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Original Article

Post-thawed semen quality and genomic variation in indigenous Indonesian buffalo breeds

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Abstract

Buffaloes (Bubalus bubalis) play a vital role in Indonesian livestock systems, but artificial insemination (AI) programs are often hindered by the decline in semen quality after cryopreservation. This study aimed to evaluate post-thawed semen performance and genetic variability among Toraja, Kalsel, and Silangit buffalo bulls, with the goal of identifying phenotypic and molecular traits linked to cryotolerance. A total of 60 frozen semen straws (20 per bull) were assessed for motility, viability, membrane integrity, and acrosome integrity using computer-assisted semen analysis (CASA) and functional assays. Genomic DNA obtained from blood samples was subjected to PCR amplification of candidate fertility genes (OPN, IGF-1, LHβ, SPAG11B, and TNP1), followed by sequence analysis to identify nucleotide mutations and deduced amino acid substitutions. Results showed that Silangit bulls had significantly greater viability (81.49%) and membrane integrity (89.70%) than Toraja and Kalsel bulls ($p < 0.001$), suggesting superior tolerance to freezing. All breeds displayed conserved G+C content (55–62%), but mutation patterns varied: OPN mutations appeared only in Toraja bulls, while LHβ and TNP1 showed higher mutation frequencies in Silangit bulls. Correlation tests revealed positive associations between LHβ mutations and sperm viability/straightness ($r = 0.999–1.000$, $p < 0.05$), while variations in OPN and SPAG11B were negatively linked to motility, acrosome integrity, and survival. These findings indicate that semen cryotolerance differences among buffalo breeds are influenced by specific genetic variations. Despite a limited sample size and narrow genomic coverage, the study highlights the value of integrating semen quality analysis with genomic tools for sire selection and improving AI success in native buffalo populations.

Keywords: Buffalo bulls, Post-thawed semen quality, Cryopreservation, Genomic variation, Indigenous breeds

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Introduction

Buffaloes (*Bubalus bubalis*) are integral to the agricultural systems of Southeast Asia, particularly Indonesia, where they serve as draught animals and a source of meat and milk. Indigenous populations such as the Toraja buffalo (South Sulawesi), the Kalsel buffalo (South Kalimantan), and the Silangit buffalo (North Sumatera) represent valuable genetic resources uniquely adapted to local environments. However, reproductive performance remains a limiting factor in genetic improvement and artificial insemination (AI) programs. In particular, semen cryopreservation frequently leads to decreased sperm motility, compromised membrane integrity, and acrosomal damage, which collectively reduce fertilization capacity and conception success (Prihandini et al., 2023). Previous studies in riverine buffaloes reported significant variation in post-thawed semen quality among breeds and even among individuals within the same breed (Khan et al., 2024). Such findings highlight the importance of evaluating semen quality traits across local Indonesian buffalo populations, which remain poorly characterized.

Cryopreservation-induced damage to spermatozoa can be quantified using several methods. Computer-assisted semen analysis (CASA) has proven to be a reliable tool for objective assessment of motility patterns (Alm-Kristiansen, 2023), while viability staining and the Hypo-Osmotic Swelling Test (HOST) provide valuable insights into plasma membrane functionality (Duran et al., 2015). Acrosome integrity, a critical determinant of fertilization success, has been extensively studied in cattle and buffaloes, with compromised acrosomes shown to directly reduce fertilization outcomes (Venkatesh at al., 2024). However, such systematic assessments of post-thawed semen quality in indigenous Indonesian buffaloes remain scarce.

While phenotypic traits are critical indicators of reproductive performance, accumulating evidence suggests that underlying genetic variation also plays a pivotal role in shaping sperm quality and fertility. Key candidate genes, including osteopontin (OPN), insulin-like growth Factor-I (IGF-1), luteinizing hormone beta (LHβ), sperm-associated antigen 11B (SPAG11B), and transition protein 1 (TNP1), have been associated with essential sperm functions such as motility, acrosome reaction, membrane integrity, and chromatin condensation (Deshmukh et al., 2021). For example, polymorphisms in *OPN* have been linked to reduced sperm motility in bulls (Hasanain et al., 2022), while *LHβ* gene variation has been associated with reproductive hormone regulation in buffaloes (Nadeem et al., 2025). Similarly, *TNP1* and *SPAG11B* are crucial for chromatin condensation and sperm maturation, with mutations leading to impaired fertility (Elango et al., 2020). Despite such advances, little is known about how these fertility-associated genes vary in Indonesian native buffaloes and how such variation correlates with semen cryotolerance.

Integrating post-thawed semen quality evaluation with genomic characterization provides a powerful approach to identify biomarkers predictive of fertility. Previous studies in cattle and riverine buffaloes demonstrated that correlations between sperm quality parameters and gene polymorphisms could be used as selection criteria in breeding programs (Carvalho et al., 2023). However, no such integrative studies have been performed in Indonesian buffalo populations, creating a critical knowledge gap.

Therefore, this study aimed to (i) compare post-thawed semen quality traits among Toraja, Kalsel, and Silangit buffalo bulls, (ii) analyze sequence variation in five candidate genes (*OPN, IGF-1, LHβ, SPAG11B,* and *TNP1*), and (iii) evaluate correlations between gene mutations, amino acid changes, and semen quality traits. By combining phenotypic and genomic analyses, this research seeks to provide novel insights into the molecular basis of semen cryotolerance, offering potential markers for genetic selection and strategies to enhance the efficiency of AI programs in Indonesian buffalo populations.

