Molecular Ecology (2006) **15**, 1873–1887 doi: 10.1111/j.1365-294X.2006.02895.x

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

THOMAS L. PARCHMAN, CRAIG W. BENKMAN* and SETH C. BRITCH†
Department of Biology, MSC 3AF, New Mexico State University, Las Cruces, New Mexico 88003-8001, USA

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*

Received 22 September 2005; revision received 19 December 2005; accepted 7 January 2006

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).

Correspondence: Thomas L. Parchman, Fax: 505-646-5665; E-mail: tparchma@nmsu.edu.
*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 mM NaCl, 100 mM 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 μg/μL.

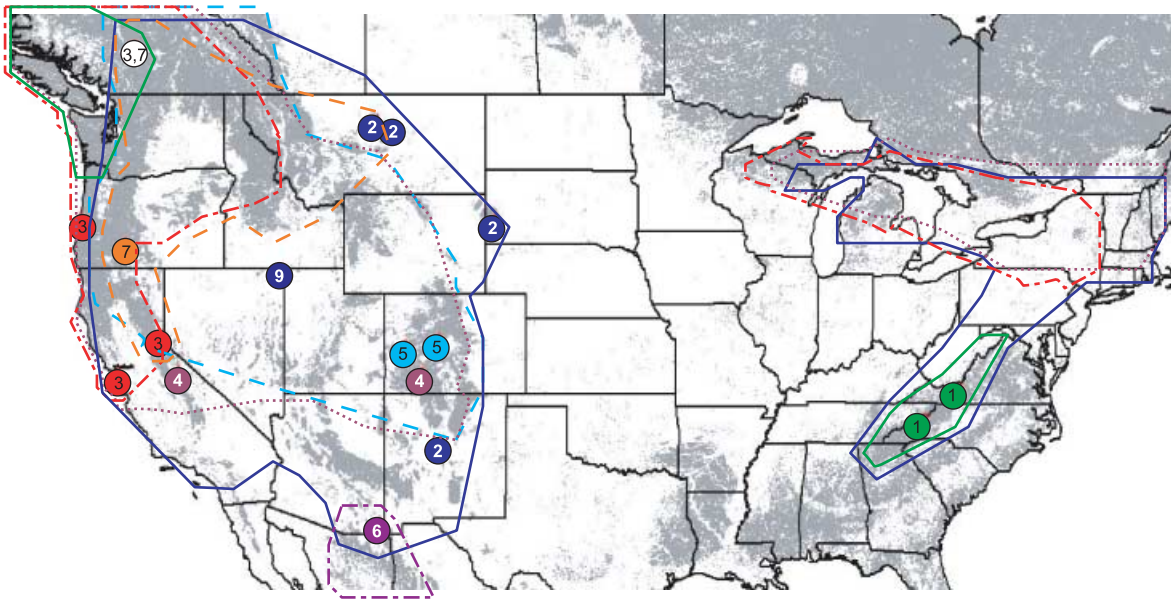

Fig. 1 Map depicting locations of capture for the different call types of red crossbills (coloured circles; the one location with two call types is not coloured; numbers refer to call type). The shaded area outlines the distribution of cone-bearing conifers and the coloured lines enclose the main areas 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 adaptor-ligation 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 µL of the diluted restriction-ligation products, 15 µL AFLP core mix (ABI), and 1 µL *Eco*RI and *Mse*I 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 µL of diluted preselective amplification product, 15 µL AFLP core mix, 1 µL of selective *Mse*I primer, and 1 µL of the fluorescently labelled *Eco*RI selective primer (all from ABI). Both *Eco*RI and *Mse*I 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 µL formamide and 0.25 µL 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 *L. curvirostra* 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 = *Eco*RI, M = *Mse*I), 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 MVSP 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 θ^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 $n \geq 4$ 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 10^6 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_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_E ranged between 0.113 and 0.158 for the different red crossbill call types (Table 2).

