Title	Estimation of multiple male mating frequency using paternity skew: An example from a grey-sided vole (Myodes rufocanus) population
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Title: Estimation of multiple male mating frequency using paternity skew: an example from a grey-sided vole (*Myodes rufocanus*) population

Running head: Estimation of multiple male mating frequency

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Abstract

Multiple male mating (MMM) causes sperm competition, which may play an important role in the evolution of reproductive traits. The frequency of multiple paternity (MP), where multiple males sire offspring within a single litter, has been used as an index of MMM frequency. However, MP frequency is necessarily lower than MMM frequency. The magnitude of the difference between MMM and MP frequency depends on litter size (LS) and fertilization probability skew (FPS), and this difference may be meaningfully large in animals with small LSs. In this study, we proposed a method to estimate MMM frequency using an individual-based model with three variables (MP frequency, LS, and FPS). We incorporated observed paternity skew data to infer a possible range of FPS that cannot be measured in free-living populations and tested the validity of our method using a dataset from a grey-sided vole (*Myodes rufocanus*) population and from hypothetical populations. MP was found in 50 out of 215 litters (23.3%) in the grey-sided vole population, while MMM frequency was estimated in 67 of 215 litters (31.2%), with a certainty range of 59 to 88 (27.4 – 40.9%). The point estimation of MMM frequency was realized, and the certainty range was limited within the practical range. The use of observed paternity skew was very effective at narrowing the certainty range of the estimate. Our method could contribute to a deeper understanding of the ecology of MMM in free-living populations.

KEYWORDS

fertilization probability skew, individual-based model, litter size, multiple male mating, multiple paternity, paternity skew

1 INTRODUCTION

Multiple male mating (MMM) is the behaviour wherein a female mates with more than one male during a single oestrous period. MMM causes sperm competition among individual males (Parker, 1970) and may play an important role in the evolution of reproductive traits, e.g., mating behaviour, penile morphology, sperm number, and sperm morphology (Birkhead & Møller, 1998). For example, larger testes have been reported in promiscuous primates in comparison to those from species where a single male monopolizes mating (Harcourt, Harvey, Larson, & Short, 1981). In the house mouse, males under sperm competition produce more motile sperm than males released from sperm competition pressure (Firman & Simmons, 2010). The coevolution between test size and sperm morphology has been suggested in rodents on the basis of evidence that species with larger testes have more streamlined spermatozoa and discussed in the context of the intensity of sperm competition (Breed et al., 2019; Pahl et al., 2018; Šandera, Albrecht, & Stopka, 2013; Varea-Sanchez, Tourmente, Bastir, & Roldan, 2016). To determine the strength of sperm competition, the prevalence of females mating with multiple males must be quantified.

It is difficult to observe the mating behaviour in cryptic species. Therefore, indirect evidence of MMM has been used in ecological studies of mammals. The mating system of this group is considered an indication of MMM, in that females are more likely to copulate with multiple males in promiscuous or polyandrous species than in monogamous or polygynous species (Harcourt et al., 1981; Kenagy & Trombulak, 1986; Møller, 1988). Relative testes size has been also used as an MMM index (Lukas & Huchard, 2015; Stockley, 2003) because frequent MMM may lead to sperm competition and encourage the evolution of large testes (Kenagy & Trombulak, 1986).

Multiple paternity (MP), in which multiple males sire offspring within a single litter, has been detected in various mammal species using molecular markers (Soulsbury, 2010) and has been considered as the evidence of MMM without direct observations of MMM behaviour. MP frequency was used as an index of female promiscuity (Bryja et al., 2008; Stockley, 2003) or the degree of sperm competition (Firman & Simmons, 2008a; Ramm, Parker, & Stockley, 2005; Soulsbury, 2010), and these authors argued that conspicuous reproductive traits, which are advantageous under scenarios of sperm competition, have evolved under high MMM conditions (Firman & Simmons, 2008a; Ramm et al., 2005; Soulsbury, 2010).

However, MP frequency is necessarily lower than or equal to MMM frequency, because not all MMM females that have mated with multiple males produce an MP litter. Even if a female mates with two or more males, all of the ova in her litter could be monopolized by one male. However, few studies have paid attention to the difference between MP and MMM frequency (Eccard & Wolf, 2009).

The difference between MP and MMM frequency is due to the frequency of single paternity (SP) litters that are produced by multiple male mating (SP_{MMM}). MMM frequency is the sum of MP frequency and SP_{MMM} frequency (Fig. 1, see also Table 1). SP frequency includes both SP_{MMM} and SP_{SMM} (SP litters that are produced by single male mating), and it is impossible to distinguish SP_{MMM} from SP_{SMM} in empirical data (Fig. 1).

Is the difference between MP and MMM frequency small enough to be ignored? The relationship between MMM and MP frequency is described as the following equation:

MP frequency = MP probability × MMM frequency (Eq. 1),

where MP probability is the probability that an MMM female produces an MP litter. MP probability is determined by the litter size (LS: the number of offspring in a litter) and the fertilization probability of males involved in an MMM litter. MP probability can be described by the following equation when a female mates with two males (Eccard & Wolf, 2009);

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

where FPS is the fertilization probability skew, defined as the highest fertilization probability in an MMM litter among categorized males based on their biological features (see Fig. 1 for a calculation example). FPS is based on the concept of "competitive skew" proposed by Dean, Ardlie, and Nachman (2006), which indicates the bias of fertilization probability associated with individual male characteristics. Dean et al. (2006) discussed this bias for mating order, testis size, sperm morphology, and others traits, but they did not explicitly explain the calculation process. Here, we redefined FPS and detailed the calculation process (Fig. 1). Small litter size and/or a highly skewed fertilization probability results in an MP probability close to zero, whereas a large LS with a small FPS results in an MP probability close to one (Appendix S1). Thus, in animals with large litter sizes, e.g., insects or fishes, the difference between MP and MMM frequency may be negligible. However, in animals with small LS, e.g., mammals and birds, the difference between MMM and MP frequency may be meaningfully large. Additionally, the effects of LS and FPS on MP probability are nonlinear (Appendix S1), so MMM frequency cannot be based on MP frequency alone. Therefore, we must estimate MMM frequency by taking MP frequency, LS, and FPS into consideration, instead of regarding MP frequency as an alternative measure of MMM frequency for animals with small LS.

It is impossible to directly measure FPS in a free-living population (Fig. 1), as FPS is calculated from all MMM litters, including SP_{MMM} litters that cannot be distinguished from SP_{SMM} (Fig. 1). Dean et al. (2006) estimated the MMM probability (the probability that MMM occurs) of a wild house mouse (*Mus musculus domesticus*) population using the observed MP frequency (33/143 litters), assuming various FPSs and found MMM probability estimates within the range of 0.2 – 1.0. This wide estimation range is not suitable for practical use in comparative studies.

Paternity skew (PS) is the highest value in the proportion of offspring sired by individual males in each MP litter (Fig. 1). PS can be obtained practically from observed MP litters, and since PS depends on FPS and LS, we can infer FPS from the observed PS and LS values.

In this study, we propose a method to estimate MMM frequencies in free-living populations considering three variables (MP frequency, LS, and FPS), and in which the observed PS was used to infer a possible range of unmeasurable FPS. We tested the validity of our method using a dataset from a grey-sided vole [*Myodes rufocanus* (Sundevall, 1846)] population and demonstrate that limiting the FPS range greatly contributes to reducing the estimated range of MMM frequency. We also show that our method could be applied to other populations under various conditions.

2 MATERIALS AND METHODS

2.1 The grey-sided vole and dataset

The grey-sided vole, *Myodes rufocanus* (Sundevall, 1846), is a small rodent species (body weight: 27–50 g; Kaneko, Nakata, Saitoh, Stenseth, & Bjørnstad, 1998) inhabiting the forests, grasslands, scrublands, and tree plantations of Hokkaido, Japan (Kaneko et al., 1998). They have a variable diet (herbs, roots, seeds, and insects) and breed from April to October (Kaneko et al., 1998).

