# Patterns of genetic variation in the adaptive radiation of New World crossbills (Aves: *Loxia*)

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## Abstract

Incipient species groups or young adaptive radiations such as crossbills (Aves: Loxia) present the opportunity to investigate directly the processes occurring during speciation. New World crossbills include white-winged crossbills (Loxia leucoptera), Hispaniolan crossbills (Loxia megaplaga), and red crossbills (Loxia curvirostra complex), the last of which is comprised of at least nine morphologically and vocally differentiated forms ('call types') where divergent natural selection for specialization on different conifer resources has been strongly implicated as driving diversification. Here we use amplified fragment length polymorphism (AFLP) markers to investigate patterns of genetic variation across populations, call types, and species of New World crossbills. Tree-based analyses using 440 AFLP loci reveal strongly supported clustering of the formally recognized species, but did not separate individuals from the eight call types in the red crossbill complex, consistent with recent divergence and ongoing gene flow. Analyses of genetic differentiation based on inferred allele frequency variation however, reveal subtle but significant levels of genetic differentiation among the different call types of the complex and indicate that between call-type differentiation is greater than that found among different geographic locations within call types. Interpreted in light of evidence of divergent natural selection and strong premating reproductive isolation, the observed genetic differentiation suggests restricted gene flow among sympatric call types consistent with the early stages of ecological speciation.

*Keywords*: adaptive radiation, AFLP, ecological speciation, genetic structure, incipient species, *Loxia curvirostra* 

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## Introduction

Evaluating the extent to which ecological and microevolutionary processes influence speciation and adaptive radiation has become a major focus of contemporary ecology and evolutionary biology (Schluter 2000; Hendry & Kinnison 2001). A burgeoning body of both theoretical and empirical research supports the importance of ecology in causing phenotypic differentiation, speciation, and adaptive radiation (Schluter 2000; Rieseberg *et al.* 2002; Coyne & Orr 2004).

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\*Present address: Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82071-3166, USA †Present address: USDA-ARS, Beneficial Insects Introduction Research Lab, 501 South Chapel Street, Newark, Delaware 19713-3814, USA Theoretical work demonstrates that reproductive isolation can readily evolve as a by-product of divergent selection on quantitative traits without geographic isolation (e.g. Dieckman & Doebeli 1999; Kondrashov & Kondrashov 1999; but see Gavrilets 2005), while empirical studies taking field, molecular, and experimental approaches provide support for the idea that divergent natural selection can promote divergence and restrict gene flow among ecologically specialized taxa (Rice & Salt 1988; Schliewen *et al.* 1994; Lu & Bernatchez 1999; Hendry *et al.* 2000; Schluter 2001; Ogden & Thorpe 2002).

Further empirical insight into how ecologically based divergent selection drives speciation is most likely to come from the study of incipient species where the processes involved in divergence and speciation are recent and ongoing (e.g. Wu *et al.* 1995; Capy *et al.* 2000; Korol *et al.* 2000; Beheregeray & Sunnucks 2001). Such groups present biologists with an opportunity to investigate directly the ecological processes influencing adaptation and the evolution of reproductive isolation. Characterizing patterns of genetic differentiation can be particularly challenging in groups diverging without geographic isolation, because ongoing gene flow can reduce or prevent differentiation at neutral loci even as traits under selection diverge, and because divergence may also be too recent for lineage sorting to have occurred (Orr & Smith 1998; Funk & Omland 2003). Such rapidly evolving, phenotypically diverse, but genetically weakly differentiated groups commonly characterize young adaptive radiations (Freeland & Boag 1999; Seehausen 2004).

Birds in the genus *Loxia* (crossbills) represent such a group where diversification is recent and ongoing (Benkman 1993, 2003; Benkman et al. 2001; Parchman & Benkman 2002), and where integrating studies of natural selection, assortative mating, and genetic structure can contribute to our understanding of speciation and adaptive radiation (Benkman 1993, 2003; Smith & Benkman in review a). Crossbills have evolved crossed mandibles as an adaptation for separating the scales of conifer cones and extracting the underlying seeds (Benkman 1987b; Benkman & Lindholm 1991), and have diversified into an array of resource specialists on different conifer species. In the New World, three species are recognized: white-winged crossbills (Loxia leucoptera), Hispaniolan crossbills (Loxia megaplaga), and red crossbills (Loxia curvirostra complex), with the last consisting of at least nine morphologically differentiated forms, each with characteristic vocalizations ('call types'; Groth 1993a; Benkman 1999). The white-winged crossbill has a small slender bill specialized for extracting seeds from between the narrow gaps of partly closed black spruce (Picea mariana) cones in the northern boreal forests (Benkman 1987b; Parchman & Benkman 2002). The Hispaniolan crossbill, until recently considered a subspecies of the white-winged crossbill (Banks et al. 2003), has apparently evolved its relatively large bill as an adaptation for feeding on seeds in the large, tough cones of Hispaniola's only cone-bearing conifer, Pinus occidentalis (Benkman 1994).

The diversity of forms in the red crossbill complex also appears to have evolved in response to selection for specialization on different conifer species or subspecies in North America (Benkman 1993, 2003; Benkman & Miller 1996; Benkman *et al.* 2001; Parchman & Benkman 2002). Studies relating bill morphology and feeding performance on the cones of different conifer species have demonstrated a match between the mean bill depths and widths of the groove in the upper palate where seeds are held during husking for many of the call types and the optima predicted from feeding experiments in aviaries, strongly implying that divergent natural selection underlies the diversity of call types and that cone and seed structure are the agents of selection (Benkman 1993, 2003). Importantly, these studies indicate that hybrids with intermediate bill morphologies should suffer reduced fitness and should be selected against (Benkman 1993, 2003), presenting an ecological mechanism for reducing gene flow between call types adapted to different resources. Such ecologically based selection against hybrids is an important prediction of ecological speciation (Schluter 2001). The maintenance of vocal and morphological variation in the face of widespread sympatry and nomadic movements of crossbills has also been used to argue that the call types represent a group of reproductively isolated sibling species (Groth 1993a).

