

Population differentiation and restricted gene flow in Spanish crossbills: not isolation-by-distance but isolation-by-ecology

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Abstract

Divergent selection stemming from environmental variation may induce local adaptation and ecological speciation whereas gene flow might have a homogenizing effect. Gene flow among populations using different environments can be reduced by geographical distance (isolation-by-distance) or by divergent selection stemming from resource use (isolation-by-ecology). We tested for and encountered phenotypic and genetic divergence among Spanish crossbills utilizing different species of co-occurring pine trees as their food resource. Morphological, vocal and mtDNA divergence were not correlated with geographical distance, but they were correlated with differences in resource use. Resource diversity has now been found to repeatedly predict crossbill diversity. However, when resource use is not 100% differentiated, additional characters (morphological, vocal, genetic) must be used to uncover and validate hidden population structure. In general, this confirms that ecology drives adaptive divergence and limits neutral gene flow as the first steps towards ecological speciation, unprevented by a high potential for gene flow.

Introduction

A long-standing debate in the study of adaptive diversification concerns the interaction between diversifying selection and homogenizing gene flow (Slatkin, 1987; Nosil, 2008; Räsänen & Hendry, 2008). Theoretical models have now demonstrated that speciation with gene flow is feasible under particular conditions (Gavrilets, 2004; Bolnick & Fitzpatrick, 2007; Van Doorn *et al.*, 2009; Weissing *et al.*, 2011), but to empirically demonstrate that initial divergence and, ultimately, speciation occurred in the face of gene flow has proven more difficult (Nosil, 2008). One approach to address this issue is to focus on the early stages of divergence, when observed patterns of divergence only reflect the effects of the primary factors driving the divergence, and not those

of later, confounding processes (Schluter, 2000; Coyne & Orr, 2004). Therefore, patterns of intraspecific geographical variation in phenotypic traits and genetic markers should provide insight into the factors that drive population differentiation and ultimately contribute to speciation (Avice, 2000; Schluter, 2000; Coyne & Orr, 2004). A typical application of the aforementioned approach tests whether gene flow decreases as populations occupy increasingly different ecological environments (Schluter, 2000; Rundle & Nosil, 2005; Nosil *et al.*, 2008; Thibert-Plante & Hendry, 2010).

Studies of adaptive population divergence benefit from examining the specific ecological variables that determine selection on the traits of interest, but this is not always straightforward (Räsänen & Hendry, 2008). Here we exploit the importance of resource specialization in crossbills to study the effect of differential resource use on crossbill population divergence. Crossbills (*Loxia curvirostra* complex, Linnaeus) are sparrow-sized songbirds that are specialized in foraging on seeds in conifer cones and that use their crossed and curved bills to

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extract seeds from between the scales of closed cones (Benkman & Lindholm, 1991). Cones of different species of conifer differ greatly in structural traits such as mass, size and scale thickness, and this variation impacts upon crossbill feeding rates (e.g. Benkman, 1993). When food is scarce, strong trade-offs in feeding efficiency result in low fitness for birds with maladaptive bills for a particular cone type, and indeed ecologically specialized populations in North America have a bill that is (near) optimal for utilizing a specific conifer as its key resource (Benkman, 1993, 2003). In addition, ecological divergence in these crossbills seems to be coupled to diagnostic flight call divergence (hence these ecologically differentiated populations are also called 'vocal types'; Groth, 1993a). Flight calls appear important in the context of mate choice and reproductive isolation by assortative mating (Groth, 1993a,b; Smith & Benkman, 2007; Snowberg & Benkman, 2007; Summers *et al.*, 2007; Edelaar, 2008a; Keenan & Benkman, 2008; Sewall, 2009).

In this article, we test whether the availability of multiple resource types (different conifer species) reliably predicts population divergence in crossbills, as argued by Benkman (2003) for North America and Edelaar (2008b) for the Himalayan region. Our study is conducted in Spain (Europe), providing an independent test of this hypothesis. Presumably due to a different set of competitors over seeds, the seed phenology and cone structure of European conifers are quite different to those of North American conifers (Farjon, 2005), with unknown consequences for crossbills. In addition, relative to comparable work from North America, we conducted our study on a smaller geographical scale and with a more extensive spatial sampling, so none of the studies from North America have included the spatial component to the detail as we do here. We focus on eastern continental Spain, because in this area four different species of pine occur naturally (see Materials and methods) that represent suitable resources that crossbills should be able to specialize upon (Massa, 1987; Benkman, 1993; Cramp & Perrins, 1994). In addition, these pines have distinctive cones that differ in size, shape and scale thickness (Farjon, 2005; pers. obs.; Fig. 1), so we would expect that selection should promote specialization by crossbills on each different pine (see also Edelaar *et al.*, 2003). Enough time for specialization is expected to have passed, because these pines have been present in Spain for about 6000 years (*P. halepensis*: Gómez *et al.*, 2005; Grivet *et al.*, 2009) or since the Tertiary (*P. sylvestris* and *P. uncinata*: Soto *et al.*, 2010), generation time is about 2 years, and crossbill bill traits are highly heritable (Summers *et al.*, 2007).

However, gene flow between populations might prevent specialization and adaptation (Slatkin, 1987; Räsänen & Hendry, 2008). Crossbills from northern Eurasia are one of the most dispersive vertebrates, with the highest natal and breeding dispersal known for any songbird (on average > 2100 km for both: Newton, 2006; see also

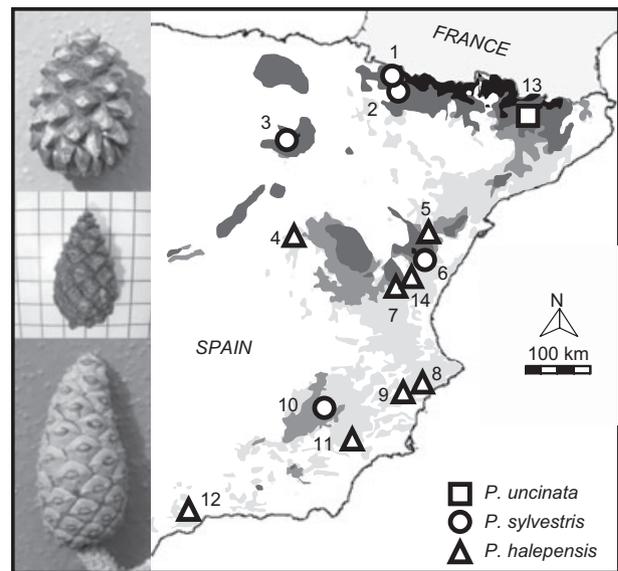


Fig. 1 Native distributions of pines and location of sites where crossbills were sampled. *Pinus uncinata* (top cone) in black, *P. sylvestris* (middle cone) in dark grey, *P. nigra* (not sampled) in medium grey, *P. halepensis* (bottom cone) in light grey. Note that all of the pine species show much geographical overlap or contact with other pine species. Localities numbered as in Table 1. Map based on species maps from the Spanish Forestry Service at <http://www.inia.es/genfored>.

