

# SCIENTIFIC REPORTS

OPEN

## A comprehensive analysis of the germline and expressed TCR repertoire in White Peking duck

Received: 15 September 2016

Accepted: 19 December 2016

Published: 30 January 2017

Zhi Yang<sup>1,2,\*</sup>, Yi Sun<sup>3,\*</sup>, Yonghe Ma<sup>1,\*</sup>, Zhenrong Li<sup>1</sup>, Yu Zhao<sup>4</sup>, Liming Ren<sup>1</sup>, Haitang Han<sup>1</sup>, Yunliang Jiang<sup>3</sup> & Yaofeng Zhao<sup>1</sup>

Recently, many immune-related genes have been extensively studied in ducks, but relatively little is known about their TCR genes. Here, we determined the germline and expressed repertoire of TCR genes in White Peking duck. The genomic organization of the duck TCR $\alpha/\delta$ , TCR $\gamma$  and unconventional TCR $\delta 2$  loci are highly conserved with their counterparts in mammals or chickens. By contrast, the duck TCR $\beta$  locus is organized in an unusual pattern, (V $\beta$ )<sub>n</sub>-D $\beta$ -(J $\beta$ )<sub>2</sub>-C $\beta$ 1-(J $\beta$ )<sub>4</sub>-C $\beta$ 2, which differs from the tandem-aligned clusters in mammals or the translocon organization in some teleosts. Excluding the first exon encoding the immunoglobulin domain, the subsequent exons of the two C $\beta$  show significant diversity in nucleotide sequence and exon structure. Based on the nucleotide sequence identity, 49V $\alpha$ , 30V $\delta$ , 13V $\beta$  and 15V $\gamma$  unique gene segments are classified into 3V $\alpha$ , 5V $\delta$ , 4V $\beta$  and 6V $\gamma$  subgroups, respectively. Phylogenetic analyses revealed that most duck V subgroups, excluding V $\beta$ 1, V $\gamma$ 5 and V $\gamma$ 6, have closely related orthologues in chicken. The coding joints of all cDNA clones demonstrate conserved mechanisms that are used to increase junctional diversity. Collectively, these data provide insight into the evolution of TCRs in vertebrates and improve our understanding of the avian immune system.

Conventional T cell receptors (TCRs) are disulfide-linked heterodimers comprising either  $\alpha$  and  $\beta$  chains or  $\gamma$  and  $\delta$  chains. All four types of TCR chains are trans-membrane molecules that contain antigen-binding variable (V) domains and membrane-proximal constant (C) domains. The V domains of TCR $\beta$  and TCR $\delta$  are assembled via somatic recombination of variable (V), diversity (D) and joining (J) gene segments, whereas the rearranged V and J segments encode the V domains of TCR $\alpha$  and TCR $\gamma$ <sup>1</sup>. Based on the combinations of TCR heterodimers, conventional T cells can be divided into two major lineages:  $\alpha\beta$  T cells and  $\gamma\delta$  T cells. The  $\alpha\beta$  T cells mainly assist in immunoglobulin (Ig) production and cytolytic T cell responses. Their  $\alpha\beta$ TCR complexes bind to the peptide antigens presented by major histocompatibility complex (MHC) or MHC-like molecules<sup>2</sup>. By contrast,  $\gamma\delta$  T cells constitute a heterogeneous T cell population with multiple functions. Some  $\gamma\delta$ TCR complexes can recognize antigens presented by MHC molecules, whereas other  $\gamma\delta$ TCRs appear to bind directly to free antigens, similar to the recognition manner utilized by Igs<sup>3</sup>. The frequencies and physiological distributions of  $\gamma\delta$  T cells differ among diverse species. In adult humans, mice and dogs,  $\gamma\delta$  T cells make up less than 5% of the peripheral T cells (“ $\gamma\delta$  low” species)<sup>3,4</sup>. However,  $\gamma\delta$  T cells constitute more than 20% of the peripheral T cells in artiodactyls, rabbits and chickens (“ $\gamma\delta$  high” species)<sup>5–8</sup>. Recently, unconventional TCR chains that use Ig-like V domains have been discovered in a few distantly related vertebrate species. These unconventional TCR chains include TCR $\delta$  that uses VH $\delta$  and is found in amphibians, birds, and duckbill platypus<sup>9–11</sup>, the NAR-TCR found in cartilaginous fish<sup>12</sup>, and the TCR $\mu$ , which is only found in nonplacental mammals<sup>13,14</sup>.

As the representative of the anseriform birds, ducks split from the related chicken approximately 65–70 million years ago<sup>15</sup>. Moreover, the duck is not only one of the most economically important waterfowl, but is also a particularly good animal model for research in immunology because it serves as a natural reservoir of influenza A

<sup>1</sup>State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, 100193, China. <sup>2</sup>Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100193, China. <sup>3</sup>Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Taian, 271018, China. <sup>4</sup>National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, 100101, China. \*These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Y.J. (email: ylijiang723@aliyun.com) or Y.Z. (email: yaofengzhao@cau.edu.cn)

viruses and carries all 16 haemagglutinin and 9 neuraminidase subtypes<sup>16</sup>. Typically, ducks do not show apparent signs of disease upon infection with many strains of highly pathogenic H5N1, making them a Trojan horse for the maintenance of H5N1 in nature<sup>17,18</sup>. Recently, the molecular basis of the natural resistance of ducks to influenza infection has become a hot topic in avian immunology. Numerous innate immune-related genes, such as *RIG-I* (also called *DDX58*)<sup>19</sup>, *RNF135*<sup>20</sup>, and gene families of *IFITM*, *BTNL*, and  $\beta$ -*defensin*<sup>21,22</sup>, as well as the repertoire, expression and function of Ig isotypes<sup>23</sup>, have been extensively studied in ducks, but little is known about the duck TCR genes. In this study, we report the detailed genomic organization and repertoire diversity of all TCR loci in White Peking duck, including three conventional TCR loci (TCR $\alpha/\delta$ , TCR $\beta$  and TCR $\gamma$ ) and the recently discovered TCR $\delta 2$  locus, providing a theoretical basis for further understanding of the avian adaptive immune system as well as the evolutionary relationships of TCRs in vertebrates.

## Results

**Genomic organization and germline repertoire of duck TCR genes.** *TCR $\alpha/\delta$  locus.* Based on the mallard TCR $\alpha$  cDNA sequence (accession number AF323922), we first identified a C $\alpha$  gene-positive BAC clone, DHS1503D01. According to the end sequence of DHS1503D01, another BAC clone, DHS1008P13, was found to overlap the 5' portion of DHS1503D01 and contain the C $\delta$  gene. An analysis of the two BAC sequences showed that the  $\delta$  locus was located within the  $\alpha$  locus, resembling the genomic organization of the TCR $\alpha/\delta$  locus in other tetrapods (Fig. 1a).

The duck C $\alpha$  and C $\delta$  genes were encoded by three exons that successively encoded the Ig domain, connecting peptide (Cp), and transmembrane-cytoplasmic (Tm-Ct) domain, all of which contained the three conserved cysteines required for intra- and inter-chain disulfide bond formation and the conserved lysine and arginine residues responsible for the interaction with other TCR dimers (Fig. 2a and b). A comparison of the amino acid sequences of duck C $\alpha$  and C $\delta$  with the corresponding sequences of other vertebrate species revealed maximum identity levels (71.6% for C $\alpha$  and 66.2% for C $\delta$ ) between the duck and chicken, but less than 35% identity between the duck and other animal species. One and five potential N-glycosylation sites were identified in duck C $\alpha$  and C $\delta$ , respectively (Fig. 2a and b). At least 68 functional J $\alpha$  segments were identified between the C $\delta$  and C $\alpha$  genes, and at least two D $\delta$  and two functional J $\delta$  segments were found upstream of the single C $\delta$  gene (Supplementary Fig. S1A and B).

