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Comparisons of Mitochondrial DNA from the Sibling Species *Heterodera glycines* and *H. schachtii*

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Abstract: Restriction fragment patterns of mitochondrial DNA from sibling species of cyst nematodes *Heterodera glycines* and *H. schachtii* were examined. Fourteen restriction endonucleases recognizing four, five, and six base-pair sequences yielded a total of 90 scorable fragments of which 10% were shared by both species. Mitochondrial genome sizes for *H. glycines* and *H. schachtii* were estimated to be 22.5–23.5 kb and 23.0 kb, respectively. A single wild type mitochondrial genome was identified in all populations of *H. glycines* examined, although other mitochondrial genomes were present in some populations. The *H. schachtii* genome exhibited 57 scorable fragments, compared with 33 identified in the *H. glycines* wild type genome. The estimated nucleotide sequence divergence between the two species was p = 0.145. This estimate suggests these species diverged from a common ancestor 7.3–14.8 million years ago.
Key words: Heterodera glycines, H. schachtii, mtDNA, molecular evolution.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, and sugarbeet cyst nematode (SBCN), *H. schachtii* Schmidt, are economically important pests throughout the world (9,21). These two species are grouped in the same taxonomic subgroup (schachtii subgroup) based on similarities in host range (22,25), morphology (10), mode of reproduction, and chromosome number (16,26).

A number of morphological features have been used to discriminate these two species. Comparative studies of cyst morphology show *H. schachtii* have shorter vulval and fenestral slit lengths and many specimens of *H. glycines* have a second underbridge which is not detected in any *H. schachtii* (17). Graney and Miller (10) studied six isolates of *H. schachtii* and two isolates of *H. glycines* and showed the most stable taxonomic features for differential diagnosis were 1) shape and slope of anterior face of subventral stylet knobs of juveniles, 2) juvenile stylet length, 3) shape and slope of the anterior face of the male dorsal stylet knobs in lateral view, 4) vulval slit lengths of cysts, 5) fenestral length of cysts, and 6) fenestral width of cysts. When the ranges of these morphological characteristics are examined between these species, however, an excessive overlap of these values makes differential diagnosis between species exceedingly difficult.

Successful hybridizations between *H. glycines* and *H. schachtii* have been reported (13,19). Miller (13) placed one juvenile of *H. glycines* with one juvenile of *H. schachtii* on a common host (*Lycopersicon esculentum* cv. Pa-1) and obtained hybrid progeny. The hybrids formed were judged to be fertile, since gravid females were recovered with viable F_2 juveniles following inoculation with F_1 juveniles.

According to agricultural surveys, no areas have yet been detected where the distribution of *H. glycines* and *H. schachtii* overlap (6; Riggs, pers. comm.). There are, however, areas where the two species are found in adjacent counties, e.g., Florida and Wisconsin.

Based on the aforementioned information, phytonematologists have viewed *H. glycines* and *H. schachtii* as sibling species. Much of the evidence supports this close taxonomic relationship, and it was even

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proposed the two species may be allopathic subspecies of the nominate *H. schachtii* (14).

In recent years, animal mitochondrial DNA (mtDNA) has proven to be a powerful tool in evolutionary and population genetic structure studies (2,4,29). The ease of purification, maternal transmission, lack of variation in gene content (gene order varies in major taxonomic groups), lack of genetic recombination, and rapid rate of evolution make this a useful system in population genetic analysis (2,5).

The relative genetic similarities between various pairs of organisms are proportional to the shared mtDNA fragments generated by restriction endonuclease digestions. If certain assumptions are made, the number of shared restriction fragments may be correlated to the number of base substitutions per nucleotide separating a given pair of organisms (12,18,27).

Estimates of mitochondrial DNA divergence have already been used to analyze relationships of diverse organisms from nearly all major taxa. The average estimated sequence divergence (p) values between conspecific individuals of mammals range between $p = 0.003$ and $p = 0.040$ (4) and for *Drosophila* spp. range between $p = 0.008$ and $p = 0.014$ (11).

