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Low support for separate species within the redpoll complex (*Carduelis flammea–hornemanni–cabaret*) from analyses of mtDNA and microsatellite markers

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ABSTRACT

The redpoll complex, consisting of three currently recognized species (*Carduelis flammea*, *C. hornemanni* and *C. cabaret*), is polytypic in biometry, morphology, physiology and behaviour. However, previous genetic work has not revealed any indications of genetic differentiation. We analysed sequence variation in the mtDNA control region, and allele frequencies of supposedly faster evolving microsatellites ($n = 10$), in an attempt to detect molecular genetic support for the three species, as well as two subspecies of *C. flammea* (ssp. *flammea* and *rostrata*), within this complex. We used samples from two subspecies of the twite (*Carduelis flavirostris*, ssp. *flavirostris* and *rufostrigata*) as outgroup. We found no structure among redpoll individuals in mtDNA haplotypes or microsatellite allele frequencies, and only marginal differences between redpoll taxa in analyses of molecular variance (AMOVAs) of predefined groups. In contrast, the two twite subspecies constituted two well-supported monophyletic groups. Our study thus strengthens previous indications of low genetic support for current redpoll taxa. Two major alternative interpretations exist. Either redpolls form a single gene pool with geographical polymorphisms possibly explained by Bergmann's and Gloger's rules, or there are separate gene pools of recent origin but with too little time elapsed for genetic differentiation to have evolved in the investigated markers. Future studies should therefore examine whether reproductive isolation mechanisms and barriers to gene flow exist in areas with sympatric breeding.

1. Introduction

Species are the basal entities in most biological studies of ecology, taxonomy and conservation (Barton, 2001). However, species descriptions are largely built on phenotypic traits whose genetic basis is often poorly known (Price, 2008). This poses a challenge to current taxonomy, especially when closely related species show little or no genetic differentiation. Does a lack of genetic differentiation imply that observed phenotypic variation is misleading for the designation of closely related species? Or could it be that the genetic analyses have missed relevant markers for species identification?

The redpoll complex (Aves: Fringillidae: *Carduelis flammea*, *C. hornemanni*, *C. cabaret*) is a group of species and subspecies with extensive morphological variation, and a controversial taxonomic issue. Some authors have considered the morphological variation in redpolls continuous, and recognized the complex as one polytypic species (e.g. Harris et al., 1965; Troy, 1985), whereas others have recognized two species; the Arctic redpoll *C. hornemanni* and the common redpoll *C. flammea* (e.g. Molau, 1985; Knox, 1988; Seutin et al., 1992). The Arctic redpoll consists of the subspecies Greenland redpoll *C. h. hornemanni* and hoary redpoll *C. h. exilipes*, and common redpoll consists of the subspecies mealy redpoll *C. f. flammea*, greater redpoll *C. f. rostrata* and Iceland redpoll *C. f. islandica* (Knox et al., 2001). Recently, the British Ornithologists' Union (BOU) Records Committee decided to elevate the subspecies lesser redpoll *C. f. cabaret* to species rank (Knox et al., 2001; Sangster et al., 2002). There is however not consensus over this decision (Dickinson, 2003; Clements, 2007). We use the term *C. cabaret* for this taxon throughout the manuscript for convenience. Breeding distributions of the different taxa are given in Fig. 1.

Redpolls have an irruptive dispersal behaviour and are found in large numbers where seed crops in birch and spruce forests are abundant (Troy, 1983). They are opportunistic breeders, and the fluctuations in population densities at specific localities indicate low return rates (Troy, 1983). A large degree of apparently geographically structured polytypism in plumage and biometrics has been found within the group, both at a local and a global scale (e.g. Molau, 1985; Knox, 1988; Seutin et al., 1995). In addition to

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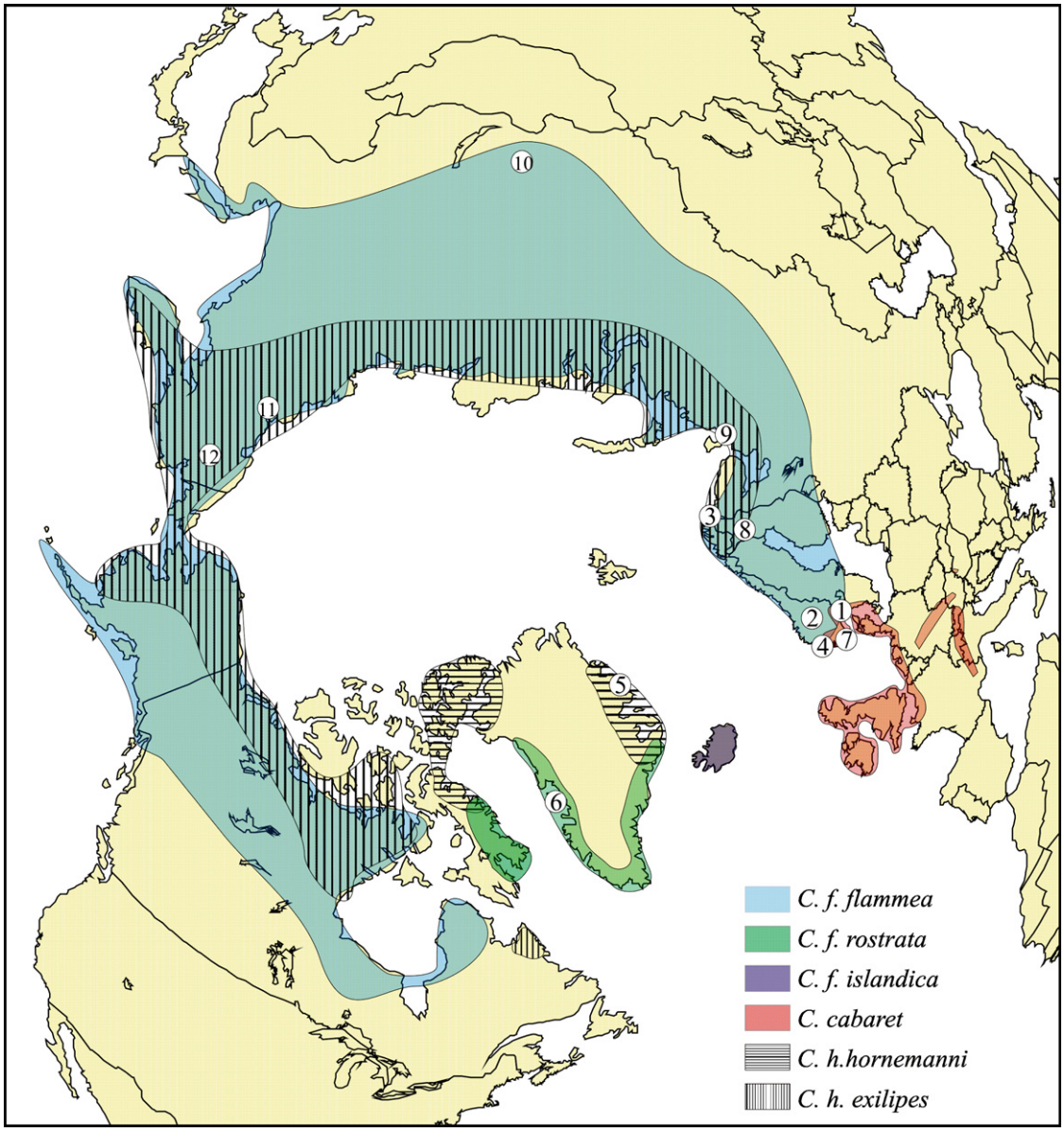

