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Research Article

Phylogeny and rapid Northern and Southern Hemisphere speciation of goldfinches during the Miocene and Pliocene Epochs

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Abstract. Mitochondrial cytochrome b (cyt b) from 25 out of 31 extant goldfinches, siskins, greenfinches and redpolls (genus *Carduelis*) has been sequenced from living samples taken around the world, specimens have also been photographed. Phylogenetic analysis consistently gave the same groups of birds, and this grouping was generally related to geographical proximity. It has been supposed that Pleistocene glaciations played a crucial role in the origin of extant diversity and distribution of Northern Hemisphere vertebrates. Molecular comparison of most extant songbird species belonging to the genus *Carduelis* does not support this assertion. The fossil record of chicken and pheasant divergence time has been used to calibrate the molecular clock; cyt b DNA dendrograms suggest that speciation in Carduelinae birds occurred during the Miocene and Pliocene Epochs (9–2 million years ago) in both the Northern and Southern Hemispheres. Only about 4% average amount of nucleotide substitution per lineage is found between the most distant *Carduelis* species; this suggests a remarkably rapid radiation when compared with the radiation of other passerine songbird genera. In addition, a continuum of small songbird speciation may be found during the Miocene Epoch in parallel with speciation of other orders (i.e. Galliformes, chicken/pheasant). Pleistocene glaciations may have been important in subspeciation (i.e. Eastern European grey-headed goldfinches/Western European black-headed goldfinches) and also in ice-induced vicariance (isolation) (i.e. siskin in Western Europe vs. siskin in Far East Asia) around the world. European isolated *Serinus citrinella* (citril finch) is not a canary, but a true goldfinch. South American siskins have quickly radiated in the last 4 million years coinciding with the emergence of the Isthmus of Panama; probably, a North American siskin related to *C. notata* invaded a suitable and varied biotope (the South American island) for *Carduelis* birds. North American goldfinches may be renamed as siskins, because they have a distant genetic relationship with European goldfinches. Genus *Acanthis* could be dropped, and thus redpolls should be separated from twite and linnet, the latter (Europeans) probably being related to American goldfinches. Also, reproductive barriers are observed between closely related species and not between other more distant ones. Finally, a tentative classification for genus *Carduelis* species is suggested.

Key words. Goldfinches; siskins; redpolls; greenfinches; *Carduelis*; *Serinus*; passerines; mitochondrial DNA.

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** The contributions by Antonio Arnaiz-Villena and Miguel Álvarez-Tejado are equal and the order of authorship is arbitrary.

Carduelis (goldfinches, siskins, redpolls and greenfinches) is a genus of finches belonging to the Fringillidae family of birds that also includes many sparrows, bramblings and chaffinches. Most of them are beautifully coloured and are widespread and familiar to birdwatchers and other people [1–3]. Evidence from molecular systematics may offer new insights about the controversial avian evolution [4–9]; arguments for early vs. a more recent speciation for songbirds (order Passeriformes) have been put forward [4–7, 9]. Late Pleistocene glaciations have been associated with passeriform speciation; fragmentation of species because of ice-driven isolation and subsequent genetic divergence and range expansion during interglacials may account for many extant songbird species [9].

In order to test this speciation-timing paradigm, we collected samples from 25 of 31 extant goldfinches (genus *Carduelis*, see table 1 for common and scientific names) throughout North and South America, Europe and Asia [3] for sequencing an orthologous gene from each species: mitochondrial cytochrome b (mt cyt b; 924 bp). Mitochondrial DNA (mtDNA) has proved to be helpful for defining evolutionary relationships among relatively distant and closely related birds and other species [10, 11]. We have also aimed to study the relatedness of these bird species in the context of paleogeography and molecular clock timing to get an overall picture of the phylogeny and time of appearance of extant *Carduelis* species [12–14].

Materials and methods

Bird samples come from species and places that are detailed in table 1. Photographs were taken with a Nikon N-90 camera equipped with a Nikon 80-200 zoom objective and automatic flash. GenBank sequence accession numbers are also given (table 1). Blood from living birds was drawn after photographing by cutting the nail of legs locally anaesthetized with lidocaine ointment. Blood was collected in EDTA at 4 °C and frozen until use. DNA was obtained, and 924 bp of the mtDNA cyt b gene was amplified with primers L14841 and H15767 as detailed in ref. 15. Polymerase chain reaction (PCR), cloning and automated DNA sequencing were performed as previously described [15, 16]. At least three clones from two different PCRs were sequenced from each species' cyt b molecule in order to assess sequencing quality. Pseudogenes were found but not included in the analysis. In the case of *Serinus citrinella*, samples from four different individuals were used, since this bird should be reclassified within the genus *Carduelis* according to the results herein obtained. The power of DNA sequencing of cyt b (or other orthologous genes) for solving taxonomy problems may be fully shown by analysing as many as possible of the closest extant species, as was done in the present work. Relationships among very close species may, however, not be solved, as is observed in the case of South American siskins, although they are statistically firmly grouped together with a high bootstrap value.