Detailed research on the link between divergent selection and the barriers causing reproductive isolation is limited to comparisons among three of the North American call types (Smith & Benkman in review), but all evidence to date indicates that different call types flock separately (Smith & Benkman, unpublished) and mate assortatively when sympatric (Groth 1993b; Smith & Benkman in review). A study of 856 breeding crossbills in Idaho revealed that call types 2, 5, and 9 pair assortatively, with estimates of the strength of premating reproductive isolation > 0.997 on a scale from 0 to 1 where 1 equals complete reproductive isolation (Smith & Benkman in review).

Although these high levels of premating reproductive isolation indicate that divergent selection for resource specialization is causing speciation in the red crossbill complex, previous studies on these and similar crossbills have found little evidence for genetic differentiation. In Europe, several crossbill species (Loxia spp.) as well as call types within red (common) crossbills (L. curvirostra complex) appear to be genetically indistinguishable. Piertney *et al.* (2001) provide evidence of complete genetic homogeneity among three currently recognized crossbill species in the UK within the mithochondrial DNA (mtDNA) control region and across seven microsatellite loci. mtDNA control region sequences show similar patterns exhibiting no differentiation between small samples of morphologically differentiated vocal types within North America and Europe, although there is a phylogeographic break between red crossbills in North America and Europe (Questiau et al. 1999). However, sample sizes of North American call types were not sufficient to detect haplotype frequency differences, and divergence among North American call types may be too recent for lineage sorting of ancestral haplotypes if; for example, most of the radiation occurred following the expansion of conifer forests after the end of the Pleistocene. The only detailed investigation of genetic variation in the New World crossbills is Groth's allozyme study (1993a), where genetic differentiation among call types was not statistically significant, perhaps due to the low levels of variability often observed in these markers. Nonetheless, the ecologically specialized call types and species of North American crossbills have yet to be subject to hierarchical analysis of genetic variation with more recently developed highly variable molecular markers.

The amplified fragment length polymorphism (AFLP) technique has recently found wide applications in studies of genetic diversity, population structure, and phylogeny reconstruction because of the relative ease of producing large numbers of highly variable markers (Mueller & Wolfenbarger 1999; Bensch & Åkesson 2005). These markers have been particularly successful in resolving phylogenetic relationships and patterns of genetic structuring for recently diverged taxa where other markers have failed to uncover variability (e.g. Albertson et al. 1999; Giannasi et al. 2001; Despres et al. 2003; Sullivan et al. 2004). Although the dominant nature of the markers produces some statistical complications, advances in procedures for the estimation of allele frequencies from dominant marker data have reduced these problems and increased the utility of AFLP analysis (Lynch & Milligan 1994; Holsinger et al. 2002). Furthermore, the large numbers of markers provided by the technique should reduce both the error in genetic distance estimation (Keim et al. 1992) and the sampling error incurred from smaller sample sizes (Travis et al. 1996). AFLP markers have been applied successfully to population genetic studies of birds (e.g. Bensch et al. 2002; Wang et al. 2003) and should be particularly appropriate for examining genetic structuring of New World crossbills.

Here we survey patterns of AFLP marker variation within and among species of New World crossbills, across eight call types of the red crossbill complex, and among different geographic locations within one of these call types. We first examine patterns of genetic similarity among white-winged and Hispaniolan crossbills, eight call types of the red crossbill complex, and common redpolls using tree-based analyses. Second, we examine patterns of genetic structure based on allele frequency variation within and among eight call types of the red crossbill complex to assess the possibility of genetic differentiation indicating a reduction in gene flow among call types.

We did not acquire samples from each call type from throughout their geographic range because the erratic temporal and spatial patterns of crossbill distribution indicate that such sampling would be both impractical and unlikely to reveal geographic structure within a call type. Crossbills move across their geographic ranges tracking fluctuations in cone crops (Newton 1972; Benkman 1987a, 1992), which are often synchronized across hundreds of kilometres of forest (Koenig & Knops 1998). Consequently, crossbills become common in a region when there is a large cone crop but then depart and remain absent from large regions where cone crops are small or absent for intervals often exceeding their approximate generation time of 3 years (Benkman et al. 2005). Such examples of great abundance of crossbills preceded by and followed by years of complete absence are well known (Griscom 1937; Godfrey 1979) and have been recorded from throughout much of North America (Munro 1919; Griscom 1937; Lawrence 1949; Bailey et al. 1953). In addition to these nomadic movements, occasional widespread cone failures cause large numbers of crossbills to move outside their usual habitats (Newton 1972; Bock & Lepthien 1976; Koenig & Knops 2001), and such movements from western North America may largely account for the occurrence of some of the call types in eastern North America (Griscom 1937, 1941; Benkman 1987a, 1993; see also Koenig & Knops 2001). These eruptions likely cause massive mortality (Newton 1972; Benkman 1987a, 1992) from which crossbills then rebound until the next eruption. The result is that most call types are unlikely to have geographic structure. The one call type for which we anticipated some geographic structure was call type 2. At least partially resident and locally adapted populations of this call type are found on two isolated mountain ranges in Montana (Siepielski & Benkman 2005) where lodgepole pine produces exceptionally stable annual seed crops (Benkman et al. 2003). Consequently, we tested for genetic differentiation between samples of call type 2 collected from the above two mountain ranges and from two other locations (Fig. 1). We also investigated the relationship between genetic and geographic distances across all populations sampled representing the red crossbill complex as well as within call type 2. Although genetic differentiation between locations sampled could arise because crossbills usually occur in flocks, these last analyses would help us address whether the genetic differentiation among call types would likely be confounded by not sampling throughout the range of each call type.

