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The genetic structure of crossbills suggests rapid diversification with little niche conservatism

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Conservatism of ecological niches can cause geographical ranges or the formation of new species to be constrained, and might be expected in situations where strong trade-offs result in ecological specialization. Here we address the flexibility of resource use in European crossbills by comparing the ecological and genetic similarities between four Mediterranean and three northern European crossbill populations, all specialized in feeding on a different resource. We used sequence data of one mitochondrial and two nuclear genes from between 211 and 256 individuals. The northern crossbills were genetically too similar to infer which population was more related to the southern ones. Crossbills from the island of Mallorca showed genetic signatures of a stable and isolated population, supporting their past treatment as a locally (co)evolving taxon, and seem to have evolved from an ecologically distinct ancestor. Previous studies in other populations also suggest that genetic similarity does not predict morphological and resource similarity. We estimate that the divergence of all western European crossbills has occurred within the last 11 000 years. Overall, it appears that crossbills can diversify rapidly and with little niche conservatism, but that such potentially reproductively isolated specialists are evolutionarily short-lived.

ADDITIONAL KEYWORDS: adaptive radiation – *Loxia curvirostra* – measures of genetic differentiation – reproductive isolation.

INTRODUCTION

The tendency of species and clades to retain their niches and related ecological traits over time is called niche conservatism (Wiens *et al*., 2010). The flexibility with which niches change over time and can be adapted to local (incl. anthropogenic) changes or new opportunities can have great impact on population persistence and rates of diversification, speciation, and geographical range evolution (reviewed in Wiens *et al*., 2010). In many different areas of ecology and conservation biology researchers have found evidence for phylogenetic conservatism in an important ecological trait (Wiens *et al*., 2010). However, equally strong examples of niche flexibility have been encountered, for example during adaptive radiation (reviewed in Schluter, 2000). While niche conservatism could be present for several reasons, one would predict it to be relatively stronger in more specialized taxa, as stronger specialization suggests stronger underlying trade-offs among functional traits.

The Holarctic avian genus *Loxia* (crossbills) might serve as an interesting test case of this expectation. These birds live almost exclusively on seeds of conifers, which they extract from between closed cone scales with their curved and crossed mandibles. However, conifers differ in traits such as cone size,

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scale thickness, and seed size, and therefore no single bill type performs best on all types of cones: larger, deeper bills are better for extracting seeds from cones with strong seed defences but are less efficient on smaller cones with weaker seed defences (Benkman, 1993, 2003). Because of these strong foraging trade-offs, it appears that each crossbill taxon is specialized in a single kind of conifer (i.e. has a different niche), and in turn that crossbill diversity and speciation is ultimately driven by conifer diversity (Benkman, 2003; Edelaar & Benkman, 2006; Edelaar, 2008a; Edelaar *et al*., 2012).

However, if bill morphology and cone morphology are so tightly linked, does this then imply that it is difficult and rare to switch between resources, and that resource switches occur mainly between very similar types of resources? For the North American crossbill taxa, the results from Parchman, Benkman & Britch (2006) show that genetically closely related taxa can have very different bill sizes, suggesting that crossbills are able to switch between very different resources. Moreover, the very low levels of genetic divergence are compatible with the recent divergence of taxa, possibly mostly post-Pleistocene, suggesting that resource switching is also relatively frequent. In addition, the very thin-billed White-winged crossbill (*L. leucoptera*) has colonized the island of Hispaniola and switched to a strongly defended pine, resulting in the evolution of the thick-billed Hispaniolan crossbill (*L. megaplaga*; Parchman, Benkman & Mezquida, 2007). While the thickness of its bill has probably evolved partly due to an arms race between crossbills and pines which may have had over 300 000 years to develop (Parchman *et al*., 2007), the resource switch from a thin-scaled, small-coned spruce to a thick-scaled, large-coned pine is striking. Overall then, it appears that switches between resources of a considerable difference have appeared frequently in American crossbills, and may have taken relatively little time.

We address here the generality of this pattern in European crossbills. In mainland Europe there are three taxonomically recognized and morphologically distinct species occurring in the north. Similar to the situation in North America, each of these species appears to be specialized on a single conifer: the two-barred crossbill (*L. bifasciata*) on Siberian larch (*Larix siberica*), the parrot crossbill (*L. pytyopsittacus*) on Scots pine (*Pinus sylvestris*), and the common crossbill (*L. curvirostra*) on Norway spruce (*Picea abies*) (Cramp & Perrins, 1994). In addition, the Scottish crossbill (*L. scotica*) of Scotland is considered a separate species (Summers, Dawson & Phillips, 2007), and mostly thought to have evolved as a specialist on Scots pine. In view of vocalizations, mating patterns, distribution, and resource use it is perhaps best considered an allopatric sister species of the Parrot crossbill.

Populations in southern Europe break this general pattern of a single, different conifer species as the food resource for each crossbill species. Local populations of common crossbills around the Mediterranean Basin feed on four different species of pine (Aleppo pine *Pinus halepensis*, Scots pine *P. sylvestris*, black pine *P. nigra* and mountain pine *P. uncinata*). This could mean that the specialization on a single resource in crossbills is not a hard rule, or that cryptic (incipient) species could be included within the common crossbill. Indeed, these Mediterranean populations using different resources show some associated differentiation in adaptive morphology, vocalizations, and genetic markers (Massa, 1987; Cramp & Perrins, 1994; Edelaar *et al*., 2012), and some island populations have received subspecific recognition (Cramp & Perrins, 1994).

In this paper we take some steps towards unravelling the evolutionary history of crossbills in Europe. Specifically, we contrast a scenario where switching between resources is relatively hard and mostly occurs between similar resources (as the existence of strong trade-offs predicts), with a scenario where switching between resources is relatively easy, including between dissimilar resources (as earlier empirical studies suggest). We do this because both scenarios have been proposed to explain the origin of the pine-feeding Mediterranean crossbills (Murray, 1978; Knox, 1990; Tyrberg, 1991). Under the first scenario it is thought that pine-feeding crossbills have had a more or less continuous distribution across Europe during times of glaciation but that they were geographically separated by the spread of broad-leaved trees across central Europe during the warmer interglaciations. Under the second scenario it is thought that the spruce-feeding common crossbill invaded Europe west of the Ural Mountains after the last glaciation (with the spread of Norway spruce) and that upon reaching Mediterranean areas it shifted resource type and started to utilize the different pines. Both hypotheses have some empirical support: Mediterranean pine-feeding crossbills are vocally more similar to the other pine-feeding crossbills from the north (Summers & Jardine, 2005; Förschler & Kalko, 2009), but morphologically more similar to the northern spruce-feeding common crossbill (Cramp & Perrins, 1994) which moreover regularly irrupts to Mediterranean areas (Tellería, Asensio & Díaz, 1999; Borrás *et al*., 2010) and probably has done so in the past.