Material and Methods

Buffalo's bull source

Three indigenous Indonesian bulls (*Bubalus bubalis*) were involved in this study, specifically the Toraja buffalo bull (from South Sulawesi), the Kalsel buffalo bull (from South Kalimantan), and the Silangit buffalo bull (from North Sumatera). The animals were obtained from Technical Implementation Unit Artificial Insemination and Semen Production, South Sulawesi Provincial Livestock and Animal Health Service; Technical Implementation Unit Artificial Insemination Center, South Kalimantan Provincial Plantation and Livestock Service; and Siborongborong Superior Livestock Breeding and Forage Center, North Tapanuli Regency, North Sumatera. All buffalo bulls used in this study were

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sexually mature (5–8 years old), representing the optimal reproductive age range for this species, and had a body weight of 450-750 kg (Kanchan and Matharoo, 2015; Ramajayan et al., 2023).

Assessment of post-thawed semen quality

Sixty straws of frozen semen (twenty straws/bull) from three bulls were used to analyze sperm motility, sperm abnormality, and sperm viability. First, the frozen semen was thawed in a water bath at 37^0C for 30 s and then put into a microtube for further analysis. Sperm motility was analyzed using CASA (computer-assisted semen analysis) based on Diansyah et al. (2025). Each sperm sample was dispensed (10 µL) onto a preheated slide and covered with a cover slip. A total of 350 sperm in a total of four fields were evaluated using the Spermvision software program (Vision version TM3.7.5 Minitube program, Germany). Viability was calculated by dripping one drop of semen and eosin solution and then mixing evenly. After drying, it is observed using a 10x40 times magnification microscope, dead spermatozoa will absorb colour, while live spermatozoa remain white (Nirmala et al., 2025).

Assessment of intact plasma membrane and intact acrosome of buffalo bulls

The integrity of the plasma membrane of sperm can be assessed using the Hypo-Osmotic Swelling Test (HOST) as described by Duran et al. (2015), which involves mixing a small volume of semen with a hypo-osmotic solution, typically with an osmolality of 150 mOsm. After incubating the mixture at 37°C for 45 minutes to 1 hour, the sperm are evaluated under a microscope. The assessment focuses on the swelling and coiling of the sperm tails, which indicates intact plasma membranes. A higher percentage of swollen spermatozoa reflects better functional integrity of the plasma membrane, making HOST a reliable and cost-effective method for evaluating sperm quality, particularly in frozen-thawed samples.

The integrity of the acrosome in sperm can be evaluated using the formol saline test as describe by Delgado et al. (2024), which involves mixing a drop of semen with a 2% formaldehyde solution in saline and incubating it at room temperature for 30 minutes. Following fixation, the sample is rinsed and examined under a microscope to assess acrosome integrity. Sperm that exhibit no green fluorescence when stained with FITC-PSA or show intact acrosomes with Giemsa stain are considered to have intact acrosomes (Purnawan et al., 2025). This method is effective for routine semen evaluation and serves as a predictive tool for fertility potential, providing valuable insights into the reproductive capabilities of livestock.

Blood sampling and DNA extraction

Blood samples (n total=18, six samples/bull) were collected, about 3 mL of blood, through the jugular vein. Blood samples were collected using a 3 mL syringe into vacuum tubes with ethylenediaminetetraacetic acid (EDTA) (Vaculab® EDTA K3, OneMed, Surabaya, Indonesia), and kept at -20°C until DNA extraction. Extraction of DNA was performed using a isolation DNA extraction kit from Quick-DNA Miniprep Plus Kit (Zymo Research Corp, California, USA).

Genomic DNA analysis

Genomic DNA was extracted from blood samples using the Quick-DNA Miniprep Plus Kit (Zymo Research, USA) according to the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). PCR amplification was performed in a 40 µL reaction mixture containing KOD One™ PCR Master Mix (Toyobo, Japan), 0.2 µM of each primer, nuclease-free water (HiMedia, India), and 20 ng of template DNA. PCR amplification was performed using a BIO-RAD thermal cycler (Bio-Rad, USA). Amplified products were assessed by electrophoresis on a 1% agarose gel (Invitrogen, USA) using the GELATO electrophoresis system (miniPCR bio, USA). PCR products yielding a single distinct band were subjected to Sanger sequencing in both forward and reverse directions. The resulting sequences were quality-checked and assembled into consensus sequences using DNA Baser v3.5.4 (Heracle Software, Lilienthal, Germany).

Gene-specific primers were designed for the full-length gene amplification. The sequence of *osteopontin* gene (OPN), *insulin-like growth factor-I* gene (IGF-1), *Luteinizing hormone beta* gene (LHβ), *Sperm Associated Antigen 11B* gen (SPAG11B), and *transition protein 1* gene (TNP1) were retrieved from the NCBI genebank. The primers were designed using Primer3 and BLAST NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) (Table 1). The conditions for amplifying amplicons were optimized.

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The PCR reaction was carried out using a 2x PCR master mix (Applied Biotechnology, Egypt). The reaction mixture was composed of 2 µl DNA template, 0.5 µl from each primer, and 25 µl master mix. PCR conditions were optimized for each primer pair (annealing temperatures ranged from 63–67 °C; extension at 72 °C for 5–10 seconds across 35 cycles). The thermo-cycling condition were done acording to the methods of Rimayanti et al. (2023). Then, 2% ethidium bromide agarose gel electrophoresis was used to detect the PCR products (Amrullah et al., 2023).

