SEQUENCE ANALYSIS OF THE D2/D3 REGION OF THE LARGE SUBUNIT RDNA FROM DIFFERENT *MELOIDOGYNE* ISOLATES

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ABSTRACT

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The phylogenetic relationships of eight *Meloidogyne* species and twelve isolates from Brazil and other countries were investigated using sequence data of the D2/D3 expansion segments of the large subunit of ribosomal DNA. The phylogenetic procedures used were maximum parsimony, maximum likelihood and neighbor-joining, using different mathematical alignment algorithms as implemented in TreeAlign and ClustalX, and different tree construction methods of TreeAlign and PAUP*. The results obtained with ClustalX alignments are robust and supported by high bootstrap values, suggesting a strong phylogenetic signal, as also supported by the obtained values of skewness parameter *g1*. Although the consensus topology of trees derived from TreeAlign alignments is more poorly resolved, the topologies obtained with different algorithms and software are congruent in dividing the species into two clusters: a heterogeneous grouping of *M. chitwoodi*, *M. exigua* (three isolates), *M. graminicola* and *M. trifoliophila*; and a much less divergent cluster with *M. arenaria* (race 2), *M. incognita*, *M. konaensis* and *M. paranaensis* (three isolates). The phylogenetic usefulness of the D2/D3 region clearly depends greatly on the evolutionary rates within the investigated lineages. For the *Meloidogyne* isolate study presented here, the D2/D3 region seems to be a more appropriate marker for relationships between species groups than between individual species.

Key words: Meloidogyne, nematodes, ribosomal DNA, phylogeny, species diagnosis, PCR, Brazil.

RESUMEN

Tenente, G. C. M. V., P. De Ley, I. Tandingan De Ley, G. Karssen and J. R. Vanfleteren. 2004. Análisis secuencias de la región D2/D3 del ADN de la sub-unidad grande ribosomal de aislados diferentes de *Meloidogyne*. Nematropica: 34:1-12.

Las relaciones filogeneticas de ocho especies de Meloidogyne y de 12 aislamientos de Brasil y de otros países fueron determinados utilizando información de secuencias de los segmentos de expansión D2/D3 de amplia unidad ribosomal de ADN. Los procedimientos filogeneticos utilizados fueron parsimonia máxima, probabilidad máxima y alineacion-vecinal. Se utilizaron diferentes algoritmos matemáticos para la alineación, implementados por “TreeAlign” y “ClustalX” y diferentes métodos de construcción de arboles, tales como “TreeAlign” y “PAUP*”. Los resultados obtenidos por alineación con “ClustalX” son sólidos y apoyados por valores altos de “bootstrap”, sugiriendo una fuerte señal filogenetica además apoyados por los valores obtenidos del parámetro de “skewness g1”. Aunque el consenso de topología derivado de los arboles con alineamientos de “TreeAlign” parece ser débilmente resuelto, las topologias obtenidas con los diferentes software de algoritmos expresan congruencia en la división de especies en dos grupos, un grupo heterogéneo que incluye: *M. chitwoodi*, *M. exigua* (tres aislamientos), *M. graminicola* and *M. trifoliophila*; y otro grupo menos divergente que incluye: *M. arenaria* (raza 2), *M. incognita*, *M. konaensis* and *M. paranaensis* (tres aislamientos). La importancia filogenetica de la región D2/D3, claramente depende en gran parte en los rangos evolu-

cionarios entre los linajes investigados. La región D2/D3 de los aislamientos de *Meloidogyne* que se presentan en este estudio parecen ser un marcador mas apropiado entre los diferentes grupos de especies que entre especies individuales.

Palabras claves: Meloidogyne, nemátodos, ADN ribosomal, filogenia, diagnosis especifica, PCR, Brasil.

INTRODUCTION

Root-knot nematodes (RKN) are the most widely distributed group of plant-parasitic nematodes and they are also among the most destructive, occurring all over the world and parasitizing many different plants (Eisenback and Triantaphyllou, 1991). Accurate species identification of RKN is necessary for effective control, and for classification and phylogenetic studies. *Meloidogyne* species are taxonomically difficult to identify and classify, even for experienced taxonomists, because current species delineations are based on subtle and often strongly overlapping characters (Esbenshade and Triantaphyllou, 1990; Tenente and Leal-Bertioli, 1999; Van Der Beek *et al.*, 1998).

