The first report of a Pelecaniformes defensin cluster: Characterization of \( \beta \)-defensin genes in the crested ibis based on BAC libraries

Hong Lan*, Hui Chen*, Li-Cheng Chen*, Bei-Bing Wang, Li Sun, Mei-Ying Ma, Sheng-Guo Fang & Qiu-Hong Wan

The Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education, State Conservation Centre for Gene Resources of Endangered Wildlife, College of Life Sciences, Zhejiang University, Hangzhou 310058, P. R. China.

Defensins play a key role in the innate immunity of various organisms. Detailed genomic studies of the defensin cluster have only been reported in a limited number of birds. Herein, we present the first characterization of defensins in a Pelecaniformes species, the crested ibis (Nipponia nippon), which is one of the most endangered birds in the world. We constructed bacterial artificial chromosome libraries, including a 4D-PCR library and a reverse-4D library, which provide at least 40 equivalents of this rare bird’s genome. A cluster including 14 \( \beta \)-defensin loci within 129 kb was assigned to chromosome 3 by FISH, and one gene duplication of AvBD1 was found. The ibis defensin genes are characterized by multiform gene organization ranging from two to four exons through extensive exon fusion. Splicing signal variations and alternative splice variants were also found. Comparative analysis of four bird species identified one common and multiple species-specific duplications, which might be associated with high GC content. Evolutionary analysis revealed birth-and-death mode and purifying selection for avian defensin evolution, resulting in different defensin gene numbers among bird species and functional conservation within orthologous genes, respectively. Additionally, we propose various directions for further research on genetic conservation in the crested ibis.

The crested ibis (Nipponia nippon) is one of the most endangered avian species in the world. It has suffered a catastrophic decline since the late nineteenth century and was thought to be extinct until 7 wild birds were rediscovered in China in 1981. Since then, this tiny population has stably expanded under great conservation efforts and is currently the only wild population in the world. However, several captive populations were established to help its recovery. By 2013, the crested ibis population in China increased to more than 1500. However, as a bottlenecked species with intensive inbreeding, recent populations suffer from low genetic diversity. The mortality of ibis embryos and nestlings is also high, and many pathogens can fatally infect the bird. Therefore, the genetic conservation and immunological function of the crested ibis require rigorous investigation.

Construction of a genomic library for the crested ibis can conserve its DNA resources and thereby promote detailed genomic research on this rare species and development of strategies for better protection. Bacterial artificial chromosomes (BACs) provide a capacity to clone genomic fragments larger than 100 kb, which is sufficient for most research on a genomic scale. Moreover, BACs can be stably transformed into bacterial hosts with low occurrence of chimerism and are more easily purified than bacteriophage \( \lambda \) cosmids vectors or yeast artificial chromosomes. Given these advantages, BACs have been widely used in the construction of DNA libraries and in genomic studies.

Defensins are a collection of small peptides (commonly less than 100 amino acids) enriched in hydrophobic and cationic residues that play a critical role in regulating the innate immune systems of a wide range of organisms including fungi, plants, invertebrates, and vertebrates. These antimicrobial peptides can protect the host against a broad spectrum of pathogens, such as bacteria, enveloped viruses, certain fungi, and parasitic protozoa, mainly by binding to and disturbing pathogen membranes, blocking viral replication cycles, or changing host cell recognition sites. Moreover, defensins are chemotactic for T cells, macrophages, mast cells, and immature dendritic cells, thereby serving as a bridge between innate and adaptive immunity.
Defensins contain 6–8 conserved cysteine residues forming 3–4 intramolecular disulfide bonds. Based on the positions of the cysteine residues and the pairing of the disulfide bridge, they are categorized into five groups: plant (C1–C8, C2–C5, C3–C6, and C4–C7), invertebrate (C1–C4, C2–C5, and C3–C6), \( \alpha \)-defensins (C1–C6, C2–C4, and C3–C5), \( \beta \)-defensins (C1–C5, C2–C4, and C3–C6), and \( \theta \)-defensins (C1–C6, C2–C5, and C3–C4), with latter three unique to vertebrates\(^{15,16,20,21}\). \( \beta \)-defensins are found in a majority of vertebrates\(^{12,14,22,23}\), while \( \alpha \)-defensins are found only in mammals, including marsupials, but are absent in some Laurasiatheria species, such as dogs and cattle\(^{24,25}\). The cyclic \( \alpha \)-defensins may be much higher due to rapid duplication\(^{28}\). Typically, a posttranslational ligation of two truncated \( \alpha \)-defensin-like precursor peptides\(^{21}\), have only been found in Old World monkeys and some apes and are defunct in gorillas, chimpanzees, and humans\(^{26}\). Based on their prevalence, the major \( \beta \)-defensins subfamilies are believed to have arisen before the bird–mammal split, followed by the appearance of \( \alpha \)-defensins after the divergence of mammals and finally \( \theta \)-defensins in the primate lineage\(^{26,27}\). In the human genome, at least 6 \( \alpha \)-defensins\(^{13}\) and 40 potential \( \beta \)-defensins were found within 5 clusters scattered on chromosome 8, 6, and 20, and the number of \( \beta \)-defensins may be much higher due to rapid duplication\(^{28}\). Typically, the human \( \beta \)-defensins contain two exons: exon 1 corresponds to the 5’ untranslated region (UTR) and signal peptide, and exon 2 corresponds to the short anionic propiece followed by the mature peptide and 3’ UTR\(^{28}\). Post-translational modifications include proteolytic cleavage of the signal peptide and propiece from the prepeptide to yield the mature peptide\(^{28,29}\).

