

The effect of multiple mating on genetic relatedness in larval aggregations of the imported willow leaf beetle (*Plagiodera versicolora*, Coleoptera: Chrysomelidae)

David E. McCauley and Regina O'Donnell

Department of General Biology, Vanderbilt University, Nashville, Tennessee 37235, USA
Mountain Lake Biological Station, Route 1, Pembroke, Virginia 24136, USA

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Summary. The effect of multiple mating on the average genetic relatedness within socially interacting sib groups was studied in the imported willow leaf beetle. Using an electrophoretically determined genetic marker, it was demonstrated that between 49 and 96% of wild caught females produce egg clutches expressing multiple sires. Following theoretical predictions of Wade (1982), this information was used to estimate the harmonic mean number of sires per family and then the average relatedness within families. Average relatedness was estimated to be 0.38. An alternate form of data analysis suggested by Pamilo and Crozier (1982) did not require any knowledge of paternity and resulted in a slightly higher estimate of relatedness, 0.44.

Introduction

The influence of both multiple mating by females and the concurrent use of multiple paternal genomes on the genetical structure of families is becoming increasingly apparent for a diversity of species (Murray 1964; McCracken and Bradbury 1981; Sassaman 1978; Hanken and Sherman 1981; Hoogland and Foltz 1982; Page and Metcalf 1982). One important consequence of multiple sires within sib groups is to reduce the average relatedness between family members below that expected for full sibs. For example, it has been shown recently that multiple mating by queens in several eusocial hymenopteran species could reduce the relatedness of female workers somewhat below the 0.75 value expected with single paternity in an outbred system (Metcalf and Whitt 1977; Lester and Selander 1981; Page and Metcalf 1982; Pamilo 1982). This, of course, restricts the conditions

under which altruistic social behavior could evolve under a family structured model of kin selection. Wade (1982) has described the theoretical effects of multiple insemination on the relatedness of both diploid and haplodiploid sib groups and shows that the average degree of relatedness within families declines with an increase in the number of sires per family, an increase in the evenness of the distribution of paternities within families, and an increase in the evenness of the distribution of numbers of sires among families. When the distribution of inseminations among females is uneven the average relatedness within family groups is determined by the harmonic rather than the arithmetic mean number of inseminations per female.

In many non-eusocial insects, immatures derived from a common mother remain aggregated for some time prior to dispersal. Often these aggregations function to facilitate feeding or predator defense. A population structure in which family members interact primarily with one another would be conducive to the operation of family structured kin selection (or trait-group selection in the parlance of Wilson 1975, 1977). Since the response to family or trait-group selection would depend, in part, on the distribution of genetic variation within and between families (i.e. the relatedness of group members), studies of the degree of multiple paternity represented in such aggregations could be instructive in helping to evaluate the potential for the operation of family structured kin selection in these species.

The imported willow leaf beetle *Plagiodera versicolora*, (Coleoptera: Chrysomelidae) displays an aggregation behavior during a portion of its larval history that is equivalent to the population structure described above. Females deposit egg clutches consisting of an average of 16 eggs on the leaves of willow trees (*Salix* spp.) (Wade and Breden, in-

preparation). Upon hatching the larvae remain tightly clustered while feeding on the epidermis and mesophyll of the willow leaves. When disturbed, the larvae exude droplets of a noxious liquid that act as a predator deterrent (Raupp 1982; Wade and Breden, in preparation). There is also some cannibalism of eggs by early hatching larvae. These aggregations persist for 3 larval instars and are then disbanded. Thus, individuals derived from the same egg clutch interact behaviorally with each other for a significant proportion of their lives and display both positive and negative social interactions. Little is known about the mating behavior and sperm usage patterns displayed by adult females and hence whether the larval aggregations consist of full or half sib groups. This is a study of the frequency of multiple sires within egg clutches derived from wild caught individual female willow leaf beetles. Estimates of the frequency of multiple paternity and the genetic composition of larval groups are then used to calculate the average relatedness within groups.

