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Microsatellite analysis detects low rate of extra-pair paternity in Tengmalm's owl, *Aegolius funereus*

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Abstract. Genetic methods enable to reveal cryptic parental contributions in reproduction, especially in socially monogamous species. The rate of extra-pair paternity is generally low in raptors and owls and its presence in some species is tightly associated with sequential polyandry which can rarely occur in years with high food availability. In this study we investigated the mating system of the Tengmalm's owl (*Aegolius funereus*) in the Ore Mountains (Czech Republic). In this area, the species usually nests in high breeding densities which could increase an opportunity for extra-pair copulations. In total, 297 individuals (54 females, 47 males and 196 juveniles) from 46 nest boxes were genotyped at seven microsatellite loci. We present results of a five-year study and four extra-pair nestlings (2.3 %) were detected which did not result from sequential polyandry.

Key words: boreal owl, mating system, microsatellites, population density

Introduction

Application of molecular approach in paternity studies revealed that genetic monogamy is relatively rare in birds. It has been shown that less than 25 % of all studied socially monogamous birds are truly genetically monogamous (Griffith et al. 2002). Several hypotheses have been proposed to explain extra-pair paternity (EPP) (e.g. Gray 1997, Neff & Pitcher 2005, Kempenaers 2007, Hasson & Stone 2011) but generally EPP is a way to maximize the reproductive success of both males and females. Whether or not an individual engages in extra-pair copulation (EPC) depends on its cost against benefit (Petrie & Kempenaers 1998, Arnqvist & Kirkpatrick 2005, Forstmeier et al. 2014).

The rate of EPP depends on many different factors e.g. breeding synchrony (Weatherhead & Yezerinac 1998, Chuang et al. 1999, Stewart et al. 2010), male parental care (Schwagmeyer et al. 1999, Møller 2000, Arnold & Owens 2002), longevity (Mauck et al. 1999, Arnold & Owens 2002), and genetic variability within a population (Dreiss et al. 2008, Gohli et al. 2013). In general, EPP rate can increase with genetic variability within a population (Petrie & Lipsitch 1994, Petrie et al. 1998) and higher breeding synchrony and can decrease with longevity and parental care (Westneat et al. 1990, Griffith et al. 2002). The most frequently discussed factor is breeding density (Birkhead & Møller 1992, Mougeot 2004, Stewart et al. 2010). It is obvious that the probability to meet potential extra-pair partners decreases at low breeding densities and EPC becomes energetically costly in this case (Birkhead & Møller 1992). Westneat & Sherman (1997), for instance, found positive correlation between breeding density and EPP in eight of 11 bird species and also many other studies revealed similar relationship (e.g. Gowaty & Bridges 1991, Bjørnstad & Lifjeld 1997, Stewart et al. 2010, but see Gyllensten et al. 1990, Moore et al. 1999, Conrad et al. 2001). Nevertheless, species, such as owls, with high investment into paternal care usually exhibit low rate of promiscuity (Birkhead & Møller 1996, Gowaty 1996, Møller 2000). Therefore the rate of EPP is rarely discussed with above-mentioned factors in these species

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(e.g. Westneat et al. 1990, Korpimäki et al. 1996, Rodriguez-Martínez et al. 2014). In owls, the highest EPP rate (6.5 %) was found in burrowing owl (*Athene cunicularia*; Johnson 1997, Roulin et al. 2004). However, this estimate could be biased because this study used small sample size of drastically decreasing and isolated population (Koopman et al. 2007).

