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DNA barcoding evidence for the North American presence of alfalfa cyst nematode, *Heterodera medicaginis*

Abstract

Specimens of *Heterodera* have been collected from alfalfa fields in Kearny County, Kansas and Carbon County, Montana. DNA barcoding with the COI mitochondrial gene indicate that the species is not *Heterodera glycines*, soybean cyst nematode, *H. schachtii*, sugar beet cyst nematode, or *H. trifolii*, clover cyst nematode. Maximum likelihood phylogenetic trees show that the alfalfa specimens form a sister clade most closely related to *H. glycines*, with a 4.7% mean pairwise sequence divergence across the 862 nucleotides of the COI marker. Morphological analyses of juveniles and cysts conform to the measurements of *H. medicaginis*, the alfalfa cyst nematode originally described from the USSR in 1971. Initial host testing demonstrated that the nematode reproduced on alfalfa, but not on soybeans, tomato, or corn. Collectively, the evidence suggests that this finding represents the first record of *H. medicaginis* in North America. Definitive confirmation of this diagnosis would require COI sequence of eastern European isolates of this species.

Key words

Alfalfa cyst nematode, COI DNA barcode, Detection, Diagnosis, *Heterodera medicaginis*, Taxonomy.
Plains state of Kansas in the US. A follow-up of these reports, utilizing morphology, host trials, and DNA barcoding using the mitochondrial gene COI along with ITS1, the transcribed spacer region between 18S and 5.8S of the nuclear ribosomal repeat region, and the heat shock protein gene Hsp90 provide supporting evidence that *Heterodera medicaginis* is present in the US.

**Materials and Methods**

**Nematode collections**

The original North American collections of suspect alfalfa cyst specimens were made in western Kansas from alfalfa fields that bordered the Arkansas River near the city of Lakin, Kearny County, KS (Table 1). Some soil was sent to the Kansas State University Diagnostic Laboratory, the University of Idaho Nematology Laboratory, and cysts were sent to the University of Nebraska and the USDA Mycology and Nematology Genetic Diversity and Biology Laboratory (MNGDBL) at Beltsville, Maryland. Additionally, several cysts containing eggs and juveniles were isolated from an alfalfa planting in Carbon County, Montana during a Cooperative Agricultural Pest Survey in 2006 (Table 1).

**Host testing**

Preliminary host testing was conducted at Kansas State University using infested field soil containing an estimated 365 *Heterodera* sp. eggs and infective second-stage juveniles (J2), as well as 325 *Meloidogyne hapla* Chitwood, 1949 J2/100 cm³. The soil was placed into 450-cm³ D40 Deepots (Stuewe and Sons Inc., Tangent, OR) and planted to either Kansas common alfalfa, an undetermined hybrid of corn, Flyer soybean, or Rutgers tomato. Nematode reproduction was determined after one and two months under greenhouse conditions. *Heterodera* females and cysts were dislodged from roots with water spray and collected on a 250-µm-pore sieve and counted. Vermiform males and J2 of *Heterodera* and *M. hapla* were collected on a 25-µm-pore sieve from one- and two-week incubations of roots in aerated water and counted.

**Morphological and microscopic analysis**

Cysts and infective juvenile stages were examined at the USDA MNGDBL and at the University of Nebraska Nematology Laboratory. Select juvenile measurements are presented in Table 2 alongside measurements from Gerber and Maas’s (1982) redescription. Images of juveniles and adult males were taken with a Leica DMLB light microscope with differential interference contrast optics and a Leica DC300 video camera. All juveniles examined at the University of Nebraska were provided an identification number which links specimen images, measurements, and placement on phylogenetic trees. Cysts were prepared for scanning electron microscopy by fixation in a 4% formalin solution followed by a graded series of alcohol to 100% ethyl alcohol prior to critical point drying and coating with gold. Images were obtained on a Hitachi S-3000N scanning electron microscope located in the Morrison Microscopy Core Research Facility at the University of Nebraska.

**Molecular analyses**

The primers used for amplification of the COI gene region were:

- **COI-F4a-Het-5′-CAGTTATATAATTCTTTTATAGCTATGCATTAATTATRATTTTTTTTYTRGTTATACC-3′.**
- **COI-R10b-Het 5′-CCAAAAAAATCTATAATCYAAATATTTACGDGG-3′.**
- The sequencing primers were **COI-F4a-Het** and for the corresponding strand, an internal primer **COI-R8-Het-5′-GAAAATGAGCTACATAATAAGTATCATGSARAACMACATC-CAAACTAGC-3′.**

After removal of the primer sequences, amplification products from the *Heterodera* specimens were 862 base pairs. GenBank sequences used in this study generally were 100 to 300 nucleotides shorter than sequences generated with the new primer set. The ITS1 primer set used in the University of Nebraska Laboratory was reported in the study of Cherry et al. (1997).

