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Original paper

Intraspecific variation in the frequency of multiple paternity in the Japanese wood mouse (*Apodemus speciosus*)

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Abstract. The magnitude of intraspecific variation in the frequency of multiple paternity, and patterns in its relationship with other variables, was investigated in wild populations of the Japanese wood mouse *Apodemus speciosus*. The genotypes of 34 females and their offspring were determined on the basis of five microsatellite loci. A high proportion of litters (61.8% = 21/34 litters) were found to have been sired by multiple males. There was high variation in the proportion of multiple paternity in association with sampling localities and/or sampling years (27.3%–78.3%), which was higher than the interspecific variation shown by the proportion of multiple paternity across all studies (40.0%–65.2%) in the genus *Apodemus* (five species). This large magnitude of intraspecific variation might potentially affect the interpretation in previous studies making interspecific comparisons, and should therefore be taken into account as an important variable.

Key words: genus *Apodemus*, litter size, local variation, microsatellite.

Multiple male mating in a single estrous period causes competition between sperms from two or more individual males (Parker 1970), and is therefore a driving force in the evolution of reproductive traits, e.g., mating behavior, penile anatomy, sperm number, and morphology (Birkhead and Møller 1998). For example, larger testes have been reported in promiscuous primates than in species in which a male monopolizes mating (Harcourt et al. 1981). Male house mice evolving under sperm competition produce greater proportions of motile sperm, compared to males without sperm competition pressure (Firman and Simmons 2010). To assess the degree to which sperm competition is important as a selection force, it is necessary to determine the prevalence of females mating with multiple males. However, multiple male mating is difficult to observe in wild mammalian populations because of their cryptic mating behavior. Instead, indirect evidence of multiple male mating (e.g., patterns of social organization) has therefore been used in ecological studies on mammals (Harcourt et al. 1981; Kenagy and Trombulak 1986; Møller 1988).

Multiple paternity, in which multiple males sire offspring within a single litter, can be detected using molecular techniques, and is considered to be evidence of multiple male mating in multiparous animals. Determining multiple paternity is thus an important aspect of studies on multiple male mating in mammalian species, although multiple male mating is not always detectable. Multiple paternity has been observed in wild populations of more than 70 mammal species in nine orders (Soulsbury 2010, with the authors' supplementary survey), and shows a large variation in frequency between species; percentages of multiple paternity determined in these reports range between 0 and 100%.

Interspecific comparisons across mammalian species suggest that higher levels of multiple paternity are associated with larger relative testes size (Ramm et al. 2005; Soulsbury 2010). These comparative analyses, however, present some difficulties; most of the genetic samples tested for multiple paternity were taken from populations for which testes size was not directly measured. If the frequency of multiple paternity were both spatially and temporally stable across different populations of a single species, intraspecific variation, which would consequently be low, could be disregarded as a factor in interspecific comparisons, and would therefore not influence

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the results. However, if the frequency of multiple paternity is shown to vary significantly between different populations of the same species, or to vary temporally—thus affecting the impact of sampling timing—then the apparent relationship between multiple paternity and testes size shown in preceding studies would need to be reconsidered. This is because the values determined by the earlier studies do not consider intraspecific variation. In addition, in most studies, the frequency of multiple paternity was assessed based on data collected during only a part of the breeding season, so these studies might not account for any temporal variation.

Temporal or spatial intraspecific variations in the frequency of multiple paternity have in fact been observed in mammals. The frequency of multiple paternity varied seasonally for some species (Bryja and Stopka 2005; Ishibashi and Saitoh 2008). Interpopulation variations in multiple paternity have also been reported in two mammalian species. In domestic cats (*Felis catus*), the proportion of multiple paternity was 76.9% (n = 52) in an urban population, whereas it was 12.9% (n = 31) and 0% (n = 13) in a rural population and a sub-Antarctic population, respectively (Say et al. 1999, 2002). In house mice (*Mus musculus domesticus*), the proportion of multiple paternity ranged from 0 to 42.9% when comparing populations in various locations, including islands (Dean et al. 2006; Firman and Simmons 2008). On the other hand, Thonhauser et al. (2014) did not find either interpopulation or seasonal variation in multiple paternity frequency in *M. musculus musculus*.

