No evidence of genetic differentiation between lesser redpolls *Carduelis flammea cabaret* and common redpolls *Carduelis f. flammea*

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The remarkable variation in plumage and morphological characters in the redpoll complex *Carduelis flammea-hornemanni* has puzzled taxonomists for several decades. In contrast, molecular studies have not revealed any genetic differentiation among the phenotypic redpoll forms. This could either be a result of high present-day gene flow or morphological differentiation following a rapid and recent population expansion. We sequenced a major portion (960 bp) of the mitochondrial control region in individuals of the two taxa *Carduelis flammea flammea* and *C. f. cabaret*. Birds were sampled on autumn migration in southern Sweden (n = 30) and on breeding areas in southern Norway (n = 11). We found 22 variable sites defining 26 different haplotypes, of which most (22/26) were singletons. The level of haplotype and nucleotide diversity was low in the two taxa and we found no evidence of genetic differentiation. A mismatch distribution was very similar to that expected from a sudden population expansion model. Our estimates suggest that the redpoll population expanded during the last glaciation episode from a small population to a long-term effective population size of 230 000 females. The findings in our study suggest that the morphological differentiation between the two taxa occurred rather recently but after the population expansion.
Key words: mtDNA, control region, population expansion, effective population size, phylogeography.

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The redpoll finch complex *Carduelis flammea-hornemanni* has received much attention from taxonomists due to its remarkable variation in plumage and morphology. Some authors have suggested one highly variable species, *flammea*, while others have suggested seven species (see Knox 1988 for review). The most widely held view recognises two species, the common redpoll (including *flammea*, *islandica*, *rostrata* and *cabaret*) and the arctic redpoll (including *hornemanni* and *exilipes*). The distribution range of the common and the arctic redpolls includes the higher latitudes of the Holarctic, where the two forms breed sympatrically over large areas (Knox 1988). Observations of intermediate birds have been put forward as evidence of hybridisation, but due to improved knowledge of plumage variation, most 'intermediate' redpolls can now be unambiguously assigned to a species identity (Molau 1985, Knox 1988). The most distinctive redpoll form, the lesser redpoll *C. flammea cabaret* was until recently restricted to Britain, Ireland and the Alps. This form has recently expanded its breeding range towards Scandinavia and is now breeding sympatrically with *flammea* in southern Norway in years when *flammea* is breeding in the lowlands (Lifjeld & Bjerke 1996). Assortative mating has been found in one area in Norway and the latest recommendation was therefore to split *cabaret* as a distinct species from *flammea* (British Ornithologists' Union 2001, Knox et al. 2001). While dif-

ferences in plumage and morphology are quite distinctive (Lindström et al. 1984, Knox 1988, Herremans 1990, Knox et al. 2001), we know little about the genetic relationship between *cabaret* and *flammea*.

Seutin et al. (1995) examined mtDNA variation in the redpoll complex by using restriction enzymes of mtDNA extracts from specimens of five taxa from both North America and Europe. They found no evidence of genetic structure related to either geographic origin or taxonomic identity. This contrasts with the clear morphological differences between the taxa, suggesting that the species complex had diverged relatively recently and that large and relatively stable population sizes had prevented mtDNA differentiation (Seutin et al. 1995). However, the number of individuals examined in that study was small and the European sample consisted of only two *flammea* and three *cabaret*. We therefore decided to investigate a larger sample of *flammea* and *cabaret* for sequence variation in the mitochondrial control region, the most variable part of the mitochondrial genome and thus the most appropriate choice for intraspecific studies (Edwards 1993, Wenink et al. 1993, Bensch & Hasselquist 1999). We make two predictions based on the result of Seutin et al. (1995). First, we expect *flammea* and *cabaret* to be undifferentiated in their mtDNA control region sequences. Second, if the populations have been stable as earlier suggested (Seutin et al. 1995), the distribution of pairwise difference between haplotypes should follow expectation at mutation-drift balance (Rogers & Harpending 1992).