The objective of this study was to compare and analyze mtDNA from two sibling nematode species, *H. glycines* and *H. schachtii.* We employed 14 restriction endonucleases which recognize four, five, and six base-pair sequences to examine sequence variability between the mitochondrial molecules. The nucleotide sequence divergence between these two heteroderids is shown to be comparable to those estimated for congeneric species of other animal taxa.

Materials and Methods

Nematodes: Populations of *H. glycines* originated from North Carolina, South Carolina, and Arkansas; the *H. schachtii* populations were from California and Kansas. Isofemale populations of *H. glycines* were cultured on *Glycine max* (L.) Merr. cv. Essex, and *H. schachtii* were cultured on *Brassica oleracea capitata* L. cv. Early Dutch. Gravid females and cysts were extracted using sugar flotation (8). Eggs and juveniles were collected using a modified sucrose gradient method (1). Residual cysts were shaken in 10 volumes of fresh 1% NaOCl/0.5 M NaOH for 10 minutes at room temperature to recover additional eggs. Composite egg and juvenile fractions were combined, washed with distilled water, and pelleted by centrifugation.

Isolation of mtDNA: The procedure used to isolate mtDNA was a modification of the method used by Lansman et al. (12). Modifications were 1) 50 μl of 25% SDS was added to the mitochondria pellet and incubated at 65 C for 10 minutes; and 2) the cesium chloride-ethidium bromide gradients were centrifuged at 20 C for 18 hours in a Beckman SW 50.1 rotor at 45,000 rpm (189,000 *g*).

Restriction endonuclease digestions: Fourteen restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD, and Boehinger Mannheim, Indianapolis, IN) which recognize four, five, and six base-pair sequences were used in digestions (Table 1). The DNA was digested with the endonucleases in a final volume of 20 μl using buffers and temperatures recommended by the manufacturer.

Molecular cloning, hybridization and electrophoresis: An *H. schachtii* mtDNA library was constructed by ligating Hind III digested mtDNA fragments with pBR322. Digested mtDNA restriction fragments fractionated by gel electrophoresis were detected with ethidium bromide staining (5 μg/ml) and by DNA-DNA hybridization using intact mtDNA of both species which was nick-translated and labeled with (^{32}P)dATP (20). The digested fragments were separated by horizontal electrophoresis (Bio-Rad, Richmond, CA) through 0.6, 0.8, or 1.2% agarose gels using a Tris-borate buffer system. Fragment sizes were estimated from Hind III digested λ DNA or Hae III cleaved ϕX 174RF DNA co-electrophoresed on the same gel. Fol-

lowing gel electrophoresis, the DNA fragments were immobilized onto nitrocellulose filters by Southern Blotting (24).

Prehybridizations were carried out in solutions of 4×SSC-10X Denhardts solution (1×SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]; 1× Denhardts is 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrollidine, and 0.1% SDS) and 10 μl/ml denatured calf thymus DNA (10 μg/ml) for 4–12 hours. Labeled probe was denatured in a 100 C water bath for 10 minutes and added to prehybridized nitrocellulose filters with 10–15 ml 4×SSC. The filters were incubated at 60 C for 24–40 hours with gentle agitation. Filters were washed four times with two washes each of 4×SSC and 2×SSC at 60 C, air dried, and autoradiographed at −70 C with Kodak X-Omat AR x-ray film with a small Cronex intensifying screen.

Restriction enzyme analysis: For each pairwise comparison of mtDNA fragments between species and populations, the total proportions of shared fragments (F) across all digestions were calculated using the formula $F = 2Nxy/(Nx + Ny)$ where Nx = number of fragments in genotype X, Ny = number of fragments in genotype Y, Nxy = number of common fragments. F values were converted to estimates of sequence divergence percentage, or number of base substitutions per nucleotide (p), by the approach of Upholt (27) and Nei and Li (18): $p = 1 - [-F + (F^2 + 8F)^{1/2}/2]^{1/n}$ where n = number of base pairs recognized per cleavage site.

