Ardeola 53(1), 2006, 1-17

CONJOINT MITOCHONDRIAL PHYLOGENETIC TREES FOR CANARIES *SERINUS* SPP. AND GOLDFINCHES *CARDUELIS* SPP. SHOW SEVERAL SPECIFIC POLYTOMIES

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SUMMARY.—*Conjoint mitochondrial phylogenetic trees for canaries* Serinus *spp. and goldfinches* Carduelis *spp. show several specific polytomies.*

Aims: Canaries and goldfinches seem to form a phylogenetic group separated from other Carduelini tribe radiations. The present study conjointly analyses for the first time the phylogenetic relationships among canaries (genus *Serinus*) and goldfinches (genus *Carduelis*), and others, and the particular case of citril finch (*Serinus citrinella*) which has been included by different authors either in the canaries or in the goldfinches group. Also, two new species: *Serinus totta* and *Serinus syriacus* have been newly DNA sequenced and studied.

Location: Eurasian, African and American canary and siskin-goldfinches species living range was surveyed. Also, island (Corsica and Sardinia) and continental (Madrid, Alps and Pyrenees) citril finch individuals were analysed.

Methods: Mitochondrial cytochrome b (mt cyt b) DNA gene was sequenced. Parsimony and genetic distance based methodologies were used for dendrograms construction. Enforced constraints were used to test the inclusion of Citril Finch within either *Serinus* or *Carduelis* groups.

Results and Discussion: Canaries and goldfinches may or may not be different genetic radiations with different evolutionary pathways. However it is confirmed that canaries are the closest Fringillidae family relatives to goldfinches, because canaries and goldfinches have conjointly been compared in the same dendrograms, also with the Fringillidae subfamilies: Peucedraminae, Emberizinae and Fringillinae (tribes Carduelini, Fringillini and Drepanidini), as indicated in Table 1. Also, each of these groups shows monophyletic and non-monophyletic supported subgroups as indicated by bootstrap values. Citril finch is definitively included within the genus *Carduelis*. This is supported by cladistic, and distance-based phylogenetic molecular analyses; it was also postulated by Bernis in 1954 based on phenetics. Citril finch island individuals seem to be more ancient than those extant in the continent. Genus *Loxia* (crossbills) is included within genus *Carduelis*. *Serinus totta* seems to cluster with the small African canaries clade and *Serinus syriacus* is included within the *Serinus pusillus*/ *Serinus alario* subgroup. Monophyly of *Carduelis* and *Serinus* is not discarded because phylogenetic tree nodes are ambiguously supported.

Key words: mtDNA, *citrinella*, *Serinus*, *Carduelis*, *totta*, *syriacus*.

RESUMEN.—*Las filogenias conjuntas de ADN mitocondrial de canarios (género* Serinus*) y jilgueros (género* Carduelis*) muestran politomías específicas.*

Objetivos: Los canarios y jilgueros parecen constituir un grupo filogenético separado de otras radiaciones de la tribu Carduelini. Este estudio analiza conjuntamente por primera vez las relaciones filogenéticas de canarios y jilgueros, y otros, y en especial el verderón serrano (*Serinus citrinella*), incluido

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por diferentes autores alternativamente dentro de uno u otro género. Además, dos nuevas especies: *Serinus totta* y *Serinus syriacus* han sido secuenciadas para su ADN y estudiadas por primera vez.

Localidad: Se han utilizado especies de canarios y jilgueros-luganos de África, Europa, Asia y América. Además, se analizaron ejemplares de verderón serrano isleños (Córcega y Cerdeña) y continentales (Madrid, Alpes y Pirineos).

Métodos: Se secuenció el gen del citocromo b mitocondrial . Se construyeron árboles filogenéticos de parsimonia y de distancias genéticas. Se hicieron también pruebas de inclusión forzada del verderón serrano para comprobar su inclusión o dentro del género *Carduelis* o del *Serinus*.

Resultados y Conclusiones: Los canarios y jilgueros pueden o no ser diferentes radiaciones genéticas con patrones evolutivos diferentes. Sin embargo, se confirma que los canarios son los parientes de la familia Fringillidae más cercanos a los jilgueros, ya que los jilgueros y los canarios se han analizado también conjuntamente en los mismos dendrogramas con las subfamilias de Fringillidae: Peucedramine, Emberizinae y Fringillinae (tribus Carduelini, Fringillini y Drepanidini), como se especifica en la Tabla 1. Además, cada uno de estos dos grupos contiene subgrupos monofiléticos y no monofiléticos débilmente soportados. El verderón serrano está definitivamente incluido dentro del género *Carduelis*. Esto está apoyado por análisis filogenéticos cladísiticos así como de distancias genéticas y ya fue propuesto por Bernis en 1954 basado en estudios fenotípicos. Los individuos de las islas parecen ser más antiguos que los continentales. Los piquituertos (género *Loxia*) están incluidos igualmente en el género *Carduelis*. *Serinus totta* parece estar incluido en el grupo de pequeños canarios africanos y *Serinus syriacus* se agrupa junto con *Serinus pusillus* y *Serinus alario*. La monofilia de *Carduelis* y *Serinus* no se puede descartar debido a que los nodos de los árboles filogenéticos están soportados de forma ambigua.

Palabras clave: mtDNA, *citrinella*, *Serinus*, *Carduelis*, *totta*, *syriacus*.

Introduction

Genus *Serinus* species (Canaries) occur in Palearctic-Afro-tropical areas. They are included within the Carduelidae bird family, and comprise thirty-two (Painter, 1968) to forty-five (Sibley and Monroe, 1990) different species. They usually thrive in Africa and some species also inhabit central and southern Europe, Turkey, Middle East and Arabia (Clement *et al.*, 1993). Most of them are small or slender finches, generally green, greenish-yellow, brown or grey with dark streaks, usually with distinctly bright rump patch and forked or notched tail (Clement *et al.*, 1993). Canaries monophyly has been questioned on the basis of behaviour and life history traits (Elzen and Khoury, 1999). Species classification based on morphology, as well as on biogeographic distribution patterns (Elzen and Khoury, 1999) recognized five monophyletic groups: *Serinus sensu stricto* (*S. alario*, *S. citrinella*, *S. canicollis*, *S. syriacus*, *S. pusillus*), *Poliospiza* (*S. leucopterus*, *S. striolatus*, *S. gularis*, *S. tristriata*), *Dendrospiza* (*S. citrinelloides*, *S. hypposticta*, *S. scotops*), *Crithagra* (*S. flaviventris*, *S. sulphuratus*, *S. donaldsoni*, *S. albogularis*), *Ochrospiza* (*S. leucopygius*, *S. reichenowi*, *S. atrogularis*, *S. citrinipectus*, *S. mozambicus*, *S. dorsostriatus*, *S. xanthopygius*). The molecular phylogeny obtained by Arnaiz-Villena *et al.* (1999) was to a great extent coincidental with these phenotypically defined groups with the exception of *Serinus citrinella* (citril finch, see below).