Molecular techniques provide new methods for identification of these nematodes (Castagnone-Sereno *et al.*, 1994; Tenente and Leal-Bertioli, 1999) and allow phylogenetic inference of relationships among species. Earlier studies have reported on the phylogenetic implications of morphological characters, enzyme data, or genomic identification techniques (e.g., Curran *et al.*, 1986; Esbenshade and Triantaphyllou 1987; Castagnone-Sereno *et al.*, 1991, 1993; Carpenter *et al.*, 1992; Piotte *et al.*, 1992; Petersen *et al.*, 1997; Williamson *et al.*, 1997; Hugall *et al.*, 1999). Phylogenetic analyses based on sequence data have advantages, such as: the large number of potential characters available for relationship inferences; the independence of nucleotide substitutions from changes in morphology; and the possibility of using sequence data for parameter inference in various models of sequence evolution, influencing the topology of the tree (Nadler, 1995). By comparison, no clearly applicable mathematical models exist for the evolution of morphological or physiological characters, imposing greater limitations on the number and sophistication of applicable tree-construction algorithms.

The ribosomal DNA (rDNA) unit is particularly popular for phylogenetic analyses because it includes highly conserved regions as well as highly variable regions, used to reconstruct phylogenetic relationships among organisms with varying degrees of relatedness (Hillis and Dixon, 1991). In the case of *Meloidogyne*, two recent studies have presented phylogenetic analyses based on different rDNA loci. Hugall *et al.* (1999) analyzed the Internal Transcribed Spacer region (ITS) but encountered substantial amounts of sequence polymorphism within individuals. Tandingan De Ley *et al.* (2002) used small subunit rDNA sequences and found that these resolved relationships between divergent lineages, but not between some closely related species of prime agricultural importance. Other loci must therefore be investigated if the evolutionary history of these species is to be unraveled.

In this paper, the authors present the first sequence data to be published of the D2/D3 expansion segments of the large subunit rDNA of *Meloidogyne* species, based on twelve isolates obtained from Brazil and other countries. Our primary purpose is to assess the potential usefulness of D2/D3 sequence data for diagnosis of various Brazilian *Meloidogyne* isolates and for phylogenetic analysis of *Meloidogyne* species in

general. A secondary goal is the assessment of two automated alignment algorithms based on very different premises.

MATERIALS AND METHODS

DNA Extraction

Twelve RKN isolates were used in this study (Table 1). For each extraction one large female was picked out of an infected root and placed on a glass slide with 20 µl of worm lysis buffer (WLB, cf Epstein and Shakes, 1995). Nematode DNA was released by cutting the female with a sterile scalpel and then pipetting the WLB and nematode fragments into a sterile microcentrifuge tube (0.5 ml) kept in ice. One µl of proteinase K (60 µg/ml, MERCK) was added, and each tube was then placed at -80°C for at least 15 min. After freezing, the tubes were incubated for 1 hr at 65°C followed by 10 min at 95°C. The tubes were then centrifuged for 5 min at 13000 rpm and the upper 5 µl of the DNA suspension were transferred to PCR reagent mixture.

Polymerase Chain Reaction (PCR)

All PCRs consisted of 25 µl reagent mixture containing: 13 µl ddH_20; 2.5 µl 10 × reaction buffer (Eurogentec); 2 µl 25 mM $MgCl_2$; 0.5 µl 10 mM dNTPs; 1 µl 25 mM primer D2A; 1 µl 25 mM primer D3B; 0.125 µl Goldstar *Taq* polymerase (Eurogentec); 5 µl template DNA. The PCR primer sequences were as follows: forward primer D2A <5'-ACA AGT ACC GTG AGG GAA AGT TG-3'> and reverse primer D3B <5'-TCC TCG GAA GGA ACC AGC TAC TA-3'> (Courtright *et al.*, 2000). The DNA amplification profile was: 4 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C; and a 10-minute polymerization at 72°C. After the DNA amplification, 5 µl of the PCR product was visualized by gel electrophoresis using 40 ml 1 × TAE buffer in 1% agarose gel (GIBCO BRL, England) stained with 0.003% ethidium bromide (0.02 µg /ml).

DNA Sequencing

To prevent interference by leftover primers and nucleotides, 3 µl of each PCR

Table 1. Overview of the different analyzed species and isolates of *Meloidogyne*, their respective natural hosts and sample origin.

