Simultaneous introgression of three POLLED mutations into a synthetic breed of Chinese cattle

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Abstract

The polled phenotype of cattle is increasingly becoming favourable mainly because of the enhanced emphasis on animal welfare, for which the causative mutations have been reported during the past years. The Shuxuan cattle are a new synthetic breed by crossing the indigenous cattle with both Simmental and Holstein semen in Sichuan of Southwest China, in which about 15% of polled individuals have newly emerged. Because official record about POLLED genotypes for the historically imported sires is unavailable, we therefore genotyped the proposed POLLED variants of P202ID, P80kbID and P219ID among 48 polled and 16 horned Shuxuan cattle. It was first revealed that all three candidate mutations have been simultaneously introgressed into Shuxuan cattle, whereas the P202ID mutation is dominant. Furthermore, one polled animal still remains to carry none of the three candidate mutations, which suggests that further mutation(s) would also exist. Additionally, we sequenced mitochondrial DNA and found that Shuxuan cattle are composed of two maternal origins of Bos taurus (65.6%) and B. indicus (34.4%); and there is no origin-biased distribution of polled phenotype. In conclusion, our study first supports the recently reported novel candidate mutation of P219ID and detects simultaneous presences of all three known POLLED mutations within a cattle breed.

Introduction

Horns are a distinctive characteristic of animals in Bovidae family and exhibit very high morphological diversity [1]. As a powerful weapon in fight against predators and amongst each other, the individuals that bear strong horns would have evolutionary advantage in the wild [2]. However, breeding hornless cattle is increasingly becoming a favourable alternative in modern husbandry systems due to the improved convenience in herd management practice. Although the genetically hornless individuals (referred to as polled phenotype) have existed
for a long time, it still remains rather common to produce hornless cattle by physical dehorning at young age [3]. Because animal welfare in relation to the physical dehorning is an increasing issue of public concern, it is anticipated that hornless cattle will be preferably produced by genetic selection of polled individuals [4].

The polled phenotype of cattle is inherited as an autosomal dominant trait, for which the POLLED locus was genetically mapped to Chromosome 1 [5–8]. During the past years, many efforts have been devoted to explore causative mutation(s) for polled phenotype in cattle [9–14] as well as the related bovine species of yak [15, 16]. Two heterogeneous mutations were first observed to perfectly segregate with polled phenotype, including the complex duplication-insertion of 202 bp fragment (P\textsubscript{202ID}) in beef or dual-purpose breeds and 80 kb fragment (P\textsubscript{80kbID}) in Holstein [10, 17]. More recently, a novel duplication-insertion event of a 219 bp fragment (P\textsubscript{219ID}) was also revealed to be responsible for polled phenotype in Mongolian Turano cattle [18]. Therefore, a total of three causative mutations have been confidently proposed to be associated with polled phenotype in cattle so far.

Although none of the three POLLED mutations is physically located into any known coding region or contains functional element, it is empirically verified recently by the genome editing technology that P\textsubscript{202ID} is a causative mutation for ontogenesis of polled phenotype in Holstein [19]. Within the mapped POLLED locus, it was first reported that there is no differentially expressed gene between the polled and horned phenotypes of cattle as being revealed by the cDNA microarray technology [20]. Subsequently, a long intergenic non-coding RNA was revealed to be ectopic expression in horn buds of polled cattle with the aid of high-throughput mRNA sequencing technology, which would be involved into the molecular regulation of horn agenesis [12]. However, direct link between these causative mutations and molecular genetic mechanism underlying polledness ontogenesis in cattle still remains to be investigated.

The Shuxuan cattle are a synthetic dual-purpose breed and mainly distributed in Sichuan Province of Southwest China by crossing the indigenous breed of Xuanhan cattle with both Simmental and Holstein semen during the past 30 years. Finally, it is estimated that the cultivated Shuxuan cattle consist of about 75% Simmental and 10% Holstein blood. In contrast to the indigenous Xuanhan cattle that are a completely horned breed, we currently observed that about 15% of Shuxuan cattle are the polled phenotype according to our field investigation. Unfortunately, no official record is available for describing the horn trait or POLLED genotype for these historically introduced sires of Simmental and Holstein. Because of allelic heterogeneity of polled phenotype as stated above, we directly genotype the proposed candidate variants for polled Shuxuan cattle in the present study and intend to reveal that which mutation(s) had been introgressed into this newly cultivated breed. The results are essential to establish the marker-assisted selection program of hornless Shuxuan cattle.