The first avian defensin genes were isolated from chicken and turkey leukocytes in the mid 1990s\(^{30}\). Since then, defensins have been increasingly studied in other avian species\(^{15,32}\), but comprehensive genomic information on defensin clusters has only been reported in chicken (14 genes)\(^{21,23}\), duck (19 genes including a pseudogene)\(^{33}\), and zebra finch (22 genes)\(^{34}\), all suggesting that only \( \beta \)-defensins are present in birds and revealing various gene duplications. Phylogenetic analysis based on the three birds revealed that the avian \( \beta \)-defensins (AvBDs) could be classified into 12 subfamilies\(^{15}\). Nine subfamilies (AvBD2, AvBD4–5, and AvBD8–13) maintain one-to-one orthologous genes in all species; two subfamilies including three members (AvBD1/AvBD3 and AvBD7) show different lineage-specific duplications among species; and one subfamily (AvBD14) is absent in zebra Finch. Accordingly, most avian defensins evolved before the bird species split from each other. In contrast to the human \( \beta \)-defensins, the avian \( \beta \)-defensins typically contain four exons: exon 1 for the first part of the 5’ UTR, exon 2 for the rest of the 5’ UTR and the signal peptide, exon 3 for the short propiece (sometimes absent) and majority of the mature peptide, and exon 4 for the C-terminus of the mature peptide and the 3’ UTR\(^{24,25}\).

In this work, we constructed genomic libraries for the crested ibis using BACs, including a routine 4D-PCR library and a simplified reverse-4D library. Based on the target BAC clones screened from the libraries, we characterized the content and organization of the defensin gene cluster, which is the first to be reported in Pelecaniformes. The genomic and evolutionary information about defensins revealed by this work will increase our understanding of the immunity of this endangered species and the historic events in avian defensin gene evolution.

**Results**

**Construction and characterization of BAC libraries.** Since the crested ibis is an endangered species, it required a routinely constructed BAC genomic DNA library, a 4D-PCR BAC library\(^{25}\), to store its genetic resource. The library contains 129312 clones conserved on 1347 96-well plates and was arrayed into 27 superpools (49 × 96 clones). The average insert size of the library was 86.5 kb, with an empty vector rate of 6.5% (Figure 1a). Through comparison with the C-value of other threskiornithidae species available in the Animal Genome Size Database (http://www.genomesize.com/), we estimated the crested ibis genome at about 1.35 Gb. Thus, the genomic coverage of this library was about 7.8 equivalents of the crested ibis genome.

Considering the relatively small insert size in the 4D-PCR library, we decided to construct a second BAC library with greater coverage. However, 4D-PCR BAC library construction is time-consuming and labor-intensive. Recently, a new type of simply arrayed BAC library, a reverse-4D BAC library, was developed for birds\(^{27}\); this method can significantly reduce the time investment and is efficient enough to

![Figure 1 | Quality and usability of BAC libraries.](image-url)
acquire positive clones covering the target genomic region. Therefore, we adjusted library construction conditions (Table 1) and built a reverse-4D BAC library for the crested ibis consisting of 1300 sub-libraries, each with an average of 400 clones. Randomly selected clones had average insert sizes of 100.9 kb and an empty vector rate of 5.6% (Figure 1a). The reverse-4D library therefore provides at least 35-fold coverage of the crested ibis genome.

Validation of the usability of the BAC libraries. We obtained two pieces of evidence to verify the usability of the crested ibis BAC libraries. First, we employed the primer pair from defensin gene AvBD7 to screen the BAC libraries and achieved five positive BACs (Figure 1b), of which 375B9 and 780E4 are from the routine 4D-PCR library while the other three (H46-1, H11-10, and H68-1) are from the simplified reverse-4D library. The overlap among BACs shows that the big BAC clones are all from the reverse-4D library, validating large inserts in the reverse-4D library. The biggest BAC, H46-1, contains a 150 kb insert spanning the target genomic region and covers all defensin genes of the crested ibis as determined from a BLAST search against the relevant genomic region of chicken defensins, providing an opportunity to characterize the crested ibis defensin cluster in detail.

Second, we used the plasmid DNA of BAC H46-1 as a probe to perform fluorescence in situ hybridization (FISH). The result shows that the crested ibis defensins physically map to chromosome 3, the same chromosome that contains the defensin clusters in the chicken and zebra finch genomes27,35, without any non-specific binding (Figure 1c). This means that, in contrast to the human and mouse defensins, which are scattered on several different chromosomes27, the avian defensins are probably restricted to only one chromosome.

