

Songbirds Conserved Sites and Intron Size of MHC Class I Molecules Reveal a Unique Evolution in Vertebrates

A. Arnaiz-Villena*, V. Ruiz-del-Valle, P. Reche, P. Gomez-Prieto, E. Lowry, J. Zamora, C. Areces, D. Rey, C. Parga and J.I. Serrano-Vela

Department of Immunology, University Complutense, the Madrid Regional Blood Center, Madrid, Spain

Abstract: Birds are considered dinosaurs that passed the 65 million years ago bottleneck. Songbirds (Passeriformes) include about half extant bird species (about 5000) and are generally the most air-thriving bird species, concordantly with their small size. Major Histocompatibility complex (MHC) molecules stimulate immune responses against microbes and its class I molecules have seven conserved residues in all vertebrates from jawed-fishes, 300 million years ago, to humans, including chickens.

All wild songbird species tested by us (n=18) and others (n= 2) differ in $\alpha 1$ domain residue 10 and $\alpha 2$ residue 96 from all other vertebrates. Amplification, cloning and sequencing were performed by standard methods. Sequences alignment were done by using PAUP and MEGA programs software. Crystallographic studies were performed by using mammal and bird MHC molecules from MPID database and other sources and showed that these changes did not significantly vary the MHC class I molecule stability in songbirds.

Further $\alpha 1$ and $\alpha 2$ domain comparisons by simple Composition Distances and Bayesian Inference showed that songbirds overall MHC class I molecules are phylogenetically more separated from mammal than other birds molecules. In addition MHC class I introns from Passeriformes (songbirds) were found to be longer than humans, chicken introns being the shortest ones.

These small mainly air-borne dinosaurs (Passeriformes) have undergone a different evolutive pathway, regarding to MHC, than all other tested vertebrates and more terrestrial birds. This may have been originated by an altogether different dinosaurs lineage origin or to adaptation to more aerial than terrestrial environment or other unknown cause. In any case, the specific changes observed in this work for class I molecules in songbirds have reached a entropic, stable solution similar to that reached by other vertebrates.

Keywords: Songbirds, MHC evolution, immunology, passerines, carduelis, serinus.

INTRODUCTION

The Major Histocompatibility Complex (MHC) comprises the most polymorphic loci in vertebrates and its molecules present antigenic peptides to clonotypic T cell receptor in order to start an immune response [1]. These proteins evolve rapidly and are quite different in primary sequence of different species [2]. However, certain amino-acid residues are conserved in MHC class I molecules from reptilian to humans for keeping the overall tertiary structure [3].

MHC class I genes have been completely sequenced ($\alpha 1$ and $\alpha 2$ protein domains [1]) and thoroughly studied in *Gallus gallus* (chicken) [4, 5], and mammals [1, 6]. The MHC locus [7] was first defined in chicken [8, 9], in particular the highly consanguineous variety 'Leghorn' [4, 10]. The chicken's MHC genetic region is considerably smaller than that of mammals —remarkably it has shorter introns [4, 11]—, and is organized quite differently [4]. Thus, a noteworthy difference is the existence of short

introns in the chicken; that supported the hypothesis that the chicken's MHC represented a 'minimal essential MHC' [4, 5]. It has been assumed that chicken (order *Galliformes*) MHC was similar to all species included in the whole class *Aves*. However, it was shown later that this was not the case (see below).

Class I MHC genes have been sequenced and studied in four songbird species: *Acrocephalus arundinaceus* (great reed warbler) [12], *Serinus canaria* (wild canary), *Serinus mozambicus* (yellow-fronted canary), and *Serinus thibetanus* (Tibetan serin) [13, 14]. These sequences showed an apparently more variable exon 3 than *G. gallus* [12-14], and that class I MHC evolution in islands was different than in sister continental species —wild canary (*Serinus canaria*) vs. Asian Tibetan serin and African yellow-fronted canary— [14].

On the other hand, mammals and dinosaurs both appeared in Triassic Epoch —about 300 million years ago (MYA)—, and both survived the 65 MYA Cretaceous extinction. Currently there are about 4200 mammal species, and 9600 dinosaur (bird) species [15, 16]. After the extinction, when all terrestrial dinosaurs disappeared, many mammals occupied these terrestrial niches recently left empty.

*Address correspondence to this author at the Departamento de Inmunología, Facultad de Medicina, Universidad Complutense de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain; Tel: +34 913017354; Fax: +34 913017356; E-mail: arnaiz@med.ucm.es

However, mammals could not compete with aerial dinosaurs (birds) [15].

We have studied class I MHC genes and their corresponding proteins in songbirds, and compared them to other birds, and to the rest of available vertebrate genes. We focused on those domains interacting with T-cell receptors and antigen peptides and subjected to variability by balancing selection ($\alpha 1$ and $\alpha 2$ domains) [1]. Particularly we were interested on highly conserved positions which are kept unchanged throughout vertebrate history [17]; some of them are related to interaction with the antigen [18, 19] or between the rest of heavy and $\beta 2$ -microglobulin chains [17]. We show that some of such “universal” conserved positions throughout vertebrate evolution bear an exception to their universality in the case of songbirds. This is relevant in an evolutionary context since birds are dinosaurs that survived the Cretaceous/Tertiary extinction about 65 MYA, but are now undergoing a new extinction mainly due to human intervention. Their habitat’s disappearance and pesticide spreading are deleterious for them, among other factors [15]. In the last 2000 years 20% or more aerial dinosaur species have disappeared by man-bound interventions [15] and about 50% of the extant species are currently being reduced by the same cause [20].

Air thriving tiny dinosaurs, mainly birds of order *Passeriformes* (songbirds) may have undergone different evolutive pressures (mainly pathogen-driven [1]) at the

MHC class I level than other vertebrates, which may include more terrestrial birds. In the present paper we have aimed to compare this key immune molecule (class I MHC) conserved sites, intron size and tertiary structure between terrestrial and air-thriving vertebrates, (mostly songbirds) to gather clues about immune system evolution and draw functional MHC conclusions in terrestrial and air-thriving vertebrates.

