



Research note: Genetic basis of the naked-neck trait in ake chickens revealed by whole-genome sequencing

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ABSTRACT

Naked-neck chickens are distributed across tropical and subtropical regions and exhibit notable resistance to heat stress. The naked-neck trait in chickens is an incompletely dominant characteristic determined by the Na gene, and significant differences in feather quantity and distribution have been observed between homozygous and heterozygous individuals. The Ake chicken, a native Chinese breed originating from southwestern Yunnan Province, is the only breed in China that possesses the naked-neck trait. The Ake chicken was developed in a relatively isolated mountain area, and the genetic basis for the determinants of the naked-neck trait in this breed remains unclear. In this study, we utilized Oxford Nanopore Technologies and next-generation sequencing to obtain genomic data from Ake chickens and other naked-neck chicken breeds from Iran and Egypt. Genome sequence alignments revealed that the mutation responsible for the naked-neck trait in Ake chickens was the same as that in naked-neck chickens from other regions, specifically, a 73-kb insertion at the end of chromosome 3. We further demonstrated that the insertion fragment was homologous to the intergenic region between the *WNT11* and *UVRAG* genes at 198 Mb on chromosome 1. The original sequence on chr 1 remained intact without any mutation. This study provides a genetic foundation for the naked-neck trait in chickens and provides a reliable molecular marker for the future breeding of heat stress-resistant chicken breeds.

Introduction

China, as a center of chicken origin and domestication, boasts a rich and diverse array of chicken breeds. Among these, the Ake chicken, which is native to Fugong County in Yunnan Province, is notable for its dual utility in meat and egg production. A distinctive characteristic of the Ake chicken is its bare, featherless neck, which extends from the lower jaw to the top of the crop (Fig. 1A); it also has fewer body feathers than other breeds. Notably, the Ake chicken is the only existing breed in China with the naked-neck trait.

Naked-neck chickens are distributed across various global regions, primarily in subtropical, tropical, and equatorial areas with hot and humid climates. The naked-neck trait in poultry has long been acknowledged; Davenport (Davenport, 1914) provided a comprehensive description of this trait, and Warren (1933) established through

experimentation that it is governed by a dominant gene, and Greenwood (1927) and Hertwig (1933) named this gene *Na* while investigating the feather characteristics of these chickens. Compared with wild-type chickens, naked-neck chickens exhibit a marked expansion of the apteria and significant diminution of the pteryla on the chest, legs, and other regions. The reduction in feathering is most pronounced on the neck, where feathers are nearly absent. The *Na* allele demonstrates incomplete dominance, allowing the phenotypic differentiation of homozygous (*NaNa*) and heterozygous (*Nana*) individuals. Heterozygous individuals display a cluster of several dozen feathers on their necks, whereas homozygous individuals either completely lack feathers or possess only a few feathers.

The application value of the naked-neck trait lies primarily in its ability to confer resistance to heat stress. Feather production in homozygous and heterozygous naked-neck chickens is reduced by 40 % and

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20–30 %, respectively, compared to that in wild-type chickens. This reduction enhances heat dissipation, thereby improving production performance metrics such as egg quantity, egg quality, and meat yield, particularly under high-temperature and high-humidity conditions (Merat, 1986). Furthermore, further studies have indicated that homozygous naked-neck chickens exhibit significant advantages in

reproductive traits, including semen volume and sperm count at all ages tested. In high-temperature environments, the proportion of abnormal embryos is significantly lower for naked-neck chickens than for normal-feathered chickens, indicating higher vitality and survival rates among these embryos (Ladjali et al., 1995).