Although information is available on multiple paternity in mammalian species, only a limited number of studies consider intraspecific variation (Bryja and Stopka 2005; Dean et al. 2006; Bryja et al. 2008; Firman and Simmons 2008; Ishibashi and Saitoh 2008; Thonhauser et al. 2014). Therefore, it is currently not possible to compare the magnitude of intraspecific and interspecific variation in the frequency of multiple paternity. In order to compare the magnitude of these variations, the frequency across various populations, or by season for a given species, needs to be investigated, and the results then compared with genetically closely related species, thus reducing the effects of the phylogenetic relationship. In this study, we focused on species of the genus *Apodemus*, because they are all common in the Palearctic region (Musser and Carleton 2005), and there is a relatively rich body of information on their multiple paternity. *Apodemus* species show interspecific variation in the frequency of multiple paternity, and intraspecific variation is also expected (e.g., Bryja et al. 2008; see next paragraph).

Multiple paternity has been reported in four *Apodemus* species, i.e., *A. uralensis* (= *A. microps*; Bryja and Stopka 2005; Bryja et al. 2008), *A. agrarius* (Baker et al. 1999; Bryja et al. 2008), *A. flavicollis* (Bryja et al. 2008; Gryczyńska-Siemiątkowska et al. 2008), and *A. sylvaticus* (Baker et al. 1999; Polechova et al. 2004; Booth et al. 2007; Bryja et al. 2008). The proportion of multiple paternity varies among studies. The highest frequency of occurrence was 100% of litters for *A. sylvaticus* (n = 5, Polechova et al. 2004), and the lowest was 30% for *A. flavicollis* (n = 10, Gryczyńska-Siemiątkowska et al. 2008). Although these values imply the existence of interspecific variation in multiple paternity, this variation must be examined in the context of intraspecific variation; any discussion about interspecific variation would be invalid if there were large intraspecific variations and focal data came from a limited area and/or period. In fact, the proportion of multiple paternity within single *Apodemus* species has been reported to show variation (33.3%–43.3% in *A. uralensis* (= *A. microps*); 58.8%–80% in *A. agrarius*; 30%–60% in *A. flavicollis*; and 50%–100% in *A. sylvaticus*). These data suggest the presence of intraspecific variation by locality and/or time of sampling (years or seasons), but the magnitude of this variation remains unclear, and needs to be assessed with respect to sample size and litter size.

The Japanese wood mouse, *Apodemus speciosus* (Temminck, 1844), is a common and endemic rodent species in Japan (Nakata et al. 2015). Individual females have home ranges that overlap with those of multiple males (Kondo 1977; Oka 1992), so multiple paternity may frequently occur. The aim of this study is to investigate multiple paternity frequency in populations of this species, taking spatial and temporal variation into consideration, and to analyze the magnitude of intraspecific variation. We investigated microsatellite genotypes of mothers and their offspring in wild populations of *A. speciosus*, and compared the intraspecific variation with interspecific variation in the genus *Apodemus*.

Materials and methods

Sample collection

Pregnant females of *A. speciosus* were collected from wild populations in Obihiro and Horokanai, Japan (Fig. 1), using Sherman-type live traps baited with oats and sunflower seeds. In Obihiro, trappings were conducted

