Microphthalmia-associated transcription factor mutations are associated with white-spotted coat color in swamp buffalo

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Summary

A candidate gene analysis of the *microphthalmia-associated transcription factor* (*MITF*) gene was used in an attempt to identify the genetic basis for a white-spotted coat color phenotype in the Asian swamp buffalo (*Bubalus bubalis carabanensis*). Ninety-three buffaloes—32 solid, 38 spotted and 23 white individuals—were Sanger-sequenced for all *MITF* exons as well as highly conserved intronic and flanking regions. *MITF* cDNA representing skin and iris tissue from six spotted, nine solid and one white buffaloes was also Sanger-sequenced to confirm detected mutations. Two independent loss-of-function mutations, a premature stop codon (c.328C>T, p.Arg110*) and a donor splice-site mutation (c.840+2T>A, p.Glu281_Leu282Ins8), both of which cause white-spotted coat color in swamp buffaloes, were identified. The nonsense mutation leads to a premature stop codon in exon 3, and likely removal of the resulting mRNA via nonsense-mediated decay pathway, whereas the donor splice-site mutation leads to aberrant splicing of exon 8 that encodes part of a highly conserved region of *MITF*. The resulting insertion of eight amino acid residues is expected to perturb the leucine zipper part in the basic helix-loop-helix leucine zipper (bHLH-Zip) domain and will most likely influence dimerization and DNA binding capacity. Electrophoretic mobility shift assay was performed using mutant and wild-type MITF proteins and showed that the mutant MITF protein resulting from the splice-site mutation decreased *in vitro* DNA binding capacity compared to wild-type MITF. White-spotted buffalo bulls are sacrificed in funeral ceremonies in Tana Toraja, Indonesia, because they are considered holy, and our results show that genetic variation causes a tie to the cultural use of these buffaloes.

Keywords *Bubalus bubalis*, donor splice-site mutation, MITF, nonsense mutation

Introduction

The domestic Asian water buffalo (*Bubalus bubalis*) is divided into two subspecies: river buffalo (*Bubalus bubalis bubalis*) and swamp buffalo or the Carabao (*Bubalus bubalis carabanensis*) (Castillo 2004). The swamp buffalo is an econom-ically important domestic animal used for meat production and as a draft animal in Indonesia as well as in other parts of South-East Asia.

The swamp buffalo exhibits several coat color phenotypes and have either solid, white or spotted coats. The solid phenotype is characterized by dark gray coat color, whereas the white phenotype appears like a dilution phenotype rather than an extreme form of white spotting. White-spotted buffalo are found exclusively in a subpopulation that exists in the Tana Toraja region, South Sulawesi, Indonesia. Buffaloes with the spotted phenotype are classi-fied according to Toraja cultural traditions into four different subclasses based on the spotted phenotype and iris color (Fig. 1).

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There is no clear information about when and why people started to keep white-spotted buffaloes, but reference is made to a cultural tradition called *Aluk To Dolo* (a rule of life that was brought to Tana Toraja by immigrants from Indochina in the years 7000 to 5000 before present). This tradition plays a key role in the funeral ceremonies, called '*Rambu Solo'*, and during these funeral ceremonies, male white-spotted buffaloes are sacrificed, according to Torajan tradition, thereby providing a 'ride to heaven' for the deceased. This tradition has continued even after 1920 when many Toraja people converted to Christianity.

The spotted phenotype implicates a defect in the migration and survival of melanocytes given that pigmentation is essentially normal in the pigmented areas. The spotted swamp buffaloes show altered pigmentation in both coat and iris (eye) color. This suggested a number of well-defined coat color genes, that is, *MITF, SOX10, EDN3, EDNRB* and *PAX3*, affecting both coat and eye pigmentation as candidate genes (Hughes *et al.* 1994; Baldwin *et al.* 1995; Edery *et al.* 1996; Read & Newton 1997; Smith *et al.* 2000; Bondurand *et al.* 2007; Pingault *et al.* 2010). We hypothesized that, among these genes, the prime candidate gene was the gene encoding the microphthalmia-associated transcription factor (*MITF*) that has been shown to be associated with coat color, eye color and hearing disorder phenotypes, known as Waardenburg syndrome and Tietz syndrome in human (Tassabehji *et al.* 1994; Smith *et al.* 2000; reviewed in Pingault *et al.* 2010) and in several other mammals, for example, mice (Steingrimsson *et al.* 2003,

2004), dogs (Karlsson *et al.* 2007; Baranowska Körberg *et al.* 2014), horse (Hauswirth *et al.* 2012) and cattle (Philipp *et al.* 2011).