Fig. 1. Worldwide breeding distribution for all recognized redpoll taxa following Knox (1988) and sampling localities. Names of localities are given in Table 1.

plumage characters and biometric measures (reviewed in Knox, 1988), *C. flammea* and *C. hornemanni* have been reported to differ in physiology (Brooks, 1968), timing of migration, nest habitats, diets, vocalizations and behaviour (Molau, 1985; Herremans, 1989). *C. flammea* and *C. cabaret* have been claimed to differ in vocal (Herremans, 1989) and behavioural (Lifjeld and Bjerke, 1996) characters, as well as in habitat selection (Knox et al., 2001). Furthermore, *C. flammea* and *C. cabaret* were found not to form mixed pairs at an event of sympatric breeding in Norway in 1994 (Lifjeld and Bjerke, 1996). The occurrence of hybrids from mixed pairs of *C. flammea* and *C. hornemanni* has been debated, but there are no conclusive observations of hybrid offspring (Molau, 1985; Knox, 1988).

Despite the extensive variation in several phenotypic characters, no studies have yet detected any genetic differences between the redpoll taxa. Marten and Johnson (1986) found no genetic differences between *C. flammea* and *C. hornemanni*, both sampled in Alaska, using 33 loci in an RFLP analysis. Seutin et al. (1995) performed an extensive mtDNA RFLP analysis, but did not find any differences between *C. flammea* individuals from North America, Europe (ssp. *flammea*) and Baffin Island (ssp. *rostrata*), *C. hornemanni* individuals from North America (ssp. *hornemanni* and *exilipes*) and *C. cabaret* individuals from Europe. Ottvall et al. (2002) sequenced a part of the mtDNA control region of *C. f. flammea* and *C. cabaret* individuals and found no genetic differentiation between the taxa. The cytochrome oxidase I (COI) gene has through the Barcoding of Life Initiative been sequenced for *C. f. flammea*, *C. hornemanni* and *C. cabaret*, and individuals of all three taxa cluster together (Hebert et al., 2004; Kerr et al., 2007; JT Lifjeld, A Johnsen, E Rindal, unpublished data).

Redpolls constitute an interesting species complex in an evolutionary perspective for several reasons. First, their opportunistic breeding behaviour makes speciation caused by geographic barriers less likely than is assumed for most other species complexes (Coyne and Orr, 2004), and opens up for hypotheses on ecological speciation. Second, redpolls are extreme in that no genetic differences have been found across the large, circumpolar distribution in arctic and temperate regions, in contrast to several other circumpolar species complexes (e.g. Questiau et al., 1999; Liebers et al., 2004). Third, the breeding range of one of the species, *C. cabaret*, has expanded during the last 50 years, from the British Isles to

the countries bordering the North Sea (reviewed in Knox, 1988), leading to potential sympatric breeding events together with *C. flammea* in southern Norway in years of high seed abundances (Lifjeld and Bjerke, 1996). This range shift may be due to a recently warmer climate. It is possible that the climate changes may affect the habitats of *C. flammea* and *C. hornemanni* too. The future development of the redpoll species is thus interesting in both a conservational and a climate change perspective.

In this study we have analysed microsatellite markers to potentially reveal genetic differences that have evolved more recently than could be detected in previous genetic studies. Microsatellites have higher mutation rates than, for example, the mtDNA control region (Hewitt, 2001), and may thus provide a higher resolution of differentiation (e.g. Eggert et al., 2004; Johnsen et al., 2006). Microsatellites are also, contrary to mtDNA, inherited through both mothers and fathers, and better reflect genetic structuring in both sexes. Furthermore, compared to previous studies, we have added new mtDNA control region sequences for *C. hornemanni*, included more breeding populations of *C. flammea* and *C. cabaret*, and added samples from the subspecies *C. f. rostrata*.

2. Methods

2.1. Sampling and DNA extraction

Blood samples or foot scrapes from museum skins were collected or granted from museum collections for 125 redpoll individuals; 56 classified as *C. flammea* (46 as ssp. *flammea*, 10 as ssp. *rostrata*), 24 classified as *C. hornemanni* (ssp. *exilipes*) and 45 classified as *C. cabaret* (Table 1). Museum skins were classified by museum curators, and blood sampled birds by bird ringers, based on plumage and morphological characters (Lindström et al., 1984; Svensson, 1992). Eight individuals of twite (*Carduelis flavirostris*), a close relative of the redpolls, were blood-sampled and used as an outgroup; four individuals from the *C. f. flavirostris* subspecies breeding in northern Europe and four from the *C. f. rufostrigata* subspecies breeding in Tibet, China (Table 1). To test for potential null alleles in the microsatellite loci, six *C. f. flammea* families including 26 chicks were sampled in Øvre Heimdalen, Norway, and analysed with the same microsatellites.

DNA was extracted from blood samples using a standard chloroform–phenol protocol (Sambrook et al., 1989) or a E.Z.N.A.® Blood DNA Kit (Omega Bio-tek), and from skin samples using a E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek) following the manufacturer's protocol.

2.2. Sequencing of the mitochondrial control region

Following Ottvall et al. (2002), a fragment of 957 base pairs of the mtDNA control region was amplified for all twite individuals and most redpoll individuals, excluding non-breeding *C. flammea* and *C. cabaret* individuals and samples analysed by Ottvall et al. (2002) from Eidsberg and Heimdalen (Table 1). For blood samples, we amplified the first half of the fragment using the primers L16743 (Tarr, 1995) and CH1 (5′CCAATAGCGCAAAAGAGCAA), and the second part with CL1 (5′CACGAGAACCGAGCTACTCAA) and H1248 (Tarr, 1995). This resulted in a 1251 base pair fragment, but because of a poly T sequence we omitted the last 294 base pairs from the analyses. For the museum skin samples we amplified four or five overlapping fragments due to degradation of the DNA, using the additional primers CL2 (5′TGTTCCTACCTACGGCC AAG), CH2 (5′TTCCTAGG TTGGGTGAGGTG), CL3 (5′GCAGAGCAGG TGTTATCTTCC), CH3 (5′TC TGGGTGTTAGGGGATTCA), LGL2, HGL2 and HCR1 (Tarr, 1995), Fig. 2. The primers not published in Tarr (1995) were designed from GenBank sequence AF416737 of *C. cabaret*.

The PCR mixture volumes were 15 μL, containing 0.6 mM dNTPs, 0.07 U/μL AmpliTaq Gold Polymerase (Applied Biosystems), 1× GeneAmp PCR Gold buffer (15 mM Tris–HCl, 50 mM KCl), 2.5 mM $MgCl_2$ solution, 0.5 μm primer and 1 or 2 μL DNA extract. The reactions were carried out under the following conditions: 5 min at 94 °C, 30 cycles of [30 s at 94 °C, 30 s at 53 °C and 2 min at 72 °C] and a final extension period of 10 min at 72 °C.