Table-1. The list of primers used in the study to amplify various fragments of OPN, IGF-1, LHβ, SPAG11B, and TNP1.

Gene	Accession ID	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')	Annealing temp.(ºC)
OPN	DQ899755.1	CTGACGACCCTGACCATTC C	ACGTAAGTGAGCACGC AGAC	67
TNP1	XM_006067232.4	GGGAGTCAAAAGAGGTGG CA	TGCCTGGTGTCGTTCAA AGT	63
SPAG11B	XM_045165130.1	GAGATGGACGGCAGATCA GG	TCAGACCTTCACCCTTG ATGC	66.7
LHβ	NM_001290957.1	GCCTGCCCTGTCTGTATCA C	GGAGACCATTGGGTCC ACTC	63

Statistical and genomic analyses

Post-thawing sperm characteristics data were presented as the mean ± standard deviation (SD). Significant differences among the Indonesian native buffalo breed groups were determined using an analysis of variance (ANOVA). For the genomic analysis, DNA sequences were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 12.0.14. This process commenced with sequence alignment, followed by the quantification of the total mutational sites and the number of amino acids in each gene. The results of this quantification were visualized using bar charts to facilitate comparison. Subsequently, a Pearson correlation analysis was performed to assess the association between post-thawing sperm characteristics and the total number of mutational sites and amino acids. The results of the correlation analysis were visualized as a heatmap for effective interpretation of the relationship patterns and strengths.

Results

Comparison of post-thawing sperm quality between local Indonesian buffalo bulls

Table-2. Post-thawed sperm quality bull between Toraja buffalo, Kalsel buffalo, and Silangit buffalo.

Characteristics	Toraja	Kalsel	Silangit	p-*value*
Total Motility (%)	67.96 ± 1.2	66.90 ± 1.13	65.03 ± 4.26	0.318
Progresive motility (%)	52.22^{a} ± 2.59	57.84^{b} ± 0.83	57.78^{b} ± 1.96	0.002
Viability (%)	54.52^{a} ± 4.58	54.88^{a} ± 2.98	81.49^{b} ± 1.52	0.000
Acrosome integrity (%)	86.70 ± 3.89	83.39 ± 2.28	84.13 ± 2.51	0.293
Membrane integrity (%)	59.85^{a} ± 1.03	70.91^{b} ± 0.44	89.70^{c} ± 1.41	0.000
DCL (µm)	87.15 ± 2.49	84.13 ± 1.98	86.40 ± 0.92	0.107
DAP (µm)	58.19^{a} ± 2.02	61.90^{ab} ± 1.38	59.67^{b} ± 0.69	0.012

DSL (μm)	$34.72^{a} \pm 0.87$	$36.75^{b} \pm 1.08$	$36.58^{b} \pm 1.13$	0.030
VCL (μm/s)	$92.90^{a} \pm 1.75$	$93.52^{a} \pm 1.97$	$79.85^{b} \pm 2.49$	0.000
VAP (μm/s)	66.94 ± 3.58	67.68 ± 1.74	63.93 ± 1.95	0.132
VSL (μm/s)	45.64 ± 1.98	46.71 ± 2.01	44.11 ± 2.29	0.250
LIN (%)	$46.36^{a} \pm 0.56$	$46.90^{a} \pm 0.8$	$60.52^{b} \pm 2.03$	0.000
STR (%)	$67.41^{a} \pm 1.07$	$67.45^{a} \pm 1.07$	$77.58^{b} \pm 1.7$	0.000
WOB (%)	0.75 ± 0.03	0.80 ± 0.04	0.75 ± 0.03	0.092

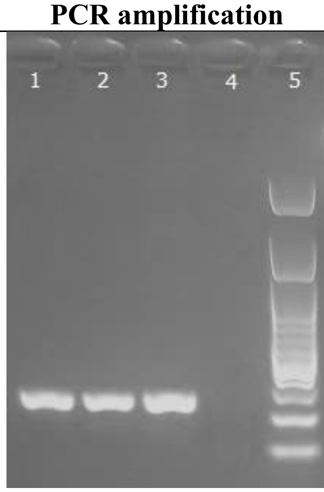
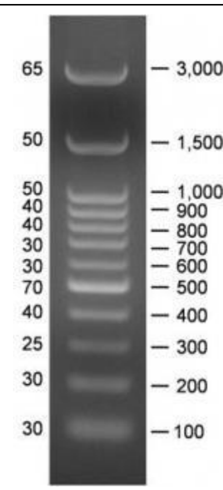
Note: Toraja= Toraja buffalo bull, Kalsel: South Kalimantan buffalo bull, Silangit: Silangit buffalo. Bull. Means within a row with different superscripts are significantly different ($p<0.05$).

As shown in Table 2, both significant and non-significant differences were present in several sperm post-thawing quality parameters. The progressive motility in Kalsel and Silangit buffaloes was found to be significantly higher ($p<0.05$) than that of Toraja buffaloes. The most pronounced differences were observed for viability and membrane integrity, wherein the Silangit buffaloes exhibited significantly higher values ($p<0.001$) compared to the other two breeds. However, no significant differences ($p>0.05$) were observed for total motility, acrosome integrity, DCL, VAP, VSL, and WOB among the three buffalo breeds.