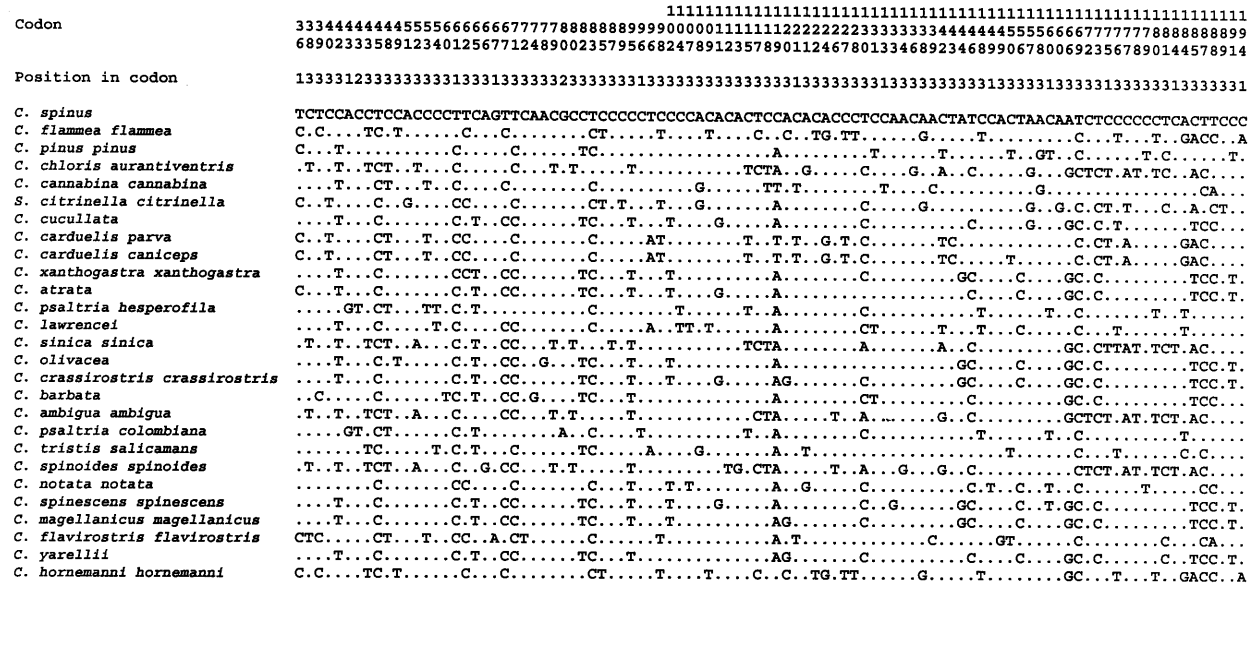
Three different methods of phylogenetic tree construction were used in order to independently confirm robustness of trees: unweighted parsimony, neighbour joining (NJ) and unweighted pair group with arithmetic mean (UPGMA); table 2 also shows that variable sites are placed mostly at third codon positions, as expected in closely related species. A matrix of genetic distances for NJ trees was obtained by the maximum likelihood method, and for UPGMA Kimura two parameter distances were used; the UPGMA tree was also used for estimating coalescence times from known outgroup divergence times [17]. Times of species divergence are only a rough estimate, particularly in our study, when only the pheasant and chicken fossil record timing is available. However, the time scale for the UPGMA tree was obtained by comparing mt cyt b of chicken [18] and pheasant [19], two species that diverged 20 MYA [20] according to combined fossil and molecular comparison calculations. The comparison yields an evolutionary rate of 0.62×10^{-9} nonsynonymous substitutions per nonsynonymous site per year and 1.7×10^{-8} synonymous substitutions per synonymous site per year, so that the overall rate is $3.97 \times 10^{-9} \pm 0.37$ [17]. This substitution rate is approximately 0.4% per million years, which leads to a rough approximation that the 4% average amount of nucleotide substitution per lineage between the most distant *Carduelis* species arose in about 10 million years (fig. 3). Slight differences may be due to the different methods used. The standard error of the pheasant-chicken distance indicates that the calibration error is about 10%. The UPGMA tree is not an exact method to infer phylogenies among species if a constant evolutionary rate does not occur. In the present study the other two types of trees would or would not validate the UPGMA tree topology [21]. In addition, the evolutionary rate within one genus (*Carduelis*) is not expected to vary greatly (indeed, this was also observed; see below). Bootstrap values are calculated as a method of testing the topological robustness of trees calculated by parsimony and NJ methods [22], and low bootstrap branches are shown because the same tree branch is obtained by at least two different tree construction methods [23]. Also, the number of variable sites, including chicken, pheasant and chaffinch sequences (327 out of 924 cyt b DNA bases) and phylogenetically informative sites (230) is sufficient to establish sound phylogenetic comparisons [21]; 924 bp are

Table 1. List of species, origin and mit cyt b sequence identification (*GenBank accession number*).

