Theoretical and empirical examinations of multiple paternity as an index of multiple male mating

（複数オス交尾の指標としてのマルチプルパタニティに関する理論的、実証的研究）

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Contents

Chapter 1

General Introduction

Multiple male mating and sperm competition

Multiple male mating (MMM) in a single estrous period causes competition among 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 sperm morphology (Birkhead and Møller 1998). For example, testes are larger in promiscuous primates than in species in which a male monopolizes mating (Harcourt et al. 1981). House mouse males under pressure from sperm competition produce greater proportions of motile sperm, compared to males without sperm competition pressure (Firman and Simmon 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, MMM of wild mammals have been observed in only a few of species e.g., some ground squirrels (Foltz and Schwagmeyer 1989; Lacey et al.1997), because of their cryptic mating behavior. Instead, indirect evidence of MMM (e.g., social mating system) has been used in ecological studies on mammals (Harcourt et al. 1981; Kenagy and Trombulak 1986; Møller 1988).

Multiple paternity as an index of multiple male mating

Multiple paternity (MP), in which multiple males sire offspring within a single litter, can be detected using molecular techniques, and is considered to be evidence of MMM in multiparous animals. Multiple paternity has been observed in wild populations of more than 70 mammal species in nine orders (Soulsbury 2010; authors supplementary survey), and shows a large interspecific variation in frequency; percentages of multiple paternity determined in these reports range between 0% and

100% (Soulsbury 2010; authors supplementary survey).

Comparative studies across species or populations reported the relationship between level of MP and testes size (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010). Those relationships have been explained by assuming that sperm competition may drive evolution of reproductive traits (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010). The explanation implies that the frequency of MP is used as an index of the frequency of MMM.

Problems in comparative analysis using frequency of multiple paternity

The comparative studies using MP frequency as an index of MMM have some deficiencies. First, these studies have not considered intraspecific variations of MP and reproductive traits. Most of samples tested for multiple paternity were taken from populations for which testes size was not directly measured. If the frequency of multiple paternity is shown to vary significantly between different populations of the same species, or to vary temporally, and the difference was larger than difference among species, then the apparent relationship between multiple paternity and testes size shown in preceding interspecific comparative studies would need to be reconsidered.

Second, they have also not considered the difference between MP frequency and MMM frequency, although the frequency of MP should be always lower than the frequency of MMM, because not all females that mated multiple males have a litter sired by multiple males. If the difference between MP frequency and MMM frequency is large and the difference rate was vary among species or populations, MP frequency could not be used as a substitute value of MMM frequency.

Conclusions about relationship between reproductive traits and sperm competition using MP frequency may become erroneous if those problems are not

considered.

Objectives of this thesis

In this study, I evidenced problems of previous studies with MP frequency as an index of MMM by showing empirical data and discussed important points that should be considered in future studies.

This thesis is composed of five Chapters. Chapter 1 is general introduction. In Chapter 2 and Chapter 3, I focused intraspecific variation and interspecific variation on frequency of MP in species of genus *Apodemus.* In Chapter 2, I investigated the magnitude of intraspecific variation in the frequency of MP in wild populations of the Japanese wood mouse, *Apodemus speciosus* (Wakabayashi et al. in press), and the results showed that intraspecific variation in MP frequency was considerably large. In Chapter 3, I investigated multiple paternity in the wild population of the small Japanese wood mouse (*A. argenteus*) in Hokkaido. In Chapter 4, I proposed a practicable method for estimation of MMM frequency in free-living populations considering three variables (MP frequency, litter size, and fertilization probability skew). I demonstrated this method using a data set of the grey-sided vole (*Myodes rufocanus*) population. Chapter 5 is general discussion. In that Chapter, I discussed about important points in future studies about frequency of multiple paternity as an index of multiple male mating frequency, and how this thesis contributes to understanding sperm competition.

Chapter 2

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

Introduction

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 Simmon 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; 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 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 2008a). 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 2008a; 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* (Gryczyńska-Siemiątkowska et al. 2008; Bryja 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. 2015a). 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 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).

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 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 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*, 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 (2008a) 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.

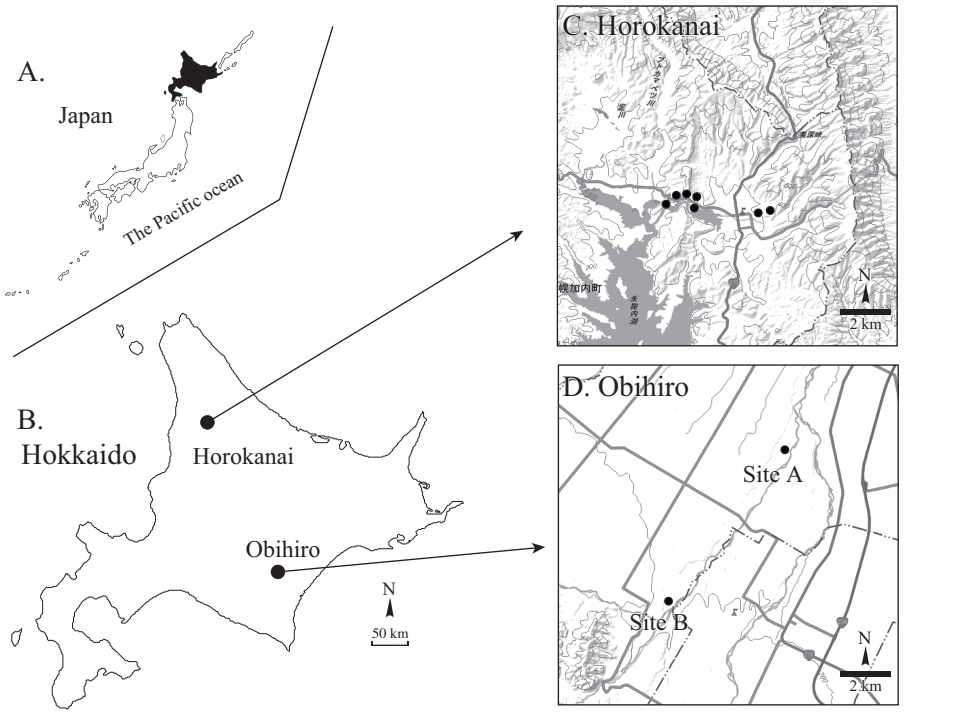


Fig. 1. Locations where sampling was conducted. A. the location of Hokkaido, Japan; B. the location of Horokanai and Obihiro in Hokkaido; C. sampling plots in Horokanai; and D. sampling sites in Obihiro.

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

Locus	Reference for initial characterization	Annealing temperature(℃)	Obihiro (N=23)				Horokanai (N=12*)			
			No. of alleles	*Ho*	*He*	Non-exclusion probability (2nd parent)	No. of alleles	*Ho*	*He*	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

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.

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)

Chapter 3

Multiple paternity in small Japanese wood mouse (*Apodemus argenteus*): A case study of a population in northern Hokkaido, Japan

Introduction

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 Simmon 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 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; authors supplementary survey) and shows a large variation in frequency among species; percentages of multiple paternity determined in these reports range between 0% and 100%.

The genus *Apodemus* has about 20 species, and they are all common in the Palearctic region (Musser and Carleton 2005). There is a relatively rich body of

information about their ecology and multiple paternity. Multiple paternity has been reported in five *Apodemus* species, i.e., *A. agrarius* (Baker et al. 1999; Bryja et al. 2008), *A. flavicollis* (Gryczyńska-Siemiątkowska et al. 2008; Bryja et al. 2008), *A. speciosus* (Wakabayashi et al. in press), *A. sylvaticus* (Baker et al. 1999; Polechova et al. 2004; Booth et al. 2007; Bryja et al. 2008), *A. uralensis* (= *A. microps*; Bryja and Stopka 2005; Bryja et al. 2008). Further data on multiple paternity are required to gain fully understanding of interspecific variation of multiple paternity in this genus.

In Japan, there are two common *Apodemus* species: the small Japanese wood mouse, *Apodemus argenteus* (Temminck, 1844) and the Japanese wood mouse, *A. speciosus* (Temminck, 1844). Both of them are endemic to Japan (Nakata et al. 2015a, 2015b). Females of *A. speciosus* have home ranges that overlap with those of multiple males (Kondo 1977; Oka 1992), suggesting polygamy or promiscuity, and a large variation of multiple paternity rate (27.3%–78.3%) was reported (Wakabayashi et al. in press). In contrast, Oka (1992) observed that home range of *A. argenteus* females overlapped with that of a single male throughout the year, suggesting monogamy. However, Ohnishi et al. (2000) observed several overlapping patterns of home ranges in breeding males and females. Although limited number of parents-offspring relationship could be detected by the kin-relationship analysis using DNA samples from wild population, the fact that some females mated different males in different mating events in same year was evidenced, (Ohnishi et al. 2000). Those suggested that the pair-bond between females and males is not absolute in *A. argenteus.* However, mating behavior in single estrous cycle has not been examined. Multiple paternity is an evidence that a female mated with multiple males. If the pair-bond in single estrous is common in *A. argenteus*, multiple paternity rate may be low, whereas if the pair-bond is limited, multiple male mating in single estrous cycle may occur, and some multiple paternity may be observed in this species.

In this study, we investigated microsatellite genotypes of mothers and their

offspring in wild populations of *A. argenteus* to reveal occurrences of multiple paternity.

Materials and methods

Sample collection

Pregnant females of *A. argenteus* were collected from wild populations in Horokanai, Japan (Fig. 1), using Sherman-type live traps baited with oats and sunflower seeds. Thirty-six pregnant females were captured from 11 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 and clipping one or two of their toes for DNA extraction, they were housed individually in separate plastic cages lined with sawdust bedding and covered by a wire lid. Food and water were provided ad libitum. 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.

Two pregnant females in 2013 died before delivery. Hence, tissue samples of their embryos were used for DNA analyses.

Microsatellite analysis

DNA was extracted from clipped toes or tips of tails using 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). the PCR reaction mixture contained the following: 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).

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. However all females delivered more than two offspring.

Microsatellite of one locus could not be amplified for one mother and two offspring from different family, although we carried out the PCR amplification several times. If litters were classified as multiple paternity using other four loci, the missing value from that locus would not have influenced our results. In a litter in which one locus of one mother could not be amplified, multiple paternity could be detected using other four loci. Other two litters, in which one locus of one offspring could not be amplified, were classified single paternity by the data from other four loci. Because only single paternal allele was detected in the problematic locus of other than the problematic offspring, and the decision that that litter was single paternity would not have changed if the problematic offspring had a new paternal allele.

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). In the three litters that had the missing value, we used other four loci for the calculation.

Statistical analysis

We used a Fisher's exact test to test frequency differences in multiple paternity between years (2013 and 2014) and the breeding seasons (spring and summer). The gestation period of *A. argenteus* have not been reported. Since the gestation period of other *Apodemus* species is 19–26 days (*A. agrarius*: 21–23 days Nowak 1999, *A. flavicollis*: 23 days: Nowak 1999, *A. semotus*: 20 days: Lin and Shiraishi 1992, *A. sylvaticus*: 23 days: Nowak 1999, *A. speciosus*: 19–26 days: Murakami 1974; Tsuchiya 1979; Oh and Mori 1998), females of *A. argenteus* that delivered before and after July 22 were defined as spring copulation (April–June) and summer copulation (July–August) mothers, respectively. In addition we compared frequency of multiple paternity of *A. argenteus* with that of *A. specious* using Fisher's exact test. We used the data of *A. specious* collected by Wakabayashi et al. (in press) in same area and same period.

