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ORIGINAL ARTICLE

Evolution of the major histocompatibility complex class I genes in *Serinus canaria* from the Canary Islands is different from that of Asian and African continental *Serinus* species

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Abstract The radiation of canaries (genus *Serinus*) occurred in Africa and Eurasia during the Miocene Epoch (9 million years ago) according to maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML) and Bayesian methodologies. *Serinus canaria* (wild canary) and *S. serinus* (European serin) together form one of the several polytomies within the genus *Serinus* phylogenetic trees. In a relatively late period, a wild ancestor of *S. canaria* invaded the Canary Islands, and these birds are the origin of all existing cage canaries, including the first genetically engineered animal: the red canary. The present analysis of the major histocompatibility complex (MHC) molecules in the Canary Islands' species *S. canaria* shows that the evolution of the MHC in this species is overall different and faster than that of continental species – namely, *S. thibetanus* (Asia) and *S. mozambicus* (Africa) – but particularly so in the peptide binding region. These data support the hypothesis that oceanic islands may be evolutionary reservoirs and not evolutionary dead ends.

Keywords Canary Islands · Evolution · Major histocompatibility complex · *Serinus canaria*

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Introduction

Maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) phylogenetic analyses have placed *Serinus canaria* and *S. serinus* together as a single group within the genus *Serinus* (Arnaiz-Villena et al. 1998, 1999; Zamora et al. 2006b). This single polytomy has been confirmed by Bayesian analysis (present article), and continental *S. thibetanus* (Asia) and *S. mozambicus* (Africa) species have been shown to belong to different and quite distant phylogenetic polytomies within genus *Serinus*. *S. canaria* shows a remarkable monomorphism on the cyt-*b* molecule throughout Macaronesia (Azores, Madeira and Canary Islands) (Dietzen et al. 2006).

The major histocompatiblity complex (MHC) comprises the most polymorphic loci in animals (Klein 1986) and plays an important role during the first steps of the immune response in vertebrates. In humans, MHC molecules (also named human leukocyte antigens, HLA) were initially regarded as class I or class II molecules based on the subset of T-lymphocytes to which these molecules present pathogenic peptides as well as the nature of such peptides.

MHC class I molecules are heterodimers in which the heavy chain (alpha) has three extracellular domains, two of which (alpha 1 and alpha 2) are polymorphic and pair in conformity with the peptide binding region (PBR)

