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Replicated population divergence caused by localized coevolution? A test of three hypotheses in the red crossbill-lodgepole pine system

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Abstract

Several lines of evidence support the hypothesis that local populations of red crossbills (*Loxia curvirostra* complex) enter into a predator-prey arms race with lodgepole pine (*Pinus contorta latifolia*) in the absence of competing pine squirrels (*Tamiasciurus hudsonicus*). Nevertheless, the alternative hypotheses that neutral evolution or factors other than squirrels have caused crossbill population differentiation have not been thoroughly tested. We compared crossbill and pine cone morphology between island populations where squirrels are absent or present, and mainland sites where squirrels are present, in order to distinguish among these hypotheses. All comparisons supported an effect of squirrel absence, not island status, on crossbill and cone morphology. Hence our results provide further evidence that strong localized coevolutionary interactions in a geographic mosaic have driven adaptive population differentiation. In addition, vocal differentiation of crossbills was related to the absence of squirrels, but not to island status. As morphological and vocal differentiation is correlated with reproductive isolation in crossbills, the geographic mosaic of coevolution also seems to promote ecological speciation.

Introduction

The comparison of species and populations as a method to extract the underlying evolutionary processes that create biodiversity has a long history of success. Using this approach it has been established that divergent selection stemming from variation between environments is a major cause for adaptive population differentiation (e.g. Schluter, 2000). It is also becoming clear that biotic interactions alone can create sufficient environmental variation to cause population differentiation. For example, within a geographic mosaic of coevolution, the coevolutionary interaction between two species varies spatially in strength or direction, depending on the composition of the rest of the community (Benkman, 1999; Thompson, 1999; Thompson & Cunningham, 2002). An increasing number of studies show how such a geographic mosaic of coevolution is reflected in the variation in ecological and evolutionary outcomes of interactions between populations (reviewed in Thompson, 2005).

An excellent example of the importance of a geographic mosaic of coevolution for the differentiation of populations is found in red crossbills (*Loxia curvirostra* complex). Crossbills are specialized for foraging on seeds in conifer cones and use their crossed mandibles to pry seeds between the scales of closed cones (Benkman & Lindholm, 1991). North American red crossbills show an adaptive radiation where each taxon is specialized for foraging on a different species of conifer (Benkman, 1993a, 2003; Benkman *et al.*, 2001; Parchman & Benkman, 2002). This extreme resource specialization goes even further for crossbill populations that have become locally adapted to geographic variation within a lodgepole pine subspecies (*Pinus contorta latifolia*) (Benkman, 1999; Benkman *et al.*, 2001, 2003; Siepielski & Benkman, 2004, 2005). Throughout most of the distribution of

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lodgepole pine, cone evolution is driven by the selective foraging of pine squirrels (*Tamiasciurus hudsonicus*), the dominant seed predator, resulting in 'squirrel-defended cones' (small, wide cones with small distal scales) (Benkman *et al.*, 2003). In turn, it has been experimentally determined that one taxon of red crossbill (type 5) has a bill depth that is optimal for foraging on these squirrel-defended cones (Benkman, 1993a; Benkman & Miller, 1996). Because red crossbills are only a marginal seed predator compared with pine squirrels, the coevolutionary interaction between crossbills and lodgepole pine is only weak or absent in the presence of pine squirrels (a coevolutionary 'coldspot', Benkman, 1999; Benkman *et al.*, 2001, 2003). However, pine squirrels are absent in some isolated populations of lodgepole pine in small mountain ranges in the plains surrounding the Rocky Mountains (hereafter lodgepole pine 'islands'). Here, red crossbills are the dominant seed predator, and their selective foraging as well as relaxation of selection by pine squirrels drives cone evolution towards different, more 'crossbill-defended cones' (large, narrow cones with large distal scales) (Benkman *et al.*, 2003). In turn, on these islands natural selection favours crossbills with deeper bills to counteract this increased defence of cones (Benkman *et al.*, 2001, 2003; Benkman, 2003), and the coevolutionary interaction between crossbills and lodgepole pine is strong (a coevolutionary 'hotspot').

Resource-driven spatially variable selection on lodgepole pine cone traits and bill depth of crossbills fits well with the observed spatial differentiation of pine and crossbill populations: replicated morphological differentiation (more crossbill-defended cones, greater bill depth) has been found in five island populations of pines and crossbills where squirrels are absent (Benkman, 1999; Benkman *et al.*, 2001, Siepielski & Benkman, 2004, 2005). In two of these (the South Hills and the adjacent Albion Mountains, Idaho), flight calls of crossbills have also differentiated (Benkman, 1999). It is believed that these differentiated calls contribute to the observed reproductive isolation between this local crossbill population and other crossbill taxa that sometimes breed in the South Hills and Albion Mountains (Smith & Benkman, unpublished data). Overall, divergent selection stemming from the geographic mosaic of coevolution between lodgepole pine and red crossbills has likely resulted in replicated population divergence and even (incipient) speciation in crossbills (Benkman, 1999; Benkman *et al.*, 2001; Smith & Benkman, unpublished data).