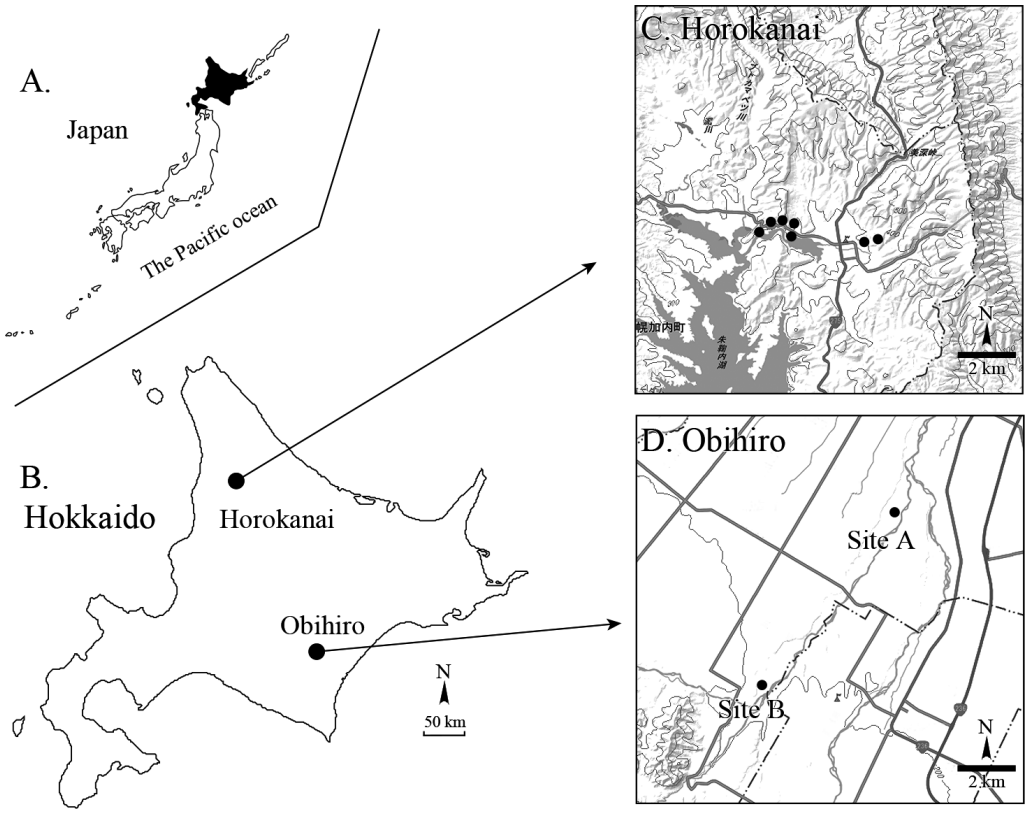

Fig. 1. Locations where sampling was conducted. A. the location of Hokkaido, Japan; B. the location of Horokanai and Obihiro in Hokkaido; C. sampling plots in Horokanai; and D. sampling sites in Obihiro. C and D were modified from the web map of The Geospatial Information Authority of Japan.

at two sites situated approximately 8 km apart. At one of the Obihiro sites (Site A), mice were captured in spring (between May and June in 2007 and 2008), while at the other site (Site B), they were captured in summer (between June and September in 2007, and August in 2008; Fig. 1D). These two trapping sites measured approximately 1 ha in 2007, and the area was extended to 1.5 ha in 2008. In Horokanai, pregnant females were captured from seven plots between May and September in 2013 and 2014 (Fig. 1C). The maximum distance between plots was approximately 4.5 km, and each plot measured approximately 0.5 ha.

After weighing the pregnant females (35 in total: 23 from Obihiro and 12 from Horokanai) and clipping one or two of their toes for DNA extraction, we housed them individually in separate plastic cages lined with sawdust bedding and covered by a wire lid. Food and water were provided ad libitum. A total of 222 offspring were delivered (134 from Obihiro females and 88 from Horokanai females). At approximately 30 days after birth, either the toes or tip of the tail of offspring were clipped to be used as tissue samples for DNA analyses.

Microsatellite analysis

DNA was extracted from clipped toes or tips of tails using the Chelex method (Walsh et al. 1991) or a DNeasy Blood and Tissue kit (Qiagen). The genotype of each mother and her offspring was determined using five microsatellite loci (Table 1). One primer of each primer pair was labeled using a fluorochrome. Microsatellite loci were amplified in a polymerase chain reaction (PCR) using the GeneAmp PCR System 9700 (Applied Biosystems). When we analyzed samples from Obihiro, the PCR reaction mixture contained the following: approximately 90 ng of DNA, 0.5 units AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1 × Taq buffer, 2 mM dNTPs, 0.25 mM of each primer, and water to a final volume of 15 μL. When we analyzed samples from Horokanai, the mixture contained approximately 90 ng of DNA, 1 × AmpliTaq Gold® 360 Master Mix (Applied Biosystems), 0.25 mM of each primer, and water to a final volume of 15 μL. PCR products were analyzed using an ABI PRISM 3100-Avant (Applied Biosystems). DNA fragments were quantified and analyzed using Gene Mapper software (Applied Biosystems).

