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Habitat area and structure affect the impact of seed predators and the potential for coevolutionary arms races

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Abstract. Both habitat patch size and structure affect the abundance and occurrence of species and thereby can affect the ecology and evolution of species interactions. Here we contrast the level of seed predation and selection exerted by Common Crossbills (*Loxia curvirostra* complex) and red squirrels (*Sciurus vulgaris*) in the extensive mountain pine (*Pinus uncinata*) forests in the Pyrenees with their level of seed predation in two small, isolated forests. Crossbills consumed 5.1 times more seeds in the Pyrenees than in the isolated forests, and six of seven cone traits under selection by crossbills were enhanced in the Pyrenees. In contrast, red squirrels tend to be uncommon in the open mountain pine forests, consuming relatively few seeds in both regions and having limited impact on both mountain pine and the interaction between crossbills and mountain pine. Resident crossbills in mountain pine forests in the Pyrenees have larger bills than in nearby forests, consistent with local adaptation by crossbills and a coevolutionary arms race between crossbills and mountain pine. The mechanisms leading to variation in the interaction between crossbills and mountain pine should be general to many systems because habitat patch size and structure often vary across the range of a species.

Key words: Common [Red] Crossbill; geographic mosaic; geographic variation; Loxia curvirostra*; mountain pine forests; phenotypic selection;* Pinus uncinata*; Pyrenees and Iberian System, Spain; red squirrel;* Sciurus vulgaris*; species interactions.*

Introduction

Much of the diversity of life began as geographic variation within species (Mayr 1963, Coyne and Orr 2004). Consequently, the processes generating such geographic variation are central to our understanding of diversity, especially when those processes can cause speciation. Although adaptation to abiotic factors has clearly contributed to geographic variation (e.g., Huey et al. 2000), there has been increasing emphasis on understanding the role of biotic interactions in generating diversity (Mittelbach et al. 2007). Likewise, there has been an upsurge in the number of studies indicating that geographic variation in the form and strength of coevolution is an important process that can generate geographic variation within species (Thompson 2005) and that such geographically divergent selection can cause speciation (Smith and Benkman 2007).

Geographic variation arising from geographically structured coevolution is well illustrated by studies of snakes and their salamander prey (Hanifin et al. 2008), weevils and the fruits they feed upon (Toju and Sota 2006), and flies and the flowers they pollinate (Anderson and Johnson 2007). Although it is often not known why such geographic variation emerges, a few studies have provided evidence that geographic variation in the occurrence and outcome of coevolution arises because of variation in the distribution of co-occurring species, including competitors that also act as strong selective agents (Benkman et al. 2001, Parchman and Benkman 2002, Craig et al. 2007, Siepielski and Benkman 2007*a*, Parchman and Benkman 2008), alternative hosts (Zangerl and Berenbaum 2003), and co-pollinators (Thompson and Fernandez 2006). In some cases, discontinuities in the distribution of other species or variation in community context arises because of historical or biogeographical factors differentially limiting dispersal (large treeless expanses preventing pine squirrels [*Tamiasciurus* spp.] but not birds from colonizing forests; Benkman et al. 2001, Siepielski and Benkman 2007*a*). In other cases, geographic variation arises because the distributions of some, but not all, interacting species span two adjacent biomes (e.g., prairie and forest; Craig et al. 2007).

Variation in habitat patch size is another general mechanism that may influence the extent to which species interact with the same set of species throughout their ranges. Many species vary in abundance as habitat patch size varies (Connor et al. 2000), and different species have different patch size thresholds below which they do not persist (Hanski 1994). Evidence that such variation affects geographic patterns of coevolution is found in crossbills (*Loxia* spp.) and the pines (*Pinus* spp.) on which crossbills feed. For example, Red

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Crossbill (*L. curvirostra* complex) population density increases logarithmically with increases in the size of isolated forest islands of lodgepole pine (*P. contorta latifolia*) east and west of the Rocky Mountains, USA. This appears to influence the strength of selection exerted by crossbills on lodgepole pine, as evidenced by the correlation between crossbill densities and the extent of seed defenses that deter crossbills (Siepielski and Benkman 2005). Because the average bill size of crossbills in these forest islands increases with seed defenses, this suggests that the extent of coevolution may vary with forest patch area (Siepielski and Benkman 2005). Moreover, only in the largest of these forest islands do we find evidence of speciation (Smith and Benkman 2007). These results are consistent with other studies on birds showing that the smallest islands lack endemic species and that, as island size increases, the proportion of endemic species increases (Price 2008).

Here, we examine the interaction between Common [Red] Crossbills and mountain pine (*Pinus uncinata*) on the Iberian Peninsula to evaluate whether evidence for coevolution is more pronounced in areas of extensive pine forest than in small, isolated forests. In addition, we further investigate whether tree squirrels (1) act to prevent coevolution between crossbills and mountain pine and (2) are important selective agents on the cone traits of mountain pine. In North America, tree squirrels in the genus *Tamiasciurus* are the dominant pre-dispersal seed predators of many conifers (Smith 1970, Benkman 1999, Parchman and Benkman 2002, Siepielski and Benkman 2007*a*), and where they are present they outcompete crossbills and drive the evolution of cone structure (Benkman et al. 2001, 2003, Parchman and Benkman 2002). However, in isolated forest areas that pine squirrels have not colonized, crossbills are resident, maintain relatively abundant populations, and have coevolved with conifers in predator–prey arms races (Benkman et al. 2001, 2003, Parchman and Benkman 2002). In Eurasia and parts of North America, *Tamiasciurus* are absent and tree squirrels in the genus *Sciurus* are the only tree squirrels present. Although the general importance of *Sciurus* as selective agents of conifers and competitors of crossbills is less well understood, some studies indicate that *Sciurus* may exert an important selective impact on cone structure and may outcompete crossbills (Mezquida and Benkman 2005, Parchman and Benkman 2008). Thus, the occurrence of *Sciurus* can give rise to a geographic selection mosaic for conifers and crossbills.

