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mtDNA PHYLOGENY OF NORTH AMERICAN *CARDUELIS PINUS* GROUP

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SUMMARY.—*mtDNA phylogeny of North American* Carduelis pinus *group.*

Aims: To uncover the genetic relationships within the *Carduelis* genera of *Carduelis dominicensis* (Antillean siskin), *Carduelis atriceps* (black-capped siskin) and *Carduelis pinus perplexus* (pine siskin *perplexus*) subspecies, and with North American *Carduelis* species, particularly *Carduelis pinus* (pine siskin) and also with Euroasiatic *Carduelis spinus* (Eurasian siskin).

Location: Males on breeding season were obtained. Antillean siskin was from Pico Duarte (Constanza, Dominican Republic) pine forest, the higher Antillean mountain; Black-capped siskin was obtained from Quetzaltenango (Guatemala highlands); Pine siskin *perplexus* was from Quetzaltenango (Guatemala highlands); *Carduelis pinus* was obtained from Dolores (Colorado, USA); and *Carduelis spinus* was taken from Madrid (Spain).

Methods: Mitochondrial cytochrome b (mt cyt-b) DNA was sequenced. Parsimony and Maximun Likelihood genetic distances based methodologies were used for dendrograms construction.

Results and Conclusions: North American Antillean siskin, black-capped siskin, pine siskin and pine siskin *perplexus* seem to form a monophyletic group together with their ancestor Eurasian siskin and separated from other North American and South American genus *Carduelis* species. This group seems to have diverged in the Pliocene Epoch. Antillean siskin is the oldest of this North American group.

Key words: atriceps, *Carduelis*, *dominicensis*, *perplexus*, phylogeny, *pinus*, siskins, *spinus*.

RESUMEN.—*La filogenia del grupo de especies norteamericanas relacionadas con* Carduelis pinus *obtenida con ADN mitocondrial.*

Objetivos: Descifrar las relaciones genéticas entre *Carduelis dominicensis* (lugano de las Antillas), *Carduelis atriceps* (lugano de copete negro), *Carduelis pinus perplexus* (lugano de los pinos *perplexus*) y *Carduelis spinus* (lugano Euroasiático).

Localidad: Se utilizaron para el estudio machos en época de cría. El lugano de las Antillas fue obtenido en el bosque de pinos de Pico Duarte (Constanza, República Dominicana), la montaña más alta de Las Antillas; el lugano de copete negro se recogió en Quetzaltenango (tierras altas de Guatemala); el lugano de los pinos *perplexus* se obtuvo en Quetzaltenango (Guatemala); el lugano de los pinos era de Dolores (Colorado, EEUU); y el lugano Euroasiático se recogió en Madrid (España).

Métodos: Se secuenció el ADN del cyt-b mitocondrial. Se utilizaron para construir dendrogramas técnicas de parsimonia, y basadas en las distancias genéticas y en la máxima verosimilitud.

Resultados y Conclusiones: Los luganos Norteamericano, Antillano, de copete negro, de los pinos y de los pinos *perplexus* parecen formar un grupo monofilético junto al ancestro común: el lugano Euroasiático, y están separados de las otras especies de *Carduelis* Norteamericanas y Sudamericanas. Este grupo parece haber divergido en el Plioceno. El lugano Antillano es el más antiguo de este grupo Norteamericano.

Palabras clave: atriceps, *Carduelis, dominicensis*, filogenia, *perplexus*, *pinus, spinus.*

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INTRODUCTION

Carduelis is a genus of finches which includes goldfinches, siskins, redpolls and greenfinches (and also crossbills; Arnaiz-Villena *et al*., 2001). It belongs to the *Fringillidae* family of birds which also includes many sparrows, bramblings and chaffinches. Most of them are beautifully coloured, widespread and familiar to birdwatchers and urban and country people (Armani, 1983; Sibley and Monroe, 1990; Clement *et al*., 1993). Many of the species comprised within this genus and other genera have recently been classified by using molecular systematics and the mitochondrial cytochrome b (mt cyt b) gene. This orthologous gene has proved to be helpful for defining evolutionary relationships among relatively distant and closely related birds, even at the subspecies level (Friesen *et al*., 1996; Questiau *et al*., 1998). In order to complete the cyt b mtDNA phylogeny of North American *Carduelis* finches and shed light about its speciation-timing three new species-subspecies have been de novo analysed, namely, Antillean siskin and black-capped siskin (Central America); *Carduelis pinus perplexus* has also been newly analysed, regardless of its uncertain phenotypic taxonomic status (subspecies or species; Howell and Webb, 1995). Pine siskin, black-capped siskin, Antillean siskin and other North American songbirds have been related to South American siskins and other Eurasian songbirds like Euroasiatic Twite and Linnet included in the genus *Carduelis* according to their mtDNA structure (Arnaiz-Villena *et al*., 1998).

Antillean siskin was discovered in Haiti in 1867 (Bryant, 1867). It thrives in Haiti and Dominican Republic pine forests and highlands, Hispaniola Island (Clement *et al*., 1993) and its phenotype resembles South American siskins (black head). Black-capped siskin was described in 1863 in Quetzaltenango, Guatemala. It is a rare (or nowadays nearly extinct) bird that thrives in Chiapas (Mexico) and Guatemala highlands (2,300 - 2,500 m; Clement *et al*., 1993). *Carduelis pinus perplexus* may or may not be a *Carduelis pinus* subspecies and is quite distinct to Pine siskin in phenotype (Fig. 2; Howell and Webb, 1995).

In the present study, a genetic analysis of these species has been carried out by studying their mitochondrial cytochrome b sequence variation aiming both, a) to relate these *Carduelis* species within North or South American *Carduelis* radiation; and b) to study the species / subspecies status of *Carduelis pinus perplexus* and to relate it with *C. pinus* and *C. atriceps*.