MATERIAL AND METHODS

Sampling and Sequencing

Class I Major Histocompatibility Complex (MHC-I) — exons 2, 3, intron 2— gene sequences from 44 taxa were analyzed. Several fish, amphibians, reptiles, non-songbird aves, songbird aves, non-primate mammals, and primates were studied, as detailed in Table 1. Distribution and GenBank sequence accession numbers are given. In the case of our own samples (wild songbirds: *Fringilla coelebs*, and species from genera *Carduelis* and *Serinus*, n =18), blood from living birds was drawn after photographing by cutting the nail of legs locally anaesthetized with lidocaine ointment. Blood was collected in EDTA at 4 °C and frozen until use. DNA was obtained, and exons 2, 3, and intron 2 of the MHC gene were amplified with primers 3’-GTTCTCCACTCC CTGGATTACC-5’ (direct) and 5’-GCGCTCCAGTCCCTT CTGCCCRTA-3’ (inverse), and subsequently cloned and sequenced, [12-14, 21, 22].

Table 1. Vertebrate Species Studied and GenBank Accession Number. -: Gap or Unknown. *: Predicted Sequence. Dark Orange: Passeriformes; Light Orange: Other Birds; Dark Blue: Primates; Light Blue: Other Mammals; Green: Reptiles; White: Fishes. Songbird Species Thriving in A (Asia), E (Europe), F (Africa), NA (North America), SA (South America), P (Pacific) [43, 44]. Note that Jawed Fish, like Zebra Fish or Carp, First Appeared on Earth Over 300 MYA

English Name	Name	GenBank #	Position	
			10	96
Zebra fish	<i>Danio rerio</i>	AAF20179	-	Gln
Carp	<i>Cyprinus carpio</i>	[3]	Thr	Gln
African clawed frog	<i>Xenopus laevis</i>	[3]	Thr	Gln
Snake	<i>Nerodia sipedon</i>	[3]	-	-
Amieva lizard	<i>Amieva amieva</i>	[3]	Thr	Gln
Cow	<i>Bos taurus</i> x <i>Bos indicus</i>	ABW70136	Thr	Gln
Dog	<i>Canis familiaris</i>	NP_001014767	Thr	Gln
Horse	<i>Equus caballus</i>	NP_001075976	Thr	Gln
Mouse	<i>Mus musculus</i>	AAV85367	Thr	Gln
Sheep	<i>Ovis aries</i>	CAJ57269	Thr	Gln
Rat	<i>Rattus norvegicus</i>	CAA74333	Thr	Gln
Greater Horseshoe Bat	<i>Rhinolophus ferrumequinum</i>	ACC68844*	Thr	Gln
Pig	<i>Sus scrofa</i>	ACA33862	Thr	Gln
Short-beaked echidna	<i>Tachyglossus aculeatus</i>	AAM54212	-	Gln
Common gibbon	<i>Hylobates lar</i>	AAB08074	Thr	Gln
Orangutan	<i>Pongo pygmaeus</i>	AAK67485	Thr	Gln
Western gorilla	<i>Gorilla gorilla</i>	CAA43100	Thr	Gln
Chimpanzee	<i>Pan troglodytes</i>	BAC78189	Thr	Gln

(Table 1) Contd.....

English Name	Name	GenBank #	Position	
			10	96
Bonobo	<i>Pan paniscus</i>	AAV59433	Thr	Gln
Human	<i>Homo sapiens</i> (HLA-A2)	BAA07530	Thr	Gln
Human	<i>Homo sapiens</i> (HLA-B)	CAA06616	Thr	Gln
Human	<i>Homo sapiens</i> (HLA-C)	CAB02408	Thr	Gln
Chicken	<i>Gallus gallus</i>	AY489160	Thr	Gln
Japanese quail	<i>Coturnix japonica</i>	D29813	Thr	Gln
Great Reed warbler	<i>Acrocephalus arundinaceus</i> ^{E+A+F}	CAA06566	Val	Leu
Black siskin	<i>Carduelis atrata</i> ^{SA}	DQ257462	Val	Leu
Black-capped siskin	<i>Carduelis atriceps</i> ^{NA}	FJ268821	Val	Leu
European goldfinch	<i>Carduelis carduelis</i> ^{E+A+F}	FJ266447	Val	Leu
Citril finch	<i>Carduelis citrinella</i> ^E	DQ257482	Val	Leu
Lawrence's goldfinch	<i>Carduelis lawrencei</i> ^{NA}	FJ314425	Val	Leu
Pine siskin	<i>Carduelis pinus</i> ^{NA}	FJ266376	Val	Leu
Eurasian siskin	<i>Carduelis spinus</i> ^{E+A+F}	FJ266399	Val	Leu
Common rosefinch	<i>Carpodacus erythrinus</i> ^{E+A}	ACL31612.1	-	Leu
Chaffinch	<i>Fringilla coelebs</i> ^{E+A}	DQ257477	Val	Leu
Yellow-rumped seedeater	<i>Serinus atrogularis</i> ^F	DQ257479	Val	Leu
African citril	<i>Serinus citrinelloides</i> ^F	DQ257484	Val	Leu
Lemon-breasted seedeater	<i>Serinus citrinipectus</i> ^F	DQ257483	Val	Leu
White-bellied canary	<i>Serinus dorsostratus</i> ^F	DQ257486	Val	Leu
Yellow canary	<i>Serinus flaviventris</i> ^F	DQ257487	Val	Leu
Streaky-headed seedeater	<i>Serinus gularis</i> ^F	DQ257489	Val	Leu
Yellow-fronted canary	<i>Serinus mozambicus</i> ^F	DQ257491	Val	Leu
Streaky seedeater	<i>Serinus striolatus</i> ^F	DQ257493	Val	Leu
Tibetan serin	<i>Serinus thibetanus</i> ^A	DQ257496	Val	Leu
Zebra finch	<i>Taeniopygia guttata</i> ^P	LOC100231469*	Val	Leu