Results

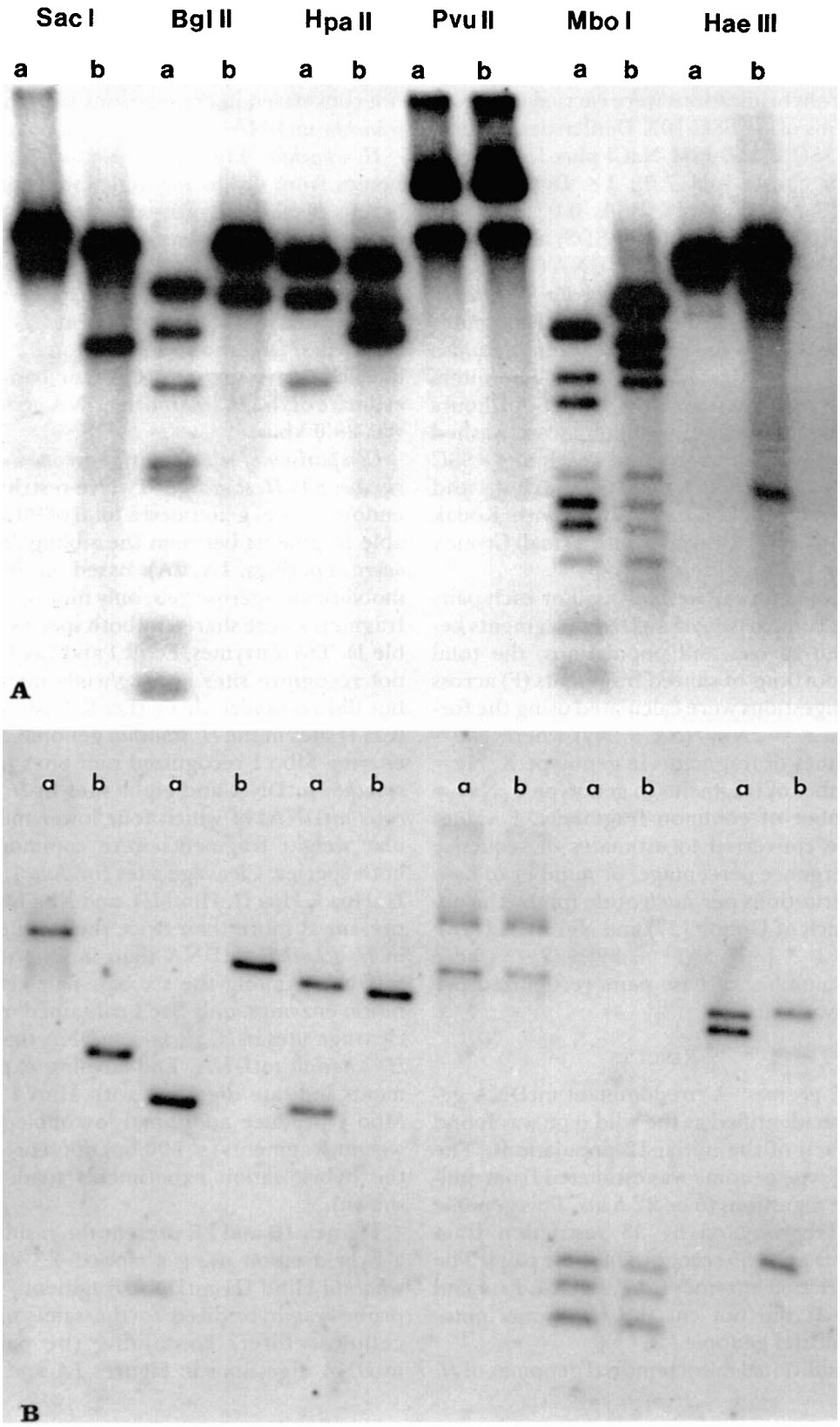
H. glycines: A predominant mtDNA genome identified as the wild type was found in each of the initial 12 populations. The wild type genome was estimated from multiple digestions to be 22.5 kb. This genome was represented by 33 restriction fragments and 165 recognizable base pairs. The restriction enzymes Cla I, EcoR I, Pst I and Pvu II did not cut the *H. glycines* mitochondrial genome.

