

## Brief report

# Extra-pair paternity in the boreal, socially monogamous Grey-headed Chickadee (*Poecile cinctus*)

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Extra-pair mating is a common alternative reproductive strategy among socially monogamous bird species, but empirical data on the genetic mating systems of most species are still lacking. Here we present the first parentage study in the Grey-headed Chickadee (*Poecile cinctus*), a socially monogamous passerine breeding at low densities in the boreal region of the Palaearctic and Nearctic. In a population in northeastern Norway sampled across six years, the probability for a brood to contain at least one extra-pair offspring was 37.5% (95% CI: 17.9–62.3%; total  $N = 16$ ) and on average 16.7% (95% CI: 6.9–35.0%; total  $N = 120$ ) of nestlings per brood were sired extra-pair.

## 1. Introduction

Females of many socially monogamous bird species regularly engage into extra-pair copulations which often leads to extra-pair paternity (EPP), i.e., part or even all offspring in a brood are not sired by the male providing parental care (reviewed by Griffith *et al.* 2002, Kempenaers & Schlicht 2010). Empirical data on the genetic mating systems of most extant bird species are, however, still lacking. This shortcoming not only hampers our in-depth understanding of a particular species' reproductive ecology, but also limits the power of comparative approaches to explain interspecific patterns of variation in mating systems

(e.g., Bonier *et al.* 2014) and avian reproductive traits (e.g., Kleven *et al.* 2009).

Many species of the Paridae (titmice and chickadees) are predominantly socially monogamous and readily accept nest boxes. They are therefore frequently used as model species for studying mating systems and the ecology and evolution of avian reproductive traits in general and EPP in particular (e.g., Otter 2007). In fact, rates of EPP have been estimated for a good number of Paridae species across different genera (see supplementary data in Biagolini-Jr. *et al.* 2017) including three *Poecile* species, for which the overall frequency of extra-pair offspring (EPO) ranged from 6.0% in the Willow Tit (*P. montanus*)

(Lampila *et al.* 2011, Orell *et al.* 1997) and 12.8% in the Black-capped Chickadee (*P. atricapillus*) (Mennill *et al.* 2004, Otter *et al.* 1998) to 17.9% in the Mountain Chickadee (*P. gambeli*) (Bonderud *et al.* 2018).

The aim of our present study was to examine, for the first time, the genetic mating system of the Grey-headed Chickadee (*Poecile cinctus*), a territorial and socially monogamous passerine breeding in the boreal region of the Palaearctic and Nearctic (Cramp & Perrins 1993).

## 2. Material and methods

### 2.1. Study species, study population and field methods

The Grey-headed Chickadee is a small (11–13 g), insectivorous, hole-nesting passerine (Cramp & Perrins 1993). It is a habitat specialist that prefers old virgin coniferous forest for breeding. Grey-headed Chickadees are predominantly sedentary with large home ranges and correspondingly low breeding densities (Cramp & Perrins 1993). They are sexually monomorphic, socially monogamous and single-brooded with clutch sizes ranging from 4–11 eggs (Cramp & Perrins 1993). Females incubate the eggs alone while both parents feed the young (Cramp & Perrins 1993).

We studied Grey-headed Chickadees in a nest box population in the Pasvik Valley (69°28'N, 29°50'W) in northeastern Norway. The study site contained 119 nest boxes on both sides along approximately 20 km of roads, with varying distances between the boxes. Annual number of Grey-headed Chickadee pairs occupying these nest boxes ranged from four to eight over a six year study period. Field work was carried out during six breeding seasons in June between 2013 and 2018. Territorial adults were trapped while they were feeding nestlings either just outside their nest box with a mist net when the birds were about to enter or inside the nest box by closing the entrance hole. We considered these adults to be the social parents of any focal brood.

Adults were banded with a numbered aluminum ring provided by the Norwegian Bird Ringing Centre at Stavanger Museum. A small droplet (~10 µL) of blood was sampled and stored in 1 mL

Queen's lysis buffer (Seutin *et al.* 1991) at 4°C until further use. Adults were sexed in the field according to the shape of their cloacal protuberance and the presence (females) or absence (males) of a brood patch (Svensson 1992). As some individuals proved difficult to sex in the field, molecular genetic analysis was used to ascertain the sex for all adult birds (see also below); all adult individuals ( $N=38$ ) proved to have been sexed correctly in the field.

Approximately 4–12 days after hatching, we collected a small (~10 µL) sample of blood by brachial vein puncture from nestlings (or tissue samples from dead nestlings) that was stored in 1 mL Queen's lysis buffer (Seutin *et al.* 1991) at 4°C until further use. Permits to capture, handle and ring the birds were issued by the Norwegian Environment Agency to OK (A-license 1082). Permits to sample blood were approved by the Norwegian Animal Research Authority (permits no. 7863 and 12088).