Characterization of the crested ibis defensin genes. Fourteen β-defensin loci clustering within a region of about 129 kb were identified in the clone H46-1 (Table 2). We also obtained the full-length cDNA sequences of these defensin genes except AvBD11 and AvBD14, which may not be expressed in the selected organs or may be only inductively expressed when exposed to certain pathogens. The cDNA sequences obtained range from 378 bp to 813 bp. By aligning the deduced amino acid sequences of the 14 crested ibis defensins, we detected high sequence similarity in the signal peptides but a very low level of conservation in the remaining regions, including the propiece and mature peptides (Figure 2a). This pattern was also observed in many other vertebrates22,27,35 and the high sequence divergence concentrated in the mature peptide among different defensin genes was suggested to be driven by accelerated adaptive selection after successive rounds of gene duplication22. Nevertheless, all the defensin genes in crested ibis exhibit six conserved cysteine residues located in the mature peptides (Figure 2a). Moreover, we noticed splicing signal variations in intron 2 (AG/CT) from AvBD12 and in intron 3 (both GT/CA) from AvBD2 and AvBD1, which coincidently correspond to the two hypervariable regions of AvBDs (i.e., the propiece and mature C-terminal, respectively) (Figure 2b–2c). Interestingly, AvBD11 showed two tandem copies of the special six-cysteine motif in the C-terminal segment with a low level of sequence identity (Figure 2a). Similar patterns were also observed for chicken AvBD1127 and lizard Anolis carolinensis AcBD1422. Obviously, these defensins have six, instead of three, intramolecular disulfide bridges, but the functional significance remains to be studied.

Notably, we also observed extensive fusion of adjacent exons in the crested ibis β-defensins (Table 2), creating three different gene organization patterns (including the numbers and locations of introns/exons) ranging from two exons (typical for human β-defensins) to four exons (typical for the avian β-defensins), as illustrated in Figure 2b. Classical AvBD genes are constituted by four exons (four-exon organization; Figure 2c), in which exon 1 corresponds to the 5'UTR, the two internal exons encode the signal, propiece (absent in some genes), and mature peptides, and exon 4 includes the C-terminal sequence of the mature peptide and the 3'UTR. In some defensins (AvBD5, 9, 10), the first two exons fused, leaving only three exons (three-exon organization). Moreover, for AvBD12 and 13, not only the first two but also the last two exons fused, resulting in “two-exon organization,” which perfectly resembles that of typical human β-defensins27. Additionally, we found one alternative mRNA splicing for AvBD5 (Table 2 and Figure 2b). Compared with the transcript AvBD5-1, the other transcript AvBD5-2 lacks the corresponding exon 1, but its exon 2 instead extends towards intron 1 for 29 bp spliced in AvBD5-1. Nevertheless, the two splice variants only differ in 5'UTR and thus share the same translated sequence.

### Table 1 | Conditions for constructing the 4D-PCR and reverse-4D libraries

| Phase                        | 4D-PCR library                  | Reverse-4D library                  |
|------------------------------|---------------------------------|-------------------------------------|
| Cell concentration in low melting point agarose | 5 × 10^6 cells/mL               | 2.5 × 10^6 cells/mL                 |
| Parameters in pulse field gel electrophoresis | pulse time 1–40 s, 6 V/cm for 13 h | 1st step: pulse time 1–40 s, 6 V/cm, 13 h |
| DNA concentration in ligation | −6 ng/μL gDNA, 0.5 ng/μL vector | 2nd step: pulse time 3–5 s, 5 V/cm, 8 h |
| Parameters in electroporation | 100 Ω, 1.5 kV, 25 μF            | ~2 ng/μL gDNA, 0.5 ng/μL vector     |
|                              |                                 | 200 μl, 1.5 kV, 25 μF               |

### Table 2 | β-defensin genes in the crested ibis

| Exon 1   | Exon 2 | Exon 3 | Exon 4 |
|----------|--------|--------|--------|
| AvBD1    | 14688–16253 | /b     | 113    | 265 |
| AvBD2    | 22992–24182 | /b     | 151    | 457 |
| AvBD10   | 68066–71073 | /b     | 133    | 133 |
| AvBD11   | 77583–81637 | /b     | 131    | 130 |
| AvBD8    | 83676–86995 | 5     | 91     | 134 |
| AvBD7    | 93568–95300 | 5     | 86     | 127 |
| AvBD12   | 101437–104896 | 66    | 79     | 121 |
| AvBD13   | 109609–112581 | 84    | 88     | 127 |
| AvBD14   | 114639–117374 | 131   | 88     | 127 |
| AvBD15   | 119977–123234 | 80    | 88     | 109 |
| AvBD16   | 125566–127120 | 224   | 87     | 127 |
| AvBD17   | 125566–126799 | /b    | 116    | 127 |
| AvBD18   | 132130–135514 | 99    | 87     | 127 |
| AvBD19   | 142664–143208 | N/A   | 55     | 125 |

*aGene locations were annotated by sequenced full-length mRNA.

*bIndicates exon fusion with the adjacent exon.

The AvBD11 and AvBD14 mRNA sequences were not obtained from the liver and spleen so that only their coding sequences were predicted using GeneWise and chicken AvBD11 and AvBD14 amino acid sequences.

*N/A means not available or not applicable (i.e., without untranslated regions).

Two AvBD5 splice variants were obtained from rapid amplification of cDNA ends.

### Comparative genomic analyses

The genomic organizations of whole β-defensin gene clusters in the chicken27, duck24, zebra finch35, and crested ibis are shown in Figure 3a–3d, and dot plot comparisons between the crested ibis and other birds are shown in
Figure 4a–4c. First, compared to the duck (19 genes within 103 kb, 0.184 genes/kb) 34, zebra finch (22 genes within 127 kb, 0.173 genes/kb) 35, and chicken (14 genes within 82 kb, 0.171 genes/kb) 27, the gene density within the \(b\)-defensin cluster is remarkably low in crested ibis (14 genes within 128 kb, 0.109 genes/kb).