Carduelis is a widespread genus that comprises about thirty one species of medium-sized finches with different bill shapes that depend on each species foraging method. They thrive throughout the Holartic and also reach the South American tip. Their African range is confined to North Africa (Clement *et al.*, 1993). Most of the *Carduelis* species were examined by mt cyt b phylogeny by Arnaiz-Villena *et al.* (1998). This study revealed the existence of several species groups, which were in general

concordant with present-day geographical distributions. Genus *Carduelis* includes: South American siskins, North American goldfinches, Eurasian greenfinches and Eurasian and Mediterranean goldfinches. *Serinus citrinella*, classified as a Canary (Clement *et al.*, 1993), was found to be a close relative to Eurasian goldfinches since both have common ancestors. Holartic redpolls appear together with genus *Loxia* (crossbills) in molecular phylogenies showing a conjoint monophyletic group (Arnaiz-Villena *et al.*, 2001).

The taxonomic status of citril finch (*Serinus citrinella*) as a canary has been questioned on molecular, behavioural and phenotypic bases (Van den Elzen, 2000; Arnaiz-Villena *et al.* 1998, 1999). However, Francisco Bernis (1954) named it *Carduelis citrinella* based on phenotypic, behavioural and biogeographic characters. A controversy has also arisen about assigning separate species status to the insular "Koenig" type, *S. c. corsicanus* (Corsica, Sardinia, and Tuscany islands) and to the mainland or "Pallas" type, *S. c. citrinella* (Pyrenees, Alps, and Spanish northern and central mountains, see Borras and Senar, 1991). Both forms show phenotypic differences (Vaurie, 1959; Clement *et al.*, 1993; Cramp and Perrins, 1994) and, particularly, the brown mantle is typical of the island species against grey mantle of the continental ones. Also, singing is different between the two citril finch forms (Chappuis, 1976; Cramp and Perrins, 1994). Pasquet and Thibault (1997) suggested that the divergence of continental and island forms may be more recent than the last connection between the mainland and the Sardinia-Corsica complex during the Messinian salinity crisis. On the other hand, they did not find significant genetic divergence of mtDNA to consider mainland and insular forms as two different species. However, Sangster (2000) pointed out that interspecific and intraspecific percentage of genetic citril finch divergence overlap.

The aim of this study is threefold: 1) to analyse the global relationships between Carduelis and Serinus genera species. Also, a conjoint analysis with species belonging to other Fringillidae family genera will also be done in order to test whether Serinus and Carduelis are the phylogenetically closest genera, 2) to definitively establishing the molecular S. citrinella inclusion within canaries or within goldfinches, 3) to assessing genetic differences between mainland and insular forms of *Serinus citrinella*.

Material and methods

Bird samples

Name of species and place of origin of the authors' own samples are given in Table 1. Table 1 footnote specify birds from other Fringillidae family used for this study and taken from the Genebank. Blood from living birds was drawn after their claws were locally anaesthetized with a lidocaine ointment and then cut and birds were also photographed. Blood was collected in ice-cold EDTA and frozen until use. 924 base pairs (from 97 to 1020) of the mitochondrial cyt b gene were amplified with primers L14841 5' - A A A A A G C T T C C A T C C A A - CATCTCAGCATGATGAAA-3' and H15767 5'- ATGAAGGGATGTTCTACTGGTTG-3' as detailed by Edwards (1991). Polymerase Chain Reaction (PCR), cloning and automatic DNA sequencing was performed as previously described (Edwards *et al.*, 1991; Arnaiz-Villena *et al.*, 1992). At least four clones from each two different PCRs were sequenced in each species; four different *S. c. corsicanus* from Sardinia and another four different *S. c. citrinella* from Madrid-Sierra were obtained and DNA sequenced by us. The rest of goldfinches and canaries origin is specified in Arnaiz-Villena *et al.* (1998, 1999). Eight cyt b clones from each specimen were sequenced in order to ensure the correct sequence acquisition. All canary and siskin cyt

TABLE 1

List of species, origin and mit cyt b sequence identification (GeneBank accesion number). § Ascents originating in northern Europe emigrated to the Antwerp region in winter. All specimens studied are male; except for the ones signed as ‖ and ♀ meaning undetermined sex and female sex respectively. Accession numbers of Fringillidae familiy species: a) subfamiliy Peucedraminae AF290139 (*Peucedramus taeniatus*). b) subfamiliy Emberizinae L78806 (*Emberiza elegans*). c) subfamiliy Fringillinae species apart from canaries and siskins: Tribe Carduelini AF342878 (*Loxia leucoptera bifasciata*), AF342876 (see table), AF342877 (see table), AF342883 (*Carpodacus erythrinus roseatus*), AF342875 (*Haematospiza sipahi*), AF342868 (*Carpodacus rubicilloides*), AF365877 (*Uragus sibiricus lepidus*), AF365878 (*Carpodacus pulcherrimus*), AF342869 (*Carpodacus thura*), AF342867 (*Carpodacus roseus*), AF342870 (*Carpodacus trifasciatus*), AF342865 (*Carpodacus mexicanus*), AF342866 (*Carpodacus nipalensis*), AF342884 (*Pyrrhula nipalensis*), AF342882 (*Pinicola enucleator*), AF342862 (*Pyrrhula erythaca*), AF342886 (*Pyrrhula pyrrhula cineracea*), AF342885 (*Pyrrhula p. iberiae*), AF342881 (*Pyrrhula p. griseiventris*), AF342872 (*Eophona personata*), AF342871 (*Eophona migratoria*), AF342880 (*Mycerobas carnipes*), AF342879 (*Mycerobas affinis*), (see Arnaiz-Villena *et al*., 2001 for details); tribe Drepanidini AF447371 (*Paroaria coronata*), AF015763 (*Oreomystis bairdi*), AF015758 (*O. mana*), AF015762 (*Pseudonestor xanthophrys*); tribe Fringillini AY495390 (*Fringilla montifringilla*), L76609 (*F. coeleb*).
*[Lista de especies, origen y número de acceso de la secuencias en el Genebank. § Ancestros originarios del norte de Europa que migraron a la región de Antwerp en invierno. Todos los especimenes estudiados son machos, excepto por los designados con ‖ y ♀ (sexo indeterminado y hembra respectivamente). Números de acceso de especies de la familia Fringillidae: a) subfamilia Peucedraminae AF290139 (*Peucedramus taeniatus*). b) subfamilia Emberizinae L78806 (*Emberiza elegans*). c) subfamilia Fringillinae (especies aparte de los géneros* Serinus *y* Carduelis*): tribu Carduelini AF342878 (*Loxia leucoptera bifasciata*), AF342876 (ver tabla), AF342877 (ver tabla), AF342883 (*Carpodacus erythrinus roseatus*), AF342875 (*Haematospiza sipahi*), AF342868 (*Carpodacus rubicilloides*), AF365877 (*Uragus sibiricus lepidus*), AF365878 (Carpodacus pulcherrimus), AF342869 (Carpodacus thura), AF342867 (*Carpodacus roseus*), AF342870 (Carpodacus trifasciatus), AF342865 (*Carpodacus mexicanus*), AF342866 (Carpodacus nipalensis), AF342884 (Pyrrhula nipalensis), AF342882 (Pinicola enucleator), AF342862 (*Pyrrhula erythaca*), AF342886 (Pyrrhula p. cineracea), AF342885 (*Pyrrhula p. iberiae*), AF342881 (*Pyrrhula p. griseiventris*), AF342872 (*Eophona personata*), AF342871 (*Eophona migratoria*), AF342880 (*Mycerobas carnipes*), AF342879 (*Mycerobas affinis*), (ver Arnaiz-Villena* et al.*, 2001); tribu Drepanidini AF447371 (*Paroaria coronata*), AF015763 (*Oreomystis bairdi*), AF015758 (*Oreomystis mana*), AF015762 (*Pseudonestor xanthophrys*); tribu Fringillini AY495390 (*Fringilla montifringilla*), L76609 (*F. coelebs*).]*