PCR amplification and optimization of target genes

According to Table 3, amplification of the four target genes (OPN, TNP1, SPAG11B, and LHb) was successfully performed from all DNA samples of Toraja buffalo, South Kalimantan buffalo, and Silangit buffalo. Verification using agarose gel electrophoresis showed the presence of a single sharp DNA band with a size consistent with the prediction for each gene: OPN (~270 bp), TNP1 (~400 bp), SPAG11B (~180 bp), and LHβ (~600 bp) (Table 3). No amplification products were detected in the negative template control (NTC), confirming the absence of contamination during the PCR process.

Table-3. PCR amplification of target genes.

No	PCR amplification	Description	Ladder reference
1.	 OPN gene	1: 507.S.8 2: 507.S.9 3: 507.S.10 4: NTC (non template control) 5: 100 bp DNA ladder Amplicon size: ~270	

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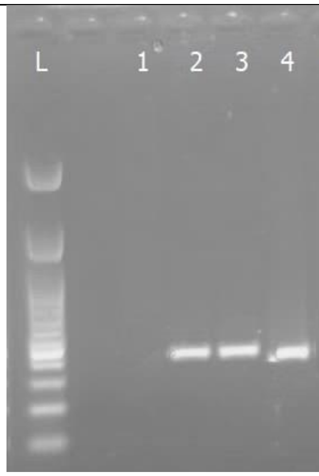
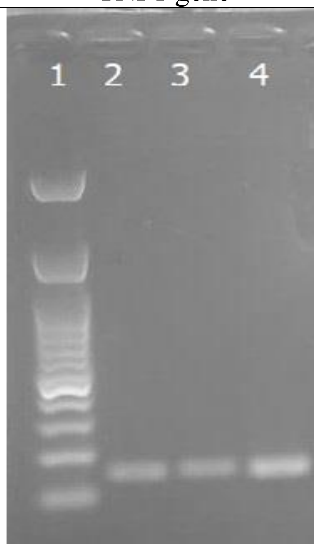
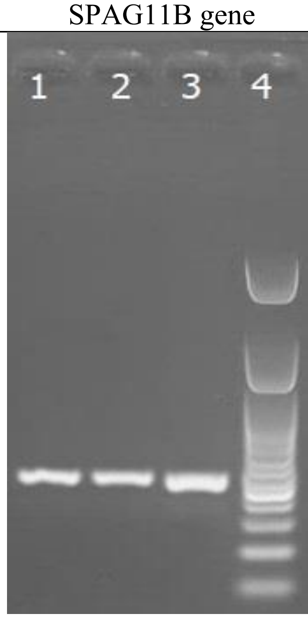
2.	 TNP1 gene	1: NTC 2: 507.S.8 3: 507.S.9 4: 507.S.10 L: 100 bp DNA ladder Amplicon size: ~400	
3.	 SPAG11B gene	1: 100 bp DNA ladder 2: 507.S.8 3: 507.S.9 4: 507.S.10 Amplicon size: ~180	
4.	 LHβ gene	1: 507.S.8 2: 507.S.9 3: 507.S.10 4: 100 bp DNA ladder Amplicon size: ~600	

Note: 507.S.8= Toraja buffalo bull, 508.S.9= Kalsel buffalo bull, 507.S.10= Silangit buffalo bull.

Analysis of nucleotide composition in OPN, IGF1, LHβ, SPAG11B, and TNP1 genes of Indonesian buffalo breeds

Nucleotide composition analysis was performed on the five candidate genes (OPN, IGF1, LHβ, SPAG11B, and TNP1) for the three buffalo groups shown in Figures 1-4. In general, the five genes showed higher guanine plus cytosine (G+C) content than adenine plus thymine (A+T), with average G+C content ranging from 55% to 62%. The OPN gene showed the highest G+C content (average 62.3%), while the SPAG11B gene had the lowest G+C content (average 55.1%). The comparative analysis results did not show any significant differences ($p > 0.05$) in the total nucleotide composition for any gene between the Toraja, South Kalimantan, and Silangit buffalo groups. This high level of sequence similarity indicates that these genes are highly conserved among local Indonesian buffalo populations. This base composition data was then used as a reference for the identification of single nucleotide polymorphisms (SNPs) in subsequent analyses.

