997). If the *TSPY-1* gene gets overexpressed then the proliferation of cells occurs and apoptosis takes place. The *SRY* gene is a small intron-less gene that is located on the short branch of the Y-chromosome. This gene produces the sex-determining region Y (*SRY*) protein, which works as a transcription factor (Kurtz *et al*. 2021). Thereby, regulating the activity of a particular gene encoded by that specific region of DNA (Anonymous 2019). Reports on molecular characterization of Nili Ravi buffalo from India are very limited. The current study has attempted to amplify two important holandric genes, namely *TSPY* (partial sequence) and *SRY* to determine the conserved nature of these gene sequences among buffaloes and other ruminants. This work attempts to amplify the 217 bp long high-mobility group (HMG) box region of the *SRY* gene and a stretch of DNA (containing partial exon 3, intron 4, and partial exon 4 using two sets of primers targeting overlapping regions) of the *TSPY* gene. The objective of the study is to report the partial sequences of the genes of the Nili Ravi buffalo (of the Indian region) and explore the evolutionary perspectives of these genes.

MATERIALS AND METHODS

Sample collection and DNA extraction: About 10 ml of peripheral blood samples were aseptically collected from three unrelated, adult, male Nili Ravi buffaloes maintained at the Directorate of livestock farm Guru Angad Dev Veterinary farm. Genomic DNA was isolated from the freshly collected peripheral blood using the

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standardized phenol-chloroform isoamyl alcohol technique (Sambrook and Russell *et al*. 2006). The quality and quantity of the extracted DNA were checked by Agarose gel electrophoresis (0.8% gel, 80V × 45 min, against 1Kb-plus DNA ladder (Promega)) and Nanodrop (Thermo-Scientific)) measurement (Fig. 1).

Primers and optimization of PCR: The primers for the present study were selected from a formerly published research paper (Mukhopadhyay *et al*. 2011) (Table 1). PCR amplification of the template DNA was carried out in a thermocycler and was programmed accordingly for each primer pair. The composition of the master mix was as follows: 2 µl of DNA template (100 ng/µL), 0.6 µl of Taq polymerase (5 U/µL), 1 µl of dNTPs and 4 µl primer (forward and reverse, each), 10 µl PCR buffer with 4 µl $MgCl_2$.

Custom sequencing of PCR products: The PCR products were sent to Kerala, India for customized Sanger sequencing (paired-end). The electropherogram of each pair of sequences was critically analysed for trimming the poor-quality termini. The forward and reverse sequences were aligned to obtain the final sequence.

Downloading homologous sequences of target genes from NCBI GenBank: The homologous gene sequences of *TSPY* and *SRY*, representing other bubaline breeds vis-a-vis divergent species were downloaded from NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nuccore). The detail of these sequences for these two genes has been adumbrated Acc. no. EU032586,DQ923704, EU350952, XM_018045416, MT768536, JQ303256, EF432554, XM_020903635, XM_043460210, XM_032474735, XM_035725886, XM_033420189, XM_030856992, XM_029233063, XM_027102774, XM_036862929, XM_043897488, XM_042926830 and Acc. No. JX668002, JX667999, JX667995, GQ259332, FJ546413, AY341337, DQ417872, DQ119747, EU294189, DQ888702, AY07914, AY079141, Z30321, EU547257, AY079146, EU016229, KF038141, KF038140, MH680934, EF100132, KC337274

Multiple sequence alignment (MSA) and evolutionary analysis: MSA of the homologous sequences (of *SRY* and *TSPY* genes) was done using the online tool Clustal Omega (Sievers *et al*. 2011; https://www.ebi.ac.uk/Tools/msa/clustalo/). Selection of best evolutionary models, construction of the phylogenetic trees, evolutionary analyses etc were done for the genes separately, using molecular evolution and genetic analysis (MEGA 7, Kumar *et al.* 2016). The best evolutionary models for the *SRY* gene and *TSPY* genes were selected based on the respective lowest Akaike Information Criterion corrected (AICc) values. MEGA 7 (https://www.megasoftware.net/) was used to construct the phylogenetic trees using the maximum likelihood method and 500 bootstrap resampling.