Materials and methods

DNA and RNA samples

Blood samples were collected from a total of 93 buffaloes, 32 solid, 38 spotted (four Lotong Boko, eight Toddi', nine Saleko, 17 Bonga) and 23 white individuals. Phenotypic data also were collected at the same time. Genomic DNA from blood samples was prepared according to standard protocols.

Skin and iris tissue samples were collected from six spotted, nine solid and one white buffaloes. Samples were stored in RNAlater (Qiagen) at 4 °C for 24 h and then kept at −80 °C until RNA extraction was performed. Tissue samples were homogenized in β-mercaptoethanol-buffer RLT (containing guanidine thiocyanate) solution. Total RNA was extracted using a standard extraction protocol (RNeasy Mini Kit; Qiagen). Total RNA was stored in RNase-free water. The quality of total RNA was evaluated using the Agilent RNA 6000 Nano Kit (Agilent Technologies).

PCR amplification and DNA sequencing

DNA quality was measured using a Nanodrop™ 1000 spectrophotometer. A total of nine exons, highly conserved
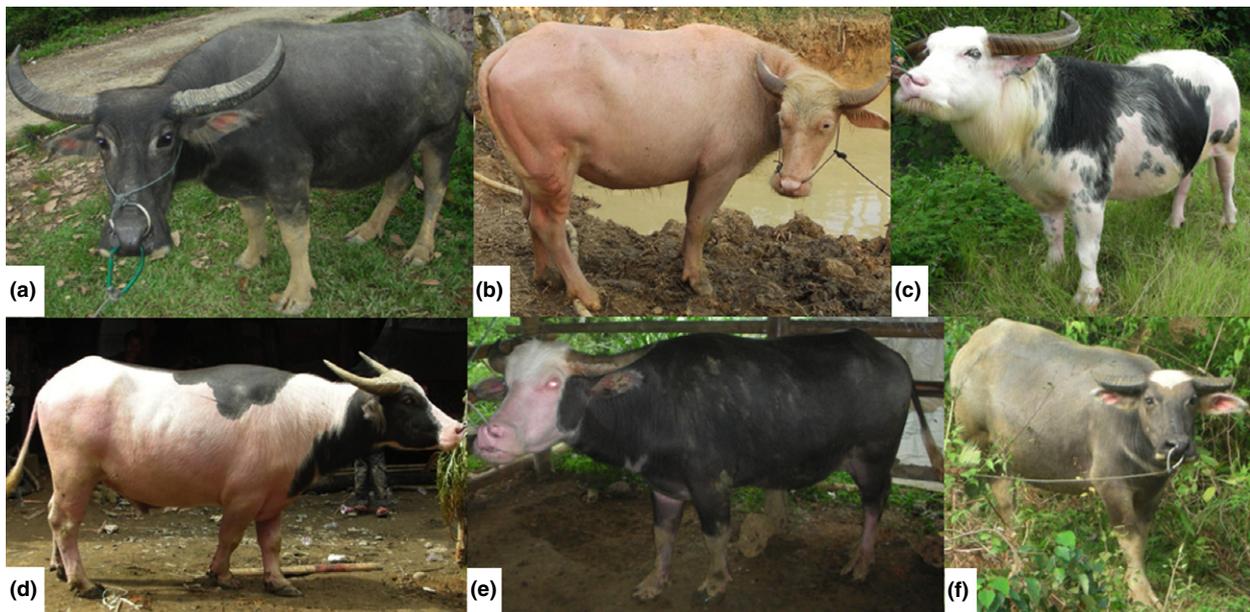

