Isolation and characterization of class I MHC genes in the giant panda (*Ailuropoda melanoleuca*)

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Artificial breeding is an important project to protect, recover and reintroduce endangered species. Knowledge of the population's genetic diversity at functional loci is important for the establishment of effective captive breeding programs. The major histocompatibility complex (MHC) genes are ideal candidate genetic markers to inform planned breeding, due to their high levels of polymorphism and importance in the main immune coding region of the vertebrate genome. In this study, we constructed BAC-based contigs and isolated six functional MHC class I genes from the giant panda (*Ailuropoda melanoleuca*), which we designated *Aime-C*, *Aime-F*, *Aime-I*, *Aime-K*, *Aime-L* and *Aime-1906*. Analyses of the tissue expression patterns and full-length cDNA sequences of these class I genes revealed that *Aime-C*, *-F*, *-I* and *-L* could be considered classical class I loci, due to their extensive expression patterns and normal exonic structures. In contrast, *Aime-K* and *-1906* appeared to be nonclassical genes based on their tissue-specific expression patterns and the presence of an abnormal exon 7 in both genes. We established techniques for genotyping exons 2 and 3 of the classical loci using locus-specific single strand conformation polymorphism (SSCP) and sequence analysis. In the Chengdu captive population, we identified one monomorphic locus (*Aime-F*) and three polymorphic loci with different numbers of alleles (4/4/4 exon 2 alleles at *Aime-C/IL* and 6/5/5 exon 3 alleles at *Aime-C/IL*). The distributions of the *Aime-C*, *-I* and *-L* alleles among members of different families were in good agreement with the known pedigree relationships, suggesting that the genotyping results are reliable. Therefore, the MHC-I genotyping techniques established in this study may provide a powerful tool for the future design of scientific breeding or release/reintroduction programs.

giant panda, MHC class I, isolation, locus-specific genotyping

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Many animal species are under threat due to human activities and habitat fragmentation, and effective management plans are needed to prevent the extinction of endangered or threatened species. Such plans may include captive breeding programs, which are widely used for the recovery and reintroduction of endangered species [1,2]. The main goal of these breeding programs is the maintenance of a stable, self-sustaining population that retains genetic diversity and experiences limited inbreeding [3,4].

To establish an effective breeding program, it is important to first learn about the genetic diversity present in

current populations [5]. Genetic analysis based on neutral markers, such as microsatellites, mitochondrial markers and single nucleotide polymorphisms, are widely used for such work [6]. These markers are useful for analyzing dispersal patterns and population histories, but are not effective for assessing the ability to adapt to environmental changes [6]. In order to assess such changes, we also need to investigate genetic diversity at functional loci such as the major histocompatibility complex (MHC) genes [7].

The MHC genes are ideal candidates for this work, due to their high polymorphism and importance in the main immune coding region of the vertebrate genome [8]. The MHC molecules can be classified into two groups: class I

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molecules, which are mainly associated with intracellular pathogens; and class II molecules, which are responsible for the presentation of extracellular pathogens [9]. The typical MHC class I molecule consists of an α chain and a β 2-microglobulin. The α chain includes three extracellular domains (α 1, α 2 and α 3), a transmembrane portion (TM) and a cytoplasmic domain (Cyt-tail) [9]. The MHC genes can be further divided into classical (class Ia) and nonclassical (class Ib) genes based on their polymorphisms, expression levels and functions [10].

The class Ib genes tend to be less polymorphic, show narrower ranges of tissue expression and have shorter cytoplasmic domains than the class Ia genes [11,12]. class Ib molecules have a range of functions [11], while class Ia molecules present endogenous peptides to CD8⁺ cells [13]. When “MHC-antigen complex” are formed and presented to the surface of the cell, T cell can be activated to remove pathogens and parasites [14].

High level of MHC polymorphism at MHC loci is maintained by coevolution of host with their parasites and pathogens [15]. Loss of alleles at MHC genes due to inbreeding depression, is thought to decrease the ability of populations to respond to pathogen [7,14,16]. Therefore, genetic variation at MHC loci is essential for captive breeding programs of endangered species.