Sequence Alignment and Statistical Analysis

A 311-bp MHC sequence and a 180-aminoacid MHC sequence from vertebrate species, including *Passeriformes*, (Table 1, Fig. 1) were analyzed; taxa with very incomplete sequences were not included in the phylogenetic analysis in order to avoid distortion of results. Sequences correspond to MHC-I $\alpha 1$ and $\alpha 2$ domains, which conform the antigen-binding groove and the TCR-binding sites (Figs. 1 and 2). Average homology and composition distance was calculated for each species. Homology was calculated as 100 x number of coincidences / number of known positions (i.e. excluding unknown ones and gaps). Alignment of consensus sequences was done using PAUP* [23] and MEGA software [24]. Sequences were numbered after human HLA-A2 [25].

Composition distance is a measure of the difference in nucleotide (or amino acid) composition for a given pair of sequences [26]. It is one half the sum of squared difference

in counts of bases (or residues). MEGA 4 computes and presents the Composition Distance per site, which is given by the total composition distance between two sequences divided by the number of positions compared, excluding gaps and missing data (complete deletion option).

Numerical results are expressed as *arithmetical mean* \pm *SEM* (n), being n the number of samples, and *SEM* the Standard Error of the Mean = standard deviation / \sqrt{n} .

3D protein structures were first obtained and studied using Swiss Model (Deep View) [27].

Structural Analyses of Classical MHC-I Molecules (Fig. 2)

Three-dimensional (3D) structures analyzed in this study consisted of 186 murine and human classical MHC-I molecules –collected from the MPID [28] database– and a

chicken classical MHC-I molecule (PDB:3BVA). Contacts and hydrogen bonds between B2M and α -chain residues in 3D-structures were obtained using the contact application implemented in the CCP4 suite [29] and the program “hbond” from the JOY package [30], respectively. Residues were considered to be in contact if they were at a distance of 0.4 nm or less. All contacts were subject to visual inspection using the program RASMOL [31].

A molecular model of the classical MHCI molecule of *T. guttata* was built by homology modelling from the 3-D coordinates of the chicken MHC class I molecule BF2*2101 (PDB: 3BVA) using the package MODELLER [32].

Accession number of the B2M and MHC-I α -chain from *T. guttata* used for modelling were XP_002192833 and XP_002186567, respectively.

Phylogenetic Analyses (Fig. 3)

Linearized Maximum Likelihood phylogram trees were constructed with PAUP* [23] and Phylip for Windows [33]. Linearized Bayesian Inference phylograms were constructed with MrBayes [34,35], using the following settings (see [33]): outgroup = *Cyprinus carpio*; model = 4by4, nucleotide substitution = 6, rates = invariable gamma distribution; 10⁶ generations; ‘burn-in’ = 25% of samples. Trees were

a

MHC-1-alpha 1

MODEL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36		
HLA-A2	Gly	Ser	His	Ser	Met	Arg	Tyr	Phe	Phe	Phr	Ser	Val	Ser	Arg	Pro	Gly	Arg	Gly	Glu	Pro	Arg	Phe	Ile	Ala	Val	Gly	Tyr	Val	Asp	Asp	Thr	Gln	Phe	Val	Arg	Phe		
Danio_riero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
Xenopus_laevi	-	-	-	-	Leu	-	Asn	Tyr	Tyr	-	Ala	-	-	Asp	Arg	Ala	Phe	-	Leu	-	Glu	-	Tyr	-	Ala	-	-	-	-	-	-	Val	Asp	Gly	Glu	Gln	Phe	Tyr
Amieva_amieva	-	-	-	-	Leu	Gln	-	-	Tyr	-	Gly	-	-	Glu	-	-	Glu	-	Leu	-	Glu	-	-	Val	-	-	-	-	-	-	Gly	Gln	Leu	-	-	Gln	Tyr	
Bos_taurus	-	-	-	-	-	-	-	-	Ser	-	Ala	-	-	-	-	-	Phe	-	-	-	-	-	Tyr	Leu	Glu	-	-	-	-	-	-	-	-	-	-	Gln	-	
Canis_familiaris	-	-	-	-	Leu	-	-	-	Tyr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Mus_musculus	-	-	-	-	-	-	-	-	Val	-	Ala	-	-	-	Leu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sus_scrofa	-	Pro	-	-	Leu	Ser	-	-	Tyr	-	Ala	-	-	-	-	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Pan_troglodytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Coturnixjaponica	-	-	-	-	-	-	-	-	Gln	-	Ala	Met	Thr	Asp	-	Por	-	Leu	-	Trp	-	Tyr	Glu	-	-	-	-	-	-	Gly	Glu	Ile	-	-	His	Tyr		
Gallus_gallus	-	Tyr	Pro	Ala	Val	His	Pro	-	Gln	-	Ala	Met	Thr	Asp	-	Por	-	Glu	-	Trp	-	Val	Thr	-	-	-	-	-	Gly	Glu	Leu	-	-	His	Tyr			
Acrocephalusarundinaceus	Val	Leu	-	-	Leu	His	-	Leu	Ser	Val	Gly	-	-	Glu	-	Ser	Por	-	Ile	-	Gln	-	Met	Glu	Met	-	-	Phe	-	-	Gly	Ile	Pro	-	-	Tyr		
Carduelis_atrata	-	-	-	-	-	-	-	-	Leu	Ser	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Thr	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr		
Carduelis_carduelis	-	-	-	-	-	-	-	-	Thr	Val	Ala	-	-	Glu	Ala	Ser	Por	-	Val	-	Gln	-	Met	Ser	Thr	-	-	Leu	-	Gly	Ile	Pro	-	-	Thr	His	Tyr	
Carduelis_lawrencei	-	-	-	-	-	-	-	-	Leu	Thr	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Thr	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr		
Carduelis_pinus	-	-	-	-	-	-	-	-	Thr	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Met	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr			
Carduelis_spinus	-	-	-	-	-	-	-	-	Thr	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Thr	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr			
Fringilla_choelebs	-	-	-	-	-	-	-	-	Leu	Thr	Val	Val	-	-	Glu	-	Ser	Por	-	Val	-	Lys	-	Met	Ser	Ile	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr		
Serinus_atrogularis	-	-	-	-	-	-	-	-	Leu	Thr	Val	Val	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Ile	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr		
Serinus_thibetanus	-	-	-	-	-	-	-	-	Leu	Thr	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Met	Ser	Thr	-	-	Leu	-	Gly	Ile	Pro	-	-	Tyr		
Taeniopygia_guttata	-	Leu	-	-	Leu	His	-	Leu	His	Val	Ala	-	-	Glu	-	Ser	Por	-	Val	-	Gln	-	Thr	Ser	Ile	-	-	Phe	-	Gly	Ile	Pro	-	-	Tyr			

