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The species status of the Corsican finch *Carduelis corsicana* assessed by three genetic markers with different rates of evolution

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ABSTRACT

Citril finches *Carduelis c. citrinella* and Corsican finches *Carduelis c. corsicana* represent two closely related forms, endemic to European mountains and some Mediterranean Islands. Their taxonomic status has recently been disputed based on the results from mitochondrial genes. We show that the use of different genetic markers may lead to different results in these two putative species. Using the mitochondrial ATP8 + 6 we found a clear difference between *C. c. citrinella* and *C. c. corsicana* (3.2%) suggesting a divergence time of 1.2 MYR. In contrast, no clear difference was found using two nuclear genes. The mismatch between the different markers suggests that the separation of *C. c. citrinella* and *C. c. corsicana* is likely to be a rather recent event, involving bottlenecks, which have enhanced the divergence in the mtDNA. Our results call for caution when using mtDNA alone for assessing times of divergence and rates of evolution. We did not find any support for the view that *C. c. corsicana* is more related to *Carduelis carduelis* than *C. c. citrinella* as proposed by previous studies.

1. Introduction

Ever since DNA sequencing became relatively accessible for phylogenetic studies, various mitochondrial genes have been used at low taxonomic levels including population studies. The relatively low effective population size of the mitochondrion makes differences between populations and species already visible at relatively short time spans. This lower effective population size makes mtDNA vulnerable to various demographic events, such as bottlenecks, which is an advantage when the purpose of the study is to analyse the history of a population, but can also cause serious problems when it comes to phylogenetic studies (reviewed by Zink and Barrowclough, 2008). In particular, since a demographic event such as a bottleneck may enhance differences between populations due to random fixation (or near so) of different haplotypes, the estimation of time since divergence might be seriously biased. Thus, there are reasons to use more slowly evolving markers such as nuclear genes to corroborate phylogenetic studies based on mitochondrial genes (Zink and Barrowclough, 2008).

The citril finch *Carduelis citrinella* is endemic to European mountains. Generally two forms are recognized, which are either treated as members of one polytypic species consisting of the two allopatric sub-species citril finch *Carduelis c. citrinella* (nominate form) inhabiting the mainland of Europe and the insular Corsican finch *Carduelis c. corsicana*, which is restricted to Corsica, Sardinia and few off-lying islands (Cramp and Perrins, 1994; Thibault and Bonaccorsi, 1999). Alternatively, the two forms are regarded as having independent evolutionary histories justifying the status as own species (Sangster et al., 2002).

Recent studies have shown that both forms vary considerably in several features. Citril finches differ from Corsican finches in some parts of their food and habitat selection (Förschler and Kalko, 2006a,b), their morphology (Förschler and Siebenrock, 2007; Förschler et al., 2008), their plumage coloration (Cramp and Perrins, 1994) and are bioacoustically clearly differentiated (Förschler and Kalko, 2007). However, all these features may also change quickly in a population level under the special and restricted habitat requirements and living conditions on islands (Blondel, 2000) and should not be overinterpretated as taxonomic criteria (see Sangster et al., 2002).

A first genetic approach to solve the problem of the two allopatric citril finch forms was presented by Pasquet and Thibault (1997), who found a genetic divergence of 2.8% in the mitochondrial cytochrome *b* gene between birds from Corsica and from the Alps/Pyrenees, and argued against the treatment of the Corsican finch as an

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independent species. Subsequently Sangster (2000) and Sangster et al. (2002) reinterpreted the results of this study and postulated a possible independent evolutionary history of the Corsican finch. In a more recent work on the phylogenies of canaries *Serinus* spp. and goldfinches *Carduelis* spp. Zamora et al. (2006) detected a genetic divergence in the mtDNA of 2.7% between the two forms, close to the previous results by Pasquet and Thibault (1997). Zamora et al. (2006) suggested that Corsican finches represent the older form and are closer related to the most recent ancestor with the goldfinch *Carduelis carduelis* than continental citril finches.

