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# THE UNUSUAL SPERM MORPHOLOGY OF THE EURASIAN BULLFINCH (*PYRRHULA PYRRHULA*) IS NOT DUE TO THE PHENOTYPIC RESULT OF GENETIC REDUCTION

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ABSTRACT.—The Eurasian Bullfinch (*Pyrrhula pyrrhula*) is unusual among passerines in that it has both an extremely unusual sperm morphology and unusually high levels of inter- and intra-male variation in sperm morphology. One possible cause of this high level of variation in sperm design is the phenotypic results of inbreeding following a bottleneck event. There are two points in time when the Eurasian Bullfinch may have been subject to a bottleneck. Many Eurasian passerines underwent a series of post-Pleistocene bottlenecks following the retreat of the glaciers. More recently, in the United Kingdom the Eurasian Bullfinch has declined severely in numbers in the past 40 years. We used universal bird microsatellite primers to directly compare the genetic diversity of the Eurasian Bullfinch with that of three fringillid finch species that display "typical" passerine sperm morphology: the European Greenfinch (*Carduelis chloris*), Common Chaffinch (*Fringilla coelebs*), and Red Crossbill (*Loxia curvirostra*). We found no evidence that the Eurasian Bullfinch has undergone a reduction in genetic variation that could account for its unusual sperm morphology. Alternative hypotheses, such as a relaxation in sperm competition, now warrant further investigation. *Received 4 December 2009, accepted 2 May 2010.*

Key words: bottleneck, Eurasian Bullfinch, genetic reduction, inbreeding, pleiomorphy, *Pyrrhula pyrrhula,* sperm abnormalities, sperm competition, sperm morphological variation, universal primers.

## La morphologie inhabituelle des spermatozoïdes de *Pyrrhula Pyrrhula* n'est pas le résultat phénotypique de la réduction génétique

RÉSUMÉ.—*Pyrrhula pyrrhula* diffère des autres passereaux en raison de la morphologie fort inhabituelle de ses spermatozoïdes et du niveau exceptionnellement élevé de variation de la morphologie des spermatozoïdes pour un même mâle et entre mâles. L'une des causes possibles de ce niveau élevé de variation dans la conception des spermatozoïdes sont les résultats phénotypiques de la consanguinité à la suite d'un événement d'étranglement. Il y a deux moments où *P. Pyrrhula* a pu être sujet à un étranglement. Plusieurs passereaux eurasiens ont été soumis à une série d'événements d'étranglement après le Pléistocène, suivant le retrait des glaciers. Plus récemment, au Royaume-Uni, les effectifs de *P. Pyrrhula* ont subit un déclin sévère au cours des 40 dernières années. Nous avons utilisé des amorces universelles de microsatellites des oiseaux afin de comparer directement la diversité génétique de *P. Pyrrhula* avec celle de trois espèces de fringillidés qui présentent une morphologie des spermatozoïdes «typique» des passereaux: *Carduelis chloris*, *Fringilla coelebs* et *Loxia curvirostra*. Nous n'avons trouvé aucune preuve que *P. Pyrrhula* ait subit une réduction de la variation génétique pouvant expliquer la morphologie inhabituelle de ses spermatozoïdes. Des hypothèses alternatives, telles que la diminution de la compétition spermatique, requièrent désormais une étude plus approfondie.

VARIATION IN SPERMATOZOAL morphology within and between males can be considerable. This is sometimes referred to as "abnormality" or "sperm pleiomorphy" and can vary between populations and species (Breed 2002, Pitnick et al. 2009). Abnormalities in sperm morphology can have serious consequences for motility and fertilizing capacity, and frequent widespread variations in sperm morphology threaten the continuation of species (Holt and Van Look 2004). Variation in sperm morphology in vertebrates can be caused by several processes, including mutation, sperm competition, and other selective events such as those following a genetic bottleneck (Moline et al. 2000, Calhim et al. 2007, Roldan and Gomendio 2009). Environmental factors (e.g., contamination of habitats with toxins) can also cause morphological variation in sperm (Wyrobek 1979, Moline et al. 2000), but these effects are usually restricted to populations rather than species (e.g., Semenza et al. 1997). Reduced sperm competition can

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potentially lead to relaxation of selective pressure for "optimal" sperm and increased variation in sperm morphology (Birkhead et al. 2005, Calhim et al. 2007, Immler et al. 2008, Kleven et al. 2008). Finally, severe selection events, such as genetic bottlenecks, have been correlated with sperm morphological variation associated with high levels of inbreeding (Packer et al. 1991, Keller and Waller 2002, Roldan and Gomendio 2009).