Mountain pine is a high-altitude conifer that is usually the dominant tree species near tree limit. Its natural distribution is mainly restricted to the Pyrenees and the western and central Alps (Fig. 1; Appendix A). This conifer produces abundant and relatively regular annual cone crops (Génard and Lescourret 1986, 1987, Clouet 2000). Such stability in food resources usually allows territorial vertebrate seed predators such as tree squirrels to maintain stable populations (Smith 1970, Wauters et al. 2008). However, previous studies found that seed predation by European red squirrels (*S. vulgaris*) was low in mountain pine forests in the Pyrenees (Lescourret and Génard 1983, 1986), suggesting a minor impact of squirrels on this pine. In contrast, crossbills are resident and relatively abundant in mountain pine forests in the Pyrenees (Génard and Lescourret 1987, Senar et al. 1993, Clouet 2000). Moreover, the characteristic structure of mountain pine cones, with thick apophyses (the part of the cone scale that is exposed in the mature closed cone) and prominent umbos (a prominence on the apophysis of the scales; Marcysiak and Boratynski 2007; Fig. 1), suggests that these traits have evolved as a defense against crossbill predation (Coffey et al. 1999, Benkman et al. 2001, 2003, Parchman et al. 2007). Indeed, crossbills in the Central Pyrenees preferentially forage on mountain pine having thinner-scaled cones, implying that crossbills exert selection favoring trees with thicker-scaled cones (Clouet and Joachim 2008). Moreover, mountain pine cone scales are thicker in the Central Pyrenees where crossbills are common and resident than in a small, low-elevation population near the French Pyrenees associated with peat bogs where crossbills are apparently absent (Clouet 2004). This is consistent with a response to selection exerted by crossbills in the Pyrenees (Clouet and Joachim 2008).

Here we build and extend upon these studies. We first quantify the intensity of seed predation by crossbills and red squirrels in several mountain pine forests on the Iberian Peninsula (Fig. 1). If red squirrels are not important seed predators in these forests, as suggested by previous studies (Lescourret and Génard 1983, 1986), we predicted that crossbills would be the main pre-dispersal seed predator and selective agent driving the evolution of seed defenses. Second, we address the hypothesis that seed predation by crossbills is less in small, isolated forests (presumably because crossbill densities are lower) and, consequently, their selective impact on cone traits should be less than in larger, extensive forests. The southwestern limit of mountain pine includes two relict isolated forests on the Iberian Peninsula (Fig. 1), where crossbills abundances tend to be lower than in the Pyrenees (Borrás and Senar 2003). Consequently, crossbills may exert weaker selection on cone traits in these isolated forests; if so, cone structure should differ from that in extensive forests, where the interaction between crossbills and pines is likely to be stronger (e.g., Siepielski and Benkman 2005). Third, we estimate the form of phenotypic selection exerted by both crossbills and red squirrels on cone traits of mountain pine, and quantify cone traits in extensive forests and in smaller, isolated forests. Based on previous studies, we predicted that differences in cone traits between extensive and small, isolated mountain pine forests would be consistent with variation in the abundances of, and selection exerted by, crossbills. This prediction would be supported if traits that act to deter crossbills, such as thick scales (Benkman et al. 2003, Clouet and Joachim 2008, Parchman and Benkman

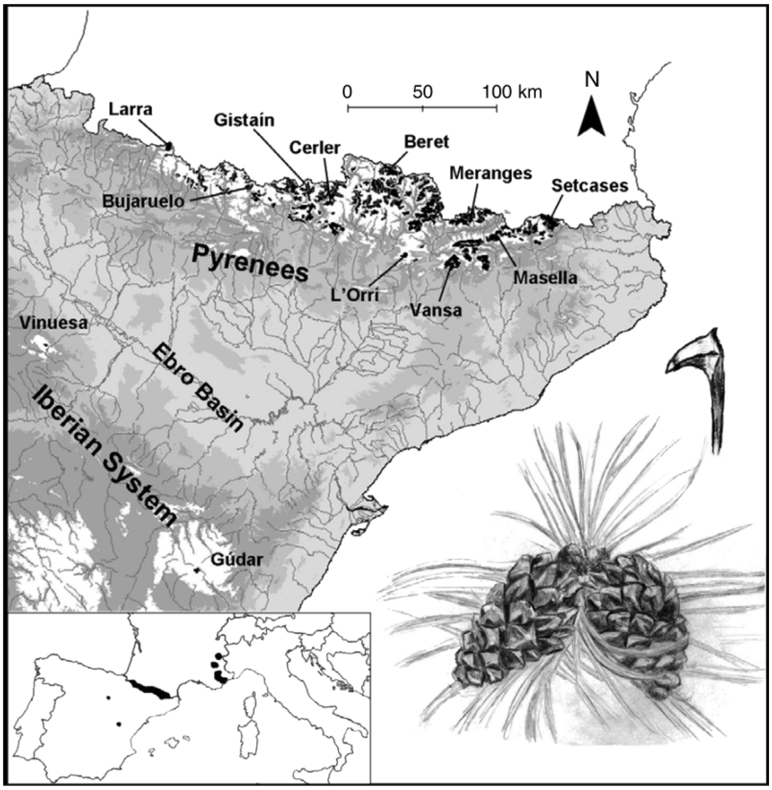

FIG. 1. The location of the study sites, with the distribution of mountain pine in black (based on Ruiz de la Torre [2002]), a drawing of two closed cones from the Pyrenees showing the distinctive thick scales (especially large apophyses), and (above and to the right of the two cones) a lateral view of a scale showing the thick apophysis and the prominent umbo projecting to the left (see *Introduction*). The lower inset shows the European distribution of mountain pine (based on Barbéro et al. [1998]). Mountain pine forests occupy 1550 km^2 on the Spanish side of the Pyrenees, and 4.2 and 4.4 km^2 on Gúdar and Vinuesa, respectively (Ruiz de la Torre 2002).

2008), are enhanced in the extensive forests of the Pyrenees where crossbills are resident and relatively abundant. Finally, we address the alternative hypothesis that differences in cone traits between extensive and small, isolated mountain pine forests are the result of abiotic factors. Specifically, we test whether variation in seed mass among mountain pine forests is consistent with variation in abiotic factors (including topographic, climatic, and geological parameters).

METHODS

Geographic variation in seed predation by crossbills and red squirrels

We quantified seed predation by crossbills and red squirrels in each of 10 mountain pine forests in the Pyrenees and two small, isolated mountain pine forests in the Iberian System (Fig. 1; Appendix B). At each site, we randomly selected 23–50 trees and counted the number of cones depredated by crossbills and squirrels at the base of trees. Common Crossbills bite off cones from the branches, extract seeds by separating and often shredding the cone scales, and then drop the cones (Castro et al. 1999). Red squirrels also remove whole cones from branches, but bite off scales to access seeds and then drop easily recognized cone cores (Mezquida and Benkman 2005). Seed predation by crossbills and squirrels is therefore readily distinguished. We measured seed predation during the shortest time period possible to minimize variation among sites, because our measure of seed predation is cumulative and will only increase during a season. We also measured seed predation as late in the season as possible before snow covered the cones on the ground to maximize the detection of seed predation.