As early as 1984, the Na locus was linked to erythrocyte antigen P

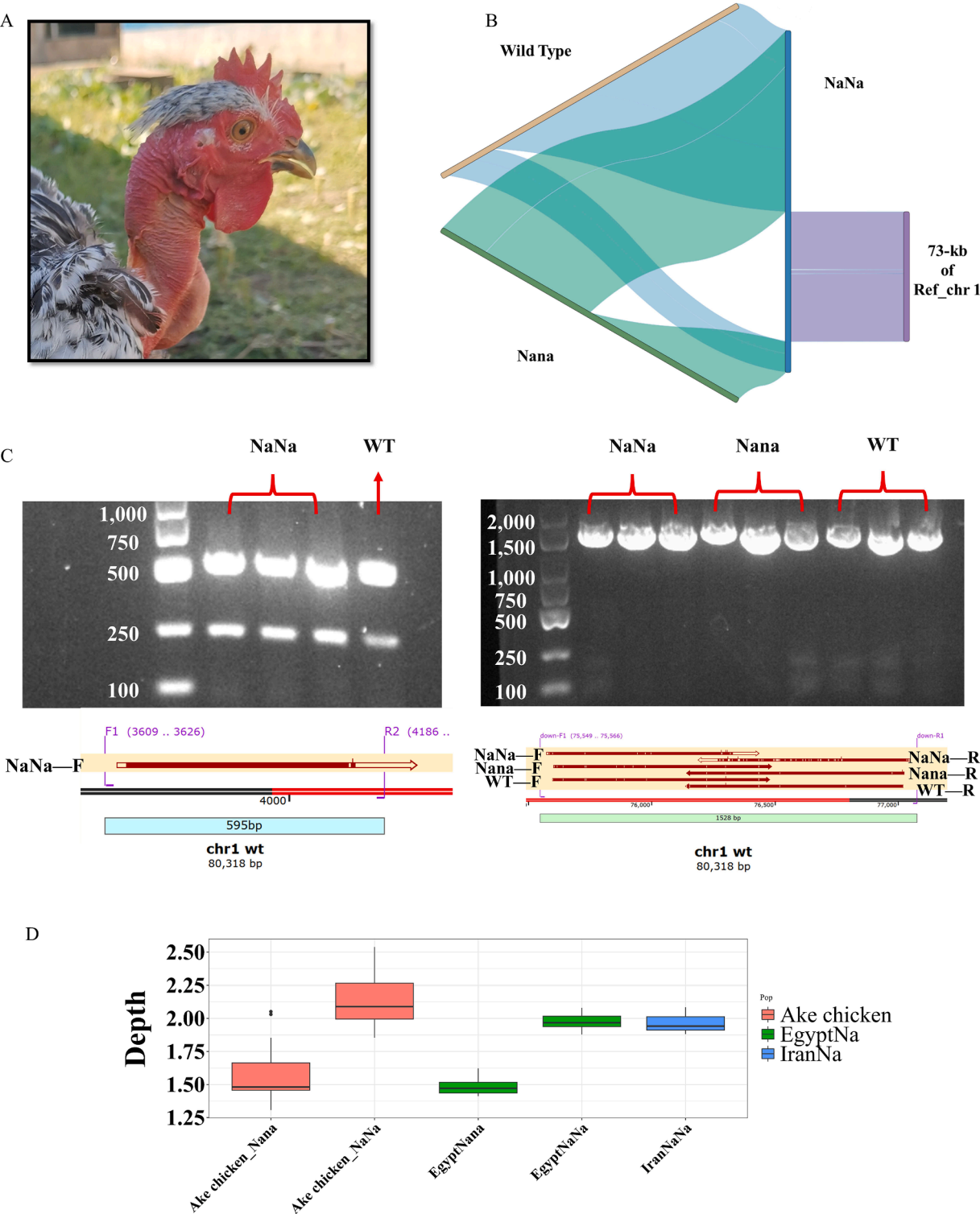


Fig. 1. Naked-neck Trait in Ake Chickens. **A.** Ake chickens with the homozygous naked-neck genotype. **B.** The homozygous naked-neck individual has a sequence at the end of chr 3 that is absent in wild-type and heterozygous individuals, and this sequence is homologous to a sequence on chr 1. **C.** PCR and Sanger sequencing results of amplification of regions upstream (left) and downstream (right) of the 73-kb sequence on chr 1. **D.** The sequence depth in homozygous naked-neck individuals was 1.5 times greater than that in heterozygous individuals.