Brunner-Munzel test was also used to assess a difference in litter size between multiple paternity and single sired litters, between years and between seasons.

Results

Frequency of multiple paternity

Thirty-six females (18 in 2013 and 18 in 2014) and their 173 offspring (93 in 2013 and 80 in 2014) were analyzed. The combined non-exclusion probability of the set of loci was lower than 1% (Table 1). Hence, the undetected multiple paternity was regarded as very low. The total frequency of multiple paternity was seven, and the percentage was 19.4% (7/36 litters). The frequency was not different significantly with that of *A. speciosus* in same area (3/11 = 27.3%; Fisher's exact test: $P = 0.679$).

Significant difference in frequencies of multiple paternity was not observed between sampling years (2013: 4 /18 = 22.2% vs. 2014: 3/18 = 16.6%; Fisher's exact test: $P = 1$), and copulation seasons (spring: 5 / 20 = 25.0% vs. summer: 2/16 = 12.5%;

Fisher's exact test: $P = 0.426$).

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 (6/7 litters), but three for one litter.

Litter size

Mean ± *SD* [range] of litter size was 4.8 ± 1.21 [3–8]. Litter size did not differ between multiple sired and single sired litters (multiple paternity: mean ± *SD* [range]=5.6 ± 1.40 [4–8] vs. non-multiple paternity: 4.6 ± 1.12 [3–8], Brunner-Munzel test: Brunner-Munzel test statistic = 1.8055, $df = 8.02$ $P = 0.109$), and seasons (spring: 5.1 ± 1.10 [4–8] vs. summer: 4.5 ± 1.32 [3–8], Brunner-Munzel test statistic = -1.6942, $df = 24.26$, $P = 0.103$). However, litter size was significantly larger in 2013 than in2014 (2013: 5.2 ± 1.20 [3–8] vs. 2014: 4.4 ± 1.19 [3–8], Brunner-Munzel test statistic = -2.3364, $df = 33.99$, $P < 0.05$).

Discussion

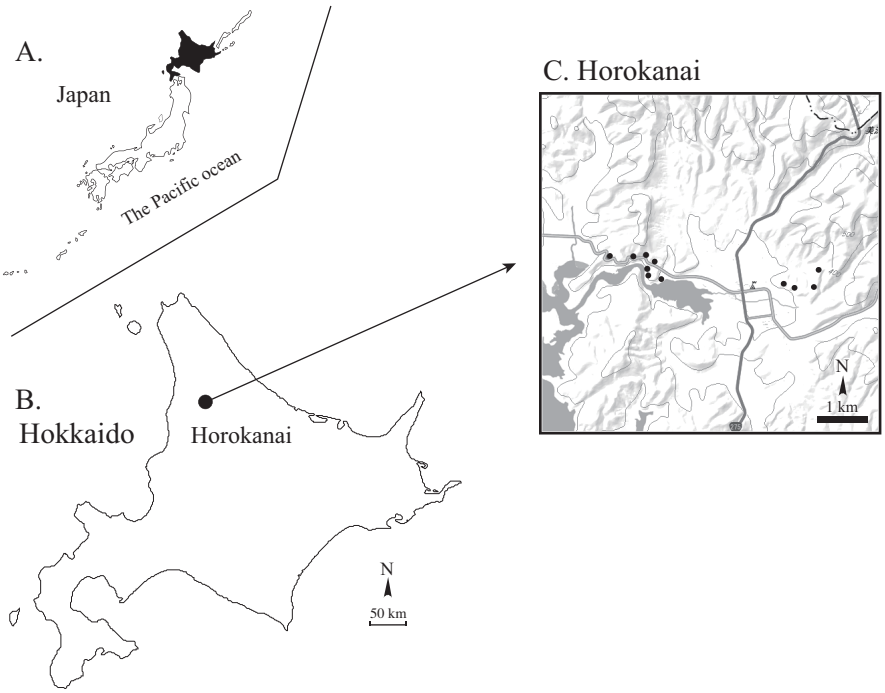
Multiple paternity and home range

Oka (1992) observed that the each home range of females overlapped with that of a single male throughout the year in *A. argenteu*, and suggested that *A. argenteus* is monogamous. Such a paring pattern was also observed in another studies (Nishikata 1982). On the other hand, the fact that home ranges of females overlapping that of multiple males was reported in other studies (Setoguchi 1981, Ohnishi et al. 2000). If the pair-bond is strong, the frequency of multiple paternity would be very low in a

population of *A. argenteus*. In this study, the proportion of multiple paternity was 19.4%. It suggests that multiple male mating occurred in at least 19.4% of mating events in the wild population of *A. argenteus* and that the pair-bond in *A. argenteus* is not absolute even in a single estrous cycle. However, this results does not directly indicate that *A. argenteus* is promiscuous, but not monogamous, because extra-pair mating in monogamous species may raise multiple paternity.

Difference of between two sympatric Apodemus species

Oka (1992) observed a difference of spatial distribution patterns of home ranges in two sympatric species, *A. argenteus* and *A. speciosus,* and suggested difference of mating system; *A. argenteus* is monogamous and *A. speciosus* is promiscuous. In this study, difference in multiple paternity frequency was not observed between these two species, and difference of mating system was could not detected by genetic paternity analysis in this area and years, at least. However, intraspecific variation of multiple paternity frequency was observed in some mammalian species (Say et al. 1999, 2002; Dean et al. 2006; Firman and Simmons 2008a) including *A. speciosus* (Wakabayashi et al. in press). In *A. argenteus,* the spatial distribution of home ranges varies among populations (Setoguchi 1981; Nishikata 1982; Oka 1992; Ohnishi et al. 2000). In order to obtain conclusive results on the difference in reproductive ecology between these species, more intensive investigations including detailed reproductive behavior with longer span in multiple populations are needed.



C was modified from the web map of The Geospatial Information Authority of Japan

Fig. 1. Locations where sampling was conducted. A. the location of Hokkaido, Japan; B. the location of Horokanai in Hokkaido; C. sampling plots in Horokanai.

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

Locus	Reference for initial characterization	Annealing temperature(℃)	N	No. of alleles	H_O	H_E	Non-exclusion probability (2nd parent)*
MSAA3	Ohnishi et al. (1998)	54	36	11	0.917	0.846	0.316
MSAA4	Ohnishi et al. (1998)	43	36	12	0.833	0.849	0.308
MSAA6	Ohnishi et al. (1998)	54	36	11	0.722	0.814	0.371
GTTC4A	Makova et al. (1998)	55	36	7	0.694	0.707	0.554
TNF(CA)	Makova et al. (1998)	60	35	14	0.886	0.856	0.285
Mean	-	-		11	0.810	0.815	$0.0057^{\#}$

N sample size; H_O and H_E, observed and expected values of heterozygosity, respectively

* Probability that an individual who is not a parent of an offspring can not be excluded from candidate parents when the first parent is known

$^{\#}$Not mean but combined non-exclusion probability of all loci

Chapter 4

Estimation of multiple male mating frequency from the frequency of multiple paternity: An example of a gray-sided vole (*Myodes rufocanus*) population

Introduction

Multiple male mating (MMM) in a single estrous period causes competition among sperms from two or more individual males (Parker 1970), and can be, 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). House mouse males under pressure from sperm competition produce greater proportions of motile sperm, compared to males without sperm competition pressure (Firman and Simmon 2010). To assess the degree to which sperm competition is as a selection force, it is necessary to determine the prevalence of females mating with multiple males.

However, MMM of wild mammals have been observed in only a few of species e.g., some ground squirrels (Foltz and Schwagmeyer 1989; Lacey et al.1997), because of their cryptic mating behavior. Instead, indirect evidence of MMM (e.g., social mating system) has been used in ecological studies on mammals (Harcourt et al. 1981; Kenagy and Trombulak 1986; Møller 1988).

Multiple paternity (MP), in which multiple males sire offspring within a single litter, can be detected using molecular techniques, and is considered to be evidence of MMM in multiparous animals. In some studies, the frequency of MP was used as an index of degree of sperm competition (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010). Those studies reported the relationship between level of MP and reproductive traits [e.g., testes size (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010) and sperm morphology (Firman and Simmons 2008a)]. However, MP frequency is always lower than MMM frequency, because not all females that mated with multiple males produce a MP litter. Even though a female mated with two of more males, one male could monopolize all ova in her litter. In this

case that litter represents single paternity (SP).

Is the difference between MP and MMM frequency small enough to be ignored? This difference is attributed to SP frequency in which a female gives a SP litter even though that female mates with two or more males (a SP litter with MMM). In fact, SP frequency consist of SP litters with MMM (SP_{MMM}) and SP litters that is produced by single male mating (SP_{SMM}), and MMM frequency is the sum of MP frequency and SP_{MMM} frequency (Fig. 1). The relationship between the MMM frequency and the MP frequency is given by the following equation:

$$\text{MP frequency} = \text{MMM frequency} \times \text{MP probability} \quad \text{(Eq. 1).}$$

The probability to be a MP litter (MP probability) is given as the probability that a female that mates with two or more males produces MP litter (in other words the probability that MMM females does not produce a SP litter). MP probability is influenced by litter size (the number of offspring in a litter) and fertilization probability (when MMM occurs, the probability that one male sires each ovum in a litter), because small litter size and skewed fertilization probability enhance SP_{MMM}. MP probability can be described as a following equation (Eccard and Wolf 2009).

$$\text{MP probability} = 1 - SP_{MMM}\text{ probability} = 1 - FPS^{LS} - (1 - FPS)^{LS} \quad \text{(Eq. 2),}$$

where LS is litter size and FPS is fertilization probability skew. Since fertilization probability ranges between zero and unity, and FPS is defined as a highest fertilization probability, FPS takes the value from 0.5 (when MMM occurs, two males have even possibility to sire an ova) to 1 (one male always monopolizes all ovum) in the case where a focal female mates with two males. Small litter size and/or skewed fertilization probability makes MP probability to be close to zero, whereas large LS and small FPS makes that probability to be close to unity (Fig. 2). Hence, in animals that deliver hundreds of offspring in same time, like some insects or fishes, the difference between MP and MMM frequency may be negligibly small. However, in animals with small LSs (e.g., mammals and birds), the difference between MMM and MP frequency may be

considerably large, particularly when the FPS is large. In addition, since the effect of LS and FPS is nonlinear (Fig. 2), it is not easy to discuss about MMM frequency simply based on MP frequency. If the difference between MP and MMM frequency is fixed or change with fixed rate in any species or populations, effects of the difference on comparative analysis may be not critical, even if the difference is large. However, the difference complexly vary by the combination of LS and FPS (Fig. 2), and the difference between MMM and MP frequency may affect comparative analyses and cause an erroneous conclusion. Therefore, we should estimate MMM frequency by considering not only MP but also LS and FPS, instead of regarding MP frequency as an alternative of MMM frequency.

