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Authors: Masters, Brian S., Johnson, L. Scott, Johnson, Bonnie G. P., Neely, Crystal J., and Williams, Karen L.

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“COMPATIBLE ALLELES” AND EXTRA-PAIR PATERNITY: CONCLUSIONS DEPEND ON THE MICROSATELLITE LOCI USED

BRIAN S. MASTERS¹, L. SCOTT JOHNSON, BONNIE G. P. JOHNSON, CRYSTAL J. NEELY, AND KAREN L. WILLIAMS

Department of Biological Sciences, Towson University, Towson, MD 21252

Abstract. Although females' mating with multiple males is widespread in socially monogamous species, its evolutionary significance is poorly understood. Tests of the hypothesis that socially monogamous females seek extra-pair copulations to increase the heterozygosity of their offspring have produced mixed results. Some of the inconsistency may be the result of technical limitations of the molecular analyses used. In a previous analysis using five microsatellite loci, we showed that in the House Wren (*Troglodytes aedon*) extra-pair sires had fewer alleles common to the population than did other nearby males that could have sired offspring but did not, suggesting a “rare male” effect (Masters et al. 2003, *Proceedings of the Royal Society of London, Series B* 270:1393–1397). We repeated the previous analysis, including an additional set of seven microsatellite loci. An analysis that used all 12 loci (both the original and additional loci) produced results suggesting that genotype affects extra-pair paternity. By contrast, an analysis using only the seven new loci alone showed no significant effects. We conclude that studies of this type are likely to be sensitive to the loci employed, particularly when relatively low numbers of loci are analyzed.

Key words: *extra-pair mating, heterozygosity, House Wren, microsatellites, Troglodytes aedon.*

“Alelos Compatibles” y Paternidad Extra-Pareja: Las Conclusiones Dependen de los Loci Microsatélites Empleados

Resumen. Aunque el apareamiento de las hembras con varios machos está ampliamente difundido en especies socialmente monógamas, su significancia evolutiva es pobremente

conocida. Las pruebas de la hipótesis de que las hembras socialmente monógamas podrían buscar copular con machos que no son su pareja social para incrementar la heterocigocidad de sus crías han producido resultados mixtos. Parte de la inconsistencia entre los estudios podría ser el resultado de limitaciones técnicas de los análisis moleculares empleados. En un análisis previo realizado con cinco loci microsatélites, demostramos que, en *Troglodytes aedon*, los machos extra-pareja que fecundaron a las hembras presentaban menos alelos comunes en la población que otros machos que podrían haber fecundado a las hembras pero no lo hicieron, lo que sugirió un efecto de “machos raros” (Masters et al. 2003, *Proceedings of the Royal Society of London, Series B* 270:1393–1397). Repetimos el análisis anterior, incluyendo un conjunto adicional de siete loci microsatélites. Un análisis basado en los 12 loci (tanto los originales como los adicionales) sugirió que el genotipo afecta la paternidad extra-pareja. En contraste, un análisis basado sólo en los siete nuevos loci no mostró efectos significativos. Concluimos que los estudios de este tipo probablemente son sensibles a los loci empleados, particularmente cuando se analiza la variación en un número de loci relativamente pequeño.

The adaptive benefits of extra-pair (EP) mating for females in socially monogamous bird species remain unclear. The “compatible alleles” hypothesis proposes that females, perhaps constrained in their selection of social mates, use EP fertilizations to increase the heterozygosity of their offspring (Brown 1997). There is strong evidence that a loss of heterozygosity affects the fitness of wild organisms, including birds, negatively (Keller and Waller 2002). This effect has been found not only in situations where individuals often mate with close relatives, e.g., in small and/or recently bottlenecked populations (e.g., Briskie and Mackintosh 2004, see also Szulkin et al. 2007), but also in larger populations where mates are not particularly close relatives (e.g., Amos et al. 2001,

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¹E-mail: bmasters@towson.edu

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Höglund et al. 2002, Spottiswoode and Møller 2003, Hawley et al. 2006, Knape et al. 2008). Therefore, it seems advantageous for females to seek mates that will increase their offspring's heterozygosity. However, studies asking whether females select EP mates on the basis of genotype have produced markedly mixed results (reviews in Akçay and Roughgarden 2007, Kempaers 2007), and a recent meta-analysis specifically failed to find support for the prediction that EP males are less related to focal females than are the males with which they pair (Akçay and Roughgarden 2007).