(*CPPP*) (Bitgood et al., 1984). In 2000, Frederique Pitel et al. located the *CPPP* gene on chromosome 3 (**chr 3**) (Pitel et al., 2000). In 2011, Pitel subsequently identified *BMP12* (*GDF7*) as the gene responsible for the naked-neck phenotype in chickens from England, Scotland, France, Mexico, and other regions (Mou et al., 2011). These authors conducted genotyping and overlapping PCR techniques to examine the regions upstream and downstream of the gene, discovering a 73-kb insertion fragment situated 260 kb downstream of the *BMP12* gene. This insertion fragment exhibited homology with the intergenic region on chromosome 1 (**chr 1**), and the sequence displayed tissue-specific synchrony in its regulatory function. Currently, the genetic basis of the naked-neck trait remains incompletely understood, and the types of mutations involved have not yet been thoroughly explored.

In this study, we employed 50 K ultralong Oxford Nanopore Technologies (ONT) sequencing to explore the genetic variation in the naked-neck trait in Ake chickens. Additionally, we integrated whole-genome resequencing data from other naked-neck chicken breeds from Iran and Egypt to determine whether the genetic variation in Ake chickens is the same as that in naked-neck chickens from other regions. This study provides technical support and a genetic foundation for the development of heat stress-resistant varieties, thereby providing a new direction for future improvements in poultry breeding.

Materials and methods

Ethics statement

All experimental protocols related to the chickens used in this study were performed in accordance with the guidelines of the Ministry of Science and Technology (Beijing, China). Ethical approval was obtained from the Animal Welfare Committee of China Agricultural University (AW71802202-1-2) and performed in accordance with the procedures of the Guide for the Care and Use of Laboratory Animals (China Agricultural University).

Sample and data sets

This study included 63 individuals from three chicken breeds: 30 Ake chicken samples collected from Fugong County in Yunnan Province, 30 Egyptian naked-neck chicken samples obtained from Egypt and data from 3 Iranian naked-neck chickens collected from the Chicken SNP Database (<https://ngdc.cncb.ac.cn/chickensd/>).

The raw sequencing data generated in this study are publicly available in the Genome Sequence Archive (GSA) of the National Genomics Data Center (NGDC) under accession number PRJCA035883.

DNA extraction

Blood samples (2 ml) were collected from the wing veins of the experimental chickens and then stored at -20°C . DNA was extracted via the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions.

ONT sequencing and genome assembly

For the ONT sequencing, the concentration and quality of the DNA were checked via 1 % agarose gel electrophoresis, NanoDrop spectrophotometry (Thermo Fisher Scientific, Lafayette, USA), and Qubit fluorometry (Invitrogen, Darmstadt, Germany). Large-fragment (≥ 30 kb) libraries were selected via the BluePippinTM System and processed via the ONT Template Prep Kit (SQK-LSK109, Oxford Nanopore Technologies, Oxford, UK). DNA fragments were end-repaired and 3'-adenylated via the NEBNext FFPE DNA Repair Mix Kit (New England Biolabs (NEB), Ipswich, MA, USA). The Nanopore sequencing adapters were ligated via the NEBNext Quick Ligation Module (E6056) (NEB). The final library was sequenced on R9 flow cells via a PromethION DNA sequencer (ONT)

and an ONT sequencing reagent kit (EXP-FLP001.PRO.6). The raw signal data were called, and the FAST5 files were converted into FASTQ files via MinkNOW (v 2.0) (ONT). Short reads (< 2 kb) and reads with low-quality bases and adapter sequences were excluded.

