J Ornithol
DOI 10.1007/s10336-013-0971-2

ORIGINAL ARTICLE

# Microsatellite variation in Rufous Hummingbirds (*Selasphorus rufus*) and evidence for a weakly structured population

**Ida E. Bailey · Gernot Segelbacher · Susan D. Healy · T. Andrew Hurly · Josephine M. Pemberton**

Received: 20 June 2012 / Revised: 17 April 2013 / Accepted: 15 May 2013


**Abstract** The Rufous Hummingbird (*Selasphorus rufus*) population is declining in some areas of North America, but not in others. The reasons for the decline are, as yet, unknown. Understanding the genetic population structure of this species could be useful in understanding its dispersal behaviour and whether particular geographical areas should be treated as separate conservation units. We tested 16 microsatellite markers designed for other hummingbird species for amplification in Rufous Hummingbirds. Using six polymorphic markers, we found that the Rufous Hummingbird population was weakly structured such that birds breeding in central British Columbia could be distinguished from those breeding on Vancouver Island and those in Alberta, each several hundred kilometres away. Whether landscape features such as the Rocky Mountains and Fraser River Valley significantly affect dispersal patterns requires further investigation.

**Keywords** *Selasphorus rufus* · Genetic structure · Microsatellite · Hummingbird

Communicated by M. Wink.

I. E. Bailey · J. M. Pemberton
Institute of Evolutionary Biology, University of Edinburgh, Kings Buildings, Edinburgh EH9 3JT, UK

*Present Address:*
I. E. Bailey (✉)
School of Biology, University of St Andrews, St Andrews KY16 9JP, UK
e-mail: ieb3@st-andrews.ac.uk

G. Segelbacher
Department of Wildlife Ecology and Management, Albert-Ludwigs-University of Freiburg, Tennenbacher Straße 4, 79106 Freiburg, Germany

S. D. Healy
School of Biology, University of St Andrews, St Andrews KY16 9JP, UK

T. A. Hurly
Department of Biological Sciences, University of Lethbridge, 4401 University Drive, Lethbridge, AB T1K 3M4, Canada

## Zusammenfassung

### Genetische Variabilität und Struktur bei der Rotrücken-Zimtelfe

Die Rotrücken-Zimtelfe (*Selasphorus rufus*), eine Kolibriart in Nordamerika, nimmt in manchen Gebieten ab, in anderen jedoch nicht. Die Ursachen des Bestandsrückgangs sind bisher jedoch weitgehend unbekannt. Um entsprechende Schutzstrategien für die einzelnen Populationen gezielt entwickeln zu können, ist ein besseres Verständnis der genetischen Struktur und damit der Wanderbewegungen zwischen den einzelnen Populationen notwendig. Insgesamt wurden in dieser Arbeit 16 Mikrosatellitenmarker anderer Kolibriarten getestet und auf ihre artspezifische Eignung hin überprüft. Sechs polymorphe Marker konnten erfolgreich für die Analysen bei *S. rufus* etabliert werden. Die Populationen zeigten nur geringe genetische Unterschiede, Individuen aus Vancover Island, British Columbia, und Alberta ließen sich jedoch unterscheiden. Welche Rolle Bergzüge wie die Rocky Mountains oder Täler wie das Fraser River Valley für das Dispersal der Art hat, sollte in zukünftigen Studien genauer analysiert werden.

## Introduction

Conservation of species, particularly those with a wide distribution, can be greatly enhanced through knowledge of

Published online: 29 May 2013

the genetic structure of their populations. For example, highly structured populations may need to be managed as a number of independent populations, whereas more panmictic populations may be considered as a single conservation unit (Esler et al. 2006). Genetic structure is the outcome of the interplay between local adaptation and the rate of effective (i.e. breeding) dispersal between natal sites, with high effective dispersal reducing structure (e.g. Walters 2000; Clark et al. 2004). While banding and recapture data provide us with information on the typical movement and dispersal behaviour of avian species, we often fail to detect rare movements among the populations that otherwise appear to be separate (Alcaide et al. 2009). Such rare movements may result in sufficient genetic population mixing to make several populations, which from banding and recapture data appear to be separate, part of a single, close to panmictic, metapopulation. Observations of such mismatches between banding and genetic data are increasingly common (Pearce and Talbot 2006).

The Rufous Hummingbird *Selasphorus rufus* is one of the most common and widespread hummingbird species in western North America. However, it appears that its population numbers have been declining by about 3 % a year for the past 30 years, equivalent to a population decline of over 50 % in that period (Healy and Calder 2006). The reasons for this decline are unknown. Furthermore, the decline is not equal across all parts of the species' breeding range (Sauer et al. 2008): it is less severe in Alaska and in eastern parts of the range including eastern Washington, northern Idaho, Montana and Alberta. As there are no data on Rufous Hummingbird population structure, it is unclear whether some areas should be treated as separate conservation units. It is possible that mountain ranges such as the Coastal and Rocky Mountains act as barriers to movement between breeding populations. Alternatively, as in other species, population differences may have arisen due to geographic isolation of costal from continental breeding populations and divergence of their migratory routes during the last glacial period (Avise and Walker 1998; Milot 2000). Either way, it is possible that populations on either side of the Rocky Mountains should be considered separately as conservation units.