The giant panda (*Ailuropoda melanoleuca*) is an endangered species endemic to China. To ensure the survival of this species, a captive breeding program was initiated in the 1980s [17]; at present, the captive populations include 328 individuals worldwide [18]. Some MHC studies have already been conducted on this rare species: MHC class II genes have been isolated [19–22] and locus-specific MHC genotyping techniques have been established [20,21]. However, only three class I MHC genes (*Aime*-128, *Aime*-152 and *Aime*-1906) have been isolated to date, and locus-specific class I MHC genotyping could not be performed due to problems with cross-locus amplification [23]. The isolation of the remaining class I MHC genes is an essential next step if we wish to accurately genotype the extremely similar functional MHC class I loci of the giant panda.

In the present work, we constructed BAC-based contig maps containing the MHC class I genes of the giant panda, and sequenced these contigs to resolve the classical and nonclassical class I genes. For each classical MHC class I gene, we developed locus-specific genotyping techniques for exons 2 and 3. We validated the reliability of these techniques in a captive population and then used them to investigate the genetic diversity of this population.

1 Materials and methods

1.1 Sampling and DNA extraction

Blood and tissue samples were collected from 26 giant pandas with clear pedigree information [24] from the

Chengdu Giant Panda Breeding Research Base (Chengdu). Blood samples were obtained during routine medical examinations and stored in liquid nitrogen. Liver and brain tissues were obtained from dead captive pandas. Genomic DNA was extracted from the blood and tissue samples using standard phenol-chloroform methods [25].

1.2 Construction of a BAC-based contig map of the MHC class I genes

Two pairs of universal primer (C1E2F3/R1, C1E3F1/R1; Table 1) were designed based on the previously determined giant panda class I cDNA sequences (EU162658, EU162659, EU162660) and class I gene sequences from other carnivores (dog: NM_001014767, NM_001014378, NM_001014379; cheetah: U07665, U07666; cat: U07667, U07668, U07669, U07670, U07672, U07673, U07674; ocelot: U07675, U07676, U07677, U07678). The universal primers were used to screen BAC pools, and the MHC class I gene-containing BACs were identified with 4D-PCR and sequencing, as previously described [26]. All of the confirmed-positive BACs were subjected to commercial end-sequencing (Majorbio, Shanghai), the obtained end sequences were repeat-masked, and non-repeat ends were identified (<http://www.repeatmasker.org>). End-primers were designed and used to build BAC-based overlapping clusters [26], and the BACs showing minimum tiling paths in the overlapping cluster were subjected to commercial BAC sequencing (Majorbio). The results were used to construct a physical map and resolve the MHC class I gene-enriched contigs. Putative functional MHC class I genes were obtained from the contigs using GENESCAN (<http://genes.mit.edu/GENSCAN>).

1.3 Expression patterns and full-length cDNA amplification of *Aime*-MHC class I genes

Over the past decade, we obtained three types of fresh samples from three different captive individuals: blood from Chenggong (studbook No. 522; living), which was obtained during a routine medical examination; liver from Jiaozizhizi (No. 577; death in 2003); and brain from Eryatouzhizi (No. 599; death in 2003). Total RNA was extracted from these tissues using the TRIzol Total RNA Isolation Kit (Invitrogen), mRNA was purified with an Oligotex mRNA Spin-Column Kit (Qiagen), and full-length cDNA was generated using a SMARTTM PCR cDNA Synthesis Kit (Clontech). Based on the coding sequences of the *Aime*-MHC class I genes predicted from the contigs using GENESCAN, we designed locus-specific primers (Table 1) and used them to detect expression in the different tissues. We then formulated locus-specific primer pairs for all of the expressed loci (Table 1; 5'UTR–exon 3 and exon 2–3'UTR) and used them to isolate the full-length cDNA sequences of the new *Aime*-MHC class I genes (i.e. excluding the known genes, *Aime*-128, -152 and -1906) [23].