As vocalizations and morphology paint opposite pictures and may show patterns of recent selection instead of evolutionary history, here we use genetic data to test these two scenarios. Previous studies on

the genetic relationships of European crossbills did not obtain resolved phylogenetic trees (apart from the phylogenetic position of the morphologically and vocally fairly distinct two-barred crossbill, which will not be treated in further detail here). Questiau *et al*. (1999) suggested that common crossbills were genetically homogenized by gene flow across Europe and perhaps beyond, as their phylogenetic tree of mtDNA haplotypes was largely unstructured. This may not come as a complete surprise given that this crossbill can display very large natal and breeding dispersal distances after their frequent mass movements in search of suitable cone crops (> 2000 km on average; Newton, 2006). Piertney, Summers & Marquiss (2001) likewise did not find any genetic differentiation among common, Scottish, and (recently established) parrot crossbills from Scotland when using mtDNA haplotypes and microsatellites: note that these three crossbills are taxonomically recognized as distinct species. In contrast to this documented lack of genetic structure, populations utilizing three different pine species on the Spanish mainland were found to be slightly but significantly genetically differentiated, in association with some vocal and morphological differentiation and independent of geographical distances among populations (Edelaar *et al*., 2012). These results suggest that association with a certain resource can provide some limitation to unrestricted gene flow (Edelaar *et al*., 2012), which could be seen as an indication that cryptic speciation may be involved. Following the argument of Parchman *et al*. (2007) for the Hispaniolan crossbill, these results may also be seen as an indication that associations with certain resources have had longer histories in the (more stable) Mediterranean area, thus suggesting that Mediterranean crossbills could be more closely related to the northern pine-feeding crossbills.

We compare the relative support for the two scenarios of resource switching using patterns of divergence in mtDNA and nuclear DNA data. Under the first scenario we would predict that the Mediterranean pine-feeding populations are genetically more similar to the northern pine-feeding taxa (parrot and Scottish crossbill). Under the second scenario we would predict that the Mediterranean pine-feeding populations are genetically more similar to the northern spruce-feeding taxon (northern common crossbill).

Figure 1. Approximate distributions and sampling sites of the European crossbill taxa included in this study. The distributions of *sylvestris* and *halepensis* are depicted as parapatric, but probably greatly overlap with altitudinal segregation. Crossbills from Central Europe (distribution not indicated on the map but connecting the range of *pytyopsittacus* with *uncinata* and *scotica*) may belong to the same population as *picea*, but here we restricted ourselves to northern samples from the distribution of *pytyopsittacus*. Sampling sites for *picea*, *pytyopsittacus*, and *uncinata* are indicated; in addition *picea* and *pytyopsittacus* previously sampled in the range of *scotica* (Piertney *et al*. 2001) are also included. Samples from *scotica*, *sylvestris*, *halepensis*, and *balearica* came from several sites (see Edelaar *et al*., 2012 for details) within their respective distributions.

METHODS

Sampling and population designation

Birds were captured with mist-nets. Each bird was ringed and its sex and age determined (Svensson, 1992). Prior to release, a small drop of blood was taken from a wing vein, and saved individually on FTA cards or in alcohol for later extraction of DNA. We sampled birds from six geographically and/or ecologically distinct populations (see Fig. 1). Birds were assigned to population by means of geography and morphology (bill measures), or for the three mainland Spanish populations by means of the dominant conifer tree at the catching site, on which they presumably were feeding at the time of catching (see Edelaar *et al*., 2012, and Piertney *et al*., 2001 for details, and Table 1 for sample sizes). To our samples of *L. curvirostra* and *L. pytyopsittacus* from Russia we added published sequences of individuals of these taxa collected in Scotland (respectively $N = 17$ and $N = 11$), as well as of *L. scotica* ($N = 17$) as a 7th population (from Piertney *et al*., 2001; GenBank accession number AY029371 and their table 2). To make the text hereafter more easy to read we label all crossbill populations according to their (sub)specific name, if available: *scotica*, *pytyopsittacus*, and *balearica*. For the remaining populations we used the

Table 1. Descriptive molecular statistics for one mitochondrial and two nuclear loci (with standard deviations in parentheses)

	halepensis	*sylvestris*	*uncinata*	*balearica*	*picea*	*pytyopsittacus*	*scotica*
(A) mtDNA control region							
No. of sequences	64	95	17	23	24	16	17
No. of haplotypes	12	16	6	5	12	7	8
Pairwise difference	2.03 (1.16)	1.81 (1.05)	1.65 (1.02)	1.56 (0.96)	2.37 (1.34)	1.46 (0.93)	1.94 (1.16)
Gene diversity	0.78 (0.043)	0.80 (0.025)	0.71 (0.11)	0.56 (0.11)	0.86 (0.050)	0.79 (0.089)	0.73 (0.11)
Nucleotide diversity	0.097 (0.061)	0.086 (0.056)	0.078 (0.054)	0.074 (0.051)	0.112 (0.071)	0.069 (0.050)	0.092 (0.062)

	halepensis	*sylvestris*	*uncinata*	*balearica*	*picea*	*pytyopsittacus*
(B) Nuclear marker 2401						
No. of sequences	120	188	44	48	6	16
No. of haplotypes	17	20	12	11	4	6
Pairwise difference	1.45 (0.89)	1.46 (0.89)	1.64 (0.96)	2.01 (1.15)	1.20 (0.88)	1.81 (1.10)
Gene diversity	0.82 (0.023)	0.81 (0.018)	0.85 (0.035)	0.88 (0.023)	0.87 (0.129)	0.83 (0.056)
Nucleotide diversity	0.072 (0.049)	0.073 (0.049)	0.082 (0.055)	0.101 (0.064)	0.060 (0.051)	0.090 (0.062)

	halepensis	*sylvestris*	*uncinata*	*balearica*	*picea*	*pytyopsittacus*
(C) Nuclear marker 12884						
No. of sequences	130	210	46	54	12	16
No. of haplotypes	6	8	4	1	1	4
Pairwise difference	0.41 (0.40)	1.07 (0.71)	0.43 (0.40)	0.00 (0.00)	0.00 (0.00)	1.25 (0.83)
Gene diversity	0.16 (0.044)	0.31 (0.041)	0.13 (0.067)	0.00 (0.00)	0.00 (0.00)	0.35 (0.15)
Nucleotide diversity	0.024 (0.025)	0.063 (0.046)	0.026 (0.026)	0.00 (0.00)	0.00 (0.00)	0.074 (0.055)