RESULTS AND DISCUSSION

Novel report on partial holandric gene sequence in Nili Ravi buffalo breed: The forward and reverse sequences were analysed along with studying the electropherogram for the discarding of the portions with noise. The overlapping fragments of TSPY have merged to produce a single sequence. The detail of the annotation of the sequences obtained (of *TSPY* and *SRY*) is given below:

>**SRY_NR_Final** (NCBI Accession Number ON427828)
GTGGTCTCGTGAACGAAGACGAAAGTTG
GCTCTAGAGAATCCCAAAATGAAAAACTCAGA
GATCAGCAAGCAGCTGGGCTATGAGTG
GAAAAGGCTTACAGATGCTGAAAAGCGCCC
ATTCTTTGAGGAGGCACAGAGACTAC
TATCCATACACT

gene <1..>155
/gene=”Sex-determining Region, Y-encoded (SRY)”
mRNA join (<1..155)
/gene=”Sex-determining Region, Y-encoded (SRY)”
/product=”Sex-determining Region, Y-encoded (SRY)”
CDS (<1..155)
/gene=”Sex-determining Region, Y-encoded (SRY)”
/codon_start=2
/product=”Sex-determining Region, Y-encoded (SRY)”
/translation=”SRERRRKLALENPKMKNSEISKQLG
YEWKRLTDAEKRPFFEEAQRLLSIH”

> **TSPY_NR_Final*** (BankIt2579742; NCBI Accession Number: ON427827)
TGATCTTTTCCTTTGGGGACAACTCCTAC
TTGTGGAACACGATGATCATTAAGGAGTATT
ACTTTGACATCACTGGTAAAACGTGGCTCTAGG
GCGGTGAGGGTAGGTGTGTAAGGAGG
TGGATGATCCCCCTGCGGAATCCGCC
CCTGTGTTCCCTGTCTTTCTGGAGGTTA
TAAGGCACGTTATTCCACTCCAGTCC
ACTGGTTCTGGGACTTTGAA

*The yellow highlighted regions in the above sequence mark the exonic region.

Annotations:
gene <1..>219

Table 1. Details of primer pairs used for amplifying the *TSPY* and *SRY* genes

Name	Sequence	Length	GC-content	Melting temp
TSPY-34-F	TCCGCCGTC CCGCTGCAAGCT	21	71.4	68.9
TSPY-34-R	CAATCCTGTTCGATTCTG GGC	21	52.4	56.1
TSPY-45-F	GTCGATCCACCCCAGTCC ACT	21	61.9	61
TSPY-45-R	ACCCCATCACCACATGTG TTT	21	47.6	57.1
SRY-HMG-F	CCATGAACGCCTTCATTG TG	20	50	54.6
SRY HMG-R	GCTCTCCGACGAGGTCGA TA	20	60	58.2

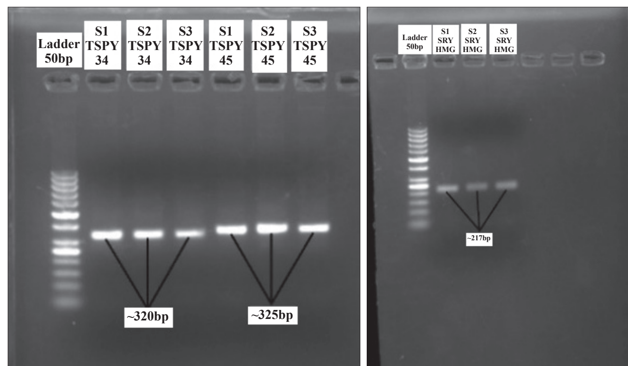

Fig. 1. Quality and integrity of the extracted DNA revealed by agarose gel electrophoresis analysis (at 80V for 45 min) for custom sequencing of *TSPY* (Left gel) and *SRY* (Right gel) gene.