### 2.2. Parentage analysis

Genomic DNA was extracted from blood using the QIAamp 96 Blood Kit (Qiagen, Hilden, Germany) and from tissue using the Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) following the protocols of the manufacturers. Sex of adult birds was determined using the universal primers P2 and P8 (Griffiths *et al.* 1998). All samples were genotyped at eight polymorphic autosomal microsatellite loci plus one Z-linked microsatellite locus (Table 1). All primers were combined into a single multiplex polymerase chain reaction (PCR) run using fluorescently-labeled forward primers and a multiplex PCR Kit (Qiagen).

PCR products were separated on an ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and allele sizes were assigned using GENEMAPPER v5.0 software (Applied Biosystems). Marker polymorphism and informativity were calculated using GenAlEx 6.5 (Peakall & Smouse 2012); for results see Table 1. Arlequin v.3.5 (Excoffier & Lischer 2010) was used to test for deviation from linkage equilibrium and a Bonferroni correction for multiple statistical tests (Rice 1989) was applied to linkage disequi-

Table 1. Characteristics of microsatellite markers used for parentage analysis and sex determination in the Grey-headed Chickadee (*Poecile cinctus*).

Locus.	Reference	<i>n</i>	#	Allele size range (bp)	$H_O$	$H_E$	$P_{HWE}$	$Freq_{NULL}$	$P_{1p}$	$P_{2p}$
Escp6	Hanotte <i>et al.</i> (1994)	38	6	106–132	0.79	0.64	0.47	−0.092	0.38	0.22
Pat MP 2-14	Otter <i>et al.</i> (1998)	38	5	122–134	0.55	0.59	0.19	0.024	0.31	0.18
Pat MP 2-43	Otter <i>et al.</i> (1998)	38	8	148–168	0.82	0.79	0.17	−0.015	0.60	0.42
PmaTGAn42	Saladin <i>et al.</i> (2003)	38	9	248–304	0.76	0.85	0.36	0.045	0.69	0.53
PmaTGAn45	Saladin <i>et al.</i> (2003)	38	8	299–348	0.34	0.33	1.00	−0.010	0.19	0.06
Titgata02	Wang <i>et al.</i> (2005)	38	14	205–241	0.82	0.85	0.65	0.019	0.71	0.55
Titgata39	Wang <i>et al.</i> (2005)	38	9	212–240	0.66	0.71	0.70	0.032	0.51	0.32
Z-054 <sup>1</sup>	Dawson <i>et al.</i> (2015)	16	4	284–290	0.56	0.61	0.78	0.028	0.36	0.20
P2/P8	Griffiths <i>et al.</i> (1998)	16♂ 22♀	1 2	325 325;384	0 1	0 1				

*n*, number of presumably unrelated adult individuals; #A, number of alleles; bp, base-pairs;  $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity;  $P_{HWE}$ , probability of deviation from Hardy-Weinberg equilibrium;  $Freq_{NULL}$ , estimated frequency of null alleles according to the Brookfield method implemented in MICRO-CHECKER (van Oosterhout *et al.* 2004) with numbers in bold indicating evidence of null alleles (due to general excess of homozygotes for most allele size classes);  $P_{1p}$ , exclusion probability assuming the mother was known;  $P_{2p}$ , exclusion probability assuming the mother was unknown; 1) Z-054 is Z-chromosome-linked and thus marker polymorphism was calculated for males only. Marker polymorphism and deviation from Hardy-Weinberg equilibrium were calculated using GenAlEx 6.5 (Peakall & Smouse 2012).

librium *p* values. None of the loci deviated significantly ( $\alpha < 0.05$ ) from Hardy-Weinberg equilibrium; there was, however, indication of null alleles (i.e., non-amplifying alleles) at locus *PmaTAGAn71*.

Furthermore, significant linkage disequilibrium was detected for one locus combination (*PmaTAGAn71–PmaTGAn45*). As locus *PmaTAGAn71* had both indications of null alleles and was in linkage with another locus, it was excluded from any downstream analyses. For the remaining seven autosomal markers, the combined exclusion probability assuming the mother was known was 0.994 and the combined exclusion probability assuming the mother was unknown was 0.950. Adding the Z-linked marker and analysing males only, the combined exclusion probabilities were 0.994 and 0.950, respectively, allowing reliable assignment of offspring paternity status as within-pair offspring (WPO) or EPO. This assessment is underscored by the fact that there were no allelic mismatches between nestlings and putative mothers.

Nestlings were considered WPO if their allele sizes matched those of the putative parents at all loci or mismatched at a single locus (with either parent). Nestlings with two or more mismatches with the putative father's allele sizes were considered EPO. There was just one case with a single allelic mismatch between a nestling and the puta-

Table 2. Frequency of extra-pair offspring (EPO) in the Grey-headed Chickadee (*Poecile cinctus*) study population.