**Materials and methods**

**Ethics statement**

No ethical approval was required in the present study because all blood samples were collected by local veterinarians for annual health inspection.

**Animals and DNA extraction**

A total of 64 Shuxuan cattle were collected in the present study, including 48 polled and 16 horned individuals (S1 Table). The polled phenotype for each cattle was accurately approved via both visual and touch inspections to avoid the scurs phenotype. The possible practice of artificial dehorning was also excluded by carefully consulting cattle breeder. However, genetic
relatedness remains unknown among all animals. The genomic DNA was extracted using Axy-Prep Genomic DNA Miniprep Kit (Axygen Bioscience, USA).

Genotyping of candidate mutations for polled phenotype

Variant $P_{202ID}$ was first genotyped using the published PCR primers and method [10], which distinguishes different genotypes through agarose gel electrophoresis of PCR products. To avoid potential false-negative PCR outcome, a primer pair was newly designed in the present study to independently amplify an overlapping fragment encompassing variant $P_{202ID}$; and for which PCR products were similarly subjected to the agarose gel electrophoresis-based genotyping. Primer sequences and genotyping methods involved in the present study are detailed in S2 Table.

Subsequently, we genotyped variant $P_{80kbID}$ according to the initially reported method by Medugorac and colleagues [10], in which the surrogate variant $P_{T1909396D2}$ (a two-base pair deletion) for $P_{80kbID}$ was used and genotyped by PCR amplification and capillary electrophoresis. Because three genotypes of variant $P_{80kbID}$ must be discriminated according to both qualitative and quantitative comparisons on the obtained signal, it would be apt to lead to false conclusion of genotyping. Therefore we further subjected this PCR amplified fragment to Sanger sequencing in parallel, by which animals in either homozygous or heterozygous states for mutant allele could be obviously distinguished from wild-type homozygote according to the presence or not of heterogeneous peaks in the chromatographic traces.

To further verify genotyping results of $P_{80kbID}$, four additionally linked variants, including the $P_{5ID}$, $P_{G1855898A}$, $P_{C1768587A}$ and $P_{G1654405A}$, were also genotyped using the Sanger sequencing approach because all of them had been proposed to segregate with $P_{80kbID}$ within a 260 kb haplotype block [10]. Subsequently, we also investigated the linkage disequilibrium by web tool of SHEsis [21] for these five genotyped variants ($P_{80kbID}$, $P_{5ID}$, $P_{G1855898A}$, $P_{C1768587A}$ and $P_{G1654405A}$). According to the recent publication [18], we further genotyped variant $P_{219ID}$ among these polled individuals in which neither $P_{202ID}$ nor $P_{80kbID}$ mutation was observed.

Mitochondrial DNA sequencing and analysis

To determine genetic composition of Shuxuan cattle, mitochondrial D-loop sequence was amplified and sequenced for all animals (S2 Table). Briefly, the PCR products were purified and partially sequenced using the forward primer, which generated sequences of ~820 bp in length. After the raw sequences were edited and truncated, we aligned them using DNASTar tool (DNAS Inc, Madison, WI, USA). Subsequently, a rooted neighbor-joining phylogenetic tree was constructed using yak (B. grunniens) as outgroup to discern matrilineal origins of B. taurus and B. indicus.

Results and discussion

We successfully genotyped candidate variants of $P_{202ID}$ and $P_{80kbID}$ for all 64 Shuxuan cattle in the present study (S1 Table). To avoid genotyping error [17], we therefore employed independent genotyping approaches for both two variants. For variant $P_{80kbID}$, we additionally investigated genotypes for the four tightly linked variants, by which the five related variants were finally revealed to be in almost complete linkage disequilibrium in Shuxuan cattle (Fig 1). This result is consistent with previous report [17] and therefore supports our genotyping reliability of $P_{80kbID}$.

