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ORIGINAL ARTICLE

Molecular evidence for extra-pair paternity and intraspecific brood parasitism in the Black-headed Gull

Radka Ležalová-Piálková

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Abstract Social monogamy is common among birds, while genetic monogamy is supposed to be rare. I have investigated the genetic mating system of the Black-headed Gull *Larus ridibundus* in which, as in seabirds and most other long-lived and socially monogamous birds, extra-pair paternity (EPP) is typically rather infrequent. The parentage of 79 chicks from 30 broods was determined based on the analysis of six microsatellite markers. In this study population, evidence of allelic inconsistencies between putative parents and chicks as a result of both EPP and intraspecific brood parasitism (ISBP) was found in 43% of nests . Extra-pair paternity was detected in 33% (10/30) of broods, and 20% (16/79) of all nestlings were sired by extra-pair males. In addition, 9% (7/79) of chicks out of five nests (17%) were not the offspring of either member of the pair, indicating ISBP. These findings reveal a moderate rate of ISBP and a high rate of EPP in the Black-headed Gull compared with other related species and shows that the Black-headed Gulls successfully participate in extra-pair copulations.

Zusammenfassung Soziale Monogamie ist bei Vögeln weitverbreitet, während genetische Monogamie vermutlich selten ist. Ich habe das genetische Paarungssystem der Lachmöwe *Larus ridibundus* untersucht, bei der, wie bei Seevögeln und den meisten anderen langlebigen und sozial monogamen Vögeln, Fremdvaterschaft (EPP) normalerweise eher selten ist. Die Elternschaft wurde mittels sechs Mikrosatellitenmarkern für 79 Küken aus 30 Bruten ermittelt. In der untersuchten Population fand ich Belege für Allelinkonsistenzen zwischen Putativeltern und Küken in 43% der Nester, was sowohl auf EPP als auch auf intraspezifischen Brutparasitismus (ISBP) zurückzuführen war. Fremdvaterschaft wurde in 33% (10/30) der Bruten nachgewiesen, und 20% (16/79) aller Nestlinge wurden von Fremdvätern gezeugt. Außerdem stammten 9% (7/79) der Küken aus fünf Nestern (17%) weder von der Mutter noch vom Vater ab, was auf ISBP hindeutet. Diese Ergebnisse lassen eine im Vergleich zu anderen verwandten Arten mittlere ISBP-Rate und eine hohe EPP-Rate erkennen und zeigen, dass Lachmöwen erfolgreich Kopulationen außerhalb des Paarbundes eingehen.

Keywords Black-headed Gull · Genetic mating system · Extra-pair paternity · Intraspecific brood parasitism · *Larus ridibundus*

Introduction

The application of molecular techniques to avian mating systems has revealed striking variations in the incidence of alternative reproductive strategies, such as extra-pair paternity (EPP) and intraspecific brood parasitism (ISBP) (Birkhead and Møller 1992; Petrie and Kempenaers 1998; Arnold and Owens 2002). Even though many hypotheses have been proposed to explain such variations (Møller and Ninni 1998; Petrie and Kempenaers 1998; Arnold and Owens 2002; Griffith et al. 2002; Westneat and Stewart

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R. Ležalová-Piálková (✉)
Department of Zoology, Faculty of Sciences,
University of South Bohemia, Branišovská 31,
370 05 České Budějovice, Czech Republic
e-mail: Radka.Lezalova@prf.jcu.cz

R. Ležalová-Piálková
Institute of Vertebrate Biology,
Academy of Sciences of the Czech Republic,
Květná 8, 60365 Brno, Czech Republic

2003; Neudorf 2004), the results from studies testing these are inconsistent. The strongest correlation with EPP was found to be phylogenetic. High EPP rates are associated with high rates of adult mortality and reduced parental care, while high ISBP rates are associated with high fecundity rates (Arnold and Owens 2002; Bennett and Owens 2002), with variation at the population or individual level more likely to be based on differences in current ecological and genetic factors (Petrie and Kempenaers 1998). Of the ecological factors affecting EPP, breeding density may be an important factor at the species level (Westneat and Sherman 1997). Colonially nesting species are predicted to have high EPP rates due to the close proximity to potential partners (Møller and Birkhead 1993).