However, islands where squirrels are absent differ in not just one but in at least two respects from Rocky Mountain (hereafter 'mainland') sites where squirrels are present: they lack squirrels but also are 'island' populations. Island populations may differ in levels of genetic drift (small, isolated populations), or may be exposed to particular environmental factors unrelated to squirrel absence but common to all islands (e.g. different climate, or absence/presence of community members other than squirrels). This makes it uncertain if indeed (only) squirrel absence is (causally) related to the observed pattern of differentiation in pine cone traits and crossbill bill depth and vocalizations on islands where squirrels are absent. Alternatively, or additionally, (i) random, neutral evolution in isolation has caused the island populations to differ from the typical mainland populations, or (ii) islands differ in some other ecological factor that causes differentiation of island populations unrelated to the absence of squirrels, calling for a further evaluation of the importance of the geographic mosaic of coevolution as a driver of population divergence.

This study capitalizes on the fact that isolated populations of lodgepole pine where squirrels are 'present' are also available for comparative studies. The absence of squirrels on some islands has been extensively used as a natural experiment to test for the effect of squirrel presence. Following Benkman (1999), we include island populations where squirrels are present as a 'natural control' for the effect of island status. In order to disentangle the effects of squirrel absence and isolation/island status as causes for the observed pine and crossbill population divergence, we compared the morphology of cones and crossbills and crossbill vocalizations between populations living on islands where squirrels are absent, populations living on islands where squirrels are present, and populations living on the mainland where squirrels are present (mainland populations where squirrels are absent are unknown). This enabled us to test if differentiation of lodgepole pine and crossbills is due to (i) neutral divergence in isolation (neutral hypothesis), (ii) some other general island effect unrelated to squirrel absence ('island hypothesis'), or (iii) squirrel absence on some islands only (the original explanation; 'squirrel hypothesis'). The neutral hypothesis assumes very weak selective forces and very weak gene flow to or from island populations, such that neutral processes (genetic drift) drive the evolution of vocalizations and morphology in random directions. This hypothesis is unlikely to be true for crossbills because most crossbills wander widely in search of cone crops and may breed wherever cone crops are large (Newton, 1972), but it has not been tested and eliminated previously. For the island and squirrel hypotheses, there may or may not be any gene flow, but the effect of selective forces is assumed to be directional and detectable above any effects of gene flow and genetic drift. The predictions for the three hypotheses regarding the differentiation of island populations in cone morphology and crossbill morphology and vocalizations are summarized in Table 1.

Benkman (1999) showed graphically that cones from lodgepole pine on islands where squirrels are present are similar to cones from mainland sites where squirrels are present, and very different from islands where squirrels are absent. For an independent set of islands where squirrels are present, we performed the first statistical test

Table 1 Predictions of the three hypotheses for differentiation of island populations of red crossbills and lodgepole pine where pine squirrels are present or absent.

	Differentiation of island populations relative to mainland	
Hypothesis	Cones/bill size	Crossbill vocalizations
Neutral evolution	Some larger, some smaller	At least some
General island effect	All larger	All
Squirrel absence	Larger only where squirrels absent	Only where squirrels absent

As neutral evolution is nondirectional and proceeds slower than evolution driven by selection, the predictions for neutral evolution include that some sites are not differentiated.

of this pattern, and show that this pattern is replicated and significant. More importantly, we showed that the same pattern is also found for bill morphology of crossbills. By showing strong support for the squirrel hypothesis, and absence of support for the neutral and island hypotheses, for both cone and crossbill morphology, this study reinforces the previous interpretation that local differences in the strength of coevolution between crossbills and lodgepole pine – due to the presence/absence of a third community member, pine squirrels – causes clear, replicated, adaptive population differentiation (Benkman, 1999; Benkman *et al.*, 2001, 2003; Siepielski & Benkman, 2004, 2005). In addition, support for the squirrel hypothesis in explaining the evolution of reproductive isolation between some crossbill populations suggests that local variation in coevolutionary interactions impedes crossbill gene flow, and may ultimately lead to (ecological) speciation.