We used Mann-Whitney *U* and Kruskal-Wallis tests to test for spatial and temporal differences in seed predation by crossbills and squirrels. Crossbill predation recorded in 2005 and 2006 at three Pyrenean forests and that recorded in 2006 and 2007 at two forests in the Iberian System were used to test for between-year differences in crossbill predation levels at each site. We analyzed crossbill and squirrel seed predation data from 2006 (and 2005 for three Pyrenean sites; Appendix B) to test whether crossbill and squirrel seed predation differed between the extensive forests in the Pyrenees and the small, isolated forests in the Iberian System. Finally, we used a Wilcoxon matched-pairs test to test for differences in seed predation by crossbills and squirrels at all sites.

Targets and form of phenotypic selection exerted by crossbills and red squirrels

We estimated selection exerted by crossbills and squirrels on cone structure by quantifying seed predation in relation to cone trait variation from 65 trees in Larra in the western Pyrenees (Fig. 1) during mid-October 2007. Although a number of studies on different species have shown that selection can vary from year to year (e.g., Grant and Grant 2002), several lines of evidence indicate that measuring selection at one site during one year is representative of the form of selection exerted by crossbills and squirrels on a given conifer. First, the form of selection exerted by crossbills has been found to be consistent between laboratory and field studies on given conifers (Parchman and Benkman 2008, Benkman and Parchman 2009). A consistent form of selection is expected because crossbills and tree squirrels feed on seeds in conifer cones in stereotypic manners (Smith 1970, Benkman 1987; C. W. Benkman, *personal observation*). Second, measures of phenotypic selection by both crossbills and squirrels from a single site during a single year have consistently provided accurate predictions of cone trait differences between regions that differ in the abundances of crossbills and squirrels (Benkman et al. 2001, 2003, Mezquida and Benkman 2005, Siepielski and Benkman 2007*a*, Parchman and Benkman 2008, Benkman and Parchman 2009).

Trees were chosen haphazardly. For each tree, we recorded the number of cones depredated by crossbills and squirrels. To calculate the proportions of cones foraged on by crossbills and squirrels, we estimated the number of cones remaining on the tree by using binoculars to count the number of cones on one side of the tree (Summers and Proctor 1999). Because we did not have a total count of cones on a tree, these calculations overestimate seed predation but should not bias our estimates of phenotypic selection.

From each tree, we used a branch cutter attached to an extendable pole to collect two mature cones without apparent deformities or signs of insect predation. We measured the following traits for each cone (see Benkman et al. 2003): maximum length and width of the closed cone, number of scales that fall along the vertical axes of the cone, cone mass without seeds, number of full seeds (i.e., filled with female gametophyte), number of empty seeds, mass of five filled seeds without their wings, thickness of five scales in the middle part of the cone, and length of three scales in the middle part of the cone. Because cones of mountain pine are asymmetric (Fig. 1), we were careful to measure scale traits, particularly scale thickness, in similar positions for all measured cones. All length measurements were made to the nearest 0.01 mm with digital calipers. All mass measurements were made to the nearest 0.01 mg with a digital scale after the cones and seeds had been oven-dried for 36 h at 60–70°C. We also calculated a composite variable, the ratio of seed mass to cone mass (a measure of the amount of energy devoted to reproduction relative to seed defense; Smith 1970, Benkman 1999), as the total seed mass in grams (number of full seeds times individual seed mass) divided by cone mass in grams. Cone measurements were averaged for each tree, which was the sample unit used in all statistical analyses unless noted otherwise. Although sampling three or four cones per tree would have been preferable, samples of two cones provide reliable estimates of most cone traits for individual pine trees because variation within trees is much less than variation among trees for most traits (Garcia et al. 2009). Indeed, a sample of 47 mountain pine trees in which four cones were measured (as suggested by Garcia et al. 2009) showed larger overall variation among trees (mean for 10 cone and seed traits: 60.3% ± 3.5% SE) than within trees (39.7% ± 3.5%). These overall percentages are similar to those found for other conifers (Garcia et al. 2009).

To determine the targets of selection by crossbills (direct selection), we used multiple regression models between relative tree fitness in relation to crossbill predation and six cone traits. Tree fitness in relation to crossbill predation was estimated as the inverse of the proportion of cones foraged on by crossbills (number of cones foraged on by crossbills divided by the total number of cones [i.e., all cones foraged on plus cones remaining on the tree]). Individual tree fitness was converted into relative fitness by dividing individual tree fitness by mean population fitness, and cone traits were standardized to zero mean and unit variance. To reduce multicollinearity, we examined correlation coefficients between traits and checked variance inflation factor scores from regression models. We removed the three cone size variables (cone length, width, and mass) because of their high variance inflation factor scores. We used a multiple linear regression model to estimate direct linear selection gradients, and we used a multiple regression model including linear as well as quadratic terms to estimate direct quadratic selection gradients (Lande and Arnold 1983). Nonlinear selection gradients were calculated by doubling quadratic regression coefficients (Stinchcombe et al. 2008).

To determine which traits were under selection due to predation by crossbills (both direct and indirect selection because of correlations between traits), we used least-squares regression analyses between relative tree fitness and each cone trait to estimate selection differentials (Lande and Arnold 1983). We tested for nonlinear selection by examining second-order regression models. We also used cubic splines (Schluter 1988) to further explore the form of selection on cone traits for which the quadratic term was significant. We followed similar procedures and analyses to estimate the targets and form of selection exerted by red squirrels on mountain pine.

Geographic variation in cone traits

For each study site (Appendix C; Fig. 1), we collected recently mature, closed cones of mountain pine during mid-October and early November as previously described. We gathered and measured cones from each of 23–45 trees at each site. One of the measured cone traits, scale thickness, was not measured in the same way in 2005 as in 2006 and 2007. Consequently, mean scale thickness values from 2005 are not included in the analyses of geographic variation. However, scale thickness was remeasured on some cones gathered from one of these sites (Larra) in 2005 and in 2006, and the averaged value was comparable to that of the other Pyrenean sites sampled (6.40 ± 0.12 mm, mean ± SE, $n = 46$ trees; see *Results*).

We used two-level nested ANOVA and principal component analysis (PCA) to characterize cone trait variation between sites in the Pyrenees and those in isolated pine forests, and among sites within each of these two regions. Most variables were ln- or square-root transformed before analyses to improve normality and reduce heteroscedasticity. A composite measure, the ratio of seed mass to cone mass, was also included in the ANOVAs. It was calculated using the total number of seeds instead of the number of full seeds, because seed abortion rates in conifers are influenced by the frequency of outcrossing (e.g., Robledo-Arnuncio et al. 2004). We performed the PCA (based on the correlation matrix) using eight cone traits: cone length, cone width, number of scales, cone mass, total number of seeds, seed mass, scale thickness, and scale length.