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)

	P	%P	H_E
L. curvirostra 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 (n = 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
L. leucoptera (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

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 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 θ^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

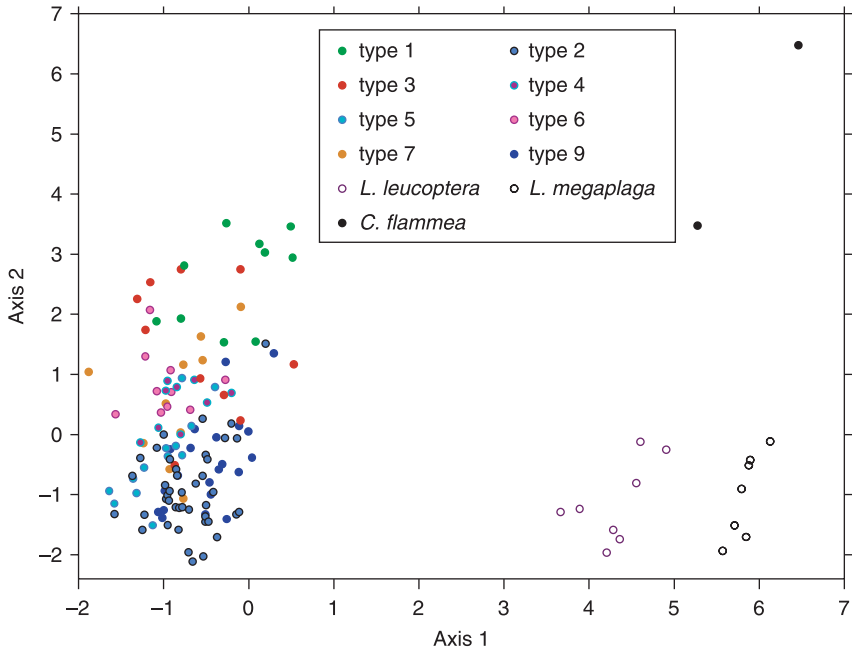

Fig. 2 Principal coordinates analysis based on Euclidian distances among the AFLP scores for all individuals included in the study. The colours for the different call types are meant to highlight the three clusters distinguished in the UPGMA dendrogram (Fig. 4): call type 1 by green, call types 2, 5, 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

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*
Among species	2	654.54	20.33	40.1	< 0.0001
Within species	138	4121.18	29.86	59.5	< 0.0001
Total	140	4775.18	50.19		

Model B

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*
Among call types	7	474.40	2.09	7.0	< 0.0001
Among samples within call type	7	243.70	1.08	3.5	< 0.0001
Within samples	109	2997.23	27.50	89.5	< 0.0001
Total	123	3715.32			

Model C

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*
Among type 2 samples	3	110.09	0.89	3.1	0.0019
Within type 2 samples	37	1045.32	28.25	97.0	< 0.0001
Total	40	1155.42	29.14		