The Eurasian Bullfinch (*Pyrrhula pyrrhula*) has, in comparison to all other passerine species examined thus far, an unusual sperm morphology (Birkhead et al. 2006; Fig. 1). Typical passerine sperm has a corkscrew-shaped head and a midpiece with a single mitochondrion twisted along its length, whereas Eurasian Bullfinch sperm has a rounded head, a very short midpiece consisting of a mitochondrial cluster, and a short flagellum (Birkhead et al. 2007). There is evidence that general sperm morphology in the Eurasian Bullfinch is not an autapomorphy (a derived trait unique to a terminal species in a clade), in that one unusual sperm trait is shared with a sister species, Beavan's Bullfinch (*P. erythaca*; Birkhead et al. 2006). A recent phylogeny of the bullfinches (Töpfer 2008) has shown that the six species of *Pyrrhula* are monophyletic. Töpfer's (2008) sampling is relatively complete, compared with other phylogenies (Arnaiz-Villena et al. 2001, Nguembok et al. 2009). Beavan's Bullfinch is paraphyletic with respect to the Red-headed Bullfinch (*P. erythrocephala*), and this clade is closest to *P. pyrrhula* (Töpfer 2008). The Pine Grosbeak (*Pinicola enucleator*) is the monotypic sister genus to *Pyrrhula* (see Töpfer 2008). The sperm head shape of the Eurasian Bullfinch is unique: Beavan's Bullfinch shares its short-midpiece trait, and the Pine Grosbeak has typical passerine sperm (Birkhead et al. 2006; Fig. 1). Owing to the difficulty of sampling, we do not have sperm morphology data on the Orange Bullfinch (*P. auranticaca*), Brown Bullfinch (*P. nipalensis*), or White-cheeked Bullfinch (*P. leucogenis*); however, compared to the sperm head shape of numerous passerines (Birkhead et al. 2006, Immler and Birkhead 2007), that of the Eurasian Bullfinch is an outlier. It is unlikely that a trait like sperm head shape, so vital to fertilization, would show such a dramatic departure from its closest relatives because of a simple neutral autapomorphy. In a survey of congeneric passerine pairs, Eurasian Bullfinch sperm was the most different from that of Beavan's Bullfinch (Birkhead et al. 2006). Birkhead et al. (2006) suggested that if the Eurasian Bullfinch were to be classified on sperm head shape alone, it might not be included within the passerines. Furthermore, this species also exhibits comparatively extreme variation in sperm morphology both between (Calhim et al. 2007) and within males (Immler et al. 2008). Phylogeny alone cannot account for the Eurasian Bullfinch's sperm morphology, given that its closest relative does not share the most unusual feature of its sperm, its rounded head shape. Therefore, we must look to other explanations for the observed sperm morphology.

High levels of variation in sperm morphology can be associated with the effects of a genetic bottleneck. The Lions (*Panthera leo*) of the Ngorongoro Crater, Tanzania, went through a severe bottleneck that followed a disease-induced population crash. The 15 founding members interbred for the next 25 years (Packer et al. 1991), and, compared with an outbred Serengeti population, the males of this population have a higher proportion of morphologically variable sperm (Wildt et al. 1987, Brown et al. 1991). Although high levels of inbreeding are known to have a deleterious effect on sperm morphology in captive vertebrates (Roldan and Gomendio 2009), the Lions of Ngorongoro Crater are one of the few wild populations to demonstrate increased variability in sperm morphology as a result of inbreeding.

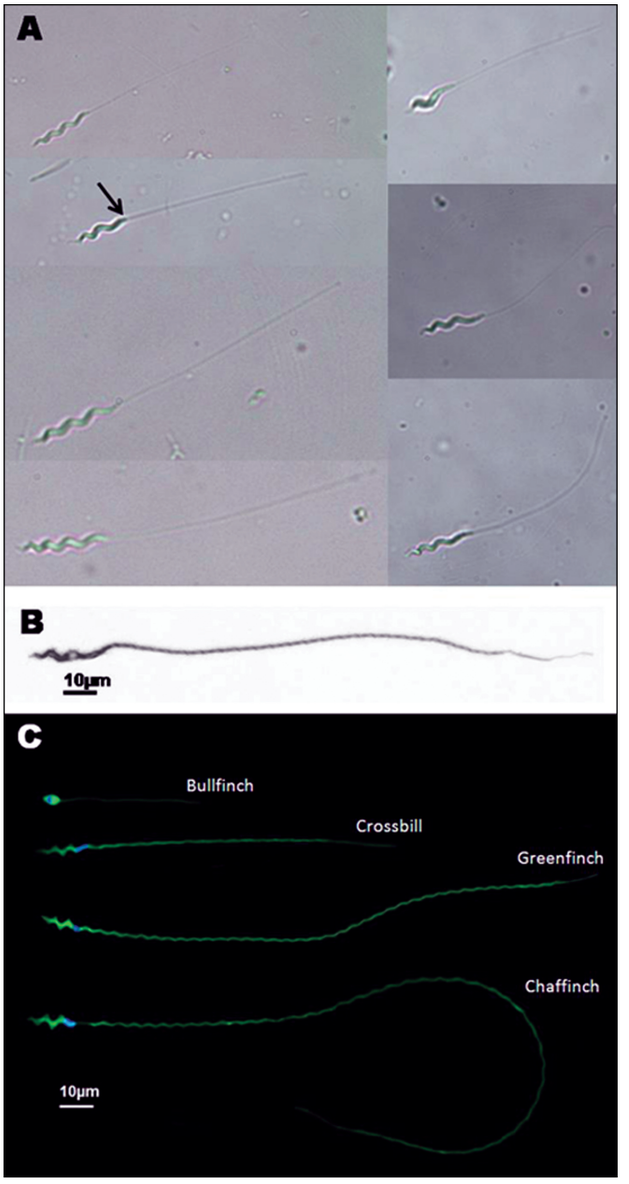


Fig. 1. (A) Photomontage of seven sperm from a single Beavan's Bullfinch under light microscopy, showing large intra-male variation, short midpiece (arrow), and typical passerine head morphology. (B) Example sperm from a Pine Grosbeak, showing typical passerine sperm morphology. (C) Fluorescent-microscopy image of sperm from the four finch species compared in the present study. Areas stained blue contain DNA, and bright green areas to the right of DNA are mitochondrial midpieces. The image has been manipulated to align the four sperm from separate photographs (image credit: S. Immler).