Females of *M. rufocanus* are polyoestrous (Saitoh, 1990) and undergo a postpartum oestrus (Kawata, 1985). In the wild, females are sexually mature after reaching 16 g body weight, and males after reaching 24 g (Nakata, 1989). However, their reproductive activities are suppressed or fail, when females cannot maintain exclusive home ranges (Kawata, 1987; Saitoh, 1981). The home ranges of males overlap extensively with one another and with those of several females (Ishibashi & Saitoh, 2008a; Kawata, 1985, 1988; Saitoh, 1985). Breeding males mate sequentially with several females during the breeding season and have a dominance structure based on body mass (Ishibashi & Saitoh, 2008a; Kawata, 1988). This vole species is considered polygynandrous (Ishibashi & Saitoh, 2008a), due to the occurrence of multiple paternity (in about 23 % of litters) and the lack of prolonged pair bonding.

The dataset was collected from an enclosed population of grey-sided voles by Ishibashi and Saitoh (2008a, 2008b). This dataset includes the 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 a range of 1 – 9. There were 50 litters sired by multiple males (i.e., the observed MP frequency (oMP) = 50, 23.3%), 48 litters sired by two males ($oMP_{2MP} = 48$, 22.3%), and two litters sired by three males ($oMP_{3MP} = 2$, 0.9%). The single paternity (SP) frequency was 165 out of 215 litters (76.7%). The average paternity skew of the MP litters was 0.6735 for two sires ($oPS_{2MP} = 0.6735$) and 0.6631 for two and three sires ($oPS_{3MP} = 0.6631$, see Appendix Table S1 for the data on oPS).

2.2 Estimation of multiple male mating frequency assuming one or two male mating

Given values for MMM frequency and FPS, the MP frequency is deterministically calculated from empirical LS data (see Eq. 1 and Eq. 2). Thus, by examining various combinations of MMM frequency and FPS, we may obtain the MMM frequencies that are likely to produce the observed MP frequency ($oMP = 50$). Although we could have used an average as the representative LS for the deterministic model, the stochastic effects of LS variation may not be negligible. This variation should be critical for animals with small LS. Additionally, the relationship between PS and FPS is not straightforward and cannot be described by a simple equation (Fig. 1); the empirical paternity is hardly predicted by a simple assumption (Dean et al., 2006; Dobson, Abebe, Correia, Kasumo, & Zinner, 2018). Therefore, we first examined the probability that *oMP* and the observed PS (oPS_{2MP}) occur simultaneously, $P(oMP \cap oPS_{2MP})$, in possible conditions of LS, MMM frequency, and FPS, using an individual-based model (IBM). By exploring $P(oMP \cap oPS_{2MP})$, the plausible combinations of MMM frequency and FPS could be determined.

We conducted an IBM analysis using four observed values from the grey-sided vole population: i.e., the sample size ($N = 215$), MP frequency ($oMP = 50$), the average PS in MP sired by two males ($oPS_{2MP} = 0.6735$), and the frequency distribution of LS. In this analysis, we assumed that all MMM females mated with two males (though this assumption was relaxed in subsequent analyses). The details of the IBM analysis are described below.

FPS is defined as the highest fertilization probability among mated males and ranges from 0.5 to 1 when a focal female mates with two males; i.e., from the lowest case where two males have an even probability of siring an offspring (FPS = 0.5) to the highest case where one male monopolizes all ova (FPS = 1). We eliminated cases in which the FPS was 1 because an MP litter cannot occur under this condition.

2.2.1 Step-1. Occurrence probability of the observed multiple paternity frequency and the observed paternity skew

To estimate the co-occurrence probability of *oMP* and oPS_{2MP}, $P(oMP \cap oPS_{2MP})$, the MP frequency and PS were obtained under various combinations of MMM frequency and FPS. MMM frequency ranged from 1 to 215, in increments of 1, and FPS ranged from 0.5 to 0.995, in increments of 0.005 (100 conditions). We had no information on the distribution of FPS, so FPS was assumed to be constant at a given value. For each of the combinations (215 × 100), hypothetical MMM litters were generated. A litter size (LS), which was randomly selected from the observed frequency distribution of LS with replacement, was assigned to each 215 hypothetical MMM litter. For each offspring in a litter, a sire was probabilistically determined from two candidate males based on a given FPS. When both candidates were selected as sires, that litter was designated as an MP litter, whereas a litter that was monopolized by a single male was designated as an SP litter (SP_{MMM}). PS was calculated for each MP litter. MP litters were counted, and PSs were averaged. These processes were repeated 10,000 times for each of the 21,500 (215 × 100) combinations.

The simulated number of MP litters and PS calculations were compared with the observed data ($oMP = 50$ and $oPS_{2MP} = 0.6735 \pm 0.0025$). When both simulated values satisfied the observed data, a focal run was considered positive. The number of positives were counted within the 10,000 runs, and the co-occurrence probability of oMP and oPS_{2MP}, $P(oMP \cap oPS_{2MP})$, was obtained (the number of positive runs / 10,000) for every combination of MMM frequency (proportion) and FPS.

2.2.2 Step-2. Estimation of multiple male mating frequency

To assess the plausibility of the MMM frequency, $P(oMP \cap oPS_{2MP})$ was summed across the full range of FPS, between 0.5 and 0.995, for each MMM frequency (proportion). This serves as a probability distribution for MMM frequencies. We considered an MMM frequency (proportion) with the largest sum of $P(oMP \cap oPS_{2MP})$ to be the most plausible for the observed population. To assess the degree of certainty of this estimation, we determined the plausible range of MMM frequencies (proportion) by excluding MMM proportions until the accumulated probability reached 2.5% of the total probabilities of both the upper and lower tails of the distribution (the shaded zone in Fig. 4). We defined this range as the certainty range of the estimate.

The codes for the IBM analysis, written in C and R language, are available as supporting information (Appendix S2).

2.3 Mating with three or more males

In the preceding sections, we assumed that all MMM females mated with two males. Under the constant FPS, the MP probability should be higher when females mate with three or more males than when only mating with two. Therefore, the MP frequency may be higher in a population in which mating with three or more males is prevailing, even though MMM frequency is the same as for a population where the number of mated males is limited to two. In other words, under the same observed value of MP frequency, the estimated MMM frequency may differ among populations with different numbers of mated males. In this section, we examined the effects of assumed numbers of mated males on the estimation of MMM frequency by using the following two approaches.

2.3.1 Effect of the number of mated males

IBM analyses were conducted for cases where MMM females could mate with three or more males using the observed values from the grey-sided vole population. Assuming that all MMM females mated with the same number of males, the number of mated males was set as three, four, or five. We specifically considered the observed MP frequency of litters sired by three males ($oMP_{3MP} = 2$), and the average paternity skew was revised to include litters sired by three males ($oPS_{3MP} = 0.6631$). MMM frequency ranged from 1 to 215 in increments of 1, and FPS was set to encompass the range of possible mated males in increments of 0.005: from 0.333 (1/3) to 0.998 (134 conditions) for three male mating, from 0.250 (1/4) to 0.995 (150 conditions) for four male mating, and from 0.200 (1/5) to 0.995 (160 conditions) for five male mating. Since FPS is the highest fertilization probability among mated males, we gave an FPS value to a dominant male as his fertilization probability, and the remaining probability (1 – FPS) was evenly allocated to subordinate males: e.g., (1 – FPS)/2 was given to a subordinate male in the case of three male mating. For each combination of MMM frequency and FPS (215 × 134 = 28,810 for three male mating, 215 × 150 = 32,250 for four male mating, and 215 × 160 = 34,400 for five male mating), the number of MP litters, averaged PSs, and the number of MP litters sired by three or more males were simulated 10,000 times. We then calculated the co-occurrence probability of oMP, oPS_{3MP}, and oMP_{3MP}, $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$, and estimated the MMM frequency (proportion) and its certainty range (as described in section 2.2).

2.3.2 A plausible number of mated males

In free-living populations, not all MMM females mate with the same number of males. Therefore, a combination of two and three (or more) male mating should be considered. IBM analyses were conducted under the seven probability scenarios of three male mating in MMM (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5), where the number of mated males was set as two or three. For example, when the probability of three male mating was 0.1 and MMM frequency was 100, 90 females mated with two males and 10 females mated with three males. MMM frequency was assigned a value from 1 to 215, in increments of 1, and FPS ranged from 0.5 to 0.995, in increments of 0.005 (100 conditions). For each combination of MMM frequency and FPS (215 × 100), the number of MP litters, averaged PSs, and the number of MP litters sired by three males were simulated 10,000 times. We calculated the co-occurrence probability of oMP, oPS_{3MP}, and oMP_{3MP}, $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$, and estimated the MMM frequency (proportion) and the certainty range (see section 2.2). The codes for the IBM analyses, written in C and R language, are available as supporting information (Appendix S3).