Nematode isolates	Natural host plant	Sample origin
M. arenaria (race 2)	Soyabean (*Glycine max*)	USA
M. chitwoodi	Potato (*Solanum tuberosum*)	The Netherlands
M. exigua	Coffee (*Coffea* sp.)	England
M. exigua (C)	Coffee (*Coffea* sp.)	Brazil
M. exigua (H)	Rubber tree (*Hevea brasiliensis*)	Brazil
M. graminicola	Rice (*Oryza sativa*)	Philippines
M. incognita	Soyabean (*Glycine max*)	USA
M. konaensis	Coffee (*Coffea arabica*)	Hawaii
M. paranaensis (isolate 61)	Coffee (*Coffea arabica*)	Brazil (Nova Esperança, Paraná)
M. paranaensis (isolate 62)	Coffee (*Coffea arabica*)	Brazil (Ourizona, Paraná)
M. paranaensis (isolate 71)	Coffee (*Coffea arabica*)	Brazil (Apucarana, Paraná)
M. trifoliophila	Clover (*Trifolium repens*)	The Netherlands

product were incubated with 0.6 µl of shrimp alkaline phosphatase (2 U/µl, USB) and 0.6 µl of exonuclease I (10 U/µl, Epicentre Technologies) for 15 min at 37°C, followed by 15 min at 80°C. Three µl of cleaned PCR product were transferred to another tube, 4 µl of Big Dye Terminator Cycle Sequencing Ready Reaction mix was added (PE Applied Biosystems, UK), 0.6 µl 5 µM primer, and 2.4 µl sterile ddH_2O with Vf 10 µl. In addition to primers D2A and D3B (see above), internal forward primer D2/F1 <5'-TTC GAC CCG TCT TGA AAC ACG-3'> and internal reverse primer D2/R1 <5'-TCC GTG TTT CAA GAC GGG TCG-3'> were used for sequencing reactions. The program used for all sequencing reactions consisted of 25 cycles of 30 sec at 96°C, 15 sec at 50°C, 4 min at 60°C. The resulting product was precipitated, supernatant was removed with a vacuum suction pipette, and the pellet was dried for 15 min under vacuum. The pellet was redissolved in loading buffer and the samples were loaded in a Perkin Elmer ABI Prism 377 DNA sequencer. The resulting sequence stretches were assembled with Auto Assembler version 1.4 (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and the obtained sequences were deposited in GenBank under Accession numbers AF435793-AF435804.

Sequence Alignments and Analyses

Alignments were constructed using two fundamentally different mathematical algorithms: parsimony-based simultaneous alignment and phylogeny optimization as implemented in TreeAlign version 1990 (Hein, 1990), *versus* progressive multiple sequence alignment as implemented in ClustalX (Thompson *et al.*, 1997). The gap penalty parameters were varied for both alignment programs (Table 2), to verify whether these parameters significantly influenced outcomes of the phylogenetic analyses. All the obtained ClustalX and TreeAlign alignments were first edited in MacClade 4.0 (Maddison and Maddison, 2000) to remove ambiguities due to unequal sequence completeness at the 5' and 3' ends. Each alignment was analyzed with ModelTest (Posada and Crandall, 1998) in order to determine the best evolutionary model(s) and to estimate appropriate parameters. Next, all alignments were used to construct unrooted trees with Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) algorithms as implemented in PAUP* 4.0b10 (Phylogenetic Analysis Using Parsimony and other methods; Swofford, 1999). Additionally, rooted trees were generated by TreeAlign in parallel with its alignment procedure (unlike ClustalX, which only generates alignments).

ModelTest selected two alternative models for each alignment (Table 2) and in each case both of these were applied using ML with heuristic search. MP analyses were performed with 3000 bootstrap replications in heuristic search, and also with exhaustive search to discover the individual most parsimonious trees. NJ analysis employed Log Determinant distance (Lockhart *et al.*, 1994) and 3000 bootstrap replications. PAUP* was used to calculate pairwise absolute distances between all twelve Operational Taxonomic Units (OTUs), for each alignment, and to determine the homogeneity of base frequencies in the different OTUs. Finally, six alignments were tested for occurrence of a molecular clock, using the two-cluster test of the online version of LINTREE (Takezaki *et al.*, 1995; http://shanghai.bio.psu.edu/lintree.html). The alignments chosen were those generated respectively by ClustalX and TreeAlign with the highest, lowest and default gap penalties listed in Table 2.