The Lion example involved an isolated subpopulation; but can a bottleneck produce a species-wide phenotypic trait? The Cheetah (*Acinonyx jubatus*) went through a "genetic reduction" (see O'Brien 1994) that has been attributed to a late-Pleistocene bottleneck that reduced genetic diversity and resulted in widespread sperm morphological variation (Menotti-Raymond and O'Brien 1993). The Hawaiian Goose (*Branta sandvicensis*) experienced a steep decline in abundance, and the species was reduced to 30 birds in about 1950 (Kear and Berger 1980). Captive birds showed low genetic variation (Paxinos et al. 2002), sperm abnormalities (Humphreys 1972), and infertility (Kear and Berger 1980). When searching for genetic signatures of bottlenecks, event timing can be uncertain even with demographic data, and multiple events are possible. Paleontological Hawaiian Goose samples showed that genetic diversity had been lost 750–900 years ago, and not as a result of the recent decline (Paxinos et al. 2002).

Eurasian Bullfinch populations have undergone a recent steep decline in abundance in the United Kingdom (Gregory et al. 2004) and a moderate decline across Europe (Hagemeijer and Blair 1997). This is a predominantly farmland species, and the recent decline has been attributed to changes in landscape use, an increase in nest failure rate, and an active control program during the 1950s and 1960s (Newton 1964, Siriwardena et al. 2001, Proffitt et al. 2004). The species was added to the UK Red List of Birds of Conservation Concern in 1996 (Gibbons et al. 1996), but its status was downgraded to "amber" in 2009 (Eaton et al. 2009). Gregory et al. (2004) estimated a 57% decline in abundance between 1970 and 2001. There is also preliminary mitochondrial evidence that the Eurasian Bullfinch, like the European Greenfinch (*Carduelis chloris*; Merilä et al. 1997), experienced a post-Pleistocene series of bottlenecks (T. Töpfer and T. R. Birkhead pers. comm.), which may have caused a genetic reduction severe enough to produce a lasting phenotypic trait of highly abnormal sperm that was then maintained by low postcopulatory sexual selection.

Eurasian Bullfinches have recently declined in Britain, and there is evidence of a Pleistocene bottleneck—the question is whether either of these events was severe enough to produce a bottleneck that caused the observed sperm morphology. A bottleneck event can be detected by a reduction in average gene diversity at many loci as assessed by variance in the expected heterozygosity under Hardy-Weinberg equilibrium (HWE; Nei and Kumar 2000) and also by the presence of fewer alleles at the same loci between species (Nei 1987). These effects have been observed using various genetic markers in comparing inbred and outbred populations (Packer et al. 1991, Akst et al. 2002; but see Reinartz et al. 2000). Although the use of the heterozygosity of neutral markers as a proxy for overall heterozygosity has been contentious, recent work has shown a strong negative relationship between the level of heterozygosity in neutral markers and the percentage of sperm morphological variability in mammals classified as "critically endangered," "endangered," or "vulnerable" (Fitzpatrick and Evans 2009). Cross-amplifying markers designed from one species in different species can entail "ascertainment bias," whereby the most closely related species is likely to amplify preferentially and falsely show greater polymorphism (Ellegren et al. 1997).

To determine whether the Eurasian Bullfinch (hereafter "bullfinch") has gone through a recent bottleneck, we used universal bird primers to directly compare microsatellite diversity among four finch species that encompass all fringillid species, therefore avoiding problems of ascertainment bias (Dawson et al. 2010). The variability of microsatellites means that we seek evidence of a recent genetic reduction, because this type of marker may not show older events. However, microsatellites are the most suitable marker, given that many examples from conservation biology show that genetic reductions assessed using microsatellites are more likely to be associated with high levels of sperm abnormality (Fitzpatrick and Evans 2009). For comparison with the bullfinch we chose the European Greenfinch (hereafter "greenfinch"), Common Chaffinch (*Fringilla coelebs*; hereafter "chaffinch"), and Red Crossbill (*Loxia curvirostra*; hereafter "crossbill"). We predicted that if the unusual shape of the bullfinch's sperm were due to the effects of a recent genetic bottleneck, this species would exhibit less genetic diversity across microsatellite loci than the related species with typically shaped sperm.

## Methods

Extracted and purified genomic DNA was provided by other researchers for our samples of greenfinches ($n = 21$), crossbills ($n = 17$), and chaffinches ($n = 20$) (see Acknowledgments). Of the three, the crossbill is the most closely related to the bullfinch, and the chaffinch is the most distantly related (Arnaiz-Villena et al. 2001, Töpfer 2008). All three comparison species have typically shaped passerine sperm (Immler and Birkhead 2007; Fig. 1) and a low incidence of intra-male sperm variation (Calhim et al. 2007, Immler et al. 2008). The greenfinch population was suspected to have gone through serial post-Pleistocene genetic bottlenecks, as assessed using mitochondrial DNA and allozymes, and the individuals that we used were from three sites—Kiev, Ukraine ($n = 8$), Oulu, Finland ($n = 7$), and Uppsala, Sweden ($n = 6$)—that were not significantly genetically different from each other (we considered them a single population; see Merilä et al. 1997). The chaffinches were from one outbred population in Sheffield, England, and the crossbills were from a single population collected from three sites in Scotland and northern England (S. Piertney pers. comm.).