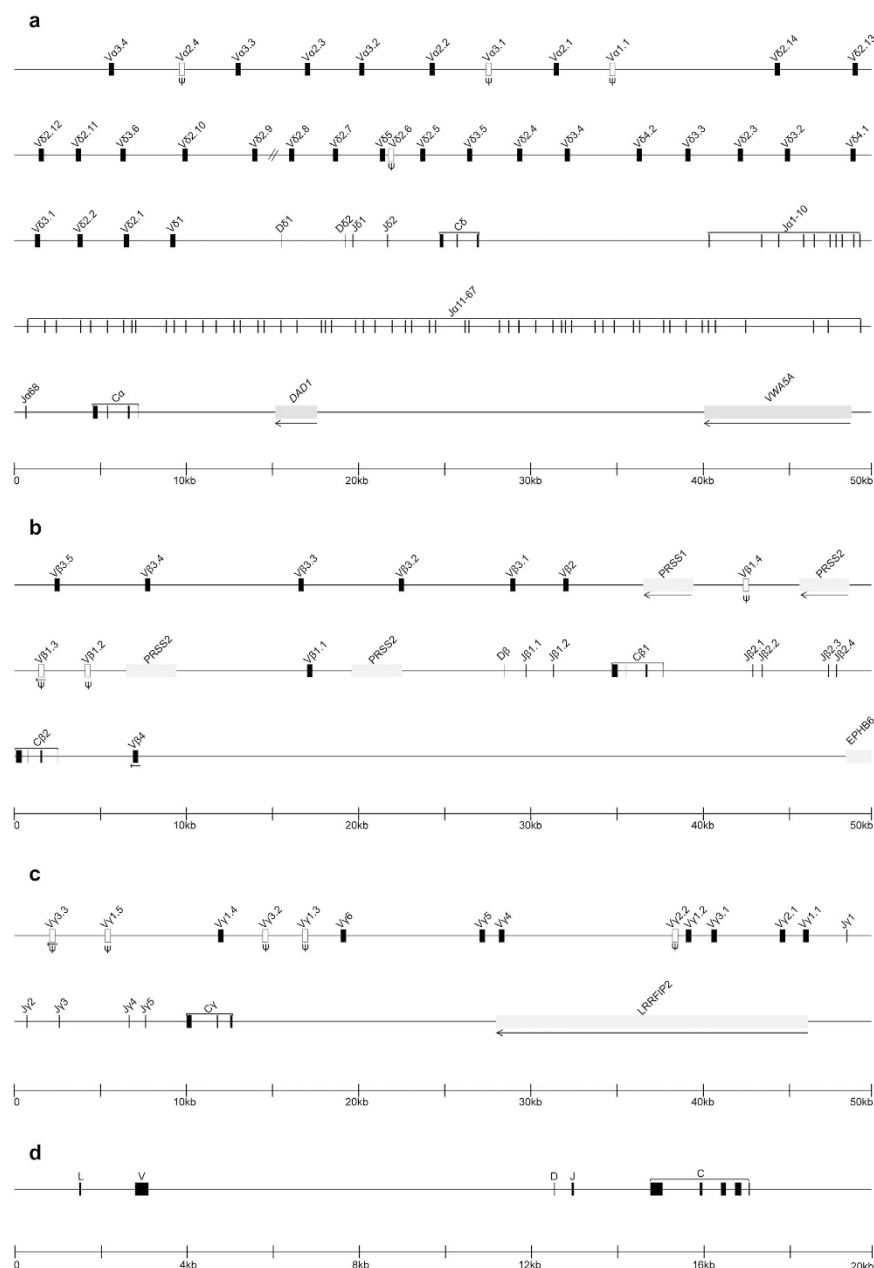
Within the BAC sequences, we further identified 33 V segments, all of which were located 5' upstream of the first D $\delta$  segment (Fig. 1a). When the nucleotide identity was compared with the V segments defined in other species, the V segments could be further categorized into one of two distinct groups, 9 V $\alpha$  at the 5' end and 24 V $\delta$  located downstream of the V $\alpha$  group. Of the 33 V segments, four were found to be pseudogenes due to non-sense mutations (V $\alpha$ 2.4 and V $\alpha$ 3.1), frameshift (V $\alpha$ 1.1) or the absence of the exon encoding the leader peptide (V $\delta$  2.6). Using 5' RACE, 40 extra V $\alpha$  and 6 extra V $\delta$  segments were detected in the cDNA clones, indicating that the current TCR $\alpha/\delta$  locus is incomplete. Based on the criterion that V segments belonging to the same subgroup should share 75% or greater nucleotide identity<sup>24</sup>, a total of 49 (9 + 40) V $\alpha$  segments could be grouped into three subgroups (V $\alpha$ 1 to V $\alpha$ 3) (Table 1) (Supplementary Fig. S2A), and the total of 30 (24 + 6) V $\delta$  segments were categorized within five subgroups (V $\delta$ 1 to V $\delta$ 5) (Table 1). The V $\delta$ 2 appeared to be the largest V $\delta$  subgroup, consisting of 20 V $\delta$  segments (Supplementary Fig. S2B). Members within a V $\alpha$  or V $\delta$  subgroup exhibited more than 76.4% or 75.1% nucleotide identity. Within a subgroup, each V $\alpha$  or V $\delta$  segment cloned from 5' RACE displayed 76.4% to 96.9% or 75.1% to 96.0% nucleotide identity with the remaining V $\alpha$  or V $\delta$  segments, respectively. The only exception is V $\alpha$ 3.16, which displayed 97.1% nucleotide identity with the pseudogene V $\alpha$ 3.1. Since the V $\alpha$ 3.16 is functional, it was considered as a novel V $\alpha$  segment.

Dot plot analyses indicated that both V $\alpha$  and V $\delta$  regions had undergone multiple duplications (Supplementary Fig. S4A). The current incomplete V $\alpha$  region originated from tandem duplications of a homology unit containing one V $\alpha$ 2 and one V $\alpha$ 3 segment (Supplementary Fig. S4B). The V $\delta$  region contained several ~4 kb repeated units, which were composed of V segments from V $\delta$ 2 and V $\delta$ 3 subgroups (Supplementary Fig. S4C).

We also performed genomic Southern blotting using probes from C and selected V subgroups. The detection of only one band with the C $\alpha$  probe verified that there was only a single copy of the C $\alpha$  gene in the duck genome (Supplementary Fig. S3A). However, one dark and two light bands were detected when the enzyme *Pst* I and the C $\delta$  probe were used, indicating that another C $\delta$ -like gene might be located outside the TCR $\alpha/\delta$  locus (Supplementary Fig. S3B), resembling the second TCR $\delta$  locus identified in chicken and zebra finch, as discussed later. The number and intensity of hybridizing bands substantiated the presence of larger number of V $\alpha$ 3 and V $\delta$ 2 segments in the genome. However, compared with the number of V segments obtained thus far, more bands were detected using the V $\alpha$ 1 and V $\delta$ 5 probes, suggesting the presence of additional germline members within the V $\alpha$  1 and V $\delta$ 5 subgroups. (Supplementary Fig. S3A and B).

*TCR $\beta$  locus.* According to the mallard TCR $\beta$  cDNA sequence (accession number AY039002), a C $\beta$  gene-positive BAC clone, DHS0801D24, was identified and sequenced. Analysis of the BAC sequence revealed that the duck TCR $\beta$  D, J, and C genes were organized in a unique pattern, D $\beta$ -(J $\beta$ )<sub>2</sub>-C $\beta$ 1-(J $\beta$ )<sub>4</sub>-C $\beta$ 2 (Fig. 1b), in contrast to the tandem-aligned D-(J)<sub>n</sub>-C clusters in most mammals or the translocon organization with a greater number of J $\beta$  genes in some teleosts.

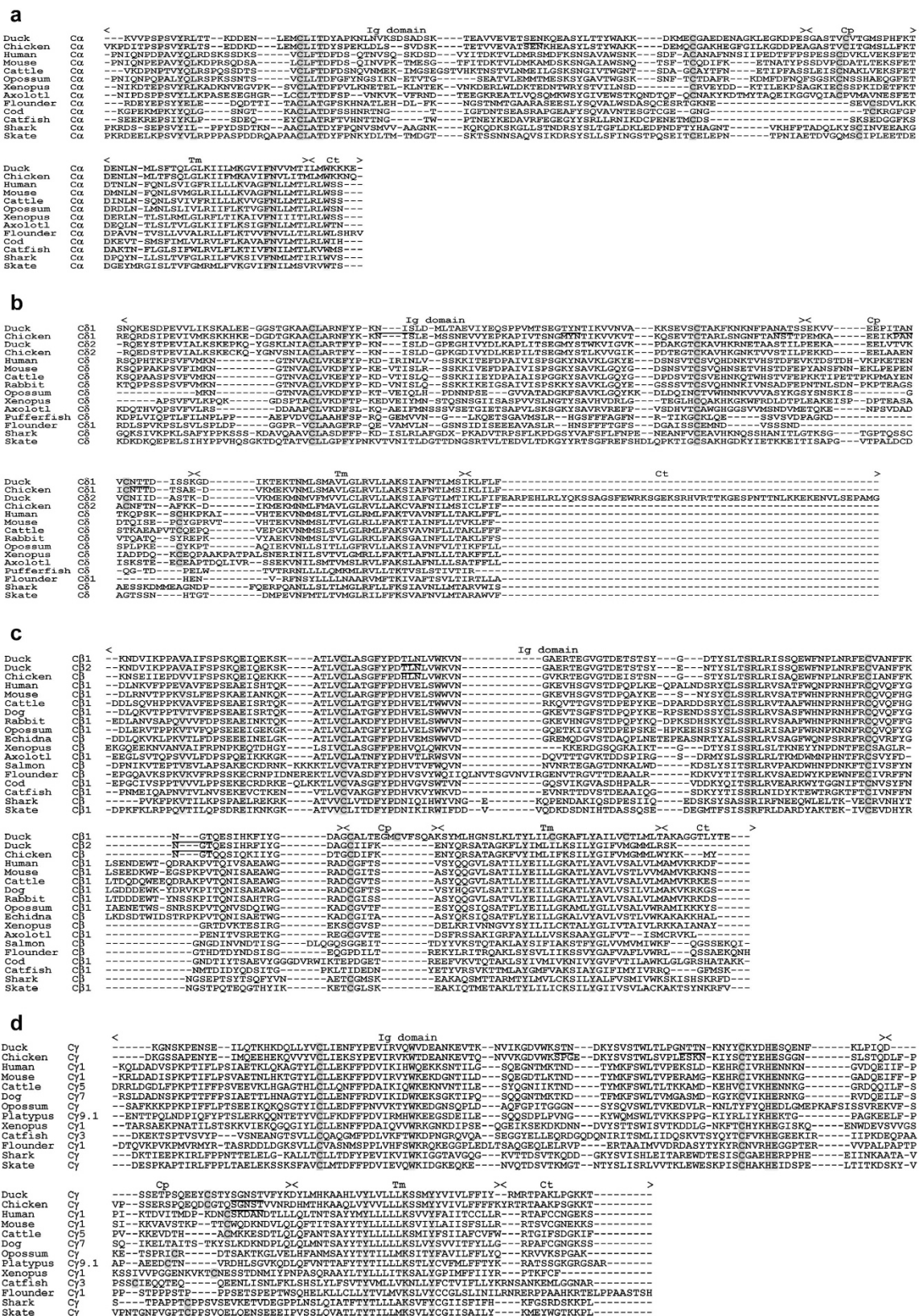
Both C $\beta$ 1 and C $\beta$ 2 genes consisted of four exons. The first exon of the two C $\beta$  genes, which encoded the Ig domain, was highly conserved with only three amino acid changes. However, the following exons were substantially divergent, with only 33% identity at the amino acid level. Maximal differences in length and nucleotide composition have been observed in exon 2, which was found to encode Cp. Exon 2 of C $\beta$ 1 encoded as many as 14 amino acids, whereas exon 2 of C $\beta$ 2 encoded only six amino acids. In C $\beta$ 2, both Tm and the cytoplasmic Ct domain were encoded by exon 3, and exon 4 contained only the 3' untranslated region (3' UTR). By contrast,



**Figure 1.** The genomic organization of duck TCR gene loci, TCR $\alpha/\delta$  (a), TCR $\beta$  (b), TCR $\gamma$  (c) and TCR $\delta 2$  (d). V, variable gene segment; D, diversity gene segment; J, joining gene segment; C, constant gene segment. V segments are named 3' to 5' with the subgroup number followed by the gene segment number if there were more than one member in the subgroup. Functional V segments are shaded, and pseudogenes are shown in hollow boxes and marked with a  $\psi$ . Non-TCR genes located in or flanking each TCR locus are shown in light grey. V segments and non-TCR genes with an opposite transcriptional orientation to the relevant C region are indicated by an arrow, and the sequence gap in (a) and (c) is marked as //.

exon 4 of C $\beta 1$  encoded eight extra amino acids, forming a longer Ct domain. In addition to the canonical cysteine required for intra- and inter-chain disulfide bond formation, C $\beta 1$  encoded three extra cysteine residues, one in the Cp domain and the other two in the Tm domain. The Ct domains of both C $\beta$  genes contained a lysine residue that was involved in the interaction with the CD3 complex. Two potential N-glycosylation sites were identified in both C $\beta 1$  and C $\beta 2$  (Fig. 2c). Southern blotting analysis further corroborated the presence of two C $\beta$  genes in the duck genome (Supplementary Fig. S3C). The single D $\beta$  segment had a 13-bp G-rich coding region that could be productively read in all three frames (Supplementary Fig. S1C). All six J $\beta$  segments were functional and shared less than 60% amino acid sequence homology (Supplementary Fig. S1C).