/gene=" Testis Specific Protein, Y-encoded" mRNA join (<1.74, 172.219)
/gene=" Testis Specific Protein, Y-encoded"
/product=" Testis Specific Protein, Y-encoded" CDS join (<1.74, 172.219)
/gene=" Testis Specific Protein, Y-encoded"
Exon 3 <1..74>
/codon_start=3
Intron 3 <75.171>
Exon 4 <172.219>
/codon_start=1
/product="Testis Specific Protein, Y-encoded"/translation=" IFSFGDNSYLWNTMIIKEYYFDITYKARYSTPV HWFWDFE"

Multiple sequence alignment (MSA): MSA was performed using Clustal Omega for *SRY* and *TSPY* from different species and places to access them.

Best model prediction: The best model predicted was based on the lowest values of BIC (Bayesian Information Criterion) and AICc (Akaike Information Criterion corrected) model selection. MEGA7 was used to do an evolutionary analysis of the sequences from diverse species. The best models for evolutionary analysis were Jukes-Cantor (JC) and Kimura 2 for *SRY* and *TSPY* genes, respectively.

Phylogenetic tree construction: Separate phylogenetic trees were constructed for *SRY* and *TSPY* genes using a maximum likelihood model with 500 bootstrap sampling (Figs. 2 and 3). Phylogenetic analysis revealed that sequences belonging to the same family or of the same order form a cluster. The trees describeed the formation of different overlays based on evolutionary changes between sequences.

Pair-wise distance: Pair-wise distance analysis revealed that our target bubaline *SRY* gene is highly divergent from the *SRY* gene of moose and different species of deer (Supplementary Fig. 1). However, the bubaline *SRY* showed a high degree of conservation. In the case of the *TSPY* sequence, the bubaline sequence is very close to other domesticated ruminant species, however, distant enough from that of deer, elk, camel, and lion (sylvian

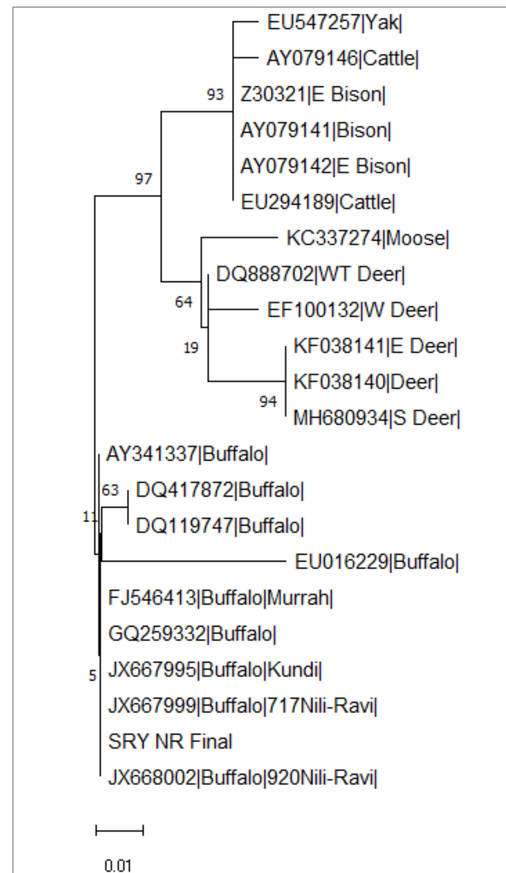

Fig. 2. Phylogenetic tree of *SRY* gene sequence, constructed by using MEGA 7 (maximum likelihood method).

species), and whales (Supplementary Fig. 2).

Evolutionary analysis: The value of dN-dS test statistic indicates whether the gene sequences have gone through positive, negative, or neutral selection. The rate of synonymous substitution per site is denoted by dS and