Species *[Especie]*	Mt cyt b sequence *[nº de acceso]*	Sample region *[Origen]*
Siskin (*Carduelis spinus*)	L76391	Madrid, Spain
Pine siskin (*Carduelis p. pinus*)	U79020	Jackson, Wyoming, USA.
Linnet (*Carduelis c. cannabina*)	L76298	Madrid, Spain
Twite (*Carduelis f. flavirostris*)	U83199	Cage bird. Antwerp, Belgium§
Common redpoll (*Carduelis f. flammea*)	L76386	Brussels, Belgium
Artic redpoll (*Carduelis hornemanni hornemmani*)	U83201	Cage bird. Antwerp, Belgium
Citril finch (*Serinus c. citrinella*)	L77872	Madrid Sierra, Spain
Citril finch (*Serinus citrinella corsicanus*)	AY583725	Sardinia, Italy
Citril finch (*Serinus citrinella citrinella*)	(Pasquet and Thibault, 1997)	Alps / Pyrenees

Species *[Especie]*	Mt cyt b sequence *[nº de acceso]*	Sample region *[Origen]*
Citril finch (*Serinus citrinella corsicanus*)	(Pasquet and Thibault, 1997)	Corsica, Italy
Goldfinch (*Carduelis carduelis parva*)	L76387	Madrid, Spain
Goldfinch (*Carduelis carduelis caniceps*)	L76388	Katmandú, Nepal
Greenfinch (*Carduelis chloris aurantiventris*)	L76297	Madrid, Spain
Oriental greenfinch (*Carduelis s. sinica*)	L76592	Szechwan, China
Black-headed greenfinch (*Carduelis a. ambigua*)	U78322	Szechwan, China
Himalayan greenfinch (*Carduelis s. spinoides*)	U79018	Katmandú, Nepal
European serin (*Serinus serinus*)	L76263	Marid, Spain
White-rumped seedeater (*Serinus leucopygius*)	L76264	Dakar, Senegal ♀
Black-throated canary (*Serinus atrogularis*)	L76267	Cape Town, South-Africa
Black-headed canary (*Serinus a. alario*)	L76276	Cape Town, South-Africa ♀
White-bellied canary (*Serinus d. dorsostriatus*)	L76278	Dar es Salam, Tanzania
Yellow canary (*Serinus flaviventris quintoni*)	L76280	Cape Town, South-Africa
Brimstone canary (*Serinus s. sulphuratus*)	L76294	Cape Town, South-Africa
African citril (*Serinus c. citrinelloides*)	L77555	Nairobi, Kenya ♀
Streaky-headed seedeater (*Serinus gularis endemoin*)	L77556	Capetown, South-Africa ‖
Fire-fronted serin (*Serinus pusillus*)	L77873	Sin Wiang, China
Yellow-fronted canary (*Serinus mozambicus*)	L76265	Dar es Salam, Tanzania
Island canary (*Serinus canaria*)	L76266	Gran Canaria. Canary Islands, Spain
Lemon-breasted seedeater (*Serinus citrinipectus*)	L78707	Maputo, Mozambique ♀
White-throated canary (*Serinus albogularis*)	L78705	Capetown, South-Africa
Cape canary (*Serinus c. canicollis*)	L78706	Capetown, South-Africa
Cape canary (*Serinus canicollis flavivertex*)	L76295	Nairobi, Kenya
Syrian serin (*Serinus syriacus*)	AY570547	Mont. Hermon, Israel ‖
Cape serin (*Serinus totta*)	AY570548	Cape Town, South-Africa
Common crossbill (*Loxia c. curvirostra*)	AF342876	Alcalá de Henares, Spain ‖
Common crossbill (*Loxia curvirostra japónica*)	AF342877	Beijing, China
Two-barred Crossbill (*Loxia leucoptera bifasciata*)	AF342878	Siberia, Russia ♀
Chaffinch (*Fringilla c. coelebs*)	L76609	Madrid, Spain

b mtDNA sequences used in this study were published and deposited in the Genbank by ourselves (Arnaiz-Villena *et al.*, 1998, 1999), with the exception of the sequences of *Serinus citrinella citrinella* from Alps and *S. citrinella corsicanus* from Corsica that were extracted directly from Pasquet and Thibault (1997). The sequences of *S. citrinella corsicanus* from Sardinia (four individuals), *S. totta* and *S. syriacus* were obtained and submitted to the GenBank for the present study.

Statistics analyses and tree construction methods

Saturation plots (not shown) were done in order to be aware of transition or transversion changes that may become saturated (multiple substitutions at single site) and then uninformative at certain divergence distances. Uncorrected pairwise divergence was used as an estimate of percent divergence (p = Nd / n, where p is the percent sequence divergence,

Nd is the number of nucleotides that differ between two sequences, and n is the total number of nucleotides compared, Nei, 1987); this gives an approximation of time since divergence. In view of these plots an *a priori* weighting based on the transition / transversion ratio would be proposed to overcome the effects of homoplasy.

Three different phylogenetic tree-constructing methodologies were used, as recommended by Härlid *et al.* (1997): 1) parsimony (Fitch, 1971), 2) Neighbour-Joining (NJ) (Saitou and Nei, 1987), and 3) Maximum Likelihood (ML) distances (Felsenstein, 1981) were also used to construct a linearized ML-based tree.

All the phylogenetic hypotheses were conducted by using the PAUP software package (Swofford, 2002). In all analyses the characters were set unordered. *Fringilla coelebs* and *Carduelis flammea* were chosen as outgroups to root trees depending on the type of analyses (Figs. 1, 2 and 3, respectively).