Marquiss *et al.*, 2008). Crossbills in search of good cone crops are known to utilize a variety of conifer species, and also in our study area, crossbills have been observed to move into areas with other pine species after local crop failures (Génard & Lescourret, 1987; Borrás *et al.*, 1996, 2010; Clouet, 2000; Borrás & Senar, 2003; pers. obs.). In addition, invasions originating from Europe north of the Pyrenees (mainly Russia) reach Spain every 3–6 years (Tellería *et al.*, 1999; Borrás *et al.*, 2010). If such population mixing is followed by reproduction, then gene flow should homogenize crossbill populations and prevent differentiation (Slatkin, 1987). In line with this expectation, prior studies support a lack of genetic structure in European crossbills (Questiau *et al.*, 1999; Piertney *et al.*, 2001), so it is not *a priori* clear that adaptive divergence can occur among Iberian crossbills.

On the other hand, even though crossbills can be very mobile, we need to consider that in general Mediterranean crossbills seem to be less dispersive than northern crossbills (Massa, 1987; Senar *et al.*, 1993; Cramp & Perrins, 1994; Clouet, 2000). Therefore, some divergence might in fact occur due to limited gene flow among distant local populations, potentially even unrelated to resource use. To disentangle the effects of resource specialization versus spatial isolation on crossbill divergence, we sampled several spatially separated populations using the same or different pine species as a food resource. We used this data set to test whether

divergence occurs due to reduced gene flow with increased geographical distance, known as 'isolation-by-distance'. We contrast this hypothesis with an adapted version of Nosil *et al.*'s (2008) hypothesis of 'isolation-by-adaptation'. Isolation-by-adaptation is described as the positive correlation between the degree of adaptive phenotypic divergence and the degree of differentiation at neutral loci, which results from the decrease in gene flow due to general barriers as adaptive divergence increases. Hence, phenotypes are used as a proxy for ecological distance among different sampling sites. In our view, this approach is vulnerable to interpretation problems with respect to causality, because limits to gene flow for unrelated reasons will facilitate the phenotypic divergence of populations under divergent selection (as also noted by Nosil *et al.*, 2008, 2009). Therefore, we used the difference between the coniferous resources as a measure of ecological distance between crossbill populations and tested for 'isolation-by-ecology', which is the positive correlation between the degree of ecological divergence and the degree of differentiation at neutral loci, which results from the decrease in gene flow due to general barriers as ecological divergence increases.

As noted previously, crossbills in search of food may feed on several species of pine. For our study area, it has been noted that not all pine species produce good cone crops each year, and that in years of low cone production birds move to other areas in search of better cone crops, either of the same or of a different pine species (Génard & Lescourret, 1987; Borrás *et al.*, 1996, 2010; Clouet, 2000; Borrás & Senar, 2003; pers. obs.). The same is also true for the specialized crossbills in North America (Benkman, 1993; Groth, 1993a), and Benkman (1993) argues that crossbills might not be very faithful to any given resource over much of the year when food is plentiful, but that resource specialization, divergent selection and population divergence mainly occur when food is scarce. This means that the species of conifer a given crossbill is feeding on at a certain time might be a poor predictor of whether that crossbill is actually specialized on that species of conifer. Hence, elucidating an unknown crossbill population structure based on resource use is challenging and may benefit from the use of additional information. We show later that indeed our ability to group crossbills improves when we consider not only tree use but also bill morphology, as confirmed by improved clustering of vocal and neutral genetic variation.

In brief, we report divergence among crossbills utilizing different resources in adaptive morphology, vocalizations and genetic markers. This divergence is not correlated with geographical distance, but is correlated with resource use. This shows that (i) ecology can be a driver of adaptive divergence and limit gene flow, even when the potential for gene flow is very large, and (ii) local variation in suitable conifers can be used to predict the presence of evolutionarily diverged crossbill populations.

Materials and methods

Study area

In the eastern half of Spain, four pine species that are suitable for crossbills occur naturally (Barbéro *et al.*, 1998; Soto *et al.*, 2010): Aleppo pine *Pinus halepensis*, Black pine *P. nigra*, Scots pine *P. sylvestris* and Mountain pine *P. uncinata*. Due to their ecological/climatic preferences, these four pines are found in a gradual cline from south to north (Fig. 1), and in a similar sequence also from sea-level (Aleppo pine) to high altitudes (Mountain pine). Because of Spain's rugged geography and associated variation in local climate, these pines grow in a mosaic-like fashion, and it is common for each pine species to find at least one other species within a fairly short distance and often forming mixed forests (Barbéro *et al.*, 1998; pers. obs.). As such, from a crossbill point-of-view, there seem to be no meaningful dispersal barriers between the different pines (Fig. 1). Palaeobotanical studies suggest that pines have been common in the area throughout the Pleistocene and Holocene, and that they have always lived geographically close/intermixed (reviewed in Rubiales *et al.*, 2010). The main temporal feature has been that during the Holocene the oceanic influence on local climates increased, resulting in a geographical reduction in pines towards the east (our study area) and the virtual disappearance of *P. uncinata* from central Spanish mountain ranges (Rubiales *et al.*, 2010; Soto *et al.*, 2010).

We sampled crossbill populations at 14 localities between 1994 and 2008 (Table 1; Fig. 1), all located in forests composed of a dominant or single pine species. At all localities, pine seeds were available and abundant, and in Spain, crossbills only use the different pine species as regular food sources (Borrás & Senar, 2003), so it is assumed that the captured crossbills were feeding on the locally dominant pine species. For Mountain pine, we obtained equal-sized samples from two localities about 5 km apart, but in view of their proximity, these were pooled in all analyses. Black pine was omitted from the analyses because we only managed to sample six birds from a single site.

Morphology

Data collection

More than 3000 birds were captured with mist nets. Each bird was ringed and its sex and age determined (Svensson, 1992). Because we expect that adaptation to the different cone types will be mainly in bill traits, we measured bill length (± 0.1 mm), bill width (± 0.1 mm) and bill depth (± 0.1 mm; Svensson, 1992). Birds were measured by Daniel Alonso or by Pim Edelaar (Table 1). There was one locality (Table 1) with data from both observers.

Table 1 Details of sampling localities for crossbills. Sample sizes for morphology and genetic markers are given separately (mt, mtDNA; nuc, nuclear genes). Observers are Daniel Alonso (DA) or Pim Edelaar (PE).