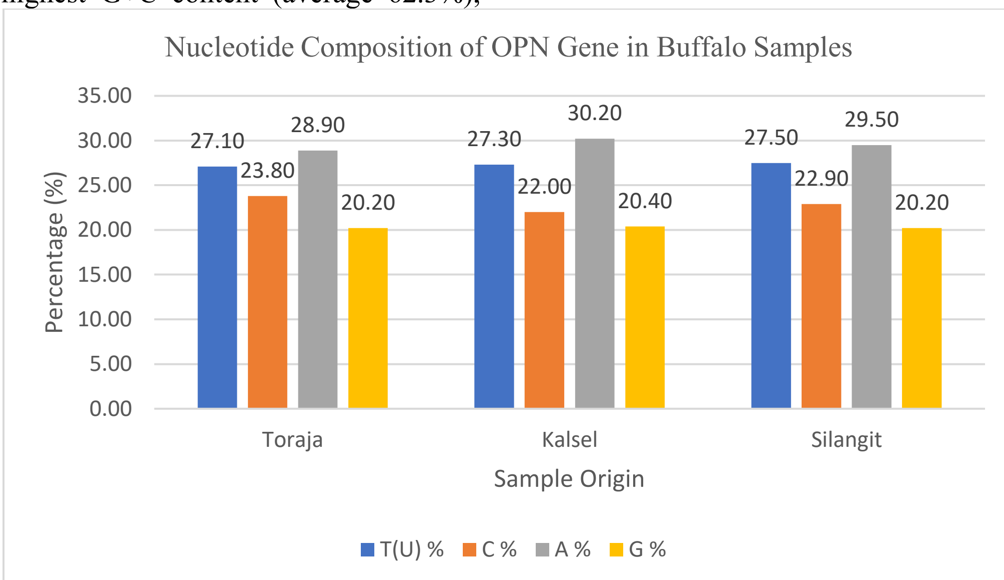


Figure-1. Nucleotide composition of OPN gene in each Indonesia local buffalo breed.

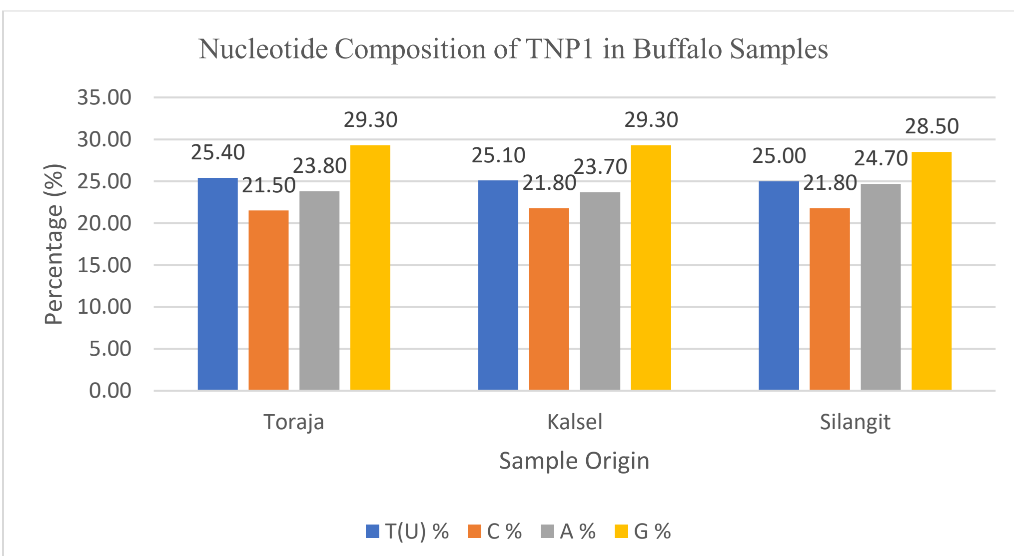


Figure-2. Nucleotide composition of TNP1 gene in each Indonesia local buffalo breed.

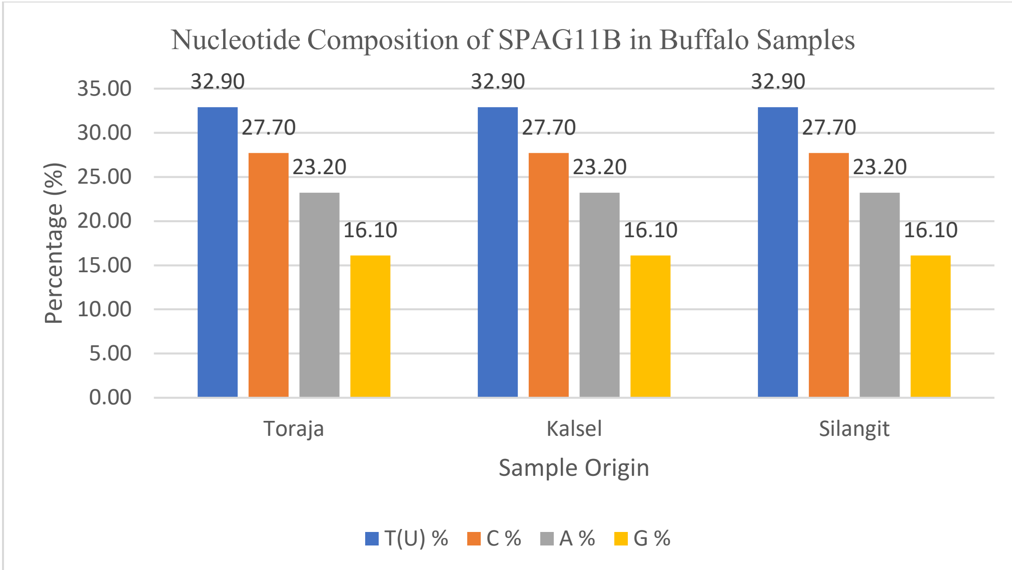


Figure-3. Nucleotide composition of SPAG11 gene in each Indonesia local buffalo breed.