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

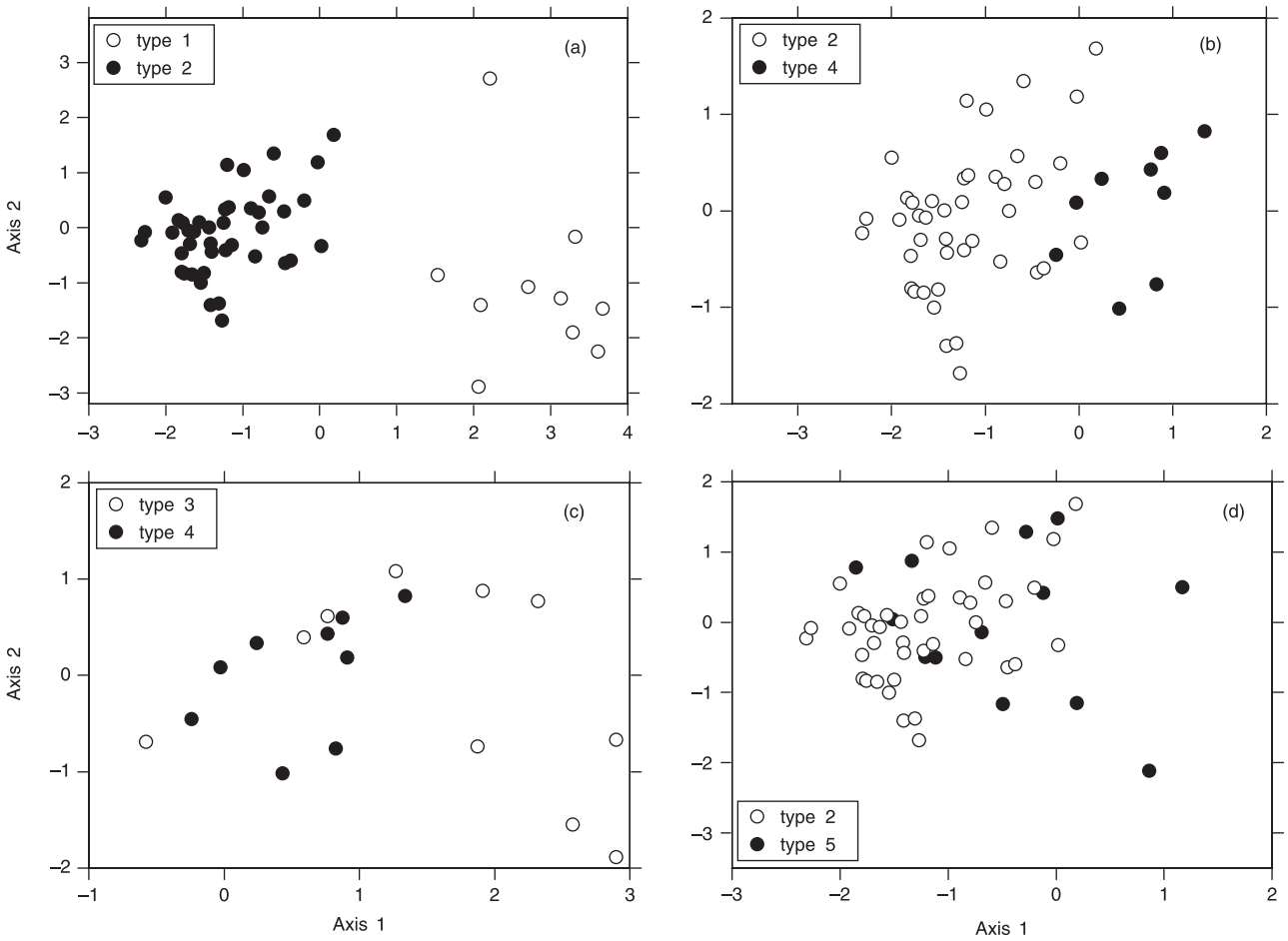

Fig. 3 Plots of the first two principal coordinate axes for an analysis conducted on the red crossbill complex alone. (a) Call types 1 and 2, (b) call types 2 and 4, (c) call types 3 and 4, and (d) call types 2 and 5.

