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New insights into polymorphisms incandidate genes associated with incidence to repeat breeder in Italian buffaloes (*Bubalus bubalis*)

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ABSTRACT: This study looked at the reproductive genes that could interact with repeat breeder incidence in Italian buffaloes. Two hundred and forty female Italian buffaloes (120 repeat breeder and 120 apparently normal) were used. Blood samples were collected from each buffalo into tubes encompassing EDTA anticoagulant to isolate DNA. PCR-DNA sequencing elicited nucleotide sequence differences between normal and repeat breeder buffaloes for the reproductive (*MFSD14A*, *PALMD*, *VPS13B*, *BMP*, *MTNR1A*, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) genes. The likelihood of dissemination of all notable nucleotide variations varied noticeably between buffalo groups with and without repeat breeders, depending on Fisher's exact test ($p < 0.01$). All of the reproductive markers under research had the exonic region mutations in repeat breeder buffaloes versus normal ones. The results may confirm the importance of these markers' nucleotide variations as candidates for repeat breeder occurrence and offer a practical buffalo management strategy.

Keyword: Italian buffaloes; candidate gene; repeat breeder.

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INTRODUCTION

Economically important for long-term food production are reproductive features, especially in monotocous animals like cattle and buffalo (Shao et al., 2021). Repeat breeding, or an extended interval between two calves, may be indicative of poor reproduction or sterility. More inseminations, veterinary care, and hormone therapies are required to address this illness, all of which have an impact on current and upcoming lactations (Biochard, 1990). Additionally, extra expenses are spent due to the culling and replacement of animals with reproduction problems (Roxström & Strandberg, 2002). The best option for reducing culling expenses, maintaining significant genetic features, and boosting farm profits is fertility improvement (Dekkers, 1991). With relation to statistical distribution, binary, interval, and continuous attributes have previously been used to classify reproductive characteristics (Berry & Evans 2014). Reproductive characteristics have been classified to make them simpler for understanding and utilizing in livestock and breeding programs (Cammack et al., 2009). These categories include features relating to ovulation, mating, and calving.

Because buffalo are mono-ovulatory, only one follicle typically grows and ovulates during the oestrus phase, which significantly reduces fertility and limits the growth of the buffalo business (Li et al., 2018). The reproductive activity of the buffaloes follows a seasonal pattern, with compromised activity in the summer and regular reproductive cyclicity and conception in the autumn and winter (Das & Khan 2010). Untangling the reproductive endocrinology of buffaloes will help researchers determine the cause of this reproductive restriction (Warriach et al., 2015). Quantitative features that affect reproduction are influenced by a variety of circumstances. Repeat breeding's occurrence in dairy farms can be reduced, and with it, the associated financial loss, by being aware of its underlying causes (Regmi&Dhakal, 2020). Repeat breeding can be caused by a wide range of factors, including insufficient fertilization, premature embryonic death, genital tract congenital or genetic faults, spermatozoa, ova or first zygote defects, microbial or traumatic inflammation, endocrine/hormonal problems, and management flaws (such as failing to identify estrus) (Yusuf et al., 2010). It is anticipated that genetic variants will influence gene expression, which will change the amount of one or more proteins, changing the phenotype of attributes (Musunuru et al., 2010; Lap-

palainen et al., 2013). A way to locate inherited components which affect susceptibility for common economic problems is marker-assisted selection (MAS) (Ashwell et al., 1997; Bishop et al., 1995). In the era of genomic assortment, excellent cow training groups that combine attributes with high-throughput genomic SNP marker data allow the use of selection strategies for emerging functional characteristics, such disease resistance (Buch et al., 2012). Another significant advantage of genetic selection is shortening of generational intervals (Schaeffer, 2006). A novel understanding of the identity of underlying genes and change in the genetic material and how they might affect the characteristic has been claimed to be gained by integrating genetic markers to find significant genes linked with target traits (Li et al., 2014). The term "transcriptome" denotes all the genes in the genome that are accurately and at high throughput during particular physiological and pathological states (Kukurba & Montgomery, 2015).

Recent genome-wide association analysis studies have focused on novel genes related to bovine reproductive performance; however, no studies have yet looked at the relationship between the SNPs in these genes and the frequency of repeat breeders (Li et al., 2018; Mahrouset al., 2022). It is important to note that more research is necessary to follow potential symptoms that can imply reproductive failure from repeat breeder because there is limited knowledge on the reproduction pathways that determine buffalo resistance to or sensitivity to repeat breeder (Li et al., 2018). The molecular alterations may also help to identify repeat breeders and provide crucial information about the interactions between the physiologies of the various reproductive pathways (Sammad et al., 2022; Heidari et al., 2019). The effectiveness of putative reproductive (*MFS14A*, *PALMD*, *VPS13B*, *BMP*, *MTNR1A*, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) genes as candidates for repeat breeder incidence prediction and tracking in Italian buffaloes was examined in this study employing PCR-DNA sequencing methods.

MATERIALS AND METHODS

Dairy buffaloes and research samples

The current study was carried out on 240 pure Italian buffaloes aged 4.5 years. Buffaloes upraised on a private farm in the province of Delta region of northern Egypt of which 120 were repeat breeder and 120 appeared to be normal. Based on breeding history and clinical indications seen by skilled vets,

both groups were selected. All of the animals were raised in stalls with free access to water. It was determined that none of the study animals had ovarian pathologies like ovarian cysts or uterine, cervical, or vaginal inflammation or infection. The buffalo cows in the control group appeared to be in good condition and had a regular estrous cycle, nonetheless, no pregnancies were achieved in the repeat breeder group notwithstanding at least three successful attempts to artificially inseminate or mate with a bull in estrus (Devkota et al., 2022). Visual observation was used to identify estrus in both groups twice daily in the morning and in the evening. All animals were checked for foetal membrane slip and/or fremitus, and rectal palpation was used to confirm pregnancy diagnoses.

Five milliliters of blood were obtained by puncturing the jugular vein of each buffalo. To obtain whole blood and recover DNA samples were placed in tubes containing EDTA as anticoagulant. The Ethical Committee approved the sample collection and animal care techniques utilized in this study, and they complied with Mansoura University's regulations. The Mansoura University Animal Care and Use Committee (MU-ACUC) gave its approval to the study's protocol (code VM.R.23.11.26).

DNA extraction and PCR amplification

DNA from the genome was extracted using total blood by means of the genetic material JET full blood genomic DNA isolation kit and the producer's directions (Thermo scientific, Vilnius, Lithuania). Using Nanodrop, DNA of good purity and concentration was considered. The following reproductive (*MFSD14A*, *PALMD*, *VPS13B*, *BMP*, *MTNR1A*, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) genes' parts of coding sites (CDS) were amplified. The oligonucleotide sequences for amplification were constructed using the PubMed *Bubalus bubalis* genome. Table 1 provides the primers utilized in the PCR.

The polymerase chain cloning mixture had been treated in a thermal cycler with a final volume of 50 µL. The succeeding elements were present in every reaction container: 19 microliters d.d. water, 4 microliters of genetic material, 1 microliters of each primer pair, and 25 microliters of the master combination (Jena Bioscience, Jena, Germany). The PCR combinations remained in use for four minutes at a starting temperature of 95 °C for unwinding. The 34 cycles included one-minute denaturation rounds

at 95 °C, one-minute annealing cycles based on the Table 1 temperature range, and 30-second for elongation at 72 °C, followed by ten minutes of final elongation. The samples were retained at 4 °C. Using agarose gel electrophoresis to obtain demonstrable outcomes and viewing PCR segment configurations underneath UV light, a gel certification technique was used.

Discovering polymorphism

Before DNA sequencing, Hamburg, Germany-based Jena Bioscience # pp-201s/Munich provided methods to purify PCR for getting rid of primer dimers, non-specific bands, and other impurities and create the predicted scope's planned amplified product (Boom et al., 1990). Using a Nanodrop (Waltham, Massachusetts, USA, UV-Vis spectrometer Q5000), adequate quality and good concentrations were obtained while measuring PCR output (Boesenberg-Smith et al., 2012). Sequence analysis of the PCR-produced amplification results has been utilized to find SNPs using normal and repeat breeder buffaloes. The PCR yields were sequenced using the Sanger et al., (1977) outlined enzyme chain terminator technique on an ABI 3730XL DNA sequencer (United States: Applied Biosystems, Waltham).

Chromas 1.45 and BLAST 2.0, the tools to evaluate results of DNA analysis (Altschul et al., 1990). Comparing the reproductive gene PCR results to the GenBank-provided reference gene sequences has revealed polymorphisms. The MEGA6 tool can detect differences in the amino acid categorizations among the examined genes according to sequence matching among the buffaloes under investigation (Tamura et al., 2007).

Statistical analysis

H_0 : Polymorphisms of reproductive genes could not interact in the incidence to repeat breeder in Italian buffaloes.

H_A : Polymorphisms of reproductive genes could interact in the incidence to repeat breeder in Italian buffaloes.

Observing the significant distribution of SNPs for the discovered genes between the examined buffaloes was conducted using Fisher's exact test analysis ($p < 0.01$). Graphpad statistical software was used to conduct the statistical analysis (Graphpad prism for Windows version 5.1, Graphpad software, Inc., San Diego, CA, USA).