## Methods

#### Genetic resources

We used samples from 142 individuals including representatives of eight call types of the red crossbill complex (Fig. 1), white-winged and Hispaniolan crossbills, and common redpolls. We used either blood taken from wild-caught birds and immediately stored in buffer (100 mм NaCl, 100 mм Tris pH 8, 100 mM EDTA) or frozen tissue samples from museum specimens; we lack samples from call type 8, the Newfoundland crossbill (Loxia curvirostra percna), which is likely extinct (Parchman & Benkman 2002). All sampled birds were previously assigned to call type based on sonograms made from recorded calls at the time of capture (see Groth 1993a; Benkman 1999). DNA was extracted from samples using QIAGEN DNeasy® tissue kits (QIAGEN) beginning with 100 µL of blood in buffer or approximately 20 mg of frozen tissue. DNA extracts were visualized for quality on 1.5% agarose gels and were quantified by flourometry using the Picogreen® DNA quantification kit (Molecular Probes) before being adjusted to a concentration of  $0.10 \, \mu g/\mu L.$ 



**Fig. 1** Map depicting locations of capture for the different call types of red crossbills (coloured circles; the one location with two call types is not coloured; numbers refer to call type). The shaded area outlines the distribution of cone-bearing conifers and the coloured lines enclose the main areas of occurrence for the correspondingly coloured call type (no lines are shown for call type 9 because it only occurs in an area smaller than the circle designating the sampling location); some of the call types occur outside of the mapped area, but their distributions are truncated at the edge of the map. The geographic ranges are based on Groth (1993a) and Adkisson (1996), the distributions of conifers important to the various call types, and on personal observations by one of the authors (C.W.B.). Sample sizes from left to right for call types with multiple samples were: call type 1 (4, 6), call type 2 (5, 14, 16, 6), call type 3 (1, 4, 3, 2), call type 4 (2, 7), call type 5 (9, 4), and call type 7 (6, 4).

### AFLP procedure

The AFLP procedure was carried out as in Vos et al. (1995) using reagents and protocols available in the AFLP plant mapping kit (ABI, Inc.). Restriction digestion and adaptorligation were carried out simultaneously on 0.5 µg of genomic DNA using the restriction endonucleases *Eco*RI and *Mse*I (NEB, Inc.). AFLP adaptor pairs (ABI) were attached to digested fragments using T4 DNA ligase (NEB). Restriction and ligation reactions were performed in 11-µL volumes and incubated for 18 h at 38 °C. Preselective amplifications were run with  $4 \mu L$  of the diluted restriction-ligation products, 15 µL AFLP core mix (ABI), and 1 µL EcoRI and MseI preselective primers (ABI). Preselective amplification primers consisted of the adaptor primer sequence with an additional nucleotide at the 3' ends. Preselective PCR conditions were 20 cycles of (94 °C, 30 s; 56 °C, 1 min; 72 °C, 2 min) with a final extension at 60 °C for 30 min. We checked 10 µL of each preselective amplification product on 1.5% agarose gels before use in selective amplifications.

Selective amplifications were run with  $3 \mu L$  of diluted preselective amplification product,  $15 \mu L$  AFLP core mix,  $1 \mu L$  of selective *MseI* primer, and  $1 \mu L$  of the fluorescently labelled *Eco*RI selective primer (all from ABI). Both *Eco*RI and *MseI* selective amplification primers had three extra nucleotides at the 3' ends in order to reduce the number of

fragments amplified to a manageable number. One microlitre of each selective amplification product was run with 8.75  $\mu$ L formamide and 0.25  $\mu$ L GeneScan 500 ROX-labelled size standard (ABI) on an ABI 3100 capillary sequencer. Forty-five selective primer combinations were screened across a sample of 14 individuals from four call types to search for AFLP fragments showing fixed differences between call types (fragments present at 100% in one call type but absent from another). After no fixed fragments were found, four primer combinations were chosen for use that consistently produced large numbers of polymorphic fragments across the sample being screened (Table 1). These combinations were not chosen with respect to differentiation at AFLP loci among the call types screened.

#### Data analysis

The presence and absence of AFLP fragments in each lane file was analysed using the programs GENESCAN and GENOTYPER 2.5 (ABI). Fragments obtained using each primer combination were scored as present or absent for each locus corresponding to a different sized fragment amplified. Analyses were limited to fragments between the sizes of 70 and 400 bp, and only unambiguously discernable loci were scored. AFLP fragments were treated as dominant marker loci with two states, presence (1) and absence (0). In order to use allele

Primer pair	No. of fragments	No. of polymorphic loci across all species (%)	No. of polymorphic loci in <i>L. curvirostra</i> complex (%)		
E-AAC/M-CTG	111	85 (76.6)	55 (50.0)		
E-AAC/M-CAG	113	90 (79.6)	61 (53.7)		
E-ACT/M-CAG	108	83 (76.8)	57 (52.8)		
E-ACT/M-CTA	108	90 (83.4)	73 (68.0)		
Total	440	348 (79.1)	246 (55.9)		

**Table 1** AFLP primer pairs used (E = EcoRI, M = MseI), the number of AFLP loci amplified, and the number and percentage of loci that were polymorphic across all species included in the study (*Loxia curvirostra*, *L. leucoptera*, *L. megaplaga*, *Carduelis flammea*) and within the *L. curvirostra* complex

frequency estimates to obtain population genetic parameter estimates, we made the assumption of Hardy–Weinberg equilibrium within populations and call types.

We assessed patterns of genetic similarity among all samples included in this study by constructing dendrograms with individuals as taxonomic units using UPGMA in PAUP version 4.0 (Swofford 2001). One thousand bootstrap replicates determined support on the different nodes. Common redpolls were used for outgroup purposes, as investigations based on mtDNA cyt b suggest redpolls and crossbills are sister taxa (Arnaiz-Villena et al. 2001). We also used principal coordinates analysis (PCO) on Euclidian distances among individual AFLP scores to visualize the clustering of the different taxa in two dimensions, as implemented in the program мvsp 3.1 (Kovach 1999). PCOs were repeated within the red crossbill complex to more closely examine clustering and overlap among the different call types. A distance-based phenogram with call types as taxonomic units was constructed based on pairwise genetic distances (Nei's D) among call types, geographically separate samples within call type 1, 2, 5, and 7, and among the different species sampled. Because call type 2 birds from the Little Rocky Mountains, Montana, were sampled in each of two years at the same location (2000, n = 5; 2001, n = 9), we separated them in the UPGMA tree to examine how variation among years at a site might compare to variation among sites within call type 2. We did not separate the different geographic samples of call types 3 due to small sample sizes (n < 4), and because these samples did not differ significantly based on  $F_{ST}$ . We obtained 1000 bootstrapped matrices of pairwise estimates of Nei's D using the program AFLP-SURV 1.0 (Vekemans 2002) and used these matrices to build UPGMA trees using the NEIGHBOR program in PHYLIP 3.6 (Felsenstein 2004). The bootstrap consensus tree was obtained using the program CONSENSE in PHYLIP 3.6.