All horned Shuxuan cattle are the wild-type homozygotes for both variants of $P_{202ID}$ and $P_{80kbID}$, which are not beyond the observations reported in previous studies [10, 12]. Both $P_{202ID}$ and $P_{80kbID}$ mutations were simultaneously detected among the polled individuals, for
which the observed frequencies of mutant alleles are 43.75% and 8.33%, respectively (Table 1). Therefore, it is concluded that the polled phenotypes in Shuxuan cattle are genetically determined by both $P_{202ID}$ and $P_{80kbID}$ mutations, which, however, is predominated by $P_{202ID}$. The results are consistent with the fact that Shuxuan cattle currently consist of about 75% Simmental and 10% Holstein blood. Furthermore, there is one animal in which both $P_{202ID}$ and $P_{80kbID}$ mutations are recombined together, which was similarly reported by Rothammer and colleagues [17].

However, there still remain six polled Shuxuan cattle that carry neither $P_{202ID}$ mutation nor $P_{80kbID}$ mutation (S1 Table and Fig 2). Although tens of cattle breeds and thousands of individuals have been comprehensively genotyped for this two candidate variants in previous studies,

![Fig 1. Linkage disequilibrium plots of the five variants of $P_{80kbID}$. Number in each cell is the pairwise Lewontin’s $D’$ (A) and $r^2$ (B), respectively.](https://doi.org/10.1371/journal.pone.0186862.g001)

Table 1. Genotypes and alleles for $P_{202ID}$ and $P_{80kbID}$ among polled individuals.

| Variants | Genotypes | Alleles |
|----------|-----------|---------|
|          | W/W       | W/M     | M/M    | W (%)  | M (%)  |
| $P_{202ID}$ | 9         | 36      | 3      | 56.25  | 43.75  |
| $P_{80kbID}$ | 44        | 0       | 4      | 91.67  | 8.33   |

W, wild-type allele; M, mutant allele.

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Fig 2. Genotyping of \(P_{80kbID}\) and the linked variants. Photos show the polled phenotypes for the six cattle that bear wild-type homozygotes for both \(P_{202ID}\) and \(P_{80kbID}\). One polled cattle (P1037) in homozygous state of mutant allele of \(P_{80kbID}\) and one horned animal (H3847) are illustratively provided in the last two rows. By the capillary electrophoresis method (\(P_{80kbID\text{-CE}}\)), a single peak at 149 bp would be visualized for the wild-type homozygote of \(P_{80kbID}\); otherwise it will result into two peaks at 147 bp and 149 bp, respectively. Sanger sequencing of the fragments encompassing \(P_{80kbID\text{-SE}}\) and \(P_{3ID}\) variants would produce heterozygous chromatograms due to presence of InDel mutation. The initially reported mutations are denoted by arrows. NULL also indicate failure in genotyping.