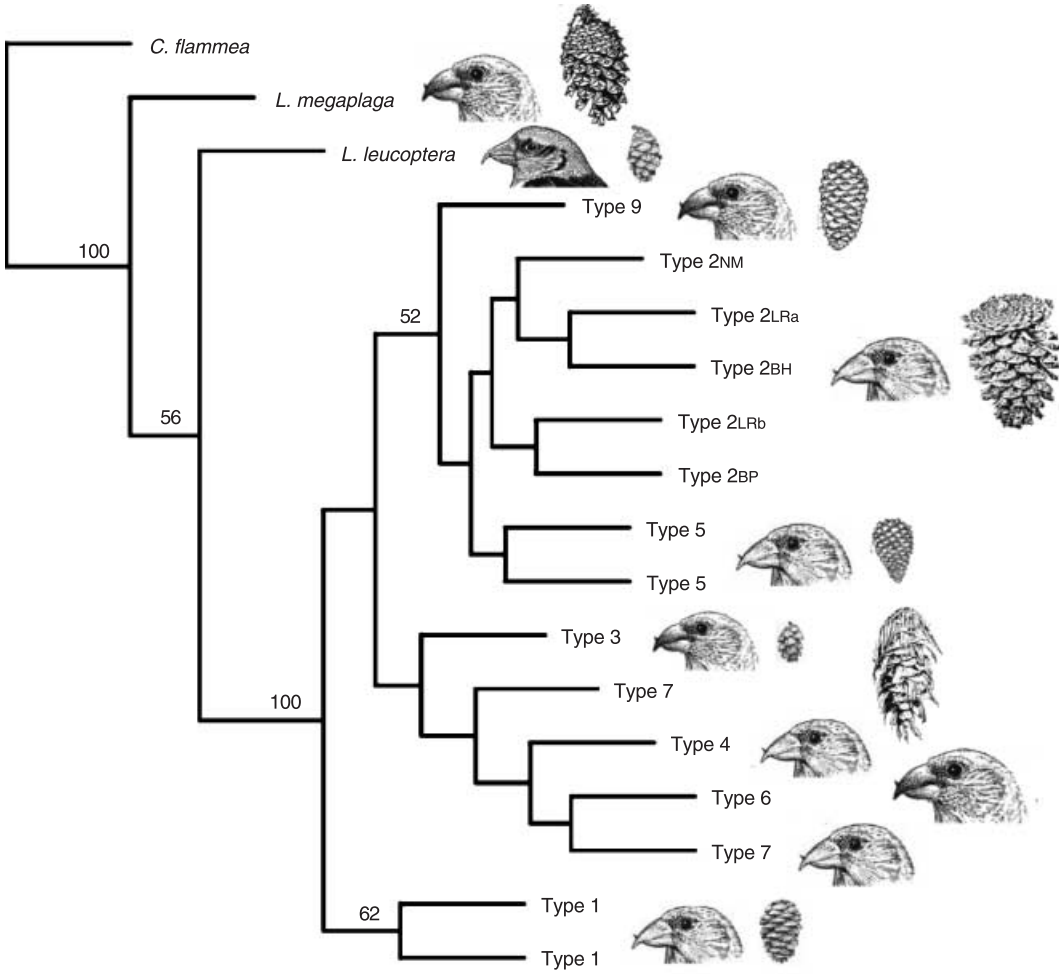

Fig. 4 UPGMA phylogram reflecting relative genetic distances based on pairwise estimates of Nei's *D* among different recognized species included in this study and eight call types of the red crossbill complex including samples from two geographic samples of call types 1, 5, and 7, and four geographic 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 ($F_{ST} = 0.057$, $P < 0.05$), and the Bears Paw Mountains, Montana ($F_{ST} = 0.041$, $P < 0.05$), and between call type 1 from North Carolina and Virginia ($F_{ST} = 0.13$, $P < 0.05$). 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_{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 white-winged 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,

stout-billed Hispaniolan crossbill that are presumably the result of divergent selection for foraging on very different food resources (Fig. 4; Benkman 1994). Of particular note was the low estimate of H_E (0.052) for the Hispaniolan crossbill, which could be due to a founder effect occurring with colonization of the island and/or a more recent population bottleneck as a result of deforestation and habitat loss on Hispaniola (Benkman 1994; Latta *et al*. 2000).

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 tree-based 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 long-term 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.

