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RESEARCH ARTICLE

Major Histocompatibility Complex Allele Persistence in Eurasia and America in the Genus *Carduelis (Spinus)* During Million Years

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Abstract:

Introduction:

Genus *Carduelis* (*Fringillidae* family) includes goldfinches, siskins, redpolls, greenfinches and crossbills. Many of the species classified within this genus and other related genera have been grouped by using molecular systematics and the mitochondrial cytochrome b (mt cyt b) gene. According to this, the Eurasian siskin (*C. spinus)* is the only one extant direct ancestor of several North American finches; North American / South American radiations may have been originated by Eurasian siskin (or extinct relative). In the present work, we aim to perform a study of transpecies and transcontinental analyses of MHC (Major Histocompatibility Complex) Class I alleles in several genus *Carduelis* / *Spinus* species in order to draw evolutionary conclusions in several wild bird species belonging to the genus *Carduelis / Spinus*.

Materials and Methods:

Blood was taken from worldwide wild bird species. Passerine phylogeny was done after analysing mtDNA with Maximun Likelihood and Bayesian dendrograms. Major histocompatibility complex alleles were obtained by standard DNA cloning and sequencing.

Results:

We found two matches between MHC-I DNA alleles from different South American siskins at DNA level. Also, it was observed that the Eurasian siskin shares a protein with pine siskin and another with three South American siskins. Eight South American siskins species also share the same MHC protein. In addition, studied songbirds MHC class I intron 2 is longer than that of *Gallus gallus*.

Conclusion:

We have drawn the following conclusions: 1) We present the first direct evidence that "Minimal Essential MHC" does not exist for birds; one of its main definition characters, *i.e.*: small intron size does not hold for songbirds. 2) We also report that MHC genes transpecies evolution exist in birds by showing also for the first time that worldwide bird species keep the same MHC protein and DNA alleles. 3) New evidences on MHC alleles conservation from Eurasian *Carduelis spinus* (most ancient) to South American siskins (most recent) during million years support that Eurasian siskin is the parental species for American Genus *Carduelis (Spinus)* species. It is uncertain whether Eurasian siskin (or extant relative) had initially an Holoartic distribution, including America.

Keywords: MHC, Major Histocompatibility Complex, *mt*DNA, *Carduelis*, *Spinus*, *Passerinae*, Songbirds, Transpecies evolution, Introns.

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1. INTRODUCTION

Genus *Carduelis / Spinus* includes goldfinches, siskins, redpolls, greenfinches and crossbills [1]. It is comprised within the *Fringillidae* family of birds together with canaries, many sparrows, bramblings and chaffinches. Most of them are familiar to birdwatchers and urban and country people [2 - 4]. Many of the species classified within genus *Carduelis* and other related genera have been grouped by using molecular systematics and the mitochondrial cytochrome b (mt cyt b) gene sequence [5 - 8]. According to this, the Eurasian siskin (*C. spinus)* appeared on Earth in the Pliocene Epoch about 5 million years ago [1, 8 - 10]. It is the one extant direct ancestor of several North American finches, which appeared around 2 million years ago [8, 10 - 12]: *C. dominicensis,* the Antillean siskin from the Caribbean high peaks of La Hispaniola Island; *C. pinus,* the pine siskin from North America; and *C. atriceps,* the Black-capped siskin from Guatemalan - Mexican altiplano [10, 11]. In addition, North American *C. tristis* and *C. notata* (also in South America) radiations may have been originated by Eurasian siskin (*C. spinus*) entrance to America [7, 8, 11, 12].

On the other hand, Major Histocompatibility Complex (MHC) is the most polymorphic loci in humans and studied vertebrates and its molecules present antigenic peptides to clonotypic T-cell receptors in order to start the immune response [13]. Classical MHC-I molecules are expressed on all nucleated cells and inhabit receptor structures that bind short peptides (antigens) derived from intracellular pathogens (like viruses) as well as peptides of individual's own body. After an antigen has been bound, the MHC-antigen complex is transported to the cell surface where it is recognized by $CD8^+$ T-cells. When the presented antigen is from a pathogen, $CD8^+$ T-cell becomes activated and the infected host cell is killed [14]. Non-classical MHC-I molecules (class-Ib) have a similar structure to classical class I molecules but they are less polymorphic and are not expressed to the same extent [15]. These class – Ib proteins evolve rapidly and are quite different in primary sequence among different vertebrate species [15, 16]. In the last years, non-classical class I MHC (HLA) genes are currently being studied: its function is immune modulation (in order to avoid autoimmunity) and immune suppression (in order to maintain mother/father proteins compatibility) and prevent fetus infection as a transplant (for an extensive review see [16]). MHC evolution is proposed to be under several kinds of selection: a) balancing selection, *i.e.*: many different alleles are maintained in the population because they may be beneficial [17], thus heterozygosis is favoured in order to maximize the range of antigens that can be recognized [18 - 20]; b) frequency-dependent selection. Rare alleles would be privileged in the prevention of rare pathogens appearance, which may unexpectedly enter into a population [13, 21]; c) a transpecific evolution; this has been observed also in humans and primates [22, 23]. Some specific alleles are markedly favoured and are preserved through speciation, so they can be found in different, genetically related species.