Some studies' failure to support the "compatible alleles" hypothesis may result, at least in part, from limitations imposed by the number and type of molecular markers used. Bartos Smith et al. (2005) used Monte Carlo simulations on artificially generated genotypes to demonstrate that heterozygosity analyses based on five to nine microsatellite loci, the number typically employed in such studies, have relatively low power of resolution. Indeed, a number of studies have made it clear that attempts to estimate overall genomic heterozygosity (i.e., the level of inbreeding) by analyzing the heterozygosity of a relatively small number of microsatellite loci is likely to be inaccurate and misleading (Balloux et al. 2004, Slate et al. 2004, DeWoody and DeWoody 2005, Hansson and Westerberg 2008). Genotypic effects may also be examined through population-level measures such as relatedness or rarity within the population, but these measures are also sensitive to the number (Koskinen et al. 2004) and even the types (Carreras-Carbonell et al. 2006, Liu et al. 2005, Olsen et al. 2004, O'Reilly et al. 2004) of loci used.

In a previous study (Masters et al. 2003), we used five microsatellite loci to examine the relationship between genotype and male success in EP mating in the House Wren (*Troglodytes aedon*). Our most prominent finding was that, in pairwise comparisons, EP males that succeeded in siring offspring with a female had fewer common alleles than did other, nearby males that could have sired EP offspring with the focal female but did not (in our population EP sires are typically males on neighboring territories; Johnson et al. 2002). This suggested that females may select, either overtly or cryptically, EP sires with rare genotypes. In theory, selection on this basis could increase the heterozygosity of the female's offspring (Charlesworth and Charlesworth 1999). Because of concerns subsequently raised regarding assessment of genotypes by relatively few loci (see above), and because studies with other species failed to replicate our results (e.g., Hansson et al. 2004, Schmoll et al. 2005, Stewart et al. 2006), we felt compelled to repeat our analyses with an additional set of loci.

METHODS

A detailed description of the study species, study site, and field and laboratory procedures appears in Masters et al. (2003). Briefly, we obtained DNA samples from within-pair (WP) males, females, offspring, and potential EP sires (i.e., males on adjacent territories; Johnson et al. 2002) at 20 nests in Wyoming. We initially typed individuals at five microsatellite loci (Table 1). For the current study, we typed individuals at seven additional loci, including Mcy4 (Double et al. 1997), Ltmr6 (McDonald and Potts 1994), Ase8 (Richardson et al. 2000), and TA-B4-2, TA-A5-15, TA-C3(B)-2, and TA-C6-7 (Cabe and Marshall 2001). We conducted all PCR amplifications by the touchdown protocol described in Johnson et al. (2002). We used a Beckman Coulter CEQ 8000 capillary sequencer and CEQ software, version 8.0, to size PCR products. We used Cervus 2.0 to analyze allele frequencies. As noted by Johnson et al. (2002), Hru3 has an apparent null allele with an estimated frequency of 0.09 in our population, and the

TABLE 1. Microsatellite loci used in this study. H_o is observed heterozygosity, H_e is expected heterozygosity, and n is number of alleles observed.

