ORIGINAL ARTICLE

COEVOLUTION BETWEEN HISPANIOLAN CROSSBILLS AND PINE: DOES MORE TIME ALLOW FOR GREATER PHENOTYPIC ESCALATION AT LOWER LATITUDE?

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Crossbills (Aves: *Loxia*) and several conifers have coevolved in predator–prey arms races over the last 10,000 years. However, the extent to which coevolutionary arms races have contributed to the adaptive radiation of crossbills or to any other adaptive radiation is largely unknown. Here we extend our previous studies of geographically structured coevolution by considering a crossbill–conifer interaction that has persisted for a much longer time period and involves a conifer with more variable annual seed production. We examined geographic variation in the cone and seed traits of two sister species of pines, *Pinus occidentalis* and *P. cubensis*, on the islands of Hispaniola and Cuba, respectively. We also compared the Hispaniolan crossbill (*Loxia megaplaga*) to its sister taxa the North American white-winged crossbill (*Loxia leucoptera leucoptera*). The Hispaniolan crossbill is endemic to Hispaniola whereas Cuba lacks crossbills. In addition and in contrast to previous studies, the variation in selection experienced by these pines due to crossbills is not confounded by the occurrence of selection by tree squirrels (*Tamiasciurus* and *Sciurus*). As predicted if *P. occidentalis* has evolved defenses in response to selection exerted by crossbills, cones of *P. occidentalis* have scales that are 53% thicker than those of *P. cubensis*. Cones of *P. occidentalis,* but not *P. cubensis*, also have well-developed spines, a known defense against vertebrate seed predators. Consistent with patterns of divergence seen in crossbills coevolving locally with other conifers, the Hispaniolan crossbill has evolved a bill that is 25% deeper than the white-winged crossbill. Together with phylogenetic analyses, our results suggest that predator–prey coevolution between Hispaniolan crossbills and *P. occidentalis* over approximately 600,000 years has caused substantial morphological evolution in both the crossbill and pine. This also indicates that cone crop fluctuations do not prevent crossbills and conifers from coevolving. Furthermore, because the traits at the phenotypic interface of the interaction apparently remain the same over at least several hundred thousand years, divergence as a result of coevolution is greater at lower latitude where crossbill–conifer interactions have been less interrupted by Pleistocene events.

KEY WORDS: Coevolutionary arms race, divergent selection, geographic mosaic of coevolution, *Loxia megaplaga*, *Pinus occidentalis*, predator–prey, speciation, species interactions.

Understanding of the coevolutionary process has grown considerably over the past decade as studies have increasingly sought to account for geographic variation in the form and outcome of species interactions. Much of this growth is owed to Thompson's (2005) geographic mosaic theory of coevolution. This theory posits that geographic variation in species interactions often gives rise to geographic mosaics of coevolutionary hot spots (areas where reciprocal selection occurs) and cold spots (areas where

it does not), and that trait remixing resulting from gene flow and other processes across such mosaics contributes to the overall dynamics and outcome of coevolution (Thompson 2005). Numerous empirical studies have provided evidence that geographic variation in coevolutionary interactions may result in geographically divergent selection causing divergence in the traits important to these interactions (Benkman et al. 2001, 2003; Brodie et al. 2002; Toju and Sota 2006; for review, see Thompson 2005). Studies of trait remixing are fewer, but gene flow is expected to constrain divergence across selection mosaics (Nuismer et al. 1999; Gomulkiewicz et al. 2000). However, when gene flow is sufficiently restricted among coevolutionary hot spots and cold spots, localized coevolution may contribute to phenotypic diversification (Benkman 1999; Buckling and Rainey 2002; Thompson 2005). Indeed, the escalatory changes in traits caused by coevolutionary arms races (Dawkins and Krebs 1979; Abrams 2000) have the potential to cause strong divergent selection between coevolutionary hot spots and cold spots (Benkman et al. 2003) capable of promoting a reduction in gene flow and driving speciation (Smith and Benkman 2007). Nonetheless, although some have inferred the role of coevolution in patterns and rates of diversification (Ehrlich and Raven 1964; Farrell 1998), none have evaluated the process across multiple species in an adaptive radiation. Extending studies of coevolutionary interactions that vary in ecological and historical context across young adaptive radiations may help to elucidate the conditions under which the geographic structuring of species interactions influences diversification.

One well-studied system in which the geographic mosaic may contribute to coevolutionary diversification involves the predator–prey interactions between crossbills (Aves: *Loxia*) and the conifers upon which they specialize. Crossbills have evolved a mandible crossing as an adaptation for prying open conifer cones and feeding on the underlying seeds (Benkman and Lindholm 1991), and have diversified into an array of species and incipient species that are morphologically and vocally differentiated (call types hereafter for red crossbills [*L. curvirostra* complex]; Groth [1993]) specialists on different conifer species (Benkman 1993, 2003). Recent studies provide evidence of coevolution between crossbills and several conifers, and indicate that the geographic mosaic of coevolution generates divergent selection (Benkman 1999; Benkman et al. 2001, 2003). For example, throughout the Rocky Mountains red squirrels (*Tamiasciurus hudsonicus*) are a dominant competitor for and predator of the seeds in lodgepole pine cones, which has evolved defenses in response to this predation (Smith 1970; Benkman 1999; Benkman et al. 2001). However, in areas where red squirrels are absent as preemptive competitors, selection by crossbills has led to the evolution of cone traits that defend seeds against crossbills (Benkman et al. 2001, 2003; Siepielski and Benkman 2005). These areas are coevolutionary hot spots for the crossbill–conifer interaction: conifers here have

evolved generally larger cones with thicker distal scales, and crossbills have evolved deeper bills in response to reciprocal selection. Patterns of bill and cone trait evolution are strikingly replicated among several coevolutionary hot spots for lodgepole pine (*P. contorta latifolia*) (Benkman 1999; Benkman et al. 2001; Edelaar and Benkman 2006) and for black spruce (*Picea mariana*) on the island of Newfoundland where tree squirrels are also absent (Parchman and Benkman 2002). Moreover, divergent selection on crossbills across coevolutionary hot spots and cold spots is capable of driving ecological speciation (Smith and Benkman 2007). This indicates that the geographic mosaic of coevolution has important consequences for crossbill diversification, and that the traits mediating and responding to reciprocal selection generally evolve in a consistent manner. Consequently, these studies can be used to frame and test hypotheses about crossbill–conifer interactions characterized by different ecological and historical contexts.