Carduelis species English (*Latin*)	Mt cyt b Sequence	Sample region
Siskin (*Carduelis spinus*)	L76391	Madrid, Spain
Pine siskin (*Carduelis pinus pinus*)	U79020	Jackson, Wyoming, USA
Red siskin (*Carduelis cucullata*)	L76299	Venezuela†
Yellow-bellied siskin (*Carduelis xanthogastra xanthogastra*)	L76389	San Jose, Costa Rica
Olivaceous siskin (*Carduelis olivacea*)	L77871	Lima, Peru
Black siskin (*Carduelis atrata*)	L76385	Sucre, Bolivia
Thick-billed siskin (*Carduelis crassirostris crassirostris*)	L77869	Mendoza, Argentina
Hooded siskin (*Carduelis magellanicus magellanicus*)	U79016	Misiones, Argentina
Yellow-faced siskin (*Carduelis yarellii*)	U83200	Recife, Brasil‡
Andean siskin (*Carduelis spinescens spinescens*)	U79017	Merida, Venezuela
Black-chinned siskin (*Carduelis barbata*)	L77868	Magallanes, Chile
Black-headed siskin (*Carduelis notata notata*)	U79019	Chiapas, Mexico
Linnet (*Carduelis cannabina cannabina*)	L76298	Madrid, Spain
Twite (*Carduelis flavirostris flavirostris*)	U83199	Cage bird. Antwerp, Belgium§
Dark-backed goldfinch (*Carduelis psaltria hesperofila*)	L76390	Sacramento, California, USA
Dark-backed goldfinch (*Carduelis psaltria colombiana*)	U78324	Maracay, Venezuela¥
American goldfinch (*Carduelis tristis salicamans*)	U79022	San Francisco, California, USA
Lawrence's goldfinch (*Carduelis lawrencei*)	L76392	San Diego, California, USA‖
Common redpoll (*Carduelis flammea flammea*)	L76386	Brussels, Belgium
Artic redpoll (*Carduelis hornemanni hornemanni*)	U83201	Cage bird. Antwerp, Belgium*
Citril finch (*Serinus citrinella citrinella*)	L77872	Madrid Sierra, Spain
Goldfinch (*Carduelis carduelis parva*)	L76387	Madrid, Spain
Goldfinch (*Carduelis carduelis caniceps*)	L76388	Katmandu, Nepal
Greenfinch (*Carduelis chloris aurantiventris*)	L76297	Madrid, Spain
Oriental greenfinch (*Carduelis sinica sinica*)	L76592	Szechwan, China
Black-headed greenfinch (*Carduelis ambigua ambigua*)	U78322	Szechwan, China
Himalayan greenfinch (*Carduelis spinoides spinoides*)	U79018	Katmandu, Nepal
Chaffinch (*Fringilla coelebs coelebs*)	L76609	Madrid, Spain
Sudan golden sparrow (*Passer luteus*)	L76714	Dakar, Senegal
Austral negrito (*Lessonia rufa*)	L77902	Puerto Natales, Chile

†Ascents from Venezuela; this particular specimen was bred in Madrid as a cage bird.
‡Ascents from Brazil, Recife, but 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 [3].
‖All specimens studied are male; the one marked with ‖ is of undetermined sex.
*Ascents probably originating in Greenland emigrated to the Antwerp region in a cold winter.
Lophura nycthemera (pheasant) and *Gallus gallus* (chicken) sequences were obtained from refs 19 and 18, respectively.

Table 2. Variable positions of the codons found in the 25 *Carduelis* and *S. citrinella* species.



Only 195 and 144 variable sites and phylogenetically informative sites, respectively, are found, when not using chicken, pheasant and chaffinch sequences.

sufficient to obtain an accurate phylogenetic tree (parsimony, NJ and UPGMA) in most cases [21] and overcome the uncertainties found in other songbird phylogenies by using only 307 bases of mt cyt b [24]. Also, DNA sequences of different lengths from the mitochondrial control region or expressed genes may be easily compared [7]. Genus *Carduelis* and its sister genus *Serinus* (canaries) are not monophyletic (not shown), according to the analysis of available extant species.

In addition, saturation diagrams were constructed by plotting percentage sequence divergence and percentage

transitions and transversions divergence [8]; transversions at the third position of codons did not reach a saturation plateau between chicken and the most recent South American siskin (fig. 3, and data not shown).

Results and discussion

Although different *Carduelis* groups are in general concordantly placed in dendrograms obtained by using parsimony (fig. 1), NJ (fig. 2) and UPGMA (fig. 3), some bootstrap values for nodes joining a few species belonging to otherwise well-established (high bootstrap) groups are low; this may mean that (i) not all extant species are tested. This may not be the case for the present *Carduelis* sample, because 25 (including *S. citrinella*) of 31 extant species were tested [2, 3]; (ii) parental species are extinct and/or each group of bootstrap-supported nodes represents separate subfamilial radiations; (iii) species (and thus DNAs) are too similar and have appeared within a short time period, as may be the case for South American siskins. The latter two may be somewhat more favoured hypotheses, and although more studies are necessary to support them, other close Fringillidae genera are definitively outgroups (these include *Serinus*, *Passer*, *Lagnosticta*, *Lonchura*, *Pyrrhula*, *Rhodopechys* and *Carpodacus*, which already existed in the early Miocene; A. Arnaiz-Villena et al., unpublished observations). Indeed, parsimony, NJ and UPGMA trees, which also included *Passer luteus* and *Lessonia rufa* (a South American suboscine)