Given the uncertainties on the species status and the evolutionary history of the Corsican finch we analysed the genetic differentiation between *C. c. corsicana* and *C. c. citrinella* and closely related finch species using three different genetic markers all with different rates of evolution. The nuclear genes are assumed to be slowest, the mitochondrial gene intermediate, and the microsatellite markers the fastest. By doing this we can obtain a timing of events, since the coalescent times of these different markers differ quite considerably (Zink and Barrowclough, 2008). In addition, since mtDNA has an effective population size that is one-fourth of the nuclear markers, it is more susceptible to demographic events which might lead to an overestimation of divergence times.

2. Material and methods

2.1. Samples and specimens

For analyses, we used 189 individuals of *C. c. citrinella* and 40 individuals of *C. c. corsicana* from all over the distribution range. Details for the samples included in the molecular phylogenetic analyses are given in Table 1.

2.2. mtDNA

We sequenced a 550-bp segment including 127 bp of the 3′-end of ATPase 8 gene and 433 bp of the 5′-end of ATPase 6 gene (ATP8 + 6) of coding mitochondrial DNA (Ödeen and Björklund, 2003). Total DNA from blood samples was extracted using Chelex (5%) extracting standard procedure (Maniatis et al., 1982). After extraction, DNA concentration was measured with a spectrophotometer. The polymerase chain reaction (PCR) amplification was carried out in 25 µl volumes using the following primers for the ATP8 + 6 regions: TLF 5′-CTTAATGATATGCCACAACTAAACCC-3′ (ATP8) and TC550R 5′-GGTCGAATAAATAGGCTAATTGTTTCGAT-3′ (ATP6). The cycle was 3 min at 94 °C, 38 cycles of 15 s at 94 °C, 15 s at 51 °C and 45 s at 72 °C plus a final 2 min at 72 °C. For short term storage we kept the PCR products in −4 °C. Amplifications were performed on an Eppendorf Mastercycler Gradient with the ramp speed set to the default 3 °C/s. for PCR amplification. To detect PCR products and to check length of the amplified fragments we run 5 µl of each sample on 2% agarose gel. Before sequencing PCR products were cleaned with ExoSAP-IT to remove unwanted primers and dNTPs. Cycle sequencing was performed by MACROGEN, Korea under BigDyeTM terminator cycling conditions. The reacted products were purified using Ethanol Precipitation and run using Automatic Sequencer 3730xl (MACROGEN company). The resulting electropherograms (ABI-files) were aligned by eye with sequencing Analysis Software BioEdit.

2.3. Nuclear DNA

We used the marker 02401 in Backström et al. (2008), which is a coding gene placed on chromosome 28, and the marker 12884, placed on chromosome 1 (Backström et al., 2008). The PCR-protocol and primers were used as in Backström et al. (2008). Processing of samples and sequencing followed the protocol for mtDNA.

2.4. Microsatellites

We used the following microsatellite primers: Ase42 (Melo and Hansson, 2006), Lox1, Lox3, Lox8, Lox 7 (Piertney et al., 1998), Pdoµ4 (Neumann and Wetton, 1996), and Cuµ04 (Gibbs et al., 1999), with the protocols in the original papers. Microsatellites were run on an ABI377 sequencer, and analysed by the softwares Genescan 3.1.2 and Genotyper 2.1 (Perkin–Elmer).

Table 1
Samples included in the molecular phylogenetic analyses, with geographic origin, and GenBank Accession Numbers for the different loci.