Table 1. Oligonucleotide primers for reproductive genes used to study genetic polymorphisms.

| Investigated marker | Sense | Antisense | Accession number | Annealing Temperature (°C) | Size of PCR product (bp) |
|---------------------|-------------------------------|------------------------------|------------------|----------------------------|--------------------------|
| <i>MFSD14A</i> | 5'-GCGAACCAGCATATCATGCTG-3' | 5'-CCAGGCCATACGCCATACCTTC-3' | XM_055584984.1 | 60 | 457 |
| <i>PALMD</i> | 5'-TGGCTTCTAGATGGAATCAGC-3' | 5'-CTATTCCTTCATTCTTTCC-3' | XM_006045619.1 | 58 | 343 |
| <i>VPS13B</i> | 5'-AATGAGCTATGTGAATCGCT-3' | 5'-ACAACTCGTCTGATCAGACTCT-3' | XM_055546577.1 | 60 | 414 |
| <i>BMP</i> | 5'-ATGGTCCCTTCTGAGCATCCTTA-3' | 5'-GTGGAGCCTCTTCTTGAAGAAG-3' | EF375880.1 | 60 | 480 |
| <i>MTNR1A</i> | 5'-GTGAGCCTGGCAGTTGCAGAC-3' | 5'-CACTCTCCATCTGACCTGAAG-3' | MF173058.1 | 56 | 465 |
| <i>TSHR</i> | 5'-ATCCGCCCTCTGGCACGCCCTAC-3' | 5'-TGGCATAAGGAATGGATTGGC-3' | KC415275.1 | 58 | 442 |
| <i>CSGALNACT1</i> | 5'-CTGCTCGCCTGGGCCCTCCCGG-3' | 5'-CGGTGGCCTGCTTGACGCCGGC-3' | XM_025275645.3 | 58 | 385 |
| <i>CADM2</i> | 5'-CGCAGCGCCGTTCTCCGCTTC-3' | 5'-GAACCATCTTATATCAGCTGCA-3' | XM_025278930.3 | 60 | 495 |
| <i>ZNF503</i> | 5'-TCTCCGCCAGGCTAACCGCCT-3' | 5'-CTTATCCGAGCCGGTTTGGAG-3' | XM_025285122.3 | 60 | 361 |
| <i>KRR1</i> | 5'-CCGACGTCGGAGAACCGAGATG-3' | 5'-CTAATGATGATATATGGATCA-3' | XM_006048659.4 | 58 | 278 |
| <i>MTPN</i> | 5'-ATGTGCGACAAAGGAGTTCATG-3' | 5'-GGCACCAGTGACATTCAGAT-3' | XM_044946911.2 | 60 | 266 |
| <i>IGFBP7</i> | 5'-AGCTGGGCGAGACCCGCGATGC-3' | 5'-GACAGAAAGCAATTTCACGCAG-3' | XM_025290001.3 | 56 | 380 |
| <i>LEP</i> | 5'-CAGTCCGTCTCCTCCAAACAGA-3' | 5'-TCAGCACCCAGGACTGAGGTC-3' | AF387814.1 | 58 | 360 |
| <i>ABCC4</i> | 5'-GCAGAAAGCGAACTTCTGCTC-3' | 5'-TATGGTTGAAC TACTCTGGTG-3' | XM_045162483.1 | 60 | 355 |

MFSD14A= Major facilitator superfamily domain containing 14A; *PALMD*= Palmdelphin; *VPS13B*= Vacuolar protein sorting 13 homolog B; *BMP*= Bone morphogenic protein; *MTNR1A*= Melatonin receptor 1B; *TSHR*= Thyroid stimulating hormone receptor; *CSGALNACT1*= Chondroitin sulfate N-acetylglucosaminyltransferase 1; *CADM2*= Cell adhesion molecule 2; *ZNF503*= Zinc finger protein 503; *KRR1*= Small subunit processome component homolog; *MTPN*= Myotrophin; *IGFBP7*= insulin like growth factor binding protein 7; *LEP*= Leptin; and *ABCC4*= ATP-binding cassette sub-family C member 4.

RESULTS

SNP variations in amplified DNA nucleotides associated with repeat breeder were found in the results of PCR-DNA sequencing on both normal and repeat breeder buffaloes for the MFSD14A (457-bp), PALMD (343-bp), VPS13B (414-bp), BMP (480-bp), MTNR1A (465-bp), TSHR (442-bp), CS-GALNACT1 (385-bp), CADM2 (495-bp), ZNF503 (361-bp), KRR1 (278-bp), MTPN (266-bp), IGFBP7 (380-bp), LEP (360-bp), and ABCC4 (355-bp) genes. The DNA sequence differences between the sequences of reference genes obtained from GenBank and the reproductive indicators examined in the studied buffaloes were used to verify each SNP that has been discovered (Figures S1–S14).

Table 2 shows the dissemination of a single base variation as well as a type of inherited change for reproductive indicators in normal and repeat breeder buffaloes. The SNPs' Fisher's exact test analysis revealed considerably different occurrences of the investigated markers in the normal and repeat breeder buffaloes ($p < 0.01$). All of the reproductive markers under research had the exonic region mutations, which resulted in different coding DNA sequences in repeat breeder buffaloes versus nor-

mal ones. Thirty SNPs were discovered using DNA sequencing of reproductive genes; 16 of them are synonymous and 15 is non-synonymous. The amino acid variations between the reproductive genes in all the research buffaloes and the reference sequences retrieved in GenBank were used to validate all observed SNPs and the corresponding amino acids (Figures S15–S28).

For the MFSD14A gene (457-bp), two observed recurrent synonymous SNPs, 165CT and 322TC, resulted in 55C and 108G respectively. One recurrent SNPs were found when the PALMD gene (343-bp) was sequenced. Synonymous mutation 36L occurred as a result of 108TC SNP. 175TC and 223TC involved a non-synonymous mutation, resulting in the substitution of the amino acid C59R, and Y75H respectively; were found in DNA sequences of the VPS13B gene (414-bp). The BMP gene (480-bp) contained three non-synonymous SNPs: 47TC, 80CA, and 184GA, which caused the amino acids V16A, P27Q, and V62I to be substituted, respectively. The nucleotide sequence of the MTNR1A gene (465-bp) revealed one recurrent synonymous SNP; 24N resulted from 72CT SNP. The 442-bp TSHR gene's DNA sequence revealed four identified

| | | |
|----------------|---|-----|
| XM_055584984.1 | GCGAACCGCAGTATCATGCTGGCCAAAAAGATCATCATTAAAGGACGGAGGCACGCCTCAA | 60 |
| H | GCGAACCGCAGTATCATGCTGGCCAAAAAGATCATCATTAAAGGACGGAGGCACGCCTCAA | 60 |
| RR | GCGAACCGCAGTATCATGCTGGCCAAAAAGATCATCATTAAAGGACGGAGGCACGCCTCAA | 60 |
| ***** | | |
| XM_055584984.1 | GGAATAGGTTCTCCTAGTGTATTATCATGCGATTATCGTCATCTTTTGGAGTTTTTCGCT | 120 |
| H | GGAATAGGTTCTCCTAGTGTATTATCATGCGATTATCGTCATCTTTTGGAGTTTTTCGCT | 120 |
| RR | GGAATAGGTTCTCCTAGTGTATTATCATGCGATTATCGTCATCTTTTGGAGTTTTTCGCT | 120 |
| ***** | | |
| XM_055584984.1 | TGGGGATTATTGACAGCACCCACCTTGGTGGTATTACATGAAACCTTCCCTAAACATACA | 180 |
| H | TGGGGATTATTGACAGCACCCACCTTGGTGGTATTACATGAAACCTTCCCTAAACATACA | 180 |
| RR | TGGGGATTATTGACAGCACCCACCTTGGTGGTATTACATGAAACCTTCCCTAAACATACA | 180 |
| ***** | | |
| XM_055584984.1 | TTTCTGATGAATGGCCTAATTCAAGGAGTAAAGGGTTTGTGTCAATTCCTCAGTGCCCT | 240 |
| H | TTTCTGATGAATGGCCTAATTCAAGGAGTAAAGGGTTTGTGTCAATTCCTCAGTGCCCT | 240 |
| RR | TTTCTGATGAATGGCCTAATTCAAGGAGTAAAGGGTTTGTGTCAATTCCTCAGTGCCCT | 240 |
| ***** | | |
| XM_055584984.1 | CTTATTGGTGCTCTTTCTGATGTTTGGGGCCGAAAATCCTTCTTGCTGCTAACAGTATTT | 300 |
| H | CTTATTGGTGCTCTTTCTGATGTTTGGGGCCGAAAATCCTTCTTGCTGCTAACAGTATTT | 300 |
| RR | CTTATTGGTGCTCTTTCTGATGTTTGGGGCCGAAAATCCTTCTTGCTGCTAACAGTATTT | 300 |
| ***** | | |
| XM_055584984.1 | TTACGCTGTGCCCAATTCTTTAATGAAAATCAGCCCATGGTGGTACTTTGCTGTTATC | 360 |
| H | TTACGCTGTGCCCAATTCTTTAATGAAAATCAGCCCATGGTGGTACTTTGCTGTTATC | 360 |
| RR | TTACGCTGTGCCCAATTCTTTAATGAAAATCAGCCCATGGTGGTACTTTGCTGTTATC | 360 |
| ***** | | |
| XM_055584984.1 | TCTGTTTCTGGGGTTTTTGCAGTGACTTTCTCCGTGGTATTGTCATATGTAGCAGATATA | 420 |
| H | TCTGTTTCTGGGGTTTTTGCAGTGACTTTCTCCGTGGTATTGTCATATGTAGCAGATATA | 420 |
| RR | TCTGTTTCTGGGGTTTTTGCAGTGACTTTCTCCGTGGTATTGTCATATGTAGCAGATATA | 420 |
| ***** | | |
| XM_055584984.1 | ACCCAAGAACATGAAAGAAGTATGGCGTATGGCCTGG 457 | |
| H | ACCCAAGAACATGAAAGAAGTATGGCGTATGGCCTGG 457 | |
| RR | ACCCAAGAACATGAAAGAAGTATGGCGTATGGCCTGG 457 | |
| ***** | | |

Figure S1. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_055584984.1| and *MFSD14A* marker (457-bp) sequences.