The predictions for genetic population structure from banding records at Rufous Hummingbird breeding sites are equivocal. Although natal dispersal is generally more common in birds than is breeding dispersal (Newton 2008), there are few data from Rufous Hummingbirds banded as juveniles and, thus, no real indication of levels of natal dispersal. From banding and recapture data of the adults, although both sexes are often faithful to their breeding sites from year to year (Finlay 2007), there are records of adults travelling fairly long distances (e.g., 87 km) within the breeding season. This indicates that at least some breeding dispersal may be occurring (Finlay 2007).

Such long-distance movements within the breeding season may partially be due to the migratory behaviour of Rufous Hummingbirds. Rufous Hummingbirds breed in western North America and Canada but migrate to Mexico and the southeast United States for the winter. Birds arrive on Vancouver Island in March and leave between May and June, whereas in Alberta they do not arrive until about this time, leaving in July and August, while central British Columbia (B.C.) birds arrive later than those from Vancouver Island but earlier than those from Alberta (Williamson 2001). Given the timing of their arrivals and departures, it seems possible that some birds from Vancouver Island may cross the Rockies into Alberta and breed again within one season but this is less likely for individuals from central B.C. population that arrive slightly later. In addition, some birds that migrate north along the coast, or some of those that follow the more inland route north, may occasionally take the alternative route and breed in a different location from the year before, adding yet another source of gene flow between populations. Given the limited data on the birds' movements, banding and recapture data alone are insufficient to infer whether or not the Rufous Hummingbird population is likely to be highly structured.

Genetic population structure due to isolation by distance or barriers to gene flow such as mountain ranges is best inferred using highly variable markers such as microsatellites (Guillot et al. 2005). Microsatellite markers are particularly useful due to their high variability (up to 50 alleles at a single locus) (Parker et al. 1998) and they are now one of the most common markers used in studies of population structure and landscape genetics (Selkoe 2006).

In this study, we used six microsatellite markers to investigate genetic structure among four breeding populations of Rufous Hummingbirds within Canada. We expected to find population differentiation between breeding populations separated by mountain ranges, e.g. birds breeding in British Columbia and those breeding in Alberta.

## Methods

### Markers

We tested 16 microsatellite markers developed for other hummingbird species for amplification and polymorphism in Rufous Hummingbirds. Six loci were developed for Amethyst–throated Hummingbird *Lampornis amethystinus* by one of us (G.S.). These markers were isolated via an enrichment protocol (Glenn 2005). The remaining ten hummingbird markers were developed from a genomic library constructed for the Broad-tailed Hummingbird *Selasphorus platycercus* and were known to at least amplify in *S. rufus* (Oyler-McCance and St. John, personal communication). Primer

sequences were obtained from S. Oyler-McCance and J. St John (University of Denver). Some of these sequences are now published (see Oyler-McCance et al. 2011).

## Samples

DNA was extracted from both tail feathers and tissue samples. In total, muscle tissue came from seven individuals, five from British Columbia and two from Alberta. Tissue samples were of breast muscle collected opportunistically from birds found dead throughout the project. Causes of death were not known for all individuals but included flying into windows of buildings and collisions with traffic. Muscle tissue was stored in 100 % ethanol. Feather samples were collected from Rufous Hummingbirds between 2007 and 2008 in Alberta (east of the Rockies) and with the help of the hummingbird-banding network, in British Columbia (west of the Rockies). To investigate the species' population structure, we genotyped feather samples from 200 individuals. Reference samples that were used during primer testing were re-run alongside these new samples to check for consistency of genotyping.

Feather samples were categorised as coming from four broad geographical areas assumed to be different local populations: Alberta, the Westcastle River valley (40 males and 19 females), Central B.C. (21 females), Eastern Vancouver Island (17 males and 42 females) and western Vancouver Island (24 males and 30 females; Fig. 1). Feathers were stored dry in paper envelopes for up to 18 months before DNA extraction.

## Extractions and amplification

Extractions from tissue samples were done using the Qiagen DNeasy tissue extraction kit and protocol with the following variations: muscle samples were lysed with proteinase K for 3 h and only one final elution step was used (200 µl), to maximise the concentration of the final DNA. For feather extractions, only the lower 1 cm of the feather shaft was used, it was lysed for over 12 h and again only one final elution step (200 µl) was used.

Microsatellite loci were amplified using a standard PCR protocol and DYAD peltier thermal cyclers (Genetic Research Instrumentation). Each 10-µl polymerase chain reaction contained 1 µl of DNA (at extraction concentration), 1 µl of each forward and backward primer (10 pmol concentration), 3.94 µl of water, 1 µl of Bioline 10× $NH_4$ reaction buffer, 1 µl of dNTP (concentration 2 mM), 0.5 µl of $MgCl_2$ (concentration 50 mM), and 0.06 µl Bioline TAQ DNA polymerase (concentration 5u/µl). The PCR program used was of the form: 94 °C for 3 min, then 35 cycles of: 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s, and finally: 72 °C for 10 min followed by 8 °C until removed from the machine.