Table 2. Alignment statistics and measures of the resulting best trees obtained with PAUP*. All g1 values are statistically significant (Hillis and Huelsenbeck, 1992), two Maximum Likelihood models were selected for each alignment using ModelTest (Posada and Crandall, 1998).

Gap opening penalty/ Gap extension penalty	Alignment parameters				Maximum parsimony results				Maximum likelihood results: Model 1 (HKY85+G for all)		Maximum likelihood results: Model 2 (GTR+G for ClustalX, GTR+I for TreeAlign)		Neighbor joining results
	Alignment width	Uninformative characters	Informative characters	g1	Best tree length	Number of best trees	Consistency index	Retention index	-Ln likelihood	Tree length	-Ln likelihood	Tree length	Tree length
ClustalX													
1/1	821	45	75	-0.624799	149	30	0.9128	0.9375	1907.2324	149	1903.00562	149	149
2/1	810	38	92	-0.659993	163	30	0.9018	0.8434	1962.01595	163	1953.52361	163	163
2/2	810	38	92	-0.658678	163	30	0.9018	0.8434	1962.01595	163	1953.52361	163	163
4/1	803	40	95	-0.661433	176	20	0.8807	0.8082	2013.22846	176	2000.85451	176	177
4/2	795	42	100	-0.650331	187	10	0.893	0.9262	2049.4866	187	2036.98359	187	188
4/4	786	37	104	-0.656065	188	10	0.8883	0.82	2040.77071	188	2029.62098	188	189
8/1	783	41	101	-0.648637	183	6	0.9126	0.8595	2000.95022	183	1989.23878	183	184
8/2	782	42	102	-0.629201	191	8	0.8953	0.8311	2049.2739	191	2035.07812	191	192
8/4	782	42	104	-0.627368	200	8	0.885	0.8123	2085.84277	200	2069.08381	200	201
8/8	778	40	104	-0.625789	198	8	0.8838	0.8112	2062.72248	198	2046.01296	198	199
15/6.66	776	36	106	-0.639459	183	2	0.929	0.9578	1966.26179	184	1951.28408	184	184
16/1	776	36	106	-0.638493	183	2	0.929	0.9577	1970.44491	184	1955.71734	184	184
16/2	776	36	106	-0.634135	183	2	0.929	0.9577	1970.44491	184	1955.71734	184	184
16/4	776	36	106	-0.634561	183	2	0.929	0.9578	1966.26179	184	1951.28408	184	184
16/8	776	40	107	-0.631734	195	2	0.9128	0.9457	2038.98728	197	2023.88428	197	196
16/16	779	41	109	-0.628798	198	1	0.9141	0.946	2068.4934	200	2052.76496	200	199
TreeAlign													
3/3	793	28	105	-0.594424	168	2	0.8929	0.9417	1945.41962	168	1932.38488	168	168
6/6	790	31	108	-0.574442	177	1	0.9096	0.9517	1979.24471	178	1962.62892	178	177
8/3	789	28	112	-0.601754	176	1	0.9091	0.9529	1990.3424	177	1976.2651	177	176
8/4	789	32	110	-0.557918	179	1	0.9106	0.9527	1991.25829	180	1976.26979	180	179
16/4	788	27	115	-0.576661	181	1	0.9061	0.8624	2007.41561	182	1993.82942	182	181
16/6	790	31	108	-0.57904	177	1	0.9096	0.8656	1979.24471	178	1962.62892	178	177

RESULTS

Sequence Characteristics

The twelve newly determined sequences of the D2/D3 region of the LSU rDNA gene ranged from 758 to 784 base pairs in size. The D2/D3 region in these isolates was composed of approximately 56% Adenosine or Thymine. The chi-square (χ^2) test indicated that base frequencies were homogeneous, and thus the resulting tree topologies inferred are not expected to suffer from compositional biases. Alignment was not complicated by the occurrence of extremely variable regions with large insertions or deletions. The pairwise distance between one OTU and its nearest relative(s) varied between 0 and 12 differences in ClustalX alignments, except for *M. chitwoodi* which had a minimum character difference of 52 differences from the other OTUs. These character differences increased as the gap penalties increased. TreeAlign was found to be prone to software hangups. It produced trees and alignments successfully for only six of the tested combinations of gap penalties (Table 2), and these alignments differed substantially from those produced by ClustalX (e.g., *M. chitwoodi* had a minimum character difference from other OTUs varying between 0 and 71 differences).