Code	Site	Province	Geographical coordinates	Species of pine (genus <i>Pinus</i>)	Year	Sample size	Sample size morphology	Observer mt/nuc
1	Sierra de Uztarroz	Navarra	42°53' N 01°00' W	<i>sylvestris</i>	1994–2008	1325	18/20	DA
2	Sierra de Leire	Navarra	42°40' N 01°08' W	<i>sylvestris</i>	1994–2008	1016	–	DA
3	Sierra de Turza	La Rioja	42°21' N 03°15' W	<i>sylvestris</i>	2007–2008	60	19/20	DA
4	Sierra Tendilla	Guadalajara	40°33' N 02°58' W	<i>halepensis</i>	2006	18	20/20	DA
5	Sierra de Ejulve	Teruel	40°46' N 00°33' W	<i>halepensis</i>	2006–2007	28	17/20	DA
6	Sierra Gúdar	Teruel	40°23' N 00°38' W	<i>sylvestris</i>	2006	43	27/31	DA
7	Sierra de Jabalambre	Valencia	39°53' N 00°58' W	<i>halepensis</i>	2007	110	25/27	DA
8	Sierra de Maimó	Alicante	38°32' N 00°35' W	<i>halepensis</i>	1998–2001+2007	251 + 25	25/25	DA + PE
9	Sierra Salina	Alicante	38°31' N 01°00' W	<i>halepensis</i>	1998–1999	67	–	DA
10	Sierra Bogarra-Prozael	Albacete	38°33' N 02°04' W	<i>sylvestris</i>	1999, 2006	50	7/7	DA
11	Sierra Espuña	Murcia	37°49' N 01°31' W	<i>halepensis</i>	2000	38	–	DA
12	Montes de Málaga	Málaga	36°49' N 04°21' W	<i>halepensis</i>	1996, 2001	118	20/20	DA
13	Eastern Pyrenees	Cataluña	42°10' N 01°32' E	<i>uncinata</i>	2007	27	17/24	PE
14	Sierra de la Espina	Valencia	40°02' N 00°38' W	<i>halepensis</i>	2007	8	–	PE
Total						3184	195/214	

Statistical treatment and standardization of the morphological data

All data were log-transformed prior to analysis to equalize means and variances. The repeated samples of locality 8 were used to correct for observer difference (all traits $P < 0.05$ for fixed factor observer effect). We used MANOVA to remove the effects of additional confounding variables on our biometric data: sex, age, year and month (Groth, 1993a; Edelaar & Van Eerde, 2010), in the presence of an effect of locality. All removed effects were highly significant (all multivariate $P < 0.001$; all univariate effects also significant at $P < 0.05$). Based on the multivariate outlier detection procedure in the software SYSTAT 8.0 (Systat Software Inc., Chicago, IL, USA), 0.62% of the corrected data were omitted.

Statistical analysis of morphological data

As individuals were sampled in different populations (localities) that were grouped as those using the same pine species, we tested for the effect of group and population within group on morphology using nested ANOVA and MANOVA (random factors). We also performed a discriminant function analysis (DFA) with resource type (pine species) as the group indicator, to determine which traits mostly discriminated among groups and to visualize how groups differ from each other.

We tested for correlation of bill morphology with geographical distance (IBD) and with ecology using partial Mantel tests, comparing three matrices of pair wise population distances for bill morphology, geographical distance and resource use. For this, we used the program MantelTester (<http://manteltester.berlios.de>, implementing Bonnet & Van de Peer, 2002). For morphological distances, we used the squared Mahalanobis distances from the DFA on the three bill traits. Geographical distances among populations were obtained using Google

Earth (<http://www.google.com/earth>). As a proxy for ecological distances among resources, we used average scale thickness of the cones, because this measure has been repeatedly shown to have strong impacts on crossbill feeding rates in captivity, on crossbill tree preferences in the wild and on patterns of cone divergence where selection exerted by crossbills varies spatially (reviewed in Mezquida & Benkman, 2010). We used an average value for each pine species calculated from our own data (3.40 mm for 10 populations of *P. halepensis*, 2.99 mm for 2 populations of *P. sylvestris*) and published values (Clouet, 2003; Mezquida & Benkman, 2005, 2010; Clouet & Joachim, 2008). This average value was assigned to all populations utilizing that particular resource, under the assumption that geographical variation in scale thickness between populations of the same conifer species is less marked than variation among conifer species (as it appears to be: pers. obs.). Note that this has a conservative effect on the test, as local scale thickness cannot be used to explain local crossbill variation. We also reran the analysis with ecological distance more coarsely expressed as 0 (same pine species used) or 1 (different pine species used). Significance of the matrix correlations was determined by 10 000 permutations.

Calls

Sample collection

Recordings of flight calls were obtained from caught individuals as well as from those flying past. All recordings were made using a high-quality digital recorder (Sound Devices 722) fitted with a parabola and mono, omnidirectional microphone (DATmic Classic, Telinga Pro). Recordings were available from site 6 (*P. sylvestris*), 8 (*P. halepensis*), 13 (*P. uncinata*) and 14 (*P. halepensis*) (see Table 1; Fig. 1).

Sample treatment and extraction of the vocal data

Several flight calls of each individual were visualized as sonograms, and a representative, good-quality flight call was cut from the recordings using AUDACITY 1.2.6 (<http://audacity.sourceforge.net/>). For released birds, the first flight call given was sometimes slightly deviant from subsequent calls (perhaps due to stress), so these calls were discarded. Sonograms were saved as .wav files with a 32 bit sample rate and a frequency bandwidth of 44 100 Hz. The tracks were uploaded in Luscinia (<http://luscinia.sourceforge.net/>), and the frequency band of the call was manually marked. If a call consisted of more than one element produced by the two sides of the syrinx, all elements were marked separately and 'stitched together' as one syllable. Harmonics were not marked. Using Luscinia, several acoustics statistics were automatically extracted: call duration, overall instantaneous peak frequency, overall peak frequency, peak frequency mean, variance, maximum and minimum, and mean frequency mean, variance, maximum and minimum.

Statistical analysis of vocal data

Similar to the morphological comparisons mentioned previously, to analyse differences in calls between groups utilizing different resources, we performed a DFA using all the call variables given above and with the three different pine species as a grouping variable. We tested by ANOVA which pairs of groups differed from each other for each of the first three discriminant functions (DFs). Because we had little replication of populations within groups, we refrained from testing partial Mantel correlations among differences in calls, geographical distance and ecology as performed for morphological differentiation.

Genetics

Data collection

DNA was collected as a small drop of blood taken from a wing vein and saved individually on FTA cards or in alcohol. We did not collect DNA in populations 9, 11 and 14 (Fig. 1). Furthermore, we *a priori* considered the two Pre-Pyrenean sampling sites 1 and 2 with *P. sylvestris* forest to be too geographically close (about 20 km across homogeneous habitat) to analyse them separately, leaving us with 10 populations for analyses.