HLA-A2	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
Danio_riero	Tyr	Tyr	-	Ser	Asn	Lys	Met	Lys	Ser	Val	-	Lys	Thr	Glu	-	Arg	-	Asn	Glu	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xenopus_laevi	Ser	-	-	Lys	Asp	Arg	Val	Glu	Ala	Ala	Thr	Gln	Trp	Met	Lys	Asp	Lys	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amieva_amieva	-	-	-	Asn	Thr	Arg	-	Glu	-	Leu	-	-	Val	Ser	-	Lys	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bos_taurus	-	-	-	-	Pro	Asn	Pro	-	-	-	-	-	-	Arg	-	Val	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Canis_familiaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mus_musculus	-	-	-	-	Glu	Asn	Pro	-	Tyr	-	-	-	-	-	Arg	-	Met	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sus_scrofa	-	-	-	-	Pro	Asn	Pro	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pan_troglodytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coturnixjaponica	-	-	Thr	Thr	Arg	-	-	Asn	Val	-	-	Thr	Glu	-	-	Lys	Ala	Gly	Ala	Val	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gallus_gallus	Asn	-	Thr	Val	Arg	Arg	-	Tyr	Val	-	-	Thr	Glu	-	-	Ala	Ala	-	Lys	Ala	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acrocephalusarundinaceus	-	-	Glu	Arg	Gly	-	-	-	-	-	-	Leu	Thr	Glu	-	-	Lys	Asp	Ala	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carduelis_atrata	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Arg	-	-	Lys	Asp	Ala	Glu	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carduelis_carduelis	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Gln	-	-	Lys	Asp	Ala	Glu	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carduelis_lawrencei	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Gln	-	-	Lys	Asp	Ala	Glu	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carduelis_pinus	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Arg	-	-	Lys	Asp	Ala	Glu	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carduelis_spinus	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Arg	-	-	Lys	Asp	Ala	Glu	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fringilla_choelebs	-	-	Glu	Arg	Gly	-	-	Lys	Val	-	Leu	Thr	Gln	-	-	Met	-	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serinus_atrogularis	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Gln	-	-	Met	-	Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serinus_thibetanus	-	-	Glu	Arg	Gly	-	-	Gln	Lys	Val	-	Leu	Thr	Gln	-	-	Lys	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Taeniopygia_guttata	-	-	Glu	Arg	Gly	-	-	-	Ala	-	-	Leu	Thr	Gln	-	-	Met	Lys	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

HLA-A2	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Danio_riero	Val	Tyr	Lys	-	-	Phe	Gln	-	-	Lys	Glu	Arg	Phe	-	-	-	-	Gln
Xenopus_laevi	Val	Phe	Lys	His	Asn	Val	Lys	-	Ala	Met	Glu	Arg	Phe	-	-	-	-	Thr
Amieva_amieva	Val	Phe	-	Gly	Asn	Ile	Asn	-	Ala	Met	Asn	Arg	-	-	-	-	-	Thr
Bos_taurus	Ser	Phe	-	-	Asn	-	Asn	-	-	-	-	-	-	-	-	-	-	-
Canis_familiaris	Arg	Tyr	-	-	-	Asp	-	-	-	-	-	-	-	-	-	-	-	-
Mus_musculus	Ser	Phe	-	-	-	Arg	-	-	Leu	-	-	-	-	-	-	-	-	Lys
Sus_scrofa	-	Tyr	-	-	Gly	-	Lys	Asn	-	-	-	-	-	-	-	-	-	-
Pan_troglodytes	-	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Asp
Coturnixjaponica	Asn	Ser	-	Ser	-	Asp	Asn	Val	Ala	Arg	Leu	-	-	-	-	-	-	Gly
Gallus_gallus	Ile	Asp	-	Glu	Asn	-	Ile	-	Gln	Arg	Arg	-	-	-	-	-	-	Thr
Acrocephalusarundinaceus	Val	Thr	Ala	Arg	Asn	-	Glu	Ile	-	-	Glu	Arg	-	-	-	-	-	Gly
Carduelis_atrata	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp	Arg	-	-	-	-	-	Gly
Carduelis_carduelis	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp	Arg	-	-	-	-	-	Gly
Carduelis_lawrencei	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp	Arg	-	-	-	-	-	Gly
Carduelis_pinus	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp	Arg	-	-	-	-	-	Gly
Carduelis_spinus	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp	Arg	-	-	-	-	-	Gly
Fringilla_choelebs	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	-	Trp	-	-	-	-	-	Thr
Serinus_atrogularis	Val	Glu	Ala	Arg	Asn	-	Glu	-	-	Gln	Asp							

(Fig. 1) Contd.....