Tengmalms owl (*Aegolius funereus*) inhabits boreal and subalpine forests in the Holarctic region, across Europe, Asia and North America (Korpimäki & Hakkarainen 2012) and its breeding density as well as its nesting success strongly vary between years, depending on the gradation cycles of its main prey, rodents (Korpimäki 1988, Zárybnická et al. 2009a, Korpimäki & Hakkarainen 2012, Zárybnická et al. 2015a). It is well known that the Tengmalm's owl male provides the complete food to his female since the beginning of courtship period and feeds their nestlings until they are three weeks old, when the female can also begin to hunt (Korpimäki 1981, Zárybnická 2009b, Zárybnická & Vojar 2013). Therefore, such costly male foraging behaviour does not allow effective female-guarding which is the primary mechanism for preventing EPC (Korpimäki et al. 1996, Arnold & Owens 2002, Kokko & Morrell 2005). In addition, some males become polygynous in years with high main prey abundance (Carlsson et al. 1987, Korpimäki 1983, 1989, 1991). Beside polygyny, sequential polyandry was also described in this species (Solheim 1983, Šindelář et al. 2015). Having abandoned the brood before they fledge, females started to nest with another partner in a different nest hole while their primary males continued rearing of the nestlings (Kondratzki & Altmüller 1976, Šindelář et al. 2015). However, most exceptions from strictly monogamous mating system were obtained on the basis of catching and ringing methods. There is only one study employing molecular genetic analysis of paternity in the Tengmalm's owl, that revealed no evidence for EPY in the Northern American population. However, most localities in that study were classified as populations of the low nesting density (Koopman et al. 2007).

The main goal of this study is to examine paternity of Eurasian Tengmalm's owl using microsatellite genetic markers. The research was going on for five years and the population density varied considerably within this period. Since nesting density can reach as high as 0.44 nests per km^2 in our study area (Zárybnická et al. 2013) and home ranges of nesting males frequently overlap (Sonerud et al. 1986), an occurrence of EPY can be expected. Hence, the level of EPP is discussed in relation to the nesting density. However, we expect that proportion of EPY will be low in general, similarly as in other owl species (e.g. Roulin et al. 2004, Hsu et al. 2006).

Material and Methods

Study area and field methods

The data were collected in the eastern part of the Ore Mountains (Czech Republic, 50°66′ N 13°58′ E, 730-960 m a.s.l.) from 2006 to 2010. The study area (100 km^2) consists of coniferous and mixed woods of different age because they were damaged by the industrial air pollution during the 1970s (more details in Zárybnická et al. 2015b). Within the study area, nest boxes were installed along forest roads in mean distance 4312.6 m ± 2333.2 m SD (300.4-12434.8 m) from each other. Adult birds primarily used the nest boxes (> 90 % nests), in this area, whereas natural cavities in solitary beech trees have been occupied very rarely (Šindelář et al. 2015). Nest boxes in the study area were checked regularly during the whole nesting season (i.e. since the beginning of March till the beginning of August). Females and nestlings were captured inside their nest boxes. Adult males were captured into the mist nets located in front of the nest box while bringing food for their social female and potential nestlings (Table 1). Blood samples (50-100 μl) were taken by venipuncture from the adults and nestlings (at the age of ca. 15-25 days) and stored in the 96 % ethanol. Finally, all the captured birds were tagged by an aluminium ring with a unique code of the Czech Bird Ringing Centre (National Museum Praha) and released immediately after sampling. In total 54 females, 47 males and 196 offspring were captured, while 7 females and 8 males were repeatedly captured over the consecutive years (Table 1). In addition, 8 (14.8 %) captured females and 7 (14.9 %) captured males were later identified as non-nesting individuals in the particular year (Table 1). The research was carried out in accordance with the current laws of the Czech Republic.

Microsatellite genotyping

The DNA was extracted from the blood samples using a DNeasy®Tissue Kit (Qiagen, Hilden, Germany). All samples were genotyped at seven microsatellite loci (Koopman et al. 2004). One tetraplex (BOOW06, BOOW13, BOOW14, BOOW19) and one triplex (BOOW04, BOOW07, BOOW18) were prepared from the fluorescently labeled primers. All PCR reactions contained 1 μl of the DNA, 5 μL of Multiplex PCR Master Mix (Qiagen), 1 μL of Q-Solution, primers

Table 1. Numbers of Tengmalm's owl nests, captured nestlings, males and females with numbers of retraps over the years in the Ore Mts. (100 km^2).