MATERIALS AND METHODS

Bird Samples

Names of species and place of origin are given in Table 1. Samples were taken in the field from males in their corresponding breeding season. Blood from living birds was drawn after one of their tarsus was locally anaesthetized (lidocain pediatric ointment; EMLA Astra Laboratories, Sweden) and then one single nail cut before releasing. Bleeding was stopped spontaneously or by a procoagulant bar touch (STAY, Mardel Labs Inc, Illinois, USA). Birds were also photographed. Two or three blood drops were collected in ice-cold EDTA and frozen until use. 924 base pairs (from 97 to 1020) of the mt cyt b gene were amplified with primers L14841 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and H15767 5'- ATGAAGGGATGTTCTACTGGTTG-3' (Arnaiz-Villena *et al.*, 1998). Polymerase Chain Reaction (PCR), cloning and automatic DNA sequencing were performed as previously described (Arnaiz-Villena *et al*., 1992; Zamora *et al*., 2005; Zamora *et al.,* 2006). At least, four clones from each of two different PCRs were sequenced from each species.

TABLE 1

List of species, origin and sequence identification (*GeneBank accesion number*).
[Lista de especies, origen e identificación de las secuencias (número de acceso del Genebank).]

Other pine siskins from different North American places were sequenced for this work (see Fig. 2: from Whitehorse, Newfoundland and Jackson). Pine siskin from Dolores (Co) DNA sequences was used for tree construction. † Ascents from Venezuela; this particular specimen was bred in Madrid as a cage bird. ‡ Ascents from Brasil, Recife, this particular specimen was bred in Reggio nell´Emilia, Italy. § Ascents originating in northern Europe emigrated to the Antwerp region in winter. ¥ Phenotypes of *C. psaltria* from Colorado and from Venezuela are not easily distinguishable. See also Clement *et al*. (1993). All specimens studied are male; except for the ones signed as || and ♀ meaning undetermined sex and female sex respectively. Chicken and pheasant sequences were obtained from refs (Desjardins and Morais, 1990) and (Kornegay *et al*., 1993), respectively.
[Para este trabajo se secuenciaron varios C. Pinus pinus *de diferentes localidades de Norteamérica (ver Fig.2: de Whitehorse, Newfoundland y Jackson). La secuencia de ADN del* C. p. pinus *de Dolores (Colorado) se utilizó para la construcción de los árboles. † Ancestros de Venezuela; este espécimen en concreto fue criado en Madrid como ave de jaula. ‡ Ancestros de Brasil, Recife, este espécimen en concreto fue criado en Reggio nell´Emilia, Italia. § Ancestros originarios del norte de Europa que emigraron a la región de Antwerp en invierno. ¥ Los fenotipos de* C. psaltria *de Colorado y de Venezuela no son fácilmente distinguibles. Ver también (Clement et al,. 1993). Todos los especimenes estudiados son machos; excepto para los asignados como || y ♀ significando sexo indeterminado y hembra respectivamente. Las secuencias del pollo y el faisán fueron obtenidas de referencias (Desjardins and Morais, 1990) y (Kornegay* et al*., 1993), respectivamente.]*

Species ***[Especies]***	**Mt cyt b sequence** ***[Secuencia Cit. b Mit.]***	**Sample region** ***[Región de la muestra]***
Siskin *Carduelis spinus*	L76391	Madrid, Spain
Pine siskin *C. pinus pinus*	AF901950	Dolores (Colorado), USA #
Pine siskin perplexus *C. pinus perplexus*	AF901951	Quetzaltenango (Guatemala)
Black capped siskin *C. atriceps*	AF342863	Quetzaltenango (Guatemala)
Antillean siskin *C. dominicensis*	AF342864	Constanza, Republica Dominicana
Red siskin *C. cucullata*	L76299	Venezuela†
Yellow–bellied siskin *C. xanthogastra xanthogastra*	L76389	San José, Costa Rica
Olivaceus siskin *C. olivacea*	L77871	Lima, Perú
Black siskin *C. atrata*	L76385	Sucre, Bolivia
Thick-billed siskin *C. crassirostris crassirostris*	L77869	Mendoza, Argentina
Hooded siskin *C. magellanicus magellanicus*	U79016	Misiones, Argentina
Andean siskin *C. spinences spinences*	U79017	Mérida, Venezuela
Yellow-faced siskin *C. yarellii*	U83200	Recife, Brasil‡
Black-chinned siskin *C. barbata*	L77868	Magallanes, Chile
Black-headed siskin *C. notata notata*	U79019	Chiapas, México
Linnet *C. cannabina cannabina*	L76298	Madrid, Spain
Twite *C. flavirostris flavirostris*		

TABLE 1 (CONT.)

Species *[Especies]*	**Mt cyt b sequence** *[Secuencia Cit. b Mit.]*	**Sample region** *[Región de la muestra]*
	U83199	Cage bird. Antwerp, Belgium§
Dark-backed goldfinch *C. psaltria hesperophila*	L76390	Sacramento, California USA
Dark-backed goldfinch *C. psaltria Columbiana*	U78324	Maracay, Venezuela ¥
American goldfinch *C. tristis salicamans*	U79022	San Francisco, California, USA
Lawrence`s goldfinch *C. lawrencei*	L76392	San Diego, California USA ‖
Common repoll *C. flammea flammea*	L76386	Brussels, Belgium
Artic redpoll *C. hornemanni hornemmani*	U83201	Cage bird. Antwerp, Belgium
Cetril finch *Serinus citrinella citrinella*	L77872	Madrid Sierra, Spain
Goldfinch *C. carduelis parva*	L76387	Madrid, Spain
Goldfinch *C. carduelis caniceps*	L76388	Katmandú, Nepal
Greenfinch *C. chloris aurantiventris*	L76297	Madrid, Spain
Oriental greenfinch *C. sinica sinica*	L76592	Szechwan, China
Black-headed greenfinch *C. ambigua ambigua*	U78322	Szechwan, China
Himalayan greenfinch *C. spinoides spinoides*	U79018	Katmandu, Nepal
Chaffinch *Fringilla coelebs coelebs*	L76609	Madrid, Spain

Statistical analyses and tree construction methods

The following calculations were carried out: a) base composition (also according to codon position); and b) number of synonymous (*dS*) and nonsynonymous (*dN*) distances by using the modified Nei-Gojobori methodology (Nei and Gojobori, 1986) considering the estimated transition/ transversion ratio via ML (Maximum Likelihood; Felsenstein, 1981). Saturation plots (not shown) were also done in order to be aware of transitional changes that may have become saturated (multiple substitutions at a single site) and thus uninformative at certain divergence times. Uncorrected pairwise divergence was used as an estimate of percent divergence [$p = n_d /n$, where p is the proportion of sequence divergence between two sequences, n_d is the number of nucleotides that differ between two sequences, and n is the total number of nucleotides compared (Nei, 1987)]; this also gives an approximation of time of species divergence. It has been used for constructing saturation plots (transition / transversion numbers against percentage of DNA sequence divergence); these results are not shown. Evolutionary rate calculations were carried out with MEGA v2.1 program (Kumar *et al*., 2001).