## Marker testing

For the six *L. amethystinus* loci, we first used unlabelled primers and identified appropriate annealing temperatures by using the same reaction mixture as above and an annealing temperature gradient PCR program: 95 °C for 5 min, then 30 cycles of 94 °C for 30 s, gradient within thermal cycler from 48 °C in row one to 60 °C in row 12 for 1 min 30 s, 72 °C for 1 min 30 s, and finally 60 °C for 30 min followed by 4 °C until removed from the machine. Visualisation of PCR products of these six markers was carried out on 4 % agarose gels stained with ethidium bromide. Only four of these six markers (Lamp1, Lamp2, Lamp4 and Lamp5) amplified and for these we obtained fluoro-labelled forward primers.

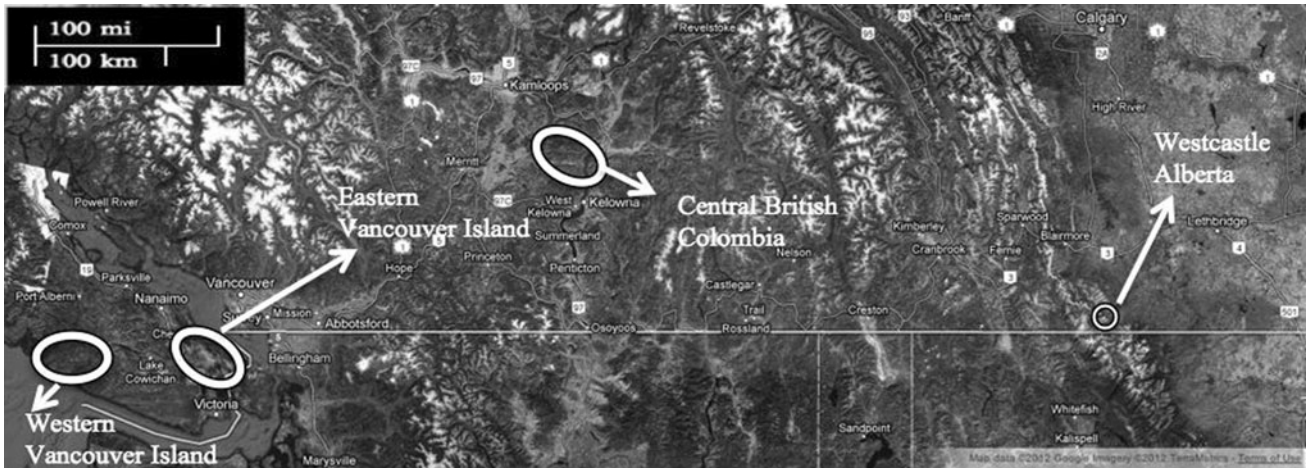


**Fig. 1** The four Canadian geographical areas from which feather samples were collected to investigate the population structure of Rufous Hummingbirds *Selasphorus rufus*. Map from Google Maps with site information and scale superimposed

For *S. platycercus* markers, we obtained primer pairs with a fluorescently-labelled forward primer and used annealing temperatures suggested by their originators (S. Oyler-McCance and J. St John, University of Denver, personal communication).

All markers successfully amplifying for *S. rufus* were tested for polymorphism using 8–12 samples from *S. rufus* from diverse locations. Amplified products were run on an ABI3730 capillary sequencer (Applied Biosystems) together with internal size standard Genescan LIZ 500 (Applied Biosystems). PCR products were diluted 1 part reaction to 100 parts water (tissue samples) and 1 part reaction to 10 parts water (feather samples). Each 10-µl GeneMapper plate well contained 1 µl of diluted PCR product and 9 µl of HiDi LIZ 500 size standard mix (1 µl size standard in 1 ml HiDi formamide). Analysis of the fragments was carried out using the software Genemapper v.4.0 (Applied Biosystems).

### Markers used to investigate population structure

Six markers were chosen to investigate population structure in *S. rufus* (Lamp4, HumB1, HumB3, HumB8, HumB9 and HumB10). These loci were chosen because they showed the highest peak heights, were easy to score and showed appreciable polymorphism. The same extraction, PCR and analysis protocols as described above were used, with the annealing temperatures indicated in Table 1.

### Analysis

Deviations from Hardy–Weinberg equilibrium for each locus and population were estimated in the program ARLEQUIN using exact tests with Bonferroni corrections made for multiple comparisons (Guo and Thompson 1992). Null allele frequencies were estimated for each locus using CERVUS 3.0 (Marshall et al. 1998). Molecular variance within and among populations and an estimate of genetic divergence among populations were calculated via $F_{ST}$ AMOVA in the program ARLEQUIN (Weir and Cockerham 1984; Excoffier et al. 1992; Schneider 2000). Analysis of molecular variance was done using 100,000 permutations. Pairwise $F_{ST}$ values between populations were calculated using 100,000 permutations for significance and 1,000 permutations for Mantel tests. Exact tests of population differentiation used 100,000 Markov chain steps and 10,000 Dememorisation Steps.

