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Extensive diversification of MHC in Chinese giant salamanders *Andrias davidianus* (Anda-MHC) reveals novel splice variants

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ABSTRACT

A series of MHC alleles (including 26 class IA, 27 class IIA, and 17 class IIB) were identified from Chinese giant salamander *Andrias davidianus* (Anda-MHC). These genes are similar to classical MHC molecules in terms of characteristic domains, functional residues, deduced tertiary structures and genetic diversity. The majority of variation between alleles is found in the putative peptide-binding region (PBR), which is driven by positive Darwinian selection. The coexistence of two isoforms in MHC IA, IIA, and IIB alleles are shown: one full-length transcript and one novel splice variant. Despite lake of the external domains, these variants exhibit similar subcellular localization with the full-length transcripts. Moreover, the expression of MHC isoforms are up-regulated upon *in vivo* and *in vitro* stimulation with *Andrias davidianus* ranavirus (ADRV), suggesting their potential roles in the immune response. The results provide insights into understanding MHC variation and function in this ancient and endangered urodele amphibian.

1. Introduction

The Chinese giant salamander *Andrias davidianus* belongs to one of the most primitive orders of urodele amphibians, the *Cryptobranchidae* (Zhang et al., 2003). It is the largest extant amphibian species, and found only in China (Wang et al., 2013). Being crown as a living fossil from 350 million years ago, and representing a transitional form that links aquatic to terrestrial organisms, it is considered to be a valuable model in studies on vertebrate evolution and biodiversity (Gao and Shubin, 2003; Robert and Cohen, 2011). The population has declined dramatically in the past five decades, and it has now been included in the list of Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2008) and in national class II protected species in China. Although the artificial breeding have been ongoing, the frequent outbreaks of infectious diseases pose a serious threat to the Chinese giant salamander population (Dong et al., 2011; Geng et al., 2011). *Andrias davidianus* ranavirus (ADRV), an emerging viral pathogen, is associated with mass mortality in farmed salamander (Chen et al., 2013; Zhang and Gui, 2012). To establish effective measures for the disease control, the development of genetic markers related to pathogen resistance is of great importance (Gui and Zhu, 2012).

One of the most ideal genetic markers in conservation programs is the major histocompatibility complex (MHC) (Sommer, 2005). It is a multigene family central to the vertebrate immune system by presenting self/non-self antigen peptides to T lymphocytes (Neefjes et al., 2011). A functional hallmark of MHC genes is extensive diversification concentrated in the peptide-binding region (PBR). MHC diversity is tightly linked to diseases resistance, and maintained through balancing selection mediated by host–pathogen co-evolution (Piertney and Oliver, 2006; Spurgin and Richardson, 2010). Hence, the well-characterized function of MHC in immune defense, alongside their outstanding diverse nature, makes them exceptional candidates to study patterns of adaptive genetic variation determining pathogen resistance, specially in species of conservation concern (Sutton et al., 2011).

To date, extensive research on MHC genes within amphibians has focused on model organisms such as the anuran *Xenopus* (Ohta et al., 2006) and the urodele axolotl (*Ambystoma mexicanum*) (Laurens et al., 2001). Compelling evidence showing high similarity in MHC structure and complexity between amphibians and mammals, and also close associations between MHC diversity and diseases resistance (Savage and Zamudio, 2011; Teacher et al., 2009). However, little information has so far been available about the functional role of MHC in the Chinese giant salamander. In our recent effort, a thymus cDNA library was constructed from Chinese giant salamander infected with ADRV. Screening for expressed sequence tags (ESTs) revealed a number of immune-related genes including those encoding MHC (Anda-MHC) and beta2-microglobuin (Anda-β2M). In this study, we embarked on investigating the variation and expression of these genes. The phylogenetic analyses were performed across vertebrate species, and the three-dimensional (3D) structures were predicted by homology modeling. The sequence polymorphism was examined, and the

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Table 1
Random sites model estimates for MHC IA, IIA and IIB alleles of the Chinese giant salamander.