FPS among males may arise from mating order effects (Levine 1967; Foltz and Schwagmeyer 1989; Lacey et al.1997; Klemme et al. 2006; Firman and Simmons 2008b), genotype of male or interactions between male and female genotypes (Edwards 1955; Levine 1967; Klemme et al. 2006; Firman and Simmons 2008b), or other male characteristics (Klemme et al. 2006). It is very hard to determine FPS in a free-living population, because it is necessary to know which female mates with multiple males. When females copulate two males that have different characteristic A and B (e.g., mating order, or sibship with the female), fertilization probability of A can be calculated as the proportion of offspring that sired by male A in all offspring across MMM litters (Fig. 1 and its legend). In this case, all litters produced by MMM should be examined, including SP_{MMM} litters. In a free-living population, however, it is practically impossible to identify which female mates with multiple males and characteristics of sires like mating order. In addition, FPS observed under laboratory conditions cannot be used for the estimation of MMM frequency for wild populations, because FPS may vary according to ecological conditions. Dean et al. (2006) estimated the probability that MMM occurs of wild house mice (*Mus musculus domesticus*) using observed MP frequency (33 / 143 litters) assuming various FPSs ranging from 0.5 to 1. As a result,

the probability that MMM occurs was estimated with a wide range (0.2-1.0). The wide range of this estimation is not suitable for a practical use to comparative analyzes.

Paternity skew (PS) is the proportion of offspring in a litter for each of sires. PS can be observed for each MP litter. It is the highest one in the proportion of offspring sired by involved males (Fig. 1). FPS should be calculated from all MMM litters including SP_{MMM} litters, while PS can be calculated based only on MP litters. Average PS of MP litters depends on fertilization probability and LS, but it is independent of MMM frequency. Thus, we could obtain PS of MP litters in an observed population and infer FPS from the observed PS of MP litters and LS.

In this study, we propose a method for estimation of MMM frequency in free-living populations considering three variables (MP frequency, LS, and FPS). We incorporated an observed PS to infer a possible range of FPS that cannot be measured in free-living populations. We demonstrated our method using a data set of the grey-sided vole [*Myodes rufocanus* (Sundevall, 1846)] population.

Materials and methods

The variables that are required to estimate MMM frequency are MP frequency, LS, and FPS (Eq. 1 and Eq. 2). MP frequency and LS are observable, but FPS is hard to measure in empirical populations. Using empirical data on a house mouse population, Dean et al. (2006) showed the possible range of the probability occurring MMM to be observed MP frequency assuming the full range of FPS. Their estimated range of MMM probability was, however, too wide for a practical use (0.2-1.0). Our new idea is to identify a possible range of FPS on the basis of observed PS of MP litters. By using a limited range of FPS the estimated range of MMM frequency could be greatly reduced.

Dataset of the gray-sided vole

The dataset of the gray-sided vole in an enclosed population that ware collected by Ishibashi and Saitoh (2008) was used. They determined parentage of 918 weaned voles (454 females and 464 males) from 215 litters ($N = 215$). The mean number of weaned offspring per litter (LS) was 4.2 with the range between 1-9. The proportion of litters with multiple sires was 23.3% (i.e., the observed frequency of MP (oMP) = 50); 48 litters (22.3%) were sired by two males and only two litters (0.9%) ware sired by three males. SP frequency was 165 litters (76.7%). The average PS of MP litters (oPS) in two sires was 0.680.

Estimation of the frequency of multiple male mating

On the assumption of various combinations of MMM frequency and FPS covering full ranges of those parameters, we can deterministically calculate MP frequency on the basis of empirical data on LS (see Eq. 1 and Eq. 2). In other words, by examining those MP frequencies, we could obtain a possible range of MMM frequencies that produce the observed MP frequency ($oMP = 50$). Although we can use an average as a representative of LS for the deterministic model, stochastic effects of LS variation may not be negligible. That variation should be critical for animals with small LS in particular. Additionally, the relationship between PS and FPS is not straightforward, and it is difficult to describe that relationship as a simple equation (Fig. 1 and its legend). Therefore, we examined the probability of oMP occurrence in possible conditions of LS, MP frequency, and FPS, using an individual based model (IBM). Another probability that we should consider was the probability that the observed PS of MP litters (oPS) occurs. By exploring the probability of the intersection of oMP and oPS $P(oMP \cap oPS)$, plausible MMM frequency and FPS could be identified.

We conducted IBM analyses using four observed values of a grey-sided vole population; i.e. sample size (N = 215), the frequency of MP (oMP = 50), the average PS of MP litters (oPS = 0.680), and the frequency distribution of LS. We assumed that a female mated with one or two males. We considered that the assumption was reasonable, because litter sired by three males was only two (4% of MP litters) in the grey-sided vole population. The R statistical software version 3.3.2 (R Core Team 2016) was used for the IBM analyses. The details of IBM are described below.

Step-1. *Probability of the occurrence of the observed frequency of multiple paternity*

To estimate the probability of oMP occurrence $P(oMP)$, MP frequencies were obtained in various conditions of MMM frequency and FPS; MMM frequency was ranged from 1 to 215 (N) in increment of 1, and FPS was ranged from 0.5 to 0.995 in the increment of 0.005 (100 conditions). For each of the combinations of MMM frequency and FPS (215 × 100), hypothetical MMM litters were generated, according to the given frequency of MMM. A litter size, which was randomly selected from the observed frequency distribution of LS with replacement, was given to each hypothetical MMM litter. For each offspring in a litter, a sire was probabilistically selected from two candidate males based on the given FPS. If both of sires were selected for a litter, that litter was regarded as a MP litter, while if a single male monopolized a litter, that litter was a SP litter. We counted MP litters (MP frequency) in the hypothetical MMM litters. These processes were repeated 1,000 times for each combination of MMM frequency and FPS. The proportion of MP frequencies that were equal to oMP in 1,000 times was obtained and defined as $P(oMP)$ for that combination.

Step-2. *Probability of the occurrence of the observed paternity skew under the observed frequency of multiple paternity*

The probability of the occurrence of the observed PS under the observed MP frequency

$P_{oMP}(oPS)$ was obtained for each FPS between 0.5 and 0.995 with the increment of 0.005 (100 conditions). Hypothetical MMM litters were generated based on assigned LSs that were randomly selected from the observed frequency distribution of LS. The paternity type (MP or SP) was determined by the same procedure of Step-1. After fifty MP litters (= *oMP*) were obtained, PS was calculated in each MP litter, and the PSs were averaged. These processes were repeated 1,000 times for each of 100 FPSs. A proportion of the average PSs that were approximately equal to *oPS* was obtained and defined as $P_{oMP}(oPS)$ of the given FPS. This approximation was done because there was very small chance that the average PS became the exactly same as *oPS*. When the difference between these values was smaller than 0.0025, these values were regarded as approximately equal.

Step-3. *Probability of the occurrence of observed frequency of multiple paternity and the observed paternity skew*

The probability of the intersection of *oMP* and *oPS* $P(oMP \cap oPS)$ is given by the following conditional probability (Eq. 3):

$$P(oMP \cap oPS) = P_{oMP}(oPS) \times P(oMP) \quad \text{(Eq. 3).}$$

For each combination of MMM frequency and FPS (215 × 100 combinations), we multiplied $P(oMP)$ Step-1 by each $P_{oMP}(oPS)$ from Step-2.

Step-4. *Estimation of frequency of multiple male mating*

To assess the plausibility of MMM frequency, we summed $P(oMP \cap oPS)$ for the full range of FPS between 0.5 and 0.995 for each MMM frequency. We considered that a MMM frequency having the largest sum of $P(oMP \cap oPS)$ was the most plausible value for the observed population. Additionally, we obtained 95% confidence interval as follows: $P(oMP \cap oPS)$ of all MMM frequencies were summed as the total $P(oMP \cap oPS)$, and we removed highest 2.5% and lowest 2.5% of MMM frequencies

in the total $P(oMP \cap oPS)$. The highest and lowest values of MMM frequencies in the remaining $P(oMP \cap oPS)$ was considered as the 95% confidence interval of estimated MMM frequency.

Results

Probability of the occurrence of the observed frequency of multiple paternity

Probability of the occurrence of the observed MP frequency, *P*(*oMP*), in the grey-sided vole population was illustrated for every combination of MMM frequency and FPS in Fig. 3. On most parameter space *P*(*oMP*) was low, while points showing higher probability than 0.1 were distributed on a J–shaped space. The highest *P*(*oMP*), 0.148, was observed, when MMM frequency was 64 (29.8%) and FPS was 0.565. The possible range of MMM frequencies, of which probability was higher than 0.05, was wide, ranging between 59 (27.4%) and 215 (100%).

Probability of the occurrence of the observed paternity skew under the observed frequency of multiple paternity

Probability of the occurrence of *oPS* under *oMP*, P_{oMP}(*oPS*), for every FPS was illustrated in Fig. 4. P_{oMP}(*oPS*) was extremely low on higher and lower sides of FPS, while highest one, 0.135, was observed when FPS was 0.725.

Probability of the occurrence of observed frequency of multiple paternity and the observed paternity skew

The probability of the intersection of *oMP* and *oPS* $P(oMP \cap oPS)$ for every combination of MMM and FPS was illustrated in Fig. 5. On most parameter space $P(oMP \cap oPS)$ was low. Space showing higher probability was greatly reduced in

comparison with $P(oMP)$ in Fig. 3. Highest $P(oMP \cap oPS)$, 0.014, was observed when the MMM frequency was 78 (36.3%) and the fertilization probability skew was 0.725.

Estimation of frequency of multiple male mating

The probability of the intersection of *oMP* and *oPS* $P(oMP \cap oPS)$ for every MMM frequency was illustrated in Fig. 6. $P(oMP \cap oPS)$ was extremely low on higher and lower sides of MMM, while highest one, 0.255, was observed when MMM frequency was 71 (33.0%). Its 95% confidential interval was from 61 (28.4%) to 100 (46.5%).

Discussion

Estimation of the multiple male mating frequency of the gray-sided vole

In this study, we estimated MMM frequency of the gray-sided vole population with the 95% confidential interval. Although the observed MP frequency was 50 (23.3%) in that population, the estimated MMM frequency was 71 (33%) and the 95% confidential interval was from 61 (28.4%) to 100 (46.5%). Point estimation of MMM frequency was realized and the range of estimation value was practically limited in this study. Hence, these estimations enable empirical discussion about MMM frequency.

Effect of incorporating an observed paternity skew of multiple paternity litters on the estimation

Dean et al. (2006) tried to estimate MMM frequency in populations of house mice (*M. musculus domesticus*), in which MP was found in 33 of 143 litters (23.1%). However, their estimation range was impractically wide (20%-100%). A cause of that wide range was the uncertainty of FPS. They had to estimate MMM frequency

assuming the full range of FPS (0.5-1.0). In fact, the estimated range of MMM proportion was also wide in the full range of FPS of this study (27.4%-100%). In this study we incorporated $P_{oMP}(oPS)$ instead of using full range of FPS. As a result, we could drastically reduce the estimation range (28.4%-46.5%). It suggests that the FPS is a necessary variable to estimate MMM frequency, and the use of observed PS is attributed to the great improvement of MMM frequency estimation in free-living populations in which FPS could not be determined.