In this high variability context which may include non-classical class I molecules [16, 23], it is relevant that some proteins of a locus or loci may remain identical in quite distant species which have diverged in a relatively long time lag [22]. This has been observed in vertebrate Major Histocompatibility Complex [24] and Plant Histocompatibility System [18]. It usually occurs when a strong positive directional evolutive selection is acting on molecules. In this respect, primate species sometimes may share the same MHC class I molecules [25]. Also, molecular analyses based on MHC class I molecules in songbirds, particularly species from genus *Serinus* and *Carduelis*, reveal that these species have two different residues in positions 10 and 96 of the molecule when they are compared with different vertebrate species [26].

In the present study, we describe new MHC songbirds molecules in genus *Carduelis* species which have radiated within million years time lag in distant Earth areas and continents. Thus, we perform a study among species, which thrive in transcontinental habitats of MHC class I alleles in several genus *Carduelis* species in order to draw evolutionary conclusions in these freely occurring wild vertebrate species belonging to the same genus (*Carduelis*).

2. MATERIALS AND METHODS

2.1. Sampling

Blood was collected from 36 individuals of wild bird *Carduelis* species (Table **1**). Distribution and cytochrome b GenBank sequence accession numbers are given in Table **1**. Blood was obtained from wild birds in their natural thriving areas, and kept at 4°C with an ethylenediaminetetraacetic acid (EDTA) solution until use [26].

Table 1. Origin and Cytochrome b GenBank accession numbers of the species analyzed in this study.

Name	Common Name	GenBank	Origin
Carduelis ambigua	Black-headed greenfinch	U78322	Szechwan, China
*Carduelis atrata**	Black siskin	L76385	Sucre, Bolivia
Carduelis atriceps	Black-capped siskin	AF342863	Quetzaltenango, Guatemala
Carduelis barbata	Black-chinned siskin	L77868	Magallanes, Chile
Carduelis cannabina	Eurasian linnet	L76298	Madrid, Spain
Carduelis carduelis caniceps	Grey-crowned goldfinch	L76388	Katmandu, Nepal
Carduelis carduelis parva	Eurasian goldfinch	L76387	Madrid, Spain
Carduelis chloris	European greenfinch	L76297	Madrid, Spain
Carduelis citrinella citrinella	Citril finch	L77872	Madrid, Spain
Carduelis citrinella corsicanus	Citril finch	AY583725	Sardinia, Italy
Carduelis crassirostris	Thick-billed siskin	L77869	Mendoza, Argentina
*Carduelis cucullata**	Red siskin	L76299	Venezuela
Carduelis dominicensis	Antillean siskin	AF342864	Constanza, Dominican Rep
Carduelis flammea	Common redpoll	L76386	Brussels, Belgium
Carduelis flavirostris	Twite	U83199	Antwerp, Belgium
Carduelis hornemanni	Hoary redpoll	U83201	Antwerp, Belgium
Carduelis lawrencei	Lawrence's goldfinch	L76392	San Diego (CA), USA
*Carduelis magellanica**	Hooded siskin	U79016	Misiones, Argentina
*Carduelis notata**	Black-headed siskin	U79019	Chiapas, Mexico
*Carduelis olivacea**	Olivaceous siskin	L77871	Lima, Peru
Carduelis pinus perplexus	Pine siskin perplexus	DQ246804	Quetzaltenango, Guatemala
*Carduelis pinus pinus**	Pine siskin	U79020	Jackson (WY), USA
Carduelis psaltria colombianus	Dark-backed goldfinch	U78324	Maracay, Venezuela
Carduelis psaltria hesperophila	Green-backed goldfinch	L76390	Sacramento (CA), USA
Carduelis sinica	Grey-capped greenfinch	L76592	Szechwan, China
*Carduelis spinescens**	Andean siskin	U79017	Merida, Venezuela
Carduelis spinoides	Black-headed greenfinch	U79018	Katmandu, Nepal
*Carduelis spinus**	Eurasian siskin	L76391	Madrid, Spain
Carduelis tristis	American goldfinch	U79022	San Francisco (CA), USA
*Carduelis xanthogastra**	Yellow-bellied siskin	L76389	San Jose, Costa Rica
*Carduelis yarrellii**	Yellow-faced siskin	U83200	Recife, Brasil
Rhodopechys obsoleta	Desert finch	AF342889	Kabul, Afghanistan
Loxia curvirostra curvirostra	Common crossbill	AF342876	Alcala de Henares, Spain
Loxia curvirostra japonica	Common crossbill	AF342877	Beijing, China
Loxia leucoptera bifasciata	Two-barred crossbill	AF342878	Siberia, Russia
Fringilla coelebs	Chaffinch	L76609	Madrid, Spain

Asterisks (*) show species whose MHC class I alleles have been analysed (see Table **2**).

2.2. DNA Extraction

DNA was isolated from whole blood with an automatic DNA extracting device *(Nucleic Acid Extraction System. QuickGene-810,* FUJIFILM) after treating samples with a commercial kit *(QuickGene Whole Blood Extraction Kit S,* FUJIFILM). DNA concentration was measured with a spectrophotometer *(ND-1000,* NANODROP), and adjusted to about 100 ng/µl. Finally, samples were stored at -20°C.