Materials and methods

We visited 27 sites to determine the morphological characteristics of the local lodgepole pine cones and crossbills and crossbill vocalizations: 12 mainland sites where squirrels were present, eight island sites where squirrels were present, and seven island sites where squirrels were absent (Fig. 1). Island populations were chosen as those populations of lodgepole pine that are separated from the more or less continuously forested Rocky Mountains by nonforested habitat lacking squirrels and crossbills. Squirrels were introduced into the Cypress Hills in 1950 and are now common (Benkman, 1999), but in the absence of a phenotypic response to squirrel presence we treat cones from this site as evolved in the absence of squirrels (as in earlier studies). Not all types of data were or could be collected at each site: Table 2 gives an overview of the study sites, the types of data collected, and sample sizes at each site. Compared with previous studies (Benkman, 1999; Benkman *et al.*, 2001, 2003; Siepielski & Benkman, 2004, 2005), all data from islands where squirrels are present is new, and much additional data from mainland sites and islands where squirrels are absent is included.

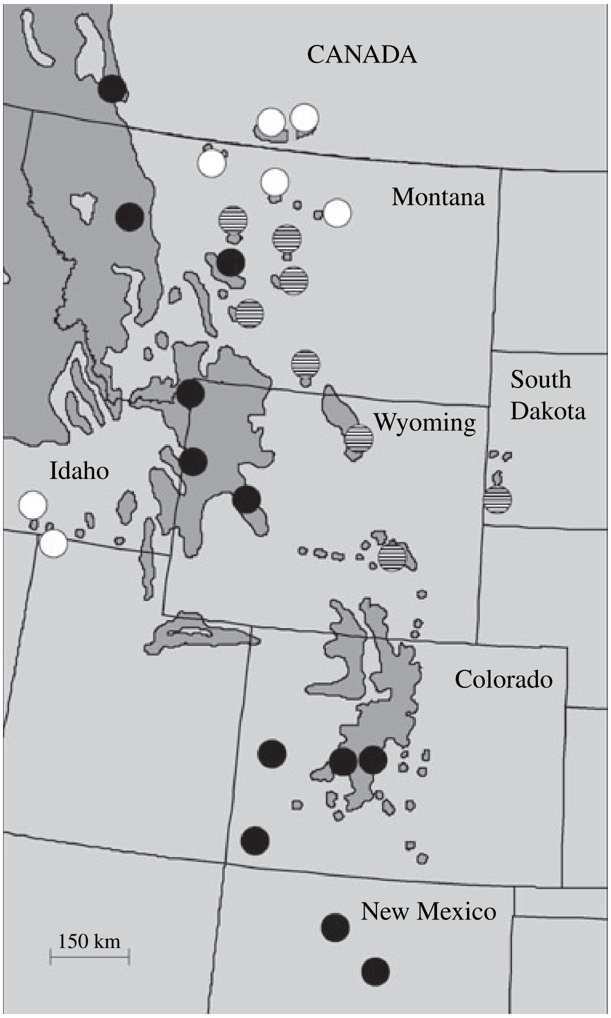

Fig. 1 Overview of the distribution of study sites (circles) and lodgepole pine (dark grey patches) in the study region. Different circle types indicate habitat type: mainland where squirrels are present (black), island where squirrels are present (black–white striped), or island where squirrels are absent (white). Black circles located outside the indicated range of lodgepole pine are sites that are connected broadly by other species of conifers, and hence these sites were included as mainland sites.

Morphological differentiation of lodgepole pine cones

We collected representative mature and closed cones from each of 13 to 43 trees per site (mean number 24.5). One (PE) or three cones (CB) were collected per tree. Prior to drying the cones (because dried cones open), we measured maximum cone length and maximum cone width to the nearest 0.01 mm with digital callipers. After drying each cone for 2 days at 60 °C, total dry mass of the cone including the seeds (full cone mass) was measured

Table 2 Overview of study sites (names and coordinates in latitude 'North' and longitude 'East'), types of data collected at each site, and sample size for assessments of cone traits, crossbill bill depth and crossbill vocal type per site, grouped by habitat type of site (mainland or island, squirrels present or absent).