Table 2. Pair wise F_{ST} values for the mitochondrial (A) and nuclear loci (B) (above diagonal: marker 2401, below diagonal: marker 12884)

	uncinata	*sylvestris*	*halepensis*	*balearica*	*pytyopsittacus*	*picea*	*scotica*
(A) mtDNA							
uncinata	–						
sylvestris	–0.010	–					
halepensis	0.002	*0.024*	–				
balearica	*0.101*	*0.046*	*0.118*	–			
pytyopsittacus	0.024	*0.102*	0.029	**0.265**	–		
picea	0.070	**0.123**	0.020	**0.237**	0.027	–	
scotica	–0.017	*0.055*	0.009	*0.199*	–0.012	0.010	–

	uncinata	*sylvestris*	*halepensis*	*balearica*	*pytyopsittacus*	*picea*
(B) Nuclear markers						
uncinata	–	–0.009	–0.004	0.005	–0.017	–0.038
sylvestris	–0.001	–	0.003	*0.024*	–0.006	–0.025
halepensis	–0.007	0.009	–	*0.028*	0.011	–0.060
balearica	0.004	0.019	0.001	–	–0.010	–0.022
pytyopsittacus	–0.008	–0.022	0.021	*0.092*	–	–0.010
picea	–0.041	–0.018	–0.036	0.000	–0.019	–

Bold figures indicate significant ($P < 0.05$) values after sequential Bonferroni adjustment for multiple testing, while figures in italics indicate values significant only without the adjustment of P values.

conifer species presumably utilized as a label. Thus, *uncinata* is the population caught on mountain pine *Pinus uncinata*; *halepensis* on Aleppo pine *P. halepensis*; *sylvestris* on Scots pine *P. sylvestris*; and *picea* on Norway spruce *Picea abies* (see Fig. 1). Because crossbills may wander widely in search of food or water, and may at times occur in forests composed of different conifers than that to which they are specialized, the dominant conifer at the catching site may be an imperfect population designator. Indeed, we found previously that birds from two Spanish sampling sites with *P. halepensis* showed the bill morphology of birds associated with *P. sylvestris*. As they independently also resembled *sylvestris* birds more in vocalizations and genetics, these birds were subsequently clustered with *sylvestris* samples (Edelaar *et al*., 2012), and we retained this reassignment in the current analyses.

It is also worth noting that we have not used individual vocalizations for a finer or potentially more accurate population subdivision into so-called 'vocal types', which has been very useful for the understanding of the population structure, ecology, and evolution of crossbills in North America (Groth, 1988, 1993; Benkman, 2003; Parchman *et al*., 2006). Despite indications that similar vocal differentiation also exists in Europe and appears evolutionarily relevant (Robb, 2000; Edelaar, Summers & Iovchenko, 2003; Edelaar, 2008b; Edelaar, van Eerde & Terpstra, 2008; Edelaar *et al*., 2012), there is currently insufficient consensus over the number of and assignment to such groups, which moreover seem to outnumber the available discrete resources for ecological specialization (Robb, 2000; Summers *et al*., 2002; Edelaar *et al*., 2003; Constantine & The Sound Approach, 2006). This is especially true for birds from Spain, where the vocal variation is surprisingly large and seems more geographically structured (Förschler & Kalko, 2009; own unpubl. data), which may be related to a much greater degree of residency as testified by high individual recapture rates stretching over multiple years (Senar *et al*., 1993; Clouet, 2000; own unpubl. data). Hence, it is currently not clear that the 'vocal type' approach to crossbill diversity will be equally successful in Europe as in North America, and testing its usefulness for genetic comparative work awaits further progress in the documentation and classification of the vocal variation. In addition, we lacked recordings for a large number of birds sampled by ourselves, as well as for the additional samples included in these analyses, so we cannot test its usefulness here. We therefore restrict our comparisons to geographical regions (northern vs. southern Europe) and ecologically defined groups of crossbills within each region. We acknowledge that this approach might oversee some finer population genetic structure if it exists, but do so under the assumption that (1) there is no finer structure present in *pytyopsittacus*, *scotica*, and *balearica* (no vocal types have been suggested in the literature), (2) vocal types

lumped within northern *picea* will be more closely related to each other than to southern vocal types from Spain, and vice versa, and (3) sampling vocal types lumped within *picea* in Spain can be ignored, and vice versa. In support of these assumptions, the classification based on resource use was very helpful in uncovering morphological and genetic population structuring among the Spanish populations (Edelaar *et al*., 2012), so this classification seems to be sufficiently robust to any error introduced by violation of the above assumptions.

Lab procedures

We sequenced a 479-bp segment of the 3′-end of the mitochondrial control region (GenBank accession nos. HQ377552–HQ377746). The lab procedures were the same as in Edelaar *et al*. (2012) and details can be found there. In short, total DNA from blood samples was extracted. We used PCR to amplify the target DNA using the universal passerine primer pair L16743 and H417. Cycle sequencing was performed by Macrogen, Korea, under BigDye™ terminator cycling conditions and run using an Automatic Sequencer 3730xl (Macrogen). The resulting electropherograms (ABI-files) were checked by eye and aligned using analysis software BioEdit (Hall, 1999). We aligned our sequences with the sequences from Piertney *et al*. (2001) using Clustal Omega (Goujon *et al*., 2010).