2.4 Validity test of estimates using hypothetical populations

To test the validity of our estimates, we conducted IBM analyses using hypothetical populations with true values for MMM frequency, FPS, and the occurrence probabilities of three male mating for MMM. The sample sizes of the hypothetical populations were set to 200 litters. The following five values were assigned to the populations as the true values; MMM frequency: 40 (20% of sample size), 80 (40%), 120 (60%), 160 (80%), and 200 (100%); FPS: 0.5, 0.6, 0.7, 0.8, and 0.9, and the probability of three male mating: 0, 0.25, 0.5, 0.75, and 1. We assumed that MMM females mated with three males at the above probability, while the remaining MMM females mated with two males. Using the frequency distribution of LS observed in the grey-sided vole population, we ran a simulation for each of the 125 conditions of true values (5 × 5 × 5) and obtained hypothetical oMP, oPS_{3MP}, and oMP_{3MP}. Then, IBM analyses were conducted assuming five conditions (0, 0.25, 0.5, 0.75, and 1) for the occurrence probability of three male mating in MMM 10,000 times for every combination of MMM frequency (200 conditions) and FPS (100 conditions). We then estimated the MMM frequency (proportion) and the certainty range for each hypothetical population. Other details of the procedure are described in section 2.3.

2.5 Difference between multiple paternity and multiple male mating

To examine the generality of the relationship between MP and MMM proportions found in the grey-sided vole population, we investigated the differences between MP and MMM proportions under various combinations of MP proportions, FPSs, and LSs, assuming that all MMM females mated with two males (to reduce our calculation load).

The difference (expected MMM proportion – MP proportion) was obtained using the following variation on Eq. 1 and Eq. 2.

$$Expected\ MMM\ proportion = \frac{MP\ proportion}{MP\ probability} = \frac{MP\ proportion}{1 - FPS^{LS} - (1 - FPS)^{LS}}$$

(Eq. 3).

Using Eq. 3 the expected MMM proportion was calculated, assuming 101 values for MP proportion between 0.0 and 1.0, and 100 values for FPS, between 0.5 and 0.995, under four different LS (4, 6, 8, and 10).

2.6 Effects of sample size on multiple male mating estimation

To elucidate the effect of sample size on the estimation of MMM, we conducted IBM analyses and obtained the certainty range of the estimated MMM frequency on various sample sizes. We used two observed values from the grey-sided vole population (i.e., oPS_{2MP} and the frequency distribution of LS). MP frequency was set as 2, 5, 7, 9, 12, 17, 23, 36, 46, or 50, corresponding to the following sample sizes of 10, 20, 30, 40, 50, 75, 100, 150, 200, and 215. Those MP frequencies were given as integers that produced the nearest proportion of MP to the observed one (0.233). Other procedures were the same as those of the basic simulation (section 2.2).

3 RESULTS

3.1 Occurrence probability of the observed multiple paternity frequency

The occurrence probability of the observed MP frequency, $P(oMP)$, was determined for every combination of MMM frequency and FPS (Fig. 2), without consideration for the occurrence probability of the observed paternity skew (oPS_{2MP}). In most parameter space, $P(oMP)$ was low, whereas the instances of probabilities greater than 0.1 were distributed in a J-shaped space. The highest $P(oMP)$, 0.126, was observed when the MMM frequency (proportion) was 64 (29.8%) and FPS was 0.550. The range of MMM frequencies (proportions) with a probability greater than 0.05 was wide, ranging between 59 (27.4%) and 215 (100%).

The co-occurrence probability of oMP and oPS_{2MP}, $P(oMP \cap oPS_{2MP})$, for every combination of MMM frequency (proportion) and FPS, is shown in Fig. 3. In most parameter space, $P(oMP \cap oPS_{2MP})$ was low, and spaces showing higher probabilities were greatly reduced in comparison to $P(oMP)$ in Fig. 2. The highest $P(oMP \cap oPS_{2MP})$, 0.014, was observed when the MMM frequency (proportion) was 72 (33.5%) and FPS was 0.685.

3.2 Estimation of multiple male mating frequency assuming two male mating

The probabilities of oMP and oPS_{2MP} co-occurrence, $P(oMP \cap oPS_{2MP})$, over the full range of FPS is shown in Fig. 3 and were summed for every MMM proportion (Fig. 4). The sum of $P(oMP \cap oPS_{2MP})$ was extremely low on the higher and lower tails of the MMM proportions, and the kurtosis was 2.59. The highest probability, 0.305, was observed when the MMM frequency (proportion) was 69 (32.1%). The certainty range defined in section 2.2.2 was between 60 (27.9%) and 94 (43.7%).

3.3 Mating with three or more males

Assuming that all MMM females mated with the same number of males (three, four, or five males), MMM frequency was estimated for the grey-sided vole population. There were no cases where oMP, oPS_{3MP}, and oMP_{3MP} simultaneously occurred; i.e., $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ was zero for all combinations of MMM frequency and FPS. The lowest average number of a simulated MP_{3MP} litters was five in runs where oMP and oPS_{3MP} were satisfied, and thus the simulated numbers of MP_{3MP} litters were always higher than oMP_{3MP} (2).

The above results suggest that the assumption that all MMM females mated with three males was too high for the MMM calculation of the grey-sided vole population. Therefore, we assumed that MMM consisted of two and three male mating and estimated MMM frequency under seven occurrence probabilities of three male mating in MMM (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5). When the occurrence probability of three male mating was 0.1, the largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ was highest (0.099, Fig. 5). The estimate of MMM frequency (proportion) was 67 (31.2%), and the certainty range was narrowest (59 – 88: 27.4% – 40.9%). The second and third highest values (0.089 and 0.065) of the largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ were observed at the occurrence probabilities of 0.15 and 0.05, respectively. The estimate of MMM frequency (the certainty range) was 67 (59 – 88), 67 (59 – 88), 67 (59 – 89), 69 (59 – 90), 72 (60 – 92), 69 or 70 (61 – 94), and 68 (61 – 97) for the seven conditions (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5), respectively. The largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ decreased as the occurrence probability of three male mating increased beyond 0.1 (Fig. 5).

3.4 Validity test

The percentage of hypothetical populations in which the true MMM frequency fell within the certainty range of the estimate was examined by the validity test. In the following analyses we excluded the cases where $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ was zero. The proportion of cases in which the true MMM frequency fell within the certainty range ranged between 87.8% and

93.2% (Table 2). When focusing on the occurrence probability of three male mating with the largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$, the proportion rose to 97.6%.

When comparing the assumed occurrence probability of three male mating with the true probability, the assumed three male mating probability with the highest value of the largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ was congruent with the true value in most hypothetical populations (76.8%).

3.5 Difference between multiple paternity and multiple male mating

Differences between MP and MMM proportions (MMM proportion – MP proportion) varied depending on the combination of LS, MP proportion, and FPS (Fig. 6). The number of combinations under which the differences were small (darker areas in Fig. 6) became smaller with decreased LS. The proportions of combinations in which the differences were smaller than 0.01 (black areas in Fig. 6) were 39.7%, 27.3%, 9.6%, and 3.3% of all possible combinations (10,100 combinations) when LS was 10, 8, 6, and 4, respectively. By contrast, the proportion of combinations under which the differences were greater than 0.1 increased from 13.0% to 31.8% with decreasing LS from 10 to 4.

The differences between MP and MMM proportions formed a negatively sloped curve in association with MP proportions and FPS (Fig. 6). The higher MP proportions were associated with the larger differences under a constant FPS value. When FPS was larger, the differences became larger, even with a lower MP proportion.

MMM proportion is not linearly related to MP proportion. Therefore, when comparing MP proportions among populations (or species), the MMM proportion of a population with a higher MP proportion is not always higher than that of a different population with a lower MP proportion. We considered two hypothetical populations as examples. In one population, LS was 4, MP proportion was 0.3, and FPS was 0.9 in one population (Population A; Fig. 6a), and in a different population (Population B; Fig. 6d), LS was 10, MP proportion was 0.6, and FPS was 0.5. The expected MMM proportions in population A and B were 0.872 and 0.601, respectively. Under these conditions, the difference between MP and MMM proportions were 0.572 and 0.001 in population A and B, respectively, and thus the MMM proportion of A (0.872 = 0.572 + 0.3) was larger than that of B (0.601 = 0.001 + 0.6). This is despite the fact that the MP proportion of A (0.3) was smaller than that of B (0.6). The inversion of the magnitude of the relationship can occur between populations in which LS is the same. Taking the third example, population C (Fig. 6a), in which LS was 4, MP proportion was 0.6, and FPS was 0.9, a small difference was observed between MP and MMM proportions (0.086). The MP proportion of A (0.3) was smaller than that of C (0.6), but the expected MMM proportion of A (0.872) was larger than that of C (0.686 = 0.086 + 0.6).