Phylogenetic inference criteria were used as implemented in PAUP software package (Swofford, 2002):

NJ (Neighbour-Joining; Saitou and Nei, 1987) and ML based trees (Maximum Likelihood; Felsenstein, 1981).

Neighbor-Joining tree was obtained by a DNA sequence matrix that calculated genetic distances by the Maximum Likelihood method, implemented in PAUP (Swofford, 2002). In order to estimate divergence times, an evolutionary rate of 0.8% substitutions per site and million years was assumed. This rate was found by Fleischer *et al*. (1998) in the Hawaiian drepanidines (subfamily *Fringillini*, tribe *Drepanidini*) based on an external geological

calibration. Bearing in mind that variation of evolutionary rate among lineages may exist, the branch lengths were estimated by ML, allowing rates to continuously change over time according to the molecular clock model by Thorne *et al*. (1998). This model has been successfully applied to several biological issues (Hasegawa *et al*., 2003) and references therein. Thorne's model allows that evolutionary rate changes continuously along time; thus, a fixed evolutionary rate for different taxa is not assumed, as it must occur very often in nature (see Fig. 1A for ML based tree and footnote for ML analysis settings). PARAMCLOCK PAUP command was used to build the ML based linearized tree. *Fringilla coelebs* was chosen as an outgroup to root the trees following evidence from other authors (Groth, 1998) and the present authors (Allende *et al*., 2001; Arnaiz-Villena *et al*., 1998; Arnaiz-Villena *et al*., 1999; Arnaiz-Villena *et al*., 2001; van den Elzen *et al*., 2001).

Parsimony trees (cladistic analysis; Fitch, 1971).

The search of the most parsimonious trees (Fig. 1B) was heuristic because the number of taxa (32) rendered an exhaustive one impractical. Characters were set unordered. *Fringilla coelebs* was chosen as an outgroup to root the trees; *Gallus gallus* (chicken) and *Lophura nycthemera* (pheasant) were also used as more distant outgroups. Parsimony settings are depicted in Fig. 1B footnote.

Enforced trees (constraint analysis)

In order to further assess the respective phylogenetic positions of Antillean siskin, black-capped siskin, pine siskin and pine siskin *perplexus* within the pinus siskin group, we constructed additional NJ trees on ML genetic distances (data not shown), adding more individuals of black-capped siskin and pine siskin (see Table 1 footnote for localities). The validity of the enforced trees (not shown) was assessed by the tree length and likelihood value (in the distance-based analysis) and by the consistency index, retention index, and tree length (in the parsimony tree). Also, the analyses of the number of substitutions per site were computed by other methods based on Kimura's 2-parameters (Li *et al*., 1985; Pamilo and Bianchi, 1993; Kumar *et al*., 2001).

RESULTS

Patterns of DNA base substitution (Tempo of evolution)

Plots for cyt b mtDNA (not shown) indicated that only third position transitions showed a clear levelling-off associated with saturation; this occurred at about 11 % uncorrected total sequence divergence (Nei, 1987), that is, among ingroup (*Carduelis* spp.) and outgroup (*Fringilla coelebs*). Therefore, it was found that five out of six data partitions (at the first, second, third codon position bases and transitions / transversions) were not saturated and were thus available to calculate correct phylogenies (results not shown; Hillis *et al*., 1994). Variable and phylogenetically informative sites were also calculated; there were 197 and 146 respectively, in the North American presently studied *Carduelis* spp. group (Table 1).

Nucleotide frequencies that were more significant than expected were detected ($P < 0.05$). These differences led to the use of the HKY85 model (Hasegawa *et al*., 1985, which assume the presence of unequal nucleotide frequencies) for the maximum likelihood genetic distances and subsequent NJ tree construction (Table 2).

The analysis of the number of substitutions per site in the presently studied genus *Carduelis* species was found to be: 0.1896 ± 0.0125 per synonymous, 0.0035 ± 0.0008 per non synonymous and 0.0618 ± 0.0005 per total sites (the number after $\pm$ is the standard deviation computed by bootstrap methodology; Felsenstein, 1985). These figures were also computed by other methods based on Kimura's 2-parameters (Li *et al.*, 1985; Pamilo and Bianchi, 1993; Kumar *et al.*, 2001). Similar results (not