Species	Locality	mtDNA ATP (550 bp)	nDNA 2401 (562 bp)	nDNA 12884 (482 bp)	Source
Carduelis citrinella	Sierra de Cazorla/South Spain	FJ817126	FJ817092	FJ817112	This study
Carduelis citrinella	Sierra de Gudar and Albaracin/Central Spain	FJ817127	FJ817098	FJ817113	This study
Carduelis citrinella	Sierra Guadarrama/Central Spain	FJ817128	-	FJ817111	This study
Carduelis citrinella	Sierra de Neila/Central Spain	FJ817129	FJ817095	-	This study
Carduelis citrinella	Navarra, W Pyrenees/North Spain	FJ817130	FJ817093	FJ817107	This study
Carduelis citrinella	Val d'Aran, C Pyrenees/North Spain	FJ817131	FJ817094	FJ817110	This study
Carduelis citrinella	Cap del Rec, E Pyrenees/North Spain	-	FJ817097	FJ817109	This study
Carduelis citrinella	Port del Compte, Pre-Pyrenees/North Spain	FJ817132	FJ817096	FJ817108	This study
Carduelis citrinella	Mont Ventoux/South France	FJ817133	FJ817102	FJ817115	This study
Carduelis citrinella	Col de Bretolet/Suisse	FJ817134	-	-	This study
Carduelis citrinella	Nordschwarzwald/Germany	FJ817135	FJ817101	FJ817114	This study
Carduelis citrinella	Nordschwarzwald/Germany	FJ817136	-	-	This study
Carduelis corsicana	Isola Capraia/Italy	FJ817137	-	-	This study
Carduelis corsicana	Tuarelli, N Corsica/France	FJ817139	FJ817100	FJ817116	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817140	FJ817099	FJ817117	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817141	FJ817103	FJ817118	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817142	-	-	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817143	-	-	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817144	-	-	This study
Carduelis corsicana	Monte Limbara, Sardinia/Italy	FJ817145	-	-	This study
Carduelis carduelis	Radolfzell/South Germany	FJ817123	FJ817104	FJ817119	This study
Carduelis chloris	Radolfzell/South Germany	FJ817124	-	-	This study
Carduelis spinus	Sierra de Neila/Central Spain	FJ817125	FJ817106	FJ817121	This study
Serinus serinus	Radolfzell/South Germany	FJ817122	-	-	This study
Loxia curvirostra	Sierra de Gudar and Albaracin/Central Spain	FJ817147	FJ817105	FJ817120	This study

Table 2
Sequence diversity in mtDNA and two nuclear markers in *C. c. citrinella* and *C. c. corsicana*. *P*-values refer to test of the difference between *C. c. citrinella* and *C. c. corsicana*.

	mtDNA		2401		12884	
	citrinella	*corsicana*	*citrinella*	*corsicana*	*citrinella*	*corsicana*
No individuals	189	40	101	23	53	12
Length	550	550	562	562	482	482
No pairwise diff.	0.145	1.013	2.57	0.996	0.975	1.61
Nuc. diversity, π	0.00026	0.00184[a]	0.00457	0.00177[c]	0.00202	0.0033
No haplotypes	5	7[b]	37	6[d]	9	7
Haplotype diversity	0.142	0.468	0.95	0.458[e]	0.591	0.833
No seg sites	4	12	16	5	10	8
Tajima D	−1.42	−1.97	−0.61	−0.78	−1.58	−1.57
P	>0.10	<0.05	>0.1	>0.1	<0.10	>0.1

[a] $P = 0.008$.
[b] $P < 0.001$.
[c] $P = 0.034$.
[d] $P = 0.037$.
[e] $P = 0.006$.

2.5. Statistical analysis

Estimates of DNA sequence diversity were obtained using the software DnaSP (Rozas et al., 2003), and microsatellite-diversity by FSTAT (Goudet, 2001). Analysis of molecular variation (AMOVA) and analysis of mismatch distributions were performed using Arlequin 2.000 (Schneider et al., 2000). Tests of differences in nucleotide diversity, number of haplotypes and haplotype diversity were performed using coalescent simulations in DnaSP. Phylogenetic analyses were done using MEGA 4 (Tamura et al., 2007). We used parsimony analyses, and Tamura-Nei distances and NJ-clustering. We also tried various other distance methods and rates of evolution but the topology was always the same. For the nuclear genes we combined the two data sets into one to get higher resolution. Bootstrap support was obtained using 1000 resampling events.

