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Incorporating Molecular Identification of *Meloidogyne* spp. into a Large-scale Regional Nematode Survey[1]

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Abstract: A regional nematode survey of potato fields was conducted in the central United States during 2002 and 2003. The survey encompassed seven states and included a morphological and molecular examination of nematodes of regulatory concern from 1,929 soil samples. No regulated pest species were recovered during this survey. *Meloidogyne* juveniles extracted from soil were identified by mitochondrial and 18S ribosomal molecular markers. Eighty-two DNA sequences representing the two marker regions for *Meloidogyne* species were submitted to GenBank to facilitate evaluation of marker variability. Sufficient 18S variation was observed among some *Meloidogyne* species to aid in identification; however, nucleotide sequence from this highly conserved region of 18S did not discriminate among *M. arenaria, M. incognita,* and *M. javanica.* The mitochondrial gene region provided greater species discrimination and revealed intraspecific variation among many isolates. One nucleotide substitution found in a subset of *M. hapla* isolates from west Texas and New Mexico affected a *Dra*I restriction site used in the PCR/RFLP diagnostic protocol. None of the mitochondrial sequence variants observed in this study compromised the PCR/RFLP identification protocol for *M. chitwoodi.* Additional sequence analysis is recommended for validation and evaluation of genetic markers used in diagnostic decisions.

Key words: 18S, *Meloidogyne* spp., *M. arenaria, M. chitwoodi, M. incognita, M. javanica,* mitochondrial DNA, molecular diagnostics, potato, quarantine nematodes.

The detection of a single specimen of *Meloidogyne chitwoodi* in a potato can result in the quarantine of a shipment of seed or table stock potatoes destined for export. Detection in soil samples will, in most cases, prevent phytosanitary certification of potatoes grown in the United States. Canada and Mexico currently will not accept seed potatoes grown in an area known to be infested with *M. chitwoodi* and require certification that potatoes are free of *M. chitwoodi, M. javanica,* and *Globodera rostochiensis* (North American Plant Protection Organization [NAPPO] Regional Standard Phytosanitary Measure Doc. 956-02201). *Meloidogyne chitwoodi* is considered an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO) and NAPPO. An A2 quarantine pest is defined as present in that area but not widely distributed there and being officially controlled.

Meloidogyne chitwoodi, the Columbia root-knot nematode, is primarily a concern due to its effect upon potato quality, and consequently the marketability of potatoes (Santo et al., 2003). Its ability to continue development in cold storage and initiate infection early in the growing season increases its damage potential. It also is a concern due to its wide host range, which includes common field crops such as corn, alfalfa, wheat, and other small grains (Nyczepir et al., 1982). This combination of potential hosts has complicated management systems based on crop rotation in the potato-growing regions of the Pacific Northwest of the United States (Ingham et al., 1999; Santo et al., 2003).

The known distribution of *M. chitwoodi* includes nine of the United States, Mexico, Argentina, Belgium, Germany, the Netherlands, Portugal, and South Africa. The origin of *M. chitwoodi* is believed to be the Pacific Northwest, based on its widespread distribution in that region. However, no biogeographic or phylogeographic studies support this belief. The species most closely related to *M. chitwoodi* is *M. fallax,* which is not known from North America. *Meloidogyne fallax* was first reported from the Netherlands (Karssen, 1996) and is thought to have been introduced recently into Australia and New Zealand (Marshall et al., 2001; Nobbs et al., 2001). Following the discovery of *M. chitwoodi* in Texas (Szalanski et al., 2001), there was concern that the species might not be localized to a single potato field in west Texas. If infected seed potatoes were the source of the Texas infestation, then the possibility for additional infestations was relatively high. In 2002 and 2003, regional potato surveys were funded through the USDA/APHIS Cooperative Agricultural Pest Survey (CAPS) program to determine the distribution of potato nematodes of regulatory and economic significance throughout the Great Plains region. This included the north-south tier of states from North Dakota to Texas, as well as Wyoming and eastern Colorado.

This paper reports the results of the 2002 and 2003 potato surveys. It also reports on the application of DNA for species-level resolution in nematode surveys. Because several *Meloidogyne* species have been reported from the survey area and soil assays rely on the detection of the infective second-stage juveniles (J2), it is important to be able to identify the juvenile stage to species. False positive identification could result in the quarantine of an entire production area, and a false negative could impact future trade relations. In this study we used two DNA genetic markers to verify species identity. During this study it became clear that ad-

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ditional reference sequence information was necessary for comparative purposes to evaluate the genetic loci used in diagnostic assays. Nucleotide sequences of *Meloidogyne* species were added to the GenBank database to increase the number of observations from a broad geographic range of isolates (Tables 1 and 2).

Materials and Methods

Nematode sampling and extraction: Officials from state departments of agriculture from the central Great Plains states (Fig. 1) were responsible for field sampling. Intensity of sampling was based on intensity of potato production and anticipated markets. Field samples were taken according to suggested National Agricultural Pest Information Service (NAPIS) protocols (http://www.ceris.purdue.edu/napis/index.html). Typically, 20 soil cores comprising a bulk field sample were collected from the plant rows. Field samples were taken post-harvest throughout the 2002 and 2003 growing seasons. Nematodes were extracted from a 100-cm^3 soil sample using a modified sieving and sugar centrifugation method (Dropkin, 1989). Heteroderid cysts were isolated on a 60-μm-pore sieve following flotation.

Nematode identification: After nematode extraction, samples were examined using a dissecting microscope at magnifications allowing preliminary assignment to genus. Nematodes of potential regulatory concern for potato commerce (*Meloidogyne* spp., *Heterodera* spp., and *Ditylenchus* spp.,) were examined by light microscopy on temporary glass slides and digital images recorded. A minimum of 20 infective juveniles of *Meloidogyne* were analyzed if numbers extracted from the sample permitted. Temporary slides were dismantled and the nematodes individually placed in a 15-μl drop of sterile water on a cover slip and crushed with a micropipet tip. The solution containing the crushed nematode was placed in individual PCR reaction tubes. A 5.0-μl portion of the solution served as DNA template for PCR reaction.