The PCR primers were used in direct sequencing. The cycle-sequencing reactions were carried out using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit, with a reaction volume of 10 μL; 1 μM primer and 5 μL PCR product. The cycle-sequencing products were run on an ABI PRISM 3100 Genetic Analyzer following the manufacturers' instructions (Applied Biosystems), aligned in SEQUENCHER 4.1.4 (Gene Codes Corporation) and edited in BIOEDIT 5.0.9 (Hall, 1999). All sequences have been deposited in GenBank (Accession Nos. EU400451–EU400542, Table 1).

2.3. Microsatellite typing

All samples were analysed with 10 variable microsatellite markers (Table 2). The PCR mixture volumes were 10 μL, containing 0.6 mM dNTPs, 0.3 U Dynazyme II DNA Polymerase (Finnzymes), 1× buffer solution (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KD and 0.1% Triton X-100; Finnzymes), 0.5 μM primer (Table 2) and 1–5 μL DNA extract. Forward primers were labelled with HEX, NED or FAM. The reactions were carried out under the following conditions: 5 min at 94 °C, 30 cycles of [30 s at 94 °C, 30 s at the annealing temperature (Table 2) and 30 s at 72 °C], and a final extension period of 10 min at 72 °C. The PCR products were run on an ABI PRISM 3100 Genetic Analyzer following the manufacturers' instructions (Applied Biosystems), and scored in GENEMAPPER 3.4 (Applied Biosystems).

2.4. Statistical analyses

Number of haplotypes, haplotype diversity and nucleotide diversity for the mtDNA control region were calculated in ARLEQUIN 3.1 (Excoffier et al., 2005). Haplotype richness was estimated by rarefaction according to El Mousadik and Petit (1996) using the program CONTRIB 1.02 (Petit et al., 1998) for the three redpoll species. We used a rarefaction size of 13. We calculated a Neighbour Joining (NJ) phylogram based on all individuals in MEGA 2.1 (Kumar et al., 2001) using Kimura 2 parameter distances. Bootstrap support was calculated with 1000 replications. The relationship between haplotypes was visualized by calculating an unrooted NJ phylogram in MEGA.

Mismatch distributions for the sequence data were calculated in DNASP (Rozas et al., 2003) to reveal potential signatures of historic sudden population expansions. An observed distribution for pairwise differences between sequences was calculated and tested against expected distributions under the scenarios of a constant population size and a sudden population growth with χ^2 tests in STATISTICA (StatSoft, 2003).

All microsatellite loci were tested for Hardy–Weinberg equilibrium and linkage disequilibrium in ARLEQUIN; sequential Bonferroni correction (Rice, 1989) was applied. Allelic richness was calculated in FSTAT 2.9.3 (Goudet, 2001).

The genetic structure among individuals was investigated for microsatellites in two analyses. First, we ran STRUCTURE 2.0 (Pritchard et al., 2000), a Bayesian-based program searching for the occurrence of independent genetic groups (K) in the dataset. We set the burn-in length to 1,00,000, followed by 1,000,000 iterations of the Markov chain Monte Carlo (MCMC). We ran both the 'admixed ancestry' model, which is recommended as starting point for analyses, and the 'not admixed ancestry' model, since this may be better at detecting subtle structure (Pritchard and Wen,

Table 1
Information on samples for mtDNA sequencing and microsatellite analyses of four redpoll taxa and two twite taxa

Taxon	Locality	Ref. to Fig. 1	Coordinates	Breeding status	Collection date	*n* mtDNA	*n* msats	Journal numbers	Accession No.
Carduelis flammea flammea	Eidsberg, Norway	1	59.52°N 11.23°E	Breeding	May 1994		4	20443–20446	
	Heimdalen, Norway	2	61.42°N 8.87°E	Breeding	May–July 1998–2003	22	30	20230–20242, 20247, 18719–34	EU400481–EU400502
	Sandfjorden, Norway	3	70.50°N 30.53°E	Breeding	July 2004	3	5	7806, 7827, 7840–42	EU400503–EU400505
	Utsira, Norway	4	59.19°N 4.53°E	Migrating	May, September, October 2004, 2005		3	8115, 8178, 18011	
	Skien, Norway		59.18°N 9.62°E	Migrating	March 1997		4	6661–6663, 6665	
C. f. rostrata	NE Greenland	5	75.0°N 20.00°W	Breeding	August–October 1906, 1914,1918, 1930	5	5	20248–20252 (DNA)/21292–94[c], 21297[b,c], 21299[c] (museum skins)	EU400510–EU400514
	Godhavn, W Greenland	6	69.25°N 53.63°W	Breeding	July–August 1990, 1994	4	4	116648, 121472–74 (ZMUC[a])	EU400506–EU400509
	Utsira, Norway	4	59.19°N 4.53°W	Migrating	September 2004	1	1	8100	EU400515
C. cabaret	Jomfruland, Norway	1	58.52°N 9.36°E	Breeding	May 2003	5	6	7112, 7117, 7150, 7193–94, 7215	EU400476–EU400480
	Utsira, Norway	4	59.19°N 4.53°E	Breeding	May–September 2003, 2004	14	14	7670, 7711, 7926, 7963–64, 7967, 7973–74, 7977, 7981, 8014, 8036–37, 8104	EU400451–EU400464
	Kvinesdal, Norway	7	58.17°N 06.54°E	Breeding	September 2003	11	11	7374–78, 7383–88	EU400465–EU400475
	Eidsberg, Norway	1	59.52°N 11.23°E	Breeding	April, May 1994		5	20225–20229	
	Falsterbo, Sweden		55.38°N 12.82°E	Migrating	April 1990		2	6594–95	
	Skien, Norway		59.18°N 9.62°E	Migrating	November–January 1995, 1996		7	6568–69, 6572–74, 6586, 20224	
C. hornemanni exilipes	Sandfjorden, Norway	3	69.08°N 29.00°E	Breeding	July 2004	2	2	7807, 7830	EU400531–EU400532
	Sør-Varanger, Norway	3	70.50°N 30.53°E	Breeding	June 1911	1	1	20260 (DNA)/452[c] (museum skin)	EU400530
	Kiruna, Sweden	8	67.87°N 20.25°E	Breeding	July 1994	1	1	946510 (NRM[a])	EU400520
	Kanin Peninsula, Russia	9	68.43°N 45.42°E	Breeding	August 1994	1	1	946649	EU400521
	Krasnojarsk, Russia	10	56.13°N 93.00°E	Breeding	February 1912	1	1	20258 (DNA)/16448[c] (museum skin)	EU400529
	Kolyma, Siberia	11	69.50°N 161.00°E	Breeding	January 1910, 1916	3	5	20253–20257 (DNA)/16317[b,c], 16319[c], 16321, 16342, 16343[c](museum skins)	EU400526–EU400528
	Chukotka, Russia	12	64.58°N 177.33°E	Breeding	July 2005	1	1	20066079[c](museum skin) (NRM[a])	EU400523
	Sør-Varanger, Norway		70.50°N 30.53°E	Migrating	November 1905		1	20259 (DNA)/440 (museum skin)	