Distance trees.- The ML-based tree (Fig. 1) was linearized bearing in mind that variation of evolutionary rate among lineages may exist. Thus, 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 was successfully applied to several biological issues (Hasegawa *et al.*, 2003; and references therein). ML analysis settings were: two substitutions types; estimated transition / transversion ratio via ML; HKY85 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. PARAMCLOCK PAUP command was used to build the ML based linearized tree. The used molecular clock was identical to the one calculated for the *Drepanidini* (Hawaiian honeycreeper birds) on both geological and mtDNA molecular bases (0.8 % DNA base substitutions per million years). This clock was used because this was a result of independent geological and molecular calculations and this *Drepanidini* evolutionary rate has been found in the closest species to the Carduelinae finches (Fleischer *et al.*, 1998).

Cladistic analysis (Parsimony).- The search of the most parsimonious trees (Fig. 2) was

FIG. 1.—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. Divergence times were estimated assuming an evolutionary rate of 0.8 % substitutions per site and million years, found by Fleischer *et al*. (1998). This rate is based on the cyt b sequence divergence of Hawaiian drepanidines, and external geological calibration points. Groups of taxa are similar to those obtained in the parsimony (see Fig. 2). 1000 replication bootstrap values are depicted in the interior part of the nodes; values lower than 5 are not shown. ML based tree scores: tree length (x 1000) = 1543.39; ln Likelihood = -5366.19865; estimated transition / transversion ratio = 4.84. ML genetic distances are depicted above the time scale (Million Years Ago).
[Árbol linearizado de Maximum Likelihood. Este árbol se construyó asumiendo que las tasas evolutivas de los linajes pudiesen ser diferentes (Thorne et al., 1998). Para la construcción del árbol se usó el comando PARAMCLOCK del programa PAUP. Los tiempos de divergencia fueron estimados asumiendo una tasa evolutiva de 0.8% substituciones por posición y millón de años (Fleischer et al.*, 1998). Esta tasa se basa en la divergencia de las secuencias de citocromo b de los mieleros de Hawai, y en puntos de calibración geológicos externos. Los agrupamientos de especies encontrados son similares a los obtenidos en el árbol de parsimonia (ver Fig. 2). Los valores de bootstrap se calcularon a partir de 1000 replicas y se muestran en el interior de cada nodo; los valores menores de 5 no se muestran. Las características del árbol son: longitud del árbol (x 1000) = 1543,39; ln Likelihood = -5366,19865; ratio estimado de transiciones / transversiones = 4,84. Las distancias genéticas Maximum Likelihood aparecen bajo la escala de tiempo (millones de años).]*

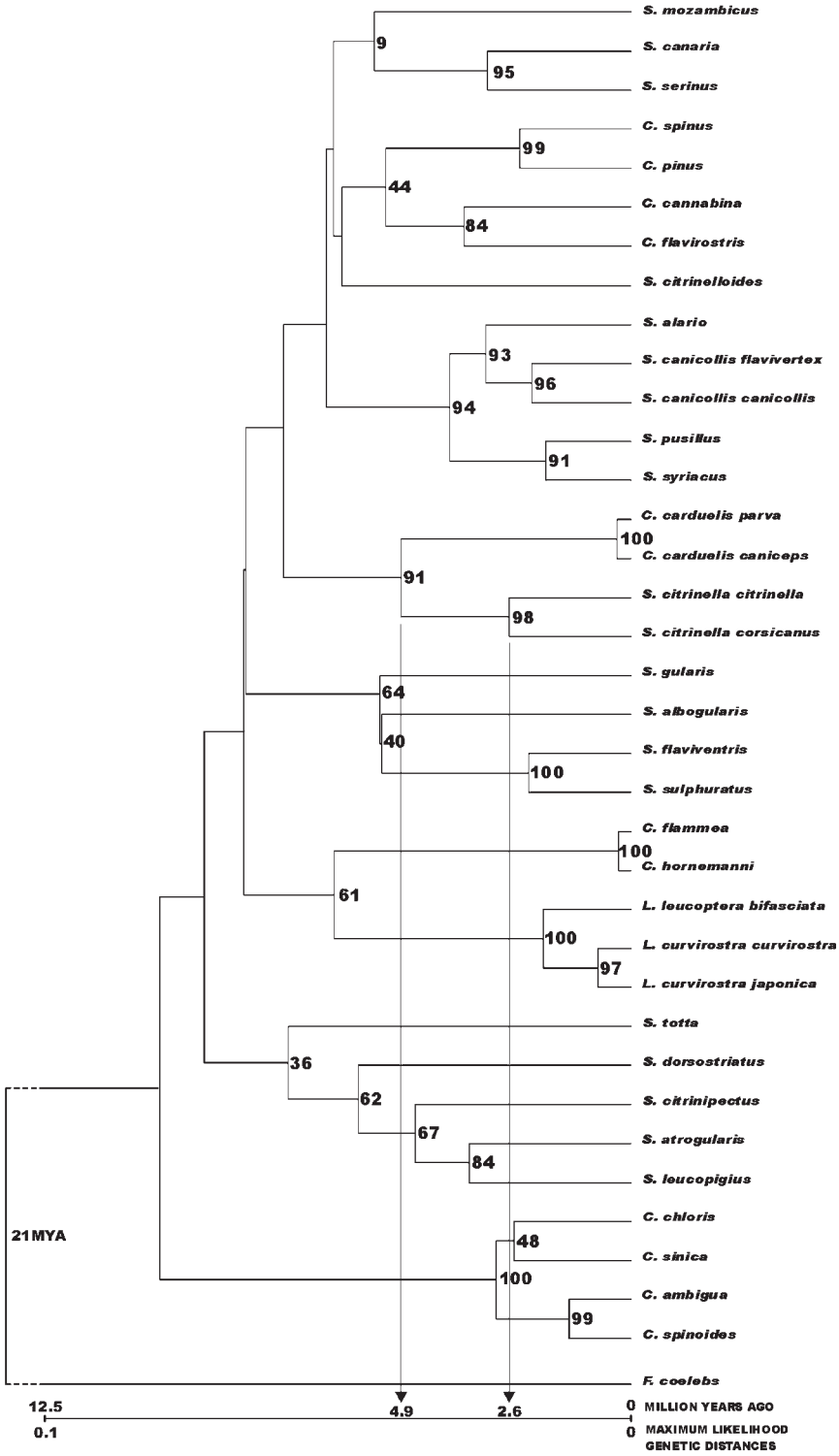

S. mozambicus
S. canaria
S. serinus
C. spinus
C. pinus
C. cannabina
C. flavirostris
S. citrinelloides
S. alario
S. canicollis flavivertex
S. canicollis canicollis
S. pusillus
S. syriacus
C. carduelis parva
C. carduelis caniceps
S. citrinella citrinella
S. citrinella corsicanus
S. gularis
S. albogularis
S. flaviventris
S. sulphuratus
C. flammea
C. hornemanni
L. leucoptera bifasciata
L. curvirostra curvirostra
L. curvirostra japonica
S. totta
S. dorsostriatus
S. citrinipectus
S. atrogularis
S. leucopigius
C. chloris
C. sinica
C. ambigua
C. spinoides
F. coelebs
9
95
99
44
84
93
96
94
91
100
91
98
64
40
100
100
61
100
97
36
62
67
84
48
100
99
21MYA
12.5
4.9
2.6
0
MILLION YEARS AGO
0.1
0
MAXIMUM LIKELIHOOD
GENETIC DISTANCES

