Melanin is the pigment of skin (coat) and hairs in all mammals. The 954 bp long MC1R gene codes for the production of MC1R or the α-MSH receptors which are present on the melanosomes. These receptors regulate the synthesis of melanin pigments viz., eumelanin or phaeomelanin leading to black and reddish to yellowish pigmentation respectively. PCR-RFLP analysis of 138 bp region of MC1R gene was conducted in two buffalo populations having distinct coat colours; Nagpuri (black) and Purnathadi (whitish). Three genotypes, EDED, EDE* and E*E* were observed at frequencies of 0.86, 0.03 and 0.11 for Nagpuri and 0.74, 0.08 and 0.18 in Purnathadi buffaloes; similarly the observed gene frequencies for ED and E* alleles were 0.87, 0.13 and 0.78, 0.22 in Nagpuri and Purnathadi buffaloes, respectively. DNA-sequencing revealed a transition mutation (A→G) at 118 bp locus confirming polymorphism, however presence in both the population failed to point towards any specific role in body coat colour of buffaloes.

**Keywords:** *Bubalus bubalis*, buffaloes, MC1R gene, coat colour gene, Nagpuri, Purnathadi

INTRODUCTION

With a total population of 108.7 million buffaloes, India ranks first in buffalo population in the world. National Bureau of Animal Genetic Resources (NBAGR), Karnal has registered seventeen breeds of buffaloes in India. In Maharashtra there are three descript breeds of buffalo from different regions of the state viz., Nagpuri (Vidarba region), Pandharpuri (Western Maharashtra) and Marathwadi (Marathwada region). Purnathadi locally known as ‘Bhuri buffaloes’, is reported to be phenotypically distinct primarily on the basis of its whitish brown coat colour. The calves born completely white with pink muzzle. The Purnathadi population predominantly found in Akola and neighbouring districts (Ali et al., 2019).

Coat colour patterns of domestic animals are of utmost importance as a breed descriptor phenotype. Melanin is the pigment responsible for coat colour in all mammals. The synthesis of melanin occurs in melanosomes present in the melanocytes on which the melanocortin 1 receptors (MC1R) are present. Consisting of a single exon; Melanocortin-1 Receptor (MC1R) gene, specifically expressed in melanocytes,
encodes for the homonymous G-protein coupled receptor involved in the regulation of the type of melanin (eumelanin or phaeomelanin) synthesized. MC1R is activated by the melanocyte stimulating hormone (MSH) secreted by the pituitary gland. Activation of these receptors leads to the production of eumelanin, catalyzed by precursor of melanin i.e. tyrosinase enzyme. A low level of tyrosinase leads to production of phaeomelanin (red), whereas high levels of tyrosinase produces eumelanin (brown or black) (Searle et al., 1968; Klungland et al., 1995). Genetic variations in this phenotype have been studied in a wide variety of species including cattle (Klungland et al., 1995), Chicken (Takeuchi et al., 1996), Horse (Marklund et al., 1996), Pigs (Kijas et al., 1998), Goat (Wu et al., 2006) and Sheep (Deng et al., 2009) but very scarce literature is available on molecular studies of buffalo coat colour inheritance.

MATERIALS AND METHODS

Coat colour pattern
The experimental animals which comprised of unrelated 34 Purnathadi buffaloes (whitish brown coat colour) and 35 Nagpuri buffaloes (Black colour).

Collection of blood samples
Blood samples of biologically unrelated Nagpuri buffaloes (n=35) and Purnathadi buffaloes (n=34) were collected from the Cattle Breeding Farm, Nagpur Veterinary College, Nagpur and the farmers’ herds from Nagpur and Wardha district of Maharashtra for Nagpuri buffaloes and Purnathadi Buffalo Unit of Post Graduate Institute of Veterinary and Animal Sciences, Akola and farmers’ herds in neighbouring talukas of Akola district of Maharashtra for Purnathadi buffaloes. Whole blood samples were aseptically collected in EDTA-vacutainers, maintaining the cold chain during transportation from the field to the laboratory. The collected blood samples were stored at -20°C in the Molecular Genetics Laboratory, Department of Animal Genetics and Breeding, PGIVAS, Akola till further processing.

DNA extraction
Genomic DNA was extracted from the respective blood samples using ‘FavorGen Prep™ Blood Genomic DNA Extraction Mini Kit.’ The purity of extracted DNA with respect to quality and quantity was ensured by gel electrophoresis and Nanophotometer respectively. DNA samples with good quality having OD (260/280) ratio within the range of 1.7 to 2.0 and DNA concentration above 30ng/µl were selected for PCR amplification.

