

Genome divergence and diversification within a geographic mosaic of coevolution

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Abstract

Despite substantial interest in coevolution's role in diversification, examples of coevolution contributing to speciation have been elusive. Here, we build upon past studies that have shown both coevolution between South Hills crossbills and lodgepole pine (*Pinus contorta*), and high levels of reproductive isolation between South Hills crossbills and other ecotypes in the North American red crossbill (*Loxia curvirostra*) complex. We used genotyping by sequencing to generate population genomic data and applied phylogenetic and population genetic analyses to characterize the genetic structure within and among nine of the ecotypes. Although genome-wide divergence was slight between ecotypes ($F_{ST} = 0.011\text{--}0.035$), we found evidence of relative genetic differentiation (as measured by F_{ST}) between and genetic cohesiveness within many of them. As expected for nomadic and opportunistic breeders, we detected no evidence of isolation by distance. The one sedentary ecotype, the South Hills crossbill, was genetically most distinct because of elevated divergence at a small number of loci rather than pronounced overall genome-wide divergence. These findings suggest that mechanisms related to recent local coevolution between South Hills crossbills and lodgepole pine (e.g. strong resource-based density dependence limiting gene flow) have been associated with genome divergence in the face of gene flow. Our results further characterize a striking example of coevolution driving speciation within perhaps as little as 6000 years.

Keywords: coevolution, divergent selection, ecological speciation, genetic differentiation, *Loxia*, population genomics

Received 31 May 2016; revision received 10 August 2016; accepted 18 August 2016

Introduction

Coevolution, the process of reciprocal adaptation by two or more species in response to reciprocal selection, is thought to be a major driver of biological diversification (Ehrlich & Raven 1964; Thompson 1994, 2005). However, demonstrating coevolution has been challenging (Gomulkiewicz *et al.* 2007). Moreover, few studies link coevolution directly to speciation and diversification (Althoff *et al.* 2014; Hembry *et al.* 2014). Coevolution between the South Hills crossbill (*Loxia curvirostra*

complex) and Rocky Mountain lodgepole pine (*Pinus contorta latifolia*) is one of the best-documented examples of coevolution (Thompson 2005; Gomulkiewicz *et al.* 2007), and of coevolution generating reproductive isolation (Althoff *et al.* 2014; Hembry *et al.* 2014). In the absence of the red squirrel (*Tamiasciurus hudsonicus*; a predispersal seed predator), crossbills in the South Hills, Idaho, USA, are resident and much more abundant and exert stronger selection on lodgepole pine cones, causing the evolution of enhanced seed defences directed at crossbills. Where red squirrels occur, they are superior competitors for the lodgepole pine seeds and crossbills are much less abundant. Under these conditions, cones evolve mostly in response to selection

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exerted by red squirrels rather than crossbills and have less crossbill-directed defence, which favours smaller beaked crossbills (Benkman 1999; Benkman *et al.* 2001, 2003). The result is a geographic mosaic of coevolution, where crossbills coevolve in an arms race with lodgepole pine in the absence of red squirrels but not in their presence (Benkman 1999; Benkman *et al.* 2001, 2003, 2013).

The South Hills crossbill is one of 10 morphologically and vocally differentiated ecotypes ('call types') of the North American red crossbill complex (Groth 1993; Benkman *et al.* 2009; Irwin 2010) that have evolved in response to selection for specialization on seeds in the cones of different conifer species (Benkman 1993; Benkman *et al.* 2003). This hypothesis was tested in past studies that quantified feeding performance for five ecotypes, including the South Hills crossbill (Type 9) and the ecotype specialized on lodgepole pine where red squirrels occur (Type 5; Benkman 1993, 2003). Feeding performance varied in relation to beak depth (influences efficiency of seed extraction from conifer cones) and groove width in the horny palate (influences seed husking ability), with each ecotype having beak traits that approximate the predicted optima for foraging on seeds of their 'key' conifer (i.e., conifers that reliably produce and hold seeds in cones; Benkman 1993, 2003). The close fit between trait means and both their predicted optima and survival selection strongly implicates resource-based divergent selection in driving this adaptive radiation (Benkman 1993, 2003).

Because divergent selection can reduce gene flow, divergent selection could lead to genetic differentiation, even in the absence of geographic isolation (Endler 1973; Nosil *et al.* 2008; Shafer & Wolf 2013). In crossbills, reproductive isolation is related to divergent selection among ecotypes, because of expected lower fitness of potentially intermediate (hybrid) phenotypes, habitat isolation, low immigrant reproduction and several forms of behavioural isolation (Smith & Benkman 2007; Snowberg & Benkman 2007, 2009; Smith *et al.* 2012). In the South Hills, the combination of well-defended lodgepole pine cones (Benkman 1999; Benkman *et al.* 2001, 2003, 2013), local adaptation by South Hills crossbills and strong density-dependent food limitation prevents all but a few individuals of the nonlocally adapted ecotypes from persisting prior to and during pairing by South Hills crossbills (Smith & Benkman 2007; see Bolnick 2011). Strong density dependence arises because of very stable seed renewal in the South Hills, unlike the episodic abundance of resources that other ecotypes experience (Benkman *et al.* 2012). This contributes to the high levels of premating reproductive isolation between the South Hills crossbill and the two other ecotypes that breed in the South Hills [0.999 on a

scale from 0 (no isolation) to 1 (complete reproductive isolation); Smith & Benkman 2007; Benkman *et al.* 2009]. However, whether local adaptation and strong contemporary premating reproductive isolation have been of sufficient duration to cause genome divergence is unknown.

The ecology and evolutionary history of the red crossbill complex have resulted in limited genetic differentiation among the ecotypes. Ecotype diversification probably occurred in the Holocene following the retreat of glaciers and expansion of conifers (Benkman 1993). The distributions of some key conifers relied upon by the ecotypes were so restricted in the late Pleistocene (e.g. coastal Douglas fir *Pseudotsuga m. menziesii* and Rocky Mountain ponderosa pine *P. ponderosa scopulorum*; Gugger *et al.* 2010; Potter *et al.* 2013) that frequent regional cone crop failures likely prevented earlier specialization by crossbills (Benkman 1993). Such recent divergence is indicated by coalescent analyses of European common (red) crossbill mtDNA haplotypes suggesting that ecotype diversification occurred rapidly over the last 11 000 years (Bjorklund *et al.* 2013). Patterns of mtDNA divergence and diversity are similar among North American ecotypes (Questiau *et al.* 1999). In addition, due to regular localized cone crop failures most ecotypes are nomadic and move long distances between natal and breeding locations, and between breeding locations (up to ~3000 km; Newton 2006), often breeding opportunistically and sympatrically in areas with abundant conifer seeds (Groth 1993; Summers *et al.* 2007). In the South Hills, multiple ecotypes breed (Smith & Benkman 2007). Such conditions and behaviour likely allowed extensive gene flow throughout the young radiation. Indeed, prior analyses using mtDNA and AFLPs found little evidence for genetic differentiation among ecotypes (Questiau *et al.* 1999; Parchman *et al.* 2006). Nonetheless, the widely sympatric occurrence of distinct ecotypes suggests that adaptation and reproductive isolation have evolved despite the large potential for homogenizing gene flow.

Recent innovations in DNA sequencing have dramatically increased our ability to address how geography, ecology and history shape genome divergence during the early phases of divergence (Alcaide *et al.* 2014; Lamichhaney *et al.* 2015; Martin *et al.* 2015). Here, we use genotyping by sequencing (GBS) to evaluate the pattern and extent of genome divergence across the North American red crossbill complex. We sampled multiple, geographically dispersed populations within each ecotype (Table S1, Fig. S1, Supporting information) to test the hypothesis that ecotypes are genetically cohesive and differentiated from one another. We tested for isolation by ecology, in the form of the evidence that genetic divergence was associated with divergent

selection (Nosil *et al.* 2008; Shafer & Wolf 2013), but anticipate little geographic structure (isolation by distance) based on the aforementioned biology of crossbills. Although our focus is on the South Hills crossbill, we include nine ecotypes to provide a broader context of divergence among closely related forms, and because the form of divergent selection and opportunities for geographic isolation are not uniquely different because of coevolution. Finally, the fossil record indicates a large reduction in the amount of lodgepole pine in the South Hills region (Mehring 1985; Davis *et al.* 1986) during a several thousand-year warm period centred around 6000 BP (Bartlein *et al.* 2014). Because this could have prevented the persistence of a local crossbill population (See Siepielski & Benkman 2005), we estimate the distribution of lodgepole pine 6000 BP to characterize the time period over which this coevolutionary interaction could have persisted.