The Black-headed Gull *Larus ridibundus*, similar to other gulls, is a long-lived, colonially breeding species with high adult survivorship (Cramp 1983). The standard clutch size of this species is three eggs. Both females and males participate in parental care throughout incubation and chick-rearing, which leads to social monogamy in this species (Cramp 1983). Given these traits, a low frequency of EPP and ISBP could be expected.

The major goal of this study was to describe the rates of EPP and intraspecific brood parasitism in the Black-headed Gull.

Materials and methods

Field methods and sampling

The study was carried out in a colony of about 3,000 pairs of Black-headed Gulls at the Jarohněvický pond in the south-eastern part of the Czech Republic (48°55′N, 17°05′E). Thirty families (chicks and both parents) were sampled during the 2004 breeding season. Adults were trapped with "walk-in-nest-traps" at randomly chosen nests from different parts of the colony, including both the center and edges and subsequently ringed with metal rings for identification. A sample of blood (approximately 50 μl) was taken from the tarsal vein of each trapped individual and mixed with 150 μl of storage buffer (0.5 M EDTA) for sex determination and paternity analysis in the laboratory.

The distribution of clutch sizes in the 30 sampled nests was one nest with four eggs, 20 nests with three eggs, seven nests with two eggs, and two nests with one egg. Eggs were individually marked with waterproof ink on the shells according to laying sequence. Each of the studied nests was enclosed within a 1.6-cm mesh wire circle about 1 m in diameter and 40 cm high. This mesh 'fence' prevented the escape of young chicks but allowed adults to access the nests.

On the first day post-hatching, approximately 25 μl of blood was obtained from the brachial or tarsal vein of chicks and mixed with 150 μl of storage buffer (0.5 M EDTA). Samples of embryos were collected from unhatched eggs. All samples were kept at −20°C until DNA extraction.

Molecular determination of sex

The sex of the social parents was determined by a PCR that amplifies an intron of the CHD1 genes on the sex chromosomes (Griffiths et al. 1998). Genomic DNA was isolated from 10 μl of the blood sample using the DNA Lego kit (Top-Bio, Prague, Czech Republic) following the manufacturer's protocol. The avian sexing primers 2550 F and 2718 R (Fridolfsson and Ellegren 1999) were used in 10 μl PCR reactions. The PCR conditions were as follows: 1 μl of 10× PCR buffer (TaKaRa, Otsu, Shiga, Japan), 2 μM of each dNTP, 2.5 pmol of each primer, and 0.5 U of *Taq* polymerase (TaKaRa). About 150 ng of genomic DNA was used as template. PCR was performed according to Griffiths et al. (1998) in a T3 thermal cycler (Biometra, Goettingen, Germany). The products were separated by electrophoresis for 45–60 min at 7–10 V/cm on a 3% agarose gel and stained with ethidium bromide.

Parentage analyses

Microsatellite typing was used to reveal the genetic relationships between parents and young. Parentage was analyzed on the basis of six polymorphic dinucleotide microsatellite loci (RBG 13, RBG 18, RBG 20, RBG 27, RBG 28, RBG 29), an analysis originally developed for the Red-billed Gull *Larus novaehollandiae scopulinus* (Given et al. 2002). These six loci exhibited little or no evidence of null alleles and showed sufficient levels of polymorphism (mean heterozygosity 0.77, mean number of alleles 11.3), making them suitable for paternity assessment in this species (combined exclusion probability: first parent 0.98, second parent 0.998) (Table 1).

Table 1 Polymorphism data for the six microsatellite markers in the Black-headed Gull *Larus ridibundus*

Locus	*k*	Allele size (bp)	He	Ho	F_{null}	NE-1P	NE-2P
RBG 13	9	205–223	0.82	0.86	0.02	0.54	0.36
RBG 18	6	170–180	0.39	0.35	0.09	0.93	0.8
RBG 20	16	169–203	0.92	0.9	0.01	0.29	0.17
RBG 27	21	185–235	0.91	0.89	0	0.33	0.2
RBG 28	7	159–173	0.76	0.75	0	0.64	0.46
RBG 29	9	125–141	0.81	0.9	0.06	0.56	0.38

Values are based on the genotypes of 60 parents and calculated using the Cervus ver. 3.0.3 software package (Field Genetics, London, UK)

k, Number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; F_{nul}, estimated frequency of the null allele; NE-1P non-exclusion probability for the first parent, NE-2P, non-exclusion probability for the second parent

Genomic DNA was extracted from 10 μl of blood sample or from 25–35 mg of embryo tissue (depending on the type of tissue) from unhatched eggs. The 10 μl PCR reactions consisted of about 100 ng of template DNA, 0.4 U *Taq* polymerase (TaKaRa), 1 μl of 10× PCR buffer (TaKaRa), 2.5 μM dNTPs, and 2.5 pmol of each primer. The forward primer was fluorescently labeled. The PCR conditions followed the protocol described by Given et al. (2002).