Methods

Data collection

Blood samples of redpolls were collected on autumn migration in 1997 and 1999 at Falsterbo Bird Observatory in southern Sweden (55º 23' N, 12º 50' E; 17 *cabaret* and 13 *flammea*) and on breeding grounds in southern Norway at Eidsberg, Østfold county (59º 31' N, 11º 14' E; 5 *cabaret* and 4 *flammea*) and Øvre Heimdalen, Øystre Slidre county (61° 25' N, 8° 52' E; 2 *flammea*) in 1993–1994 (Fig. 1). Individuals were identified to subspecies from plumage and morphological characters (Lindström et al. 1984, Svensson 1992) and for this study we only considered taxon-diagnostic specimens.

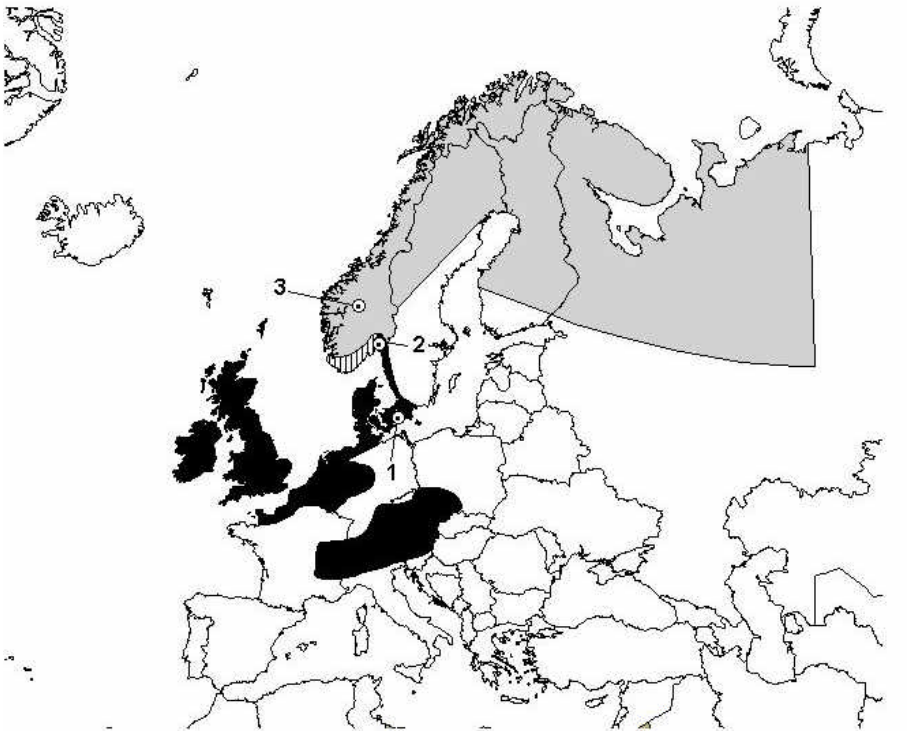

Figure 1. Map showing the European breeding range of the redpoll subspecies *Carduelis f. flammea* (grey) and *C. f. cabaret* (black) and the three sites for sample collection: 1) Falsterbo, 2) Eidsberg and 3) Øvre Heimdalen. In 'irruption' years, the southern limit of the distribution of *flammea* in Norway reaches the distribution of *cabaret* (stippled).

Molecular analyses

Genomic DNA was extracted from blood samples using a standard proteinase k phenol/chloroform procedure. We used the primers L16743 and H1248 (Tarr 1995) to amplify the control region. Polymerase chain reactions (PCR) were performed in volumes of 25 µl and included 25 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM $MgCl_2$, 1 X PCR buffer (Perkin Elmer), 0.6 µM of each primer and 0.5 units of Taq DNA polymerase. The PCRs were run using the following conditions: 30 s at 94 °C, 30 s at 53 °C, 45 s at 72 °C (35 cycles). Before the cyclic reactions the samples were incubated at 94 °C for 3 min, and after completion at 72 °C for 10 min. Fragments were sequenced directly from both ends and with an internal primer L437 (Tarr 1995), using dye terminator cyclic sequencing (big dye) and loaded on an ABI PRISM™ 310 (Perkin Elmer). A total length of 960 bp covering domain II and III was sequenced in each of 41 individuals (Haplotype #1 in GenBank AF416737).