PCR amplification
PCR amplification of a region flanking 138 bp of MC1R gene was achieved using forward 5’ CAAGAACCGCAACCTGCACT 3’ and reverse 5’ GCCTGGGTGGCCAGGACA 3’ primers reported by Klungland et al. (1995); Russo et al. (2007) for cattle. The primer sequences were confirmed from NCBI after successfully blast on the complete coding sequence (954 bp) of Bubaline MC1R gene (Accession number: KM011853, Goud et al., 2014) using the NCBI-Primer BLAST software. The amplification protocol reported by Klungland et al. (1995), with an initial denaturation at 94°C for 3 minutes followed by 35 subsequent cycles of denauration 94°C for 15 seconds, primer annealing at 63°C for 35 seconds and extension at 72°C for 35 seconds and an additional step for final extension at 72°C for 10 minutes was followed. Successful amplification of the PCR products was confirmed
by running the PCR amplicons on a 2% agarose gel against a standard 50 bp (SRL make ‘ProxiO’) DNA ladder.

**PCR-RFLP of the 138 bp fragment of MC1R gene**

Restriction endonuclease *MspI (Morexella species)* (recognition site – CCGG) earlier reported by Russo *et al.* (2007) was used for RFLP of the amplified PCR amplicons. The obtained RE digested products were electrophoresed at standard gel electrophoresis conditions on a 4 % agarose gel against a 50 bp DNA ladder. RFLP product analysis using UV-Transilluminator revealed fragments of 138 bp, 118 bp and 20 bp; based on which, the samples were genotyped as E^ED^, E^E^* and E^E*.

**Single nucleotide polymorphism (SNP) screening**

Representative samples of each genotype were outsourced to ‘GenOmbio Technologies Pvt. Ltd.’ for DNA sequencing. Chromatogram of the raw sequences of the studied MC1R (138 bp) gene were visualized using software (Chromas, version 2.6.6, Technelysium Pty., Ltd) and FASTA files were generated. The sequences were edited and aligned thereafter by Mega-X software (Kumar *et al.*, 2018). The sequenced samples were aligned with a reference sequence of NCBI (accession number: KM011853) using ClustalW multiple sequence alignment tool (Larkin *et al.*, 2007) to screen and analyse the SNPs.

**RESULTS AND DISCUSSIONS**

**RFLP analysis of the Bubaline MC1R gene**

The PCR-RFLP of all sixty-nine samples by *MspI* restriction endonuclease, resulted in fragmentation of the 138 bp PCR product into the uncut 138 bp (Figure 1), assigned as homozygous dominant E^ED^ genotype; the 138 bp and 118 and 20 bp bands of heterozygous E^E^* genotype and bands of 118 bp and 20 bp were assigned E^E* genotype. Similar findings of 138, 118 and 20 bp on *MspI* digestion by PCR-RFLP analysis were also reported by Russo *et al.* (2007) in Italian cattle breeds. In the present study, genotype frequencies of E^ED^, E^E^* and E^E* were 0.86, 0.03 and 0.11 for Nagpuri and 0.74, 0.08 and 0.18 in Purnathadi buffaloes, respectively. Similarly, the observed gene frequencies for E^D^ allele were 0.87 and 0.78, and for E^*^ allele were 0.13 and 0.22 in Nagpuri and Purnathadi buffaloes, respectively. Despite the differences in coat colour, the Nagpuri and Purnathadi buffaloes showed similar polymorphic pattern at the studied MC1R locus (138 bp). Many researchers, (Klungland *et al.*, 1995; Sasazaki *et al.*, 2005; Russo *et al.*, 2007; Guastella *et al.*, 2011) conducted PCR-RFLP studies to explore the MC1R gene in Cattle, Sheep, Goat and other domestic animals but very scarce literature is available on *Bubaline* MC1R gene. Perhaps, the only published research of MC1R gene in buffaloes is by Miao *et al.* (2010) and reported monomorphic pattern of MC1R gene in Nili-Ravi and Murrah buffaloes, which is contrary to the present findings.

**Analysis of sequenced samples**

Sequencing of the studied 138 bp MC1R gene revealed a SNP at position 118 of the PCR amplicons. A transition mutation A→G was observed in three of the eight
Figure 1. PCR-RFLP fragments of MC1R gene digested by MspI showing bands of 138 bp and 118 bp. M: Molecular maker (Standard 50 bp DNA ladder). N1 to N7: RFLP products of Nagpuri buffalo MC1R gene.

Figure 2. Chromotogram showing mutation A→G at position 118 bp (a) ‘A’ nucleotide (wild type) (b) ‘G’ SNP (transition mutation).
The polymorphism thus confirmed; was due to A→G (CCAG to CC↓GG) transition at base position 118, which altered the MspI restriction site as depicted in the chromatogram in Figures 2. Thus, the genotypes observed by the PCR-RFLP method were confirmed as EDED (AA) 138bp, EDE'(AG) 138, 118, 20 bp and E'E' (GG) as 118 and 20 bp.

It can thus be concluded that A118G transition of 138 bp allele of MC1R gene is insufficient for explaining the existing coat colour variations observed in Nagpuri and Purnathadi buffaloes. However, gene expression analysis of complete coding sequence of the MC1R gene (954 bp) and screening of other relevant loci responsible for epistatic interactions causing coat colour variation may validate the underlying molecular mechanism of coat colour variation in buffaloes.

REFERENCES