Because the above-mentioned studies are based on a restricted set of ecological conditions, further studies are needed to determine the importance of coevolution in the adaptive radiation of crossbills. For example, lodgepole pine and black spruce produce cone crops of similar size from year to year and have serotinous or semi-serotinous cones that hold seeds for several to many years (Burns and Honkala 1990). Because they represent highly stable food resources, this allows vertebrate seed predators such as *Tamiasciurus* to maintain stable populations and to be both strong competitors and selective agents on cone structure (Benkman et al. 2001, 2003). This stability has also allowed crossbills to establish resident populations in which tree squirrels are absent, setting the stage for consistent local reciprocal selection in coevolutionary hot spots (Benkman et al. 2003). Most crossbills, however, specialize on conifers that produce variable cone crops from year to year and shed all of their seeds within the first year (Benkman 1993, 2003). Such annual fluctuations in seed production and availability presumably limit local population densities and selective impacts of relatively sedentary predators such as tree squirrels and insects (Hulme and Benkman 2002; Kelly and Sork 2002). However, crossbills track fluctuations in cone crops through nomadic movements (Newton 1972; Benkman 1987a), and therefore may still have strong selective impacts on the cone structure of fluctuating conifers.

Here we evaluate whether crossbills coevolve with fluctuating conifers, and thus whether coevolution is likely to be a general process in the adaptive radiation of crossbills, by testing the hypothesis that the Hispaniolan crossbill (*L. megaplaga*) owes its distinctive bill morphology to coevolution with Hispaniolan pine (*P. occidentalis*) (Fig. 1; Benkman 1994; Latta et al. 2000). It typically produces large cone crops in a given year followed by two or more years of little or no cone production in a given region (Benkman 1994), and thus represents a food resource that varies
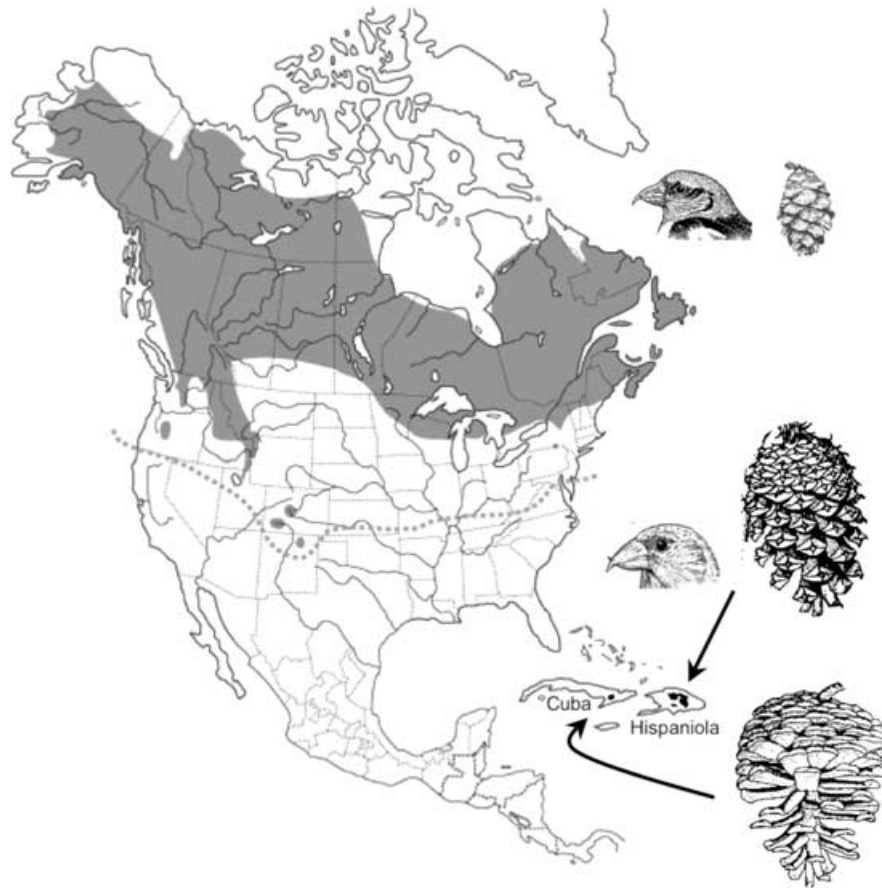
Figure 1. The geographic distributions of *L. l. leucoptera* in North America (dotted line represents how far south it occasionally moves; from Benkman 1992), *P. cubensis* in Cuba, and *P. occidentalis* in Hispaniola. Black spruce (*Picea mariana*) occurs throughout most of the range of *L. l. leucoptera*. Drawings of crossbills and cones are to the right of their respective geographic distributions (*L. l. leucoptera* and black spruce are above *L. megaplaga* and *P. cubensis*) and are drawn to relative scale. *P. occidentalis* and *P. cubensis* cones were drawn by R. Wise and are reproduced from Farjon and Styles (1997).

considerably in local abundance. It is also Hispaniola's sole native cone-bearing conifer.