Locus	H_o	H_e	n
Original loci used by Masters et al. (2003)			
Fhu2	0.76	0.78	10
Hru3	0.68	0.81	11
Hru6	0.40	0.43	11
Pca3	0.49	0.47	8
Poccl	0.42	0.42	6
New loci used in the current study			
Ase8	0.48	0.55	10
Ltmr6	0.90	0.91	18
Mcy4	0.91	0.93	23
TA-A5-15	0.62	0.65	8
TA-B4-2	0.84	0.82	9
TA-C3(B)-2	0.93	0.94	26
TA-C6-7	0.65	0.63	6

alleles for this locus deviated significantly from Hardy–Weinberg equilibrium. All other loci in this study did not deviate from Hardy–Weinberg equilibrium, and estimated frequencies of null alleles were low (<0.05). We stored all DNA samples at -20°C , and there was no evidence of sample degradation between the two studies. In particular, we repeated the analysis at Fhu2 during the second study with no apparent decline in allele number or signal quality.

Following Masters et al. (2003), we used the relatedness measure of Queller and Goodnight (1989) to quantify genetic relatedness and the possession of alleles common within the population. Relatedness is determined by the extent to which individuals share alleles, and particular alleles are weighted by their frequency in the population. In this measure, a value of 1 indicates genetic identity, with lower values indicating decreased relatedness. To determine the extent to which males possessed alleles common within the population, we followed Queller and Goodnight (1989) and compared male genotypes with a hypothetical genotype composed of the most common allele(s) found in this population at each locus. In this case lower values indicate a greater genetic rarity. We refer readers to Masters et al. (2003) for further details.

The allelic diversity of three loci (Mcy4, Ltmr6, and TA-C3(B)-2) used in this study was greater than that of any used by Masters et al. (2003), with no single allele being substantially more frequent than the others. We combined the three most common alleles at these loci and considered them as a single allele for use in the hypothetical common genotype.

We compared the genotypes of cuckolded WP males and identified EP sires with regard to degree of relatedness to WP females and possession of alleles common within the population. In 17 of 20 cases, there was at least one male on adjacent territories (with nests within 200 m of the focal pair's nest) that we excluded as sires of EP young. In these cases, we compared the genotypes of the excluded neighbor(s) to the genotypes of both the actual EP sire(s) and the WP male. For any nest for which there was more than one EP sire or more than one excluded neighbor, we used mean values for all males in the analyses. We used paired t -tests for statistical comparisons of individuals' genotypes. Following up on the previous study,

TABLE 2. Pairwise comparison of genotypes of within-pair (WP) males, extra-pair (EP) sires, and neighboring males that were excluded as EP sires at nests of House Wrens. For comparisons between EP sires and WP males, $n = 20$. For comparisons involving excluded neighbors, $n = 17$. Shown are mean values ($\pm$ SE) for each class of males as a group and the mean difference between individuals in different classes in pairwise comparisons at focal nests. Values for males of different classes were compared by paired t -tests. All P values are one-tailed.

