

SHORT COMMUNICATION

Multiple paternity in wild populations of invasive *Rattus* species

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Abstract: Multiple paternity within litters has been recorded among a variety of small mammal species, including some species of rodents. Although multiple mating has been observed in wild *Rattus* populations, whether such mating results in litters with multiple paternities has not been established previously. For studies involving invasive species, it is useful to be aware of the level of genetic diversity a single pregnant invader can bring to a population. Multiple paternity is a means of providing additional genetic diversity to founding populations of rats on islands, which might improve population fitness. We used a genetic approach to confirm that multiple paternity occurs in wild populations of two rat species (*Rattus norvegicus* and *R. rattus*) in New Zealand. This was accomplished by genotyping litters of embryos in pregnant females, and subtracting the known maternal alleles to find the number of paternal alleles necessary to form the litter. The number of paternal contributors cannot be overestimated by this method, befitting a conservative approach to the detection of multiple paternity, but can be underestimated. We used simulations to investigate the level of underestimation likely under two possible scenarios involving multiple paternity.

Keywords: founder; genetics; microsatellite; *Rattus norvegicus*; *Rattus rattus*

Introduction

Rattus norvegicus (Norway rats) and *R. rattus* (ship rats) are two of the three species of *Rattus* classified as invasive (Howald et al. 2007). Following their introduction to New Zealand by Europeans, both species have had a devastating impact on native species (Bell 1978; Atkinson 1985; Towns et al. 2006). The establishment and maintenance of pest-free island sanctuaries are important conservation goals, so investigation of island invasion processes by rats is of particular interest for conservation in New Zealand (Russell et al. 2008b).

Island populations founded by a small number of invaders are vulnerable to the adverse effects of low genetic diversity. Low genetic diversity reduces the survival of individuals, through inbreeding depression, and limits the ability of the population to resist disease and adapt to environmental conditions (Jamieson 2009; Russell et al. 2009a). If pregnant females are capable of carrying offspring with multiple fathers, a single pregnant invader might be carrying the genes of three or more founders. Such multiple paternity would act against inbreeding depression and increase the chance of the population establishing and surviving in the long term.

Population establishment by pregnant females is considered a serious threat for many island sanctuaries. In northern New Zealand, female rats are capable of pregnancy throughout the year (Innes 2001; Innes et al. 2001). It is not known whether wild rats avoid mating with siblings or parents; however, for some species of rodents there is evidence that even small kinship differences can affect mating behaviour (Ryan & Lacy 2003). Multiple paternity would decrease relatedness within a litter, and might therefore lead to more breeding between litter-mates as well as better survival of their offspring. It is potentially a significant factor in enabling rat populations to establish from a very small number of invaders, perhaps just one pregnant female. The purpose of this study was to determine whether multiple paternity occurs in wild populations of *R. norvegicus* and *R. rattus* in New Zealand.

The mating behaviour of *R. norvegicus* varies according to population density. At low densities, males hold territories and guard groups of females for exclusive mating (Calhoun 1963; Waterman 2007). At high densities, the social structure shifts to a despotic system where territories are ill-defined and males rank themselves in dominance, generally according to age (Barnett 1958; Lott 1984;

Waterman 2007). In this situation, males are unable to defend females for exclusive mating, and roving bands of males will attempt to mate with any female that comes into oestrus, resulting in multiple mating (Calhoun 1963; Robitaille & Bovet 1976). Although multiple mating raises the possibility of multiple paternity, it does not necessarily lead to multiple paternity. It might be that only the fittest male's sperm is selected to fertilise the female's eggs.

The mating behaviour of *R. rattus* has received less attention than that of *R. norvegicus*. Ewer (1971) and Corbet and Southern (1977) describe similar density-dependent behaviour to *R. norvegicus* among *R. rattus* populations that were commensal with humans. However, Hooker and Innes (1995) found that wild *R. rattus* in New Zealand that were non-commensal with humans tended to prefer solitude. Males did not maintain groups of females in their territories, even when population density was low. In this social arrangement, it is more difficult for a male to ensure he mates exclusively with particular females.