Table 1 The primers used to isolate *Aime*-MHC class I genes^{a)}

Application	Primer name		Primer sequence (5'→3')	Size (bp)	T _a (°C)
BAC pool screening	C1E2-	F3	GTGGACGACACGCAGTTCG	190	62.0
		R1	CGGCCTCGCTCTGGTTGTAGTAGC		
	C1E3-	F1	GCGCGGATTACATCGCCCTGAAC	186	63.0
		R1	CGCGCTGCAGCGTCTCCTTCC		
End location	1611G3-T7	F	CTGCACCCCGCCCTCTA	257	55.5
		R	TCCCCCTGCCTGTGTTC		
	1611G3-RP2	F	CACCCCTGCTGCCTTTCTCCTC	233	59.2
		R	TCTCCCGCCCTTAGTTCCAGTGAT		
	1470D3-T7	F	AGGCGCTGAAGAAATAAAAACTA	338	53.1
		R	CTTCTCCTGCCCCGCTGACTCCA		
	1470E1-T7	F	TGCCGGCACCCATAGTAG	385	56.0
		R	GGCCATTTTCCCTCATTTT		
	1470E1-RP2	F	ATATCCCCAGGTGTAGAGTGTGAC	280	55.5
		R	AGGGGGAGTGGGAGAGGGAGAA		
	1470E3-RP2	F	CCCCCTCGCTTGGCATACTTCTGT	339	60.5
		R	AGCGGGTGTGTGGCGGGTGTC		
	897E9-T7	F	GGGGCAGAAGCAAAGGAAGACA	351	59.5
		R	AGGCCGGGCTCAGGGTAAG		
	897E9-RP2	F	CGGCAGTAAGACAGGAAAGGAAC	401	58.0
		R	GTAACCCCGGGGCTCAGTCA		
Expression detection	<i>Aime</i> -C-	Fe	GGACCGGGAGACGCGGATCAT	876	60.5
		Ru	TGCATCACTCAGTCCCCATG		
	<i>Aime</i> -F-(i.e. 152)	cF	GACCGGGAGACGCGGAAT	815	61.5
		cR	TCCCCACAAGGCAGCTCA		
	<i>Aime</i> -I-(i.e. 128)	cF	CCGAGTGGACTTGACAGACCGCC	832	59.5
		cR	CATCACTCAGTCCCCACG		
	<i>Aime</i> -K-	Fe	GAGGCCGGCTCTCACACTATG	836	60.0
		Ru	AAGAGAACTCAGGGGTGTGTGATG		
	<i>Aime</i> -L-	Fe	GCTCTCACACCATCCAGGA	784	62.0
		Ru	CATCACTCAGTCCCCACG		
	<i>Aime</i> -1906-	cF	GGAAGGGTCTCACACCTC	849	54.5
		cR	TTATGCTAATAGGAACATATA		
Isolation of the full-length cDNA sequence	<i>Aime</i> -C-	Fu	CCCGCCAGTGGACCACTGTC	428	61.5
		Re	CAGCCGTACATCCACTGGTA		
		Fe	—	876	60.5
		Ru	—		
	<i>Aime</i> -K-	Fu	TCTCCCCAGAACCTGGAG	572	59.0
		Re	TCCAGGTAGTTCCTGAGG		
		Fe	—	784	62.0
		Ru	—		
	<i>Aime</i> -L-	Fu	CACGGTTTCGGGTATAAG	455	59.5
		Re	AGGTCACAGCCGTGCATCTC		
		Fe	—	784	62.0
		Ru	—		

a) T_a, annealing temperature for each primer pair.

1.4 Locus-specific amplification of the classical *Aime*-MHC class I loci

For the classical *Aime*-MHC class I genes, we analyzed exons 2 and 3, which encode the highly variable $\alpha 1$ and $\alpha 2$ domains of the class I proteins. Given the highly similar intronic sequences adjacent to exons 2 and 3 of the classical genes, we used a nested-PCR genotyping technique (Table 2): the first round consisted of a locus-specific amplification that yielded fragments >350 bp, while the second round used common primers to produce fragments that were <350 bp, and therefore suitable for single strand conformation polymorphism (SSCP) analysis [21].