The program TFPGA (Miller 1997) was used to estimate the percentage of polymorphic loci and unbiased expected heterozygosities ( $H_{E'}$ , Nei 1978) to determine genetic diversity within species, call types, and geographic samples within call type 2. Pairwise estimates of  $F_{ST}$  among call types were obtained using ARLEQUIN version 2.0 (Schneider *et al.* 2000). The significance of these estimates was tested by comparing observed  $F_{ST}$  estimates with a null distribution created by 1000 random permutations of the data set (Excoffier et al. 1992). Pairwise estimates of the F-statistic analogue  $\theta^{B}$  (Holsinger *et al.* 2002) were also obtained via a Bayesian approach that accounts for uncertainty in the magnitude of inbreeding within populations using the program HICKORY version 1.0 (Holsinger & Lewis 2003). Although available sample sizes were very small for call types other than type 2, we also obtained estimates of  $F_{ST}$  among geographically separate samples within call types 1, 2, 5, and 7 for comparisons where  $n \ge 4$  per geographic sample. Hierarchical structuring of genetic variation among and within call types and species was also examined using analysis of molecular variance (AMOVA; Excoffier et al. 1992) in ARLEQUIN version 2.0. Three AmovA models were analysed: one with variation partitioned among and within species of crossbills; the second with variation partitioned among call types, among geographically separate samples within call type, and within samples; and the third with variation partitioned within and among samples of call type 2 from different geographic locations. We used the nested AMOVA mentioned above rather than a model with variation partitioned only among and within different geographic samples across all call types, because each separate geographic sample represented only one call type and we lack samples of different call types from the same location. Mantel tests (Mantel 1967) were conducted to test for a relationship between geographic and genetic distance among all geographically separate samples of red crossbills as well as within call type 2 crossbills sampled from the Little Rocky and Bears Paw Mountains, Montana, the Black Hills, South Dakota, and the Sandia Mountains, New Mexico (Fig. 1). Mantel tests were run using matrices of pairwise estimates of genetic distance (Nei's D), and geographic distance among crossbills (regardless of call type) sampled from different geographic areas [geographic distances obtained from MapSource (Garmin, Inc.)] in ARLEQUIN version 2.0.

We also used a model-based Bayesian clustering method for multilocus genotype data to infer population structure and to assign individuals to population without a priori information on population origin as implemented in the program STRUCTURE (Pritchard *et al.* 2000). AFLP data were prepared for input into STRUCTURE using AFLP-SURV 1.0. We used a burn-in period of 20 000 iterations and collected data for 10<sup>6</sup> iterations using the no-admixture model. We used these analyses to infer the number of populations (*k*) and to assign individuals to these populations without a priori information on call type origin by running the simulations for a series of k = 1 through k = 12 and inputting the full AFLP data set for the red crossbill call types. We ran simulations three times and obtained similar results on each run.

## Results

## AFLP patterns and polymorphism

The four AFLP selective primer combinations generated 440 unambiguous markers ranging in size from 80 to 400 bp. Three hundred forty-eight bands (79.1%) were polymorphic across 142 individuals spanning four species, while 246 bands (55.9%) were polymorphic within the red crossbill complex (Table 1). We scored a similar number of bands for each of the four AFLP selective primer combinations used, and levels of polymorphism among species and call types of the red crossbill complex were similar for each primer combination (Table 1). The observed patterns of polymorphism were very similar to those reported in a recent AFLP study of house finch (Carpodacus mexicanus) populations and the closely related purple finch (Carpodacus *purpureus*) and Cassin's finch (*Carpodacus cassini*) (Wang et al. 2003). No AFLP markers exhibited fixed differences between any of the call types (markers fixed as present in one call type and absent in another). However, six loci were fixed between red crossbills and all other crossbills, seven loci were fixed between white-winged and Hispaniolan crossbills, and 17 loci exhibited fixed differences between all crossbills and common redpolls. Estimates of  $H_{\rm E}$  were similar for red crossbills (0.166), white-winged crossbills (0.168), and common redpolls (0.137), but were lower for Hispaniolan crossbills (0.052) (Table 2). Estimates of  $H_{\rm E}$  ranged between 0.113 and 0.158 for the different red crossbill call types (Table 2).

#### Genetic variation among species

UPGMA trees constructed with individuals as taxonomic units had high (> 50%) bootstrap support for the nodes separating common redpolls, white-winged, Hispaniolan, and red crossbills. However, there was no tendency for individuals of the same call type to cluster together and almost all nodes had lower than 50% bootstrap support. Due to the large number of taxa and because there was no resolution of branching patterns or evidence of clustering in the red crossbill complex, we do not present these trees here. The PCO analyses revealed patterns consistent with tree-building analyses. Common redpolls, white-winged, Hispaniolan, and red crossbills formed completely nonoverlapping clusters although there is considerable overlap within the red crossbill complex (Fig. 2). Similarly, AMOVAS with variation **Table 2** Number of loci polymorphic (*P*), percentage of loci polymorphic (*P*, 95% criterion), and Nei's (1978) unbiased expected heterozygosity ( $H_E$ ) for species and call types included in this study based on genotypes of 440 AFLP loci. Estimates were obtained using TFPGA (Miller 1997). Sample sizes are represented by (*n*)

	Р	%P	$H_{\rm E}$
<i>L. curvirostra</i> complex ( $n = 125$ )	264	56.0	0.166
Type 1 $(n = 10)$	146	33.3	0.126
Type 2 ( $n = 41$ )	185	42.1	0.157
Bears Paw Mountains, MT ( $n = 5$ )	113	25.7	0.107
Little Rocky Mountains, MT ( $n = 14$ )	154	34.4	0.139
Black Hills, SD ( $n = 6$ )	115	26.2	0.113
Sandia Mountains, NM ( $n = 16$ )	170	38.7	0.153
Type 3 ( <i>n</i> = 10)	151	34.4	0.122
Type 4 $(n = 9)$	142	32.3	0.129
Type 5 ( $n = 13$ )	139	31.7	0.131
Type 6 ( $n = 10$ )	109	24.9	0.113
Type 7 ( $n = 10$ )	117	26.7	0.116
Type 9 ( $n = 22$ )	173	39.4	0.158
<i>L. leucoptera</i> $(n = 8)$	189	43.1	0.168
L. megaplaga $(n = 7)$	58	13.2	0.052
C. flammea ( $n = 2$ )	122	27.8	0.137

partitioned among and within the three species of crossbills suggest that a large amount of variation (40.1%) was due to differences among species (Table 3A).