Second, a series of gene duplication events were identified in these species (replicated blocks, Figure 4a–4c), including several species-specific duplications and one common duplication (\(AvBD1\) vs. \(AvBD3\)), also supported by the relative gene locations (Figure 3a–3d). The detection of two \(AvBD7\) copies (\(AvBD6\) and \(AvBD7\)) in chicken (Figure 4a), seven \(AvBD3\) copies (\(AvBD3S1\), \(AvBD3A–3F\)) in duck (Figure 4b), and three \(AvBD1\) copies (\(AvBD123–AvBD125\)) and nine \(AvBD3\) copies (\(AvBD115–AvBD122\)) in zebra finch (Figure 4c) were all in accordance with previous reports 27,34,35.

Third, \(AvBD14\), which is absent from zebra finch 35, was identified in the chicken 27 and duck 34 as well as the crested ibis. In fact, we found that Hwamei (Passeriforme) also lost \(AvBD14\) (unpublished data). Furthermore, the phylogenetic analysis in the next section shows that \(AvBD14\) is a single copy orthologous gene among these three birds (Figure 5). Thus, we predicted that the absence of this gene may be exclusive to Passeriformes. Collectively, this study reveals that gene gain and loss have occurred widely during the evolution of the avian defensin gene clusters. Except for the different gene duplications found in the four birds, \(b\)-defensin gene clusters are generally conserved with nearly identical gene order and orientation ranging from \(AvBD13\) to \(AvBD14\) on the gene map (Figure 3a–3d) with considerable sequence homology (unbroken diagonal lines, Figure 4a–4c).

Interestingly, we noticed that the GC content of the regions corresponding to the subfamily \(AvBD1/AvBD3\) in all species and the subfamily \(AvBD7\) in chicken, where gene duplication events occurred, is generally greater than in non-duplicated areas of each species, especially in duck (Figure 3b) and zebra finch (Figure 3c). We further compared the average GC content between the duplicated and non-duplicated regions in each bird and found significant differences (\(P < 0.005\)) (Supplementary Table S1). Earlier studies reported a positive correlation between duplication/recombination and GC content 39,40, which implies that the frequent duplication...
events in certain avian defensins, especially in the AvBD1/AvBD3 subfamily, might be associated with the high GC content in these regions. The AvBD5 region shows the highest GC content in chicken, zebra finch, and crested ibis but has an obviously low GC percentage in duck, suggesting this segment has species-specific GC content variation. Actually, besides the duplicated defensin gene regions, we also detected significantly higher \( P, 0.005 \) GC content in the tRNA clusters located between AvBD10 and AvBD11 in the four species (Figure 3a–3d) (Supplementary Table S1), within which extensive duplications were also detected by dot plots (Figure 4a–4c). According to the predicted results, these tRNA genes (4 in chicken, 8 in duck, 5 in zebra finch, and 7 in crested ibis) are mainly responsible for transferring tryptophan (hydrophobic) and arginine (cationic), which are typical for defensins 12, 20, indicating the coevolution of defensin and tRNA genes via physical proximity.

**Evolutionary analyses.** In Bayesian and maximum-likelihood (ML) phylogenetic analyses (Figure 5), genes from the same subfamilies were clustered with relatively high posterior probabilities (or bootstrap values), most of which were \( >90\% \) (bootstrap values \( >90\% \)). Meanwhile, except for \( 1:1:1:1 \) orthologs in the AvBD2, 4, 5, 8, 9, 10, 11, 12, and 13 clusters, the other clusters exhibit single-copy orthologs among 2–3 birds and/or extra paralogs in the remaining species (Figure 5). Based on the strong orthologous signals and the frequent gene gain and loss, we propose that the avian defensin genes evolved by the birth-and-death process, which was suggested to be an important evolutionary mode for multigene families, especially for immune system genes 41, and has been reported for the mammalian 42 and reptilian \( \beta \)-defensins 22.

In both phylogenetic trees, the AvBD1 and AvBD3 lineages are grouped into a large subfamily, which further supports the historical gene duplication event detected in Figure 4a–4c. For AvBD1, chicken and duck present one copy each while crested ibis and zebra finch show two and three copies, respectively (Figure 5). Nonetheless, the three zebra finch AvBD1s are grouped into one intra-species cluster while the crested ibis AvBD1s and 1f are separated into one basal primitive branch of the all-AvBD1 clade and another group with the chicken and duck AvBD1s (Figure 5), suggesting one ancient duplication in the crested ibis AvBD1 and two recent duplications in the zebra finch AvBD1. For AvBD3, we noticed a sharp contrast in gene distribution pattern among species; chicken and crested ibis have only one copy AvBD3 while duck and zebra finch have many duplicated AvBD3s (Figure 5). In addition, AvBD6 is unique to chicken and shows a high degree of pairing with Gagua-AvBD7.
Figure 4 | Dot plot analysis of defensin clusters. Crested ibis vs. (a) chicken, (b) duck, and (c) zebra finch. Positions of the genes for each species are indicated on the corresponding axes (colors as in Figure 3). Defensin (red boxes) and tRNA cluster (purple boxes) duplications are depicted as replicated blocks between syntenic regions.
Figure 5 | Phylogenetic relationship of avian β-defensins. AvBD genes from chicken (Gallus gallus, Gaga), zebra finch (Taeniopygia guttata, Tagu), duck (Anas platyrhynchos, Anpl), and crested ibis (Nipponia Nippon, Nini; grey). Numbers beside nodes indicate maximum-likelihood bootstrap supports/Bayesian posterior probabilities (values > 50 only). The AvBD12 lineage branch is truncated by a dash. Green lizard (Anolis carolinensis, Anca) AcBD15 is included as the outgroup.
(Figure 5), indicating that the AvBD6 gene was duplicated from AvBD7 recently and exclusively in Galliformes. As a result, gene duplication further enlarged and diversified the repertoire of β-defensins in different birds.