Examining the interaction between the Hispaniolan crossbill and pine is valuable for two other reasons. First, in previous studies we detected the effects of coevolution between crossbills and conifers by comparing areas with and without tree squirrels. However, many of the differences in cone structure between these areas were the result of selection and relaxation of selection by tree squirrels (e.g., Benkman et al. 2001). We eliminate this potential confounding variable by comparing two species of pines on islands that tree squirrels have not colonized: *P. occidentalis* and its sister species *P. cubensis* (Adams and Jackson 1997; Gernandt et al. 2005) that occur in similar habitats 500 km to the west in the Sierra Maestra, Cuba, where crossbills are absent (Fig. 1). Second, the Hispaniolan crossbill and *P. occidentalis* have co-occurred and potentially coevolved for much longer than the crossbills and conifers that we have previously studied in the North Temperate Zone. Here, crossbill–conifer coevolution began less than 10,000 years ago after conifer and seed predator distributions shifted fol-

lowing the last glacial retreat (Benkman et al. 2001; Parchman and Benkman 2002). The continual rearrangement of conifer distributions in the North Temperate Zone during cyclical Quaternary glacial cycles (Bennett 1990; Jansson and Dynesius 2002) may commonly limit the time periods over which local interactions coevolve before communities and resulting patterns of reciprocal selection are rearranged. In contrast, the distributions of plants and other taxa at lower latitudes, and thus the geographic structuring of their interactions, have generally been less affected by glacial cycles (Colinvaux et al. 1996; Dynesius and Jansson 2000). Genetic data suggest that Hispaniolan crossbills diverged from their probable sister taxa, white-winged crossbills (*Loxia leucoptera leucoptera*) of the northern boreal forests in North America (Fig. 1), to a far greater extent than the above-mentioned call types of red crossbills, which are polyphyletic and display only subtle allele frequency differentiation (Parchman et al. 2006). In addition, pines on Caribbean islands have had their present distributions for much longer time periods than those in northern habitats (Adams and Jackson 1997). These findings indicate that Hispaniolan crossbills

and pines have had the potential to coevolve for a considerably longer time (e.g., $\gg$10,000 years) than crossbills and conifers in previous studies. This may also, for example, account for *P. occidentalis*, *P. cubensis*, and the Hispaniolan crossbill evolving into and being recognized as distinct species.

Our goals were to determine (1) if resource variability limits crossbill–conifer coevolution, (2) if the same traits continue to experience and evolve in response to reciprocal selection in the absence of preemptive competitors, and (3) if greater levels of trait divergence driven by coevolution characterize an interaction occurring at lower latitude and persisting for a longer period of geologic time. Greater trait divergence would be expected if, for example, the same traits remain the targets of reciprocal selection over such longer periods of time. First, we quantify variation in the cone and seed traits of *P. occidentalis* and *P. cubensis*, and interpret this in the context of previously conducted analyses on the form of phenotypic selection exerted by crossbills to test the hypothesis that *P. occidentalis* has evolved defenses directed at crossbills. We then evaluate whether the large-billed Hispaniolan crossbill (Fig. 1) evolved in response to reciprocal selection for foraging on *P. occidentalis*. Because high heritabilities are known for the cone and seed traits of conifers (Verheggen and Farmer 1983) and for the bill structure of crossbills (Summers et al. 2007), evolutionary responses to selection should occur. Finally, we use sequence variation spanning three mitochondrial genes to produce a phylogenetic hypothesis for the genus *Loxia* and to estimate the time since the Hispaniolan crossbill diverged from its sister taxa as an estimate of the upper limit of time crossbills have been isolated and have potentially coevolved on Hispaniola.

Methods

ARE DIFFERENCES IN CONE TRAITS BETWEEN *P. OCCIDENTALIS* AND *P. CUBENSIS* CONSISTENT WITH SELECTION EXERTED BY CROSSBILLS?

We compared cone and seed traits of *P. occidentalis* and *P. cubensis* to determine if the traits enhanced in *P. occidentalis* were the ones consistently found to be under selection (direct or indirect) by crossbills in previous studies. Selection was estimated using univariate selection gradients (Lande and Arnold 1983) that were based on the relationship between relative tree fitness quantified in the wild, and the cone and seed traits of *P. contorta latifolia* in the Little Rocky Mountains, Montana (Benkman et al. 2003), *P. ponderosa scopulorum* in the Black Hills, South Dakota (T. Parchman and C. W. Benkman, unpubl. data), and *P. nigra* on the island of Cyprus (T. Parchman and C. W. Benkman, unpubl. data). Relative tree fitness was estimated as inversely related to the proportion of cones foraged on by crossbills (crossbills are exclusively conifer seed predators) and standardized by the mean fitness of trees in the population.

We collected *P. occidentalis* cones during January 2004 in Sierra de Bahoruco ($n = 27$ trees) and Valle Nuevo national parks ($n = 22$ trees) in the Dominican Republic. Cones of *P. cubensis* were sampled during August 2005 at two sites in the Sierra de Nipe at Estación Pinares de Mayarí ($n = 18$ trees) and El Gurugú ($n = 22$ trees) in Cuba. Five to 10 mature, closed cones without apparent deformities or insect damage were collected from trees chosen haphazardly under the constraint that they could be reached with a nine-meter extendable pruning tool. The following measurements were made on two cones from each tree: cone length, cone width at the widest point of a closed cone, cone mass, length (measured from the scale tip to the distal end of the seed scars) and thickness of three scales from the middle third of the cone, mass of five seeds filled with kernel (female gametophyte and embryo), number of full seeds, and number of empty seeds (seed coat developed but lacking kernel). Measurements were taken in a standard manner (see Benkman et al. 2003 for further details). The mean values of the cone traits measured for each tree were used in subsequent analyses because trees were the experimental units. Because cones of *P. cubensis* had opened prior to arriving in our laboratory, several traits were measured differently for this pine. Instead of counting the number of seeds from individual cones, the entire number of seeds was counted for cones from each tree and divided by the total number of cones obtained for that tree. Cones of *P. cubensis* were reclosed with moisture after all other measurements were taken to measure cone width.

Two-level nested analysis of variance (ANOVAs) were conducted using proc GLM in SAS version 9.1 (SAS, Cary, NC) on log-transformed data to test for differences in the cone traits between pine species and among sites within species. The assumption of normality was met for residuals for all variables (Shapiro-Wilk's test), but variances were often unequal between *P. occidentalis* and *P. cubensis* (inspection of residual plots and Levene's test). Because there were no problematic patterns evident in residual plots (e.g., variance increasing as a function of the mean), we analyzed data with standard two-level nested ANOVAs. To determine the traits most strongly distinguishing the cones of *P. occidentalis* from those of *P. cubensis,* we used discriminant functions analysis based on seven cone and seed traits (cone length, width, and mass; scale length and thickness; seed mass; and total number of seeds). In the above multivariate analyses, we used the total number of seeds instead of the number of full seeds because the number of full seeds is influenced by the frequency of out-crossed pollen (Smith et al. 1988).