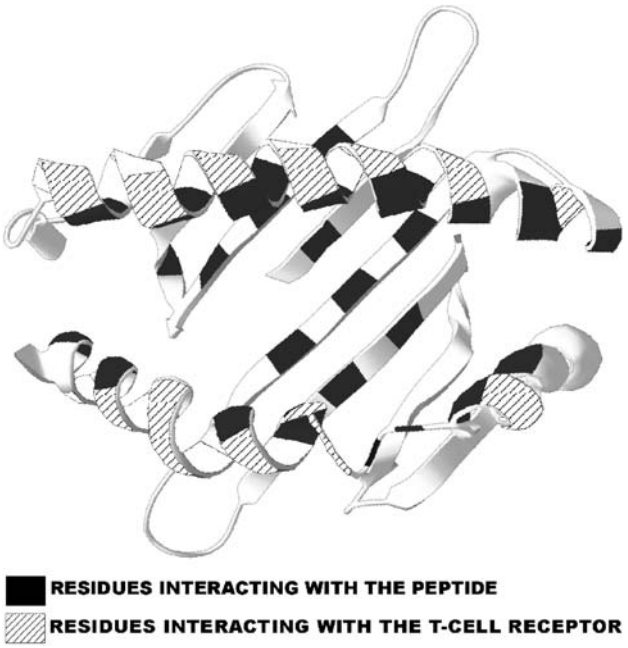

Fig. 1 Representation of a major histocompatiblity complex (MHC) class I protein deduced from exon 2 and exon 3 gene sequencing (Schwede et al. 2003) showing a shell-like structure which accommodates microbe peptides and presents them to the T lymphocyte receptor in order to start an immune response. Twenty-seven *black squares* represent residues interacting with microbe peptides, and 15 *striped squares* show residues interacting with the T lymphocyte receptor, which are different between *Serinus canaria* (wild canary from the Canary Islands; allele Secan-F*0101) and *Serinus mozambicus* (Mozambique serin, thriving in Equatorial continental Africa; allele Semoz-F*0201). The differential residues between the two *Serinus* species are peptide-binding amino acids (positions 2, 15, 17, 19, 51, 55, 58, 59, 62, 65, 66, 69, 72, 73, 76, 85, 87, 89, 103, 105, 132, 136, 142, 146, 149, 157, 161) and T cell receptor interacting amino acids (positions 50, 54, 57, 61, 64, 68, 74, 134, 135, 144, 145, 148, 152, 153, 156)

(Bjorkman et al. 1987) (Fig. 1). The PBR is thought to be subject to balancing selection for variability, which is the cause of the very high polymorphism of the MHC molecules (Hughes and Nei 1988). Different pathogenic epitopes would be the evolutive force causing the balancing selection (Hughes and Nei 1988).

MHC class I molecules have only been studied in three species of songbirds, namely, *Acrocephalus sechellensis* (Richardson and Westerdahl 2003), *A. arundinaceus* (Westerdahl et al. 1999) and *Passer domesticus* (Bonneaud et al. 2004), but with a different aim and in a different context to the one pursued in the present investigation. Here, we have studied the very different structure and evolution of MHC class I molecules in the wild canary (*S. canaria*) from the Canary Islands relative to the continental *Serinus* species Tibetan serin (*S. thibetanus*) and the Mozambique serin (*S. mozambicus*). All three species belong to the same species radiation (Arnaiz-Villena et al. 1999). Our aim was to assess the differences in MHC molecules between the canary of the Canary Islands and the continental canaries and, based on the results, infer evolutionary consequences; we did not study phylogenies because MHC molecules are very polymorphic and subject to strong evolutive forces and, consequently, provide inadequate data for inferring phylogenies (Sato et al. 2001).

Methods

Sampling

Three species of genus *Serinus* have been used to compare the structure and evolution of MHC class I molecules: *S. canaria* (wild canary), *S. mozambicus* (Mozambique serin) and *S. thibetanus* (Tibetan serin). The geographic distribution of *S. canaria* comprises the Canary, Madeira and Azores Islands; it has given rise to all cage canaries and to the first genetically engineered animal: the red canary (Birkhead 2004). Three individuals were sampled in Gran Canaria (Canary Islands, Spain). *S. thibetanus* thrives in the Eastern Himalayas at altitudes of 2800–4000 m a.s.l., and *S. mozambicus* thrives in most of the sub-Saharan African continent at various altitudes. These were sampled (three of each) in Szechwan (China) and Dar es Salam (Tanzania), respectively.

DNA extraction, amplification and sequencing

Blood was drawn from a cut in one claw of living birds following treatment with a local anesthetic (lidocaine ointment), collected in EDTA, cooled at 4°C and frozen until use. DNA was purified using a standard phenol-chloroform methodology (Sambrook et al. 1989). A PCR was performed to amplify a 803-bp fragment comprising exon 2 (alpha 1 domain), intron 2, and exon 3 (alpha 2 domain) of bird MHC class I molecules, as described by Westerdahl et al. (1999). Cloning and automatic DNA sequencing procedures were as previously described (Arnaiz-Villena et al. 1992). To assess the extent of MHC class I polymorphism in the *Serinus* spp. sequences, at least eight clones from each of two different PCRs were sequenced from each species, and an average of five of these were used to obtain the allelic sequences. Allele names were assigned following the guidelines proposed by Klein (Klein et al. 1990).