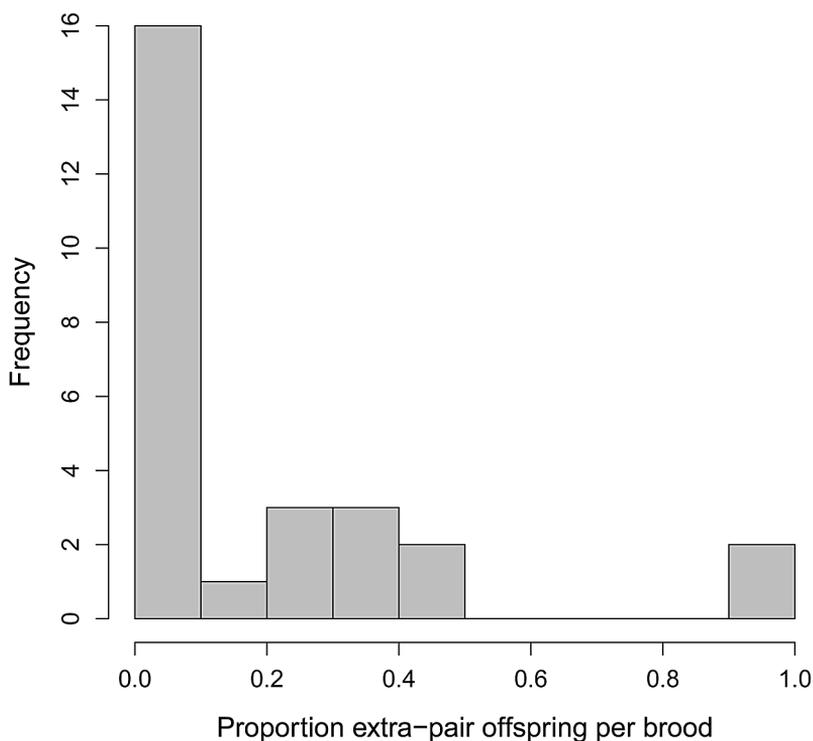
Year	Broods with EPO	Percentage EPO
2013	0.0% of 4	0.0% of 38
2014	50.0% of 4	7.7% of 26
2015	66.7% of 3	50.0% of 20
2016	40.0% of 5	14.7% of 34
2017	40.0% of 5	17.9% of 39
2018	50% of 6	21.7% of 46

tive father (at locus *Titgata02*). In this case, the offspring allele differed in size by a single repeat unit (four base pairs) compared to that of the closest-sized allele of the putative father. As microsatellite mutations typically occur by addition or deletion of a single repeat unit (e.g., Anmarkrud *et al.* 2008), we assumed this allelic mismatch to result from a mutation and considered the nestling as sired within-pair.

### 2.3. Statistical analysis

Across the six study years, we obtained data on offspring paternity for 203 nestlings from 27 broods of 23 different social pairs involving 20 different social mothers and 16 different social fa-

Fig. 1 Frequency distribution of the proportion of extra-pair offspring per brood (total  $N = 203$  offspring) for 27 Grey-headed Chickadee (*Poecile cinctus*) broods of 23 different pairs involving 20 different social mothers and 16 different social fathers sampled across six consecutive breeding seasons.



thers. We describe patterns of EPP based on the full data set of 27 broods, but restrict statistical modelling for obtaining population-level estimates including confidence intervals to a reduced data set of 16 broods in which each individual is represented only once to avoid pseudoreplication. For the latter data set, we used generalised linear models (GLMs) with logit link and binomial or quasibinomial errors to model i) the probability that a brood contained at least one EPO and for ii) estimating the proportion of EPO per brood (the latter using the R function *cbind* to create the independent variable as a column-bind matrix of the number of EPO and the number of WPO, respectively).

We estimated these figures including corresponding 95% Wald confidence intervals by fitting the grand mean as the only effect. Quasibinomial instead of binomial errors were assumed for the proportion of EPO per brood because inspection of the dispersion parameter indicated overdispersion. We refrained from using year of study as an additional effect due to the low number of broods per year. We used R 3.5.3 (R Core Team 2019) for all computations.

### 3. Results

There were no allelic mismatches between nestlings and putative mothers and hence, there were no indications of intra-specific brood parasitism. In the full data set, 11 (40.7%) out of 27 broods contained at least one EPO and overall 34 (16.7%) out of 203 nestlings were not sired by the male providing parental care (Table 2). The mean number of allelic mismatches between EPO and the male providing parental care was 3.7 (range: 2–6). There were two broods (of different pairs) in which all nestlings (six and seven, respectively) had been sired extra-pair (Fig. 1). Extra-pair sires were identified for 18 (52.9%) of the 34 EPO which could be assigned to three different genetic fathers: 12 EPO in three different broods over three years to one male who himself also held a territory (occupied a nest box and sired WPO) in each of the three years; and two times three EPO in one brood each to each of two other males that were not recorded breeding in a nest box in the respective years. The maximum number of extra-pair sires in a focal brood was two (one case).