Descriptive statistics and data analyses

We tested for structuring among the four redpoll samples (*flammea* and *cabaret* from Sweden and Norway, respectively) by calculating ϕ_{ST} statistics using the program Arlequin 2.0 (Excoffier et al. 1992). Significance of variance components between populations was tested with a randomisation procedure provided in the program. We used MEGA (Kumar et al. 1993) to calculate the relationship between haplotypes and visualised these in an un-rooted neighbour-joining tree using Jukes and Cantor's distance.

Nucleotide diversity (π), D statistics (Tajima 1989) and the parameters used to estimate changes in effective population size (θ and τ) were calculated using the program DnaSP 2.52 (Rozas & Rozas 1997). The rate of molecular evolution (s) for the control region is not known, but for various species of birds has been suggested to be between 2 % and 20 % per million years (Baker & Marshall 1997). Because its rate relative to the cytochrome *b* gene is variable and taxon specific (Ruokonen & Kvist 2002) and perhaps much higher than inferred from indirect phylogenetic evidence (Lambert et al. 2002), we report the result calculated for low (2 %), intermediate (10 %) and high (20 %) rates. As in Seutin et al. (1995) we assumed the generation time (g) for redpolls to be 2 years.

At equilibrium, θ equals $2uN$ where θ is the mean number of pairwise differences, u the mutation rate of the sequence (total number of mutations per sequence per generation) and N is the female effective population size. According to Watterson (1975), a population of constant size is expected to have a mismatch distribution following

$$Fi = \theta^i / [(\theta + 1)^{i+1}]$$

where Fi is the proportion of haplotypes differing by i substitutions and θ is estimated from the data as the observed mean of pairwise differences (Rogers & Harpending 1992).

The pairwise distribution of differences between haplotypes may reveal historical changes in the effective population size (Rogers 1995). The simple situation for which Rogers & Harpending (1992) developed their calculations assumes that an initial population of size N_0 is at equilibrium and suddenly changes to a new population size of N_1, and that this is observed from sequence data t generations later. The time since population size change can be derived assuming that $\tau = 2ut$ and $u = \mu L$ (μ is the mutation rate per nucleotide and generation, L is the length of the studied DNA sequence).

From the estimates of θ and τ we used DnaSP 2.52 (Rozas & Rozas 1997) to calculate the expected distribution of pairwise differences under the hypothesis of a sudden population expansion that happened t generations ago. We estimated the population size before expansion assuming that $\theta_0 = 2uN_0$ and that $\theta_0 = (v - m)^{½}$, where v is the variance and m is the mean of the observed pairwise number of differences (Rogers & Harpending 1992). An estimate of the present population size can be obtained using the fact that the vertical intercept (F_0) of the observed distribution of pairwise differences roughly equals $1 / (1 + \theta_1)$ (Rogers & Harpending 1992).

Results

A total of 26 different haplotypes were detected in the 41 sequenced individuals (Table 1). Most haplotypes (22/26) were detected in single individuals only. There were 22 variable sites of which only one involved a

Table 1. Variable sites in the control region among redpolls from Sweden and Norway. The sites are numbered relative to positions in haplotype #1 (GenBank AF416737).