Statistical analyses

Mitochondrial cytochrome *b* DNA sequences from genus *Serinus* and genus *Carduelis* species (Arnaiz-Villena et al. 1999; Zamora et al. 2006b) were used for phylogenetic

calculations (see Table 1). MP, NJ and ML dendrograms were constructed, as previously described (Arnaiz-Villena et al. 1999; Zamora et al. 2006b), using the PAUP4.0 computer program (Swofford 2002) (results not shown). A Bayesian analysis was performed with MrBayes ver. 3.1.2 software (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) (Fig. 2).

MHC DNA sequences were visually aligned and partitioned into two data sets corresponding to PBR and non-PBR coding regions. For each partition, synonymous, non-synonymous, and overall maximum likelihood genetic distances were calculated in pairs among *S. canaria*, *S. mozambicus* and *S. thibetanus* using the MEGA computer program (Kumar et al. 2001) (Fig. 3). A three-dimensional model of MHC-I alpha 1 and alpha 2 domains (Fig. 1) was constructed using Swiss-Model software (Schwede et al. 2003).

Results and discussion

Avian Bayesian phylogenetic analysis (Fig. 2) was carried out in addition to the MP, NJ and ML calculations (not shown). The results are basically in agreement with those obtained previously with other methodologies (Arnaiz-Villena et al. 1999; Zamora et al. 2006b). *S. serinus*

Table 1 List of species used in the phylogenetic calculations. GenBank accession numbers of the mitochondrial cytochrome *b* DNA sequences are shown

Scientific name	Common name	GenBank	Sample region
Fringilla coelebs (outgroup)	Chaffinch	L76609	Madrid, Spain
Carduelis ambigua	Black-headed greenfinch	U78322	Szechwan, China
Carduelis carduelis caniceps	Goldfinch	L76388	Katmandu, Nepal
Carduelis carduelis parva	Goldfinch	L76387	Madrid, Spain
Carduelis chloris	Greenfinch	L76297	Madrid, Spain
Carduelis flammea	Common redpoll	L76386	Brussels, Belgium
Carduelis hornemanni	Arctic redpoll	U83201	Antwerp, Belgium
Carduelis pinus	Pine siskin	U79020	Jackson (WY), USA
Carduelis sinica	Oriental greenfinch	L76592	Szechwan, China
Carduelis spinoides	Himalayan greenfinch	U79018	Katmandu, Nepal
Carduelis spinus	Siskin	L76391	Madrid, Spain
Serinus alario	Black-headed canary	L76276	Cape Town, South Africa
S. albogularis	White-throated canary	L78705	Cape Town, South Africa
S. atrogularis	Black-throated canary	L76267	Cape Town, South Africa
S. canaria	Wild canary	L76266	Gran Canaria, Spain
S. canicollis canicollis	Cape canary	L78706	Cape Town, South Africa
S. canicollis flavivertex	Cape canary	L76295	Nairobi, Kenya
S. citrinella citrinella	Citril finch	L77872	Madrid, Spain
S. citrinella citrinella	Citril finch	Pasquet and Thibault (1997)	Alps/Pyrenees
S. citrinella corsicanus	Citril finch	Pasquet and Thibault (1997)	Corsica, France
S. citrinella corsicanus	Citril finch	AY583725	Sardinia, Italy
S. citrinelloides	African citril	L77555	Nairobi, Kenya
S. citrinipectus	Lemon-breasted seedeater	L78707	Maputo, Mozambique
S. dorsostriatus	White-bellied canary	L76278	Dar es Salam, Tanzania
S. flaviventris	Yellow canary	L76280	Cape Town, South Africa
S. gularis	Streaky-headed seedeater	L77556	Cape Town, South Africa
S. leucopygius	White-rumped seedeater	L76264	Dakar, Senegal
S. mozambicus	Mozambique serin	L76265	Dar es Salam, Tanzania
S. pusillus	Fire-fronted serin	L77873	Sin Wiang, China
S. serinus	European serin	L76263	Madrid, Spain
S. sulphuratus	Brimstone canary	L76294	Cape Town, South Africa
S. syriacus	Syrian serin	AY570547	Mont Hermon, Israel
S. thibetanus	Tibetan serin	L76279	Szechwan, China
S. totta	Cape serin	AY570548	Cape Town, South Africa