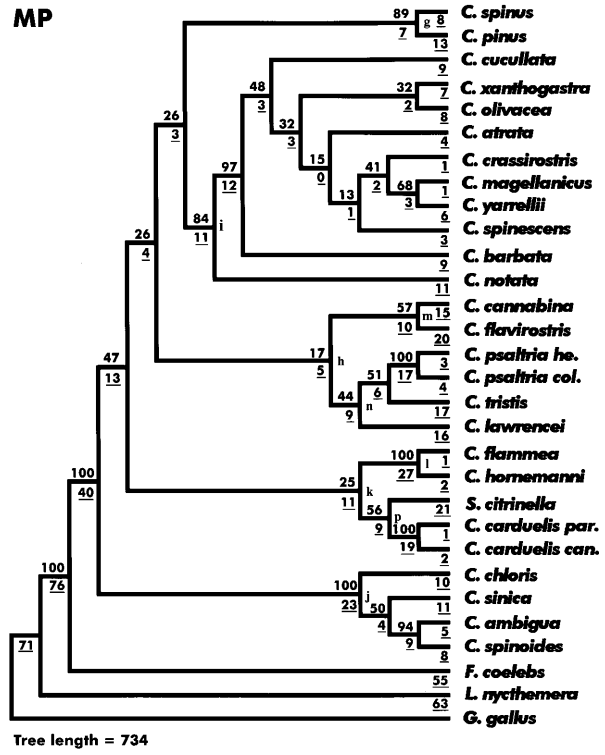


Figure 1. Phylogenetic tree. Maximum unweighted parsimony heuristic search was used (PAUP) [28]. Thirty-two trees which were equally parsimonious were obtained; 21 of them were generated because of changes in South American bird branches, which radiated about 5–3 MYA and have very similar mt cyt b sequences and show low bootstrap values. Consistency and retention indexes are 0.6 and 0.7, respectively. The majority rule bootstrap consensus tree based on 924 bases of cyt b genes from 25 *Carduelis* species (including *S. citrinella*) is shown. The transition/transversion ratio used was 2/1; the observed 8/1 ratio made no difference. First, second and third codon bases were used unweighted. Parsimony was used unweighted because weighting is only recommended for greater amounts of evolutionary divergence, which are not expected among the relatively closely related species analysed, all belonging to one genus [21]. Parsimony bootstrap analysis was done with 1000 replications and values (in percent) shown above branches. The number of events is shown underlined below branches. Letters **g**, **h**, **i**, **j**, **k** and **l** point out the most significant nodes which are commented on the text. *he.*, *hesperofila*; *col.*, *colombiana*; *par.*, *parva*; *can.*, *caniceps*.

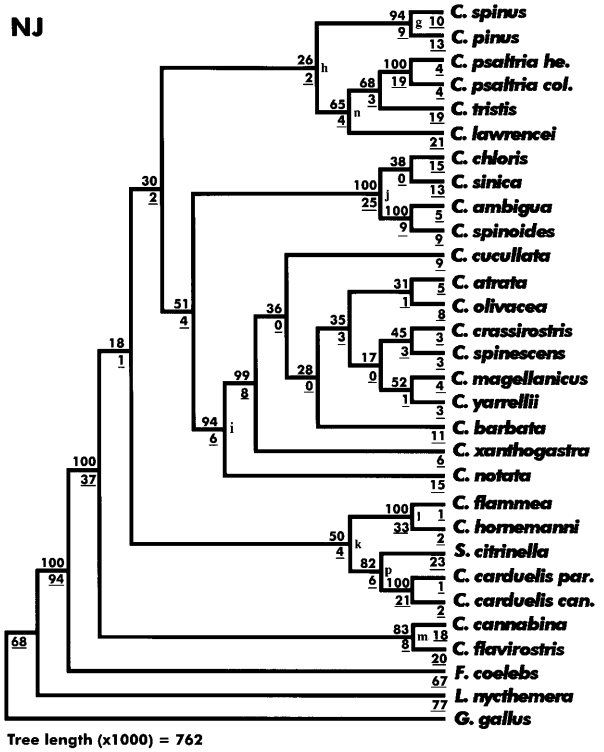


Figure 2. Neighbour-joining bootstrap tree (1000 replications) based on 924 bases of cyt b genes from 25 *Carduelis* species. Bootstrap values are shown above branches; branch lengths ($\times$ 1000) are shown underlined below branches. The evolutionary model used was 'the minimum evolution'. Transition/transversion ratio used was 2/1. Distance matrices were calculated based on maximum likelihood analysis [28]. Cyt b from chaffinch (*Fringilla coelebs*), pheasant (*Lophura nycthemera*) and chicken (*Gallus gallus*) were also used as outgroups. Letters **g**, **h**, **i**, **j**, **k** and **l** point out the most significant nodes which are commented on the text. *he.*, *hesperofila*; *col.*, *colombiana*; *par.*, *parva*; *can.*, *caniceps*.