For *M. chitwoodi* identification, the first of two PCR amplifications was conducted with primer set C2F3/1108 (5′ GGTCAATGTTCAGAAATTTGTGG 3′ and 5′ TACCTTTGACCAATCACGCT 3′) located in the COII and 16S ribosomal mitochondrial genes, respectively (Powers and Harris, 1993). PCR reaction master mix consisted of 1.5 units of JumpStart *Taq* Polymerase (Sigma #D-6558, St. Louis, MO) in a 1× dilution of the 10× stock buffer (Sigma #P-2192), Mg^{+2} at 3.0 mM final concentration, dNTP each at 200 μM final concentration, and each primer at 0.36 μM final concentration. From the master mix, 25.0 μl was aliquoted to a PCR tube containing 5.0 μl nematode template and mixed thoroughly. Amplification conditions included a modified hot-start, an initial denaturation at 94 °C for 2 minutes, followed by 45 cycles of denaturation at 94 °C for 15 seconds, annealing at 55 °C for 15 seconds, 30 °C/minute ramp to extension, and extension at 72 °C for 30 seconds. A final extension step was conducted for 2 minutes at 72 °C.

The initial C2F3/1108 PCR amplification products (5.0 μl of each mixed with 1.0 μl loading buffer [gel dye]) were separated on a 1.0% agarose gel made with Agarose Low EEO Electrophoresis Grade (Fisher Scientific, St. Louis, MO) and 0.5× TBE buffer. Size standards used were 1 kb or 100 bp ladders (Gibco/BRL, Rockville, MO) at approximately 0.2 μg DNA/gel lane. Electrophoresis was conducted in 0.5× TBE running buffer for 100 volt-hours, stained with ethidium bromide (EtBr) during the run, and results recorded on Polaroid 667 film (Fuller & Albert, Fairfax, VA).

The size of individual amplification products determined subsequent assays as follows (Fig. 2):

1. C2F3/1108 amplification products of 1.0 kb were designated as *M. arenaria* (Powers and Harris, 1993).
2. C2F3/1108 amplification products of approximately 1.5 kb were digested with *Hinf*I. If products of 1.15 kb and 0.35 kb were produced, the specimen was designated *M. incognita*. If no digestion occurred, the specimen was designated *M. javanica* (see Blok et al., 2002 for a discussion of this digestion pattern).
3. If the amplification product was 0.52 kb, it was subjected to a *Dra*I digestion: The expected digestion products indicative of *M. chitwoodi* were 260 bp, 120 bp, 85 bp, and 40 bp (Powers and Harris, 1993).
4. If digestion of C2F3/1108 amplification products indicated *M. chitwoodi* was present, a second amplification was conducted on the original DNA. For verification of *M. chitwoodi*, the 18S primer set (18sl.2/18sr2b: 5′ GGCGATCAGATACCGCCCTAGTT 3′ and 5′ TACAAAGGGCAGGGACGTAAT 3′) produced a 636-bp amplification product that included the primers. Digestion with *Alu*I results in a 350-, 115-, 85-, 50-bp set of fragments, a pattern apparently unique for *M.* chitwoodi (Powers and Mullin, unpubl. data).

To assess additional variation within this gene region, 18S DNA was amplified from specimens representing a wide geographic range (Table 1). Amplified templates of *Meloidogyne* isolates used for comparison were cleaned and sent to Davis Sequencing, Inc. (Davis, CA) and added to the GenBank sequence database. PCR products were cleaned in Microcon-100 Centrifugal Filter Devices (Fisher Scientific, St. Louis, MO). All survey reports were sent to NAPIS http://www.ceris.purdue.edu/napis/.

Results

Nematodes were extracted from 1,929 soil samples from the central Great Plains during summer and fall of 2002 and 2003 (Fig. 1). Seventy-eight of the samples contained at least one *Meloidogyne* juvenile. The highest number of infective J2 of *Meloidogyne* recovered from an individual soil sample was 65. Fifteen of the samples in

TABLE 1. 18S Sequence variants among isolates of *Meloidogyne* species collected in a regional survey of potato fields.