For orthologous genes among the four birds, the pattern of purifying selection was supported by a mean ω (overall dS/dD ratio) < 1 in all defensin loci and further supported by all the pairwise calculated ω < 1, with exception for comparison of AvBD2 between the crested ibis and zebra finch (Table 3). This implies that the β-defensins may have mainly evolved under relaxed purifying selection in Aves. Furthermore, the signal peptide coding regions generally have higher ω values than the mature peptides, indicating that the mature peptides have relatively greater sequence conservation within each gene lineage, which may be critical for maintaining specific structure or function for each orthologous defensin gene.

### Discussion

The present study constructed a 4D-PCR library with a relatively small insert size and a reverse-4D library with an elevated average insert size, which is attributed to our condition optimization (Table 1). First, we decreased the number of cells embedded in the low melting point agarose in order to reduce the DNA concentration and increase separation efficiency for small DNA fragments by pulse field gel electrophoresis (PFGE), thus decreasing the amount of short fragments and facilitating inclusion of large DNA fragments into vectors. Second, compared to the PFGE process in the construction of 4D-PCR library, we performed an additional PFGE step for 8 hours (3–5 s pulses at 5 V/cm), which further eliminated small DNA fragments. Third, the electrical resistance was increased to 200 Ω, thereby elongating the pulse time for electrotansformation, which may promote the uptake of large BACs.

Due to the difficulty of acquiring samples from endangered and rare species, it is important to store genetic resources in genomic DNA libraries. Thus, this study provides optimized conditions of library construction and increases the convenience of building large-insert BAC libraries for other species. Because very few molecular or genomic studies have been performed in the crested ibis, the BAC libraries presented here are not only key for this work but also provide a solid foundation for future species protection based on genomic features of the crested ibis.

In this study, we observed extensive fusion in the crested ibis β-defensins (Figure 2b and Table 2), ranging from the classical four-exon structure (Figure 2c) to mammal-like two-exon organization. In chicken, only AvBD12 presents fusion of the last two exons (resultant three-exon structure), while all the other defensin genes consist of four exons. In the green lizard, at least seven different gene organization patterns also ranging from two to four exons were reported, including the exact “four-exon organization” (AcBD7, 12, and 26), “three-exon organization” (most AcBDs) and “two-exon organization” (AcBD17, 21, 22, 31, and 30b). Such variability of gene organization has also been reported in many invertebrates and was proven to be a result of exon-shuffling during evolution. Exon-shuffling by intronic recombination plays an important role in rapid construction of new genes encoding multidomain proteins during evolution. It was previously speculated that the two-exon human β-defensins may evolve through exon-shuffling, and this method is presumably adaptive since it allows β-defensins to mobilize faster to efficiently defend against pathogens.

We also observed two splice variants for the crested ibis AvBD5 (Figure 2a–2b). Such alternative splice patterns have been revealed in the green lizard (AcBD13a/b and AcBD18a/b), and the authors suggested that the first exons in both variants probably associate with different proximal promoters, which may contribute to tissue- or time-specific gene expression regulation. Actually, four different alternative splice patterns were found for the green lizard β-defensins, which is undoubtedly a critical mechanism for generating new molecular diversity in β-defensin genes. Thus, exon fusion and alternative splicing might play special roles in defensin evolution and innate immunity of the crested ibis.

The Bayesian and ML trees show that the crested ibis AvBD1xz has a closer phylogenetic relationship to the AvBD1 genes of the other three birds, while AvBD11f forms a basal primitive branch of the whole AvBD1 lineage (Figure 5). This implies that after the duplication between ancestral AvBD1 and AvBD3 lineages, the AvBD1 was further duplicated before the four birds diverged, which generated the two copies AvBD1xz and AvBD1f1. However, AvBD1f1 was later lost during the subsequent avian evolution history and has so far only been identified in the crested ibis genome. Consequently, the crested ibis defensins provide insight into ancient avian defense structure; in addition to the previously suggested 13 ancestral avian β-defensins including AvBD1–5 and AvBD7–14, we propose that the ancient Neognathae may also have AvBD1f1, which needs verification in other avian orders.