Multiple paternity has not previously been established at the genetic level for wild populations of *R. norvegicus* or *R. rattus*. Heiberg et al. (2006) inferred multiple paternity in *R. norvegicus* removed from the wild, but they did not use known maternal genotypes. Instead, parents were assigned probabilistically by choosing the male–female pair that most likely contributed their alleles to a juvenile rat. The natural breeding behaviour of the rats might also have been affected by being kept under experimental conditions. In this study, we genotyped litters of embryos inside pregnant females, together with the corresponding maternal tissue, to determine the number of paternal alleles required to form the litter. This approach will confirm the occurrence of multiple paternity if there is some genetic locus at which more than two paternal alleles are required.

Methods

Sampling

Rattus norvegicus were trapped on the island of Pakihi (114 ha) in the Hauraki Gulf, Auckland, New Zealand, as part of a related study. Thirty Tomahawk live-traps and 20 Victor snap-traps baited with chocolate paste were deployed over the summers of 2004 to 2006, returning 49 rats (Russell et al. 2008a). Three visibly pregnant female

rats had their uteri removed so that a genetic analysis of the mothers and their embryos could be conducted. The uteri, still containing embryos, were stored in 70% ethanol prior to DNA extraction. Only the largest embryos from each uterus were selected, since DNA extraction from smaller embryos risked extracting maternal uterine tissue, or contaminating the sample with maternal DNA from embryos that were in the process of being resorbed.

Rattus rattus were sampled from Puketi Forest (7345 ha) in Northland, New Zealand, between 21 November and 2 January 2005. All traps were Victor snap-traps baited with white chocolate. The number of traps set varied between 325 and 1076 per night over the trapping period, capturing 147 ship rats in total (J. Taillon 2005, unpubl. data). The uteri of pregnant females were stored in 70% ethanol. Three of the uteri with the largest embryos were examined as part of this study. Again, only the largest embryos were examined, to avoid the possibility of maternal contamination.

Genotyping

DNA was extracted using the DNeasy Tissue Kit (Qiagen). Microsatellite markers characterised for the *R. norvegicus* genome mapping (D10Rat20, D11Mgh5, D12Rat76, D15Rat77, D16Rat81, D18Rat96, D19Mit2, D20Rat46, D2Rat234, D5Rat83 and D7Rat13) were used (Jacob et al. 1995), 11 for *R. norvegicus* samples and 10 for *R. rattus* samples. Amplicons from locus D12Rat76 for *R. rattus* did not show the characteristic variability and stutter seen for microsatellites, suggesting D12Rat76 did not amplify a microsatellite on the *R. rattus* genome. Consequently, this locus was discarded for *R. rattus* specimens. To avoid physical linkage, markers were chosen on different chromosomes. Each forward locus primer was labelled with fluorescent dyes before amplification by polymerase chain reaction (PCR). PCR was performed in 10- μ L volumes, containing 10 ng of DNA, 0.1 μ M of forward primer labelled with 5' fluorescent labels, 0.2 μ M of reverse primer, 0.2 μ M of each dNTP, 0.2 units Platinum Taq DNA polymerase (Invitrogen), and 1X reaction buffer with 1.5 mM MgCl₂. PCR products were pooled with Genescan 400HD [ROX] Size Standard for a single run using an ABI Prism 3730 Genetic Analyzer capillary electrophoresis system (Applied Biosystems). Amplification size was scored using GENESCAN ANALYSIS 3.7 and GENOTYPER 3.7.

Determining paternity

Each set of data comprised the maternal microsatellite genotype and the associated embryo genotypes. Assuming no genotyping error, paternal alleles were inferred by eliminating known maternal alleles from each embryo's genotype. In the case where the maternal and embryo genotypes at a locus were heterozygous and identical, it was not possible to determine which of the offspring's alleles was contributed by the mother and which by the father. If one of these alleles was already shown to be paternally contributed for another embryo, we selected this allele as the paternally contributed allele. This choice favours fewer paternal contributors, suiting a conservative approach. If both alleles were already required to be paternal alleles, the choice was inconsequential. Such detection of multiple paternity is guaranteed to be conservative; if multiple fathers share a common allele, that allele cannot be used as evidence of more than one paternal contributor.