2.3. MHC and mtDNA Cyt b Amplification, Cloning and Sequencing

Sequences of the MHC molecule most variable region (exon 2, intron 2, exon 3) were amplified by the polymerase chain reaction (PCR) method [27] in 42 cycles (10 s at 95°C, 30 s at 65°C, 60 s at 72°C) using an EPPENDORF thermocycler and *AmpliTaq DNA Polymerase* (APPLIED BIOSYSTEMS) [28]. Primers were used as described in [26]. Fragments of about 850 base pairs were obtained; some of them were purified by electrophoresis in a 2% agarose gel in order to verify the amplification process. Sequencing was performed using the Sanger method [29], using the same primers as for amplification, plus an internal primer: 5'- GGATTGATGTGGCTCCAAGG-3'. Ambiguities due to

heterocygosity were solved by cloning in competent *Escherichia coli* cells. An average of 12 different cloned sequences per individual were obtained. Amplification and sequencing of cyt b gene 924 base pairs (bp) was performed as previously described [30].

Table 2. MHC class I DNA alleles found in wild *Carduelis* individuals. GenBank accession numbers are also shown.

Species	Common Name (Number of Analysed Individuals in Brackets)	Alleles	GenBank
Carduelis spinus	Eurasian siskin (14)	Casp-F*0101	FJ266399
		Casp-F*0102	FJ266400
		Casp-F*0103	FJ266401
		Casp-F*0106	FJ266404
		Casp-F*0201	FJ266409
		Casp-F*0202	FJ266410
		Casp-F*0203	FJ266411
		Casp-F*0301	FJ266412
		Casp-F*0401	FJ266414
		Casp-F*0402	FJ266415
		Casp-F*0403	FJ266416
		Casp-F*0501	FJ266421
		Casp-F*0502	FJ266422
		Casp-F*0503	FJ266423
		Casp-F*0504	FJ266424
		Casp-F*0505	FJ266425
		Casp-F*0601	FJ266426
		Casp-F*0701	FJ266427
		Casp-F*0702	FJ266428
		Casp-F*0901	FJ266434
Carduelis pinus	Pine siskin (6)	Capi-F*0101	FJ266376
		Capi-F*0201	FJ266379
		Capi-F*0301	FJ266381
		Capi-F*0401	FJ266383
		Capi-F*0501	FJ266384
		Capi-F*0601	FJ266385
		Capi-F*0701	FJ266388
		Capi-F*0803	FJ266391
Carduelis atrata	Black siskin (8)	Caat-F*0101	FJ266350
		Caat-F*0102	FJ266351
		Caat-F*0201	FJ266354
		Caat-F*0202	FJ266355
		Caat-F*0301	FJ266359
		Caat-F*0302	FJ266360
		Caat-F*0401	FJ266361
		Caat-F*0402	FJ266362
		Caat-F*0501	FJ266365
		Caat-F*0601	FJ266367
		Caat-F*0901	FJ266371
		Caat-F*1002	FJ266373
Carduelis notata	Black-headed siskin (1)	Cano-F*0101	DQ257468
		Cano-F*0201	DQ257469
Carduelis spinescens	Andean siskin (1)	Caspe-F*0101	DQ257472
Carduelis olivacea	Olivaceous siskin (1)	Caol-F*0101	DQ257470
		Caol-F*0201	DQ257471
Carduelis cucullata	Red siskin (1)	Cacu-F*0101	DQ257465
		Cacu-F*0102	DQ257466

(Table 2) contd.....

Species	Common Name (Number of Analysed Individuals in Brackets)	Alleles	GenBank
Carduelis xanthogastra	Yellow-bellied siskin (1)	Caxa-F*0101	DQ257473
		Caxa-F*0201	DQ257474
Carduelis yarrellii	Yellow-faced siskin (1)	Caya-F*0101	DQ257475
		Caya-F*0201	DQ257476
Carduelis magellanica	Hooded siskin (1)	Cama-F*0101	DQ257467

Black-headed siskin (*C. notata*)

This bird is found in Middle America, Central Mexico and Nicaragua. It is observed in conifer and oak forests and lower edges of cloud forests, both in summer and in winter [4]. This siskin or a related ancestor was thriving on Earth about 3 million years ago (Fig. **1**) and it probably is the extant ancestor of South American siskin radiation [11]. Two different alleles were found in this species in 1 individual (Table **2**).

Black Siskin (*C. atrata*)

It thrives in Andes from central Peru to western Argentina. Specifically inhabits in puna grassland, rocky slopes, crags, gullies and hillsides, both in winter and in summer [4]. The time of appearance on Earth of this species is approximately 500,000 years ago (Fig. **1**), being part of South American siskin radiation group; its extant ancestor which gave rise to South American siskin radiation is *C. notata* [11]. In the present work, twelve different alleles were found in 8 different individuals (Table **2**).