The crested ibis β-defensins, first characterized in this study, will be helpful for protecting this endangered bird in the two following aspects. In recent years, there has been an increasing number of reports regarding infectious diseases in the crested ibis, including septicemia, newcastle, Salmonella infection, Escherichia coli infection, avian influenza, and others, which implies that this rare bird is at increasingly high risk under variable pathogen pressures. Identification of defensin genes responsive to certain pathogens has been conducted in many avian studies but never in crested

### Table 3 | dS/dD values calculated for orthologous β-defensin genes in chicken, zebra finch, duck, and crested ibis

| Gene       | Crested ibis vs. Chicken | Crested ibis vs. Duck | Crested ibis vs. Zebra finch | Chicken vs. Zebra finch | Duck vs. Zebra finch | All | Signal peptide | Mature peptide |
|------------|--------------------------|-----------------------|-----------------------------|-------------------------|----------------------|-----|----------------|----------------|
| AvBD2      | 0.253                    | 0.167                 | 1.925                      | 0.197                   | 0.191                | 0.260 | 0.244         | 0.239          |
| AvBD4      | 0.533                    | 0.480                 | 0.732                      | 0.707                   | 0.484                | 0.586 | 0.984         | 0.461          |
| AvBD5      | 0.339                    | 0.278                 | 0.190                      | 0.426                   | 0.407                | 0.279 | 1.062         | 0.135          |
| AvBD7      | 0.357                    | 0.412                 | 0.480                      | 0.157                   | 0.464                | 0.353 | 0.541         | 0.273          |
| AvBD8      | 0.200                    | 0.200                 | 0.285                      | 0.242                   | 0.238                | 0.245 | 1.053         | 0.167          |
| AvBD9      | 0.256                    | 0.824                 | 0.158                      | 0.482                   | 0.184                | 0.303 | 0.122         | 0.371          |
| AvBD10     | 0.264                    | 0.096                 | 0.248                      | 0.285                   | 0.414                | 0.240 | 0.624         | 0.168          |
| AvBD11     | 0.325                    | 0.433                 | 0.171                      | 0.518                   | 0.222                | 0.302 | 0.609         | 0.232          |
| AvBD12     | 0.411                    | 0.148                 | 0.655                      | 0.301                   | 0.309                | 0.294 | 0.798         | 0.188          |
| AvBD13     | 0.291                    | 0.443                 | 0.341                      | 0.290                   | 0.384                | 0.345 | 0.395         | 0.311          |
| AvBD14a    | 0.292                    | 0.513                 | /                          | 0.423                   | /                    | 0.410 | 0.644         | 0.204          |
| Mean       | 0.320                    | 0.363                 | 0.517                      | 0.366                   | 0.330                | 0.329 | 0.643         | 0.250          |

*dS/dD values of AvBD14 were only calculated among chicken, duck, and crested ibis.

The dS/dD values greater than 1 are in bold.
This study determines crested ibis β-defensin genes in detail and thus makes it possible to conduct pathogen-associated analysis and further help this endangered bird better cope with pathogen invasion. Although the number of crested ibis individuals has expanded greatly, the severe threat of high mortality rates during the pre- and post-hatch periods, largely caused by pathogen infection, has emerged due to extreme inbreeding. This could be mainly attributed to the immature immune system of the developing embryos and newly hatched chicks, since components of the adaptive response are in a naïve stage. Many avian studies have demonstrated that avian defensins can protect embryos and nestlings from potential pathogenic assault and promote the early transition from an innate immune response to an adaptive one. Thus, some studies utilized defensin genes as genetic markers to select for resistance to certain infections and thereby enhance the innate immune response via selective breeding in poultry, while some others adopted artificially regulated defensin expression via maternal diet, for example, to control the selective colonization of birds and thereby suppress pathological micrornas. These findings all provide conservation implications for scientists and managers, e.g., methods for using defensin-based genetic management and dietary regulation to reduce the early mortality rates of crested ibis, especially in captive populations.

Methods

Ethics statement. The samples used in this study, including blood, liver and spleen, were collected from two captive individuals with permission from the Crested Ibis Breeding Centers (CIBC) and the Department of Wildlife Conservation, State Forestry Administration. The blood sample was obtained from the wing vein of a female individual bred in CIBC, Louguantai County, Shaanxi Province, China and was performed in cooperation with technical staff from the center with utmost care. The female individual bred in CIBC, Louguantai County, Shaanxi Province, China and post-hatch periods, largely caused by pathogen infection, has thus made it possible to conduct pathogen-associated analysis and thereby enhance the innate immune response via selective breeding in poultry, while some others adopted artificially regulated defensin expression via maternal diet, for example, to control the selective colonization of birds and thereby suppress pathological micrornas. These findings all provide conservation implications for scientists and managers, e.g., methods for using defensin-based genetic management and dietary regulation to reduce the early mortality rates of crested ibis, especially in captive populations.

Construction of the BAC Library. A routine BAC genomic DNA library was generated with DNA from the blood sample. The construction of this library was based on a previous protocol with a few modifications using a pC1BAC vector (Epigenesis Technologies). The library was arranged for rapid screening by 4D-PCR. A reverse-4D BAC library was generated and characterized following a previous protocol, with some conditions optimized (Table 1). Three different restriction enzymes (MboI, EcoRI, and HindIII) were used separately to form DNA libraries using the MboI restriction enzyme. The library was arrayed for rapid screening (Epicentre) and MboI restriction enzyme. The library was arrayed for rapid screening (Epicentre) and MboI restriction enzyme. The library was arrayed for rapid screening (Epicentre) and MboI restriction enzyme. The library was arrayed for rapid screening (Epicentre) and MboI restriction enzyme.