Using ammonium acetate precipitation, we extracted genomic DNA from whole blood from 23 bullfinches captured in the Sheffield region between 2007 and 2008. The DNA of chaffinches (which had been in long-term storage) and bullfinches was assessed for concentration using a fluorometer (FLUOstar Optima, BMG Labtechnologies, Offenburg, Germany), and concentrations were standardized to 10 ng $\mu L^{-1}$. We tested 34 universal bird primers (Dawson et al. 2010) on each of the four species, using Zebra Finch (*Taeniopygia guttata*) DNA as a positive control. Multiplex polymerase chain reactions (PCRs) were performed for no more than three primer sets in a reaction for all 34 loci. Any loci that failed in a multiplex were rerun in a single-primer PCR (with the exception of loci TG1-040, TG1-011, and TG13-016 in the crossbill, which failed as multiplexes but were not tested singly because of insufficient DNA). See Dawson et al. (2010) for PCR methods and microsatellite running conditions. Data were collated and assessed using GENEMAPPER, version 3.7 (Applied Biosytems, Foster City, California; see Dawson et al. 2010).

*Locus statistics.*—A potential issue with cross-amplification of primers designed for other species is null alleles (e.g., Castro et al. 2006, Panova et al. 2008). We had no pedigree data to test for the

presence of null alleles. Instead, we used (1) high rate of amplification (≥0.82), (2) nonsignificant results for tests of deviation from HWE after correction with a B-Y false-discovery-rate method (Benjamini and Yekutieli 2001; see Narum 2006), (3) little discrepancy between observed and expected heterozygosity, and (4) test results of estimated null allele frequency from CERVUS, version 2.0 (Marshall et al. 1998, Kalinowski et al. 2007), to indicate absence of null alleles. In the absence of pedigree data, the loci were treated with caution and those that failed any of the four tests for any species were discarded. There were no significant results, after correction for multiple tests, in tests for linkage disequilibrium between loci. This left us with 22 microsatellite loci that were polymorphic in at least one species. Locus statistics were calculated using MSA, version 4.05 (Dieringer and Schlötterer 2003). Tests for deviation from HWE and of linkage disequilibrium were performed using GENEPOP, version 1.2 (Raymond and Rousset 1995).

We assessed the data using BOTTLENECK (Cornuet and Luikart 1996), which estimates the distribution of heterozygosity expected from the observed number of alleles (*k*; Appendix) for each locus and population under consideration. The program performs three tests to detect heterozygosity excess, which is a genetic signature of a bottleneck event. The assumptions of such tests are that each sample is representative of a well-defined population with no immigration and no population substructure. We used mean $F_{IS}$ between species across loci to estimate population substructure (Weir and Cockerham 1984). BOTTLENECK assumes selective neutrality of genetic markers, and microsatellites are selectively neutral.

Microsatellites are often highly variable and may evolve rapidly after an older bottleneck event but will show the result from a recent bottleneck. To look for recent genetic reductions, we compared allelic richness and variance in the expected heterozygosity between species using a resampling method to account for differences in sample sizes between species. The resampling function in MSA was used to randomly choose a sample for each species that equaled the smallest species sample size. This was repeated 1,000 times to calculate allelic range (*k*) and genetic variance for each species after each bootstrap. The 1,000 reconstructed data points were averaged, and a one-way analysis of variance (ANOVA) was performed using MINITAB (Minitab, State College, Pennsylvania).

TABLE 1. Average $F_{IS}$ values and 95% confidence intervals (CI) across 22 universal bird microsatellite loci among the four comparison species. All loci in all species did not deviate from Hardy-Weinberg equilibrium.

| Species | Mean $F_{IS}$ | 95% CI |
|---|---|---|
| European Greenfinch | 0.2251 | ±0.1543 |
| Red Crossbill | 0.2328 | ±0.1213 |
| Common Chaffinch | 0.2187 | ±0.1399 |
| Eurasian Bullfinch | 0.1182 | ±0.1537 |

## RESULTS

*Locus statistics*.—The 22 loci that we used were moderately polymorphic, ranging from 1 to 17 alleles (*k*), with a mean $k = 4.0$ across species (Appendix). This varied among species, but no single species was noticeably less diverse. The mean estimated *k* across species was 3.3 using the stepwise mutational model of microsatellite evolution (SMM; Ohta and Kimura 1973), and 4.4 using the infinite alleles model (IAM; Kimura and Crow 1964). Allele size increments indicated that microsatellite conformation varied little between species. Only four loci showed a mutation in some but not all species, which produced a single repeat unit in an otherwise dinucleotide repeat microsatellite locus. This mutation was observed four times in different loci in bullfinches, three times in crossbills, and once in greenfinches, demonstrating that most species were susceptible to these mutations, which can make scoring and allele counts less straightforward (Appendix).