HAS THE HISPANIOLAN CROSSBILL EVOLVED IN RESPONSE TO INCREASED SEED DEFENSES?

To evaluate whether morphological evolution in the Hispaniolan crossbill is consistent with adaptation in response to reciprocal selection resulting from increased defenses in *P. occidentalis*, we

characterized patterns of bill depth and length variation in crossbills. Measurements were made of wild-caught and museum specimens (all made by CWB; see Benkman et al. 2005 for methods and repeatabilities) of Hispaniolan crossbills, New World white-winged crossbills (*L. l. leucoptera*), Old World two-barred crossbills *(Loxia leucoptera bifasciata)*, and six call types of New World red crossbills (Groth 1993; Benkman 1999). Because males are generally larger than females and sample sizes differ between the sexes for some of the call types, we present weighted means of the male and female bill depths and bill lengths. Total sample sizes are as follows for each taxon: Hispaniolan crossbill *L. megaplaga* (*n* = 3)*, L. l. leucoptera* (*n* = 107), *L. l. bifasciata* (*n* = 6), call type 2 (bill depth *n* = 155, bill length *n* = 133), call type 3 (bill depth *n* = 44, bill length *n* = 43), call type 4 (*n* = 12), call type 5 (bill depth *n* = 34, bill length *n* = 30), call type 8 (*n* = 12), and call type 9 (bill depth *n* = 141, bill length *n* = 140).

PHYLOGENETIC HYPOTHESIS OF CROSSBILL RELATIONSHIPS AND ESTIMATION OF DIVERGENCE TIMES

We used DNA sequences from three mitochondrial genes (cytochrome b [1242 bp], control region [1140 bp], and NADH subunit six [519 bp]) previously deposited in GenBank (J. Groth, unpubl. data) to generate a phylogenetic hypothesis for the genus *Loxia* and to date the divergence of the Hispaniolan crossbill. We used sequences representing the Hispaniolan crossbill, *L. l. leucoptera*, and *L. l. bifasciata* (GenBank accession numbers AF171661, AF171660, and AF171655, respectively), sequences representing three North American red crossbill taxa (call type designations following Groth [1993] in parentheses), *L. c. pusilla* (call type 2) (AF171662), *L. c. minor* (call type 3) (AF171658), and *L. c. stricklandi* (call type 6) (AF171663), one Old World red crossbill, *L. c. curvirostra* (AF171657), and the hoary redpoll *Carduelis hornemanni* (AF171659) for use as an outgroup. The sequences were easily aligned manually due to the low variation among taxa and the lack of insertion and deletion events.

We performed combined analyses with sequences partitioned by codon position and allowed variable rates at each position, because the three DNA sequences share the same genealogical history and we failed to reject the null hypothesis of partition homogeneity (*P* = 0.20) with sequences partitioned by gene using PAUP (Swofford 2003). We estimated phylogenetic trees using Bayesian methods implemented in MrBayes (Huelsenbeck and Rhonquist 2001), and with parsimony and maximum likelihood using PAUP. For Bayesian and maximum-likelihood analyses, we used the general time reversible (GTR) model of sequence evolution with among site rate variation following a gamma distribution. Because likelihood ratio tests (Felsenstein 1981) failed to reject the hypothesis of a molecular clock (χ^2 = 6.75, df = 7, *P* =

0.34), we enforced the molecular clock where appropriate in phylogenetic analyses. Clade support was evaluated with posterior probability values from the Bayesian analysis and 1000 bootstrap replicates from the parsimony analysis.

We calculated the percent divergence among sequences representing the Hispaniolan crossbill and *L. l. leucoptera* for the three genes combined. Sequence divergence was converted to absolute time using a conventional avian mtDNA clock of 2% sequence evolution between a pair of lineages per million years, which is commonly applied in studies of avian mtDNA (Lovette 2004). Although rate variation is known for avian mtDNA (Garcia-Moreno 2004), and we are aware of problems with molecular clock-based dating, this approach should be appropriate for our goal of roughly estimating the upper limit of time since the Hispaniolan crossbill split from *L. l. leucoptera*. The use of a different calibration or clock rate (e.g., 2.12% [Fleischer et al. 1998]) would not affect the qualitative nature of our results.

Results

ARE DIFFERENCES IN CONE TRAITS BETWEEN *P. OCCIDENTALIS* AND *P. CUBENSIS* CONSISTENT WITH SELECTION EXERTED BY CROSSBILLS?

Univariate regression analyses from other studies indicate that the form of phenotypic selection exerted by crossbills was consistent across several traits for *P. contorta latifolia*, *P. ponderosa*, and *P. nigra*, favoring the evolution of longer, wider, and heavier cones with thicker and longer cone scales (Table 1).

Two-level nested ANOVAs revealed significant differences in some, but not all, of the cone traits of *P. occidentalis* and *P. cubensis*. Cones from *P. occidentalis* were wider and had thicker scales (Fig. 2), but were lighter and had shorter scales and lighter

Table 1. Cone traits under selection from crossbills in previous studies of *P. contorta latifolia* (Benkman et al. 2003), *P. ponderosa* (T. Parchman and C. W. Benkman, unpubl. data), and *P. nigra* (T. Parchman and C. W. Benkman, unpubl. data). Pluses or minuses refer to increases and decreases, respectively, predicted to occur in the traits in response to selection exerted by crossbills and the observed trait changes in *P. occidentalis* with respect to *P. cubensis* (Table 2; using sequential Bonferroni correction). Traits detected as under selection in laboratory experiments but not in the wild are indicated in parentheses.

