On the Species Status of the Root-Knot Nematode *Meloidogyne ulmi* Palmisano & Ambrogioni, 2000 (Nematoda: Meloidogynidae)

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On the species status of the root–knot nematode *Meloidogyne ulmi* Palmisano and Ambrogioni, 2000 (Nematoda: Meloidogynidae)

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Summary

The root–knot nematode *Meloidogyne ulmi* is synonymised with *Meloidogyne mali* based on morphological and morphometric similarities, common hosts, as well as biochemical similarities at both protein and DNA levels. *M. mali* was first described in Japan on *Malus prunifolia* Borkh.; and *M. ulmi* in Italy on *Ulmus chenmoui* Cheng. Morphological and morphometric studies of their holo- and paratypes revealed some important similarities in certain characters as well as some general variability in some others. Host test also showed that besides the two species being able to parasitize the type hosts of the other, they share some other common hosts. Our study of the esterase and malate dehydrogenase isozyme phenotypes of some *M. ulmi* population gave a perfectly comparable result to that already known for *M. mali*. Finally, phylogenetic study of their SSU and LSU rDNA sequence data revealed that the two are not distinguishable at DNA level. All these put together, leave strong evidences to support the fact that *M. ulmi* is not a valid species, but a junior synonym of *M. mali*. Brief discussion on the biology and life cycle of *M. mali* is given. An overview of all known hosts and the possible distribution of *M. mali* in Europe are also presented.

**Keywords**: Morphological, morphometrics, esterase, malate dehydrogenase, Japan, Italy, Mierenbos, *Malus prunifolia, Ulmus chenmoui*, SSU rDNA, LSU rDNA.
The genus *Meloidogyne* comprises all root–knot nematodes. It contains over 100 described species (Karssen & Moens, 2006). Its members are without a doubt the most widely distributed of all plant–parasitic nematodes (Sasser, 1977). This widespread distribution and their economic importance are primarily the reasons why the genus has been the subject of more research than any other plant–parasitic nematodes, including the cyst–forming nematodes (Sasser & Carter, 1982). Despite the numerous studies about their biology and taxonomy, their identification to the species level still pose a huge challenge to many diagnosticians (Blok & Powers, 2009) mostly because of the very small inter–specific morphological variation (Jepson, 1987).

In 2000, Palmissano and Ambrogioni described from Italy the root–knot nematode, *Meloidogyne ulmi*, from *Ulmus chenmoui* Cheng on which it was found to induce large galls. For many years, elm remained the only known host of *M. ulmi*. According to the authors, the tree at the type locality was introduced from the Netherlands as part of a breeding programme focussed on resistance to the Dutch elm disease (DED). The Netherlands, like many other countries in Europe, North America and New Zealand, has for years been battling against the notorious Dutch elm disease, caused by sac fungi named *Ophiostoma ulmi* (Buisman) Melin & Nannf. (1934) and *O. novo–ulmi* Brazier 1991. These fungi are vectored by elm bark beetles, *Scolytus multistriatus* Marsham, 1802, *Scolytus schevyrewi* Semenov Tjan–Shansky, 1902 or *Hylurgopinus rufipes* Eichhiff, 1868, which deposit their eggs in the bark of the elm tree. Once the fungi infect the xylem of the tree, the latter closes all its stomata as an immune response to these fungi. The response from the tree results in the termination of sap flow within the tree, usually leading to its death (Heybroek *et al.*, 2009).

It was for this reason that the former Dutch phytopathological laboratory “Willie Commelin Scholten” (WCS), based in Baarn, was mandated with the research on Dutch elm disease. This marked the first ever breeding programme on elm for selection of resistant cultivar. Later on the breeding programme was moved to Wagningen at the former institute the Dorschkamp Research Institute for Forestry & Landscape Planning; and in this programme, elm trees from all over the world were tested. Trial field “Mierenbos”, a part of the Dorschkamp Research Institute for Forestry & Landscape Planning, was used for growing and improving resistant elm cultivars. It was from this trial field that resistant elm seedlings were sent to ten other European countries at the end of the breeding programme, among others, Italy in 1992 (Heybroek, 1993).
The first observation of galls on elm trees was already in 1960 at Baarn, and the associating nematode was diagnosed as *M. arenaria* (Neal, 1889) Chitwood, 1949 by the Dutch Plant Protection Service (Oostenbrink, 1961). Interestingly, about that same period, a *Meloidogyne* species found parasitizing apple trees in Japan was also inadvertently misidentified as *M. arenaria*, because this nematode species, like the one on elm bore some resemblance to *M. arenaria* perineal patterns (Itoh et al., 1969). This Japanese species would later be described and named *M. mali* by Itoh et al. (1969). In his comprehensive study on the host range of *M. mali*, Toida (1979) associated this species with several other plant species belonging to different families, particularly the Rosaceae. Following its description, several studies have also been conducted on its taxonomy, ecology, damage and control (Inagaki, 1978), SEM studies of male and second–stage juvenile head morphology, and morphological variability of its different populations (Okamoto et al., 1983). At the trial field “Mierenbos”, the first report of galling symptoms on *Ulmus* trees was in 1979 (Brinkman, 1980). Presently, all the *Ulmus* trees there are infected with *Meloidogyne* and are showing severe symptoms of root galling (Karssen et al., 2008 & Karssen, 2009).