Andean Siskin (*C. spinescens*)

This species lives in northern South America, mainly it is found in areas such as low bushes, forests, open hillsides, subtropical and paramo zones in scrub [4]. This bird appeared on Earth approximately 500,000 years ago (Fig. **1**) and it is a descent of *C. notata*, being part of South American siskin radiation [8, 11]. Only one allele is found in 1 individual (Table **2**).

Olivaceous Siskin (*C. olivacea*)

This siskin lives in South America, especially in areas from Ecuador to northern Peru and Bolivia. It is observed in forest edges of the subtropical zone [4]. Olivaceous siskin appeared on Earth about 1.2 million years ago (Fig. **1**). Two different alleles were found in 1 individual (Table **2**).

Red Siskin (*C. cucullata*)

This bird can be found in northern Venezuela and Colombia, in forests, dry scrubs and grassy areas with scattered trees [4]. Red siskin belongs to South American siskin group and appeared on Earth 1 million year ago (Fig. **1**, Table **2**) from the common ancestor of this group of siskins, the Black-headed siskin (*C. notata)* or an extinct relative. Two different alleles were found in 1 individual (Table **2**).

Yellow-Bellied Siskin (*C. xanthogastra*)

This species inhabits in Central and northern South America, mainly in tropical and subtropical forest edges and pastures [4]. It appeared on Earth approximately 1.2 million years ago considering our phylogenetic results shown in Fig. (**1**). Two different alleles were found in 1 individual in our present analyses.

Yellow-Faced Siskin (*C. yarrellii*)

This siskin may be observed in northern Brazil, in areas such as, lowland humid forests, woodland and edges of plantations [4]. It appeared on Earth 500,000 years ago being part of South American siskins (Fig. **1**). Two different alleles were found in 1 individual of this species (Table **2**).

Hooded Siskin (*C. magellanica*)

It thrives in South America. It may be observed in woods, groves or plantations, edges of cultivation, scrubs, parks and large gardens from coastal lowlands to tropical and subtropical zones [4]. This bird as well as Yellow-faced siskin appeared on Earth 500,000 years ago (Fig. **1**). Only one allele is found in 1 individual in the present study (Table **2**).

2.4. Phylogenetic Analyses

MEGA 5.0 software [31] was used to align MHC sequences and translate DNA sequences into protein, for each individual and clone. Different alleles were identified and characterized manually.

Regarding to cyt-b gene, sequences were further analyzed with MEGA 5.0 as described [31]. Phylogenetic dendrograms were obtained using Maximum Likelihood (ML) methodology [32] with PAUP* v. 4.0b10 program [33] and Bayesian Inference (BI) methodology using MrBayes program [34, 35]. Model test v. 3.7 [36] was used to find out a DNA substitution model that fits the data best. Also, best model was used prior to both ML and BI analyses. Linearized ML dendrograms were obtained with PAUP* v. 4.0b10 [33] with the estimated branch length [37] which assumes that the rates among the evolutionary lineages may not be constant. Tree calculation strategy consisted of a heuristic search with NNI (Nearing Neighbour Interchange) swapping algorithm. Robustness of nodes was assessed by 1000 bootstrap replicates in the ML analyses. The parameters rates defining the model of evolution were allowed to change in the BI analysis after each generation in order to increase the likelihood of resulting trees. Therefore, none of the parameters were *a priori* fixed. In BI analyses, two independent runs (with one cold and three heated chains each) were performed along with 5 million generations. Trees were sampled every 100 generations and the first 12,500 samples were discarded as 'burn-in'. Split frequencies average standard deviation approached to zero being around 0.01 at the end of the analysis. Posterior probability values (ppv) indicate the robustness of the nodes in the BI. In Phylogenetic analyses chaffinch (*Fringilla coelebs)* (family *Fringillidae*, subfamily *Fringillinae*), was used as outgroup.

3. RESULTS

3.1. Phylogenetic Trees and Age of Appearance on Earth

The peopling of America continent by genus *Carduelis* species could be carried out by three rapid radiations according to extant present day species: A Mesoamerican goldfinch radiation, a North American siskin radiation and a South American siskin radiation [11]. In this work, species from South American and North American groups have been studied. The age of appearance on Earth of the extant ancestor of North American group, Eurasian siskin (*C. spinus*) is approximately 5 million years ago [8, 11, 12] (Figs. **1** and **2**). It is suggested that this bird passed to America through Beringia/Aleutian Islands [38], since there have been sightings of these birds in areas near these islands. However, it is possible that *C. spinus* entering to America came also through the East Coast (Greenland, Iceland, Newfoundland): both East and West entering may have possible [11]. Eurasian siskin (or extinct close relative) evolves to Antillean siskin *(C. dominicensis)* during the Pliocene Epoch due to a geographical isolation after reaching Antillean area. Pine siskin *(C. pinus)* seems to be the descendent of Antillean siskin (Figs. **1** and **2**). Regarding the South American siskin group, the Black-headed siskin *(C. notata)* is suggested to be as extant ancestor of this group. South American radiation occurred probably after 3 million years ago, and *C. notata* or an extinct ancestor passed to South America from Mexican mountains, probably after Isthmus of Panama emerged [11]. This fact favoured the invasion of mesothermal plants from the Rocky Mountains to Andean Spine causing the expansion of this species *Carduelis / Spinus* genus and triggering the radiation [11].