Amplifications were carried out in 10- μ L reactions containing 20 ng of DNA template, 5 μ L of 2 $\times$ GC I buffer with MgCl_2 (TaKaRa), 0.2 $\mu\text{mol/L}$ of each primer, 0.2 mmol/L dNTP, and 0.25 U Ex-Taq polymerase (TaKaRa). For the first-round PCR reaction, the following amplification conditions were used: 5 min at 95°C followed by 22–25 cycles of 30 s at 95°C, 30 s at the annealing temperature (Table 2), and 1 min at 72°C, with a final extension period of 5 min at 72°C. The products of the first-round PCR were used as the templates for the second-round PCR, which started with 5 min of denaturation at 95°C, followed by 30–35 cycles of 30 s at 95°C, 30 s at the annealing temperature, and 30 s at

72°C, and ended with an elongation step at 72°C for 5 min. Negative control reactions were amplified in each case to rule out possible contamination.

1.5 MHC genotyping

We used SSCP analysis to screen the PCR fragments, because this method is highly sensitive to variation and can detect differences of only a single base pair [27]. The obtained PCR products were mixed with equal volumes of gel loading buffer, denatured at 95°C for 3–4 min, chilled on ice, and then electrophoresed at 35 W on 12% nondenaturing polyacrylamide gels for 8–10 h at 4°C. The resulting DNA bands were visualized by silver staining.

1.6 Data analysis

All sequences were edited and aligned using the DNASTAR software package (DNASTAR, Inc.). The antigen binding sites (ABS), identified by comparison to human sequences [28], were used to assess sequence variability.

Allele frequencies and deviations from Hardy-Weinberg equilibrium (HWE) for each locus were tested using exact tests, as implemented in GenePop 4.0 [29]. Observed (H_O) and expected (H_E) heterozygosities were calculated using

Table 2 Locus-specific primers used to amplify exons 2 and 3 from the *Aime*-MHC class I genes

Locus	Primer name	Primer sequence (5'→3')	Size (bp)	T_a (°C)
<i>Aime</i> -CE2	1611E21F ^{a)}	GCTCTCCCCACTCAGTA	600	60.1
	1611E21R ^{a)}	CAGCCGTACATCCACTGGTA		
<i>Aime</i> -CE3	1611E34F ^{a)}	ATGACGTATTCTACACCGGC	1127	64.1
	1611E33R ^{a)}	TGCAGGTCTAAGAGGGAGAGCGCT		
<i>Aime</i> -FE2	152E2A3 ^{a)}	GCCCTGCTCTCCCCACTCAG	496	65.3
	152E2A4 ^{a)}	CGGGGGTTCTTGAGAGTTGGGGC		
<i>Aime</i> -FE3	152E3A1 ^{a)}	AGTCCGAGCGTTGCCCAACTCTC	787	59.5
	152E3A2 ^{a)}	AGTAATGGCCCTGAGTAAGGTTTC		
<i>Aime</i> -IE2	128E22F ^{a)}	CCTGCTCTCCCCAACGCG	614	62.4
	128E22R ^{a)}	GTCACAGCCGTGCATCCA		
<i>Aime</i> -IE3	128E31F ^{a)}	CCGAGTGGACTIONGACACCGCC	1131	62.5
	128E32R ^{a)}	GTCCGGGGTTTCTGAAGAAGAACG		
<i>Aime</i> -LE2	1300E22F ^{a)}	GGGAGAAGGGTCGGGCGGGAC	535	62.2
	1300E21R ^{a)}	AGGTCACAGCCGTGCATCTC		
<i>Aime</i> -LE3	1300E31F ^{a)}	GCTCTCACACCATCCAGGA	887	62.1
	1300E31R ^{a)}	GTCGGGGGTTTCTGAAGAAGAATC		
<i>Aime</i> -C/F/IE2	C1E2B1 ^{b)}	TCAGCCCCTCCGCGCCCGCAG	312	66.3
	C1E2B2 ^{b)}	GACCCGGGCGCGTCGCTCAC		
<i>Aime</i> -C/F/IE3	C1E3B1 ^{b)}	TCGCTCTGTCGGGCGGGGCCAG	349	65.6
	C1E3B2 ^{b)}	AGCCAGCCCCAGCGAGGGG		

a) and b) indicate the first and second primer pairs used for the nested PCR, respectively. The same second-round primer pairs were used for all four classical loci.

the program GENETIX4.05 [30].