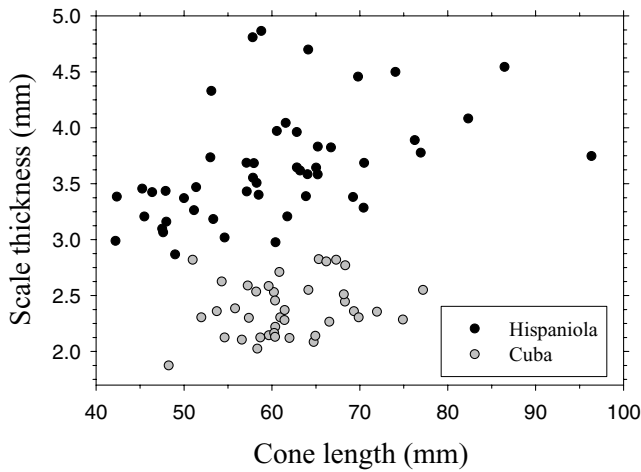| Cone trait	*P. contorta latifolia*	*P. ponderosa*	*P. nigra*	Change in *P. occidentalis*
Cone length	+	(+)	+	
Cone width	+	+	+	
Cone mass	+	+	+	
Scale length	+	(+)	+	−
Scale thickness	+	+	(+)	+

Figure 2. Scale thickness in relation to cone length for *P. occidentalis* on Hispaniola (*n* = 40 trees) and *P. cubensis* on Cuba (*n* = 39 trees).

seeds (Table 2). In previous studies (e.g., Benkman et al. 2001, Parchman and Benkman 2002), cone trait differences were consistently highly significant ($P < 0.0001$), so that we did not consider statistical corrections for multiple comparisons. However, in this study, many of the differences were marginal. After employing a sequential Bonferroni correction to adjust significance levels for individual statistical tests, only scale thickness, scale length, and seed mass differed significantly between the pine species (Table 2). The canonical discriminant functions correctly classified all of the trees representing the two different species of pine (Wilk's lambda = 0.121, $P < 0.001$). Based on the canonical discriminant functions, cones from the different species were most strongly distinguished by scale thickness (Table 2), which is the cone trait showing the greatest proportional difference between the two pines (Table 2) and is also consistently under selection by crossbills (Table 1).

Table 2. Mean cone measurements for *P. cubensis* and *P. occidentalis*. *P*-values are based on two-level nested ANOVAs (log-transformed data). The ratio of seed mass/cone mass is individual seed mass in grams times the number of seeds per cone divided by cone mass in grams. Percent change is the difference between trait means for *P. occidentalis* and *P. cubensis* divided by the mean of *P. cubensis*, multiplied by 100. Comparisons significant after sequential Bonferroni correction are in bold. The final column presents standardized canonical coefficients from discriminant functions analysis.

Measurement	*P. cubensis*		*P. occidentalis*	
	Pinares de Mayarí	El Gurugú	Bahoruco	Valle Nuevo
Cone length (mm)	60.35	62.92	60.57	59.79
Cone width (mm)	26.79	28.44	28.9	29.86
Cone mass (gm)	13.02	15.57	13.63	12.56
Scale length (mm)	15.27	16.56	12.33	12.06
Scale thickness (mm)	2.42	2.35	3.56	3.76
Seed mass (mg)	13.4	15	12.5	11.5
Number of full seeds	22.81	24.51	26.89	16.8
Number of empty seeds	14.54	16.15	19.79	18.02
Number of seeds total	37.35	40.66	46.69	34.82
Seed mass/cone mass	0.039	0.039	0.045	0.035
Number of trees sampled	18	22	27	22

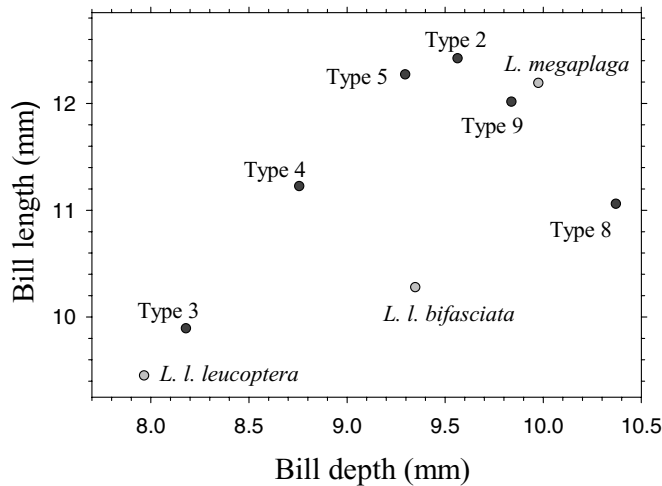| Differences among sites within species			Differences between *P. occidentalis* and *P. cubensis*			Percent change between species	Standardized canonical coefficients
F (df=3, 87)	P	Percent of total	F (df=1, 3)	P	Percent of total		
0.39	0.679	0	1.26	0.265	1.9	−2.4	0.033
1.97	0.146	4	4.73	0.032	5.6	6.4	0.059
2.23	0.114	5	4.77	0.031	5.2	−8.4	−0.334
1.39	0.256	0.7	68.41	**<0.001**	59.9	−23.4	−0.904
1.19	0.309	0.1	282.9	**<0.001**	86.4	53.5	2.153
3.34	0.04	7	20.71	**<0.001**	26.3	−15.5	−0.076
6.56	0.002	20.2	2.45	0.121	0	−7.7	
0.38	0.685	0	5.17	0.026	9.7	−9.4	
6.49	0.002	19.9	0.35	0.557	0	4.5	0.338
4.71	0.012	14.4	0.03	0.858	0	2.6	

Figure 3. Bill length in relation to bill depth for nine crossbill taxa. Values represent the mean of the two means for males and females of each taxon.

HAS THE HISPANIOLAN CROSSBILL EVOLVED IN RESPONSE TO INCREASED SEED DEFENSES?

The Hispaniolan crossbill (*L. megaplaga*) has diverged in bill size from *L. l. leucoptera,* especially in relation to the differences between other morphologically specialized taxa of New World red crossbills (Figs. 1 and 3).