	S Norway		Migrating	February, October 1982, 2007	3	3	19940–41, 20261 (DNA)/11326 (museum skin)		EU400525, EU400533–EU400534
	Sweden		Migrating	February, March 1998, 2005	2	2	20006068, 20056127 (NRM[a])		EU400522, EU400524
	Falsterbo, Sweden	55.38°N 12.82°E	Migrating	October, November 1990	4	6	119816–21 (ZMUC[a])		EU400516–EU400519
C. flavirostris/*flavirostris*	Heimdalen, Norway	61.42°N 8.87°E	Breeding	May 1994	2	2	6908–09		EU400535–EU400536
	Tromsø, Norway	69.67°N 18.93°E	Breeding	July 2006	1	1	17678		EU400538
	Hornøya, Norway	70.38°N 31.15°E	Breeding	June 2006	1	1	16795		EU400537
C. flavirostris rufostrigata	Damshung, Tibet, China	30.50°N 91.08°E	Breeding	July 2006	4	4	17008, 17011, 17034, 17036		EU400539–EU400542
Total					92	133			

Samples are from the Natural History Museum in Oslo if not otherwise stated.
[a] ZMUC, Zoological Museum University of Copenhagen; NRM, Swedish Museum of Natural History.
[b] The first 85 bp (including two polymorphic sites) did not amplify.
[c] The last 224 bp (including four polymorphic sites) did not amplify.

2004). We also used both 'independent' and 'linked allele frequencies'; the first works well when allele frequencies in the different populations are reasonably different from each other, the second potentially improves clustering for closely related populations (Pritchard and Wen, 2004). No prior population origin information was used. We ran five repeats for each K; $K = 1–10$.

Second, we conducted a principal coordinate analysis (PCO) in GENALEX 6 (Peakall and Smouse, 2006), and the two first axes were plotted to visualize the genetic structure of individuals. Genetic distances were calculated as outlined in the GENALEX 6 guide for codominant data. Only individuals scored for all ten microsatellite loci were included, as preliminary analyses revealed that missing values had a great influence on the results.

We investigated the degree of difference between the taxa with AMOVAs and pairwise Φ_{ST} values (control region) and F_{ST} values (microsatellites) in ARLEQUIN. The Kimura 2 parameter model was used in the Φ_{ST} calculations. For microsatellites, individuals with less than eight scored loci were excluded to avoid exclusion of loci in the AMOVA; ARLEQUIN allows only 5% missing data for each locus.

3. Results

3.1. Marker characteristics

An alignment consisting of 73 redpoll mtDNA control region sequences of 957 base pairs (excluding incomplete sequences from museum skin samples) contained 23 polymorphic sites; 16 were parsimoniously informative (Table 3). Three sites had three variants (A/G/T, C/T/A), no other transversion occurred. We found 34 haplotypes, 25 haplotypes occurred in single individuals (Appendix A). Overall nucleotide diversity was 0.00295.

The 954 base pair control region fragments from the twites varied in five sites, four were parsimoniously informative. All polymorphic sites were transitions. Genetic distance (Kimura 2 parameter) between redpolls and twites was 0.056.

Number of alleles at the 10 microsatellite loci ranged from 8 to 84 in the redpoll dataset, and observed heterozygosity ranged from 0.452 to 0.872 (Table 2). When all populations were pooled, two loci (CtC101 and LTR6) were not in Hardy–Weinberg equilibrium after sequential Bonferroni correction. These deviations from Hardy–Weinberg were probably not caused by missing alleles (null alleles) as no such were detected for these loci in the family material. Null alleles were on the other hand demonstrated for another locus; three chicks in three different families lacked one CtA8 allele. The candidate alleles for inheritance from the parents were more than 350 base pairs long in all three cases and may have been disfavoured in the PCR. CtA8 was also the most diverse locus, with 84 alleles. As a high number of alleles may underestimate differences between populations (Hedrick, 1999), all genetic structure analyses were performed both including and excluding the four most variable loci with more than 30 alleles; CtA8, CtD108, LOX1 and LOX2 (Table 2). Lox1 was in linkage disequilibrium with CtD108 and CtA8; no other loci were in linkage disequilibrium after Bonferroni correction.

For the *C. flavirostris* individuals, one microsatellite locus was monomorphic (FhU2) and one did not amplify (Lox1).

3.2. Within-taxon diversity

Carduelis flammea, *C. cabaret* and *C. hornemanni* did not differ significantly in microsatellite allelic richness (Friedman ANOVA: $\chi^2_{10,2} = 5.0$, $p = 0.082$) or observed heterozygosity ($\chi^2_{10,2} = 2.6$, $p = 0.27$), Table 4. The taxa did also not differ much in mtDNA haplotype or nucleotide diversity, or haplotype richness, and the pattern of variation among taxa in the different measures was not consistent (Table 4).

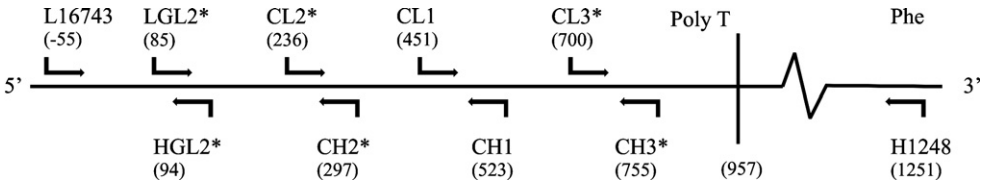

Fig. 2. Primers used for sequencing of the mtDNA control region in redpolls (*Carduelis flammea*, *C. hornemanni*, *C. cabaret*) and twites (*C. flavirostris*). Primers marked with asterisks were used for museum skin samples only. Numbers in parentheses correspond to base pair positions in GenBank sequence AF416737. The last 294 base pairs are excluded from the analyses because of a poly T sequence ending at position 957. Phe = tRNA phenylalanine. See text for primer references.

