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Genetic Analysis of Esterase Polymorphism in the Soybean Cyst Nematode, *Heterodera glycines*[1]

P. R. ESBENSHADE AND A. C. TRIANTAPHYLLOU[2]

Abstract: The genetic basis of esterase polymorphism in *Heterodera glycines* was investigated through controlled matings and analysis of F_1 and F_2 progeny. Three nematode lines, each fixed for a different esterase phenotype, were isolated and purified through repeated directional selection and inbreeding. Each phenotype was characterized by its distinct pair of closely spaced bands of esterase activity. Single-female single-male crosses were conducted according to a modified agar-plate mating technique. F_1 progeny were homogeneous, exhibiting both parental esterase phenotypes (codominant heterozygotes) but no hybrid bands. Approximately 1,500 F_2 progeny segregated in a 1:2:1 ratio for expression of the esterase phenotypes of the female parental line, the heterozygote, and the male parental line. Apparently the three esterase phenotypes correspond to three codominant alleles of a single esterase locus. Reciprocal crosses gave similar results, suggesting no maternal inheritance.
Key words: esterase, genetics, *Glycine max, Heterodera glycines,* hybridization, inheritance, isozyme, soybean cyst nematode.

Genetic studies of plant-parasitic nematodes have been limited in number and scope. The main reasons may have been the difficulty in carrying out controlled single-pair crosses and the lack of identifiable mutant forms that could serve as markers for genetic analysis. Most genetic studies have involved cyst nematodes, although some work has been done with *Ditylenchus* and *Pratylenchus* spp. In many of these experiments, mass matings and crude procedures for carrying out controlled crosses have been employed. The main objective of such studies has been to determine the inheritance of the ability of the nematodes to reproduce on resistant host plants (9,10,16-18,22-24). Selection experiments have also been used to estimate the number and type of genes controlling nematode parasitism (12,13). No specific genes for parasitism have been identified thus far, but their existence has been speculated in several cases (4,9,16,19,22). Indeed, the genetic analysis of nematode parasitism on plants has proven to be rather difficult because of the inherent complexity of the interaction between host and parasite.

Recent application of electrophoretic techniques in the study of enzymatic variation in plant-parasitic nematodes has opened the way for more successful genetic studies (6). The genetics of many enzymes and their interrelationships can now be studied accurately because miniaturized electrophoretic techniques permit determination of the activity of one or more enzymes from the extract of a single female nematode. This capability allows researchers to carry out dihybrid and trihybrid crosses and thus obtain information about the linkage relationships of a number of enzymatic loci. Genetic linkage maps can be constructed, and such maps can be helpful in providing markers for future genetic studies of more complex biological characteristics, such as host specificity and parasitism, which are more difficult to analyze independently.

Nonspecific esterases have shown extensive variation in *Meloidogyne* spp., and their activity can easily be determined in individual females of various cyst nematodes (6). Preliminary studies have indicated extensive polymorphism in field populations of *H. glycines.* They have also demonstrated that the esterase phenotype of an individual female can be determined after her eggs have been mechanically removed from her body. Such eggs can be used subsequently

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

as inoculum to obtain progeny of the same female. These features suggest that the inheritance of esterase activity can be studied precisely through appropriate single-pair crosses and analysis of F_1 and F_2 or backcross progeny. This paper reports the basic methodology employed in carrying out single-pair crosses between inbred lines of *H. glycines* and provides electrophoretic data elucidating the mode of inheritance of nonspecific esterases in this nematode.

Materials and Methods

Nematode populations obtained from three soybean fields in North Carolina were maintained on susceptible soybeans (*Glycine max* (L.) Merr. cv. Lee) under greenhouse conditions. Homogenates of about 200 females from each population were individually electrophoresed and stained for nonspecific esterases to detect esterase polymorphisms.