We sequenced a 479-bp segment of the 3'-end of the mitochondrial control region for 195 birds (Table 1; GenBank accession no. HQ377552–HQ377746). Total DNA from blood samples was extracted using the standard Chelex (5%) extracting procedure (Maniatis *et al.*, 1982) for samples stored in alcohol or following Smith & Burgoyne's (2004) procedure for samples stored on FTA cards. After extraction, DNA concentration was measured with a spectrophotometer. We used PCR to amplify the target DNA using the universal

passerine primer pair L16743 and H417 (Tar, 1995). The PCR cycle was as follows: 10 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 50 °C and 90 s at 72 °C plus a final 5 min at 72 °C, using (25 µL reactions) 100 µM dNTP, 2 mM MgCl₂, 1 unit AmpliTaq Gold (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) and 25 pmol of each primer. Amplifications were performed on an Eppendorf Mastercycler Gradient with the ramp speed set to the default 3 °C s⁻¹ for PCR amplification and 1 °C s⁻¹ for cycle sequencing. To detect PCR products and to check length of the amplified fragments, we ran 5 µL of each sample on a 2% agarose gel. Before sequencing, PCR products were cleaned with ExoSAP-IT to remove unwanted primers and dNTPs. Cycle sequencing was performed by Macrogen Inc. (Seoul, South Korea) under BigDye™ terminator cycling conditions. The reacted products were purified using ethanol precipitation and run using an Automatic Sequencer 3730xl (Macrogen Inc.). The resulting electropherograms (ABI-files) were checked by eye and aligned using Analysis Software BroEdrr (Hall, 1999).

In addition, for 214 individuals we sequenced about 500 bp of intronic DNA for each of the nuclear markers 2401 (GenBank accession no. HQ377747–HQ377947) and 12884 (GenBank accession no. HQ377948–HQ378155), which are genes located on chromosomes 1 and 28, respectively (Backström *et al.*, 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 earlier.

Statistical analysis of genetic data

To calculate basic statistics such as the number of haplotypes, gene diversity, nucleotide diversity, Tajima's *D* and the number of pair wise differences, we used the software Arlequin 3.11 (Excoffier *et al.*, 2005). In the nuclear sequences, there were variable sites (SNPs) that were totally linked and after collapsing those we had in total 30 presumably unlinked SNPs. We tested for pair wise linkage disequilibrium within each population using Arlequin. After sequential Bonferroni correction (within each population), we found 6% significant cases of linkage disequilibrium, close to the expected 5% of significant tests. Linkage was not common neither consistent among sites or populations, so we treat the different SNPs as effectively unlinked. Note that sampling populations with a hidden structure will elevate the incidence of linkage disequilibrium (Wahlund-effect). As the SNP data consisted of loci where the alternative alleles were rare, we tried to use the data more efficiently in terms of information content by creating haplotypes using the EM-algorithm in Arlequin. By doing this, we can use the information of likely gametic phases across loci in a more efficient way, even if the actual gametic phase is not known (see Excoffier & Slatkin, 1995 for details).

To visualize the relatedness and distribution of mtDNA haplotypes, we constructed a haplotype median-joining network using NETWORK 4.5.1.6 (<http://www.fluxus-engineering.com>) based on Bandelt *et al.* (1999) and Network Publisher 1.1.0.7 (<http://www.fluxus-engineering.com>).

We calculated and tested pair wise and global F_{ST} across resource types for both mitochondrial and nuclear genetic data using Arlequin and GenePop (Raymond & Rousset, 1995; Rousset, 2008), both for pooled populations within resources and by a hierarchical AMOVA. As F_{ST} is negatively related to marker variability (Jost, 2008), this complicates comparisons across markers or populations that differ in variability. We therefore also calculated Jost's unbiased estimate of D (using the equations from Jost, 2008), which takes into account within-population variability. Similarly, AMOVA can be misinformative when only haplotype identity is used and markers differ in variability (Meirns, 2006). We therefore performed AMOVA using both haplotype frequencies and Kimura-2 parameter genetic distances among haplotypes. By using the haplotype frequencies, we partition the variance in haplotype frequencies at different levels, regardless of the difference between the haplotypes. By using genetic distances, we also use the information about the absolute difference between haplotypes, which corrects for differences in marker variability due to variation in mutation rate (Kronholm *et al.*, 2010; Whitlock, 2011; Edelaar *et al.*, 2011) assuming populations have reached mutation–gene flow equilibrium. Where relevant, we combined probabilities over loci using the method of Whitlock (2005).

In a similar fashion as for morphological differentiation, we calculated the independent (partial Mantel) correlations between genetic, geographical and ecological differentiation, using the online program IBD WEBSERVICE 3.16 (Jensen *et al.*, 2005). Matrices used for geographical and ecological distances are the same as described before. For genetic distances, we constructed two * two = four different kinds of matrices of genetic differentiation: two for each type of marker (nuclear haplotypes or mtDNA haplotypes) and two for F_{ST} versus Jost's D as measures of genetic differentiation. Following Rousset (1997) for a two-dimensional sampling design, geographical distance was log-transformed and genetic distance was expressed as $X/(1 - X)$, where X is the metric of genetic differentiation (F_{ST} or D) for each pair wise comparison. Significance was again assessed by 10 000 permutations. Population 10 was omitted from this analysis because of small sample size ($n = 7$).

Results

Morphology

Birds using the different pine species differed significantly in bill morphology ($F_{6,6118} = 5.75$, $P < 0.001$, nested

MANOVA), even when the differences between different populations were taken into account ($F_{21,8784} = 12.03$, $P < 0.001$). The discriminant function analysis of crossbills utilizing different pines showed that the first factor accounted for 96.5% of the variance. The factor structure shows that this dominant discriminant function displays robustness of the bill (standardized factor coefficients: bill depth = 0.78, bill width = 0.37, bill length = -0.68). The squared Mahalanobis distances were significant in all pair wise comparisons (not shown).

