

Amplified fragment length polymorphism (AFLP) markers reveal extra-pair parentage in a bird species: the bluethroat (*Luscinia svecica*)

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Abstract

We tested the use of amplified fragment length polymorphism (AFLP) to assess the frequency of extra-pair parentage in a bluethroat (*Luscinia svecica namnetum*) population. Thirty-six families totalling 162 nestlings were analysed. Using a combination of three primer pairs, we reached an exclusion probability of 93% for the population. This probability can reach 99% considering families independently. We revealed that extra-pair fertilizations are very common: 63.8% of all broods contain at least one extra-pair young, totalling 41.9% of all young analysed. However, with the technique and the three primer pairs used it was not possible to attribute the parentage exclusions to extra-pair paternity, maternity or both. As brood parasitism has never been reported in this species, it seems likely that the exclusions are due to extra-pair males. This study shows that dominant AFLP markers can be useful for studying the mating system of taxa for which no microsatellite primers are available. This technique allows the approximate estimation of parentage exclusions despite the fact that it is not possible to know which parent has to be excluded.

Keywords: AFLP, exclusion probability, extra-pair parentage, *Luscinia svecica*, mating system, sexual selection

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Introduction

There has been a growing interest in studying mating systems in animals thanks to the development of molecular techniques that allow a more accurate measurement of individual fitness. Especially in birds, in which most of the species were considered monogamous, DNA markers have revealed the occurrence of extra-pair parentage in broods of socially monogamous species. Therefore, by giving the true measure of breeding success, they allow some of the hypotheses of sexual selection, especially female choice, to be tested. Indeed, males have the opportunity to maximize their fitness through extra-pair fertilizations, but females may to some extent play an important role in the outcome of the fertilizations by

controlling the sperm that will fertilize their eggs. Females may solicit and engage in extra-pair copulations to gain fitness: by obtaining 'good genes' for their descendants, such as resistance against parasites, or increasing the genetic variability of their offspring, or compensating for the possible low fertility of their social partner, or also obtaining some help through parental care or other direct benefits, such as access to resources in good territories (Birkhead & Møller 1992; Kempenaers & Dhondt 1993). Thus, extra-pair fertilizations occur in many species, including social monogamous ones. Their frequency among bird species can range from nonexistent to substantial even at the intraspecific level (Lifjeld *et al.* 1991, 1993; Yamagishi *et al.* 1992; Fleischer *et al.* 1994; Hasselquist *et al.* 1995; Brün *et al.* 1996; Dunn & Cockburn 1996).

Most of the studies dealing with paternity assessment in birds use multilocus DNA fingerprinting with microsatellite probes (e.g. Gibbs *et al.* 1990; Hartley *et al.* 1995;

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Hasselquist *et al.* 1995; Westneat 1995; Krokene *et al.* 1996) or more rarely single-locus minisatellite fingerprints (Gyllenstein *et al.* 1990; Burke *et al.* 1991; Hanotte *et al.* 1991; Dixon *et al.* 1994) or microsatellite profiles (A. Johnsen *et al.* unpublished). The use of minisatellite probes is relatively easy but it requires large amounts of genomic DNA. Conversely, microsatellite loci are revealed via a polymerase chain reaction (PCR) that allows the use of very small amounts of DNA. However, specific primers have to be defined by cloning microsatellite loci, which is time-consuming and is constrained by the fact that primers cannot always cross-amplify in related taxa.

The amplified fragment length polymorphism (AFLP) technique is based on the double amplification via PCR of a subset of restriction fragments from a total digest of genomic DNA (Vos *et al.* 1995). DNA is cut with two enzymes (a rare and a frequent cutter according to the length of their restriction site). Each fragment is ligated to adaptors (not recreating the restriction site) that serve as a binding site for primers with one additional selective nucleotide included at the 3' end, which probes the internal sequence of the fragment. The selected fragments are amplified in a first PCR reaction (preselective). The PCR product is then used as the template for a second amplification (selective) using primers with three additional selective nucleotides included at the 3' end. Subsets of fragments possessing the complementary sequence to the primer elongation are amplified. This limits the number of scorable markers on the gel. The result is a multilocus fingerprint-like pattern, which can be scored on an automated sequencer by the use of fluorescent primers. This technique is mainly used in plant mapping and studies of diversity in crops or wild plants (Majer *et al.* 1996; Schondelmaier *et al.* 1996; Travis *et al.* 1996; Escaravage *et al.* 1998). AFLP data on animals are still scarce (Otsen *et al.* 1996; Vos & Kuiper 1996; Ajmone-Marsan *et al.* 1997; Questiau *et al.* 2000).

The bluethroat (*Luscinia svecica namnetum*) is a territorial passerine bird defined as socially monogamous, with paternal care (food provisioning) (Glutz von Blotzheim & Bauer 1994). The males have a conspicuous courtship display and a colourful throat ornament. They guard their mates intensively during the fertile period (Johnsen & Lifjeld 1995). Sexual selection is believed to be strong considering the conspicuous male courtship display, the variance in male sexual traits, and the strong mate-guarding behaviour by males. The opportunities for extra-pair copulations are abundant and have actually been reported in another bluethroat subspecies (Krokene *et al.* 1996). At the start of this study, six microsatellites from other species that were reported to cross-amplify with the bluethroat and the pied flycatcher (Hanotte *et al.* 1994; Primmer *et al.* 1996) were tested. These few did not detect sufficient polymorphism (a maximum of three alleles for 10 unrelated

individuals). It was decided to test whether the AFLP markers could be used to study parentage exclusion in that bird species.