Limitations of this method

A key variable of our method is PS in order to narrow the estimated range of MMM frequency. The dataset of gray-sided voles used in this study was collected in the enclosed population, not only mothers but also fathers of offspring could be identified, and PS of MP litters could be determined. However, genotypes of candidate fathers are not always utilizable in wild populations, and MP has been analyzed sometimes only data on mother and offspring genotypes. For example, Dean et al. (2006) analyzed MP using the data on pregnant females and their embryos without identifying fathers. It is because, paternal care is absence is in many mammal species, and identification of candidate fathers is difficult in wild population, unlike some bird species that form a pair-bond. In fact, MP has been analyzed without identifying a father in more than 30 studies for wild mammal populations (approximately 30% of wild mammal MP studies; authors survey). However, PS can be estimated from genotypes of mothers and offspring and the allele frequency of a population by some software. For example, GERUD (Jones 2001, 2005) was used for PS calculation in some wild rodent populations (Booth et al. 2007, Firman and Simmons 2008a). Therefore, PS may be determined in most studies on multiple paternity.

An enough number of MP litters is also critical to obtain reliable $P_{oMP}(oPS)$. When the number of MP litters is small, the variance of estimated PS is large, and the

variation of $P_{oMP}(oPS)$ between FPSs is too small to identify the most possible FPS (Fig. 7). When the proportion of MP is low like the gray-sided vole population, a large number of litters is required to ensure the enough number of MP litters. On the other hand, when the proportion of MP is high enough, required sample size become small.

In this study, we assumed that females mated with two males when MMM occurred. In the gray-sided vole population, litter sired by three males was only two (4% of MP litters). Hence the assumption seems to be realistic. However, effects of the number of mating males on the estimation of MMM frequency is not known. The evaluation of the applicability of this method to populations, in which litters sired by three or more males are frequent, is subject of future investigation.

For future use of this method

Our method requests three variables, i.e., MP frequency, LS and PS. It is not hard to obtain those variables by conventional sampling. When MP frequency in a population is determined, the frequency distribution of LS is usually obtained, and PS of MP litters can be estimated using genotype of mothers and offspring and the allele frequency of the population. Sufficient sample size is critical to obtain reliable $P_{oMP}(oPS)$ in our method. When MP frequency is small, the variation of $P_{oMP}(oPS)$ between FPSs is too small to narrow the range of estimated MMM frequency (Fig. 7). A necessary sample size may be different depending on the proportion of MP litters. In this study, we could estimate MMM frequency based on 50 MP litters in the total of 215 litters. Paternity of wild multiparous mammal populations have been analyzed in more than 100 studies, and multiple paternity has been observed in more than 70 mammal species in nine orders (Soulsbury 2010; authors supplementary survey). However, sample sizes are mostly 30 litters or less, and the number of study of which sample size is 200 litters or more is only two (Foltz 1981; Ishibashi and Saitoh 2008). Therefore, studies with a large sample size that supply an enough number of MP litters are strongly

encouraged. The relationship between the number of MP litters (sample size × MP proportion) and the estimation of MMM frequency has not been examined systematically. Such studies are also encouraged to determine a recommended sample size for MP studies.

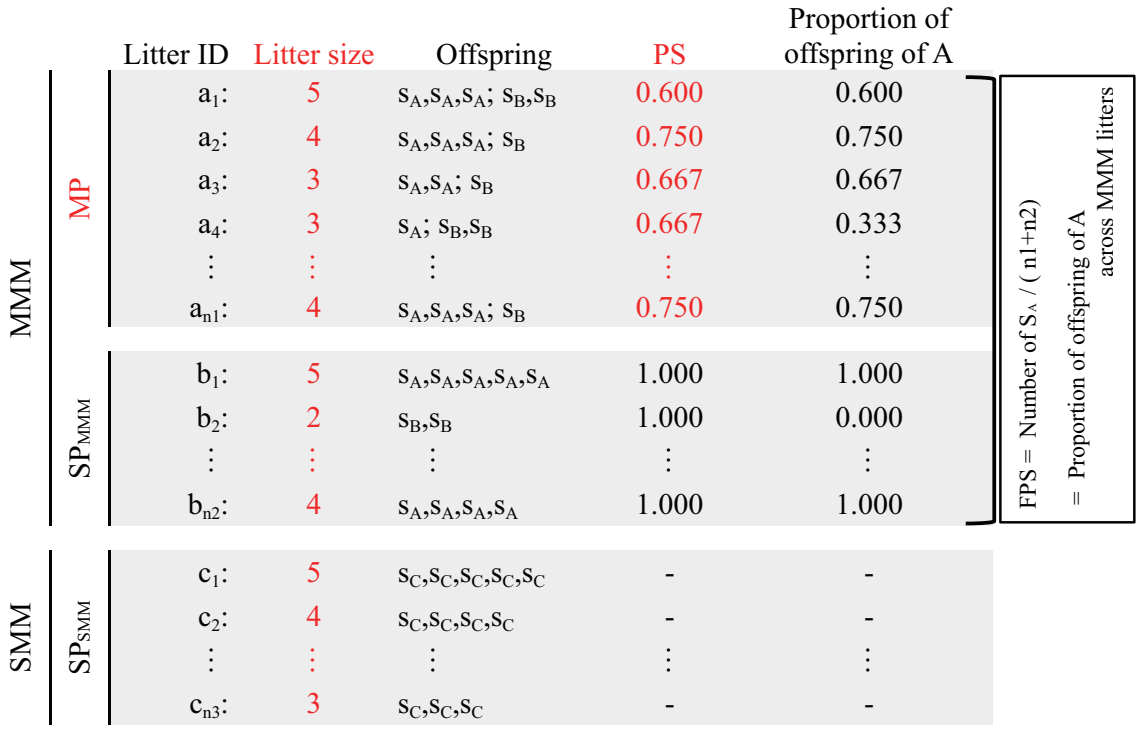


Fig. 1. A schematic example of the contents of litters. Observable variables are shown by red, while black letters represent the values that cannot be directly measured. Observed litters consist of multiple paternity litters (MP) and single paternity litters (SP). Further, SP litters consist of two types of litters; ones derived from multiple male mating (SP_{MMM}) and the others from single male mating (SP_{SMM}), although they are not distinguishable. The frequency of multiple male mating (MMM) is the sum of MP and SP_{MMM} frequency (MMM = MP + SP_{MMM}). In this example (n1 + n2 + n3) litters were observed. Of those observed litters n1 litters were MP (a_1 … a_{n1}), while (n2 + n3) litters were SP. Although n2 and n3 litters were SP_{MMM} (b_1 … b_{n2}) and SP_{SMM} (c_1 … c_{n3}), respectively, they were not distinguished. MMM females were assumed to mate with two males (A and B), where A and B had different characteristic (e.g., mating order, or sibship with a focal female). A and B may be different individuals among different litters. For example, in the litter a_1 a mother mated with male A and B, and A and B sired three and two offspring, respectively. In this case, paternity skew (PS) is

calculated as 0.600. In the litter b_1 a mother also mated with male A and B, but A monopolized that litter and PS is 1.000. In the litters of SP_{SMM} mothers mated with single male (e.g., C) and their PSs should not be considered. Fertilization probability skew (FPS) is the proportion of offspring sired by A (or B) in the sum of MMM litters. In this case A sired 21 of 30 offspring (0.700) and B sired 9 of 30 offspring (0.300). Since FPS is defined as a higher value, it is 0.700 in this population. In the litter a_4, male B sired more offspring than male A, and PS is 0.667 by B but not 0.333 by A, because PS is defined as a higher value. Therefore, PS is not always the same as a proportion of offspring of A. Since the relationship between PS and FPS is non-straightforward, their relationship cannot be described as a simple equation.

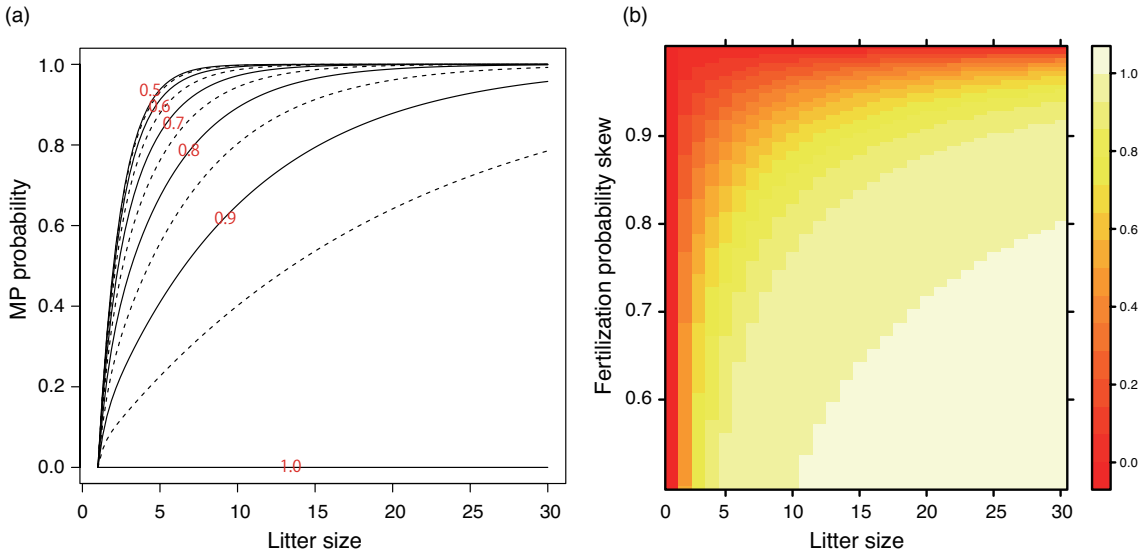


Fig. 2. Effects of litter size (LS) and fertilization probability skew (FPS) on the occurrence of multiple paternity. Probability to be multiple paternity (MP probability) is calculated with 30 LSs based on Eq.2 in the main text assuming 11 FPSs between 0.5 and 1.0. (A) The relationship between MP probability and litter size under the 11 conditions of FPS. FPSs are shown by red. (B) MP probability under various combinations of FPS and LS. Lighter color indicates higher probability to be multiple paternity (MP probability).