In addition, we sequenced about 500 bp of intronic DNA for each of the nuclear markers 2401 (GenBank accession nos. HQ377747–HQ377947) and 12884 (GenBank accession nos. HQ377948–HQ378155), which are genes located on chromosomes 1 and 28, respectively (Backström, Fagerberg & Ellegren, 2008). The PCR-protocol and primers followed Backström *et al*. (2008). Processing of samples, products, sequencing, and scoring of heterozygotes followed the protocol for mtDNA as described above.

Statistics

To calculate basic statistics such as the number of haplotypes, gene diversity, nucleotide diversity, and the number of pairwise differences we used the software Arlequin 3.5.1.3 (Excoffier & Lischer, 2010). As linkage among single nucleotide polymorphisms (SNPs) within our short nuclear sequences is likely to be strong, and recombination is even absent for mtDNA, we created haplotypes from our sequences using the coalescent-based Bayesian PHASE-algorithm implemented in DnaSP (Librado & Rozas, 2009; see Stephens, Smith & Donnelly, 2001 for details). Linking SNPs into haplotypes has also been shown to improve power of detection of population structure (Gattepaille & Jacobsson, 2012).

We compared the genetic composition of populations by calculating pairwise F_{ST} in Arlequin. For this we used the matrix of pairwise nucleotide differences among haplotypes, i.e. we used information on both haplotype genetic distances and haplotype frequencies. This metric is in more recent years often referred to as Phi_{ST} (e.g. Kronholm, Loudet & De Meaux, 2010). One alternative approach would be to calculate the conventional F_{ST} statistic using the frequencies of haplotypes only. However, this is problematic for two reasons: (1) F_{ST} based on allele frequencies alone decreases with increasing within-population heterozygosity, which makes comparisons among populations/markers/studies problematic (Jost, 2008), and (2) alternative metrics to F_{ST} likewise based on allele frequencies such as Hedrick's G'_{ST} (Meirmans & Hedrick, 2011) and Jost's D_{EST} (Jost, 2008) will be affected by sequence length, making comparisons between markers and studies problematic (Pennings, Achenbach & Foitzik, 2011). This is because longer sequences are more likely to differ, up to the point that each individual may well have a unique haplotype, by which time population differentiation can no longer be determined. Whether based on haplotype identity or haplotype distance, F_{ST} is in fact not an index of population differentiation (in the sense of the degree to which populations do not share alleles) but a fixation index (Jost, 2008; Edelaar & Björklund, 2011). Therefore, we will not interpret F_{ST} values as indicating genetic differentiation among populations as is often done (for which metrics such as Jost's D_{EST} are more appropriate), but as differential fixation of haplotypes among populations. F_{ST} increases with less dispersal between populations and smaller population sizes. Negative values can occur with unbiased estimation procedures, e.g. when differential fixation is absent in the samples (Meirmans & Hedrick, 2011).

Using these pairwise F_{ST} values, we constructed a nearest-neighbour tree in Phylip 3.69 (Felsenstein, 2005) for the marker(s) that show clear population structure. Here negative values were converted to zeros, although the differences in the resulting tree were very minor and did not change the structure of the tree.

To visualize the relatedness and distribution of mtDNA haplotypes we constructed a haplotype reduced median-joining network (Bandelt, Forster & Röhl, 1999) using Network 4.6.1.0 (http://www.fluxus-engineering.com) designed for this purpose, with $\varepsilon = 10$, and with a maximum parsimony post-processing (Polzin & Daneschmand, 2003). The reduced median-joining algorithm has been shown to work well for network reconstruction (Woolley, Posada & Crandall, 2010). We tried a number of options but the result was the same in all cases. As our nuclear haplotypes can recombine, calculation of

a nuclear network was not undertaken as it is less certain to approach the true process of haplotype evolution.

Using Arlequin, we performed mismatch analyses whereby the observed frequency distribution of pairwise haplotype differences is compared with the distribution expected under certain historical demographic events (for details see Arlequin manual). We tested two models (recent demographic and spatial expansion), which are compared with the data in terms of their fit by means of the sum-of-squared-deviations (SSD) and the significance values obtained by 1000 simulations in Arlequin. A significant result means that a model is rejected.

We used Mesquite 2.73 (Maddison & Maddison, 2010) to estimate Slatkin & Maddison's (1989) *s*, which estimates the minimum number of migration events based on phylogenetic information. Finally, we used BEAST 1.7.4 (Drummond *et al*., 2012) to make inferences of past demographic history. We used Trace 1.7 (Rambaut & Drummond, 2007) to make lineage-through-time plots, where lineages are not species but individual haplotypes. This analysis can give important information on the history of the population by analysing the rate of coalescence of the individual haplotypes. If the population has been stable then the rate of coalescence would be stable over time, whereas if the population has expanded recently we expect a rapid and non-linear rate of coalescence. These plots show how lineages coalesce backwards in time, where time is measured in units of mutations per site per million years. Plots were based on 10^9 trees with the first 10^8 trees discarded (burn-in), and sampling every 10^4 steps. To put a time scale on the results we used the mutation rate of the avian control region derived by Millar *et al*. (2008) for the Adélie penguin. It is well known that the mutation rate of this gene is variable across taxa (Ruokonen & Kvist, 2002). To account for uncertainty in mutation rates we used the 95% confidence interval from Millar *et al*. (2008) and created a distribution of mutation rates from which we sampled and calculated the time of coalescence. Because the distribution is asymmetric we used a gamma distribution scaled to fit the intervals given in Millar *et al*. (2008). As we have no information on the mutation rates of the nuclear genes we only present the lineage-through-time plots. Estimation of time since divergence and gene flow using Ima2 (Hey, 2010) gave uninformative and probably unreliable results (flat posteriors), and the results obtained showed the signs of violating the basic assumptions (most importantly constant population sizes and constant rate of gene flow over time) of the model as explained by Hey (2010), a problem also pointed out recently by several authors (e.g. Becquet & Przeworski, 2009; Gaggiotti, 2011; Strasburg & Rieseberg, 2011). The same problems were found when trying to estimate θ ($= 4N_e\mu$) and migration rates using Lamarc (Kuhner, 2006) and hence we will not present those values as the uncertainty is too high for these estimates.