Further analysis of population structure was carried out using two Bayesian methods. Firstly, we analysed the data with the program STRUCTURE 2.3 using a Bayesian clustering algorithm (Falush et al. 2007). This program assumes there are $K$ populations each with a characteristic set of allele frequencies at each locus. Hardy–Weinberg and linkage equilibriums of loci is assumed within populations. Individuals are assigned probabilistically to a population, or populations (in the case of an admixed individual), resulting in a number of clusters of individuals as close as possible to Hardy–Weinberg and linkage equilibriums (Pritchard et al. 2000). The most likely number of populations ($K$) in the dataset was estimated independently in five replicates of $K = 1$–6 to allow detection of any populations structuring between sites within the four sampling areas. The model was run using a burn-in period of $2 \times 10^4$ and a run of $5 \times 10^5$ Markov chain Monte Carlo steps, under the standard model of admixed ancestry and the model of correlated allele frequency ($\lambda = 1$). The occurrence of null alleles was estimated simultaneously, using the recessive alleles model. We also used the function LOCPRIOR. LOCPRIOR uses sampling location information as prior information to aid clustering and is designed for use on datasets where population structure is too weak to be found using standard STRUCTURE models (Hubisz et al. 2009).

At values of $F_{ST} < 0.03$, such as over half the $F_{ST}$ values calculated between populations in this study, STRUCTURE tends to underestimate the probable number of clusters of individuals (Latch et al. 2006). Therefore, we used an additional clustering program BAPs (BAPs 3.1; Corander et al. 2004), as, in contrast to STRUCTURE, BAPs is more likely to identify population structure when $F_{ST}$ <0.03. BAPs works in a similar way to STRUCTURE but uses a stochastic optimization algorithm in place of a Markov chain Monte Carlo randomisations to infer the best model and runs much faster than STRUCTURE (Corander et al. 2006). We used the 'groups of individuals' model. This model uses both individual genotypes and the a priori information about the allele frequencies of sample groups (in this case the sampling region) to infer population clusters (Corander et al. 2006). We modelled the data in this way using both mixture models where individuals are assigned completely to one of the identified clusters and admixture models where an individual can be assigned entirely to one group or partially to several groups (Corander and Marttinen 2006; Corander et al. 2006).

## Results

### Marker testing: levels of amplification and polymorphism

Of the 16 markers initially tested, the 10 developed for *S. platycercus* all (100 %) amplified, while 4 of the 6 markers (66 %) developed for *L. amethystinus* amplified in *S. rufus* (Table 1). Nine (90 %) of the *S. platycercus* markers (HumB1, HumB2, HumB3, HumB6, HumB7, HumB8,

**Table 1** A summary of test results for Rufous Hummingbirds *Selasphorus rufus* using microsatellite markers developed for other hummingbird species

| Locus | GenBank accession no. | TA | *A* | *n* | Allele sizes (bp) | $H_o$ | Comment | Primers sequences | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Lamp1** | | **56** | 2 | 11 | 145, 149 | 0.09 | | F: GGAAAGGCTTTATTGGAGCA R: CCACACCACTTGACCAACAG | Gernot Segelbacher, University Freiburg |
| **Lamp2** | | **56** | 7 | 9 | 138, 142, 143, 147, 151, 155, 159 | 0.67 | High stutter | F: TGCACTGTTGCTTCACAGGT R: TGAAAACACAATATGATAGGTCAGG | Gernot Segelbacher, University Freiburg |
| Lamp3 | | – | 0 | 11 | – | – | | F: TTGGGTATGGCCTACTTTGG R: GGAGCATCCATCACTGTCCT | Gernot Segelbacher, University Freiburg |
| **Lamp4** | | **56** | 3 | 11 | 113, 115, 117 | 0.55 | | F: GCTACAAGGCTTGGCAGAAC R: TTCCAGACCACAGAGTAACACAA | Gernot Segelbacher, University Freiburg |
| Lamp5 | | 58–59 | 1 | 11 | 181 | 0 | | F: TCCATCTAGCTTCCTCTCCA R: CAGGAAGTGCCCTTAGGATG | Gernot Segelbacher, University Freiburg |
| Lamp6 | | 59–60 | 0 | 11 | – | – | | F: CAAGGGGGTTAGATTTTTGTTG R: CTGGAATTCGCCCTTGTTTA | Gernot Segelbacher, University Freiburg |
| **HumB1** | **HQ316946** | **60** | 3 | 8 | 137, 141 | 0 | | Provided | Oyler-McCance et al. (2011) |
| )**HumB2** | **HQ316947** | **60** | 3 | 8 | 142, 146, 150 | 0.75 | Poor amplification | Provided | Oyler-McCance et al. (2011) |
| **HumB3** | **HQ316948** | **60** | 3 | 8 | 150, 152, 156 | 0.63 | | Provided | Oyler-McCance et al. (2011) |
| **HumB6** | **HQ316949** | **60** | 10 | 8 | 144, 147, 150, 162, 168, 171, 174, 180, 183, 210 | 0.88 | Poor amplification | Provided | Oyler-McCance et al. (2011) |
| **HumB7** | | 52 | 2 | 8 | 152, 155 | 0.38 | High stutter | Provided (unpublished data) | S. Oyler-McCance and J. St John, University of Denver |
| **HumB8** | **HQ316950** | **58** | 5 | 8 | 123, 126, 129, 132, 135 | 0.75 | | Provided | Oyler-McCance et al. (2011) |
| **HumB9** | **HQ316951** | **60** | 7 | 8 | 96, 102, 106, 108, 116, 122, 124 | 0.88 | | Provided | Oyler-McCance et al. (2011) |
| **HumB 10** | **HQ316952** | **52** | 8 | 8 | 116, 121, 130, 133,139, 142, 146, 167 | 0.50 | | Provided | Oyler-McCance et al. (2011) |
| HumB 14 | | 60 | 1 | 8 | 188 | 0 | | Provided (unpublished data) | S. Oyler-McCance and J. St John, University of Denver |
| **HumB 15** | **HQ316955** | **60** | 7 | 8 | 132, 140, 146, 150, 154, 158, 162 | 0.50 | Poor amplification | Provided | Oyler-McCance et al. (2011) |