Acknowledgements

We thank J. Groth, K. Winker, S. Latta, T. Hahn, and P. Edelaar for assistance in obtaining samples. We thank C. Cicero and the late N.K. Johnson at the Museum of Vertebrate Zoology, UC Berkeley and K. Winker and J. Maley at the Museum of Ornithology, University of Alaska, Fairbanks, for providing tissue samples as well as helpful discussion. National Science Foundation grants (DEB-0212271 and DEB-0344503) and a National Geographic Grant (Grant 6820-00) to C.W.B., and an AMNH Frank Chapman Memorial Fund award and an Environmental Protection Agency GRO Fellowship to T.L.P. supported this work. The manuscript was improved by the comments of A. Buerkle, D. McDonald, T. Price, A. Siepielski, M.D. Sorenson, members of the Howard laboratory at New Mexico State University and five anonymous reviewers. D. Althoff and K. Kay graciously provided advice and guidance during the early stages of AFLP data analysis. D. Bailey provided encouraging and helpful discussion and support and helped run principal coordinates analyses with MVSP on his computers. G. Beauvais graciously created the map for Fig. 1. We especially thank D.J. Howard for both encouraging us to use AFLPs and for providing the initial funds (National Science Foundation DEB-011613 and DEB-0316194 to DJH) for a preliminary study.

References

Adkisson CS (1996) Red crossbill (*Loxia curvirostra*). In: *The Birds of North America*, No. 256 (eds Poole A, Gill F), pp. 1–24. The Academy of Natural Sciences, Philadelphia, and The American Ornithologists' Union, Washington, D.C.

Albertson RC, Markert JA, Danley PD, Kocher TD (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences, USA*, **96**, 5107–5110.

Arnaiz-Villena A, Guillén J, Ruiz-del-Valle V *et al.* (2001) Phylogeography of crossbills, bullfinches, grosbeaks, and rosefinches. *Cellular and Molecular Life Sciences*, **58**, 1159–1166.

Bailey AM, Neidrach RJ, Baily AL (1953) *The Red Crossbills of Colorado*. Museum Pictorial 9, Denver Museum of Natural History, Denver, Colorado.

Banks RC, Cicero C, Dunn JL *et al.* (2003) Forty-fourth supplement to the American Ornithologists' Union checklist of North American birds. *Auk*, **120**, 923–931.

Beheregeray LB, Sunnucks P (2001) Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Molecular Ecology*, **10**, 2849–2866.

Benkman CW (1987a) Food profitability and the foraging ecology of crossbills. *Ecological Monographs*, **57**, 251–267.

Benkman CW (1987b) Crossbill foraging behavior, bill structure, and patterns of food profitability. *Wilson Bulletin*, **99**, 351–368.

Benkman CW (1992) White-winged crossbill. In: *The Birds of North America,* No. 27 (eds Poole A, StettenheimP, Gill F), pp. 1–20. The Academy of Natural Sciences, Philadelphia, and The American Ornithologists' Union, Washington, D.C.

Benkman CW (1993) Adaptation to single resources and the evolution of crossbill (*Loxia*) diversity. *Ecological Monographs*, **63**, 305–325.

Benkman CW (1994) Comments on the ecology and status of the Hispaniolan crossbill (*Loxia leucoptera megaplaga*), with recommendations for its conservation. *Caribbean Journal of Science*, **30**, 250–254.

Benkman CW (1999) The selection mosaic and diversifying co-evolution between crossbills and lodgepole pine. *American Naturalist*, **153**, S75–S91.

Benkman CW (2003) Divergent selection causes the adaptive radiation of crossbills. *Evolution*, **57**, 1176–1181.

Benkman CW, Lindholm AK (1991) The advantages and evolution of a morphological novelty. *Nature*, **349**, 519–520.

Benkman CW, Miller RE (1996) Morphological evolution in response to fluctuating selection. *Evolution*, **50**, 2499–2504.

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 co-evolution between crossbills and lodgepole pine. *American Naturalist*, **162**, 182–194.