Haplotype	Position			*cabaret*		*flammea*		Total
	1222223333 7902679037 7487729039	3344555668 8837189369 3540407284	99 44 89	Fbo	Norway	Fbo	Norway	
#1	GACCAACCCA	ACTATCATTA	TT	4	0	1	1	6
#2	. . . . .	. . . . CT	. .	0	1	0	0	1
#3	. . . . .	. . . . C . . C . .	. .	0	0	0	1	1
#4	. . . . .	. . . . . C . .	. .	1	0	0	0	1
#5	. . . . . T .	. . . . . C . .	. .	1	0	0	0	1
#6	. . . T	. . . . . C . .	. .	0	0	1	0	1
#7	. . T	. . . . . C . .	. .	0	0	1	0	1
#8	. . T	. . . . C . . C . G	. .	0	1	0	0	1
#9	. . T . . G . . .	. . . . C . . C . G	. .	0	0	1	0	1
#10	. . . . .	. . . . . CC .	. .	0	1	0	0	1
#11	. . . . .	. . . . . CCG	. .	0	0	1	0	1
#12	A	. . . . . C . G	. .	1	0	0	0	1
#13	A . . . . . -	. . . . . C . .	. .	0	0	1	0	1
#14	. . . . .	. . . . . G	. .	0	0	1	0	1
#15	. . . . .	. . . . . C . G	. .	3	1	1	1	6
#16	. . . . . T . . .	. . . . . C . G	. .	1	0	0	0	1
#17	. . . . . T . .	. . . G . . . C . G	. .	0	0	1	0	1
#18	. . . . G	. . . . . C . G	. .	0	0	1	0	1
#19	. . . . .	. T . . . . . C . G	. .	0	0	1	0	1
#20	. G	. . . . . TGC . .	. .	0	0	1	0	1
#21	AG	. . . . . T . C . G	CC	1	0	0	0	1
#22	. G	. . . . . T . C . G	. .	2	1	1	0	4
#23	. G	. . C . . T . C . G	. .	3	0	0	0	3
#24	. . T	. . . . . C . G	. .	0	0	0	1	1
#25	. . . . .	G . . . . . C . G	. .	0	0	0	1	1
#26	. . . . .	. . . . . A . C . G	. .	0	0	0	1	1
Total				17	5	13	6	41

transversion, the remaining being transitions. The number of haplotypes was similar in the two subspecies (17 in *flammea* and 12 in *cabaret*) as was the level of nucleotide diversity (*flammea* π = 0.00303; *cabaret* π = 0.00311). The overall nucleotide diversity was 0.00317 (s.d. = 0.00023). The estimates of Tajimas's (1989) D statistic were negative for both *flammea* (–1.61) and *cabaret* (–0.31) but not significantly different from the neutral mutation hypothesis (P > 0.05).

We found no evidence of genetic structuring when comparing the samples from southern Sweden and Norway (AMOVA; *flammea* ϕ_{ST} = –0.08, n.s.; *cabaret* ϕ_{ST} = –0.01, n.s.). Moreover, there was no sign of differentiation between the two subspecies (ϕ_{ST} = 0.01, n.s.). The absence of genetic structure between either sampling regions or subspecies is obvious from the neighbour joining tree (Fig. 2) in which individuals from the two subspecies appear equally scattered. Because we did not find any evidence of mtDNA differentiation between *flammea* and *cabaret*, we pooled the data for the following analyses.

The mismatch distribution (Fig. 3) was very similar to expectation from the sudden population expansion model (χ^2_8 = 3.34, n.s.) and significantly different from a constant population size model (χ^2_8 = 32.3, P < 0.001). Using a divergence rate of 10 % per million years for the control region, our estimates of τ (2.78), θ_0 (0) and θ_1 (20.0) suggested that the population expanded 63 000 years ago from a very small population to a long-term effective population size of 230 000 females. A