Underestimation of multiple paternity

While our method for detecting multiple paternity cannot produce a false positive result, it is possible to underestimate the incidence of multiple paternity. To investigate the extent of underestimation, we performed a series of simulations. For each simulation, one maternal genotype and a fixed number of paternal genotypes were constructed according to the sample allele frequencies found in the relevant study area. In one scenario, each male fertilised a similar number of embryos in a litter, while in another scenario, one male fertilised the majority of the embryos. Eight embryo genotypes were constructed by randomly selecting one allele from each parent at each locus, to which we applied the method for detecting multiple paternity. For each simulation scenario we performed 10 000 simulations, recording

the estimated number of paternal contributors per simulation, and compared these estimates with the true number.

Results

Rattus norvegicus from Pakihi Island

The first of the three sets of embryos was successfully typed at all 11 loci for the mother and eight embryos. A minimum contribution of three paternal alleles was required at each of five loci. This would require a contribution to the litter from at least two fathers (Table 1a).

In the second set, four embryos and the maternal tissue were successfully typed at 10 loci. From this set of data, multiple paternity was identified at two loci, each requiring the contribution of three paternal alleles, again suggesting a minimum of two paternal contributors (results not shown).

The final set comprised a mother and seven embryos. The maternal sample and two of the embryos failed to be genotyped at two loci, and another embryo failed at four loci. Based on the available data, there was no evidence of multiple paternity in this litter (results not shown).

Rattus rattus from Puketi Forest

All three sets of *R. rattus* samples from Puketi Forest were successfully genotyped at 10 loci. The samples provided four, five, and six embryos, respectively. One of the samples provided no evidence of multiple paternity. Each of the other two samples identified multiple paternal contributions at five loci. One of these is shown in Table 1b. For both sets of embryos, we detected a minimum of three paternally contributed alleles, requiring a minimum of two paternal contributors.

Simulation results

The results in Table 2 indicate that the overall detection rate of multiple paternity was high, especially as the true number of fathers increased. However, if one male fertilised the majority of the embryos, the detection rate decreased.

For both reference populations, the conservative method was prone to underestimate the true number of paternal contributors. For instance, in the *R. norvegicus* population, we would rarely estimate more than two paternal contributors, regardless of the true number of contributors or how paternity was spread. This is because our reference sample for *R. norvegicus* allele frequencies had little genetic diversity, due to a low sample size and genetic homogeneity within the local population of Norway rats on Pakihi Island.

The six litters in our study yielded estimates of one or two paternal contributors each. However, the simulation results showed that these results would also be common if the true number of paternal contributors was higher.

Discussion

We have confirmed multiple paternity in wild *R. norvegicus* and *R. rattus*. By detecting a high incidence of multiple paternity in opportunistically acquired pregnant females from a mainland and an island source in this study, we can reasonably deduce that multiple paternity is common in wild *R. norvegicus* and *R. rattus* populations in New Zealand.

Dean et al. (2006) proposed that a single locus that suggests multiple paternity should not be taken as conclusive evidence on its own, as there are possible explanations for more than two paternal alleles being required without the incidence of multiple paternity. For instance, there could be genotyping error in the form of allelic dropout or null alleles, or mutation during gametogenesis (Dakin & Avise 2004). These events are rare, however, so the chance that such events occur for two or more loci are deemed effectively negligible. Because we identified up to five loci requiring genetic contributions from two or more males, we are confident we have confirmed multiple paternity in wild populations of *R. norvegicus* and *R. rattus*.

Table 1. Examples of maternal and embryo genotypes at loci requiring two or more paternal contributors.

(a) Pregnant female *R. norvegicus* from Pakihi Island with sample ID PK25 and eight of her embryos. Six loci requiring only one paternal contributor have been omitted.