**Amplification conditions**

Nematodes amplified at the UNL Nematology Laboratory were individually smashed in 18 µL of sterile H₂O with a transparent microfuge micropipette tip on a coverslip, added to a 0.5 mL microfuge tube and stored at −20°C until needed. Amplification conditions were as follows: denaturation at 94°C for 5min, followed by 45 cycles of denaturation at 94°C for 30s, annealing at 48.0°C or 50.0°C for 30s, and extension at 72°C for 90s with a 0.5°C per second ramp rate to 72°C. A final extension was performed at 72°C for 5min as described by Powers et al. (2014) and Olson et al. (2017). PCR.
Table 1. Collection data for specimens used in this study. Specimens 1 to 15 were examined as fixed specimens.

| Specimen ID | Species       | Locality            | Host     | Marker | GenBank Accession No. |
|-------------|---------------|---------------------|----------|--------|-----------------------|
| 1           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 2           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 3           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 4           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 5           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 6           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 7           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 8           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 9           | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 10          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 11          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 12          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 13          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 14          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| 15          | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | N/A    |                        |
| P169028     | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | 18S    | AY912048               |
| N838        | *H. schachtii*  | Goshen County, Wyoming | Soybean | COI    | MK093062               |
| N839        | *H. schachtii*  | Goshen County, Wyoming | Soybean | COI    | MK093063               |
| N864        | *H. schachtii*  | Goshen County, Wyoming | Soybean | COI    | MK093064               |
| N4143       | *Meloidodera sp.*| Big Thicket National Preserve, Texas | Beech | COI    | MK093163               |
| N4178       | *Meloidodera sp.*| Big Thicket National Preserve, Texas | Water oak | COI    | MK093159               |
| N7083       | *H. avenae*     | Rio Grande County, Colorado | Barley | COI    | MK093164               |
| N7095       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093160               |
| N7096       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093162               |
| N7243       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS1   | MK093168               |
| N7244       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093169               |
| N7244       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS1   | MK093180               |
| N7245       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093170               |
| N7246       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093171               |
| N7247       | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI    | MK093172               |

(Continued)
DNA barcoding evidence for the North American presence of alfalfa cyst nematode, *Heterodera medicaginis*