Figure 1 Variations of coat and eye color phenotype in swamp buffalo. (a) Solid coat color with black iris. (b) White coat color with pigmented iris. (c) Spotted coat color, *Saleko* type, with white iris color; individual shown was identified as a carrier of the nonsense mutation. (d) Spotted coat color, *Lotong Boko* type, with black iris; individual classified not to carry either of the two mutations. (e) Spotted coat color, *Bonga* type, with white iris; individual identified to be a carrier of the donor splice-site mutation. (f) Spotted coat color, *Toddi'* type, with black iris; individual identified as carrier of the donor splice-site mutation; the type of spotted coat color was classified according to Toraja culture classification system.

intronic regions and flanking regions based on sequence conservation as defined by comparison with 29 mammals (Lindblad-Toh *et al.* 2011) were amplified using PCR primer pairs (Table S1) designed based on the *Bos taurus* genome reference, NM_001001150.2 (UCSC Genome Browser assembly ID: October 2011, Baylor Btau_4.6.1/bosTau7). PCR amplifications were prepared in 25-µl reactions, which included 1× PCR buffer II, 2.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.06 U of AmpliTaq® Gold (Applied Biosystems), 0.5 µM of forward and reverse primers and 10 ng of genomic DNA. The PCR profile included an initial denaturation step at 94 °C for 10 min, followed by 10 touchdown cycles (denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, followed by extension at 72 °C for 30 s), 30 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s) and an additional extension step of 7 min at 72 °C. PCR products were purified with ExoCiAP mix containing alkaline phosphatase, Exo1 buffer and Exo1 at 37 °C for 1 h and 85 °C for 15 min. The purified PCR products were sequenced using Sanger sequencing. Nucleotide sequences were aligned using CODON CODE ALIGNER (Codon Code Corporation), which was also used for SNP calling. Sequencing data for both genomic DNA and cDNA (see below) were deposited in the European Nucleotide Archive (ENA) (Study accession: HG917419–917433), available at www.ebi.ac.uk/ena and Table S2.

cDNA sequencing

Reverse-transcription PCR was performed to produce cDNA from total RNA samples. The RT-PCR protocol included 5 mM of $MgCl_2$, 1 mM of dNTP, 1× PCR Buffer II, 0.8 U of RNase inhibitor, 1 µM of RNA oligo (dT), 5 U of MiLV-RT enzyme, RNase-free water and 2 µg of RNA samples. The PCR profile included initiation at 25 °C for 10 min, amplification at 45 °C for 80 min and extension at 15 °C for 5 min. cDNA sequences were generated using Sanger sequencing, and CODON CODE ALIGNER was used for sequence analysis. Primer pairs were designed based on the cattle (*Bos taurus*) genome assembly NM_001001150.2 (UCSC Genome Browser assembly ID: October 2011, Baylor Btau_4.6.1/bosTau7).

Transcription/translation system for PCR-generated DNA

PCR-generated DNA was translated into protein using the TnT® T7 Quick Kit (Promega). The TnT® quick master mix was thawed and other components were thawed at room temperature and thereafter stored on ice. The final volume of each reaction was 50 µl and included 40 µl of TnT® quick master mix, 1 µl of 1 mM methionine, 0.5 µg of PCR-generated DNA template, 1 µl of T7 TnT® PCR enhancer and nuclease-free water. The reaction was incubated at 30 °C for 90 min and stored at −80 °C.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed using a LightShift™ Chemiluminescent EMSA Kit (Thermo Scientific). Probe and competitor DNAs used in EMSA included wild-type *MITF* (5′-CCAACATGTGCACTC CAC-3′) and mutant *MITF* (5′-CCAAACTGGTCACTCCAC-3′). The wild-type MITF probe was labeled with biotin using a Biotin 3′ End DNA Labeling Kit (Thermo Scientific). EMSAs were performed by incubating wild-type and mutant MITF proteins in binding buffer (10 mM of Tris-HCl, pH 7.5, 50 mM of NaCl, 1 mM of dithiothreitol, 50 ng/µl of poly (dIdC), 1 mM of EDTA, 5% glycerol, 5 mM of $MgCl_2$, 50 mM of KCl and 4 pmol of unlabeled *MITF* DNA) on ice for 20 min. Thereafter, 20 fmol Biotin End-Labeled MITF DNA was added to each reaction with a total volume of 20 µl, and the reactions were incubated at room temperature for 20 min. Then, 5 µl of 5× loading buffer was added to each 20-µl binding reaction. MITF proteins were separated from DNA by electrophoresis through 5% Criterion TBE gel from Bio-Rad at 120 V for 70 min and then transferred to a nylon membrane at 100 V for 60 min. Transferred DNA was cross-linked for 15 min with the membrane face down on a trans-illuminator equipped with 312-nm bulbs. The membrane was exposed using a CCD camera.