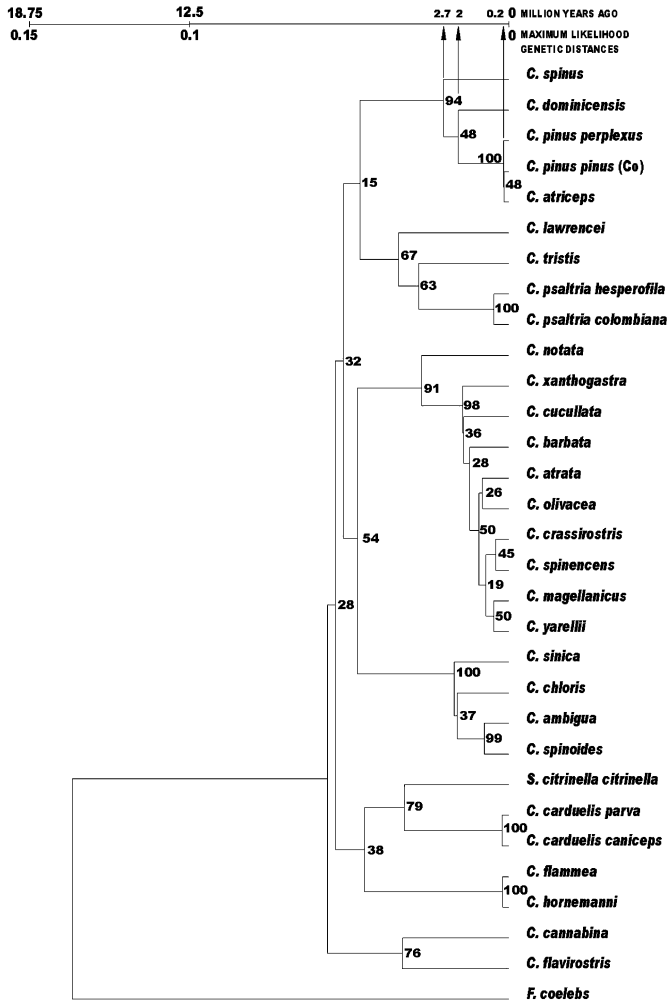

FIG. 1A.—ML (Maximum Likelihood) based tree. This linearized tree was constructed by assuming that evolutionary rates between lineages may be different (Thorne *et al*., 1998). PARAMCLOCK PAUP command was used for tree building. Groups of taxa are similar to those obtained in the parsimony and Neighbour-Joining on Maximum Likelihood dendrograms (Fig. 1B and not shown respectively). Genus *Carduelis* speciation appears to have occurred during the Miocene and Pliocene Epochs in both the northern and southern hemispheres. Studied bird species and subspecies details are shown in Table 1. Bootstrap values (1000 replications) are depicted in the interior part of the nodes. Co (Colorado), MYA (Million Years Ago). ML analysis settings were: two substitutions types; an estimated transition / transversion ratio via ML; HKY85 nucleotide substitution model; empirical nucleotide frequencies; none assumed proportion of invariable sites and gamma distribution of rates at variable sites, divided in four categories as done by Yang (1994) for mitochondrial DNA sequences. Negative branch lengths were allowed. ML based tree scores: tree length (x1000) = 894.53; ln Likelihood= -3993.9410 estimated transition / transversion ratio = 5.029949. ML (x1000) genetic distances are depicted above the time scale (Million Years Ago).

FIG. 1A.—*[Árbol de ML (Máxima verosimilitud). Este árbol linearizado fue construido asumiendo posibles variaciones en las tasa evolutiva entre linajes, (Thorne* et al., *1998). El comando PARAMCLOCK PAUP se utilizó para la construcción del árbol. Los grupos de taxones son similares a aquellos obtenidos en los dendogramas de parsimonia (Fig. 1B) y de Unión de Vecinos con distancias genéticas de Máxima Verosimilitud (no mostrado). La especiación del género* Carduelis *parece haber ocurrido durante el Mioceno y el Plioceno en ambos hemisferios Norte y Sur.Informacion relevante sobre las especies y subespecies de aves estudiadas están detalladas en la tabla 1. Los valores de Bootstrap (1000 repeticiones) están representados en la parte interior de los nodos. Co (Colorado), MYA (Millón de Años). Para el análisis de ML se utilizaron dos tipos de sustituciones; un ratio estimado de transiciones / transversiones; el modelo de sustitución nucleotídica HKY85; frecuencias nucleotídicas empíricas; no se asumió ninguna proporción de sitios invariables y se consideró una distribución gamma de la tasa de sustitución, dicha distribución se dividió en cuatro categorías como propuso Yang (1994), para secuencias de ADN mitocondrial. Se permitieron longitudes de ramas negativas. Las características del árbol basado en ML fueron: longitud del árbol (x1000) = 894.53; logaritmo neperiano de la verosimilitud = -3993.9410; ratio estimado de transiciones / transversiones = 5.029949. Las distancias genéticas ML (x1000) están representadas encima de la escala de tiempo (Millones de años).]*

shown) have been obtained using other methods based on Kimura's 2-parameters (see Material and Methods section).

Phylogeny

Maximum likelihood based tree, distance based and parsimony trees

In general, and except for the slight differences due to the different methods used, the ML genetic distances based tree (Fig. 1A), a NJ on ML distances (not shown) tree and the parsimony tree (Fig. 1B) reflected the same branching pattern. The pine siskin monophyletic group showed the same type of branching in the ML based tree (Fig. 1A) and NJ (not shown) trees: first siskin, followed by Antillean siskin, and the more terminal taxa were pine siskin and black-capped siskin (Fig. 1A). However, the latter three species were grouped in a polytomy in the parsimony tree (Fig. 1B) due to the lack of

TABLE 2

Uncorrected *p* distance (x1000) matrix showing the closest distance between *C. pinus perplexus* and *C. atriceps* (value in bold typing).
[La matriz de distancias p sin corregir (x1000) muestra la distancia más corta entre C. pinus perplexus *y* C. atriceps *(valor en negrita).]*

	C. spinus	***C. dominicensis***	***C. p. pinus***	***C. p. perplexus***	***C. atriceps***
C. spinus	-				
C. dominicensis	24.89	-			
C. p. pinus	23.81	24.89	-		
C. p. perplexus	21.65	22.73	21.60	-	
C. atriceps	22.73	23.81	3.25	**1.08**	-

