Journal of Nematology 22(4):452–456. 1990.

Demonstration of Multiple Mating in *Heterodera glycines* with Biochemical Markers[1]

A. C. Triantaphyllou and P. R. Esbenshade[2]

Abstract: Controlled crosses of *Heterodera glycines* were carried out by placing one or more virgin females of known esterase phenotype on an agar plate and adding, at various time intervals, one or more males of different esterase phenotypes. Progeny (second-stage juveniles) of such crosses were propagated on soybeans, and 30 days later young females were subjected to electrophoretic analysis to determine their esterase phenotype. Esterase phenotypes that represented the heterozygous state of the maternal and paternal genomes confirmed the hybrid nature of the progeny and identified their male parent. When each of 74 females was given the opportunity to mate successively with two males of different esterase phenotypes, 43 mated with a single male and 31 mated with both males. One female mated with three males, i.e., with a male of its own population (sib mating) and the two males provided for the cross. Inseminated females could mate for a second time soon after, or as late as 24 hours after, their first mating. When single males were given the opportunity to mate with many females, about equal numbers of them inseminated zero, one, two, or three females. In greenhouse tests, 12 females were given the opportunity to mate with many males of three different esterase phenotypes. Two females mated with one and possibly more males of the same phenotype, and 10 females mated with males of two different esterase phenotypes. In conclusion, multiple mating appears to be a common behavior of males and females of *H. glycines*.

Key words: biochemical marker, cross, electrophoresis, enzyme, esterase, *Heterodera glycines*, multiple mating, soybean cyst nematode.

In sedentary, obligatorily amphimictic nematode species, such as *Heterodera glycines* Ichinohe, the rate of reproduction in a population depends, to a large extent, upon successful insemination of the females. When the number of males is small, e.g., under light infestations, an increased number of females may become inseminated if each male has the capacity to mate with more than one female. Furthermore, the genetic variation and, consequently, the adaptability of the offspring of a given female will be greater if such a female is inseminated by more than one male. Knowledge about mating patterns, therefore, is essential for understanding and explaining rates of reproduction and genetic potential of nematode populations, especially in light infestations.

There is no direct evidence that multiple matings occur in plant-parasitic nematodes. Some investigations, however, have suggested indirectly that multiple matings occur in *Globodera rostochiensis* (Woll.) Behrens and *Heterodera schachtii* Schmidt (3,5).

In this study, we conducted controlled crosses between males and females of *H. glycines* of known esterase phenotypes. We then used esterase phenotypes as genetic markers to identify the male parent of the offspring resulting from such crosses. The results direclty demonstrated that multiple matings do occur in *H. glycines*.

Materials and Methods

Three inbred isolates of *H. glycines* (esterase phenotypes AA, BB, and CC), which had been selected for homozygosity for three esterase alleles (a, b, and c) (1), were used in this study. Crosses were attempted between females of a given esterase phenotype and males of the same or different esterase phenotype. Tests were performed in two experimental settings: on agar plates in the laboratory and on soybean, *Glycine max* (L.) Merr., seedlings growing under greenhouse conditions.

Received for publication 20 February 1990.

[1] Paper No. 12564 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7643. This study was supported in part by the United States Department of Agriculture Grant 87-CRCR-12379. Use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned.

[2] Professor and former Laboratory Research Specialist, Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614.

Laboratory tests

Virgin females, 24 days old, were removed from roots of soybeans growing under hydroponic conditions and placed into small depressions on the surface of 1.5% agar containing 20 ppm streptomycin sulfate in 6-cm petri plates (1). Males were collected from the same hydroponic cultures by sieving.