North American siskin group and South American siskin group are closely related. Both were separated almost 5 million years ago (Fig. **1**). However, the common precursor of South and North American radiations, namely, the link between Eurasian siskin (*C. spinus)* and Black-headed siskin (*C. notata)* is missing, as well as the common ancestor of all three groups (North, Meso and South radiations) [11], unless that *C. spinus* was the American siskin ancestor and several links are missed [12].

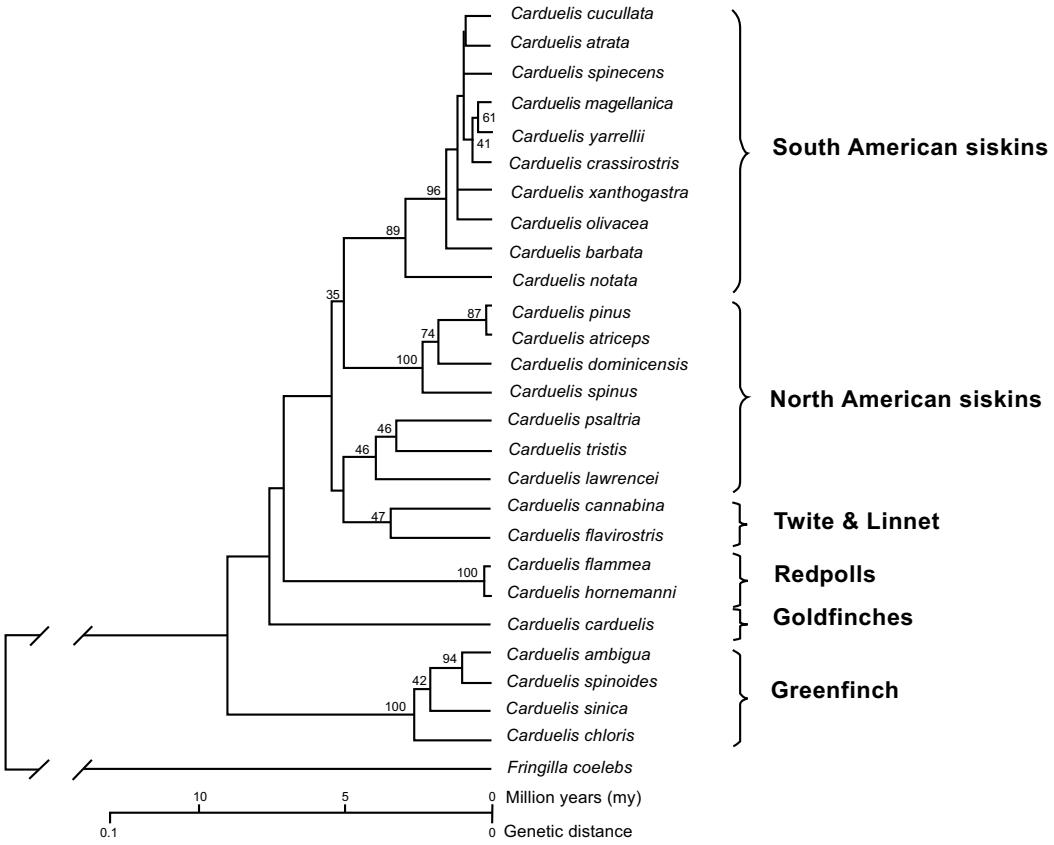

Fig. (1). Linearized Maximum likelihood dendrogram based on mitochondrial cytochrome b DNA sequences. Fringilla coelebs was chosen as outgroup.