| Accession | Species       | Location                      | Type     | Region          | Barcode | GenBank Accession |
|-----------|---------------|-------------------------------|----------|-----------------|---------|------------------|
| N7248     | *H. trifolii* | Rio Arriba County, New Mexico | Vineyard | COI              | MK093173 |
| N7250     | *H. trifolii* | Rio Arriba County, New Mexico | Vineyard | COI              | MK093174 |
| N7251     | *H. avenae*   | Akershus, Norway               | Oats     | COI              | MK093175 |
| N7253     | *H. avenae*   | Akershus, Norway               | Oats     | COI              | MK093176 |
| N8306     | *H. schachtii*| Platte County, Wyoming          | Bean     | COI              | MK093165 |
| N8876     | *H. medicaginis* | Kearny County, Kansas       | Alfalfa  | COI              | MK093166 |
| N8877     | *H. medicaginis* | Kearny County, Kansas      | Alfalfa  | COI              | MK093161 |
| N8881     | *Heterodera* sp. | Dąbrówka, Poland        | Peat meadow | COI              | MK093167 |
| P119063   | *H. medicaginis* | Carbon County, Montana    | Alfalfa  | COI              | MK093053 |
| P119064   | *H. schachtii* | Big Horn County, Montana      | Alfalfa  | COI              | MK093054 |
| P125039   | *H. schachtii* | Platte County, Wyoming        | Bean     | COI              | MK093055 |
| P125063   | *H. schachtii* | Laramie County, Wyoming       | Sugar beet | COI              | MK093056 |
| P145076   | *H. schachtii* | Platte County, Wyoming        | Sugar beet | COI              | MK093057 |
| P150049   | *H. avenae*   | Montana                       | Barley   | COI              | MK093058 |
| P150069   | *Vittatidera* zeaphila | Tennessee    | Corn     | COI              | MK093060 |
| P164043   | *H. avenae*   | Montana                       | Barley   | COI              | MK093059 |
| P200034   | *Cactodera* sp. | Sheridan County, Nebraska  | Potatoes-wheat | COI              | MK093061 |
| P231089   | *H. glycines* | Unknown                       | Soybean  | COI              | MK093049 |
| P213017   | *H. glycines* | Unknown                       | Soybean  | COI              | MK093050 |
| P231088   | *H. glycines* | Unknown                       | Soybean  | COI              | MK093051 |
| P231091   | *H. schachtii* | Unknown                      | Soybean  | COI              | MK093052 |
| P243062   | *H. glycines* | Richardson County, Nebraska   | Soybean  | COI              | MK093086 |
| P243063   | *H. glycines* | Richardson County, Nebraska   | Soybean  | COI              | MK093087 |
| P243064   | *H. glycines* | Richardson County, Nebraska   | Soybean  | COI              | MK093088 |
| P243069   | *H. glycines* | Seward County, Nebraska       | Soybean  | COI              | MK093077 |
| P243080   | *H. glycines* | Lancaster County, Nebraska     | Soybean  | COI              | MK093078 |
| P243083   | *H. glycines* | Lancaster County, Nebraska     | Soybean  | COI              | MK093079 |
| P243085   | *H. glycines* | Cuming County, Nebraska        | Soybean  | COI              | MK093080 |
| P243086   | *H. glycines* | Cuming County, Nebraska        | Soybean  | COI              | MK093081 |
| P243094   | *H. glycines* | Nebraska                      | Soybean  | COI              | MK093084 |
| P243096   | *H. glycines* | Nebraska                      | Soybean  | COI              | MK093085 |
| P244003   | *H. glycines* | Cedar County, Nebraska        | Soybean  | COI              | MK093095 |
| P244004   | *H. glycines* | Cedar County, Nebraska        | Soybean  | COI              | MK093096 |
| P244020   | *H. glycines* | Holt County, Nebraska         | Soybean  | COI              | MK093089 |
| P244021   | *H. glycines* | Holt County, Nebraska         | Soybean  | COI              | MK093090 |
| P244041   | *H. glycines* | Buffalo County, Nebraska      | Soybean  | COI              | MK093093 |
| P244042   | *H. glycines* | Buffalo County, Nebraska      | Soybean  | COI              | MK093094 |
| P244060   | *H. glycines* | Burt County, Nebraska         | Soybean  | COI              | MK093091 |
| P244062   | *H. glycines* | Burt County, Nebraska         | Soybean  | COI              | MK093092 |
| P244065   | *H. glycines* | Stanton County, Nebraska      | Soybean  | COI              | MK093082 |
| P244066   | *H. glycines* | Stanton County, Nebraska      | Soybean  | COI              | MK093083 |
| P244072   | *H. glycines* | Cass County, Nebraska         | Soybean  | COI              | MK093097 |
| P244074   | *H. glycines* | Cass County, Nebraska         | Soybean  | COI              | MK093098 |
| P244076   | *H. schachtii* | Big Horn County, Montana    | Pinto bean | COI              | MK093065 |

(Continued)
| Accession Number | Taxon     | Location                          | Stage     | Genbank Accession Number |
|------------------|-----------|-----------------------------------|-----------|--------------------------|
| P244077          | H. schachtii | Big Horn County, Montana          | Pinto bean | COI MK093066             |
| P244078          | H. schachtii | Big Horn County, Montana          | Black bean | COI MK093067             |
| P244079          | H. schachtii | Big Horn County, Montana          | Black bean | COI MK093068             |
| P244081          | H. schachtii | Park County, Wyoming              | Pinto bean | COI MK093069             |
| P244082          | H. schachtii | Park County, Wyoming              | Pinto bean | COI MK093070             |
| P244084          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093071             |
| P244085          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093072             |
| P244086          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093073             |
| P244087          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093074             |
| P244088          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093075             |
| P244089          | H. schachtii | Platte County, Wyoming            | Pinto bean | COI MK093076             |
| P245019          | H. glycines | Alabama                           | Soybean   | COI MK093127             |
| P245023          | H. glycines | Alabama                           | Soybean   | COI MK093128             |
| P245025          | H. glycines | Alabama                           | Soybean   | COI MK093129             |
| P245027          | H. glycines | Alabama                           | Soybean   | COI MK093130             |
| P245029          | H. glycines | Alabama                           | Soybean   | COI MK093131             |
| P245033          | H. glycines | Alabama                           | Soybean   | COI MK093119             |
| P245035          | H. glycines | Alabama                           | Soybean   | COI MK093132             |
| P245038          | H. glycines | Alabama                           | Soybean   | COI MK093133             |
| P245041          | H. glycines | Alabama                           | Soybean   | COI MK093120             |
| P245043          | H. glycines | Alabama                           | Soybean   | COI MK093134             |
| P245044          | H. glycines | Cedar County, Nebraska            | Soybean   | COI MK093109             |
| P245046          | H. glycines | Cedar County, Nebraska            | Soybean   | COI MK093110             |
| P245089          | H. glycines | Oconee County, Georgia            | Soybean   | COI MK093111             |
| P245090          | H. glycines | Oconee County, Georgia            | Soybean   | COI MK093112             |
| P245094          | H. glycines | Burke County, Georgia             | Soybean   | COI MK093135             |
| P245095          | H. glycines | Burke County, Georgia             | Soybean   | COI MK093136             |
| P246006          | H. glycines | Boone County, Missouri            | Soybean   | COI MK093121             |
| P246008          | H. glycines | Boone County, Missouri            | Soybean   | COI MK093137             |
| P246010          | H. glycines | Livingston County, Missouri       | Soybean   | COI MK093138             |
| P246014          | H. glycines | Knox County, Missouri             | Soybean   | COI MK093113             |
| P246016          | H. glycines | Knox County, Missouri             | Soybean   | COI MK093114             |
| P246018          | H. glycines | Atchison County, Missouri         | Soybean   | COI MK093139             |
| P246021          | H. glycines | Atchison County, Missouri         | Soybean   | COI MK093122             |
| P246033          | H. glycines | Fulton County, Ohio               | Soybean   | COI MK093123             |
| P246035          | H. glycines | Fulton County, Ohio               | Soybean   | COI MK093140             |
| P246042          | H. glycines | Sandusky County, Ohio             | Soybean   | COI MK093124             |
| P246044          | H. glycines | Sandusky County, Ohio             | Soybean   | COI MK093115             |
| P246047          | H. glycines | Sandusky County, Ohio             | Soybean   | COI MK093116             |
| P247094          | H. glycines | Lee County, Arkansas              | Soybean   | COI MK093141             |
| P247096          | H. glycines | Lee County, Arkansas              | Soybean   | COI MK093142             |
| P247099          | H. glycines | Lee County, Arkansas              | Soybean   | COI MK093143             |
| P248001          | H. glycines | Lee County, Arkansas              | Soybean   | COI MK093144             |
| P248004          | H. glycines | Washington County, Arkansas       | Soybean   | COI MK093145             |
| P248006          | H. glycines | Washington County, Arkansas       | Soybean   | COI MK093146             |
| P248009          | H. glycines | McLeod County, Minnesota          | Soybean   | COI MK093147             |
DNA barcoding evidence for the North American presence of alfalfa cyst nematode, *Heterodera medicaginis*