Genotypic parameter	Males compared		Mean difference	<i>t</i>	<i>P</i>
(a) Results with the 5 original loci used by Masters et al. (2003)					
	EP sires	WP males			
Relatedness to WP females	-0.06 ± 0.08	0.06 ± 0.06	0.12 ± 0.08	-1.47	0.08
Rarity in population	-0.14 ± 0.07	0.16 ± 0.09	-0.14 ± 0.13	-2.38	0.014
	EP sires	Neighbors			
Relatedness to WP females	-0.10 ± 0.08	0.10 ± 0.07	-0.19 ± 0.09	-1.66	0.058
Rarity in population	-0.15 ± 0.08	0.04 ± 0.06	-0.19 ± 0.09	-2.24	0.02
	WP males	Neighbors			
Relatedness to WP females	-0.01 ± 0.06	0.10 ± 0.07	-0.11 ± 0.1	-1.06	0.15
Rarity in population	0.06 ± 0.1	0.04 ± 0.06	0.02 ± 0.12	0.17	>0.95
(b) Results with the 7 new loci					
	EP sires	WP males			
Relatedness to WP females	-0.16 ± 0.05	-0.13 ± 0.07	-0.03 ± 0.06	-0.52	0.30
Rarity in population	0.0 ± 0.1	-0.12 ± 0.1	0.12 ± 0.15	0.71	>0.95
	EP sires	Neighbors			
Relatedness to WP females	-0.17 ± 0.05	-0.06 ± 0.07	-0.11 ± 0.1	-1.41	0.089
Rarity in population	0.0 ± 0.11	-0.01 ± 0.08	0.01 ± 0.15	0.07	>0.95
	WP males	Neighbors			
Relatedness to WP females	-0.11 ± 0.08	-0.06 ± 0.07	-0.05 ± 0.12	-0.43	0.33
Rarity in population	-0.10 ± 0.12	-0.01 ± 0.08	-0.09 ± 0.15	-0.65	0.26
(c) Results with all 12 loci combined					
	EP sires	WP males			
Relatedness to WP females	-0.11 ± 0.04	-0.03 ± 0.05	-0.08 ± 0.07	-1.53	0.07
Rarity in population	-0.10 ± 0.04	0.02 ± 0.06	-0.08 ± 0.08	-1.36	0.09
	EP sires	Neighbors			
Relatedness to WP females	-0.13 ± 0.04	0.03 ± 0.05	-0.16 ± 0.07	-2.78	0.007
Rarity in population	-0.12 ± 0.04	0.02 ± 0.04	-0.14 ± 0.07	-1.91	0.037
	WP males	Neighbors			
Relatedness to WP females	-0.06 ± 0.05	0.03 ± 0.05	-0.09 ± 0.08	-1.16	0.13
Rarity in population	0.01 ± 0.06	0.02 ± 0.04	-0.01 ± 0.08	-0.14	0.44

we specifically tested whether EP sires were less related to females, or less likely to possess common alleles, than excluded neighbors or WP males, thus tests are one-tailed. Likewise, we used one-tailed comparisons to ask specifically whether excluded neighbors were more related to females and more likely to possess common alleles than were WP males. P -values given are not corrected for multiple comparisons.

RESULTS

Analysis at the five original loci indicated that EP sires were less likely to have alleles that were common in the population than were either WP males or neighbors that were excluded from paternity (Table 2a). Extra-pair sires also tended to be less related to focal females than were either WP males or excluded neighbors. By contrast, analysis at the set of seven new loci gave no indication that EP sires were less likely to have common alleles than were within-pair males and excluded neighbors; indeed, the trends were in the opposite direction (Table 2b), although not significantly so (both $P > 0.2$ if two-tailed tests were used). Extra-pair sires were less related to focal females than WP

males or excluded neighbors. This trend was more apparent in comparisons between EP sires and neighbors, but in neither case was the difference significant (Table 2b).

When the data from the original analysis at five loci were combined with the data from the seven additional loci, results were qualitatively similar those obtained with just the five original loci (Table 2c). Specifically, EP males were less likely to possess common alleles than were either WP males or excluded neighbors (although this was now only a trend with regard to WP males). In addition, EP sires again showed a strong tendency to be less related to focal females than were WP males. Extra-pair sires were significantly less likely to be related to females than were excluded neighbors, a result that, in the previous study, was only a strong trend.

DISCUSSION

Analyses previously reported by Masters et al. (2003) showed a significant difference between EP sires and both WP males and excluded, neighboring males (i.e., potential EP sires) with regard to possession of alleles that are common in the population,

and a (nonsignificant) trend for EP sires to be less related to females than were either WP males or excluded neighbors. Analysis of the same samples at the new set of seven loci revealed the same trend for EP sires to be less related to females than were WP males and excluded neighbors. With these new loci, however, there was no indication that EP males were more likely than their WP counterparts to have rare alleles. Clearly, an analysis based solely on the seven loci described in this paper would conclude that there is no evidence to suggest that genotype is significant for EP fertilizations in our Wyoming population of House Wrens, while analysis at the five original loci did. We do not see how to interpret this other than to conclude that the analysis is sensitive to the loci used.