Species designation	Origin	Sequence source	Isolate[a]	Stage	GenBank acc. #[b]	Sequence type[c]
Meloidogyne arenaria	Unknown	GenBank	Govan	Unknown	U42342	A
	Georgia	GenBank	Race 2	Unknown	AF535867	A
	Texas	Current study	Special B	Juvenile	AY757832	B
	Texas	Current study	TXB717	Juvenile	AY757833	B
	Texas	Current study	TXB723	Juvenile	AY757834	B
Meloidogyne chitwoodi	Netherlands	GenBank	Lok A	Unknown	AF442195	A
	Washington	Current study	WAMC24	Juvenile	AY757835	A
	Washington	Current study	WAMC25	Juvenile	AY919153	A
	Colorado	Current study	SLV1402	Juvenile	AY146458	A
	Colorado	Current study	SLV1602	Juvenile	AY919154	A
	Oregon	Current study	ORMC8	Juvenile	AY919155	A
	Oregon	Current study	ORMC11	Juvenile	AY757836	A
	Oregon	Current study	ORMC12	Juvenile	AY919156	A
	California	Current study	CAMC2	Juvenile	AY757837	A
	Unknown	Current study	VBA3-1A	DNA	AY757838	A
	Portugal	Current study	VBA4 Port	DNA	AY757839	A
	New Mexico	Current study	NM-03 CD	Juvenile	AY919157	A
	New Mexico	Current study	NM-03 CE	Juvenile	AY757840	A
	Texas	Current study	CSS TX-04	Juvenile	AY757841	A
	Texas	Current study	CSS TX-07	Juvenile	AY919158	A
	Idaho	Current study	BP-01	Juvenile	AY757842	B
	Idaho	Current study	BP-02	Juvenile	AY919159	B
Meloidogyne fallax	Unknown	Current study	VBA2-1A	DNA	AY757843	A
Meloidogyne graminicola	Philippines	GenBank	WB	Unknown	AF442196	A
	Florida	Current study	Home FL-03 G	Juvenile	AY757844	B
	Florida	Current study	Home FL-03 H	Juvenile	AY919160	B
Meloidogyne graminis	Arizona	Current study	McL J2 F	Juvenile	AY757845	A
	Arizona	Current study	McL J2 H	Juvenile	AY919165	A
	Texas	Current study	GD454 N	Juvenile	AY757846	A
	Texas	Current study	GD454 P	Juvenile	AY919166	A
	Texas	Current study	TXB441 G	Juvenile	AY757847	B
	Kansas	Current study	KonVAA-129	Juvenile	AY919167	C
	Kansas	Current study	KonVIIEE-84	Juvenile	AY919168	C
	Texas	Current study	TXB441 I	Juvenile	AY757848	D
Meloidogyne hapla	Netherlands	GenBank	C4900	Unknown	AF442194	A
	Arizona	Current study	AZMH	Juvenile	AY757849	A
	Unknown	Current study	DodMH	Juvenile	AY757850	A
	Wyoming	Current Study	WY E	Juvenile	AY757851	A
	Wyoming	Current study	WY J	Juvenile	AY919169	A
	Texas	Current study	TXB80	Juvenile	AY757852	A
	Texas	Current study	TXB606	Juvenile	AY757853	A
	Texas	Current study	TXB607 R	Juvenile	AY757854	A
	Texas	Current study	TXB607 S	Juvenile	AY919170	A
	New Mexico	Current study	NM A3	Juvenile	AY757855	A
	Texas	Current study	TXB83	Juvenile	AY757856	A
	Texas	Current study	TXB85	Juvenile	AY757857	A
	Texas	Current study	TXB86 MhB	Juvenile	–	A
	New Mexico	Current study	NM03 B1	Juvenile	AY919171	A
	New Mexico	Current study	NM03 E	Juvenile	AY757858	A
	Unknown	Current study	MH97-13-05	Male	–	A
	Unknown	Current study	MH97-13-06	Juvenile	AY757859	A
	Connecticut	Current study	CT04-01	Male	AY757860	A
	Connecticut	Current study	CT04-02	Juvenile	AY919172	A
	Michigan	Current study	MI02-03	Male	AY757861	A
	Rhode Island	Current study	RI5-03	Male	AY919173	A
	Rhode Island	Current study	RI5-05	Juvenile	AY757862	A
	New York	Current study	NY3-07	Juvenile	AY919174	A
	New York	Current study	NY3-08	Juvenile	AY757863	A
	Texas	Current study	TXB869 MhB	Juvenile	AY757864	A
	Texas	Current study	TXB873 1B MhB	Juvenile	AY919175	A
	Texas	Current study	TXB873 2B MhB	Juvenile	AY919176	A
	Texas	Current study	TXB873 7B MhB	Juvenile	AY757865	A
	Texas	Current study	TXA198 1B MhB	Juvenile	AY757866	A
	Texas	Current study	TXA198 2B MhB	Juvenile	AY919177	A
Meloidogyne haplanaria	Texas	Current study	AnM 7	Juvenile	AY757867	A
	Texas	Current study	AnM 8	Juvenile	AY919178	A

TABLE 1. *Continued.*

Species designation	Origin	Sequence source	Isolate[a]	Stage	GenBank acc. #[b]	Sequence type[c]
Meloidogyne incognita	Unknown	GenBank	Unknown	Unknown	U81578	A
	Georgia	GenBank	Hussey	Unknown	AF535868	B
	Texas	Current study	TXB754	Juvenile	AY757869	C
	Texas	Current study	TXB862 C	Juvenile	AY757868	D
	Texas	Current study	TXB862 D	Juvenile	AY919179	D
	Texas	Current study	TXB864	Juvenile	AY757870	D
Meloidogyne javanica	China	GenBank	Unknown	Unknown	AF442193	A
	Unknown	Current study	Gov RR	Juvenile	AY919180	A
	Unknown	Current study	Gov SS	Juvenile	AY757871	A
	Unknown	Current study	JC-10 UU	Juvenile	AY919181	A
	Unknown	Current study	JC-10 WW	Juvenile	AY757872	A
Meloidogyne mayaguensis	Puerto Rico	Current study	PR2180-1	Juvenile	AY757873	A
	Puerto Rico	Current study	PR2180-2	Juvenile	AY919182	A
	Florida	GenBank	FL4 A	Juvenile	AY446965	A
	Florida	GenBank	FL4 C	Juvenile	AY446966	A
	Florida	GenBank	FL5 C	Juvenile	AY446967	A
	Florida	GenBank	FL5 D	Juvenile	AY446968	A
Meloidogyne partityla	New Mexico	Current study	NMN	Juvenile	AY919183	A
	New Mexico	Current study	NMO	Juvenile	AY757874	A
	Texas	Current study	Pecan BB	Juvenile	–	B
	Texas	Current study	Pecan CC	Juvenile	–	B

[a] More information about nematode isolates can be obtained at: http://nematode.unl.edu/meldds.htm
[b] Isolates with a (–) have not been submitted to GenBank at time of publication.
[c] Letters refer to sequence variants for the 18S genetic marker.

which *Meloidogyne* J2 were detected contained only a single specimen. Many of the juveniles appeared starved or dead. No *Meloidogyne* species were recovered from samples in Colorado, Kansas, Minnesota, Nebraska, or North Dakota. *Meloidogyne* J2 were extracted from a single sample from Wyoming and from 77 Texas samples. No *Globodera* spp. or *Ditylenchus destructor* were detected in any samples.