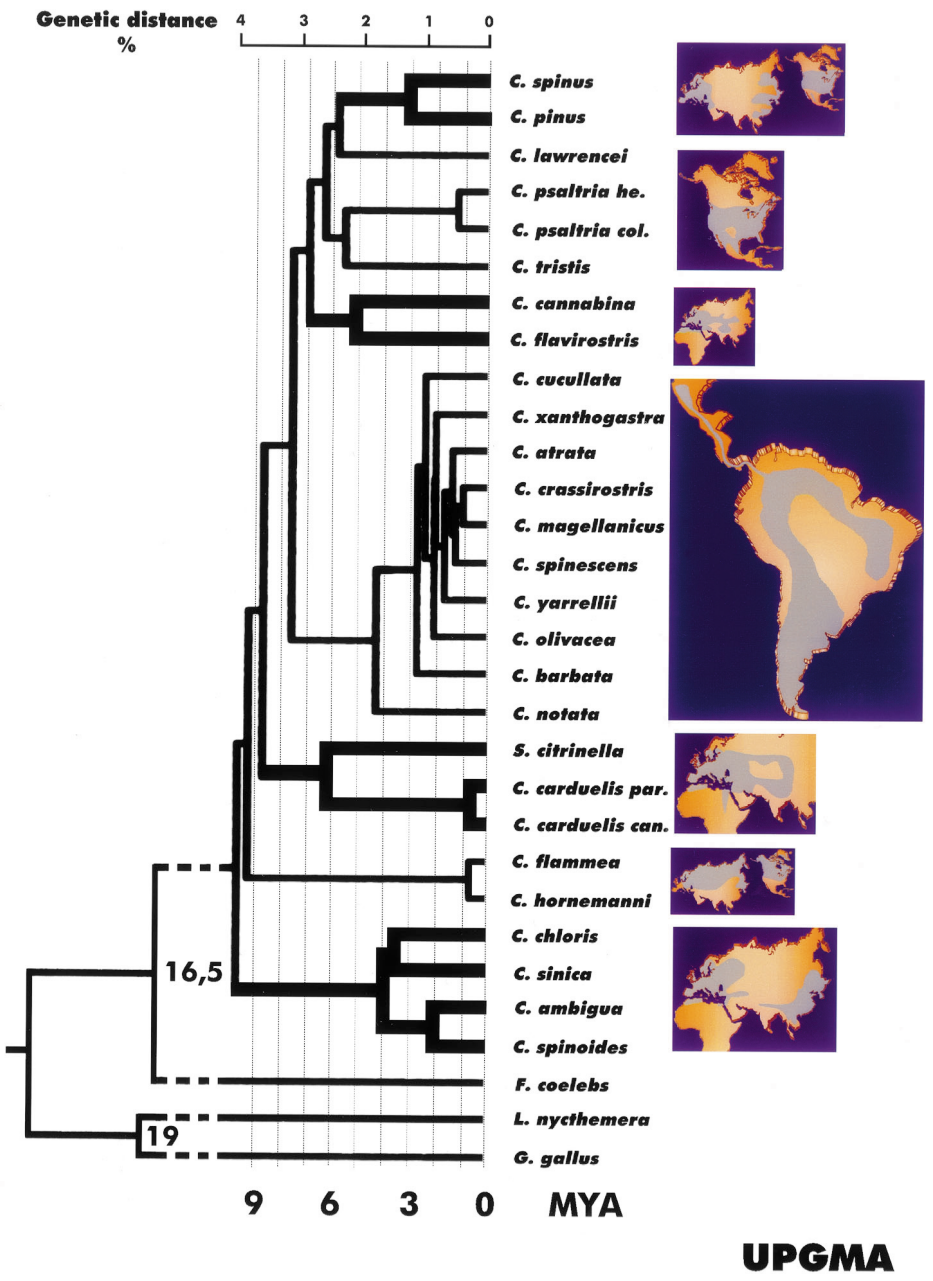


Figure 3. Approximate calculations on the time of appearance of genus *Carduelis* lineage based on the chicken/pheasant amount of nucleotide substitution per lineage and their postulated times of appearance. Times were calculated from the estimated time of pheasant (*L. nycthemera*) and chicken (*G. gallus*) appearance and their respective cyt b substitution rate comparisons; see ref. 17. UPGMA dendrograms and their corresponding Kimura biparametric distance matrices were obtained using PAUP-4d50 and the PHYLIP package (version 3.5c), kindly provided by D. L. Swofford (1996) and by J. Felsenstein, respectively. UPGMA methodology tends to perform poorly if the assumption of equal rate of cyt b evolution among species does not reflect their actual evolution. However, it seems to perform correctly in the closely related bird species used for this work since groups of taxa are similar to those obtained in NJ and parsimony dendrograms (see figs 1 and 2). Bird species and subspecies used are detailed in table 1; MYA, million years ago. Geographic distribution is depicted to show graphically that *Carduelis* speciation occurred during the Miocene and Pliocene in both the Northern and Southern Hemispheres. Thicker and thinner branches have also been drawn to facilitate visualizing the bird groups.

(table 1), showed the same groups of *Carduelis* species. Sequences show 327 variable and 230 phylogenetically informative sites (including chicken, pheasant and chaffinch sequences) that are sufficient to obtain sound results [21]. In general, established bird groups within dendrograms are geographically related. It is also remarkable that only 4% average amount of nucleotide substitution per lineage is found between the most distantly related of the 25 *Carduelis* species (see fig. 3). This suggests a relatively fast radiation for *Carduelis* species, compared with other documented songbird radiations (i.e. genus *Zonotrichia*, 4.1% for only 7 species; genus *Pipilo*, 6.4% for only 6 species [25, 26]).

Analysis of nonsynonymous first and second codon position differences and third codon transversions was not useful for our study, since all species belong to one genus. Indeed, the number of events in the parsimony tree is low, and the branch lengths in the NJ tree are also short. Also, the variable sites occur mostly at the third position of codons. Table 2 shows the bases at variable positions of the codon within the analysed *Carduelis* species and *S. citrinella*. The analysis in table 3 further confirms that most variable sites are found at the third position of codons, as expected.