Materials and methods

DNA sequencing, assembly and variant calling

We sequenced DNA from 219 red crossbills representing nine morphologically and vocally differentiated ecotypes, as well as 12 white-winged crossbills (*L. l. leucoptera*; Table S1, Fig. S1, Supporting information). We utilized a GBS protocol that we have used in previous studies (Gompert *et al.* 2012; Nosil *et al.* 2012; Parchman *et al.* 2012, 2013), and generated three lanes of single-end 100-base sequencing on an Illumina HiSeq 2000 at the National Center for Genome Resources (Santa Fe, NM, USA). We used Perl scripts to remove contaminant DNA, trim barcodes and match barcodes to individual sample information. We first used SEQMAN NGEN 3.0.4 (DNASTAR) to perform a de novo assembly for a subset of 30 million reads sampled randomly from the sequencing data for all individuals. The purpose of this step was to produce a consensus GBS reference of the genomic regions represented in our libraries. Because our library preparation method produces reads identical in length from genomic regions beginning with EcoRI cut sites, reads typically align neatly into rectangular contigs, the consensus sequences of which represent a reference of genomic regions sampled by GBS. After removing low-quality or overassembled contigs, we generated a reference of 349 865 contig consensus sequences. We then aligned all reads for each individual onto the GBS reference using BWA 0.7.8 (Li & Durbin 2009). We used SAMTOOLS 1.19 and BCFTOOLS 1.19 (Li *et al.* 2009) to identify bi-allelic single nucleotide polymorphisms (SNPs), and only called variants when 98% of the individuals had at least one read. We used this high threshold here to obtain SNPs with higher

coverage and genotypes having relatively high levels of statistical certainty. While we could have called more SNPs with a lower threshold, we were interested mostly in genome-wide parameter estimates for this study and hope to generate more comprehensive resequencing data for locus-specific analyses in the future. We randomly selected a single variant from each contig to increase the independence of loci, and limited analyses to loci with minor allele frequencies >0.03 for population genetic analyses. Further details on assembly and variant calling are in the Supporting information.

Phylogenetic analyses

Phylogenetic analyses included the 219 red crossbills, and 12 white-winged crossbills for use as an outgroup. We first generated a second data set of SNPs present in alignments of both crossbill species using BWA, SAMTOOLS and BCFTOOLS. While calling variants, we disregarded insertions and deletions, and only considered SNPs when 95% of the individuals had at least one read at that locus. We used a custom Perl script to produce a multiple alignment by defining the DNA state of each individual and variant as the genotype with the highest likelihood. Heterozygotes were coded using IUPAC ambiguities (i.e. M for A/C, R for A/G, W for A/T, S for C/G, Y for C/T and K for G/T), and loci with too much uncertainty (i.e. equal likelihoods for the three genotypes) were encoded as missing data. This resulted in a multiple alignment of 238 615 positions and 231 individuals. We inferred maximum-likelihood (ML) trees using EXAML 2.0.4 (Stamatakis & Aberer 2013), and executed 25 independent ML searches using as starting points 25 parsimony trees inferred using PARSIMONATOR 1.0.3 (Stamatakis 2014). We conducted ML inferences using a GTR + Γ substitution model and performed 500 bootstrap replicates using EXAML with a GTR substitution model using the CAT approximation. We produced the bootstrapped ML analysis with RAXML 8.0.20 (Stamatakis 2014) and used parsimonator as before to obtain starting trees from every alignment. We summarized the ML analyses using RAXML in two different ways: (i) drawing bootstrap support values onto the best-supported ML tree and (ii) computing a bootstrap consensus tree using the majority rule extended criterion.

Population genetic analyses

We used analyses based on allele frequencies and genotype probabilities to quantify patterns of genetic structure within and among the red crossbill ecotypes using the 18 385 high-coverage SNPs described above. We estimated population allele frequencies and genotype

probabilities based on genotype likelihoods estimated with *BCFTOOLS* using a hierarchical Bayesian model (Gompert *et al.* 2012). This model treats population allele frequencies and individual genotypes as unknown model parameters and utilizes Markov Chain Monte Carlo (MCMC). We used this model to estimate allele frequencies and genotype probabilities for each geographically separate sample within each ecotype (Table S1, Supporting information). These 22 population samples included multiple samples from within five of the ecotypes (types 2, 3, 4, 5 and 7) that were from geographically distant regions (Fig. S1, Supporting information). We ran MCMC chains for 20 000 steps, discarded 5000 as burn-in and recorded every fifth step. We first summarized genotypic variation across all individuals of the red crossbill complex using principal components analysis (PCA). We generated a genetic covariance matrix based on the genotype point estimates for each bird and performed the PCA on this genetic covariance matrix using the *PRCOMP* function in *R* (R Core Team, 2013). We tested for significant differentiation between ecotypes and for significant differentiation among populations within ecotypes using permutational multivariate analysis of variance (*PERMANOVA*; Anderson 2001) based on Euclidian distances of the first two principal components using the *vegan* package in *R* (Oksanen *et al.* 2013).

We used allele frequency estimates to calculate Nei's genetic distance (Nei's *D*; Nei 1972) among ecotypes and among all samples (populations) within ecotypes. We calculated pairwise Hudson's *F_{ST}* (Hudson *et al.* 1992) based on estimated allele frequencies at all loci for each ecotype and each population, using code written in *R*. We generated ML estimates of the folded-site allele frequency spectrum, nucleotide diversity (π) and expected heterozygosity as indicators of genetic variation within each ecotype using the expectation maximization algorithm of Li (2011) as implemented in *SAMTOOLS* and ran the algorithm for 20 iterations for each population.

We further investigated hierarchical patterns of genetic structure across the ecotypes and populations within ecotypes using a hierarchical Bayesian model that is similar to the correlated allele frequency model of *STRUCTURE* (Pritchard *et al.* 2000; Falush *et al.* 2003). We used this model (hereafter *ENTROPY*, described in Gompert *et al.* 2014) to characterize population structure and estimate admixture proportions for individuals in the absence of information on sample origin. Importantly, *ENTROPY* allows for stochastic variation in sequence coverage across individuals and loci and estimates allele frequency and genotype probability parameters along with admixture proportions. Similar to the admixture model in *STRUCTURE*, *ENTROPY* assumes that the genome of each

individual consists of loci with ancestry from one of *k* ancestral populations and makes no a priori assumptions about the population or cluster origin of individual samples. Admixture proportions, which represent the fraction of an individual's genome inherited from each of the *k* clusters, are estimated for each individual. In addition, *ENTROPY* generates estimates of deviance information criterion (DIC) as a metric for model choice and comparison; models with lower DIC values are those that fit the data better (Gompert *et al.* 2014).

To facilitate the convergence and stabilization of MCMC chains, we initialized individual admixture proportions in the chains using probabilities of cluster membership based on *k*-means clustering of the principal component scores (equivalent to a no-admixture model; Falush *et al.* 2003). Specifically, we used *k*-means clustering (*KMEANS* package in *R*) based on the principal components estimated from genotypes in a linear discriminant analysis (*LDA* package in *R*; Jombart *et al.* 2010). This provided reasonable starting values of *q* to initialize MCMC and ensured proper mixing and convergence of MCMC chains. Importantly, this approach uses genotypic data without reference to sample origin and does not constrain posterior sampling. We ran *ENTROPY* separately for predefined values of *k* = 1–9 and ran five independent chains for each *k*. Each chain used the probability of cluster membership as mean expectation for the admixture proportion *q*, but random deviates with a precision scalar of 20 were drawn from a Dirichlet distribution to initialize *q* for each chain. We used an upper value of 9 for *k*, representing the number of ecotypes included in our analysis. We ran each MCMC chain for 80 000 steps following 60 000 steps that were discarded as burn-in and saved every 10th step. We estimated posterior medians, and 95% credible intervals for parameters of interest. We checked for mixing and convergence of posterior parameter estimates by plotting MCMC steps for different parameter sets and inspected mixing during the burn-in period and convergence among chains.

The localities sampled are geographically separated and could have allele frequencies that differ due to genetic drift, with population homogenization due to migration declining with distance, leading to isolation by distance. Likewise, divergent selection could reduce gene flow and potentially lead to differences in population allele frequencies (Endler 1973; Nosil *et al.* 2008; Shafer & Wolf 2013). To investigate the extent to which allele frequency differences can be attributed to geographic and phenotypic distances between populations, we modelled pairwise genetic distances (Nei's *D*) between populations as a function of geographic and phenotypic distances (difference in mean beak depth) between populations. Beak depth data (sample

sizes for males and females in parentheses) were from Groth (1993) for ecotypes 1 (39 and 33) and 7 (5 and 1), unpublished measurements of live birds and museum specimens by CWB for ecotypes 2 (226 and 149), 3 (47 and 34), 4 (25 and 15), 5 (61 and 31) and 6 (150 and 71), Benkman *et al.* (2013) for ecotype 9 (471 and 335) and Irwin (2010) for ecotype 10 (54 and 35). Phenotypic distances were calculated based on differences between ecotype means. The use of mean trait values should be conservative, because it provides less power to detect patterns than analyses based on measurements from each individual. Geographic distances were calculated based on Haversine distances, as implemented in the R package *FOSSIL* (Vavrek 2011). Geographic and phenotypic distances were normalized (transformed to Z-scores) so that their coefficients would be on the same scale. Genetic distances were logit-transformed and centred on the mean so as to not be bounded by zero and one. We used a Bayesian linear model that did not require all observations in the response variable to be independent, but instead modelled random effects for all population pairs (Clarke *et al.* 2002; Gompert *et al.* 2014) and over all coefficients for geographic and genetic distances, and separately for phenotypic and genetic distances. The model was specified in JAGS (version 3.4; Plummer 2003), and samples were gathered from the R interface to JAGS (RJAGS; R core Team 2013). After discarding 2000 steps as burn-in, we obtained 2000 samples of the posterior distributions from each of three chains, by retaining every fifth iteration of 10 000 MCMC steps. All chains were inspected graphically for adequate convergence and mixing.