Name of site	Coordinates (North–East)	Number of cones measured (*n* = trees)	Number of crossbills measured	Number of crossbills vocally assessed	Vocal types encountered
Mainland sites where squirrels are present					
Aspen	39.12–106.72	–	9 type 5	>20	5
Teton Pass	43.50–110.95	–	9 type 2, 8 type 5	17	2, 5
Grand Mesa	39.03–108.00	–	12 type 5	>20	5
Jemez Mountains	35.89–106.31	–	9 type 2	>20	2
San Juan Mountains	37.70–108.72	–	32 type 2	>40	2
Sandia Mountains	35.20–106.43	–	27 type 2	>40	2
Wood Lake	47.25–112.47	14	4 type 2	>10	2, 4
Crow's Nest	49.62–114.46	28	–	–	–
Little Belt	46.95–110.75	22	–	8	2, 5
Twin Lakes	39.08–106.38	23	–	–	–
Wind River	42.59–109.46	24	–	–	–
Yellowstone	44.42–111.06	–	–	> 10	2, 4, 5
Islands where squirrels are present					
Black Hills	44.10–103.53	15	18 type 2	100's	2, 4
Pryor Mountains	45.10–108.27	18	24 type 2	> 50	2, 4
Big Snowy Mountains	46.49–109.29	–	11 type 2	> 100	2, 4
Bighorn Mountains	44.16–106.56	15	4 type 2	> 50	2
Crazy Mountains	46.11–110.24	13	2 type 2	> 30	2, 5
Highwood Mountains	47.24–110.34	–	2 type 2	> 20	2
Judith Mountains	47.12–109.13	–	2 type 2	> 10	2
Medicine Bow	42.36–105.41	20	–	> 10	2, 4, 5
Islands where squirrels are absent					
Bears Paw	48.10–109.40	19	43 type 2	> 50	2, 7
Little Rocky Mountains	47.56–108.31	38	50 type 2	> 100	2
Cypress Hills West	49.64–110.23	29	–	–	–
Cypress Hills East	49.66–109.50	30	–	–	–
Sweet Grass Hills	48.56–111.32	–	–	4	2
South Hills	42.10–114.16	43	300 'SH', 4 type 2	100's	2,5,'SH'
Albion Mountains	42.19–113.36	41	14 'SH', 7 type 2	> 50	2,4,5,'SH
Total *n*		392	589		

Vocal types measured or observed per site are given, and are identified by their number (type 1–7, and 'SH' for the locally differentiated type that is found in South Hills and Albion Mountains, Southern Idaho).

to the nearest 0.1 mg with a digital scale. Other traits previously found to be under selection, such as scale thickness, number of seeds per cone, and total seed mass (Benkman *et al.*, 2001, 2003) were not measured for all sites, so we did not include those traits in the analyses. For sites where seeds were weighed separately from the cone, we added the estimated total mass of seeds (number of seeds times individual seed mass) to the empty cone mass to estimate full cone mass. Based on tree means, we calculated for each site the average cone length, width and mass as the basic statistics for analysis, to avoid pseudo-replication and domination of the analysis by sites with more observations.

Previous studies (Benkman, 1999; Benkman *et al.*, 2001, 2003) have shown that cones from islands where squirrels are absent differ in mass and relative width from cones from island and mainland sites where squirrels are present. Using a new set of islands where squirrels are present, we determined which of the three hypotheses presented above (Table 1) was supported as an explanation for the differentiation in cone morphology by testing for a squirrel effect and an island effect on cone mass and the ratio of cone width to cone length using a two-factor ANOVA.

Morphological differentiation of crossbills

Crossbills were caught with mist nets at drinking or feeding sites with the help of caged decoys. Bill depth was measured at the level of the nostril, perpendicular to the cutting edge, to the nearest 0.01 mm with digital callipers. Measurements by each author were very repeatable [repeatability within each author calculated following Lessells & Boag (1987), for birds measured at least several days (PE: $F_{8,9} = 18.5$, $r = 0.90$) or one or more years apart (CB: $F_{10,11} = 7.67$, $r = 0.77$)]. After measuring, the birds were released and their flight calls recorded (see below).

Because we were interested in testing alternative hypotheses for the vocal and morphological characteristics of the locally differentiated crossbills from two lodgepole pine islands where squirrels are absent (South Hills and Albion Mountains), we limited our analysis of morphology to this locally differentiated type and vocal types 2 and 5 from Groth (1993). These latter two vocal types are the most likely ancestors of the locally differentiated crossbills. First, they are the genetically most closely related types (Parchman *et al.* in press, Benkman & Britch, unpublished data on AFLP markers). In support of this, of all sympatrically occurring vocal types, types 2 and 5 are the types that are morphologically most similar to the locally differentiated type. Second, type 5 is the type that is specialized on lodgepole pine on the mainland where squirrels are present (Benkman, 1993a; Benkman & Miller, 1996). While type 2 is specialized on ponderosa pine (Benkman, 1993a), it is the only other type that has resident populations living in island populations of lodgepole pine where squirrels are absent (Little Rocky and Bears Paw mountains, Montana; Siepielski & Benkman, 2005), i.e. it has the ability to live on seeds of lodgepole pine alone. Hence, the ability of only types 2 and 5 to feed exclusively on seeds of lodgepole pine further suggests that one or both types are ancestral to the locally differentiated type feeding on lodgepole pine on islands where squirrels are absent.