Materials and methods

Samples

This study was conducted during two breeding seasons (1995, 1996) in a population of bluethroats breeding in the Guérande saltmarsh (47°20'N, 2°25'W), France. We focused on 36 pairs (16 in 1995 and 20 in 1996) in which samples were collected from both parents and offspring. Each bird was banded with coloured rings for individual recognition in addition to a numbered metal ring. Of these 36 families, only three were second broods of pairs for which we also had the first brood. We considered them as independent families for the paternity analysis. For the parents, blood samples were taken from the brachial vein and stored in Queen's lysis buffer (Seutin *et al.* 1991) until DNA extraction. Growing feathers containing large amounts of pulp were plucked from nestlings when 7–8 days old and were stored in 80% ethanol. This method took less time and was less invasive than blood sampling.

DNA isolation

A 3 mm piece of the base of a growing feather or 400 µL of the mix of blood plus lysis buffer were digested in 400 µL of a proteinase K solution (10 mM Tris-HCl, pH 8.0, 2 mM ethylene diamine tetra acetic acid (EDTA), 10 mM NaCl, 1% sodium dodecyl sulphate (SDS), 10 mg/mL dithiothreitol (DTT), 0.5 mg/mL proteinase K). DNA extraction was carried out twice with an equal volume of phenol-chloroform and once with chloroform as described in Taberlet & Bouvet (1991). DNA was precipitated in ethanol and diluted in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) following a standard procedure (Sambrook *et al.* 1989).

AFLP procedure

AFLPs were resolved according to the AFLP™ Plant Mapping Kit protocol (Perkin Elmer). No more than 55 ng of DNA from blood samples was used to eliminate potential enzymatic reaction inhibitors in the restriction-ligation reaction. Between 60 and 200 ng of DNA from the growing feathers was used. Enzymatic digestion with *MseI* and *EcoRI*, frequent and rare cutter, respectively, and ligation of the adaptors at 37 °C for 2 h in a final volume of 11 µL were performed at the same time. The reaction was then diluted to 200 µL in TE_{0.1} (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Four microlitres of the diluted restriction-ligation DNA were added to 1 µL of preselective

primers with one nucleotide extension at the 3' end and 15 µL of AFLP Core Mix supplied in the kit. The preselective PCR was run as follows: 2 min at 72 °C followed by 35 cycles with the following cycle profile: 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C. The amplification reaction was diluted 20 times with TE_{0.1}. Three microlitres of the preamplified diluted DNA were mixed to 15 µL of AFLP Core Mix, 5 pmol of selective *MseI* primer and 1 pmol of fluorescent *EcoRI* selective primer. Among the 64 possible primer combinations provided in the kit, 46 were tested and three primer pairs were chosen because of their clean and reproducible patterns and their acceptable polymorphism (see Results): B = *MseI*-CTA and *EcoRI*-ACT (FAM-blue), G = *MseI*-CAC and *EcoRI*-AAG (JOE-green) and Y = *MseI*-CAG and *EcoRI*-ACC (NED-yellow). The selective amplification began with one cycle with a 2 min denaturation step at 94 °C, 30 s annealing at 65 °C and 2 min elongation at 72 °C. Eight cycles followed with 10 s denaturation at 94 °C, 30 s annealing with the temperature reduced each cycle by 1 °C from 64 °C to 57 °C, and elongation 2 min at 72 °C. The PCR was continued for 30 cycles (10 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C).

Two microlitres for B, 3 µL for G, 6 µL for Y selective amplification were added to 3 µL of loading buffer containing 2.4 µL of deionized formamide, 0.44 µL of blue dye and 0.16 µL of Genescan-500 ROX-labelled size standard and concentrated under vacuum until the final volume was 2.5 µL. The loading mixture was denatured and run on an ABI PRISM™ 377 DNA sequencer (Perkin Elmer) in a 5% Long Ranger™ gel (FMC) for 5 h.

Data analysis

Scorable fragments. Each amplified fragment was analysed on electrophoregrams showing the fluorescence detected as a function of time. All peaks were scored for presence/absence in each individual using the GENESCAN™ 2.0.2 analysis software in the 50–500 bp range. In each pattern the fluorescence was not distributed homogeneously, the height of peaks being larger for the small fragments and going down according to the increasing fragment size. We thus considered all peaks with a height above at least 150 fluorescent units for small fragments and above 50 for the second part of the profiles. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.6 bp. We considered each fragment position as a dominant locus with two states: presence, absence.

To test the validity of the technique, 154 replicates were conducted for the B primer pair (130 from the same digest, 16 from the same extract and eight from two different extracts) totalling 148 individuals (+ six repeated a third time). The rate of reproducibility can be expressed

as the number of repeatable peaks out of the total number of peaks: this reached 98.8%. This value was achieved for the three kinds of replicates (same digests, same extracts, and different extracts). This margin of error was not detrimental to the overall exclusion estimations. The replicates were conducted extensively for only one primer pair.