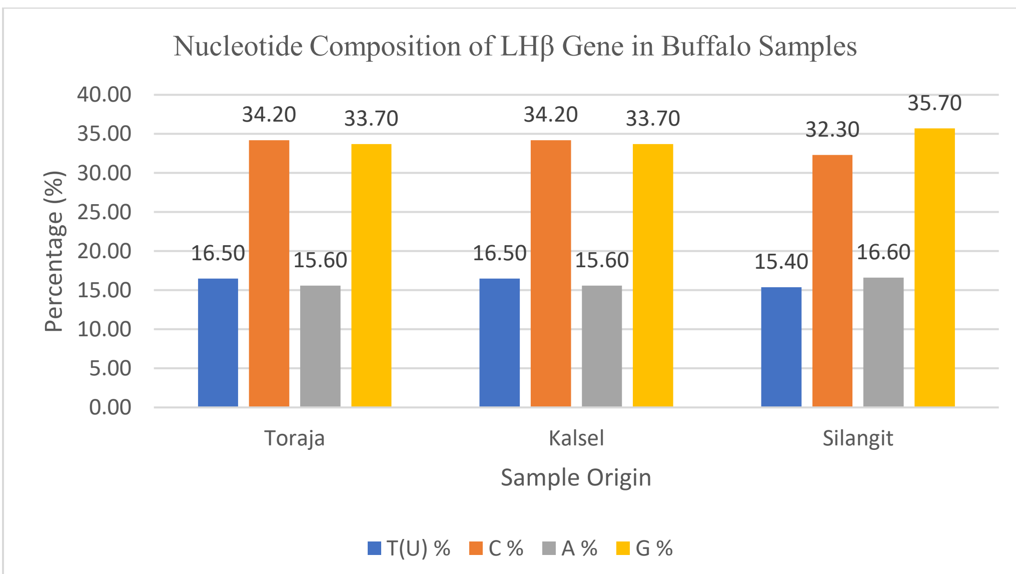


Figure-4. Nucleotide composition of LHβ gene in each Indonesia local buffalo breed.

Quantification of total sites mutation and total amino acid in OPN, IGF1, LHβ, SPAG11B, and TNP1 genes of Indonesian buffalo breeds

The quantification of total mutation sites and total amino acids is presented in Figure 5 and Figure 6, respectively. The OPN gene shows that only Toraja buffaloes have 3 mutation sites, while South Kalimantan and Silangit buffaloes have no gene mutations. The opposite occurs in the LHβ and TNP1 genes. The total number of mutation sites for these two genes is highest in Silangit buffaloes (34 sites in the LHβ gene and 25 sites in the TNP1 gene). In contrast to the SPAG11B gene, this gene experienced high and identical mutations (94 mutation sites) in all Indonesian local buffalo breeds, indicating that the SPAG11B gene region could be a hotspot of variation or a highly polymorphic site in the three buffalo breeds.

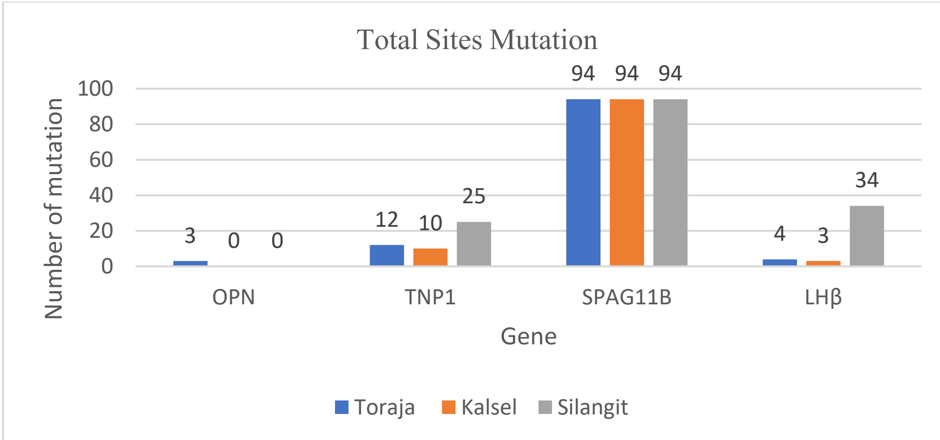


Figure-5. Total sites mutation of OPN, TNP1, SPAG11B, and LHβ gene in each Indonesia local buffalo breed.

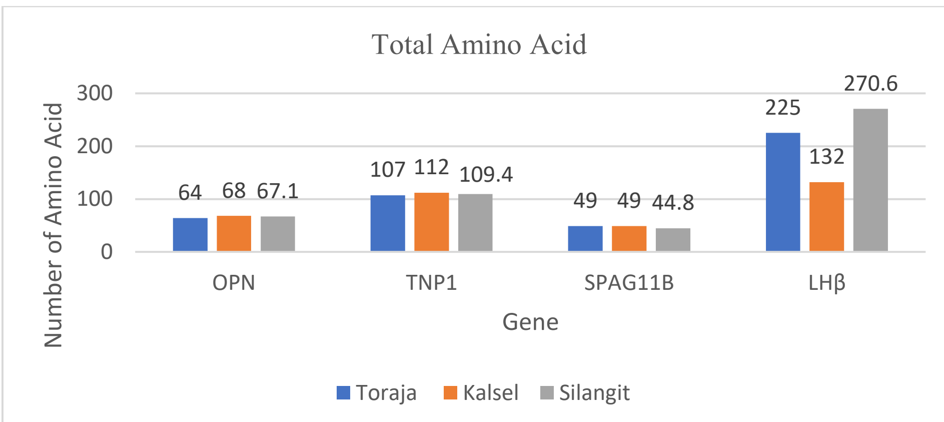


Figure-6. Total amino acid of OPN, TNP1, SPAG11B, and LHβ gene in each Indonesia local buffalo breed.