Plotting the distributions of the discriminant function scores for the different populations (Fig. 2), two major groups that differ in bill shape are observed: one group with a long and slender bill (low DF scores), and the other group with a short and thick bill (high DF scores). There was virtually no overlap in 95% confidence intervals among populations belonging to these different bill shape groups, but almost complete overlap among populations belonging to the same bill shape group (Fig. 2). The long, slender bill shape was mostly found in populations feeding on *P. halepensis* (similar bill shape as crossbills from the island of Mallorca, where only *P. halepensis* occurs – not shown), whereas the short, thick bill shape was mostly found in populations feeding on *P. sylvestris* or *P. uncinata*. There were, however, three exceptions to this pattern: birds feeding on *P. halepensis* in Teruel and Valencia had bill shapes similar to the populations feeding on *P. sylvestris*, and birds from

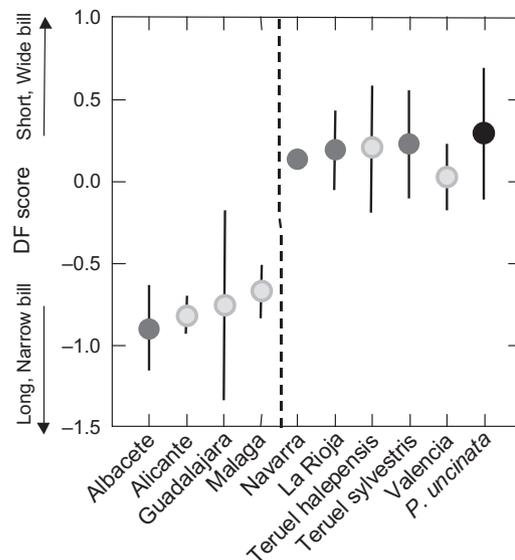


Fig. 2 Crossbill populations fall into two bill shape groups. Plotted are mean and 95% confidence intervals for scores on the dominant root from the canonical analysis based on the three bill traits. Dot colour indicates dominant pine species at catching site: pale grey for *Pinus halepensis*, dark grey for *P. sylvestris* and black for *P. uncinata*. The dashed line separates the two groups. (The confidence interval for Navarra is smaller than the symbol size).

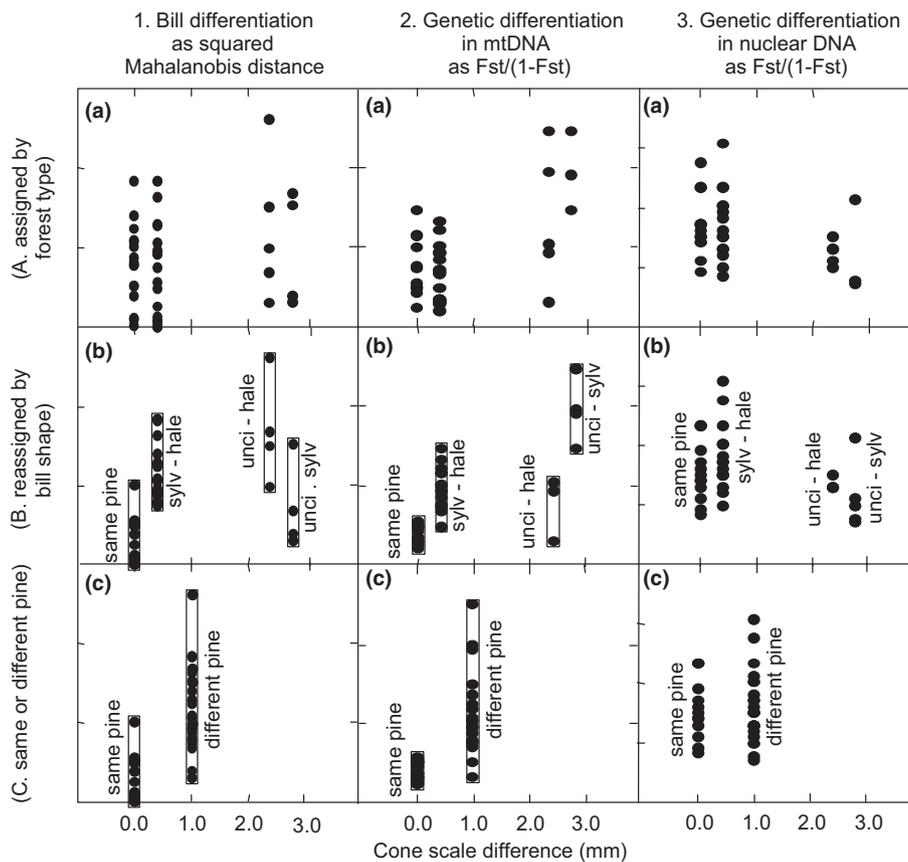


Fig. 3 Patterns of differentiation for all possible pair wise comparisons among 10 crossbill populations utilizing the pine species *P. uncinata* (unci), *P. halepensis* (hale) or *P. sylvestris* (sylv) as a food resource. Plotted are differentiation in bill morphology and two different genetic markers against the differences in cone scale thickness of these pines (0 = same pine). Panels a: initial data grouping based on dominant pine species at the catching site. Panels b: after reassigning three populations to an alternative resource based on bill shape (see Fig. 2 and text). Panel c: as b, but with resources only expressed as being the same (0) or a different (1) pine species. Boxes around data points are used to stress biologically important patterns. Average values of differentiation among populations using different pines are in Table 3.

Albacete feeding on *P. sylvestris* had a typical *P. halepensis* bill shape. As stated in the introduction, this could be due to birds temporarily utilizing abundant resources different from the one they are evolutionarily specialized upon. If this is the case, then just utilizing dominant pine species as the criterion for grouping would introduce assignment error. If indeed crossbills are ecologically specialized to different resources, and bill shape represents a functional adaptation to resource specialization, then we would expect that unrelated traits such as genetics and calls will show a higher degree of differentiation if we incorporate bill shape as an additional distinguishing criterion for assigning populations to a certain resource. Hence, in some of the analyses presented later, we used two alternative classifications: one only according to the dominant pine species where the birds were captured, and one where these three mismatching populations have been reassigned to an alternative resource based on their bill shape. Indeed, when we reassigned these populations to a resource based on

their bill shape, relationships improved. For morphology, recalculating DF scores according to this reassignment changed the factor structure slightly but not its interpretation.

The Mantel tests showed that initially there was a weak correlation between bill morphological and geographical distance (corrected for cone scale distances: $r = 0.27$, $P = 0.07$), but not between bill morphological and cone scale distances (corrected for geographical distances: $r = 0.15$, $P = 0.29$; Fig. 3-1a). Similar results were obtained when the difference in resource use is expressed as 0 or 1 ($r = 0.30$, $P = 0.049$ and $r = 0.06$ and $P = 0.32$, respectively). When the three populations were reassigned to a resource based on their bill shape, the pattern of morphological versus ecological differentiation improved (Fig. 3-1b). However, it is clear that there is no linear correlation between morphological differentiation and ecological differentiation as based on scale thickness. We therefore reran the Mantel correlations on the reassigned data set using only cone difference (0 or 1)

Table 2 Squared Mahalanobis distances (D^2) in vocalizations among crossbills utilizing different pine species as a food resource. Crossbills were allocated to a certain pine species in two ways: based on dominant pine species at the catching site (Naive allocation) or when also taking into account bill shape (Informed allocation – see text). Degrees of freedom are 11 and 77.