Electrophoresis: Each white female nematode was transferred to the well of a depression glass slide resting on a bed of ice. It was thoroughly macerated with the blunt end of a glass rod and then mixed with a small drop of 20% (w/v) sucrose and 2% (v/v) Triton X-100 solution. The crude nematode homogenate was absorbed onto a wick (1.5 × 11 × 0.2 mm) cut out of a sheet of Whatman 3 MM Chr chromatography paper and frozen at −20 C until needed. Electrophoresis was conducted in 7% native polyacrylamide or isoelectric focusing gels (IEF, pH 5–8), and esterases were detected using α-naphthyl acetate as substrate (7).

Selection and inbreeding of nematodes: Mature cysts were collected from soybean plants grown in the greenhouse. Each cyst was crushed to release eggs and juveniles that were used to inoculate a Lee soybean seedling. Approximately 40 days later the roots were washed free of soil and white females were transferred individually to dishes containing a small quantity of water. Each female was mechanically crushed to release the eggs. The female body was transferred to the well of a depression glass slide and processed for determination of its esterase phenotype with polyacrylamide gel electrophoresis. If the esterase phenotype of the female was of the simplest possible form observed in this study, i.e., consisting of a pair of closely spaced bands, then eggs from that female were propagated on another soybean seedling. If the phenotype was more complex, e.g., three-banded or four-banded, then heterozygosity was assumed and the eggs were discarded. Selection continued in each subsequent generation until all females of a selected line (family) exhibited the same simple esterase phenotype, presumably representing the homozygous state of the particular gene involved.

Controlled matings: Controlled matings between a female and a male of different esterase types were conducted on agar plates using a modification of the technique employed earlier for various cyst nematodes (8,9,11,15,16,22,25).

Fifty freshly hatched juveniles from an inbred line were used to inoculate a Lee soybean seedling growing in an 11-cm-d pot in an environmental chamber set for 29 C, 16-hour day and 26 C, 8-hour night periods. Seven days later the plants were carefully washed to remove all soil from the roots. Each plant was suspended in a quart jar with its roots totally immersed in half-strength Hoagland's solution. The jars were wrapped with aluminum foil to exclude light and thus reduce algal growth. The solution was continuously aerated by bubbling air from an aquarium pump and changed at 2-day intervals. Males emerging from the roots sank to the bottom of the jars and were retrieved daily by sieving. They were stored at 13 C for later use in mating experiments. Some males were recovered as early as 15 days after inoculation. Approximately 18 days from inoculation, young females were carefully removed from the roots and washed in a solution of 40 ppm streptomycin sulfate. Each female was transferred to a small depression made with a needle on the surface of 1.5% agar containing 20 ppm streptomycin sulfate in a 6-cm plastic petri dish. The following day, a single male from a

different inbred line was placed on the surface of the agar close to the female. The dishes containing nematodes were maintained in an incubator at 26 C in the dark. Twenty days later the female was removed from the agar, placed in a dish with a small amount of water, and crushed to release the F_1 eggs and juveniles. Eggs and juveniles were used to inoculate a Lee soybean seedling. Approximately 35 days later the soybean plant was washed and the white F_1 females were removed from the roots. Each female was placed in a dish with a small quantity of water, and the posterior half of its body was carefully punctured with a sharp dissecting knife. Most of the embryonated eggs and second-stage juveniles were thus released in the water. The female body was removed and processed for determination of its esterase phenotype. Eggs and juveniles from females heterozygous for esterases were used to inoculate a single soybean plant in order to obtain F_2 progeny. Eggs and juveniles of an occasional homozygous female were discarded, since it was assumed that such a female was not an F_1 female but the result of an accidental insemination of its mother by a male of the maternal line.