b	MHC-1-alpha 2																																			
	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126
HLA-A2	Gly	Ser	His	Thr	Val	Gln	Arg	Met	Tyr	Gly	Cys	Asp	Val	Gly	Ser	Asp	Trp	Arg	Phe	Leu	Arg	Gly	Tyr	His	Gln	Tyr	Ala	Tyr	Asp	Gly	Lys	Asp	Tyr	Ile	Ala	Leu
Danio_riero	.	Ile	.	.	Ile	.	Glu	Glu	Trp	Asp	Asp	Glu	Thr	.	Ala	Thr	Asn	.	Phe	Tyr	.	Asp	Ser	.	.	.	Glu	.	Phe	Val	Tyr	.
Xenopus_laevi	.	Thr	.	Met	.	.	Trp	Glu	Leu	.	Asp	.	Gly	Ser	.	Ile	.	.	Asp	.	His	Val	.	.	Arg	Glu	Phe	Phe	.	.	.	
Amieva_amieva	.	Leu	.	.	Trp	.	Trp	Glu	Leu	Arg	Gly	.	Gly	Ser	.	Lys	Gly	.	.	Ser	.	Phe	Gly	.	.	Arg	.	Phe	Val	.	.	
Bos_taurus	Leu	.	Trp	.	Ser	Pro	.	Gly	.	Leu	Arg	.	.	.	Phe	Met	.	Gly	.	.	Arg	.	.	Leu	.	.	
Canis_familiaris	Arg	.	Trp	Leu	.	Pro	.	Gly	.	Gly	.	Leu	.	.	.	Ser	.	Asp	.	.	Ala	
Mus_musculus	Ile	.	Val	Ile	Ser	.	.	Glu	Gly	.	Leu	Gln	Cys	
Sus_scrofa	Tyr	.	Ser	Tyr	Leu	.	Pro	.	Gly	Leu	Leu	Arg	Ala	
Pan_troglodytes	Ile	.	Ile	Gly	Arg	.	Asp
Coturnixjaponica	Trp	Ile	Leu	Asp	.	Gly	Thr	.	Thr	.	.	.	Asn	Arg	.	Phe	.	Val	Phe	.	
Gallus_gallus	Trp	Ile	Leu	Glu	Gly	.	Gly	Pro	.	Ile	.	.	Tyr	.	Met	.	.	Arg	.	Phe	Thr	.	Phe	.	.	
Acrocephalusarundinaceus	.	Leu	.	.	.	Leu	.	Val	Ser	.	.	Glu	Leu	.	.	.	Gly	Ser	.	Val	.	.	Ser	Glu	Arg	His	Gly	.	Arg	.	Phe	.	Ser	Phe	.	
Carduelis_atrata	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Carduelis_carduelis	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	His	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Carduelis_lawrencei	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Carduelis_pinus	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Carduelis_spinus	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Fringilla_choelebs	.	Phe	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Serinus_atrogularis	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Serinus_thibetanus	.	Leu	.	.	Leu	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Ile	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	His	Leu	Ser	Phe	.	
Taeniopygia_guttata	.	Leu	.	.	Ala	Leu	Trp	Val	.	.	.	Leu	Leu	.	.	.	Gly	Ser	.	Val	.	.	Ser	Ser	Arg	Leu	Gly	.	Trp	.	Phe	Leu	Ser	Phe	.	

HLA-A2	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	158	157	158	159	160	161	162
HLA-A2	Lys	Glu	Asp	Leu	Arg	Ser	Trp	Thr	Ala	Ala	Asp	Met	Ala	Ala	Gln	Thr	Thr	Lys	His	Lys	Trp	Glu	Ala	Ala	His	Val	Ala	Glu	Gln	Leu	Arg	Ala	Tyr	Leu	Glu	Gly
Danio_riero	Asp	Thr	Glu	Glu	Trp	Val	Tyr	Val	Pro	Ser	Val	Phe	Tyr	.	Leu	.	Thr	Gln	.	Asn	Pro	Glu	Val	Asn	.	.	Arg	Asn	Lys	Asn	.	.	Ser	Ile	.	
Xenopus_laevi	Asp	Lys	Glu	Thr	Leu	Thr	Ser	Glu	.	.	Val	.	Ser	.	.	.	Asp	Leu	Glu	Ala	Met	Asn	Gln	Gly	Arg	Lys	Phe	.	.	Asn	.	
Amieva_amieva	Asn	Gly	Glu	Thr	Glu	.	.	Ile	.	Arg	Gly	Thr	.	.	His	Asp	.	Asn	.	.	.	Lys	.
Bos_taurus	Asn	Thr	.	.	.	Ile	.	Arg	Arg	Gly	Thr	.	.	His	Asp	.	Asn
Canis_familiaris	Asn	.	.	.	Lys	Thr	Leu	Ile	Gln	.	Gly	Glu	.	Arg	Thr	.	
Mus_musculus	Asn	Thr	.	.	.	Ile	.	Arg	Thr	.	Asn	.	.	Arg	Arg	.	Ser
Sus_scrofa	Asn	Ile	.	Arg	Ala	.	.	Arg	.	Gln	Gln	.
Pan_troglodytes	Asp	Lys	.	Thr	Met	Thr	Phe	.	.	Val	Pro	Glu	.	Val	Pro	.	.	Arg	Glu	Gly	Asp	Tyr	.	Arg	Gln	Lys	His	
Coturnixjaponica	Asp	Lys	Gly	Thr	Met	Thr	Phe	.	.	Val	Pro	Glu	.	Val	Pro	.	.	Arg	Glu	Gly	Ser	Glu	Pro	.	Arg	Trp	Lys	Asn	.	.	Glu	
Gallus_gallus	Asp	Leu	Glu	Ser	Gly	Arg	Phe	Val	.	.	Ser	.	Glu	Ile	.	Arg	Arg	His	Glu	Gly	Ile	.	.	Arg	Lys	Thr	Asn	.	.	.	Glu	
Acrocephalusarundinaceus	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Carduelis_atrata	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Ala	Asn	.	Lys	His	
Carduelis_carduelis	Asp	Ser	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Carduelis_lawrencei	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Carduelis_pinus	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Carduelis_spinus	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	Ile	.	Arg	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Met	Asn	.	Lys	His	
Fringilla_choelebs	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Serinus_atrogularis	Asp	Pro	Lys	Ser	Gly	Lys	Phe	Val	Pro	.	Asn	Ser	Ser	.	Glu	.	Asn	Gly	Lys	His	.	.	Glu	Gly	Ile	Glu	Val	.	Arg	Trp	Thr	Asn	.	Lys	His	
Serinus_thibetanus	Asp	Leu	Gly	Ser	Gly	Lys	Phe	Leu	.	.	Ser	.	.	Glu	Ile	.	Arg	Arg	Arg	.	.	.	Glu	Asp	Met	.	.	Arg	.	Lys	Asn	.	Lys	His	.	
Taeniopygia_guttata	Asp	Leu	Gly	Ser	Gly	Lys	Phe	Leu	.	.	Ser	.	.	Glu	Ile	.	Arg	Arg	Arg	.	.	.	Glu	Asp	Met	.	.	Arg	.	Lys	Asn	.	Lys	His	.	