3.6 Effects of sample size on the estimation range

The effects of sample size on the certainty range of an estimated MMM proportion were analysed using the dataset from the grey-sided vole population. The certainty range became narrower with an increase in sample size (Appendix S4). For smaller sample sizes, the reduction rate of the certainty range width became higher. This relationship was well described by the negatively sloped curve obtained from a quadratic formula ($y = 0.844 - (8.4 \times 10^{-3})\,x + (2.5 \times 10^{-5})\,x^2$, $F_{2,7} = 178.7$, $P < 0.001$, adjusted $R^2 = 0.975$). When the sample size was larger than 100, the curve was flat, and the certainty range became narrower than 0.2 when the sample size was 200.

4. DISCUSSION

4.1 Multiple male mating estimation

MMM frequency (proportion) was estimated to be 68 (31.2%) and the certainty range defined in section 2.2.2 was between 59 (27.4%) and 88 (40.9%) for the studied population of grey-sided voles, in which the observed MP frequency was 50 (23.3%) with 48 oMP_{2MP} and 2 oMP_{3MP}. This model assumed that 90% of MMM females mated with two males and 10% mated with three males. The estimated MMM proportion was 1.34 times larger than the observed MP proportion.

Dean et al. (2006) estimated MMM frequency (proportion) for a population of house mice (*M. musculus domesticus*), in which MP was found in 33 of 143 litters (23.1%). However, they could not obtain a point estimate for the MMM proportion, and their estimation range was impractically wide (20 – 100%). A cause for this insufficient estimation was the uncertainty of FPS; they had to estimate MMM frequency (proportion) assuming the full range of FPS (0.5 – 1.0).

To narrow the estimation range, the range of FPSs should be limited. The estimation range of MMM proportions in the grey-sided vole population was also wide (27.4 – 100%) when using the full range of FPS (0.5 – 0.995) in this study. However, by limiting FPS to the values that were likely to produce both oMP and oPS_{2MP}, we obtained a point estimate of the MMM proportion (32.1%) and greatly reduced the certainty range to 15.8% (27.9 – 43.7%), on the assumption that all MMM females mated with two males. Further, the certainty range narrowed to 13.5% (27.4 – 40.9%) with the point estimate of 31.2% on the assumption that 90% and 10% of MMM females mated with two and three males, respectively. FPS is a necessary variable to estimate MMM frequency (proportion), and the limitation of FPS to only the possible range can greatly improve the estimation of MMM frequency (proportion). The use of *oPS* contributed to this improvement by limiting the range of FPS.

4.2 Number of mating males

Three observed values (oMP, oPS_{3MP}, and oMP_{3MP}) did not simultaneously occur with the assumption that all MMM females mated with three (or more) males. The simulated MP_{3MP} was always larger than the oMP_{3MP}. These results indicate that this assumption was inadequate for the grey-sided vole population. MMM may consist of two and three male mating, and three male mating may not be so common in this population.

We tested this prediction under the seven scenarios for the occurrence probability of three male mating in MMM (Fig. 5). The estimates of MMM frequency varied, but the variation was small. The largest sum of $P(oMP \cap oPS_{3MP} \cap oMP_{3MP})$ was highest, and the certainty range was narrowest, when the occurrence probability of three male mating was assumed to be 0.1. Our prediction was, therefore, supported.

The observed ratio of litters sired by three males (MP_{3MP}) in MP litters was 0.04 (2 / 50). The ratio of MMM with three males should be higher than 0.04. In this study, the plausible ratio of MMM with three males was around 0.1, which was close to the observed ratio of MP_{3MP}. The largest number of males involved in the observed MPs of the grey-sided vole population was three, and we have obtained the reasonable estimate of MMM frequency and its certain range by assuming the largest number of males involved in MMM to be three. It is practical to assume the largest number of males involved in MP as the largest number of males involved in MMM.

MP probability may increase with the probability of three male mating. The MMM proportion is the inversely related to MP probability (Eq. 3), so lower MMM frequencies are expected under higher MP probability, which is caused by the higher probability of three male mating. However, the estimate of MMM frequency seemed to increase along with the probability of three male mating in our analyses. The observed MP with three male mating (oMP_{3MP}) was small (2) in comparison to oMP_{2MP} (48) in the grey-sided vole population. To reconcile the low frequency of oMP_{3MP} with a high occurrence probability of three male mating, a high FPS is required because it raises the proportion of offspring that a dominant male sires in a litter and decreases the number of sires in an MP litter. A high FPS also enhances the chance that a dominant male monopolistically sires a litter and increases the frequency of single paternity derived from multiple male mating (SP_{MMM}). Therefore, to keep the observed MP proportion, MMM must be estimated at a high proportion. MMM frequency (proportion) tends to increase with increasing FPS (Fig. 2).

4.3 Validity and generality of this method

We could estimate MMM frequency under the conditions of the grey-sided vole population. To test the validity and the generality of our method, we made 125 hypothetical populations with various combinations of true values for MMM frequency, FPS, and the occurrence probability of three male mating and estimated MMM frequency. In all conditions, the certainty range included the true MMM frequency with high probability (Table 2). In particular, 97.6% of the true MMM frequencies fell within the certainty range when focusing on populations with the most plausible occurrence probability of three male mating. The consideration of three male mating contributed to improving the certainty of the MMM estimation.

These results suggest that our method produces a highly valid MMM estimation and can be applied to populations with various conditions beyond those of the grey-sided vole population.

4.4 Difference between multiple paternity and multiple male mating

The differences between MP and MMM proportions varied according to the combinations of MP proportion, FPS, and LS (Fig. 6). This variation may lead to inconsistencies in the magnitude of relationships between MP and MMM proportions. A population (or species) with a small LS and a high FPS can have a large MMM proportion, even when its MP proportion is small. By contrast, a population (or species) with a large LS and a small FPS may have an MMM proportion close to its MP proportion. Inconsistencies can occur when comparing these populations (or species). For example, the expected MMM proportion was higher in population A (0.872) than in population B (0.601), even though the MP proportion for population A (0.3) was smaller than that for B (0.6) (Fig. 6).

This inconsistency may require revisions to the previous comparative analyses of MP frequency (proportion). Previous comparative studies have explained the variation in reproductive traits by the variation of MP proportion instead of MMM proportion (Firman & Simmons, 2008a; Ramm et al., 2005; Soulsbury, 2010). These studies showed that larger testes size were associated with higher levels of MP and interpreted this as an indication that higher sperm competition under higher MMM frequency may cause the evolution of larger testes due to their advantages on fertilization. However, if the inconsistencies between MP and MMM proportions are common, the observed relationships between MP proportion and reproductive traits should be reconsidered using estimates for MMM proportion instead of MP proportion.

If FPS is highly variable, the inconsistencies between MP and MMM proportions may not be negligible in natural populations. Mating order has been shown to influence fertilization probability. In mammals, there is variation in fertilization probability associated with mating order (Ginsberg & Huck, 1989). First-mated males are reported to hold an advantage in four species; the proportion of offspring sired by first males is 86% for rabbits (Dziuk, 1965), 91.1% for the Arctic ground squirrel, *Spermophilus parryii plesius* (Lacey, Wieczorek, & Tucker, 1997), 57.5% for the Columbian ground squirrel, *Urocitellus columbians* (Raveh et al., 2010), and 80.6% for the sandy inland mouse, *Pseudomys hermannsburgensis* (Firman, 2014). In the agile antechinus (*Antechinus agilis*), second-mated males sire significantly more offspring (69.5% of offspring) than first males (Kraaijeveld-Smit, Ward, Temple-Smith, & Paetkau, 2002), while in the bank vole (*Myodes glareolus*), the number of offspring sired by first- or second-mated males do not significantly differ (Ratkiewicz & Borkowska, 2000). In contrast to mammals, birds are more consistent, with an advantage conferred to the second-mated male (reviewed by Birkhead & Møller, 1992).