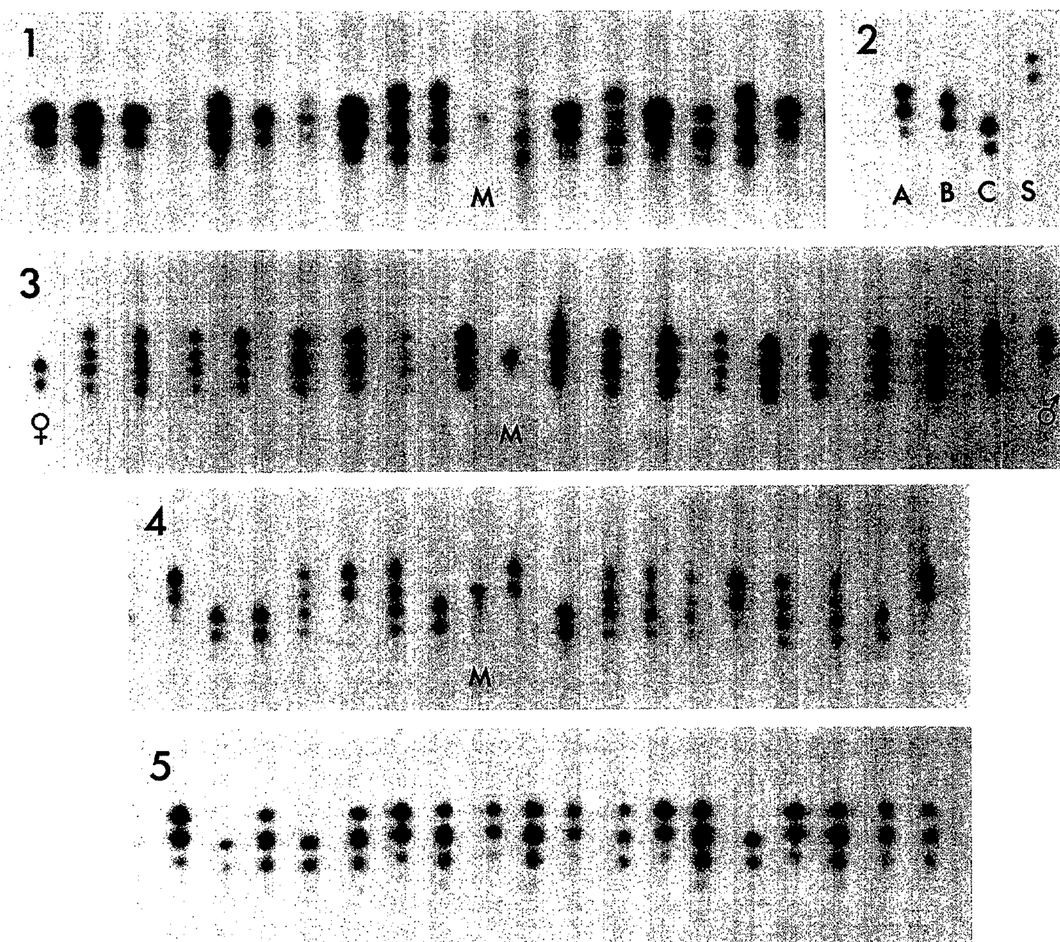
Results

Three field populations were included in the preliminary survey that aimed at identification of esterase polymorphism in *H. glycines.* All three populations were found to be polymorphic. Most females of each population had complex esterase banding patterns, with three, four, or more bands (Fig. 1). Some females had a simple pattern consisting of two closely spaced bands. The latter females could be classified in three categories depending on the relative migration rate of the double bands. The three categories were later recognized as distinct phenotypes and were named A, B, and C according to increasing migration rate toward the anode (Fig. 2). The Camden population had females of all three phenotypes. For this reason, it was chosen for study of the genetic basis of its esterase polymorphism.

Mostly, progeny of a female with a given phenotype—A, B, or C—would exhibit a variety of esterase patterns; some would be of the same type as their mother and others would have many bands. In order to obtain a nematode family homogeneous for a particular phenotype, single-female isolations had to be made for four to seven consecutive generations. In each generation only the progeny (families) of females with the particular phenotype were propagated individually. A total of 30 such inbred families were derived in the course of these studies. Each family was homogeneous for one of the three esterase phenotypes—A, B, or C. Families of the same phenotype were later combined together to obtain the three basic populations that represented the phenotypes A, B, and C. These populations were used in the genetic studies of inheritance of esterases.