| | | |
|----------------|---|-----|
| XM_006045619.4 | TGGCTTCTAGATGGAATCAGCAGTGGAAAAAGAGCAGGAAGAGATGAAGAAGCAAAATCAA | 60 |
| H | TGGCTTCTAGATGGAATCAGCAGTGGAAAAAGAGCAGGAAGAGATGAAGAAGCAAAATCAA | 60 |
| RR | TGGCTTCTAGATGGAATCAGCAGTGGAAAAAGAGCAGGAAGAGATGAAGAAGCAAAATCAA | 60 |
| XM_006045619.4 | CAAGACCAGCACCAGATCCAGGTTCTAGAACAAAGTATCCTCAGACTTGAGAAAGAGATC | 120 |
| H | CAAGACCAGCACCAGATCCAGGTTCTAGAACAAAGTATCCTCAGACTTGAGAAAGAGATC | 120 |
| RR | CAAGACCAGCACCAGATCCAGGTTCTAGAACAAAGTATCCTCAGACTTGAGAAAGAGATC | 120 |
| XM_006045619.4 | CAAGATCTTGAAAAGGCTGAACTGCAAAATCTCAACCAATGAAGAAGCAATTTTAAAGAAA | 180 |
| H | CAAGATCTTGAAAAGGCTGAACTGCAAAATCTCAACCAATGAAGAAGCAATTTTAAAGAAA | 180 |
| RR | CAAGATCTTGAAAAGGCTGAACTGCAAAATCTCAACCAATGAAGAAGCAATTTTAAAGAAA | 180 |
| XM_006045619.4 | CTGAATCAGTTGAGAGGACAAACAGACATAATAAGGTCTGTGAAGGTGGAAAAGGAA | 240 |
| H | CTGAATCAGTTGAGAGGACAAACAGACATAATAAGGTCTGTGAAGGTGGAAAAGGAA | 240 |
| RR | CTGAATCAGTTGAGAGGACAAACAGACATAATAAGGTCTGTGAAGGTGGAAAAGGAA | 240 |
| XM_006045619.4 | GAAACATCAGGAGAGTCAGTTGAAGACATCTATGCTAATATCCCGACCTTCCAAATCC | 300 |
| H | GAAACATCAGGAGAGTCAGTTGAAGACATCTATGCTAATATCCCGACCTTCCAAATCC | 300 |
| RR | GAAACATCAGGAGAGTCAGTTGAAGACATCTATGCTAATATCCCGACCTTCCAAATCC | 300 |
| XM_006045619.4 | TACATACCTTCCAGGTTAAGGAAGGAAAGAAATGAAGGAATAG | 343 |
| H | TACATACCTTCCAGGTTAAGGAAGGAAAGAAATGAAGGAATAG | 343 |
| RR | TACATACCTTCCAGGTTAAGGAAGGAAAGAAATGAAGGAATAG | 343 |

Figure S2. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb| XM_006045619.4| and *PALMD* marker (343-bp) sequences.

| | | |
|----------------|---|-----|
| XM_055546577.1 | AATGAGCTATGTGAATCGCTACATCAAGAACTTAAAGCCGTCAGATCTACAGCTTTTCGCT | 60 |
| H | AATGAGCTATGTGAATCGCTACATCAAGAACTTAAAGCCGTCAGATCTACAGCTTTTCGCT | 60 |
| RR | AATGAGCTATGTGAATCGCTACATCAAGAACTTAAAGCCGTCAGATCTACAGCTTTTCGCT | 60 |
| XM_055546577.1 | ATGGGGTGGAGACGTTGGTTCTCAGCAAGCTGGAGTTAAATTTGGATGTGCTGGAGCAGGA | 120 |
| H | ATGGGGTGGAGACGTTGGTTCTCAGCAAGCTGGAGTTAAATTTGGATGTGCTGGAGCAGGA | 120 |
| RR | ATGGGGTGGAGACGTTGGTTCTCAGCAAGCTGGAGTTAAATTTGGATGTGCTGGAGCAGGA | 120 |
| XM_055546577.1 | ATTGAAATTACCAATTCACATTTTTTAAGTGGACATATTCAATGAAGTGGAGATCCATGTACC | 180 |
| H | ATTGAAATTACCAATTCACATTTTTTAAGTGGACATATTCAATGAAGTGGAGATCCATGTACC | 180 |
| RR | ATTGAAATTACCAATTCACATTTTTTAAGTGGACATATTCAATGAAGTGGAGATCCATGTACC | 180 |
| XM_055546577.1 | ATGGACAAAACCTGGGTTCCAGAACCAAGTGGTAATTACCATCAATACAATGGAATGCATTTT | 240 |
| H | ATGGACAAAACCTGGGTTCCAGAACCAAGTGGTAATTACCATCAATACAATGGAATGCATTTT | 240 |
| RR | ATGGACAAAACCTGGGTTCCAGAACCAAGTGGTAATTACCATCAATACAATGGAATGCATTTT | 240 |
| XM_055546577.1 | GAAACTTAAAGATGGAATACAGGATGATCATGAAAGCTGTGGTTCTAATTCTACCAACCG | 300 |
| H | GAAACTTAAAGATGGAATACAGGATGATCATGAAAGCTGTGGTTCTAATTCTACCAACCG | 300 |
| RR | GAAACTTAAAGATGGAATACAGGATGATCATGAAAGCTGTGGTTCTAATTCTACCAACCG | 300 |
| XM_055546577.1 | TAGTACTACTGAGAACACAAAACATCAGCAAAACCCGAGAAATTCAACAGGCCACTCC | 360 |
| H | TAGTACTACTGAGAACACAAAACATCAGCAAAACCCGAGAAATTCAACAGGCCACTCC | 360 |
| RR | TAGTACTACTGAGAACACAAAACATCAGCAAAACCCGAGAAATTCAACAGGCCACTCC | 360 |
| XM_055546577.1 | CACAGATCCTGATTGCCACCAGGCTATGTACAGAGTCTGATCAGACGAGTTGT | 414 |
| H | CACAGATCCTGATTGCCACCAGGCTATGTACAGAGTCTGATCAGACGAGTTGT | 414 |
| RR | CACAGATCCTGATTGCCACCAGGCTATGTACAGAGTCTGATCAGACGAGTTGT | 414 |

Figure S3. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_055546577.1| and *VPS13B* marker (414-bp) sequences.

frequent synonymous SNPs; 87CT, 126TC, 177CT, and 372CT, which were associated with the amino acids 29S, 42T, 59I, 42S and 124V.

For the *CSGALNACT1* gene (385-bp), one synonymous SNP was found; the results of 135GAwas45T. Three recurrent synonymous SNPs; 105GA, 168TC,

and 354GT cleared during DNA sequencing of the *CADM2* (495-bp) gene, which resulted in the amino acids 50R, 56N, and 118T, respectively. One recurrent non-synonymous SNP, 217GC, was elaborated using DNA sequencing of the *ZNF503* gene (361-bp), which led to the substitution of the amino acid

```

EF375880.1  ATGGTCCTTCTGAGCATCCTTAGAATCCTTCTTCTTTGGGGACTGGTGCTTTTATGGAA 60
H           ATGGTCCTTCTGAGCATCCTTAGAATCCTTCTTCTTTGGGGACTGGTGCTTTTATGGAA 60
RR          ATGGTCCTTCTGAGCATCCTTAGAATCCTTCTTCTTTGGGGACTGGCGCTTTTATGGAA 60
*****

EF375880.1  CATAGGGTCCAAATGACACCGGTAGGGCAGCCCTCTATTGCCCACTGCCTGAGGCCCT 120
H           CATAGGGTCCAAATGACACAGGTAGGGCAGCCCTCTATTGCCCACTGCCTGAGGCCCT 120
RR          CATAGGGTCCAAATGACACCGGTAGGGCAGCCCTCTATTGCCCACTGCCTGAGGCCCT 120
*****

EF375880.1  ACCTTGCCCTGATTGAGGAGCTGCTAGAAGAAGCCCTGGCAAGCTGCAGAGGAAGCCG 180
H           ACCTTGCCCTGATTGAGGAGCTGCTAGAAGAAGCCCTGGCAAGCTGCAGAGGAAGCCG 180
RR          ACCTTGCCCTGATTGAGGAGCTGCTAGAAGAAGCCCTGGCAAGCTGCAGAGGAAGCCG 180
*****

EF375880.1  CGGGTCTTAGGGCATCCCTTACGGTATATGCTGGAGTTGTACCACCGTTGAGCTGACGCA 240
H           CGGATCTTAGGGCATCCCTTACGGTATATGCTGGAGTTGTACCACCGTTGAGCTGACGCA 240
RR          CGGGTCTTAGGGCATCCCTTACGGTATATGCTGGAGTTGTACCACCGTTGAGCTGACGCA 240
*****

EF375880.1  AGTGGACACCCTAGGGAAAACCGCACCATTGGGGCCACCATGGTGAGGCTGGTGAGGCCA 300
H           AGTGGACACCCTAGGGAAAACCGCACCATTGGGGCCACCATGGTGAGGCTGGTGAGGCCA 300
RR          AGTGGACACCCTAGGGAAAACCGCACCATTGGGGCCACCATGGTGAGGCTGGTGAGGCCA 300
*****

EF375880.1  CTGGCTAGTGTAGCAAGGCCTCTCAGAGGCTCCTGGCACATACAGACCCCTGGACTTTTCT 360
H           CTGGCTAGTGTAGCAAGGCCTCTCAGAGGCTCCTGGCACATACAGACCCCTGGACTTTTCT 360
RR          CTGGCTAGTGTAGCAAGGCCTCTCAGAGGCTCCTGGCACATACAGACCCCTGGACTTTTCT 360
*****

EF375880.1  CTGAGACCAAACCGGGTAGCATACCAACTAGTCAGAGCCACTGTGGTTTACCGCCATCAA 420
H           CTGAGACCAAACCGGGTAGCATACCAACTAGTCAGAGCCACTGTGGTTTACCGCCATCAA 420
RR          CTGAGACCAAACCGGGTAGCATACCAACTAGTCAGAGCCACTGTGGTTTACCGCCATCAA 420
*****

EF375880.1  CTTACCTAAGTCTTCCACCTCTCCTGCCATGTGGAGCCCTGGGTCCAGAAAAGCCCA 480
H           CTTACCTAAGTCTTCCACCTCTCCTGCCATGTGGAGCCCTGGGTCCAGAAAAGCCCA 480
RR          CTTACCTAAGTCTTCCACCTCTCCTGCCATGTGGAGCCCTGGGTCCAGAAAAGCCCA 480
*****