Besides mating order, the following factors are also known to influence fertilization probability: the timing of mating (Cheng, Burns, & Mckinney, 1982; Dziuk, 1965; Huck et al., 1989; Kraaijeveld-Smit et al., 2002), dominance relationships between males (Klemme, Eccard, & Ylönen, 2006), and genotypes of males or interactions between male and female genotypes (Edwards, 1955; Firman & Simmons, 2008b; Levine, 1967). Therefore, inter-specific and/or intra-specific variation in FPS may not be so small, and the inconsistency between MP and MMM proportions should not be ignored in natural populations. Accurate estimation of the MMM frequency (proportion) is crucial for the reliable assessment of sperm competition.

4.5 Study design for this method

Our method required three variables: MP frequency, LS, and PS. A key variable was PS, which narrowed the estimated range of the MMM frequency. In the dataset used in this study, both the mothers and fathers of offspring were identified, and PS was determined. For wild populations, the genotypes of all candidate fathers are not always available, so MP is sometimes analysed with only mother and offspring genotypes (Booth, Montgomery, & Prodoehl, 2007; Bryja et al., 2008; Dean et al., 2006; Firman & Simmons, 2008a; Thonhauser, Thoß, Musolf, Klaus, & Penn, 2014). In these studies, a litter is classified as MP when three or more paternal alleles are observed. PS cannot be directly determined in these studies, but it can be estimated by using a software, e.g., COLONY, GERUD, PARENTAGE, and PEDIGREE (see Jones, Small, Paczolt, & Ratterman, 2010 for review). For example, GERUD (Jones, 2001, 2005) has been used to calculate PS for some wild populations (Booth et al., 2007; Borkowska & Ratkiewicz, 2010; Firman & Simmons, 2008a). Thus, PS can be obtained in most studies of MP.

In this study, we estimated MMM proportions based on 50 MP litters out of a total of 215 litters. MP has been observed in more than 70 mammal species in 10 orders (see Table S1). However, there are only two studies where the sample size exceeded 200 litters (Foltz, 1981; Ishibashi & Saitoh, 2008a, 2008b); most studies have included 30 litters or less. For the grey-sided vole population considered in this study, when the sample size was less than 30 litters, the certainty range was wider than 0.6 (Appendix S4). This range is too large to compare MMM proportions between species or populations, as ranges larger than 0.5 always overlap. When the sample size was more than 100 litters, the certainty range was less variable (Appendix S4). This suggests that sampling efforts are critical for reducing the certainty range to a reasonable width. Therefore, we recommend sampling at least 100 litters for populations with similar reproductive features to grey-sided voles. Of course, this sample size recommendation is a challenging limitation of our methods. To relax this, an additional paternity-related variable should be considered. This study demonstrated the use of the observed mean of PS as an effective way to narrow the certainty range of the MMM frequency estimate. Although the observed mean is a representative statistics, other statistics (e.g., SD, skewness, and kurtosis) could also be used. The distribution of observed PS showed a low kurtosis (–0.796, Appendix Table S1) and may be associated with specific FPSs. Detailed analyses of the effects of FPS on the distribution of PS may contribute to improving the methods for narrowing the possible FPS range and relaxing the sample size request.

The MP frequency (proportion) also affects the certainty range of MMM frequency (proportion) estimates because MMM frequency (proportion) cannot be smaller than MP frequency (proportion). When MP frequency (proportion) is high, the possible range of MMM frequencies (proportions) is limited, regardless of sample size. For example, when MP proportion is 0.8, the maximum range of MMM proportion is between 0.8 and 1.0. Preliminary investigations are recommended to determine a sufficient sample size.

4.6 Biological implications

Inter- (Ramm, Parker, & Stockley, 2005; Soulsbury, 2010) and intra-specific variations in MP proportions (Bryja & Stopka, 2005; Dean, et al., 2006; Firman & Simmons, 2008a; Ishibashi & Saitoh, 2008a; Say et al., 1999; Say, Devillard, Natoli, & Pontier, 2002) have been widely documented. In most of these studies, the differences in MP proportions are considered a result of the differences in MMM proportions. However, the MP proportion is determined not only by the MMM proportion but also by LS and FPS. Therefore, we should reconsider the explanatory power of the MP proportion. When a high MP proportion is observed, the population undoubtedly has a high MMM proportion, but a low MP proportion does not always indicate a low MMM proportion. The combination of a high FPS and a small LS can result in a low MP proportion, even though many females exhibit MMM (Fig. 6). Based on our methods, we can discuss the effects of MMM proportion, FPS, and LS, individually, on variation in MP proportions. Therefore, our method contributes to a deeper understanding of MP proportion variation.

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DATA ACCESSIBILITY

The program codes for the individual-based model are available in Appendix S2 and S3, including the data to run the program. Information on published studies related to multiple paternity, from which data used in this study were obtained, is also available in another appendix (Table S2).

AUTHOR CONTRIBUTION

H. W. and T. S. designed this study. T. S. provided biological expertise for the grey-sided vole and the dataset. H. W. completed the simulation programming and ran simulations and downstream analyses. Both authors contributed to drafting and editing the manuscript and equally worked as the corresponding authors.

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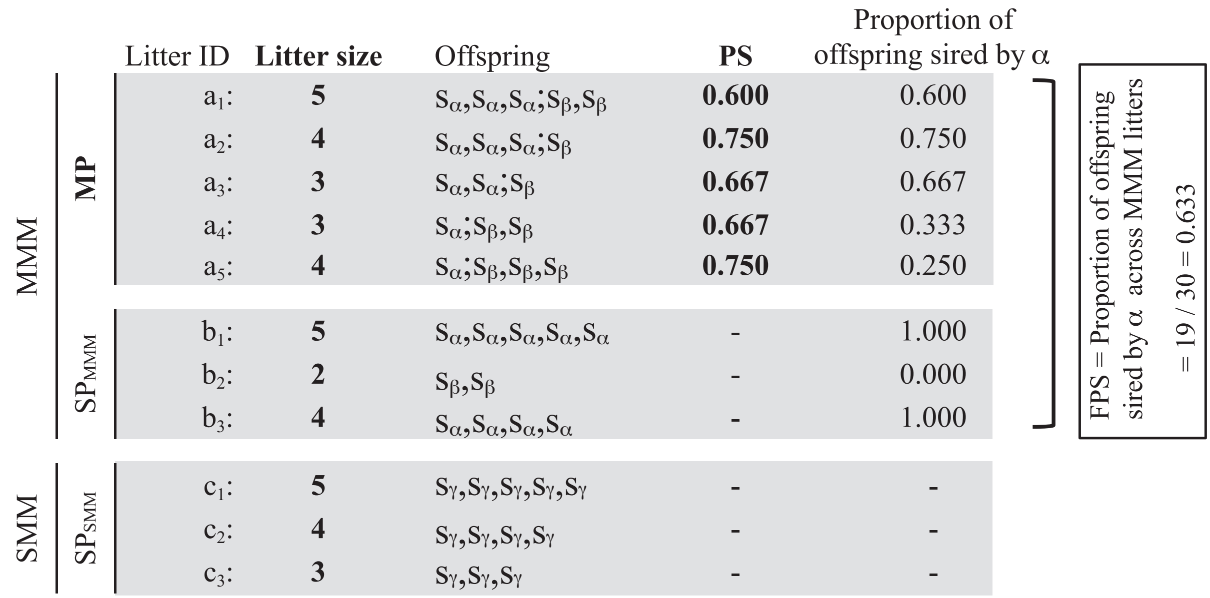
Table 1. List of abbreviations for variables in the estimation of multiple male mating frequency.

Abbreviation		Definition
Root variable	Derivatives	
MMM		Multiple male mating / The behaviour that a female mates with more than one male during a single estrous period.
SMM		Single male mating / The behaviour that a female mates with single male during a single estrous period.
LS		Litter size / Number of offspring in a litter.
FPS		Fertilization probability skew / The highest fertilization probability among mated males. It is practically impossible to determine it in an empirical population (see Fig. 1).
MP		Multiple paternity / The phenomenon in which multiple males sire offspring within a single litter.
	oMP	Observed mltiple paternity frequency
	oMP_{3MP}	Observed multiple paternity frequency sired by three males
SP		Single paternity / The phenomenon in which single male sires all offspring of a litter.
	SP_{MMM}	Single paternity derived from multiple male mating
	SP_{SMM}	Single paternity derived from single male mating
PS		Paternity skew / The highest value in proportions of offspring sired by involved males in each multiple paternity litter. It is observable in an empirical population (see Fig. 1).
	oPS	Observed paternity skew / Average paternity skew of multiple paternity litters.
	oPS_{2MP}	Observed paternity skew calculated based on multiple paternity litters sired by two males
	oPS_{3MP}	Observed paternity skew calculated based on multiple paternity litters sired by two and three males

Table 2. Summary of the results of multiple male mating (MMM) frequency estimation for 125 hypothetical populations.