RESULTS

In total we found 21 segregating sites and 34 haplotypes for the mitochondrial control region. Nuclear marker 2401 yielded 20 segregating sites and 29 haplotypes, whereas nuclear marker 12884 gave 17 segregating sites and only 11 haplotypes (Table 1, Supporting Information Tables S1–S3). Highest pairwise nucleotide difference between two haplotypes was 10 for mtDNA, 7 for nuclear marker 2401, and 10 for nuclear marker 12884. Gene diversity differed between loci: it was highest in nuclear marker 2401, slightly lower in the mitochondrial control region, and very low in nuclear marker 12884 (Table 1). Molecular diversity of the control region was quite a bit lower in *balearica*, and highest in *picea* (Table 1).

The three most common mtDNA haplotypes differed in frequency among populations (Supporting Information Table S1). Haplotype 1 was by far the most common haplotype in the *balearica* birds, occurred in the three Spanish mainland populations at lower frequencies, but was absent from the northern populations. Haplotype 2, by contrast, was rare in the *balearica* birds but reached high frequencies in all other populations, in particular in *uncinata* and *scotica* where it was the most abundant haplotype. Haplotype 6 was found in all populations, but at a fairly low frequency, except in *picea* where it was more common. With respect to nuclear marker 2401, the three most common haplotypes also differed in frequency among populations but not to the same extent as the mitochondrial haplotypes (Supporting Information Table S2). The most common haplotype (Haplotype 2) had a high frequency in all populations except in *balearica*. Haplotype 3 was absent from *picea*, but was fairly common in all other populations. The third haplotype was most common in *picea*, with lower frequencies in the other populations and especially so in *balearica*. Nuclear marker 12884 was much less variable (Table 1 and Supporting Information Table S3), and Haplotype 1 dominated in all populations and even appears to be fixed in *balearica* and *picea*.

These haplotype frequency differences among populations are reflected in the values of F_{ST} obtained using haplotype pairwise nucleotide differences (Table 2). These values were highest for the mitochondrial control region. In particular, *balearica* showed high and statistically significant F_{ST} values when compared with all other populations, especially when

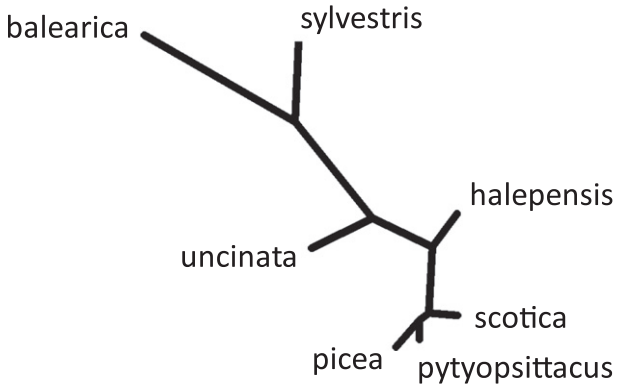

Figure 2. Unrooted nearest-neighbour tree based on pairwise mtDNA F_{ST} values.

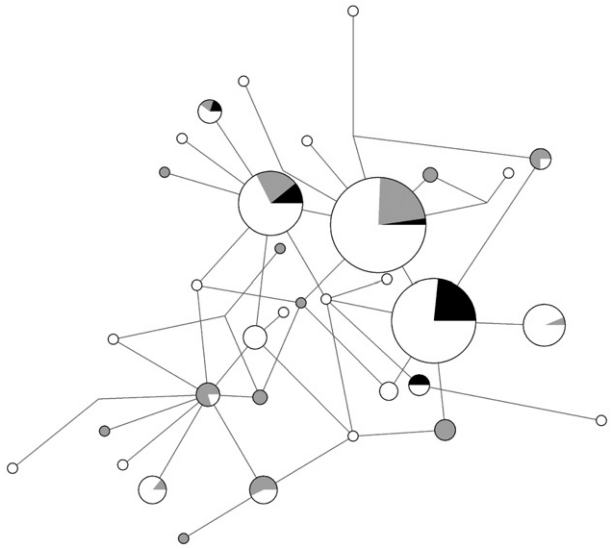

Figure 3. Mitochondrial haplotype network. Colours indicate the frequencies of three groups: white for mainland Spain (*uncinata*, *sylvestris*, and *halepensis*), black for insular *balearica*, grey for northern Europe (*picea*, *pytyopsittacus*, and *scotica*). All connecting lines represent a mutation step of one base, except for the bottom right line (two bases different).

compared with the northern populations and least when compared with *sylvestris*. The Spanish mainland populations showed very little differential fixation amongst each other, although it was significant for the comparison between *sylvestris* and *halepensis*. Likewise, the three northern populations showed small and statistically non-significant differential fixation. F_{ST} was higher for the comparison between northern and Spanish mainland populations, and especially so when comparing northern populations with *sylvestris*. Moreover, the three Spanish mainland populations generally showed higher F_{ST} values when compared with *picea* than when compared with *pytyopsittacus* and *scotica*. The unrooted nearest-neighbour tree (Fig. 2) gives a clear overall summary of these results: at one end the three northern taxa are very close to each other, *uncinata* and *halepensis* are the two Spanish taxa that most resemble the northern taxa, while *sylvestris* is more similar to *balearica* which forms the other end of the unrooted tree.

Differential fixation between populations was much lower for the two nuclear markers, and mostly was statistically non-significant, with three exceptions: *balearica* showed differential fixation when compared with *sylvestris* and *halepensis* for marker 2401, and also when compared with *pytyopsittacus* for marker 12884.

The mitochondrial haplotype network did not show a pattern consistent with geography (Fig. 3). In contrast, we found some very common haplotypes separated by only a few steps, and a large number of haplotypes derived from these, again separated mostly by a single step from the main haplotypes.

The mismatch distributions using mtDNA showed three things. First, the majority of the taxa have a mismatch distribution that fits well with the sudden expansion and/or the spatial expansion model (Table 3; Fig. 4). Second, although *picea* follows the same pattern, the peak is displaced to a mean of around 3 (Fig. 4). Third, *balearica* does not fit the demographic expansion model at all ($P = 0.0001$, Table 3), whereas the deviation from the spatial expansion model is not statistically significant despite being four times larger than for any other population (Table 3; Fig. 4). The mismatch distributions for nuclear marker 2401 showed that the distributions of *balearica*, *sylvestris*, and *halepensis* are significantly different from both the sudden expansion and the spatial expansion model, although the deviations appear small (Table 3, Fig. 4). The mismatch distributions for nuclear marker 12884 did not suggest deviations from the demographic or spatial expansion model.