To estimate the time of the split between *C. c. citrinella* and *C. c. corsicana* we used the software Ima (Hey and Nielsen, 2007). We applied a burn-in period of 500,000 steps, and then sampled 5000 genealogies with 100,000 steps in between. In this way we reduced the problem of autocorrelation among genealogies. The ESS-values ranged between 1445 and 287,000. Since the estimates of time since split and effective population sizes are scaled by mutation rate, we used a value of 0.45 substitutions/site/100 MYR, which translates to 2.5×10^{-6} per gene and generation (Lockridge Mueller, 2006), for the mitochondrial data (550 bp), and 2.5×10^{-8} per site and generation (Nachmann and Crowell, 2000), which translates to 2.6×10^{-5} for the combined data set of nuclear data (1044 bp).

3. Results

3.1. mtDNA

For all parameters estimated, *C. c. corsicana* had more sequence variability than *C. c. citrinella* (Table 2). For example, the nucleotide diversity (π) in *C. c. corsicana* was about seven times larger than in *C. c. citrinella* ($P = 0.008$). The sampling effort was much greater (almost five times more samples) for *C. c. citrinella* than for *C. c. corsi-*

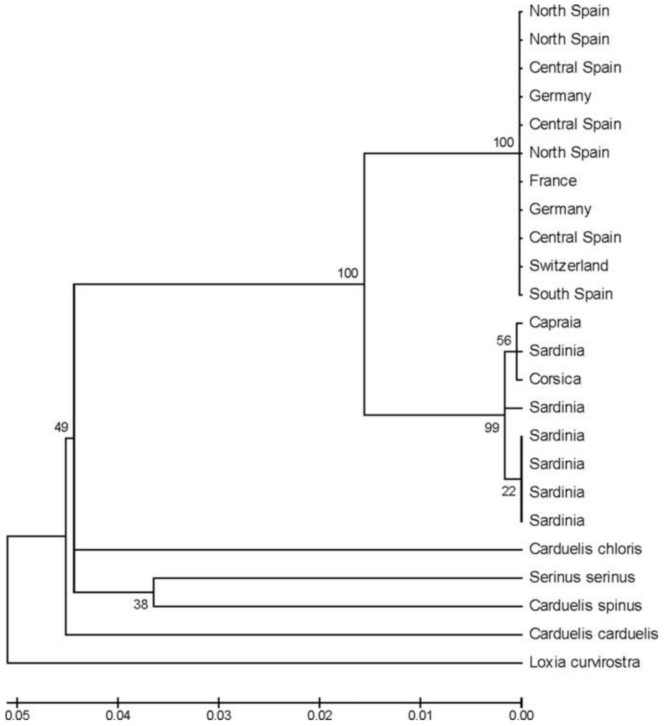

Fig. 1. Phylogenetic relationships based on mtDNA. Numbers refer to bootstrap support.

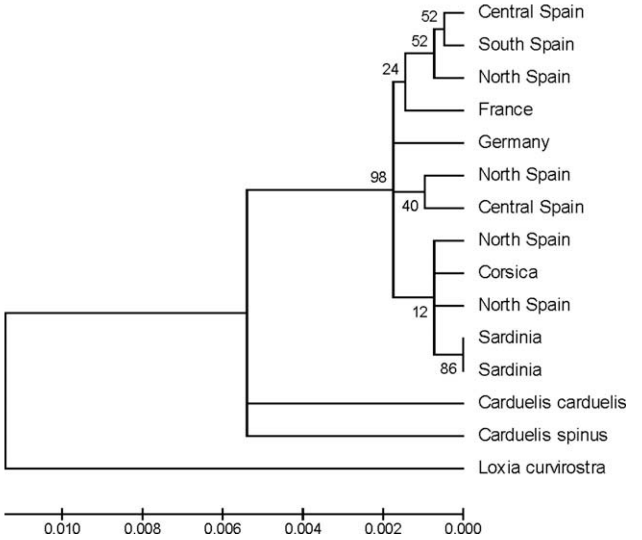

Fig. 2. Phylogenetic relationships based on the combined nuclear data set. Numbers refer to bootstrap support.