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| Polled phenotype | \(P_{80kbID\text{-CE}}\) | \(P_{80kbID\text{-SE}}\) | \(P_{3ID}\) | \(P_{G1855898A}\) | \(P_{C1768587A}\) | \(P_{G1654405A}\) |
|------------------|-------------------------|-------------------------|-------------|----------------|----------------|----------------|
| P1025            | ![Image](image1)         | ![Image](image2)        | ![Image](image3)       | ![Image](image4)       | ![Image](image5)       | ![Image](image6)       |
| P1083            | ![Image](image7)         | ![Image](image8)        | ![Image](image9)       | ![Image](image10)      | ![Image](image11)      | ![Image](image12)      |
| P7760            | ![Image](image13)        | ![Image](image14)       | ![Image](image15)      | ![Image](image16)      | ![Image](image17)      | NULL             |
| P0040            | ![Image](image18)        | ![Image](image19)       | ![Image](image20)      | ![Image](image21)      | ![Image](image22)      | NULL             |
| P1041            | ![Image](image23)        | ![Image](image24)       | ![Image](image25)      | ![Image](image26)      | ![Image](image27)      | NULL             |
| P1099            | NULL                     | ![Image](image28)       | ![Image](image29)      | ![Image](image30)      | ![Image](image31)      | ![Image](image32)      |
| P1051            | ![Image](image33)        | ![Image](image34)       | ![Image](image35)      | ![Image](image36)      | ![Image](image37)      | NULL             |
| H3847            | ![Image](image38)        | ![Image](image39)       | ![Image](image40)      | ![Image](image41)      | ![Image](image42)      | NULL             |
only one Holstein sire in polled phenotype was finally revealed to carry neither of them [10, 11, 17]. Accordingly, we initially speculated that there would still be unknown mutation(s) associated with the polled phenotype in Shuxuan cattle. During the preparation of this manuscript, however, we found that Medugorac and colleagues [18] much recently reported the third candidate mutation (P219ID) of polled phenotype independently observed in Mongolian Turano cattle. Therefore, the six polled Shuxuan cattle were again subjected to genotyping of the novel P219ID, by which we newly found that five cattle are heterozygous genotypes for this mutation. However, one animal (P0040) still remains to be wild-type homozygote for the variant P219ID. The Mongolian Turano cattle are mainly distributed in North Eastern Asia and suggested to contain a specific mitochondrial DNA haplogroup of T4 that would have been locally domesticated [22]. We subsequently classified the two polled Shuxuan cattle of B. taurus which also carry P219ID mutation and revealed that they belong to T2 rather than T4 haplogroup. Additionally, there is no official record for the possible paternal introgression of gene pool of Mongolian Turano cattle into Shuxuan cattle. Therefore, the presence of P219ID mutation in Shuxuan cattle can’t be explained by the acknowledged breeding process. An alternative possibility is that P219ID mutation would be also present in other breeds, which had ever been imported into the Shuxuan cattle.

Although all three proposed POLLED mutations were simultaneously detected in Shuxuan cattle, there still remains a polled animal that doesn’t carry any one of them yet. This observation raises our presumption that unknown causative mutation(s) of polled phenotype in cattle would also exist. In fact, it would have become conclusive that both P202ID and P80kbID are perfectly associated with polled phenotype after both of them had been comprehensively genotyped in a large panel of European cattle breeds [17]. However, it becomes again an open question due to novel finding of the third candidate mutation of P219ID in Mongolian Turano cattle [18]. More importantly, all the three variants of P202ID, P80kbID and P219ID are referred to as the mutation type of fragment duplication-insertion and also adjacently located within a ~270 kb block. It would therefore be reasonable to deduce that similar mutation within or around this block region also would likely cause the polled phenotype in cattle, which should be investigated in the future.

In addition to the genetic variants, a phenotypic character of atypical eyelashes was also proposed to be perfectly associated with the polled phenotype in cattle [12]. Unfortunately, we did not examine this visual phenotype for all individuals sampled in the present study. Another critical question is the molecular mechanism underlying phenotypic ontogenesis of polledness in cattle, to which a few studies have been specially paid via investigating the differentially expressed genes between polled phenotype and wild-type [12, 13, 20]. However, only limited evidence has been effectively elucidated possibly because of low detection power of microarray-based gene expression profiling platform [20] or insufficient biological samples subjected to high-throughput mRNA sequencing [12]. Additionally, it would be very difficult to determine appropriate sampling time points due to the complexity of prenatal horn bud dynamic development [13, 23].

The Chinese cattle are evolutionarily composed of two matrilineal origins of B. taurus and B. indicus [24]. The indigenous cattle breeds that are distributed in central China, such as Sichuan and Shaanxi provinces, would be essentially mixed by this two components [25]. Therefore, we intended to know whether the presence of polled phenotype in Shuxuan cattle is unevenly distributed between B. taurus and B. indicus. By sequencing mitochondrial DNA, a total of 42 and 22 individuals were classified into the B. taurus and B. indicus, respectively (Fig 3). Furthermore, all 48 polled Shuxuan cattle consist of 31 B. taurus and 17 B. indicus individuals, which herein suggest that there is no matrilineal origin-biased distribution of polled phenotype.
Based on the estimated proportion of 15% of polled individuals in Shuxuan cattle, it can be computed that the total frequency of polled alleles is 8.6% for all the three candidate variants. Furthermore, about 75% of the mutant alleles of polled phenotype are currently carried in heterozygous state. In order to breed a stable polled population in Shuxuan cattle, therefore, the direct selection based on phenotype or even on the genotype would take a long time or result into considerable genetic loss because the polledness trait is inherited in the dominant manner. Fortunately, a recent study first proposed that approach of genomic selection could speed up the selection progress of polled population [26], which could be applied to Shuxuan cattle in the future.