The Maddison–Slatkin *s*, which estimates the minimum number of migration events, is around 60 using all populations, and when the populations are merged into three groups (Spanish mainland, Northern Europe and *balearica*) it drops to around 21. The lineages-through-time plots indicate a very recent and rapid diversification (Fig. 5A) using the mtDNA data. The scaling with a distribution of mutation rates indicates a median of the start of the expansion of around 3700 years ago, with a 95% interval ranging between 550 and 11 000 years ago (Fig. 5B). The pattern of coalescence was very similar for the nuclear gene 2401 (Fig. 5C), supporting the mtDNA data of a very recent and fast expansion.

Table 3. Results from the mismatch analysis for one mitochondrial and two nuclear loci

	uncinata	*sylvestris*	*halepensis*	*balearica*	*pytyopsittacus*	*picea*	*scotica*
mtDNA control region							
Demographic SSD	0.006	0.005	0.009	**0.42**	0.001	0.007	0.009
P	0.82	0.53	0.54	**0.0001**	0.91	0.47	0.83
Spatial SSD	0.007	0.006	0.010	0.040	0.001	0.006	0.008
P	0.74	0.34	0.57	0.33	0.82	0.60	0.78
Nuclear marker 2401							
Demographic SSD	0.005	0.004	0.006	0.015	0.002	0.054	
P	0.24	*0.025*	*0.040*	*0.020*	0.83	0.25	
Spatial SSD	0.005	0.005	0.006	0.015	0.002	0.054	
P	0.15	**0.007**	*0.017*	*0.012*	0.75	0.23	
Nuclear marker 12884							
Demographic SSD	0.002	0.010	0.002	No variation	0.017	No variation	
P	0.25	0.37	0.31	–	0.37	–	
Spatial SSD	0.001	0.006	0.001	–	0.013	–	
P	0.27	0.52	0.36	–	0.46	–	

'Demographic' and 'Spatial' refer to models of demographic expansion and spatial expansion, respectively. As a measure of fit to the model the sum-of-squared deviations (SSD) is used. Bold figures indicate significant ($P < 0.05$) deviations between data and model predictions after sequential Bonferroni adjustment for multiple testing, while figures in italics indicate values significant only without the adjustment of *P* values.

DISCUSSION

We found a fairly large number of both nuclear and mitochondrial haplotypes and our markers showed good levels of molecular diversity, although nuclear marker 12884 was much less diverse and provided hardly any insight into population structure. F_{ST} values generally were much higher for the mitochondrial control region, and this is probably due to the four times smaller effective population size (haploid, maternal inheritance only), allowing for greater levels of genetic drift. Population structure for the mtDNA marker was quite pronounced (Table 2), and the unrooted nearest-neighbour tree (Fig. 2) gives a good overall view of this result.

This structure allows us to evaluate the support for our two different evolutionary scenarios for the existence of pine-feeding Mediterranean crossbills: a shared ancestry with the northern pine-feeding *pytyopsittacus* and *scotica* that supports resource conservatism, or a shared ancestry with the northern spruce-feeding *picea* that supports a recent and fairly large resource switch. Interestingly, the pattern we encountered does not fit either scenario, as the northern taxa are basically undifferentiated. This strong genetic similarity was also reported by Piertney *et al*. (2001), and their remarkable result is confirmed here after adding more individuals from the continent, adding two nuclear markers, and re-analysis. We found that the mitochondrial haplotypes form a network rather than a distinct tree and showed a pattern typical for a recent expansion with a few very common haplotypes and a star-like pattern of rare ones separated by one or two mutations (Fig. 3). We also found large estimates of gene flow, and estimated a very recent and rapid increase in the number of lineages (Fig. 5). All of these results support that these crossbills form a newly branching group. The whole group seems to have increased quite rapidly in terms of both population size and geographical range recently (Table 3, Figs 4, 5), the estimated timing depending on the method used and estimates of mutation rates. Perhaps our estimates of divergence could be further improved by analysis of microsatellite variation (but see Piertney *et al*., 2001), although it seems most likely that European crossbill populations are not characterized by long-term associations with specific food resources: the present-day associations seem relatively recent. The especially low F_{ST} values among the northern taxa could be due to more recent divergence and/or very large effective population sizes. Alternatively or in addition, there might be sufficient gene flow between these taxa to keep them homogenized (Piertney *et al*., 2001; but see Summers *et al*., 2007). Our attempts to time the divergence of the southern populations point towards recent resource switches, but whether this was from an ancestral pine- or spruce-feeding crossbill remains unknown.

Either way, it is striking that the Mediterranean crossbills are much more genetically structured than the northern taxa. This is especially true for *balearica*, which showed the highest F_{ST} values.