Amplification products were run on an ABI Prism 3,100 automatic sequencer (Applied Biosystems, Foster City, CA), and Gene Mapper software (Applied Biosystems) was used to size the alleles. The number of alleles, observed and expected heterozygosity, parentage exclusion probability for the first and second parents, and theoretical frequency of null alleles for each locus were estimated with Cervus ver. 3.0.3 software (Kalinowski et al. 2007) (Table 1). Allele frequencies were used to calculate the cumulative probability of resemblance ($P_{\mathrm{R}Cum}$) for specific parent–offspring cases where ambiguous mismatches occurred (Ibarguchi et al. 2004).

Results

The paternity of 79 chicks and 60 adults from 30 broods was assessed. Offspring that possessed a microsatellite allele that did not match the putative father at two or more loci were considered to be extra-pair young (EPY), and an offspring that did not share an allele with either of their social parents was considered to be a case of ISBP. No case of allelic mismatch between the offspring and their putative parents was found in 17 of the 30 nests sampled (57%). Thus, all social parents were true genetic parents in these nests. However, in 13/30 (43%) nests, allelic inconsistencies between putative parents and chicks were detected, resulting from both EPP and ISBP. More specifically, in 10/30 (33%) nests, 17/79 [21.5%; 95% confidence interval (CI) 0.13–0.32] chicks shared the same allele at each locus with their mother but were mismatched with the social father in at least two loci (Table 2). The number of mismatching loci in extra-pair chicks ranged from two to five (Table 3). In three cases in which the chick and its putative father differed only in two loci, I estimated that the likelihood that mismatched loci were caused by mutation. Cumulative probabilities of resemblance ($P_{\mathrm{R}cum}$; Ibarguchi et al. 2004) were calculated for these three chicks, excluding loci with the mismatched alleles. In two cases, the probabilities of sharing alleles by chance were so high (0.2×10^{-3} and 1.3×10^{-3}, respectively) that mismatches due to mutation could be ruled out. In the third case, the cumulative probability of resemblance was much lower (5×10^{-6}), suggesting that this was a case of mutation and the chick could be reassigned as being legitimate. I considered the remaining 16/79 (20%; 95% CI 0.12–0.31) chicks to be extra-pair since in all cases social mothers were also true genetic mothers.

Chicks which did not match either of their social parents were found in two of the ten nests with EPY and in three other nests. Altogether, 7/79 (9%; 95% CI 0.04–0.17) chicks out of 5/30 (17%) nests were considered to result from intraspecific brood parasitism (Table 2). The frequency of mismatching loci in these parasitic chicks ranged from three to six loci (Table 3).

Differences between chicks and parents at a single locus were found in another five chicks; in four cases, the chicks differed from their social father, and in one case the chick mismatched at one locus with their mother. The cumulative probabilities of resemblance were calculated for these five chicks, excluding loci with the mismatched alleles. In four of five cases, the probabilities of sharing alleles by chance were so low (4.08×10^{-6} to 7.01×10^{-7}) that mutation is the most probable reason for the mismatches. In the last case, the probability of sharing alleles was higher (1×10^{-2}) due to the male and chick sharing common alleles (Table 3). All of those five chicks were considered as being within-pair offspring.