Markers that were both polymorphic and showed strong clear peaks are highlighted in bold. Of these Lamp2, HumB2, HumB6 and HumB15 suffered from problems (see comments) when large numbers of samples were tested and so were not used

*TA* Annealing temperature, *A* number of alleles, *n* number of individuals, $H_o$ observed heterozygosity

HumB9, HumB10, HumB15) and three (50 %) of the *L. amethystinus* markers (Lamp1, Lamp2 and Lamp4) were polymorphic in *S. rufus* (Table 1). Of the markers that amplified, the number of alleles (in a sample of 8–12 individuals) ranged from 1 to 10 [mean = 4.43 ± 0.77 (SE); of those that were polymorphic the number of alleles ranged from 2 to 10 mean = 5.00 ± 0.78 (SE)]. Following further testing, data for six markers were taken forward for numerical analysis (for identities see Table 2).

## Population structure

The six loci chosen for investigation of population structure amplified in 139–185 of the 190 samples used and the number of alleles ranged from 6 to 25 (Table 2). However, this still left 1.2–18.0 % missing data for each locus. Null allele frequency estimates ranged from 0.083 to 0.249.

Across the 190 samples tested, 165 gave a clear genotype at three or more of the six markers. These 165 samples were split among the four geographical regions as follows: Alberta (45 individuals), Central B.C. (17 individuals), Eastern Vancouver island (53 individuals) and Western Vancouver Island (50 individuals). Only data from these 165 individuals were used in the analysis of within-population Hardy–Weinberg equilibrium and analysis of population structure in ARLEQUN.

In all populations, locus HumB10 deviated significantly from Hardy–Weinberg equilibrium (Table 2). Loci Lamp4, HumB3, HumB8 and HumB9 deviated from Hardy–Weinberg equilibrium in at least one population, in all cases due to fewer than expected heterozygotes. Locus HumB1 was in Hardy–Weinberg equilibrium in all populations except in Central B.C. where it was monomorphic.

Analysis in ARLEQUIN revealed that most genetic variation was found within populations, but there was a small, significant amount of variation among populations (Table 3).

There was a slight but significant differentiation between birds breeding on Vancouver Island and those breeding on the mainland ($F_{ST} = 0.081$–$0.015$, $p < 0.001$–$0.006$; alpha = 0.008 following Bonferroni correction for multiple comparisons; Table 4). There was no difference between birds breeding on the east and west of Vancouver Island ($F_{ST} = 0.003$, $p = 0.369$). While the birds breeding in Central B.C. tended to differ from those breeding in Alberta, this was not significant ($F_{ST} = 0.025$, $p = 0.013$).

The most likely number of population clusters of individuals (populations) identified by STRUCTURE 2.2 was $K = 1$ [average $\ln P(X|K) = -2762.28$]. For values of $K > 1$, the $\ln P(X|K)$ values decreased and the variation among the independent runs increased (adjusted $R^2 = 0.82$, $t = 11.35$, $p < 0.01$; Fig. 2). It therefore seems unlikely that the birds sampled came from more than one population. There was no clear genetic distinction among any of the sampling sites with all individuals being partially assigned to all populations. Removing the locus that deviated most severely from Hardy–Weinberg equilibrium (HumB10) from the analysis did not affect the most likely number of population clusters of individuals identified. The LOCPRIOR model from STRUCTURE 2.3 also identified $K = 1$ as the most likely number of clusters.

The same data were analysed using BAPs 3.1. The model was run both with and without admixture for ten iterations and the same number of $K$ ($K = 1$–6). Both of these models predicted two populations, with all the birds from Vancouver Island and the birds from Alberta in one population and the birds from Central B.C. forming the second population. The mixture model predicted two populations [Log(marginal likelihood) of optimal

**Table 2** Summary of information for six loci across 190 samples and from the 165 individuals from four sampling sites with more complete data

| Locus | Lamp4 | HumB1 | HumB3 | HumB8 | HumB9 | HumB10 |
|---|---|---|---|---|---|---|
| Summary across whole population (190 samples) | | | | | | |
| *N* alleles | 7 | 6 | 10 | 16 | 11 | 25 |
| *N* individuals amplified | 155 | 185 | 148 | 161 | 139 | 171 |
| Null allele frequency estimate (from CERVUS) | 0.134 | 0.145 | 0.123 | 0.065 | 0.083 | 0.249 |
| % missing data | 14.4 | 1.2 | 12.0 | 9.0 | 18.0 | 6.0 |
| Area | Hardy–Weinberg test *p* values from ARLEQUIN | | | | | |
| Summary within populations (165 samples) | | | | | | |
| West VCI ($N = 50$) | **<0.01** | 0.06 | 0.86 | 0.28 | **0.01** | **<0.01** |
| East VCI ($N = 53$) | 0.12 | 1.00 | **0.01** | **0.03** | 0.06 | **<0.01** |
| Central B.C. ($N = 17$) | 1.00 | Mono | **<0.01** | 0.44 | 0.24 | **<0.01** |
| Alberta ($N = 45$) | **0.05** | 1.00 | **0.01** | 0.11 | **0.04** | **<0.01** |