To detect bottlenecks, it is important that each sample does not have cryptic population substructure; therefore, we estimated mean $F_{IS}$ between species across loci (Weir and Cockerham 1984; Table 1). These values, coupled with genotypic frequencies that match Hardy-Weinberg expectations in all loci, indicated that our samples from each species represented a single genetic population.

*Bottleneck detection*.—Locus numbers differed between species because we used only polymorphic loci in our estimate (Table 2). All BOTTLENECK results were nonsignificant for heterozygosity excess, a sign of a bottleneck event. The "mode-shift indicator" looks at the distribution of alleles for each locus in each species,

TABLE 2. Summary of results from the program BOTTLENECK (IAM = infinite alleles model, SMM = stepwise mutational model of microsatellite evolution, and *T2* = standardized differences test statistic). The results do not demonstrate a significant heterozygosity (*H*) excess indicative of a recent bottleneck (Cornuet and Luikart 1996).

| Species | Loci (*n*) | Evolutionary model | Sign test for *H* excess | | | Standardized differences | | Wilcoxon sign test (one-tailed) for *H* excess (*P*) |
|---|---|---|---|---|---|---|---|---|
| | | | Observed | Expected | *P* | *T2* | *P* | |
| European Greenfinch | 20 | IAM | 10.97 | 8 | 0.132 | –0.896 | 0.185 | 0.806 |
| | | SMM | 11.61 | 2 | >0.001 | –5.864 | >0.001 | 0.999 |
| Red Crossbill | 19 | IAM | 10.49 | 7 | 0.083 | –1.932 | 0.026 | 0.977 |
| | | SMM | 10.96 | 3 | >0.001 | –7.349 | >0.001 | 0.999 |
| Common Chaffinch | 21 | IAM | 11.08 | 9 | 0.243 | –0.844 | 0.199 | 0.730 |
| | | SMM | 11.79 | 6 | 0.009 | –5.441 | >0.001 | 0.998 |
| Eurasian Bullfinch | 17 | IAM | 9.03 | 8 | 0.396 | –0.432 | 0.333 | 0.573 |
| | | SMM | 9.62 | 4 | 0.006 | –4.295 | >0.001 | 0.999 |

and an L-shaped allele distribution indicates no recent bottleneck events (Luikart et al. 1998). Each population had the L-shaped distribution. The sign tests showed no positive difference under IAM or SMM for any population. The standardized differences test, which evaluates the significance of the magnitude of heterozygosity excess or deficiency, showed negative standard deviates and, hence, heterozygosity deficiency in each population. Finally, the one-tailed Wilcoxon sign-rank tests for heterozygosity excess were all nonsignificant (Cornuet and Luikart 1996; see Table 2).

*Genetic diversity.*—The results of the one-way ANOVA for the resampled mean allelic range ($k$) across loci for each species were nonsignificant ($F = 0.18$, df = 3, $P = 0.911$), which indicates that there were no differences in number of alleles amplified among the four species. The one-way ANOVA for the resampled mean genetic diversity across loci for each species was also nonsignificant ($F = 0.15$, df = 3, $P = 0.930$), which indicates that there were no differences in expected heterozygosity among the four species.

## Discussion

Our microsatellite data provided no evidence for a recent bottleneck event in any of the four fringillid species. Our direct comparison of genetic diversity among the four species demonstrated no significant differences in observed microsatellite polymorphism or heterozygosity. This indicates that the Eurasian Bullfinch is no less diverse than any of the other fringillid species examined; therefore, there is no evidence that it has undergone a recent genetic reduction.

Although bullfinch numbers have declined recently in the United Kingdom, it has not apparently been severe enough to produce an identifiable signature in the genome. Likewise, the postulated post-Pleistocene bottleneck events have not left a genetic sign, but this was not unexpected given the high mutation rate of microsatellites. There is a critical window of time between a bottleneck and the search to find its effects when bottlenecks are most likely to be detected using molecular techniques. Migration and dispersal can quickly erase the genetic signature of even steep declines in abundance (see Busch et al. 2007). This may be one reason why we found no evidence, using microsatellites, of a recent genetic reduction in the bullfinch. However, cases in which there are phenotypic results of inbreeding, such as high levels of sperm morphological variability, are also generally accompanied by a genetic signature of the bottleneck event (e.g., Humphreys 1972, Packer et al. 1991, Menotti-Raymond and O'Brien 1993, Paxinos et al. 2002, Gage et al. 2006). Therefore, the unusual sperm head shape and high degree of variation in bullfinch sperm morphology are unlikely to be due to the result of bottlenecking and inbreeding.