cana due its larger distributional range. Despite the difference in sampling effort, all measurements of sequence diversity were several times larger in the smaller *C. c. corsicana* sample (Table 2). The mean sequence distance within *C. c. citrinella* was 0.00172, and within *C. c. corsicana* 0.00258. This should be contrasted to a between-group sequence distance of 0.0361, which is 21 times larger than the distance within *C. c. citrinella* and 14 times larger than the mean distances within *C. c. corsicana*.

Using the AMOVA approach we found that the variation among groups accounted for 77.9% of total variation, and variation within groups for the remaining 22.1%. This translates to an F_{ST} of 0.779 ($P < 0.001$). The phylogenetic analysis show two distinct clusters,

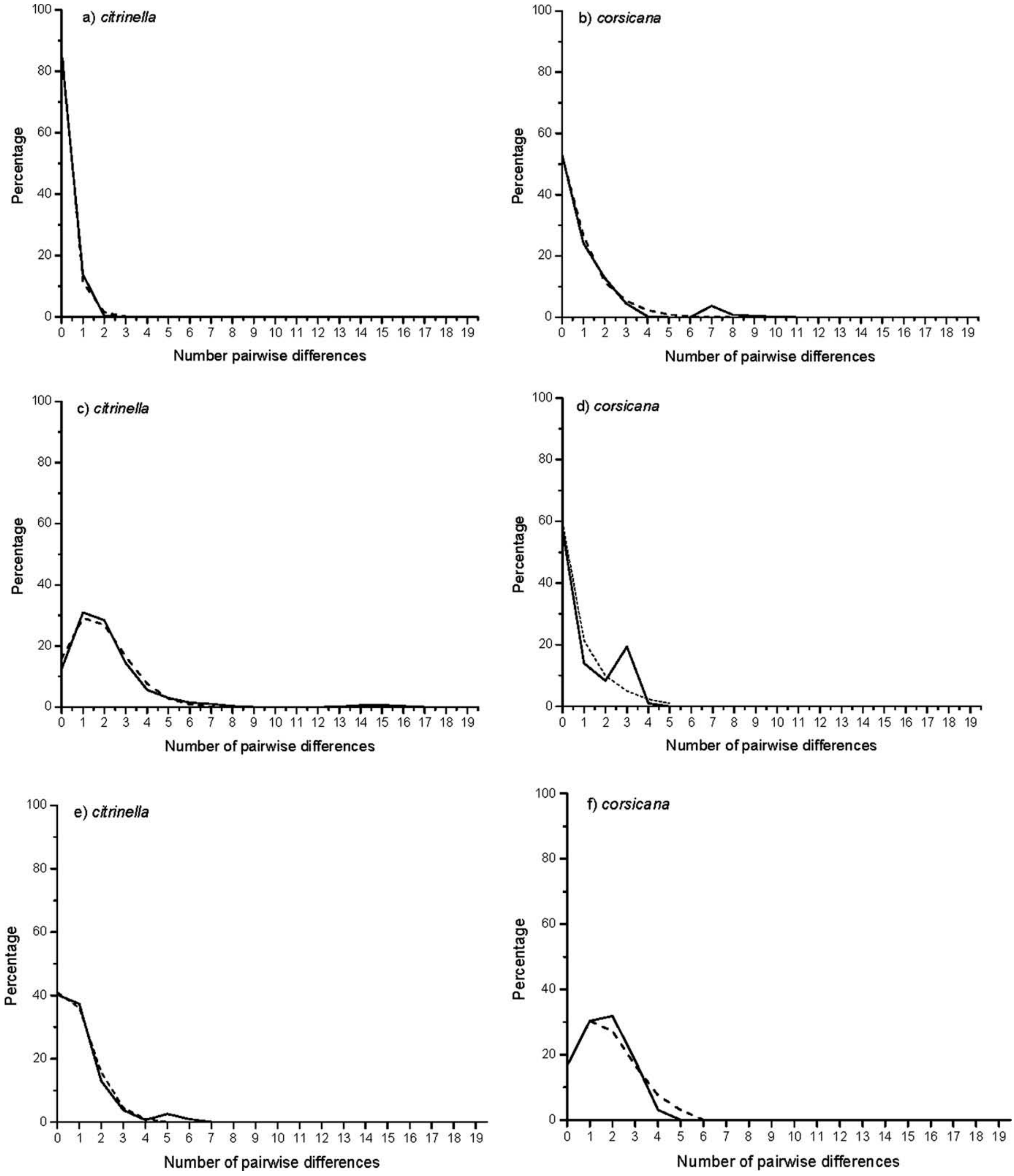

Fig. 3. Mismatch distributions for mtDNA (a and b) and the two nuclear marker (2401, c and d) and 12884, e and f). Unbroken lines show the observed distribution, and the dotted lines the expected using an expansion model.

regardless of method and models, with the mainland *C. c. citrinella* in one cluster and *C. c. corsicana* in the other cluster, with approximately 3.2% sequence divergence, both well separated from the outgroups (approximately 15% sequence divergence; Fig. 1) supported by a very high bootstrap value. This indicates that *C. c. citrinella* and *C. c. corsicana* diverged around 1.2 MYR ago, and they split from the common ancestor of other *Carduelis* around 6 MYR ago.

The estimate of the splitting time using Ima gave a result of $t\mu = 0.0015$, which gives a time of split about 600 generations ago. However, the likelihood function was rather flat, which means a large confidence interval. The time to MRCA was 0.0275 (90% 0–0.0605), which translates to about 11,000 generations 0–24,000). The effective population size ($4N_e\mu$) of *C. c. citrinella* was 9.9×10^{-5} (0–0.0252), and for *C. c. corsicana* 2.7×10^{-4} (0–0.0292). This corresponds to N_e values of 10 for *C. c. citrinella* and of around 30 for *C. c. corsicana*, but the upper range is in the order of 3000. The number of migration events was estimated to be one in each direction (range 0–3), and estimated to have occurred 200 generations ago (upper range about 2000 generations).

The mismatch distribution was not different from a sudden expansion model in neither *C. c. citrinella* ($P = 0.33$; Fig. 3a) nor *C. c. corsicana* ($P = 0.75$; Fig. 3b).

3.2. Nuclear DNA

All estimates of sequence diversity were higher in the *C. c. citrinella* sample than in the *C. c. corsicana* sample in both markers and in many cases the differences were significant (Table 2). The mean sequence distance in 2401 in *C. c. citrinella* was 0.0032, and in *C. c. corsicana* 0.00179. This should be contrasted to the between-group sequence distance of 0.0028, i.e. the amount of divergence is about equal within and between groups. In the nuclear gene 12884 the mean sequence distance within *C. c. citrinella* was 0.00196, and in *C. c. corsicana* 0.00262. The between-group sequence divergence in this marker was 0.00254, i.e also in this marker was the divergence about equal within and between groups.

The AMOVA showed that 13.6% of the variation was between groups ($F_{ST} = 0.136$, $P < 0.001$), and the remaining 86.4% was found within populations for gene 2401. For the gene 12884 4.7% of the variation was between populations ($F_{ST} = 0.047$, $P = 0.092$). We combined the data sets for the phylogenetic analysis and found a deep split between *C. c. citrinella* and *C. c. corsicana* and the outgroups (0.2% divergence) with a very high bootstrap support, but no split between *C. c. citrinella* and *C. c. corsicana* (Fig. 2). This corresponds to a divergence time of around 0.7 MYR of *C. c. citrinella*–*C. c. corsicana* from the common ancestor of the *Carduelis* analysed here.