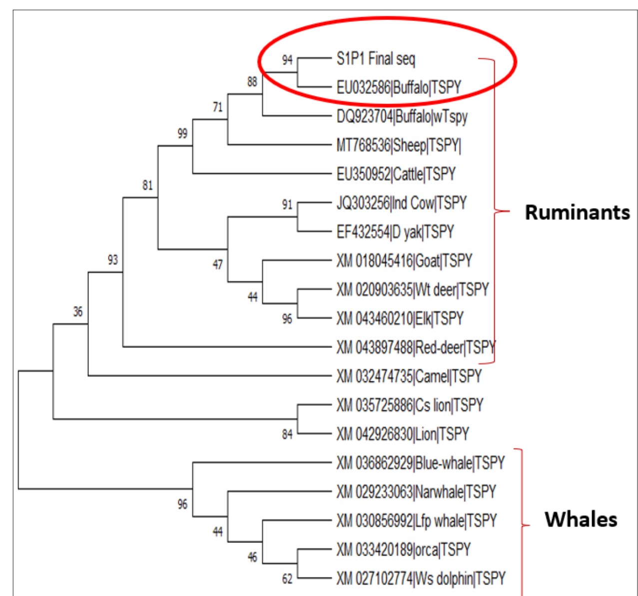

Fig. 3. Phylogenetic tree of *TSPY* gene sequence, constructed by using MEGA 7 (maximum likelihood method).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
1		SRY_NR_Final	JX668002|Buffalo|920Nili-Ravi	JX667999|Buffalo|717Nili-Ravi	JX667995|Buffalo|Kundi	GQ259332|Buffalo|	FJ546413|Buffalo|Murrah	AY341337|Buffalo|	DQ417872|Buffalo|	DQ119747|Buffalo|	EU294189|Cattle|	DQ888702|WT_Deer|	AY079142|E_Bison|	AY079141|Bison|	Z30321|E_Bison|	EU547257|Yak|	AY079146|Cattle|	EU016229|Buffalo|	KF038141|E_Deer|	KF038140|Deer|	MH680934|S_Deer|	EF100132|W_Deer|	KC337274|Moose|
2	SRY_NR_Final		0.000	0.000	0.000	0.000	0.000	0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
3	JX668002|Buffalo|920Nili-Ravi|	1.000		0.000	0.000	0.000	0.000	0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
4	JX667999|Buffalo|717Nili-Ravi|	1.000	1.000		0.000	0.000	0.000	0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
5	JX667995|Buffalo|Kundi|	1.000	1.000	1.000		0.000	0.000	0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
6	GQ259332|Buffalo|	1.000	1.000	1.000	1.000		0.000	0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
7	FJ546413|Buffalo|Murrah|	1.000	1.000	1.000	1.000	1.000		0.000	-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
8	AY341337|Buffalo|	1.000	1.000	1.000	1.000	1.000	1.000		-1.004	-1.004	0.993	0.399	0.993	0.993	0.993	0.778	1.416	-0.578	1.763	1.763	1.763	0.770	1.225
9	DQ417872|Buffalo|	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	0.784	0.132	0.784	0.784	0.784	0.578	1.237	-0.783	1.602	1.602	1.602	0.569	1.050
10	DQ119747|Buffalo|	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.784	0.132	0.784	0.784	0.784	0.578	1.237	-0.783	1.602	1.602	1.602	0.569	1.050
11	EU294189|Cattle|	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.217	0.217		0.990	0.000	0.000	0.000	-1.004	1.013	0.399	1.410	1.410	1.410	1.221	1.592
12	DQ888702|WT_Deer|	0.345	0.345	0.345	0.345	0.345	0.345	0.345	0.448	0.448	0.162		0.990	0.990	0.990	0.774	1.413	-0.169	1.801	1.801	1.801	0.688	1.212
13	AY079142|E_Bison|	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.217	0.217	1.000	0.162		0.000	0.000	-1.004	1.013	0.399	1.410	1.410	1.410	1.221	1.592
14	AY079141|Bison|	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.217	0.217	1.000	0.162	1.000		0.000	-1.004	1.013	0.399	1.410	1.410	1.410	1.221	1.592
15	Z30321|E_Bison|	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.217	0.217	1.000	0.162	1.000	1.000		-1.004	1.013	0.399	1.410	1.410	1.410	1.221	1.592
16	EU547257|Yak|	0.219	0.219	0.219	0.219	0.219	0.219	0.219	0.282	0.282	1.000	0.220	1.000	1.000	1.000		0.692	0.242	1.223	1.223	1.223	1.040	1.428
17	AY079146|Cattle|	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.109	0.109	0.157	0.080	0.157	0.157	0.157	0.245		0.846	1.761	1.761	1.761	1.592	1.918
18	EU016229|Buffalo|	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.345	1.000	0.345	0.345	0.345	0.405	0.200		1.216	1.216	1.216	0.227	0.687
19	KF038141|E_Deer|	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.056	0.056	0.081	0.037	0.081	0.081	0.081	0.112	0.040	0.113		0.000	0.000	1.599	2.227
20	KF038140|Deer|	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.056	0.056	0.081	0.037	0.081	0.081	0.081	0.112	0.040	0.113	1.000		0.000	1.599	2.227
21	MH680934|S_Deer|	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.056	0.056	0.081	0.037	0.081	0.081	0.081	0.112	0.040	0.113	1.000	1.000		1.599	2.227
22	EF100132|W_Deer|	0.221	0.221	0.221	0.221	0.221	0.221	0.221	0.285	0.285	0.112	0.246	0.112	0.112	0.112	0.150	0.057	0.411	0.056	0.056	0.056		1.403
23	KC337274|Moose|	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.148	0.148	0.057	0.114	0.057	0.057	0.057	0.078	0.029	0.247	0.014	0.014	0.014	0.082	