Correlation between total mutation sites, total amino acids, and the quality of Indonesian local buffalo sperm

Based on Table 4 and Table 5 heatmap Pearson Correlation shows the relationship between genetic variation. Blue indicates a positive relationship, and red indicates a negative relationship. The LHβ gene has a positive and significant correlation with viability characteristics ($r = 0.999^*$, $p<0.05$) and STR ($r = 1.000$, $p<0.05$), indicating that more LHβ gene mutations will induce higher sperm viability and efficient straight movement. The opposite occurs in the OPN gene, which shows a strong and significant negative correlation with total motility ($r= -0.998^*$, $p<0.05$), indicating that more OPN gene mutations cause sperm to become less active, which can be detrimental to sperm movement. Furthermore, the SPAG11B gene cannot be applied (N/A) because the number of mutations in this gene is exactly the same in all three local Indonesian buffalo breeds, so there are no differences that can be correlated.

Furthermore, Table 5 showed that amino acid changes in the SPAG11B gene had a perfect negative correlation ($r = -1.000$) and were significant ($p<0.01$) with viability and STR characteristics, which means that even the slightest change in SPAG11B would severely damage the sperm's ability to survive and move straight after the freezing process. The same applies to the OPN gene, which shows a perfect and highly significant negative correlation ($r = -1.000$, $p<0.01$) with acrosome integrity, which plays a crucial role in the enzymatic process of penetrating the oocyte layer. Amino acid changes in OPN can directly damage the crucial components involved in fertilization.

Table-4. Heatmap Pearson Correlation results of total sites mutation and sperm quality traits.

Gene	Total Motility (%)	Progresive motility (%)	Viability (%)	Acrosome integrity (%)	Membrane integrity (%)	DCL (µm)	DAP (µm)	DSL (µm)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (%)	STR (%)	WOB (%)
OPN	-0.998*	-0.985	-0.508	0.983	-0.775	0.716	-0.884	-0.967	0.572	-0.731	-0.765	-0.551	-0.493	-0.655
TNP1	0.454	0.542	0.991	-0.213	0.884	0.364	-0.085	0.142	-0.978	-0.343	-0.295	0.983	0.993	-0.441
LHβ	0.536	0.619	0.999*	-0.304	0.925	0.274	0.009	0.235	-0.993	-0.253	-0.204	0.996	1.000*	-0.354
SPAG11B	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Note: Red indicates a negative correlation, while blue indicates a positive correlation between the number of mutations and sperm quality. The color intensity reflects the correlation strength (r). Asterisks denote significance levels as follows: *p < 0.05 and **p < 0.01."

Table-5. Heatmap Pearson Correlation results of total amino acid and sperm quality traits.

Gene	Total Motility (%)	Progresive motility (%)	Viability (%)	Acrosome integrity (%)	Membrane integrity (%)	DCL (µm)	DAP (µm)	DSL (µm)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (%)	STR (%)	WOB (%)
OPN	0.959	0.925	0.311	-1.000**	0.621	-0.849	0.964	0.999*	-0.383	0.861	0.885	0.360	0.295	0.802
TNP1	0.815	0.752	-0.014	-0.936	0.333	-0.975	0.998*	0.959	-0.063	0.979	0.988	0.037	-0.031	0.952
LHβ	-0.124	-0.022	0.747	0.372	0.470	0.823	-0.629	-0.437	-0.694	-0.811	-0.780	0.712	0.758	-0.868
SPAG11B	-0.560	-0.641	-1.000**	0.331	-0.935	-0.246	-0.038	-0.263	0.996	0.225	0.176	-0.998*	-1.000**	0.327

Note: Red indicates a negative correlation, while blue indicates a positive correlation between amino acid identity and sperm quality. The color intensity reflects the correlation strength (r). Asterisks denote significance levels as follows: *p < 0.05 and **p < 0.01.

Discussion

The comparative evaluation of post-thawed semen quality among Toraja, Kalsel, and Silangit buffalo bulls revealed distinct breed-specific differences in cryotolerance (Table 2). While total motility, acrosome integrity, and certain kinematic traits such as VAP and VSL did not differ significantly, progressive motility, viability, and membrane integrity showed clear variation. Silangit bulls demonstrated markedly higher viability (81.49%) and membrane integrity (89.70%) compared with Toraja and Kalsel bulls, suggesting a superior ability to withstand osmotic and thermal stress during cryopreservation. This pattern is consistent with earlier studies in riverine buffaloes and cattle, which demonstrated that sire and breed differences significantly influence post-thaw sperm survivability (Vale et al., 2022). The relatively lower progressive motility in Toraja bulls supports the hypothesis that cryoinjury compromises flagellar function and mitochondrial ATP generation, as also reported in previous work on buffalo sperm (Maulana et al., 2024).