### Genetic structuring in the red crossbill complex

Estimates of the Bayesian F-statistic analogue  $\theta^{B}$  (Holsinger & Lewis 2003) and  $F_{ST}$  were similar for all pairwise comparisons; we present only  $F_{ST}$  estimates for simplicity. Genetic differentiation across the red crossbill complex as a whole was highly significant ( $F_{ST} = 0.09$ , P < 0.001), and only 3 of 28 pairwise estimates of  $F_{ST}$  between call types were not significant, all of which pertained to call type 7 (Table 4). AMOVA models focused on the red crossbill complex revealed most of the variation to be within geographic samples (89.5% for all call types, 97% for call type 2), but also indicated significant levels of genetic differentiation among call types and among geographic samples within call types. Seven per cent of the variation was due to differences among call types while 3.5% was due to differentiation among geographic samples within call types (Table 3B). When call type 1 (the only samples from east of the Great Plains; Fig. 1) was removed from this analysis, 5.0% of the variation was due to differences among call types (P < 0.001) while 2.4% was due to differentiation among samples within call types (P < 0.001). The different call types of the red crossbill complex also overlapped considerably in PCO space when viewed together, although individual birds of the same call type formed clusters, and several pairs of call types (only one shown, Fig. 3a) formed



**Fig. 2** Principal coordinates analysis based on Euclidian distances among the AFLP scores for all individuals included in the study. The colours for the different call types are meant to highlight the three clusters distinguished in the UPGMA dendrogram (Fig. 4): call type 1 by green, call types 2, 5, and 9 by shades of blue and call types 3, 4, 6, and 7 by shades of red; similar colours were used for the call types in Fig. 1.

**Table 3** Analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) performed in ARLEQUIN version 2.0 (Schneider *et al.* 2000) based on 440 AFLP loci amplified in the species and call types of crossbills included in this study. Model A has the variation partitioned among and within species of crossbills. Model B has the variation partitioned among call types, among geographically separated samples within call types, and within such samples of the *Loxia curvirostra* complex. Model C has the variation partitioned among and within geographically separated samples of call type 2 Model A

	C	<b>V</b> 7	Deventere	
1.6	Sum or	variance	Percentage	D
d.f.	squares	components	of variation	Р
2	654.54	20.33	40.1	< 0.0001
138	4121.18	29.86	59.5	< 0.0001
140	4775.18	50.19		
	Sum of	Variance	Percentage	
d.f.	squares	components	of variation	Р
7	474.40	2.09	7.0	< 0.0001
7	243.70	1.08	3.5	< 0.0001
109	2997.23	27.50	89.5	< 0.0001
123	3715.32			
	Sum of	Variance	Percentage	
d.f.	squares	components	of variation	Р
3	110.09	0.89	3.1	0.0019
37	1045.32	28.25	97.0	< 0.0001
40	1155.42	29.14		
	d.f. 2 138 140 d.f. 7 7 109 123 d.f. d.f. 3 37 40	d.f. Sum of squares   2 654.54   138 4121.18   140 4775.18   0 4775.18   0 5000 of squares   7 474.40   7 243.70   109 2997.23   123 3715.32   0 4.f.   3 110.09   37 1045.32   40 1155.42	d.f. Sum of squares Variance components   2 654.54 20.33   138 4121.18 29.86   140 4775.18 50.19   Variance components   d.f. Sum of squares Variance components   7 474.40 2.09   7 243.70 1.08   109 2997.23 27.50   123 3715.32 27.50   3 110.09 0.89   37 1045.32 28.25   40 1155.42 29.14	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$

**Table 4** Pairwise estimates of Nei's unbiased genetic distance (above diagonal) and  $F_{ST}$  (below diagonal) between all species and call types. Estimates of Nei's unbiased genetic distance and  $F_{ST}$  were obtained using TFPGA version 1.3 (Miller 1997) and ARLEQUIN version 2.0 (Schneider *et al.* 1999), respectively. Significant  $F_{ST}$  values (P < 0.01) are in bold; 1000 permutations were used to test the significance of  $F_{ST}$  estimates

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 9	L. leucoptera	L. megaplaga	C. flammea
Type 1	0	0.044	0.020	0.033	0.040	0.040	0.033	0.036	0.117	0.169	0.334
Type 2	0.226	0	0.031	0.019	0.012	0.022	0.024	0.012	0.099	0.151	0.317
Type 3	0.057	0.123	0	0.018	0.027	0.019	0.016	0.024	0.117	0.159	0.337
Type 4	0.133	0.050	0.028	0	0.022	0.013	0.012	0.021	0.113	0.154	0.348
Type 5	0.211	0.031	0.107	0.052	0	0.024	0.024	0.017	0.111	0.165	0.345
Type 6	0.183	0.100	0.031	0.033	0.091	0	0.014	0.026	0.113	0.160	0.346
Type 7	0.173	0.084	0.025	0.020	0.081	0.002	0	0.026	0.122	0.161	0.348
Type 9	0.189	0.041	0.089	0.052	0.034	0.098	0.080	0	0.092	0.146	0.320
L. leucoptera	0.383	0.318	0.350	0.338	0.368	0.380	0.369	0.314	0	0.109	0.249
L. megaplaga	0.589	0.523	0.565	0.579	0.589	0.610	0.588	0.498	0.380	0	0.342
C. flammea	0.615	0.594	0.604	0.613	0.644	0.651	0.628	0.594	0.462	0.733	0



**Fig. 3** Plots of the first two principal coordinate axes for an analysis conducted on the red crossbill complex alone. (a) Call types 1 and 2, (b) call types 2 and 4, (c) call types 3 and 4, and (d) call types 2 and 5.