PHYLOGENETIC RELATIONSHIPS OF CROSSBILLS AND ESTIMATED DIVERGENCE TIME FOR THE HISPANIOLAN CROSSBILL

Trees estimated using Bayesian, parsimony, and maximum-likelihood analyses reveal identical topologies and suggest that the Hispaniolan crossbill is sister to *L. l. leucoptera* (Fig. 4). However, support for the clade including the Hispaniolan crossbill and *L. l. leucoptera* is not strong, and in analyses based on individual genes, one of the three genes (cytochrome b) produced a tree with a conflicting topology at this node. Sequence divergence between
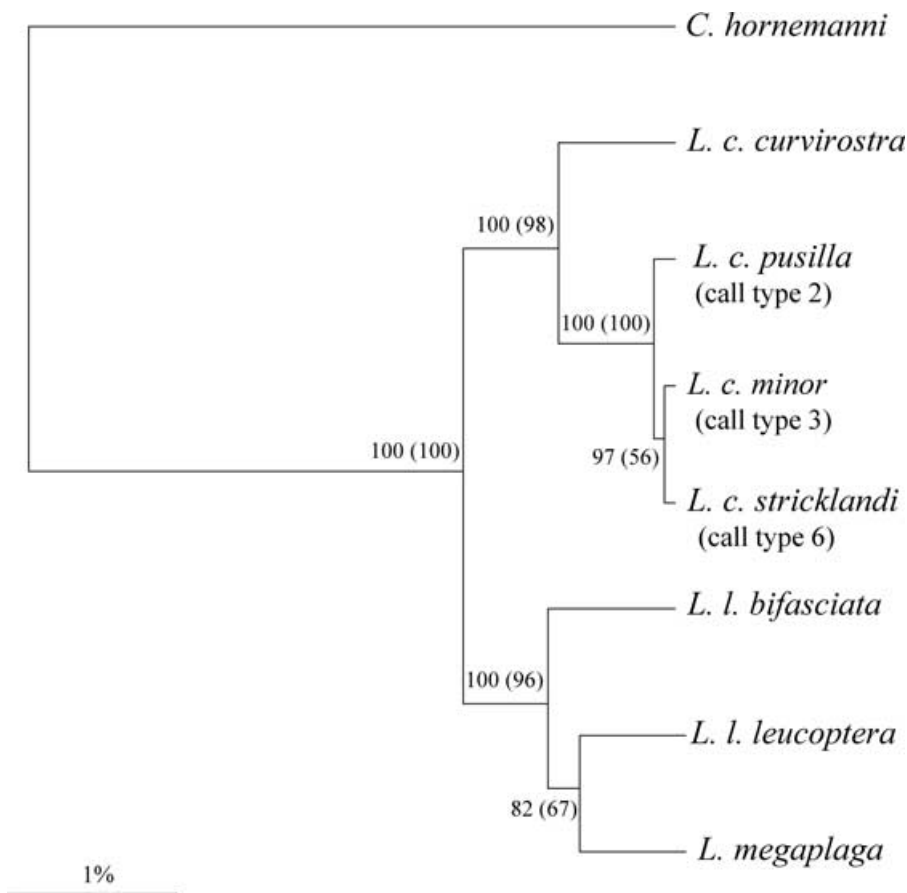

Figure 4. Bayesian phylogenetic tree with a molecular clock enforced. *Loxia leucoptera bifasciata* and *L. c. curvirostra* are Old World crossbills, whereas *L. megaplaga* (the Hispaniola crossbill), *L. l. leucoptera*, *L. c. pusilla*, *L. c. stricklandi*, and *L. c. minor* are New World crossbills, with *Carduelis hornemanni* as an outgroup. The bar in the lower left represents 1% sequence divergence. Values at the nodes represent posterior probabilities from Bayesian analyses and those in parentheses represent bootstrap support based on 1000 replicates in parsimony analyses.

the Hispaniolan crossbill and *L. l. leucoptera* is 1.38%. If we assume a rate of 2% sequence evolution per million years, the split between the Hispaniolan crossbill and *L. l. leucoptera* dates to roughly 680,000 years ago. Because the coalescence of mitochondrial genes is expected to predate population divergence (Edwards and Beerli 2000), this should be viewed as a slight overestimate of the upper limit of the time since this divergence occurred.

Discussion

The evolution of much thicker cone scales in *P. occidentalis* compared to *P. cubensis* is consistent with the hypothesis that *P. occidentalis* has evolved defenses in response to selection exerted by crossbills on Hispaniola. Furthermore, because the Hispaniolan crossbill likely evolved from an ancestor that was morphologically similar to the small-billed *L. l. leucoptera,* the direction and magnitude of bill evolution in the Hispaniolan crossbill suggests that it adapted reciprocally in response to increased seed defenses. This implies that coevolution is responsible for patterns of trait divergence on both sides of this interaction. In addition, our results suggest that annual resource fluctuation does not impede crossbill–conifer coevolution, that the same traits remain the targets of reciprocal selection over long periods of time, and consequently that coevolution can give rise to greater levels of trait divergence when interactions persist for longer time periods. Below we discuss these findings in light of other studies on the contribution of coevolution to crossbill diversity.

EVOLUTION OF *P. OCCIDENTALIS* IN RESPONSE TO CROSSBILL PREDATION

Previous studies of phenotypic selection exerted by crossbills on pines suggest that crossbills consistently favor the evolution of longer, wider, and heavier cones with thicker and longer cone scales (Table 1). Moreover, these traits are elevated in a consistent manner in isolated areas where crossbills and conifers have coevolved (Benkman et al. 2001; Parchman and Benkman 2002; T. Parchman and C. W. Benkman, unpubl. data), with the exception of Aleppo pine (*P. halepensis*) where the only elevated trait was scale thickness (Mezquida and Benkman 2005). Like Aleppo pine, the comparison of *P. occidentalis* to *P. cubensis* revealed considerable differences in only scale thickness (Table 2). All other traits either did not differ significantly in these two pine species, or they differed by a much smaller percentage (Table 2) and inconsistently relative to the expected direction of change (Table 1). For example, one of the traits that did differ was seed mass (Table 2); a trait on which we have not detected selection in previous studies (Parchman and Benkman 2002; Benkman et al. 2003). We have no explanation for why *P. cubensis* has larger seeds than *P. occidentalis,* but suspect this difference may be related to selection during the seedling stage (Westoby et al. 1996). Seed mass tends to be

correlated with scale length (e.g., *P. contorta latifolia,* McGinley et al. 1990; Benkman et al. 2003) presumably because heavier seeds require longer seed wings for effective wind dispersal, and thus have longer associated cone scales (Benkman 1995). It is not surprising therefore that scale length is also longer in *P. cubensis* than in *P. occidentalis* (Table 2).