The nonrepeatable fragments occurred primarily in nondiagnostic sites (31 of 38 (81.5%), G₁, William's correction = 14.88, $P < 0.01$). The nonrepeatable fragments occurring in diagnostic sites (seven of 38 peaks, in seven nestlings) were not detrimental for the detection of extra-pair fertilizations. All the seven nestlings, except one, were considered unambiguously either as legitimate offspring (no other diagnostic peak found) or conversely, as extra-pair young (more than two diagnostic peaks found in the profiles of the two other primer pairs).

The nonrepeatable fragments were not distributed equally among loci (G₂₂, William's correction = 43.33, $P < 0.01$). Sixty-eight per cent of all nonrepeatable peaks were found in six loci out of the 23 polymorphic ones. Thus, these loci were considered with caution when diagnostic peaks occurred. Moreover, the 38 nonrepeatable peaks were distributed among 26 individuals. This means that some individuals could have several nonrepeatable peaks in their B profile (on average 1.4 ± 1.0 (mean \pm standard deviation (SD))), but these mismatches were not detrimental for the detection of extra-pair fertilizations. Only one nestling had up to five nonrepeatable peaks and its profiles in all repetitions differed largely from each other. This could be due to degraded DNA or to the presence of enzyme inhibitors limiting the power of enzymatic digestion–ligation. This bird was not considered subsequently in the analysis.

We checked for a possible correlation between peaks (or between loci) between all pairwise comparisons of loci for all individuals, where the state at each locus is encoded by '1' for the presence of a peak and by '0' for the absence. For that purpose, we calculated an index of correlation in migration (I.C.) for all loci i and j with $i \neq j$ as:

$$\text{I.C.} = \frac{\sum_{n=1}^N | \text{state at the } i\text{th locus } (n) - \text{state at the } j\text{th locus } (n) |}{N}$$

with N being the total number of individuals.

This index value can rise from zero to one. A value of one between two fragment positions would mean that when a peak appears at the first position, another peak is not seen at the second position and vice versa. A value of zero would suggest a consistent comigration of two fragments. No correlation in the migration of bands was detected in the three primer pair profiles (no detected 0 or 1; average index for B = 0.49 ± 0.11 (mean \pm SD), for G = 0.48 ± 0.17 , for Y = 0.49 ± 0.11). We thus considered

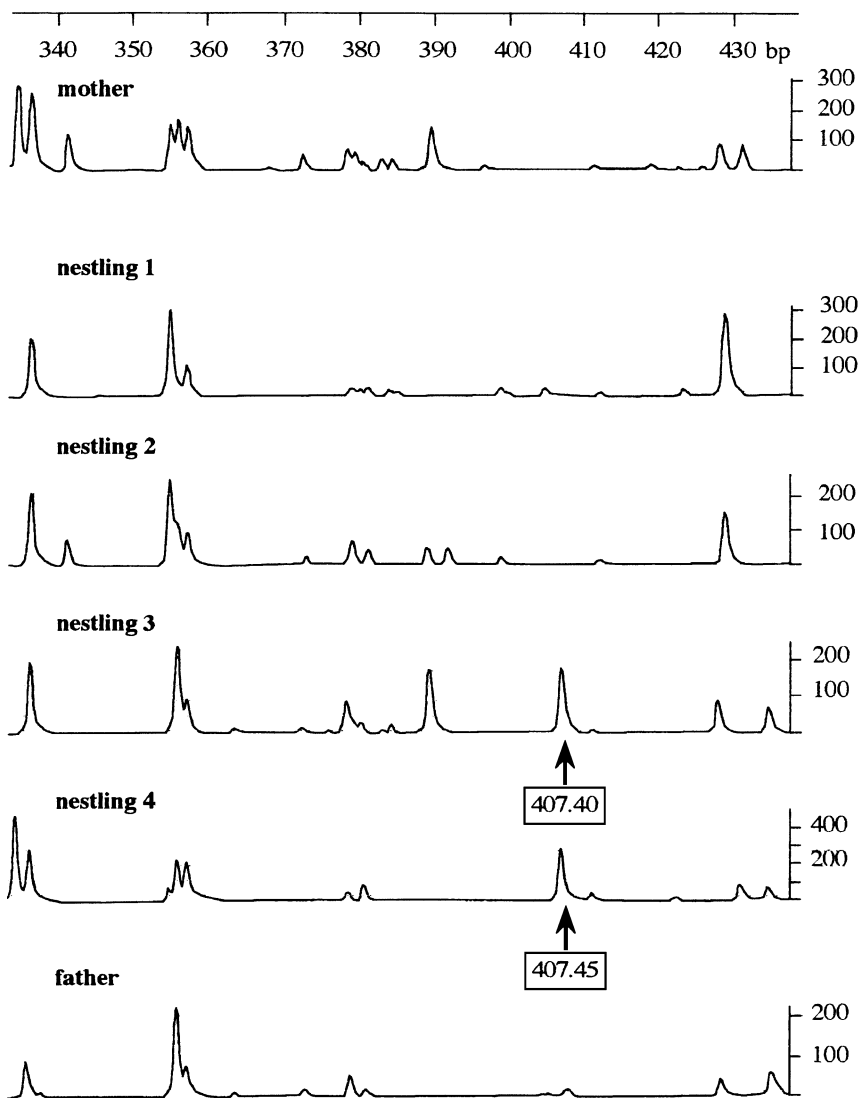


Fig. 1 Portion of an amplified fragment length polymorphism (AFLP) profile obtained with the *MseI*-CTA/*EcoRI*-ACT primer pair (B in the text) for a bluethroat family. A 407 bp fragment is considered diagnostic for nestlings 3 and 4 because it is absent in both parents. This could be the result of an extra-pair fertilization; however, at least one other diagnostic peak has to be found to confirm these two cases of extra-pair paternity. Note that the small peak in the father's profile, near in size to the diagnostic fragment, is another peak with a 2 bp difference in size.

each locus as 'independent'. We considered also the AFLP loci as markers with Mendelian inheritance. However, we outline that we did not make a true segregation analysis because it is difficult to check for Mendelian inheritance for such a small number of offspring per pair in this wild passerine bird and also because heterozygotes cannot be identified.