Siskins. Both parsimony (fig. 1) and NJ dendrograms (fig. 2) separate several groups which are in general concordant with present-day geographical distributions; however, there are some important exceptions. Node **g** groups siskin (*C. spinus*, Eurasia) and pine siskin (*C. pinus*, North America, fig. 4). The common ancestor of these sister species probably originated somewhere in the Northern Hemisphere; they are probably phylogenetically related to North American goldfinches (*C. tristis*, *C. lawrencei* and *C. psaltria*) and possibly to the Eurasian linnet (*C. cannabina*) and twite (*C. flavirostris*, figs. 2, 3 and 5). These last two species are grouped by node **h** together with North American goldfinches in the parsimony tree and as a separate lineage in NJ trees (figs 1 and 2); however, the UPGMA dendrogram groups them with North American goldfinches (fig. 3). Siskin and pine siskin may have diverged from a species which lived throughout the Northern Hemisphere probably about 3 MYA; the Bering Strait was not a geographical barrier during long periods in the last 2 MY (fig. 3; ref. 12). This latter group may also be related to another group which comprises a surprising mixture of North American and Eurasian species (node **h**, parsimony tree and fig. 3). Eurasian species are *C. cannabina* (linnet) and its sister species *C. flavirostris* (twite), which do not appear to form a group (so-called *Acanthis*) with *C. flammea* (common redpoll) and *C. hornemanni* (Arctic redpoll), as has been proposed [1]. Also, the lineage leading to both redpolls which inhabit northern areas may have appeared approximately 9 MYA, while that leading to twite and linnet may have arisen about 2 MY later (fig. 3).

North American *Carduelis* finches should not be named goldfinches, since they have only a distant molecular relationship with European goldfinches; instead they should be included among siskins. *C. tristis* (American goldfinch) and *C. psaltria* (lesser goldfinch) are also close relatives. The first is found in North America, including parts of Mexico, and the second is confined to the western United States and Mexico (*C. ps. hesperofila*, fig. 5, green back) and to the Southwest United States and Central America (*C. ps. psaltria*) and Central America and northern South America (*C. ps. colombiana*, figs 5 and 6); the two latter subspecies show a deep dark back, and hybrids among the three subspecies with the corresponding phenotypic variability have been observed [3]. Although the geographical distribution and phenotype of *C. psaltria* suggest that it could have shared a recent common ancestor with the South American siskins, our results indicate that *C. psaltria* is a North American bird which has colonized South American habitats and has undergone the corresponding phenotypic changes (darker in head and back, figs 5 and 6). An ancestor of *C. psaltria* and *C. tristis* may have existed around 5 MYA; *C. psaltria* subspecies may have originated relatively recently, about 1 MYA. This subspeciation may have occurred after the closing of the Isthmus of Panama (5–3 MYA; 12–14). Some authors suggest that siskins originated in North America and may have later dispersed to Asia, Europe and South America [27]. Also, the oldest and precursor siskin has been proposed to be *C. pinus* [26]; our results would not contradict these hypotheses. According to figure 3, the earliest siskin species are both *C. pinus* and *C. spinus* ancestors together with *C. lawrencei* (6 MYA); *C. tristis* and *C. psaltria* lineages would be slightly more recent (about 5 MYA). However, if the Eurasian twite and linnet are considered akin to North American siskins (figs 1 and 3), they or their ancestors would be the oldest (6.5 MYA) and precursor siskins. It is uncertain but possible that linnet and twite were once living in North America and that pine siskin and siskin existed both in America and

Table 3. Matrix of observed substitutions for first and second positions (below diagonal) and third position (above) in cyt b codons from five *Carduelis*, one *Fringilla* (see also ref. 29) and *Gallus* species (see table 1 for scientific names).

	1	2	3	4	5	6	7
1 Himalayan greenfinch	57	61	66	61	105	140	
2 Black siskin	5		48	47	57	95	127
3 Linnet	6	5		44	44	87	125
4 American goldfinch	2	3	4		52	94	132
5 Goldfinch	4	5	6	4		93	130
6 Chaffinch	18	17	20	16	16		123
7 Chicken	51	49	52	48	49	51	

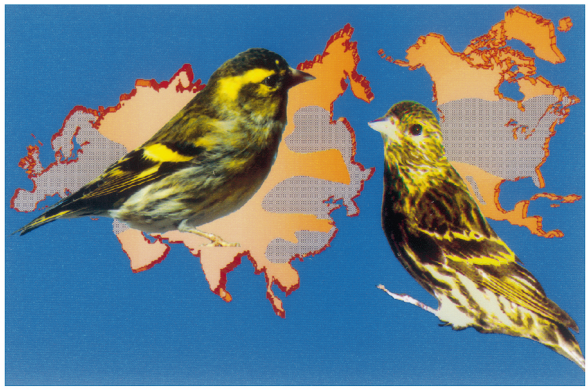


Fig. 4

Fig. 5

Fig. 6

Fig. 7

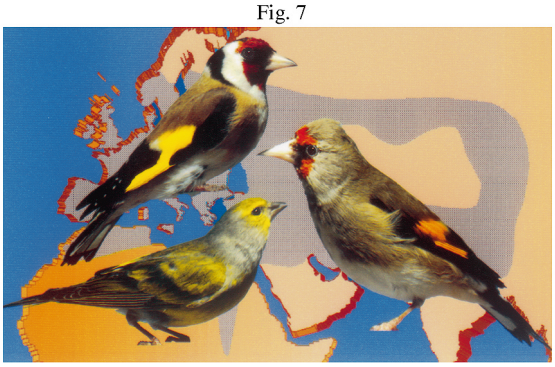


Fig. 8

Fig. 9

Eurasia; Bering Strait land bridges have occurred intermittently and may have facilitated exchanges [12].