Abiotic factors as potential selective pressures on cone traits

To test whether abiotic factors explained geographic differences in seed mass, which is the only cone trait likely to be influenced by abiotic factors (Westoby et al. 1996), we used a general linear model with mean individual seed mass for each site as the response variable and elevation, bedrock type, and the first two principal components extracted from a PCA of 12 climatic parameters as predictors. We obtained the bedrock type for each site from a geological map (IGME 1994). Three general categories were included: limestone, sandstone, and quartzite. We estimated the following 12 climatic parameters for each site: annual, spring, summer, autumn, and winter precipitation in millimeters; annual, summer, and winter average temperature in degrees centigrade; average temperature of the warmest month and average temperature of the coldest month in degrees centigrade; sum of the 12 monthly potential evapotranspiration estimates; and annual water balance (Thornthwaite and Marther 1957), considering 250 mm as the highest value for monthly soil water storage. We used the models developed by Sánchez-Palomares et al. (1999) to estimate these climatic parameters for each site. These models included information from 2605 weather stations, covering the period from 1974 to 1990, and estimate climatic parameters as a function of elevation, geographical position (coordinates x and y of the Universal Transversal Mercator projection, Hayford ellipsoid), and hydrographical basin or sub-basin in which the site is located.

RESULTS

Geographical patterns of seed predation by crossbills and red squirrels

Seed predation by crossbills was 5.1 times higher in the Pyrenees than in the two isolated forests in the Iberian System ($U = 6880.5$, $P < 0.001$; Fig. 2A), and overall seed predation by crossbills was 3.3 times higher than that by squirrels (Wilcoxon matched-pairs test, $T = 7718.5$, $n = 414$ trees, $P < 0.001$). Although seed predation by crossbills recorded in 2006 varied among sites in the Pyrenees (Kruskal-Wallis test, $H = 77.0$, $P < 0.001$; Fig. 2A), the amount of crossbill seed predation did not differ between 2005 and 2006 at three Pyrenean sites (Larra: Mann-Whitney U test, $U = 1096.5$, $P = 0.83$, Cohen's d effect size $= 0.08$; Bujaruelo: $U = 584.5$, $P = 0.10$, $d = 0.54$; Gistaín: $U = 875$, $P = 0.09$, $d = 0.35$). In contrast, seed predation by crossbills tended to vary between years (2006 and 2007) in the two isolated forests (Gúdar: $U = 557.5$, $P = 0.056$, $d = 0.59$; Vinuesa: $U = 544$, $P = 0.041$, $d = 0.46$). These results are consistent with previous findings that crossbills are resident and more abundant in the Pyrenees than in the isolated ranges, where they are more nomadic (Senar et al. 1993, Clouet 2000). Levels of seed predation by squirrels did not differ between the Pyrenees and the two small, isolated forests ($U = 9164.5$, $P = 0.48$; Fig. 2B).

Phenotypic selection exerted by crossbills and red squirrels

The linear multiple regression model including six cone traits (highest correlation between traits, $r = 0.33$) indicated that scale thickness was the target of selection for crossbills, favoring the evolution of thicker scales (Table 1). The model that included the quadratic terms also showed that scale thickness was the target of selection and that the nonlinear selection gradient for this trait was significant (Appendix C). Nonlinear univariate selection differentials were also significant for cone width and mass and for scale thickness and length (Appendix C), although examination of cubic splines indicated that selection on each trait was directional and not stabilizing. This is illustrated for scale thickness in Fig. 3. Selection (direct and indirect combined) exerted by crossbills (Table 1) favored trees that produced larger cones with more, thicker, and longer scales, as well as heavier seeds.

More selection coefficients were significant for crossbills than for squirrels and those for crossbills were, on average, over 16 times larger than those for squirrels (Tables 1 and 2). Multiple regression models suggested that selection exerted by red squirrels favored trees

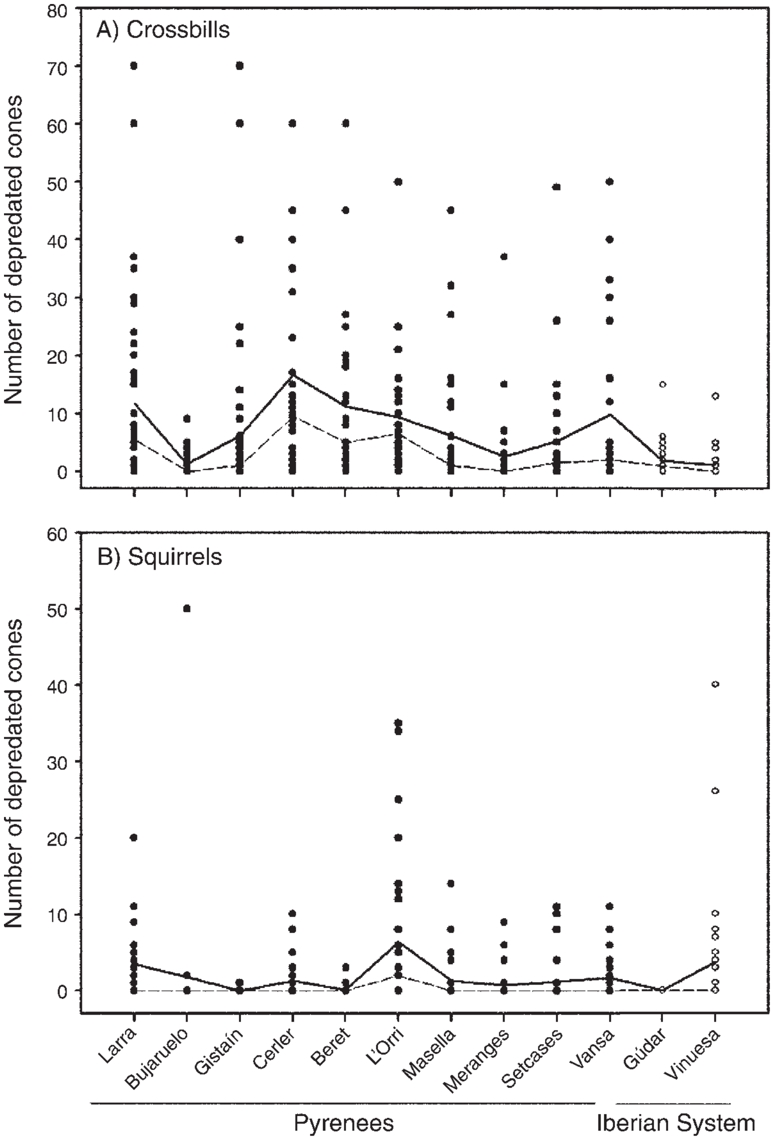

FIG. 2. Number of mountain pine cones foraged on (A) by crossbills and (B) by red squirrels in 10 sites in the Pyrenees (solid circles) and two sites in small, isolated forests in the Iberian System (open circles). The solid lines connect the site means, the dashed lines connect the site medians, and the circles represent one or more trees. Sample sizes at each site ranged from 23 to 50 trees.

having cones with more and shorter scales (Table 2). The number of scales was the only trait that was significantly correlated with fitness in the pairwise linear regressions (Table 2). Nonlinear univariate selection differentials were only significant for the number of full and empty seeds (Appendix D), with cubic splines indicating weak disruptive selection (not shown).