Groups	Model	*P*	*lnL*	Parameter estimates	LRT	2Δ*lnL*	Positively selected sites
MHC IA	M0: one-ration	1	−2684.2561	κ = 2.6518, ω = 0.8073			
	M1: nearly neutral	1	−2648.4741	κ = 2.4850, p_0 = 0.5622, p_1 = 0.4378, ω_0 = 0.0000, ω_1 = 1.0000			
	M2: positive selection	3	−2626.1863	κ = 2.8482, p_0 = 0.5182, p_1 = 0.3740, p_2 = 0.1078, ω_0 = 0.0000, ω_1 = 1.0000, **ω_2 = 6.3411**	M2 vs M1	44.5757	90L, **122V**, **124Y**, 129R, 139F, **182E**, 189E, 193A, 196K
	M3: discrete	5	−2625.6300	κ = 2.8574, p_0 = 0.5823, p_1 = 0.3465, p_2 = 0.0712, ω_0 = 0.0000, ω_1 = 1.5611, **ω_2 = 8.2568**	M3 vs M0	117.2522	
	M7: beta	2	−2649.3795	κ = 2.5164, p = 0.0051, q = 0.0051			
	M8: beta & omega	4	−2626.2206	κ = 2.8415, p_0 = 0.8871, p_1 = 0.1129, p = 0.050, q = 0.0075, **ω = 6.0823**	M8 vs M7	46.3178	90L, **122V**, **124Y**, 129R, 139F, **182E**, 189E, 193A, 196K
MHC IIA	M0: one-ration	1	−1394.5989	κ = 2.8839, ω = 1.0114			
	M1: nearly neutral	1	−1394.3163	κ = 2.8043, p_0 = 0.1782, p_1 = 0.8218, ω_0 = 0.0000, ω_1 = 1.0000			
	M2: positive selection	3	−1392.9339	κ = 2.8416, p_0 = 0.9488, p_1 = 0.0000, p_2 = 0.9488, ω_0 = 0.7422, ω_1 = 1.0000, **ω_2 = 5.8360**	M2 vs M1	2.7648	9P, 18G, 32I, 87M
	M3: discrete	5	−1392.9339	κ = 2.8416, p_0 = 0.4599, p_1 = 0.4889, p_2 = 0.0512, ω_0 = 0.7422, ω_1 = 0.7422, **ω_2 = 5.8360**	M3 vs M0	3.3300	
	M7: beta	2	−1394.3779	κ = 2.8398, p = 0.0473, q = 0.0050			
	M8: beta & omega	4	−1392.9349	κ = 2.8417, p_0 = 0.9492, p_1 = 0.0508, p = 99.0000, q = 33.9983, **ω = 5.8612**	M8 vs M7	2.8859	9P, 18G, 32I, 87M
MHC IIB	M0: one-ration	1	−1533.1951	κ = 3.0802, ω = 2.9798			
	M1: nearly neutral	1	−1533.7786	κ = 2.5054, p_0 = 0.4423, p_1 = 0.5578, ω_0 = 0.0000, ω_1 = 1.0000			
	M2: positive selection	3	−1506.2896	κ = 3.6550, p_0 = 0.4120, p_1 = 0.5011, p_2 = 0.0870, ω_0 = 1.0000, ω_1 = 1.0000, **ω_2 = 27.5920**	M2 vs M1	54.9780	42A, **59L**, **78F**, **79V**, **98I**, **101D**, **102A**, **103R**, **109Y**,161S
	M3: discrete	5	−1506.1608	κ = 3.7159, p_0 = 0.1577, p_1 = 0.7646, p_2 = 0.0777, ω_0 = 0.0000, ω1 = 1.6150, ω_2 = **34.7115**	M3 vs M0	54.0686	
	M7: beta	2	−1533.9187	κ = 2.4607, p = 0.0050, q = 0.0050			
	M8: beta & omega	4	−1506.2896	κ = 3.6550, p_0 = 0.9131, p_1 = 0.0870, p = 2.8720, q = 0.0050, **ω = 27.5919**	M8 vs M7	55.2581	42A, **59L**,**78F**, **79V**, **98I**, **101D**, **102A**, **103R**, **109Y**, 161S