Table 1. Summary of microsatellite loci and results of allele frequency analysis

Locus	Reference for initial characterization	Annealing temperature (°C)	Obihiro (n = 23)				Horokanai (n = 12*)			
			No. of alleles	H_O	H_E	Non-exclusion probability (2nd parent)	No. of alleles	H_O	H_E	Non-exclusion probability (2nd parent)
MSAA3	Ohnishi et al. (1998)	50	12	0.609	0.891	0.258	8	1.000	0.891	0.295
MSAA6	Ohnishi et al. (1998)	54	13	0.826	0.834	0.333	12	0.917	0.928	0.224
CAA2A	Makova et al. (1998)	60	11	0.913	0.867	0.299	7	1.000	0.851	0.360
GTTD9A	Makova et al. (1998)	55	6	0.652	0.692	0.575	5	0.833	0.790	0.473
TNF(CA)	Makova et al. (1998)	57	9	0.783	0.732	0.433	8	0.667	0.764	0.462
Mean	–	–	10.2	0.757	0.803	0.0064[#]	8	0.883	0.845	0.0052[#]

*: Including one female that delivered only two offspring.
[#]: Not mean but combined non-exclusion probability of all loci.

Multiple paternity analysis

Allelic diversity, heterozygosity, and non-exclusion probabilities when the first parent is known for markers were calculated using CERVUS 3.0 software (Kalinowski et al. 2007). A litter was classified as exhibiting multiple paternity when three or more paternal alleles at a locus were observed within the litter after subtracting maternal alleles. Multiple paternity cannot be detected using this method for litter sizes of two or less, which were therefore excluded from the multiple paternity analysis. This applied to only one female, caught at Horokanai, that delivered two offspring. The data collected from that female were used for allele frequency analyses, but not multiple paternity or litter size analysis. All other females delivered more than two offspring.

The least possible number of fathers was calculated from genotypes of a mother and offspring in a litter using GERUD v2.0 (Jones 2001, 2005).

Statistical analysis

We used a Fisher's exact test to determine the significance of the difference in frequency of multiple paternity between Obihiro and Horokanai samples. A generalized linear model (binomial distribution, logit link) was used to analyze the effect of multiple variables on the occurrence of multiple paternity, setting the paternity type of each litter (multiple paternity or not) as a binary dependent variable, and sampling location and season of copulation (spring or summer) as explanatory variables. Since the gestation period of *A. speciosus* is 19–26 days (Murakami 1974; Tsuchiya 1979; Oh and Mori 1998), females that delivered before and after July 22 were defined as spring copulation (April–June) and summer copulation (July–August) mothers, respectively. The effect of explanatory variables was assessed using a likelihood ratio test based on the change in deviance (ΔD) in the backward elimination approach.

A Wilcoxon rank sum test was also used to assess the significance of the difference in litter size between the Obihiro and Horokanai samples, and between multiple paternity litters and single sired litters.

Results

Frequency of multiple paternity

34 females (23 from Obihiro and 11 from Horokanai; these figures exclude one female that delivered only two offspring) and their 220 offspring (134 from Obihiro and 86 from Horokanai) were analyzed. The combined non-exclusion probability of the set of loci was lower than 1% in both of localities (Table 1); hence, the undetected multiple paternity was regarded as very low. The total frequency of multiple paternity was 21, and the percentage was 61.8% (21/34 litters). The frequency of multiple paternity was significantly higher in samples from Obihiro (18/23 = 78.3%) than in samples from Horokonai (3/11 = 27.3%; Fisher's exact test: $P < 0.01$; Table 2). The generalized liner model showed that sampling locality had a significant effect on the occurrence of multiple paternity, but not season (Table 3).

