**MOLECULAR EVIDENCE FOR EXTRA-PAIR PATERNITY AND INTRASPECIFIC
BROOD PARASITISM IN THE BLACK-HEADED GULL**

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Running headline: EPP and ISBP in the Black-headed Gull

Abstract

Social monogamy is common among birds, while genetic monogamy is supposed to be rare. I investigated the genetic mating system of the Black-headed Gull *Larus ridibundus*, where, as in seabirds and most other long-lived and socially monogamous birds, extra-pair paternity (EPP) is typically rather infrequent. Parentage was determined using six microsatellite markers for 79 chicks from 30 broods. In this study population, I found evidence of allelic inconsistencies between putative parents and chicks in 43% of nests, resulting from both EPP and intraspecific brood parasitism (ISBP). Extra-pair paternity was detected in 33 % (10/30) of broods, and 20% (16/79) of all nestlings were sired by extra-pair males. Furthermore, 9% (7/79) of chicks out of 5 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 compared with other related species, and shows that Black-headed Gulls successfully participate in extra-pair copulations.

Key words Black-headed Gull, genetic mating system, extra-pair paternity, intraspecific brood parasitism, *Larus ridibundus*

Zusammenfassung

Molekularer Nachweis von Fremdvaterschaft und intraspezifischem Brutparasitismus bei der Lachmöwe *Larus ridibundus*

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.

Introduction

The application of molecular techniques to avian mating systems has revealed striking variation 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 this variation (Petrie and Kempenaers 1998; Møller and Ninni 1998; Arnold and Owens 2002; Griffith et al. 2002; Westneat and Stewart 2003; Neudorf 2004), there is still inconsistency in the results. The strongest correlation with EPP was found 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). Variation at the population or individual level is more likely to be based on differences in current ecological and genetic factors (Petrie and Kempenaers 1998). Of ecological factors, 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 because proximity to potential partners is high (Møller and Birkhead 1993).

The Black-headed Gull, like 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 extra-pair parentage and intraspecific 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 breeding season 2004. Adults were trapped with "walk-in-nest-traps" at randomly chosen nests from different parts of the colony, including both the centre and edges, and 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 (0.5M EDTA), for later sex determination and paternity analysis.

The distribution of clutch sizes in the thirty sampled nests size was: 4 eggs (n=1), 3 eggs (n=20), 2 eggs (n=7) and one egg (n=2). 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 fence prevented the escape of young chicks but allowed adults to access the nests.

On the first day after hatching, approximately 25 µl of blood was obtained from the brachial or tarsal vein of chicks and mixed with 150 µl of storage buffer (0.5M 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 social parents was determined by a polymerase chain reaction that amplifies an intron of the CHD1 genes on the sex chromosomes (Griffiths et al. 1998). Genomic DNA was isolated from 10 µl of blood sample using the DNA Lego Kit (Top Bio) 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: 1µl of 10X PCR buffer (TaKaRa), 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 Biometra thermal cycler. The products were separated by electrophoresis for 45-60 min at 7-10 V/cm using a 3% agarose gel stained with ethidium bromide.

Parentage analyses

Microsatellite typing was used to reveal the genetic relationships between parents and young. Parentage was analysed using 6 polymorphic dinucleotide microsatellite loci (RBG 13, RBG 18, RBG 20, RBG 27, RBG 28, RBG 29) 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).

Genomic DNA was extracted from 10 µl of blood sample or from 25-35 mg of

embryo tissue (depending on type of tissue) from unhatched eggs. The 10 µl PCR reactions consisted of about 100 ng of template DNA, 0.4 units of *Taq* Polymerase (TaKaRa), 1 µl of 10x PCR buffer (TaKaRa), 2.5 µM dNTPs and 2.5 pmol of each primer. The forward primer was fluorescently labelled. The PCR conditions followed the protocol described by Given et al. (2002).

Amplification products were run on an ABI Prism 3100 automatic sequencer, and Gene Mapper software (Applied Biosystems Inc) 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 3.0.3 software (Kalinowski et al. 2007) (Table. 1). Allele frequencies were used to calculate the cumulative probability of resemblance P_{RCum} for specific parent-offspring cases where ambiguous mismatches occurred (Ibarguchi et al. 2004).