Upstream of the D $\beta$  gene, we identified ten V $\beta$  segments. Among them, three were pseudogenes due to in-frame stop codon. As in mammals, a single V $\beta$  gene (V $\beta 4$ ) with an inverted transcriptional orientation was located 3' downstream of C $\beta 2$  (Fig. 1b). The current TCR $\beta$  locus is also incomplete because two extra V $\beta$



**Figure 2.** Alignment of deduced amino acid sequences of TCR C regions in selected vertebrates, Cα (a), Cδ (b), Cβ (c) and Cγ (d). The different domains of the C region are indicated above the sequences. Dashes indicate gaps. Canonical amino acids are shaded, and the potential N-linked glycosylation sites are underlined.

|            | Subgroup     | Genome             | cDNA    |
|------------|--------------|--------------------|---------|
| V $\alpha$ | V $\alpha$ 1 | 1 (0) <sup>a</sup> | 4 (4)   |
|            | V $\alpha$ 2 | 4 (3)              | 16 (15) |
|            | V $\alpha$ 3 | 4 (3)              | 20 (19) |
| V $\delta$ | V $\delta$ 1 | 1 (1)              | —       |
|            | V $\delta$ 2 | 14 (13)            | 6 (6)   |
|            | V $\delta$ 3 | 6 (6)              | —       |
|            | V $\delta$ 4 | 2 (2)              | —       |
|            | V $\delta$ 5 | 1 (1)              | —       |
| V $\beta$  | V $\beta$ 1  | 4 (1)              | —       |
|            | V $\beta$ 2  | 1 (1)              | —       |
|            | V $\beta$ 3  | 5 (5)              | 2 (2)   |
|            | V $\beta$ 4  | 1 (1)              | —       |
| V $\gamma$ | V $\gamma$ 1 | 5 (3)              | 1 (1)   |
|            | V $\gamma$ 2 | 2 (1)              | —       |
|            | V $\gamma$ 3 | 3 (1)              | 1 (1)   |
|            | V $\gamma$ 4 | 1 (1)              | —       |
|            | V $\gamma$ 5 | 1 (1)              | —       |
|            | V $\gamma$ 6 | 1 (1)              | —       |

**Table 1. Number of V gene segments found in the genome and cDNA analyses.** <sup>a</sup>The numbers of functional genes are indicated in parenthesis.

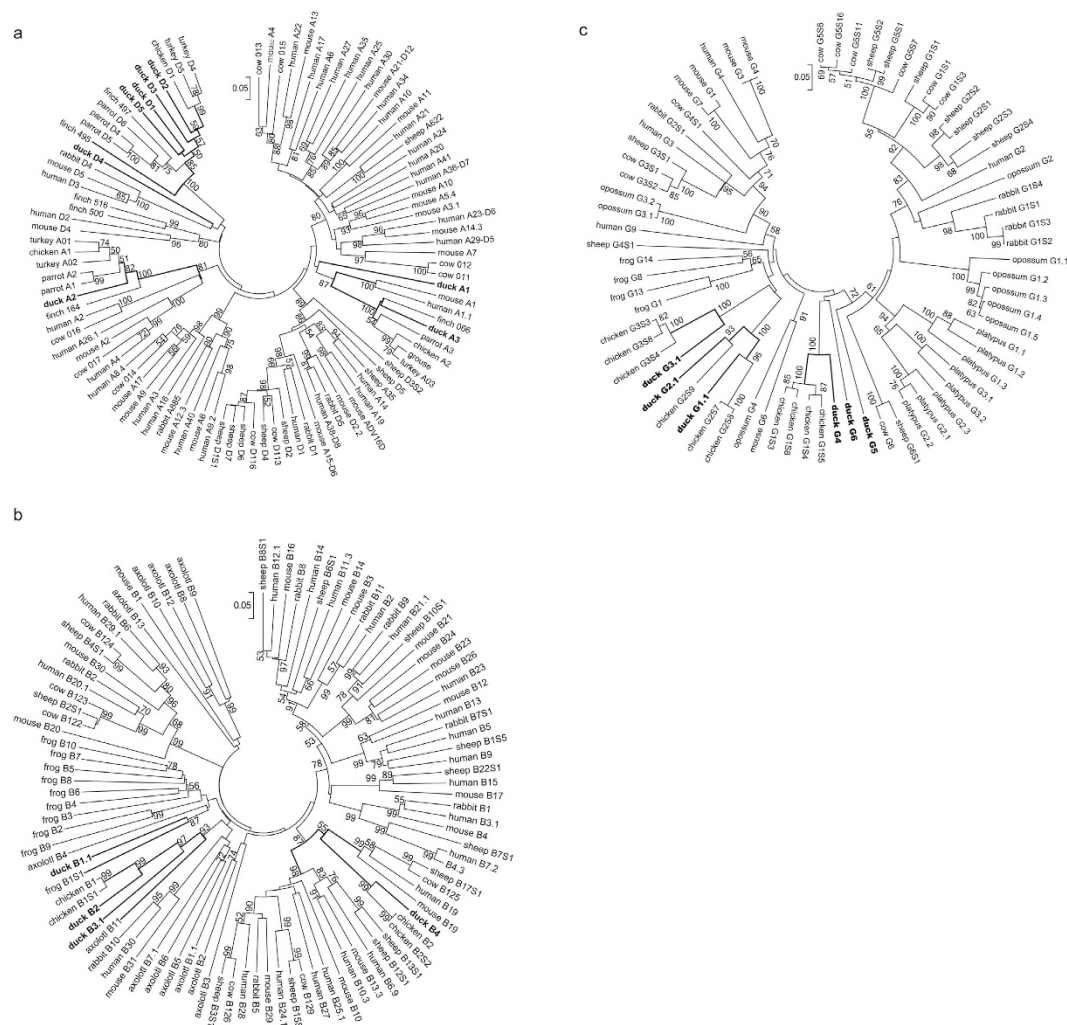
segments, designated as V $\beta$ 3.6 and V $\beta$ 3.7, were cloned from 5' RACE PCR. The total 13 duck V $\beta$  segments could be grouped into four subgroups (V $\beta$ 1 to V $\beta$ 4) (Table 1) (Supplementary Fig. S2C). Members within a V $\beta$  subgroup shared more than 91.4% nucleotide identity. The two V $\beta$  segments cloned from 5' RACE exhibited 91.4% and 95.3% nucleotide identity with the remaining V $\beta$ 3 members, respectively. Dot-plot matrix showed two regions containing tandem duplications, one corresponding to V $\beta$ 3 subgroup and the other comprising of three copies of a homology unit, in which a *PRSS2* gene and a V $\beta$ 1 segment are located (Supplementary Fig. S4D). Southern blotting analysis substantiated the presence of larger number of V $\beta$ 3 segments and smaller number of V $\beta$ 2 segments in the genome (Supplementary Fig. S3C).

**TCR $\gamma$  locus.** The BAC clone DHS0702G12 was isolated using primers designed to amplify the first exon of the mallard C $\gamma$  cDNA (accession number AF378702). BAC end sequencing demonstrated that this clone likely encompassed most of the duck TCR $\gamma$  locus. Shotgun sequencing of this BAC clone provided three contigs, contig 14 (5,853 bp), contig 34 (5,335 bp), and contig 53 (183,893 bp), which were located sequentially 5' to 3' but did not overlap.

The duck TCR $\gamma$  locus exhibited a translocon organization. A single C $\gamma$  gene containing three exons was identified in BAC clone DHS0702G12 and was also detected in the genomic Southern blotting assay (Fig. 1c and Supplementary Fig. S3D). Exon 1 encoded the extracellular Ig domain, which contained two conserved cysteine residues that were required for intra-chain disulfide bond formation and three N-glycosylation sites. Exon 2 encoded a short Cp containing the single conserved cysteine that formed the inter-chain disulfide bond with TCR C $\delta$ , and exon 3 encoded the Tm, a positively charged Ct, and the 3'UTR regions. As expected, pairwise alignments showed that the duck TCR C $\gamma$  chain exhibits the highest amino acid identity (67.1%) with the chicken TCR C $\gamma$  chain, but low amino acid identity (less than 30%) with those of other vertebrates (Fig. 2d).

Thirteen V $\gamma$  segments were identified in BAC clone DHS0702G12. Of them, five were pseudogenes due to an in-frame stop codon (Fig. 1c). Two extra V $\gamma$  segments, designated as V $\gamma$ 1.6 and V $\gamma$ 3.4, respectively, were cloned by 5' RACE PCR, suggesting that there are at least two germline V $\gamma$  segments located 5' upstream of the V $\gamma$ 3.3 in the genome. All 15 duck V $\gamma$  segments could be divided into six subgroups (V $\gamma$ 1 to V $\gamma$ 6) based on the same criterion applied for TCR $\alpha/\delta$  and TCR $\beta$  (Table 1) (Supplementary Fig. S2D). Members within a V $\gamma$  subgroup shared more than 79.3% nucleotide identity. The V $\gamma$ 1.6 or V $\gamma$ 3.4 displayed 81.0% to 93.3% or 78.9% to 89.6% nucleotide identity with the remaining V $\gamma$ 1 or V $\gamma$ 3 segments, respectively. Dot plot analysis indicated no duplicated units longer than 2 kb in the current V $\gamma$  region (Supplementary Fig. S4E). The results of Southern blotting of the representative V $\gamma$  subgroups are shown in Supplementary Fig. S3D. The number and intensity of hybridizing bands substantiated the presence of larger number of V $\gamma$ 1 segments and smaller number of V $\gamma$ 5 segments in the genome. Between V $\gamma$ 1.1 and C $\gamma$ , five functional J $\gamma$  segments were identified (Supplementary Fig. S1D).