Past forest distribution estimation

Random Forests (Breiman 2001) is a nonparametric classification and regression tree approach that we used to model the distribution of lodgepole pine, because of its past success when true absence data are available, and its ability to identify nonlinear relationships and interaction terms (Cutler *et al.* 2007). We used the USFS Forest Inventory and Analysis data (O'Connell *et al.* 2014) for 3406 presences and 10 855 absences of lodgepole pine in Idaho, Montana, Wyoming, Colorado and Utah to estimate the distribution of Rocky Mountain lodgepole pine. This was augmented with 50 000 'likely-absence' points randomly placed within cells classified as 'unforested' in the LANDFIRE Forest Canopy Cover data set; this likely-absence data set was reduced by 59 points by eliminating all points within 1 km of lodgepole pine presence points to allow for error or lack of precision in the canopy cover layer, resulting in a total of 60 796 absences.

Covariates used for modelling were the BIOCLIM set (Hijmans *et al.* 2005) and elevation (Gesch *et al.* 2002). BIOCLIM values are similar to climate predictors used to model the distribution of tree species in other studies, including lodgepole pine, by other researchers (Boucher-Lalonde *et al.* 2012; Bell *et al.* 2014), but have the advantage of having been modelled for the mid-Holocene (6000 BP). Covariates were resampled to 1 km spatial resolution for modelling. We iteratively generated 100 models, each of which used a subsample of the absence data so that there were three times the number of absence points as presence points. This was done because Random Forests performs poorly when classes are highly imbalanced (Chen *et al.* 2004). The 100 models were combined into a single classification model. Summary statistics and graphs of covariance convergence for the subsampled absence data were used to evaluate the stability of the model. The out-of-bag (Breiman 2001) error rate in predicting known presences and absences was 9%. This model was applied to the historical distribution of climate variables in the BIOCLIM data sets to predict the past distribution of lodgepole pine. We estimated the relative amount of lodgepole pine forest 6000 BP compared with the current amount based on the combined area and relative probabilities of occurrence during the two time periods.

Results

After removing barcodes from the raw reads, and discarding contaminant reads, we retained 321 627 388 reads representing all 231 individuals. Initial de novo assembly placed 24 352 918 reads into 403 678 contigs; the 349 865 highest quality contigs from this assembly were used as a GBS reference. We subsequently aligned reads from all individuals to this reference using BWA. After using SAMTOOLS and BCFTOOLS to call variant sites, discarding loci with minor allele frequency <0.03, and randomly sampling a single SNP per contig, we retained a final set of 18 385 SNPs (mean coverage per individual per locus of 7.2×) for population genetic analyses across the red crossbill complex. Phylogenetic analyses were based on a set of 238 615 SNPs that were called in the alignments of red crossbills and white-winged crossbills, as described above and in the Supporting information.

Phylogenetic analyses revealed topologies with white-winged crossbills and red crossbills each forming strongly supported monophyletic groups (Fig. 1), consistent with previous studies (Questiau *et al.* 1999; Parchman *et al.* 2006). In contrast to past studies (Parchman *et al.* 2006), South Hills crossbills formed a strongly supported monophyletic group and were the only monophyletic red crossbill lineage (Fig. 1). Individuals

of the remaining ecotypes were dispersed throughout the tree and showed no evidence of clustering, with the exception of Type 6, for which bootstrap support was weak (Fig. 1). Type 6 is the only ecotype that could be considered allopatric to the other ecotypes, as it is confined mainly to Mexico south of the other ecotypes (Groth 1993). Consistent with phylogenetic analyses, population genetic analyses based on 18 385 SNPs revealed low levels of genome-wide genetic differentiation between the different ecotypes, as indicated by small Nei's D and F_{ST} estimates (mean F_{ST} = 0.021, range: 0.011–0.035; Table S2, Supporting information).

Despite low levels of divergence, genetic differentiation among the ecotypes and similarity among geographically dispersed samples within individual ecotypes was evident in the PCA (Fig. 2), where the first two principal components differed significantly among ecotypes (PERMANOVA, $F_{8, 210}$ = 1220; R^2 = 0.98; P < 0.001). South Hills crossbills were the most distinct in these analyses and were separated from other ecotypes along PC1, while the remaining ecotypes were separated mostly along PC2 (Fig. 2). The four smallest ecotypes (types 1, 3, 4 and 10) have the highest PC2 scores (Fig. 2), three of which are found mostly in the Pacific Northwest (types 3, 4 and 10), whereas Type 1 is the one ecotype found mostly in eastern North America (Fig. S1, Supporting information; Groth 1993). Intermediate-sized ecotypes are found in the middle cluster, including the two most abundant ecotypes in the Rocky Mountain region (types 2 and 5) and Type 7, which is uncommon but found within the geographic ranges of

types 2 and 5 in the northern Rocky Mountains and west to the Cascades (C. W. Benkman, personal observations; Groth 1993). The largest ecotype is Type 6, which has the smallest PC2 scores (Fig. 2) and occurs mostly in Mexico, allopatric to the other ecotypes. Geographically dispersed samples from within the same ecotype overlapped extensively in PC space, indicating genetic cohesiveness within ecotypes.

Support for the distinctiveness of the South Hills crossbill and genetic similarity of geographically dispersed samples within each of the ecotypes was also found in Bayesian clustering analyses (ENTROPY; Gompert *et al.* 2014). DIC values were similar from k = 2 through k = 6 (Table S3, Supporting information), and all five models led to conclusions consistent with PCAs above. Inspection of MCMC chains indicated sufficient mixing and convergence only for k ≤ 6 models. Subtle allele frequency differences among some of the clusters likely caused problems with the mixing of the MCMC chains for k > 6 models. We highlight results from k = 2, 3 and 5. In the k = 2 model, South Hills crossbills were assigned to one cluster, whereas all other individuals were assigned to the other (Fig. 3A). The k = 3 model also assigned South Hills crossbills to a single cluster and assigned types 1, 3, 4 and 10 to a second cluster, and types 2, 5, 6 and 7 to a third (Fig. 3B). The k = 5 model assigned individuals to clusters that largely reflect the four nonoverlapping groups of ecotypes in the PCA (Fig. 2), with types 5, 6 and 9 each assigned to their own clusters, types 1, 3, 4 and 10 assigned to one cluster, and types 2 and 7 assigned to the fifth cluster (Fig. 3C).

We detected no evidence for isolation by distance. Pairwise genetic distances were unrelated to geographic distances (Fig. 4A), both for pairs of samples within the same ecotype and between all 22 geographically separate samples [the credible interval for the slope for the relationship between genetic and geographic distance included zero; slope for full analysis: 1.5×10^{-6} ; 95% credible or equal-tail probability interval (ETPI): -1.8×10^{-5} to 4.2×10^{-5}]. In contrast to the lack of isolation by distance, ecotype beak depth tended to decrease with increasing PC2 values (Fig. 2), and these groupings of ecotypes were detected using ENTROPY (Fig. 3). In addition, genetic distance tended to increase with increasing beak depth divergence among ecotypes (Fig. 4B), but this pattern was not statistically significant (slope: 0.212; 95% ETPI: -0.008 to 0.439).