Table 2
Marker information for 10 microsatellite loci analysed for redpolls (*Carduelis flammea*, *C. hornemanni*, *C. cabaret*) and twites (*C. flavirostris*)

Locus	Annealing temp. (°C)	# Alleles	Observed heterozygosity	Allele range	Reference
CtA8	58	84	0.832	260–568	Tarvin (2006)
CtA105	55	8	0.452	111–123	Tarvin (2006)
CtC16	55	27	0.868	179–251	Tarvin (2006)
CtC101	58	14	0.609	218–277	Tarvin (2006)
CtC105	58	9	0.833	249–297	Tarvin (2006)
CtD108	58	43	0.870	193–282	Tarvin (2006)
LOX1	55	82	0.771	285–483	Piertney et al. (1998)
LOX2	52	45	0.872	185–400	Piertney et al. (1998)
LTR6	55	21	0.785	185–228	McDonald and Potts (1994)
FhU2 (PTC3)	58	19	0.760	119–144	Ellegren (1992)

3.3. Genetic structure

A NJ phylogram for all individuals sequenced on the control region revealed no structure among the redpoll taxa (not shown). The individuals in the outgroup (*C. flavirostris*) constituted a monophyletic clade with 99% bootstrap support, and the two subspecies *C. f. flavirostris* and *C. f. rufostrigata* clustered with 81% and 95% support, respectively. All other clusters had a bootstrap support lower

Table 3
Variable sites in the mtDNA control region in 73 redpoll individuals (*Carduelis flammea*, *C. hornemanni*, *C. cabaret*) with complete sequences (11 museum skin samples excluded)

Haplotype	Position																							
	7 7	8 4	1 9 0	1 9 2	1 9 3	1 9 4	2 0 8	2 2 7	2 6 7	2 7 2	3 0 0	3 0 4	3 1 5	3 3 3	3 8 5	3 8 8	4 3 4	5 1 4	5 1 8	5 8 0	6 3 2	8 9 4	9 4 1
#1	G	T	T	C	C	A	C	C	A	A	C	C	A	C	C	T	T	T	T	C	T	A	A
#2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.
#3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.
#4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
#5	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.
#7	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
#8	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.
#14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
#15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#18	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#22	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C	G	.
#23	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	T	C	G	.
#24	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#27	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#28	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	C	G	.
#29	A	.	.	T	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	C	G	.
#30	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	G	.
#31	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#32	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
#33	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	C	.	A	C	G	.
#34	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#35	.	.	.	.	.	.	.	T	.	.	T	.	.	T	.	.	.	.	.	.	C	G	.
#36	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
#37	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	.	.	.	C	G	.
#38	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	C	G	.
#39	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	G	.
#40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	G	.
#41	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	C	G	.
#42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.
#43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	G	.
#44	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	C	G	.
#45	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.
#46	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	G	.
#47	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	G

Haplotype #1–#26 are identical to Ottvall et al.'s (2002) haplotypes carrying the same names.

Table 4
Measures of genetic diversity for four redpoll taxa and two twite taxa in mtDNA control region sequences and 10 microsatellite markers

Taxon	mtDNA control region							Microsatellites		
	n	# Haplotypes	Haplotype diversity	Haplotype richness	SE	Nucleotide diversity	SE	*n*	Allelic richness	Observed heterozygosity
C. flammea	**35**	**22**	**0.867**	**8.96**	**0.0081**	**0.0024**	**0.00025**	**56**	**14.19**	**0.763**
C. f. flammea	25	20	0.973		0.0044	0.0029	0.00036	46		0.778
C. f. rostrata	5[a]	4	0.900		0.0720	0.0017	0.00063	10		0.662
C. cabaret	**30**	**16**	**0.922**	**7.40**	**0.0053**	**0.0032**	**0.00035**	**45**	**13.45**	**0.783**
C. hornemanni	**13**[a]	**8**	**0.910**	**7.46**	**0.0190**	**0.0025**	**0.00044**	**24**	**13.10**	**0.730**
C. flavirostris	**8**	**3**	**0.679**		**0.0430**	**0.0027**	**0.00064**	**8**		**0.694**
C. f. flavirostris	4	1	0			0		4		0.510
C. f. rufostrigata	4	2	0.500		0.133	0.0005	0.00035	4		0.875

Bold type indicates species status *C. flammea* and *C. flavirostris* include all samples for both listed subspecies.
[a] Incomplete sequences from museum samples excluded.

than 64%, and all clusters contained individuals from two or all three redpoll species. A NJ phylogram showing the relationship between haplotypes, and frequency of individuals carrying the different haplotypes, is given in Fig. 3; exact numbers are given in Appendix A.

We found no genetic structure in the individual-based analyses with microsatellites either. In STRUCTURE, for 'no admixture' and 'independent allele frequencies', the likelihood scores increased for each K until $K = 5$, with a relatively large increase from $K = 2$ to $K = 3$, indicating that the true number of groups is 3 (Pritchard and Wen, 2004). For $K = 3$, *C. flavirostris* individuals (the outgroup) constituted one cluster together with four redpoll individuals (two *C. hornemanni* and two *C. flammea* individuals) and the rest of the redpoll individuals were assigned 1/3 to one and 2/3 to the other of the two remaining clusters. There was no consistency in which individual assigned 2/3 to which cluster with respect to taxonomic adherence. When excluding *C. flavirostris*, the likelihood scores increased until $K = 4$ for 'no admixture' and 'independent allele frequencies', but the individuals still assigned arbitrarily to the different clusters regarding taxonomic adherence. For the 'admixture' model and 'correlated allele frequencies' the likelihood scores were highest for $K = 1$. No more structure was found when excluding non-breeding individuals that could have been misdiagnosed or when excluding loci with more than 30 alleles.

A lack of structure within the redpoll complex was also found in the PCO analysis of microsatellites, where individuals from

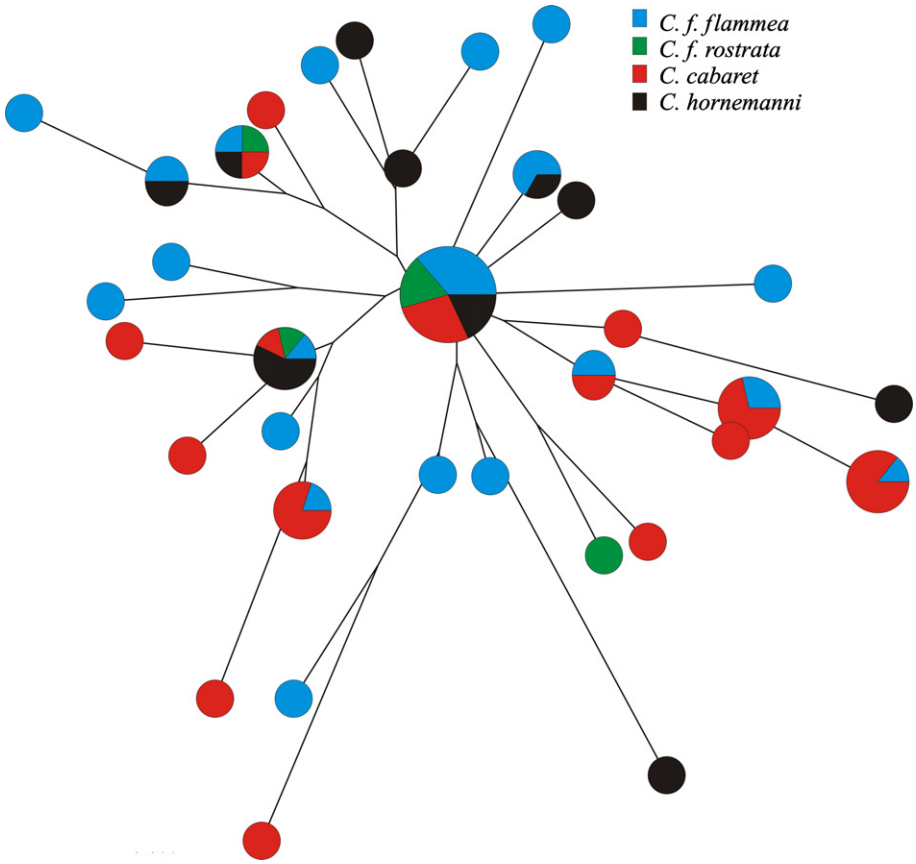

Fig. 3. Neighbour joining tree for mtDNA control region haplotypes with pie charts indicating frequencies of individuals from four redpoll taxa carrying the haplotypes. Details are given in Appendix A.