```

Figure S4. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|EF375880.1| and *BMP* marker (480-bp) sequences.

```

MF173058.1  GTGAGCCTGGCAGTTGCAGACCTGCTGGTGGCCGTGTATCCGTACCCCTTGGCGCTGGCG 60
H           GTGAGCCTGGCAGTTGCAGACCTGCTGGTGGCCGTGTATCCGTACCCCTTGGCGCTGGCG 60
RR          GTGAGCCTGGCAGTTGCAGACCTGCTGGTGGCCGTGTATCCGTACCCCTTGGCGCTGGCG 60
*****

MF173058.1  TCTATAGTTAACGATGGGTGGAGCCTGAGCTCCCTGCATTGCCAACTTAGTGGCTTCCTG 120
H           TCTATAGTTAATGATGGGTGGAGCCTGAGCTCCCTGCATTGCCAACTTAGTGGCTTCCTG 120
RR          TCTATAGTTAACGATGGGTGGAGCCTGAGCTCCCTGCATTGCCAACTTAGTGGCTTCCTG 120
*****

MF173058.1  ATGGGCTTGAGCGTCATCGGGTCCGTTTTCAATATCACGGGAATTGCCATCAACCGCTAT 180
H           ATGGGCTTGAGCGTCATCGGGTCCGTTTTCAATATCACGGGAATTGCCATCAACCGCTAT 180
RR          ATGGGCTTGAGCGTCATCGGGTCCGTTTTCAATATCACGGGAATTGCCATCAACCGCTAT 180
*****

MF173058.1  TGCTGCACTGCCACAGCCTCAGATACGACAAGCTGTATAGCAGCACAATTCCTCTGC 240
H           TGCTGCACTGCCACAGCCTCAGATACGACAAGCTGTATAGCAGCACAATTCCTCTGC 240
RR          TGCTGCACTGCCACAGCCTCAGATACGACAAGCTGTATAGCAGCACAATTCCTCTGC 240
*****

MF173058.1  TACGTGTTCTGATATGGCTGCTGACGTTGCTGGCGATCGTGCCCAACCTGTGTGTGGGG 300
H           TACGTGTTCTGATATGGCTGCTGACGTTGCTGGCGATCGTGCCCAACCTGTGTGTGGGG 300
RR          TACGTGTTCTGATATGGCTGCTGACGTTGCTGGCGATCGTGCCCAACCTGTGTGTGGGG 300
*****

MF173058.1  ACCCTGCGGTACGACCCGAGGATCTATTCTTGTACCTTCACACAGTCCGTGAGCTCAGCC 360
H           ACCCTGCGGTACGACCCGAGGATCTATTCTTGTACCTTCACACAGTCCGTGAGCTCAGCC 360
RR          ACCCTGCGGTACGACCCGAGGATCTATTCTTGTACCTTCACACAGTCCGTGAGCTCAGCC 360
*****

MF173058.1  TACACGATCGCCGTGGTGGTGTTCATTTCATAGTTCCAATGCTCGTAGTCATCTTCTGT 420
H           TACACGATCGCCGTGGTGGTGTTCATTTCATAGTTCCAATGCTCGTAGTCATCTTCTGT 420
RR          TACACGATCGCCGTGGTGGTGTTCATTTCATAGTTCCAATGCTCGTAGTCATCTTCTGT 420
*****

MF173058.1  TACCTGAGAATCTGGGCCTGGTTCTTCAGGTCAGATGGAGAGTG 465
H           TACCTGAGAATCTGGGCCTGGTTCTTCAGGTCAGATGGAGAGTG 465
RR          TACCTGAGAATCTGGGCCTGGTTCTTCAGGTCAGATGGAGAGTG 465
*****

```

Figure S5. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|MF173058.1| and *MTNR1A* marker (465-bp) sequences.


```

KC415275.1  ATCCGCCCTCTGGCAGCCCTACGTCATCATGCTGGGGGGCTGGGTTTGCTGCTTCCTGCTC 60
H          ATCCGCCCTCTGGCAGCCCTACGTCATCATGCTGGGGGGCTGGGTTTGCTGCTTCCTGCTC 60
RR          ATCCGCCCTCTGGCAGCCCTACGTCATCATGCTGGGGGGCTGGGTTTGCTGCTTCCTGCTC 60
*****
KC415275.1  GCCTTGCTCCCTTTGGTGGGAATAAGCAGCTATGCCAAGGTGAGCATCTGCCTGCCCATG 120
H          GCCTTGCTCCCTTTGGTGGGAATAAGCAGCTATGCCAAGGTGAGCATCTGCCTGCCCATG 120
RR          GCCTTGCTCCCTTTGGTGGGAATAAGTAGCTATGCCAAGGTGAGCATCTGCCTGCCCATG 120
*****
KC415275.1  GACACTGAGACTCCTCTTGCCCTGGCGTACATTATCCTCGTTCTGTTACTCAACATCGTT 180
H          GACACTGAGACTCCTCTTGCCCTGGCGTACATTATCCTCGTTCTGTTACTCAACATCGTT 180
RR          GACACCGAGACTCCTCTTGCCCTGGCGTACATTATCCTCGTTCTGTTACTCAACATCGTT 180
*****
KC415275.1  GCCTTTATCATCGTCTGTGCTGTACGTGAAGATCTACATCACAGTCCGAAATCCCCAC 240
H          GCCTTTATCATCGTCTGTGCTGTACGTGAAGATCTACATCACAGTCCGAAATCCCCAC 240
RR          GCCTTTATCATCGTCTGTGCTGTACGTGAAGATCTACATCACAGTCCGAAATCCCCAC 240
*****
KC415275.1  TACAACCGGGGGACAAAGATACTAGAATTGCCAAAAGGATGGCTGTGTGATCTTCACT 300
H          TACAACCGGGGGACAAAGATACTAGAATTGCCAAAAGGATGGCTGTGTGATCTTCACT 300
RR          TACAACCGGGGGACAAAGATACTAGAATTGCCAAAAGGATGGCTGTGTGATCTTCACT 300
*****
KC415275.1  GACTTCATGTGCATGGCCCAATCTCTTTCTACGCTCTGTGCGCCCTTAAGAACAAGCCT 360
H          GACTTCATGTGCATGGCCCAATCTCTTTCTACGCTCTGTGCGCCCTTAAGAACAAGCCT 360
RR          GACTTCATGTGCATGGCCCAATCTCTTTCTACGCTCTGTGCGCCCTTAAGAACAAGCCT 360
*****
KC415275.1  CTCATCACCGTCAACCAATTCAAAATCTTGCTGGTCTCTTCTACCCACTTAACCTCTGT 420
H          CTCATCACCGTCAACCAATTCAAAATCTTGCTGGTCTCTTCTACCCACTTAACCTCTGT 420
RR          CTCATCACCGTTAACCAATTCAAAATCTTGCTGGTCTCTTCTACCCACTTAACCTCTGT 420
*****
KC415275.1  GCCAATCCATTCTCTATGCCA 442
H          GCCAATCCATTCTCTATGCCA 442
RR          GCCAATCCATTCTCTATGCCA 442
*****

```

Figure S6. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|KC415275.1| and *TSHR* marker (442-bp) sequences.

```

XM_025275645.3  CTGCTCGCCTGGGCTCCCGGGTGGTGGTCTTGCTCGTCTTCTCTGCTGCGTGGCGTCT 60
H              CTGCTCGCCTGGGCTCCCGGGTGGTGGTCTTGCTCGTCTTCTCTGCTGCGTGGCGTCT 60
RR              CTGCTCGCCTGGGCTCCCGGGTGGTGGTCTTGCTCGTCTTCTCTGCTGCGTGGCGTCT 60
*****
XM_025275645.3  GTCCTCTACATGCTGGCCTGCACGCCGAGGGGCGATGATGAGCGCCAGGGGCTGCCAGG 120
H              GTCCTCTACATGCTGGCCTGCACGCCGAGGGGCGATGATGAGCGCCAGGGGCTGCCAGG 120
RR              GTCCTCTACATGCTGGCCTGCACGCCGAGGGGCGATGATGAGCGCCAGGGGCTGCCAGG 120
*****
XM_025275645.3  GCCAACGGCCCCACGGGACGGGCGGCTCCAGGCGGCGGTGCTCGGCTGGGAGGAGCGT 180
H              GCCAACGGCCCCACGGGACGGGCGGCTCCAGGCGGCGGTGCTCGGCTGGGAGGAGCGT 180
RR              GCCAACGGCCCCACGGGACGGGCGGCTCCAGGCGGCGGTGCTCGGCTGGGAGGAGCGT 180
*****
XM_025275645.3  CACCGCGACCAAGTGGGAGCCTCAAGAGGCAATTCGCGAGCTGCAGGAGGAGCTGCAA 240
H              CACCGCGACCAAGTGGGAGCCTCAAGAGGCAATTCGCGAGCTGCAGGAGGAGCTGCAA 240
RR              CACCGCGACCAAGTGGGAGCCTCAAGAGGCAATTCGCGAGCTGCAGGAGGAGCTGCAA 240
*****
XM_025275645.3  GAGCGGAGCGAGCAGCTGAAGGCGCGCAGCAGCTGGCGGGGAGCCCGCGGGTGCCCCG 300
H              GAGCGGAGCGAGCAGCTGAAGGCGCGCAGCAGCTGGCGGGGAGCCCGCGGGTGCCCCG 300
RR              GAGCGGAGCGAGCAGCTGAAGGCGCGCAGCAGCTGGCGGGGAGCCCGCGGGTGCCCCG 300
*****
XM_025275645.3  GGCAGGGCCCAGGCGACCTGCTGGCCTTCCTGCGCTCGCAGGTGGACAGGGCGGAGGTG 360
H              GGCAGGGCCCAGGCGACCTGCTGGCCTTCCTGCGCTCGCAGGTGGACAGGGCGGAGGTG 360
RR              GGCAGGGCCCAGGCGACCTGCTGGCCTTCCTGCGCTCGCAGGTGGACAGGGCGGAGGTG 360
*****
XM_025275645.3  CACGCCGGCGTCAAGCAGGCCACCG 385
H              CACGCCGGCGTCAAGCAGGCCACCG 385
RR              CACGCCGGCGTCAAGCAGGCCACCG 385
*****