FIG. 2.—Parsimony tree. Bootstrap (1000 replicates) and branch length values are above and underlined below the branches respectively. Tree length = 742; consistency index = 0.36; retention index = 0.54. *[Árbol de parsimonia. Los valores de bootstrap (1000 réplicas) y longitudes de rama aparecen respectivamente sobre y bajo las ramas. Longitud del árbol = 742; índice de consistencia = 0,36; índice de retención = 0,54.]*

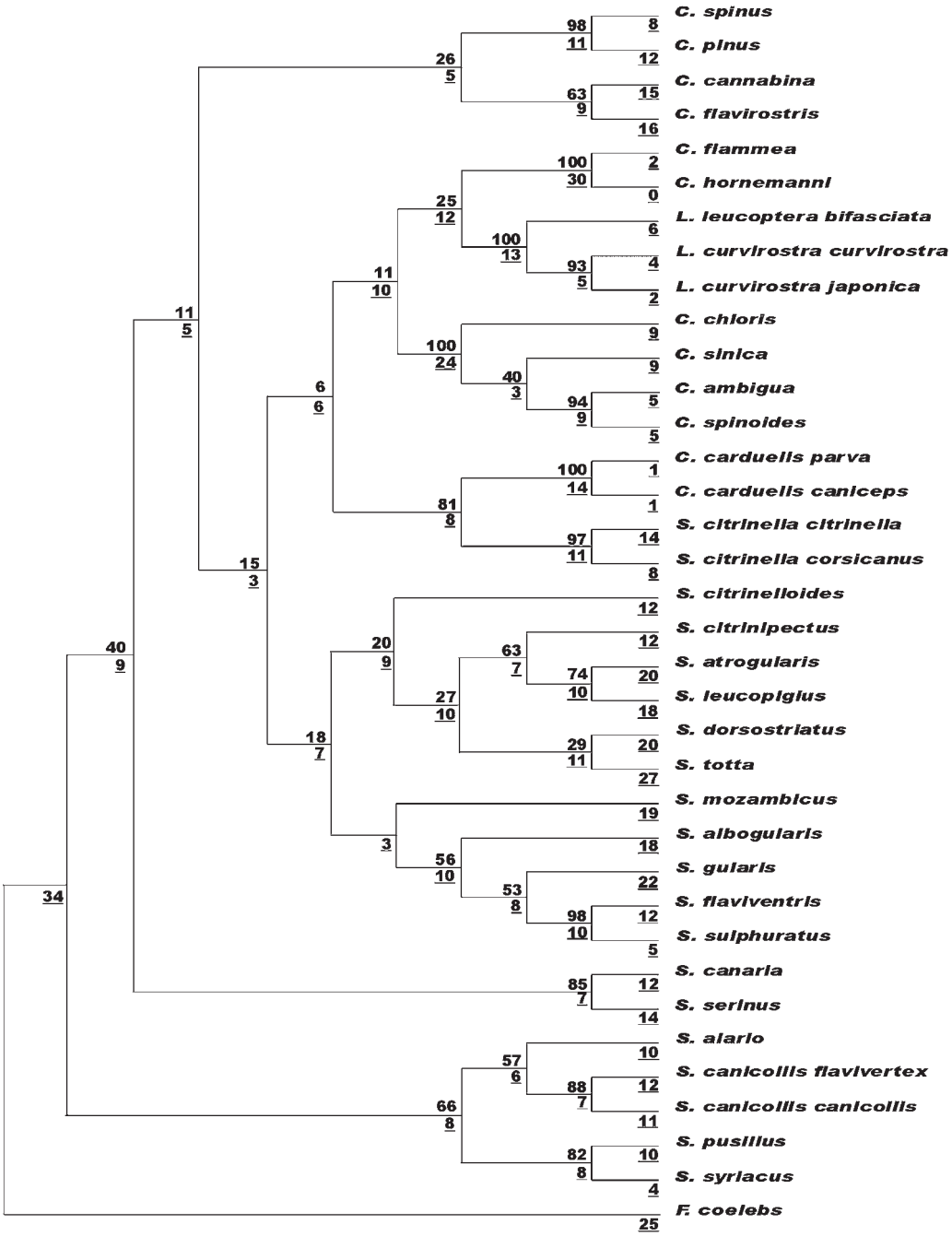


TABLE 2

Enforced constraints on NJ (Neighbour-Joining) upon ML (Maximum Likelihood) distances and parsimony support that *S. citrinella* is included within the genus *Carduelis*. * Statistically significant differences for the Winning Sites Test, Templeton Test and Kishino Hasegawa Test. [1] Both *Serinus c. citrinella* and *S. citrinella corsinanus* mtDNA sequences were used for these calculations. [2] Both *Carduelis c. parva* and *Carduelis carduelis caniceps* mtDNA sequences were used for these calculations. [3] *Loxia c. curvirostra*, *L. curvirostra japonica* and *L. leucoptera bifasciata* mtDNA sequences were used for these calculations. [4] The following species mtDNA sequences were used for these calculations: *S. serinus, S. leucopygius, S. atrogularis, S. a. alario, S. d. dorsostriatus, S. flaviventris quintoni, S. sulphuratus sulphuratus, S. c. citrinelloides, S. gularis endemoin, S. pusillus, S. mozambicus, S. canaria, S. citrinipectus, S. albogularis, S. c. canicollis, S. canicollis flavivertex, S. syriacus* and *S. totta*.
[Las constricciones forzadas sobre árboles NJ (Neighbour-Joining) con distancias ML (Maximum Likelihood) y sobre árboles de parsimonia apoyan la inclusión de S. citrinella *en el género* Carduelis*.* * *Diferencias estadísticamente significativas (P < 0,0001) para el test de "Winning Sites", test de Templeton y test de Kishino y Hasegawa. [1] Las secuencias de mtDNA de* Serinus c. citrinella *y* S. citrinella corsinanus *fueron utilizadas para estos cálculos. [2] Las secuencias de mtDNA de* Carduelis c. parva *y* Carduelis carduelis caniceps *fueron utilizadas para estos cálculos. [3] Las secuencias de mtDNA de* Loxia c. curvirostra, L. curvirostra japonica *and* L. leucoptera bifasciata *fueron utilizadas para estos cálculos. [4] Las secuencias de mtDNA de la siguientes especies fueron utilizadas para estos cálculos:* S. serinus, S. leucopygius, S. atrogularis, S. a. alario, S. d. dorsostriatus, S. flaviventris quintoni, S. sulphuratus sulphuratus, S. c. citrinelloides, S. gularis endemoin, S. pusillus, S. mozambicus, S. canaria, S. citrinipectus, S. albogularis, S. c. canicollis, S. canicollis flavivertex, S. syriacus *y* S. totta.*]*