| Accession | Species     | County          | Crop      | gene         | GenBank Accession |
|-----------|-------------|-----------------|-----------|--------------|------------------|
| P248010   | *H. glycines* | McLeod County, Minnesota | Soybean | COI          | MK093125         |
| P248013   | *H. glycines* | Wilken County, Minnesota | Soybean | COI          | MK093117         |
| P248016   | *H. glycines* | Wilken County, Minnesota | Soybean | COI          | MK093118         |
| P248017   | *H. glycines* | Nicollet County, Minnesota | Soybean | COI          | MK093148         |
| P248019   | *H. glycines* | Nicollet County, Minnesota | Soybean | COI          | MK093149         |
| P248023   | *H. glycines* | Dodge County, Minnesota | Soybean | COI          | MK093099         |
| P248024   | *H. glycines* | Dodge County, Minnesota | Soybean | COI          | MK093100         |
| P248026   | *H. glycines* | Red Lake County, Minnesota | Soybean | COI          | MK093101         |
| P248027   | *H. glycines* | Red Lake County, Minnesota | Soybean | COI          | MK093102         |
| P248029   | *H. glycines* | Redwood County, Minnesota | Soybean | COI          | MK093150         |
| P248031   | *H. glycines* | Redwood County, Minnesota | Soybean | COI          | MK093151         |
| P248035   | *H. glycines* | Dakota County, Minnesota | Soybean | COI          | MK093103         |
| P248036   | *H. glycines* | Dakota County, Minnesota | Soybean | COI          | MK093104         |
| P248040   | *H. glycines* | Waseca County, Minnesota | Soybean | COI          | MK093105         |
| P248041   | *H. glycines* | Waseca County, Minnesota | Soybean | COI          | MK093106         |
| P248058   | *H. cf. urticae* | Faulkner County, Arkansas | Chickweed | COI        | MK093155         |
| P248059   | *H. cf. urticae* | Faulkner County, Arkansas | Chickweed | COI        | MK093156         |
| P248060   | *H. trifolii* | Washington County, Arkansas | Clover    | COI         | MK093157         |
| P248061   | *H. trifolii* | Washington County, Arkansas | Clover    | COI         | MK093158         |
| P248066   | *H. glycines* | Gentry County, Missouri | Soybean  | COI         | MK093107         |
| P248068   | *H. glycines* | Gentry County, Missouri | Soybean  | COI         | MK093108         |
| P248071   | *H. glycines* | Mercer County, Missouri | Soybean  | COI         | MK093152         |
| P248073   | *H. glycines* | Mercer County, Missouri | Soybean  | COI         | MK093153         |
| P248086   | *H. glycines* | Sussex County, Delaware | Soybean  | COI         | MK093154         |
| P248087   | *H. glycines* | Sussex County, Delaware | Soybean  | COI         | MK093126         |
| 105A13    | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI         | MK093177         |
| 105A14    | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI         | MK093178         |
| 105A15    | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | COI         | MK093179         |
| Hsp90_3530 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | Hsp90      | MH798843         |
| Hsp90_3531 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | Hsp90      | MH798844         |
| Hsp90_3532 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | Hsp90      | MH798845         |
| Hsp90_3533 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | Hsp90      | MH798846         |
| Hsp90_2996 | *H. trifolii* | Alexandria, Egypt | Sugar beet | Hsp90 | MK095224 |
| Hsp90_2997 | *H. trifolii* | Alexandria, Egypt | Sugar beet | Hsp90 | MK095225 |
| Hsp90_2994 | *H. trifolii* | Alexandria, Egypt | Sugar beet | Hsp90 | MK095222 |
| Hsp90_2995 | *H. trifolii* | Alexandria, Egypt | Sugar beet | Hsp90 | MK095223 |
| Hsp90_2998 | *H. trifolii* | Alexandria, Egypt | Sugar beet | Hsp90 | MK095226 |
| HetITS105 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093184         |
| HetITS106 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093185         |
| HetITS107 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093186         |
| HetITS108 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093187         |
| HetITS109 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093188         |
| HetITS110 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093189         |
| HetITS111 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093190         |
| HetITS112 | *H. medicaginis* | Kearny County, Kansas | Alfalfa  | ITS         | MK093191         |
Table 2. Morphological data on juveniles, all measurements in µm.