Year	Males	Retraped males*	Females	Retraped females*	Nestlings	Nests
2006	15	0	19	0	73	18
2007	5	3	7	1	38	7
2008	6	4	11	2	23	7
2009	8	2	8	2	15	6
2010	13	5	9	5	47	8
Total	47	14	54	10	196	46

* Males 402, 404, 542, 849 were retraped one time, males 66, 92 were retraped two times, males 504, 507 were retraped three times. Females 20, 66, 311, 850 were retraped one time, females 67, 91, 402 were retraped two times.

Table 2. Characteristics of microsatellite loci for the Tengmalm's owl population of adults (n = 101) from the Ore Mts. (CR). The loci in bold were used in the parental analyses.

Locus	k	HO	HE	HW	F (Null)	FC (µM)
BOOW04	**11**	**0.741**	**0.729**	**NS**	**–0.0116**	**0.50**
BOOW07	**10**	**0.840**	**0.827**	**ND**	**–0.0116**	**0.30**
BOOW13	4	0.284	0.544	***	0.3097	0.05
BOOW14	**2**	**0.062**	**0.060**	**ND**	**–0.0073**	**0.05**
BOOW18	**20**	**0.753**	**0.867**	**NS**	**0.0683**	**0.10**
BOOW19	**2**	**0.556**	**0.503**	**NS**	**–0.0526**	**0.40**
BOOW06	**2**	**0.062**	**0.060**	**ND**	**–0.0073**	**0.20**

k = number of alleles at the locus, HO = observed heterozygosity, HE = expected heterozygosity, HW = significance of deviation from Hardy-Weinberg equilibrium (NS = not significant, ND = not determined, *** = significant at the 0.1 % level), F (Null) = estimated null allele frequency, FC (µM) = final concentration of primers in PCR multiplex.

in various concentrations (Table 2) and ddH$_2$O to a volume of 10 µL. PCR amplifications were conducted using the following conditions: an initial denaturation step of 15 min at 95 °C followed by 40 cycles of 30 sec at 94 °C, 90 sec at 57 °C, 60 sec at 72 °C, and final extension of 30 min at 60 °C. PCR products were separated by capillary electrophoresis on ABI Prism 3130 Genetic Analyser and their length was analysed using GeneMapper 3.7. software (both Applied Biosystems, Waltham, MA, U.S.A.).

Statistical analyses

General characteristics of used microsatellite loci in our data sample were computed using the Cervus 3.0.7 (Kalinowski et al. 2007) software on the basis of all adult individual's genotypes (without recapture individuals, n = 77): deviation from Hardy-Weinberg equilibrium, observed and expected heterozygosity and a null allele (i.e. non-amplified) frequency for each locus and overall loci (Table 2). Although the mean allele number per locus was 7.8, there were strong differences among loci reaching from two up to twenty alleles per locus (Table 2). We found a significant deviation from Hardy-Weinberg equilibrium at locus BOOW13 which may be caused by high proportion of null alleles (30.97 %, Table 2). This locus was therefore excluded from the subsequent analyses. Based on these six loci, combined total non-exclusion probability for the first and second parent was 0.108 and 0.026 respectively.

Parentage of the Tengmalm's owl was also assigned using Cervus 3.0.7 including nesting females (n = 46), their nestlings (n = 171) and candidate fathers (n = 47). The simulation of parentage analysis was carried out with 100000 cycles and 1 % error rate. The parentage analysis was done for each year separately. Social father was not captured in six nest boxes, therefore these 25 nestlings with unknown genotype of the social father (12.8 %) did not enter the parentage analysis.

To check possible egg dumping first, the maternity analysis was carried out and candidate mother was considered as genetic mother with 95 % confidence in 145 (84.8 %) offspring. In 26 offspring (15.2 %) candidate mother was assigned as genetic mother with lower confidence due to one mismatch with their putative mother. All mismatches, however, were found at homozygous loci where occurrence of null allele cannot be excluded. Therefore the possibility of egg dumping was excluded and obtained mother identity from the previous step (putative mothers) was used for a paternity analysis.