	Parsimony scores [Resultados del análisis de parsimonia]		
S. citrinella[1]	Tree length *[Longitud del árbol]*	Consistency Index *[Índice de Consistencia]*	Retention Index *[Índice de Retención]*
without constraints (Fig. 2) *[sin constricciones (Fig. 2)]*	742	0.358	0.544
with C. carduelis[2] - non constricted *[con C. carduelis[2] sin constricción]*	742	0.358	0.544
with genus Loxia[3] *[con el género Loxia[3]]*	758*	0.351	0.528
grouped within genus Serinus[4] *[agrupada dentro del género Serinus[4]]*	755*	0.352	0.531
with S. mozambicus *[con S. mozambicus]*	752*	0.354	0.534

NJ and ML scores *[resultados del árbol de Neighbour Joining con distancias Maximum Likelihood]*

heuristic because of the number of taxa (36) rendered unpractical an exhaustive search. Constrictions were also used in order to obtain the best phylogenetic position (*Serinus* or *Carduelis*) for Citril Finch (Table 2).

Enforced constricted trees.- Three different tests were used to statistically assess the differences in the support of characters between the constrained and unconstrained parsimony trees: Winning Sites Test (Prager *et al.*; 1988), Templeton Test (1983) and the Kishino and Hasegawa Test (1989).

Confidence of analyses.- Bootstrap values were calculated with 1000 replicates for test-

TABLE 2 (Continuación)

S. citrinella[1]	-Ln Likelihood	MES [Minimum Evolution Score]	Negative branch lengths [Longitudes de rama negativas]
without constraints (Fig. 1) *[sin constricciones (Fig. 1)]*	5295.04745	0.92305	no
with *C. carduelis*[2] - non constricted *[con* C. carduelis[2] *sin constricción]*	5295.04745	0.92305	no
with genus *Loxia*[3] *[con el género* Loxia[3]*]*	5323.62912	0.94634	yes
grouped within genus *Serinus*[4] *[agrupada dentro del género* Serinus[4]*]*	5312.99986	0.94180	yes
with *S. mozambicus* *[con* S. mozambicus*]*	5319.37214	0.94270	yes

ing the topology robustness of trees (Felsenstein, 1985). In order to further assess node robustness in the ML-based linearized tree the statistical confidence probability (CP) of a particular sequence cluster was also calculated (Takezaki *et al.*, 1995); CP obtained results will not be shown in the phylogenetic tree (Fig. 1) since they are equivalent to the bootstrap values.

RESULTS AND DISCUSSION

General patterns of DNA base substitution

Saturation plots for cyt b mtDNA (not shown) indicated that only third position transitions showed a clear levelling-off associated with saturation; this occurred at 11 % uncorrected total sequence divergence, that is, between ingroup (*Carduelis*, *Loxia* and *Serinus* spp.) and outgroup (*Fringilla coelebs*). Therefore, it was concluded that five out of six data partitions (at the first, second, third codon position bases and transitions/ transversions) were not saturated and were thus suitable to infer correct phylogenies (Irwin *et al.*, 1991; Hillis *et al.*, 1994). Variable and phylogenetically informative sites were 288 and 210 respectively, when the presently, studied *Carduelis-Loxia* / *Serinus* group (Arnaiz-Villena *et al.*, 2001) was analysed using *F. coelebs* as an outgroup; if no outgroup is considered sites numbers were 249 and 213, respectively. This variability within the cyt b gene was theoretically sufficient to establish sound phylogenetic relationships according to the number of observed parsimony-informative sites (Hillis *et al.*, 1994).

The cyt b gene nucleotide distribution pattern, that is, the A, C, G and T percentages at the first, second and third codon position of the birds under study was similar to that found in previous analyses of this gene for other birds (Hackett, 1996; Krajewski and King, 1996) and mammals (Irwin *et al.*, 1991): a) the four bases had similar frequencies at the first codon positions; b) fewer G residues and more T residues were seen at the second position and c) the bias against G and T was high at the third codon position (Edwards *et al.*, 1991). Thus, a correct phylogeny may be inferred from the parsimony analysis. The overall bias in base composition is similar in all studied species (24.3 % T, 34.2 % C, 27.7 % A and 13.9 % G). Therefore, the parsimony and NJ methodologies seem to be adequate for all our species under testing (Lockhart *et al.*, 1994). Notwithstanding, differences among the overall A, G, C, and T base

frequencies (%) were found to be significant (χ^2, $P < 0.005$); these differences led to using HKY85 model (that assumes the presence of unequal nucleotide frequencies, Hasegawa *et al.*, 1985) for the Maximum Likelihood analysis and subsequent NJ tree construction (Figs. 1 and 3A; Table 2). Nearly all observed DNA sequence differences were silent substitutions, as expected (Kocher *et al.*, 1989). Thus; 77.59 % of the third codon positions were not conserved among species, as it has been shown in other analysis for this gene, which evolves relatively rapidly under strong functional constraints (Irwin *et al.*, 1991); The variability for the first and second codon positions were 12.98 % and 2.59 %, respectively.

The estimated divergence time obtained by assuming the Drepanidini (Fleischer *et al.*, 1998) evolutionary rate gave approximately the same times of divergence already described: all canary/ goldfinch radiations appeared in the middle/ late Miocene (Arnaiz-Villena *et al.*, 1998, 1999, 2001).

Dendrograms and enforced constraint analyses

Firstly, canaries and goldfinches grouped together in all distance and cladistic trees and separated from all available Fringillidae species listed in Table 1 and Table 1 footnote (results not shown).