Only birds with fully crossed mandibles and with at least some adult feathers were included in the analyses, because birds in full juvenile plumage could still be growing (Groth, 1993). Testing for an age-effect after this procedure showed that birds moulting out of juvenile plumage did not differ significantly in bill depth from adult-plumaged birds. Prior to analysis, we checked for the need of correction for a number of variables that could obscure the pattern of interest. In a stepwise backward ANOVA explaining variation in bill depth, the variable ‘year’ ($F_{7,364} = 0.52$, $P = 0.82$) and ‘direction of mandible crossing’ ($F_{1,373} = 1.05$, $P = 0.31$) were not significant, so no correction was necessary. We corrected bill depth of both sexes (males downward and females upward: males had 0.23 mm deeper bills, $F_{1,543} = 94.2$, $P < 0.0001$) in order to obtain the intermediate, ‘sex-less’ average crossbill [birds of unknown sex, determined following Phillips (1977) and Svensson (1992), were excluded from all analyses]. We used the same correction factor for both observers, as the interaction term for observer × sex was nonsignificant ($F_{1,542} = 0.10$, $P = 0.75$). The average measurements differed significantly between the two observers (PE 0.34 mm larger than CB, $F_{1,543} = 65.5$, $P < 0.0001$), probably because of slightly different positions of measuring bill depth (basal and distal end of nostril respectively). Because of this observer difference, we give statistical results both for each observer separately and for observers combined. For the analysis of the combined dataset, we corrected for the observer difference by calculating the average difference between the means at five sites where both observers measured an independent sample of the population. Repeatability between observers for these five-paired sites was high (after correction for constant observer difference: $F_{4,5} = 9.45$, $r = 0.81$). To aid comparison with earlier studies, we left the measurements of CB uncorrected but corrected the measurements of PE downward. For the five sites sampled by each observer, we used the mean of the average values obtained by each observer.

Similar to the analysis of cone morphology, we tested for a squirrel effect and an island effect on bill depth using a two-factor ANOVA. We compared the data from crossbills from island sites with mainland type 2 and 5 crossbills separately, because it is not yet fully clear which type is the closest relative to the locally differentiated crossbills from islands where squirrels are absent. We used average bill depth per call type per site as the basic statistic, to avoid pseudo-replication and domination of the analysis by sites with more observations. In the South Hills and Albion Mountains, both the locally differentiated crossbill and type 2 crossbills were caught and measured. As there does not seem to be a resident population of type 2 on these islands where squirrels are absent (Smith & Benkman, unpublished data), these type 2 crossbills were treated in the analyses as immigrant birds originating from mainland areas where squirrels were present. This has a conservative effect on the statistical effect of presence of squirrels on bill depth, if only type 2 crossbills with relatively large bills remain, survive or reproduce at these sites with very large, crossbill-defended cones (cf. Siepielski & Benkman, 2005). In order to reduce sampling variance, three sites where only two adult birds of known sex were measured were excluded from these analyses.

Vocal differentiation of crossbills

At all sites, both free-flying and captured crossbills were recorded with Sennheiser shotgun microphones and Marantz tape recorders. Sonograms of flight calls were prepared with Canary software and visually compared with published sonograms of the eight different crossbill taxa (vocal types) described by Groth (1993) for North America, and one additional vocal type described by Benkman (1999). These calls mainly differ in structure, including presence, position and slope of frequency modulations and additional elements of higher frequency (Groth, 1993). Calls are normally easily identified, so each observer analysed their own recordings. Some recordings ($n = 52$) were a bit harder to identify for PE, and these were also identified by CB and Julie Smith, leaving only one individual unidentified (and excluded from analysis). Out of 611 caught birds, 22 could not be identified with certainty and were excluded from analysis: 13 gave no or weak calls, and nine gave deviating (stress or social

Table 3 Statistical results of two-factor ANOVA of lodgepole pine cone traits and crossbill bill depth. For crossbills, tests were performed separately for comparisons with type 2 and 5 mainland crossbills, and separately for each of two observers as well as for both observers combined.