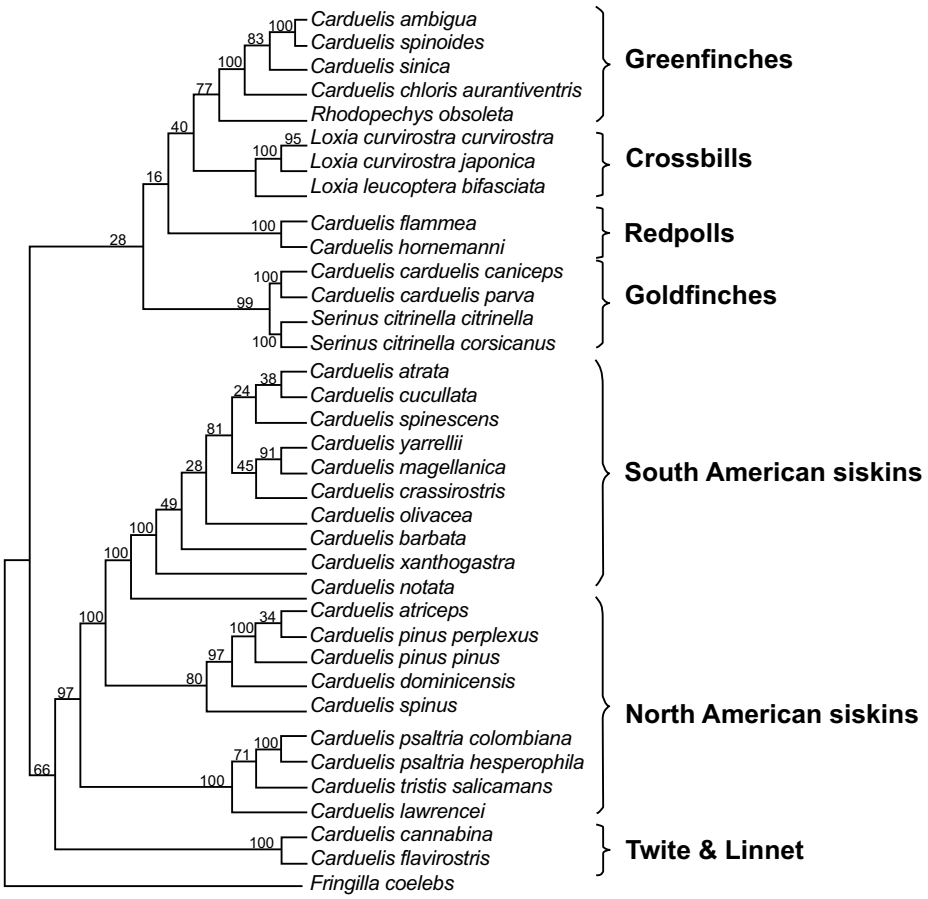

Fig. (2). Linearized Bayesian dendrogram based on mitochondrial cytochrome b DNA sequences. Fringilla coelebs was chosen as outgroup.

3.2. New MHC Alleles Found in Genus *Carduelis*

Once collected all MHC DNA sequences, they were classified according to DNA coding groups of similar or identical proteins. Then, a new provisional nomenclature of alleles was carried out according to the proposal of J. Klein [39], (Fig. (**3**) footnote). The MHC locus was generically identified with the letter F, referring to chicken class I genes (BF complex), since found alleles have been compared to human and other species class I molecules and found to belong to class I type of MHC molecules [26]. MHC class I molecules often different genus *Carduelis* species have been studied in the present work. Details of appearance on Earth and habitat of South American siskins are accounted in Table **2** footnote (low case letter).

Eurasian siskin (C. spinus) thrives in Paleartic and Oriental forests and conifer woodland in summer, whereas in winter it is observed in common weedy areas, plantations and gardens [4]. Eurasian siskin appeared on Earth 5 million years ago: this is observed in the linearized Maximum Likelihood dendrogram (Fig. **1**). It is the extant ancestor of the North American goldfinch group [1, 7, 8, 10, 12]. In this study, twenty different alleles of *C. spinus* were found in 14 individuals (Table **2**). This species may have been ancestor of all North American siskins [1, 7, 8, 10, 12]. Its close relative *Pine siskin (C. pinus)* inhabits in all North America and goes South to Guatemala. It may be observed in conifer forests, plantations, thickets and shrubs [4]. This bird is living on Earth since 200,000 years ago, as it is deduced from Fig. (**1**). Eight different alleles of pine siskin were found in 6 individuals in this present study (Table **2**).

DNA alleles were compared with each other (Table **2**). Similarities were found: Caat-F*0101 *C. atrata* allele also appears in *C. olivacea* (Caol -F*0201) and Caat-F*0401 *C. atrata* allele is present in other 6 species of South American siskins, such as *C. spinescens* (Caspe-F*0101), *C. xanthogastra* (Caxa-F*0101), *C. olivacea* (Caol-F*0101), *C. notata* (Cano-F*0101), *C. magellanica* (Cama-F*0101) and *C. cucullata* (Cacu-F*0102).