Although South Hills crossbills stand out in the phylogenetic and population genetic analyses (Figs 1–3), point estimates of genome-wide differentiation were not greater than in pairwise comparisons among all of the other ecotypes (Fig. 5A). Instead, the upper tails of the F_{ST} distributions for the South Hills crossbill had higher

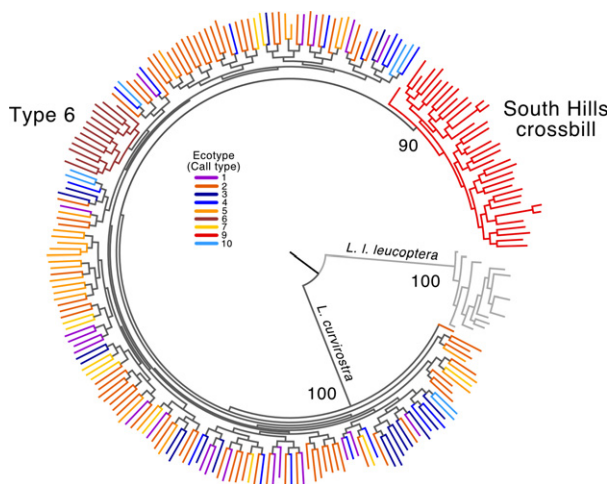


Fig. 1 A maximum-likelihood tree for the 219 red crossbills (*Loxia curvirostra*) and 12 white-winged crossbills (*L. l. leucoptera*) based on 238 615 SNPs. Bootstrap support values on the nodes are based on 500 bootstrap replicates and are only shown for major nodes having >75% support; bootstrap support for monophyly of Type 6 was 10.

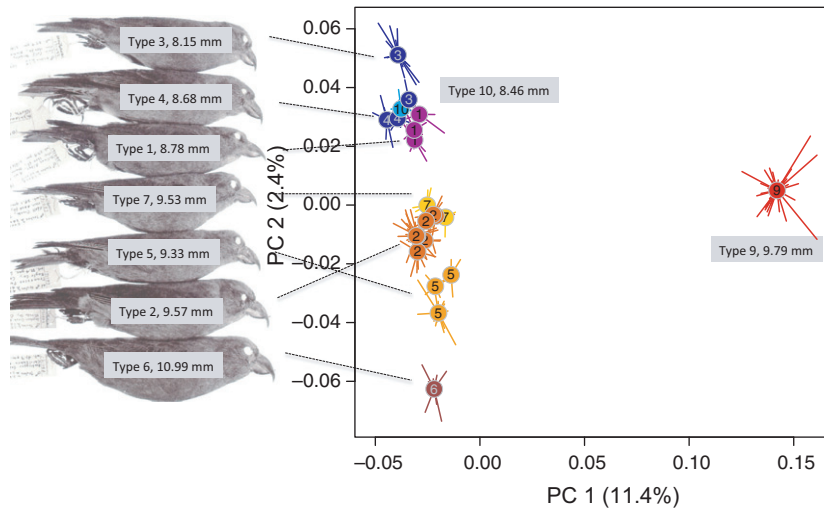


Fig. 2 Genotypic variation (based on 18 385 SNPs) among individuals summarized by the first two principal components from a PCA of the matrix of genotype covariances between individuals. Lines connect individual PC values to the mean for each sampled population, with the mean represented by circles. Numbers and colours correspond to ecotypes (call types), and different geographically separated samples from a given ecotype have the same number and colour. All geographically separate samples within an ecotype, with the exception of Type 7, overlap in PC1-PC2 space. To the left, are representative study skins and the corresponding mean beak depth of seven of the ecotypes (photograph from Groth 1993). Dotted lines connect the specimen images to their ecotype's mean PC values.

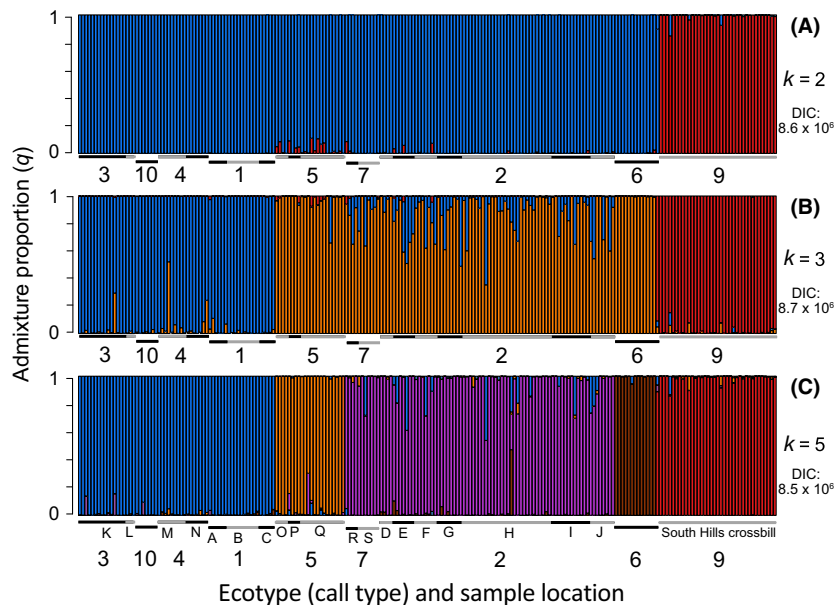


Fig. 3 Admixture proportion estimates (q) from the hierarchical Bayesian model implemented in ENTROPY. Each vertical bar represents a bird, and bars are coloured to reflect the posterior medians of each individual's admixture proportions for each of k clusters. Results with k equal to 2, 3 and 5 are shown. Numbers along the abscissa represent ecotype, and letters for geographically separate populations correspond to those given in Table S1 (Supporting information). The grey and black bars indicate boundaries between population samples.

densities (more loci with especially high F_{ST}) (Fig. 5B), and these loci differentiated South Hills crossbills in PCA and Bayesian clustering analyses. For example, in pairwise comparisons between South Hills crossbills and other ecotypes, there was a strong relationship between locus-specific F_{ST} and the strength of PC1 loading (Fig. 5C), a pattern that does not exist in comparisons among the other ecotypes (Fig. 5D). Similarly, the

0.8 quantiles of the genome-wide F_{ST} distributions were higher for pairwise analyses involving South Hills crossbills (Fig. 5B). Thus, elevated divergence in a restricted number of genomic regions, rather than mean genome-wide genetic divergence, distinguished South Hills crossbills in PCA and ENTROPY analyses.

Estimates of nucleotide diversity indicate that South Hills crossbills harbour lower levels of genetic diversity

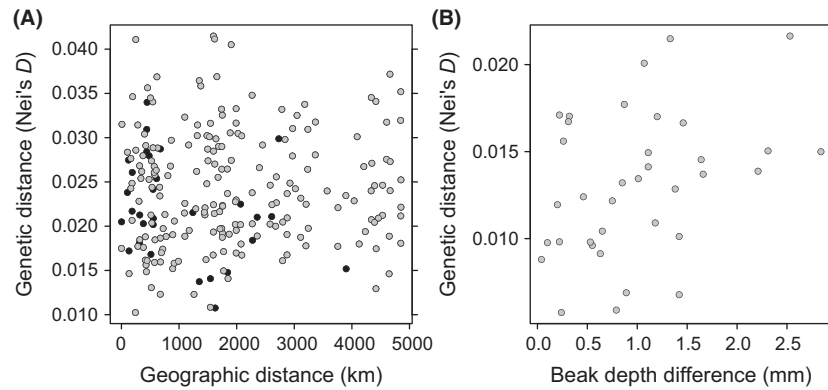


Fig. 4 (A) Genetic distances were unrelated to geographic distances between geographically separate samples within ecotypes (black symbols) and between all 22 samples, and (B) genetic distances between ecotypes did not increase significantly with increasing beak depth divergence among ecotypes (B includes only between-ecotype comparisons). Estimates of the effect of geographic distance or beak depth on genetic distance are for appropriately transformed variables (see 'Materials and methods'), rather than the untransformed values in the plots.

than the other ecotypes (Fig. 7), which could reflect the fact that South Hills crossbills reside in only $\sim 70 \text{ km}^2$ of lodgepole pine forest (Fig. 6) and likely have a much smaller effective population size than other ecotypes. Our historical reconstruction of lodgepole pine distribution in the region where South Hills crossbills occur suggests that there was little pine forest available only 6000 years ago during a period of warming (Fig. 6B, D). Unless the South Hills crossbill and pine began coevolving elsewhere and subsequently codispersed to the South Hills, these results suggest that coevolution and genome divergence occurred within the last 6000 years.

Discussion

Although levels of genetic differentiation were low, many ecotypes correspond to genetically cohesive groups that are differentiated from other such groups (Fig. 2). The low levels of genetic differentiation in our results and those of previous studies (Parchman *et al.* 2006; Bjorklund *et al.* 2013) are consistent with the ecotype diversification occurring recently, in the face of gene flow, or both. Glacial advances during the Pleistocene caused severe reductions in habitat that likely eroded ecotype diversity before glacial retreats allowed a vast expansion of conifers and an ensuing diversification of ecotypes (Benkman 1993; Dynesius & Jansson 2000). The absence of isolation by distance (Fig. 4A), consistent with ecotype nomadism and opportunistic breeding, indicates that genetic differentiation was not dependent on geographic distance or isolation. Instead, our results highlight the importance of adaptation to alternative conifer species (Benkman 1993, 2003) in contributing to reproductive isolation and genetic differentiation. These results contrast with the evidence that

divergence without geographic isolation appears uncommon in birds (Price 2008). Although this difference might be attributable to the use of much smaller sets of genetic markers in past studies (but see Poelstra *et al.* 2014; Mason & Taylor 2015), strong reproductive isolation as a by-product of adaptation to alternative resources (Smith *et al.* 1999, 2012; Smith & Benkman 2007) distinguishes crossbills from most bird species (Price 2008).