Fig. 4. Table of Negative selection using MEGA7 software of different species and places of *SRY*. The test statistic (dN-dS) is shown above the diagonal and the corresponding; level of significance (p-value) is shown below diagonal.

		S1P1_Final_seq	EU032586|Buffalo	DQ923704|Buffalo	EU350952|Cattle|	XM_018045416|G	MT768536|Sheep	JQ303256|Ind_Co	EF432554|D_yak|	XM_020903635|W	XM_043460210|E	XM_032474735|C	XM_035725886|C	XM_033420189|o	XM_030856992|L	XM_029233063|N	XM_027102774|W	XM_036862929|E	XM_043897488|F	XM_042926830|L
2	S1P1_Final_seq		1.01	1.13	1.94	-0.41	1.11	0.14	0.14	0.07	-0.15	0.47	-0.16	-0.36	-0.15	-0.22	-0.31	-0.35	0.32	-0.29
3	EU032586|Buffalo|TSPY	0.16		1.52	2.19	-0.16	1.42	0.39	0.39	0.32	0.11	0.72	0.08	-0.11	0.10	0.03	-0.06	-0.11	0.63	-0.04
4	DQ923704|Buffalo|wTspy	0.13	0.06		2.42	-0.33	1.49	0.41	0.41	0.17	-0.33	0.91	-0.02	-0.42	-0.20	-0.51	-0.36	-0.36	0.37	-0.42
5	EU350952|Cattle|TSPY	0.03	0.02	0.01		0.79	0.65	0.74	0.74	1.06	0.68	1.94	1.44	0.81	0.89	0.85	0.76	0.71	1.38	0.87
6	XM_018045416|Goat|TSPY	1.00	1.00	1.00	0.22		1.03	0.42	0.54	-1.55	-0.87	0.57	-0.71	-0.99	-1.00	-0.90	-1.01	-1.38	1.29	0.20
7	MT768536|Sheep|TSPY|	0.13	0.08	0.07	0.26	0.15		0.79	0.79	0.93	0.52	1.26	0.78	0.58	0.79	0.72	0.62	0.57	1.17	0.55
8	JQ303256|Ind_Cow|TSPY	0.44	0.35	0.34	0.23	0.34	0.21		-1.00	-1.31	0.45	1.34	-0.41	-0.99	-0.77	-0.67	-0.89	-0.48	1.47	0.07
9	EF432554|D_yak|TSPY	0.44	0.35	0.34	0.23	0.29	0.21	1.00		-1.46	0.33	1.22	-0.52	-1.11	-0.88	-0.78	-1.01	-0.60	1.33	-0.04
10	XM_020903635|Wt_deer|TSPY	0.47	0.37	0.43	0.15	1.00	0.18	1.00	1.00		-0.40	-0.21	-1.62	-1.31	-1.32	-1.21	-1.44	-1.05	1.38	-0.95
11	XM_043460210|Elk|TSPY	1.00	0.46	1.00	0.25	1.00	0.30	0.33	0.37	1.00		0.73	-0.68	-0.73	-0.54	-0.46	-0.65	-0.31	0.56	-0.39
12	XM_032474735|Camel|TSPY	0.32	0.24	0.18	0.03	0.29	0.11	0.09	0.11	1.00	0.23		-0.66	-0.84	-0.58	-0.48	-0.72	-0.25	1.21	-0.11
13	XM_035725886|Cs_lion|TSPY	1.00	0.47	1.00	0.08	1.00	0.22	1.00	1.00	1.00	1.00	1.00		-2.66	-2.38	-1.84	-2.53	-2.14	-0.22	0.16
14	XM_033420189|orca|TSPY	1.00	1.00	1.00	0.21	1.00	0.28	1.00	1.00	1.00	1.00	1.00	1.00		-1.42	-0.45	-1.00	-0.47	0.54	-0.96
15	XM_030856992|Lfp_whale|TSPY	1.00	0.46	1.00	0.19	1.00	0.21	1.00	1.00	1.00	1.00	1.00	1.00	1.00		0.19	-1.00	0.18	0.67	-0.71
16	XM_029233063|Narwhale|TSPY	1.00	0.49	1.00	0.20	1.00	0.24	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.43		-0.15	0.25	0.53	-0.59
17	XM_027102774|Ws_dolphin|TSI	1.00	1.00	1.00	0.23	1.00	0.27	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		-0.17	0.65	-0.85
18	XM_036862929|Blue-whale|TSP	1.00	1.00	1.00	0.24	1.00	0.28	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.43	0.40	1.00		0.65	-0.63
19	XM_043897488|Red-deer|TSPY	0.38	0.27	0.36	0.09	0.10	0.12	0.07	0.09	0.08	0.29	0.12	1.00	0.29	0.25	0.30	0.26	0.26		1.05
20	XM_042926830|Lion|TSPY	1.00	1.00	1.00	0.19	0.42	0.29	0.47	1.00	1.00	1.00	1.00	0.43	1.00	1.00	1.00	1.00	1.00	0.15	