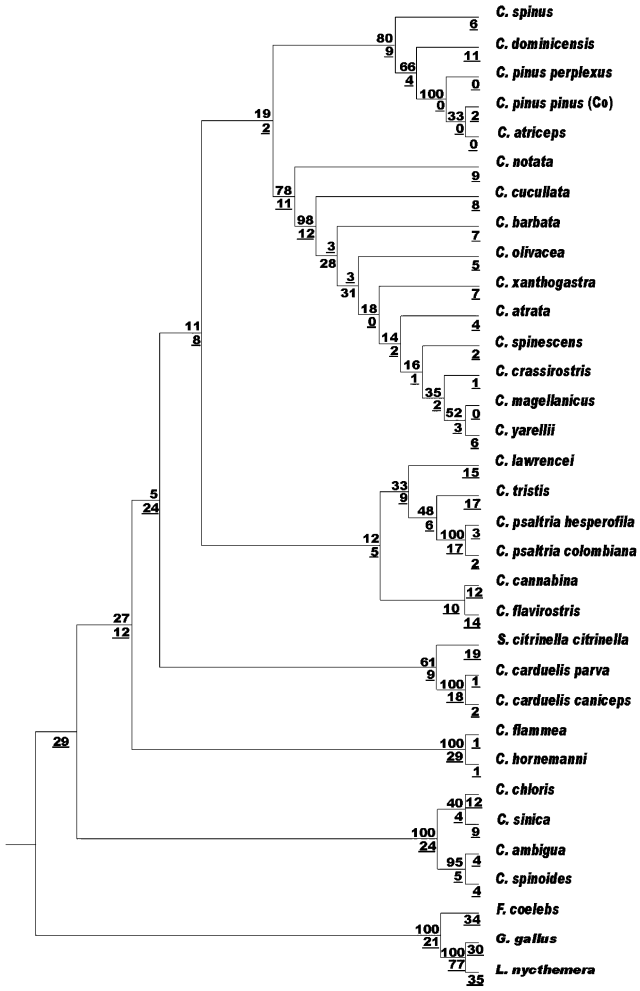

FIG. 1B.—[Parsimony tree. Bootstrap (1000 replications) (Felsenstein, 1985) and branch lengths values are above and underlined below the branches respectively. The addition of sequences was determined by the closest stepwise addition method. TBR (Tree Bisection and Reconnection) branch swapping was set in order to increase the probability of finding the optimum trees. The scores for the most parsimonious trees were: tree length = 625; consistency index = 0.515; retention index = 0.690. Co (Colorado).
[Árbol de Parsimonia. Los valores de Bootstrap (1000 repeticiones) (Felsenstein, 1985) y de longitud de rama están encima y debajo (subrayados) de las ramas respectivamente. La adición de secuencias fue determinada por el método closest stepwise addition. *Para aumentar la probabilidad de encontrar el árbol óptimo se utilizó el método de intercambio de ramas* (branch swapping) TBR (*Tree bisection and Reconnection*). *Los resultados para el árbol más parsimonioso fueron: longitud de árbol= 625; índice de consistencia = 0.515; índice de retención = 0.690. Co (Colorado).]*

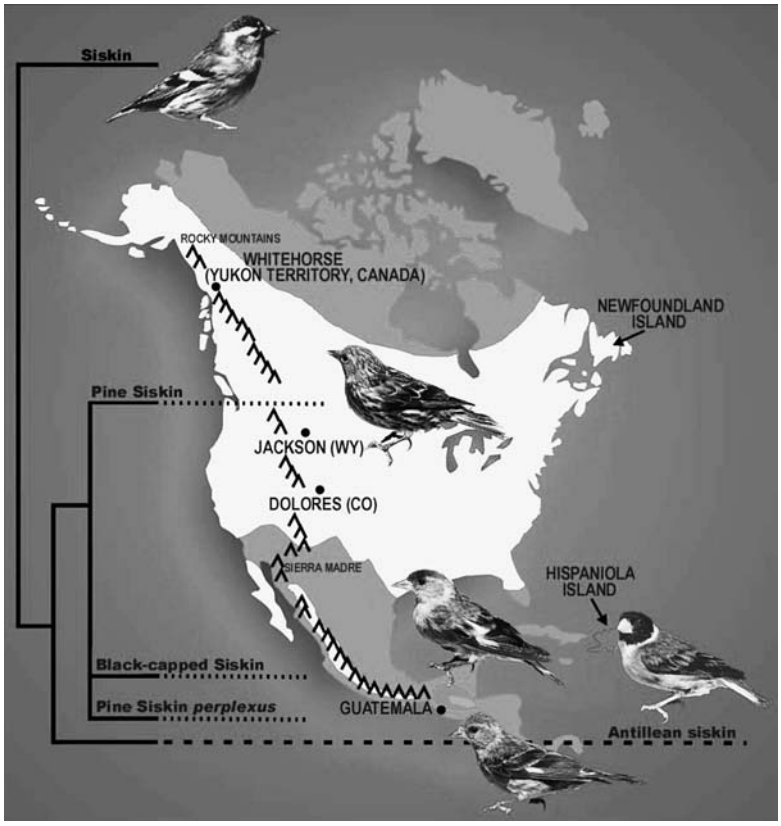

FIG. 2.—Geographic distribution and photographs (males) of the studied species. Antillean siskin (red) thrives in mountain pine forests of Haiti and Santo Domingo (Hispaniola Island) above 1,500 m. Black-capped siskin (blue) is an uncommon bird and thrives in Guatemala and Chiapas (Mexico) highlands between 2,350 and 3,000 m. Pine siskin *perplexus* (orange) has a similar but more restricted distribution than Guatemalan Siskin in Guatemala and Chiapas (Mexico) highlands. Pine siskin (yellow) lives in Southern Alaska and Canada, USA and Mexico; Eurasian siskin distribution is not shown but it is now found in Europe, Mediterranean, Central Asia; after a discontinuity it appears again in Mongolia, China, Japan and Eastern Siberia (see Arnaiz-Villena *et al*., 1998; Clement *et al*., 1993). Dendrogram topology is extracted from Fig. 1B. Photographs were taken by A. Arnaiz-Villena. GeneBank accession: Pine siskin (Jackson, Wy, USA) -DQ246805-; Pine siskin (St. John`s, Newfoundland, Canada) -DQ246809-; Pine siskin (Whitehorse, Yukon, Canada) -DQ246807-.

[Distribuciones geográficas y fotografías (machos) de las especies estudiadas. Carduelis dominicensis *(rojo),* C. atriceps *(azul),* C. pinus perplexus *(naranja) y* C. pinus pinus *(amarillo); el rango de distribución geográfica del lugano Euroasiático no se muestra (véase Arnaiz-Villena* et al., *1998*; *Clement* et al., *1993). La topología del dendrograma se extrajo de la Fig.1B. Las fotografías fueron tomadas por A. Arnaiz-Villena. Números de acceso al GeneBank: lugano de los pinos (Jackson, Wy, USA) -DQ246805-; Lugano de los pinos (St John´s de Terranova, Canada) -DQ246809-; Lugano de los pinos (Whitehorse, Yukon, Canada) -DQ246807-.]*

enough parsimonious informative sites for resolving the branching pattern.