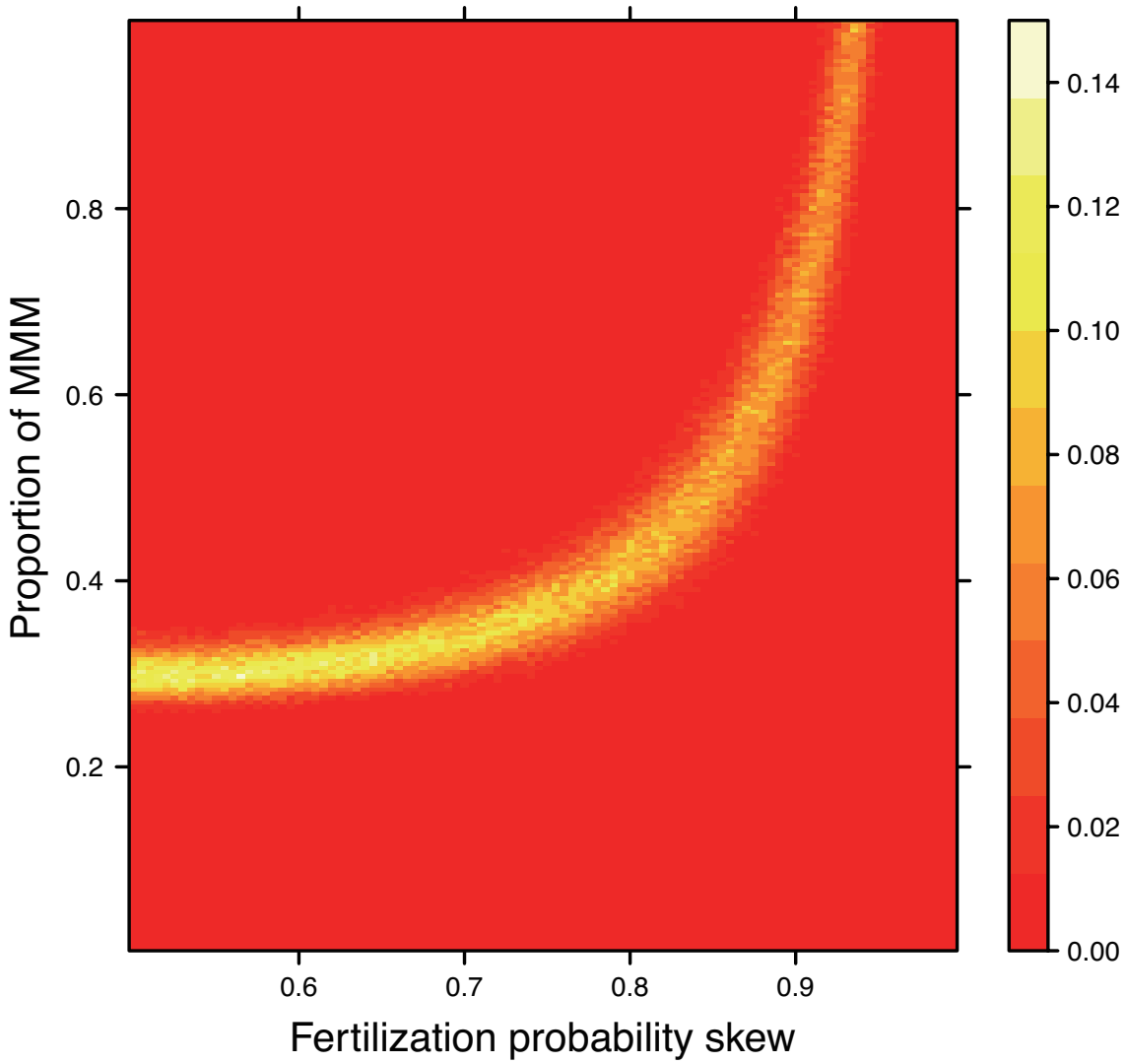


Fig. 3. The probability that the frequency of multiple paternity (50 litters / 215 litters) observed in the grey-sided vole population occurs for each combination of multi male mating proportion and fertilization probability skew. Lighter color indicates higher probability.

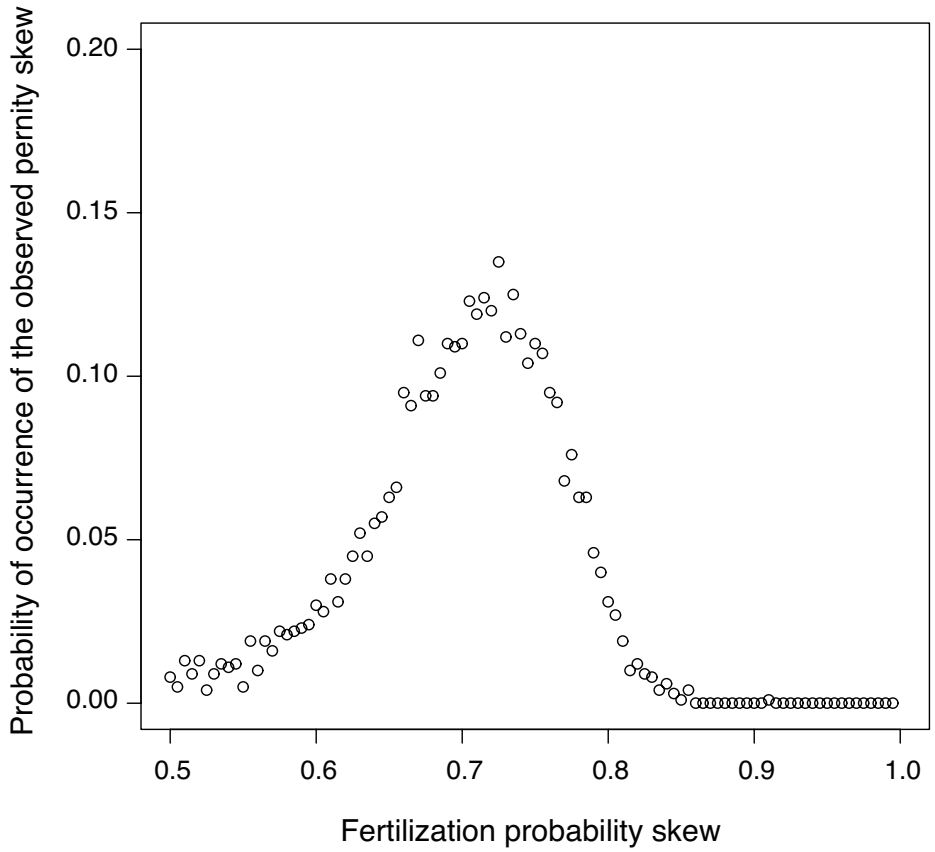


Fig. 4. The probability of the occurrence of the observed paternity skew under the observed frequency of multiple paternity under the full range of fertilization probability skew. A proportion of paternity skews that were approximately equal to the observed paternity skew (0.6800 ± 0.0025) was given for each fertilization probability skew under the assumption that the observed frequency of multiple paternity (50 litters) occurred in the grey-sided vole population.

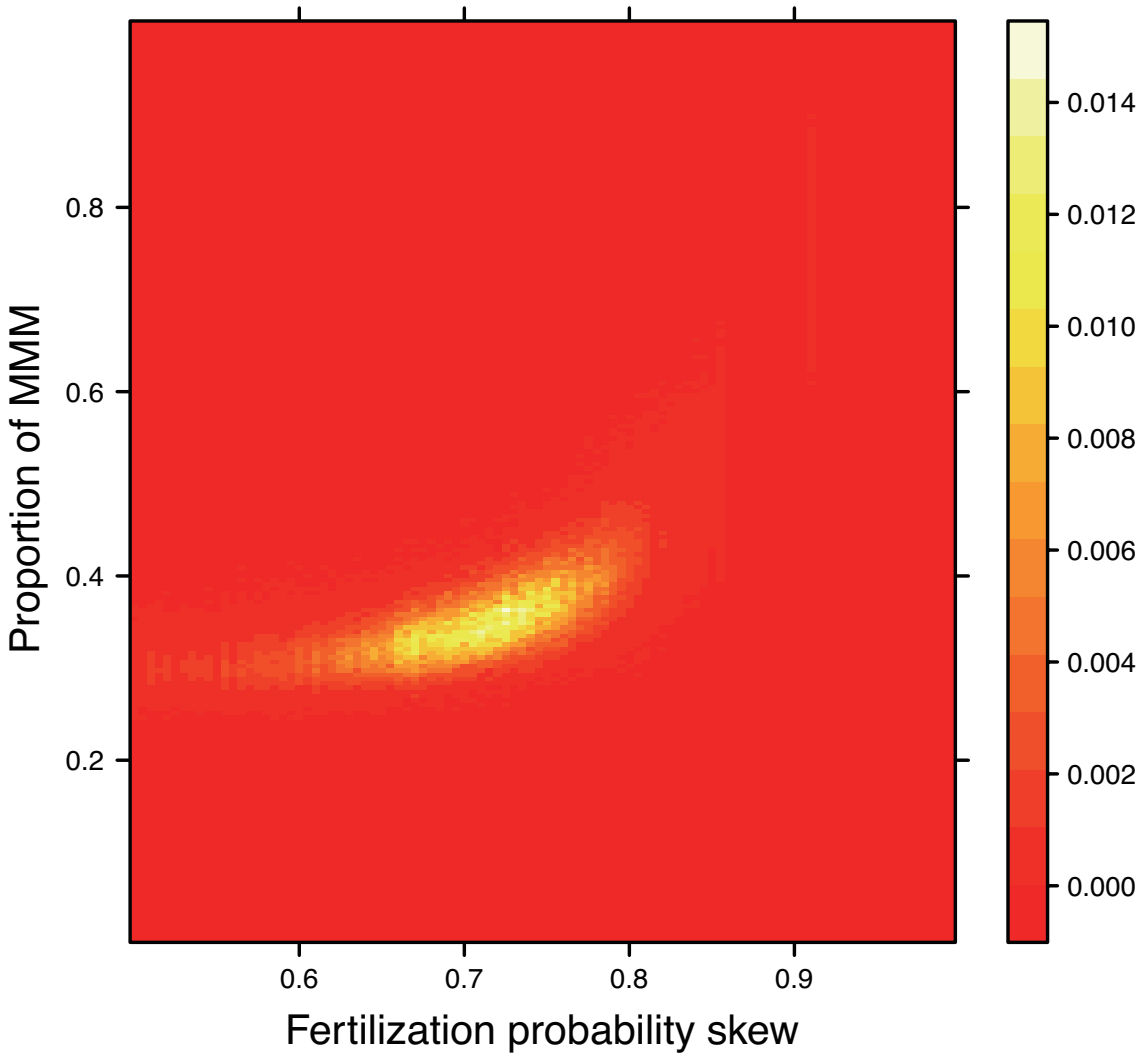


Fig. 5. The probability being observed frequency of multiple paternity (50 litters / 215 litters) and the observed paternity skew (0.6800 ± 0.0025) for each combination of multiple male mating proportion and fertilization probability skew. Lighter color means that the probability is high.