Assumed occurrence probability of three male mating in MMM	Number of hypothetical populations of which MMM frequency could be estimated*	Number of hypothetical populations of which the certainty range of MMM included true MMM frequency	Proportion of hypothetical populations in which the certainty range of MMM included true MMM frequency
0.00	27	25	92.6% (25/27)
0.25	85	79	92.9% (79/85)
0.50	103	96	93.2% (96/103)
0.75	99	92	92.9% (92/99)
1.00	90	79	87.8% (79/90)

* Not all simulated populations satisfied given conditions for *oMP*, oPS_{3MP} and oMP_{3MP}. This indicates that the combination of MMM frequency, FPS, and the probability of three male maintg in MMM given to an unsuccessful simulation was unlikely to occur.



		Litter ID	**Litter size**	Offspring	**PS**	Proportion of offspring sired by α
MMM	**MP**	a_1:	**5**	$S_\alpha,S_\alpha,S_\alpha;S_\beta,S_\beta$	**0.600**	0.600
		a_2:	**4**	$S_\alpha,S_\alpha,S_\alpha;S_\beta$	**0.750**	0.750
		a_3:	**3**	$S_\alpha,S_\alpha;S_\beta$	**0.667**	0.667
		a_4:	**3**	$S_\alpha;S_\beta,S_\beta$	**0.667**	0.333
		a_5:	**4**	$S_\alpha;S_\beta,S_\beta,S_\beta$	**0.750**	0.250
	$\mathrm{SP_{MMM}}$	b_1:	**5**	$S_\alpha,S_\alpha,S_\alpha,S_\alpha,S_\alpha$	-	1.000
		b_2:	**2**	S_β,S_β	-	0.000
		b_3:	**4**	$S_\alpha,S_\alpha,S_\alpha,S_\alpha$	-	1.000
SMM	$\mathrm{SP_{SMM}}$	c_1:	**5**	$S_\gamma,S_\gamma,S_\gamma,S_\gamma,S_\gamma$	-	-
		c_2:	**4**	$S_\gamma,S_\gamma,S_\gamma,S_\gamma$	-	-
		c_3:	**3**	$S_\gamma,S_\gamma,S_\gamma$	-	-

FPS = Proportion of offspring sired by α across MMM litters

= 19 / 30 = 0.633

Fig. 1

FIGURE 1 A schematic of multiple male mating and its consequence in different types of litters. Observable variables are shown in bold letters, while other letters represent values that cannot be directly measured. Examined litters consist of multiple paternity litters (MP) and single paternity litters (SP). SP litters include two types of litters: those derived from multiple male mating ($\mathrm{SP_{MMM}}$) and those from single male mating ($\mathrm{SP_{SMM}}$), although they are not empirically distinguishable. The number of multiple male mating (MMM) is the sum of MP and $\mathrm{SP_{MMM}}$ (MMM = MP + $\mathrm{SP_{MMM}}$). In this example, of the observed litters (11 = 5 + 3 + 3 in total), five are MP (a_1 … a_5), three are $\mathrm{SP_{MMM}}$ (b_1, b_2, and b_3), and the remaining three are $\mathrm{SP_{SMM}}$ (c_1, c_2, and c_3). MMM females are assumed to have mated with two males (α and β). α (or β) may be a different individual from a different litter, but individuals named α (or β) have a common feature, e.g., the first-mated male (or the second-mated male). In litter a_1, a female mates with males α and β, and α and β sire three and two offspring, respectively. In this case, the paternity skew (PS), which is the highest value in the proportion of offspring sired by an individual male in an MP litter, is calculated to be 0.600 (see also Appendix S1, in which the observed paternity skews (oPS) are presented). In litter b_1, a female mates with males α and β, but α monopolizes that litter. In the litters of $\mathrm{SP_{SMM}}$, females mate with a single male (e.g., γ). Litters of $\mathrm{SP_{MMM}}$ and $\mathrm{SP_{SMM}}$ are, by definition, excluded from the PS calculation – averaged PS was 0.687. In MMM litters, α sires 19 of 30 offspring (0.633), β sires 11 of 30 offspring (0.367). Fertilization probability skew (FPS), which is the highest fertilization probability among males with a certain feature in an MMM litter, is 0.633. This is the proportion of offspring sired by α (i.e., males with an advantageous feature and a larger fertilization probability) in MMM litters. In litter a_4, male β sires more offspring than male α, and the PS is 0.667 by β. The proportion sired by α (0.333) is not adopted by the definition of PS. Therefore, PS is not always the same as the proportion of offspring from α.

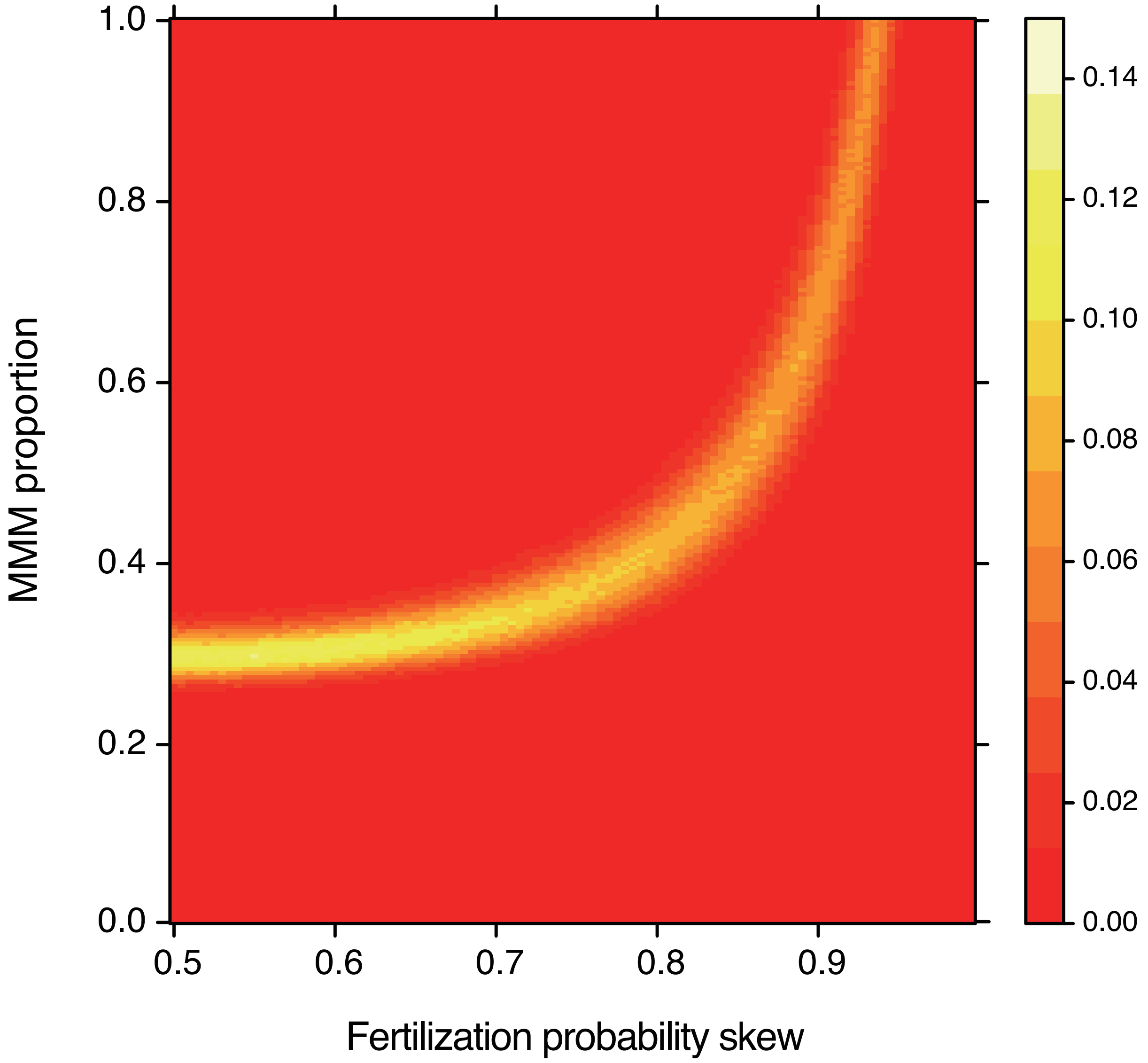


Fig. 2

FIGURE 2 Occurrence probability of the observed frequency of multiple paternity (*oMP*, 50 litters/215 litters) for each combination of multiple male mating proportion and fertilization probability skew. Lighter colours indicate higher probabilities.