Geographic variation in cone structure

Cones from the Pyrenees were larger and heavier, had more, thicker, and longer scales, and had a lower proportion of seed mass to cone mass than did those from the Iberian System (Table 3; Appendix E). These differences in cone traits between the two regions were consistent with higher levels of predation by crossbills in the Pyrenees than in the Iberian System (Fig. 2), and with the form of selection (direct and indirect) exerted by crossbills (Table 1). Six of the seven traits under selection (Table 1) differed significantly in the predicted direction (Table 3). Scale thickness, which was the target of selection by crossbills (Table 1), was the trait showing the greatest proportional change between regions (24% thicker in Pyrenees than in the Iberian System). Seed mass was the only cone trait under selection by crossbills (Table 1) that did not differ between the Pyrenees and the Iberian System (Table 3). Seed mass was also the one trait that we predicted would be under selection by abiotic factors. Most traits also showed differences

TABLE 1. Multivariate and univariate selection coefficients for phenotypic selection exerted by crossbills on mountain pine (*Pinus uncinata*) in Larra, western Pyrenees, Spain ($n = 65$ trees).

	Multiple linear regression		Pairwise linear regressions	
Cone and seed traits	$\beta \pm$ SE	P	$\beta' \pm$ SE	P
Cone length (mm)			0.226 ± 0.036	<**0.001**
Cone width (mm)			0.218 ± 0.037	<**0.001**
Number of scales	0.041 ± 0.036	0.253	0.101 ± 0.044	**0.026**
Cone mass (g)			0.216 ± 0.037	<**0.001**
Number of full seeds	−0.019 ± 0.036	0.598	0.031 ± 0.046	0.500
Number of empty seeds	0.061 ± 0.036	0.091	0.073 ± 0.045	0.110
Individual seed mass (mg)	0.035 ± 0.038	0.364	0.110 ± 0.044	**0.014**
Seed mass/cone mass			−0.085 ± 0.045	0.062
Scale thickness (mm)	0.194 ± 0.037	<**0.001**	0.227 ± 0.036	<**0.001**
Scale length (mm)	0.072 ± 0.041	0.086	0.160 ± 0.041	<**0.001**

Notes: Multivariate selection gradients were estimated using multiple linear regression (β) for six cone traits (highest correlation between traits, $r = 0.33$). The multivariate full model was significant ($R^2 = 0.51$, $F_{6,58} = 10.1$, $P < 0.001$). Univariate selection differentials were estimated using pairwise linear regressions (β'). Significant values ($P < 0.05$) are shown in bold.

among sites within regions, but these differences generally accounted for less variation than that observed between regions (Table 3).

The first three principal components accounted for 78% of the total variation in cone traits. The first principal component represented variation in cone size (highest factor loadings: number of scales = 0.80, cone length = 0.77); the second principal component represented variation in scale thickness and cone width (scale thickness = 0.91, cone width = 0.74); and the third principal component represented variation in seed mass (0.89). Centroids of mean PC1 and PC2 scores indicated that cones from the Pyrenees were wider and had thicker scales than those from the small, isolated forests, irrespective of cone size (Fig. 4A). A bivariate plot of scale thickness in relation to cone length further indicated that cones from isolated forests were not simply smaller versions of cones from the Pyrenees (Fig. 5); this is consistent with the direction of selection exerted by crossbills and their higher predation intensities in the Pyrenees (Table 1, Fig. 3). The slopes of the regressions between scale thickness and cone length did not differ between the Pyrenees and the small, isolated forests (ANCOVA, $F_{1,261} = 0.62$, $P = 0.43$), but their intercepts did differ (ANCOVA, $F_{1,262} = 45.75$, $P < 0.001$); the Pyrenees had relatively thick scales (Fig. 5). Centroids of mean PC1 and PC3 scores showed that some populations had heavier seeds, regardless cone size, but the differences were not consistent between the two regions (Fig. 4B).

Relationships between abiotic factors and seed mass

The first principal component (PC1) extracted from the PCA of 12 climatic parameters accounted for 51% of the variation and loaded positively by autumn, winter, spring, and annual precipitation, and annual water

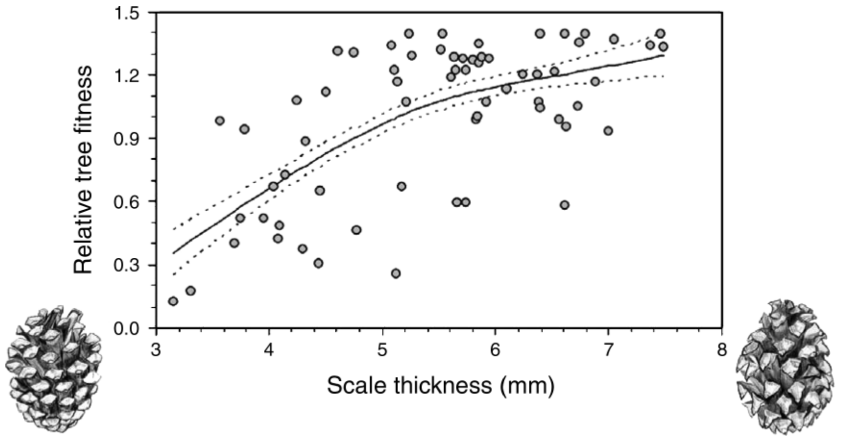

FIG. 3. The relationship between relative tree fitness because of seed predation by crossbills and scale thickness in Larra, western Pyrenees ($n = 65$ trees). Relative tree fitness was estimated as the inverse of the proportion of cones foraged on by crossbills divided by mean population fitness (see *Methods: Targets and form of phenotypic selection exerted by crossbills and red squirrels*). The solid curve represents a cubic spline, and the dashed curves represent ±SE based on 50 bootstrap replicates. The drawings of the two cones represent the variation in scale thickness from thinner scales on the left to thicker scales on the right. The cones are open to aid in the illustration of scale thickness.

Table 2. Multivariate and univariate selection coefficients for phenotypic selection exerted by red squirrels on mountain pine in Larra, western Pyrenees ($n = 65$ trees).