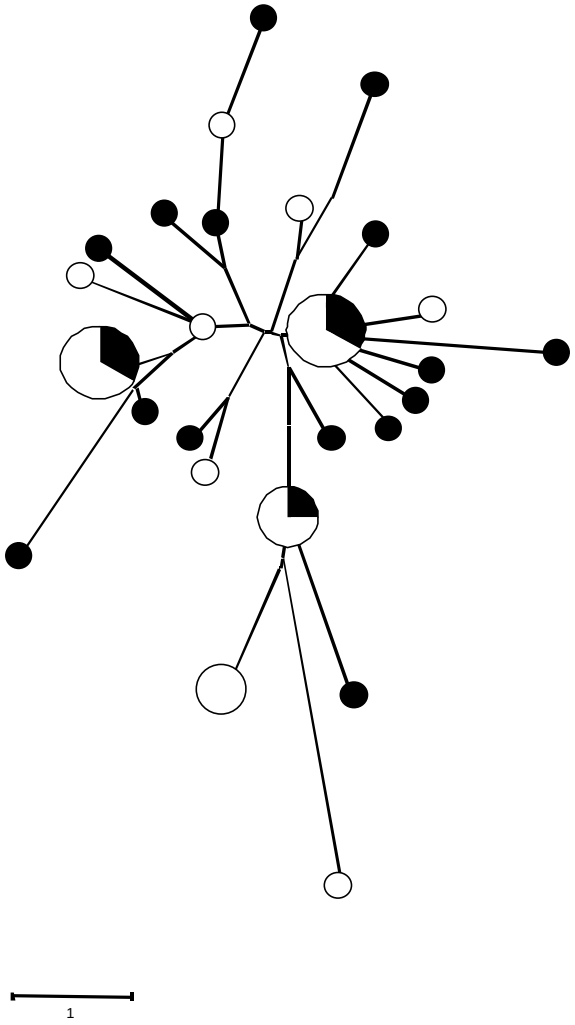

Figure 2. Unrooted neighbour-joining phylogram of redpoll mtDNA haplotypes (n = 26) based on control region sequences (960 bp). Sizes of circles correspond to number of observed individuals (1–6) per haplotype. Filled = *flammea*, open = *cabaret*.

lower divergence rate (2 %) would put the expansion time back to 310 000 years BP and a higher divergence rate (20 %) as recently as 32 000 years BP. Hence, the population expansion appears to have occurred within the period of the last glaciation cycle, although there is considerable uncertainty in the estimate because of our poor knowledge of molecular evolution in control region sequences.

Discussion

Variation in mitochondrial control region sequences suggests that the two redpoll taxa *flammea* and *cabaret* are panmictic. A potential limitation of our study is that samples were collected at only three localities, all situated within a limited part of the *flammea*/*cabaret* breeding range. Our results agree, however, with the finding of Seutin et al. (1995), where redpoll individuals from almost the whole breeding range in the Holarctic were included. Seutin et al. (1995) estimated the mean divergence (π) between lineages to be 0.295 %, very similar to our estimate of 0.317 %. Moreover, we could not find any genetic structuring between the samples from the breeding area in Norway and from the migration locality at Falsterbo. It is therefore likely that our results would hold, had we used samples collected from a larger area of the species' breeding range.

Strong differentiation in morphological traits combined with the lack of genetic differentiation, parallels many other studies of mtDNA variation among bird populations (Zink & Dittmann 1993, Greenberg et al. 1998). Such a pattern could be a result either of high levels of current gene flow and strong selection on adaptive traits to maintain differences, or alternatively, adaptive evolutionary change in morphology occurring too recently for neutral mtDNA to diverge. These two mechanisms may also act in concert to an uncertain degree.

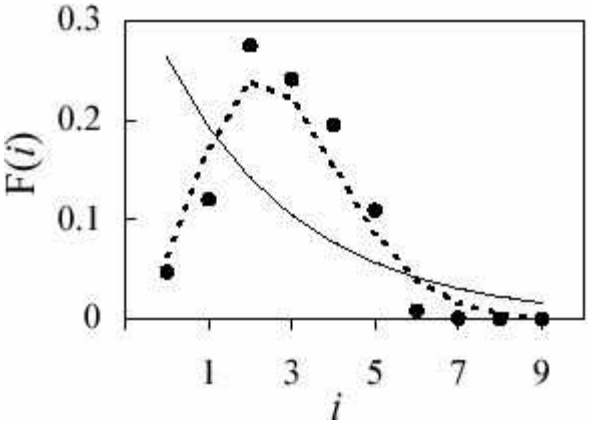

Figure 3. Frequency distribution of pairwise nucleotide differences between mtDNA haplotypes (mismatch distribution in redpolls). The solid line indicates the expected distribution under a constant population size model, and the stippled line the expected distribution under a population increase model.

Present-day gene flow

The traditional view is that subspecies and recently differentiated sister species of birds have evolved in allopatric habitat refugia during Pleistocene glaciation cycles (e.g. Avise & Walker 1998, Klicka & Zink 1999). For example, *cabaret* might have been confined to the Alps of Central Europe and *flammea* in a different habitat pocket somewhere else in Eurasia or in North America. If this was the case, however, the subspecies would have accumulated at least some differences in mtDNA haplotype frequencies. The question is therefore; could post-glacial gene flow have eliminated differences in haplotype frequencies to the level of apparent panmixis presently observed between *cabaret* and *flammea*?