The ONT reads were aligned to the GRCg7b (https://ftp.ensembl.org/pub/release-110/fasta/gallus_gallus/dna/) reference genome using minimap2 (v 2.20), and the resulting alignment files were sorted using SAMtools (v 1.17). The output BAM files were transferred to bigWig files by deeptools (v 3.2.0). IGV software (v 2.16.0) was used to inspect the ONT long-read alignments. NextDenovo software (v 2.5.2) was used with default parameters to assemble the long reads.

Whole-genome sequencing, reads mapping, and depth statistics

Sequencing libraries were constructed from at least 1 mg of genomic DNA following the TruSeq Nano Sample Prep Kit protocol (Illumina Inc., San Diego, CA, USA). Paired-end libraries with an average insert size of approximately 350 bp were sequenced on an Illumina HiSeq X Ten platform (Illumina) by Berry Genomics Co. Ltd. (Beijing, China), yielding 150-bp reads with a target sequencing depth of $10\times$ coverage per genome.

Sequence reads were filtered via fastp (v 0.12.3). The clean reads were aligned to the GRCg7b (https://ftp.ensembl.org/pub/release-110/fasta/gallus_gallus/dna/) reference genome via BWA-MEM (v 0.7.17), and the resulting alignment files were sorted using SAMtools (v 1.17).

Synten analysis

MUMmer (v 4.0.0) was used to conduct collinearity analysis on the genomes of single individuals representing each of the three genotypes to verify the integrity and accuracy of the chromosome assembly and to perform interindividual chromosome sequence alignment. We then visualized the results via NGenomeSyn (v 1.41).

PCR and agarose gel electrophoresis

We designed primers upstream of 240 bp and downstream of 1.2 kb from the target sequence, conducted standard PCR, performed agarose gel electrophoresis, and subsequently conducted Sanger sequencing. The PCR mixtures contained 10 μM of each barcoded specific primer in a 20 μl reaction (Taq $2\times$ Easy Master Mix). The PCR1 program consisted of initial denaturation (2 min at 94°C) followed by 5 to 10 cycles of denaturation (15 s at 94°C), annealing (30 s at 60°C), and extension (6 min at 65°C) and a final extension (5 min at 65°C).

Results and discussion

ONT sequencing results

Due to the incomplete dominance of the naked-neck trait in Ake chickens, which results in distinct phenotypes for each of the three genotypes, we selected one sample from each of the three genotypes for ONT sequencing.

The output data for each sample exceeded 81 GB, with an average depth greater than $30\times$. After the genome assembly process, 137 scaffolds were generated from the homozygous naked-neck individual (NaNa), with the longest scaffold measuring 105,197,613 bp. For the heterozygous individual (Nana), 135 scaffolds were obtained, the longest of which was 105,524,787 bp. Additionally, 124 scaffolds were obtained for the wild type, with the longest measuring 149,445,342 bp. The genome sizes of the three genotypic representatives surpassed 1,000 Mb, with NaNa having a genome size of 1,008.82 Mb, Nana having a genome size of 1,027.43 Mb, and the wild type having a genome size of 1,023.35 Mb.

The collinearity results between individuals of the three genotypes

and galgal7b revealed that the autosomal assemblies were relatively complete, except for that of chr 1, which was composed of several scaffolds.

Variations on chromosome 3

To identify the causative mutations underlying the naked-neck trait in Ake chickens, we conducted a collinearity analysis on the individual genome assembly results of the three genotypes in comparison to those of galgal7b. This analysis detected various structural variations (SVs), including gaps (GAP), duplications (DUP), breakpoints (BRK), inversions (INV), jumps (JMP) and sequence mismatches (SEQ, such as SNPs and indels). The results are detailed in Table 1.