Benkman CW, Colquit JS, Gould WR, Fetz T, Keenan PC, Santisteban L (2005) Can selection by an ectoparasite drive a population of red crossbills from its adaptive peak? *Evolution*, **59**, 2025–2032.

Bensch S, Åkesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, **14**, 2899–2914.

Bensch S, Helbig AJ, Solomon M, Siebold I (2002) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. *Molecular Ecology*, **11**, 473–481.

Bock CE, Lepthien LW (1976) Synchronous eruptions of boreal seed-eating birds. *American Naturalist*, **110**, 559–571.

Capy P, Veuille M, Paillette M, Jallon JM, Vouidibio J, David JR (2000) Sexual isolation of genetically differentiated sympatric populations of *Drosophila melanogaster* in Brazzaville, Congo: the first step towards speciation? *Heredity*, **84**, 468–475.

Cousyn C, De Meester L, Colbourne JK, Brendonck L, Verschuren D, Volckaert F (2001) Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proceedings of the National Academy of Sciences, USA*, **98**, 6256–6260.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Despres L, Gielly L, Redoutet B, Taberlet P (2003) Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. *Molecular Phylogenetics and Evolution*, **27**, 185–196.

Dieckmann U, Doebeli M (1999) On the origin of species by sympatric speciation. *Nature*, **400**, 354–357.

Dynesius M, Jansson T (2002) The fate of clades in a world of recurrent climate change: Milankovitch oscillations and evolution. *Annual Review of Ecology and Systematics*, **33**, 741–777.

Endler JA (1995) Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution*, **10**, 22–29.

Excoffier L, Smouse PE, Quatro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Farjon A, Styles BT (1997) *Pinus (Pinaceae)*. Flora Neotropica, Monograph 75. New York Botanical Garden, New York.

Felsenstein J (2004) *PHYLIP: Phylogeny Inference Package, Version 3.6*. Department Genetics, University of Washington, Seattle, Washington. Distributed by the author.

Freeland JR, Boag PT (1999) The mitochondrial and nuclear genetic homogeneity of the phenotypically diverse Darwin's ground finches. *Evolution*, **53**, 1553–1563.

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, **34**, 397–423.

Gavrilets S (2005) Adaptive speciation: it is not that easy: a reply to Doebeli *et al*. *Evolution*, **59**, 696–699.

Giannasi N, Thorpe RS, Malhotra A (2001) The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*. *Molecular Ecology*, **10**, 419–426.

Godfrey WE (1979) *The Birds of Canada*. National Museum of Natural Sciences, Ottawa, Canada.

Grant PR, Grant BR (1992) Hybridization among bird species. *Science*, **256**, 193–197.

Grant BR, Grant PR (1993) Evolution of Darwin's finches caused by a rare climatic event. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **251**, 111–117.

Griscom L (1937) A monographic study of the red crossbill. *Proceedings of the Boston Society of Natural History*, **41**, 77–210.

Griscom L (1941) Second flight of the Sitka crossbill to Massachusetts. *Auk*, **58**, 411–413.

Groth JG (1988) Resolution of cryptic species in Appalachian red crossbills. *Condor*, **90**, 745–760.

Groth JG (1993a) *Evolutionary Differentiation in Morphology, Vocalizations, and Allozymes among Nomadic Sibling Species in the North American Red Crossbill (Loxia curvirostra) Complex*. University of California Publications in Zoology, No. 127. Berkeley, California.

Groth JG (1993b) Call matching and positive assortative mating in red crossbills. *Auk*, **110**, 398–401.

Hendry AP, Kinnison MT (2001) An introduction to microevolution: rate, pattern, process. *Genetica*, **112–113**, 1–8.

Hendry AP, Wenburg JK, Bentzen P, Volk EC, Quinn TP (2000) Rapid evolution of reproductive isolation in the wild: evidence from introduced Salmon. *Science*, **290**, 516–519.