One fragment specific to females in the B primer pair was excluded from the paternity analysis but was useful in the sex determination of young (Questiau *et al.* 2000).

Extra-pair paternity (EPP). We looked for all diagnostic sites where a peak was absent in both parents (= recessive homozygotes) and present in one nestling or more (Fig. 1). We have assumed that there is no brood parasitism in this species (see Discussion). The presence of an extra peak is thus assumed to result from an extra-pair

fertilization with the sperm of another male (or several) possessing the fragment at this locus. We considered a nestling as an extra-pair young when at least two diagnostic peaks were found among all of its three profiles. This threshold reduces scoring errors that may result when using a single diagnostic marker. In studies using minisatellite patterns, an extra-pair young is usually defined by the presence of at least three novel bands taking into account the high mutation rate of minisatellites. In AFLP, mutations occur in restriction sites, primer elongation binding sites or possibly in the amplified region (detectable if they are insertions/deletions). The mutation rate is lower compared with minisatellites and two diagnostic peaks seem to be sufficient for detecting an extra-pair young. Moreover, in order to estimate the expected number of mismatches in case of EPP, we took one legitimate nestling per family ($n = 30$), except in the

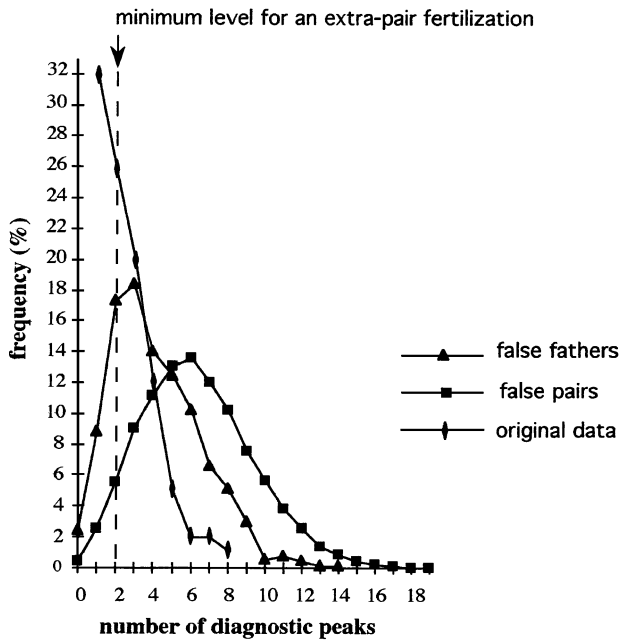


Fig. 2 Distribution of the number of mismatching peaks in young in our data and for young for which fathers or pairs other than the social one has been attributed. Note that for our data, the high number of 'one mismatch' is due both to true diagnostic peaks (i.e. undetected extra-pair fertilization according to the minimum level of acceptance) and to artefactual peaks.

families with complete extra-pair parentage. We allocated each nestling 'false' fathers by taking the AFLP profiles of each male of 1995 and 1996, except its own social father. Overall, 811 'false' father–nestling relationships have been simulated. The social mother has been considered as the true mother. We then counted the number of diagnostic peaks. The number of peaks ranged from 0 to 14 with a mean of 4.1 ± 2.4 (Fig. 2). We decided to use two diagnostic peaks as discriminant for paternity exclusion. For this threshold the estimated error to consider a case of EPP as a true paternity is 11.2%. A level of three mismatches would increase this error too much (28.5%). However, under these conditions, the level of EPP will be underestimated anyway. A test with false pairs has also been carried out to estimate the number of diagnostic peaks in case of false paternity and maternity (see Discussion).

Considering the acceptable rate of reproducibility (98.8%), the presence of diagnostic peaks is probably not due to incomplete digestion and/or ineffective amplification. When incomplete digestion or ineffective amplification occurred this was obvious, because the number of peaks in the profile was divided by two or by three. In only four cases was the sample extracted again and the overall procedure repeated.

We did not use the similarity index ($D = 2n_{ab}/n_a + n_b$ where n_{ab} is the number of fragments shared between

individuals a and b, and n_a and n_b represent the total number of peaks in the profiles of individuals a and b, respectively) to corroborate our results on extra peaks. Indeed, the distribution of the similarity index calculated for all pairwise comparisons of adults considered as nonrelatives (0.55 ± 0.09 , mean \pm SD), greatly overlapped that for first-order relatives (mother/offspring, father/offspring and offspring/offspring when extra-pair young were discarded) (0.51 ± 0.08). Even by considering only loci ($n = 20$) with markers at intermediate frequency (≈ 0.5), the overlap between the two distributions remained too large (background band sharing = 0.48 ± 0.15 and 0.66 ± 0.14 for nonrelatives and first-order relatives, respectively), that greatly limited the utility of such a calculation for studying relatedness (see Lynch & Milligan (1994)).