2 Results

2.1 Construction of MHC class I contigs and identification of *Aime*-MHC class I loci

Using primer pairs C1E2F3/R1 and C1E3F1/R1, we isolated 16 positive BACs, 7 of which were used to form two contigs of minimum BAC tiling paths (Figure 1). GENESCAN predicted six MHC class I genes from these two contigs. Three were new and the remaining three corresponded to the previously reported *Aime*-128, -152 and -1906 loci [23]. For the purposes of this study, we designated the genes in the first contig as *Aime*-C, *Aime*-F, *Aime*-I, *Aime*-K and *Aime*-L (Figure 1(a)), and retained the name *Aime*-1906 for the single gene found in the second contig (Figure 1(b)). *Aime*-I, *Aime*-F, and *Aime*-1906 corresponded to *Aime*-128, -152 and -1906 loci respectively [18].

Analysis of the tissue expression patterns of these cDNAs showed that both *Aime*-K and *Aime*-1906 were expressed in brain and liver, but not in blood, whereas the other four genes were expressed in all three tested tissues (Figure 2). Furthermore, analysis of the full-length cDNA sequences for *Aime*-C, -F, -I, -K, -L and -1906 revealed that *Aime*-K lacked exon 7 and *Aime*-1906 had a prestop codon in exon 7, while the other four genes presented normal exonic structures (Figure 3). Therefore, we classified *Aime*-K and *Aime*-1906 as nonclassical genes and *Aime*-C, -F, -I and -L as classical loci. Accordingly, we used the latter four genes to establish locus-specific genotyping.

2.2 Establishment of a locus-specific genotyping technique

Genotyping of exons 2 (270 bp) and -3 (276 bp) from the

four classical *Aime*-MHC class I loci was performed. We found that *Aime*-F was monomorphic, while *Aime*-C, -I and -L were polymorphic. No more than two sequences were present in any animal, indicating that our gene-specific primers did not produce cross-locus amplification. Furthermore, we obtained perfect genotype matches for all parent-offspring pairs, indicating that the utilized primers did not yield null alleles, and therefore represented the desired intra-locus conserved regions.

2.3 Genetic variability of *Aime*-MHC class I genes

In total, we obtained 4/4/4 exon 2 alleles and 6/5/5 exon 3 alleles from *Aime*-C/-I/-L, respectively (Table 3). All of the alleles were confirmed in either two individuals or two PCR reactions from the same individual. Consistent with our expression data (see above), none of the alleles contained any deletion, insertion or stop codon, suggesting that all of the detected sequences should represent functional molecules. Some of the alleles were highly frequent (e.g.,

Figure 1 Two contigs representing the *Aime*-MHC class I region. The black and gray diamonds indicate the BAC ends used for primer design and repeats that were not used for primer design, respectively. The black rectangles represent the *Aime*-MHC class I genes.

Figure 2 Expression levels of six *Aime*-MHC class I genes in different tissues. B1, blood; Br, brain; and Lv, liver.

Signal peptide

Aime-C	--MQVMART	-LVLLSGAL	AVTETWA	[27]
Aime-F	--SNLLR	-		[27]
Aime-I	--T	-		[27]
Aime-K	--LT	-V	N	[27]
Aime-L	--G	-		[27]
Aime-1906	MW.A.SRP	L.L	TL.K.S	[27]

$\alpha 1$ domain

	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
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Figure 3 Amino acid alignments deduced from the *Aime*-MHC class I cDNA sequences. Dots and gaps indicate identities and missing amino acids, respectively, compared with the first sequence. Crosses represent putative peptide-binding sites, as determined using the method of Bjorkman et al. [28].