Materials and methods

Determination of multiple paternal representation among the offspring of a given mother requires a genetic marker with multiple alleles that can be scored easily for the mother and her young. When the genotypes of the mother and a group of her offspring are known, the presence of at least 3 different paternally derived alleles among the offspring is required to prove multiple paternity for that family. The marker used in this study was the enzyme phosphoglucosyltransferase (PGM) that was shown to have 4 common alleles when resolved using standard starch gel electrophoresis. These alleles will be symbolized A, B, C, and D, the sequence representing slowest to fastest migration rates displayed by the electromorphs during electrophoresis. The PGM genotypes of both adults and larvae were resolved on a tris-maleate buffer system described by Selander et al. (1971).

Imported willow leaf beetles used in this study were collected as adults in June and July 1983 from willow trees growing along Sinking Creek and its tributaries in Giles County, Virginia. Single adult females were collected, brought back to the laboratory, and maintained in petri dishes lined with damp paper towels and stocked with fresh willow leaves. Those females who deposited eggs in the dishes were frozen until their genotype could be determined. Larvae hatching from the egg masses were maintained on willow leaves for several instars until they had achieved sufficient size for electrophoretic studies. Paternal alleles identified in this way were derived from matings in the field prior to collection of the mothers. Twenty such families, ranging in size from 5–15 larvae were used in this study. In addition, a random sample of 232 adults was collected from the field site so that an accurate estimate of the allele frequencies at the PGM locus could be made.

Results

The allelic and genotypic frequencies found for the PGM locus are presented in Table 1. There is no

Table 1. Absolute genotypic and relative allelic frequencies for the PGM locus as calculated from 232 adult willow leaf beetles

Genotype									
AA	AB	AC	AD	BB	BC	BD	CC	CD	DD
17	29	3	46	33	13	54	1	5	31
$p_A=0.24, q_B=0.35, r_C=0.05, s_D=0.36$									

significant deviation from the Hardy-Weinberg expectation ($G=10.12, df=6, P>0.10$) in this four allele system. The maternal genotypes and the genotypes of all offspring scored for the 20 families are presented in Table 2 along with those paternal alleles that can be identified. It can be seen that 3 or more paternal alleles are positively identified in 8, or 40%, of the families. Because a female could use the sperm of several males with identical PGM genotypes, and because paternal contributions can not be identified when the mother and offspring are identical heterozygotes, this technique can not detect all of the cases of multiple paternity. Furthermore, because family size is finite the larvae sampled in a particular family may not express the genetic diversity present in the male gametes stored by the mother. We follow the statistical techniques of Birdsall and Nash (1973), and Merritt and Wu (1975) for adjusting the observed frequency of multiple mating to account for cases of multiple mating not detected by the particular genetic system used as a marker.

The techniques set an upper bound and lower bound on the estimate of the proportion of families that have multiple sires by assuming two contrasting mating systems. The lower bound on the estimate of the proportion of families displaying multiple sires rests on the assumption that a female either mates once or so many times that it is very likely that 3 different paternal alleles would be represented among the large group of males contributing to any multiply sired family. In this case the lower bound for the estimate is simply the proportion of families determined to contain 3 different paternal alleles. When a mother and her offspring are identical heterozygotes, however, maternal and paternal allelic contributions can not be determined and the total number of paternal alleles represented in the sample can not be determined exactly. From Table 2 it can be seen that many mothers are heterozygotes, as expected in a 4 allele system. In some families 3 paternal alleles can still be detected, in others only 1 or 2 paternal alleles can be detected unambiguously. In families nos. 4, 10, and 19 we find mothers and a class of offspring

Table 2. The PGM genotypes of 20 adult female willow leaf beetles and their offspring. Known and inferred (*parenthesis*) paternal alleles are identified