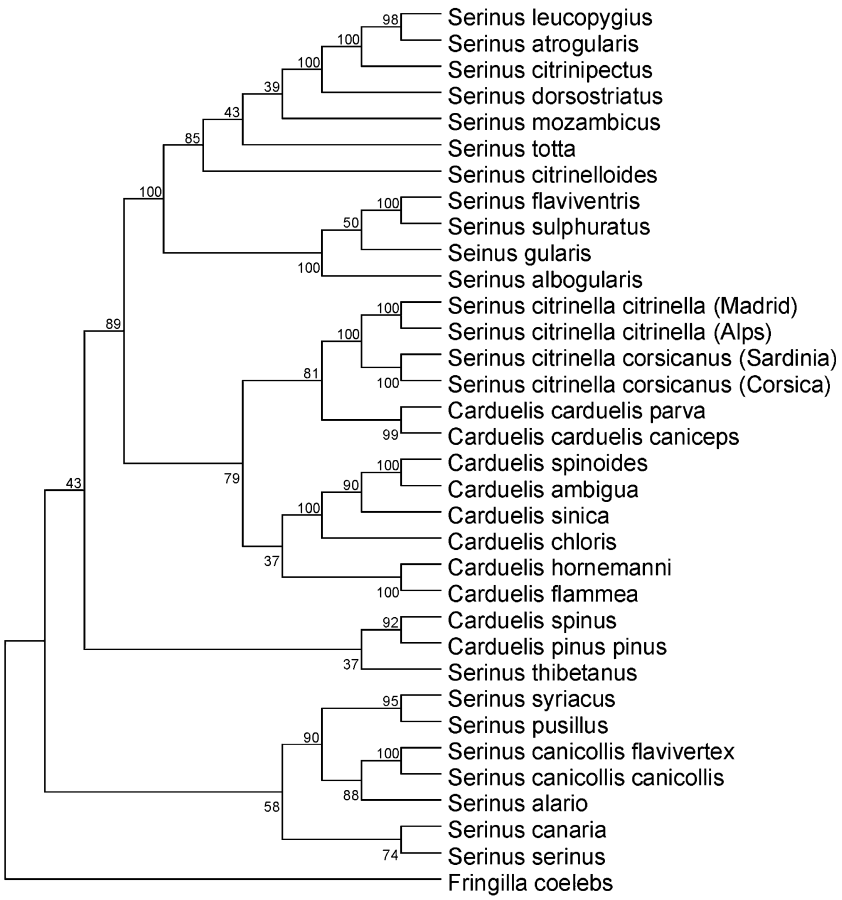

Fig. 2 Bayesian phylogenetic analysis of genus *Serinus* and genus *Carduelis* species based on cytochrome *b* DNA sequences. The posterior probability values (×100) are indicated *above the branches* for each node, ranging from 0 (minimum) to 100 (maximum). *Serinus canaria* (from Canary Islands), *S. mozambicus* (from the African continent) and *S. thibetanus* (from the Asian continent) can be seen to belong to different evolutive groups. The model of evolution used to compute the Bayesian analysis was GTR + I + G, which considers six different ratio of changes between nucleotides, a proportion of invariable sites and a gamma distribution. A total of 1,250,000 generations were run, sampling every 100 generations and discarding the first 3125 samples. See Table 1 for sequences accession numbers in GenBank

(European serin) and *S. canaria* (wild canary), which thrive in Europe and the Mediterranean area, respectively, form a single evolutionary group within genus *Serinus*. *S. mozambicus* (Mozambique serin) forms another evolutionary group with the small African canaries, and the Asian *S. thibetanus* (Tibetan serin) seems to form a single species evolutionary group by itself, probably because of the disappearance of sister species, supported by the fact that species of a similar appearance and geographically close do not exist.