Additional mitochondrial genomes of *H. glycines* were found in some populations. These genomes differed from the wild type by discrete size polymorphisms and were not considered in comparisons with the *H. schachtii* mtDNA.

H. schachtii: The *H. schachtii* mtDNA genomes from California and Kansas populations displayed consistent size and stoichiometric banding patterns indicating the presence of a single genome. The *H. schachtii* mtDNA genome was represented by 57 restriction fragments and at least 308 base pairs. Digestion with Pst I and Pvu II did not cleave the mtDNA. Our best size estimate of the *H. schachtii* mtDNA genome was 23.0 kb.

Comparison of mitochondrial genomes of H. glycines and H. schachtii: Twelve restriction endonucleases generated a total of 90 scorable fragments between the sibling *Heterodera* spp. (Figs. 1A, 2A). Based on similar mobilities in agarose gels, only nine of these fragments were shared by both species (Table 1). Two enzymes, EcoR I and Cla I, did not recognize sites in *H. glycines* mtDNA but did recognize three (EcoR I) and one (Cla I) sites in the *H. schachtii* genome. The enzyme Mbo I recognized nine sites in *H. schachtii* mtDNA and eight sites in *H. glycines* mtDNA, of which four lower molecular weight fragments were common to both species. Cleavage sites for Ava I, Bgl II, Hpa I, Hpa II, Hind III, and Xba I were present at more than twice the frequency in *H. schachtii* mtDNA than in *H. glycines* mtDNA. Among the six base-pair recognition enzymes, only Sac I contained more cleavage sites in *H. glycines* mtDNA than in *H. schachtii* mtDNA. End-labeling experiments indicate digestion with Hinf I and Mbo I produce additional low molecular weight fragments (< 500 bp) not scored in the hybridization experiments (data not shown).

Figures 1B and 2B present the results of a hybridization using a cloned 4.3 kb *H. schachtii* Hind III mtDNA fragment. This probe was hybridized to the same nitrocellulose filters containing the paired mtDNA digestions in Figures 1A and 2A.


Sac I
Bgl II
Hpa II
Pvu II
Mbo I
Hae III
a b a b a b a b a b a b
A
a b a b a b a b a b a b
B

Only the subset of fragments from both species that contain DNA homologous to the 4.3 kb probe gave a signal. In these paired-species comparisons, the DNA homologous to the probe was most often on separate restriction fragments of different electrophoretic mobilities. One Xba I fragment and three Hinf I fragments scored homologous for both species on the basis of similar mobilities also hybridized with the probe. This result supports the interpretation of fragment homology derived from restriction patterns.

Discussion

The similarities in morphology, cytology, and host range make species differentiation between *H. glycines* and *H. schachtii* difficult. Results of mtDNA restriction site analysis indicate that these two species are genetically divergent.

The estimated p value of p = 0.145 (F = 0.198) between these two sibling nematode species is comparable to the p value (p = 0.13–0.17) estimated for sibling species of mice, *Peromyscus polionotus* and *P. maniculatus* (3). Estimates of p obtained from analysis of mtDNA from eight species comprising the *Drosophila melanogaster* subgroup indicate a slightly lower estimate of nucleotide sequence divergence (p = 0.015–0.10) occurs for sibling and nonsibling species (23). Solignac et al. (23) estimated the nucleotide sequence divergence between the nonsibling *D. yakuba* and *D. melanogaster* to be p = 0.071, which corresponds closely to the p = 0.072 estimated by Wolstenholme and Clary (30) based on nucleotide sequencing of a 4,869-bp sequence for the two species. The p value estimated between *H. glycines* and *H. schachtii* exceeds the ranges estimated for three sea urchin *Strongylocentrotus* species (p = 0.06–0.11) (28).