Population genomic analyses indicate that the South Hills crossbill (Type 9) was the most genetically distinct ecotype (Figs 2 and 3A), and it was the only monophyletic ecotype in phylogenetic analyses (Fig. 1). This pattern of genetic differentiation indicates that previously documented patterns of divergent selection, adaptation (Benkman 1999; Benkman *et al.* 2003), and reproductive isolation (Smith & Benkman 2007; Benkman *et al.* 2009) associated with a local coevolutionary arms race have contributed to genome divergence. Our results show that, rather than overall genome-wide divergence, elevated genetic differentiation in a small number of genomic regions characterizes divergence in the South Hills crossbill (Fig. 5). This pattern is expected when adaptive divergence occurs in the face of gene flow (Peccoud *et al.* 2009; Feder *et al.* 2012), although such a pattern could also arise from selective sweeps in the absence of reproductive isolation (Cruickshank & Hahn 2014). Our findings are consistent with recent studies of other vertebrate taxa with phenotypic differentiation and reproductive isolation (Poelstra *et al.* 2014; Malinsky *et al.* 2015; Mason & Taylor 2015), where differentiation is restricted to few genomic regions across a background of genomic homogeneity. While GBS data offer a coarse assessment of patterns of ecotype genome divergence, whole genome resequencing

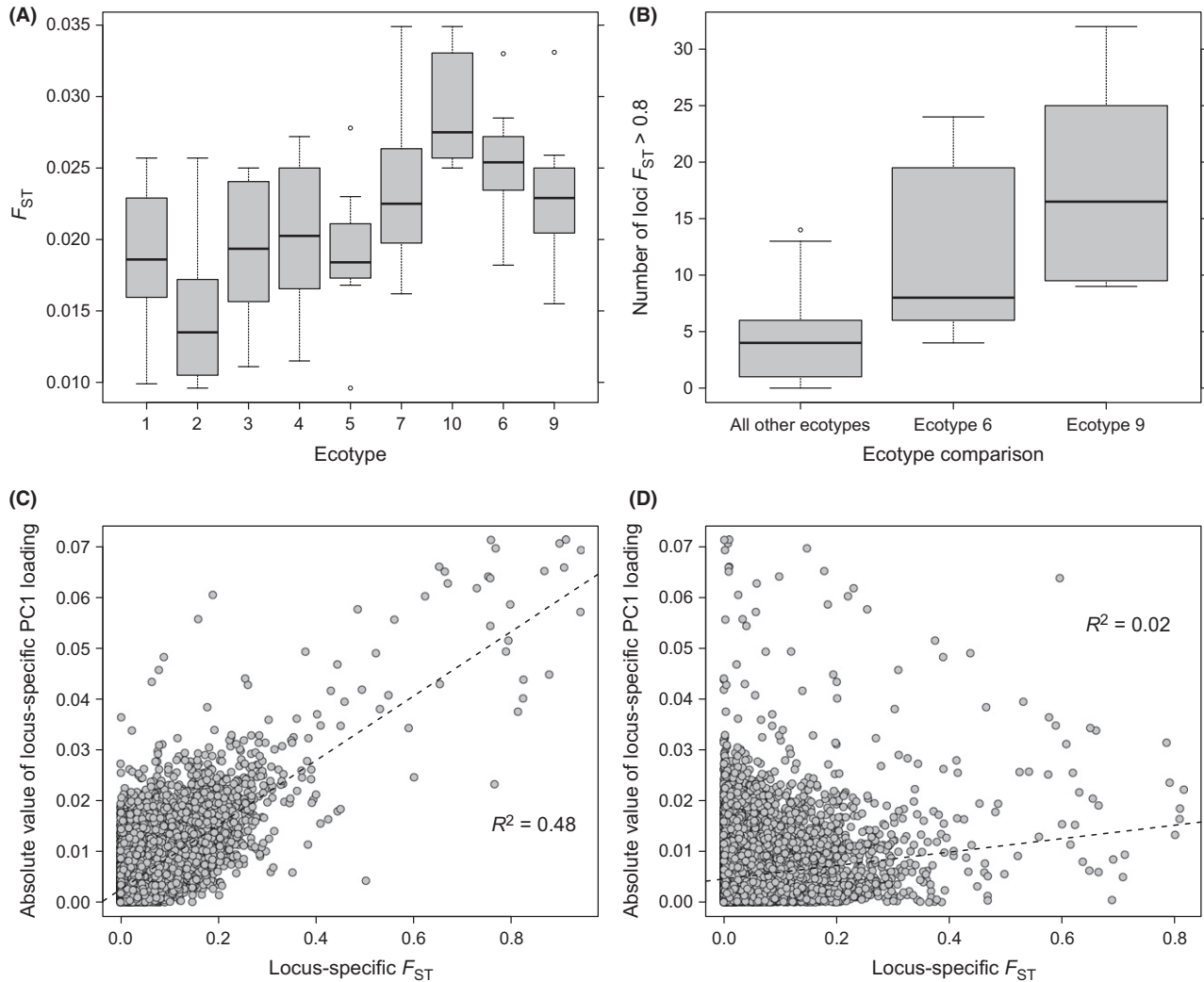


Fig. 5 Ecotypes 6 and 9, shown on the right in A and B, do not have particularly large pairwise F_{ST} estimates relative to those including only the other ecotypes (A), but they do have more numerous locus-specific F_{ST} estimates > 0.8 (of 18 385 loci) (B) in comparison with pairwise estimates between the other ecotypes (ecotype 6 vs. all others: Wilcoxon pairwise test, $Z = 2.48$, $P = 0.013$; ecotype 9 vs. all others: $Z = 3.57$, $P = 0.0004$; ecotype 6 vs. 9: $Z = 0.16$, $P = 0.16$; similar patterns were found for $F_{ST} > 0.9$, but are not shown). Analyses of PC1 loadings in relation to locus-specific F_{ST} estimates for pairs of ecotypes revealed much stronger relationships when ecotype 9 was included (C: ecotypes 2 and 9 shown; $P < 2.2 \times 10^{-16}$) than when it was excluded (D: ecotypes 2 and 10 shown; $P < 2.2 \times 10^{-16}$). Box plots (A, B) show minimum, first quartile, median, third quartile and maximum. Dashed lines (C, D) represent least-squares linear regressions.

could eventually resolve the size and organization of genomic regions involved in divergence and speciation.

Genome divergence in the South Hills crossbill could be influenced by geographic isolation, strength of reproductive isolation and effective population size. South Hills crossbills are not geographically isolated, as other nomadic ecotypes regularly move through and breed in the South Hills annually (Smith & Benkman 2007; Benkman *et al.* 2009). Moreover, the large scale over which we were unable to detect isolation by distance (Fig. 4A), suggests that 150 km of forestless area separating the South Hills from the vast forests to the north is unlikely

to affect the opportunity for gene flow. Alternatively, reproductive isolation is potentially stronger in the South Hills than elsewhere, because strong density-dependent food limitation (Benkman *et al.* 2012) and cones with elevated defences against crossbills make it more difficult for nonlocally adapted ecotypes to persist and breed (Smith & Benkman 2007). Our test of isolation by ecology was not statistically significant (Fig. 4B), indicating that increasing divergent selection alone did not result in an increase in genetic differentiation. However, as implied above, our measure of divergent selection (beak depth divergence) does not capture certain