Number of fathers for a litter

The least possible number of fathers, which was calculated from genotypes of a mother and her offspring, was two in most multiple paternity litters (19/21 litters), but three for two litters in samples from Obihiro.

Litter size

The average litter size in the Obihiro sample (mean ± SD = 5.8 ± 1.11: range = 4–8) was significantly lower

Table 2. Frequency of multiple paternity in Obihiro and Horokanai, and other related data

Locality	Year	Site	Mothers	Multiple paternity litters	Multiple paternity (%)
Obihiro	2007	A	6	4	66.7
		B	6	6	100.0
		A+B	12	10	83.3
	2008	A	4	2	50.0
		B	7	6	85.7
		A+B	11	8	72.7
		Total	23	18	78.3
Horokanai	2013	–	8	1	12.5
	2014	–	3	2	66.7
		Total	11	3	27.3
	Total		34	21	61.8

Table 3. Results of a generalized linear model (binomial distribution, logit link function), evaluating separately the effects of sampling locality and season on the occurrence of multiple paternity

Term	Estimate	*SE*	*df*	*ΔD*	*P*
Sampling locality + Season			*2*	10.264	0.006
(intercept)	0.678	0.637			
Sampling locality; Horokanai	–2.651	0.966	1	9.709	0.002
Season; summer	1.261	0.935	1	2.006	0.157

The Nagelkerke's pseudo R-squared (Nagelkerke 1991) of the fundamental model is 0.3542.

than in the Horokanai sample (7.8 ± 2.04: range = 5–10, Wilcoxon rank sum test: $Z = -2.576$, $P < 0.01$). The size of litters sired by multiple males was significantly higher than that of the single sired litters from Obihiro (6.1 ± 1.00 [4–8] vs. 5.0 ± 1.22 [4–7], $Z = 1.881$, $P < 0.05$). Litter size did not differ between multiple sired and single sired litters in the Horokanai sample (multiple paternity: 7.0 ± 1.73 [6–9] vs. non-multiple paternity: 8.1 ± 2.17 [5–10], $Z = -0.836$, $P = 0.45$).

Discussion

Intraspecific variation in multiple paternity

In this study, a significant difference in multiple paternity frequency was observed between Obihiro and Horokanai samples. However, we were not able to separate the effect of the sampling locations from that of sampling years, because we did not investigate multiple paternity frequency of both populations in the same year. Therefore, the cause of the observed difference between these two localities can be considered as combined effects of locality and sampling year. Spatial and/or temporal variations in environmental conditions may lead to variations in mating behavior among populations of the same species. A long-term study including multiple locations is required in order to separate effects of locality from temporal effects.

Multiple paternity and litter size

In stochastic terms, a larger litter is more likely to show multiple paternity than a smaller litter. Intraspecific variation in litter size is commonly observed in mammalian species (Conaway et al. 1974; Whorley and Kenagy 2007; Bywater et al. 2010), and frequency of multiple paternity is reportedly dependent on litter size (Eccard and Wolf 2008). However, the variation of multiple paternity between the Obihiro and Horokanai samples observed in this study could not be explained by the variation of litter size. The proportion of multiple paternity was found to be higher in the Obihiro sample than in the Horokanai sample, but litter size was smaller in the Obihiro sample.

The relationship between multiple paternity and litter size differed between the Obihiro and the Horokanai samples. In Horokanai, where mice had larger litter size, no association was observed between litter size and paternity type (whether or not a litter exhibited multiple paternity). Conversely, in Obihiro, where litter size was smaller, litters sired by multiple males were larger than those sired by single males. Why did the relationship between litter size and paternity type differ between the two locations? This relationship may be masked in samples from Horokanai because of the low frequency of multiple paternity and small sample size. If most females copulated with multiple males, and the sample size were large enough, then it should be possible to detect this relationship. Populations from Obihiro may have satisfied these conditions; most females (over 78.3%) copulated with multiple males, and the sample size was 23. In contrast, populations from Horokanai may not have satisfied these conditions; the proportion of multiple paternity was low (27.3%), and the sample size was only 11. Since multiple paternity is a much less frequent characteristic in the Horokanai population, stochastic effects may have masked the effect of litter size on multiple paternity occurrence.