**Fig. 4** UPGMA phylogram reflecting relative genetic distances based on pairwise estimates of Nei's *D* among different recognized species included in this study and eight call types of the red crossbill complex including samples from two geographic samples of call types 1, 5, and 7, and four geographic samples of call type 2 (BP, Bears Paw Mountains; NM, New Mexico; BH, Black Hills; LR, Little Rocky Mountains, with the samples taken in 2000 and 2001 distinguished as LRa and LRb, respectively). Values at the nodes represent bootstrap support based on 1000 replicates; values < 50% are not shown. A representative head and, where known, a cone of the conifer on which each crossbill specializes is shown. Heads and cones are from figures in Benkman (1987b, 1999), Parchman & Benkman (2002) and Farjon & Styles (1997), with bill sizes and cones altered to reflect relative sizes among the different crossbills and conifers, respectively. Cones from top to bottom are: *Pinus occidentalis, Picea mariana, Pinus contorta latifolia* from South Hills, *Pinus ponderosa scopulorum, Pinus contorta latifolia, Tsuga heterophylla, Pseudotsuga menziesii menziesii,* and *Picea rubens.* Call type 4 is associated with *Pseudotsuga m. menziesii.* 

nonoverlapping clusters when the analysis was restricted to red crossbills.

Only three of nine comparisons of different geographic samples within call types revealed significant genetic differentiation, which contrasts with the relatively high number of comparisons that were significant between call types (25 of 28 comparisons; Fisher's exact test, P = 0.002). The significant within-call-type comparisons were between call type 2 in the Black Hills, South Dakota, and both the Sandia Mountains, New Mexico ( $F_{\rm ST} = 0.057$ , P < 0.05), and the Bears Paw Mountains, Montana ( $F_{\rm ST} = 0.041$ , P < 0.05), and between call type 1 from North Carolina and Virginia ( $F_{\rm ST} = 0.13$ , P < 0.05). AMOVA examining variation among

samples of call type 2 from different geographic locations (Fig. 1) indicates that the vast majority of variation is found within location (97%) but that a significant amount of variation (3.1%) was due to differences among locations, which was lower than the 7% explained by differences among call types (Table 3). Finally, different geographic samples within call types 1, 2, and 5 grouped together in the UPGMA dendrogram (Fig. 4), suggesting genetic continuity within these call types, whereas the two geographically separate samples of call type 7 did not group together (Fig. 4), perhaps not surprisingly as call type 7 did not differ significantly from call types 3, 4, and 6 based on  $F_{\rm ST}$  (Table 4).

Mantel tests examining the relationship between genetic and geographic distances for all red crossbills revealed a significant relationship between genetic and geographic distances ( $r^2 = 0.26$ , P = 0.012). However, such geographic structure was related in large part to the nonrandom locations of the samples (Fig. 1 e.g. call type 1 only from the east). After call type 1, which was the only call type sampled east of the Great Plains (Fig. 1), was removed, a relationship was no longer detected ( $r^2 = 0.03$ , P = 0.09). When the Mantel test was applied to the four geographic samples of call type 2, no relationship between genetic and geographic distances was evident ( $r^2 = 0.02$ , P = 0.66). Moreover, two samples of call type 2 taken 1 year apart at the same location in the Little Rocky Mountains, Montana, differed ( $F_{ST} = 0.05$ , P < 0.05) as much as the other geographically separated samples of call type 2 (Fig. 4). This finding, along with the absence of a correlation between genetic and geographic distance within call type 2 and among all locations across western North America (Fig. 1) indicates that the genetic differences among samples of red crossbills do not fit an isolation-by-distance scenario, and that differentiation between call types was not likely an artefact of sampling location.

The UPGMA dendrogram, based on pairwise genetic distances treating species, call types, and geographically separate samples within several call types as taxonomic units, separates call type 1 from the remaining call types that were separated into two groups consisting of call types 3, 4, 6, and 7 in one, and call types 2, 5, and 9 in the other, with geographically separate samples within call types 1, 2, and 5, but not call type 7, grouping together (Fig. 4). The PCO using only red crossbills shows varying levels of overlap between call types (Fig. 3) consistent with the relationships shown in the UPGMA dendrogram (Fig. 4). Call type 1 is the most distinctive call type and overlaps very little in PCO plots with any other call type. For example, call types 1 and 2, which were the first call types described where they co-occur and breed in the Appalachian Mountains (Groth 1988), show no overlap in the PCO plot (Fig. 3a). Although call type 1 is found in western North America (Fig. 1; Groth 1993a), the distinctiveness of call type 1 in this study is likely influenced by it being the only call type sampled east of the Great Plains. Two other recognizable groupings of call types comprising call types 3, 4, 6, and 7 and the second comprising call types 2, 5, and 9 (Fig. 4) also overlap little. For example, call types 2 and 4 show almost no overlap in the PCO plot (Fig. 3b). In contrast, call types within one of the above groups show higher overlap in the PCO plots. For instance, call types 3 and 4 (Fig. 3c) and call types 2 and 5 (Fig. 3d) overlap considerably.

Analyses run in STRUCTURE revealed genetically distinctive groups, but did not result in the assignment of individuals of a call type to the same cluster at high rates. The natural logarithm of the probability of the data was lowest with k = 1 (ln = -12021.5), and highest with k = 7 (ln = -11051.1), indicating the red crossbill complex represents, at most, seven distinctive groups. This roughly corresponds to the number of call types in the complex (call type 7 appears to be not differentiated from other call types), but is far from corresponding to the number of geographically separate samples. However, assignment of individuals from each call type to unique clusters was not clear (except for call type 1) and many individuals were inferred to be of admixed ancestry. This may reflect subtle genetic differentiation among call types as well as relatively small sample sizes for many of the call types (for similar results see Sefc *et al.* 2005).