Significant (after Bonferroni correction) within-population HWE *p* values are highlighted in bold and are all due to an excess of homozygotes

partition = −2882.08, probability of two clusters = 1; Fig. 3a]. The admixture model assigned all but 2 of the 165 individuals to one of two populations with a probability of 1, and the remaining 2 individuals to both populations with probabilities of 0.10 and 0.12 (Fig. 3b). Excluding locus HumB10 did not qualitatively change the results.

## Discussion

We identified six markers that are useful for genotyping *S. rufus*. No genetic structure was detected using the commonly-used Bayesian approach implemented in STRUCTURE, but some structure was found using both a traditional method ($F_{ST}$) and a sensitive Bayesian approach (BAPs). First, the genotypes of Rufous Hummingbirds breeding on Western and Eastern Vancouver Island could not be distinguished and the Vancouver Island (VI) population can tentatively be treated as a single population. Both the sensitive methods found that the VI birds were genetically different to the birds breeding in central British Columbia (B.C.). Alberta birds were distinguishable from central B.C. birds using BAPs, but the $F_{ST}$ between these groups was not significant after Bonferroni correction, while Alberta birds and VI birds were indistinguishable by both methods. This lack of clarity is probably due to the very high level of within-population variation compared to among-population variation. In addition, it is important to note that, with such low $F_{ST}$ values, both clustering algorithms used in the software packages BAPs and STRUCTURE may have difficulties in identifying the number of clusters correctly (Latch et al. 2006). BAPs is more likely to overestimate the number of clusters and STRUCTURE to underestimate them (Latch et al. 2006). However, taking results of both Bayesian clustering methods and the $F_{ST}$ analysis together, it seems probable that there are at least two genetically distinguishable sub-populations. Clarifying the situation with such weak population structure would require analysis of the samples with more markers than were available to this study. When $F_{ST}$ is 0.05 or more, a sample size of 20 individuals per population is sufficient, but when $F_{ST}$ is around 0.01, as in this study, closer to 100 individuals per population is advised (Kalinowski 2005).

It seems probable, as is generally accepted for many other North American migrant bird species, that Rufous Hummingbird population differentiation occurred due to geographic isolation during the Pleistocene glaciation (Avise and Walker 1998). Postglacial range expansion may then have led to secondary contact and increased gene flow among populations (e.g., Swainson's Thrush *Catharus ustulatus*; Ruegg 2007). The apparent weak but significant population structure that still exists among regions suggests that the adult breeding site fidelity, recorded in the banding data at around 12 % (Finlay 2007) and levels of natal dispersal (unknown) are sufficiently low to maintain population structure among the geographically more distant populations. However, enough individuals must be moving between populations at smaller geographic scales, e.g., on Vancouver Island, to maintain gene flow and a panmictic population. This movement may be due to the dispersal of juveniles, the movement of non-territorial males throughout the season, or to females re-locating within and between seasons.

Large-scale gene flow among *S. rufus* populations may also be due to migration patterns. It is possible that some

**Table 3** Sources of variation in microsatellite molecular data based on analysis in ARLEQUIN

| Source of variation | *df* | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among populations | 3 | 13.225 | 0.036 | 2.41 |
| Within populations | 330 | 488.913 | 1.482 | 97.59 |
| Total | 333 | 502.138 | 1.518 | |

$F_{ST}$ among populations = 0.0241, $p < 0.001$

**Table 4** Pairwise differentiation among populations ($F_{ST}$, below diagonal) and probability tests for allele frequency differences ($p$ value above diagonal)

| Population | Western VCI | Eastern VCI | Central B.C. | Alberta |
|---|---|---|---|---|
| Western VCI | – | 0.369 ± 0.001 | <0.001 ± 0.000 | 0.004 ± 0.000 |
| Eastern VCI | 0.003 | – | <0.001 ± 0.000 | 0.006 ± 0.000 |
| Central B.C. | **0.081** | **0.068** | – | 0.013 ± 0.000 |
| Alberta | **0.018** | **0.015** | 0.025 | – |

Bonferroni correction for six tests (alpha = 0.008). $F_{ST}$ values significantly different from zero after Bonferroni correction are in bold