**TCR $\delta$ 2 locus.** Based on the previously reported cDNA sequence encoded by the duck TCR $\delta$ 2 locus (accession number AF415216)<sup>10</sup>, we obtained the complete genomic sequence of this locus in a BAC clone, DHS0901N17. The duck TCR $\delta$ 2 locus spanned approximately 16 kb and had a conserved organization similar to that of chicken, containing a single cluster of VH $\delta$ , D $\delta$ , J $\delta$ , and C $\delta$  genes (Fig. 1d). The VH $\delta$  gene was flanked by typical 3' 23-RSS, and the D gene had 5' 12-RSS and 3' 23-RSS, which were canonically used in the TCR $\delta$ . The J $\delta$  gene had 5' 12-RSS and a conserved splice site at the 3' end. The C $\delta$ 2 gene consisted of five exons. Exon 1 encoded the extracellular Ig domain, exon 2 encoded a short Cp, and the last three exons together encoded the Tm and a long Ct containing

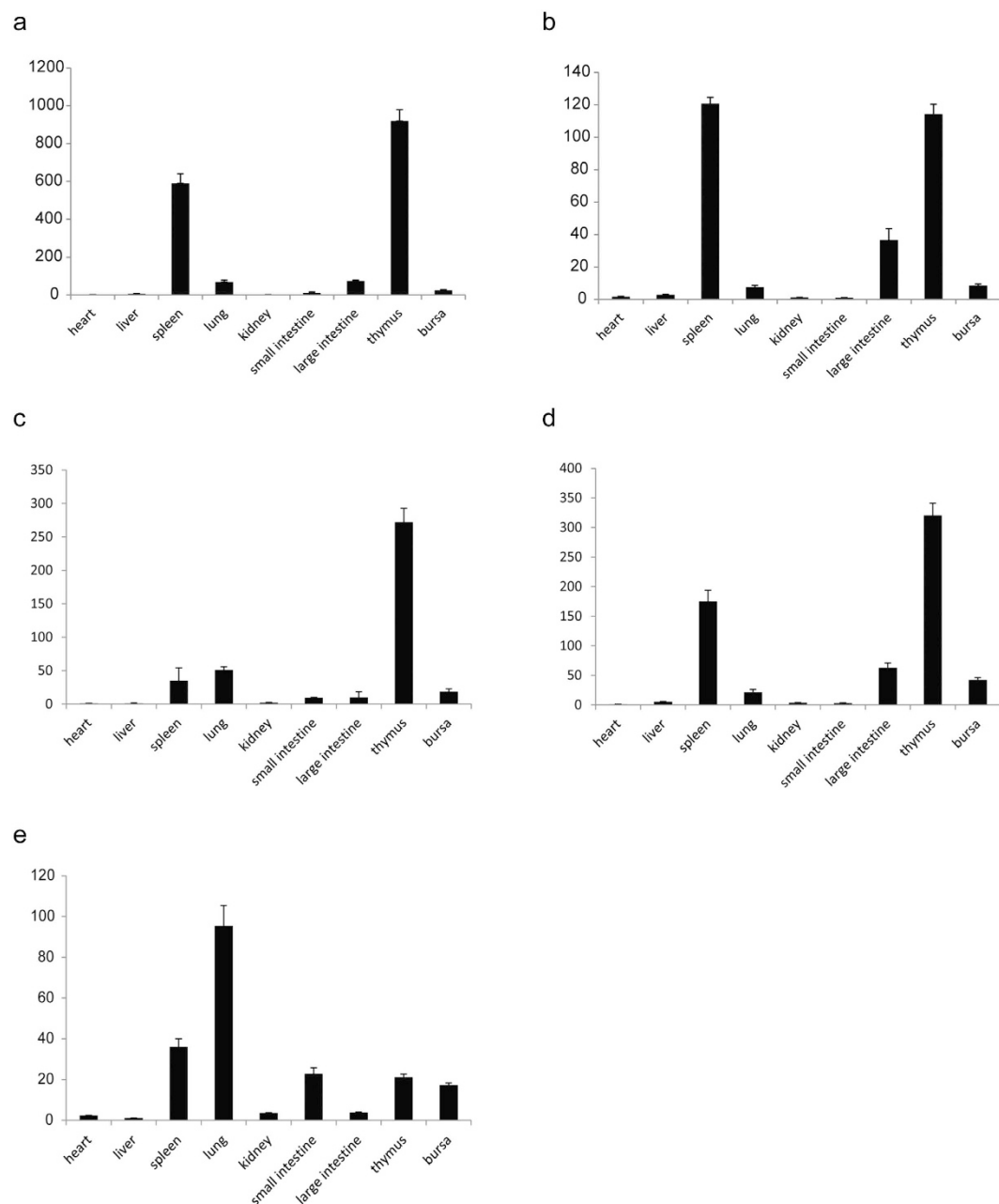


**Figure 3.** Phylogenetic analyses of the TCR V gene segments from representative mammalian, avian and amphibian species, V $\alpha$  and V $\delta$  (a), V $\beta$  (b) and V $\gamma$  (c). The unrooted trees were constructed using the neighbour-joining method with nucleotide sequences corresponding to FR1 through FR3. Duck V gene segments are indicated in bold. Numbers next to the branches show the percentages of the nodes in 1,000 bootstrap replicates.

62 amino acids, as well as the 3'UTR regions (Fig. 2b). To determine whether duck has more than one TCR $\delta$ 2 locus in its genome, Southern blotting was performed using one probe from VH $\delta$  and one probe from exon 1 of C $\delta$ 2. Because the enzyme sites of *Pvu* I and *Pst* I were located in the VH $\delta$  sequence, one dark and one light band were detected using the VH $\delta$  probe (Supplementary Fig. S3E). We also found one dark and one light band using the enzyme *Pst* I and the C $\delta$  probe (Supplementary Fig. S3E). The single dark band corresponded to the actual C $\delta$  2 gene, but the single light band seemed to be the conventional C $\delta$  gene, in which exon 1 shares 59.5% nucleotide identity with that of the C $\delta$ 2 gene (Fig. 2b).

**Phylogenetic analyses of duck V $\alpha$ , V $\delta$ , V $\beta$ , and V $\gamma$  gene segments.** As shown in Fig. 3a, the duck V $\alpha$ 2 and V $\alpha$ 3 subgroups were closely related to the chicken (and zebra finch) V $\alpha$ 1 and V $\alpha$ 2 subgroups, respectively, and orthologous genes have also been found in mammals. The duck genes from the V $\delta$ 1, V $\delta$ 2, V $\delta$ 3, and V $\delta$ 5 subgroups fell in the same phylogenetic clade with the V $\delta$ 1 subgroup of chicken as well as the V $\delta$ 1 and V $\alpha$ 3 subgroups of zebra finch, but this clade was distinct and specific for birds. However, the duck V $\alpha$ 1 and V $\delta$ 4 subgroups did not clearly cluster with any V $\alpha$  or V $\delta$  genes from other birds or mammals (Fig. 3a).

Although duck V $\beta$  genes belonging to subgroup V $\beta$ 2 and V $\beta$ 3 were classified as distinct subgroups, both subgroups fell in the same phylogenetic clade as the chicken V $\beta$ 1 subgroup, and all were derived from a common ancestral gene that was also present in amphibians. The duck V $\beta$ 1 subgroup lacked orthologues in chicken and mammals but demonstrated a clear relationship with amphibian V $\beta$  genes. Conversely, the duck V $\beta$ 4 was closely related to the V $\beta$  genes from chicken V $\beta$ 2 and many mammalian V $\beta$  subgroups but lacked a known orthologue in amphibians, suggesting its emergence after the separation of amphibians (Fig. 3b).



**Figure 4.** Quantitative real-time PCR analyses of the relative expression levels of duck TCR genes in different tissues, TCR $\alpha$  (a), TCR $\delta$  (b), TCR $\beta$  (c), TCR $\gamma$  (d), and TCR $\delta 2$  (e). The duck *EF1A1* gene was chosen as an internal control. The y-axis shows the normalized fold changes in expression, and the nine tissues are listed below the x-axis.

In contrast to V $\beta$  genes, all duck V $\gamma$  subgroups showed a high specificity to birds, except the V $\gamma 6$  subgroup, which formed a weakly supported group (72% support) with the clade containing all mammalian V $\gamma$  genes. The duck V $\gamma 1$  and V $\gamma 2$  subgroups clustered with chicken V $\gamma 2$ , and the V $\gamma 3$  and V $\gamma 4$  subgroups clustered with chicken V $\gamma 3$  and V $\gamma 1$ , respectively. The V $\gamma 5$  subgroup appeared to have evolved separately in duck or anseriform species because it did not clearly cluster with any V $\gamma$  genes from other tetrapods (Fig. 3c).

**Expression of duck TCR genes in various tissues.** The expression pattern of duck TCR genes in different tissues was assessed by quantitative real-time PCR. TCR $\alpha$ ,  $\gamma$ , and  $\delta 1$  were highly expressed in the thymus and spleen, and relatively weakly in the large intestine, lung, and bursa, but they were barely detectable in other tissues (Fig. 4a,b and d). TCR $\beta$  was only expressed at high levels in the thymus; it was expressed at much lower levels in other tissues, including the spleen (Fig. 4c). Unexpectedly, TCR $\delta 2$  was expressed at the highest levels in the lung but relatively weakly in lymphoid tissues, including the spleen, small intestine, thymus, and bursa (Fig. 4e), indicating that the TCR $\delta 2$  may play a crucial role in the tolerance of ducks to avian influenza viruses.