Experiment 1: This experiment was designed to clarify whether a female can be inseminated by two or more males. A single virgin female of a certain esterase phenotype (e.g., phenotype CC) was transferred to each petri plate. About 12–15 hours later, one male of a different esterase phenotype (e.g., phenotype AA) was placed on the agar close to the female. Its behavior was observed under the stereoscope at 3–5-minute intervals. Initiation of mating was assumed when the male made contact and coiled around the female. Twenty minutes after initiation of mating by the first male, three additional males of a different esterase phenotype (e.g., phenotype BB) were added to each plate. In a supplementary test the second group of males was added 1, 2, 12, and 24 hours after initiation of mating with the first male. Twenty-one days later, each female was transferred to a small dish containing water and crushed to release eggs and juveniles, which were used to inoculate a 'Lee' soybean seedling growing in a 10-cm-d pot. Thirty-five days later, females of the F_1 generation were washed from the roots and collected on a 250-μm-mesh sieve. The esterase phenotype of each female was determined following electrophoresis to reveal the male parent. Seventy-four such controlled crosses of individual females and 1,095 progeny were analyzed electrophoretically in this experiment.

Experiment 2: This experiment was designed to estimate the number of females that can be inseminated by a single male. Ten females of a certain esterase phenotype (e.g., phenotype AA) were evenly spaced in a 1.5-cm-d circle on the surface of the agar in a petri plate. Twelve to fifteen hours later, a single male of a different esterase phenotype (e.g., phenotype BB) was placed on the agar in the center of the circle. Twenty-one days later, each female from a petri plate was transferred individually to a dish containing a small amount of water where it was crushed to release eggs and juveniles. Females without eggs were assumed to be uninseminated and were discarded. The number of females with eggs was recorded and the eggs and juveniles of each female were used to inoculate a Lee soybean. Thirty-five days later, adult females (F_1 progeny) were collected from the roots and subjected to electrophoresis to determine their esterase phenotype and verify their hybrid nature. The experiment was replicated 10 times.

Greenhouse test

Fifteen soybean seedlings growing in 10-cm-d pots under greenhouse conditions were inoculated with 200 second-stage juveniles of an isolate of *H. glycines* homozygous for a given esterase phenotype (e.g., phenotype AA). Twelve days later and at 2-day intervals thereafter, one plant was washed free of soil and the young females recovered from its roots were smeared on slides and stained with orcein for cytological observations (5). The presence of sperm in the spermatothecae of a female indicated insemination by a male of its own population (sib mating). When approximately 10% of the females recovered from a plant had sib mated (90% were still virgin), about 200 males of phenotype BB and an equal number of males of phenotype CC were added to two of the remaining plants in the greenhouse. Twenty-five days later, these plants were washed free of soil and 10 large cysts extracted from their roots were used individually to inoculate an equal number of soybean seedlings. Thirty-five days after inoculation, the plants were washed free of soil. Females recovered from each plant, representing the F_1 progeny of individual cysts, were subjected to electrophoretic analysis to determine their esterase phenotype and identify their male parent. This test was repeated twice.

TABLE 1. Multiple mating in *Heterodera glycines* females under laboratory conditions.

Parental esterase phenotypes†			Number of females (and phenotypes of progeny) that mated			Total progeny studied
Female	1st male	2nd male	1st male	2nd male	Both males	
AA	BB	CC	10 (AB)	6 (AC)	6 (AB + AC)	181
AA	CC	BB	0 (AC)	6 (AB)	4 (AC + AB)	172
BB	AA	CC	7 (BA)	4 (BC)	9 (BA + BC)	393
CC	AA	BB	6 (CA)	2 (CB)	10 (CA + CB)	
					1 (CC + CA + CB)‡	330
CC	BB	AA	0 (CB)	2 (CA)	1 (CB + CA)	19
Total			23	20	31	1,095

† Letters designate esterase phenotypes of the parental stocks and those of the first-generation progeny. Presumably they correspond to the allelic constitution of the genotype of each individual. Some phenotypes are illustrated in Figures 1–3.

‡ The presence of the CC phenotype in some of the progeny of this female indicates that she had mated with a male of her own type (CC) before she was used for this mating test.