Enforced trees (constraint analysis)

These analyses resulted in a lack of geographic structure of the pine siskin according to their cyt b sequences, and also in a close relationship between pine siskin *perplexus* and black-capped siskin. Furthermore, pine siskin *perplexus* and black-capped siskin would seem to be "sister" species according to the number of nucleotide differences (see Table 2). When Antillean siskin was enforced to group with black-capped siskin, distance-based and parsimony trees (not shown) rendered a lower likelihood value and a higher tree length.

Discussion

Phylogeny of North American Carduelis *(siskins)*

Siskin, North American Antillean siskin, black-capped siskin, pine siskin and pine siskin *perplexus* show a monophyletic group separated from other North and South American *Carduelis* spp. (Figs. 1A and 1B). This has been analysed in the results section. The new mt cyt-b DNA sequences belong to the new species *Carduelis pinus*, *Carduelis atriceps*, *Carduelis dominicensis* and *Carduelis pinus perplexus* (see Genbenk accession numbers in Table 1), and the three pine siskins from different locations (Genbank accession numbers in Fig. 2 footnote). Variability within the cyt-b gene was sufficient to stablish phylogenetic relationships according to the number of observed parsimony-informative sites (146) (Hillis *et al*., 1994). Nearly all sequence differences were silent substitutions, as expected (Kocher *et al*., 1989): 64.3 % of the third codon positions were not conserved among species, as it has previously been shown for this gene (evolving relatively rapidly under strong functional constraints). The variability for the first and second codon positions was 9.7 % and 2.3 %, respectively, as expected (Arnaiz-Villena *et al*., 1998; Arnaiz-Villena *et al*., 1999; Arnaiz-Villena *et al*., 2001; Allende *et al*., 2001).

Carduelis spinus (siskin)

Eurasian siskin *C. spinus* seems to be a close relative to North American *Carduelis* species. This phylogenetic pattern would not fit with its Eurasian distribution range. However, siskin and pine siskin "react" to each other in captivity (Cramp and Perrins,1994), are closely related (Arnaiz-Villena *et al*., 1998; Vaurie, 1959), and are thought to form a superspecies (Cramp and Perrins, 1994; Mayr and Short, 1970) The answers as to why Eurasian siskin does not thrive nowadays in North America and how it gave rise to *C. dominicensis* remain unclear and open to debate. Central to these issues is the fact that West Indies have been continuously colonized primarily by birds from Central America and more recently from South America (Bond, 1948; Bond, 1963; Gill, 1995; Lanyon, 1967). Based on this premises, it is tentatively suggested that easternmost Asian *Carduelis spinus* passed to America through the Beringia / Aleutian Islands. After this, during the Pliocene Epoch, *C. spinus* invasions from an undetermined area of the North American East coast reached the Antilles and evolved as a geographical isolate resulting in the present *C. dominicensis*. Although phenotypic differences between *C. spinus* and *C. dominicensis* are evident (Fig. 2), they may be primarily not entirely based on genetic differences, but also on distinctive environmental forces (James, 1983) and/or are controlled by genes with a higher evolutionary rate (Ball *et al*., 1988). The effect of directional selection due to adaptation to new environment

and genetic drift may be responsible for the very different phenotype. With regard to the present day absence of *C. spinus* in North America, *C. spinus* has been recorded in the American part of the Bering Strait and in the Aleutian Islands; these have been considered escapes from captivity (Clement *et al*., 1993).

Carduelis dominicensis (Antillean siskin)

This species was first described by Bryant (1867). The species is monotypic (with no subspecies), endemic to mountain pine forest of Hispaniola Island (Haiti and Dominican Republic; Clement *et al*., 1993), which are the highest mountains of the Caribbean Islands. The timing for the appearance of Antillean siskin seems to be 2 MYA (Fig. 1A), in the Pliocene Epoch, about the time of the Panama's Isthmus closure. The geographical distribution of Antillean siskin is the most peripheral of the group and relatively close to that of black-capped siskin (see Fig. 2). Taking into account the position of Antillean siskin in dendrograms (Figs. 1A and 1B), it could seem that it is this North American group extant ancestor. Bootstraps not being 100 % may mean that extinct species are lacking for analyses. The enforced NJ tree, which clusters together Antillean siskin and black-capped siskin, yielded poorer tree scores (not shown). Also, a phylogenetic placement of this species within South American siskins was discarded (Figs. 1A and 1B). It seems that Antillean siskin would be the oldest of the North American birds within this group, and that had given rise to pine siskin. Most of the *Carduelis dominicensis* particular traits (black head and neck, yellow breast) are shared with other forest or highland birds like *Carduelis notata* (black-headed siskin), *Linurgus olivaceus* (oriole finch) and *Mycerobas* genus spp. (Clement *et al*., 1993). Thus, these head and colour traits in Antillean siskin might be due to convergent evolution on a highland forest.

Carduelis pinus pinus (pine siskin), *C. pinus perplexus* (pine siskin perplexus) and *C. atriceps* (black-capped siskin)

Pine siskin was first described by Wilson (1810). This species thrives in North America from Alaska, South to Guatemala (Clement *et al*., 1993). mtDNA from pine siskin taken at Dolores (Colorado) was arbitrarily chosen for tree building and calculations (Figs. 1A and 1B), because other single samples from distant sites within the Pine siskin range had almost identical sequences (Fig. 2). Its origin could be postulated about 200,000 years ago, in the late Pleistocene (Fig. 1A). This species has already been described as a sister taxa of the *Carduelis spinus* (Eurasian siskin; Arnaiz-Villena *et al*., 1998). Their common ancestor most likely originated in the northern Hemisphere (Arnaiz-Villena *et al*., 1998).