A MHC class I NJ tree of all African and non-African canaries showed a trans-species evolution and an extremely limited polymorphism in terms of the MHC class I genes (Arnaiz-Villena et al. 1999; unpublished results). However, the Canary Islands' *S. canaria* appears in a branch that is separate from that of all other Eurasian and African canaries, and even outside the outgroup (*Fringilla coelebs* or chaffinch) MHC class I molecules. This is why it is not possible that the effect described in the following paragraphs is due to genus *Serinus* intra-specific MHC variation. The results from our analysis of three individuals per species provide enough evidence to draw conclusions since the trans-species evolution of alleles has already been shown in several *Serinus* species – namely, the same MHC allele has been found in different species. In other words, monomorphism of these molecules in different genus *Serinus* species has been found (results not shown). In contrast, the Canary Islands' *S. canaria* shows an altogether different MHC allele from that of both *S. mozambicus* and *S. thibetanus* (Fig. 3).

Four new avian MHC class I alleles have been described: one in *S. canaria* (Secan-F*0101); one in *S. thibetanus* (Sethi-F*0101); and two in *S. mozambicus* (Semoz-F*0101 and Semoz-F*0201). All sequence partitions in which distances were computed between the three species showed equivalent values, either with Semoz-F*0101 or Semoz-F*0201 (not shown). Therefore, only results from comparisons between Semoz-F*0101, Sethi-F*0101 and Secan-F*0101 will be shown (Fig. 3).

MHC class I ML genetic distances between both Asian (*S. thibetanus*) and African (*S. mozambicus*) continental species alleles were lower than when each of these continental species were compared with the Canary Islands' species (*S. canaria*). This was true for both the PBR (subjected to variability by selection) and for the non-PBR region (Parham et al. 1988a) (Fig. 3). Also, when the variability percentages within the PBR and non-PBR regions of the continental species (S. *mozambicus* and

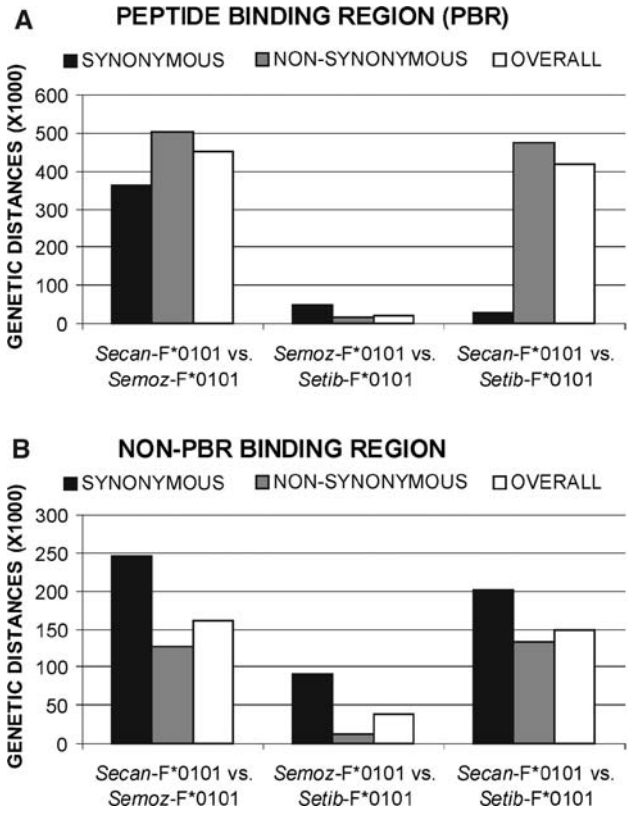

Fig. 3 Synonymous, non-synonymous and overall maximum likelihood genetic distances within PBR (**a**) and non-PBR (**b**) coding regions among *S. canaria*, *S. mozambicus* and *S. thibetanus* alleles (Secan-F*0101, Semoz-F*0101 and Sethi-F*0101, respectively). For the calculations, PBR included residues pointing into the PBR as well as those pointing to the T cell receptor and pointing away from the site (Parham et al. 1988b). The GenBank accession numbers of these new sequences are: DQ257480 (Secan-F*0101), DQ257496 (Sethi-F*0101) and DQ257491 (Semoz-F*0101)

S. thibetanus) are taken to be the expected distribution, the observed differences in percentage variability between the Islander *S. canaria* and each continental bird species show significant differences both at the PBR and non-PBR binding region ($P < 0.005$, chi-square test) (data not shown).