	Multiple linear regression		Pairwise linear regressions	
Cone and seed traits	β ± SE	P	β′ ± SE	P
Cone length (mm)			−0.016 ± 0.035	0.642
Cone width (mm)			0.014 ± 0.035	0.701
Number of scales	0.116 ± 0.035	**0.001**	0.083 ± 0.034	**0.017**
Cone mass (g)			−0.005 ± 0.035	0.891
Number of full seeds	−0.022 ± 0.035	0.537	−0.039 ± 0.035	0.267
Number of empty seeds	−0.026 ± 0.035	0.456	−0.033 ± 0.035	0.349
Individual seed mass (mg)	0.057 ± 0.037	0.130	0.019 ± 0.035	0.593
Seed mass/cone mass			−0.043 ± 0.035	0.217
Scale thickness (mm)	−0.018 ± 0.036	0.616	−0.003 ± 0.035	0.937
Scale length (mm)	−0.082 ± 0.040	**0.045**	−0.058 ± 0.034	0.099

Notes: Multivariate selection gradients were estimated using multiple linear regression (β) for six cone traits (highest correlation between traits, $r = 0.33$). The multivariate full model was significant ($R^2 = 0.22$, $F_{6,58} = 2.7$, $P = 0.022$). Univariate selection differentials were estimated using pairwise linear regressions (β′). Significant values ($P < 0.05$) are shown in bold.

balance (loadings > 0.94). The second principal component (PC2) accounted for 30% of the variation and loaded positively by evapotranspiration, average temperature of the coldest month, winter temperature, and annual temperature (loadings > 0.85). We did not detect significant effects on seed mass for any of the abiotic factors included in the general linear model (elevation: $F_{1,6} = 0.12$, $P = 0.74$; PC1: $F_{1,6} = 0.69$, $P = 0.44$; PC2: $F_{1,6} = 0.92$, $P = 0.37$; bedrock: $F_{1,6} = 0.70$, $P = 0.53$; full model: $R^2 = 0.39$, $F_{5,11} = 0.76$, $P = 0.61$).

DISCUSSION

Our results indicate that mountain pine in the Pyrenees is evolving defenses in response to seed predation and selection exerted by crossbills. In contrast, squirrels appear to have little evolutionary impact on mountain pine and we found no evidence that they alter the interactions between crossbills and mountain pine, presumably because squirrels are uncommon in these high-elevation forests. We will discuss (1) why squirrels are not important seed predators of mountain pine in subalpine habitats, (2) the evidence for a geographic selection mosaic in relation to patch size and isolation, (3) reciprocal adaptations in mountain pine and crossbills, and (4) alternative sources of selection.

Why red squirrels are not important seed predators of mountain pine

European red squirrels mostly rely on conifer seeds from late summer to early spring (Moller 1983, Wauters and Dhondt 1987, Wauters 2000). Consequently, the abundance and year-to-year availability of tree seeds have a large impact on red squirrel populations (Wauters and Dhondt 1987, Wauters et al. 2008). Because mountain pine produces relatively abundant and regular cone crops (Génard and Lescourret 1986, 1987, Clouet 2000), forests of mountain pine (the only tree species in our study areas) should support abundant red squirrel populations. However, we found that the intensity of seed predation by red squirrels, which indirectly reflects their abundance, was low in these forests, supporting earlier studies in the Pyrenees (Lescourret and Génard 1983, 1986).

Mountain pine forests tend to be open (Ruiz de la Torre 2006), particularly at higher elevations (Camarero et al. 2000, Ninot et al. 2008), so that the risk of predation should be high for red squirrels (Lima et al.

Table 3. Mean cone measurements (weighted means) for seven sites in the Pyrenees and two small, isolated sites in the Iberian System.

			Between regions			Among sites		
Cone and seed traits	Pyrenees	Iberian System	$F_{1,7}$	P	% of total	$F_{7,256}$	P	% of total
Cone length (mm)	48.59	44.75	30.7	**<0.001**	12.8	11.9	**<0.001**	23.7
Cone width (mm)	29.55	28.37	8.2	**0.005**	5.5	2.5	**0.018**	4.5
Number of scales	8.97	8.55	9.4	**0.002**	8.4	1.0	0.462	0.0
Cone mass (g)	9.42	7.66	32.2	**<0.001**	17.4	7.9	**<0.001**	15.8
Number of full seeds	38.23	35.62	1.5	0.220	0.0	6.3	**<0.001**	15.2
Number of empty seeds	6.20	6.30	0.3	0.616	0.0	2.4	**0.021**	4.6
Individual seed mass (mg)	9.21	9.50	1.7	0.194	0.0	4.8	**<0.001**	11.4
Seed mass/cone mass	0.043	0.051	17.5	**<0.001**	13.2	2.6	**0.012**	4.6
Scale thickness (mm)	6.55	5.28	71.9	**<0.001**	41.1	3.0	**0.005**	3.7
Scale length (mm)	18.29	17.59	5.9	**0.016**	0.0	7.6	**<0.001**	18.4

Notes: The F and P values are based on univariate two-level nested ANOVAs comparing the two regions and the sites within regions. Significant values ($P < 0.05$) are shown in bold.

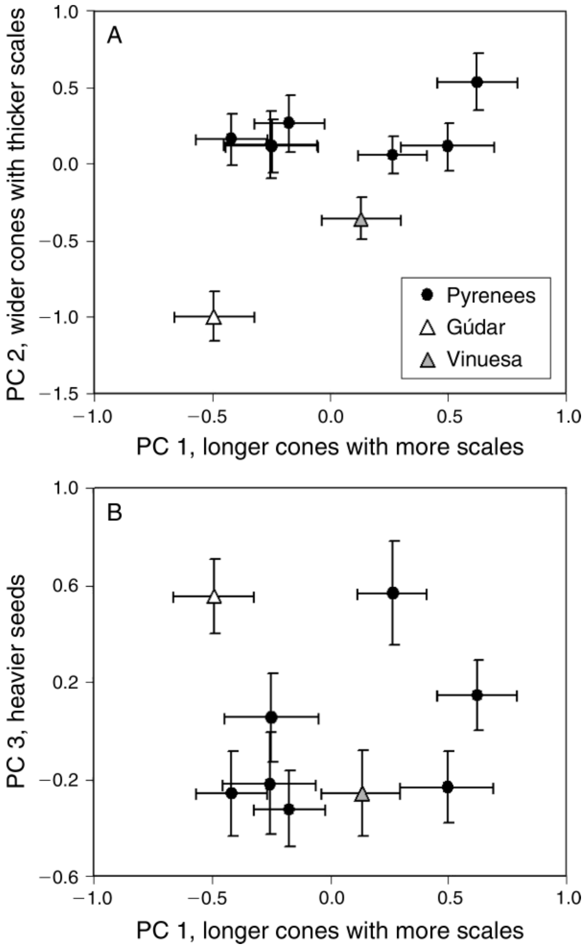

FIG. 4. Variation in mountain pine cone structure among seven sites in the Pyrenees (circles) and two sites in small isolated forests in the Iberian System (triangles) in relation to (A) the first two principal components and (B) the first and third principal components of eight cone traits. PC scores for each site (mean ± SE) are shown. Pyrenean study sites from left to right correspond to: Meranges, Masella, Vansa, Setcases, Cerler, Beret, and L'Orri.