Amplification products were generated from *Meloidogyne* J2 from 76 of the 78 root-knot nematode positive samples. In the 15 samples represented by a single juvenile, 14 produced an amplification product with the mitochondrial primer set (C2F3/1108). Mitochondrial amplification products of 520 bp, 1.0 kb, and 1.5 kb were observed among *Meloidogyne* juveniles (Fig. 3). Nematodes from 15 of the 78 *Meloidogyne* positive soil samples produced initial amplification products of 520 bp, requiring restriction digestion for *M. chitwoodi* identification.

Digestion of the amplified 520-bp product with *Dra*I generated three different profiles, none conforming to the expected digestion profile of *M. chitwoodi* (Fig. 4). The predominant digestion pattern corresponded to the predicted pattern for *M. hapla,* and two patterns from Texas samples were generated that were previously not observed in our laboratory in *Meloidogyne* restriction digestions. One of the two new patterns conformed to the predicted pattern of *M. graminis* based on DNA sequence information from known *M. graminis* isolates recovered from turf in Arizona (AY757886). Subsequent DNA sequence analysis of the mitochondrial and 18S markers revealed 100% identity with the *M. graminis* isolates from turf. The second new *Dra*1 pattern appeared to be a variant of *M. hapla.* The mitochondrial sequence diverged from "typical" *M. hapla* by a single nucleotide that eliminated a *Dra*1 restriction site (Fig. 4). The 18S sequence from this *M. hapla* variant was identical to other *M. hapla* isolates from North America (Table 1). *Meloidogyne hapla* isolates from Arizona, Connecticut, Michigan, New Mexico, New York, Rhode Island, Texas, Washington, and Wyoming were included in this comparison.

Restriction digestion of 18S provided a second marker for verification of species determination initially based on mitochondrial restriction patterns. Figure 5 displays an *Alu*I digestion pattern of the 640-bp 18S amplification product from selected *Meloidogyne* species including *M. chitwoodi.* The presence of a 350-bp fragment in 18S digestions distinguished *M. chitwoodi* (plus *M. fallax*) from the other *Meloidogyne* species in the analyses that produced an initial 520-bp amplification product with the mitochondrial primer set. Although no *M. chitwoodi* were observed during this survey, the 18S-primer set was used in control amplifications of *M. chitwoodi* DNA from confirmed positive identifications. These control specimens represented isolates from California, Colorado, Idaho, New Mexico, Oregon, and Washington. Overall in this survey, four nominal *Meloidogyne* species were recovered from potato fields in the central U.S. region. *Meloidogyne hapla,* recorded from Wyoming, was the only *Meloidogyne* species observed outside Texas. *Meloidogyne incognita, M. arenaria, M. hapla,* and *M. graminis* J2 were also observed in potato soils, although their reproduction could be associated with weeds or a previous crop.

Other plant-parasitic nematodes were commonly re-

TABLE 2. Mitochondrial sequence variants among isolates of *Meloidogyne* species collected in a regional survey of potato fields.

Species designation	Origin	Sequence source	Isolate[a]	Stage	GenBank acc. #[b]	Sequence type[c]	Sequence length
Meloidogyne chitwoodi	Washington	Current study	WAMC25 N	Juvenile	AY757875	A	521
	Washington	Current study	WAMC25 O	Juvenile	AY757876	A	
	Unknown	Current study	2002VBA3-1B	DNA	AY757881	A	
	Portugal	Current study	VB Port	DNA	AY757882	A	
	Texas	Current study	Lub 67A	Juvenile	–	B	522
	Oregon	Current study	ORMC11 M	Juvenile	AY757877	C	520
	Oregon	Current study	ORMC11 N	Juvenile	AY757878	C	
	New Mexico	Current study	NM03 C	Juvenile	AY757879	C	
	New Mexico	Current study	NM03 F	Juvenile	AY757880	C	
Meloidogyne fallax	Unknown	Current study	VB A2-1B	DNA	AY757883	A	522
Meloidogyne graminicola	Florida	Current study	Home FL-03 7B	Juvenile	AY757884	A	531
	Florida	Current study	Home FL-03 8B	Juvenile	AY757885	A	
Meloidogyne graminis	Arizona	Current study	McL J2 E	Juvenile	AY757886	A	540
	Arizona	Current study	McL J2 F	Juvenile	–	A	
Meloidogyne hapla	Arizona	Current study	AZMH	Juvenile	AY757887	A	528
	Unknown	Current study	DodMH	Juvenile	AY757888	A	
	Texas	Current study	TXB86	Juvenile	–	B	529
	Texas	Current study	TXB869	Juvenile	AY757891	B	
	Texas	Current study	TXB873 A	Juvenile	AY757892	B	
	Texas	Current study	TXB873 B	Juvenile	AY757893	B	
	Texas	Current study	TXA 198	Juvenile	AY757894	B	
	New Mexico	Current study	NM03h A	Juvenile	AY757895	B	
	New Mexico	Current study	NM03h B	Juvenile	AY757896	B	
	Unknown	Current study	MH97-13 5	Male	AY757897	B	
	Unknown	Current study	MH97-13 6	Juvenile	AY757898	B	
	New Mexico	Current study	NM A3 15	Juvenile	–	C	529
	New Mexico	Current study	NM A3 16	Juvenile	AY757890	D	529
	Wyoming	Current study	WY 25	Juvenile	AY757889	E	529
	Michigan	Current study	MI2-03	Male	AY757901	E	
	New York	Current study	NY3-08	Juvenile	AY757904	E	
	Wyoming	Current study	WY26	Juvenile	–	F	528
	Connecticut	Current study	CT4-01	Male	AY757899	G	528
	Connecticut	Current study	CT4-02	Juvenile	AY757900	G	
	Rhode Island	Current study	RI5-03	Male	AY757902	H	528
	Rhode Island	Current study	RI5-05	Juvenile	AY757903	H	
Meloidogyne haplanaria	Texas	Current study	AnM 6	Juvenile	AY757905	A	540
	Texas	Current study	AnM 7	Juvenile	AY757906	A	
Meloidogyne incognita	Texas	Current study	TXB402 M	Juvenile	–	A	1627
	Texas	Current study	TXB402 Q	Juvenile	–	A	
Meloidogyne mayaguensis	Unknown	Blok lab	VB Mm	Unknown	AJ421396	A	705
	Puerto Rico	Current study	PR2180-1	Juvenile	AY757907	B	704
	Puerto Rico	Current study	PR2180-2	Juvenile	–	B	
	Florida	GenBank	FL2 O	Juvenile	AY446971	C	704
	Florida	GenBank	FL2 Q	Juvenile	AY446972	C	
	Florida	GenBank	FL3 L	Juvenile	AY446973	D	705
	Florida	GenBank	FL3 N	Juvenile	AY446974	D	
	Florida	GenBank	FL1 A	Juvenile	AY446969	E	705
	Florida	GenBank	FL1 B	Juvenile	AY446970	E	
	Florida	GenBank	FL5 A	Juvenile	AY446977	F	705
	Florida	GenBank	FL5 C	Juvenile	AY446978	F	
	Florida	GenBank	FL4 E	Juvenile	AY446975	F	
	Florida	GenBank	FL4 G	Juvenile	AY446976	F	
Meloidogyne partityla	New Mexico	Current study	NM A1 A	Juvenile	AY757908	A	528
	New Mexico	Current study	NM A1 B	Juvenile	AY757909	A	
	Texas	Current study	TX AA	Juvenile	–	B	527