Since combined total non-exclusion probability for the second parent was relatively low, a social male was considered as genetic father if it was assigned by Cervus software as a candidate father with at least 80 % confidence or it showed maximum of two mismatches at homozygous loci or one mismatch at heterozygous loci with genotype of putative offspring. These mismatches can be ascribed to the presence of allelic drop-out, null alleles or high frequency of

Table 3. Numbers of nestlings with unknown genotype of the social father, nestlings and extra-pair nestlings of Tengmalm's owl in relation to the nesting density (Ore Mts., Czech Republic).

Year	Density (nests/km^2)	Nestlings	Nestlings with unknown genotype of the social father	Extra-pair nestlings
2006	0.343	66	7	1
2007	0.110	26	12	0
2008	0.140	19	4	2
2009	0.170	13	2	1
2010	0.130	47	0	0

mutations resulting in 2-4 bp shifts. In addition, young relatedness in all nests was analysed in Colony 2.0.6.2 (Wang 2004, Jones & Wang 2010) as an alternative approach to assess parentage. In total, 10 runs were performed using null allele frequency which was estimated by Cervus software. Genotypes of all candidate adults were included in the analysis and all offspring were assigned to their biological mothers. The sibling relatedness analysis was done for each year separately.

Results

In total, 46 nest boxes (Table 1) were genotyped at six loci. The Genotype of the putative father was known for 171 juveniles and it fully corresponded to the genotype of 128 nestlings (74.9 %). One mismatch was found in 38 nestlings (22.2 %), two mismatches were observed in three cases (1.8 %) and three in two cases (1.2 %). The social partner was assigned as the most probable father in 130 nestlings (76.0 %). However, 37 juveniles cannot be considered as EPY due to possible occurrence of null alleles or by a shift of 2-4 base pairs. In addition, in these 37 cases, the social father had similar LOD scores and the same number of mismatching loci as the most likely male assigned by Cervus. According to our predefined criteria, we found four EPY in total (2.3 %).

The four proved EPY came from three different nest boxes and three different years. One EPY occurred in the year of the highest breeding density (i.e. 2006, Table 3), whereas the others were found in years with a moderate breeding density (i.e. two in 2008 and one in 2009). No evidence for EPP was found in 2007 and 2010 (Table 3).

The results of the sibling relatedness analysis were consistent with those from Cervus program. In the nest boxes where EPY were not detected by Cervus all young were full-siblings in the clutches. In two nest boxes where EPY were detected by Cervus, young were shown to be unrelated to its nest mates using Colony. In one nest box with only two young, both extra-pair, Colony shows that both of them sharing the same father and mother in the clutch. In nests where social father was not captured, all young were detected as full-siblings in the Colony runs.

Discussion

Mating systems is extraordinarily variable among bird species, ranging from absolute genetic monogamy to polygamy or promiscuity. The previous study revealed no EPY in the Tengmalm's owl, though the paternity was investigated across extensive geographic region (Koopman et al. 2007). Our research was going on for five years in one population and provides the first evidence for EPP in this species.

In our study, we used microsatellite loci directly developed for the Tengmalm's owl in North America (Koopman et al. 2004). We found relatively low polymorphism of these microsatellite loci in our population of interest. Furthermore, one locus had relatively high estimate of null allele frequency and Hardy-Weinberg equilibrium was not possible to calculate for other 3 loci. The lower utility (i.e. null allele occurrence) of the markers for our population of interest could be caused by relatively large genetic distance between the North American and Eurasian subspecies of the Tengmalm's owl (Koopman et al. 2005). However, in the Scandinavian population (i.e. within the same subspecies), heterozygosity deficiency was found only at one locus and all loci were in Hardy-Weiberg equilibrium (Koopman et al. 2004). Therefore, low variability of microsatellites loci can be better explained by a possible bottleneck which was probably caused by significant decrease and consecutive expansion of Tengmalm's owl population in the Czech Republic during the 20th century (Šťastný et al. 2006).