As explained below, two convex clusters (in other words, the clusters could never be resolved as polyphyletic, regardless of potential root placement) of OTUs were obtained. These clusters are henceforth referred to as A and B (Fig. 1). Depending on gap penalties, cluster A had pairwise distances between its OTUs ranging from 1 to 81 differences. Within this cluster, from 1 to 9 nucleotide differences were detected between the three isolates of *M. exigua*. The pairwise differences among the OTUs in cluster B were much lower, varying from 0 to 9 differences.

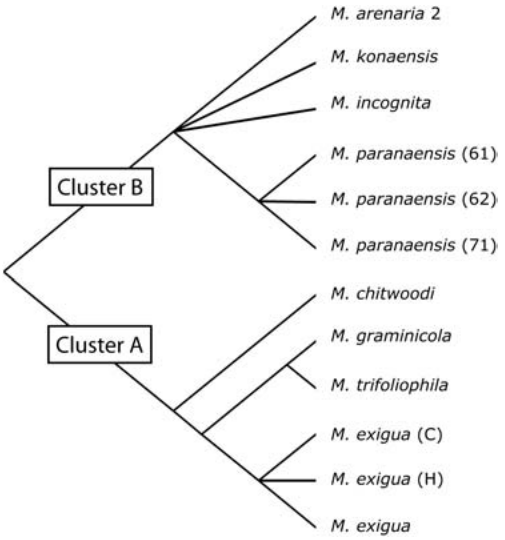

Fig. 1. Strict consensus trees of all topologies produced with PAUP* based on ClustalX alignments and rooted in accordance with the small subunit phylogenies of Tandingan De Ley *et al.* (2002).

Alignment Characteristics and Signal Strength

The phylogenetic analyses of the twelve OTUs produced results that differed in some aspects according to their alignment origins (TreeAlign or ClustalX), but were similar in overall topology. A strong phylogenetic signal was present in our data, as also supported by the obtained values of *g1* (Table 2). In the case of cluster B, however, only 4 parsimony informative characters were present, reflecting the very high degree of similarity between the majority of included sequences. As a result, ClustalX alignments with low gap penalties yielded up to 30 best trees with MP (Table 2), typically differing from each other in topology of cluster B only. The molecular clock hypothesis was rejected by LINTREE for all six tested alignments, with moderate to very high significance (data not shown).

Tree Topologies Obtained with ClustalX Alignments

All of the trees produced resolved the three *M. paranaensis* populations as a convex clade in Cluster B, while the three *M. exigua* populations were placed as a convex clade in Cluster A. Additionally, *M. graminicola* and *M. trifoliophila* were consistently placed as sister taxa, and formed a convex clade with *M. exigua*. This arrangement of convex groups was largely supported by high bootstrap values. Within cluster B, however, relationships between *M. arenaria*, *M. konaensis*, *M. incognita* and *M. paranaensis* were not resolved consistently across different alignments and algorithms. A strict consensus tree of all topologies produced from the ClustalX alignments is shown in Fig. 1, and an example of topology produced with MP from one alignment is shown in Fig. 2.

Tree Topologies Obtained with TreeAlign Alignments

Neither the populations of *M. paranaensis* nor those of *M. exigua* were consistently resolved as convex groups in trees produced by TreeAlign itself, or in trees produced in PAUP* from TreeAlign alignments. A strict consensus tree of all topologies was largely unresolved, except for the occurrence of clusters A and B (data not shown). Trees produced by TreeAlign itself were consistently rooted between clusters A and B. As noted above, trees produced in PAUP* with TreeAlign alignments were analyzed without assigning outgroups.