HLA-A2	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	179	180
HLA-A2	Thr	Cys	Val	Glu	Trp	Leu	Arg	Arg	Tyr	Leu	Glu	Asn	Gly	Lys	Glu	Thr	Leu	Gln	Arg	Thr
Danio_riero	Glu	.	Ile	.	.	.	Glu	Lys	.	Met	Gln	Tyr	.	.	Ser
Xenopus_laevi	Ile	.	Ile	.	Asp	.	Lys	Lys	.	.	Ser	Tyr	.	Gln
Amieva_amieva	Phe	.	Ile	.	.	.	Gln	Lys	.	.	Arg	Tyr	.	Asn	Lys
Bos_taurus	Glu
Canis_familiaris	Met
Mus_musculus	Lys
Sus_scrofa	Leu	.	.	.	Ser	.	Glu	.	.	.	Met	.	.	.	Asp
Pan_troglodytes
Coturnixjaponica	.	.	.	Gln	His	Val
Gallus_gallus	Val
Acrocephalusarundinaceus	Glu	.	Pro	.	.	.	Gln	.	His	Val	Arg	Tyr	.	Gln	Lys
Carduelis_atrata	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Carduelis_carduelis	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Carduelis_lawrencei	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Carduelis_pinus	Leu	.	Pro	.	Ser	.	Lys	.	Ile	Gly
Carduelis_spinus	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Fringilla_choelebs	Arg	.	Pro	Gly	Ser	.	Gln	Lys	.	Ile	Arg
Serinus_atrogularis	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Serinus_thibetanus	Leu	.	Pro	.	Ser	.	Lys	.	Val	Gly
Taeniopygia_guttata	Lys	.	Pro	.	.	.	Lys	.	Val	Gly	Tyr	.	Gln	Lys	Glu	.	Glu	.	Lys	.

Fig. (1). MHC sequences. 1a. Alpha-1 chain aminoacid sequences. **1b.** Alpha-2 chain aminoacid sequences. Specifically conserved residues (10, 96) in class I MHC molecules in Passerine birds thriving in a world-wide range. Non-Passerine birds and other vertebrates show Thr and Gln at positions 10 and 96 (boxed), respectively, since 300 MYA, while songbirds bear Val and Leu (boxed). Numbering of positions is referred to HLA-A2 molecule [3,37,45]. Zebra finch sequence is deduced from [38]. Common rosefinch $\alpha 2$ domain sequence is taken from [39].

displayed with MEGA, treated with CorelDraw [36] for esthetic purposes, and exported to “tiff” format.

RESULTS AND DISCUSSION

Songbirds Bear Val10 Instead of Thr10 at $\alpha 1$ Chain and Leu96 Instead of Gln96 at $\alpha 2$ Chain

Seven positions in vertebrates had been shown to be conserved in MHC class I $\alpha 1$ and $\alpha 2$ protein chains from

jawed fishes to humans (from Devonian Epoch, 300 MYA, until present) [3]. These conserved residues are: Thr10, Asp29, and Asn86 in the $\alpha 1$ chain, and Gln96, Gly100, Cys101, and Cys164 in the $\alpha 2</$