Pine comparison	Naive allocation	Informed allocation
<i>P. halepensis</i> – <i>P. sylvestris</i>	4.80 ($P < 0.0001$)	8.19 ($P < 0.0001$)
<i>P. sylvestris</i> – <i>P. uncinata</i>	2.52 ($P < 0.0001$)	4.98 ($P < 0.0001$)
<i>P. uncinata</i> – <i>P. halepensis</i>	4.39 ($P = 0.013$)	3.38 ($P = 0.0002$)

to test for the effect of ecology (Fig. 3-1c). This yielded a strong correlation between morphological and ecological distance (corrected for geographical distance: $r = 0.66$, $P = 0.0002$), but only a very weak and nonsignificant correlation between morphological and geographical distance (corrected for ecological distance: $r = 0.07$, $P = 0.35$).

Calls

Birds using the different pines differed significantly in terms of calls (Wilks' lambda = 0.47, $P = 0.00001$). The Mahalanobis distances among pairs of groups were in all cases significant (Table 2). The two eigenvalues accounted for 64% and 36% of the variance, respectively. A one-way ANOVA showed that the three groups differed in scores on both the first ($F_{2,87} = 25.7$, $P < 0.00001$) and the second root ($F_{2,87} = 14.3$, $P < 0.00001$). When we reassigned the three putatively mismatching populations based on bill shape as mentioned previously, the difference in calls between birds using different pines increased in significance (Wilks lambda = 0.31, $P < 0.00001$, Fig. 4). Accordingly, the Mahalanobis distances among pairs of groups generally became substantially larger, and the P -values smaller (Table 2). Jackknifed classification success also improved remarkably when the reassigned allocation was used for classification: from 50% to 72% for *P. halepensis*, from 50% to 76% for *P. sylvestris* and from 66% to 70% for *P. uncinata* (overall success from 58% to 72%).

Genetics

We found a total of 27 mtDNA haplotypes (Table S1). The number of pair wise differences and nucleotide diversity did not differ obviously between the groups (Table S3). We found 17 SNPs in the nuclear gene 12884 and 21 in 02401. Collapsing linked SNPs left us with 30 unlinked SNPs in total. Mean expected heterozygosity (H) was very low (*uncinata* 14 polymorphic loci, mean $H = 0.059$, $SD = 0.10$; *sylvestris* 17 polymorphic loci, mean $H = 0.055$, $SD = 0.092$; *halepensis* 25 polymorphic loci, mean $H = 0.061$, $SD = 0.088$). There were never more than two alleles at each SNP. The alternative allele

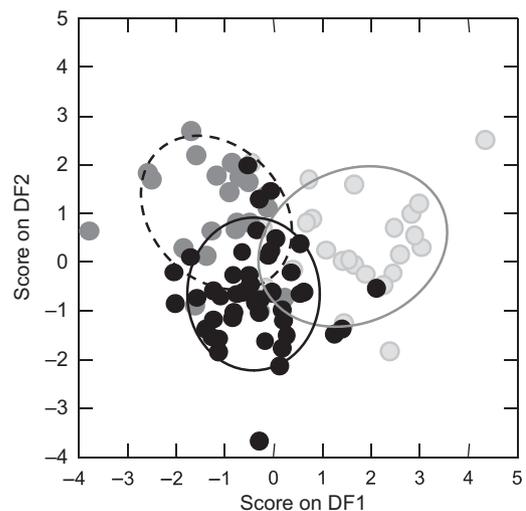


Fig. 4 Crossbills using different pines as a food resource differ in vocalizations, as shown by the clustering by resource of scores on the first and second discriminant function. Results are after allocation to resource is adjusted with respect to bill shape (see Fig. 2 and text).

never reached a relative frequency higher than 0.25, but often was found at relative frequencies well below 0.10. This results in a data set of low power, and almost no structure could be found using the SNPs directly. We therefore used the estimated haplotypes (see Materials and methods). We found in total 56 haplotypes in the nuclear data set (Table S2). The number of pair wise differences and nucleotide diversity did not differ obviously between the groups. The number of pair wise differences was similar to that of mtDNA, but the nucleotide diversity indices were overall smaller (Table S3).

The pair wise F_{ST} values were higher for mtDNA haplotypes than for nuclear haplotypes (Table 3). In general, F_{ST} values were low but significant, except between *P. uncinata* and *P. halepensis*. The values of Jost's D were between 2.3 and 6.7 times higher than the corresponding F_{ST} values but varied mostly in parallel and were again higher for mtDNA than for nuclear DNA. When we combined the two marker types (following Whitlock, 2005), we found that pair wise comparisons were significant except for the difference between *P. sylvestris* and *P. uncinata*, which neared significance (Table 3c).

The mtDNA haplotype network (Fig. 5) shows that haplotypes were closely related and differed mostly by only one, or rarely two, bp from the nearest haplotype. It also shows how haplotypes from each resource are found across the network.

The ANOVA initially found no significant variation at the resource level for either mtDNA or nuclear DNA. However, if the three putatively mismatching populations were reassigned according to their bill shape, a

Table 3 Pair wise F_{ST} (below diagonal) and Jost's D (above diagonal) for crossbills utilizing different pine species as a food resource, for each marker type separately (a, b), and values combined over all markers (c). The 95% confidence intervals determined by bootstrap, and significant results based on exact tests in Arlequin (at $P < 0.05$) in bold.

Pine	<i>P. halepensis</i>	<i>P. sylvestris</i>	<i>P. uncinata</i>
(a) MtDNA haplotypes ($^1P = 0.18$, $^2P = 0.078$, $^3P = 0.0088$)			
<i>P. halepensis</i>	–	0.13 (0.048, 0.34)	0.12 (0.0034, 0.39)
<i>P. sylvestris</i>	0.021 ¹ (0.0093, 0.052)	–	0.30 (0.022, 0.60)
<i>P. uncinata</i>	0.053 ² (0.019, 0.23)	0.10 ³ (0.021, 0.32)	–
(b) Nuclear haplotypes ($^1P < 0.001$, $^2P = 0.28$, $^3P = 0.35$)			
<i>P. halepensis</i>	–	0.033 (0.022, 0.13)	0.025 (0.024, 0.23)
<i>P. sylvestris</i>	0.0049 ¹ (0.004, 0.015)	–	0.024 (0.0087, 0.26)
<i>P. uncinata</i>	0.0055 ² (0.0051, 0.024)	0.0061 ³ (0.0046, 0.031)	–
(c) Combined mean values over markers. P -values are combined probabilities from the two separate tests ($^1P = 1.2 \times 10^{-4}$, $^2P = 0.079$, $^3P = 0.026$)			
<i>P. halepensis</i>	–	0.060	0.092
<i>P. sylvestris</i>	0.009 ¹	–	0.14
<i>P. uncinata</i>	0.020 ²	0.035 ³	–