Node **i** groups a branch of South American siskins (fig. 6) which show very close molecular and phenotypic relationships (particularly colour distribution and bill shape) and probably originated quite rapidly after the Isthmus of Panama emerged (fig. 6). This is an example of a quick siskin radiation probably due to rapid dispersal into the South American island (isolated between 95 and 5–3 MYA) which had recently joined the north; although small birds can fly long distances [9], it is possible that the South American *Carduelis* radiation occurred only when mesothermal plants (genus *Carduelis* food) from the Rockies invaded the Andean spine after the emergence of the Isthmus of Panama [14].

Greenfinches (figure 7). Greenfinches (*C. chloris*, *C. sinica*, *C. ambigua* and *C. spinoides*) seem to be the earliest lineage belonging to the genus *Carduelis* (fig. 3); their ancestors probably originated around 9 MYA [12–14] coinciding with when Mediterranean Sea started drying up and the climate around the sea also got drier. Only *C. chloris* (greenfinch) lives in Europe and North Africa. Greenfinches are the *Carduelis* finches closest to the outgroup *Fringilla coelebs* (chaffinch) in Figures 1 and 3, and *C. ambigua* and *C. spinoides* may have originated from the same ancestor by ice-caused vicariance during the last 2 MY glaciations, although their ancestors could have existed 9 MYA (figs 1–3).

Goldfinches (figure 8). Classical goldfinches comprise the European black-headed goldfinch (*C. carduelis parva*, *britannica* etc.) and the Asian (grey-headed) goldfinch (*C. carduelis caniceps*, *subulata* etc.). Both grey- and black-headed subspecies include variants with a marked degree of local differences in size, beak, shape and colours. *S. citrinella* (citril finch, classified as a canary) is a relict of a few islands (Corsica, Sardinia) and central and southern Europe mountains; it has been found to be a close relative to goldfinches and may be classified as such, since both have common ancestors. This bird should be renamed *C. citrinella* (figs 1–3). The ancestor of these three species may have appeared around 6 MYA. Colour, body and bill shape of *C. citrinella* (citril finch) are very different from the typical goldfinches (fig. 8); however, its singing, which is delivered in short repeated phrases with metallic notes, is similar to that of classical goldfinches and the type of flight (light and undulating) resembles that of the goldfinch [3 and our own observations]. Colour and shape of beak have been shown to evolve very rapidly in birds, even in a range of hundreds of years [9]. Grey- and dark-headed goldfinch subspecies have probably split during Pleistocene glaciations less than 1 MYA, when eastern (grey-headed) and western (black-headed) goldfinch ancestors may have become isolated (fig. 3) probably by ice-caused vicariance events. In general, Pleistocene glaciations are shown not to be related to *Carduelis* or *Serinus* (A. Arnaiz-Villena et al., unpublished observations) speciation around the world (including the Southern Hemisphere); this is also found by Klicka and Zink for North America by studying other passerine genera [7].

Redpolls (figure 9). Both redpolls, *C. flammea* and *C. hornemanni* (Arctic redpoll), are now considered to be sister subspecies [2, 3] and should be separated from other *Carduelis* finches because (i) they are the only *Carduelis* species which live all along the Holoarctic region, i.e. North Eurasia and North America; (ii) they

Figure 4. Northern siskins. These sister species are grouped within node **g** in figures 1 and 2. They breed nearly as far north as redpolls. Siskin is now the Eurasian species, and pine siskin lives in North America. Grey area, habitat.

Figure 5. North American siskins and brown siskins (Eurasian). Both groups of birds are likely to have shared common ancestors and are grouped both in UPGMA (fig. 3) and parsimony (fig. 1, node **h**) dendrograms, although they show low bootstrap values. Brown Eurasian siskins: linnet (above) and twite (below). North American siskins: from upper left, clockwise: lesser goldfinch, *C. psaltria hesperofila*, green-backed (California); American goldfinch; lesser goldfinch, dark-backed, (Colorado, central and northern South America); Lawrence's goldfinch (young). Cyt b DNA sequences of *C. psaltria psaltria* and *C. psaltria colombiana* (both dark-backed) are identical, except for intraspecific small variations (unpublished observations); in the figure the second subspecies is represented and not *C. ps. psaltria*. Grey area, habitat.

Figure 6. South American siskins. They are tightly grouped under node **i** (figs 1 and 2), and bootstrap values are not very high among them because they show very similar DNA cyt b sequence and have radiated in the last 5 MY (fig. 3). A genuine North American bird, the dark-backed lesser goldfinch, has successfully colonized northern South America (see text). From left to right and from top downwards: black-headed siskin, red siskin, Andean siskin, dark-backed lesser goldfinch, yellow-faced siskin, black siskin, yellow-bellied siskin, olivaceous siskin, thick-billed siskin, hooded siskin and black-chinned siskin. Bird positions in figure roughly correspond to their habitats. Yellow bellied siskin ranges from Central America through Bolivia [see ref. 3].