*VCI* Vancouver Island

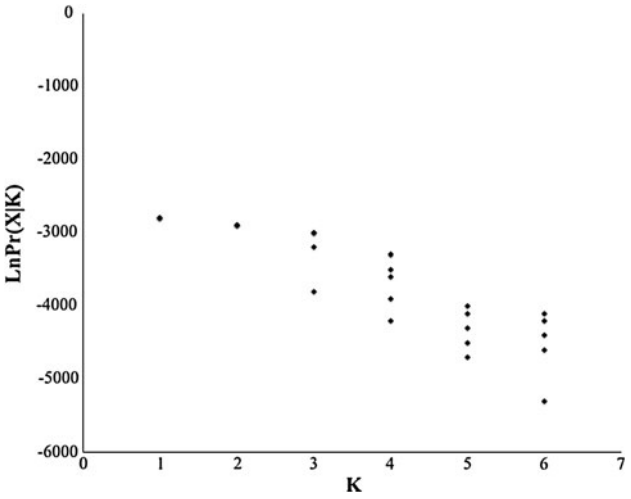


**Fig. 2** The likelihood of each value of LnPr($X|K$) for five independent runs of $K = 1$–6. Analysis was conducted using a burn-in period of $2 \times 10^4$ and a run of $5 \times 10^5$ Markov chain Monte Carlo steps, under the standard model of admixed ancestry, correlated allele frequency ($\lambda = 1$) and no prior population information in the program STRUCTURE 2.2

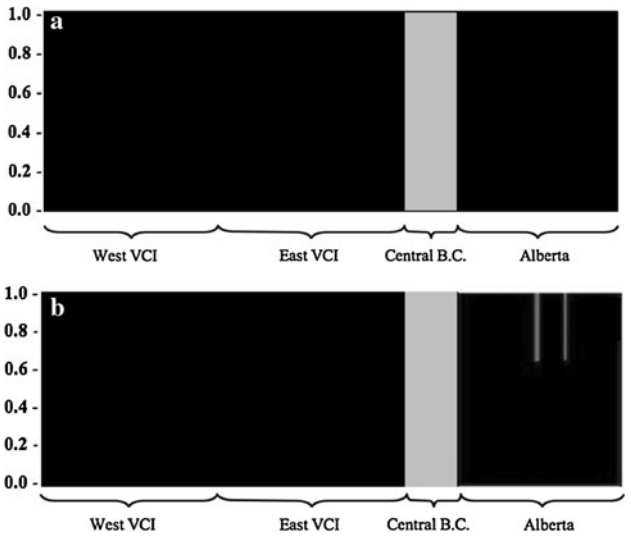


**Fig. 3** **a** The assignment of each individual from each geographic location ($n = 4$) to one of each simulated cluster of genotypes ($K = 2$), using a mixture model in BAPs. **b** The estimated probability, of each individual ($n = 165$), belonging completely or partially to each cluster of genotypes ($K$) using an admixture model (with $K = 2$) in BAPs. The *vertical lines* each represent an individual. In each case, the *vertical lines* are either *black*, *grey* or both representing the proportion of that individual assigned to each cluster (genotype cluster one = *black*, and genotype cluster two = *grey*). Partial assignment to both clusters is only possible in the admixture model. Individuals are grouped by location. *VCI* Vancouver Island

birds from the west, particularly those that fail to breed early in the season and those males who typically leave the breeding grounds earlier than females, move on and make a second attempt further along the migration route (see Aebischer and Potts 1994; Rohwer et al. 2009, for examples of double breeding in Quail *Coturnix coturnix*, Yellow-billed Cuckoo *Coccyzus americanus*, Cassin's Vireo *Vireo cassinii*, Yellow-breasted Chat *Icteria virens*, Hooded Oriole *Icterus cucullatus* and Orchard Oriole *Icterus spurius*). Migration by western individuals along a more easterly route in some years or vice versa cannot be ruled out, but, given the banding evidence that the majority of birds move along the west coast, this seems less likely.

Migration behaviour and the timing of breeding may also explain the rather odd result that, while the central population is distinct from the Vancouver and Alberta populations, the latter two do not clearly differ. The timing of the departure from Vancouver Island could mean that these birds move to more easterly breeding grounds in Alberta to breed again, while the central B.C. birds, which start their first breeding attempt slightly later, would not have time to do so. Alternatively, birds breeding in Central B.C. may belong to the small percentage of birds that take a more inland migration route than the general population (Healy and Calder 2006). It is not clear whether such movements are age- or sex-specific as the banding and recapture data analysed thus far contain too few examples of individual bird movements for useful comment. To clarify the degree of differentiation among populations and their extent, a study including samples from more geographical locations would be necessary.

Among the loci screened in up to 165 hummingbirds, several loci deviated significantly from Hardy–Weinberg equilibrium due to a deficit of heterozygotes. However, the exclusion of the locus most severely affected by this (H10) from analyses of population structure did not significantly affect the results. The deficit of heterozygotes is most probably due to the presence of null alleles or to poorly amplifying alleles, which can be a problem when amplifying DNA from samples containing low quantities of DNA, such as feather extractions. Further genotyping errors can arise due to the generation of false alleles during PCR (Navidi et al. 1992; Gerloff et al. 1995; Taberlet et al. 1996; Gagneux et al. 1997; Segelbacher 2002).

In conclusion, we found six markers that could be useful for genotyping *S. rufous*. Using these, we found that the breeding population of Rufous Hummingbirds in Canada has very weak genetic structure that is not clearly related to geographical barriers such as the Rocky Mountains and more probably arose due to population isolation during the last glaciation. These genetic data add usefully to the banding data, which allowed us to determine which locations the birds move between, as it now appears that at least some of those movements result in gene flow among populations. More work is necessary using improved protocols, more samples and more markers before we can say if these movements are in any way influenced by landscape structure.