Statistical analysis

Statistical analyses were performed using the statistical program VASSARSTATS (http://faculty.vassar.edu/lowry/VassarStats.html). A 2 × 2 table of allele frequencies was used for Fisher's exact test.

Results

Nucleotide sequencing of the *MITF* gene was performed using PCR products obtained from genomic DNA prepared from wild-type (solid), white and white-spotted buffaloes. Sequencing data were deposited in European Nucleotide Archive (ENA) (Study accession: HG917419–917433), available at www.ebi.ac.uk/ena and Table S2. We identified a total of 14 SNPs and calculated their allele frequencies in solid and white-spotted buffaloes. The genetic association between genotype and phenotype was estimated, and two of these SNPs independently showed a strong association with the white-spotting phenotype (Table S2). One of the SNPs constitutes a nonsense mutation (c.328C>T) causing a premature translational stop (p.Arg110*) at position chr22: 32 322 242 (NM_001001150.2) in *MITF* exon 3 (Fig. 2), whereas the other mutation (c.840+2T>A) introduces a donor splice-site mutation (p.Glu281_Leu282Ins8) at
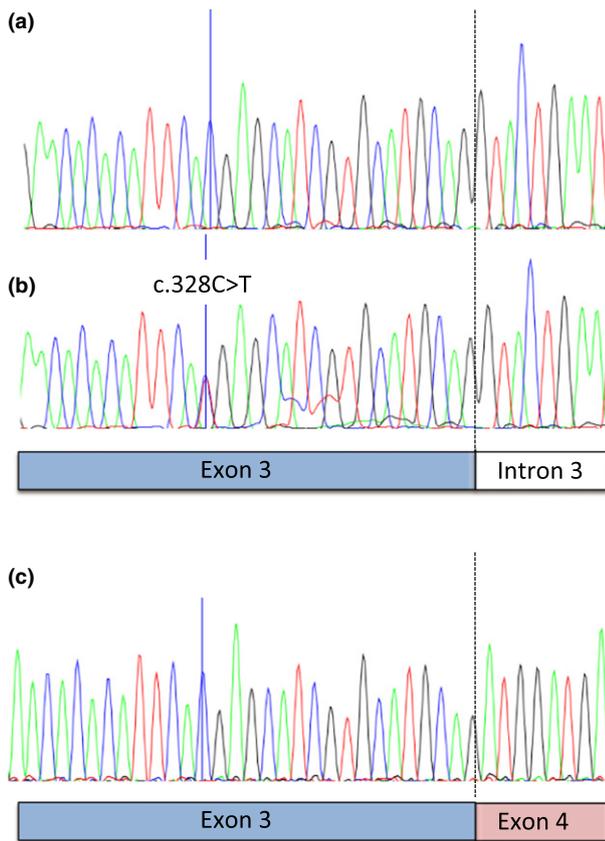
(a)