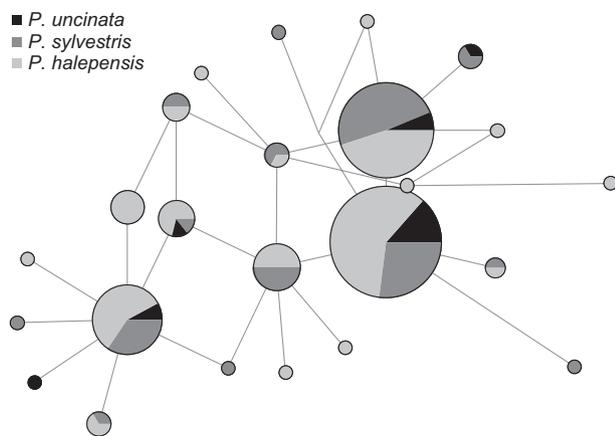


Fig. 5 MtDNA haplotype network, showing haplotype relatedness and haplotype sharing across the groups of crossbills utilizing different pines as a food resource. Dots size increases with sample size, and colour indicates group identity. Lengths of links (mutation steps) are normally one, but are two for the two right-most haplotypes.

significant amount of variation was found at the resource level both for the mtDNA haplotypes (3.61%, $P = 0.013$, using haplotype frequencies) and for the nuclear haplotypes (0.67%, $P = 0.033$, using haplotype distances).

The partial Mantel correlation between log-geographical distance (corrected for the correlation with ecology) and any metric of genetic differentiation was never close to significant in any of the analyses (P -values between 0.21 and 0.95, and correlations often even weakly negative). Partial correlations (corrected for log-geographical distance) between genetic distance and ecological distance as measured by scale thickness differences were never significant for the nuclear DNA haplotypes ($P > 0.86$; see Fig. 3-3a), but were always significant for mtDNA differentiation whether expressed as F_{ST} ($P = 0.041$; see Fig. 3-2a) or as Jost's D ($P = 0.047$).

For the mtDNA data, significance increased remarkably after the three putatively mismatching populations were reassigned to a resource based on their bill shapes (F_{ST} : $P = 0.001$, Fig. 3-2b; Jost's D : $P = 0.003$).

However, from Fig. 3-2b it is also clear that the pattern of genetic and ecological differentiation as expressed by cone scale thickness differences is not linear (as earlier for morphological differentiation). Using only cone difference (0 or 1) to test for the effect of ecology on genetic differentiation, we found a strong correlation between mtDNA genetic distance and ecology (corrected for log-geographical distance: for F_{ST} , $r = 0.63$, $P = 0.002$, Fig. 3-2c; for Jost's D , $r = 0.66$, $P = 0.003$), but no correlation between mtDNA genetic distance and log-geographical distance (corrected for ecology: for F_{ST} , $r = -0.07$, $P = 0.60$; for Jost's D , $r = -0.11$, $P = 0.73$). For nuclear DNA genetic distances, we found no correlation with ecology (all $P > 0.22$, Fig. 3-3c) or geographical distance (all $P > 0.94$).

Discussion

Crossbill divergence: isolation-by-distance versus isolation-by-ecology

We found statistically significant crossbill population structure in relation to differential resource use in all measured aspects: morphology, vocalizations and neutral genetics. The differentiation in bill shape (Figs 2 and 3) provides support for the hypothesis that trade-offs in feeding efficiency on different resources result in ecological specialization and adaptive population differentiation (Benkman, 1993, 2003; see also Massa, 1987; Clouet, 2003; Edelaar, 2008b). The interpretation that this divergence is adaptive and selection-driven is strengthened by the observation that morphological differentiation among populations using different pines is greater than that of populations using the same pine, irrespective of the amount of neutral genetic divergence (Fig. 6,

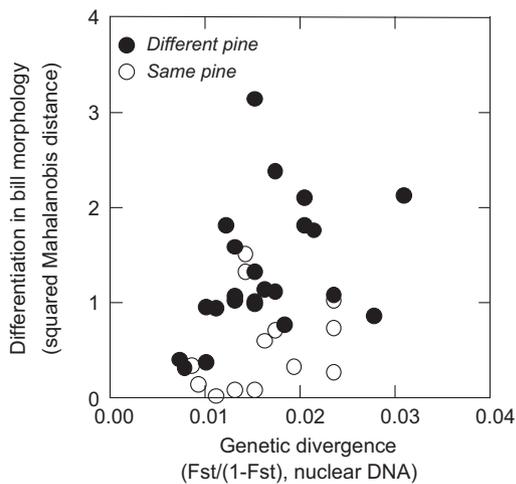


Fig. 6 Crossbill populations utilizing different pines as a food resource had more divergent bills than populations utilizing the same pine, irrespective of the magnitude of neutral genetic divergence.

following Orr & Smith, 1998; see also Edelaar & Björklund, 2011). In addition, the bill depth of the different groups of crossbills differed by about 0.2 mm (average corrected bill depths: 10.76 mm for crossbills using *P. uncinata*, 10.54 mm for crossbills using *P. sylvestris*, and 10.31 mm for crossbills using *P. halepensis*), which is very similar to the difference in bill depth among some ecologically highly specialized North American crossbills (Benkman, 1993, 2003). These results confirm, extend and generalize earlier indications that divergence in morphology among some populations of Spanish crossbills is due to differential resource use (Alonso *et al.*, 2006; Borrás *et al.*, 2008). This result also confirms the results obtained from other geographical areas: if several suitable conifer species are present, evolutionarily diverged crossbill populations are found (North America: Benkman, 2003; Himalayas: Edelaar, 2008b). This leads us to conclude that, as a pattern, ecological opportunity consistently drives crossbill diversification.

Our data show that such diversification is not driven by limited gene flow among geographically distant populations (which could happen if Spanish crossbills are very resident and resources are geographically separated). Based on the partial Mantel correlations, the observed divergence in morphology and neutral genetics is not related to geographical isolation among distant populations. We expected *a priori* that genetic differentiation in Spain, if any, would show a north to south cline, with greatest differentiation thus between *P. uncinata* and *P. halepensis* birds. In fact, these are genetically quite similar, and geographically intermediate birds utilizing *P. sylvestris* appear to be more distinct (Fig. 3-2b). Hence, the observed differentiation of crossbills utilizing different resources is not confounded by the spatial structuring of these resources or our sampling (*P. uncinata* in the

north, *P. sylvestris* more central, *P. halepensis* more in the south). Apparently differentiation was also not prevented by unrestricted homogenizing gene flow among populations utilizing different but neighbouring resources. The greatest genetic differentiation is found between *P. sylvestris* and *P. uncinata* birds, even though the mountain-top, mosaic-like forests of *P. uncinata* are surrounded by forests of *P. sylvestris* (Fig. 1), creating great scope for homogenizing gene flow. This scope for gene flow is not only currently present, but must have been absent historically, because these two species have abutting ecological and climatological requirements, have co-occurred in the region since the Tertiary and have been found to have lived next to each other in the Pyrenees and several central Spanish mountain ranges during the Pleistocene (Barbéro *et al.*, 1998; Rubiales *et al.*, 2010; Soto *et al.*, 2010). The encountered strongest genetic differentiation between *P. uncinata* and *P. sylvestris* birds, despite their long-term parapatric/sympatric occurrence, is perhaps the most convincing exponent of our results that neutral genetic differentiation in mainland Spanish crossbills is not driven by geographical isolation. We therefore conclude that the observed differentiation of crossbills and the partial limitation of gene flow are due to utilization of different, spatially contiguous/overlapping resources, despite a high potential for homogenizing gene flow.