We found a 73-kb sequence at the end of chr 3 of the naked-neck individual that was not present in the wild-type or heterozygous individuals. Notably, the insertion position of this sequence was consistent between the wild-type and Nana individuals. Moreover, this sequence was compared with the sequence on chr 1 (Fig. 1B). This is a feature that was not observed in the other detected SVs, indicating that this 73-kb sequence is the only mutation that meets the criteria for the Na mutation. The original sequence on chr 1 comprises 72,385 bp, which, after mapping to chr 3, occupy a position of 73,704 bp. The length of the insertion region at the end of chr 3 identified in this study was 73,715 bp, with an additional 11 bp, precisely matching the findings of Pitel's team (Mou et al., 2011). This evidence indicates that the mutation responsible for the naked-neck trait in Ake chickens is the same as that observed in other naked-neck chicken breeds.

Variations on chromosome 1

According to the findings of Pitel's team, the location of the naked-neck gene on chr 3 was identified, and the inserted fragment exhibited homology with the intergenic region between the WNT11 and UVRAG genes at the 198 Mb position on chr 1 (Mou et al., 2011). However, it remains unclear whether this mutation is a translocation or a duplication.

To investigate whether the homologous segment on chr 1 is missing in naked-neck individuals, we first inspected the ONT long-read alignments using IGV. The analysis revealed continuous read coverage on both sides of the 73-kb region on chr 1, indicating that the flanking regions are uninterrupted. Subsequently, we conducted PCR to amplify segments of chr 1 from the three genotypes. Primers were designed for the regions upstream and downstream of the segment in question and used to amplify DNA samples from the three genotypes, which were subsequently subjected to Sanger sequencing. The results revealed that the same continuous fragments in both the upstream and downstream regions were amplified from samples from all three genotypes. Furthermore, these sequences were largely consistent with the reference genome sequence (Fig. 1C).

NGS data from 63 individuals across three breeds, comprising 30 Ake chickens, 3 subjected to sequencing depth analysis. The results indicated that across the three breeds, the depth of the 73-kb sequence was 1.5 times greater in homozygous naked-neck individuals than in heterozygous individuals within the same breed (Fig. 1D). This finding suggested that the naked-neck mutations in these three breeds are identical. Furthermore, this result provides evidence that this 73-kb sequence mutation is a duplication rather than a translocation.

The Ake chicken, a naked-neck breed discovered in China, presents phenotypic characteristics that are essentially identical to those of naked-neck chickens from other regions. In the 1980s, China imported numerous chicken breeds from abroad, resulting in the introduction of more than 30 breeds to meet the domestic demand for chicken meat and eggs, thereby possibly facilitating the influx of naked-neck genes.

In conclusion, this study utilized ONT data and revealed that the

Table 1
Number of structural variation types in the three genotypes.

SV Type	NaNa	Nana	Wild type
GAP	6,804	6,940	6,785
DUP	7,310	6,677	6,189
BRK	1,873	1,645	1,399
INV	205	247	254
JMP	1,729	1,767	1,936
SEQ	479	500	440

naked-neck mutation in Ake chickens is characterized by a 73-kb insertion at the end of chr 3, which is derived from chr 1. This finding is consistent with findings in other naked-neck chickens. Furthermore, NGS data and PCR analyses revealed no differences in chr 1 among various breeds and genotypes of naked-neck chickens, suggesting that the original sequence on chr 1 remains intact in naked-neck individuals. This evidence confirms that the naked-neck trait of Ake chickens is attributed to a 73-kb insertion in the chromosomal duplication rather than a translocation. Our findings not only elucidate the genetic basis of the naked-neck trait in Ake chickens but also provide a valuable molecular marker for practical breeding applications. The use of Na is a potential solution due to the animals' high temperature tolerance. However, the benefit will be even greater if we combine additional strategies (Fernandes et al., 2023). In future breeding strategies, genomic selection utilizing this unique molecular marker can be combined with other favorable production traits to develop heat-resistant chicken breeds, thereby improving productivity in tropical and sub-tropical regions.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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