The observed high variability of the PBR of vertebrate MHC molecules is generally regarded to be due to the effect of a heterozygous individual's advantage for presenting more microbe peptides to T lymphocytes and for coping better with a higher number of possible immune responses to a wider range of microbes (balancing selection). In the present avian model, we use the wild canary, whose ancestor colonized the island quite recently (in the last three million years), a long time after the Asian (*S. thibetanus*) and African (*S. mozambicus*) continental species or their ancestors existed (about 9 and 5 million years ago, respectively) (Arnaiz-Villena et al. 1998, 1999; Zamora et al. 2006a). This recent colonization is consistent with results reported elsewhere with other molecules (Dietzen et al. 2006).

When the PBR and non-PBR residues of the Canary Islands' canary are compared to those of the continental African species *S. mozambicus*, the former has undergone 27 changes in the PBR residue and 15 changes in the non-PBR residue (those interacting with the T lymphocyte receptor) (Fig. 1). This result may fit with the variability in the MHC molecule being due to balancing selection, because residue changes are faster in the PBR (Figs. 1, 3). The observed effect is probably be due to a very different set of microbes existing in the Canary Islands in comparison with the African and Asian continents (Figs. 1, 3). However, it cannot be excluded that other types of selection have been acting on the Island canary MHC molecule, since it also is rapidly accumulating changes in non-PBR MHC molecule residues. In fact, the most frequent changes in the Canary Islands' *S. canaria* MHC whole molecule are not third codon base substitutions, but rather at the second and third positions (not shown). Consequently, the island environment is also associated with MHC molecule changes that may be due to environmental changes other than microbes (that only act at the PBR). It is not possible to disregard, for example, the existence of frequency-dependent selection due to the relatively (to continents) small populations of the Canary Islands or other types of selection (Filardi and Moyle 2005). In support of this is the finding that many changes are occurring in the Island molecule (Figs. 1, 3) and not only at the PBR (about double PBR). However, the Island molecule shows about a fourfold amount of synonymous DNA divergence at the PBR relative to the African molecule (Mozambique serin) in relation to the Asian molecule (Tibetan serin) (Fig. 3). This may be due to both the older divergence of the Asian canary (9 million years ago) relative to the African canary (5 million years ago) and the effect of evolutive pressures for effective (non-synonymous) residue variation at the PBR (Arnaiz-Villena et al. 1999).

The conclusions that can be drawn from our observations are: (1) this overall divergence in the MHC molecule of the Canary Islands' canaries (compared to divergence between continental, Asian and African ones) (Fig. 3) is in accordance with these islands being ''evolutionary reservoirs'', as also found for other birds (Filardi and Moyle 2005); (2) the very similar MHC molecules of continental *S. thibetanus* and *S. mozambicus* versus the Canary Islands' *S. canaria* at synonymous, non-synonymous and overall residue substitutions at the microbe or PBR (Fig. 3a) suggest that the observed differences are indeed due to different evolutive forces in the Canary Islands than in the continent, probably related to microbes and inbreeding (Sato et al. 2001).

We have also shown the homogeneity of cyt-*b* of *S. canaria* throughout the Azores, Madeira and Canary

Islands (Dietzen et al. 2006). Further studies are necessary to confirm this homogeneity on the MHC molecules, although more environmentally enforced variability may be expected (Sato et al. 2001) despite the probable recent introduction of the bird in the Atlantic islands (Dietzen et al. 2006).

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