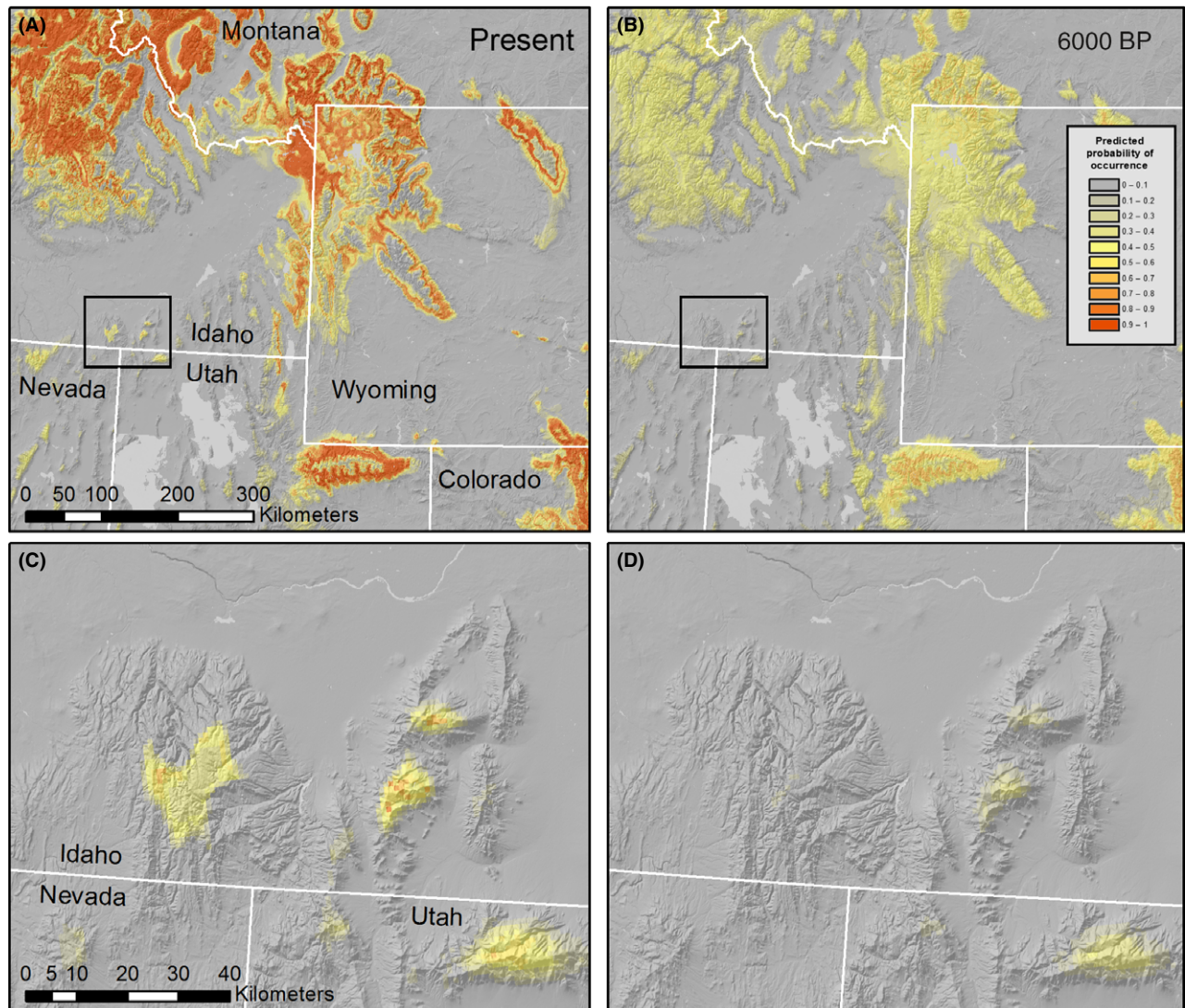


Fig. 6 The distribution of Rocky Mountain lodgepole pine based on Random Forests models that infer the probability of occurrence. A and C (inset in A enlarged) show the current distribution predicted by the models, which matches the actual distribution well except in Nevada, and within the area of Utah in C, where lodgepole pine does not occur. B and D (inset in B enlarged) represent the predicted lodgepole pine distribution 6000 BP. The amount of lodgepole pine forest in the South Hills and Albion Mountains, Idaho, 6000 BP is estimated to have been 86% less than its current abundance in these two mountain ranges where South Hills crossbills currently reside. Lodgepole pine did not occur in northwest Utah 6000 BP (Mehring 1985) contra D.

elements of ecology such as strength of density dependence that likely affect reproductive isolation (Bolnick 2011). Furthermore, given the heterogeneity of differentiation across the genome (Nosil *et al.* 2009), average genome-wide divergence is a poor measure for such analyses when divergence occurs with gene flow. An additional factor that could contribute to the greater genetic distinctiveness of the South Hills crossbill is its small effective population size, as genetic drift could increase relative genetic differentiation (F_{ST}). Indeed, ecotype-level estimates of heterozygosity (π) are lowest for the South Hills crossbill (Fig. 7), consistent with a

small effective population size [our current (October–November 2015) total population estimate is $N_c \sim 4000$ birds; N. Behl and C. W. Benkman, unpublished data]. Small population size, in addition to geographic isolation, has likewise been suggested to contribute to the relatively elevated levels of genetic divergence of the crossbill endemic to the Aleppo pine forests (*P. halepensis*) on the island of Mallorca (*L. c. balearica*; Bjorklund *et al.* 2013).

Reconstructions of historical lodgepole pine distribution using classification models (Random Forests) based on BioClim data were consistent with palaeobotanical

studies (Mehring 1985; Davis *et al.* 1986) indicating that pine forest was sparse during several thousand years of warming centred on 6000 BP in the region where South Hills crossbills currently reside (Fig. 6). Given the current South Hills crossbill population size, such a large (~86%) reduction in the amount of lodgepole pine would likely have prevented a distinct crossbill population from persisting in this region (Siepielski & Benkman 2005). This conclusion is supported by the evidence for exceptionally high temperatures 5000–7000 BP (Bartlein *et al.* 2014) conducive to lodgepole pine experiencing frequent catastrophic fires (Westerling *et al.* 2011), which would further reduce habitat for crossbills.

Thus, the South Hills crossbill either diverged as it coevolved with lodgepole pine in the South Hills during the last 6000 years, or it diverged elsewhere then subsequently colonized the expanded lodgepole pine forests of the South Hills. The latter is unlikely, as our reconstructions of historical lodgepole pine distributions for 22 000 BP (not shown) and palaeobotanical studies (Mehring 1985) provide no indication that large forested areas occurred in this region at an earlier time. Furthermore, coevolving crossbills and pines were unlikely to have moved to the South Hills from elsewhere, because to the east and north red squirrels are widespread and lodgepole pine cones there reflect strong selection exerted by red squirrels suggesting a history of interaction (Benkman 1999; see Arbogast *et al.* 2001). To the west and south, lodgepole pine does not and has not occurred (Wells 1983). Finally, the high level of reproductive isolation in the South Hills crossbill is

related to both the local resource characteristics that have evolved in response to the absence of red squirrels (Benkman & Siepielski 2004; Benkman *et al.* 2012) and crossbill-pine coevolution resulting in strong density dependence and local adaptation (Smith & Benkman 2007).

Conclusions

Coevolution has often been invoked to explain patterns of macroevolutionary diversification (Ehrlich & Raven 1964; Thompson 2005; Jablonski 2008), and some components of coevolution have been documented in numerous natural populations (Thompson 1994, 2005). However, clear examples of reciprocal selection and adaptation driving speciation (the link between coevolution as a micro- and macroevolutionary process) are largely lacking (Althoff *et al.* 2014; Hembry *et al.* 2014). Past studies on the South Hills crossbill have provided strong evidence for the role of coevolution in driving morphological divergence and reproductive isolation (Benkman 1999, 2003; Benkman *et al.* 2001, 2013; Smith & Benkman 2007). Our results indicate that the high contemporary measures of premating reproductive isolation (Smith & Benkman 2007; Benkman *et al.* 2009) reflect a longer term barrier to gene flow. Moreover, the nomadic behaviour of crossbills, their common sympatric occurrence and the absence of isolation by distance across all ecotypes (Fig. 4A) suggest that local coevolution rather than geographic isolation per se is responsible for the high levels of reproductive isolation for the South Hills crossbill. Model-based reconstructions of the past lodgepole pine distribution in the South Hills region (Fig. 6) and other lines of the evidence indicate that the ancestors of South Hills crossbills became resident and began coevolving with lodgepole pine more recently than 6000 BP. If the South Hills crossbill evolved so recently, it could represent one of the fastest examples of speciation in birds (Price 2008). Unfortunately, it is likely that this most genetically differentiated New World red crossbill lineage will go extinct within this century due to climate change and loss of suitable habitat (Santisteban *et al.* 2012; Benkman in press).

The evidence presented here for genetic differentiation associated with resource specialization in the absence of clear geographic isolation is rare, but similar to that seen in host races of insects (Mallet 2008; Pécoud *et al.* 2009; Nosil 2012; Powell *et al.* 2013). More generally, there is growing evidence that reproductive isolation can arise in part as a by-product of adaptation to alternative resources (i.e. ecological speciation; Schluter 2000; Nosil 2012). Crossbills might be unusual among birds, which diverge primarily in allopatry

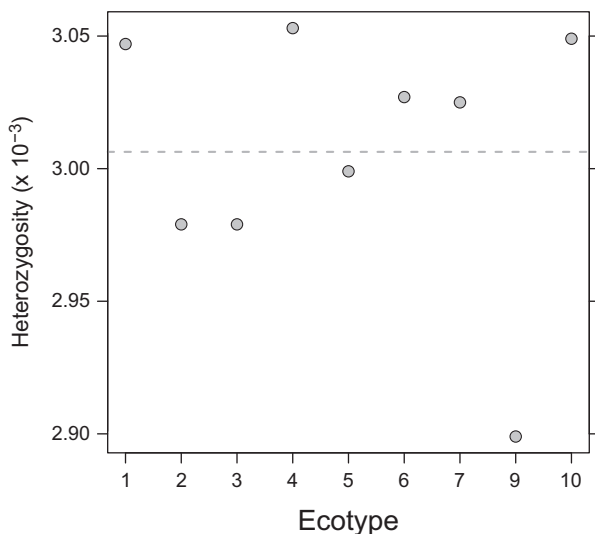


Fig. 7 Ecotype 9 (South Hills crossbills) exhibits much lower genetic diversity (likelihood estimates of heterozygosity) than the other ecotypes. The dashed line represents the overall mean.