Family no.	Mother	Offspring	Paternal alleles
1	AD	2AA, 1AB, 2AC, 1AD, 2BD, 3CD, 2DD	A, B, C, D
2	AB	1AD, 5BB, 3BD	B, D
3	DD	11AD, 1CD, 3DD	A, C, D
4	AD	2AB, 5AD, 2BD, 1DD	B, D, (A)
5	BD	1AB, 1BB, 1BC, 2BD, 1CD, 4DD	A, B, C, D
6	BB	4BB, 2BD	B, D
7	BB	1AB, 9BB, 1BD	A, B, D
8	BB	4AB, 6BB	A, B
9	AD	1AB, 2AC, 2AD, 4CD, 1DD	B, C, D, (A)
10	BC	5AC, 4BB, 2BC	A, B, (C)
11	AA	12AA	A
12	BB	5BB, 4BD	B, D
13	BD	4AB, 4AD, 1BB, 4BD, 1CD	A, B, C, (D)
14	BD	2AD, 1BB, 2BD, 1DD	A, B, D
15	DD	5DD	D
16	BD	6BD, 2DD	D, (B)
17	BD	2AD, 7BB, 2BD, 2DD	A, B, D
18	AD	3AA, 4AD, 3DD	A, D
19	AD	1AA, 3AB, 2AD, 4BD	A, B, (D)
20	AB	4AD, 1BB, 4BD	B, D

that are identical heterozygotes. These offspring contain an allele not assigned to the fathers through some other offspring in that group. It is possible that the heterozygous offspring in question could have received this allele from either the mother or some unknown father. For each of these 3 families the probability that at least one copy of this allele could have been derived paternally was calculated as follows. Assuming random union of gametes drawn from a heterozygous female and from the general population of males we can calculate the proportion of offspring who are genotypically identical to their mother and who derived the allele in question from their father. For example, in family no. 4 the AD heterozygotes are identical to their mothers and the A allele is not identified as a paternal contribution through other genotypic classes of offspring. We calculate that 40% of AD heterozygotes derived from an AD mother and random male gametes would have gotten the A allele from their fathers. Since there are 5 AD heterozygotes in this family, the probability that they all derived their A alleles from their mother is 0.6^5 or the probability that at least one of the 5 A alleles comes from a male is 0.92. The net result of this correction when applied to all 3 fami-

lies was to increase the lower bound on the proportion of multiply sired families to 0.49.

Calculation of the upper bound assumes that only two males can contribute to any multiply sired family. In that case we can simply identify all possible genotypic combinations of two males that would provide less than 3 different paternal PGM alleles to the offspring. The cumulative frequency of these combinations can be calculated for the system under study, given an accurate estimate of the appropriate allele frequencies. In this study, 49% of families sired by two random males would not be detected by the PGM system. Thus, the upper bound on the estimate of the proportion of families with multiple sires is $(0.49/(1-0.49)) = 0.96$.

In addition, when family sizes are small, these bounds should be further adjusted to account for the probability that all possible paternal genotypes stored by the female are not expressed in the particular offspring sampled (Birdsall and Nash 1973; Merritt and Wu 1975). We ignore this correction here because, in a system with 4 alleles present at the observed frequencies, we calculate that over 95% of the females actually storing sperm expressing 3 or more PGM alleles would express them in an average sized sample of 10 offspring. This simplification results in a slight underestimate of the true level of multiple paternity.

Discussion

These results can be used to estimate the average relatedness (\bar{r}) within larval aggregations present in the field. Wade (1982) shows that with multiple inseminations

$$\bar{r} = (1/2) \times (1/2 + (1/2 \bar{H})) \quad (1)$$

where \bar{H} is the harmonic mean number of sires. Because the harmonic mean is included in this equation, \bar{r} is very sensitive to the distribution of numbers of mates among females and less so to the arithmetic mean number of mates. The distribution of sires among females in the willow leaf beetles was estimated above in two ways giving two quite different distributions of numbers. Under one assumption there were two classes of females, 51% of whom mated once and 49% who mated many times. Recall that the harmonic mean is the reciprocal of the average of the reciprocals. Note also that we approximate the reciprocal of some large number of mates as zero. This gives a harmonic mean number of mates (\bar{H}) of 1.96 $(1/(0.49 \times 0 + 0.51 \times 1))$ and an average relatedness