| Specimen No. | Length | Stylet | Tail | Hyaline |
|--------------|--------|--------|------|---------|
| 1            | 440    | 25     | –    | 25      |
| 2            | 421    | 25     | –    | 21      |
| 3            | 425    | 25     | 45   | 25      |
| 4            | 420    | 25     | 50   | 25      |
| 5            | 421    | 24     | 43   | 22.5    |
| 6            | 420    | 23     | 46   | 23      |
| 7            | 447    | 25     | 45   | 28      |
| 8            | 475    | 24     | 52   | 25.5    |
| 9            | 462    | 25     | 45   | 22.5    |
| 10           | 395    | 25.5   | 45   | 25      |
| 11           | 491    | 25     | –    | 25.5    |
| 12           | 485    | 25     | 50   | 21      |
| 13           | 413    | 25     | 48   | 25      |
| 14           | 410    | 26     | 40   | 20      |
| 15           | 415    | 25     | 45   | 23      |
| NID 7095     | 462.5  | 25     | 50   | 27      |
| NID 7096     | 442.5  | 24     | 51   | 30      |
| NID 7243     | 477.5  | 25     | 52.5 | 30      |
| NID 7244     | 455    | 26     | 50   | 25      |
| NID 7245     | 450    | 26     | 49   | 22.5    |
| NID 7246     | 425    | 25     | 48   | 27.5    |
| NID 7247     | 452.5  | 25     | 47.5 | 27.5    |
| NID 8876     | 472    | 27     | 52   | 28      |
| NID 8877     | 470    | 26     | 50   | 32      |
| Mean         | 445.0  | 25.1   | 47.8 | 25.3    |
| SD           | 27.2   | 0.8    | 3.2  | 3.0     |
| Maximum      | 491    | 27     | 52.5 | 32      |
| Minimum      | 395    | 23     | 40   | 20      |

Gerber and Maas (1982) *n = 100*

|                | Mean     | Stylet  | Tail     | Hyaline   |
|----------------|----------|---------|----------|-----------|
| *Heterodera medicaginis* | 462 (417-512) | 25 (24-26) | 51.8 (41-60) | 28.5 (22-33) |
| *H. glycines* | 439±6.7 (375-490) | 23±0.01 (22-24) | 50.4±1.0 (42-59) | 26.6±0.7 (20-33) |
| *H. schachtii* | 452±49.7 | 25.6±0.11 | 48.5±0.73 | 27.1±0.61 |
Table 3. Host trial for alfalfa cyst nematodes.