Carduelis pinus perplexus is resident below the Mexican Isthmus in the highlands (2,000-3,500 m) from northern Chiapas to western Guatemala (van Rossen, 1938). Our specimen was captured in Quetzaltenango (Guatemala highlands). Pine siskin p*erplexus* is quite different in appearance from pine siskin and studies are necessary to determine its taxonomic status (Fig. 2; Howell and Webb, 1995). There has been an attempt to add more taxonomic molecular data in the present paper and pine siskin *perplexus* would seem to be a "sister" species to black-capped siskin based on genetic distances (Table 2).

Black-capped siskin was first described by Salvin (1863). This bird is monotypic (with no different subspecies), from the highlands of Chiapas, southeast Mexico, south to the western highlands of Guatemala. Pine siskin *perplexus* is grouped with black-capped siskin in the *p* genetic distance matrix (Table 2) and in analyses that include several individuals of pine siskin and Black-capped siskin (not shown).

Finally, it appears that siskin, Antillean siskin, pine siskin, pine siskin *perplexus* and

black-capped siskin form a monophyletic group separated from other North American *Carduelis* finches (*C. lawrencei*, *C. tristis*, *C. psaltria*, which seem to be closer to other European *Carduelis:* twite and linnet -Arnaiz-Villena *et al*., 1998-). Antillean siskin is not genetically related to South American siskins.

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BIBLIOGRAPHY

ALLENDE, L. M., RUBIO, I., RUIZ-DEL-VALLE, V., GUILLEN, J., MARTINEZ-LASO, J., LOWY, E., VARELA, P., ZAMORA, J. and ARNAIZ-VILLENA, A. 2001. The Old World sparrows (genus Passer) phylogeography and their relative abundance of nuclear mtDNA pseudogenes. *Journal of Molecular Evolution*, 53: 144-154.

ARBOGAST, B. S. and SLOWINSKI, J. B. 1998. Pleistocene speciation and the mitochondrial DNA clock. *Science*, 282: 1995a.

ARMANI, G. C. 1983. *Guide des Passereaux Granivores*. Paris.

ARNAIZ-VILLENA, A., ALVAREZ-TEJADO, M., RUIZ-DEL-VALLE, V., GARCIA-DE-LA-TORRE, C., VARELA, P., RECIO, M. J., FERRE, S. and MARTINEZ-LASO, J. 1998. Phylogeny and rapid northern and southern hemisphere speciation of goldfinches during the Miocene and Pliocene epochs. *Cellular and Molecular Life Sciences*, 54: 1031-1041.

ARNAIZ-VILLENA, A., ALVAREZ-TEJADO, M., RUIZ-DEL-VALLE, V., GARCIA-DE-LA-TORRE, C., VARELA, P., RECIO, M. J., FERRE, S. and MARTINEZ-LASO, J. 1999 Rapid radiation of canaries (Genus *Serinus*). *Molecular Biology and Evolution*, 16: 2-11.

ARNAIZ-VILLENA, A., GUILLEN, J., RUIZ-DEL-VALLE, V., LOWY, E., ZAMORA, J., VARELA, P., STEFANI, D. and ALLENDE, L. M. 2001. Phylogeography of crossbills, bullfinches, grosbeaks, and rosefinches. *Cellular and Molecular Life Sciences*, 58: 1159-1166.

ARNAIZ-VILLENA, A., TIMON, M., CORELL, A., PEREZ-ACIEGO, P., MARTIN-VILLA, J. M. and REGUEIRO, J. R. 1992. Primary immunodeficiency caused by mutations in the gene encoding the CD3-gamma subunit of the T-lymphocyte receptor. *The New England Journal of Medicine*, 327: 529-533.

ASHTON, K. G. 2002. Patterns of within-species body size variation of birds: strong evidence for Bergmann's rule. *Global Ecology and Biogeography*, 11: 505-523.

BALL, R. M. JR., FREEMAN, S., JAMES, F. C., BERMINGHAM, E. and AVISE, J. C. 1988. Phylogeography population structure of the red-winged Blackbirds assessed by mitochondrial structure. *Proceedings of the National Academy of Sciences U S A*, 85: 1558-1562.

BOND, J. 1948. Origin of the bird fauna of the West Indies. *Wilson Bulletin*, 60: 207-229.

BOND, J. 1963. Derivation of the Antillean avifauna. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 115: 79-98.

BRODKORB, P. 1971. Origin and evolution of birds. In, D. S. Farner and J. R. King (Eds.): *Avian Biology*, pp. 19-55. Academic Press. New York.

BRYANT, H. 1867. A list of the birds of Santo Domingo, with descriptions of some new species. *Proceedings of the Boston Society of Natural History*, 11: 89-98.

CHIAPPE, L. M. 1995. The first 85 million years of avian evolution. *Nature*, 378: 349-355.

CLEMENT, P., HARRIS, P. and DAVIES, J. 1993. *Finches and Sparrows*. Christopher Helm. London.

CRAMP, P. and PERRINS, C. M. (Eds.) 1994. *Complete Birds of the Western Paleartic*. Vol. VIII. Oxford University Press, New York.

DESJARDINS, P. and MORAIS, R. 1990. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. *Journal of Molecular Biology*, 212: 599-634.

FEDUCCIA, A. 1995. Explosive Evolution in Tertiary Birds and Mammals. *Science*, 267: 637-638.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, 17: 368-376.

FELSENSTEIN, J. 1985. Confidence limits of phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-795.

FITCH, W. M. 1971. Toward defining the course of evolution, minimum change from a specific tree topology. *Systematic Zoology*, 20: 406-415.

FLEISCHER, R. C., MCINTOSH, C. and TARR, C. L. 1998. Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Molecular Ecology*, 7: 533-545.

FRIESEN, V. L., MONTEVECCHI, W. A., BAKER, A. J., BARRETS, R. T. and DAVIDSON, W. S. 1996. Population differentiation and evolution in the common guillemot Uuria aalge. *Molecular Ecology*, 5: 793-805.

GILL, F. B. 1995. *Ornithology*. W. H. Freeman and Company, New York.