Exclusion probability. Because the markers are considered dominant, we had to assume that the population is in Hardy–Weinberg equilibrium to calculate the 'allele frequencies'. We considered only the adult population for the allele frequency calculations. As the allele frequency estimator (eqn 2a) of Lynch & Milligan (1994) gave the same results as the classical Hardy–Weinberg proportions, we decided to use the proportion of individuals with no peak for a given locus as the genotypic frequency of the recessive homozygotes (q^2), with q being the estimation of the frequency of the allele 'absence' in the population for that locus. We defined p as the frequency of the allele 'presence' with $p = 1 - q$. We focused only on polymorphic loci with $q^2 > 3/N$, with N being the number of adult individuals, as recommended by Lynch & Milligan (1994).

Exclusion probabilities were calculated: (i) at the population level; and (ii) for each family taken independently by using the allele frequencies calculated earlier for the adult population. At the population level, we used all loci and the equation of Chakraborty *et al.* (1974) with at least two diagnostic markers relevant for the detection of an extra-pair young. First, we calculated Q , the cumulative probability of exclusion for at least one diagnostic marker, for k markers:

$$Q = 1 - \prod_{i=1}^k (1 - \pi_i)$$

The exclusion probability P on at least two diagnostic peaks is:

$$P = Q - \sum_{i=1}^k \pi_i \prod_{\substack{j=1 \\ j \neq i}}^k (1 - \pi_j)$$

with π_i being the probability of exclusion based upon the i th marker, that is the probability of having no peak in both parents ($q_i^2 \times q_i^2$) and one allele 'present' p_i in a nestling (obligatory heterozygote as the putative mother is supposed to have sired all the young in her nest):

$$\pi_i = q_i^2 \times q_i^2 \times p_i$$

For each family, this probability can be calculated using a π_i value of p_i just for the i th favourable locus (that is no peak in both parents).

Results

In total, 162 nestlings were analysed in 36 families.

We used a total of 81 polymorphic loci (23 for B out of a total of 56 positions, 24 for G out of 87 positions and 34 for Y out of 89 peaks) with a frequency of peaks for each locus ranging from 0.16 to 0.92 (mean \pm SD = 0.53 ± 0.25) for the population (all the adults). The mean number of peaks scored in each individual was 41 ± 5 (mean \pm SD) for the combination of the three primer pairs. The allele frequency (p_i) ranged from 0.06 to 0.71.

In 23 broods (63.8%), we revealed at least one nestling issued from an extra-pair mating, totalling 41.9% of all young. A male (or several) other than the attendant one sired all the young in six broods (16.6%). The number of diagnostic peaks for the extra-pair young ranged from two to eight with a mean of 3.2 ± 1.4 . In 31 nestlings out of 162 (19.1%) there was a single mismatched peak that was not considered as diagnostic according to the level of acceptance defined in Materials and methods. Those nestlings were considered legitimate. Figure 3 shows the proportional distribution of extra-pair young in each brood.

For the combination of the three primer pairs, the global exclusion probability for the population considering all loci was 0.93. For each primer pair taken separately the exclusion probability was 0.33, 0.29 and 0.61 for B, G and Y, respectively. Figure 4 shows the exclusion probability for each primer pair and different combinations of two to three primer pairs. For each family, the exclusion probability, calculated only for the loci where 'absence' is

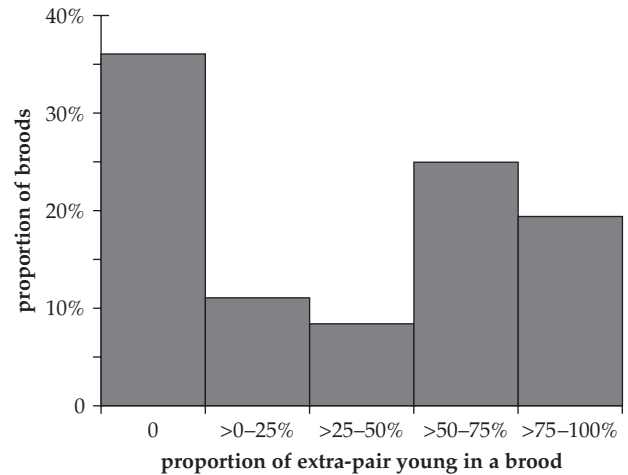


Fig. 3 Distribution of extra-pair young in bluethroat broods.

recorded for both parents, ranged from 0.79 to 0.99 with a mean of 0.93 ± 0.05 . Seventy-five per cent of all families had an exclusion probability above 0.90 (Fig. 5).

Discussion

The aim of this study was to assess the potential utility of AFLP markers for studying extra-pair parentage in a bird species. To our knowledge, this is the first time these markers have been used for this purpose. In this study, we assessed the number of extra-pair young in broods. Nevertheless, compared with single-locus methods with codominant alleles, we were confronted with some limitations to the analysis of extra-pair fertilizations. A diagnostic peak reveals an extra-pair young, but in practice it is not possible with this technique and the three primer pairs used to exclude maternity, paternity or both. For that aim, the similarity index was of little use as the overlap between the similarity distributions between relatives