Scale thickness is the most intuitive seed defense against crossbills; greater scale thickness requires crossbills to exert greater forces to spread apart the scales to access seeds (Benkman et al. 2001; Parchman and Benkman 2002). Scale thickness has also been consistently related to foraging rates in captivity (Benkman 1987b; Benkman et al. 2001; Parchman and Benkman 2002) and tree preferences in the wild (Summers and Proctor 1999; Benkman et al. 2003). These results all point to scale thickness as the key defensive trait evolving to deter crossbill predation. In addition, the cones of *P. occidentalis* have exaggerated prickly spines on the distal end of the scale, a trait absent from the cones of *P. cubensis* (Fig. 1). Aviary experiments have shown that the presence of such spines depresses the feeding rates of crossbills (Coffey et al. 1999). The elevation of this trait in *P. occidentalis* suggests it evolved as a seed defense in response to selection exerted by crossbills.

Cone trait divergence between coevolutionary hot spots and cold spots detected in previous studies was due in part to selection and relaxation of selection by tree squirrels (Benkman et al. 2001; Parchman and Benkman 2002; Mezquida and Benkman 2005). For example, tree squirrels preferentially harvest cones with a higher ratio of seed mass to cone mass (Smith 1970; Benkman 1999; Benkman et al. 2001; Siepielski and Benkman 2007). In addition, many conifers including limber pine (*Pinus flexilis*) and whitebark pine (*P. albicaulis*) (Siepielski and Benkman 2007), lodgepole pine (Benkman et al. 2001), and black spruce (Parchman and Benkman 2002) have evolved lower ratios of seed mass to cone mass where tree squirrels are present compared to where they are absent. In contrast, we have not detected crossbills exerting selection on seed mass to cone mass ratios (Benkman et al. 2001, 2003; Parchman and Benkman 2002). In previous studies, it was reasoned that cones became larger in areas where crossbills were more important selective agents because selection from crossbills favored larger and thicker scales, and also because relaxation of selection from tree squirrels favors more seeds per cone. Larger scales and more seeds per cone (i.e., more scales) in turn would result in larger overall cone size because of trait correlations (Benkman et al. 2001; Parchman and Benkman 2002). The lack of consistent divergence in cone size-related traits in this study (Table 2) suggests that the relaxation of selection from tree squirrels had a considerable influence on these traits in previous studies. In the only other comparison between areas with and without crossbills where squirrels were absent altogether (Aleppo pine on the Balearic Islands), scale thickness was also the only

trait that exhibited appreciable divergence consistent with an evolutionary response to selection exerted by crossbills (Mezquida and Benkman 2005).

RECIPROCAL ADAPTATION IN THE HISPANIOLAN CROSSBILL

The bill depth of crossbills is correlated with feeding performance and fitness, and crossbills generally evolve deeper bills for foraging on larger, tougher conifer cones with thicker scales (Benkman 1993, 1999, 2003; Benkman et al. 2001). Moreover, in areas where tree squirrels are absent and conifers exhibit elevated defenses against crossbills, crossbills have repeatedly evolved larger and deeper bills (Benkman et al. 2001, 2003; Parchman and Benkman 2002; Mezquida and Benkman 2005; Siepielski and Benkman 2005; Edelaar and Benkman 2006). The Hispaniolan crossbill has a bill that is 25% deeper than that of its sister taxa *L. l. leucoptera* (Figs. 1 and 3). The Hispaniolan crossbill also is convergent with the South Hills crossbill (call type 9, Fig. 3), which evolved similarly in response to a coevolutionary arms race with another species of pine (Benkman 1999; Benkman et al. 2001, 2003). Evidence for the evolution of *P. occidentalis* cone defenses in response to crossbill predation, together with the evolution of a large bill in the Hispaniolan crossbill, implies that these taxa have coevolved. This indicates both that coevolution is not limited to crossbills specialized on highly stable resources and that the presence and absence of crossbills is sufficient for generating divergent selection between conifer populations.

The evidence that cone scale thickness continues to increase in response to selection exerted by crossbills, and crossbills evolving even larger bills, indicates that this crossbill–conifer interaction can be characterized as phenotypic escalation. Presumably scale thickness continues to increase until the tree experiences counter selection as a result of trade-offs that limit further increases in scale thickness (Benkman 1999). Variation in the strength of selection exerted by crossbills and trade-offs experienced by the tree presumably contribute to the variation in scale thickness among some populations of conifers (e.g., Siepielski and Benkman 2005). We do not know whether the interaction between the Hispaniolan crossbill and pine will continue to escalate or if it is at equilibrium. However, we do not expect that selection by crossbills on a quantitative trait such as scale thickness would ever by itself eliminate crossbills as seed predators. If such a defense becomes increasingly effective, then selection by crossbills will weaken and trade-offs will counter further increases in the defense. In contrast, selection exerted by other seed predators such as tree squirrels (*Sciurus* spp.) on larger coned pines (e.g., *P. halepensis* [Mezquida and Benkman 2005] and *P. ponderosa ponderosa* [T. Parchman and C. W. Benkman, unpubl. data]) appears to cause the evolution of even larger cones that crossbills increasingly avoid.