There is no evidence of present gene flow between *flammea* and *cabaret* but, before the northward expansion of *cabaret* into Scandinavia, gene flow was probably very restricted. This is because the breeding ranges of *flammea* and *cabaret* were separated by >300 km (Hagemeijer & Blair 1997). After years of irruptive migration, *flammea* has occasionally been found to breed south of its normal breeding range (Cramp & Perrins 1994) when gene flow might have occurred. Within *flammea* Seutin et al. (1995) found no geographic structure between Europe and North America, suggesting a large panmictic population including both continents. Although redpolls are agile birds with a nomadic dispersal pattern, a substantial present-day gene flow between North America and Eurasia seems rather unlikely. Hence, the similarity between *cabaret* and *flammea* in Europe, and between *flammea* in Europe and North America, calls for a common explanation that does not depend on high levels of present-day gene flow.

Population size change

An alternative explanation to the incongruent pattern of molecular and morphological variation in *flammea* and *cabaret* is that adaptive evolutionary change in morphology occurred too recently for neutral mtDNA to diverge. Such a pattern is particularly likely to arise following a rapid population increase and range expansion from a bottlenecked population, a scenario that redpolls and many other species of higher latitudes have experienced after the retreat of the last ice age (Rogers 1995, Hewitt 1996, Merilä et al. 1997, Zink 1997). This is because neutral alleles are less likely to get lost due to genetic drift in growing populations (Otto & Whitlock 1997) whereas adaptive traits rapidly evolve in the newly colonised habitats (Schluter & Nagel 1995). In support of this, we rejected the population 'equilibrium' model as pairwise sequence differences gave a good fit to expectations from the 'sudden expansion' model (Rogers & Harpending 1992). The marked peak in the distribution of pairwise differences at a distance of two mutations is consistent with a model of a small population ($\theta_0 = 0$) that has experienced a large population expansion (Marjoram & Donnelly 1994). According to Harpending (1994), a large and stationary population should generate a ragged distribution of pairwise sequence differences. The smooth curve observed in our data set from redpolls suggests that the population has been expanding since the bottleneck.

The population bottleneck, followed by the population increase, implies that the redpoll habitats were greatly affected at some point during the Pleistocene. The common redpoll prefers subalpine birch forests and bushy tundra (Cramp & Perrins 1994), habitats that may have increased in range during glaciated periods, and one can speculate whether this habitat increase promoted the population expansion. Seutin et al. (1995) concluded that the different redpoll taxa radiated in large and stable populations. In contrast, our data on haplotype and nucleotide diversity in redpolls are not consistent with a population showing long-term demographic stability. However, the present analyses cannot establish whether the population expansion preceded the differentiation of the two taxa. The similar haplotype frequencies strongly suggests, however, that they differentiated after the population expansion, and hence in relatively large populations towards the second half of the last glaciation.

Species status

Based on the variation in mtDNA there is no reason to rank *flammae* and *cabaret* as distinct species. The lack of genetic differentiation, however, does not exclude the presence of reproductive barriers between them. Hybridisation has not yet been conclusively established but such observations are difficult in secretive birds such as redpolls. Assortative mating between *flammea* and *cabaret* found by Lifjeld & Bjerke (1996) supports

their species status. On the other hand, more birds with intermediate plumage and morphology have been captured at Falsterbo Bird Observatory in recent years (observations by GW). Of the redpolls caught in autumn 1999, about 10 % of individuals could not be identified to taxon. These unidentified individuals showed a *cabaret*-like plumage but a morphology similar to *flammea* or vice versa. We believe that these intermediate birds are likely to have been of hybrid origin because *cabaret* is the most distinctive form of the redpolls and should not cause any identification problems (Herremans 1990, Knox et al. 2001). Alternatively, the variation in plumage and morphology within *flammea* is more extensive than presently appreciated (Svensson 1992, Knox et al. 2001) and includes both small individuals and phenotypes with plumage colouration resembling *cabaret*. To our knowledge, this has never been suggested previously and we find it very unlikely as an explanation for the intermediate phenotypes captured at Falsterbo. While we are waiting for more detailed studies on sympatrically breeding *flammea* and *cabaret*, we recommend that the two taxa should be treated as subspecies.

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