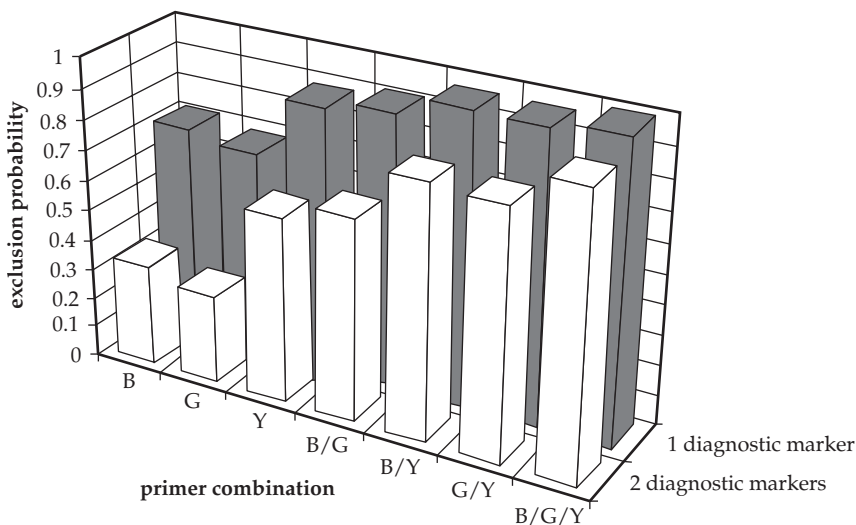


Fig. 4 Exclusion probability for the three primer pairs taken independently, by two or together considering a single diagnostic marker as relevant for detecting an extra-pair paternity (probability Q , see text) or considering at least two diagnostic markers (probability P).

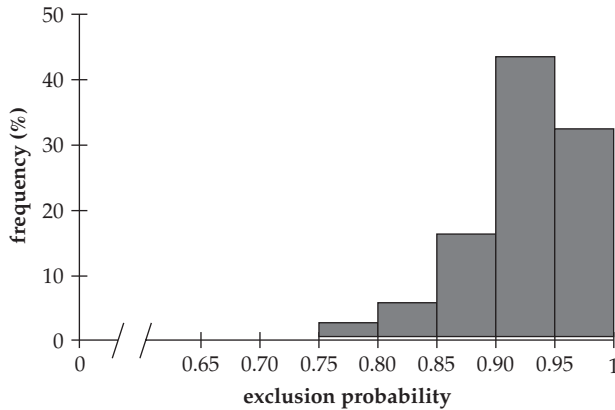


Fig. 5 Distribution of the exclusion probabilities for each blue-throat family for the combination of the three primer pairs.

and nonrelatives was too great (see Lynch & Milligan (1994)). In order to calculate the exclusion probabilities, we have assumed that one parent (male or female) is excluded. This assumption allows us to consider an extra-pair young as a heterozygote for the diagnostic locus and to calculate relatively easily the probability of a single allele 'presence' at this locus. We preferred to cast doubt on paternity because it is more likely than brood parasitism. One can argue that we cannot be sure that there is no brood parasitism in this population. However, it has never been reported in the bluethroat. The absence of brood parasitism has been confirmed by Krokene *et al.* (1996) in a genetic study of the mating system with minisatellites in another subspecies. Our own observations of nests from the beginning of the laying period confirmed that females lay one egg per day. We never observed the appearance of more than one egg in a day that would indicate brood parasitism. Moreover, the number of diagnostic peaks can theoretically reach 14 for the three profiles (B + G + Y) in the case of a single illegitimate parent (see the test with the 'false' fathers in Materials and methods). The presence of eight diagnostic peaks (the maximum detected) is compatible with a case of extra-pair fertilization due to a single unfaithful parent. We think that if brood parasitism had indeed occurred we would have revealed a larger number of diagnostic peaks unless the resident male had fertilized the parasite female. To verify this assumption, we have simulated false pairs by taking one legitimate nestling per nest ($n = 30$) to whom all possible adult pairs were allocated. A total of 24 533 parentage tests were performed. The mean number of diagnostic peaks revealed for both illegitimate parents was 6.3 ± 2.9 and was significantly larger than the number of diagnostic peaks in the case of false paternity despite a large overlap of the two distributions ($T = 4.35$; $df = 58$; $P = 0.0001$) (Fig. 2).

Assuming that extra-pair young are due to EPP, the exclusion probabilities can be calculated: with a

combination of three primer pairs, this probability can be increased from 1.5 to 3 times compared with the use of a single primer pair. Given the exclusion probability for each family and at the population level (93%), AFLP markers were efficient in giving an approximate estimate of the importance of EPP within this population. However, the exclusion probabilities are relatively low compared with other markers such as microsatellites. We could increase this level by adding other primer pairs which would detect suitable polymorphisms.

Due to the dominant nature of the AFLP markers and the number of primer pairs used in this study, it seems difficult to assign paternity from amongst a number of potential males (Wetton *et al.* 1995; Double *et al.* 1997). First, we did not analyse all the potential sires (other territorial males or floaters); second, the dominant nature of the marker prevents the identification of heterozygotes. If several males are potential sires it may not be possible to discriminate amongst them. The number of diagnostic peaks may have to be increased, as well as the sampling effort. This requires the use of additional primer pairs, which is time-consuming and expensive. However, this method can give an initial estimate of the importance of extra-pair fertilizations in taxa where no codominant markers, such as microsatellites, are available. If the question is whether extra-pair parentage occurs or not in a population, or a species that has never been investigated, AFLP are sufficient. If more detail is required concerning the true sire, more discriminating codominant markers are needed.