```

Figure S7. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_025275645.3| and *CSGALNACT1* marker (385-bp) sequences.

| | | |
|----------------|---|-------|
| XM_025278930.3 | CGCAGCGCCGTTCTCCGCTTCTACAGTGTCTGCGGGCTCCTGCTACAAGCGGCTGCTTCA | 60 |
| H | CGCAGCGCCGTTCTCCGCTTCTACAGTGTCTGCGGGCTCCTGCTACAAGCGGCTGCTTCA | 60 |
| RR | CGCAGCGCCGTTCTCCGCTTCTACAGTGTCTGCGGGCTCCTGCTACAAGCGGCTGCTTCA | 60 |
| ***** | ***** | ***** |
| XM_025278930.3 | AAGAATAAAGTTAAAGGCAGCCAAAGGGCAGTTTCCACTCACACAGAATGTAACGGTTGTT | 120 |
| H | AAGAATAAAGTTAAAGGCAGCCAAAGGGCAGTTTCCACTCACACAGAATGTAACGGTTGTT | 120 |
| RR | AAGAATAAAGTTAAAGGCAGCCAAAGGGCAGTTTCCACTCACACAGAATGTAACGGTTGTT | 120 |
| ***** | ***** | ***** |
| XM_025278930.3 | GAAGGCGGAAGTGCATTTTGACCTGCAGGGTTGATCAAAATGATAATACCTCCCTCCAG | 180 |
| H | GAAGGCGGAAGTGCATTTTGACCTGCAGGGTTGATCAAAATGATAATACCTCCCTCCAG | 180 |
| RR | GAAGGCGGAAGTGCATTTTGACCTGCAGGGTTGATCAAAATGATAATACCTCCCTCCAG | 180 |
| ***** | ***** | ***** |
| XM_025278930.3 | TGGTCAAATCCAGCTCAACAGACTCTGTACTTTGATGACAAGAAAGCCTTAAGAGACAAT | 240 |
| H | TGGTCAAATCCAGCTCAACAGACTCTGTACTTTGATGACAAGAAAGCCTTAAGAGACAAT | 240 |
| RR | TGGTCAAATCCAGCTCAACAGACTCTGTACTTTGATGACAAGAAAGCCTTAAGAGACAAT | 240 |
| ***** | ***** | ***** |
| XM_025278930.3 | AGAATCGAGCTGGTTTCGGGCTTCTGGCATGAAGTATCAGCGTCAGTGTGTCTCT | 300 |
| H | AGAATCGAGCTGGTTTCGGGCTTCTGGCATGAAGTATCAGCGTCAGTGTGTCTCT | 300 |
| RR | AGAATCGAGCTGGTTTCGGGCTTCTGGCATGAAGTATCAGCGTCAGTGTGTCTCT | 300 |
| ***** | ***** | ***** |
| XM_025278930.3 | CTGTCTGATGAAGGACAGTACACCTGTTCTTTATTTACAATGCCTGTCAAACGTCCAAG | 360 |
| H | CTGTCTGATGAAGGACAGTACACCTGTTCTTTATTTACAATGCCTGTCAAACGTCCAAG | 360 |
| RR | CTGTCTGATGAAGGACAGTACACCTGTTCTTTATTTACAATGCCTGTCAAACGTCCAAG | 360 |
| ***** | ***** | ***** |
| XM_025278930.3 | GCCTATCTCACTGTCTGGGTGTTCTTGAGAAACCTCAAATTAGTGGATTTTCATCCCCA | 420 |
| H | GCCTATCTCACTGTCTGGGTGTTCTTGAGAAACCTCAAATTAGTGGATTTTCATCCCCA | 420 |
| RR | GCCTATCTCACTGTCTGGGTGTTCTTGAGAAACCTCAAATTAGTGGATTTTCATCCCCA | 420 |
| ***** | ***** | ***** |
| XM_025278930.3 | GTTATGGAGGGAGACTTGATGCAGCTGACTTTGTAACATCTGGTAGTAAACCTGCAGCT | 480 |
| H | GTTATGGAGGGAGACTTGATGCAGCTGACTTTGTAACATCTGGTAGTAAACCTGCAGCT | 480 |
| RR | GTTATGGAGGGAGACTTGATGCAGCTGACTTTGTAACATCTGGTAGTAAACCTGCAGCT | 480 |
| ***** | ***** | ***** |
| XM_025278930.3 | GATATAAGATGGTTC | 495 |
| H | GATATAAGATGGTTC | 495 |
| RR | GATATAAGATGGTTC | 495 |
| ***** | ***** | ***** |

Figure S8. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_025278930.3| and *CADM2* marker (495-bp) sequences.

| | | |
|----------------|--|-------|
| XM_025285122.3 | TCTCCGCCAGGCTAACCGCCTGCCCATCAAGGTGCTGAAGATGCTGACGGCAGGACTGG | 60 |
| H | TCTCCGCCAGGCTAACCGCCTGCCCATCAAGGTGCTGAAGATGCTGACGGCAGGACTGG | 60 |
| RR | TCTCCGCCAGGCTAACCGCCTGCCCATCAAGGTGCTGAAGATGCTGACGGCAGGACTGG | 60 |
| ***** | ***** | ***** |
| XM_025285122.3 | CCATATTTTGCACCTGAGTACCTGCAGCCCCTGCCTTCCACTCCCGTCAGCCCCATAGA | 120 |
| H | CCATATTTTGCACCTGAGTACCTGCAGCCCCTGCCTTCCACTCCCGTCAGCCCCATAGA | 120 |
| RR | CCATATTTTGCACCTGAGTACCTGCAGCCCCTGCCTTCCACTCCCGTCAGCCCCATAGA | 120 |
| ***** | ***** | ***** |
| XM_025285122.3 | GCTCGATGCCAAGAAGAGCCCGCTGGCGCTGTTGGCCCAACATGCTCGCAGATCGGGAA | 180 |
| H | GCTCGATGCCAAGAAGAGCCCGCTGGCGCTGTTGGCCCAACATGCTCGCAGATCGGGAA | 180 |
| RR | GCTCGATGCCAAGAAGAGCCCGCTGGCGCTGTTGGCCCAACATGCTCGCAGATCGGGAA | 180 |
| ***** | ***** | ***** |
| XM_025285122.3 | GCCCGACCCCTCGCCCTCGTCCAAACTCTCCTCAGTGGCCTCCAAACGGGGGTGGCAGGG | 240 |
| H | GCCCGACCCCTCGCCCTCGTCCAAACTCTCCTCAGTGGCCTCCAAACGGGGGTGGCAGGG | 240 |
| RR | GCCCGACCCCTCGCCCTCGTCCAAACTCTCCTCAGTGGCCTCCAAACGGGGGTGGCAGGG | 240 |
| ***** | ***** | ***** |
| XM_025285122.3 | CGGTGCCGCGCGCGCGCGGGGCGACAGGACGCCAAGTCGGGCCCCCTCAAGCTGAG | 300 |
| H | CGGTGCCGCGCGCGCGCGGGGCGACAGGACGCCAAGTCGGGCCCCCTCAAGCTGAG | 300 |
| RR | CGGTGCCGCGCGCGCGCGGGGCGACAGGACGCCAAGTCGGGCCCCCTCAAGCTGAG | 300 |
| ***** | ***** | ***** |
| XM_025285122.3 | CGACATCGGCGTGAAGACAAGTCGAGTTTCAAGCCGTACTCCAAACCCGGCTCGGATAA | 360 |
| H | CGACATCGGCGTGAAGACAAGTCGAGTTTCAAGCCGTACTCCAAACCCGGCTCGGATAA | 360 |
| RR | CGACATCGGCGTGAAGACAAGTCGAGTTTCAAGCCGTACTCCAAACCCGGCTCGGATAA | 360 |
| ***** | ***** | ***** |
| XM_025285122.3 | G | 361 |
| H | G | 361 |
| RR | G | 361 |
| ***** | * | ***** |