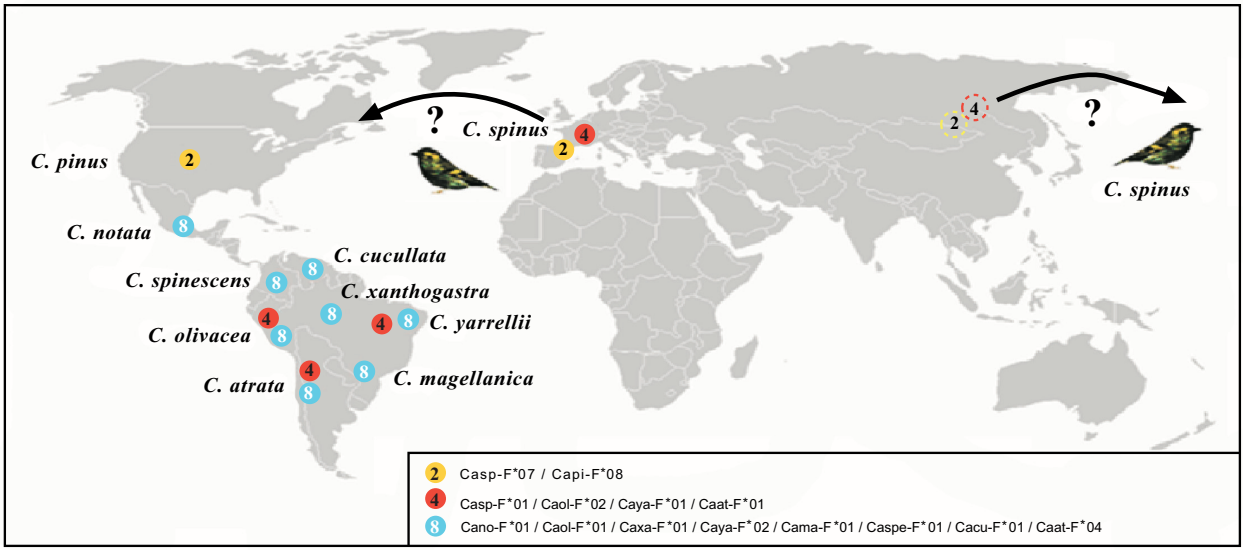

Fig. (3). Map which shows the geographic location of species which share MHC-I proteins. Proteins of different species are named as follows: *Carduelis spinus (Casp-F*)*; *Carduelis pinus (Capi-F*)*; *Carduelis notata (Cano-F*)*; *Carduelis spinescens (Caspe-F*)*; *Carduelis olivacea (Caol-F*)*; *Carduelis atrata (Caat-F*)*; *Carduelis magellanica (Cama-F*)*; *Carduelis yarrellii (Caya-F*)*; *Carduelis xanthogastra (Caxa-F*)* and *Carduelis cucullata (Cacu-F*)*. Color of the circles indicates the three different proteins found and number inside each circle indicates species that share each protein (Fig. **4**).

Fig. (4). *Carduelis spinus* (left side) and *Carduelis atrata* (right side). *Carduelis spinus* appeared on Earth 5 MYA and *Carduelis atrata* appeared on Earth 0.5-1 MYA [8, 10 - 12].

When MHC protein alleles are compared, it is observed that *C. spinus* shared a allele with *C. pinus* (Casp-F*07 = Capi-F*08) and another one with *C. atrata* (Casp-F*01 = Caat-F*01) (Fig. **3**). Furthermore, if South American siskins proteins are considered, it is observed that Casp-F*01 protein is the same one than Caol-F*02 (*C. olivacea*) and Caya-F*01 (*C. yarrellii*). Fig. (**3**) shows one the most shared protein (blue) that is present in 8 species of South American siskins; the most worldwide extended protein (red) appears both in Europe and in South America. And finally, there are 3 species which share 2 proteins (*C. olivacea*, *C. atrata* y *C. yarrellii*, blue and red).

Therefore a total of 2 DNA allele sequences present in 7 different species were found: (*C. atrata* (Caat-F*0401), *C. spinescens* (Caspe-F*0101), *C. xanthogastra* (Caxa-F*0101), *C. olivacea* (Caol-F*0101), *C. notata* (Cano-F*0101), *C. magellanica* (Cama-F*0101), *C. cucullata* (Cacu-F*0102)) and in 2 (*C. atrata* (Caat-F*0101) and *C. olivacea* (Caol - F*0201)) and 3 protein allele sequences are showed in 8 different species (*C. notata* (Cano-F*01), *C. olivacea* (Caol-F*01), *C. xanthogastra* (Caxa-F*01), *C. yarrellii* (Caya-F*02); *C. magellanica* (Cama-F*01), *C. spinus* (Casp-F*01); *C. cucullata* (Cacu-F*01); *C. atrata* (Caat-F*04)), 4 (*C. spinus* (Casp-F*01), *C. olivacea* (Caol-F*02), *C. yarrellii* (Caya-F*01), *C. atrata* (Caat-F*01)) and 2 (*C. spinus* (Casp-F*07), *C. pinus* (Capi-F*08)). South American siskins are

the species that most sequences have in common, and the Eurasian siskin (*C. spinus*) shares proteins with the pine siskin (*C. pinus*) and even South American siskins *C. atrata*, *C. olivacea* and *C. yarrellii* (Fig. **3**). A preliminary comparison of these DNA sequences with those found in *Passer domesticus* class I sequences [40] do not show close relatedness with this other species.

4. DISCUSSION

4.1. The Extant Ancestor: Eurasian siskin (*C. spinus*)

Eurasian siskin migratory behaviour is unpredictable each year: its North to South migrations do not always follows the same longitudinal patterns (it is "irruptive") [4]. Nowadays, this bird does not thrive in America, but it lives in easternmost and westernmost Eurasia, being a gap between Central Russia and its easternmost range. It is possible that Eurasian siskin was thriving in Eurasia and also in North America about Pliocene / Pleistocene Epoch limits, approximately 2 million years ago. Later, the Eurasian siskin might have advanced to Caribbean Islands and to Mexican mountains and Guatemalan-Mexican altiplano. About 200,000 years ago, the Eurasian siskin might have given rise to pine siskin (*C. pinus*) in Mexican sierras and to Antillean siskin (*C. dominicensis*), nowadays isolated in Hispaniola Island [11,41]; documented rainfall variations in the Caribbean during the Pleistocene, however, could have also affected distribution of these birds [42]. This may be a typical example of adaptive radiation originated by a North to South migration barrier and provincialism that drove evolution to create these new finch species. Last Wisconsin Glaciation ended and North American ice melted about 12,000 years ago: pine siskin would have followed the ancestral North to South migrations observed today and covered all North America. It might occupy American niches of Eurasian siskin which could not reach America from Asia during the last 2 million years because of an extant thick ice shield. Neither could it later because of species competition by ecologic niche with its descent pine siskin [12].