Fig. 5. Table of Negative selection using MEGA7 software of different species and places of *TSPY* gene. The test statistic (dN-dS) is shown above the diagonal and the corresponding; level of significance (p-value) is shown below diagonal.

the non-synonymous substitution rate is represented by dN, respectively. The codon-based test of purifying selection is considered, if the null hypotheses for the test for neutral selection (dN=dS) gets rejected and the alternate hypotheses (dN<dS) are accepted.

The results indicate that the *SRY* genes of bubaline origin (irrespective of breeds) have made a single clad. Interestingly, the *SRY* genes of cattle and bison are forming a separate and distant clad concerning that of buffalo. Besides, the *SRY* genes of moose and deer are even closer to the *SRY* of cattle. So, it can infer that the evolution of bubaline SRY has experienced some selection pressure (purifying selection) that has carved its sequence features (Fig. 4). Besides, using bubaline *SRY* for sex determination needs to be revisited while using cattle SRY sequence.

However, the story of the *TSPY* gene in cattle and buffalo is a bit different, as the sequence similarity between all ruminant *TSPY* is high and the *TSPY* of marine whales is different. It is understood that divergent evolution has contributed to the variable sequence features between ruminant and marine mammals (Fig. 5).

SRY gene is conserved among buffalo breeds divergent between buffalo and deer. The TSPY gene is highly divergent from the *TSPY* gene of ruminants and aquatic animals. The analysis reviewed that the *SRY* gene has experienced (negative) selection in Eld's deer, Deer, South China Sika deer, and concerning cattle. *TSPY* gene has also experienced the (negative) selection in cattle, camels, and aquatic animals. The conserved regions of these male-specific genes could be potentially used for sex determination (Qin *et al*. 2022).

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