	Lodgepole pine cone		Crossbill bill depth					
			Type 2			Type 5		
Effect	Mass	Ratio width to length	PE	CB	Combined	PE	CB	Combined
Squirrel presence	$F_{1,13} = 74.9$ $P<0.0001$	$F_{1,13} = 10.7$ $P = 0.006$	$F_{1,8} = 10.1$ $P = 0.013$	$F_{1,6} = 14.2$ $P = 0.009$	$F_{1,12} = 16.4$ $P = 0.002$	$F_{1,8} = 8.70$ $P = 0.018$	$F_{1,5} = 8.68$ $P = 0.032$	$F_{1,8} = 12.4$ $P = 0.008$
Island status	$F_{1,13} = 0.01$ $P = 0.95$	$F_{1,13} = 0.19$ $P = 0.67$	$F_{1,8} = 0.01$ $P = 0.92$	$F_{1,6} = 3.19$ $P = 0.12$	$F_{1,12} = 1.98$ $P = 0.18$	$F_{1,8} = 2.43$ $P = 0.16$	$F_{1,5} = 0.63$ $P = 0.46$	$F_{1,8} = 1.60$ $P = 0.24$

context-related) 'jittering' calls that could not be identified [from six different sites, maximum two per site: these were never the majority of all recorded birds at a site (see Table 2), which would otherwise suggest a localized population of a previously unknown vocal type]. We tested for a squirrel effect and an island effect on vocal differentiation using Fisher exact tests.

Results

Table 3 summarizes the results of the ANOVA of cone traits and bill depth. Cones were heavier (Fig. 2a) and relatively narrower (Fig. 2b) where squirrels are absent. When comparing crossbills from islands with either type 2 and 5 crossbills from the mainland, and for both observers separately (Fig. 3a) or combined (Fig. 3b), crossbills had consistently deeper bills at sites where squirrels were absent.

Free-flying crossbills at all mainland and island sites where squirrels were present ($n = 9$ and 8 sites respectively) gave calls that could all be categorized as belonging to one of the vocal types previously described by Groth (1993). The vocal types we encountered (predominantly type 2, with small percentages of types 4 and 5 at some sites; see Table 2) are common and found throughout much of western North America, and have all been reported before for sites where squirrels were present (Groth, 1993). In addition, all captured crossbills from islands where squirrels were present also belonged to type 2. Hence, vocalizations of both free-flying and captured crossbills provided no evidence for the existence of vocally differentiated crossbill populations at any site where squirrels were present. In contrast, at two of the five island sites where squirrels were absent (South Hills and Albion Mountains), the calls of about 60–95% of all individuals assessed deviated from the calls of vocal types recorded elsewhere, and conformed to the calls described by Benkman (1999) as a locally differentiated vocal type that is not known to occur anywhere else. Based on these data, there was no effect of 'island status' on vocal differentiation of crossbills (Fisher exact test: 0 of 9 mainland sites were differentiated, and 2 of 13 island sites were differentiated; $P = 0.51$), but there was a near-significant effect of 'squirrel presence' (Fisher exact test: 0 of 17 sites where squirrels were present were differentiated, and 2 of 5 sites where squirrels were absent were differentiated; $P = 0.086$).

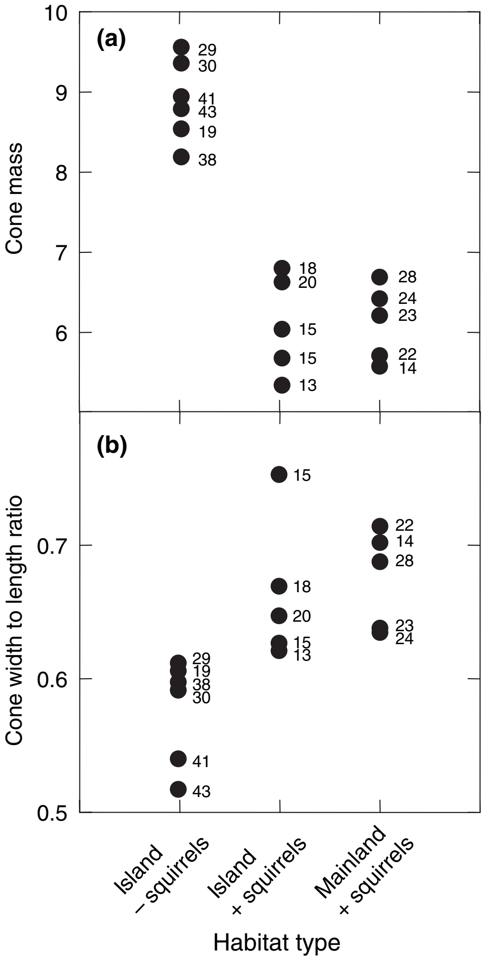

Fig. 2 Average cone mass (a), or average ratio of cone width to cone length (b), for lodgepole pine populations that differ in isolation and presence of pine squirrels. For each population the number of trees sampled is given.