C. flammea, *C. cabaret* and *C. hornemanni* overlapped completely (Axes 1 and 2 are plotted in Fig. 4; Axis 3 did not reveal more structure). *C. flavirostris* individuals were completely separated from the others, and the two subspecies were completely separated on axes 1 and 2, except from one *C. f. flavirostris* individual grouping with the *C. f. rufostrigata* individuals (Fig. 4). No more structure appeared when the *C. flavirostris* samples were excluded, or when non-breeding individuals were excluded. The exclusion of the four loci with more than 30 alleles, or the five loci not amplified for the museum skin samples (increasing the number of *C. hornemanni* and *rostrata* individuals), did also not reveal any more structure in the PCO analysis.

We found small but significant differences between the taxa in AMOVAs. When excluding incomplete control region sequences from museum skin samples, 5.1% of the variation was explained by the three taxa *C. flammea*, *C. hornemanni* and *C. cabaret* ($p = 0.0078$, number of samples are given in Appendix B). In pairwise comparisons, *C. cabaret* differed from *C. hornemanni* and *C. flammea*, significantly so from *C. flammea* after Bonferroni correction (Table 5). The two *C. flammea* subspecies (*rostrata* and *flammea*) did not differ ($\Phi_{ST} = -0.05$, $p = 0.9$).

Analyses of shorter sequences (648 base pairs) enabling inclusion of 11 museum skin samples gave similar Φ_{ST} values, as did analyses excluding samples from non-breeding birds (Appendix B).

For microsatellites, 0.62% of the variation was explained by the three redpoll species ($p = 0.0039$). Pairwise differences between all pairs of species were non-significant after Bonferroni correction (all p values >0.021, adjusted $\alpha = 0.017$, Table 5). In a locus-by-locus AMOVA, overall F_{ST} values ranged from 0 (CtA105) to 0.011 (CtD108). The two *C. flammea* subspecies (*rostrata* and *flammea*) did not differ significantly ($F_{ST} = 0.019$, $p = 0.073$). Exclusion of loci with more than 30 alleles, and exclusion of samples of non-breeding individuals, gave similar values (Appendix B). Similar values were also found when excluding five microsatellite loci, enabling the inclusion of all museum skin samples (Appendix B).

To conclude on the analyses of genetic structure, the redpoll taxa were found to differ slightly in frequency of mtDNA haplotypes and microsatellite alleles, but no structure was found in analyses without predefined groups.

Table 5
Pairwise Φ_{ST} values for mtDNA (below diagonal; excluding museum skin samples [957 bp]) and F_{ST} values for microsatellites (above diagonal; all 10 loci)

	C. cabaret	*C. flammea*	*C. hornemanni*
C. cabaret		0.0046	0.0085
C. flammea	**0.0590**		0.0086
C. hornemanni	0.0843	−0.0140	

Bold type indicates significance after sequential Bonferroni correction.

A mismatch distribution analysis on the redpoll samples (all taxa pooled) revealed a signature that differed from the expected distribution for a population with a constant population size ($\chi^2_6 = 35.4$, $p < 0.001$), and did not differ from the expected distribution for an exponentially increasing population size ($\chi^2_6 = 1.1$, $p = 0.99$, Fig. 5).

4. Discussion

Our results show basically no genetic divergence within the redpoll complex, either with the relatively rapidly evolving control region of mtDNA or even faster evolving microsatellites. The taxa are not separated in individual-based analyses, and AMOVAs reveal very small differences. The lack of structure is particularly striking when compared to the outgroup (the twite), which was not only clearly separated from the redpolls in both mtDNA and microsatellites, but also divided into two reciprocally monophyletic groups in mtDNA, corresponding to the two recognized subspecies. Our results are in concordance with previous genetic studies on the redpoll complex. Seutin et al. (1995) found no differentiation between most recognized taxa in an RFLP study, Ottvall et al. (2002) found no differences between *C. flammea* and *C. cabaret* in mtDNA control region sequences, and Kerr et al. (2007) and JT Lifjeld, A Johnsen, E Rindal (unpublished data) found no differentiation between *C. flammea*, *C. cabaret* and *C. hornemanni* in COI sequences.

Three hypotheses can explain the low level of genetic structure among redpoll taxa: (1) There is a single gene pool, (2)

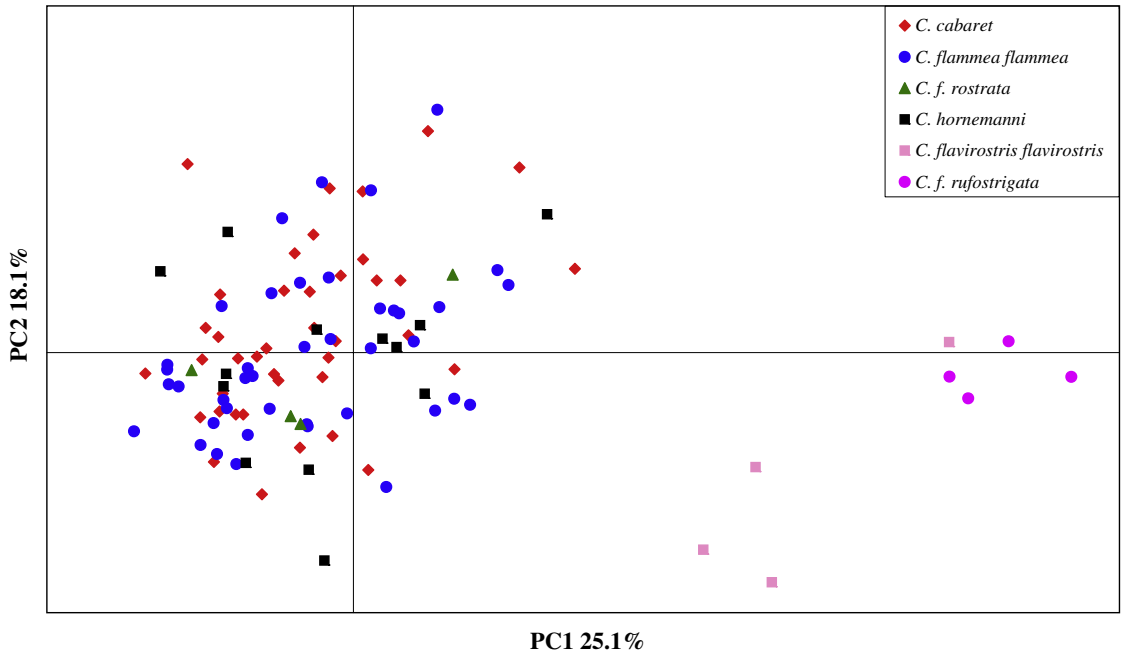

Fig. 4. Principal coordinate analysis plot for individuals from four redpoll taxa and two twite taxa analysed with 10 microsatellite loci.