Figure S9. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_025285122.3| and *ZNF503* marker (361-bp) sequences.

```

XM_006048659.4    CCGACGTCGGAGAACCGAGATGAATCTGAACCTCTCACTGTTCCAGATGGTTGGAAGGAG    60
H                  CCGACGTCGGAGAACCGAGATGAATCTGAACCTCTCACTGTTCCAGATGGTTGGAAGGAG    60
RR                 CCGACGTCGGAGAACCGAGATGAATCTGAACCTCTCACTGTTCCAGATGGTTGGAAGGAG    60
*****
XM_006048659.4    CCAGCTTTCTCCAAAGAGGACAATCCCAGAGGACTTCTGGAGGAGAGCAGTTTTGCAACT    120
H                  CCAGCTTTCTCCAAAGAGGACAATCCCAGAGGACTTCTGGAGGAGAGCAGTTTTGCAACT    120
RR                 CCAGCTTTCTCCAAAGAGGACAATCCCAGAGGACTTCTGGAGGAGAGCAGTTTTGCAACT    120
*****
XM_006048659.4    TTGTTTCCGAAATACAGAGAGGCTTACTTGAAAGAGTGCTGGCCATTGGTACAGAAAGCC    180
H                  TTGTTTCCGAAATACAGAGAGGCTTACTTGAAAGAGTGCTGGCCATTGGTACAGAAAGCC    180
RR                 TTGTTTCCGAAATACAGAGAGGCTTACTTGAAAGAGTGCTGGCCATTGGTACAGAAAGCC    180
*****
XM_006048659.4    TTGAATGAACATCATGTTAATGCCACCTGGACCTGATTGAGGGCAGCATGACTGTCTGC    240
H                  TTGAATGAACATCATGTTAATGCCACCTGGACCTGATTGAGGGCAGCATGACTGTCTGC    240
RR                 TTGAATGAACATCATGTTAATGCCACCTGGACCTGATTGAGGGCAGCATGACTGTCTGC    240
*****
XM_006048659.4    ACCACCAAGAAGACATTGTATCCATATATCATCATTAG    278
H                  ACCACCAAGAAGACATTGTATCCATATATCATCATTAG    278
RR                 ACCACCAAGAAGACATTGTATCCATATATCATCATTAG    278
*****

```

Figure S10. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_006048659.4| and *KRR1* marker (278-bp) sequences.

```

XM_044946911.2    ATGTGCGACAAGGAGTTCATGTGGGCCCTGAAAAACGGAGACCTAGATGAGGTGAAAGAC    60
H                  ATGTGCGACAAGGAGTTCATGTGGGCCCTGAAAAACGGAGACCTAGATGAGGTGAAAGAC    60
RR                 ATGTGCGACAAGGAGTTCATGTGGGCCCTGAAAAACGGAGACCTAGATGAGGTGAAAGAC    60
*****
XM_044946911.2    TATGTGGCCAAGGGAGAAGATGTCAACCGGACACTAGAAGGTGGAAGAAAGCCTCTTCAT    120
H                  TATGTGGCCAAGGGAGAAGATGTCAACCGGACACTAGAAGGTGGAAGAAAGCCTCTTCAT    120
RR                 TATGTGGCCAAGGGAGAAGATGTCAACCGGACACTAGAAGGTGGAAGAAAGCCTCTTCAT    120
*****
XM_044946911.2    TATGCAGCAGATTGTGGACAGCTTGAAATCCTGGAATTTCTGCTGCTGAAAGGAGCAGAT    180
H                  TATGCAGCAGATTGTGGACAGCTTGAAATCCTGGAATTTCTGCTGCTGAAAGGAGCAGAT    180
RR                 TATGCAGCAGATTGTGGACAGCTTGAAATCCTGGAATTTCTGCTGCTGAAAGGAGCAGAT    180
*****
XM_044946911.2    ATTAATGCTCCAGATAAACATCATATCACACCTCTTCTGTCTGCCGTCTATGAAGGTCAT    240
H                  ATTAATGCTCCAGATAAACATCATATCACACCTCTTCTGTCTGCCGTCTATGAAGGTCAT    240
RR                 ATTAATGCTCCAGATAAACATCATATCACACCTCTTCTGTCTGCCGTCTATGAAGGTCAT    240
*****
XM_044946911.2    GTTTCCTGCGTGAAATTGCTTCTGTG    266
H                  GTTTCCTGCGTGAAATTGCTTCTGTG    266
RR                 GTTTCCTGCGTGAAATTGCTTCTGTG    266
*****

```

Figure S11. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_044946911.2| and *MTPN* marker (266-bp) sequences.

G73R. The nucleotide sequence of the *KRR1* gene (278-bp) revealed one synonymous SNP; 56A resulted from 168GA SNP. Two recurrent SNPs in the *MTPN* gene (266-bp) were discovered using DNA sequencing; 45AG and 189TC, resulted in synonymous mutations, 15L and 63A, respectively. Three recurrent non-synonymous SNPs were found in the *IGFBP7* gene (380-bp); 38GC, 107TC, and 161AC changed the amino acids R13P, V36A and Q54P, respectively. Two recurrent synonymous SNPs, 93GA, and 216GA induced amino acids 31L, and 72P were found in the *LEP* gene (360-bp). Four recurrent SNPs in the *ABCC4* gene (355-bp) were discovered using DNA sequencing; one of these, 294CT resulted in

synonymous mutations, 98I. While the non-synonymous mutation brought about by the SNPs 65CT, 109AG and 306GT led to the substitution of the amino acids S22L, R37G, and L102F, respectively

DISCUSSION

Efficiency of investigated reproductive genes as candidates for repeat breeder incidence

For the effective exploitation of disease-resistant cattle or the complete eradication of ill animals, knowledge of the genes, it is necessary to understand the fundamental mutations and interactions that contribute to resistance (Pal & Chakravarty, 2020). The reproductive (*MFSD14A*, *PALMD*, *VPS13B*, *BMP*,

| | | |
|----------------|---|-----|
| XM_025290001.3 | AGCTGGGCGAGACCCGCGATGCAITGCGGCTGCTGCCCGGTGTGCGCCCGCGGCGAGGGCG | 60 |
| H | AGCTGGGCGAGACCCGCGATGCAITGCGGCTGCTGCCCGGTGTGCGCCCGCGGCGAGGGCG | 60 |
| RR | AGCTGGGCGAGACCCGCGATGCAITGCGGCTGCTGCCCGGTGTGCGCCCGCGGCGAGGGCG | 60 |
| XM_025290001.3 | AGCCGTGCGGGGTGGCGGCGCCGCGAGGGGGCACTGCGCGCCGGGTATGGAGTGCCTGA | 120 |
| H | AGCCGTGCGGGGTGGCGGCGCCGCGAGGGGGCACTGCGCGCCGGGTATGGAGTGCCTGA | 120 |
| RR | AGCCGTGCGGGGTGGCGGCGCCGCGAGGGGGCACTGCGCGCCGGGTATGGAGTGCCTGA | 120 |
| XM_025290001.3 | AGAGCCGCAAGAGGCGGAAGGGTAAGCCGGGGCAGCAGCAGGCGGCCCGGTTGTGAGCG | 180 |
| H | AGAGCCGCAAGAGGCGGAAGGGTAAGCCGGGGCAGCAGCAGGCGGCCCGGTTGTGAGCG | 180 |
| RR | AGAGCCGCAAGAGGCGGAAGGGTAAGCCGGGGCAGCAGCAGGCGGCCCGGTTGTGAGCG | 180 |
| XM_025290001.3 | GCGTGTGTGTGTGCAAGAGCCGCTACCCCGTGTGCGGCAGCAGCGGTGTACCTACTCCA | 240 |
| H | GCGTGTGTGTGTGCAAGAGCCGCTACCCCGTGTGCGGCAGCAGCGGTGTACCTACTCCA | 240 |
| RR | GCGTGTGTGTGTGCAAGAGCCGCTACCCCGTGTGCGGCAGCAGCGGTGTACCTACTCCA | 240 |
| XM_025290001.3 | GCGGCTGCCAGCTGCGCGCCGCCAGCCTCAGGGCCGAGAGCCGCGGGGAGAAGGCCATCA | 300 |
| H | GCGGCTGCCAGCTGCGCGCCGCCAGCCTCAGGGCCGAGAGCCGCGGGGAGAAGGCCATCA | 300 |
| RR | GCGGCTGCCAGCTGCGCGCCGCCAGCCTCAGGGCCGAGAGCCGCGGGGAGAAGGCCATCA | 300 |
| XM_025290001.3 | CCCAGGTGAGCAAGGGCACTGCGAGCAAGGTCTTCCATCGTGACACCCCCAAGGACA | 360 |
| H | CCCAGGTGAGCAAGGGCACTGCGAGCAAGGTCTTCCATCGTGACACCCCCAAGGACA | 360 |
| RR | CCCAGGTGAGCAAGGGCACTGCGAGCAAGGTCTTCCATCGTGACACCCCCAAGGACA | 360 |
| XM_025290001.3 | TCTGGAATGTCACTGGTGCC | 380 |
| H | TCTGGAATGTCACTGGTGCC | 380 |
| RR | TCTGGAATGTCACTGGTGCC | 380 |

Figure S12. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_025290001.3| and *IGFBP7* marker (380-bp) sequences.

| | | |
|------------|---|-----|
| AF387814.1 | CAGTCCGTCTCCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCCT | 60 |
| H | CAGTCCGTCTCCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCCT | 60 |
| RR | CAGTCCGTCTCCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCCT | 60 |
| AF387814.1 | CTCCTGAGTTTGTCCAAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGT | 120 |
| H | CTCCTGAGTTTGTCCAAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGT | 120 |
| RR | CTCCTGAGTTTGTCCAAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGT | 120 |
| AF387814.1 | CTGCCTTCCAGAAATGTGGTCCAAATATCTAATGACCTGGAGAACCTCCGGGACCTTCTC | 180 |
| H | CTGCCTTCCAGAAATGTGGTCCAAATATCTAATGACCTGGAGAACCTCCGGGACCTTCTC | 180 |
| RR | CTGCCTTCCAGAAATGTGGTCCAAATATCTAATGACCTGGAGAACCTCCGGGACCTTCTC | 180 |
| AF387814.1 | CACCTGCTGGCCGCTCCAGAGAGCTGCCCTTGCCGACAGGTGAGGGCCCTGGAGAGTTTG | 240 |
| H | CACCTGCTGGCCGCTCCAGAGAGCTGCCCTTGCCGACAGGTGAGGGCCCTGGAGAGTTTG | 240 |
| RR | CACCTGCTGGCCGCTCCAGAGAGCTGCCCTTGCCGACAGGTGAGGGCCCTGGAGAGTTTG | 240 |
| AF387814.1 | GAGAGCTTGGGCGTCTGCTGGAAGCCTCCCTCTACTCCACCGAGGTGGTGGCCCTGAGC | 300 |
| H | GAGAGCTTGGGCGTCTGCTGGAAGCCTCCCTCTACTCCACCGAGGTGGTGGCCCTGAGC | 300 |
| RR | GAGAGCTTGGGCGTCTGCTGGAAGCCTCCCTCTACTCCACCGAGGTGGTGGCCCTGAGC | 300 |
| AF387814.1 | CGGCTGCAGGGGTCACTACAGGACATGTTGCGGCAGCTGGACCTCAGTCCTGGGTGCTGA | 360 |
| H | CGGCTGCAGGGGTCACTACAGGACATGTTGCGGCAGCTGGACCTCAGTCCTGGGTGCTGA | 360 |
| RR | CGGCTGCAGGGGTCACTACAGGACATGTTGCGGCAGCTGGACCTCAGTCCTGGGTGCTGA | 360 |

Figure S13. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|AF387814.1| and *LEP* marker (360-bp) sequences.