The analysis of time since population split using Ima gave an estimate of $t\mu = 7.5 \times 10^{-7}$, which gives a time of the split of 130 generations. Also with this data set, the interval was very large. Other parameters were close to the ones obtained using mtDNA, but the intervals were large despite very extensive sampling.

The mismatch distribution was not different from a sudden expansion model in neither *C. c. citrinella* ($P = 0.99$; Fig. 3c), nor *C. c. corsicana* ($P = 0.35$, Fig. 3d) in gene 2401, and the same was true for gene 12884 ($P = 0.40$, *C. c. citrinella*; Fig. 3e, and $P = 0.70$, *C. c. corsicana*, Fig. 3f).

3.3. Microsatellites

The gene diversity was in general very high in both *C. c. citrinella* and *C. c. corsicana* (Table 3), as expected when many populations have been pooled. Neither gene diversity nor allelic richness differed between the two populations. Overall F_{ST} was 0.051 ($P = 2 \times 10^{-21}$, combined over loci). Using this marker, we also found a significant divergence between the Corsican and the Sardinian populations ($F_{ST} = 0.015$, $P = 0.0092$).

Table 3
Comparison of microstallite variation between *C. c. citrinella* and *C. c. corsicana*.

Locus	*citrinella*		*corsicana*	
	Gene diversity	Allelic richness	Gene diversity	Allelic richness
Lox1	0.768	8.2	0.804	9.0
Lox3	0.982	23.6	0.984	22.0
Lox7	0.984	23.7	0.965	19.5
Lox8	0.972	18.5	0.981	22.7
Ase42	0.175	3.1	0.575	4.5
Pdoμ4	0.638	2.9	0.638	9.0
CuU04	0.926	17.9	0.926	15.3
Mean	0.778	14.0	0.839	14.6

4. Discussion

We used three different markers with different rates of evolution to understand the divergence between two putative species of the citril finch. All three markers were congruent with regard to the divergence as such, but they differed considerably with regard to the extent of divergence. The use of markers with different rates of evolution allows for a rough understanding of the timing of the split between these populations. The slowest evolving marker (the nuclear genes) showed a significant differentiation using the AMOVA, but the differentiation was not sufficient to get support for reciprocal monophyly of *C. c. citrinella* and *C. c. corsicana*. The mtDNA-data, on the other hand, show very strong differentiation, and a tree with reciprocal monophyly of *C. c. citrinella* and *C. c. corsicana*, and no detectable differentiation within the groups. The microsatellites, which are supposed to have the highest mutation rate, also show a significant differentiation between *C. c. corsicana* and *C. c. citrinella*, but also a differentiation among populations within each group. In contrast to Zamora et al. (2006) we found no support for the view that *C. c. corsicana* is closer related to the common ancestor with *C. carduelis* than to *C. c. citrinella*. Instead, we found strong support for reciprocal monophyly, and we suggest that the conclusion made by Zamora et al. (2006) might be the result of the exclusive use of mitochondrial genes combined with small sample sizes.

The estimate of the time of the split show that this is most likely to be a very recent event, involving bottlenecks and low effective population sizes. This conclusion is also supported by the mismatch analyses. These demographic effects are most prominent in the mtDNA-data as is expected due to the lower effective population size of mitochondrial genes. The sequence divergence is 3.2%, which suggests a split around 1.2 MYR ago, a result that is not at all supported by the nuclear data, and the difference between these two markers is an order of magnitude different with regard to time of divergence. Using the relationship $F_{ST}(nuclear) = 1 - e^{0.25*\ln(1-F_{ST}(mt))}$ (Zink and Barrowclough, 2008), then we would expect the divergence of the nuclear markers to be in the order of 0.31, while in reality the divergence is close to an order of magnitude lower.