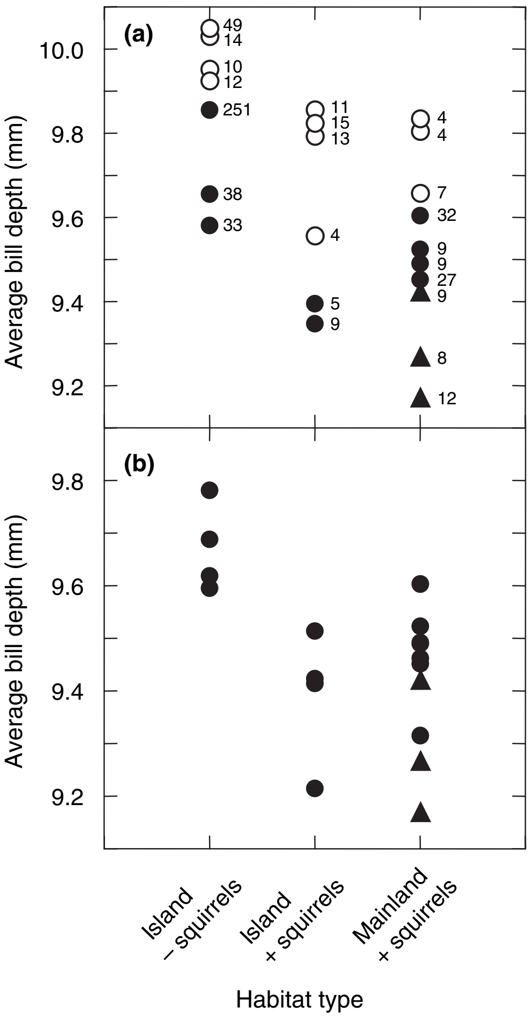

Fig. 3 Average bill depth of red crossbills (dots, type 2; triangles type 5) from populations that differ in isolation and presence of pine squirrels, as measured (a) separately by PE (open symbols) or CB (filled symbols), and (b) observers combined. For each population the number of crossbills measured is given.

Discussion

Previous studies that measured selection on cone and bill traits have demonstrated that in the absence of pine squirrels, bill and cone traits of red crossbills and lodgepole pine are best explained as reciprocal adaptations resulting from their strong coevolutionary interaction (Benkman, 1999; Benkman *et al.*; 2001, 2003; Siepielski & Benkman, 2004, 2005). However, in the comparisons of crossbill populations underlying these studies, the absence of squirrels and population island status were correlated. Hence, differentiation of crossbills could alternatively have been driven by neutral processes, or by other, unknown factors also correlated with population island status. By comparing both lodgepole pine cones and – for the first time – crossbills between mainland areas where squirrels were present, islands where squirrels were absent, and islands where squirrels were present, we could statistically tease apart the effects of island status and squirrel presence on the differentiation of lodgepole pine cone and crossbill morphology, and crossbill vocalizations. While a correlation between squirrel presence and traits of cones and crossbills by itself does not necessarily prove that squirrels are causally involved in the creation of these patterns, the design of these tests does have the ability for refute, or further confirm, previous studies documenting that a coevolutionary arms race between crossbills and lodgepole pine has caused substantial population differentiation, and even incipient speciation.

We found that cones from island sites where squirrels were absent were larger and relatively narrower than cones from both island and mainland sites where squirrels are present. This pattern is consistent with previous studies (Benkman, 1999; Benkman *et al.*, 2001, 2003), and this study adds to these by presenting previously unavailable statistical support for cone similarity between mainland sites and islands where squirrels are present. The significant effect of squirrel presence but not island status on cone size and shape does not support either the neutral or island hypothesis, but does support the squirrel hypothesis.

Similar to cones, crossbill bill depth showed a significant effect of squirrel presence, both for each observer separately and in the combined dataset, and this was true whether comparing the crossbills from islands with type 2 or type 5 mainland (ancestral) crossbills. In all cases crossbills from islands where squirrels were absent had deeper bills, supporting the squirrel hypothesis but not the neutral and island hypotheses. None of the tests showed a significant effect of island status on bill depth, hence there is no support for the alternative hypotheses.

These significant effects of squirrel presence on the differentiation of cone traits and bill depth are in line with the observations for vocal differentiation of crossbill populations: the only populations with vocalizations deviating from the mainland were encountered on islands where squirrels are absent (South Hills and Albion Mountains, see Benkman, 1999). Siepielski & Benkman (2005) recorded crossbills on three islands where squirrels are present (new data from two of these included in the present analysis), and also did not observe crossbills with deviating calls. There was no statistical support for a general island effect on vocal differentiation, but we did find near-significant statistical support for a squirrel effect on vocal differentiation. Therefore the importance of the absence of squirrels for morphological population differentiation of lodgepole pine and crossbills also seems to apply to vocal differentiation of crossbills, in line with the predictions in Table 1.