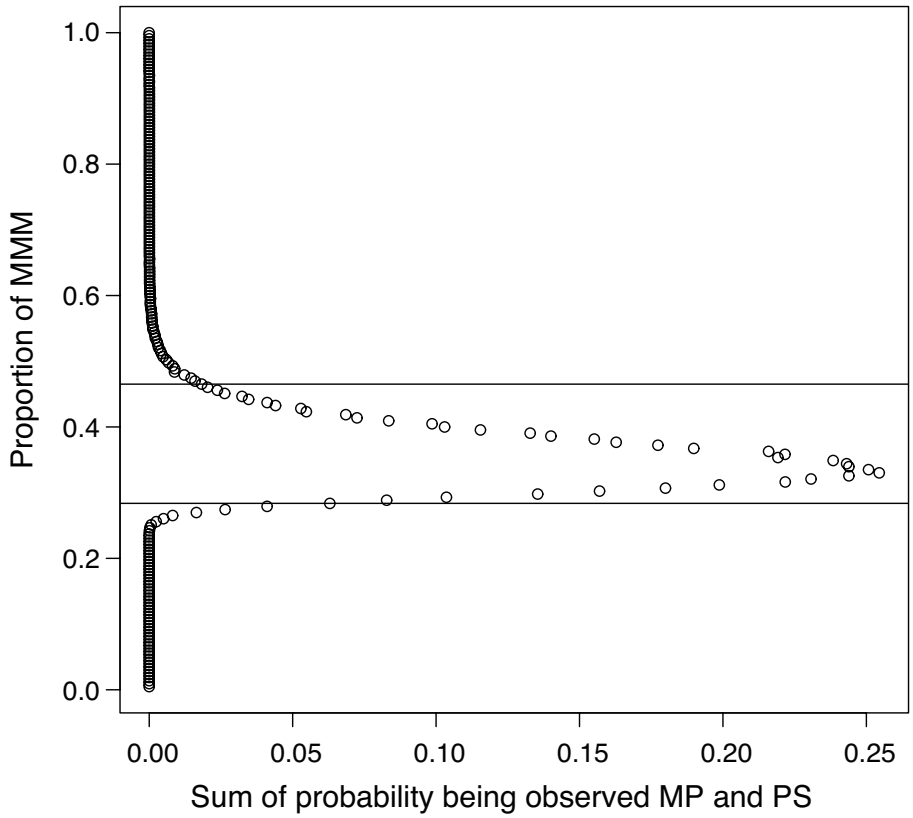


Fig. 6. Sum of probabilities being observed frequency of multiple paternity (50 litters / 215 litters) and the observed paternity skew (0.6800 ± 0.0025) for each multiple male mating proportion. The range between lines is the 95% confidence interval of estimated multiple male mating proportion.

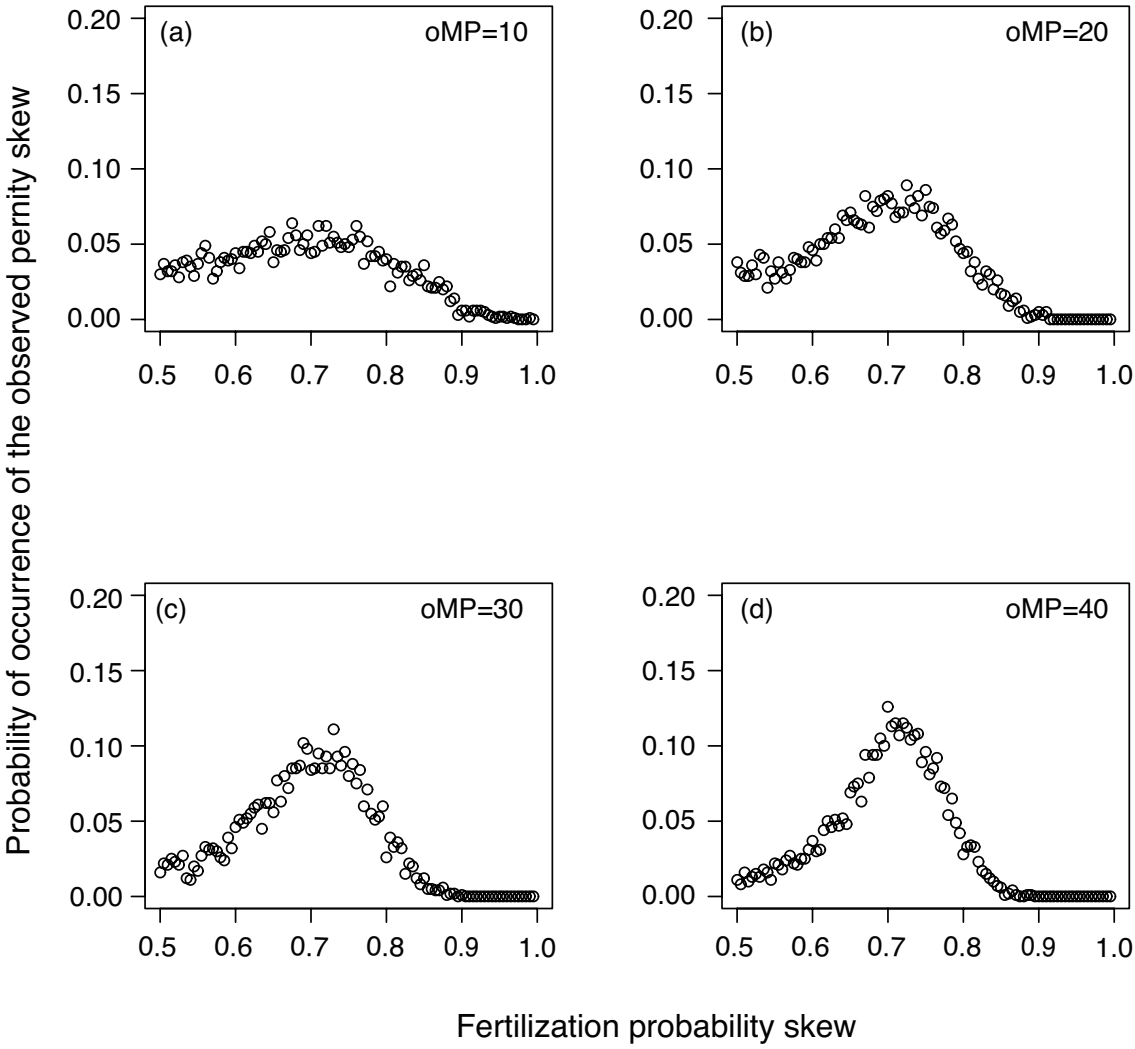


Fig. 7. The effect of multiple paternity frequency on the probability of the occurrence of the observed paternity skew. The probability of the occurrence of the observed paternity skew under four frequencies of multiple paternity (a: 10 litters, b: 20 litters, c: 30 litters, and d: 40 litters) under the full range of fertilization probability skew. A proportion of paternity skews that were approximately equal to the observed paternity skew (0.6800 ± 0.0025) was given for each fertilization probability skew. The probability was calculated using the frequency distribution of litter size observed in the grey-sided vole population.

Chapter 5

General Discussion

The frequency of multiple paternity (MP) has conventionally been used as an index of degree of sperm competition (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010). Those studies reported a positive correlation between level of MP and testes size (Ramm et al. 2005; Firman and Simmons 2008a; Soulsbury 2010). However, as I showed in this thesis, there are some debatable points in studies on MP frequency in free-living populations.

First, the magnitude of intraspecific variation in MP frequency has not been fully examined. In the chapter 2 and 3, I presented that interspecific variation was considerably large and might potentially affect the interpretation in previous studies, which showed the positive correlation between level of MP and testes size by interspecific comparisons, and interspecific variation of MP should therefore be taken into account.

Second, a difference between multiple male mating (MMM) frequency and MP frequency have not been considered. In the chapter 4, I presented that the difference may be too large to ignore in mammal species that have small litter sizes and proposed a method for the estimation of MMM frequency using observed MP frequency and paternity skew.

In future researches of MP frequency, what kind of points should we concern with sperm competition? And how do the results of this thesis contribute to understanding sperm competition in wild mammal population?

Effect of sample size on the multiple male mating frequency estimation

In the chapter 4, I suggested that a necessary sample size may be different depending on the proportion of MP litters. How many litters are required? I illustrated effects of sample size and MP proportion on MMM frequency estimation under possible

fertilization probability skew using observed frequency distribution of litter sizes and the observed paternity skew of the gray-sided vole population (Fig. 1, Fig. 2, Fig. 3).

MP proportion and sample size mainly affected the position and the thickness of J-shaped zones (MMM with high occurrence probability of the observed MP frequency), respectively (Fig. 1). MP proportion determined the vertical position of the J-shaped zones; MMM with high occurrence probability of the observed MP frequency became higher with higher MP proportion (Fig. 1). Larger sample sizes made J-shaped zones thinner; i.e., sample size is one of determinants of the estimation range of MMM (Fig. 1).

The occurrence probability of the observed paternity skew (PS) showed a clear peak against fertilization probability skew (FPS) under high MP frequency (= sample size × MP proportion) (Fig. 2). In Chapter 4 I demonstrated that the range of MMM estimation was determined by the value and the range of FPS. Therefore, higher MP frequency is preferable, because it could narrow the range of FPS.

The relationship between MMM estimation and FPS is non-linear (Fig. 1). When FPS was low, MMM estimation was less variable, and that means that effects of FPS on MMM estimation was minor under low FPS. In contrast, when FPS was high, the range of MMM estimation became wide; e.g., in Fig. 1l MMM, estimation range between 0.49 and 0.79, when FPS 0.925. Therefore, the absolute value of FPS is critical for the range of MMM estimation.

A large sample contributes to narrowing the range of FPS. Since effects of FPS on MMM estimation was the J-shaped, in the range of low FPS effects of sample size on the range of MMM estimation was minor. In contrast, when FPS was high, the range of MMM estimation became very sensitive to FPS variation. Therefore, a large sample size is essentially required to reduce the range of MMM estimation.

As a result, the estimation range of MMM frequency is changed intricately by the combination of sample size, MP proportion and observed PS, and sufficient sample

size for each case could not be determined easily. Therefore I recommend preliminary research with small sample size (about 30 litters) to acquire prior knowledge about MP proportion and PS. If MP proportion in preliminary researches was high, additional sampling may be not necessary. If low MP proportion was observed, more sampling efforts is necessary. If MP proportion and PS was similar to the gray-sided vole population, 100 litters is target value of sampling to estimate MMM frequency (Fig 3k).

What is the MP frequency meaning?

In previous studies, MP frequency used as an index of MMM frequency. However, MP frequency and MMM frequency is different as I demonstrated in Chapter 4. MP frequency is determined by not only MMM frequency but also litter size and FPS. To discuss about what MP frequency represents in connection with MMM frequency, I calculated the occurrence probability of the observed MP frequency of populations of *A. specious* in Obihiro and *A. argenteus* in Horokanai, which were analyzed in Chapter 2 and Chapter 3, considering litter size in comprehensive combination of MMM frequency and FPS (Fig. 4).

In *A. specious* in Obihiro, MP frequency was high (18 / 23 litters = 78.3%). To be such high MP frequency, high MMM frequency combined with not so high FPS is essential (Fig. 4a). On the other hands, MP frequency of *A. argenteus* was low (7 / 36 litters=19.4%). Conditions that produced such low MP frequency could be roughly categorized to two patterns. One is a combination of low MMM frequency and low FPS, while the other is a combination of high MMM frequency and high FPS (Fig. 4b). Those suggest that discussion about variation of MP frequency means discussion about variation of combination of MMM frequency and FPS. Especially, a low MP frequency not always represents a low MMM frequency. Thus, there could remain the possibility

that sperm competition occurred frequently, even if a low MP frequency was observed.