The agar-plate, single-female single-male mating technique proved to be quite efficient, yielding 72% successful matings. Less than 2% of these matings were accidental inseminations of females by males of their own population (sib matings). They must have occurred before the transfer of the females to the agar plates. Females of the F_1 generation resulting from such accidental sib matings were easily identified because they exhibited the esterase phenotype of the female parent. These females were discarded. All other females of the F_1 generation expressed the esterase phenotypes of both parents and were considered true F_1 progeny. The number of F_1 progeny obtained from single-pair matings ranged from 5 to 39 with an average of 12. The number of F_2 progeny, obtained following propagation of all the F_1 progeny of each single-pair mating, ranged from 6 to 235, with an average of 79.

Segregation data of crosses (including two reciprocal crosses) involving all possible combinations of the three esterase phenotypes observed in the basic populations are presented in Table 1. The F_1 progeny were heterozygotes, codominantly expressing the phenotypes of both parents (Fig. 3). The 1,496 F_2 progeny expressed



FIGS. 1–5. Esterase phenotypes of individual females of *Heterodera glycines.* 1) Females from a field population originating from Camden County, North Carolina. Lane M represents a female of *Meloidogyne hapla* used as a control. 2) The three phenotypes A, B, and C of the nonsegregating, basic populations derived through selection and inbreeding. Lane S represents a population of *H. schachtii.* 3) Phenotypes of the parental and the F_1 progeny of a cross between a CC female (♀) and an AA male (♂). All intermediate lanes represent heterozygous F_1 progeny, except for lane M which is the control, a female of *Meloidogyne hapla.* 4) Phenotypes of the F_2 progeny of the same cross as in Figure 3. Notice segregation into homozygous A, homozygous C, and heterozygous AC phenotypes. 5) Phenotypes of F_2 progeny from a cross between a CC female and a BB male. Notice that the phenotypes of heterozygotes are three-banded with a larger middle band formed by the fusion of the lower band of phenotype B and the upper band of phenotype C.

the phenotypes of the female parent, the hybrid, and the male parent (Figs. 4, 5). The calculated relative frequency of the three phenotypes fit within comfortable probability levels in a 1:2:1 ratio expected for two alleles for each cross involving two esterase phenotypes. A single esterase locus with three alleles—*a, b,* and *c*—is indicated by the data from crosses representing the three phenotype combinations. Reciprocal crosses gave similar results and, therefore, provided no evidence for maternal inheritance.

DISCUSSION

The present study has demonstrated that single-pair crosses by the agar-plate mating procedure can provide reliable data for genetic analysis of esterases in *H. glycines.* The same procedure can be employed in the study of the genetics of additional polymorphic enzymes, as well as morphological

TABLE 1. Segregation data of esterase polymorphism in *Heterodera glycines.*

Parental phenotypes (♀ × ♂)	No. of offspring and phenotypes				χ^2†	*P* value
	F₁	F₂				
A × B	23 AB	153 AA	333 AB	180 BB	2.18	0.30–0.50
B × A	6 AB	46 AA	124 AB	65 BB	3.79	0.10–0.25
A × C	47 AC	73 AA	151 AC	68 CC	0.51	0.70–0.80
C × A	22 AC	29 AA	59 AC	30 CC	0.02	> 0.95
C × B	13 BC	42 BB	96 BC	47 CC	0.53	0.70–0.80

† Within cross χ^2 with 2 df tested against a 1:2:1 expectation.

and behavioral traits that are expressed as two or more distinct phenotypes. Indeed, this procedure allows for dihybrid and trihybrid crosses to be carried out successfully and thus opens the way for more detailed genetic studies of plant-parasitic nematodes.