BAC screening and sequencing. Nine pairs of universal primers were used to perform PCRs with the crested ibis genomic DNA as template. Agarose gel electrophoresis on PCR products indicated that four primer pairs (primers for AvBD7, 7, 11, and 12) had derived products, among which the product of primers for AvBD7 (5’ to 3’ sequences: F: TTGATGCTTTTCTGGTTGAATGAC, R: TCACCTTTTTCGACAGAAAGATA) produced a single band of the expected size. To further confirm this identification, the product amplified by AvBD7 primers was processed by (1) isolation by agarose gel electrophoresis and purification by DNA gel extraction kit (Axygen), (2) ligation into pMD18-T vector (TaKaRa) and transformation into E. coli DH5α competent cells (TaKaRa), and (3) sequencing of the cloned products. Sequences were identified by BLAST as AvBD7 from the crested ibis.

BAC clones containing β-defensin loci were subsequently screened from both BAC libraries using the AvBD7 primers above. The PCR screening processes were performed as previously described. Insert sizes of positive clones were estimated by electrophoresis, and BAC end sequencing was performed. The end sequences of these β-defensin-containing clones were aligned with the available chicken β-defensin genomic sequences from NCBI to preliminarily determine whether the BAC clone contained the entire defensin cluster. Finally, the BAC clone was shotgun subcloned, sequenced, and assembled by commercial sequencing service (Majorbio). The full BAC sequence was deposited in GenBank (accession number: KM098134).

Chromosome localization of the crested ibis β-defensins. Metaphase chromosomes were prepared from the peripheral blood of the crested ibis according to the method described by Moorhead et al., and the BAC clone H46-1 was used as a probe. FISH was performed following the previously reported protocol. One hundred well-spread and distinct metaphases were karyotyped by scanning 15 slides, and the chromosome sizes were measured using ImageJ, including the total length of the macrochromosomes and the arm ratio (long arm/short arm, q/p). Chromosome numbering was performed according to the reported karyotype ideogram of crested ibis and the criteria recommended by Levan et al.

Sequence analyses. According to the order and orientation as well as the clustering results of phylogenetic analysis described below, the crested ibis defensin genes were named following the nomenclature proposed previously. The mRNA was extracted from liver, spleen, and brain of the pre- and post-hatch periods, largely caused by pathogen infection, has thus made it possible to conduct pathogen-associated analysis and thereby enhance the innate immune response via selective breeding in poultry, while some others adopted artificially regulated defensin expression via maternal diet, for example, to control the selective colonization of birds and thereby suppress pathological micrornas. These findings all provide conservation implications for scientists and managers, e.g., methods for using defensin-based genetic management and dietary regulation to reduce the early mortality rates of crested ibis, especially in captive populations.

1. Ding, C. Q. Research on the crested ibis. 1–388 (Shanghai Scientific and Technical Publishing House, 2008) in China.
2. Chen, S. L. et al. Phylogenetic and pathogenic analyses of two virulent Newcastle disease viruses isolated from Crested Ibis (Nipponia nippon) in China. Virus Genes 46, 447–453 (2013).
3. Zhang, B., Fang, S. G. & Xi, Y. M. Low genetic diversity in the endangered crested ibis Nipponia nippon and implications for conservation. Bird Conserv Int 14, 183–190 (2004).
4. Li, X. H. & Li, D. M. Current state and the future of the crested ibis (Nipponia nippon): a case study by population viability analysis. Ecol Res 13, 323–333 (1998).
5. Fan, G. L. et al. The histopathological observations of young crested ibis infected with Escherichia coli. Chinese J Zoooll 39, 44–46 (2005).
6. Xi, Y. M., Wood, C. L., Lu, B. Z. & Zhang, Y. M. Prevalence of a septicemia disease in the crested ibis (Nipponia nippon) in China. Avian Dis 51, 614–617 (2007).
7. Li, L. J. et al. Isolation and identification of LPAIV from crested ibis. Prog Vet Med 32, 42–44; 10.3969/j.issn.1007-5038.2011.02.010 (2011) (in Chinese).
8. He, Y. L. et al. The pathological observation of crested ibis death of Salmonellosis. Acta Vet Sci Chin 17, 1–14 (1988).
9. Shizuya, H. et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. P Natl Acad Sci USA 89, 8794–8797 (1992).
10. Osoegawa, K. et al. An improved approach for construction of bacterial artificial chromosomes. Genomics 52, 1–8 (1998).
11. Zhang, G. L. & Sunkara, L. T. Avian antimicrobial host defense peptides: from biology to therapeutic applications. Pharmaceuticals 7, 220–247 (2014).
12. Cuperus, T., Coorens, M., van Dijk, A. & Haagsman, H. P. Avian host defense peptides. Dev Comp Immunol 41, 352–369 (2013).
13. Klokstra, M. E. & Chang, T. L. Defensins in innate antimicrobial immunity. Nat Rev Immunol 6, 447–456 (2006).
14. Froy, O. & Gurevitz, M. Arthropod and mollusk defensins—evolution by exon-shuffling. Trends Genet 19, 684–687 (2003).
15. Thomma, B. P., Cammue, B. P. & Thevissen, K. Plant defensins. Planta 216, 197–202 (2002).
16. Lehrer, R. I. & Ganz, T. Defensins of vertebrate animals. Curr Opin Immunol 14, 96–102 (2002).
17. Chen, H. Q. et al. Recent advances in the research and development of human defensins. Peptides 27, 931–940 (2006).
18. Brogdon, K. A. Antimicrobial peptides: pore formers or metabolite inhibitors in bacteria. Nat Rev Microbiol 3, 238–250 (2005).