**Conclusion**

In the present study, we first reported that all three proposed mutations of polled phenotype of cattle are simultaneously introgressed into a synthetic cattle breed in China. However, one polled animal was revealed to carry none of these candidate mutations, which therefore suggests that further causative mutation(s) of the polled phenotype in cattle would also exist.

**Supporting information**

S1 Table. Sample information and genotyping results of $P_{80kbID}$ and the related variants.

(XLSX)

S2 Table. Primers, PCR amplification and genotyping methods used in the present study.

(DOCX)

**Author Contributions**

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References

1. Lundrigan B. Morphology of horns and fighting behavior in the family Bovidae. J Mammal. 1996; 77(2):462–75.
2. Schafberg R, Swalve HH. The history of breeding for polled cattle. Livest Sci. 2015; 179:54–70.
3. Cozzi G, Gottardo F, Brecic M, Contiero B, Iriang N, Knerim U, et al. Dehoming of cattle in the EU Member States: A quantitative survey of the current practices. Livest Sci. 2015; 179:4–11.
4. Windig JJ, Hoving-Bolink RA, Veerkamp RF. Breeding for polledness in Holstein cattle. Livest Sci. 2015; 179:96–101.
5. Drögemüller C, Wöhlke A, Mömke S, Distl O. Fine mapping of the polled locus to a 1-Mb region on bovine chromosome 1q12. Mamm Genome. 2005; 16(8):613–20. https://doi.org/10.1007/s00335-005-0016-0 PMID: 16180143
6. Georges M, Drinkwater R, King T, Mishra A, Moore SS, Nielsen D, et al. Microsatellite mapping of a gene affecting horn development in Bos taurus. Nat Genet. 1993; 4(2):206–10. https://doi.org/10.1038/ng0693-206 PMID: 8348158
7. Sehnmutz SM, Marquess FLS, Berryere TG, Moker JS. DNA marker-assisted selection of the polled condition in Charolais cattle. Mamm Genome. 1995; 6(10):710–3. PMID: 8563169
8. Brenneman RA, Davis SK, Sanders JO, Burns BM, Wheeler TC, Turner JW, et al. The polled locus maps to BTA1 in a Bos indicus × Bos taurus cross. J Hered. 1996; 87(2):156–61. PMID: 8830095
9. Seichter D, Russ I, Rothammer S, Eder J, Förster M, Medugorac I. SNP-based association mapping of the polled gene in divergent cattle breeds. Anim Genet. 2012; 43(5):595–8. https://doi.org/10.1111/j.1365-2052.2011.02302.x PMID: 22497248
10. Medugorac I, Seichter D, Graf A, Russ I, Blum H, Göpel KH, et al. Bovine polledness—an autosomal dominant trait with allelic heterogeneity. PLoS One. 2012; 7(6):e39477. https://doi.org/10.1371/journal.pone.0039477 PMID: 22737241
11. Glatzer S, Merten NJ, Dierks C, Wöhlke A, Philipp U, Distl O. A Single nucleotide polymorphism within the Interferon Gamma Receptor 2 gene perfectly coincides with polledness in Holstein cattle. PloS One. 2013; 8(6):e67992. https://doi.org/10.1371/journal.pone.0067992 PMID: 23805331
12. Allais-Bonnet A, Grohs C, Medugorac I, Krebs S, Djari A, Graf A, et al. Novel insights into the bovine polled phenotype and horn ontogenesis in Bovidae. PloS One. 2013; 8(5):e63512. https://doi.org/10.1371/journal.pone.0063512 PMID: 23717440
13. Wiedemar N, Tetens J, Jagannathan V, Menoud A, Neuenschwander S, Bruggmann R, et al. Independent Polled mutations leading to complex gene expression differences in cattle. PLoS One. 2014; 9(3):e93435. https://doi.org/10.1371/journal.pone.0093435 PMID: 24671182
14. Capitan A, Allais-Bonnet A, Pinton A, Guienne BM, Bourhis DL, Grohs C, et al. A 3.7 Mb deletion encompassing ZEB2 causes a novel polled and multisystemic syndrome in the progeny of a somatic mosaic bull. PloS One. 2012; 7(11):e49084. https://doi.org/10.1371/journal.pone.0049084 PMID: 23152892
15. Liu WB, Liu J, Liang CN, Guo X, Bao PJ, Chu M, et al. Associations of single nucleotide polymorphisms in candidate genes with the polled trait in Datong domestic yaks. Anim Genet. 2014; 45(1):138–41. https://doi.org/10.1111/age.12081 PMID: 24033474
16. Liang C, Wang L, Wu X, Wang K, Ding X, Wang M, et al. Genome-wide association study identifies loci for the polled phenotype in yak. PloS One. 2016; 11(7):e0158642. https://doi.org/10.1371/journal.pone.0158642 PMID: 27389700
17. Rothammer S, Capitan A, Mullaart E, Seichter D, Russ I. The 80-kb DNA duplication on BTA1 is the only remaining candidate mutation for the polled phenotype of Friesian origin. Genet Sel Evol. 2014; 46(1):44.
18. Medugorac I, Graf A, Grohs C, Rothammer S, Zagdsuren Y, Gladyr E, et al. Whole-genome analysis of introgressive hybridization and characterization of the bovine legacy of Mongolian yaks. Nat Genet. 2017; 49(3):470–5. https://doi.org/10.1038/ng.3775 PMID: 28135247