1985, Brown 1999), which use their tree-climbing ability to escape predators. In fact, red squirrels are uncommon in mountain pine forests located at higher elevations and tend to only occupy more dense forests at lower elevations (Lescourret and Génard 1986). Red squirrels are also less abundant in subalpine forests dominated by Scots pine (*Pinus sylvestris*) in southern Spain and arolla pine (*Pinus cembra*) in the Alps than in lower elevation forests (Castro et al. 1999, Wauters et al. 2008). Similarly, red squirrels are more important seed predators in dense than in more open stands of Scots pine (Summers and Proctor 1999). Relatively low red squirrel abundances lead to weak interactions between squirrels and mountain pine, as our results on seed predation and phenotypic selection indicate (Fig. 2, Table 2). Furthermore, as tree spacing increases, other factors such as predation risk are likely to become more important than cone traits in determining tree preferences of foraging red squirrels; this would weaken selection on cone traits. This may explain, for example, why selection by red squirrels was not detected near tree limit in arolla pine, but was detected at lower elevations in Scots pine (Molinari et al. 2006), where tree densities were over 50% higher (Wauters et al. 2008).

Mountain pine evolution in response to phenotypic selection by crossbills

Mountain pine in the Pyrenees shows elevated seed defenses in response to selection exerted by crossbills, which are the main vertebrate pre-dispersal seed predator of mountain pine (Lescourret and Génard 1983, 1986). Scale thickness, in particular, is the target of selection (Table 1) and is the most enhanced cone trait in the Pyrenees relative to the Iberian System. Similarly, crossbills were found to avoid foraging on mountain pine with thicker cone scales in the Central Pyrenees (Clouet and Joachim 2008) and scales were thicker there, on average, than in isolated low-elevation peat bogs, where crossbills do not seem to occur (Clouet 2004). Thick cone scales act to deter crossbills, which use their crossed mandibles to spread apart scales to reach underlying seeds (Benkman 1987). The thicker the scale, the more difficult it is to spread them apart. Large umbos or spines at the tips of cone scales also have been shown to be effective deterrents to foraging crossbills (Coffey et al. 1999); presumably such benefits to mountain pine account for their extremely large apophyses and umbos (Figs. 1 and 3). The effectiveness of thick cone scales is further supported by other studies showing that scale thickness is consistently related to crossbill foraging rates in captivity (Benkman 1987, Benkman et al. 2001, Parchman and Benkman 2002, Benkman and Parchman 2009), crossbill tree preferences in the wild (Summers and Proctor 1999, Benkman et al. 2003, Clouet and Joachim 2008, Parchman and Benkman 2008), and patterns of cone divergence where selection exerted by crossbills varies (Benkman et al. 2001, Parchman and Benkman 2002, 2008, Mezquida and Benkman 2005, Parchman et al. 2007, Clouet and Joachim 2008, Benkman and Parchman 2009).

Variation in the evolution of seed defenses in mountain pine in response to selection exerted by crossbills appears to be due to differences in the level of seed predation by crossbills between the Pyrenees and in both small, isolated ranges in the Iberian System (our study) and low-elevation peat bogs near the French Pyrenees (Clouet 2004). Consistent with our measures of seed predation by crossbills, the small, isolated forest patches of mountain pine seem to support fewer, and probably more nomadic, crossbills (Borrás and Senar 2003) than do the more extensive forests in the Pyrenees, where crossbills are abundant and maintain resident populations (Génard and Lescourret 1987, Senar et al. 1993, Clouet 2000). Likewise, crossbill abundance has increased as the area of isolated lodgepole pine forests

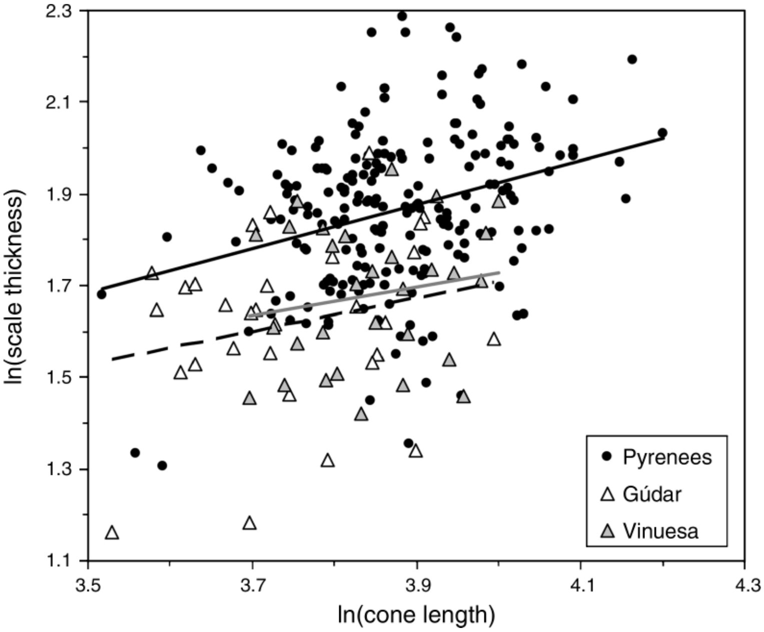

FIG. 5. Scale thickness in relation to cone length (log-transformed data) for cones from the Pyrenees and two small, isolated forests (Gúdar and Vinuesa) in the Iberian System. Each symbol represents the mean of one tree. The lines represent the best-fit linear regressions for trees from the Pyrenees (solid line: $R^2 = 0.10$, $F_{1,203} = 22.2$, $P < 0.001$) and two small, isolated forests in the Iberian System (dashed line for trees from Gúdar and gray solid line for trees from Vinuesa are shown for illustrative purpose; the best-fit linear regression for trees from both sites [$R^2 = 0.07$, $F_{1,58} = 4.2$, $P = 0.044$] has been used for between-areas comparison; see *Results: Geographic variation in cone structure*).

increased in western North America (Siepielski and Benkman 2005). Presumably where tree squirrels are absent or uncommon, variation in forest area and degree of isolation explains much of the variation in crossbill abundance, which in turn determines the levels of seed predation, the strength of selection that crossbills exert, and the differences in cone traits between the Pyrenees and the small, isolated forests in the Iberian System.