|                      | Crop       | Alfalfa | Corn  | Soybean | Tomato |
|----------------------|------------|---------|-------|---------|--------|
| **Month 1**          |            |         |       |         |        |
| Root weight (g)      |            | 0.04    | 0.29  | 0.21    | 0.16   |
| **Heterodera**       |            |         |       |         |        |
| Females and cysts    |            | 0       | 0     | 0       | 0      |
| Cysts/g              |            | 0       | 0     | 0       | 0      |
| **Heterodera infective juveniles** | | 70     | 0     | 0       | 0      |
| J2/g                 |            | 1,750   | 0     | 0       | 0      |
| **Meloidogyne J2 infective juveniles** | | 210    | 4     | 10      | 3,100  |
| Root incubation      |            | 5,250   | 14    | 48      | 19,375 |
| J2/g                 |            | 292     | 0     | 0       | 0      |
| **Heterodera/Meloidogyne males** | | 130    | 0     | 20      | 0      |
| Root incubation      |            | 3,250   | 0     | 95      | 0      |
| Males/g              |            | 130     | 0     | 56      | 520    |
| **Month 2**          |            | 0.69    | 0.95  | 0.54    | 0.23   |
| Root weight (g)      |            |         |       |         |        |
| **Heterodera**       |            |         |       |         |        |
| Females and cysts    |            | 52      | 0     | 0       | 0      |
| Cysts/g              |            | 75      | 0     | 0       | 0      |
| **Heterodera infective juveniles** | | 292     | 0     | 0       | 0      |
| J2/g                 |            | 423     | 0     | 0       | 0      |
| **Meloidogyne J2 infective juveniles** | | 0      | 0     | 308     | 12,040 |
| Root incubation      |            | 0       | 0     | 570     | 52,348 |
| J2/g                 |            | 0       | 0     | 56      | 520    |
| **Heterodera/Meloidogyne males** | | 136    | 0     | 104     | 2,261  |
| Root incubation      |            | 197     | 0     | 104     | 2,261  |
| Males/g              |            | 197     | 0     | 104     | 2,261  |

Nematodes analyzed in the Beltsville lab were smashed in worm extraction buffer and extracts prepared as described by Skantar et al. (2012). The ITS and 28S rDNA regions were amplified using primers TW81 and AB28, and D2A and D3B, respectively (Skantar et al., 2012). COI was amplified with primers...
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- P231098 Heterodera glycines North Carolina
- P244003 Heterodera glycines Cedar County, Nebraska
- P244009 Heterodera glycines McLeod County, Minnesota
- P244010 Heterodera glycines McLeod County, Minnesota
- P244017 Heterodera glycines Nicollet County, Minnesota
- P244019 Heterodera glycines Nicollet County, Minnesota
- P244023 Heterodera glycines Dodge County, Minnesota
- P244024 Heterodera glycines Dodge County, Minnesota
- P244026 Heterodera glycines Red Lake County, Minnesota
- P244027 Heterodera glycines Red Lake County, Minnesota
- P244029 Heterodera glycines Redwood County, Minnesota
- P244035 Heterodera glycines Dakota County, Minnesota
- P244040 Heterodera glycines Waseca County, Minnesota
- P244041 Heterodera glycines Waseca County, Minnesota
- P244055 Heterodera glycines Burke County, Georgia
- P244057 Heterodera glycines Dakota County, Minnesota
- P244061 Heterodera glycines Mercer County, Missouri
- P244065 Heterodera glycines Rally County, Nebraska
- P244066 Heterodera glycines Gentry County, Missouri
- P244068 Heterodera glycines Gentry County, Missouri
- P244071 Heterodera glycines Mercer County, Missouri
- P244073 Heterodera glycines Mercer County, Missouri
- P244086 Heterodera glycines Sussex County, Delaware
- P244087 Heterodera glycines Sussex County, Delaware
- HM640930 Heterodera glycines GB
- KC172914.1 Heterodera glycines GB
- P244062 Heterodera glycines Burt County, Nebraska
- P244065 Heterodera glycines Stanton County, Nebraska
- P241307 Heterodera glycines Virginia
- P241369 Heterodera glycines Arkansas
- P243062 Heterodera glycines Richardson County, Nebraska
- P243063 Heterodera glycines Richardson County, Nebraska
- P243064 Heterodera glycines Richardson County, Nebraska
- P243069 Heterodera glycines Seward County, Nebraska
- P243060 Heterodera glycines Lancaster County, Nebraska
- P243083 Heterodera glycines Lancaster County, Nebraska
- P243085 Heterodera glycines Cumming County, Nebraska
- P243086 Heterodera glycines Cumming County, Nebraska
- P243094 Heterodera glycines Nebraska
- P243096 Heterodera glycines Nebraska
- P244004 Heterodera glycines Cedar County, Nebraska
- P244020 Heterodera glycines Holt County, Nebraska
- P244021 Heterodera glycines Holt County, Nebraska
- P244041 Heterodera glycines Buffalo County, Nebraska
- P244042 Heterodera glycines Buffalo County, Nebraska
- P244060 Heterodera glycines Burt County, Nebraska
- P244066 Heterodera glycines Stanton County, Nebraska
- P244072 Heterodera glycines Cass County, Nebraska
- P244074 Heterodera glycines Cass County, Nebraska
- P245019 Heterodera glycines Alabama
- P245023 Heterodera glycines Alabama
- P245025 Heterodera glycines Alabama
- P245027 Heterodera glycines Alabama
- P245029 Heterodera glycines Alabama
- P245033 Heterodera glycines Alabama
- P245035 Heterodera glycines Alabama
- P245038 Heterodera glycines Alabama
- P245041 Heterodera glycines Alabama
- P245043 Heterodera glycines Alabama
- P245044 Heterodera glycines Cedar County, Nebraska
- P245046 Heterodera glycines Cedar County, Nebraska
- P245098 Heterodera glycines Oconee County, Georgia
- P245090 Heterodera glycines Oconee County, Georgia
- P245094 Heterodera glycines Burke County, Georgia
- P244006 Heterodera glycines Boone County, Missouri
- P244008 Heterodera glycines Boone County, Missouri
- P244010 Heterodera glycines Livingston County, Missouri
- P244014 Heterodera glycines Knox County, Missouri
- P244016 Heterodera glycines Knox County, Missouri
- P244018 Heterodera glycines Atchison County, Missouri
- P244021 Heterodera glycines Atchison County, Missouri
- P244033 Heterodera glycines Fulton County, Ohio
Het-CoxIF and HetCox-1R according to Subbotin et al. (2017). Partial Hsp90 fragments were amplified with primers U288 and L1110 (Skantar et al., 2012). PCR products were cleaned with the Monarch DNA Gel Extraction Kit (NEB, Ipswich, MA). ITS, COI, and Hsp90 amplicons were cloned using the Strataclone PCR Cloning Kit (Agilent, Santa Clara, CA) according to manufacturer’s instructions. Plasmid clones of DNA were prepared with the Monarch Plasmid Miniprep Kit (NEB) and sequenced by Macrogen, Inc.