Although a characterization of which features of microsatellite loci predict effective analysis in studies of EP mating is beyond the scope of this paper and this data set, some aspects of our analysis bear discussing. In analyses of either set of loci alone, EP sires tended to be less related to focal females than did WP males, and WP males tended to be less related to focal females than did neighbors excluded from paternity. However, these trends, while consistent in both sets, rise to significance only when both data sets are combined, and then only for the comparison of the extremes of the continuum, i.e., EP sires vs. excluded neighbors (Table 2c). Thus the correlation we have observed between relatedness and EP paternity is apparent only with the analysis of the larger number of loci obtained from combining both sets. By contrast, the analysis for genetic rarity gives a very different pattern. In the first set of alleles analyzed, EP sires had significantly fewer common alleles than did either WP males or excluded neighbors, while WP males had more common alleles than did excluded neighbors. In the second set of alleles, this pattern is reversed. Extra-pair sires actually had more common alleles than did either WP males or excluded neighbors, while WP males had fewer common alleles than did excluded neighbors. It is possible that associations between genetic rarity and EP mating success are most likely to be detected with certain types of loci. The two sets of loci that we studied do appear to be qualitatively different. The first set contains loci with fewer alleles, and the observed heterozygosity is considerably lower than that observed in the second set of alleles (Table 1). Additional studies are needed to determine whether this pattern is typical.

Both of the measures used here, relatedness to focal females and possession of common alleles, are population-level measures. Empirical analysis has demonstrated that population studies based on five or seven alleles are so limited as to be vulnerable to stochastic noise, and even a modest increase to 12 loci can result in a substantial increase in power of analysis (Koskinen et al. 2004). Taken in this context, our results are not surprising, and we would expect a more meaningful analysis with 12 loci rather than with either five or seven. Our results provide a caveat for all studies of this nature. Some studies, like the present one, have shown an association between genotype and EP mating success, whereas others have not (reviews in Akçay and Roughgarden 2007, Kempenaers 2007). This inconsistency might reflect differences among species in underlying biology; however, it is also possible that some results are an artifact of technical limitations. Such limitations include the number and nature of molecular markers used. A recent meta-analysis lists 22 studies that have tested the compatible-genes hypothesis to explain the occurrence of EP mating by females (Akçay and Roughgarden 2007). Of the 13 studies that used microsatellites (the rest employed multilocus minisatellites), the median number of loci employed was six. Use of a similarly limited number of loci is typical in studies testing

the hypothesis that females use EP mating to increase their offspring's heterozygosity (Kempenaers 2007). Given the results we describe in this paper, what can we really conclude from these studies?

In future studies, researchers should not only maximize the number of loci used but also recognize that some loci will be more informative than others (e.g., Liu et al. 2005). Ironically, the loci most preferred for paternity studies (i.e., those with many alleles) might be less useful in revealing population-level effects, such as degree of relatedness (Olsen et al. 2004, O'Reilly et al. 2004, Carreras-Carbonell et al. 2006). It seems likely that increasing the number of loci analyzed increases the probability of including loci sensitive to particular patterns of variation, and in the absence of knowledge of the utility of loci for particular analyses, this alone is a reason why the use of few loci is inappropriate.

We believe that until the molecular approaches used to determine mating patterns have been validated with regard to their ability to resolve intra- and inter-population variation (as was done, for instance, by Foerster et al. 2006), it will be difficult to draw conclusions about the role of heterozygosity and genetic distance in choice of EP mates. The effects of relatedness, if they exist at all, may be quite subtle, and at present we have little insight into the probability of type II errors for many of these studies. Given all of this, and the empirical example of this study, it is difficult at this point to interpret the outcomes of many of the studies currently in the literature on the effect of genotype on EP mating.

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