How long crossbills have exerted stronger selection on mountain pine in the Pyrenees than in the Iberian System is unknown. However, the large difference in scale thickness between the two regions (24% thicker in the Pyrenees) suggests that differences in selection have occurred for a considerable amount of time. For example, 12–15% differences in scale thickness have been found between coevolutionary hotspots and coldspots (sensu Thompson 2005) for crossbills and conifers in North America, where divergence between areas probably began less than 10 000 years ago (Benkman et al. 2001, Parchman and Benkman 2002). In contrast, a 53% difference in scale thickness of Hispaniolan pine relative to Cuban pine occurs in the Greater Antilles, where coevolution on Hispaniola has driven divergence for about 550 000 years (Parchman et al. 2007). Of course, factors other than time will also influence the extent of divergence, but these patterns suggest that phenotypic divergence between forests in the Pyrenees and in the Iberian System probably began more than 10 000 years ago, which is consistent with recent genetic evidence (Dzialuk et al. 2009). Indeed, such divergence may have been occurring for considerably longer because climatic oscillations associated with major glacial events during the Quaternary led to expansions and contractions of the area occupied by mountain pine (Benito-Garzón et al. 2007), which could have periodically reduced the differences in selection exerted by crossbills between the two regions.

Coevolution between mountain pine and common crossbills

Our characterization of mountain pine cone structure and analyses of phenotypic selection exerted by crossbills, in addition to similar analyses by Clouet (Clouet 2004, Clouet and Joachim 2008), indicate that mountain pine has evolved defenses to deter crossbill seed predation, especially in the Pyrenees. The evolution of thicker scales in mountain pine should, in turn, favor deeper bills in crossbills, as in other conifer–crossbill coevolutionary hotspots (Benkman et al. 2001, 2003, Parchman and Benkman 2002, Mezquida and Benkman 2005, Parchman et al. 2007, Benkman and Parchman 2009). Deeper bills allow crossbills to exert greater forces to separate overlapping scales and retrieve the underlying seeds, and hence to improve feeding performance and ultimately fitness (Benkman 1993, 2003, Benkman et al. 2003). Moreover, reciprocal adaptations between mountain pine and crossbills are expected to evolve because cone traits in conifers, as well as bill structure in crossbills, are known to have high heritabilities (Singh and Chaudhary 1993, Matziris 1998, Summers et al. 2007). As expected, bill depths are significantly greater in crossbills captured in mountain pine forests in the Pyrenees when compared to crossbills captured in black pine (*P. nigra*) forests only 10–60 km away (Borrás et al. 2008). Indeed, the difference in bill depths between crossbills in these two forest types approaches 0.2 mm (Borrás et al. 2008), which is only slightly smaller than the differences between reproductively isolated call types of Red Crossbills in western North America (Smith and Benkman 2007). This suggests local adaptation of (resident) crossbills to mountain pine and coevolution of mountain pine and crossbills in a predator–prey arms race in the Pyrenees.

Other causes of geographic variation

Our study is based on correlative evidence, so we considered alternative hypotheses that could account for the observed differences. Seed mass is a trait that is likely to experience selection by abiotic factors, because seedlings that grow from larger seeds are more likely to survive dry or unfavorable conditions (Westoby et al. 1996). Moreover, climatic variables have been associated with mountain pine recruitment (Camarero et al. 2005, Camarero and Gutiérrez 2007). In addition, seed mass is correlated with other cone traits (e.g., seed mass correlated with cone length, $r = 0.30$; with cone width, $r = 0.30$; with cone mass, $r = 0.27$; with scale thickness, $r = 0.27$; and with scale length, $r = 0.33$). Thus, differences in cone structure could also partly be the result of variation in seed mass. However, we failed to detect an abiotic variable or interaction among these variables that could account for the observed variation in seed mass among sites.

Conclusions

The geographic variation in mountain pine cone structure most likely arises because crossbills interact more weakly with mountain pine in the small, isolated forests than in large forests such as those in the Pyrenees. Because population densities often vary with the size of habitat patches (Connor et al. 2000), as well as between islands and continents (e.g., Buckley and Jetz 2007), we anticipate that the strength of species interactions will commonly vary among habitats of different sizes. In previous studies, it was found that isolated areas sufficiently large to allow crossbills to persist and adapt ($\sim$80–100 km^2 for lodgepole pine; probably larger for most other conifers) provide conditions that favor coevolution between crossbills and conifers (Siepielski and Benkman 2005). As the area of isolated forest decreased, evidence for coevolution declined, apparently because crossbills cannot persist for extended periods of time and their low densities result in weak selection on the conifers (Siepielski and Benkman 2005). In the most extensive forests, other species, tree squirrels in particular, are more likely to be present and dominate the interaction, so that coevolution between conifers and crossbills is suppressed relative to more isolated areas without squirrels (Benkman 1999, Benkman et al. 2001, 2003, Parchman and Benkman 2002, 2008).

Tree squirrels do not appear to exert much selection on mountain pine or to prevent crossbills from coevolving with mountain pine in the Pyrenees. In contrast, tree squirrels are dominant seed predators (and are probably more abundant) in forest types that are less open; there they impede coevolutionary interactions between conifers and crossbills (Benkman et al. 2001, Mezquida and Benkman 2005, Parchman and Benkman 2008) and between pines and the birds that disperse their seeds (Siepielski and Benkman 2007*a*, *b*). Similarly, antagonists dominate and overwhelm mutualisms involving plants where they occur in dense patches, but not where they occur in smaller, low-density patches (McDade and Kinsman 1980, Yu et al. 2001). Because the density and occurrence of many species often vary because of variation in patch size and isolation (Hanski 1994, Connor et al. 2000, Buckley and Jetz 2007) and vegetation structure (e.g., Bro-Jørgensen 2008), our findings, in addition to helping us understand the processes contributing to the diversification of crossbills, should provide guidance for future studies into the processes contributing to the diversification of many taxa.

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APPENDIX A

Photograph of mountain pine cones in the Pyrenees (*Ecological Archives* E091-059-A1).

APPENDIX B

Information on the year, sampling period, and type of data recorded in the study sites in the Pyrenees and the Iberian System (*Ecological Archives* E091-059-A2).

APPENDIX C

Multivariate and univariate nonlinear selection coefficients for phenotypic selection exerted by crossbills on mountain pine in the Pyrenees (*Ecological Archives* E091-059-A3).

APPENDIX D

Multivariate and univariate nonlinear selection coefficients for phenotypic selection exerted by red squirrels on mountain pine in the Pyrenees (*Ecological Archives* E091-059-A4).

APPENDIX E

Mean mountain pine cone measurements for sites in the Pyrenees and the Iberian System (*Ecological Archives* E091-059-A5).