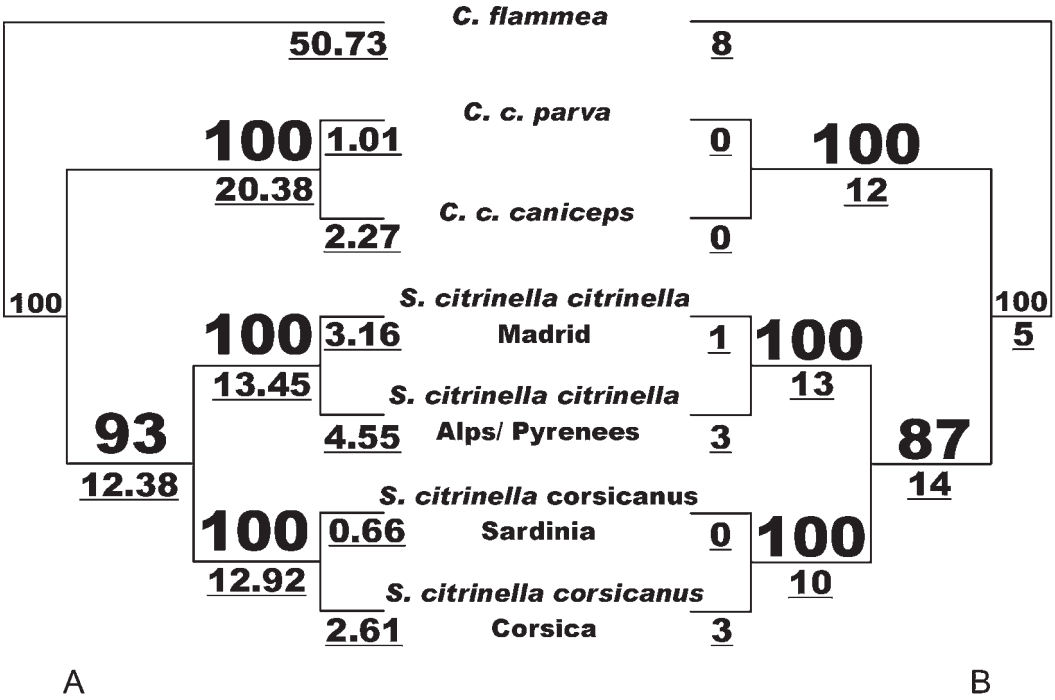
ML-based and parsimony trees (Figs. 1 and 2, respectively) showed a clear well-supported inclusion of both *S. citrinella* forms (from Madrid and Sardinia) into the goldfinches cluster. *Loxia* species were also included into the genus *Carduelis* within the redpolls group, as previously described (Arnaiz-Villena *et al.*, 2001). The branching pattern found in the parsimony analysis did not vary (regarding to citril finch position) when an estimated transition / transversion ratio was used to weight the third base codon positions (not shown). The enforced inclusion of *S. mozambicus* (Pasquet and Thibault, 1997) or *S. serinus* as *S. citrinella* sister taxa (or simply including the latter within the genus *Serinus*) showed a lower likelihood and a minimum evolution score values; also, negative branch lengths appeared in the distance analysis, together with longer tree lengths, and lower consistency indexes in the parsimony analysis (Table 2). Furthermore, Winning Sites Test, Templeton Test and Kishino and Kasegawa Test rendered statistically significant differences between constrained and unconstrained parsimony topologies. Hence, these calculations strongly supported the inclusion of citril finch within genus *Carduelis*.

European goldfinches C. carduelis *and citril finch*

Analyses were also focused on the goldfinches / *Serinus citrinella* group (Figs. 3A and 3B). The sequences obtained by Pasquet and Thibault (1997) from *S. citrinella* (individuals from Alps and Pyrenees) as well as from Corsica were conjointly studied with our own data on Sardinian and Madrid-Sierra samples. *C. flammea* (a redpoll) was chosen as an outgroup. 88 and 51 out of the 924 cyt b mtDNA bases were found to be variable and informative respectively. Only five positions (amino acids) are variable at the cyt b protein molecule: all of them are placed out of the Qo and Qi "reactive" centres and occur within the transmembrane region.

Total percentage divergence between mainland *S. citrinella* from Madrid and the insular one *S. c. corsicanus* from Sardinia was found to be 2.81 %; this value was close to the previously described by Pasquet and Thibault (1997) (2.7 %) between Corsica and Alps/ Pyrenees forms, see Table 3, and Figure 4 for morphological differences. Both distance-based and parsimony analyses gave the same tree topology (Figs. 3A and 3B), consisting of a well-supported differentiation of two clusters: one corresponding to the main-

FIG. 3A.—(Left) Neighbour-Joining (NJ) tree upon Maximum Likelihood (ML) distances and 3B (right) parsimony tree faced each other showing equal evolutive relationships between mainland / insular *S. citrinella* forms and *C. carduelis.* Bootstrap analyses were done with 1000 replications for the parsimony and NJ trees respectively and corresponding values (percentages) are over the branches. Number of steps and branch lengths are underlined below the branches in the parsimony and NJ trees respectively. NJ and ML scores: tree length and minimum evolution score (x 1000) = 124.11; -Ln Likelihood = 1798.73767; estimated transition / transversion ratio = 5.81. Parsimony scores: tree length = 69; consistency index = 0.783; retention index = 0.805. See table 3 that is fully concordant with these results.
[3A (izquierda) Árbol NJ (Neighbour-Joining) construido sobre distancias ML (Maximum Likelihood) y 3B (derecha) árbol de parsimonia que muestran las mismas relaciones evolutivas entre individuos S. citrinella *continentales e insulares y* C. carduelis*. El análisis de bootstrap se realizó con 1000 réplicas; los valores (porcentajes) aparecen encima de las ramas. Los números de pasos y longitudes de rama aparecen respectivamente subrayados debajo de las ramas en el árbol de parsimonia y NJ. Características del árbol NJ: longitud del árbol (x 1000) = 124,11; -Ln Likelihood = 1798,73767; ratio estimado de transiciones / transversiones = 5,81. Características del árbol de parsimonia: Longitud del árbol = 69; índice de consistencia = 0,783; índice de retención = 0,805. Ver tabla 3 que apoya estos resultados.]*



land forms of *S. citrinella* (Alps/ Pyrenees and Madrid) and another one which groups the *S. c. corsicanus* from Sardinia and Corsica islands. Citril finch seems to have diverged from European goldfinches about 5 MYA and later (2.6 MYA), the divergence between mainland and insular *S. citrinella* forms may have occurred (Fig. 1). Both genetic distances and number of steps in NJ and parsimony analyses respectively (Figs. 3A and 3B) show that the insular forms (subspecies) of *S. citrinella* share the most recent ancestor with *C. carduelis*, in contrast to the continental forms.

TABLE 3

Uncorrected "p" genetic distance (x 100) (See Fig. 3A)
[Distancias genéticas "p" sin correcciones (x 100) (ver Fig. 3A), p: proporción de posiciones nucleotídicas que difieren entre dos secuencias]

	C. flammea	*C .c. parva*	*C. c. caniceps*	*S. c. citrinella* Madrid	*S. c. corsicanus* Sardinia	*S. c. citrinella* Alps/Pyrenees	*S .c. corsicanus* Corsica
C. flammea	----						
C. c. parva	6.061	----					
C. c. caniceps	6.619	0.325	----				
S. c. citrinella Madrid	6.619	4.437	4.545	----			
S. c. corsicanus Sardinia	6.602	4.004	4.113	2.814	----		
S. c. citrinella Alps / Pyrenees	6.387	4.765	4.873	0.785	3.032	----	
S. c. corsicanus Corsica	6.928	4.222	4.331	3.139	0.325	2.709	----