In order for the p values to be significant, a correlation between the number of shared fragments (F) in two digestion profiles and the nucleotide sequence divergence was generated under the assumptions listed by Lansman et al. (12). Attempts at estimating p with F values less than 0.25 should be approached with caution, since small variations in estimating F will result in large differences in p (2). Whereas the total proportion of shared fragments in *H. glycines* and *H. schachtii* (F − 0.198) may compromise the estimate of p, it undoubtedly underscores the genetic divergence between these two species. Previous electrophoretic studies of soluble proteins and isozyme phenotypes also indicate these species are well differentiated at the biochemical level (unpubl. data).

Currently there is considerable controversy in the application of molecular rates of evolution to date divergence (15). Estimates of mitochondrial DNA evolution rates range from 0.13% nucleotide divergence per lineage per million years for certain sea urchin lineages (28) to approximately 5.0% for Hawaiian *Drosophila* (7). A provisional rate of 0.5–1.0% for primate mtDNA has been proposed (15). If this provisional mtDNA rate applies to *Heterodera* spp., then *H. glycines* and *H. schachtii* diverged 7.3–14.5 million years ago. Regardless of the rate chosen to estimate the time of divergence, these values indicate the two species have been established over a prolonged time period, and perhaps they diverged sometime before the Pliocene.

In order to increase our knowledge of phylogeny and speciation of nematodes, more diverse species consisting of various

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FIG. 1. A) Restriction fragment profiles of mtDNA from *Heterodera schachtii* (a) and *H. glycines* (b) separated in 0.8% agarose and detected by DNA hybridization with nick-translated total mtDNA from *H. glycines* and *H. schachtii*. Sac I, Bgl II, Hpa II, Pvu II, Mbo I, Hae III. Pvu II did not recognize any sites for both species. B) The same nitrocellulose filter hybridized with a nick-translated 4.3 kb cloned Hind III mtDNA fragment of *Heterodera schachtii*. Mbo I lanes a and b display three conserved fragments for both species that have equal electrophoretic mobility and hybridize with the cloned probe.