**Acknowledgments** Financial support was provided by NERC and the Natural Sciences and Engineering Research Council of Canada. We thank Cam Finlay and members of the British Columbia Hummingbird banding network for collecting samples, Jonathan Moran for sharing samples with us, Judith St John and Sara Oyler-McCance for the use of their primers and Jaime Garcia-Moreno for his support and for providing the samples of *Lampornis amethystinus* that G.S. used to developed the six loci for that species which we have subsequently tested in *Selasphorus rufus*.

## References

Aebischer NJ, Potts GR (1994) Quail *Coturnix coturnix*. Birdlife International, Cambridge

Alcaide M, Serrano D, Tella JL, Negro JJ (2009) Strong philopatry derived from capture-recapture records does not lead to fine-scale genetic differentiation in lesser kestrels. J Anim Ecol 78:468–475

Avise JC, Walker D (1998) Pleistocene phylogeographic effects on avian populations and the speciation process. Proc R Soc Lond B 265:457–463

Clark RG, Hobson KA, Nichols JD, Bearhop S (2004) Avian dispersal and demography: scaling up to the landscape and beyond. Condor 106:717–719

Corander J, Marttinen P (2006) Bayesian identification of admixture events using multilocus molecular markers. Mol Ecol 15:2833–2843

Corander J, Waldmann P, Marttinen P, Sillanpaa MJ (2004) BAPs 2: enhanced possibilities for the analysis of genetic population structure. Bioinformatics 20:2363–2369

Corander J, Marttinen P, Mantyniemi S (2006) A Bayesian method for identification of stock mixtures from molecular marker data. Fish Bull 104:550–558

Esler D, Iverson SA, Rizzolo DJ (2006) Genetic and demographic criteria for defining population units for conservation: the value of clear messages. Condor 108:480–483

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: applications to human mitochondrial DNA restriction data. Genetics 131:479–491

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Finlay JC (2007) Nine years of banding and recapture of hummingbirds in Southern British Columbia. Br Columbia Birds 15:9–23

Gagneux P, Boesch C, Woodruff DS (1997) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. Mol Ecol 6:861–868

Gerloff U, Schlotterer C, Rassmann K, Rambold I, Hohmann G, Fruth B, Tautz D (1995) Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan-paniscus*). Mol Ecol 4:515–518

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. Methods Enzymol 395:202–222

Guillot G, Estoup A, Mortier F, Cosson JF (2005) A spatial statistical model for landscape genetics. Genetics 170:1261–1280

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Healy SD, Calder WA (2006) Rufous hummingbirds (*Selasphorus rufus*). In: Pool A (ed) The birds of North America online. Cornell Lab of Ornithology, Ithaca

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Res 9(5):1322–1332

Kalinowski ST (2005) Do polymorphic loci require large sample sizes to estimate genetic distances? Heredity 94:33–36

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes OE (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. Conserv Genet 7:295–302

Marshall TC, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655

Milot E, Gibbs HL, Hobson KA (2000) Phylogeography and genetic structure of northern populations of yellow warbler (*Dendroica petechia*). Mol Ecol 9(6):667–681

Navidi W, Arnheim N, Waterman MS (1992) A multiple-tubes approach for accurate genotyping of very small DNA samples by using pcr—statistical considerations. Am J Hum Genet 50:347–359

Newton I (2008) The migration ecology of birds. Academic Press, Oxford, UK

Oyler-McCance SJ, Fike JA, Talley-Farnham T, Engelman T, Engelman F (2011) Characterization of ten microsatellite loci in the Broad-tailed Hummingbird (*Selasphorus platycercus*). Conserv Genet Res 3:351–353

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA (1998) What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79:361–382

Pearce JM, Talbot SL (2006) Demography, genetics, and the value of mixed messages. Condor 108:474–479

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Rohwer S, Hobson KA, Rohwer VG (2009) Migratory double breeding in Neotropical migrant birds. Proc Natl Acad Sci 106:19050–19055

Ruegg K (2007) Divergence between subspecies groups of Swainson's thrush (*Catharus ustulatus ustulatus*) and *C. U. swainsoni*. Ornithol Monogr 63:67–77

Sauer JR, Hines JE, Fallon J (2008) The North American breeding bird survey, results and analysis 1966–2007. Version 5.15.2008 USGS Patuxent Wildlife Research Center, Laurel, MD. http://www.mbr-pwrc.usgs.gov/bbs/bbs.html

Schneider S, Roessli D, Excoffier L (2000) Arlequin v.2.000: a software for population genetics analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Segelbacher G (2002) Noninvasive genetic analysis in birds: testing reliability of feather samples. Mol Ecol Notes 2:367–369

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologist: a practical guide to using and evaluating microsatellite markers. Ecol Lett 9(5):615–629

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24:3189–3194

Walters JR (2000) Dispersal behavior: an ornithological frontier. Condor 102:479–481

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370

Williamson SL (2001) Hummingbirds of North America. Houghton Mifflin, Boston