(Price 2008), but the mechanisms underlying their diversification could prove general considering the tremendous diversity of host-specific insects (Mallet 2008) and that coevolution is thought to be a major driver of diversification (Thompson 2005; Althoff *et al.* 2014; Hembry *et al.* 2014). In particular, geographic variation in the coevolutionary process has been documented as an important source of divergent selection for many interactions (Thompson 2005, 2009). In cases where such divergent selection generates reproductive isolation, the geographic mosaic of coevolution could contribute prominently to ecological speciation.

Acknowledgements

This research was funded by NSF grants to C.A.B., the Robert B. Berry Chair to C.W.B. and UNR Start-Up Funds to T.L.P. We thank Carla Cicero, Museum of Vertebrate Zoology at The University of California at Berkeley, for providing tissue samples that Jeff Groth had collected, Pim Edelaar for additional samples and Mark Andersen for conducting the forest distribution modelling. We thank Pim Edelaar, Darren Irwin, Joshua Jahner, Patrik Nosil, Cody Porter and Katie Wagner for comments on drafts of the manuscript.

References

- Alcaide M, Scordato ES, Price TD, Irwin DE (2014) Genomic divergence in a ring species complex. *Nature*, **511**, 83–85.
- Althoff DM, Segraves KA, Johnson MTJ (2014) Testing for coevolutionary diversification: linking pattern with process. *Trends in Ecology & Evolution*, **29**, 82–89.
- Anderson M (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.
- Arbogast BS, Browne RA, Weigl PD (2001) Evolutionary genetics and Pleistocene biogeography of North American tree squirrels (*Tamiasciurus*). *Journal of Mammalogy*, **82**, 302–319.
- Bartlein PJ, Hostetler SW, Alder JR (2014) Paleoclimate. In: *Climate Change in North America* (ed. Ohring G), pp. 1–51. Springer International Publishing, Cham, Switzerland.
- Bell DM, Bradford JB, Lauenroth WK (2014) Early indicators of change: divergent climate envelopes between tree life stages imply range shifts in the western United States. *Global Ecology and Biogeography*, **23**, 168–180.
- Benkman CW (1993) Adaptation to single resources and the evolution of crossbill (*Loxia*) diversity. *Ecological Monographs*, **63**, 305–325.
- Benkman CW (1999) The selection mosaic and diversifying coevolution between crossbills and lodgepole pine. *The American Naturalist*, **154**, S75–S91.
- Benkman CW (2003) Divergent selection drives the adaptive radiation of crossbills. *Evolution*, **57**, 1176–1181.
- Benkman CW (in press) The natural history of the South Hills crossbill in relation to its impending extinction. *The American Naturalist*, **188**.
- Benkman CW, Siepielski AM (2004) A keystone selective agent? Pine squirrels and the frequency of serotiny in lodgepole pine. *Ecology*, **85**, 2082–2087.
- Benkman CW, Holimon WC, Smith JW (2001) The influence of a competitor on the geographic mosaic of coevolution between crossbills and lodgepole pine. *Evolution*, **55**, 282–294.
- Benkman CW, Parchman TL, Favis A, Siepielski AM (2003) Reciprocal selection causes a coevolutionary arms race between crossbills and lodgepole pine. *The American Naturalist*, **162**, 182–194.
- Benkman CW, Smith JW, Keenan PC, Parchman TL, Santisteban L (2009) A new species of the red crossbill (Fringillidae: *Loxia*) from Idaho. *Condor*, **111**, 169–176.
- Benkman CW, Fetz T, Talluto MV (2012) Variable resource availability when resource replenishment is constant: the coupling of predators and prey. *The Auk*, **129**, 115–123.
- Benkman CW, Smith JW, Maier M, Hansen L, Talluto MV (2013) Consistency and variation in phenotypic selection exerted by a community of seed predators. *Evolution*, **67**, 157–169.
- Bjorklund M, Alonso D, Edelaar P (2013) The genetic structure of crossbills suggests rapid diversification with little niche conservatism. *Biological Journal of the Linnean Society*, **109**, 908–922.
- Bolnick DI (2011) Sympatric speciation in threespine stickleback: why not? *International Journal of Ecology*, **2011**, 942847. doi:10.1155/2011/942847.
- Boucher-Lalonde V, Morin A, Currie DJ (2012) How are tree species distributed in climatic space? A simple and general pattern. *Global Ecology and Biogeography*, **21**, 1157–1166.
- Breiman L (2001) Random forests. *Machine Learning*, **45**, 5–32.
- Chen LJ, Lee DS, Song ZP, Suh HS, Lu BR (2004) Gene flow from cultivated rice (*Oryza sativa*) to its weedy and wild relatives. *Annals of Botany*, **93**, 1–7.
- Clarke R, Rothery P, Raybould A (2002) Confidence limits for regression relationships between distance matrices: estimating gene flow with distance. *Journal of Agricultural, Biological, and Environmental Statistics*, **7**, 361–372.
- Cruickshank TE, Hahn MW (2014) Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. *Molecular Ecology*, **23**, 3133–3157.
- Cutler DR, Edwards Jr TC, Beard KH, Cutler A, Hess KT (2007) Random forests for classification in ecology. *Ecology*, **88**, 2783–2792.
- Davis OK, Sheppard JC, Robertson S (1986) Contrasting climatic histories for the Snake River Plain, Idaho, resulting from multiple thermal maxima. *Quaternary Research*, **26**, 321–339.
- Dynesius M, Jansson R (2000) Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proceedings of the National Academy of Sciences USA*, **97**, 9115–9120.
- Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. *Evolution*, **18**, 586–608.
- Endler J (1973) Gene flow and population differentiation. *Science*, **179**, 243–250.
- Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, **164**, 1567–1587.
- Feder JL, Egan SP, Nosil P (2012) The genome of speciation-with-gene-flow. *Trends in Ecology & Evolution*, **28**, 342–350.
- Gesch D, Oimoen M, Greenlee S, Nelson C, Steuck M, Tyler D (2002) The national elevation dataset. *Photogrammetric Engineering and Remote Sensing*, **68**, 5–32.