**Diversity of conventional TCR transcripts in duck.** Based on the results of 5' RACE PCR, a total of 142 TCR $\alpha$ , 76  $\delta$ , 42  $\beta$ 1, 43  $\beta$ 2, and 102  $\gamma$  cDNA clones were sequenced, and after removing duplicates, 134  $\alpha$ , 75  $\delta$ , 42  $\beta$ 1, 43  $\beta$ 2, and 102  $\gamma$  remaining clones were considered unique. These clones were analysed for the use of V, D, and J gene segments and overall CDR3 diversity.

**TCR $\alpha$ .** Of 134 unique TCR $\alpha$  cDNA clones, 112 clones were deemed potentially functional based on their complete ORFs. In general, members of subgroup V $\alpha$ 3 (75 clones) appeared to be more frequently utilized than those of subgroups V $\alpha$ 1 (29 clones) and V $\alpha$ 2 (30 clones). However, excluding V $\alpha$ 3.4, none of the germline V $\alpha$  segments presented in the BAC sequence were found in the 5' RACE clones (Supplementary Fig. S5A). For the potentially functional clones, the length of CDR3 was  $28.4 \pm 6.2$  bp, encoding 4 to 16 amino acid residues with an average of 9.5 residues (Supplementary Figs S5A and S6A).

**TCR $\delta$ .** Among 75 unique TCR $\delta$  cDNA clones, 57 clones had an intact ORF. Forty-nine clones utilized 20 V $\alpha$  segments, of which nine were also used by TCR $\alpha$ . Notably, none of the functional members belonging to subgroup V $\alpha$ 1 were used in the TCR $\delta$  rearrangement, and in contrast to TCR $\alpha$ , all of the germline V $\alpha$  segments identified in the BAC sequence, excluding the pseudo V $\alpha$ 3.1, participated in TCR $\delta$  rearrangement, indicating that TCR $\alpha$  and TCR $\delta$  have different usage preferences for the V $\alpha$  segments (Supplementary Fig. S5B). In the remaining 26 clones containing V $\delta$  segments, members of the subgroup V $\delta$ 2 (18 clones) were more frequently used, whereas members of the subgroup V $\delta$ 4 were not observed (Supplementary Fig. S5B). There appeared to be a J $\delta$  usage preference. The J $\delta$ 1 segment, which has a more conserved heptamer in its RSS, accounted for more than two-thirds (57 clones) of the expressed J $\delta$  repertoire (Supplementary Fig. S5B). Most VJ junctions contained either one (10 clones for D $\delta$ 1 and 27 clones for D $\delta$ 2) or both (31 clones) D $\delta$  segments. Among them, N and P nucleotide additions between different gene segments were common. However, the remaining seven clones demonstrated evidence for N nucleotide additions but no D segment incorporation, indicating extensive trimming of D or direct V $\alpha$ / $\delta$ -J $\delta$  recombination during rearrangement. For the potentially functional clones, the length of CDR3 was  $34.2 \pm 9.0$  bp and encoded 5 to 19 amino acid residues, with an average of 11.5 residues (Supplementary Figs S5B and S6B).

**TCR $\beta$ .** Of the 42 unique TCR $\beta$ 1 and 43  $\beta$ 2 cDNA clones, 74 clones were considered to be potentially functional. Both TCR $\beta$ 1 and  $\beta$ 2 showed a similar usage pattern of V $\beta$  segments. A total of 55 clones (24 of  $\beta$ 1 and 31 of  $\beta$ 2) used the V segments from subgroup V $\beta$ 3, which contained the most germline members. Notably, the most frequently used V segment was V $\beta$ 2, the single member of subgroup 2, accounting for more than 20% of the expressed V $\beta$  repertoire in both  $\beta$ 1 (12 clones) and  $\beta$ 2 (9 clones), whereas V $\beta$ 1.1, the only functional segment from subgroup 1, was not observed in the cDNA of either  $\beta$ 1 or  $\beta$ 2 (Supplementary Fig. S5C). Similar to TCR $\delta$ , TCR $\beta$  also demonstrated a biased usage of J $\beta$  segments, especially  $\beta$ 1, which utilized J $\beta$ 1.2 more frequently than J $\beta$ 1.1 (37 vs. 6 clones) (Supplementary Fig. S5C). Due to the single D $\beta$  segment, the CDR3 length of TCR $\beta$  was  $30.9 \pm 7.1$  bp, encoding 5 to 17 amino acid residues (average of 10.2 residues) (Supplementary Figs S5C and S6C). The features described above for TCR $\delta$  junctions were also found in the TCR $\beta$  junctions (Supplementary Fig. S5C).

**TCR $\gamma$ .** Among 102 unique TCR $\gamma$  cDNA sequences, 93 clones displayed an intact ORF. All potentially functional V $\gamma$  segments identified in the BAC sequence, excluding V $\gamma$ 5, were found in the cDNA clones. Members of subgroup 1 (49 clones) and 3 (37 clones) were preferentially used, especially V $\gamma$ 1.6 and V $\gamma$ 3.4, which were not located on the BAC sequence but each contributed to approximately 20% (20 clones) of the expressed V $\gamma$  repertoire (Supplementary Fig. S5D). For all potentially functional clones, the average length of CDR3 was  $24.1 \pm 8.4$  bp, encoding 2 to 16 amino acid residues with an average of 8 residues (Supplementary Figs S5D and S6D).

**Diversity of duck TCR $\delta$ 2 transcripts.** The total RNA of thymus tissue as well as the primers complementary to VH $\delta$  and C $\delta$ 2 (Supplementary Table S1) were used in RT-PCR to investigate the junctional diversity of the duck TCR $\delta$ 2 transcripts. A total of 18 TCR $\delta$ 2 cDNA clones were sequenced, and after removing the duplicates, the remaining 16 clones were considered unique. The junctional diversity of the duck TCR $\delta$ 2 repertoire was characterized by clear P nucleotide additions to the 3' ends of both V and D regions in almost all TCR $\delta$ 2 clones. For 13 productive rearranged clones, the average length of CDR3 was  $36.6 \pm 6.1$  bp, encoding 9 to 14 amino acid residues with an average of 11.5 residues (Supplementary Figs S5E and S6E).

## Discussion

Compared with TCR $\alpha$ / $\delta$  and TCR $\gamma$  gene loci, the germline repertoire of the TCR $\beta$  locus has been extensively studied in many vertebrates. Among all mammals studied to date, the genomic organization of the TCR $\beta$  locus is highly conserved, with a pool of V $\beta$  genes positioned at the 5' end and several tandem repeated D $\beta$ -(J $\beta$ )<sub>4-7</sub>-C $\beta$  clusters followed by a single V gene with an inverted transcriptional orientation located at the 3' end<sup>25-31</sup>. C $\beta$  genes within each mammalian species maintain a high degree of sequence similarity in the coding region but present high divergence in the 3'UTR, indicating that the C $\beta$  genes have undergone concerted evolution by intra-species homogenization using gene conversion<sup>28,30,32,33</sup>. However, the genomic organization of the TCR $\beta$  locus and concerted evolution of the C $\beta$  genes that seems to be conserved in mammals are not present in other vertebrate species, especially in teleosts. The TCR $\beta$  locus of zebrafish resembles that observed in mammals, but the D gene is absent from the second D $\beta$ -(J $\beta$ )<sub>n</sub>-C $\beta$  cluster<sup>34</sup>. The TCR $\beta$  locus of channel catfish (*Ictalurus punctatus*) is arranged in a typical translocon organization containing a single D $\beta$  gene followed by a total of 29 J $\beta$  genes and two tandem C $\beta$  genes<sup>35</sup>. Notably, the sequence similarity of C $\beta$  isotypes within a single teleost species varies considerably. In the Japanese flounder (*Paralichthys olivaceus*) and Atlantic cod (*Gadus morhua*), different C $\beta$  isotypes show more

| Species     | V $\alpha$ <sup>a</sup>             | V $\beta$                 | V $\gamma$              | V $\delta$       | Reference |
|-------------|-------------------------------------|---------------------------|-------------------------|------------------|-----------|
| Human       | 54 (44–47), 41 (33–35) <sup>b</sup> | 64–67 (40–48), 30 (21–23) | 12–15 (4–6), 6 (2)      | 3 (3), 3 (3)     | 61        |
| Mouse       | 98 (73–84), 23 (19)                 | 35 (21–22), 31 (19)       | 7 (7), 5 (5)            | 6 (5), 5 (4)     | 61        |
| Cattle      | >300 (?), >38 (>33)                 | >134 (>79), 24 (19)       | 17 (17), 10 (10)        | >100 (?), 6 (6)  | 28,62,63  |
| Rabbit      | —                                   | 75 (59), 24 (20)          | 10 (10), 4 (4)          | —                | 30,64     |
| Dog         | —                                   | 37 (20), 25 (16)          | 16 (8), 8 (4)           | —                | 29,65     |
| Opossum     | 68 (56), 41 (33)                    | 36 (27), 28 (21)          | 9 (9), 4 (4)            | 6 (4), 6 (4)     | 31        |
| Platypus    | 89 (83), 17 (16)                    | —                         | >15 (?), 3 <sup>d</sup> | 10 (10), 2 (2)   | 11,66     |
| Chicken     | 60 (50), 2 (2)                      | >10 (?), 2 <sup>d</sup>   | >24 (?), 3              | 36 (32), 1 (1)   | 45,67–69  |
| Zebra finch | >10 (>10), 3 (3)                    | —                         | —                       | 4 (4), 4 (4)     | 10        |
| Duck        | ~49 (~44), 3 (3)                    | ~13 (~11), 4 (4)          | ~15 (~10), 6 (6)        | ~30 (~29), 5 (5) | —         |

**Table 2. Numbers of TCR V segments and subgroups in selected mammals and birds.** <sup>a</sup>The V $\alpha$  segments include the V $\alpha$  expressed in either TCR $\alpha$  and/or TCR $\delta$  chains. <sup>b</sup>Numbers preceding the comma are the V segments, and numbers following the comma are the V subgroups. The numbers of functional segments or subgroups are shown in brackets. <sup>c</sup>The numbers of V segments were deduced based on the numbers of hybridizing bands with probes of specific subgroups using genomic Southern blotting. <sup>d</sup>The numbers of subgroups were deduced using cDNA sequences. “—” Indicates that no relevant information was available.