What does high intraspecific variation of MP frequency in *A. speciosus* means?

As I described, MP frequency is produced by the combination of MMM frequency and FPS. High MP frequency in *A. specious* that sampled in Obihiro represents high MMM frequency and not so high FPS. How about in Horokanai? In Hrokanai, MP frequency was low (3/11 litter = 27.3 %). That indicates low MMM frequency and low FPS, or high MMM frequency and high FPS. Thus, there is still the possibility that MMM frequency was high in Horokanai. On the other hands, MMM frequency in Obihiro not so different from MP frequency. Those suggest the probability that intraspecific variation in MMM frequency could be smaller than intraspecific variation in MP frequency in *A. specious*.

In wild populations, it is difficult to observe MMM directory. Thus, MP frequency is a good tool to study sperm competition. In this thesis, I discussed some debatable points in previous studies about MP frequency, i.e., intraspecific variation in MP frequency and a difference between MMM and MP frequency. Using the method of MMM frequency estimation proposed in Chapter 4 it is possible to discuss MMM frequency and FPS separately in wild populations. Therefore, I strongly recommend that future researches of multiple paternity are carried out in wild populations with sufficient sample size and data of paternity skew.

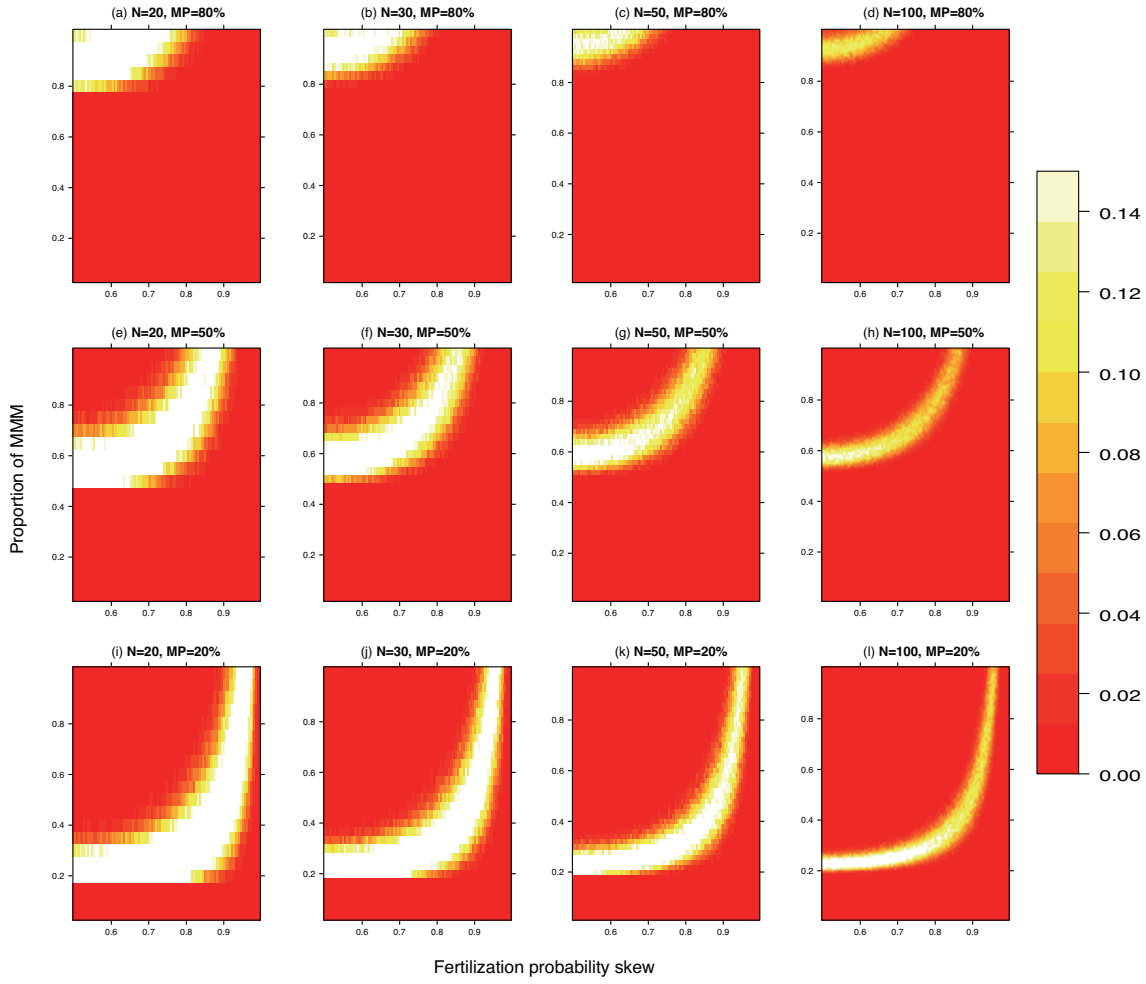


Fig. 1. The effect of the combination of sample size and proportion of multiple paternity on the probability that each observed multiple paternity frequency occurs for each combination of multiple male mating proportion and fertilization probability skew. The probability calculated using 12 combinations of four sample sizes (N; 20, 30, 50, and 100) and three proportions of multiple paternity (MP; 20%, 50%, 80%). Lighter color indicates higher probability.

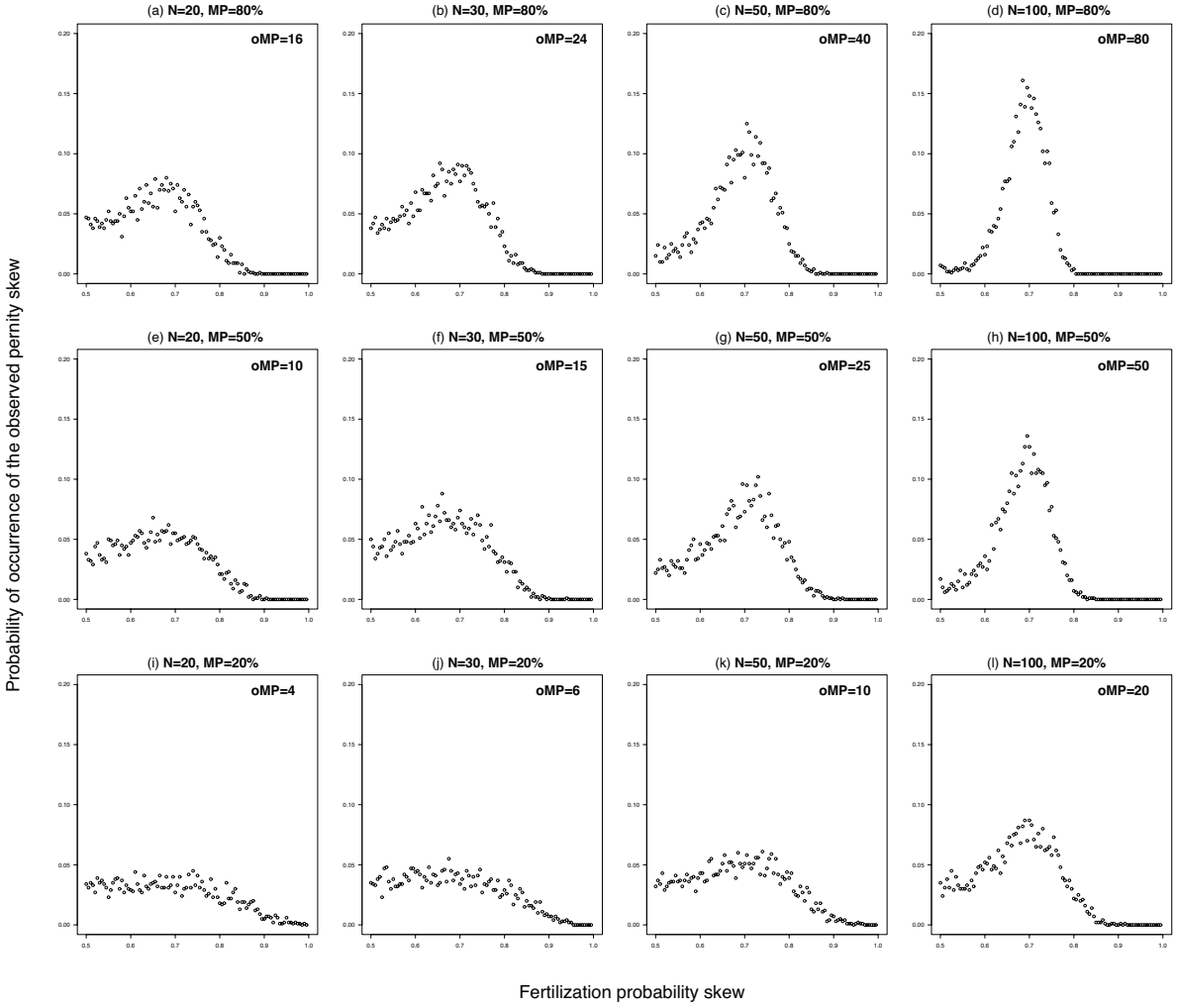


Fig. 2. The effect of the combination of sample size and proportion of multiple paternity on the probability of the occurrence of the observed paternity skew. The probability of the occurrence of the observed paternity skew (0.6800 ± 0.0025) under 12 combinations of four sample sizes (N; 20, 30, 50, and 100) and three proportions of multiple paternity (MP; 20%, 50%, 80%) under the full range of fertilization probability skew. oMP is observed multiple paternity frequency.