Data storage

Nucleotide sequences have been submitted to GenBank and the Barcode of Life Database (BOLD).

Phylogenetic analysis

Hsp90 sequences obtained for the Kansas population were aligned with partial Hsp90 genomic DNA sequences from other cyst nematode species (new or from GenBank) using the MAFFT algorithm within Geneious 10.2.6. (https://www.geneious.com). The sequence data set was analyzed with Bayesian interference (B) using the MrBayes module within Geneious under the model GTR with rate variation set to invgamma, 6 gamma categories, and outgroup set to Globodera pallida (Stone, 1973) Behrens, 1975. The Markov-Chain Monte Carlo (MCMC) values were set to 1×10^6 chain length, subsampling frequency 1,000, four heated chains, and a burn-in length of 10,000. Two runs were performed for each analysis. Topologies were used to generate a 50% majority rule consensus tree.

ITS1 and COI phylogenetic trees were constructed under maximum likelihood (ML) criteria in MEGA version 6. Sequences were edited using CodonCode Aligner version 8.0.1 (http://www.codoncode.com/) and aligned using MUSCLE within MEGA version 6 (Tamura et al., 2013). The gap opening penalty was set at −400 with a gap extension penalty of −200. For the COI tree, the General Time Reversible Model with Gamma distributed rates with Invariant sites (GTR + G + I) was determined to be the best substitution model by Bayesian information criterion using the best fit substitution model tool in MEGA 6.0., while the ITS1 tree used HKY. Both ML trees used a ‘use all sites’ option for gaps and 200 bootstrap replications to assess clade support.

Results

Host trial results indicated that among the crop species tested, only alfalfa was a suitable host for the Kansas alfalfa Heterodera population (Table 3). Mature females and cysts were recovered from alfalfa roots at two months after planting, but Heterodera J2 were recovered from alfalfa root incubations at both trial periods. In contrast, Meloidogyne J2 were recovered in large numbers from tomato roots, with lower numbers recovered from alfalfa and soybean roots.

Figure 1 presents a maximum likelihood tree based on 862 base pairs of the COI mitochondrial gene from 154 specimens of heteroderid species. In total, 13 sequences from isolates collected from soil underneath alfalfa plantings form a well-supported homogeneous group that is a sister group to Heterodera glycines. The specimens from alfalfa are distinct from H. glycines at 42 nucleotide sites, with a mean pairwise P-distance (raw distance) of 4.7%. In total, 34 of the 42 nucleotide substitutions are at third-base pair positions in the COI gene. Three substitutions result in amino
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Figure 2: Maximum likelihood tree inferred from the ITS1 gene. Bootstrap support values above 50% are labeled in red. GenBank accession specimen from Russia is boxed in yellow.
Figure 3: Phylogenetic relationships of *Heterodera* species as inferred from analysis of a partial Hsp90 gene using Bayesian inference. Posterior probabilities more than 50% are given next to nodes. Newly obtained sequences are shown in bold.