2△*lnL*: Log likelihood difference between models using the c2-test, P: number of free parameters for the ω ratios, κ: transition/transversion rate, ω: Ratio of non-synonymous to synonymous nucleotide substitution, p_n: proportion of sites that fall into the ω_n site class, p, q: shape parameters of the β function (for models M7 and M8), Positively selected sites with posterior probability >0.95 are in bold.

selection pattern was test to explore the underlying mechanisms shaping MHC diversity. The expression profiles of MHC genes upon stimulation with ADRV were investigated to elucidate their immune signification. Additionally, three alternative transcripts lacking the extracellular domains (α1/α2, α2, and β2) were isolated from MHC IA, IIA and IIB, respectively. The expression and subcellular distribution patterns were further compared between the full-length and truncated transcripts. Our study provide important genetic information for conservation of the endangered Chinese giant salamander.

2. Materials and methods

2.1. Salamanders, cells, and virus

Chinese giant salamanders weighing about 30 g were obtained from a farm in Jiangxi, China. The salamanders were maintained at 22 °C in an aerated freshwater tank for 2 weeks, and no clinical signs were observed prior to experiment. Chinese giant salamanders thymus (CGST) cells were cultured at 20 °C in medium 199 with 10% FBS, and *Epithelioma papulosum* cyprini (EPC) cells were maintained at 25 °C in medium 199 with 10% FBS. *Andrias davidianus* ranavirus (ADRV) was isolated from the Chinese giant salamanders and propagated in EPC cells as described previously (Chen et al., 2013).

Table 2
Primers used in this study.

Primer name	Sequence (5′–3′)	Usage
β-Actin-F	CCACTGCTGCCTCCTCTT	Real-time PCR
β-Actin-R	GCAATGCCTGGGTACATG	
MHC IA-F1	GGACTTCATCAGCCTCCACA	
MHC IA-R1	AGGTTCCGTCAGGGTTCG	
β2M-F1	TTTGCTCCTGCTGGTGGT	
β2M-R1	AGATAAGGGTGTTCGGTTTT	
MHC IIA-F1	CTGCTGTCACCGTGTTCC	
MHC IIA-R1	ACTGGTTGCGTGTTCTTCA	
MHC IIB-F1	CACTTCCTGAACGGCTCG	
MHC IIB-R1	CGGGCGGGTAAAAGTCT	
MHC IA-F2	ATGACGTCCCGGACCACTCT	Complete ORF of MHC
MHC IA-R2	GGCGGAGGCGGTGCTGGA	
MHC IIA-F2	ATGGCTGCAGTCCGGTGC	
MHC IIA-R2	TTTCATTCTTCTTCTGTGACTTGGG	
MHC IIB-F2	ATGCGTCCCCCCCTCAATCC	
MHC IIB-R2	TAGGCAACATCATTCTGAGGGA	
MHC IA-F3	CCCTCGAGATGACGTCCCGGACCACT	Subcellular localization
MHC IA-R3	CGCGGATCCGGCGGAGGCGGTGCTGGA	
β2M-F2	CCCTCGAGCTATGGGTACCACTGTGAGG	
β2M-R2	CGGAATTCCAGCTTGGGATCCCAGGTGTG	
MHC IIA-F3	CCCTCGAGATGGCTGCAGTCCGGTGC	
MHC IIA-R3	CGGGATCCTTTCATTCTTCTTCTGTG	
MHC IIB-F3	CCCTCGAGCTATGCGTCCCCCCCTCAATCC	
MHC IIB-R3	CGGGATCCGGCAACATCATTCTGAGGGA	