[a] More information about nematode isolates can be obtained at: http://nematode.unl.edu/meldds.htm
[b] Isolates with a (–) have not been submitted to GenBank at time of publication.
[c] Letters refer to sequence variants for the mitochondrial genetic marker.

covered from potato fields in the central Great Plains region. Ranked according to frequency of occurrence in soil samples, these were: *Pratylenchus* (present in 33% of samples), *Tylenchorhynchus* (29%), *Ditylenchus* (22%), *Quinisulcius* (21%), *Mesocriconema* (16%), and *Xiphimena* (13%). *Cactodera* juveniles and cysts were recovered frequently from Texas soils (present in 21% of Texas samples) but otherwise found only in a single sample from Kansas and two samples from Nebraska. There was no evidence that the *Cactodera* species reproduced on potato although, based on their common occurrence in potato fields, further investigation is warranted.

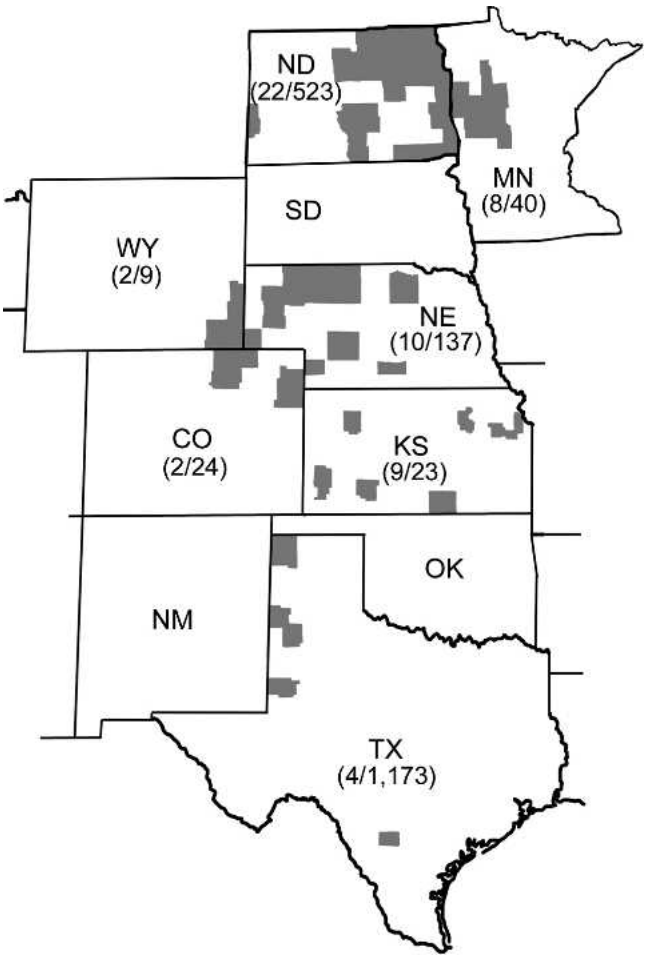

FIG. 1. Location of counties surveyed in 2002 and 2003. The numbers in parenthesis indicate number of counties/number of samples.

Sequence analysis of the 18S gene region from *Meloidogyne* reference samples displayed little intraspecific variation (Table 1). The *M. arenaria* sequences from Texas specimens differed by a single nucleotide from GenBank accessions U42342 and AF535867, and were identical to sequences from nematodes identified as *M. incognita* sequence type "C" from Texas and GenBank accession AF442193, an isolate of *M. javanica* from China. The Texas *M. arenaria* specimens had a 1.0-kb mitochondrial amplification product, and the *M. incognita* sequence type "C" mitochondrial product was 1.5 kb. There was no 18S sequence variation among 15 *M. chitwoodi* isolates, although a single deletion was observed in two specimens extracted from a single store-bought potato from Idaho. *Meloidogyne fallax* produced an 18S sequence identical to the majority of *M. chitwoodi* isolates. *Meloidogyne graminis* with four 18S sequence types appeared the most variable for this marker. Thirty *M. hapla* specimens from eight U.S. states and the Netherlands produced identical 18S sequence. Table 3 presents the sequence variants for each of the *Meloidogyne* 18S sequence types observed in this study.