Figure 7. Greenfinches. Greenfinch (upper left and then clockwise), which probably recently separated from Oriental greenfinch, black-headed greenfinch and Himalayan greenfinch. They are grouped together tightly in node **j** (figs 1 and 2) and probably represent the oldest *Carduelis* finch lineage (fig. 3). Grey area, habitat.

Figure 8. Goldfinches are grouped under node **p** (figs 1 and 2). Western European subspecies have a dark head, and eastern European and western Asian subspecies have a grey head. *Serinus* (should be *Carduelis*) *citrinella* is closely related to these 'true' goldfinches [30]; it is older than classical Goldfinches (figs 1–3) and is confined to fragmented habitats in European mountains: Alps, Central Spanish Sierra, Corsica and Sardinia. *C. carduelis parva* (above), *C. carduelis caniceps* (right) and *S./C. citrinella* (below). Grey area, habitat.

Figure 9. Redpolls (figs 1 and 2, node **l**). Both of them live in the northern regions of the planet with seasonal southward migrations. Left: Arctic redpoll. Right: Common redpoll. Grey area, habitat.

do not clearly group together within any other *Carduelis* subgroup (fig. 3), although they may be related to goldfinches by node **k** (figs 1 and 2); (iii) they are evolutionarily distant from linnet and twite, which are siskins according to parsimony and UPGMA dendrograms (figs 1 and 3); thus genus *Acanthis*, which include twite, linnet and redpolls, may not exist. It is likely that both redpoll subspecies also separated during Pleistocene glaciations around 500,000 years before present. This date has already been suggested [27]; their ancestors may have lived in the Miocene about 9 MYA.

Simultaneous speciation of passerines and other genera during Miocene and Pliocene Epochs. In conclusion, the estimated divergence time for most of genus *Carduelis* species suggests that they appeared in a range of time between the Miocene and Pliocene; there is no evidence for a divergence time consistent with late Pleistocene origin. This radiation was intermingled in time with *Serinus* species radiation (A. Arnaiz-Villena et al., unpublished observations). Other authors using different methods (other genes and only molecular clock timing) also support the inference that North American songbirds do not have a Pleistocene origin [7]. Our approach yielded 230 phylogenetically informative sites (also taking into account chicken, pheasant and chaffinch sequences) from the mtDNA cyt b gene delineating the *Carduelis* species phylogeny, and a mixed external fossil and molecular record divergence time (pheasant/chicken) was used to calibrate our dendrograms. The common view that small songbirds, Passeriformes (i.e. chaffinches and goldfinches), must have closer speciation times among themselves when compared with bigger birds (i.e. Galliformes) does not hold up. The chaffinch divergence time is closer to that inferred for chicken and pheasant than to divergence times inferred for *Carduelis*. Thus, a continuum of small songbird speciation may be found during the Miocene and Pliocene in parallel with the speciation of other orders. However, it is possible that certain *Carduelis* birds classically considered as subspecies originated during Pleistocene glaciations; the divergence time calculated for *C. carduelis* subspecies (grey-headed Asian and black-headed European goldfinch) is less than 800,000 years. Also, late glaciations may have separated western European siskins (*C. pinus*) from the Far East subspecies by an ice- induced vicariance event. In conclusion, the lack of evidence found by others [7] for Pleistocene speciation in North American songbirds has also been found in the present study not only in North America but also in Eurasia and South America. Indeed, the analysis of most extant species of genus *Serinus* supports the same conclusions, and includes the African Southern Hemisphere for *Serinus* (A. Arnaiz-Villena et al., unpublished observations). A continuum of the appearance of the Carduelinae species during the Miocene and Pliocene Epochs may be postulated; its start coincided with dramatic changes in climate when the eastern Mediterranean Sea (Thetis Sea) was closing [12–14]. Pleistocene glaciations may have induced further speciation events but on a scale lower than previously thought [9]. For example, subspeciation of grey- and black-headed *Carduelis carduelis* (true goldfinches) and *C. ambigua* and *C. spinoides* speciation may also have originated during the last 2 MY glaciations, although their ancestors could have existed about 9 MYA (see above). Although inferences of the divergence time of species may be taken as only a rough estimate, it is remarkable that the calculations shown in the present paper are concordant with other partial ones published for Carduelinae and Passerinae [8, 27].

Classification. Finally, a tentative classification might be proposed for genus *Carduelis* that takes into account our molecular phylogenetic data and also geographical and gross phenotypic data, that is body size. We suggest the following (habitat range shown in photographs has been taken from ref. 3):

a. **Northern siskins**: fig. 4. b. **North American siskins**: fig. 5. c. **Brown siskins** (fig. 5: possibly related to North American siskins): twite (Eurasia), linnet (Eurasia). Genus *Acanthis* including twite, linnet and redpolls could be abandoned as a taxonomic unit. d. **South American siskins**: fig. 6. e. **Goldfinches**: fig. 8. f. **Redpolls**: fig. 9. g. **Greenfinches**: fig. 7.

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