(b)

c.328C>T

| Exon 3 | Intron 3 |

(c)

| Exon 3 | Exon 4 |

Figure 2 Sanger sequencing of gDNA and cDNA in solid and spotted buffaloes revealed a heterozygous nonsense mutation in exon 3 in *MITF*. (a) Wild-type gDNA sequence in solid buffaloes. (b) A nonsense mutation (NM_001001150.2) causing a premature translational stop c.328C>T, p.Arg110* at genomic position chr22: 32 322 242 was identified in *MITF* exon 3 in spotted buffaloes. Exon–intron borders in exon 3 are shown. (c) Only wild-type sequence could be detected at cDNA level in spotted buffaloes carrying the c.328C>T mutation, suggesting nonsense-mediated decay where only the wild-type mRNA exists.

position chr22: 32 297 683 (NM_001001150.2) in intron 8 (Fig. 3).

Twenty-three of 38 spotted individuals (60.5%) were heterozygous for the nonsense variant, whereas all solid and all white individuals were homozygous for the wild-type allele. The nonsense variant showed a highly significant association with white spotting ($P = 1.3 \times 10^{-7}$; Table 1). To confirm that *MITF* mRNAs were expressed from the mutant alleles detected in genomic DNA, *MITF* cDNAs derived from mRNA that was isolated from skin and irises of six different spotted individuals were analyzed (Table 1). RT-PCR analysis was performed on cDNA made from these mRNA preparations. In mRNA prepared from skin obtained from Saleko type spotted buffaloes carrying the nonsense variant (Fig. 1c), mRNA molecules containing the nonsense variant were undetectable, suggesting that this mutant transcript is degraded by the nonsense-mediated decay pathway (Maquat 2004).

Seven of 38 spotted individuals (18.4%) and one of the 32 solid buffaloes (3.1%) were heterozygous for the splice-site variant ($P = 0.05$, Table 1). The presence of mRNA expressed in spotted individuals carrying the splice-site variant allele was confirmed by RT-PCR analysis performed on mRNA prepared from skin obtained from Bonga type individuals (Fig. 1e). The capacity of the mutant MITF protein produced by aberrant splicing to bind *in vitro* to the well-established MITF target site (5′-CACGTG-3′) was evaluated by EMSAs using *in vitro* translated mutant and wild-type MITF protein. The mutant protein showed a lower efficiency in binding to the MITF binding site *in vitro* (Fig. 4). Schematic views of the wild-type and mutant MITF proteins are shown in Fig. S1.

Discussion

Here we have identified two *MITF* mutations associated with the white-spotted coat color phenotype in swamp buffaloes. As expected for loss-of-function mutations in an essential gene, either one of the two mutations was found only in the heterozygous form. *MITF* mRNA carrying the nonsense mutation was undetectable in our mRNA preparations from iris and skin, most likely because such mRNAs are degraded by the nonsense-mediated mRNA decay pathway. The mRNA containing the splice-site variant was detected at similar levels as the wild-type *MITF* mRNA. There was considerable phenotypic variability in spotted coat color among the 23 animals that were heterozygous for the nonsense variant. Some individuals showed massive spotted color, whereas others had only a very small white spot on the forehead. This phenotypic variation is likely caused by genetic variation at loci interacting with *MITF*.

There are many examples of *MITF* mutations associated with hypopigmentation and white-spotted phenotypes in different mammalian species. In German Fleckvieh cattle, a dominant and recessive lethal p. Arg210Ile missense mutation has been identified as responsible for hypopigmentation, heterochromia iridis, colobomatous eyes and bilateral hearing loss (Philipp *et al.* 2011). Three variant mutations, a proximal melanocyte-specific *M* promoter replacing a thymine with 11 nucleotides, a small deletion in exon 5 and a *de novo* missense mutation in exon 6, were detected causing splashed white and macchiato in horses (Hauswirth *et al.* 2012). In white-spotted dogs, genome-wide association studies identified a region overlapping the melanocyte-specific *MITF*-M promoter to be associated with white coat color (Karlsson *et al.* 2007). Subsequent functional studies revealed that regulatory mutations located in the *MITF*-M promoter influenced transcription where a simple repeat polymorphism was shown to be a key factor underlying white spotting (Baranowska Körberg *et al.* 2014).