MTNRI1A, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) genes in repeat breeder and healthy Italian buffaloes were identified in this study utilizing sequenced amplified PCR products. The findings show that there are differences between the SNPs involving the two categories. The assessed buffaloes had a considerable

nucleotide polymorphism dispersion ($p < 0.01$), according to the Fisher's exact test. It is crucial to emphasize that, when compared to the relevant datasets obtained from GenBank, the variations discovered and materials readily accessible in this context offer fresh information on the markers under consideration.

| | | |
|----------------|--|-------|
| XM_045162483.1 | GCAGAAGGCGAAGCTTCTGCTCGCGCCTGTTTGTCTGGTGGCTAAACCCCTTGTTTAAAT | 60 |
| H | GCAGAAGGCGAAGCTTCTGCTCGCGCCTGTTTGTCTGGTGGCTAAACCCCTTGTTTAAAT | 60 |
| RR | GCAGAAGGCGAAGCTTCTGCTCGCGCCTGTTTGTCTGGTGGCTAAACCCCTTGTTTAAAT | 60 |
| ***** | ***** | ***** |
| XM_045162483.1 | TGGTCACAAACGGAATAGAACAGATGACATGACTCGGTGCTTCCAGAAGATCGCTC | 120 |
| H | TGGTCACAAACGGAATAGAACAGATGACATGACTCGGTGCTTCCAGAAGATCGCTC | 120 |
| RR | TGGTTACAAACGGAATAGAACAGATGACATGACTCGGTGCTTCCAGAAGATCGCTC | 120 |
| **** | ***** | ***** |
| XM_045162483.1 | CCAGTGCCTTGGAGAAGAGCTGCAAGGGTACTGGGATCAGAAGTTAAGAGAGCCAGAA | 180 |
| H | CCAGTGCCTTGGAGAAGAGCTGCAAGGGTACTGGGATCAGAAGTTAAGAGAGCCAGAA | 180 |
| RR | CCAGTGCCTTGGAGAAGAGCTGCAAGGGTACTGGGATCAGAAGTTAAGAGAGCCAGAA | 180 |
| ***** | ***** | ***** |
| XM_045162483.1 | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 240 |
| H | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 240 |
| RR | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 240 |
| ***** | ***** | ***** |
| XM_045162483.1 | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 300 |
| H | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 300 |
| RR | GGATGGACAGGAACCCCTCTTTAGTGAAGCAATCGTAAAGTGTACTGGAATCCTATTT | 300 |
| ***** | ***** | ***** |
| XM_045162483.1 | AATTTGGGAATGTTTACATTTCTTGAGGAAGGCACCAGAGTAGTTCAACCCATA | 355 |
| H | AATTTGGGAATGTTTACATTTCTTGAGGAAGGCACCAGAGTAGTTCAACCCATA | 355 |
| RR | AATTTGGGAATGTTTACATTTCTTGAGGAAGGCACCAGAGTAGTTCAACCCATA | 355 |
| ***** | ***** | ***** |

Figure S14. Analysis of nitrogenous bases matching for DNA in healthy and repeat breeder buffaloes using GenBank gb|XM_045162483.1| and *ABCC4* marker (355-bp) sequences.

In recent studies, unique genes that are specifically related to the prevalence of bovine repeat breeders have been discovered using genome-wide association analysis for our investigated markers (Li et al., 2018; Mahrous et al., 2022), however, no research have yet looked into the relationship between repeat breeder risk and the SNPs in these genes. The gene sequences from the *Bubalus bubalis* employed in our study, which was published in PubMed, are the first to show this connection. The variance of the reproductive (*MFSD14A*, *PALMD*, *VPS13B*, *BMP*, *MTNR1A*, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) markers and how they relate to repeat breeder in Italian buffaloes have not, to our knowledge, been the subject of any prior research. However, the candidate gene technique has been used to monitor the validity of bovine fertility features. For instance, Egyptian buffaloes showed a connection between *IGF-I* genetic variations and issues with reproduction (Ramadan et al., 2018). Buffaloes with *MTNR1A* gene polymorphisms had seasonal suppression of fertility (Rangashamaiah et al., 2022). The frequency of repeat breeding in Egyptian buffaloes have been investigated in relation to the polymorphisms of the *LEP*, and *BMP* genes (Mahrous et al., 2022; El-Debaky et al., 2020). Buffalo genetic variations in the *FSHR* and *ESR* genes and were correlated with the frequency of repeat breeders (Fouda et al., 2021). *LHR* gene polymorphisms and Egyptian buffalo fertility was also investigated (Sosa et al., 2016). *CYP19A1*

gene polymorphisms were associated with anoestrus susceptibility and inactive ovaries in water buffaloes when a candidate gene approach to anoestrus was investigated (El-Bayomi et al., 2018; Abbaset al., 2014). Also discovered in Murrah buffaloes were polymorphisms in the *HSP70* gene, which may be related to post-partum anoestrus (Kumar et al., 2017).

The primary source of adaptation and selection is mutation (Chu & Wei, 2019). All of the reproductive markers that were being studied in this situation had exonic region mutations, which led to altered coding DNA sequences in repeat breeder buffaloes compared to healthy ones. Using DNA sequencing of reproductive genes, 30 SNPs were found; 16 of them are synonymous, and 15 are non-synonymous. Non-synonymous mutations alter protein sequences, and people who have these mutations are frequently the subject of natural selection (Chu, & Wei, 2019). The encoded amino acid at the mutant location is altered by genetic variation brought on by non-synonymous SNPs, which can result in structural and functional alterations in the mutated protein (Dakal et al., 2017). For a very long time, it was believed that selection on synonymous mutations was either nonexistent or very weak (Chu & Wei, 2019). In order to accurately characterize the investigated reproductive genes at the molecular level and to understand the physiological differences in reproductive performance between normal and repeat breeder buffaloes, our study discovered polymorphisms on the basis

Table 2. Single base differential dissemination as well as category of inherited alteration for reproductive markers in normal and repeat breeder buffaloes.

| Gene | SNPs | Normal n = 120 | Repeat breeder n = 120 | Total n = 240 | kind of inherited alteration | Amino acid order and sort |
|-------------------|-------|-------------------|---------------------------|------------------|---------------------------------|------------------------------|
| <i>MFSD14A</i> | C165T | - | 72 | 72/240 | Synonymous | 55 C |
| | T322C | - | 85 | 85/240 | Synonymous | 108 G |
| <i>PALMD</i> | T108C | 91 | - | 91/240 | Synonymous | 36 L |
| <i>VPS13B</i> | T175C | 48 | - | 48/240 | Non-synonymous | 59 C to R |
| | T223C | - | 63 | 63/240 | Non-synonymous | 75 Y to H |
| | T47C | - | 98 | 98/240 | Non-synonymous | 16 V to A |
| <i>BMP</i> | C80A | 58 | - | 58/240 | Non-synonymous | 27 P to Q |
| | G184A | 76 | - | 76/240 | Non-synonymous | 62 V to I |
| <i>MTNR1A</i> | C72T | 49 | - | 49/240 | Synonymous | 24 N |
| | C87T | - | 83 | 83/240 | Synonymous | 29 S |
| <i>TSHR</i> | T126C | - | 38 | 38/240 | Synonymous | 42 T |
| | C177T | 69 | - | 69/240 | Synonymous | 59 I |
| | C372T | - | 103 | 103/240 | Synonymous | 124 V |
| <i>CSGALNACT1</i> | G135A | 47 | - | 47/240 | Synonymous | 45 T |
| | G150A | 91 | - | 91/240 | Synonymous | 50 R |
| <i>CADM2</i> | T168C | 63 | - | 63/240 | Synonymous | 56 N |
| | G354T | 39 | - | 39/240 | Synonymous | 118 T |
| <i>ZNF503</i> | G217C | - | 98 | 98/240 | Non-synonymous | 73 G to R |
| <i>KRR1</i> | G168A | 107 | - | 107/240 | Synonymous | 56 A |
| <i>MTPN</i> | A45G | 46 | - | 46/240 | Synonymous | 15 L |
| | T189C | - | 104 | 104/240 | Synonymous | 63 A |
| | G38C | - | 58 | 58/240 | Non-synonymous | 13 R to P |
| <i>IGFBP7</i> | T107C | - | 112 | 112/240 | Non-synonymous | 36 V to A |
| | A161C | 92 | - | 92/240 | Non-synonymous | 54 Q to P |
| <i>LEP</i> | G93A | 71 | - | 71/240 | Synonymous | 31 L |
| | G216A | - | 86 | 86/240 | Synonymous | 72 P |
| | C65T | - | 89 | 89/240 | Non-synonymous | 22 S to L |
| <i>ABCC4</i> | A109G | - | 109 | 109/240 | Non-synonymous | 37 R to G |
| | C294T | - | 74 | 74/240 | Synonymous | 98 I |
| | G306T | 93 | - | 93/240 | Non-synonymous | 102 L to F |

Single base difference dispersal for reproductive markers in normal and repeat breeder buffaloes showed a highly significant variation ($p < 0.01$) according to Fisher's exact analysis.