4.2. Number of Alleles

A detailed genetic map of the MHC in songbirds has not been obtained, so nothing can be said about the precise number of genes that composes it and its proximity on the chromosome. In spite of this, some gene numbers have been postulated [43]. In this work we have not analyzed such a characteristic and we have found no more than two different MHC alleles *per* single individual of the studied species of these particular genes. Therefore, our findings fit with detection of one paternal and one maternal gene belonging to a single locus.

In species such as *Coturnix japonica* at least four classic class I genes have been found with a high variety of alleles [44 - 46]; this also is the case of goose [47]. Duck has five classic class I genes although only two of them seem to actively work [48]. In songbirds, class I sequences have been studied in very few species. In the great reed warbler [49, 50] a high genetic MHC variability has been described compared with chicken (*Gallus gallus*) while in South American siskins [51] and in canaries [52, 53] only one gene with a low variability has been found.

4.3. MHC Transpecies Evolution in Birds

A transpecific gene existance occurs in several mammals MHC, like apes [25, 51]. This phenomenon usually occurs when speciation happens quickly, while gene differentiation has not yet taken place. It could also mean that the MHC naturally adapts to habitat of species and select alleles to combat characteristic antigens / pathogens thriving in the area, and does not need to generate an unlimited polymorphism as in the case of "artificial" MHC, where there are a high number of alleles and numerous immunological disorders that appear to be associated with the HLA system, such as autoimmune processes [51, 52]. Human, laboratory mouse and chicken are considered "artificial" vertebrate models because all have originated through a bottleneck and subsequent relatively high inbreeding, which enhances crossover at meiosis and thus to excessive MHC diversity [22, 51, 52].

Phylogenetic analysis of MHC sequences from the species studied in the present work allowed to visualizing more clearly the phenomenon of trans-specificity since, two matches between MHC-I alleles from different South American siskin species were found at DNA level. It was also found that the Eurasian siskin shares a MHC protein allele with the pine siskin (North-Central America range) and another protein allele with three South American siskin species. Eight South American siskins species, including parental *C. notata*, also share another MHC protein among themselves (Fig. **3**). All MHC genes transmit their alleles to the descendant species, but the most common fact is that the allelic identity between the ancestral species and their descendants get lost due to balancing selection diversification. The antiquity of studied MHC alleles goes back no longer than four - five millions years, when South American siskins and North

American goldfinches species were separated (Figs. **1** and **2**) [11].

A closest relationship between the Eurasian siskin and the Pine siskin had been found [8, 10, 11]. This is further supported by the fact of sharing one MHC protein allele. Eurasian siskin (o extant relative) could have been a species widely spread around Europe, Asia and America that could be have led to both North American goldfinches and South American siskins radiations. Otherwise, it could have only originated North American goldfinches radiations and these could have originated the South American siskin radiation [11, 12].

In conclusion, our data on mitochondrial cytochrome-b combined with the first evidence of trans-species MHC evolution so far described in birds, suggests that the Eurasian siskin is the extant ancestor of all North and South American *Carduelis* species [11, 12].

4.4. MHC Large Intron Size in Passerines

This set of wild bird species studied (Table **1** [26],) has given the first direct evidence that one of the main characters of "Minimal Essential Bird MHC" postulated for birds [54] is in fact not universal for birds.

MHC class I introns from presently and previously [26] studied songbirds are longer than humans. Chicken introns are the shortest ones [26]. In addition, MHC class I genes introns 2 were homologous on 38.3% to human class I MHC and 35% to *Gallus gallus* one. MHC class I intron 2 had an average of 304 bp in songbirds, 238 bp in human and 288 bp in *Gallus gallus* [26]. This is also a direct evidence that the main postulate of "Minimal Essential MHC" for birds only applies to *Gallus gallus*, and not to Passerines [54]. Additional information may be found in references [55, 56].The fact that songbirds other than *Carduelinae* family (*i.e.*: *Acrocephalus arundinaceus* and *Taeniopygia guttata,* zebra finch [26]) are also different at residues 10 and 96 at MHC class I proteins than all other vertebrates (including *Gallus gallus*) may indicate that our observations of on large intron size and different conserved 10 and 96 residues on MHC proteins could be extended to songbird family (*i.e.*: about half of the about 10,000 avian extant species [3]). Passerine evolutionary pathway may be altogether different from that of other birds [26].

CONCLUSION

"Minimal Essential MHC" concept is not valid for birds.Trans specific evolution on MHC wild birds is observed and it also supports that *Carduelis spinus* (Eurasian siskin) or extinct relative is parental species of all American *Genus Carduelis (Spinus)* species.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This project was approved by University Complutense Ethics Committee.

HUMAN AND ANIMAL RIGHTS

The reported experiments in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animal (http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf) published by the National Academy of Sciences, The National Academies Press, Washington DC, United States of America.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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