The absence of *MITF* mutations in the white buffalo confirms our hypothesis that these do not represent an extreme form of white spotting. Importantly, among the
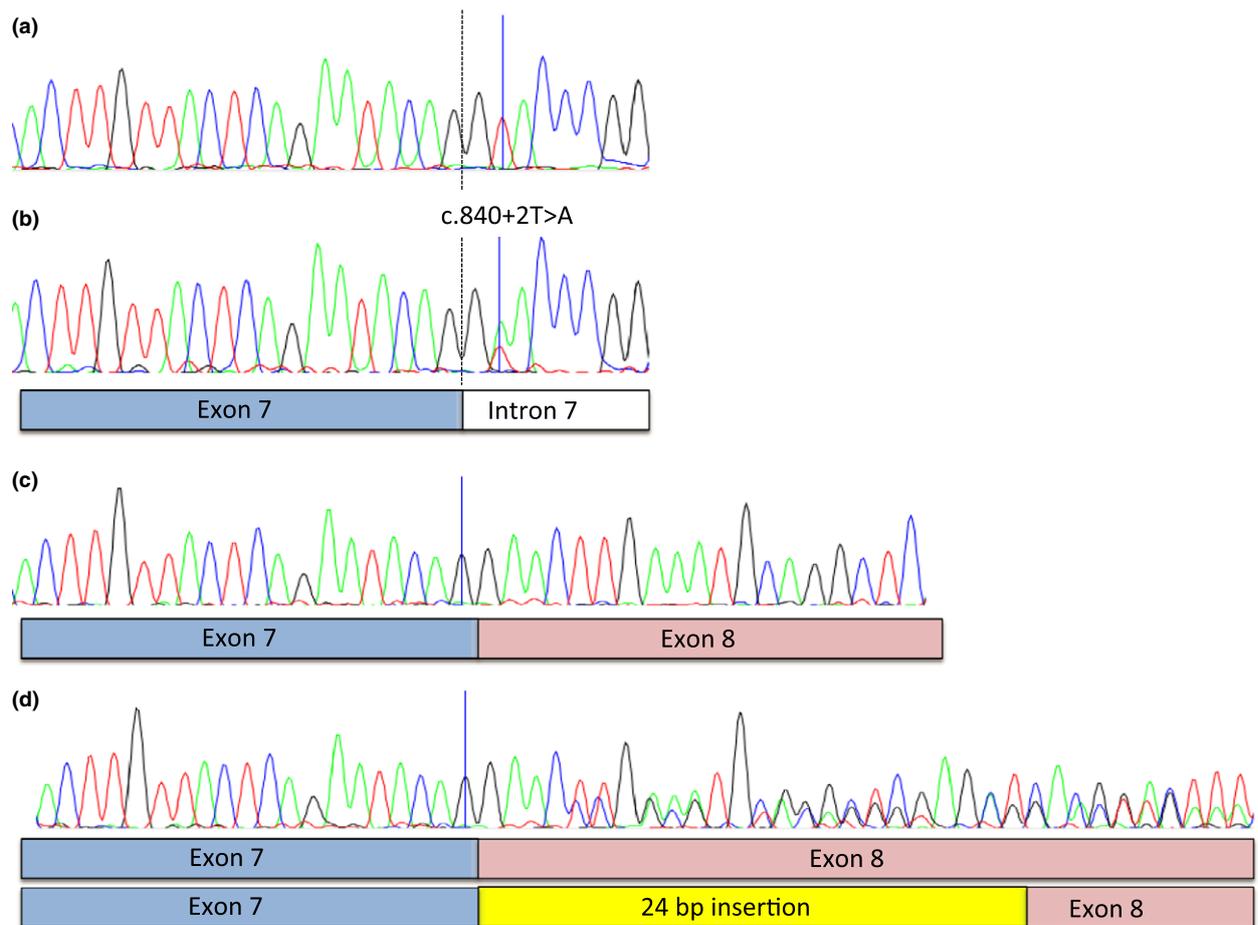
(a)

(b)

c.840+2T>A

Exon 7	Intron 7

(c)