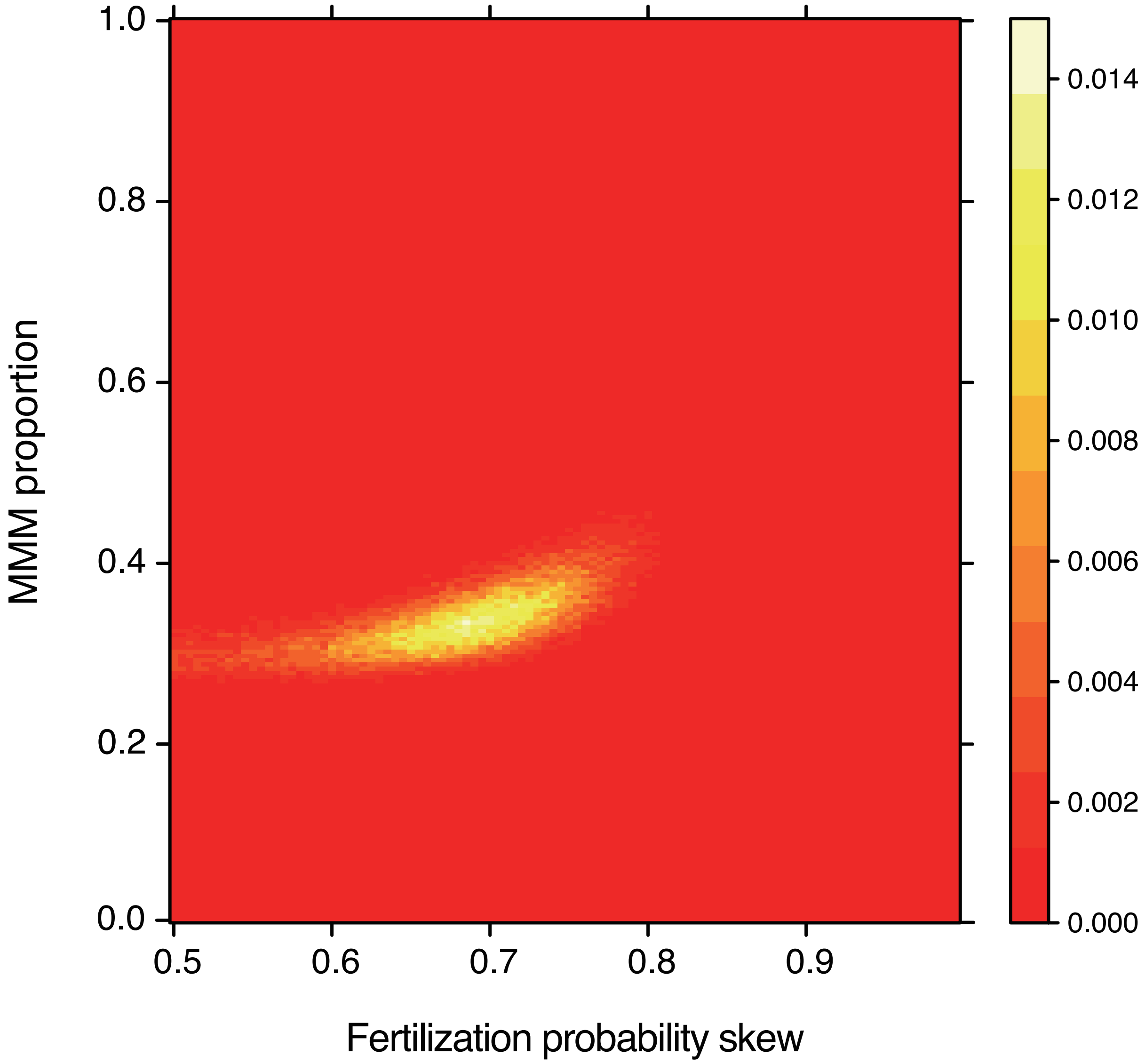


Fig. 3

FIGURE 3 Probability that the observed frequency of multiple paternity (*oMP*, 50 litters/215 litters) and observed paternity skew (*oPS*, 0.6735 ± 0.0025) simultaneously occur for each combination of multiple male mating proportions and fertilization probability skew. Lighter colours indicate higher probabilities.

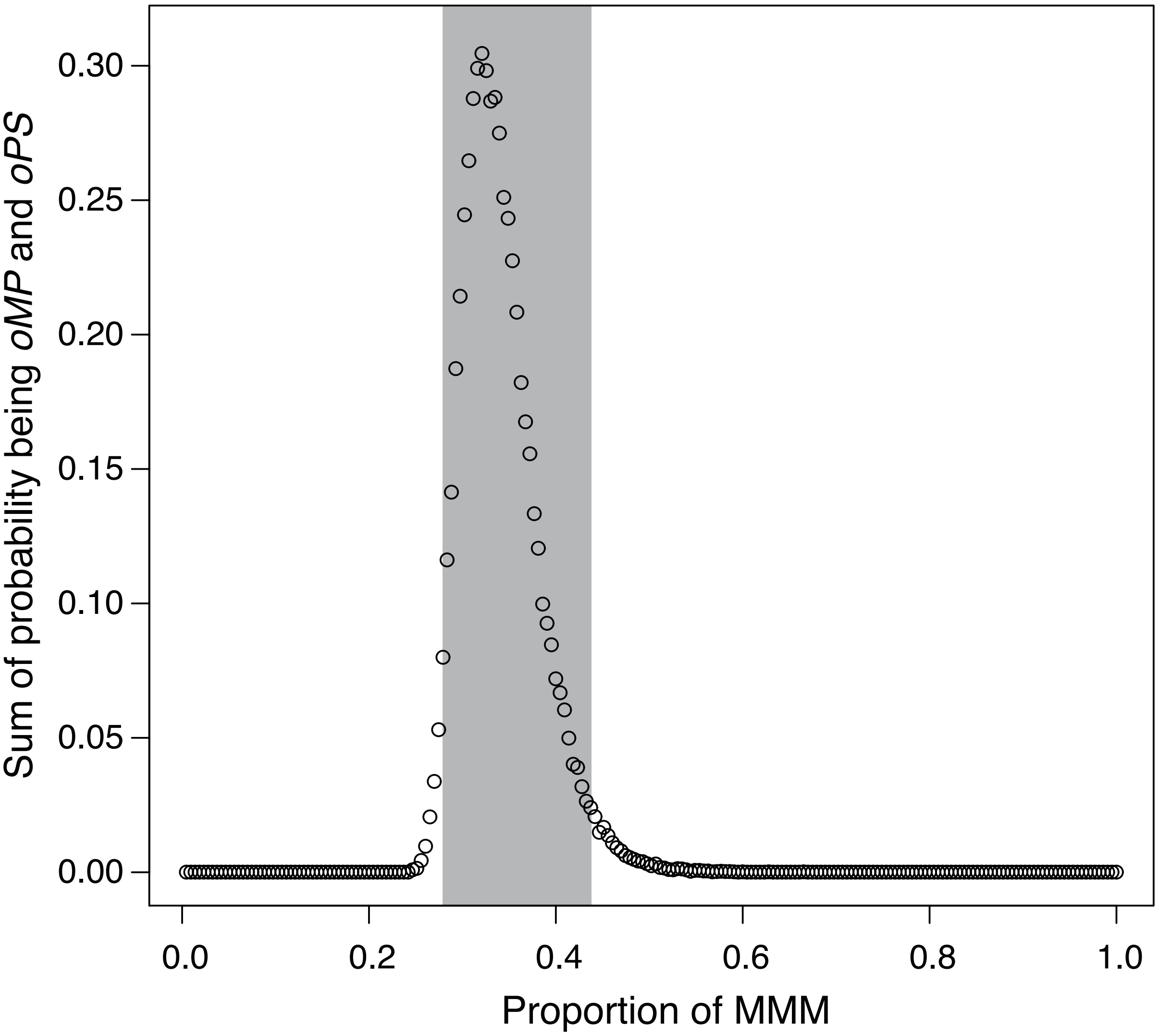


Fig. 4

FIGURE 4 The sum of the probabilities that the observed frequency of multiple paternity (*oMP*, 50 litters/215 litters) and the observed paternity skew (*oPS*, 0.6735 ± 0.0025) simultaneously occur for each multiple male mating (MMM) proportion. The shaded zone is the certainty range, which was obtained by excluding MMM proportions until the accumulated probability reaches 2.5% of the total probabilities both for the upper and lower tails.