		D10Rat20	D11Mgh5	Locus D12Rat76	D19Mit2	D2Rat234
Maternal genotype	PK25	116, 116	230, 248	99, 101	213, 213	107, 111
Embryo genotypes	PK25.1	116, 118	248, 248	99, 101	213, 213	107, 115
	PK25.2	116, 118	230, 230	99, 99	213, 213	107, 111
	PK25.3	110, 116	228, 248	99, 101	205, 213	109, 111
	PK25.4	116, 118	230, 248	99, 101	213, 213	107, 111
	PK25.7	114, 116	230, 248	91, 99	205, 213	111, 115
	PK25.8	116, 118	230, 230	101, 101	183, 213	111, 115
	PK25.9	116, 118	230, 248	101, 101	213, 213	107, 115
	PK25.10	110, 116	228, 248	99, 101	205, 213	107, 107
Paternal alleles	110, 114, 118	228, 230, 248	91, 99, 101	183, 205, 213	107, 109, 115	

(b) Pregnant female *R. rattus* from Puketi Forest with sample ID PukA130 and six of her embryos. Five loci requiring only one paternal contributor have been omitted.

		D15Rat77	D16Rat81	Locus D18Rat96	D19Mit2	D20Rat46
Maternal genotype	PukA130	232, 238	165, 165	234, 238	223, 223	185, 185
Embryo genotypes	PukA130a	232, 238	157, 165	234, 234	223, 231	165, 185
	PukA130b	232, 238	157, 165	238, 238	223, 231	185, 185
	PukA130c	238, 250	155, 165	234, 238	223, 231	165, 185
	PukA130d	232, 254	155, 165	232, 234	223, 229	185, 187
	PukA130e	238, 250	155, 165	234, 238	223, 231	185, 185
	PukA130f	232, 238	165, 165	234, 238	223, 223	185, 185
Paternal alleles	232/238, 250, 254	155, 157, 165	232, 234, 238	223, 229, 231	165, 185, 187	

Table 2. Number of paternal contributors detected using the conservative approach when two, three, or four fathers have produced the litter. Two scenarios are considered: where each father sires roughly equal proportions of the litter, or where one dominant father sires the majority of the litter. Results are expressed as percentages from 10 000 simulations. Parental genotypes were simulated using the allele frequencies associated with each of the study areas. Percentages for the correct number of paternal contributors are shown in bold.

	Pakihi				Puketi			
<i>Two fathers</i>								
Estimated no. fathers	1	2			1	2		
4:4 paternity ratio	5.9	94.1			0	100		
7:1 paternity ratio	23.7	76.4			0.1	99.9		
<i>Three fathers</i>								
Estimated no. fathers	1	2	3		1	2	3	
3:3:2 paternity ratio	0.8	97.0	2.2		0	34.5	65.5	
6:1:1 paternity ratio	4.3	95.7	0		0	100	0	
<i>Four fathers</i>								
Estimated no. fathers	1	2	3	4	1	2	3	4
2:2:2:2 paternity ratio	0.2	93.9	5.9	0	0	8.1	88.7	3.2
5:1:1:1 paternity ratio	0.9	97.4	1.7	0	0	42.7	57.4	0

The simulation study indicates that it is possible to detect multiple paternity successfully even when the genetic structure of a population is fairly homogeneous. By way of example, there was an average of only 3.36 different alleles per locus for the reference population of adult *R. norvegicus* on Pakihi Island. However, it should be borne in mind that if the multiple paternal contributors to a litter are closely related, their genotypes are likely to be more in common than two unrelated males from the same population. In this situation, there is a greater chance of not detecting multiple paternity within a litter. Although it is possible that the paternal contributors to each embryo set we studied were related, we still successfully detected multiple paternity in four of the six sets.

Multiple paternity is an important consideration when studying island colonisation by invasive rats, in particular when estimating the number of founders and founder effects (Russell et al. 2009a, b). Taking multiple paternity into account, the hypothesis of a “single pregnant invader” cannot be discounted solely on the basis of detecting more than four alleles in the descendant population. The increased genetic diversity introduced by pregnant female invaders carrying litters sired by several males could lead to an overestimation of the number of founders or rate of invasion if multiple paternity is not taken into consideration.

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