than 85% amino acid identity<sup>36,37</sup>. Conversely, in both zebrafish and catfish, the sequences of two C $\beta$  isotypes are substantially different, sharing only 36% identity at the amino acid level<sup>34,35</sup>. Such multiple divergent C $\beta$  isotypes have also been observed in bicour damselfish (*Stegastes partitus*)<sup>38</sup>, as well as an urodele amphibian Mexican axolotl (*Ambystoma mexicanum*)<sup>39</sup>. Before this study, chicken was the only other bird for which the sequences of the TCR $\beta$  D-J-C region had been reported. The locus contains a single D $\beta$ , 4 J $\beta$  genes and a seemingly single C $\beta$  gene<sup>40</sup>. In this study, we determined the complete sequence of the duck TCR $\beta$  locus, which is arranged in an unusual fashion, similar to that of the zebrafish, with a single D $\beta$  gene followed by two tandem-aligned (J $\beta$ )<sub>n</sub>–C $\beta$  clusters. The absence of the 2nd D $\beta$  gene in ducks may have occurred as an independent event and happens to form a functional genotype that is similar to that of zebrafish. Another attractive feature of duck TCR $\beta$  lies in the sequence conservation of each domain between the two C $\beta$  genes. The Ig domains of the two C $\beta$  are well-conserved, whereas the following Cp, Tm, and Ct domains differ remarkably. This special distribution of C $\beta$  identity has not been reported in any other vertebrates, in which the sequence identity is high (>80%) or low (<50%) throughout the whole coding region of the different C $\beta$  genes. Furthermore, the coding sequence of the Ct domain is entirely included within exon 3 of C $\beta$ 2 but separated into two exons by intron 3 in C $\beta$ 1, suggesting that the two C $\beta$  genes might be the result of an ancient duplication that occurred long before the speciation of *Anas*.

The birth/death hypothesis has been postulated as an evolutionary mechanism of V genes from both Ig and TCR loci<sup>41</sup>. Recently, a phylogenetic analysis of genomic V-gene repertoires, which were extracted from mammals and reptiles with available WGS sequences, indicated that V genes from Ig and TCR loci might have markedly different evolutionary pathways. The Ig V genes undergo more pronounced birth/death processes, thereby permitting the frequent duplication of specific V subgroups that could directly recognize rapidly changing antigens in the external environment. By contrast, the V genes from the TCR $\alpha$  and TCR $\beta$  loci, which consist of multiple subgroups (Table 2) with relatively low duplication permissiveness throughout evolution, appeared to have undergone a co-evolution process with MHC molecules, resulting in natural evolutionary pressures<sup>42–44</sup>. As shown in Table 2, the most striking feature of duck V $\alpha$  and V $\beta$  genes is the presence of fewer subgroups in comparison to mammals. The same feature are also observed in the V $\alpha$  and V $\beta$  genes of chicken and zebra finch<sup>10,45,46</sup>. According to the co-evolution hypothesis, there might be some evolutionary connections between the diversity of V $\alpha$ /V $\beta$  subgroups and the number of expressed classical MHC loci. A larger number of expressed MHC genes would result in the positive selection of a more diverse TCR repertoire, but too many expressed MHC class I genes would also reduce the T cell repertoire during negative selection. Currently, the precise numbers of MHC class I and/or MHC class II genes have been ascertained in only a few birds. The chicken *MHC-B* locus contains two classical MHC class I genes (*BF1* and *BF2*) and two classical MHC class II B genes (*BLB1* and *BLB2*). However, only *BF2* and *BLB2* are dominantly expressed at the RNA and protein levels<sup>47</sup>. Similarly, among the five MHC class I genes in duck, only *UAA* is a dominantly expressed classical MHC class I gene; the others are the weakly expressed *UDA* and unexpressed pseudogenes (*UBA*, *UCA*, and *UEA*)<sup>48</sup>. Furthermore, in the genome sequence of zebra finch, only one functional MHC class I gene has been identified<sup>49</sup>. The above examples suggest that the evolution of fewer V $\alpha$ /V $\beta$  subgroups is probably due to the dominant expression of a single classical MHC class I gene in these avian species, providing an opportunity for the co-evolution of both MHC and TCR genes with associated roles in presenting and recognizing antigens.

As summarized in Table 2, many more functional germline V $\delta$  genes have been identified in “ $\gamma\delta$  high” species than “ $\gamma\delta$  low” species, indicating that the germline diversity of the V $\delta$  gene is directly proportional to the percentage of peripheral  $\gamma\delta$  T cells in mammals and chicken. Furthermore, three important points are relevant to the V $\delta$  genes. First, the subgroup numbers of V $\delta$  genes show no significant differences between the “ $\gamma\delta$  high” and “ $\gamma\delta$  low” species. Second, an enormous expansion of the germline repertoire of some V $\delta$  subgroups is a striking feature observed in “ $\gamma\delta$  high” species. For example, the V $\delta$ 1 subgroup of cattle, sheep, and pig contains at least 52<sup>50</sup>, 40<sup>51</sup>, and 31<sup>52</sup> members, respectively. Finally, the single V $\delta$  subgroup of chicken, which contains as many as 36 members<sup>45</sup>, falls into a bird-specific clade without any mammalian counterparts in the phylogenetic analysis.

| Species           | TCR $\alpha$             | TCR $\beta$  | TCR $\gamma$ | TCR $\delta$ | Reference |
|-------------------|--------------------------|--------------|--------------|--------------|-----------|
| Duck              | 4, 16 (9.5) <sup>a</sup> | 5, 17 (10.2) | 2, 16 (8.0)  | 5, 19 (11.5) | —         |
| Human             | 6, 12 (9.2)              | 6, 12 (9.5)  | 1, 12 (7.2)  | 8, 21 (14.5) | 53        |
| Mouse             | 6, 12 (8.5)              | 4, 13 (8.9)  | 4, 11 (8.8)  | 6, 19 (12.7) | 53        |
| Japanese flounder | 7, 15 (11.2)             | 7, 15 (11.2) | 5, 10 (8.5)  | 9, 17 (13.3) | 36        |
| Nurse shark       | 3, 12 (8.0)              | 0, 19 (9.6)  | 6, 12 (9.1)  | 4, 27 (9.8)  | 54        |

**Table 3. CDR3 length of TCR chains in selected vertebrates.** The mean length is bracketed. The CDR3 length was defined as four amino acids less than the number of amino acid residues between the J region–encoded GXG triplet, where G is glycine and X is any amino acid, and the nearest preceding V region–encoded cysteine. <sup>a</sup>Range of CDR3 length: minimum, maximum.

Taken together, these findings suggest that the V $\delta$  genes evolved following birth/death pathways similar to those that gave rise to Ig because antigen recognition by both Ig and the  $\gamma\delta$  TCR complex is not MHC-restricted<sup>3</sup>. Although the distribution of T-cell populations in birds except the chicken remains to be determined, the presence of such a large number of V $\delta$  genes as well as the expansion of the V $\delta$ 2 subgroup suggest that the duck probably belongs to the “ $\gamma\delta$  high” species.

The length distribution of the CDR3 loop has been used as a metric in assessments of the possible range of binding paratopes generated by a given TCR type and has been analysed in human, mouse<sup>53</sup>, Japanese flounder<sup>36</sup>, and nurse shark<sup>54</sup>, albeit the data of the latter two species were reported from a limited sample size (Table 3). In the human, mouse, and nurse shark, the CDR3 $\delta$  loops display a much broader length distribution than in the other three TCR types because of the presence of multiple D gene segments (for the mouse and human) that can join together, as well as the numerous N nucleotides (for the nurse shark) inserted into the V-D and D-J junctions. Notably, although 0 to 2 putative D $\delta$  segments were shown to incorporate into a single CDR3 $\delta$ , the duck CDR3 $\delta$  loop lengths are 5–19 amino acid residues, similar to the ranges for the other TCR types of duck. However, the CDR3 $\gamma$  loops in the human, mouse, Japanese flounder, and nurse shark display a narrow length distribution (1–12, 4–11, 5–10, and 6–12, respectively), whereas the duck CDR3 $\gamma$  loops exhibit a broader distribution with 2–16 amino acid residues, which is far beyond the range exhibited by the listed counterparts. Given that the  $\gamma\delta$ TCR can interact with diverse ligands in various ways, it is likely that the broad length distribution of CDR3 $\gamma$  compensates for the narrow length distribution of CDR3 $\delta$  in ducks. The CDR3 $\alpha$  and CDR3 $\beta$  loops of human, mouse, and Japanese flounder have, on average, very similar lengths (9.2 vs. 9.5, 8.5 vs. 8.9, and 11.2 vs. 11.2, respectively). The average lengths of the duck CDR3 $\alpha$  and CDR3 $\beta$  loops show a tendency similar to the three species, although the average CDR3 $\alpha$  loops appear to be 0.7 amino acid residues shorter than CDR3 $\beta$  (9.5 vs. 10.2). However, the duck CDR3 $\alpha$  and CDR3 $\beta$  loops, ranging from 4–16 and 5–17 amino acid residues, display much wider distribution than those of the three species (6–12, 6–12 and 7–15 for CDR3 $\beta$ , as well as 6–12, 4–13, and 7–15 for CDR3 $\alpha$ ). This indicates that duck CDR3 $\alpha$  and CDR3 $\beta$  may have increased flexibility and are therefore better suited to recognize a larger number of antigenic conformations presented by MHC molecules.

## Methods

**Animal, DNA and RNA isolation and reverse transcription.** A White Peking Duck aged 90 days post-hatching was purchased from Beijing Jinxin Duck Centre. Genomic DNA was extracted from blood cells following a routine phenol-chloroform protocol. Total RNA was isolated from various tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was conducted using M-MLV reverse transcriptase (Invitrogen, Beijing, China) with an oligo(dT) adapter primer NotI-d(T)18 (Supplementary Table S1). Animal care was in accordance with the guidelines of China Agricultural University for animal welfare. All animal experiments in the present study were approved by the Animal Care and Use Committee of China Agricultural University.

**Bacterial artificial chromosome (BAC) genomic library.** The White Peking Duck BAC (bacterial artificial chromosome) genomic library was constructed by Majorbio Co. Ltd., Shanghai, China. The BAC library was divided into two sub-libraries, each of which was prepared using blood cell genomic DNA that had been partially digested with the restriction enzymes *Hind* III or *Bam* HI. Each sub-library was composed of 49,152 clones, which were placed into 16 superpools of 8 × 384-well plates. Using pulsed-field gel electrophoresis analysis of 185 clones that were randomly selected from two sub-libraries, the average insert sizes were estimated to be 152 kb.