19. Carlson DF, Lancto CA, Zang B, Kim ES, Walton M, Oldeschulte D, et al. Production of hornless dairy cattle from genome-edited cell lines. Nat Biotechnol. 2016; 34(5):479–81. https://doi.org/10.1038/nbt.3560 PMID: 27153274

20. Mariasegaram M, Reverter A, Barris W, Lehnert SA, Dalrymple B, Prayaga K. Transcription profiling provides insights into gene pathways involved in horn and scurs development in cattle. BMC Genomics. 2010; 11(1):370.

21. Shi Y, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 2005; 15(2):97–8. https://doi.org/10.1038/sj.cr.7290272 PMID: 15740637

22. Mannen H, Kohno M, Nagata Y, Tsuji S, Bradley DG, Yeo JS, et al. Independent mitochondrial origin and historical genetic differentiation in North Eastern Asian cattle. Mol Phylogenet Evol. 2004; 32(2):539–44. https://doi.org/10.1016/j.ympev.2004.01.010 PMID: 15223036

23. Wiener DJ, Wiedeman N, Welle MM, Droegemueller C. Novel features of the prenatal horn bud development in cattle (Bos taurus). PloS One. 2015; 10(5):e0127691. https://doi.org/10.1371/journal.pone.0127691 PMID: 25993643

24. Lai SJ, Liu YP, Liu YX, Li XW, Yao YG. Genetic diversity and origin of Chinese cattle revealed by mtDNA D-loop sequence variation. Mol Phylogenet Evol. 2006; 38(1):146–54. https://doi.org/10.1016/j.ympev.2005.06.013 PMID: 16054846

25. Chen S-Y, Liu Y-P, Wang W, Gao C-Z, Yao Y-G, Lai S-J. Dissecting the matrilineal components of Tongjiang cattle from southwest China. Biochem Genet. 2008; 46(3–4):206–15. https://doi.org/10.1007/s10528-008-9144-z PMID: 18246422

26. Gaspa G, Veerkamp RF, Calus MP, Windig JJ. Assessment of genomic selection for introgression of polledness into Holstein Friesian cattle by simulation. Livest Sci. 2015; 179:86–95.