within sibships (\bar{r}) of 0.38. Under the other assumption, 96% of the females mate twice and 4% mate once. This gives a harmonic mean number of mates of 1.92 ($1/(0.96 \times 0.5 + 0.04 \times 1)$) and again a relatedness of 0.38. Thus, the two extreme interpretations of the distribution of mates required to set limits on the numbers of families with multiple mating result in nearly identical estimates of the average relatedness.

Pamilo and Crozier (1982) suggest a quite different statistical procedure for estimating the average relatedness within populations given the genotypes of the interacting individuals and a 2 allele system. Here genotypes are assigned numerical values according to an additive relationship between arbitrarily numbered alleles. Thus, if the 2 alleles were numbered 1 and 2 a heterozygote would receive a genotypic score of 3. An average genotypic value (Y) is then calculated for each aggregation and regressed on the genotypic values calculated for each individual in the aggregation (X values). This is done for each aggregation considered in the study so as to provide multiple Y values. Pamilo and Crozier suggest that either individuals or aggregations can be weighted equally in the analysis. The estimate of the average relatedness within aggregations is the regression coefficient (b) calculated from the above data. Because individual X values contribute to the calculation of their paired Y value, a correction term for the regression coefficient is also provided by the authors. The corrected estimate $E(b)$ is calculated as

$$E(b) = (b - 1/n) \times (n/(n-1)) \quad (2)$$

where b is the linear regression coefficient calculated directly from the data and n is the average sample size per family.

Pamilo (1984) suggests several statistical techniques for estimating relatedness when there are more than 2 alleles at the marker locus. One of these is to assign numeric values to all alleles and compute additive genotypic values. These are used in a regression analysis similar to that described above. When sample sizes are small, however, the weighting scheme can influence the estimate of \bar{r} . Here, Pamilo (1984) suggests rotating the sequence of allelic weightings to include all possible sequences and averaging them. This procedure was applied to the data set because, in estimating relatedness by a procedure quite different from that considering estimates of multiple paternity, we can test some of the assumptions inherent in our data analysis by comparing the concordance of the results derived by the two methods. First, allelic

values of 1, 2, 3, 4 were assigned to alleles A, B, C, and D respectively. Weighting all individuals equally results in a regression coefficient of 0.43. The corrected value and thus the estimate of average relatedness is 0.36. Then estimates of \bar{r} were derived from all 23 additional combinations of the 4 alleles and the 4 weighting values (A=1, B=2, C=4, D=3, etc.). The average of all of the estimates of \bar{r} derived from the allelic weighting method is 0.44.

It is not clear how to assign confidence limits to an estimate of average relatedness derived in this way. Suffice it to say that the estimates derived from the estimate of polyandry and the regression technique agree moderately well. In Wade (1982) the influence of multiple mating on relatedness is determined by the distribution of sires within females as well as between females. When one male predominates in fertilizing the eggs laid by a multiply mated female the harmonic mean number of mates drops and relatedness increases relative to an even distribution of fertilization rates among the same number of sires. While nothing is known of the sperm precedence rules in *Plagioderia versicolora*, fertilization predominance by one male in multiply mated females is the rule for many insect species (Thornhill and Alcock 1983). If partial sperm precedence is the rule for *P. versicolora* then an estimate of \bar{r} derived from measures of polyandry would be expected to be low. This could explain the discrepancy between the estimate of 0.38 derived from the harmonic mean number of mates and the estimate of 0.44 that makes no assumptions about the mating system.