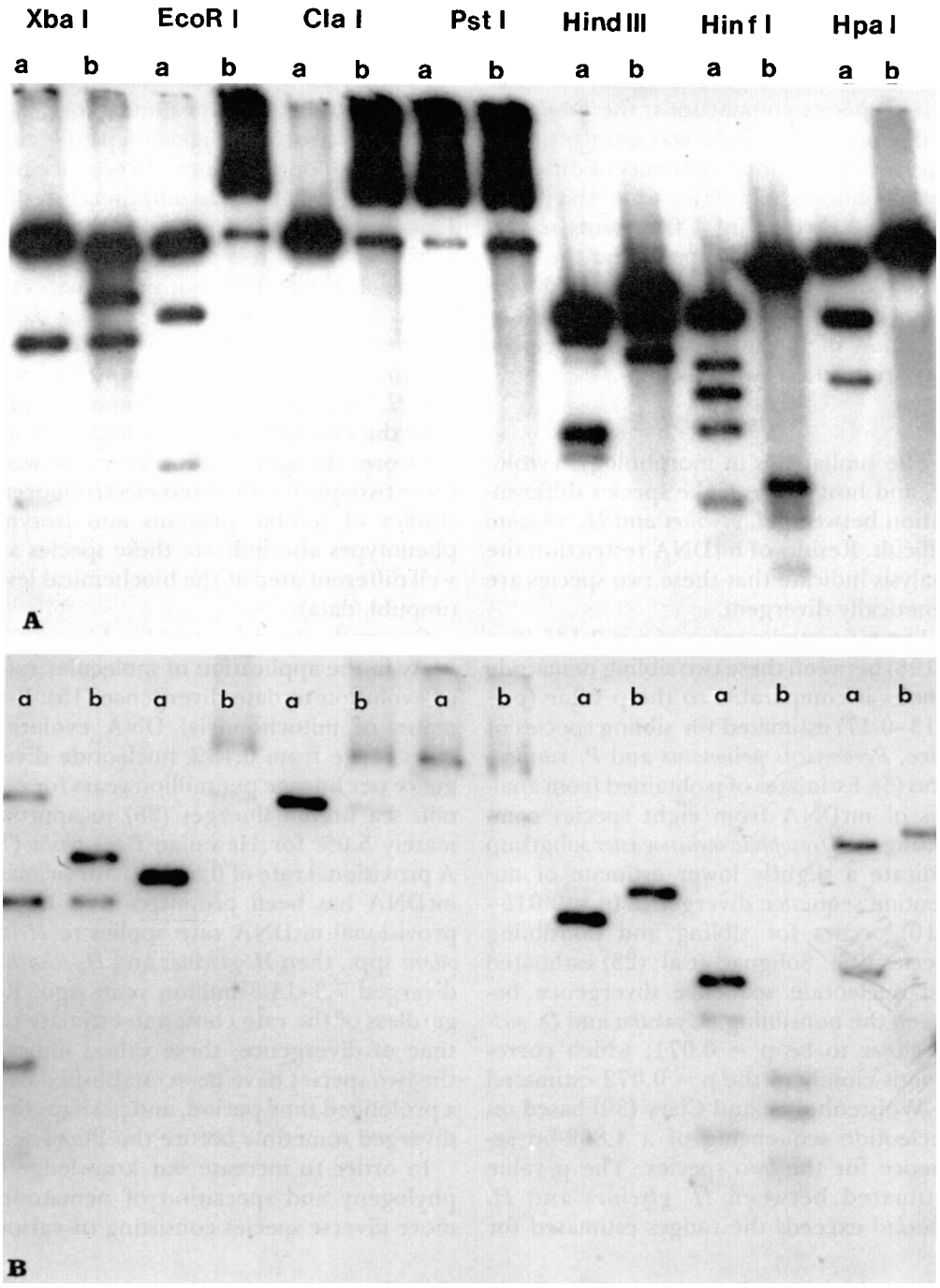

FIG. 2. A) Restriction fragment profiles of mtDNA from *Heterodera schachtii* (a) and *H. glycines* (b) separated in 0.8% agarose and detected by DNA hybridization with nick-translated total mtDNA from *H. glycines* and *H. schachtii*. Xba I, EcoR I, Cla I, Pst I, Hind III, Hinf I, Hpa I. EcoR I and Cla I did not recognize any sites for *H. glycines*. Pst I did not recognize any sites for both species. B) The same nitrocellulose filter hybridized with the 4.3 kb cloned *Heterodera schachtii* probe mentioned in Figure 1B. A single Xba I fragment displays the only conserved fragment to hybridize with the probe in these digestions.

TABLE 1. Restriction endonucleases and number of scored fragments analyzed in comparing mtDNA from *Heterodera glycines* (SCN) and *H. schachtii* (SBCN).

Endonucleases	Recognition sequence	Scored fragments		
		SCN Nx	SBCN Ny	Shared Nxy
Ava I	5′–CPyCGPuG–3′	1	2	0
Bgl II	5′–AGATCT–3′	2	7	0
Cla I	5′–ATCGAT–3′	0	1	0
EcoR I	5′–GAATTC–3′	0	3	0
Hae III	5′–GGCC–3′	3	2	0
Hind III	5′–AAGCTT–3′	4	8	0
Hinf I	5′–GANTC–3′	5	12	4
Hpa I	5′–GTTAAC–3′	1	3	0
Hpa II	5′–CCGG–3′	3	3	0
Mbo I	5′–GATC–3′	8	9	4
Pst I	5′–CTGCAG–3′	0	0	0
Pvu II	5′–CAGCTG–3′	0	0	0
Sac I	5′–GAGCTC–3′	3†	1	0
Xba I	5′–TCTAGA–3′	3	6	1
Total		33	57	9

† The lower molecular weight fragment was identified as a doublet from single and multiple digestion experiments.

modes of reproduction and larger sample sizes are needed for more accurate assessments. The precision of this type of analysis will provide nematologists and population geneticists with mtDNA markers for distribution, dispersal, hybridization, and introgression studies.

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