In contrast to minisatellite fingerprints, the double amplification permits the use of small DNA starting quantities (at least 50 ng of good-quality DNA is sufficient for AFLP but is probably unsuitable for minisatellite profiling). However, we used growing feathers and are not sure that mature feathers would yield enough DNA to be used in AFLP. Moreover, given the large number of polymorphic fragments that can be obtained with several primer combinations, this method can be used simultaneously for other purposes such as investigating population genetic structure (Travis *et al.* 1996; Escaravage *et al.* 1998) or sex determination (Questiau *et al.* 2000).

By using two diagnostic peaks as the criteria to detect a case of EPP, we revealed a high number of extra-pair fertilizations at the population level. This result can be compared to the high rate of EPP reported in several species such as the willow warbler *Phylloscopus trochilus* (Fridolfsson *et al.* 1997) and the tree swallow *Tachycineta bicolor* (Lifjeld *et al.* 1993). Krokene *et al.* (1996) studied another bluethroat population in Norway with minisatellite probes and found 35% of all nests with at least one extra-pair young, and 20% of all young not being sired by their putative father. The large difference between the two

populations is not necessarily surprising. Large variation within the same species has already been reported for the willow warbler for which Gyllensten *et al.* (1990) found no case of EPP in one population whilst 50% of the broods contained at least one extra-pair offspring in another population (G. Bjørnstad and J. T. Lifjeld, personal communication). In the Guérande saltmarsh, the density of birds is high (mean: 7 pairs/10 ha for the two breeding seasons) compared with the Norwegian population (3.8 pairs/10 ha; Anthonisen *et al.* 1997). Despite Westneat & Sherman's (1997) general finding that there seems to be no evidence of a correlation between density and extra-pair fertilization frequencies in birds, density may be important in individual cases and particularly in bluethroat. Males probably spend more time in defending their territory against a potentially large number of intruders than in guarding their female. Additional behavioural studies are needed to test this hypothesis. Moreover, the spatial structure of the marsh with a tight network of high embankments reducing mate-guarding efficiency, can provide females the opportunity to seek extra-pair copulations more or less discreetly. Smiseth & Amundsen (1995) have shown that radio-tracked bluethroat females regularly entered territories of neighbouring males. Mate guarding can thus be considered as less efficient in this landscape structure. The Guérande population is actually isolated from the other subspecies and is believed to have undergone a recent bottleneck (Questiau *et al.* 1998) which could explain the high similarity index found in this population. Contrary to the expectation of Petrie & Kempnaers (1998) that low frequencies of EPP would be expected in such populations, we found a higher rate of extra-pair fertilization than in the Norwegian population. Thus, the variation in the frequency of extra-pair offspring may be more related to ecological factors rather than to differences in genetic diversity. However, the comparison is difficult as the techniques used are not the same. It would be interesting to compare the different techniques in the same population to test their congruence.

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References

- Ajmone-Marsan P, Vecchiotti-Antaldi G, Bertoni G, Valentini A, Cassandro M, Kuiper M (1997) AFLP™ markers for DNA fingerprinting in cattle. *Animal Genetics*, **28**, 418–426.
- Anthonisen K, Krokene C, Lifjeld JT (1997) Brood division is associated with fledging dispersion in the bluethroat (*Luscinia s. svecica*). *Auk*, **114**, 553–561.
- Birkhead TR, Møller AP (1992) *Sperm Competition in Birds. Evolutionary Causes and Consequences*. Academic Press, London.
- Brün J, Winkel W, Epplen JT, Lubjuhn T (1996) Elternschaftsnachweise bei Trauerschnäppern *Ficedula hypoleuca* am Westrand ihres mitteleuropäischen Verbreitungsareals. *Journal für Ornithologie*, **137**, 435–446.
- Burke T, Hanotte O, Bruford MW, Cairns E (1991) Multilocus and single-locus minisatellite analysis in population biological studies. In: (eds Burke T, Dolf G, Jeffreys AJ, Wolff R) *DNA Fingerprinting: Approaches and Applications*, pp. 154–168. Birkhäuser, Basel.
- Chakraborty R, Shaw M, Schull WJ (1974) Exclusion of paternity: the current state of the art. *American Journal of Human Genetics*, **26**, 477–488.
- Dixon A, Ross D, O'Malley SLC, Burke T (1994) Paternal investment inversely related to degree of extra-pair paternity in the reed bunting. *Nature*, **371**, 698–700.
- Double MC, Cockburn A, Barry SC, Smouse PE (1997) Exclusion probabilities for single-locus paternity analysis when related males compete for matings. *Molecular Ecology*, **6**, 1155–1166.
- Dunn PO, Cockburn A (1996) Evolution of male parental care in a bird with almost complete cuckoldry. *Evolution*, **50**, 2542–2548.
- Escaravage N, Questiau S, Pornon A, Doche B, Taberlet P (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology*, **7**, 975–982.
- Fleischer RC, Tarr CL, Pratt TK (1994) Genetic structure and mating system in the palila, an endangered Hawaiian honeycreeper, as assessed by DNA fingerprinting. *Molecular Ecology*, **3**, 383–392.
- Fridolfsson A-K, Gyllensten UB, Jacobsson S (1997) Microsatellite markers for paternity testing in the willow warbler *Phylloscopus trochilus*: high frequency of extra-pair young in an island population. *Hereditas*, **126**, 127–132.
- Gibbs HL, Weatherhead PJ, Boag PT, White BN, Tabak LM, Hoysak DJ (1990) Realized reproductive success of polygynous red-winged blackbirds revealed by DNA markers. *Science*, **250**, 1394–1397.
- Glutz von Blotzheim U, Bauer KM (1994) *Handbuch der Vögel Mitteleuropas*, Vol. 11. Aula, Wiesbaden.
- Gyllensten UB, Jacobson S, Temrin H (1990) No evidence for illegitimate young in monogamous and polygynous warblers. *Nature*, **343**, 168–170.
- Hanotte O, Burke T, Armour JAL, Jeffreys A (1991) Hypervariable minisatellite DNA sequences in the Indian Peafowl *Pavo cristatus*. *Genomics*, **9**, 587–597.
- Hanotte O, Zanon C, Pugh A, Greig C, Dixon A, Burke T (1994) Isolation and characterization of microsatellite loci in a passerine bird: the reed bunting *Emberiza schoeniclus*. *Molecular Ecology*, **3**, 529–530.
- Hartley IR, Davies NB, Hatchwell BJ, Desrochers A, Nebel D, Burke T (1995) The polygynandrous mating system of the alpine accentor, *Prunella collaris*. II. Multiple paternity and parental effort. *Animal Behaviour*, **49**, 789–803.
- Hasselquist D, Bensch S, von Schantz T (1995) Low frequency of extrapair paternity in polygynous great reed warbler, *Acrocephalus arundinaceus*. *Behavioral Ecology*, **6**, 27–38.