Considerably more nucleotide variation was observed in sequences from the mitochondrial amplification product (Table 2). The large number of insertions and deletions, together with the high A/T content made sequence alignment difficult. For example, the variation among mitochondrial sequence types A-C for *M. chitwoodi* included two regions in which A was repeated either eight or nine times and T was repeated nine or 10 times. The T-to-A transversion that deleted a *Dra*I site (TTTAAA recognition sequence), observed in isolates of *M. hapla* sequence type "B" from New Mexico and Texas (Fig. 4), was located within a region of the 16S mitochondrial gene in which 107/109 nucleotides were either A or T. Variation in this gene region is the subject of a wider molecular survey of mitochondrial evolution (Powers, unpubl. data).

Discussion

No new observations of *M. chitwoodi* or other nematode species of regulatory concern were recorded in this survey of central U.S. potato fields. Since phytosanitary certification required species identification of *Meloidogyne,* the survey provided an opportunity to test molecular protocols on infective juveniles extracted from field soils. In many cases these juveniles were not in optimum condition, making initial morphological assessment of species identity difficult. In the 15 samples where a single juvenile represented the only available specimen for analysis, successful amplification of 14/15 of the specimens illustrated the robustness of the molecular protocols. Furthermore, the ability to conduct multiple amplifications from a single juvenile provided additional confidence in species identification. The precision of the protocol, however, comes at a price. At a minimum, a single PCR amplification and gel was sufficient to exclude the presence of *M. chitwoodi* in a sample. However, in cases where the initial amplification with the mitochondrial primer set produced a 520-bp product, restriction digestion and a second gel followed PCR. If the first digestion indicated the presence of *M. chitwoodi,* we sought added verification by the application of a second genetic marker. We used restriction digestion of the 18S amplification product (Fig. 5). This marker was selected because of the extensive supporting 18S sequence available in GenBank. A quicker and more cost-effective marker may be the specific primer set developed by Williamson et al. (1997). Even though multiple specimens are typically analyzed in a molecular assay, additional amplification and digestion steps can easily raise the cost of a single assay beyond $100. Ultimately, rapid and inexpensive DNA Barcoding will replace PCR/RFLP as a preferred diagnostic method (Powers, 2004).

Following the completion of this survey we received notice that one shipment of potatoes from a surveyed field had been rejected at an international border. Agents at a border station had reportedly detected *M.*

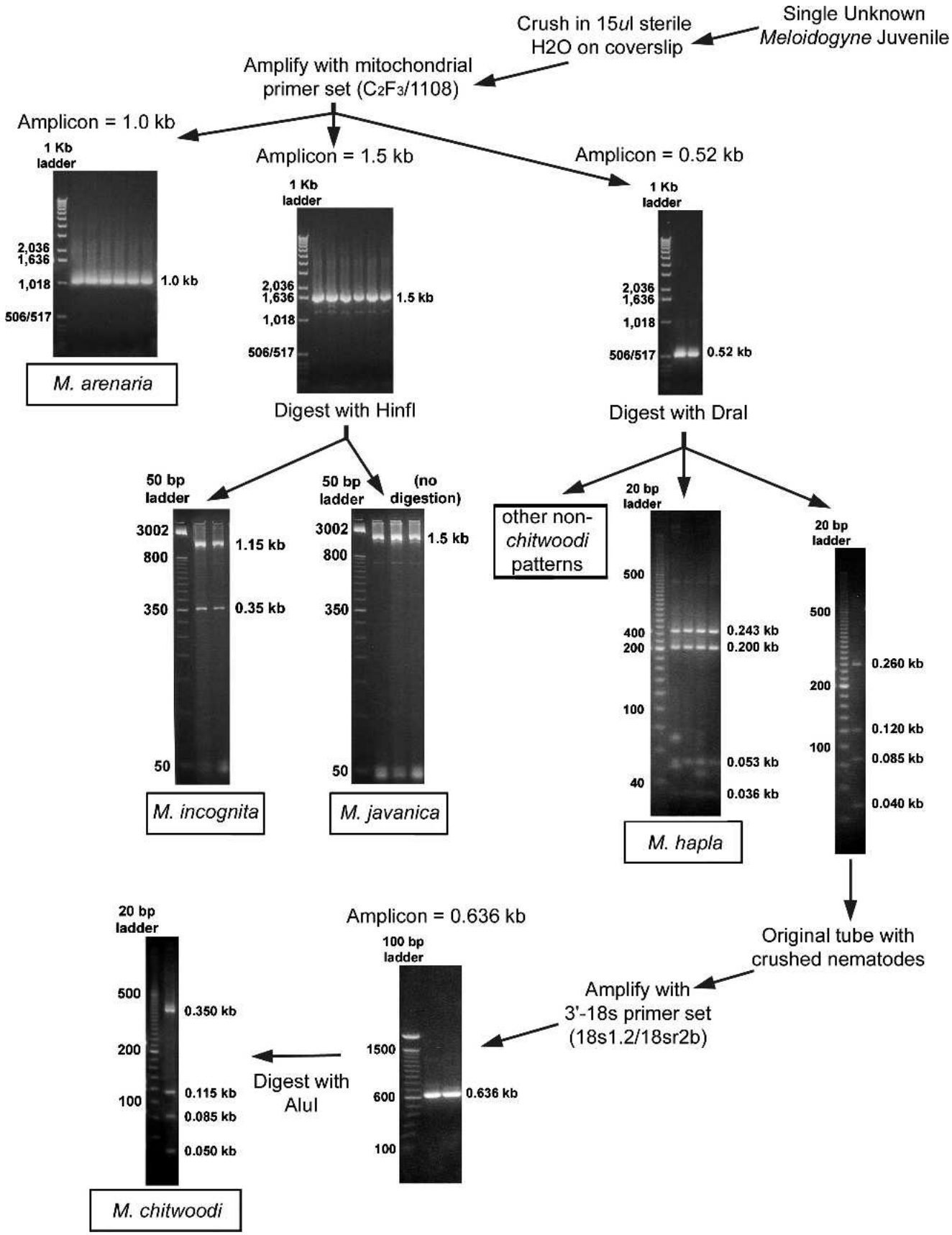

FIG. 2. Flow chart depicting the PCR/RFLP steps used to identify *Meloidogyne* species in this study.