DISCUSSION

General Features of the Obtained Topologies

The rDNA cistron provides an excellent target for phylogenetic analysis because it is present in many copies in the genome of an organism. From the extensive LSU rDNA gene, the D2/D3 region was chosen because it consists of a tandem of 2 variable regions alternating with more conserved parts of the gene. However, as will be discussed below, these variable regions did not always prove distinctive enough to differentiate between all the included *Meloidogyne* spp. Comparisons made between the tree topologies obtained with different algorithms (TreeAlign, ClustalX and PAUP*) are concordant in overall grouping of the included OTUs into two clusters. Cluster A contains all of the meiotic parthenogenetic *Meloidogyne* spp. studied, while cluster B includes all of the mitotic parthenogenetic species sequenced. The results presented here are largely consistent with the identification and characterization of *Meloidogyne* spp. using enzymatic relationships (Esbenshade and Triantaphyllou, 1987), repeated-DNA homologous probe methods (Piotte *et al.*, 1992), PCR methods (Powers *et al.*, 1997; Williamson *et al.*, 1997), and specific probes (Petersen *et al.*, 1997). Likewise, our results are consistent with some existing phylogenies deduced from repetitive DNA analysis (Castagnone-Sereno *et al.*, 1993, Van Der Beek *et al.*, 1998), and rDNA comparisons made by Blok *et al.* (1997) and Hugall *et al.* (1999).

Species Groups and Limits of Resolution

One of our incentives for analyzing the present dataset was the possible usefulness of D2/D3 data in confirming or rejecting the validity of *M. paranaensis*, as well as assessing the relationship between two *M. exigua* populations from coffee and one population from rubber trees. Based on the analysis of small subunit rDNA sequences by Tandingon, De Ley *et al.* (2002), we can place the root with some confidence between clusters A and B in our