- Johnsen A, Lifjeld JT (1995) Unattractive males guard their mates more closely: an experiment with bluethroats (Aves, Turdidae: *Luscinia s. svecica*). *Ethology*, **101**, 200–212.
- Kempnaers B, Dhondt AA (1993) Why do females engage in extra-pair copulations? A review of hypotheses and their predictions. *Belgium Journal of Zoology*, **123**, 93–103.
- Krokene C, Anthonisen K, Lifjeld JT, Amundsen T (1996) Paternity and paternity assurance behaviour in the bluethroat, *Luscinia s. svecica*. *Animal Behaviour*, **52**, 405–417.
- Lifjeld JT, Dunn PO, Robertson RJ, Boag PT (1993) Extra-pair paternity in monogamous tree swallows. *Animal Behaviour*, **45**, 213–229.
- Lifjeld JT, Slagsvold T, Lampe HM (1991) Low frequency of extra-pair paternity in pied flycatchers revealed by DNA fingerprinting. *Behavioral Ecology and Sociobiology*, **29**, 95–101.
- Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**, 91–99.
- Majer D, Mithen R, Lewis BG, Vos P (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research*, **100**, 1107–1111.
- Otsen M, den Bieman M, Kuiper MTR *et al.* (1996) Use of AFLP markers for gene mapping and QTL detection in the rat. *Genomics*, **37**, 289–294.
- Petrie M, Kempnaers B (1998) Extra-pair paternity in birds: explaining variation between species and populations. *Trends in Ecology and Evolution*, **13**, 52–58.
- Primmer CR, Møller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology*, **5**, 365–378.
- Questiau S, Eybert MC, Guginskaya AR, Gielly L, Taberlet P (1998) Recent divergence between two morphologically differentiated subspecies of bluethroat (Aves: Muscicapidae: *Luscinia svecica*) inferred from mitochondrial DNA sequence variation. *Molecular Ecology*, **7**, 239–245.
- Questiau S, Escaravage N, Eybert MC, Taberlet P (2000) Nestling sex ratios in a wild population of bluethroat (*Luscinia svecica*) inferred from AFLP™ analysis. *Journal of Avian Biology*, in press.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Schondelmaier J, Steinrücken G, Jung C (1996) Integration of AFLP markers into linkage map of sugar beet (*Beta vulgaris* L.). *Plant Breeding*, **115**, 231–237.
- Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82–90.
- Smiseth PT, Amundsen T (1995) Female Bluethroats (*Luscinia s. svecica*) regularly visit territories of extrapair males before egg laying. *Auk*, **112**, 1049–1053.
- Taberlet P, Bouvet J (1991) A single plucked feather as a source of DNA for bird genetic studies. *Auk*, **108**, 959–960.
- Travis SE, Maschinski J, Keim P (1996) An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology*, **5**, 735–745.
- Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.
- Vos P, Kuiper M (1996) AFLP™. In: (eds Caetano-Anolles G, Gresshoff PM) *DNA Markers: Protocols, Applications and Overviews*. J. Wiley & Sons Inc., UK.
- Westneat DF (1995) Paternity and paternal behaviour in the red-winged blackbird, *Agelaius phoeniceus*. *Animal Behaviour*, **49**, 21–35.
- Westneat DF, Sherman PW (1997) Density and extra-pair fertilizations in birds: a comparative analysis. *Behavioral Ecology and Sociobiology*, **41**, 205–215.
- Wetton JH, Burke T, Parkin DT, Cairns E (1995) Single-locus DNA fingerprinting reveals that reproductive success increases with age through extra-pair paternity in the house sparrow. *Proceedings of the Royal Society London B*, **260**, 91–98.
- Yamagishi S, Nishiumi I, Shimoda C (1992) Extrapair fertilization in monogamous bull-headed shrikes revealed by DNA fingerprinting. *Auk*, **109**, 711–721.

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