Results

I assessed paternity for 79 chicks and 60 adults from 30 broods. Offspring that possessed a microsatellite allele that did not match the putative father at two or more loci were considered extra-pair young (EPY), and offspring that did not share an allele with either of their social parents were considered to be a case of intraspecific brood parasitism. In 17 out of 30 sampled nests (57%), I found no case of allelic mismatch between the offspring and their putative parents. 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 I found that 17/79 (21.5%; 95% c.i.: 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 loci (Table.3). In three

cases, where the chick and its putative father differed only in two loci, I estimated the likelihood that mismatched loci were caused by mutation. Cumulative probabilities of resemblance (P_{Rcum} ; 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}) 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 legitimate. I considered the remaining 16/79 (20%; 95% c.i.: 0.12-0.31) chicks to be extra-pair, since in all cases social mothers were also true genetic mothers.

Moreover, in two of the ten nests with EPY and in three other nests I found chicks which did not match either of their social parents. Altogether 7/79 (9%; 95% c.i.: 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 3 to 6 loci (Table.3).

Finally, in another five chicks I detected differences between chicks and parents at a single locus; in four cases 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, 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 those five chicks were considered as within pair offspring.

Discussion

Black-headed Gulls in the study population successfully participate in extra-pair copulations – 33% of nests contained at least one extra-pair young (EPY). This level of

EPP is relatively high in comparison to 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 EPF rates for Laridae species has been low, ranging 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 Common Gull (*Larus canus*; Bukacinska 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. Extra-pair paternity could arise by two means, either as the result of extra-pair copulations (EPC), or rapid mate-switching (Birkhead and Møller, 1992, Martins et al. 2002). In gulls, EPCs are common (Mills 1994, Bukacinska 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.

I collected no behavioural data on mate-switching, so that I can not exclude the possibility that the two nests where all three chicks were found to be sired by other than the social father (Table.2) could be cases of mate-switching or, alternatively, cases of putative father infertility. The replacement of a mate might happen rapidly due to a large supply of potential mates and strong competition for mates during the early stage of breeding. In the remaining 8 nests, one (5 nests) or two (3 nests) EPYs were detected, probably resulting from successful EPCs (Table.2). However, to unequivocally explain this high rate of EPP, other, especially behavioural studies will be necessary. Furthermore, the accuracy of the estimate could be affected by used 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. The magnitude of error around my estimate of EPP level in studied population is 19%, which suggest that further sampling would increased the accuracy of the estimate.

Moreover, I found a quite high rate of ISBP, especially considering the life-history

strategy of gulls. In five (17%) out of 30 sampled nests, at least one chick was found that did not match either of their social parents. This rate of ISBP should not be affected by possible chick adoption, since the studied nests were enclosed with mesh wire and chicks were sampled within a couple of hours after 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 61 species of gulls, intraspecific parasitism has been documented in another four species (*Larus argentatus*, *L. canus*, *L. delawarensis* and *L. minutus*; in Yom-Tov 2001 and 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 >1 egg. These results are much higher than 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 finding the real frequencies of ISBP in birds (Grønstøl et al. 2006).

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337

338

339 **Table 1**

340

341 **Table.1** Polymorphism data for six microsatellite markers in the Black-headed Gull.
 342 Values are based on the genotypes of 60 parents and calculated using CERVUS 3.0.3. *k* -
 343 the number of alleles, size (*bp*)- range of allele size in base-pairs, *He* – expected
 344 heterozygosity, *Ho* – observed heterozygosity, *F_{null}* – estimated frequency of the null
 345 allele, *NE-1P* – non-exclusion probability for the first parent, *NE-2P* – non-exclusion
 346 probability for the second parent.

Locus	<i>k</i>	size (<i>bp</i>)	<i>He</i>	<i>Ho</i>	<i>F_{null}</i>	<i>NE-1P</i>	<i>NE-2P</i>
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
RBG20	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

Table 2

Table.2 Distribution of chicks with extra-pair paternity (EPP) and parasitic chicks (ISBP) across the nests. n = number of analysed nests.

		No. of chicks with extra- pair parentage in nest			Total (chick/nests)	n
		1	2	3		
No. of nests with determined	EPP	6	2	2	16/10	30
	ISBP	4	-	1	7/5	30

Table 3

366 **Table.3** Frequency of chicks with different number of mismatched loci. PR_{cum} -
 367 cumulative probability of resemblance of sharing alleles by chance for parent-offspring
 368 cases with low number of mismatching loci, nd – not defined.

No. of mismatched loci	No. of chicks	PR_{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 (max. 5)	14	nd	EPP
> 3 (max. 6)	7	nd	ISBP

369