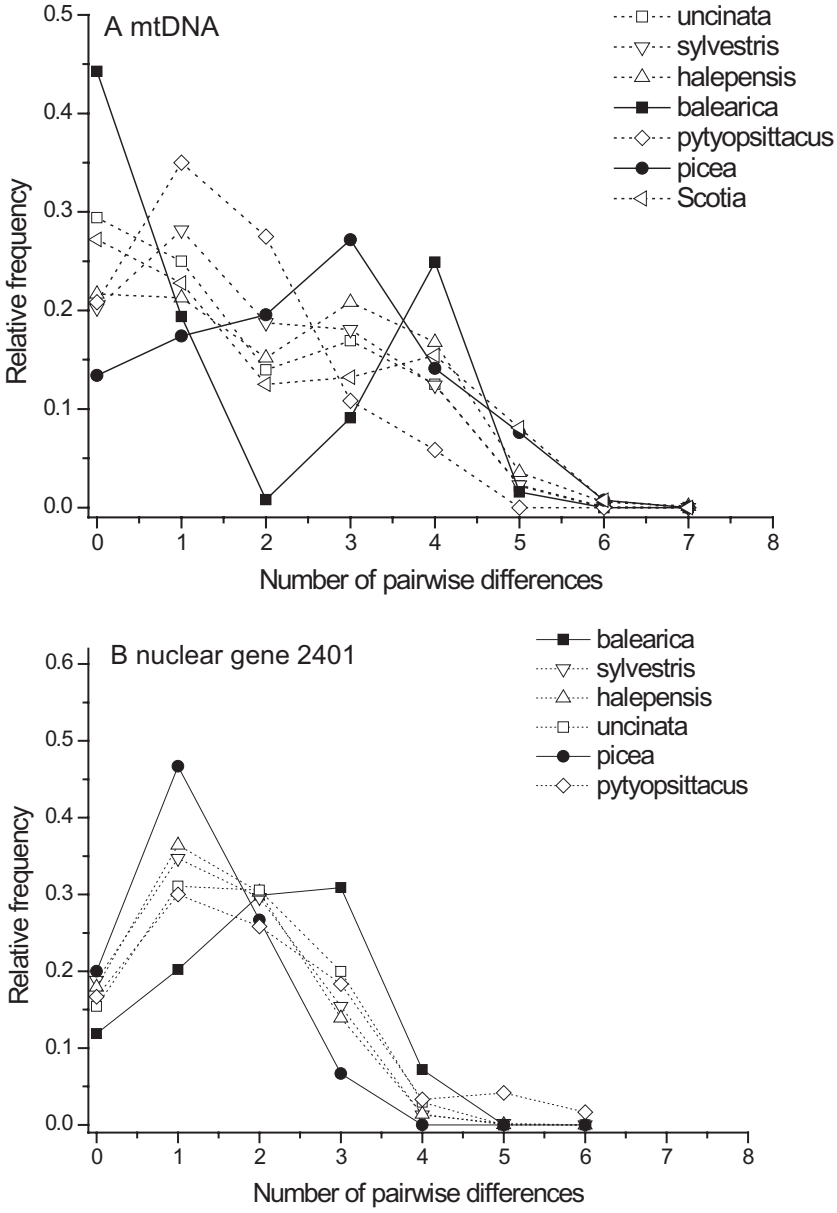

Figure 4. Mismatch distribution of mitochondrial (A) and nuclear haplotypes (B).

Balearica has a reduced genetic diversity, does not fit the demographic expansion model, and does not show high frequencies of private haplotypes. It therefore appears that its higher levels of differential fixation are probably the effect of higher levels of genetic drift in a stable, small population together with demographic isolation. This in turn fits well with its ecology: it is believed to be a resident island taxon which feeds on a pine that has a fairly reliable annual cone crop, and moreover produces closed cones that stay on trees for several years (known as serotony), which evens out any annual fluctuations in cone crop. Our genetic data support that *balearica* is an isolated population, which in turn supports the possibility that this taxon is engaged in a coevolutionary arms race with the pine it feeds on (Mezquida & Benkman, 2005). Interestingly, northern *picea* has been recorded several times to reach the *balearica* population (based on vocalizations), and it is quite probable that it does so frequently and in good numbers (having reached Iceland, North Africa, and even the Canary Islands: Cramp & Perrins, 1994). The quite high levels of differential fixation of *balearica* suggest that these immigrant northern birds effectively do not (or hardly) contribute to the genetic pool of *balearica*, which would suggest that these taxa are to some