#### Discussion

Our investigation of AFLP marker variation across New World members of the genus Loxia revealed greater genetic structuring based on allele frequency variation than was evident in previous studies based on allozymes (Groth 1993a) and mtDNA (Questiau et al. 1999), but did not provide evidence of monophyletic clustering among individual birds representing different call types of the red crossbill complex. Such an absence of monophyletic clustering of individual birds in tree-based analyses is not surprising and is consistent with previous studies on crossbills utilizing allozymes, mtDNA, and microsatellites (Groth 1993a; Questiau et al. 1999; Piertney et al. 2001); as well as with probable recent divergences and low levels of hybridization among some of the call types. Nonetheless, analyses based on allele frequency variation provided evidence of genetic differentiation among call types. Below we discuss this variation in light of previous and ongoing studies on the role of natural selection and premating reproductive isolating barriers in contributing to diversification in the red crossbill complex.

#### Genetic variation among species

As expected, the different species included in this study were clearly separated in UPGMA trees treating individuals as taxonomic units based on AFLP variation (not shown). These results, consistent with principal coordinates analyses (Fig. 2) and mtDNA control region sequence variation (J. Groth, unpublished data in GenBank), indicate that whitewinged and Hispaniolan crossbills are sister taxa. The recent elevation of the Hispaniolan crossbill to species status (Banks *et al.* 2003) is supported by the presence of seven AFLP markers that were fixed between white-winged and Hispaniolan crossbills and by the clear groupings depicted in our PCO plots (Fig. 2). Further supporting such a distinction are the substantial morphological differences between the small, slender-billed white-winged crossbill and the large,

### Genetic structuring in the red crossbill complex

The absence of fixed differences between call types in our screening of approximately 4000 AFLP markers is not surprising in light of other published studies on genetic variation in both New and Old World crossbill complexes (Questiau et al. 1999; Piertney et al. 2001). We interpret the lack of monophyly among the different call types in treebased analyses treating individual birds as taxonomic units to be the result of recent divergence and ongoing introgression rather than a lack of phylogenetic signal due to the number of markers in our AFLP data set (Funk & Omland 2003). In addition, mtDNA cyt b sequence variation suggests that subspecific differentiation of allopatric Old World crossbills occurred in the Pleistocene (Arnaiz-Villena et al. 2001), suggesting that call-type divergence in the red crossbill complex is similarly recent. Such patterns commonly characterize phylogenetic analyses of incipient species groups or recent adaptive radiations with low levels of ongoing gene flow among diverging taxa (Freeland & Boag 1999; Beheregeray & Sunnucks 2001; Seehausen 2004).

Nevertheless, analyses based on allele frequency variation inferred from our AFLP data set allowed us to reject the null hypothesis of no genetic structuring among the call types (Tables 3 and 4). Such genetic structuring is also evident from the PCOs confined to the red crossbill complex (Fig. 3). Significant estimates of  $F_{ST}$  for almost all pairwise comparisons between call types (Table 4) reveal that all but one of the call types are significantly genetically differentiated from one another, with estimates of genetic differentiation among call types (mean  $F_{ST} = 0.091$ ) in the low to moderate range (Wright 1978). Although analyses of molecular variance reveal that most of the genetic variation in the AFLP data set resides within call types, a significant amount of variation (7% of the total) is still due to differences among call type. This fine-scale genetic differentiation among call types is biologically meaningful because the sympatric distributions and nomadic movements of crossbills provide ample opportunity for gene flow that would erase or impede genetic differentiation in the absence of some degree of reproductive isolation. This interpretation is consistent with recent work revealing strong premating reproductive isolation among three call types based on habitat, temporal, and behavioural isolating barriers (Smith & Benkman in review).

The finding that differentiation among geographically separate samples within call types is less pronounced than that seen among call types further supports this conclusion. For example, pairwise  $F_{ST}$  estimates among call types were more often significant than similar comparisons among geographic locations within call types, and AMOVAS also indicated a higher percentage of variation resulting from differences among call types than from differentiation among different geographic locations within call types (Table 3B). In addition, different geographic samples for call types 1, 2, and 5 grouped together in the UPGMA dendrogram (Fig. 4), suggesting genetic continuity within these call types (e.g. Sefc et al. 2005). On the other hand, the different geographic samples of call type 7 did not group together, but this is not surprising given that call type 7 is the least differentiated call type (Table 4). Moreover, this is the only call type in the Northwest for which we have been unable to predict a conifer on which it might specialize (see Benkman 1993). Finally, two groups of call type 2 crossbills captured at the same netting location in 2000 and 2001 were as different from one another as other call type 2 samples taken hundreds of kilometres apart (Fig. 4). These results suggest that geographic genetic structuring within a call type is limited by high vagility and erratic nomadism, and are consistent with Groth's (1993a) findings of no morphological differentiation among call type 2 crossbills across North America. This is also consistent with the results of Mantel tests suggesting no correlation between geographic and genetic distance for different samples of call type 2. Although these inferences would be strengthened by having larger sample sizes and more thorough geographic sampling within each call type, our understanding would likely not be improved by sampling multiple geographic locations within each call type because nomadic movements result in a majority of birds of a given call type occupying a small fraction of their range each year with the foci of occurrence shifting geographically like a kaleidoscope from year to year. This nomadism, along with the results we present here, suggests that the genetic structuring we detected among call types is unlikely to be confounded by the geographic source of capture.

Many studies have demonstrated that divergent natural selection can produce rapid evolution and phenotypic divergence, often in the absence of discernable differentiation at neutral DNA (Orr & Smith 1998; Schneider *et al.* 1999; Cousyn *et al.* 2001). Presumably due to high vagility, such patterns have been especially common in passerine birds (Seutin *et al.* 1995; Freeland & Boag 1999). Previous studies on crossbills in Europe are evidence that large morphological differences can exist in the face of ongoing gene flow and indicate that divergence in crossbills in some cases occurs despite gene flow (Piertney *et al.* 2001; Marquiss & Rae 2002). However, interpreted in light of known divergent selection (Benkman 2003) and premating reproductive isolation (Smith & Benkman in review), the evidence of fine-scale genetic

structuring presented here suggests gene flow among call types may be restricted as expected during incipient ecological speciation (e.g. Wake 1997; Ogden & Thorpe 2002; Sorenson *et al.* 2003). In addition, the very low levels of genetic differentiation among some of the call types (e.g. call types 2, 5, and 9) despite morphological differentiation may suggest that adaptation via natural selection for resource specialization may not be greatly impeded by gene flow (Orr & Smith 1998; Piertney *et al.* 2001). This is further supported by the absence of a correlation between genetic and morphological differentiation in Mantel tests ( $r^2 = 0.02$ , P = 0.66; using bill depths of the different call types in Appendix D from Groth 1993b as a measure of morphological differentiation).