Discussion

The Black-headed Gulls of the study population successfully participated in extra-pair copulations—33% of nests contained at least one EPY. This level of EPP is relatively high in comparison to that reported for related species and other colonially breeding seabirds (e.g. Mauck et al. 1995; Gilbert et al. 1998; Lorentsen et al. 2000; Griggio et al. 2004). Previously measured variation in extra-pair fertilization rates for Laridae species has been low, ranging

Table 2 Distribution of chicks with EPP and parasitic chicks (ISBP) across the nests

Number of nests with determined:	Number of chicks with extra-pair parentage in the nest			Total (chick/nests)	*n*
	1	2	3		
EPP	6	2	2	16/10	30
ISBP	4	–	1	7/5	30

EPP extra-pair paternity, *ISBP* intraspecific brood parasitism, *n*, number of analyzed nests

Table 3 Frequency of chicks with a different number of mismatched loci

Number of mismatched loci	Number of chicks	$P_{\mathrm{R}cum}$	Classified as
1	5	1×10^{-2} to -7.01×10^{-7}	True parentage
2	1	5×10^{-6}	True parentage
2	2	0.2×10^{-3} and 1.3×10^{-3}	EPP
>3 (maximum 5)	14	ND	EPP
>3 (maximum 6)	7	ND	ISBP

$P_{\mathrm{R}cum}$, Cumulative probability of resemblance of sharing alleles by chance for parent–offspring cases with a low number of mismatching loci, *ND* not defined

from zero in the Western Gull (*Larus occidentalis*; Gilbert et al. 1998), Black-legged Kittiwake (*Rissa tridactyla*; Helfenstein et al. 2004), Common Tern (*Sterna hirundo;* Griggio et al. 2004), and Little Auk (*Alle alle*; Lifjeld et al. 2005), to 8.3% in the Common Gull (Bukaciński et al. 1998). These findings are in line with the prediction of low EPP rates in species with a long reproductive lifespan, such as seabirds.

In contrast, my data suggest that Black-headed Gulls have an unexpectedly high rate of EPP. This EPP could arise by two means, namely, as the result of extra-pair copulations or from rapid mate-switching (Birkhead and Møller 1992; Martins et al. 2002). Extra-pair copulations are common in gulls (Mills 1994; Bukaciński et al. 1998). In Black-headed Gulls, copulation activity has not yet been studied in detail; Frantová (2007) found a low frequency of forced copulations (1.8%) but did not distinguish between within-pair and unforced extra-pair copulations.

No data were collected on behavioral data on mate-switching. Consequently, there is the possibility that the two nests in which all three chicks were found to be sired by a male other than the social father (Table 2) could be cases of mate-switching; alternatively, cases of putative father infertility cannot be excluded. The replacement of a mate may occur quickly due to the large supply of potential mates and strong competition for mates during the early stage of breeding. In the remaining eight nests, one (5 nests) or two (3 nests) EPY were detected, probably resulting from successful extra-pair copulations (Table 2). However, to unequivocally explain this high rate of EPP, additional studies, behavioral studies in particular, will be necessary. Furthermore, the accuracy of the estimate could be affected by sample size (Griffith et al. 2002). Griffith et al. (2002) suggest that approximately 200 offspring is a reasonable sample size, limiting the magnitude of error around the estimate. In the present study, the magnitude of error around the estimate of EPP level in the studied population is 19%, which suggests that further sampling would have increased the accuracy of the estimate.

The rate of ISBP was also found to be high in the study population, especially given the life-history strategy of gulls. In five (17%) of the 30 nests sampled, at least one chick was found that did not match either of the social parents. This rate of ISBP should not have been affected by possible chick adoption, since the studied nests were enclosed with mesh wire and chicks were sampled within a couple of hours of hatching. Multiple parentage in these clutches most probably occurred through nest parasitism. The possibility that some of the mixed clutches represented female–female pairs or quasi-parasitism can be excluded, since the social parents trapped at each nest were always a male and female, and neither of them matched potentially parasitic chicks.

Among the 61 species of gulls studied to date, intraspecific parasitism has been documented in another four species (*Larus argentatus*, *L. canus*, *L. delawarensis*, and *L. minutus*; in Yom-Tov 2001; Duda et al. 2008) as well as in the Black-headed Gull (Duda et al. 2008; Ležalová-Piálková and Honza 2008). Duda et al. (2008) revealed the maternity in a population of Black-headed Gulls in Poland by protein fingerprinting and found multiple maternity in 34% of nests with more than one egg. These results are much higher then those obtained from a previous study of ISBP based on the regular monitoring of nests in the same Black-headed Gull population as the current study (Ležalová-Piálková and Honza 2008). In this previous analysis, we found a parasitism rate of 10%, which now seems to be an underestimation. These studies allow an interesting comparison between three different methodological approaches to the question of ISBP, showing that a molecular approach is essential for determining the real frequencies of ISBP in birds (Grønstøl et al. 2006).

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