Holsinger KE, Lewis PO (2003) *HICKORY: A Package for the Analysis of Population Genetic Data, Version 1.0*. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut. Distributed by the authors.

Holsinger KE, Lewis PO, Dey DK (2002) A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology*, **11**, 1157–1164.

Johnson NK, Cicero C (2004) New mtDNA data affirm the importance of Pleistocene speciation in North American birds. *Evolution*, **58**, 1122–1130.

Keim P, Bevis W, Schupp J, Freestone R (1992) Evaluation of soybean RFLP markers in adaptive germplasm. *Theoretical and Applied Genetics*, **85**, 205–212.

Koenig WD, Knops JMH (1998) Scale of mast seeding and tree-ring growth. *Nature*, **396**, 225–226.

Koenig WD, Knops JMH (2001) Seed-crop size and eruptions of North American boreal seed-eating birds. *Journal of Animal Ecology*, **70**, 609–620.

Kondrashov AS, Kondrashov FA (1999) Interactions among quantitative traits in the course of sympatric speciation. *Nature*, **400**, 251–354.

Korol A, Rashkovestsky E, Iliadi K, Michalak P, Ronin Y, Nevo E (2000) Non-random mating in *Drosophila melanogaster* laboratory populations derived from closely adjacent ecologically contrasting slopes at 'Evolution Canyon'. *Proceedings of the National Academy of Sciences, USA*, **97**, 12637–12642.

Kovach WL (1999) *A Multivariate Statistical Package for Windows*, version 3.1. Kovach Computing Services, Pentraeth, UK.

Latta SC, Sondreal ML, Brown CR (2000) A hierarchical analysis of nesting and foraging habitat for the conservation of the Hispaniolan white-winged crossbill (*Loxia leucoptera megaplaga*). *Biological Conservation*, **96**, 139–150.

Lawrence LK (1949) The red crossbill at Pimisi Bay, Ontario. *Canadian Field-Naturalist*, **63**, 147–160.

Lu G, Bernatchez L (1999) Correlated trophic specializations and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): support for the ecological speciation hypothesis. *Evolution*, **53**, 1491–1505.

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**, 91–99.

Magurran A (1998) Population differentiation without speciation. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **353**, 275–286.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, **27**, 209–220.

Marquiss M, Rae R (2002) Ecological differentiation in relation to bill size amongst sympatric, genetically undifferentiated crossbills *Loxia* spp. *Ibis*, **144**, 494–508.

Miller MP (1997) *Tools for Population Genetic Analyses (TFPGA), Version 1.3. A Windows program for the analysis of allozyme and molecular population genetic data*. Distributed by the author.

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution*, **14**, 389–393.

Munro JA (1919) Notes on the breeding habits of the red crossbill in the Okanagan Valley, British Columbia. *Condor*, **21**, 57–60.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Newton I (1972) *Finches*. Collins, London.

Ogden R, Thorpe RS (2002) Molecular evidence for ecological speciation in tropical habitats. *Proceedings of the National Academy of Sciences, USA*, **99**, 13612–13615.

Orr MR, Smith TB (1998) Ecology and speciation. *Trends in Ecology & Evolution*, **13**, 502–506.

Parchman TL, Benkman CW (2002) Diversifying coevolution between crossbills and black spruce on Newfoundland. *Evolution*, **56**, 1663–1672.

Piertney SB, Summers R, Marquiss M (2001) Mitochondrial and microsatellite DNA homogeneity among phenotypically diverse crossbill taxa in the UK. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **268**, 1511–1516.

Pritchard JK, Stevens 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.

Rice WR, Salt GW (1988) Speciation via disruptive selection on habitat preference — experimental evidence. *American Naturalist*, **131**, 911–917.

Rieseberg LH, Widmer A, Arntz AM, Burke JM (2002) Directional selection is the primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences, USA*, **99**, 12242–12245.