**BAC screening and sequencing.** Positive BAC clones covering the duck TCR $\alpha/\delta$ ,  $\beta$ ,  $\gamma$  and  $\delta$ 2 loci were isolated from the BAC library via PCR-based screening with primers (Supplementary Table S1) designed based on the available TCR mRNA constant sequences of mallard from GenBank. For TCR $\alpha/\delta$ , the first positive BAC clone was sequenced from both ends, and the end sequences were used to design primers (Supplementary Table S1) for the next round of screening to determine the BAC clone overlap. The positive BAC clones were subjected to shotgun sequencing and assembled using the next-generation sequencing platform by BGI (Beijing, China).

**Identification of germline V, D, J and C gene segments.** To determine the locations of the V gene segments, BAC sequences were screened using the IgBLAST algorithm (<http://www.ncbi.nlm.nih.gov/igblast/>) by similarity to homologues from human and mouse. V gene segments are named 3' to 5' with the subgroup number followed by the gene segment number if there was more than one member in this subgroup. The D and J gene segments were annotated by searching the recombination signal sequences (RSS) using FUZZNUC (<http://>

embossgui.sourceforge.net/demo/fuzznuc.html) and the conserved motif FGXG encoded by the J segments manually. The exon-intron organization of the C regions was searched manually by comparing the cDNA sequence encoding the complete C region for each TCR with the duck genomic sequences. Non-TCR genes located in or flanking each TCR locus were identified using GENSCAN (<http://genes.mit.edu/GENSCAN.html>).

**5' RACE.** The 5' RACE System for Rapid Amplification of cDNA Ends (version 2.0, Life Technologies/Gibco BRL, Gaithersburg, MD, USA) was applied to thymus total RNA to obtain the expressed repertoire of each TCR type as well as the novel expressed V segments that were not located on the BAC clones. Specific primers for each constant region of the TCR $\alpha/\delta$ ,  $\beta$  and  $\gamma$  loci are listed in Supplementary Table S1. The resulting PCR products were cloned into the pMD-19T vector (TaKaRa, Dalian, China) and sequenced.

**3' RACE.** The cDNA sequences encoding the complete C region of each TCR, including the immunoglobulin domain, Cp, Tm, Ct and 3'UTR, were obtained by nested 3' RACE PCR using thymus cDNA. Specific primers for each TCR gene were derived from the V region sequences. For the first round of PCR, sense primer located closer to the 5' end of the cDNA (Supplementary Table S1) were paired with the antisense primer RT-P1. For the second round of PCR, a nested primer located 3' to the original primer (Supplementary Table S1) was paired with antisense primer RT-P2, and a dilution of the first PCR was used as the template. The resultant PCR products were cloned into the pMD-19T vector and sequenced.

**Southern blotting.** Genomic DNA was digested with different restriction enzymes and loaded into a 0.9% agarose gel, electrophoresed for 6 h, and transferred to a positively charged nylon membrane (Roche, Germany) for hybridization. The conserved C $\alpha$ , V $\alpha$ 1, V $\alpha$ 2, C $\delta$ , V $\delta$ 2, V $\delta$ 5, C $\beta$ , V $\beta$ 2, V $\beta$ 3, C $\gamma$ , V $\gamma$ 1, V $\gamma$ 6, V $\delta$  and C $\delta$  sequences from White Peking duck were used as probes. These cDNA fragments were labelled using a PCR DIG Probe Synthesis Kit (Roche, Beijing, China) using the primers listed in Supplementary Table S1. Hybridization and detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Beijing, China) according to the manufacturer.

**Detection of gene expression in different tissues by quantitative real-time PCR.** The cDNA samples from nine tissues (heart, liver, spleen, lung, kidney, small intestine, large intestine, thymus and bursa) were used to determine the expression of TCR $\alpha/\delta$ ,  $\beta$ ,  $\gamma$  and  $\delta$ 2 by quantitative real-time PCR. PCR was performed using a LightCycler 480 and LightCycler 480 SYBR Green I Master Mix (Roche, Beijing, China). Each sample was run in triplicate. The White Peking Duck *EF1a1* gene was used as the internal control. The PCR consisted of the following conditions: 95 °C for 10 min; 35 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s; and a final extension at 72 °C for 7 min. The PCR primers are listed in Supplementary Table S1. The relative expression levels of the gene were determined using the  $2^{-\Delta\Delta C_t}$  method by comparing the values with the internal control.

**Sequence analyses.** DNA and protein sequence editing, alignments, and comparisons were performed using the DNASTAR Lasergene software suite<sup>55</sup> and Boxshade software ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Dot plot analyses of the V regions of TCR $\alpha/\delta$ , TCR $\beta$  and TCR $\gamma$  loci were conducted with the dotter program<sup>56</sup>. For a given TCR type, if the V region (corresponding to FR1 through FR3) of a cDNA clone shared less than 97% nucleotide identity with the germline V segments identified in the BAC as well as V regions of every other cDNA clone, the V region was considered a novel V segment<sup>57,58</sup>. The CDR3 of the rearranged TCR V domain was defined as the region between the J region-encoded FGXG motif and the nearest preceding V region-encoded cysteine, according to the IMGT unique numbering system<sup>59</sup>. The length of CDR3 was defined as four amino acids less than the number of amino acid residues between the J region-encoded GXG triplet, where G is glycine and X is any amino acid, and the nearest preceding V region-encoded cysteine as described in ref. 53.

**Phylogenetic analyses.** The nucleotide sequences corresponding to FR1 through FR3 of all V genes were aligned for tree construction using ClustalW. Phylogenetic trees were constructed in MEGA version 5.10<sup>60</sup> using the neighbour-joining method with 1,000 bootstrap replicates. The GenBank accession numbers of all sequences used are listed in Supplementary Table S2.

## References

- Litman, G. W., Rast, J. P. & Fugmann, S. D. The origins of vertebrate adaptive immunity. *Nat Rev Immunol* **10**, 543–553 (2010).
- Scott-Browne, J. P. *et al.* Evolutionarily conserved features contribute to alphabeta T cell receptor specificity. *Immunity* **35**, 526–535 (2011).
- Chien, Y. H., Meyer, C. & Bonneville, M. gammadelta T cells: first line of defense and beyond. *Annu Rev Immunol* **32**, 121–155 (2014).
- Faldyna, M., Sinkora, J., Knotigova, P., Leva, L. & Toman, M. Lymphatic organ development in dogs: major lymphocyte subsets and activity. *Vet Immunol Immunopathol* **104**, 239–247 (2005).
- Hein, W. R. & Mackay, C. R. Prominence of gamma delta T cells in the ruminant immune system. *Immunol Today* **12**, 30–34 (1991).
- Sawasdikosol, S. *et al.* Selection of rabbit CD4- CD8- T cell receptor-gamma/delta cells by *in vitro* transformation with human T lymphotropic virus-I. *J Exp Med* **178**, 1337–1345 (1993).
- Sowder, J. T., Chen, C. L., Ager, L. L., Chan, M. M. & Cooper, M. D. A large subpopulation of avian T cells express a homologue of the mammalian T gamma/delta receptor. *J Exp Med* **167**, 315–322 (1988).
- Chen, C. H., Gobel, T. W., Kubota, T. & Cooper, M. D. T cell development in the chicken. *Poult Sci* **73**, 1012–1018 (1994).
- Parra, Z. E., Ohta, Y., Criscitiello, M. F., Flajnik, M. F. & Miller, R. D. The dynamic TCRdelta: TCRdelta chains in the amphibian *Xenopus tropicalis* utilize antibody-like V genes. *Eur J Immunol* **40**, 2319–2329 (2010).
- Parra, Z. E., Mitchell, K., Dalloul, R. A. & Miller, R. D. A second TCRdelta locus in Galliformes uses antibody-like V domains: insight into the evolution of TCRdelta and TCRmu genes in tetrapods. *J Immunol* **188**, 3912–3919 (2012).
- Parra, Z. E., Lillie, M. & Miller, R. D. A model for the evolution of the mammalian t-cell receptor alpha/delta and mu loci based on evidence from the duckbill Platypus. *Mol Biol Evol* **29**, 3205–3214 (2012).