MFSD14A= Major facilitator superfamily domain containing 14A; *PALMD*= Palmdelphin; *VPS13B*= Vacuolar protein sorting 13 homolog B; *BMP*= Bone morphogenic protein; *MTNR1A*= Melatonin receptor 1B; *TSHR*= Thyroid stimulating hormone receptor; *CSGALNACT1*= Chondroitin sulfate N-acetylgalactosaminyltransferase 1; *CADM2*= Cell adhesion molecule 2; *ZNF503*= Zinc finger protein 503; *KRR1* = Small subunit processome component homolog; *MTPN*= Myotrophin; *IGFBP7*= insulin like growth factor binding protein 7; *LEP*= Leptin; and *ABCC4*= ATP-binding cassette sub-family C member 4. A = Alanine; C = Cysteine; F = Phenylalanine; G = Glycine; H = Histidine; I = Isoleucine; L = Leucine; N= Asparagine; P= Proline; Q= Glutamine; R = Arginine; S = Serine; T = Threonine; V = Valine; and Y= Tyrosine.

of translated DNA sequence to be of greater value than intronic parts.

Role of investigated genes in reproduction

A protein-coding gene called major facilitator superfamily domain containing 14A (MFSD14A) has product homology to the family of solute carrier proteins (Doran et al., 2016). Additionally, MFSD14A functions as a component of cellular fundamentals, a molecular controller of transporter action, and a biological tool involved in trans-membrane transport (Sreedharan et al., 2011). The nuclear transfer transcripts in pig blastocyst stage were discovered to be negatively controlled by *MFSD14A* (Whitworth, et al., 2011), and the gene may also play a role in mouse spermatogenesis (Doran, 2016). Two SNPs in the *MFSD14A* gene have been linked to characteristics of buffalo milk production (Liu et al., 2018). A cytosolic protein involved in the phosphorylation of p53 is encoded by the palmdelphin (*PALMD*) (Moioli et al., 2013). The variance in milk yield is likely to be caused by the *PALMD* gene (Seo et al., 2016; Moioli, et al., 2013). *PALMD* polymorphisms had a significant impact on back fat thickness in a research to identify probable causative mutations associated to pig production attributes (Martínez-Montes et al., 2017). These *PALMD* functional studies suggest that it may be a key target gene that regulates variables related to reproductive performance.

The eye, hematological system, and central nervous system all develop and function as a result of the potential trans-membrane protein that the vacuolar protein sorting 13 homolog B (*VPS13B*) gene encodes (Liu et al., 2018). Protein transport has been identified as the primary biological utility of VPS13B, and it may play a role in vesicle-mediated transport as well as protein sorting within cells. In a prior investigation, *VPS13B* was discovered inside a QTL linked to the shape of the legs in dairy cattle (Van den Berg et al., 2014). Additionally, it was hypothesized that attributes related to milk production in Holstein cattle as well as female fertility were linked to the genomic region on BTA14 encompassing *VPS13B* (Capitan et al., 2014).

The secreted factor known as transforming growth factor (TGF) was initially discovered to influence cell proliferation (Roberts & Sporn, 1990). The proteins can be divided into two main groups according to cell signaling pathways: activins/transforming growth factors and bone morphogenetic proteins/growth differentiation factors (Chang et al., 2002). Among the BMP family memberships expressed in-

side the uterus, ovary, granulosa cells, and oocyte are BMP 2, 3, 4, 5, 6, 7 and 15 (Tanwar& McFarlane, 2011; Sun et al., 2010; Otsuka et al., 2001). They provide significant biological tasks as controllers of ovarian follicle growth, female reproductive tract discrepancy, blastocyst embedding in the uterus, and organogenesis and morphogenesis during embryo expansion (Kishigami & Mishina, 2005). The *BMP* gene has been the subject of numerous investigations in mammals, including cattle (Baloza et al., 2017), sheep (Ibrahim, 2019), and goats (Latifah & Saa, 2021; Ortiz et al., 2015). The blastocyst rate was shown to be substantially correlated with *BMP* SNPs by Lari et al., (2012).

Photoperiod and melatonin have been identified as the hypothalamus level modulators of the reproductive axis in a number of ovine investigations (Karsch et al., 2013). The pineal gland produces melatonin hormone in direct proportion to the length of darkness (Malpaux et al., 2001). The nighttime plasma melatonin content was highest in winter and lowest in summer in the Mediterranean buffaloes that had a seasonal reproductive tendency (Parmeggiani et al., 1994). Subcutaneous insertion of sustained-release melatonin implants in buffaloes during summer anestrus lengthens the daily presence of melatonin, simulating the effect of short days, and has been extensively investigated for the activation of the reproductive axis (Kavita et al., 2018). Only the melatonin receptor 1A gene (*MTNR1A*) is connected to reproductive seasonality of the G-protein couple receptors melatonin receptor 1A gene (*MTNR1A*) and melatonin receptor 1B gene (*MTNR1B*), to which melatonin binds (Dubocovich et al., 2020).

Animal reproduction research have used hormones and antibiotics as therapies because hormones are crucial to animal reproduction (Refsdal, 2000). Seasonal cycles of body weight and reproduction are significantly influenced by thyroid hormones (Barrett et al., 2007). By chance, buffalo are a species that go into estrus seasonally. Thyroid hormone resistance (THR) has also been shown to be highly correlated with the THR mutation (Işik et al., 2013). The genes involved in the metabolic pathways that impact animal fertility have already been extensively studied (Morfeld & Brown, 2014). One of these genes, *CSGALNACT1*, is necessary for healthy cartilage development (Watanabe et al., 2010). The *CSGALNACT1* gene was hypothesized to affect bovine follicle growth by altering the degree of expression that controls glucose metabolism (Li et al., 2018). Buffalo reproductive efficiency was discovered to be

correlated with cell adhesion molecule 2 (*CADM2*) (Song et al., 2013). One SNP at the bovine *CADM2* gene was connected to the number of days between conception and the first calving as well as the first and second calving (Li et al., 2018).

The target genes for buffalo reproductive features include *ZNF503*, *KRR1*, and *MTPN* (Lappalainen et al., 2013). *ZNF503* enhances cell invasion and proliferation in mammary epithelial cells, which has a key role in embryogenesis (Shahi et al., 2015; Chang et al., 2013). *KRR1* has been linked to polycystic ovarian syndrome (PCOS) in European populations (Day et al., 2015). *MTPN* is crucial for the development of cells and skeletal muscles (Makinaet al., 2015) and is associated with the process of antigen recognition, a crucial step in the immune reaction (Wang & Wang, 2012). The elevated expression of these three genes in buffalo granulosa cells raises the possibility that they took part in dominant follicle selection (Li et al., 2018). *IGFBP7* and follistatin share sequence similarity (Kato, 2000), the latter of which was once thought to be an inhibitor of FSH secretion (Jorgezet et al., 2004) and is essential for ovarian and folliculogenesis (Muttukrishna et al., 2004). Additionally, *IGFBP7* was discovered to be expressed in the granulosa cells of the large antral follicles of the pig ovary (Wandji et al., 2002) and the bovine corpus luteum (Casey et al., 2004). Therefore, we assume that *IGFBP7* is in some way responsible for the regulation of follicle development and ovulation (Li et al., 2018).

The versatile peptide hormone leptin, which is mostly generated by adipocytes, plays a crucial role in reproduction alongside controlling energy expenditure and body weight (Wiles et al., 2014). Leptin and its receptors have been discovered to be expressed in buffalo ovaries. Contrary to the widely held idea that leptin primarily affects the neuroendocrine component of reproduction, this clearly demonstrates that leptin directly engages in ovarian activity (Reshma et al., 2016). According to Fu et al., (2016), the ovary has leptin receptors that can control steroidogenesis and improve the oocyte's ability to support later embryonic development. The *ABCC4* gene was linked to the number of services per conception (NSC). Since it affects prostaglandin efflux from cells, this gene appears to be crucial to support pregnancy in the endometrium of pregnant cows (Spencer et al., 2013) and pigs (Seo et al., 2014). Prostaglandin plays a number of roles in re-

production, including conceptus implantation and maternal recognition of pregnancy—processes that are intimately connected to NSC. The expression of *ABCC4* was substantially linked with residual feed intake (RFI) in Angus cattle and was up-regulated in animals with high RFI (Chen et al., 2012). Nucleotide sequence variants in *ABCC4* was linked to marbling score in Nelore cattle (Feitosa et al., 2014). The recently proposed theory is that *ABCC4*, which is highly polymorphic and acts in fundamental metabolic pathways, may have effects on reproductive and meat quality (De Camargo et al., 2015).

CONCLUSIONS

Using PCR-DNA sequencing, single nucleotide variations (SNPs) in the genes were found for reproductive (*MFSD14A*, *PALMD*, *VPS13B*, *BMP*, *MTNR1A*, *TSHR*, *CSGALNACT1*, *CADM2*, *ZNF503*, *KRR1*, *MTPN*, *IGFBP7*, *LEP*, and *ABCC4*) indicators in normal and repeat breeder Italian buffaloes. These special functional variations present a promising opportunity to lower the occurrence of repeat breeders by using genetic markers in conjunction with normal wellbeing during buffalo selection. Future approaches to dealing with repeat breeders may be facilitated based on the gene domains here.

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CONFLICT OF INTEREST

There are no competing interests, according to the authors.

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