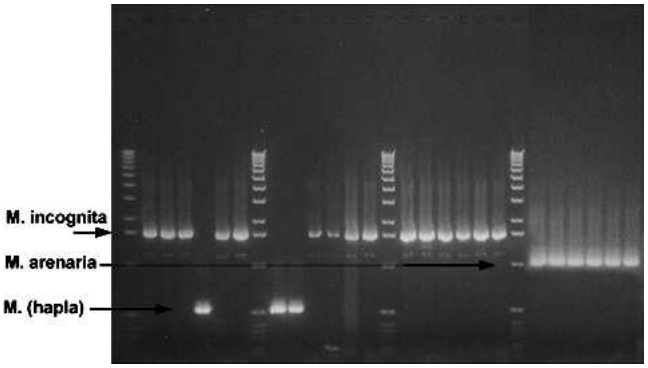

FIG. 3. Three size classes of *Meloidogyne* mitochondrial amplification products observed during the survey. Products of either 520 bp, 1.0 kb, or 1.5 kb were observed among *Meloidogyne* juveniles.

hapla and *M. chitwoodi* in tubers. The soil assay had reported no *Meloidogyne* species present, although an adjacent field managed by the same producer reported the presence of 1 *M. hapla* juvenile/100 cm^3 soil. As a follow-up procedure, 50 potatoes were extracted from the storage bin housing the original shipment. These potatoes were washed, peeled, and sliced with each step digitally recorded. Although there were no external symptoms of *Meloidogyne* infection, two potatoes were observed to contain two small lesions with mature *Meloidogyne* females, eggs, and juveniles (http://nematode.unl.edu/melhapsy.htm). Juveniles from the egg masses were determined to be *M. hapla* by DNA restriction fragment analysis and DNA sequencing. This incident illustrates the difficulties associated with surveys when sampling protocols are near the limit of detection. Sampling efficiency needs to be improved and the cost of molecular assays lowered before molecular approaches can be applied routinely in large-scale surveys.

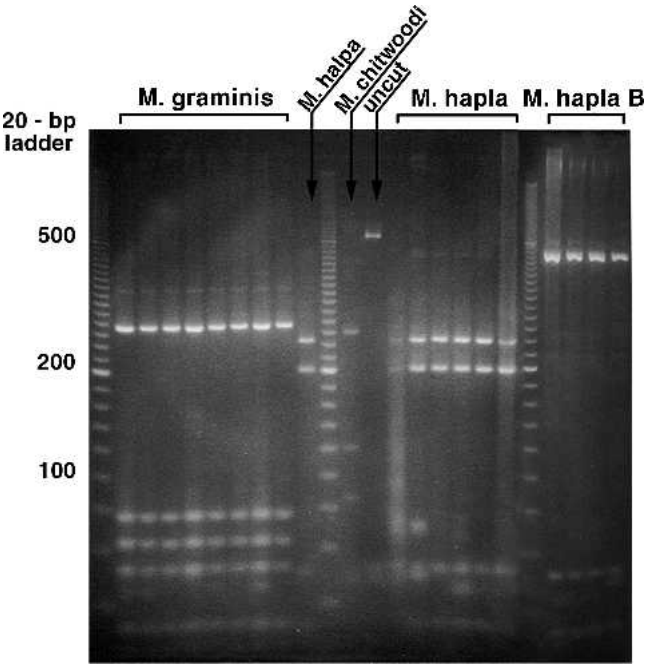

FIG. 4. *Dra*I digestion products of the amplified 520-bp product. *Meloidogyne chitwoodi* positive control is a reference juvenile extracted from an infected potato from New Mexico. *M. hapla* "B" corresponds to *M. hapla* mitochondrial sequence type B observed in New Mexico and Texas.

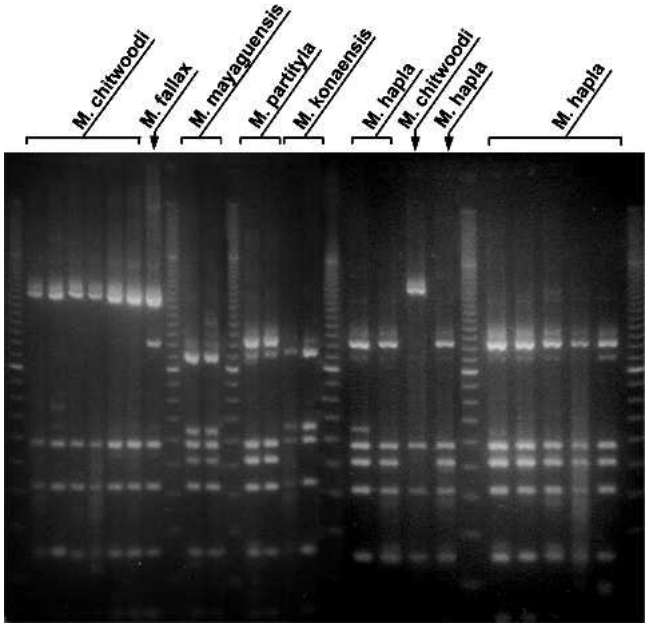

FIG. 5. *Alu*I digestion pattern of the 640-bp 18S amplification product from selected *Meloidogyne* species. The 350-bp fragment observed in *M. chitwoodi* and *M. fallax* was unique for those species in these comparisons.