19. Grigat, J., Soruri, A., Forssmann, U., Riggert, J. & Zwirner, J. Chemoattraction of macrophages, T lymphocytes, and mast cells is evolutionarily conserved within the human alpha-defensin family. J Immunol 179, 3958–3965 (2007).

20. Selsted, M. E. & Ouellette, A. J. Mammalian defensins in the antimicrobial immune response. Nat Immunol 6, 551–557; 10.1038/nili2006 (2005).

21. Tang, Y. Q. et al. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated α-defensins. Science 286, 498–502 (1999).

22. Dalla Valle, L., Benato, F., Maistro, S., Quinzani, S. & Albardi, L. Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard Anolis carolinensis. Dev Comp Immunol 36, 222–229; 10.1016/j.dci.2011.05.004 (2012).

23. Maxwell, A. I., Morrison, G. M. & Dorin, J. R. Rapid sequence divergence in mammalian β-defensins by adaptive evolution. Mol Immunol 40, 413–421; 10.1016/s0161-5800(03)00160-3 (2003).

24. Bruhn, O., Paul, S., Tetens, J. & Thaller, G. The repertoire of equine intestinal α-defensins. BMC genomics 10, 631; 10.1186/1471-2164-10-631 (2009).

25. Belov, K. et al. Characterization of the opossum genome provides insights into the evolution of the mammalian immune system. Genome Res 17, 982–991 (2007).

26. Nguyen, T. X., Cole, A. M. & Lehrer, R. I. Evolution of primate β-defensins: a serpentine path to a sweet tooth. Peptides 24, 1647–1654 (2003).

27. Xiao, Y. J. et al. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5, 56; 10.1186/1471-2164-5-56 (2004).

28. Pazgier, M., Hoover, D. M., Yang, D., Lu, W. & Lukasiew, J. Human β-defensins. Cell Mol Life Sci 63, 1294–1313 (2006).

29. Schutte, B. C. et al. Discovery of five conserved β-defensin gene clusters using a computational search strategy. P Natl Acad Sci USA 99, 2129–2133 (2002).

30. Evans, E. W., Beach, G. G., Wunderlich, J. & Harmon, B. G. Isolation of antimicrobial peptides from avian heterophils. J Leukocyte Biol 56, 661–665 (1994).

31. Wang, R. Q. et al. Identification and characterization of an avian β-defensin orthologue, avian β-defensin 9, from quails. Appl Microbiol Biol 87, 1395–1405 (2010).

32. Ma, D. Y. et al. Functional analysis and induction of four novel goose (Anser cygnoides) avian β-defensins in response to salmonella enteritidis infection. Comp Immunol Microbiol Infect Dis 35, 197–207 (2012).

33. Lynn, D. J. et al. Avian beta-defensin nomenclature: a community proposed update. ImmunoLett 110, 86–89 (2007).

34. Huang, Y. H. et al. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. Nat Genet 45, 776–783 (2013).

35. Hellgren, O. & Ekblom, R. Evolution of a cluster of innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. Mol Ecol Resour 11, 686–692 (2011).

36. Brogden, K. A. Antimicrobial peptides: pore formers or metabolite inhibitors in bacteria. Nat Rev Microbiol 3, 238–250 (2005).

37. Hasenstein, J. R. & Spichal, I. Probiotics manipulate host cytokine response and induce antimicrobial peptides. Folia Microbiol 51, 507–510 (2006).

38. Hellgren, O. & Sheldon, B. C. Locus-specific protocol for nine different innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. Mol Ecol Resour 11, 686–692 (2011).

39. Moorshead, P. S., Nowell, P. C., Mellman, W. J., Batts, D. M. & Hungerford, D. A. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 20, 613–616 (1960).

40. Wang, Q. H. et al. Genome analysis and signature discovery for diving and sensory properties of the endangered Chinese alligator. Cell Res 23, 1091–1105 (2013).

41. Abramoff, M. D., Magalhães, P. J. & Ram, S. J. Image processing with Image. Biophotonics Int 11, 36–43 (2004).

42. Liu, L. Y., Wang, B. G., Guo, X. C. & Li, F. L. Chromosomal isolation of antimicrobial peptides from avian heterophils. J Leukocyte Biol 55, 661–665 (1994).

43. Trebicjaský, J. & Spichal, I. Probiotics manipulate host cytokine response and induce antimicrobial peptides. Folia Microbiol 51, 507–510 (2006).

44. Hasenstein, J. R., Zhang, G. L. & Lamont, S. J. Analyses of five gallinacin genes and the salmonella enterica serovar Enteritidis response in poultry. Infect Immun 74, 3375–3380 (2006).

45. Hasenstein, J. R. & Lamont, S. J. Chicken gallinacin gene cluster associated with Salmonella invasion in advanced intercellular response. Avian Dis 51, 561–567 (2007).