Table 3 Genotype frequencies and heterozygosities of the four classical MHC class I loci^{a)}

Locus	N_a	01	02	03	04	05	06	H_o	H_E
Exon 2	<i>Aime-C</i>	4	0.173	0.423	0.365	0.038	—	0.654	0.669
	<i>Aime-F</i>	1	1.000	—	—	—	—	—	—
	<i>Aime-I</i>	4	0.865	0.019	0.058	0.058	—	0.269	0.249
	<i>Aime-L</i>	4	0.231	0.077	0.365	0.327	—	0.769	0.714
Exon 3	<i>Aime-C</i>	6	0.058	0.115	0.365	0.250	0.038	0.173	0.769*
	<i>Aime-F</i>	1	1.000	—	—	—	—	—	—
	<i>Aime-I</i>	5	0.019	0.750	0.096	0.058	0.077	—	0.308
	<i>Aime-L</i>	5	0.077	0.019	0.231	0.327	0.346	—	0.769

a) N_a is the number of alleles. The predominant allele is marked in bold. Asterisks denote significant deviations from HWE ($P < 0.05$).

*Aime-CE2*02*, *Aime-IE2*01*, *Aime-LE2*03* and 04, *Aime-CE3*03*, *Aime-IE3*02*, and *Aime-LE3*04* and 05), with two showing frequencies > 0.5 (*Aime-IE2*01* and *Aime-IE3*02*).

Similar levels of heterozygosities were observed at exon 2 (0.269–0.769) and exon 3 (0.308–0.769) for *Aime-C/I-L*. The HWE test showed no significant deviation for exons 2 and 3 at any locus with the exception of *Aime-CE3*. We found higher than expected heterozygosity at *Aime-IE2*, *Aime-LE2* and *Aime-LE3*, and lower than expected heterozygosity at *Aime-CE2*, *Aime-CE3* and *Aime-IE3* (Table 3).

2.4 Sequence variation among the *Aime*-MHC class I genes

The alignment of exon 2 (270 bp) nucleotide sequences revealed 23/17/12 variable sites for *Aime-C/I-L*, respec-

tively, corresponding to 14/10/18 variable amino acids in the $\alpha 1$ domain. The alignment of exon 3 (276 bp) nucleotide sequences yielded 20/14/8 variable sites for *Aime-C/I-L* respectively, corresponding to 9/7/10 variable amino acids in the $\alpha 2$ domain (Figure 4). The mean number of variable amino acids in the $\alpha 1$ domains of the three polymorphic loci (14) was more than that in the $\alpha 2$ domains (8.7). Furthermore, the variable amino acids were concentrated in the ABS of both domains, suggesting that most of the variation was localized to the sites responsible for antigen recognition and presentation (Figure 4).

3 Discussion

The isolation of MHC class I genes can be complicated by

Figure 4 Amino acid alignments deduced from classical *Aime*-MHC class I alleles of the $\alpha 1$ (A) and $\alpha 2$ (B) domains. Dots and gaps indicate identities and missing amino acids, respectively, compared with the first sequence. Crosses represent putative peptide-binding sites, as determined using the method of Bjorkman et al. [28].

the high similarities among orthologous and paralogous members [31]; hence many studies on MHC variation have been conducted across multiple MHC class I genes [32,33]. However, such studies are unable to assign alleles to the individual loci, often making it impossible to identify true genotypes and recognize complex processes occurring at individual loci (e.g., selection pressure, mate behavior, and associations between MHC genotype and parasite load) [13,34]. Here, we successfully isolated six class I genes and developed a set of high-resolution genotyping primers for proper genotypic analysis of the giant panda. Using these primers, we found that the alleles previously identified for the so-called *Aime*-128 locus in Pan et al. [23] actually represented two loci: *Aime*-I and *Aime*-L. This also indicated that our locus-specific technique was effective.

In total, we identified 13 exon 2 alleles and 17 exon 3 alleles for the four classical *Aime*-MHC class I genes in 26 individuals, indicating that the giant panda retains a relatively high variability in its adaptive immune system. Sequence alignment reveals that the variable amino acids were mainly concentrated in the ABS positions (Figure 4), suggesting that polymorphism of *Aime*-MHC class I genes could be attributed to the requirement of antigen recognition and presentation.

In conclusion, we herein successfully isolated six functional MHC class I genes from the giant panda and established an effective locus-specific genotyping technique for the four classical MHC class I genes. This may provide a new viewpoint for isolating MHC genes for other species, and our data on genetic diversity may aid in captive breeding of the Chengdu population. Furthermore, our results

provide a good reference for better understanding the giant panda's living situation and may facilitate the future establishment of detailed conservation strategies.

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