Schliewen UK, Tautz D, Paabo S (1994) Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature*, **368**, 629–632.

Schluter D (2000) *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.

Schluter D (2001) Ecology and speciation. *Trends in Ecology & Evolution*, **16**, 372–380.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: A Software for Population Genetics Data Analysis. Version 2.0*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland. Distributed by the authors.

Schneider CJ, Smith TB, Larison B, Moritz C (1999) A test of alternative models of diversification in tropical rainforests: ecological gradients vs. rainforest refugia. *Proceedings of the National Academy of Sciences, USA*, **96**, 13869–13873.

Seehausen O (2004) Hybridization and adaptive radiation. *Trends in Ecology & Evolution*, **19**, 198–207.

Sefc KM, Payne RB, Sorenson MD (2005) Genetic continuity of brood-parasitic indigobird species. *Molecular Ecology*, **14**, 1407–1419.

Seutin G, Ratcliffe LM, Boag PT (1995) Mitochondrial DNA homogeneity in the phenotypically diverse redpoll finch complex Aves: Carduelinae: *Carduelis flammea-hornemanni*. *Evolution*, **49**, 962–973.

Siepielski AM, Benkman CW (2005) A role for island area in the geographic mosaic of coevolution between crossbills and lodgepole pine. *Journal of Evolutionary Biology*, **18**, 1042–1049.

Smith JW, Benkman CW (In review) Ecological divergence causes reproductive isolation in red crossbills.

Smith JW, Benkman CW (Unpublished) Causes and consequences of assortative flocking by call type in red crossbills.

Sorenson MD, Sefc KM, Payne RB (2003) Speciation by host switching in brood parasitic indigobirds. *Nature*, **424**, 928–930.

Sullivan JP, Lavoue S, Arnegard ME, Hopkins CD (2004) AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution*, **58**, 825–841.

Swofford DL (2001) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer Associates, Sunderland, Massachusetts.

Travis SE, Maschinski J, Keim P (1996) An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology*, **5**, 735–745.

Vekemans X (2002) *AFLP-SURV, Version 1.0. Laboratoire de Génétique et Ecologie Végétale*. Université Libre de Bruxelles, Belgium. Distributed by the author.

Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Wake DB (1997) Incipient species formation in salamanders of the *Ensatina* complex. *Proceedings of the National Academy of Sciences, USA*, **94**, 7761–7767.

Wang Z, Baker AJ, Hill GE, Edwards SV (2003) Reconciling actual and inferred population histories in the house finch (*Carpodacus mexicanus*) by AFLP analysis. *Evolution*, **57**, 2852–2864.

Weir JT, Schluter D (2004) Ice sheets promote speciation in boreal birds. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **271**, 1881–1887.

Wright S (1978) *Evolution and the Genetics of Populations*, Vol. 4. *Variability within and Among Natural Populations*. University Chicago Press, Chicago, Illinois.

Wu C-I, Hollocher H, Begun D, Aquadro C, Xu Y, Wu M-L (1995) Sexual isolation in *Drosophila melanogaster*: a possible case of incipient speciation. *Proceedings of the National Academy of Sciences, USA*, **92**, 2519–2523.

Thomas Parchman is a doctoral student in the laboratory of Dr. Craig Benkman, where his dissertation research is focused on the determining the contribution of geographically structured coevolution between crossbills and conifers to the adaptive diversification of crossbills. His general interests are in integrating studies of natural selection and genetic variation in the study of microevolutionary process, coevolution, and speciation. Dr. Craig Benkman is a professor in the Department of Zoology and Physiology at the University of Wyoming. His research has focused on linking resource availability to various aspects of behavior, ecology, and evolution. The major focus of current work in his laboratory is on the role of resource specialization and coevolution in the evolution of crossbill diversity. Dr. Seth Britch is currently a Research Entomologist with the USDA Agricultural Research Service studying host range evolution and speciation in parasitoid wasps.