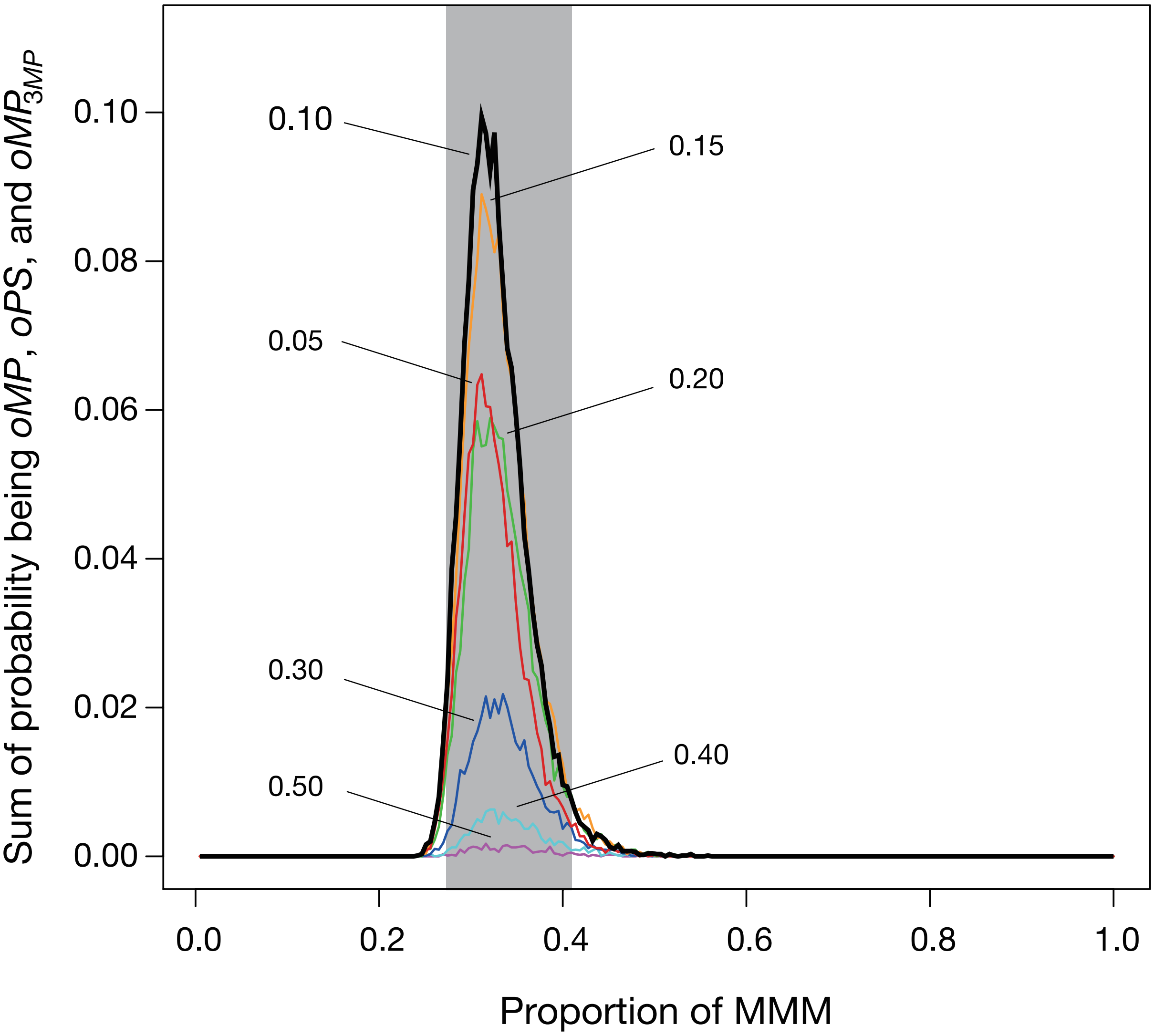


Fig. 5

FIGURE 5 The effect of three male mating on multiple male mating (MMM) frequency estimation. The sum of the probabilities that the observed frequency of multiple paternity (*oMP*, 50 litters/215 litters), the observed paternity skew (oPS_{3MP}, 0.6631 ± 0.0025), and the observed frequency of multiple paternity sired by three males (oMP_{3MP}, 2 litters/215 litters) simultaneously occur was calculated for seven occurrence probability scenarios of three male mating in MMM (0.05; red, 0.1; black, 0.15; orange, 0.2; green, 0.3; blue, 0.4; light blue, and 0.5; purple). The shaded zone is the certainty range with the assumption that the occurrence probability was 0.1.

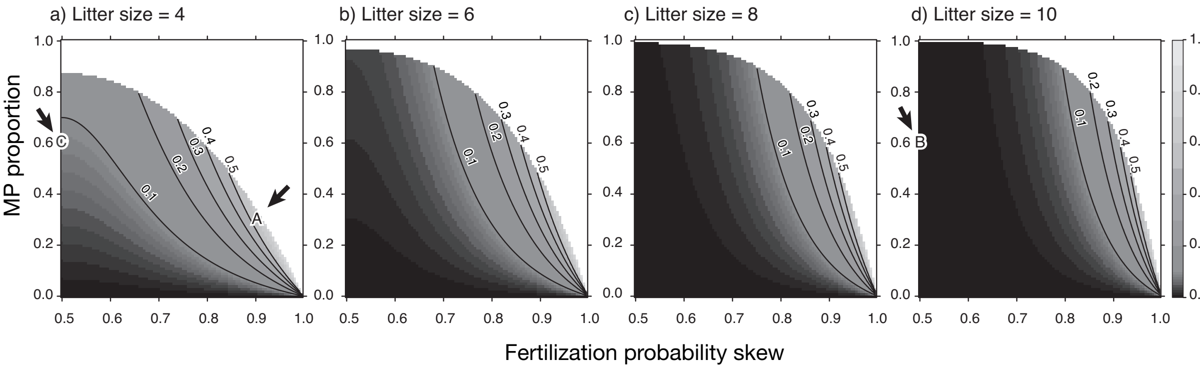


Fig. 6

FIGURE 6 Difference between multiple paternity (MP) and multiple male mating (MMM) proportions (expected MMM proportion – MP proportion) in various combinations of MP proportion, fertilization probability skew (FPS), and litter size (LS). The expected MMM proportion was calculated for four cases of LS (a, 4; b, 6; c, 8; d, 10) using Eq. 3 in the main text. This calculation assumes the combinations of 100 values of FPS between 0.5 and 0.995, and 101 values of MP proportion between 0.0 and 1.0. Darker areas show smaller differences between MP and MMM proportions. Letters with arrows in the panels indicate three hypothetical populations (A, B, and C), which are discussed in the main text. In population A, LS is 4, MP proportion is 0.3, and FPS is 0.9. In population B, LS is 10, MP proportion is 0.6, and FPS is 0.5. In population C, LS is 4, MP proportion is 0.6, and FPS is 0.5. Blank areas indicate unrealistic combinations of MP proportions and FPS where the expected MMM proportion exceeds its limitation (1.0). A large MP proportion was unlikely to occur under high FPSs. Under some combinations of large MP proportions and high FPSs, the expected MMM proportion exceeded 1.0. For example, when LS was 4, MP proportion was 0.9, and FPS was 0.9, the expected MMM proportion was obtained by Eq. 3 as follows:

$$Expected\ MMM\ proportion = \frac{MP\ proportion}{MP\ probability} = \frac{MP\ proportion}{1 - FPS^{LS} - (1 - FPS)^{LS}}$$

$$\frac{0.9}{1 - 0.9^4 - (1 - 0.9)^4} = 2.62.$$