The significant albeit low levels of differentiation in neutral markers (Table 3; Fig. 3) suggests that neutral gene flow is partly limited among these ecologically specializing populations, supporting the evolution of a certain level of reproductive isolation. This result was robust to whether we used scale thickness or cone difference as proxy for ecological difference, whether or not some populations were reassigned to an alternative resource based on bill shape, and whether we used F_{ST} or Jost's D as a measure of genetic differentiation. Finally, the presence of some degree of reproductive isolation is also supported by the divergence in flight calls among the crossbills utilizing different pines (Table S2; Fig. 4), either as a consequence of reproductive isolation (drift/neutral divergence in calls) and/or as a driver of reproductive isolation (assortative mating by calls).

Several mechanisms might contribute to divergence despite the potential for gene flow. Apart from natural selection on feeding performance (Benkman, 1993, 2003), reduced performance on a nonoptimal resource will also reduce an individual's ability to obtain a mate, because condition-dependent sexually selected traits such as song rate, plumage quality or courtship feeding will be negatively affected by a lower food intake rate (see Snowberg & Benkman, 2009 for an example in crossbills). This interaction between ecology, local performance and sexually selected traits can reduce gene flow among differentiating populations, even up to the point that this synergy between natural and sexual selection enables sympatric speciation (Van Doorn *et al.*,

2009; Weissing *et al.*, 2011). On the other hand, depending on their bill traits and local performance, some dispersing crossbills may decide to settle on a given resource, whereas others move on and will settle on a different resource which is better suited for their bill traits. Such Matching Habitat Choice (Edelaar *et al.*, 2008) will bring together phenotypically similar individuals and therefore should promote population differentiation. This is especially so if pair formation and breeding occur within the local habitat, as is sometimes true for crossbills. Hence, the movement of individuals and the mixing of populations do not necessarily need to result in realized gene flow and the mixing of gene pools (Hendry *et al.*, 2001).

Resource use and crossbill divergence

Our analyses of morphology suggested that bill shapes come in two different kinds, which are broadly related to observed resource use. Based on this observation, we interpreted bill shape to represent a functional adaptation to differential resource use. In that case, we apparently observed crossbill populations utilizing three alternative resources, but where each crossbill population is not 100% faithful to the resource they specialize on. Studies from North America show that this is often the case because resource choice depends on the relative profitabilities, and that broader resource use does not preclude specialization on a single resource on evolutionary time scales (Benkman, 1993; Groth, 1993a). By reassigning three putatively mismatching populations to a resource based on their bill shape, we obtained improved discrimination between resource groups for vocalizations (results from DFA and ANOVA) and for genetic markers (results from AMOVA and partial Mantel correlations). In principle, the observation that a given resource was sometimes utilized by birds with a different kind of bill type could suggest that morphology is not related to resource specialization. However, the improved discrimination between groups for both vocalizations and genetics after reassigning such mismatching populations provides independent, multiple support for our initial interpretation that bill shape is indeed related to resource use and serves as an informative additional predictor of resource group membership. As such, these findings are in line with the hypothesis that different cones select for different kinds of bills. They also validate our decision to reassign mismatching populations to alternative resources based on bill shape and show that when actual resource use is not a 100% reliable predictor for long-term resource specialization, other types of information should be utilized to uncover hidden population structure. Finally, these mismatching populations reinforce our claim that movements between resources are not uncommon and that therefore the potential for gene flow is large. In fact, such movements are expected to result in some degree of gene flow, which could explain the rather low levels of

genetic differentiation and the incomplete vocal differentiation we encountered.

We did not find (not shown) genetic isolation-by-ecology when ecological distance was based on adaptive phenotypes (Mahalanobis distances of bills) as a proxy for differences in ecology, as advocated by Nosil *et al.* (2008, 2009). A similar lack of genetic isolation-by-ecology based on bill depth in crossbills was encountered by Parchman *et al.* (2006). This suggests that bill morphology in crossbills is evolutionarily labile, shows very little phylogenetic signal and is prone to convergent evolution. The upshot is that this suggests it is also prone to rapid, unconstrained adaptation to novel resources.

Conclusion

A geographically induced reduction in gene flow is expected to promote adaptive population divergence (reviewed in Räsänen & Hendry, 2008). However, geographical isolation does not have to be a prerequisite for divergence. We encountered a pattern of isolation-by-ecology without isolation-by-distance in Spanish crossbills utilizing three distinct pine species as a food resource. Similar patterns (although often tested using different methodologies) have been found in various taxonomic groups (reviewed in Dres & Mallet, 2002; Bolnick & Fitzpatrick, 2007; Nosil *et al.*, 2008, 2009). Also in birds, differentiation among sympatric or parapatric populations may be less rare than previously believed, and both functional and neutral genetic divergences have now been encountered not only in the absence of a geographical barrier but also in the presence of ecological differences (Grapputo *et al.*, 1998; Parchman *et al.*, 2006; Senar *et al.*, 2006; Ryan *et al.*, 2007; Badyaev *et al.*, 2008; Evans *et al.*, 2009; Rolshausen *et al.*, 2009; De Leon *et al.*, 2010). Together with these studies, our results indicate that (i) ecological opportunity drives biological diversification and that (ii) the potential for gene flow frequently might be less of a hindrance to adaptive divergence and the initiation of reproductive isolation than is often asserted (Coyne & Orr, 2004; Räsänen & Hendry, 2008).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Absolute and relative frequencies of mtDNA control region haplotypes as encountered in Spanish crossbills utilising different pines (genus *Pinus*) as a food resource.

Table S2 Absolute and relative frequencies of inferred haplotypes of nuclear genes 2401 and 12884 as encountered in Spanish crossbills utilising different pines (genus *Pinus*) as a food resource.

Table S3 Mean number and standard deviation of pair wise differences (k) and nucleotide diversity (π) for mtDNA and the derived nuclear haplotypes from the SNP-data.

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