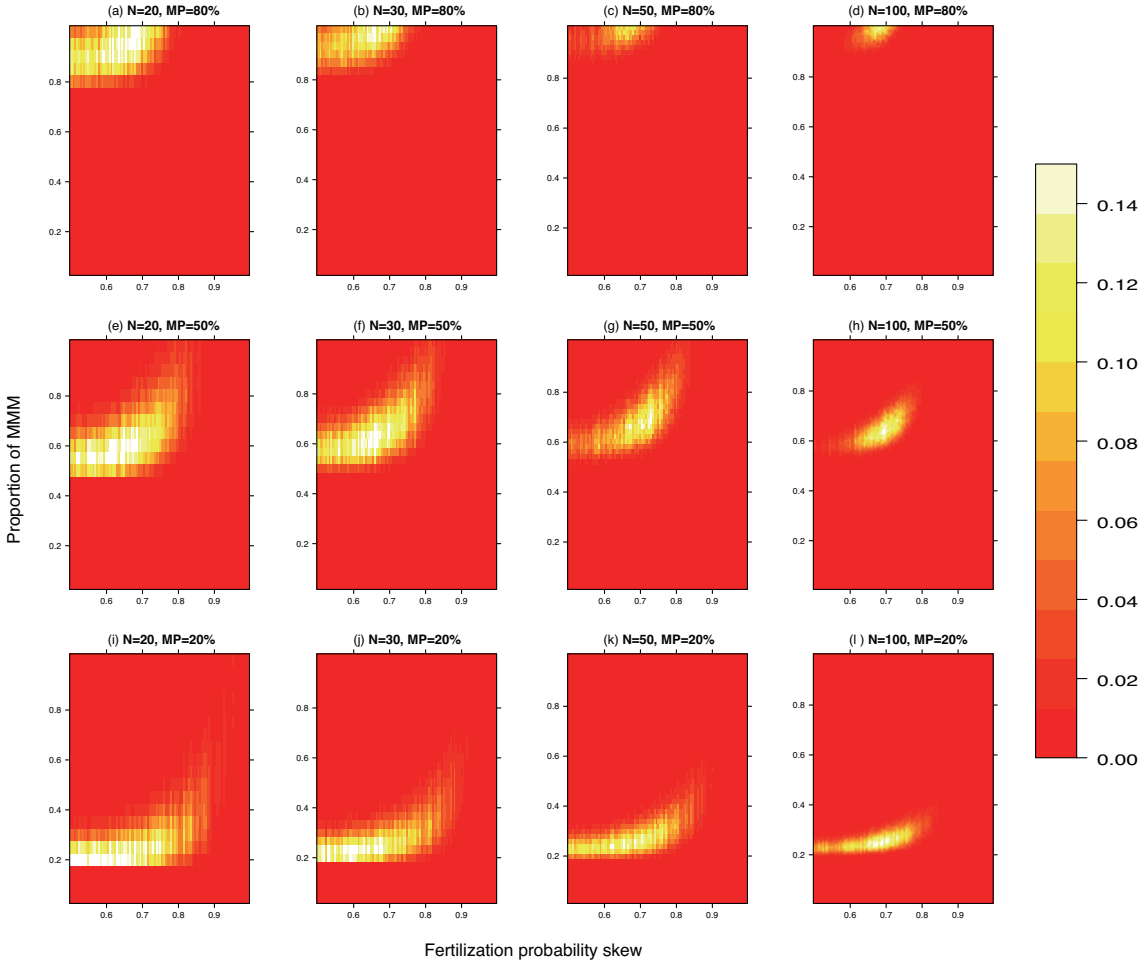


Fig. 3. The effect of the combination of sample size and proportion of multiple paternity on the probability being each observed frequency of multiple paternity and the observed paternity skew (0.6800 ± 0.0025) for each combination of multiple male mating proportion and fertilization probability skew. The probability calculated using 12 combinations of four sample sizes (N; 20, 30, 50, and 100) and three proportions of multiple paternity (MP; 20%, 50%, 80%). Lighter color means that the probability is high.

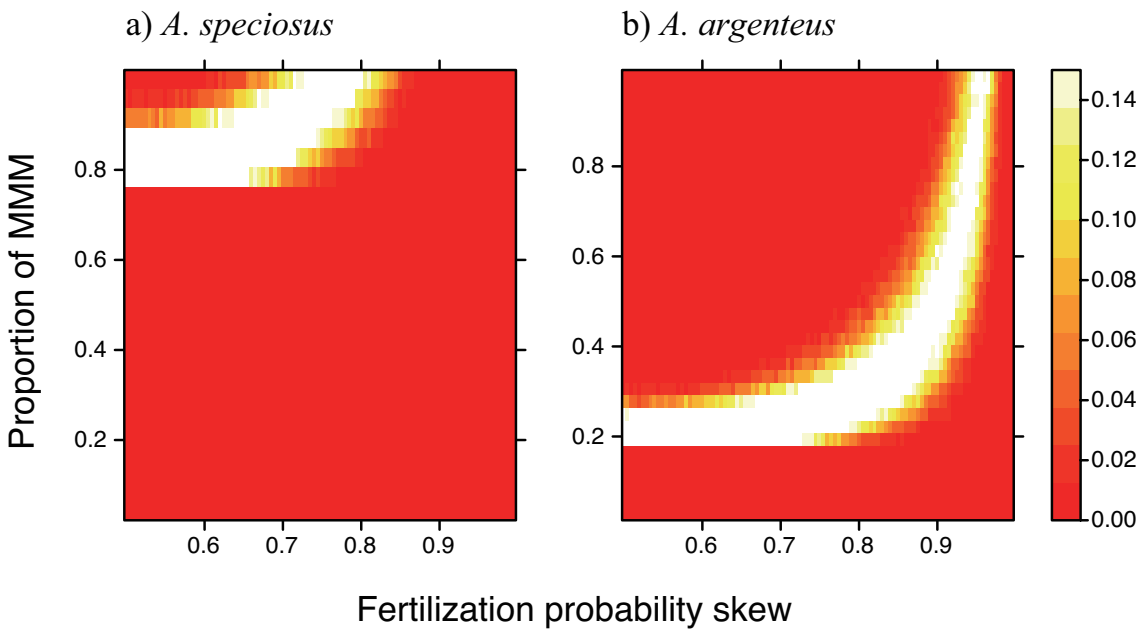


Fig. 4. The probability that the frequency of multiple paternity observed in two *Apodemus* species populations occurs for each combination of multiple male mating proportion and fertilization probability skew. Lighter color indicates higher probability. The observed multiple paternity proportion was (a); 78.3% (18 / 23 litters) in *A. speciosus* sampled from Obihiro, and (b); 19.4% (7 / 36 litters) in *A. argenteus* sampled from Horokanai.

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Summary (日本語)

複数オス交尾は精子を通じたメスをめぐるオス間の競争を生み出す。近年このような交尾後の性淘汰が、交尾前性淘汰と同様に、繁殖形質の進化をもたらす強い淘汰圧となることが明らかになってきている。交尾後性淘汰の強さを評価するためには複数オス交尾頻度を知ることは必須の知見である。しかし、野生下の動物、特に繁殖行動を直接観察することが困難な哺乳類では、複数オス交尾頻度を知ることは非常に難しい。分子生物学的手法の発達により、マルチプルパタニティ(一腹の子の父親が複数いること)が多様な種で観察されるようになると、複数オス交尾頻度の指標としてマルチプルパタニティ頻度を用いて、野外個体群の交尾後性淘汰が議論されるようになった。例えば、幾つかの分類群における種間比較によって、高いマルチプルパタニティ頻度が観察された種では、大きい精巣を持つ傾向があることが明らかになった。複数オス交尾が起こった場合、より多くの精子をメスに送り込んだオスが競争に有利であるため、大きな精巣が進化したと考えられている。このように実際にマルチプルパタニティ頻度を用いて、交尾後性淘汰が議論されているが、マルチプルパタニティ頻度の種間比較分析にはいくつかの問題点がある。まず、種内変異が十分に検討されていないこと、また複数オス交尾頻度とマルチプルパタニティ頻度の違いについて十分に検討されていないことが挙げられる。本研究では、野ネズミ類に注目し、複数オス交尾の指標としてのマルチプルパタニティを、実際の野外個体群におけるデータを用いつつ、理論的にも考察した。

第１章では、哺乳類におけるマルチプルパタニティ研究の現状をまとめ、これまでの研究の問題点を整理した。

第２章では、日本に広く分布する固有種であるアカネズミ(*Apodemus speciosus*)に注目した。これまで哺乳類における種間比較分析によって、マルチプルパタニティ頻度と精巣サイズなどの繁殖形質に関係性が見られることがわかっている。しかしこれらの研究で用いられたデータは、マルチプルパタニティ頻度と精巣サイズなどのデータを異なる個体群で採取したもの、またマルチプルパタニティ頻度を繁殖期中の一部の時期に測定したものであることがほとんどであり、マルチプルパタニティの種内における空間的、時間的変異を考慮に入れたものではなかった。種内変異が大きければ、過去の種間比較分析の結果の再検討が必要とされる。本章ではマルチプルパタニティ頻度の種内変異の大きさを検討するため、アカネズミのマルチプルパタニティを北海道内の２地点で調査し、比較した。その結果、アカネズミのマルチプルパタニティ頻度の種内変異は非常に大きく、その大きさはアカネズミ属内のマルチプルパタニティ頻度の種間変異を超えるものであった。このことから、今後の種間比較では、種内変異を考慮に入れた分析を行うことが交尾後性淘汰のより良い理解につながることを示した。

第３章では、アカネズミと同様に日本に広く分布する固有種であるヒメネズミ(*A. argenteus*)に注目し、その北海道におけるマルチプルパタニティ頻度を明らかにした。この値は、今までマルチプルパタニティ頻度が報告されているア

カネズミ属の種の中で、最も小さい値であった。

第 4 章では、マルチプルパタニティ頻度を用いた、より実用的な複数オス交尾頻度の推定方法を検討した。複数のオスと交尾したメス、全てがマルチプルパタニティになるわけではないため、マルチプルパタニティ頻度をそのまま複数オス交尾頻度の代替値として用いると、過小評価になってしまう。その過小評価の度合いは、交尾オス間の受精確率の偏りと一腹産子数に大きく影響されるため、一腹産子数の小さい哺乳類では特に、過小評価の影響は深刻である。複数オス交尾頻度推定を試みたこれまでの研究では、野外での測定が難しい受精確率の偏りについては、任意の値を網羅的に与えるにとどまっており、推定値の幅も大きく、手法の改善が求められている。本章では、エゾヤチネズミの充実した野外データを基に、マルチプルパタニティ頻度、一腹産子数および受精確率の偏りを考慮に入れて複数オス交尾頻度を推定した。この方法では IBM モデルに基づいたシミュレーションを用いて、観察したマルチプルパタニティ頻度が起こりうる、複数オス交尾頻度および受精確率の偏りを求めた。受精確率の偏りは、マルチプルパタニティ腹における父性の偏り(一腹の子のうち、多く子を残せたオスの子の割合)の観察値が起こりうる範囲に限定することで、推定幅の縮小を図った。エゾヤチネズミ個体群のマルチプルパタニティの実測頻度は 215 腹中 50 腹 (23.3%)であった。複数オス交尾頻度の推定値は、受精確率の偏りを考慮しない場合、59 (27.4%)～215 (100%)であった。しかし、父性の偏りの実測値を用いて、受精確率の偏りを限定することで、複数オス交尾頻度の推定値は 61 (28.4%)～100 (46.5%)となり、推定幅を大幅に小さくすることができた。また点推定も可能となり、この場合推定値は 71 (33.0%)であった。父性の偏りの実測値を用いて受精確率の偏りを限定するこの方法は、受精確率の偏りを測定できない野外個体群における複数オス交尾頻度の推定には非常に有効な手段である。また、複数オス交尾頻度の推定幅を狭めるためには、十分な量のサンンプルサイズが必要であり、その必要量はマルチプルパタニティ頻度に依存する。そのため、今後のマルチプルパタニティの研究では、父性の偏り及びサンプルサイズを考慮に入れた野外調査が必要である。

第 5 章では、アカネズミ属のマルチプルパタニティ頻度と精巣サイズのデータを例に用いた種間比較について、また複数オス交尾頻度に対するサンプルサイズの影響について考察し、今後の野生哺乳類におけるマルチプルパタニティ研究の注意点についてまとめた。

本論文では、現状でのマルチプルパタニティ頻度研究の問題点が明らかになった。しかし幾つかの注意点を考慮に入れることにより、マルチプルパタニティ研究は今後の野生下における交尾後性淘汰の理解のための重要な手掛かりとなることを示した。