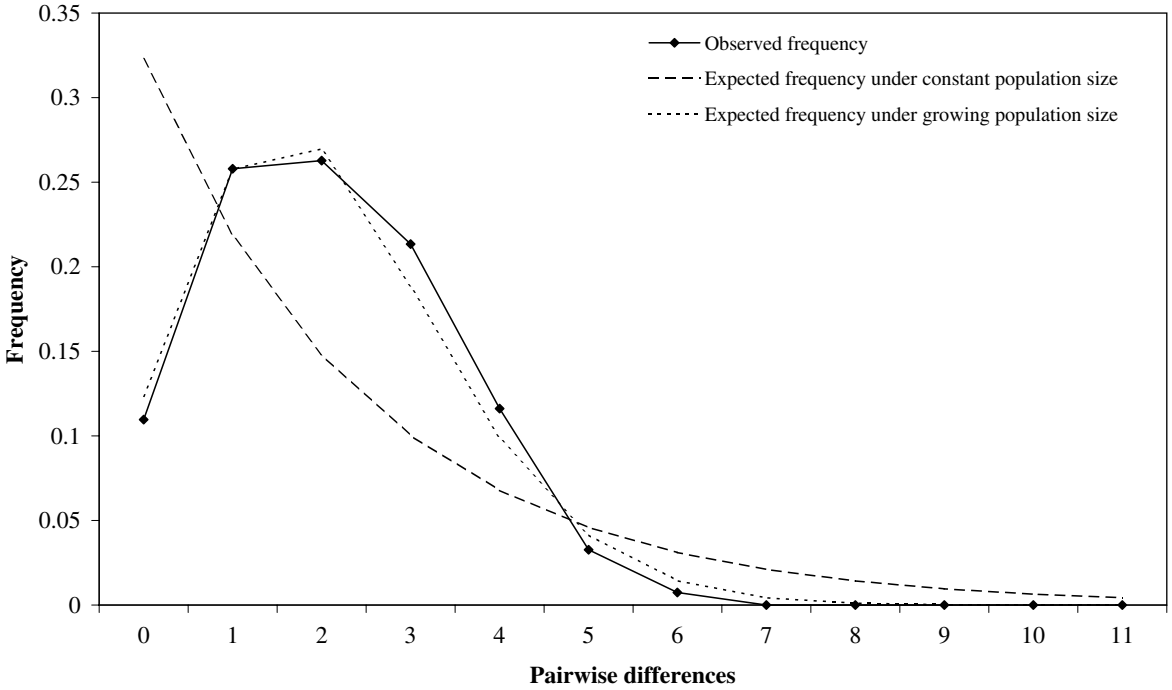

Fig. 5. Observed and expected distributions of pairwise differences between mtDNA control region sequences in redpolls (*Carduelis flammea*, *C. hornemanni*, *C. cabaret*) for models of constant and growing population sizes.

there are two or more gene pools, with incomplete lineage sorting, or (3) there are two or more gene pools, with genetic exchange between them (Ball and Avise, 1992; Ottvall et al., 2002). The first hypothesis depicting a single gene pool can be argued to contradict the phenotypic clusters claimed to exist within the redpoll complex (Molau, 1985; Knox, 1988; Herremans, 1990). However, phenotypic clusters may exist within species. First, morphological polytypism within single species can arise from phenotypic plasticity (Price, 2008). Habitat or diet, for example, may affect phenotype to a large extent (Slagsvold and Lifjeld, 1985; Leafloor et al., 1998). Second, morphological polytypism within species could be caused by one major locus with pleiotropic effects (one gene affects several phenotypic characters) or epistatic effects (two or more gene loci interact and affect the phenotype in a stronger way than two non-interacting loci would have) (Seutin et al., 1995). In, for example, bananaquit (*Coereba flaveola*), snow goose (*Anser c. caerulescens*) and Arctic skua (*Stercorarius parasiticus*), melanistic polymorphisms have been shown to be controlled by one single locus (Mundy, 2005). Pleiotropic or epistatic effects causing morphological differences are possible in redpolls, but it seems unlikely that all the reported correspondingly varying characters (see Section 1) are affected by such mechanisms. More likely, the differences reported between phenotypic groups in redpolls represent selectively active traits. If there is just one gene pool, ecological selection gradients, i.e. strong selection pressures differing between e.g. habitats, latitudes or altitudes, could maintain the differences in phenotypic characters (Harris et al., 1965). Strong selection on differing traits counteracting the effect of gene flow has, for example, been shown in little greenbulls (*Andropadus virens*) breeding in different ecotone habitats in the rainforest (Smith et al., 1997). Hendry et al. (2001) have shown that even weak selection pressures may oppose the effects of gene flow and create phenotypic differentiation between populations.

Bergmann's rule states that populations in cooler climates tend to be larger than populations of the same species living in warmer climates, apparently in order to decrease the body surface relative to body size (Mayr, 1970). In accordance with this, the smallest redpoll species, lesser redpoll, has the most southern distribution of the redpolls. Gloger's rule says that populations in cool and dry habitats are less heavily pigmented than populations in warm and humid areas (Mayr, 1970). This has been suggested to be linked to dark feathers' higher resistance to bacterial degradation compared to light feathers, and that there is higher occurrence of bacteria in humid environments (Burtt and Ichida, 2004). Arctic redpoll is palest among the redpolls and lives in the coldest and northernmost habitats. The observed polytypic variation in redpolls is thus largely consistent with both these rules of clinal phenotypic variation.

The second hypothesis explaining the lack of neutral genetic differences between redpoll taxa describes a situation with two or more gene pools, where genetic drift has not yet created genetic differences between them. This could be due to, for instance, recent divergence of the lineages, combined with differences in evolutionary rates in phenotypic traits and neutral genetic loci (Orr and Smith, 1998; Price, 2008). Strong or rapid natural or sexual selection on phenotypic traits may increase the evolutionary rate of phenotypic characters compared to neutral genetic loci. This has been demonstrated for genetically similar subspecies in, for example, yellow wagtail (*Motacilla flava*, Ödeen and Björklund, 2003), bluethroat (*Luscinia svecica*, Questiau et al., 1998; Zink et al., 2003; Johnsen et al., 2006), common grackle (*Quiscalus quiscula*, Zink et al., 1991), song sparrow (*Melospiza melodia*, Zink and Dittmann, 1993) and swamp sparrow (*Melospiza georgiana*, Greenberg et al., 1998). Demography may also influence relative differences in evolutionary rate between phenotypic characters and neutral genetic loci (Avise, 2000). When, for example, populations expand in size in a short time period, genetic drift may not be strong enough to eliminate neutral alleles (Otto and Whitlock, 1997; Avise, 2000). Also, if the population expands into new habitats (Schluter and Nagel, 1995), or gene flow is not homogeneous (Garant and Kruuk, 2005), adaptive traits may evolve

rapidly, creating an even larger discrepancy between phenotypic adaptive traits and neutral genetic markers. Redpolls did apparently experience a population expansion at some point in time, as seen in this study (Fig. 5), and also shown in Ottvall et al. (2002). Life history traits also influence the rate and strength of genetic drift. The highly nomadic behaviour of redpolls (Cramp and Perrins, 1994) potentially creates a large effective population size. Fixation of alleles then takes much longer time (Avise, 2000).