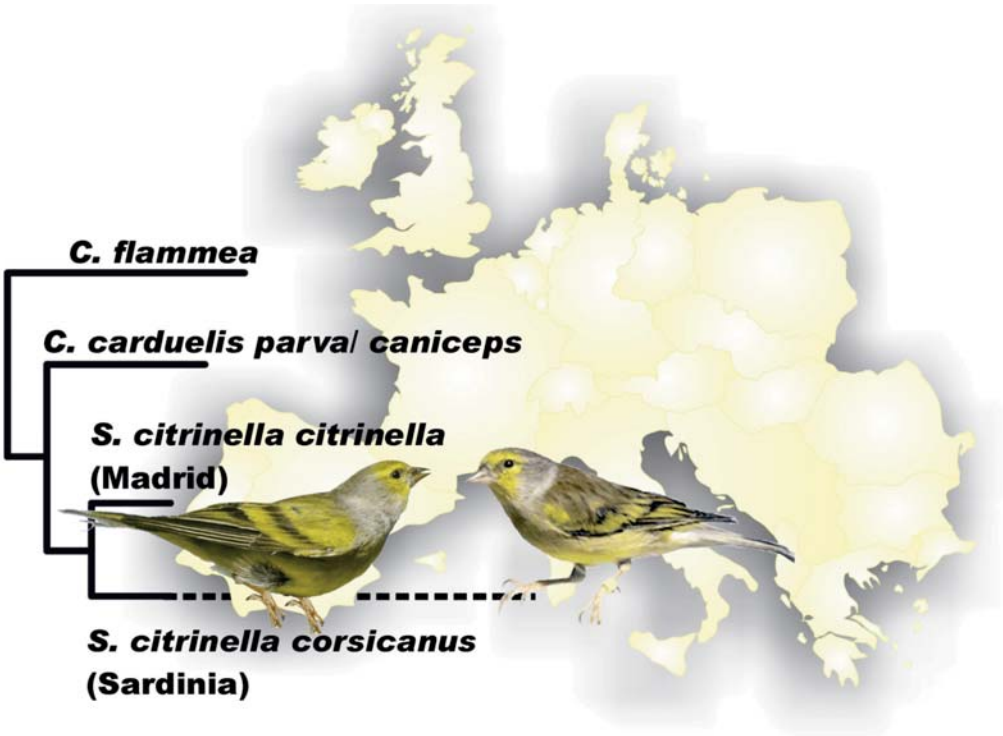
In summary, all analyses supported that a common ancestor for citril finch and its sister species, the european goldfinch (*Carduelis carduelis*), there existed (Arnaiz-Villena *et al.*, 1998, 2001).

Phylogeography and taxonomy

Figures 1 and 2 do not seem to support the monophyly of traditionally recognised *Serinus* and *Carduelis* genera; they rather support that different genera subgroups have evolved in parallel (basal polytomies). Thus, both genera may have been artificially classified by only using phenotypic methodologies and, on the other hand, subgroups that had phenotypically been identified might be valid (Arnaiz-Villena *et al.*, 1998, 1999; Van den Elzen *et al.*, 2001). Also, some of the observed polyphyly may also be an artefact due to lack of species sampled and/or to non-analyzed extinct species or to cyt b informative insufficiency. Thus, monophyly of both genera should not be completely discarded.

The island citril finch forms are closer to the hypothesized goldfinch / citril finch common ancestor, this is confirmed by both distance and cladistic methodologies (Figs. 3A and 3B; Table 3; however, more citril finch individuals need to be studied). It is possible that some intermediate species between these two sister groups are now extinct. In the case that *Carduelis* and *Serinus* radiations had taken place in the Miocene Epoch (Fig. 1, Arnaiz-Villena *et al.*, 1998, 1999), the divergence of both citril finch forms could have been coincidental with the Messinian salinity crisis (Pasquet and Thibault, 1997). Thus, an evolutionary hypothesis could be that an ancestor evolved to a form of citril finch (*S. c. corsicanus*) in Corsica or Sardinia about 5 MYA when the Gibraltar Strait closed, the Mediterranean Sea dried up and an arid climate established (Messinian crisis). Only salty mountains and very salty small lakes there existed in the western Mediterranean basin (Maldonado, 1985). The Mediterranean Sea filled with water again about half million years later. It may be postulated that an ancestral finch (to the citril finch forms) got isolated in the Sardinian or Corsican mountains dur-

FIG. 4.—Parsimony tree over Europe map showing the phenotypic differences between mainland (left) and islands (right) *S. citrinella* subspecies. Mainland: Spanish northern and central mountains and Alps. Islands: Corsica, Sardinia and other Tuscany Islands. Dendrogram is taken from Figure 3B.
[Árbol de parsimonia sobre el mapa de Europa que muestra las diferencias fenotípicas entre individuos continentales (izquierda) e isleños (derecha) de las subespecies de S. citrinella*. Continente: montañas del norte y centro de España. Islas: Córcega, Cerdeña y otras islas de la Toscana. El dendrograma ha sido extraído de la Fig. 3B.]*



ing the arid period (Maldonado, 1985) having the only available source of non-salty drinking water there. The island citril finch could have resulted from a vicariant speciation there, keeping brown back, like its sister species (the european goldfinch), and probably like a common ancestor(s) (see Figs. 3A and 3B; Table 3). Later, *S. c. corsicanus* could have reached the continental mountains, Alps and others, through the "Toscana islands" and would have later lost the brown back, becoming the *S. c. citrinella* form. This may have also reached the Iberian mountains. Other hypotheses are not discarded. Discriminating numbers that would support this hypothesis are not very high, but remain concordant, in the genetic distances data (Table 3), in the distance dendrograms (Fig 3A) and in the cladistic dendrograms (Fig. 3B).

The present and previous studies (Arnaiz-Villena *et al.*, 1998, 1999, 2001) are consistent with *Serinus* and *Carduelis* / *Loxia* being two different genera which tend to cluster separately. Paraphyletic groups within the genera may be due to the absence of extinct and a few extant species. *S. thibetanus* and *S. striolatus* are placed within genus Serinus in all cladistic and distance molecular dendrograms (Arnaiz-Villena *et al.*, 1998, 1999).

Finally, Carduelinae finches include both *Serinus* (canaries) and *Carduelis* (goldfinches-siskins) genera, which seem to be the closest relatives among Fringillidae species and also among the tribe Carduelini species (not shown). Each of these groups are split in several monophyletic subgroups (some of them are not supported by bootstrap values); even more, groups of canaries and goldfinches mtDNA phylogenies may be intermingled (own preliminary results). However, whether lack of evidences for *Carduelis* and *Serinus* monophyly is masked by lack of phylogenetic resolution of cyt b gene and/or lack of species sampling (extant or extinct) also remains as a possibility. *S. c. corsicanus* (island citril finch) seems to have evolved firstly (at the Messinian salinity crisis time) from the european goldfinches radiation; the continental citril finch forms may have later appeared. Also, the newly an analysed species (for this work) *Serinus totta* seems to cluster with the small African canaries (Arnaiz-Villena *et al.*, 1999) and the newly analysed species *Serinus syriacus* is included within the *Serinus pusillus* / *Serinus alario* group of canaries, as expected (Arnaiz-Villena *et al.*, 1999).

ACKNOWLEDGEMENTS.—This work was supported by grants from the Spanish Ministry of Science PM-1999-23, BMC-2001-1299 and from Mutua Madrileña Automovilista (2004 and 2005).

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[Recibido: 09-02-05]
[Aceptado: 25-05-05]