In the reduced (non-pseudoreplicated) data set

of 16 broods, the probability for a brood to contain at least one EPO was 37.5% (95% CI: 17.9–62.3%) and on average 16.7% (20/120; 95% CI: 6.9–35.0%) nestlings per brood were EPO. Among six broods with at least one EPO, on average 46.5% (20/43) of nestlings had been sired extra-pair.

#### 4. Discussion

Molecular genetic analyses during the last three decades have revealed that social monogamy does not equal genetic monogamy in birds (reviewed by Griffith *et al.* 2002, Kempnaers & Schlicht 2010). In our study of the socially monogamous Grey-headed Chickadee, we found that extra-pair mating was a common reproductive strategy, as approximately 40% of the broods contained extra-pair offspring and around 17% of offspring per brood were EPO on average. It should be noted, however, that about half of all EPO were sired by just three males, hence the incidence of EPP might be driven by a few individuals in our study population.

Among 132 passerine species with a predominantly socially monogamous mating system, the median frequency of EPO per brood was 15.3% (data extracted from supplementary material of Biagolini-Jr. *et al.* 2017). Our point estimate of the frequency of EPO in the Grey-headed Chickadee was thus close to the average level among these passerine species although substantial uncertainty in our estimates is reflected by relatively wide confidence intervals. Our estimates were also comparable to those of congeners (see introduction) and also to other Paridae species across different genera (e.g., Great Tits (*Parus major*), Lubjuhn *et al.* (2007), Coal Tits (*Pariparus ater*), Schmoll *et al.* (2009), and African Blue Tits (*Cyanistes tene-riffae*), Garcia-Del-Rey *et al.* (2012)).

It should be noted though that evidence from Blue Tits suggests that the probability for an egg to be sired extra-pair may decline with the laying sequence (Magrath *et al.* 2009). In the case of nestling mortality, hatching asynchrony may disadvantage hatchlings from eggs laid late in the laying sequence, which could potentially lead to an upward bias of EPO frequencies when broods suffering mortality are sampled relatively late during the

nestling period. Due to logistical reasons, we unfortunately have no information on clutch sizes or brood sizes at hatching.

The Grey-headed Chickadee is a boreal species typically breeding in low densities (Cramp & Perrins 1993) which likely decreases extra-pair mating opportunities, thereby potentially increasing the costs of extra-pair mating behavior for both sexes (reviewed in Birkhead & Møller 1992). Comparisons of multiple study plots with high versus low nest box densities may help to elucidate the effect of breeding densities on patterns of EPP in the study species.

In two broods of different pairs, parentage analysis revealed that all genotyped nestlings had been sired extra-pair. One of these two males sired WPO in three previous years, while for the other male we only have paternity data from the one year. The latter male did also not sire any EPO. Total within-pair paternity loss may be due to male functional infertility, which has been observed in 2–4% of males in other passerines (Lifjeld *et al.* 2007), including a Paridae species (Schmoll & Kleven 2016). Further research is required to determine the frequency of male infertility among Grey-headed Chickadees and to evaluate whether this could be a factor contributing to selection for female extra-pair mating behaviour as suggested by the fertility insurance hypothesis (Wetton & Parkin 1991)

In summary, our results provide the first estimate of the frequency of EPP in the Grey-headed Chickadee which now is available for comparative work. We found that extra-pair mating is a common reproductive strategy in a Norwegian population of Grey-headed Chickadees.

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#### Lapintiaisen parin ulkokuoliset parittelut

Parin ulkokuoliset parittelut ovat yleinen lisääntymisstrategia sosiaalisesti monogaamisilla linnuilla. Empiirinen aineisto on kuitenkin puutteellinen

monilla lajeilla. Tässä tutkimuksessa esitetään ensimmäistä kertaa aineistoa lapintiaisen geneettisestä isyydestä. Lapintiaisen on sosiaalisesti monogaaminen varpuslintu, joka pesii alhaisissa tiheyksissä koko havumetsävyöhykkeellä. Koillis-Norjassa olevasta lapintiaispopulaatiosta kerättiin aineistoa kuuden vuoden ajalta ja havaittiin, että 37.5 %:n todennäköisyydellä (95 % luottamusväli: 17.9–62.3 %, otoskoko 16) pesässä oli yksi parin ulkopuolinen poikanen, ja keskimäärin 16.7 % (luottamusväli 6.9–35 %, otoskoko 120) pesän poikasista oli parin ulkopuolisista partitteleista.

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