- Gompert Z, Lucas LK, Nice CC, Fordyce JA, Forister ML, Buerkle CA (2012) Genomic regions with a history of divergent selection affect fitness of hybrids between two butterfly species. *Evolution*, **66**, 2167–2181.
- Gompert Z, Lucas LK, Buerkle CA, Forister ML, Fordyce JA, Nice CC (2014) Admixture and the organization of genetic diversity in a butterfly species complex revealed through common and rare genetic variants. *Molecular Ecology*, **23**, 4555–4573.
- Gomulkiewicz R, Drown DM, Dybdahl MF *et al.* (2007) Dos and don'ts of testing the geographic mosaic theory of coevolution. *Heredity*, **98**, 249–258.
- Groth JG (1993) *Evolutionary Differentiation in Morphology, Vocalizations, and Allozymes Among Nomadic Sibling Species in the North American Red Crossbill (Loxia curvirostra) Complex*. Vol. 127. University of California Press, Berkeley, CA, USA.
- Gugger PF, Sugita S, Cavender Bares J (2010) Phylogeography of Douglas-fir based on mitochondrial and chloroplast DNA sequences: testing hypotheses from the fossil record. *Molecular Ecology*, **19**, 1877–1897.
- Hembry DH, Yoder JB, Goodman KR (2014) Coevolution and the diversification of life. *The American Naturalist*, **184**, 425–438.
- Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.
- Hudson RR, Slatkin M, Maddison W (1992) Estimation of levels of gene flow from DNA sequence data. *Genetics*, **132**, 583–589.
- Irwin K (2010) A new and cryptic call type of the red crossbill. *Western Birds*, **41**, 10–25.
- Jablonski D (2008) Biotic interactions and macroevolution: extensions and mismatches across scales and levels. *Evolution*, **62**, 715–739.
- Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, **11**, 94.
- Lamichhaney S, Berglund J, Almén MS *et al.* (2015) Evolution of Darwin's finches and their beaks revealed by genome sequencing. *Nature*, **518**, 371–375.
- Li H (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, **27**, 2987–2993.
- Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, **25**, 1754–1760.
- Li H, Handsaker B, Wysoker A *et al.* (2009) The sequence alignment/map format and SAMtools. *Bioinformatics*, **25**, 2078–2079.
- Malinsky M, Challis RJ, Tyers AM *et al.* (2015) Genomic islands of speciation separate cichlid ecomorphs in an East African crater lake. *Science*, **350**, 1493–1498.
- Mallet J (2008) Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **363**, 2971–2986.
- Martin CH, Cutler JS, Friel JP, Denig CT, Coop G, Wainwright PC (2015) Complex histories of repeated gene flow in Cameroon crater lake cichlids cast doubt on one of the clearest examples of sympatric speciation. *Evolution*, **69**, 1406–1422.
- Mason NA, Taylor SA (2015) Differentially expressed genes match bill morphology and plumage despite largely undifferentiated genomes in a Holarctic songbird. *Molecular Ecology*, **24**, 3009–3025.
- Mehring Jr PJ (1985) Late-Quaternary pollen record from the interior Pacific Northwest and northern Great Basin of the United States. In: *Pollen Records of Late-Quaternary North America Sediments* (eds Bryant Jr WM, Holloway RG), pp. 167–189. American Association of Stratigraphy and Palynology Foundation, Dallas, Texas.
- Nei M (1972) Genetic distance between populations. *The American Naturalist*, **106**, 283–392.
- Newton I (2006) Movement patterns of common crossbills *Loxia curvirostra* in Europe. *Ibis*, **148**, 782–788.
- Nosil P (2012) *Ecological Speciation*. Oxford University Press, Oxford, UK.
- Nosil P, Egan SP, Funk DJ (2008) Heterogeneous genomic differentiation between walking-stick ecotypes: “isolation by adaptation” and multiple roles for divergent selection. *Evolution*, **62**, 316–336.
- Nosil P, Funk DJ, Ortiz-Barrientos D (2009) Divergent selection and heterogeneous genomic divergence. *Molecular Ecology*, **18**, 375–402.
- Nosil P, Gompert Z, Farkas TE *et al.* (2012) Genomic consequences of multiple speciation processes in a stick insect. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 5058–5065.
- O'Connell BM, LaPoint EB, Turner JA, Ridley T, Pugh SA, Wilson AM, Waddell KL, Conkling BL (2014) The Forest Inventory and Analysis database. In: *Database description and user guide version 6.0 for phase 2*. pp. 182 US Department of Agriculture Forest Service, Raleigh, NC.
- Oksanen J, Blanchet F, Kindt R *et al.* (2013) *Vegan: Community Ecology Package*. R package version 2.0-9.
- Parchman TL, Benkman CW, Britch SC (2006) Patterns of genetic variation in the adaptive radiation of New World crossbills (Aves: *Loxia*). *Molecular Ecology*, **15**, 1873–1887.
- Parchman TL, Gompert Z, Mudge J, Schilkey F, Benkman CW, Buerkle CA (2012) Genome-wide association genetics of an adaptive trait in lodgepole pine. *Molecular Ecology*, **21**, 2991–3005.
- Parchman TL, Gompert Z, Braun MJ *et al.* (2013) The genomic consequences of adaptive divergence and reproductive isolation between species of manakins. *Molecular Ecology*, **22**, 3304–3317.
- Peccoud J, Ollivier A, Plantegenest M, Simon JC (2009) A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences USA*, **106**, 7495–7500.
- Plummer M (2003) JAGS: a program for analysis of Bayesian graphical models using Gibbs sampling. In: *Proceedings of the 3rd international workshop on distributed statistical computing*. Vol. 124.
- Poelstra JW, Vijay N, Bossu CM *et al.* (2014) The genomic landscape underlying phenotypic integrity in the face of gene flow in crows. *Science*, **344**, 1410–1414.
- Potter KM, Hipkins VD, Mahalovich MF, Means RE (2013) Mitochondrial DNA haplotype distribution patterns in *Pinus ponderosa* (Pinaceae): range-wide evolutionary history and

- implications for conservation. *American Journal of Botany*, **100**, 1562–1579.
- Powell THQ, Hood GR, Murphy MO *et al.* (2013) Genetic divergence along the speciation continuum: the transition from host race to species in *Rhagoletis* (Diptera: Tephritidae). *Evolution*, **67**, 2561–2576.
- Price T (2008) *Speciation in Birds*. Roberts and Company Publishers, Greenwood Village, Colorado.
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.
- Questiau S, Gielly L, Clouet M, Taberlet P (1999) Phylogeographical evidence of gene flow among common crossbill (*Loxia curvirostra*, Aves, Fringillidae) populations at the continental level. *Heredity*, **83**, 196–205.
- R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Santisteban L, Benkman CW, Fetz T, Smith JW (2012) Survival and population size of a resident bird species are declining as temperature increases. *Journal of Animal Ecology*, **81**, 352–363.
- Schluter D (2000) *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford, UK.
- Shafer A, Wolf JB (2013) Widespread evidence for incipient ecological speciation: a meta-analysis of isolation-by-ecology. *Ecology Letters*, **16**, 940–950.
- Siepielski AM, Benkman CW (2005) A role for habitat area in the geographic mosaic of coevolution between red crossbills and lodgepole pine. *Journal of Evolutionary Biology*, **18**, 1042–1049.
- Smith JW, Benkman CW (2007) A coevolutionary arms race causes ecological speciation in crossbills. *The American Naturalist*, **169**, 455–465.
- Smith JW, Benkman CW, Coffey K (1999) The use and misuse of public information by foraging red crossbills. *Behavioral Ecology*, **10**, 54–62.
- Smith JW, Sjöberg SM, Mueller MC, Benkman CW (2012) Assortative flocking in crossbills and implications for ecological speciation. *Proceeding of the Royal Society B: Biological Sciences*, **279**, 4223–4229.
- Snowberg LK, Benkman CW (2007) The role of marker traits in the assortative mating within red crossbills, *Loxia curvirostra* complex. *Journal of Evolutionary Biology*, **20**, 1924–1932.
- Snowberg LK, Benkman CW (2009) Mate choice based on a key ecological performance trait. *Journal of Evolutionary Biology*, **22**, 762–769.
- Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**, 1312–1313.
- Stamatakis A, Aberer AJ (2013) Novel parallelization schemes for large-scale likelihood-based phylogenetic inference. In: *Parallel & Distributed Processing (IPDPS), 2013 IEEE 27th International Symposium*, pp. 1195–1204. IEEE Computer Society, New York.
- Summers RW, Dawson RJ, Phillips RE (2007) Assortative mating and patterns of inheritance indicate that the three crossbill taxa in Scotland are species. *Journal of Avian Biology*, **38**, 153–162.
- Thompson JN (1994) *The Coevolutionary Process*. University of Chicago Press, Chicago, Illinois.
- Thompson JN (2005) *The Geographic Mosaic of Coevolution*. University of Chicago Press, Chicago, Illinois.
- Thompson JN (2009) The coevolving web of life. *The American Naturalist*, **173**, 125–140.
- Vavrek MJ (2011) Fossil: palaeoecological and palaeogeographical analysis tools. *Palaeontologia Electronica*, **14**, 16.
- Wells PV (1983) Paleobiogeography of montane islands in the Great Basin since the last glaciopluvial. *Ecological Monographs*, **53**, 341–382.
- Westerling AL, Turner MG, Smithwick EAH, Romme WH, Ryan MG (2011) Continued warming could transform greater Yellowstone fire regimes by mid-21st century. *Proceedings of the National Academy of Sciences USA*, **108**, 13165–13170.

T.L.P., C.A.B. and C.W.B. designed research; T.L.P. generated and assembled data; T.L.P., C.A.B. and V.S.C. analysed data; and T.L.P. and C.W.B. wrote paper with contributions from C.A.B. and V.S.C.

Data accessibility

A detailed description of our genotyping by sequencing protocol, a matrix of genotype probabilities and .bam files from BWA assemblies are available at the Dryad digital repository (<http://dx.doi.org/10.5061/dryad.65d97>).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Methods.

Table S1 The number of individuals sampled of *Loxia curvirostra* ecotypes, including geographic locations of separate samples within each ecotype, as well as white-winged crossbills *Loxia leucoptera*.

Table S2 Pairwise estimates of Nei's *D* (upper triangle) and *F_{ST}* (lower triangle) among *Loxia curvirostra* ecotypes (call types).

Table S3 Deviance information criterion (DIC) estimates for entropy models run for *k* = 2 through *k* = 9. Lower estimates of DIC reflect better model fit.

Fig. S1 The map illustrates sampling localities for red crossbill (*Loxia curvirostra* complex). Individual points refer to geographically separate collection locations and correspond with Table S1. Circles of the same number and color represent different geographical samples from a given ecotype.