DIVERSIFYING COEVOLUTION BETWEEN THE HISPANIOLAN CROSSBILL AND *P. OCCIDENTALIS*

Our results suggest the Hispaniolan crossbill diverged from *L. l. leucoptera* after their common ancestor diverged from the Old World *L. l. bifasciata* (Fig. 4), although this clade is not strongly supported in phylogenetic analyses (Fig. 4). We suspect that this common ancestor would have initially evolved a small, slender bill for specializing on black spruce that forms extensive forests in the boreal region of North America. Given that coevolution between *L. l. leucoptera* and black spruce was likely minimal (Parchman and Benkman 2002), it is from this *L. l. leucoptera*-like ancestor that we suspect the Hispaniolan crossbill evolved after colonizing Hispaniola. The large bill size difference between the Hispaniolan crossbill and *L. l. leucoptera* (Figs. 1 and 3) highlights the substantial adaptive divergence of the Hispaniolan crossbill. Once crossbills isolated on the island had diverged in morphology due to specialization and coevolution, sufficient ecological barriers to interbreeding with *L. l. leucoptera* would have been in place (Smith and Benkman 2007). Predator–prey coevolution could essentially be viewed as driving allopatric divergence and ecological speciation in this manner (Schluter 2000).

Estimates of divergence time based on mtDNA suggest that the Hispaniolan crossbill diverged from *L. l. leucoptera* and perhaps evolved in isolation on the island for nearly 680,000 years. This roughly corresponds to the time period when Quaternary glaciations began to increase in intensity (Barendregt and Irving 1998), and when the distributions of northern conifers, where the ancestral *L. l. leucoptera*/ *L. megaplaga* presumably resided, shifted considerably to the south (Williams 2003). This may have facilitated the colonization of Hispaniola, where a presumably weakly defended *P. occidentalis* (i.e., *P. cubensis* like) would have been the only conifer available as a food resource for crossbills. Given that *P. occidentalis* must have been on Hispaniola since the initial divergence of the Hispaniolan crossbill, it is likely that *P. occidentalis* has diverged from *P. cubensis* for at least 680,000 years and perhaps up to 1.6 million years based on genetic data. We infer the upper time limit because the sequences spanning the cpDNA genes matk and rbcl (2818 bp) are identical for *P. occidentalis* and *P. cubensis* (Gernandt et al. 2005) and it takes roughly 500,000 to 1.6 million years for one mutation to occur across these genes (Willyard et al. 2006; A. Liston, pers. comm.).

The increased intensity of climate change resulting from periodic glacial episodes in North Temperate Zone habitats compared to more southern latitudes is a possible factor contributing to both the difference in the time span over which these interactions coevolve and, consequently, their extent of phenotypic divergence. The repeated occurrence of glacial episodes at approximately 100,000-year intervals during the Quaternary (Lisiecki and Raymo 2005) has continually rearranged species distributions and communities (Bennett 1990), with this effect increasing

with latitude (Dynesius and Jansson 2000; Jansson and Dynesius 2002). Many populations at northern latitudes experienced cyclic periods of local extinction, recolonization, and remixing (Huntley and Birks 1983; Bennett et al. 1991). These dynamics presumably eroded and recreated microevolutionary change that occurred in response to species interactions (Bennett 1990) as well as increasing gene flow among historically separated populations, all of which would act to limit adaptive divergence and speciation across the geographic mosaic of coevolution. In contrast, plants and other taxa at lower latitudes, especially those on oceanic islands (Cronk 1997), retained more stable, long-term, and restricted distributions (Colinvaux et al. 1996; Jansson and Dynesius 2002). Consequently, species interactions at lower latitudes may have retained their geographic structure with populations experiencing divergent selection for longer time periods and divergence between populations less constrained by gene flow. This is consistent with phenomena including the higher numbers of local endemic species in the tropics, lower levels of vagility and smaller range sizes in tropical taxa (Jansson and Dynesius 2002), and deeper genetic divergences between lower latitude populations (Martin and McKay 2004).

Because seed predators and conifers examined in our previous studies were from more northern latitudes and their current distributions formed only after the last glacial retreat, localized coevolution has been restricted to the last 10,000 years (Benkman et al. 2001; Parchman and Benkman 2002). The relatively low levels of morphological and genetic differentiation among crossbills in coevolutionary hot spots and cold spots with lodgepole pine (compare call types 9 and 5, respectively, in Fig. 3) are consistent with the scenario that this divergence has arisen recently and in the face of gene flow (Parchman et al. 2006). In contrast, the Hispaniolan crossbill and *P. occidentalis* likely coevolved for a much longer time period, and these taxa exhibit more pronounced morphological divergence. For example, scale thickness in *P. occidentalis* has diverged to a greater extent (53% increase, Table 2) than the same trait in coevolutionary hot spots for lodgepole pine (12% [Benkman et al. 2001]), Aleppo pine (12% [Mezquida and Benkman 2005]), and black spruce (13% [Parchman and Benkman 2002]). Such findings indicate that coevolution may have a stronger effect on the evolution of long-term adaptive diversity, and even speciation at lower latitudes, when interactions retain their geographic structuring for longer time periods. Consequently, our study suggests an example of how biotic interactions may play a stronger role in the origin of diversity at lower than higher latitudes (Schemske 2002; Mittelbach et al. 2007).

Conclusions

Our results in combination with previous studies reveal replicated changes in reciprocally adaptive traits of crossbills and conifers as a result of predator–prey interactions. Such a replication strongly implicates coevolution as being responsible for divergence, and as an important process driving the evolution of crossbill diversity. The same traits, which we document as causing and responding to reciprocal selection in interactions evolving over only the last 10,000 years, remain those evolving in response to reciprocal selection over much longer periods of time. This is important because it indicates that selection in these interactions does not occur on one set of traits until they evolve to some level, followed by refocused selection on another set of traits. Furthermore, this suggests that coevolutionary arms races are more likely to drive trait divergence and speciation when populations are allowed to coevolve over longer periods of time. Patterns of increased defenses in prey at lower latitudes are well documented and have been discussed in the context of higher predation intensities or higher primary productivity in the tropics (Vermeij 1978; Coley and Aide 1991). Our study highlights how localized coevolution may play a more prominent role in driving divergence when geographically structured coevolution persists for longer time periods at lower latitudes, which may also contribute to geographic patterns of defenses, offenses, and species diversity.

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Associate Editor: P. Abbot