In summary, our findings are entirely consistent with, and add to, previous studies showing that divergent selection because of local variation in food resources as a result of spatially varying coevolutionary interactions has caused significant population differences in conifer and crossbill morphology and vocalizations (Benkman, 1999; Benkman *et al.*, 2001; Parchman & Benkman, 2002; Benkman *et al.*, 2003; Mezquida & Benkman, 2005; Siepielski & Benkman, 2004, 2005). (i) At all sites where squirrels are lacking, cones are more crossbill-defended, (ii) at all such sites that are currently holding crossbill populations, bill depth is large, and (iii) at only such sites, crossbill calls are deviating. Lodgepole pine and crossbills from previously unstudied islands where squirrels are present did not show similarly crossbill-defended cones, deep bills and deviating calls, refuting the alternative explanations that large cones, deep bills and deviating calls on islands where squirrels are absent were caused (i) by neutral evolution in isolation, or (ii) by other unknown but general ecological differences between islands and mainland habitats, unrelated to squirrel presence.

Support for ecological speciation driven by the geographic mosaic of coevolution

The coevolutionary process is commonly claimed as an important cause behind biodiversity (e.g. Ehrlich & Raven, 1964; Van Valen, 1973; Vermeij, 1987, 1994). However, limited empirical support is available for this claim. According to the hypothesis of ecological speciation, the evolution of reproductive isolation between populations (speciation) is ultimately the result of divergent natural selection caused by differences in the environment (Schluter, 2000, 2001). Divergent selection arising from a geographic mosaic of coevolution may hence drive speciation (Thompson, 2005), but incipient speciation within the red crossbill-complex is the only example to date that indicates that reproductive isolation indeed evolves because of divergent natural selection across a geographic mosaic of coevolution (Benkman, 1999; Benkman *et al.*, 2001, 2003; Siepielski & Benkman, 2005).

Crossbill vocalizations are learned from parents (Groth, 1993), do not seem to depend on morphology (Groth, 1993), and remain distinct in sympatry (Groth, 1993; Smith & Benkman, unpublished data), suggesting reproductive isolation between the different vocal types. Vocally and morphologically differentiated populations from South Hills and Albion Mountains show a high degree of assortative mating in the field with respect to closely related (ancestral) immigrant vocal types 2 and 5 (Smith & Benkman, unpublished data), supporting ‘current’ reproductive isolation. In addition, they are genetically differentiated in AFLP markers (Parchman *et al.* in press, Benkman & Britch, unpublished data) despite significant immigration and sympatric breeding of other vocal types, supporting ‘historical’ reproductive isolation. Therefore the distinct crossbills from South Hills and Albion Mountains are (at least partially) reproductively isolated from other vocal types. Apparently, adaptation to specific resources such as crossbill-defended cones is the basis of ecological speciation in crossbills (Benkman, 2003), although the exact mechanisms have not been determined yet. While we have no detailed quantitative measurements of reproductive isolation involving crossbills from islands where squirrels are present, we can think of no selective advantage favouring reproductive isolation between crossbills from islands where squirrels are present and crossbills from mainland sites where squirrels are present. Indeed, we found that these crossbills do not differ either vocally or morphologically, strongly suggesting that reproductive isolation between them is absent. Hence, effects of neutral evolution or general island factors do not explain the observed reproductive isolation of crossbills from islands where squirrels are absent. Instead, the near-significant correlation between the absence of squirrels and reproductive isolation provides additional support for the hypothesis that a geographic mosaic of coevolution is not only a driver of adaptive population differentiation, but also a driver of ecological speciation.

Because of the unending nature of change in a coevolutionary arms race (West-Eberhard, 1983; Abrams, 2000, Thompson, 2005), stronger and stronger divergent selection may be a common feature in a geographic mosaic of coevolution, making the geographic mosaic of coevolution a potentially important driver of ecological speciation and biodiversity. Unfortunately, geographic mosaics of coevolution are eroding because of introductions and extinctions, destroying ongoing population differentiation and speciation (Benkman, 1989, 1993b; Parchman & Benkman, 2002). Establishing whether population divergence and speciation in geographic mosaics of coevolution is a commonly occurring process (as suggested above) may thus prove to be not only of fundamental interest but also of high conservation importance, if we wish to preserve evolutionary (Benkman, 1999, 2003; Siepielski & Benkman, 2004, 2005) and ecological (Benkman & Siepielski, 2004) diversity.

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