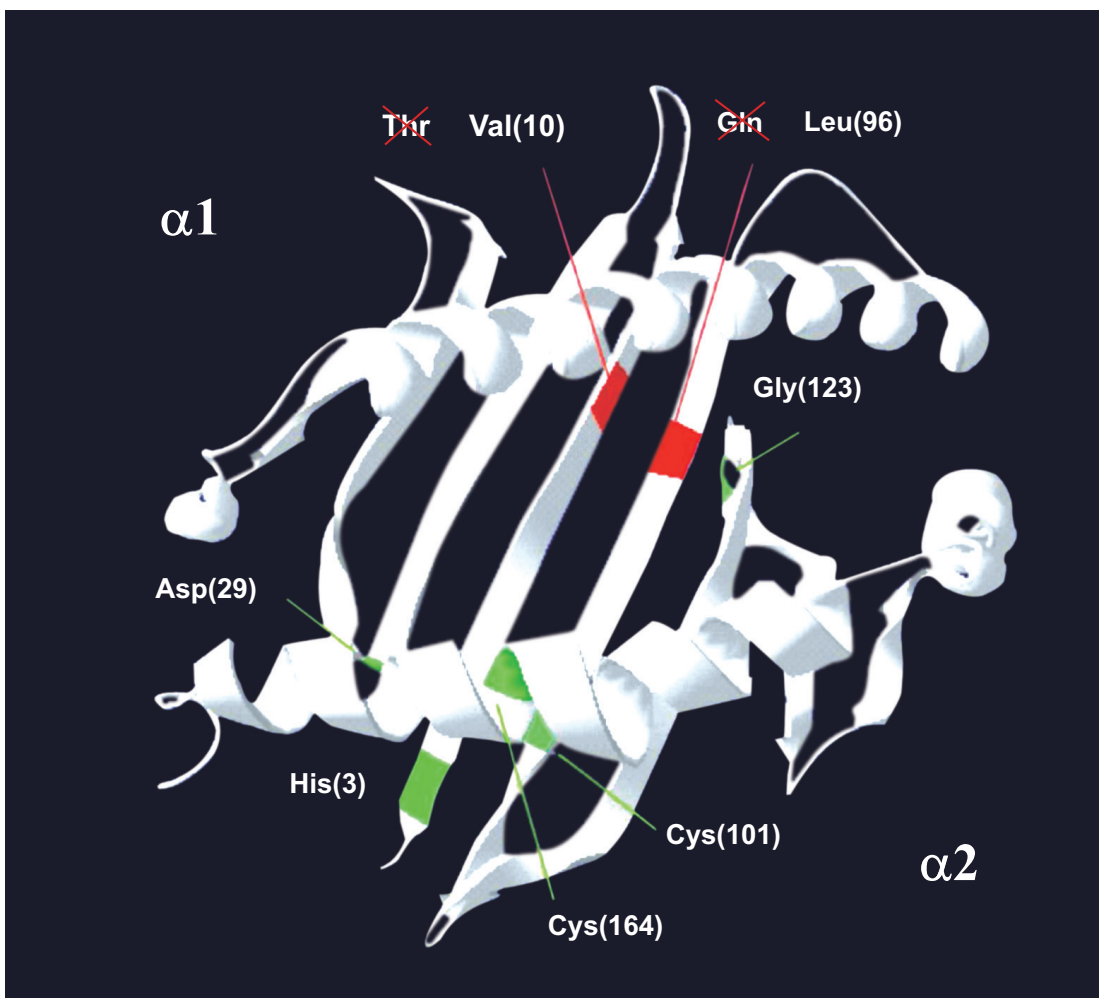


Fig. (2). The MHC-I molecule. Three-dimensional model of domains α -1 and α -2 of a songbird Class I histocompatibility molecule. Green: five positions conserved in vertebrates [3], including chicken and pheasant. Red: the two conserved positions (10 and 96) showing a different aminoacid in studied songbirds compared to the rest of vertebrates. In brackets: aminoacid position. See Table 1, Fig. (1).

[3,37]. However, some of these conserved residues at such a wide time-evolutionary scale are not kept in half of the extant birds: songbirds (*Passeriformes*) tested so far; they bear Val10 instead of Thr10 on α 1 chain and Leu96 instead of Gln96 on α 2 chain (Figs. 1 and 2, Table 1). All Passerine birds' MHC sequences, except those of warbler and zebra finch predicted sequence [38] have been obtained by us. All continents, latitudes and environments are represented with the songbird studied species (Table 1).

Songbirds preserve different MHC class I constant residues than other vertebrates, but also than other, more terrestrial birds, like chicken (*G. gallus*) and Japanese quail (*Coturnix japonica*) (Table 1, Fig. 1).

A phylogenetic classification of birds, partly based on DNA hybridization was put forward by Sibley and Alquist [16]. Our own studies have shown that *Passeriformes* (4600 species) of *Serinus* and *Carduelis* Genera bear these two aminoacid changes, unlike other extant bird species such as *G. gallus* (Table 1, Figs. 1 and 2). Also, other *Passeriformes*, like *Taeniopygia guttata* (zebra finch) MHC class I obtained from a whole genome linkage map [38] and *Acrocephalus arundinaceus* (great reed warbler) [12] are compared; Common rosefinch α 2 sequence is taken from [39].

Interactions of both differential conserved songbird residues in a tridimensional MHC molecule are as follows:

1. Side chains of residues Thr10 and Gln96 in all vertebrates do not interact with each other or with peptide (see Methodology).
2. Side chains of both residues interact with β 2-microglobulin (B2M) and appear to contribute to the overall structure of MHC class I molecules.
3. In terrestrial vertebrate residues (including chicken and quail birds):
 - a. Gln96 establishes two hydrogen bonds with B2M atoms: one with His31 and another one with Trp60.
 - b. Thr10 is nested between residues Met54 and Phe62 of B2M; in addition, Thr10 is interacting with a trapped H₂O molecule (see Methodology).
4. Specific songbird residues (Table 1, Fig. 2)
 - a. Change to Leu96 will result in a loss of the two stabilizing hydrogen bonds with B2M