Until now the main difficulty in controlled crosses conducted with plant-parasitic nematodes was the frequent fertilization of females by males of their own population (9,16,25). As a result of such sib matings, the F_1 generation contains a mixture of hybrid progeny as well as nonhybrid progeny of the maternal population. For this reason, previous genetic studies with cyst nematodes frequently seemed to suggest maternal inheritance of the trait under consideration. The procedures employed in this study have minimized the chances for sib matings and made available a higher percentage of virgin females for the agar-plate crosses (more than 98% versus 85–90% in other investigations). Furthermore, by determining the esterase pattern of the females of the F_1 generation and eliminating those females that had been derived through sib matings, we have been able to propagate solely the true F_2 juveniles. This aspect is essential for a meaningful genetic analysis of the F_2 generation or any backcross. Until a more reliable procedure for obtaining virgin females of cyst nematodes is developed, verification of the hybrid nature of the F_1 progeny through enzymatic or other markers will be necessary for any accurate genetic study. Enzymatic markers have an advantage over most other genetic markers because of the codominant nature of inheritance of enzymes, a feature that permits easy identification of the phenotypes of the heterozygotes as well as both homozygotes.

The esterase phenotype obtained following simple maceration of a female and subsequent electrophoresis of the homogenate was characteristic of the phenotype of the body tissues of that female and was not influenced by the phenotype of the eggs present in her gonoducts. This fact was of critical importance in these studies. It permitted definitive identification of the esterase phenotypes of females of the F_1 and F_2 generations without any modifications attributable to the eggs of the F_2 and F_3 generations, respectively. Future investigations with cyst nematodes that maintain many eggs in their bodies need to take into consideration the possibility that other enzymes may not be expressed in a similar manner as esterases. For some enzymes, the phenotype of a female may represent a combination of the phenotypes of the female itself and those of its progeny.

Sufficient numbers of F_2 progeny were obtained in these experiments to permit satisfactory statistical evaluation of the segregation ratios of esterase phenotypes. We believe, however, that the number of F_1 progeny can be increased by extending from 18 to 24 days the time females are allowed to feed on the host before they are transferred to the agar plates. Green et al. (9) reported increased numbers of progeny and a higher frequency of successful mating when older females of *Globodera rostochiensis* and *Heterodera schachtii* were used in crosses.

The genetic analysis of this study was based on electrophoretic data from female

progeny only. The esterase phenotypes of individual males could not be determined with any degree of certainty. We assumed that the phenotypes of males were similar to those of females of the same allelic constitution. Furthermore, we assumed that there were no meiotic-drive effects that would result in different segregation ratios for males and females in the F_2 generation.

The esterase phenotypes of the F_1 females or the heterozygous females of the F_2 generation contained only the protein bands of the phenotypes of the homozygous parents. They did not show any hybrid bands. This observation suggests that the esterase molecule in the soybean cyst nematode is a monomer in spite of its double-banded expression in the homozygous state. The esterases of *Caenorhabditis elegans* and several other animals are also monomeric (1,14,20).

Each esterase allele was expressed as a pair of closely spaced bands in both 7.5% native or IEF gels. The two bands suggest the presence of slightly different protein molecules that appear to be coded by the same allele. A similar, double-banded expression has been observed for the glycerol-3-phosphate dehydrogenase gene in the fruit fly, *Drosophila melanogaster* (2), and a multibanded phenotype is expressed by the esterase-4 gene in tomato, *Lycopersicon esculentum* (21). The prevalent interpretation of such multibanded phenotypes is that a single allele may be expressed differently in various tissues (2). Because in our study homogenates of whole nematodes were used for electrophoresis, the two protein bands may indeed represent slightly different proteins produced in separate tissues of the nematode body under the genetic control of a single allele (3,5). It is also possible that the difference between the two proteins may result through post-transcriptional or post-translational modifications.

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