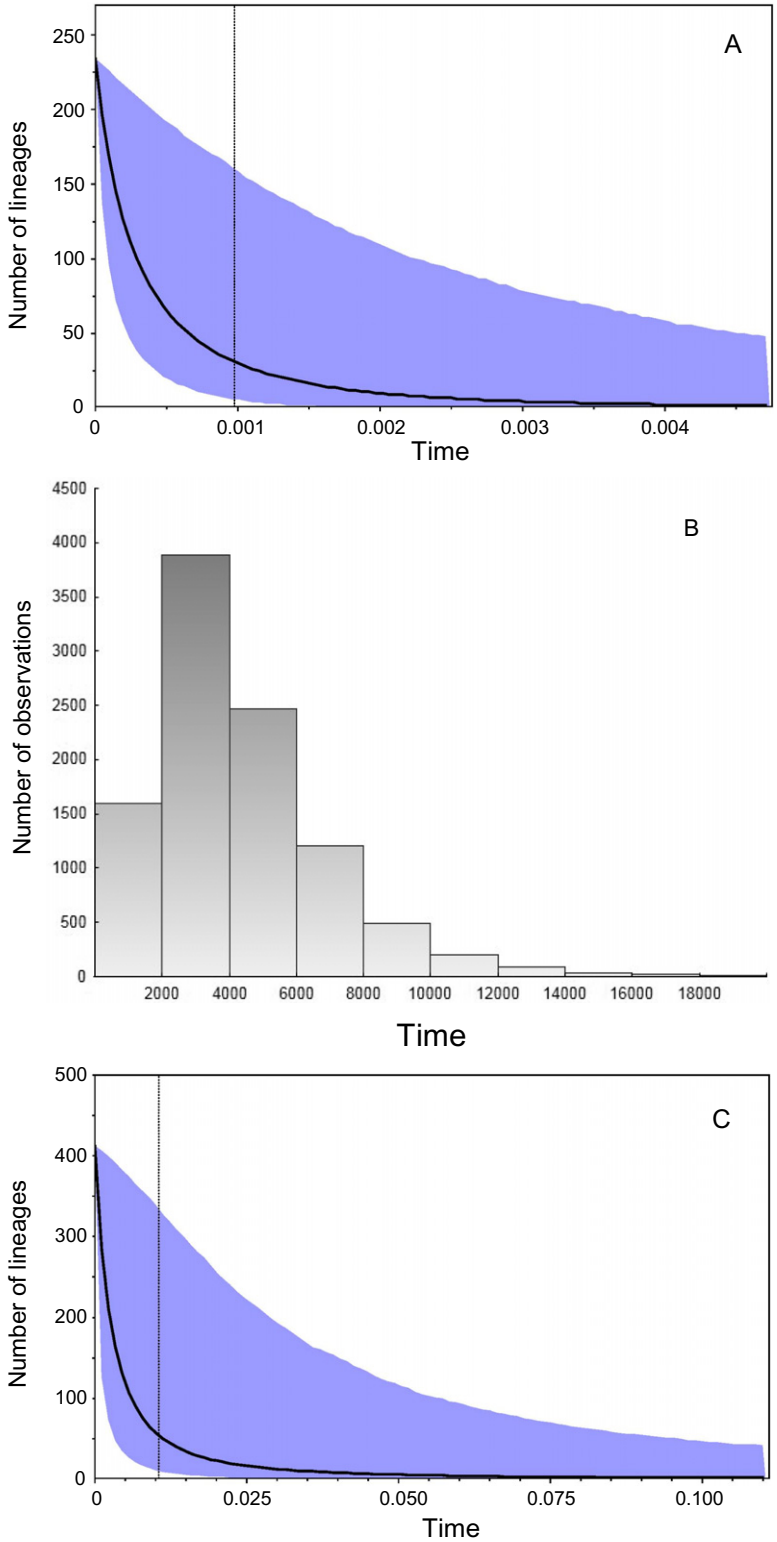

Figure 5. Rates of coalescence of individual haplotypes. A, C, the number of individual haplotypes for the mitochondrial control region and nuclear gene 2401, respectively, in relation to time. The coloured interval is the 95% Bayesian credible interval. Time 0 is the present and the *x*-axis is measured in time before present scaled by mutation rate. The vertical line is the lower 95% tree height. B, histogram of inferred times of coalescence for the mitochondrial haplotypes, taking into account uncertainty in mutation rate estimates.

extent reproductively isolated. This is in line with the conclusion of Benkman (2003) and Edelaar *et al*. (2012) that resource diversity drives crossbill diversity, but more data on abundance and (if it occurs) breeding and mating patterns of northern *picea* on Mallorca are necessary to draw firm conclusions.

It is intriguing that the mainland population that is genetically closest to *balearica* is *sylvestris*, and not *halepensis*, even though *halepensis* and *balearica* use the same species of pine and are also geographically closer to each other (Fig. 1). Although care should be taken when interpreting a tree of a single locus as this may not represent the actual pattern of population divergence (Maddison, 1997), this pattern suggests that the island of Mallorca was colonized by *sylvestris* birds instead of *halepensis* birds. The pine that *sylvestris* feeds on is the most irregular producer of cone crops in Spain and hence *sylvestris* birds are probably more likely to explore new areas in search of food. Also, *sylvestris* has relatively longer wings than *halepensis* (Alonso *et al*., 2006), which increases flying ability. Both factors support that *sylvestris* indeed may have been the founder of *balearica*. If so, it is another example of a fairly recent switch between resources in crossbills.

Compared with the northern taxa, the mainland Spanish populations also are fairly distinct (Table 2, Fig. 2), although to a lesser extent than *balearica*. Several non-exclusive explanations might fit this pattern of higher F_{ST} values: greater residency (Massa, 1987; Senar *et al*., 1993; Cramp & Perrins, 1994; Clouet, 2000) and smaller effective population sizes in Mediterranean populations resulting in higher rates of genetic drift, greater habitat stability at lower latitudes during glacial cycles resulting in greater times since divergence (Parchman *et al*., 2007), and differential adaptation to distinct resources resulting in reduction in gene flow from other Spanish populations (Edelaar *et al*., 2012) and from frequently irrupting *picea*. Regarding the third explanation, it is striking that F_{ST} values are generally higher for comparisons between mainland Spanish populations and *picea* (which is frequently recorded as an immigrant: Tellería *et al*., 1999; Borrás *et al*., 2010) than for comparisons between mainland Spanish populations and *pytyopsittacus* and *scotica* (which have never been recorded in Spain). Hence, despite the greater potential for genetic mixing with *picea*, we do not find a genetic signal of this, which supports some degree of reproductive isolation between all mainland Spanish populations and northern *picea*. As with *balearica*, field data or genetic data from many additional loci are necessary to confirm this tentative conclusion through direct or indirect (simulation) observations, but a similar suggestion of reproductive isolation between *uncinata* and *picea* was made by Clouet (2000), based on the absence of an increase in breeding densities in *uncinata* habitat after a massive irruption of *picea*.

However, the generally low F_{ST} values might also be taken to indicate that there are limitations to speciation in this system. The process of ecological speciation is driven by specialization to different niches, aided by assortative mating and loss of hybrid fitness if populations overlap geographically (e.g. Schluter, 2001; Rundle & Nosil, 2005; Hendry, 2009; Sobel *et al*., 2010). Ecological speciation takes time, but is faster and more likely under allopatric than under sympatric conditions and when niches have a high dimensionality (summarized by Nosil & Harmon, 2009; Nosil, Harmon & Seehausen, 2009). Introgression from irrupting *picea* (basically temporarily changing distributions from allopatric towards sympatric) might homogenize Mediterranean populations and prevent further phenotypic adaptation to distinct resources and the associated evolution of reproductive isolation. In addition, it appears that in some cases the only dimension of ecological separation in crossbills is related to cone size, so it is possible that this is not enough for divergence to proceed to full speciation, as the niche difference and total divergent selection simply is too weak for the evolution of strong pre- or postzygotic reproductive isolation. The end result is some differentiation persisting over time but with considerable and continued mixing of lineages (Gavrilets, 2004; Hendry, 2009; Nosil & Harmon, 2009; Nosil *et al*., 2009).

Overall, our results resemble those of Parchman *et al*. (2006). They used amplified fragment length polymorphism (AFLP) markers to try to understand the genetic relationships between nine morphologically and vocally divergent crossbill forms in the New World. They found significant F_{ST} values between most of the types, but the phylogenetic resolution of the types was very poor (largest bootstrap value was 0.62), even though some of these forms are confirmed to be reproductively isolated (Smith & Benkman, 2007). This suggests that the history of the Western European crossbills is similar to the history of the American crossbills, i.e. a rapid expansion around or after the last glaciation and subsequent specialization on different food sources. This process then leads to a clear ecological, vocal, and morphological differentiation and in some cases (confirmed or suspected) reproductive isolation, but very shallow genetic structure. Crossbills in general seem to specialize, diverge, and speciate rapidly (perhaps aided by but not restricted to geographical isolation and residency) yet show no or little genetic structure. However, the conifer species they specialize upon have in many cases been around in the same areas for much longer times (see, for example, Edelaar *et al*., 2012). This suggests that

in this system ecological speciation may be rampant and rapid (although still largely underrecorded), yet may only result in a transient species flock that is characterized by equally high extinction rates.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Haplotype frequencies (%, sample size in parentheses) per population for the mitochondrial control region.

Table S2. Haplotype frequencies (%, sample size in parentheses) per population for nuclear marker 2401.

Table S3. Haplotype frequencies (%, sample size in parentheses) per population for nuclear marker 12884.