Magnitude of intraspecific variation

Table 4 summarizes findings on the proportion of multiple paternity for the species in the genus *Apodemus*,

Table 4. Proportions of multiple paternity litters, sample size, and sampling locality for five species of *Apodemus*, extracted from published articles and this study

Species	Locality	Sample size	Multiple paternity (%)	Multiple paternity (%) across all studies	Reference
A. speciosus	Obihiro, Japan	23	78.3	61.8	This study
	Horokanai, Japan	11	27.3		This study
A. agrarius	Northern Ukraine	10	80.0	63.6	Baker et al. (1999)
	Southeastern Slovakia	34	58.8		Bryja et al. (2008)
A. flavicollis	Southern Czech Republic & Southeastern Slovakia	25	60.0	51.4	Bryja et al. (2008)
	Northeastern Poland	10	30.0		Gryczyńska-Siemiątkowska et al. (2008)
A. urarensis (= *A. microps*)	Southern Moravia, Czech Republic	24	33.3	40.0	Bryja and Stopka (2005)
	Southern Moravia, Czech Republic & Southeastern Slovakia	46	43.5		Bryja et al. (2008)
A. sylvaticus	Northern Ukraine	6	50.0	65.2	Baker et al. (1999)
	The suburb of the city of Prague, Czech Republic	5	100.0		Polechova et al. (2004)
	Northern Ireland	13	53.8		Booth et al. (2007)
	Southern Moravia, Czech Republic	22	68.2		Bryja et al. (2008)

from this and earlier studies. The interspecific variation in the proportion of multiple paternity, calculated using frequencies of multiple paternity across all studies for each species, ranged between 40.0% and 65.2%. The intraspecific variation for *A. speciosus* (27.3%–78.3%) was larger than this interspecific variation. Therefore, for comparisons of multiple paternity, using a value from a particular study as representative for a given species might lead to incorrect conclusions. Some studies compared multiple paternity frequencies from a particular study as a representative value for a given species with relative testis sizes from another population of that species (e.g., Ramm et al. 2005; Soulsbury 2010). In addition to the intraspecific variation in multiple paternity frequency, testis size may also show intraspecific variation. Therefore, conclusions about correlation between frequency of multiple paternity and testes size might become erroneous if intraspecific variation is not taken into consideration.

Multiple male mating and sperm competition

The frequency of multiple male mating is always larger than the frequency of multiple paternity. In this study, 61.8% or more of the mating events comprised multiple male mating in wild *A. speciosus* populations. Although the true frequency of multiple male mating is unknown, this result suggests that sperm competition frequently occurs in *A. speciosus*. Sperm competition drives the evolution of reproductive traits (Birkhead and Møller 1998). Larger testes size in polyandrous taxa has been reported across various mammalian classes, in comparison with those of monandrous mammals (Gomendio et al. 1998). It is possible that frequent occurrences of sperm competition in *A. speciosus* may lead to the selection of larger testes producing more sperms in this species. In fact, males of *A. speciosus* have larger testes than expected. The mass of their testes is 3.17% of body mass (testes mass = 1.366 g, body mass = 43.1 g, $n = 24$; unpublished data), and 2.4 times as large as the expected mass obtained from the general equation for rodents (i.e., expected testes mass = $0.031 \times \text{body mass}^{0.77}$; Kenagy and Trombulak 1986).

The large intraspecific variation in the frequency of multiple paternity observed in this study suggests that the selection force may vary spatially and/or temporally, even in the same species, and thus a correlation between multiple paternity frequency and testes size would be expected within a species. A good example is provided by a study by Firman and Simmons (2008) on island populations of the house mouse (*M. musculus domesticus*). The authors collected samples to assess multiple paternity and testes size in the same sampling period from each of their study populations, and found that the frequency of multiple paternity was a predictor of testes size in the study populations. Further research that focuses on intraspecific variation in multiple paternity frequency and testes size is required to understand how reproductive traits reflect variations in selection forces.

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