Molecular analysis provided further insights into the genetic basis of these phenotypic differences. PCR amplification of *OPN, TNP1, SPAG11B,* and *LHβ* yielded single, distinct bands of expected size (Table 3), confirming that these fertility-associated genes are conserved across breeds. Nucleotide composition analysis (Figures 1–4) revealed consistently higher G+C content (55–62%), which is characteristic of genes under functional constraint and is associated with greater genomic stability (Raza et al., 2023). Although base composition did not differ significantly among the three buffalo breeds, sequence analysis revealed breed-specific mutational profiles. For instance, *OPN* mutations were exclusive to Toraja bulls, while Silangit bulls exhibited the highest number of mutational sites in *LHβ* (34 sites) and *TNP1* (25 sites) (Figure 5). By contrast, *SPAG11B* displayed a consistently high number of mutations across all breeds (94 sites), suggesting that this gene is highly polymorphic within local buffalo populations.

The functional interpretation of these variations was supported by amino acid sequence analysis (Figure 6) and correlation results (Tables 4 and 5). Mutations in *LHβ* showed strong positive correlations with viability ($r = 0.999$, $p < 0.05$) and STR ($r = 1.000$, $p < 0.05$), indicating that sequence variability in this gene may enhance reproductive hormone signaling and support spermatogenesis. This is consistent with reports in buffaloes and cattle showing that *LHβ* polymorphisms influence gonadotropin function and reproductive efficiency (Reen et al., 2019). In contrast, *OPN* mutations were strongly and negatively correlated with total motility ($r = –0.998$, $p < 0.05$) and acrosome integrity ($r = –1.000$, $p < 0.01$), suggesting detrimental effects on sperm activity and fertilization potential. These findings agree with earlier studies demonstrating that *OPN* plays an essential role in sperm–oocyte binding and zona pellucida penetration (Gonçalves et al., 2008).

The most striking result was observed for *SPAG11B*, where amino acid substitutions were perfectly and negatively correlated with sperm viability and STR ($r = –1.000$, $p < 0.01$). As *SPAG11B* encodes an epididymal protein essential for sperm maturation and antimicrobial defense (Solanki et al., 2023), excessive polymorphism in this gene may impair post-epididymal sperm function, thereby compromising survival and directional motility after thawing. This interpretation is in line with previous findings in cattle that linked *SPAG11B* variants to reduced sperm maturation efficiency (Mukherjee et al., 2023). Together, these results suggest that *LHβ* and *SPAG11B* represent critical determinants of cryotolerance in buffalo semen, whereas *OPN* variation may be associated with reduced fertilization potential.

By combining phenotypic evaluation with molecular characterization, the present study highlights that differences in semen cryotolerance among Indonesian buffalo breeds are underpinned by specific genetic signatures. The superior performance of Silangit bulls may be partially explained by favorable mutational patterns in *LHβ* and *TNP1*, while detrimental *OPN* mutations in Toraja bulls may contribute to reduced post-thaw motility. These findings align with earlier work demonstrating that integrating molecular markers with semen quality assessment improves sire selection in cattle breeding programs (Khan et al., 2024) Implementing such strategies in Indonesian buffalo populations could accelerate genetic improvement while preserving the reproductive potential of indigenous breeds.

Several limitations of this study should be noted. The sample size was restricted to three bulls (60 straws of semen), limiting the generalizability of the findings to broader populations. Only five candidate genes were examined, whereas other loci such as *PRM1, HSP70,* and *CAT* are also known to influence sperm freezability and should be incorporated into future analyses. Furthermore, the correlations identified here are associative and cannot establish causality without validation through gene expression, transcriptomic, or proteomic studies. Environmental factors such as nutrition, heat stress, and health status, which are known modulators of semen quality, were not considered in the present design and may interact with genetic variation. Expanding the sample size, applying multi-omics approaches, and validating results across multiple breeding centers will be critical steps toward confirming the utility of these markers in applied breeding and artificial insemination programs.

Conclusion

This study demonstrates that post-thawed semen quality differs significantly among Indonesian native buffalo breeds, with Silangit bulls showing superior viability and membrane integrity compared with Toraja and Kalsel bulls (Table 2). Molecular characterization confirmed the presence of conserved fertility-associated genes, yet revealed breed-specific variation, including *OPN* mutations unique to Toraja bulls and extensive mutational sites in *LHβ* and *TNP1* in Silangit bulls (Figures 5 and 6). Correlation analyses established that *LHβ* variability was positively associated with sperm viability and linearity (Tables 4 and 5), whereas *OPN* and *SPAG11B* mutations were negatively associated with motility, acrosome integrity, and survival. Collectively, these findings suggest that *LHβ* and *SPAG11B* represent promising genetic markers of cryotolerance, while *OPN* variation may compromise fertilization potential. Although limited by a small sample size and the focus on a restricted number of genes, the results provide novel evidence that breed-specific genetic variation contributes to semen cryosurvival. These insights establish a foundation for marker-assisted selection and the integration of genomic tools into buffalo breeding and artificial insemination programs in Indonesia.

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Ethical Approval Statement

The Research Ethics Commision of National Research and Innovation Agency, approved this study with the number: 050/KE.02/SK/03/2023.

Contribution of Authors

Utomo B, Khan FA, Said S & Santoso S: Contributed to supervising the experiment, as well as the conception and design of the study.
Rizal M, Hasan F, Nurlatifah A & Diansyah AM: Contributed to the conception and design of the study and edited the manuscript.
Amrullah NK & Amrullah MF: Were responsible for data collection and drafting the manuscript.
Rimayanti R & Amrullah MF: Contributed to data analysis and drafting the manuscript.
Rimayanti R, Restiadi TI & Mulyati S: Provided critical review, suggestions and editing of the manuscript.

All authors read and approved final draft of the manuscript.

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