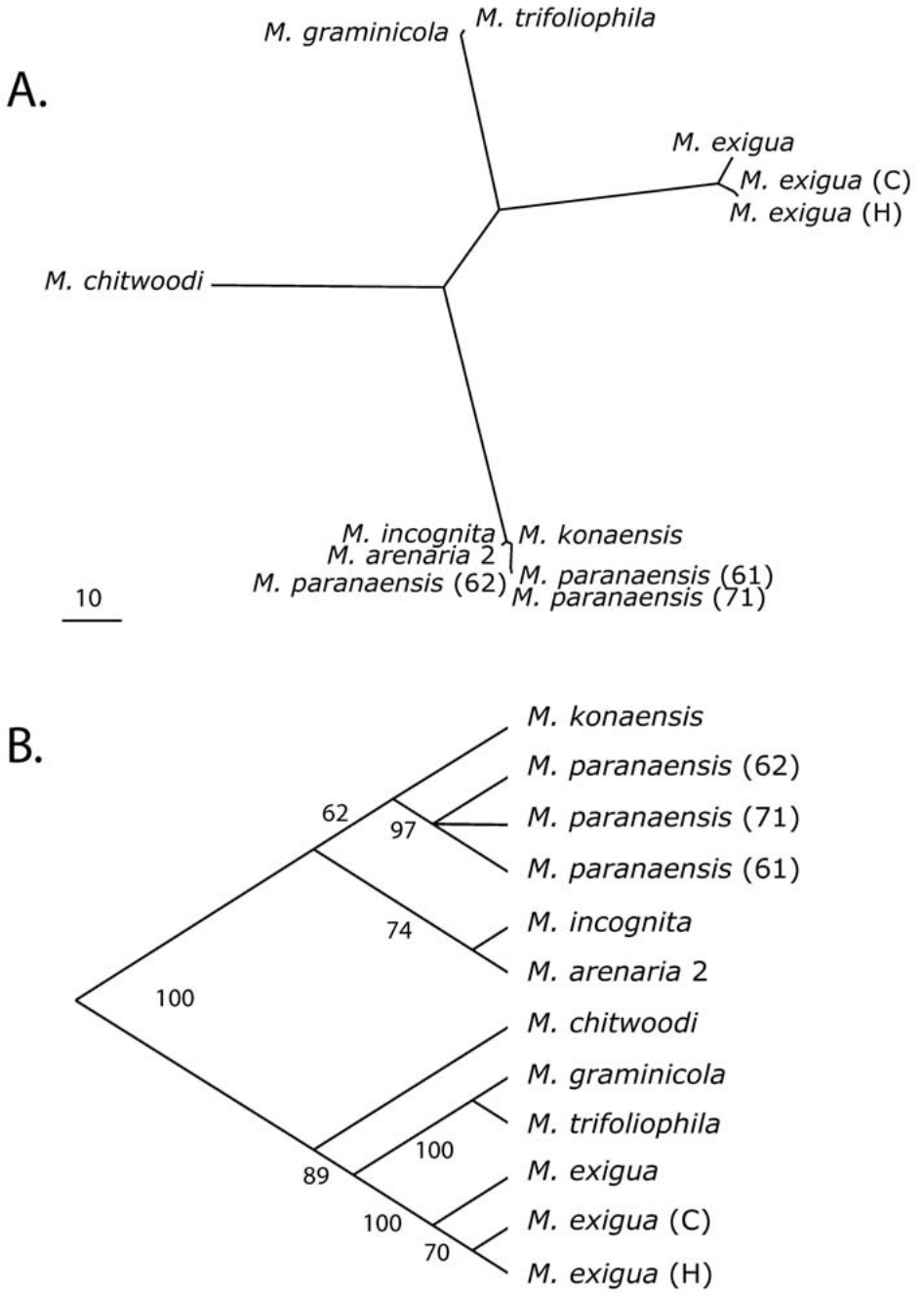

Fig. 2. Example of one particular tree topology produced with PAUP* based on ClustalX alignments. A. Unrooted exhaustive search Maximum Parsimony phylogram derived from the ClustalX alignment with default penalty settings (gap opening penalty = 15, gap extension penalty = 6.66); scale bar equals 10 differences. B. The same tree shown as a cladogram rooted in accordance with the small subunit phylogenies of Tandingan De Ley *et al.* (2002); numbers next to branches are MP bootstrap values from a corresponding heuristic search with 3000 replicates.

unrooted D2/D3 trees derived from ClustalX alignments. According to this position of the root, the three isolates of *M. paranaensis* form a monophyletic clade in all trees derived from ClustalX alignments, independent of the phylogenetic method chosen. The *M. exigua* isolates also cluster together monophyletically in topologies based on ClustalX alignments. In contrast, neither species is consistently resolved as a monophyletic clade in trees derived from TreeAlign. We interpret this as evidence for poorer performance by TreeAlign compared to ClustalX, and not as grounds for assuming that any of the isolates studied might actually represent a species other than *M. paranaensis* or *M. exigua*.

Usefulness of D2/D3 Sequence Data in Meloidogyne: A First Evaluation

The results obtained can be considered fairly robust in the constructed nucleotide sequence alignments and the obtained tree topologies. In more detail, it seems that the alignments and tree construction method of ClustalX and PAUP* (combined) produce evolutionary histories that are more consistent with existing species diagnoses than those obtained with TreeAlign. The latter algorithm enforces a molecular clock and is therefore susceptible to artefacts with a dataset like ours, in which the molecular clock hypothesis does not apply. Our analyses include the recently proposed species *M. paranaensis* (Carneiro *et al.*, 1996), represented here by three isolates obtained from municipalities that are between 20 and 100 km apart (Table 1). Although the ClustalX alignments support *M. paranaensis* as a separate clade, the mitotic parthenogenetic species *M. arenaria* (race 2), *M. incognita*, *M. konaensis*, *M. paranaensis* are actually all extremely similar to one another in D2/D3 sequence (as is e.g., evident from the branch lengths in Fig. 2A). It is therefore possible that at least some of these are synonymous species, but we consider it more likely that the D2/D3 region is simply too conserved for the phylogenetic analysis of mitotic parthenogenetic *Meloidogyne* species.

Our results contrast with those obtained by De Ley *et al.* (1999) with D2/D3 sequences of morphologically cryptic *Acrobeloides* species, which nevertheless exhibited 31-45 differences despite being nearly indistinguishable under the light or scanning electron microscope. Addressing much more divergent nematode taxa, Litvaitis *et al.* (2000) applied D3 sequence data for inference of phylogenetic relationships among adenophorean groups. However, they may have investigated evolutionary divergences that are too distant in the past, rather than too recent, especially when using sequences only about half as long as our own data.

The diagnostic and phylogenetic usefulness of the D2/D3 region clearly depends on how quickly or slowly this region evolved within the investigated taxa. In our study, evolutionary rates were higher for some nematode lineages than for others, and/or lineages diverged longer ago, and so more substitution differences are likely to have accumulated in this region (cf. the results obtained by Blaxter *et al.*, 1998 for small subunit rDNA). As for the *Meloidogyne* study presented here, D2/D3 appears to be a more appropriate marker for relationships between species groups than between individual species. Compared to ITS, the apparent absence of polymorphisms is a methodological advantage, but diagnostic resolution of D2/D3 is insufficient to discriminate between some of the most closely related, most problematic and economically most damaging species. Although good phylogenetic resolution can be obtained for more distantly related species, relationships within species groups cannot

always be resolved, and it may be more appropriate to analyze these with multiple loci and/or with longer sequence stretches.

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