Exon 7	Exon 8

(d)

Exon 7	Exon 8	
Exon 7	24 bp insertion	Exon 8

Figure 3 Sanger sequencing of gDNA and cDNA in solid and spotted buffaloes revealed a heterozygous donor splice-site mutation in intron 7 in *MITF* resulting in 24-bp insertion (a) Wild-type gDNA sequence in solid buffaloes. (b) A donor splice-site mutation (NM_001001150.2) c.840+2T>A, p.Glu281_Leu282Ins8 at position chr22: 32 297 683 in intron 7 was identified in spotted buffaloes. Exon–intron borders in exon 7 are shown. (c) Wild-type cDNA sequence including exon borders of exons 7 and 8. (d) The heterozygous splice-site mutation resulted in one wild-type cDNA allele and one cDNA allele containing an insertion of 24 extra bp (eight amino acids).

Table 1 Genotype frequencies of the *MITF* nonsense variant c.328C>T, p.Arg110* (chr22: 32 322 242[1]) and the splice-site variant c.840+2T>A, p.Glu281_Leu282Ins8 (chr22: 32 297 683[1]) in swamp buffaloes with different coat color phenotypes.

Phenotype	Nonsense variant				Splice-site variant			
	CC	CT	TT	P^2	TT	AT	AA	P^2
Solid	32	0	0		31	1	0	
Spotted	15	23	0	1.3×10^{-7}	31	7	0	0.05
White	23	0	0		23	0	0	

[1]Nucleotide positions are given according to the cattle reference genome assembly NM_001001150.2 (UCSC Genome Browser assembly ID: October 2011, Baylor Btau_4.6.1/bosTau7).
[2]Fisher's exact test (one-tailed) comparing the allele frequencies in solid and spotted animals.

spotted individuals, none of them were homozygous for the mutations. According to Mendelian segregation, 25% of calves produced from matings between two spotted buffaloes will be either homozygous recessive or composite heterozygotes that may lead to embryonic lethality, at least for animals that are homozygous for the nonsense mutation.

The mutations detected using genomic DNA was confirmed by sequencing cDNA derived from mRNA that was isolated from skin and iris of six different spotted individuals. The splice-site mutation leads to aberrant splicing of exon 8, and the resulting protein translated from such aberrantly spliced mRNA is expected to disturb the leucine zipper part in the basic helix-loop-helix zipper (bHLH-Zip) domain of MITF and will most likely influence dimerization and DNA binding capacity. MITF belongs to the bHLH-Zip transcription factor family and the bHLH-Zip domain is highly conserved among both vertebrates and invertebrates (Steingrimsson *et al.* 2004). There was a clear trend that individuals that carry the splice-site variant show a less pronounced white-spotting phenotype compared with most of the animals carrying the nonsense variant, suggesting that this is a partial loss-of-function allele,

Figure 4 Binding affinity of mutant vs. wild-type MITF protein on electrophoretic mobility shift assay. (a) DNA only, (b) 2 μl of wild-type protein, (c) 4 μl of wild-type protein, (d) 6 μl of wild-type protein, (e) 2 μl of mutant protein, (f) 4 μl of mutant protein, (g) 6 μl of mutant protein, (h) 2 μl of wild-type protein + 2 μl mutant protein, (i) 3 μl of wild-type protein + 3 μl mutant protein, (j) 4 μl of wild-type protein + 4 μl of mutant protein, (k) 4 μl of wild-type protein + 400-fold molar excess of unlabeled wild-type DNA.
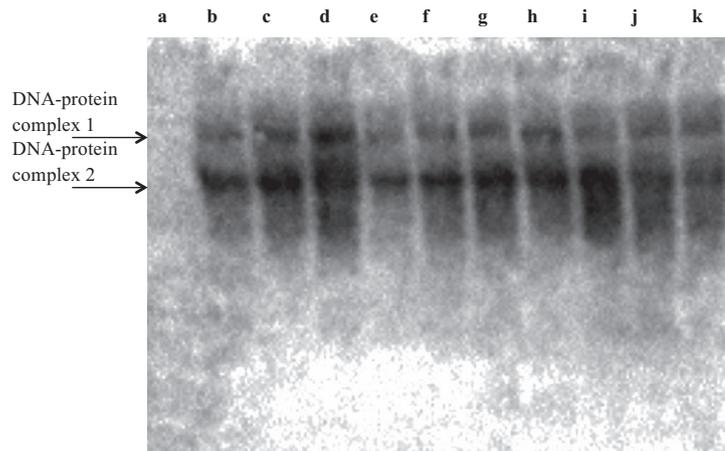
whereas the nonsense mutation results in a null allele. The observation of a solid colored buffalo carrying the splice-site variant suggests either that this is due to a phenotype misclassification or that this mutation is not fully penetrant.