Even though we did not find evidence of geographic genetic structuring within call types that could confound our interpretation of structuring between call types, there was evidence that differentiation across the complex as a whole may contain the signature of geography. For example, the wide separation of call type 1 from other call types in PCO space and the UPGMA tree may be confounded by the fact that call type 1 was the only call type sampled east of the Great Plains (Fig. 1). However, call type 1 commonly co-occurs with call type 2 in the Appalachian Mountains (Groth 1988) and has been found with call type 3 along the Pacific coast (Fig. 1; T. P. Hahn, personal communication; CWB, personal obsservation). Samples of call type 1 from a wider geographic area could help clarify these patterns. In addition to call type 1, there are two fairly distinct clusters of call types (Fig. 4: call types 3, 4, 6, and 7 and call types 2, 5, and 9) where call types within these clusters have little overlap in the PCO plots with call types in the other cluster (Fig. 3b). In contrast, the overlap within each of these clusters is much higher (Fig. 3c, d) and genetic distances among call types much smaller (Table 4), indicating that call types within these groups diverged more recently and/ or exhibit higher levels of ongoing hybridization than call types in different clusters. Indeed, the call types within the three groupings depicted in the UPGMA dendrogram tend to have more similar geographic distributions (Fig. 1), which may indicate some signature of geography in patterns of genetic variation across the complex. For example, call types 2 and 5 specialize on ponderosa pine (Pinus ponderosa scopulorum) and lodgepole pine (Pinus contorta latifolia), respectively (Fig. 4), and are the most common crossbills in the Rocky Mountains, while call types 3 and 4 specialize on western hemlock (Tsuga heterophyla) and Douglas-fir (Pseudotsuga menziesii menziesii), respectively (Fig. 4), and are more common in the Pacific Northwest (Benkman 1993). These patterns are generally consistent with those suggested from Groth's (1993a) analyses of allozyme variation among the call types with the exception of call type 6, which Groth's data grouped with call types 2 and 5. The placement of the very large-billed call type 6, which occurs in the Sierra Madre of Mexico, with the large-billed call types 2 and 5, whose distributions extend into the southern Rocky Mountains (Fig. 1), seems more logical than our findings.

While it is apparent that the different call types in the red crossbill complex currently experience sympatric or nearly sympatric conditions (Fig. 1), delineating the biogeographical context within which divergence originated among call types is difficult. Some of the youngest sister species in the boreal avifauna have been inferred to have speciated during the late Pleistocene (Johnson & Cicero 2004; Weir & Schluter 2004) in refugia restricted to the Pacific Northwest, Rocky Mountains, and northeastern regions of North America (Weir & Schluter 2004). The three groupings of call types depicted in the UPGMA dendrogram (Fig. 4) could represent groups that were isolated in these refugial forests where they diverged allopatrically before renewed contact when further hybridization and diversification contributed to the patterns of genetic structuring and morphological diversity we currently observe. In addition, the conifers specialized on by some of the call types exhibiting lower levels of genetic differentiation from one another (e.g. call types 2 and 5, and call types 3 and 4; Fig. 4) regularly exist in mixed or a mosaic of stands, which for nomadic crossbills could set the stage for sympatric divergence via resource specialization.

Nevertheless, our estimates of genetic differentiation are by no means large and whether complete reproductive isolation and speciation will be the end result for any of the call types is uncertain (Magurran 1998). Short- and longterm fluctuations in resource availability (e.g. fluctuations in conifer cone crops) and distribution may ultimately limit speciation by relaxing ecological selection against phenotypically intermediate hybrids and thereby increasing gene flow (Grant & Grant 1992, 1993). In addition, the distributions and abundances of the conifer species specialized on by crossbills were dramatically affected by cycles of glaciations during the Quaternary (Dynesius & Jansson 2002). Such ephemeral habitats or resources have been speculated to limit speciation in Trinidadian guppies (*Poecilia reticulata*), a species renowned for showing rapid adaptive evolution (Endler 1995). Indeed, the red crossbill complex may represent Beta cladogenesis (cf. Dynesius & Jansson 2002), instead of the initial stages of an adaptive radiation, where divergence during the interglacials is mostly erased or reticulated at the end of such periods when resource distributions and availability may change drastically. Previously evolved red crossbill diversity on continents could be wiped out during glacial episodes when the ranges of conifers change and contract before interglacial periods see another burst of diversification and/or hybridization, which would be consistent with the low levels of genetic differentiation seen among the call types. Such an ebb and flow of diversification and hybridization may limit the process of speciation in red crossbills, leaving the group indefinitely as a highly variable polytypic species complex where genetic differentiation remains subtle and adaptive divergence may be rapid.

## Conclusions

Our investigation of AFLP variation among New World crossbills revealed a hierarchical continuum of genetic structuring ranging from little to no differentiation among geographically separated samples within a call type to low to moderate differentiation among call types to patterns of fixed differentiation at numerous loci among recognized species. These results highlight the utility of AFLP analysis applied to animal taxa at the early stages of adaptive radiation (Bensch & Åkesson 2005). The patterns of genetic variation within and among the vocally and morphologically diverse call types of the red crossbill complex are consistent with the differentiation we would expect to see in a group of young incipient ecological species exhibiting high levels of vagility and sympatry. While we do not conclude that speciation has occurred between any of these call types, our results do suggest that patterns of divergent natural selection (Benkman 1993, 2003) and premating reproductive isolation (Smith & Benkman in review) revealed by previous studies of the red crossbill complex are potentially important to the formation of long-term diversity in the group.

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