That multiple mating by females can greatly reduce the relatedness of family groups has become generally recognized by those concerned with the evolution of social behaviors (Hamilton 1964; West Eberhard 1975; Page and Metcalf 1982). However, the influence of the evenness of the distribution of paternities among females has not been as widely noted. High levels of multiple mating do not necessarily reduce average relatedness significantly. As shown by Wade (1982) and demonstrated empirically here, it is the harmonic rather than the arithmetic mean number of mates that should be estimated when calculating average relatedness in family groups. This information was obtained here without any real knowledge of the distribution of sires within and between mothers or of the arithmetic mean number of mates. The contrasting mating systems hypothesized in order to set upper and lower bounds on the proportion of multiple sired families would produce radically different arithmetic mean numbers of mates per fe-

male. The properties of the harmonic mean are such, however, that the two very different distributions of matings result in quite similar harmonic means and thus measures of average relatedness. In the case of the willow leaf beetles, the relatively high rate of multiple mating and the possibility that some females could mate many times (inflating the arithmetic mean number of mates) would have a surprisingly minor influence on the response to any family level selection.

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References

- Birdsall OA, Nash D (1973) Occurrence of successful multiple insemination of females in natural populations of deer mice (*Peromyscus maniculatus*). *Evolution* 27:106–110
- Hamilton WD (1964) The genetical evolution of social behavior, II. *J Theor Biol* 7:17–52
- Hanken J, Sherman PW (1981) Multiple paternity in Belding's ground squirrel litters. *Science* 212:351–353
- Hoogland JL, Foltz DW (1982) Variance in male and female reproductive success in a harem-polygynous mammal, the black-tailed prairie dog (Sciuridae: *Cynomys ludovicianus*). *Behav Ecol Sociobiol* 11:155–163
- Lester RJ, Selander RK (1981) Genetic relatedness and the social organization of *Polistes*. *Am Nat* 117:147–166
- McCracken GF, Bradbury JW (1981) Social organization and kinship in the polygynous bat *Phyllostomus hastatus*. *Behav Ecol Sociobiol* 8:11–34
- Merritt RB, Wu BJ (1975) On the quantification of promiscuity (or "*Promyscus*" *maniculatus*). *Evolution* 29:575–578
- Metcalfe RA, Whitt GS (1977) Intra-nest relatedness in the social wasp *Polistes metricus*. A genetic analysis. *Behav Ecol Sociobiol* 2:339–351
- Murray J (1964) Multiple mating and effective population size in *Cepaea nemoralis*. *Evolution* 18:283–291
- Page RE, Metcalfe RA (1982) Multiple mating, sperm utilization, and social evolution. *Am Nat* 119:263–281
- Pamilo P (1982) Multiple mating in *Formica* ants. *Hereditas* 97:37–45
- Pamilo P (1984) Genotypic correlation and regression in social groups: multiple alleles, multiple loci and subdivided populations. *Genetics* 107:307–320
- Pamilo P, Crozier RH (1982) Measuring genetic relatedness in natural populations: methodology. *Theor Popul Biol* 21:171–193
- Raupp MJ (1982) Spatial distribution and seasonal abundance of the Imported Willow Leaf Beetle, *Plagioderma versicolora* Laich: The effects of plant nutrition and defense, physical factors, and activities of competitors and predators. Dissertation, University of Maryland, College Park, Md
- Sassaman C (1978) Mating systems in porcellionid isopods: Multiple paternity and sperm mixing in *Porcellio scaber* Latr. *Heredity* 41:385–397
- Selander RK, Smith MH, Yang SY, Johnson WE, Gentry JB (1971) Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). *Stud Genet Univ Texas* 6:49–90
- Thornhill R, Alcock J (1983) The evolution of insect mating systems. Harvard, Cambridge
- Wade MJ (1982) The effect of multiple inseminations on the evolution of social behaviors in diploid and haplo-diploid organisms. *J Theor Biol* 95:351–368
- West Eberhard MJ (1975) The evolution of social behavior by kin selection. *Q Rev Biol* 50:1–33
- Wilson DS (1975) A theory of group selection. *Proc Natl Acad Sci* 72:143–146
- Wilson DS (1977) Structured demes and the evolution of group-advantageous traits. *Am Nat* 111:157–185