An important consideration for any molecular-based diagnostic method is protocol validation (Hübschen et al., 2004). In the potato survey, we attempted to examine the specificity of the protocol across a broad range of *Meloidogyne* isolates. We encountered more variation than expected, although none of the variants compromised the PCR-RFLP method used to identify *M. chitwoodi*. Nonetheless, there are still many *Meloidogyne* species that have not been examined. Until more comparative studies are completed, we suggest that molecular identification protocols incorporate more than a single genetic marker in diagnostic assays.

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TABLE 3. Nematode 18S sequence types identified in Table 1.

Species designation	Sequence type	Sequence length	63	64	67	68	69	70	210	217	312	339	359	362	364	365	366	368	369	371	372	373
Meloidogyne arenaria	A	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
	B	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
Meloidogyne chitwoodi	A	636	G	A	A	T	A	–	A	A	T	G	T	A	A	T	T	T	T	T	T	T
	B	635	G	A	A	T	A	–	A	A	T	G	T	A	A	T	T	T	T	T	T	–
Meloidogyne fallax	B	636	G	A	A	T	A	–	A	A	T	G	T	A	A	T	T	T	T	T	T	T
Meloidogyne graminicola	A	637	G	A	A	T	A	A	A	A	T	G	T	A	A	T	T	A	T	T	A	T
	B	639	G	A	A	T	A	A	A	A	T	G	T	A	A	T	T	A	T	T	A	T
Meloidogyne graminis	A	637	G	A	A	T	A	–	G	A	T	G	C	A	A	C	A	A	T	T	T	T
	B	636	G	A	A	T	A	–	G	A	T	G	C	A	A	C	A	A	T	T	T	T
	C	636	G	A	A	T	G	–	G	A	T	G	C	A	A	C	A	A	T	T	T	T
	D	636	G	A	A	T	A	–	G	A	T	G	C	A	A	C	A	G	T	T	A	T
Meloidogyne hapla	A	636	G	–	T	C	A	–	G	A	T	G	C	A	G	C	A	A	T	T	T	A
Meloidogyne haplanaria	A	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
Meloidogyne incognita	A	637	A	A	A	T	A	–	G	A	T	G	C	G	A	C	A	A	A	T	T	A
	B	637	A	A	A	T	A	–	G	G	T	G	C	G	A	C	A	A	A	T	T	A
	C	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
	D	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
Meloidogyne javanica	A	637	A	A	A	T	A	–	G	A	T	G	T	G	A	C	A	A	C	C	T	T
Meloidogyne mayaguensis	A	637	A	A	A	T	A	–	G	A	T	G	C	G	A	C	A	A	C	C	T	T
Meloidogyne partityla	A	635	G	–	T	C	A	–	G	A	T	G	C	A	G	C	A	A	T	T	A	A
	B	635	G	–	T	C	A	–	G	A	C	A	C	A	G	C	A	A	T	T	A	A

Species designation	Sequence type	Sequence length	374	375	377	378	379	380	381	382	384	385	405	406	407	410	505	509	518	550	573	596
Meloidogyne arenaria	A	637	A	–	T	G	T	A	T	A	A	G	G	C	G	C	A	T	C	G	C	T
	B	637	A	–	T	G	T	A	T	A	A	G	G	C	G	C	A	T	C	G	C	G
Meloidogyne chitwoodi	A	636	–	–	A	A	T	A	T	A	A	A	G	T	G	T	T	C	C	G	C	G
	B	635	–	–	A	A	T	A	T	A	A	A	G	T	G	T	T	C	C	G	C	G
Meloidogyne fallax	B	636	–	–	A	A	T	A	T	A	A	A	G	T	G	T	T	C	C	G	C	G
Meloidogyne graminicola	A	637	A	A	A	A	T	A	–	–	A	A	G	T	G	T	T	C	C	G	C	G
	B	639	A	A	A	A	T	A	T	A	A	A	G	T	G	T	T	C	C	G	C	G
Meloidogyne graminis	A	637	T	–	T	G	T	A	T	T	G	G	G	T	G	C	G	C	T	G	C	G
	B	636	–	–	T	G	T	A	T	T	G	G	G	T	G	C	G	C	T	G	C	G
	C	636	–	–	T	G	T	A	T	T	G	G	G	T	G	C	G	C	T	G	C	G
	D	636	–	–	T	G	T	A	T	T	G	G	G	T	G	C	G	C	T	G	C	G
Meloidogyne hapla	A	636	C	–	T	G	T	A	T	T	G	G	G	C	T	C	A	C	T	G	C	G
Meloidogyne haplanaria	A	637	T	–	T	G	T	A	T	A	A	G	A	C	G	C	A	T	C	G	C	G
Meloidogyne incognita	A	637	A	–	T	G	T	A	T	A	A	G	G	T	G	C	A	C	C	G	C	G
	B	637	A	–	T	G	T	A	T	A	A	G	G	T	G	C	A	C	C	G	C	G
	C	637	A	–	T	G	T	A	T	A	A	G	G	C	G	C	A	T	C	T	C	G
	D	637	A	–	T	G	T	A	T	A	A	G	G	C	G	C	A	T	C	G	C	G
Meloidogyne javanica	A	637	A	–	T	G	T	A	T	A	A	G	G	C	G	C	A	T	C	G	C	G
Meloidogyne mayaguensis	A	637	A	–	T	G	T	A	T	A	A	G	G	T	G	C	A	C	C	G	C	G
Meloidogyne partityla	A	635	–	–	T	G	C	T	T	T	G	G	G	C	T	C	A	C	C	G	T	G
	B	635	–	–	T	G	C	T	T	T	G	G	G	C	T	C	A	C	C	G	T	G

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