Low number of EPY is usually detected in owl species. For instance, Rodriguez-Martínez et al. (2014) found only 1.5 % of EPY in 565 broods (674 juveniles) in burrowing owl (*Athene cunicularia*) despite its high population density in urban areas. Only one young (0.5 %) from 54 broods (211 juveniles) was sired by another male than social father in the barn owl (*Tyto alba*, Roulin et al. 2004). Similarly, the paternity analysis of the 200 nestlings (108 broods) revealed only two EPY (1.5 %) in Lanyu scops owl (*Otus elegans botelensis*), though EPC was frequently observed in the population with high nesting density (Hsu et al. 2006). No evidence for EPP has been found in other owl species (e.g. eastern screech-owl, Lawless et al. 1997; long-eared owl, Marks et al. 1999; little owl, Müller et al. 2001; flammulated owl, Arsenault et al. 2002); including the Tengmalm's owl, where 109 juveniles (32 broods) have been genotyped from a large study area in the north-eastern part of the U.S.A. and Alaska (Koopman et al. 2007). In contrast, four EPY (2.3 %) from 46 broods were found in our population. Nevertheless it should be noted that in the Swiss population of the barn owl it was found that EPY can result from the sequential polyandry, i.e. females desert their first brood and produce the second one (Oring 1986, Korpimäki et al. 2011, Šindelář et al. 2015) with a male of lower fitness than the first partner, hence EPY are sired by the first male i.e. the male with higher fitness (Roulin 2002, Henry et al. 2013). Although female desertion also occurs in the Tengmalm's owl (Korpimäki & Hakkarainen 2012, Šindelář et al. 2015), we can conclude that our four EPY are probably not the case of sequential polyandry because all young came most likely from the first clutches of the entire nesting period.

EPP is traditionally discussed in relationship with nesting density because nesting in proximity simply provides more opportunities for EPC (Birkhead & Møller 1992, Mougeot 2004, Stewart et al. 2010). Tengmalm's owl nesting density in our study area is higher in comparison with other regions and significantly vary between years (e.g. Zárybnická et al. 2015a, Zárybnická unpublished data). Nevertheless, due to relatively short termed study and low annual variation in breeding density we are not able to directly test the relationship between the nesting density and occurrence of EPY but our results suggest that the EPP is not associated with the breeding density, because only one EPY occurred in the year of the highest breeding density (i.e. in 2006), whereas the nesting density during the following years (i.e. 2007-2010) was comparably lower. No relationship between EPP and population density was also found in North-American subspecies of Tengmalm's owl (Koopman et al. 2007). Lower rates of EPP are expected in the population exhibiting low genetic variability due to the reduced potential for good gene advantage of female promiscuity (Petrie et al. 1998, Gohli et al. 2013). However, sufficient data to test this hypothesis are missing for our model species. Nevertheless, low proportion of EPY in our population is similar to the other owl studies (barn owl, Roulin et al. 2004; Lanyu scops owl, Hsu et al. 2006; tawny owl, Saladin 2007). Therefore, we believe that the low occurrence of EPY in the Tengmalm's owl is rather associated with high effort in the parental care and high adult survival rate. It is clear that paternal care of the male is crucial for breeding success of the Tengmalm's owl because the male delivers food to their offspring till 6-8 weeks after they fledge (Newton 1979, Korpimäki 1981, Eldegard & Sonerud 2010). Therefore, the female cannot risk loss or reduction of the parental care due to EPC (Birkhead & Møller 1996). Males of species with longer life spans usually do not tolerate higher rate of EPP (Mauck et al.1999, Wink & Dyrcz 1999) and mortality of the Tengmalm's owl adults is relatively low (mean annual survival is 67 % for individuals older than one year, Korpimäki & Hakkarainen 2012). In conclusion, our study provides the first evidence for EPP in the Tengmalm's owl. Since polymorphism of used microsatellite loci was low in this study, further effort to assess the Tengmalm's owl paternity, population structure or demography should include development of more variable markers. Long-term study with larger sample size is needed for detailed analysis of other explanatory variables potentially affecting reproductive success in this bird species.

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