The four finch species did not differ significantly in numbers of alleles or expected heterozygosity when we directly compared loci amplified by universal bird primers. This could be interpreted as being at odds with results obtained for the greenfinch by Merilä et al. (1997), who detected a signature of bottlenecking using other molecular markers. However, the fact that universal bird microsatellite loci yielded no evidence for such an effect when we compared the four fringillid species indicates that microsatellites are best used to detect recent genetic reductions. There is evidence that many European passerines have gone through a period of postglacial bottlenecks during the Pleistocene (Blondel and Mourer-Chauviré 1998). It is possible that all four finch species went through a series of bottlenecks during the Pleistocene and the microsatellites are too variable to demonstrate a lasting effect. Merilä et al. (1996, 1997) found that both allozyme and mitochondrial DNA evidence pointed to a reduction in genetic diversity in greenfinches from northern Europe (the origin of our samples) compared with those from southern Europe. However, this reduction was indicated by lower individual heterozygosity in northern greenfinches while there was no difference in mean heterozygosity across loci, mean number of alleles, or proportion of polymorphic loci (Merilä et al. 1996), which is similar to our results. This concordance of microsatellite-derived results indicates that our novel approach of using universal bird microsatellite primers is appropriate for this kind of inquiry. While avoiding the problems of ascertainment bias, we have performed the first direct comparison of genetic diversity across a range of species that are close phylogenetic relatives and ecologically relevant to each other. Furthermore, there is no indication of a genetic reduction that would demonstrate a lasting effect from the recent steep decline in bullfinch numbers across the United Kingdom and the moderate declines across Europe. These recent events have not left a signature in the microsatellite allelic diversity of the bullfinch that has been linked to severe sperm abnormalities in other species (Fitzpatrick and Evans 2009), despite an estimated 57% recent decline in bullfinch abundance in the United Kingdom (Gregory et al. 2004).

If the unusually shaped sperm of the bullfinch has not been caused by postbottleneck selection and is not a phylogenetic effect, what alternatives are there? A reduced level of sperm competition between males could cause high inter- and intra-male variability in sperm morphology (Birkhead et al. 2006, 2007). Given the extremely small testes of the bullfinch (0.29% of body mass compared with 0.65–2.2% in other fringillids; Birkhead et al. 2006), sperm competition theory predicts that the bullfinch should be genetically monogamous (Møller and Briskie 1995, Calhim and Birkhead 2007). Although there are no paternity studies that demonstrate genetic monogamy in wild bullfinch populations, the relatively small size of their sperm and high intra-ejaculate variation in morphology are consistent with an interpretaton of weak postcopulatory selection on sperm (Birkhead et al. 2006, Calhim et al. 2007, Immler et al. 2008). Experimentally imposed monogamy and subsequent reduced sperm competition have produced pronounced and heritable morphological alterations in reproductive traits in a dung beetle (*Onthophagus taurus*; Simmons and García-González 2008). We suggest that reduction of sperm competition in the Eurasian Bullfinch is the reason for its widely varying sperm morphology and unusual sperm head shape. This hypothesis remains to be tested.

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APPENDIX. Locus statistics for each of 22 universal bird-microsatellite loci amplified from four species of fringillid finches (*k* = number of alleles, SMM = stepwise mutational model, IAM = infinite alleles model, *H*o = observed heterozygosity, and *H*e = expected heterozygosity). Allele size increments (number of base pairs [bp] per repeat) with question marks indicate either that only one allele was amplified or that amplified alleles were very far apart and that, therefore, there was not enough available information to determine the motif; "1 mut" indicates a single-step mutation. Nonsignificant *P* values for $F_{IS}$ indicate that loci did not deviate from Hardy-Weinberg equilibrium after correction for multiple tests (significant at $P < 0.0121$; see text). Greenfinch = European Greenfinch, crossbill = Red Crossbill, chaffinch = Common Chaffinch, and bullfinch = Eurasian Bullfinch.