Therefore, the deep divergence in the mtDNA could be a result of a very recent divergence, enhanced by a strong bottleneck with a subsequent rapid lineage sorting due to the low effective population size of mitochondrial genes. The very recent time of split and the low effective population sizes suggested by Ima support this conclusion. This calls for caution when using mtDNA alone for assessing times of divergence and rates of evolution. Since mtDNA is particularly affected by demographic events due to its lower effective population size, this might lead to substantial over-

Table 4
Comparison of data on life history traits, vocalisation, morphology and genetics from *C. c. citrinella and C. c. corsicana.*

Life history traits		
Niche	*citrinella* restricted to conifer forest Niche expansion in *corsicana* into more open tree heath macchia	Förschler and Kalko (2006a)
Conifer dependence	*citrinella* dependent on pine seeds *corsicana* partially independent of pine seeds	Förschler and Kalko (2006b)
Vocalisation		
Song divergence	Differentiation with longer, slower and more segmented songs in *corsicana*	Förschler and Kalko (2007)
Call divergence	Differentiation with shorter and more frequency-modulated calls in *citrinella*	Förschler and Kalko (2007)
Morphology		
Flight apparatus	More pointed wings in migratory *citrinella*	Förschler and Siebenrock (2007); Förschler et al. (2008)
Feeding apparatus	Smaller bills in *corsicana*	Förschler and Siebenrock (2007)
Hind limb	Stronger legs and stronger feet in *citrinella*	Förschler and Siebenrock (2007)
Mitochondrial DNA		
Cytochrome *b*	2.8% Divergence	Pasquet and Thibault (1997)
ATP region	3.2% Divergence	This study

estimation of divergences and divergence times as is the case in our study.

The results implicate a strong and relatively recent bottleneck. In many species bottlenecks occurred during the last glaciation (e.g. Merilä et al., 1997), but in the case of the citril finch it seems to be more recent. The reason for the bottleneck is probably related to the abrupt changes in the composition of the vegetation by 5900–6000 BP, when in only 20–100 years the great extensions of *Pinus nigras cf.*, which is a main food source for the citril finches (Borras et al., 2003), were substituted by various *Quercus* species (Carrión and van Geel, 1999; Carrión et al., 2001). In this way, the availability of suitable breeding areas for the citril were greatly reduced. The reason for the drastic change in vegetation was a general climatic change with rapidly increasing temperatures, but perhaps more important, large-scale fires (Carrión and van Geel, 1999; Carrión, 2003). Our data did not allow for a precise timing of the citril finch bottleneck, but at least it is consistent with the vegetational history of the Mediterrean region. (This does not mean that other putative causes, such as epidemics, may have had an impact as well).

Our results show a clear genetic differentiation of *C. c. citrinella* and *C. c. corsicana* in the mitochondrial ATP region. This differentiation is even higher than the one observed by Pasquet and Thibault (1997) in the cytochrome *b* gene (2.8%). The two populations differ clearly in terms of habitat selection, especially the evident niche expansion on the islands, including differences in food selection (Förschler and Kalko, 2006a,b), morphology (Förschler and Siebenrock, 2007; Förschler et al., 2008) and clear bioacoustical differentiation (Chappuis, 1976; Förschler and Kalko, 2007) (see Table 4). Following a phylogenetic (lineage) species concept, we argue that *C. c. citrinella* and *C. c. corsicana* should be treated as different species despite their very recent divergence, and the incomplete lineage sorting of nuclear genes, since they have a set of diagnostic features and share a clear lineage sorting with regard to mtDNA. We therefore follow the taxonomic suggestions by Sangster (2000) and Sangster et al. (2002) to treat the two Citril finch taxa as two independent species and members of the super-species *Carduelis* [*citrinella*].

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