Acid changes. The alfalfa specimens plus *H. glycines* form a group that is paired with *H. schachtii* Schmidt, 1871, with the three species constituting a well-supported clade (91 bootstrap value) that is joined to a second well-supported clade (bootstrap support, 98%) of other members of the *H. schachtii* group that include *H. cicero* Vovlas, Greco, and Di Vito, 1985, *H. daverti* Wouts & Sturhan, 1979, and *H. trifolii* Goffart, 1932. All six species within the *H. schachtii* group form a clade with a bootstrap support value of 100.

The ITS1 tree (Fig. 2) provides less clarity on the distinction between *Heterodera medicaginis* and *H. glycines* due to the sequence heterogeneity within both species. Depending on alignment parameters associated with gap creation and extension for ITS1, the diagnostic signal for this marker may be obscured. Most *H. glycines* sequences retrieved from GenBank cluster together apart, albeit with weak bootstrap support, from the suspected *H. medicaginis* sequences, including the single reference sequence from Russia (AF274391.1). Several GenBank *H. glycines* ITS se-
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Sequences of questionable identity fall outside the groupings of either species (LC208694.1, LC208695.1).

Four partial Hsp90 sequences were obtained from the Kansas population (Table 1). Although no Hsp90 sequence was available from a reference population of *H. medicaginis*, Bayesian analysis of partial Hsp90 genomic DNA showed that the Kansas population formed a distinct clade from *H. glycines* and other species from the Schachtii group (Fig. 3), giving further support for identification of this population as *H. medicaginis*.

The 28S sequence from the Kansas population was identical to *H. glycines* GenBank accession numbers (LC208677, KY795945, KY795944, KY795943, KX790324, GU475087, and DQ328692) and therefore did not discriminate *H. medicaginis*. The 28S sequence was submitted to GenBank (Accession No. MH793872) for future phylogenetic studies.

Measurements of J2 specimens are reported in Table 2 and illustrated (Fig. 4A-F). The J2 has four lines in the lateral field and a tail with a finely rounded terminus (Fig. 4E-G). The male anterior end, posterior end, and entire body length showing spicules are also depicted (Fig. 4H–J). Morphologically both cysts and juveniles conformed to *Heterodera medicaginis* except for one cone mount, which displayed molar-shaped bullae typical of *H. schachtii*. That cone, however, was slightly folded. Contrary to the redescriptions of *H. medicaginis*, the underbridge was moderately well-developed, about 100 mm long, with branches and heavily scattered bullae (Fig. 5B–D). Cysts were mostly oval, wide to lemon-shaped, brown in color, and with a cyst wall displaying a zig-zag pattern with few punctations (Fig. 6C–E). Cysts were ambifenestrate (Fig. 5A), the entire fenestra length ranging from 40 to 47 µm, fenestra width 30 to 32.5 µm or more, vulva

Figure 4: *Heterodera medicaginis* juvenile and male specimens. (A–G), juvenile specimens; (H–J), male specimen. (A) NID 7095, entire body; (B) NID 7243, entire body; (C) NID 7095, anterior; (D) NID 7243, anterior; (E) NID 7095, tail; (F) NID 7243, tail; (G) NID 7243, lateral lines; (H) PNID 169028, anterior; (I) PNID 169028, tail; (J) PNID 169028, entire body.
slit long with length 38 to 52 µm. The vulva to anus distance was 50 µm (Fig. 6E).

**Discussion**

A cyst nematode reproducing on alfalfa has been observed along the Arkansas River in western Kansas. A DNA record from a molecular survey from a Montana alfalfa field suggests the distribution may be wider than a single river valley in Kansas. DNA barcoding data by COI sequence rules out the identity of these cyst nematodes on alfalfa as being *Heterodera glycines*, *H. schachtii*, *H. trifolii*, or either of the two other members of the *H. 'schachtii' group.* Additionally, the morphology and measurements of juvenile and cyst stages are consistent with those of *H. medicaginis*. Cyst cone structure is more elaborate than was described in Gerber and Maas’s (1982) redescription. Initial greenhouse host-reproduction trials using infested field soil are also consistent with the limited host-range of *H. medicaginis*. The presence of *Meloidogyne hapla* in these soils suggests that damage estimates should take both species into consideration. Collectively, these data support the identity of these North American cyst specimens as *Heterodera medicaginis*, the alfalfa cyst nematode. DNA sequence of the COI gene from Russian specimens will be necessary to definitively make the connection between US and confirmed eastern European isolates.

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Figure 6: SEM micrographs of Heterodera medicaginis cysts from Kansas. (A) entire cyst 1, partial view of cyst 2; (B) entire cyst 3; (C) anterior region and excretory pore of cyst 1; (D) anterior region of cyst 3; (E) vulva and anus of cyst 2; (F) vulva of cyst 2 (left) and cyst 1 (right).

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