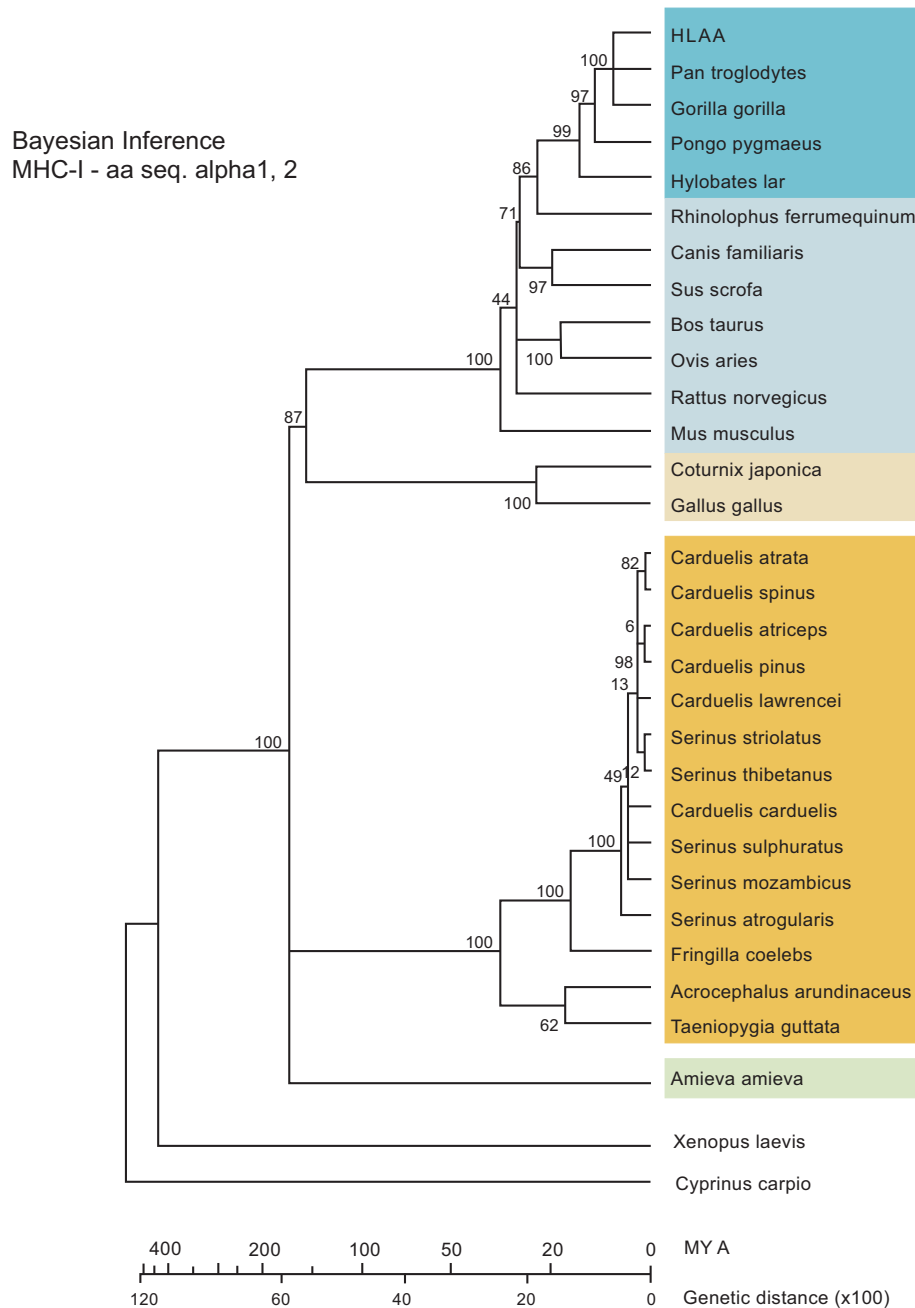


Fig. (3). Linearized Bayesian Inference phylogram. It is built from alpha-1 and alpha-2 aminoacid sequences. Dark orange: *Passeriformes*; light orange: other birds; dark blue: primates; light blue: other mammals; green: *Amievia* lizard; white: other vertebrates. Three different vertebrate groups are found: one for reptiles, one for mammals and more “terrestrial” birds, and one for more “aerial” birds. Time divergence of lineages has been found more reliable, if molecules other than selection pressured MHC ones are used. (i.e: mtDNA molecules which are thought to be subjected to constant evolutive pressures).

- b. Change to Val10 may compensate the previous change regarding the B2M attachment affinity to the α chain, because Van der Waals interactions with B2M’s Met54 and Phe62 arise; moreover, the trapped H₂O molecule will be released with a subsequent entropy gain (see Methodology).

B2M contributes to stability of the quaternary structure of the groove [17]. Therefore, and assuming that B2M residues in *Passerines* are equivalent as the B2M in humans (which is indeed concluded because of deduced zebra finch

B2M sequence [17, 38]), it seems that these couple of changes will also maintain solid relationships between MHC class I α and β molecules in songbirds.

On the other hand (Fig. 1), sequences from songbird’s $\alpha 1$ and $\alpha 2$ domains seem to be overall different from other vertebrates. Considering composition distance [24] from HLA-A2 (not shown), it is found that it is much higher to songbirds (0.93 ± 0.08 , $n=11$, $p < 0.00001$), than to other vertebrates (0.43 ± 0.08 , $n=19$) or to reptiles/fishes (0.34 ± 0.07 , $n=16$). Distance from songbirds to the rest of vertebrates is

1. Are songbirds direct descents of dinosaurs, while chicken is not, or are they descents of an altogether different dinosaur lineage? There is not sufficient dinosaur evolution details to answer this question, although non-flying dinosaurs had color feathers [42], remarking that feathers may have not evolved for flight but for mating choice or other reasons.
2. Are songbirds different to all other extant vertebrates, including other more terrestrial birds like chicken, in MHC class I molecules because they have been selected for advantages in mostly air life environment?

These questions may be answered as more data become available. However, chaffinch (*Fringillinae*), zebra finch (*Estrildinae*), great reed warbler (*Sylviidae*) and *Carduelinae* (common rosefinch, canaries, goldfinches) are relatively quite distant for justifying a generalization within *Passerines*, which will possibly have exceptions when more data are available.

Recently, zebra finch genome has been deduced by automated computational analysis from a predicted genomic sequence (NW_002197937) [38]. Although MHC class I genes were not specifically “sequenced”, a part of genomic DNA (XP_002186567) seems to be a class I antigen (Table 1). In addition, this zebra finch outlined genome shows how different chicken chromosomes [7] are from zebra finch’s: many more gene placements than expected are rearranged, or in other words, they are found in different chromosome or linkage groups. This further stresses the large difference in genetic homology between two types of birds: a galliform (chicken [16]) and a Passerine or songbird (zebra finch [16]). This is in concordance with the conserved MHC class I residues: chicken resembles other vertebrates while zebra finch resembles all available songbirds (Table 1, Figs. 1 and 2).

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ABBREVIATION

MHC = Major histocompatibility complex

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