| Locus | Species | Allele size increments (bp) | *k* | Estimated *k* (SMM) | Estimated *k* (IAM) | *H*o | *H*e | Estimated null allele frequency | $F_{IS}$ | *P* value ($F_{IS}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| TG01-077 | Greenfinch | 1 | 3 | 3.64 | 4.94 | 0.48 | 0.55 | 0.0050 | 0.13 | 1 |
| | Crossbill | 1 | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Chaffinch | ? | 2 | 1.43 | 1.46 | 0 | 0.10 | 0 | 1 | — |
| | Bullfinch | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| TG01-114 | Greenfinch | 2 | 3 | 2.41 | 2.78 | 0.29 | 0.32 | 0.04 | 0.11 | 0.57 |
| | Crossbill | 2 | 2 | 1.68 | 1.76 | 0.18 | 0.17 | –0.04 | –0.08 | 1 |
| | Chaffinch | ? | 2 | 1.41 | 1.44 | 0 | 0.20 | 0.09 | 1 | — |
| | Bullfinch | 2 | 2 | 2.16 | 2.41 | 0.22 | 0.26 | 0 | 0.17 | 0.41 |
| TG01-147 | Greenfinch | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Crossbill | 2 | 3 | 3.49 | 4.56 | 0.47 | 0.54 | –0.03 | 0.12 | 1 |
| | Chaffinch | 2 | 4 | 4.87 | 7.19 | 0.65 | 0.70 | 0.014 | 0.05 | 0.26 |
| | Bullfinch | ? | 2 | 1.37 | 1.40 | 0 | 0.09 | 0 | 1 | — |
| TG01-148 | Greenfinch | 2 | 5 | 4.91 | 7.33 | 0.86 | 0.70 | –0.13 | –0.25 | 0.29 |
| | Crossbill | 2 | 8 | 7.90 | 11.31 | 0.76 | 0.85 | –0.03 | 0.08 | 0.01 |
| | Chaffinch | 2 | 5 | 3.26 | 4.21 | 0.3 | 0.49 | 0.13 | 0.39 | 0.20 |
| | Bullfinch | 2 | 5 | 5.08 | 7.72 | 0.48 | 0.71 | 0.12 | 0.32 | 0.46 |
| TG02-078 | Greenfinch | 1 | 4 | 3.77 | 5.19 | 0.29 | 0.57 | 0.19 | 0.50 | 0.17 |
| | Crossbill | 1 | 3 | 1.70 | 1.78 | 0.18 | 0.17 | –0.04 | –0.06 | 1 |
| | Chaffinch | 1 | 5 | 2.09 | 2.30 | 0.17 | 0.26 | –0.04 | 0.35 | 1 |
| | Bullfinch | 1 | 2 | 1.87 | 2.00 | 0.13 | 0.20 | 0.20 | 0.33 | 0.22 |
| TG02-088 | Greenfinch | 2 (1 mut) | 12 | 10.38 | 15.15 | 0.86 | 0.89 | –0.03 | 0.03 | 0.22 |
| | Crossbill | 2 (1 mut) | 9 | 6.87 | 9.99 | 0.71 | 0.81 | 0.01 | 0.12 | 0.89 |
| | Chaffinch | 2 | 7 | 6.60 | 10.07 | 0.75 | 0.8 | –0.04 | 0.05 | 0.07 |
| | Bullfinch | 2 (1 mut) | 11 | 9.18 | 14.16 | 0.78 | 0.87 | 0.01 | 0.09 | 0.08 |
| TG02-120 | Greenfinch | 2 | 2 | 1.58 | 1.64 | 0.14 | 0.14 | –0.03 | –0.06 | 1 |
| | Crossbill | 2 (1 mut) | 5 | 2.94 | 3.61 | 0.29 | 0.45 | 0.06 | 0.33 | 0.56 |
| | Chaffinch | 2 | 2 | 1.21 | 1.22 | 0.05 | 0.05 | –0.01 | –0.01 | — |
| | Bullfinch | 2 (1 mut) | 5 | 2.24 | 2.52 | 0.22 | 0.28 | –0.05 | 0.21 | 1 |
| TG03-002 | Greenfinch | 1 | 9 | 6.10 | 9.40 | 0.81 | 0.78 | –0.16 | –0.06 | 0.46 |
| | Crossbill | 2 | 4 | 4.04 | 5.54 | 0.53 | 0.62 | 0.07 | 0.13 | 0.34 |
| | Chaffinch | 2 | 4 | 2.58 | 3.04 | 0.32 | 0.37 | –0.09 | 0.13 | 1 |
| | Bullfinch | 2 | 3 | 2.65 | 3.17 | 0.35 | 0.37 | –0.002 | 0.05 | 0.09 |
| TG03-031 | Greenfinch | ? | 1 | 1 | 1 | 0 | 0 | 0.16 | n.d. | 0.21 |
| | Crossbill | ? | 1 | 1 | 1 | 0 | 0 | –0.01 | n.d. | — |
| | Chaffinch | 2 | 2 | 2.6 | 3.08 | 0.26 | 0.37 | 0 | 0.28 | — |
| | Bullfinch | 2 | 2 | 1.24 | 1.25 | 0.06 | 0.06 | 0 | –0.02 | — |
| TG03-098 | Greenfinch | 1 | 3 | 3.67 | 4.99 | 0.43 | 0.56 | 0.10 | 0.22 | 0.04 |
| | Crossbill | 2 | 7 | 4.42 | 6.20 | 0.41 | 0.66 | 0.05 | 0.37 | 0.20 |
| | Chaffinch | 1 | 7 | 6.54 | 9.97 | 0.75 | 0.80 | –0.01 | 0.05 | 0.40 |
| | Bullfinch | 1 | 5 | 5.17 | 7.96 | 0.52 | 0.72 | 0.11 | 0.27 | 0.10 |
| TG04-004 | Greenfinch | 2 | 2 | 1.20 | 1.21 | 0.048 | 0.05 | –0.01 | –0.01 | — |
| | Crossbill | 2 | 8 | 6.34 | 9.25 | 0.82 | 0.79 | –0.08 | –0.05 | 0.99 |
| | Chaffinch | 2 | 3 | 1.99 | 2.16 | 0.15 | 0.23 | 0.19 | 0.35 | 0.11 |
| | Bullfinch | 2 | 3 | 1.55 | 1.61 | 0.13 | 0.13 | –0.02 | –0.04 | 1 |
| TG04-012 | Greenfinch | 2 | 4 | 3.29 | 4.28 | 0.48 | 0.50 | –0.08 | 0.03 | 0.44 |
| | Crossbill | 2 | 4 | 4.32 | 6.03 | 0.47 | 0.65 | 0.15 | 0.27 | 0.33 |
| | Chaffinch | 1 | 6 | 5.37 | 8.08 | 0.65 | 0.73 | 0.02 | 0.11 | 0.71 |
| | Bullfinch | 2 (1 mut) | 5 | 4.39 | 6.47 | 0.74 | 0.65 | –0.08 | –0.16 | 0.95 |
| TG04-012a | Greenfinch | ? | 2 | 1.40 | 1.42 | 0 | 0.09 | 0 | 1 | — |
| | Crossbill | 4 | 4 | 2.34 | 2.65 | 0.24 | 0.32 | –0.06 | 0.25 | 1 |
| | Chaffinch | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Bullfinch | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |

(*Continued*)

APPENDIX. (Continued)

| Locus | Species | Allele size increments (bp) | *k* | Estimated *k* (SMM) | Estimated *k* (IAM) | *Ho* | *He* | Estimated null allele frequency | $F_{IS}$ | *P* value ($F_{IS}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| TG04-041 | Greenfinch | 2 | 3 | 1.99 | 2.16 | 0.15 | 0.23 | –0.03 | 0.35 | 1 |
| | Crossbill | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Chaffinch | 2 | 2 | 1.21 | 1.22 | 0.05 | 0.05 | –0.01 | –0.01 | — |
| | Bullfinch | ? | 3 | 2.05 | 2.25 | 0 | 0.24 | 0 | 1 | — |
| TG04-061 | Greenfinch | 1 | 5 | 3.54 | 4.76 | 0.33 | 0.54 | 0.15 | 0.38 | 0.20 |
| | Crossbill | 1 | 5 | 4.03 | 5.51 | 0.65 | 0.62 | –0.12 | –0.07 | 0.52 |
| | Chaffinch | 1 | 4 | 3.68 | 4.98 | 0.55 | 0.56 | –0.04 | 0.01 | 0.42 |
| | Bullfinch | 1 | 6 | 5.92 | 9.31 | 0.87 | 0.76 | –0.12 | –0.15 | 0.34 |
| TG05-030 | Greenfinch | 2 | 4 | 2.80 | 3.41 | 0.38 | 0.40 | –0.10 | 0.05 | 1 |
| | Crossbill | 2 | 3 | 1.91 | 2.05 | 0.12 | 0.22 | –0.02 | 0.46 | 1 |
| | Chaffinch | 2 | 2 | 3.25 | 4.20 | 0.5 | 0.49 | –0.02 | –0.03 | 1 |
| | Bullfinch | 2 | 2 | 1.37 | 1.40 | 0.09 | 0.09 | –0.01 | –0.03 | 1 |
| TG05-046 | Greenfinch | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Crossbill | ? | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| | Chaffinch | ? | 2 | 2.81 | 3.42 | 0.45 | 0.41 | –0.06 | –0.12 | 1 |
| | Bullfinch | 2 | 1 | 1 | 1 | 0 | 0 | 0 | n.d. | — |
| TG05-053 | Greenfinch | 1 | 8 | 6.72 | 10.39 | 0.95 | 0.80 | –0.14 | –0.20 | 0.07 |
| | Crossbill | 1 | 4 | 2.13 | 2.35 | 0.18 | 0.27 | –0.04 | 0.35 | 1 |
| | Chaffinch | 1 | 4 | 4.68 | 6.78 | 0.63 | 0.68 | 0.002 | 0.06 | 0.30 |
| | Bullfinch | 1 | 5 | 4.06 | 5.81 | 0.48 | 0.61 | –0.02 | 0.20 | 0.68 |
| TG06-009 | Greenfinch | 2 | 3 | 1.59 | 1.65 | 0.05 | 0.14 | –0.01 | 0.65 | — |
| | Crossbill | ? | 2 | 1.47 | 1.50 | 0 | 0.11 | 0 | 1 | — |
| | Chaffinch | 2 | 3 | 2.64 | 3.14 | 0.45 | 0.38 | –0.12 | –0.22 | 1 |
| | Bullfinch | 2 | 4 | 3.13 | 4.02 | 0.39 | 0.46 | 0.03 | 0.15 | 0.30 |
| TG13-009 | Greenfinch | 2 | 4 | 1.97 | 2.13 | 0.10 | 0.22 | 0.29 | 0.57 | 0.08 |
| | Crossbill | 2 | 3 | 1.91 | 2.05 | 0.12 | 0.22 | –0.02 | 0.46 | 1 |
| | Chaffinch | 2 | 3 | 2.68 | 3.21 | 0.25 | 0.38 | –0.07 | 0.34 | 1 |
| | Bullfinch | 2 | 2 | 1.37 | 1.40 | 0.09 | 0.09 | –0.01 | –0.03 | 1 |
| TG13-016 | Greenfinch | 1 | 4 | 1.97 | 2.13 | 0.05 | 0.22 | –0.01 | 0.79 | — |
| | Crossbill | 1 | 4 | 2.13 | 2.35 | 0.18 | 0.27 | –0.04 | 0.35 | 1 |
| | Chaffinch | ? | 3 | 1.62 | 1.68 | 0.05 | 0.14 | –0.01 | 0.65 | — |
| | Bullfinch | 1 | 1 | 1 | 1 | 0 | 0 | — | n.d. | — |
| TG22-001 | Greenfinch | 2 | 7 | 5.74 | 8.70 | 0.7 | 0.76 | 0.03 | 0.07 | 0.52 |
| | Crossbill | 2 (1 mut) | 13 | 9.90 | 13.30 | 0.75 | 0.89 | 0.07 | 0.14 | 0.23 |
| | Chaffinch | 2 | 8 | 5.74 | 8.60 | 0.63 | 0.76 | 0.09 | 0.16 | 0.30 |
| | Bullfinch | 2 (1 mut) | 17 | 14.44 | 19.22 | 0.95 | 0.93 | –0.02 | –0.04 | 0.89 |