The small, yet significant, differences found between the redpoll taxa in the ANOVAs support this hypothesis of two or more redpoll gene pools. A plausible mechanism for creating such separate redpoll gene pools is ecological speciation. Ecological speciation is divergent selection on traits between populations in different environments, leading to reproductive isolation (Schluter, 2001). The clearest example of ecological differentiation or speciation in birds is divergent selection on bill sizes and shapes. In Darwin's finches (*Geospiza* spp.), selection for bill size (together with introgressive hybridization) has probably facilitated convergent evolution (Grant et al., 2004). Two island dwelling species of *Nesospiza* buntings with different bill depths evolved most likely through ecological speciation (Ryan et al., 2007). A third example is the crossbill species complex (*Loxia* spp.), in which described taxa differ predominantly in size and bill morphology (Knox, 1976; Cramp and Perrins, 1994). These differences have probably been created through local adaptations to fluctuating resources (Benkman, 2003). Differences in vocalization characters have been found, also below the species level (e.g. Groth, 1988; Edelaar et al., 2008), and these may have helped in assortative mating between bill morphs (Snowberg and Benkman, 2007; Edelaar, 2008). Ecological speciation is thus a plausible mechanism creating separate gene pools in the crossbills.

The crossbill species complex bears several resemblances to the redpoll complex. Crossbills feed on conifer seeds, which fluctuate in northern areas, and birds living in this region are therefore, like redpolls, nomadic (Cramp and Perrins, 1994; Questiau et al., 1999). Also a low level of genetic differentiation is apparent in the crossbill system. Although North American and European crossbills were demonstrated to be reciprocally monophyletic in a study on mtDNA control region sequences (Questiau et al., 1999), no phylogeographic structure or genetic groups corresponding to species or subspecies taxonomy within North American or European crossbills was detected. A second study found no differentiation between the three species breeding within the UK (*L. curvirostra*, *L. pytopsittacus, L. scotica*) in mtDNA control region sequences or in microsatellites (Piertney et al., 2001). However, strong genetic support was found for three North American species in an AFLP study, and eight call types (vocally differentiated forms) of red crossbills (*L. curvirostra* complex) were subtly, but significantly, differentiated in analyses of genetic differentiation based on inferred allele frequency variation (Parchman et al., 2006).

Redpolls may have experienced a similar situation as the crossbills. The selection pressures were probably not the same for the two complexes, since the most conspicuous differences between redpoll species are plumage characters and size (Knox, 1988). Selection pressures for the redpolls may have varied over habitats and environmental gradients; cf. Gloger's and Bergmann's rules. As for crossbills, redpoll taxa have been reported to differ in non-breeding communication calls (Molau, 1985; Herremans, 1989), and this may have facilitated assortative mating in sympatry (Lifjeld and Bjerke, 1996). Thus, ecological speciation seems plausible also in redpolls.

The third hypothesis explaining a lack of genetic differentiation between redpoll taxa suggests present gene flow between two or more gene pools. However, the occurrence of two or more genetic groups was not detected in any analysis in either investigated marker, and the branches in the mitochondrial DNA tree are not particularly long (Fig. 3). This would have been expected from a scenario of previous isolation and divergence with subsequent gene flow. The third hypothesis can thus be dismissed. However, discerning between hypothesis one and two is difficult. One way to test this is to study reproductive barriers. If there is one redpoll gene pool, we expect no barriers, but if there are several gene pools, we expect some form of reproductive isolation. Depending on how far the speciation process has progressed, premating, prezygotic and/or postzygotic barriers can be expected.

In conclusion, our results are in line with previous molecular studies of the species complex, and thus provide no genetic support for the current species or subspecies taxonomy. However, molecular data alone should not be used for designating species status (Knox et al., 2001; Helbig et al., 2002; Sangster et al., 2002), and we acknowledge that there might have been missed the relevant genetic markers to distinguish gene pools. We also note that there is a lack of empirical support for the existence of reproductive isolating mechanisms between the phenotypic morphs in areas of sympatric breeding. We would therefore encourage more studies of morphology and breeding behaviour and their underlying genetics in hybrid zones or areas of sympatric breeding.

Acknowledgments

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Appendix A. Distribution of mtDNA control region haplotypes in populations of redpolls. Haplotypes are given in Table 3

Haplotype	*C. f. flammea*		*C. f. rostrata*	*C. cabaret*			*C. hornemanni*						Total
	Norway		Greenland	Norway			Norway		Sweden			Russia	
	Heimdalen	Finnmark		Kvinesdal	Utsira	Jomfruland	Finnmark	S Norway	S Sweden	N Sweden	Falsterbo	Kanin Peninsula	
#1	1			1	2	1							5
#2					1								1
#3	1												1
#4	1		1	1			1	1	1		1		7
#5					1								1
#7	1							1					2
#8				1									1
#14	2										1		3
#15	4		2		2	1	1					1	11
#18				1									1
#22	2			3	2								7
#23	1			2	2	2							7
#24	1		1		1					1			3
#27					1								1
#28											1		1
#29					1								1
#30					1								1
#31			1										1
#32						1							1
#33								1					1
#34	1			1									2
#35	1												1
#36	1												1
#37	1												1
#38	1												1
#39	1												1
#40				1									1
#41	1												1
#42											1		1
#43	1												1
#44										1			1
#45		1											1
#46		1											1
#47		1											1
Total	22	3	5	11	14	5	2	3	1	2	4	1	73

Museum skin samples with short sequences (due to degraded DNA) are not included.

Appendix B. Overall Φ_{ST} values for mtDNA control region sequences (a) and F_{ST} values for microsatellites (b) for various datasets of redpolls

a)

Sequence lengths	*n* individuals	Φ_{ST}	*p*
957 bp	30 *C. flammea* (5 of these ssp. *rostrata*), 30 *C. cabaret*,13 *C. hornemanni*	0.051	0.0078
648 bp including museum skins	34 *C. flammea* (9 of these ssp. *rostrata*), 30 *C. cabaret*,18 *C. hornemanni*	0.073	0.00098
957 bp breeding individuals	29 *C. f. flammea*, 30 *C. cabaret*, 7 *C. hornemanni*	0.055	0.012

b)

Number of loci	*n* individuals	F_{ST}	*p*
10 loci	51 *C. flammea* (5 of these ssp. *rostrata*), 45 *C. cabaret*,16 *C. hornemanni*	0.0062	0.0039
6 loci <30 alleles	50 *C. flammea* (5 of these ssp. *rostrata*), 44 *C. cabaret*,16 *C. hornemanni*	0.0064	0.040
5 loci including museum skins	55 *C. flammea* (10 of these ssp. *rostrata*), 42 *C. cabaret*,24 *C. hornemanni*	0.0062	0.011

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