GROTH, J. G. 1998. Molecular Phylogenetics of Finches and Sparrows: Consequences of Character State Removal in Cytochrome b sequences. *Molecular Phylogenetics and Evolution*, 10: 377-390.

HACKETT, S. J. 1996. Molecular phylogenetics and biogeography of tanagers in the genus Ramphocelus (Aves). *Molecular Phylogenetics and Evolution*, 5: 368-382.

HASEGAWA, M., KISHINO, H. and YANO, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, 22: 160-174

HASEGAWA, M., THORNE, J. L. and KISHINO, H. 2003. Time scale of eutherian evolution estimated without assuming a constant rate of molecular evolution. *Genes and Genetic Systems*, 78: 267-283.

HEDGES, S. B., PARKER, P. H., SIBLEY C. G. and KUMAR, S. 1996. Continental breakup and the ordinal diversification of birds and mammals. *Nature*, 381: 226-229.

HILLIS, D. M., HUELSENBECK, J. P. and CUNNINGHAM, C. W. 1994. Application and accuracy of molecular phylogenies. *Science*, 264: 671-677.

HOWELL, S. and WEBB, S. 1995. *A Guide to the Birds of Mexico and Northern Central America*. Oxford University Press, Oxford.

JAMES, F. C. 1970. Geographic size variation in birds and its relationship to climate. *Ecology*, 51: 365-390.

JAMES, F. C. 1983. Environmental Component of Morphological-Differentiation in Birds. *Science*, 221: 184-186.

KLICKA, J. and ZINK, R. 1997. The importance of recent ice ages in speciation: a failed paradigm. *Science*, 277: 1666-1669

KOCHER, T. D., THOMAS, W. K., MEYER, A., EDWARDS, S. V., PAABO, S., VILLABLANCA, F. X. and WILSON, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences USA*, 86: 6196-6200.

KORNEGAY, J. R., KOCHER, T. D., WILLIAMS, L. A. and WILSON, A. C. 1993. Pathways of lysozyme evolution inferred from the sequences of cytochrome b in birds. *Journal of Molecular Evolution*, 37: 367-379.

KUMAR, S., TAMURA, K., INGRID, B. and NEI, M. 2001. *MEGA2: Molecular Evolutionary Genetics Analysis software, version 2.0*. Arizona State University, Tempe, Arizona, USA.

LANYON, W. E. 1967. Revision and probable evolution of the *Myiarchus* flycatchers of the West Indies. *Bulletin of the American Museum of Natural History*, 136: 329-370.

LI, W., WU, C. and LUO, C. 1985. A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. *Molecular Biology and Evolution*, 2: 150-174.

MAYR, E. and SHORT, L. 1970. Species taxa of North American birds. *Publications of the Nuttall Ornithological Club*, 9: 1-127

NEI, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.

NEI, M., and GOJOBORI, T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution*, 3: 418-426.

PAMILO, P. and BIANCHI, N. 1993. Evolution of the Zfx and Zfy, genes: Rates and interdependence between the genes. *Molecular Biology and Evolution*, 10: 271-281.

QUESTIAU, S., EYBERT, M. C., GAGINSKAYA, A. R., GIELLY, L. and TABERLET, P. 1998. Recent divergence between two morphologically differentiated subspecies of bluethroat (Aves: Muscicapidae: Luscinia svecica) inferred from mitochondrial DNA sequence variation. *Molecular Ecology*, 7: 239-245.

SAITOU, N. and NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phylo-

genetic trees. *Molecular Biology and Evolution*, 4: 406-425.

SALVIN, O. 1863. Description of thirteen new species of birds discovered in Central America. *Proceedings of the Zoological Society of London*, 186-192.

SIBLEY, C. G. and MONROE, B. L. JR. 1990. *Distribution and Taxonomy of Birds of the World*. Yale University Press, New Haven and London.

SWOFFORD, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods) version 4*. Sinauer Associates, Sunderland, Massachusetts.

THORNE, J. L., KISHINO, H. and PAINTER, I. S. 1998. Estimating the Rate of Evolution of the Rate of Molecular Evolution. *Molecular Biology and Evolution*, 15: 1647-1657.

VAN DEN ELZEN, R., GUILLEN, J., RUIZ-DEL-VALLE, V., ALLENDE, L. M., LOWY, E., ZAMORA, J. and ARNAIZ-VILLENA, A. 2001. Both morphological and molecular characters support speciation of South American siskins by sexual selection. *Cellular and Molecular Life Sciences*, 58: 2117-2128.

VAN ROSSEN, A. J. 1938. *Bulletin of the British Ornithologists' Club*, 58: 134-135.

VAURIE, C. 1959. *The Birds of the Palearctic Fauna. A sistematic reference. Order Passeriformes*. H.F.and G.Witherby Limited, London.

WILSON, A. 1810. *The Natural History of the Birds of the United States: Illustrated with Plates Engraved and Colored from Original Drawings taken from Nature.*

YANG, Z. 1994. Estimating the pattern of nucleotide substitution. *Journal of Molecular Evolution*, 39: 105-111.

ZAMORA, J., LOWY, E., RUIZ-DEL-VALLE, V., MOSCOSO, J., SERRANO-VELA, J. I., RIVERO-DE-AGUILAR, J. and ARNAIZ-VILLENA, A. 2006. *Rhodopechys obsoleta* (desert finch): a pale ancestor of greenfinches (*Carduelis* spp.) according to molecular phylogeny. *Journal of Ornithology*, 147: 448-456.

ZAMORA, J., MOSCOSO, J., RUIZ-DEL-VALLE, V., LOWY, E., SERRANO-VELA, J. I., IRA- CACHAFEIRO, J. and ARNAIZ-VILLENA, A. 2006. Conjoint mitochondrial phylogenetic trees for canaries (*Serinus* spp.) and goldfinches (*Carduelis* spp.) show several specific polytomies. *Ardeola*, 53: 1-17.

ZINK, R. M. 1996. Comparative phylogeography in North American birds. *Evolution*, 50: 308-317.

ZINK, R. M. and KLICKA, J. 2006. The tempo of avian diversification: a comment on Johnson and Cicero. *Evolution International Journal of Organic Evolution*, 60: 411-412.

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