12. Criscitiello, M. F., Saltis, M. & Flajnik, M. F. An evolutionarily mobile antigen receptor variable region gene: doubly rearranging NAR-TCR genes in sharks. *Proc Natl Acad Sci USA* **103**, 5036–5041 (2006).
13. Parra, Z. E. *et al.* A unique T cell receptor discovered in marsupials. *Proc Natl Acad Sci USA* **104**, 9776–9781 (2007).
14. Wang, X., Parra, Z. E. & Miller, R. D. Platypus TCRmu provides insight into the origins and evolution of a uniquely mammalian TCR locus. *J Immunol* **187**, 5246–5254 (2011).
15. Jarvis, E. D. *et al.* Whole-genome analyses resolve early branches in the tree of life of modern birds. *Science* **346**, 1320–1331 (2014).
16. Dugan, V. G. *et al.* The evolutionary genetics and emergence of avian influenza viruses in wild birds. *PLoS Pathog* **4**, e1000076 (2008).
17. Hulse-Post, D. J. *et al.* Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc Natl Acad Sci USA* **102**, 10682–10687 (2005).
18. Kim, J. K., Negovetich, N. J., Forrest, H. L. & Webster, R. G. Ducks: the “Trojan horses” of H5N1 influenza. *Influenza Other Respir Viruses* **3**, 121–128 (2009).
19. Barber, M. R., Aldridge, J. R. Jr., Webster, R. G. & Magor, K. E. Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci USA* **107**, 5913–5918 (2010).
20. Magor, K. E. *et al.* Defense genes missing from the flight division. *Dev Comp Immunol* **41**, 377–388 (2013).
21. Huang, Y. *et al.* The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* **45**, 776–783 (2013).
22. Smith, J. *et al.* A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* **16**, 574 (2015).
23. Magor, K. E. Immunoglobulin genetics and antibody responses to influenza in ducks. *Dev Comp Immunol* **35**, 1008–1016 (2011).
24. Giudicelli, V. & Lefranc, M. P. Ontology for immunogenetics: the IMGT-ONTOLOGY. *Bioinformatics* **15**, 1047–1054 (1999).
25. Rowen, L., Koop, B. F. & Hood, L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* **272**, 1755–1762 (1996).
26. Glusman, G. *et al.* Comparative genomics of the human and mouse T cell receptor loci. *Immunity* **15**, 337–349 (2001).
27. Bosc, N. & Lefranc, M. P. The mouse (*Mus musculus*) T cell receptor beta variable (TRBV), diversity (TRBD) and joining (TRBJ) genes. *Exp Clin Immunogenet* **17**, 216–228 (2000).
28. Connelley, T., Aerts, J., Law, A. & Morrison, W. I. Genomic analysis reveals extensive gene duplication within the bovine TRB locus. *BMC Genomics* **10**, 192 (2009).
29. Mineccia, M. *et al.* New insight into the genomic structure of dog T cell receptor beta (TRB) locus inferred from expression analysis. *Dev Comp Immunol* **37**, 279–293 (2012).
30. Antonacci, R., Giannico, F., Ciccicarese, S. & Massari, S. Genomic characteristics of the T cell receptor (TRB) locus in the rabbit (*Oryctolagus cuniculus*) revealed by comparative and phylogenetic analyses. *Immunogenetics* **66**, 255–266 (2014).
31. Parra, Z. E. *et al.* Comparative genomic analysis and evolution of the T cell receptor loci in the opossum *Monodelphis domestica*. *BMC Genomics* **9**, 111 (2008).
32. Rudikoff, S., Fitch, W. M. & Heller, M. Exon-specific gene correction (conversion) during short evolutionary periods: homogenization in a two-gene family encoding the beta-chain constant region of the T-lymphocyte antigen receptor. *Mol Biol Evol* **9**, 14–26 (1992).
33. Antonacci, R. *et al.* Organization, structure and evolution of 41kb of genomic DNA spanning the D-J-C region of the sheep TRB locus. *Mol Immunol* **45**, 493–509 (2008).
34. Meeker, N. D. *et al.* Characterization of the zebrafish T cell receptor beta locus. *Immunogenetics* **62**, 23–29 (2010).
35. Zhou, H., Bengten, E., Miller, N. W., Clem, L. W. & Wilson, M. The T cell receptor beta locus of the channel catfish, *Ictalurus punctatus*, reveals unique features. *J Immunol* **170**, 2573–2581 (2003).
36. Nam, B. H., Hirano, I. & Aoki, T. The four TCR genes of teleost fish: the cDNA and genomic DNA analysis of Japanese flounder (*Paralichthys olivaceus*) TCR alpha-, beta-, gamma-, and delta-chains. *J Immunol* **170**, 3081–3090 (2003).
37. Wermenstam, N. E. & Pilstrom, L. T-cell antigen receptors in Atlantic cod (*Gadus morhua* L.): structure, organisation and expression of TCR alpha and beta genes. *Dev Comp Immunol* **25**, 117–135 (2001).
38. Kamper, S. M. & McKinney, C. E. Polymorphism and evolution in the constant region of the T-cell receptor beta chain in an advanced teleost fish. *Immunogenetics* **53**, 1047–1054 (2002).
39. Fellah, J. S., Durand, C., Kerfourn, F. & Charlemagne, J. Complexity of the T cell receptor Cbeta isotypes in the Mexican axolotl: structure and diversity of the VDJCbeta3 and VDJCbeta4 chains. *Eur J Immunol* **31**, 403–411 (2001).
40. Shigeta, A. *et al.* Genomic organization of the chicken T-cell receptor beta chain D-J-C region. *J Vet Med Sci* **66**, 1509–1515 (2004).
41. Nei, M. & Rooney, A. P. Concerted and birth-and-death evolution of multigene families. *Annu Rev Genet* **39**, 121–152 (2005).
42. Olivieri, D. N., von Haefen, B., Sanchez-Espinel, C., Faro, J. & Gambon-Deza, F. Genomic V exons from whole genome shotgun data in reptiles. *Immunogenetics* **66**, 479–492 (2014).
43. Olivieri, D. N. & Gambon-Deza, F. V genes in primates from whole genome sequencing data. *Immunogenetics* **67**, 211–228 (2015).
44. Olivieri, D. N., Gambon-Cerda, S. & Gambon-Deza, F. Evolution of V genes from the TRV loci of mammals. *Immunogenetics* **67**, 371–384 (2015).
45. Parra, Z. E. & Miller, R. D. Comparative analysis of the chicken TCRalpha/delta locus. *Immunogenetics* **64**, 641–645 (2012).
46. Lahti, J. M. *et al.* Two distinct alpha beta T-cell lineages can be distinguished by the differential usage of T-cell receptor V beta gene segments. *Proc Natl Acad Sci USA* **88**, 10956–10960 (1991).
47. Davison, F., Kaspers, B. & Schat, K. A. 161–164 (Academic Press in an imprint of Elsevier, 2008).
48. Moon, D. A., Veniamin, S. M., Parks-Dely, J. A. & Magor, K. E. The MHC of the duck (*Anas platyrhynchos*) contains five differentially expressed class I genes. *J Immunol* **175**, 6702–6712 (2005).
49. Balakrishnan, C. N. *et al.* Gene duplication and fragmentation in the zebra finch major histocompatibility complex. *BMC Biol* **8**, 29 (2010).
50. Herzig, C. T., Lefranc, M. P. & Baldwin, C. L. Annotation and classification of the bovine T cell receptor delta genes. *BMC Genomics* **11**, 100 (2010).
51. Antonacci, R. *et al.* Artiodactyl emergence is accompanied by the birth of an extensive pool of diverse germline TRDV1 genes. *Immunogenetics* **57**, 254–266 (2005).
52. Yang, Y. G., Ohta, S., Yamada, S., Shimizu, M. & Takagaki, Y. Diversity of T cell receptor delta-chain cDNA in the thymus of a one-month-old pig. *J Immunol* **155**, 1981–1993 (1995).
53. Rock, E. P., Sibbald, P. R., Davis, M. M. & Chien, Y. H. CDR3 length in antigen-specific immune receptors. *J Exp Med* **179**, 323–328 (1994).
54. Criscitiello, M. F., Ohta, Y., Saltis, M., McKinney, E. C. & Flajnik, M. F. Evolutionarily conserved TCR binding sites, identification of T cells in primary lymphoid tissues, and surprising trans-rearrangements in nurse shark. *J Immunol* **184**, 6950–6960 (2010).
55. Burland, T. G. DNASTAR's Lasergene sequence analysis software. *Methods Mol Biol* **132**, 71–91 (2000).
56. Sonnenhammer, E. L. & Durbin, R. A dot-matrix program with dynamic threshold control suited for genomic DNA and protein sequence analysis. *Gene* **167**, GC1–10 (1995).
57. Arden, B., Clark, S. P., Kabelitz, D. & Mak, T. W. Human T-cell receptor variable gene segment families. *Immunogenetics* **42**, 455–500 (1995).

58. Arden, B., Clark, S. P., Kabelitz, D. & Mak, T. W. Mouse T-cell receptor variable gene segment families. *Immunogenetics* **42**, 501–530 (1995).
59. Lefranc, M. P. *et al.* IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev Comp Immunol* **27**, 55–77 (2003).
60. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739 (2011).
61. *IMGT Repertoire* <http://www.imgt.org/IMGTrepertoire/> (2016).
62. Reinink, P. & Van Rhijn, I. The bovine T cell receptor alpha/delta locus contains over 400 V genes and encodes V genes without CDR2. *Immunogenetics* **61**, 541–549, doi: 10.1007/s00251-009-0384-9 (2009).
63. Conrad, M. L. *et al.* The genomic sequence of the bovine T cell receptor gamma TRG loci and localization of the TRGC5 cassette. *Vet Immunol Immunopathol* **115**, 346–356 (2007).
64. Massari, S., Ciccarese, S. & Antonacci, R. Structural and comparative analysis of the T cell receptor gamma (TRG) locus in *Oryctolagus cuniculus*. *Immunogenetics* **64**, 773–779 (2012).
65. Massari, S. *et al.* The deduced structure of the T cell receptor gamma locus in *Canis lupus familiaris*. *Mol Immunol* **46**, 2728–2736 (2009).
66. Parra, Z. E., Arnold, T., Nowak, M. A., Hellman, L. & Miller, R. D. TCR gamma chain diversity in the spleen of the duckbill platypus (*Ornithorhynchus anatinus*). *Dev Comp Immunol* **30**, 699–710 (2006).
67. Tjoelker, L. W. *et al.* Evolutionary conservation of antigen recognition: the chicken T-cell receptor beta chain. *Proc Natl Acad Sci USA* **87**, 7856–7860 (1990).
68. McCormack, W. T., Tjoelker, L. W., Stella, G., Postema, C. E. & Thompson, C. B. Chicken T-cell receptor beta-chain diversity: an evolutionarily conserved D beta-encoded glycine turn within the hypervariable CDR3 domain. *Proc Natl Acad Sci USA* **88**, 7699–7703 (1991).
69. Six, A. *et al.* Characterization of avian T-cell receptor gamma genes. *Proc Natl Acad Sci USA* **93**, 15329–15334 (1996).

## Acknowledgements

We thank Dr. Jing Wang from China Agricultural University for Dotplot analysis. This work was supported by grants from the National Foundation of Natural Sciences of China (No. 31272433 and No. 31472085).

## Author Contributions

Y.F.Z. conceived the hypothesis and provided funding support and supervision for this work. Y.L.J. designed the experiments and provided supervision for this work. Z.Y. designed and performed most of the experiments. Y.S. designed the experiments and wrote the paper. Y.H.M. performed the experiments and analysed most of the data. Z.R.L. and Y.Z. performed the experiments and analysed data. L.M.R. collected the samples. H.T.H. contributed reagents, materials and analytic tools. All authors have read and approved the final manuscript.

## Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Yang, Z. *et al.* A comprehensive analysis of the germline and expressed TCR repertoire in White Peking duck. *Sci. Rep.* **7**, 41426; doi: 10.1038/srep41426 (2017).

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017