RESULTS AND DISCUSSION

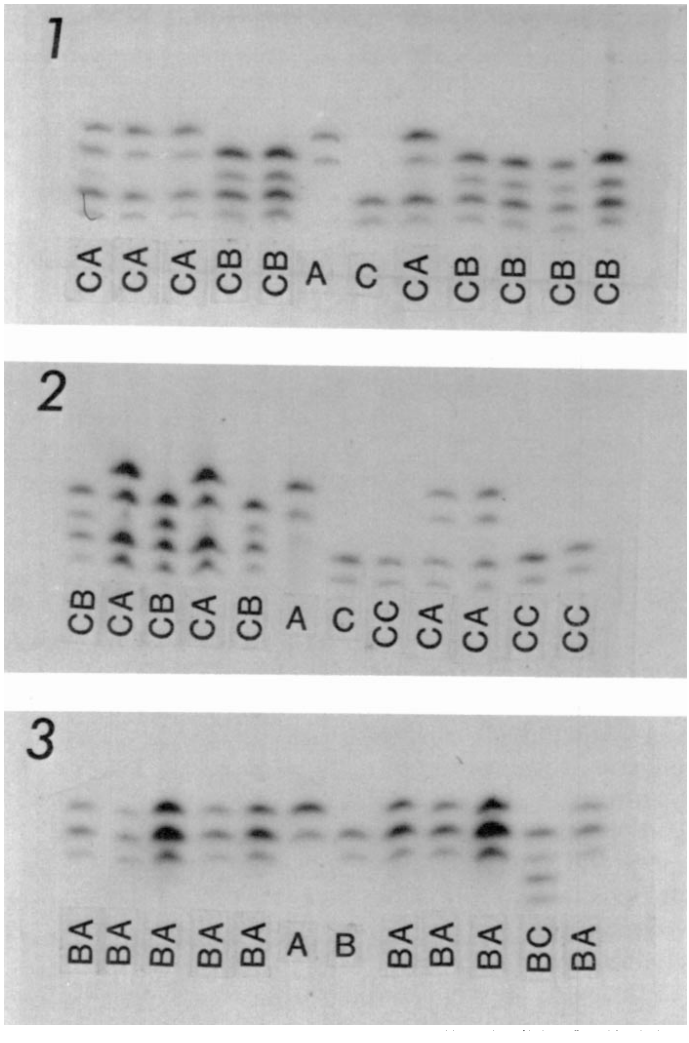
Of 400 attempted crosses between individual females and males of two different esterase phenotypes, 74 were successful. Esterase phenotypes of the progeny of successful crosses indicated that 23 females had been inseminated by the first male, 20 by the second male, and 31 by both males (Table 1; Figs. 1–3). One female of the last group had mated with three males. Its progeny exhibited three esterase phenotypes (Table 1; Fig. 2), one of which represented the homozygous state of the female's genotype. This female apparently had mated with a male of her own population (sib mating) before she was exposed to the other two types of males provided for the cross.

In the supplementary test, when a second group of males was added to the agar plate at intervals of 1, 2, 12, and 24 hours after initiation of the first mating of a female, multiple matings occurred at all intervals tested. This observation suggests that females were receptive to males and could be inseminated for a second time soon after, or at least as late as 24 hours after, their first mating. A copulation plug that would prevent a second mating apparently was not formed during the same period.

In the second laboratory experiment, a single male was given the opportunity to successively mate with 10 females on the same agar plate. Five of the 15 males tested failed to mate with any of the females. Four males inseminated only one female each, as evidenced by the number of females that produced progeny (eggs and juveniles). Three males inseminated two females each. In all cases, progeny of the inseminated females exhibited esterase phenotypes that represented the heterozygous state of the maternal and paternal genotypes. This observation confirms that they were indeed hybrid progeny and not the result of sib mating that could have occurred earlier; sib mating would have resulted in homozygous progeny of the maternal phenotype and would indicate that the females used in the test were not virgin. The remaining three males inseminated three females each. Again, the progeny of each female were tested for esterase phenotype and found to be heterozygous, expressing both the maternal and paternal phenotypes.