To assess the *in vitro* DNA–protein interaction effect of the mutant MITF protein, we performed EMSAs using MITF-specific consensus oligonucleotides and *in vitro* translated mutant and wild-type MITF protein. Two specific complexes were detected, most likely representing dimers and multimers. The mutant protein was shown to bind with lower efficiency compared to wild-type protein (Fig. 4, lanes b vs. e; c vs. g). Thus, the mutant MITF protein maintained its *in vitro* binding profile but with decreased binding efficiency. Increasing amounts of *in vitro* translated mutant MITF protein was added to *in vitro* translated wild-type MITF to evaluate whether mutant MITF could interfere with the binding activity of wild-type MITF, but we could not ascertain that mutant MITF had any negative effect on wild-type MITF target interaction. Most likely, the insertion of the eight amino acid residues negatively influences dimerization capacity because the insertion is located immediately before the third leucine in the leucine zipper domain (Fig. S1). Dimerization of MITF is required for its ability to bind DNA (Steingrimsson *et al.* 2004). In mice, the DNA binding domain of Mitf (Mitf^mi, Mitf^mi-wh, Mitf^mi-b, Mitf^mi-H, Mitf^mi-enu5, Mitf^mi-bc2 and Mitf^mi-ew) was affected by semi-dominant mutations (Steingrimsson *et al.* 2004).

Moreover, eight of 38 spotted individuals did not carry any of the two *MITF* mutations that were identified in our study (Table 1) and none were composite heterozygous for the two mutations as expected for loss-of-function mutations. Thus, there must be at least one additional mutation affecting white spotting in this population of swamp buffaloes.

In conclusion, we have identified two independent *MITF* mutations responsible for white-spotting phenotypes in swamp buffaloes. The fact that at least two mutations are causing white spotting in swamp buffaloes is consistent with the huge cultural importance these animals have had.

Strong positive selection over a considerable time period has led to the accumulation of multiple mutations causing a white-spotting phenotype. This is an example of the cultural importance of white color in domesticated animals. Another famous example is white horses caused by the Greying-with-age in *Syntaxin 17*, which have had a huge impact on human culture worldwide (Rosengren Pielberg *et al.* 2008).

Ethics statement

The Swedish Board of Agriculture has given the permission to import (Dnr 6.2.18-2166/13) and use biological samples from *Bubalus bubalis* and to use the animal byproducts for research purposes (Dnr 38-10019/12; Dnr 38-9492/12). All animal work has been conducted according to the national and international guidelines for animal welfare. Veterinary division in North Toraja, South Sulawesi, Indonesia, has approved this study (Permit number 553/10/P3/01-10).

Acknowledgements

Competing interests

A patent application has been filed and is pending.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Schematic representation of *MITF* exons. The estimated positions of the encoded helix-loop-helix DNA binding domain (HLH DBD) and the leucine zipper part in the basic helix-loop-helix zipper (bHLH-Zip) domain in the wild-type individual. The splice-site mutation is expected to disturb the leucine zipper part in the bHLH-Zip domain because of the eight additional amino acid residues in exon 8.

Table S1 Primers used to amplify gDNA.

Table S2 Identified SNPs in swamp buffalo *MITF* gene (chr22:32 291 319–32 324 807).