In the greenhouse test, esterase phenotypes of the F_1 progeny of 12 females indicated that two of them (females 3 and 7, Table 2) had mated with one and possibly more males of the same phenotype. All other females mated with males of two different phenotypes. None of the females mated with males of all three phenotypes. This observation cannot exclude the possibility that females can mate with more than two males. Only 20 (in one case 40) progeny of each female were examined in these tests; therefore, progeny of the third phenotype may have been missed because of low frequency of occurrence.

This study provides the first confirmed



FIGS. 1–3. Polyacrylamide gels showing the esterase phenotypes of the progeny of three females of *Heterodera glycines* mated with males of different esterase phenotypes. The two central lanes in each gel are the controls, i.e., females homozygous for phenotypes A, B, or C. 1) Phenotypes of parents (not illustrated); female CC, males AA and BB. Phenotypes of progeny CA and CB. 2) Phenotypes of parents; female CC, males AA and BB. Phenotypes of progeny CA, CB and CC. The presence of progeny with the CC phenotype indicates that this female had mated with a male of its own type (sib mating) before she was used for the test. 3) Phenotypes of parents; female BB, males AA and CC. Phenotypes of progeny BA and BC.

record of multiple matings occurring in a plant-parasitic nematode. Confirmation has been made possible by the use of biochemical markers (esterase phenotypes) genetically characterized in a previous investigation (1). Thus, the three esterase phenotypes (AA, BB, and CC) in the present study had been identified as three codominant alleles at a single esterase locus of the *H. glycines* genome. By crossing a female of known esterase phenotype (e.g., AA) with males of a number of different esterase phenotypes (e.g., AA, BB, and CC), we could identify the male parent of each one of her progeny. Progeny of the AA phenotype would represent sib matings, whereas, progeny of the AB and AC phenotypes would represent matings with BB and CC males, respectively.

The subject of multiple mating in various organisms has received much attention in the last 15 years because of its impor-

TABLE 2. Multiple mating in *Heterodera glycines* females under greenhouse conditions.

Female parent	Parental esterase phenotypes			Number and phenotypes of female (F_1) progeny		
	Female	Sib males	Added males	Sib matings	Outcrossings	
1	AA	AA	BB + CC	16 AA	4 AB	
2	AA	AA	BB + CC	20 AA	20 AB	
3	AA	AA	BB + CC	12 AA		
4	AA	AA	BB + CC	12 AA	8 AB	
5	AA	AA	BB + CC	11 AA	9 AB	
6	CC	CC	AA + BB	1 CC		19 CB
7	CC	CC	AA + BB			20 CB
8	CC	CC	AA + BB	5 CC	15 CA	
9	CC	CC	AA + BB	2 CC	18 CA	
10	CC	CC	AA + BB	2 CC		18 CB
11	CC	CC	AA + BB		17 CA	3 CB
12	CC	CC	AA + BB	2 CC	18 CA	

Letters designate esterase phenotypes that presumably correspond to the allelic constitution of the genotype of each individual male or female. Some phenotypes are illustrated in Figures 1–3.

tance in evolutionary theory regarding maximization of male fitness and interpretation of the genetics of polymorphism (8). Multiple paternity in a litter or brood has been demonstrated in natural populations of mammals, snakes, salamanders, fish, and arthropods, as well as in laboratory populations of birds and insects (4). In most cases, multiple paternity is inferred from the presence, in a single brood, of two or more electrophoretically detectable parental alleles at a given enzyme locus.

The use of biochemical markers to identify parenthood of progeny, as employed in the present study, can have a wide application in many other types of investigations with nematodes. Especially in studies on the inheritance of parasitism, crosses between isolates with different parasitic capabilities can be reliably controlled by monitoring the enzyme phenotypes of the original isolates and those of the progeny in each successive generation. Inability to verify the parenthood of individual progeny resulting from controlled crosses has been a serious obstacle in the past in carrying out genetic studies of nematode parasitism on plants (7).

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