In 2006, root samples of the dying type host apple containing *M. mali* from the type locality in Japan sent by Dr. Takayuki Mizukubo were received at the Dutch Plant Protection Service. About the same time, galled root samples of the type host of *M. ulmi* were obtained from Italy. They were propagated and maintained on the *Ulmus x hollandica* variety “Wredei”. Juveniles isolated from the Japanese apple root samples were used for sequencing and the resulting SSU rRNA sequence was discovered to be almost identical to that of *M. ulmi* (Holterman et al., 2009) Additionally, isozyme phenotypes of *M. ulmi* population from the trial field in Wageningen were also compared to that of *M. mali* from Japan (Karssen, unpublished; Sakai & Mizukubo, 2009). Those also revealed similar patterns of esterase and malate dehydrogenase to that obtained for *M. mali* (Sakai & Mizukubo, 2009). With these observed similarities, a closer look needed to be taken into these two species. Based on the evidences available to us now, we postulate that *M. ulmi* entered Europe as *M. mali* through elm rootstocks imported from Japan. Supporting this is the report that *M. mali*, in addition to its numerous hosts, can also infect *Ulmus davidiana* var. *japonica* (Toida, 1979).

The original description of *M. ulmi* differentiates it from *M. mali* on the basis of characters that generally show high intraspecific variations. With the original description being the only paper written about *M. ulmi*, all the known features so far are ones from the original
description. On *M. mali*, however, there have been quite a lot of research on the hosts, life cycle, ecology, detailed morphology, as well as their variations within species (Toida, 1979; Inagaki, 1978; Okamoto et al., 1983).

The objectives of this current research, therefore, are:

1. To evaluate the morphological similarities between *M. mali* and *M. ulmi*;
2. To search for other host plants, than *Ulmus* sp, present at the trail field “Mierenbos”;
3. To test *M. ulmi* on selected host plants on which *M. mali* is already known to reproduce;
4. Analyze their biochemical similarities, at the protein and DNA levels.

**Materials and methods**

**MORPHOLOGY AND MORPHOMETRICS**

Paratype slides of *Meloidogyne ulmi* used for morphological and morphometric studies were obtained from Dr. Z.A. Handoo of USDA. In addition to these we obtained *M. mali* specimens on slides taken to the USDA by Dr. Ichinohe during his visit in 1958 as well as additional specimens of males, second–stage juveniles and females stored in formalin that were only recently isolated from root samples sent to USDA by Ichinohe during that same visit. Also, by courtesy of Dr. Hiromichi Sakai and Shigeyuki Sekimoto, some paratypes that were deposited at the National Agriculture and Food Research Organization, Agricultural Research Center (Kannondai, Tsukuba, Ibaraki, Japan), the then Central Agricultural Experiment Station were also obtained. All slides were observed using compound light microscope (DM 2500, LEICA) equipped with differential interference contrast (DIC), and camera (DC 300F, LEICA) for taking images. Comparisons of morphological and morphometric characters were based on the most differential characters, previously used by Karssen (2002).
**Table 1.** The various forms of type specimens of *Meloidogyne mali* and *M. ulmi* studied and their sources

| Form       | Meloidogyne mali | Meloidogyne ulmi |
|------------|------------------|------------------|
|            | Sex/stage        | Source           | Sex/stage | Source               |
| Holotype   | 1 female         | Shigeyuki Sekimoto | –         | –                    |
| Allotype   | 1 male           | Shigeyuki Sekimoto | 2 PP & 1  | Dr. Zafar A. Handoo  |
| Paratype   | 17 perineal      | Dr. Zafar A. Handoo | anterior part | Dr. Zafar A. Handoo |
| Paratype   | 3 males          | Dr. Zafar A. Handoo | 2 males   | Dr. Zafar A. Handoo  |
| Paratype   | 4 juveniles      | Dr. Zafar A. Handoo | 3 juveniles | Dr. Zafar A. Handoo  |
| Paratype   | 1 male           | Dr. Hiromichi Sakai |           |                      |
| Paratype   | 1 juvenile       | Dr. Hiromichi Sakai |           |                      |

**HOST TEST**

This test is a combination of sampling undertaken in 2011 and 2012 at “Mierenbos” on several plant species and a subsequent greenhouse experiment involving some important plant species already associated with *M. mali* in previous studies (Itoh *et al.*, 1969; Toida, 1979). Host herein is defined as a plant the nematode can penetrate and reproduce on.

**Table 2.** Plant species included in the host plant test with *M. ulmi* in the greenhouse

| Family       | Genus + species                       |
|--------------|---------------------------------------|
| Brassicaceae | *Brassica oleracea* L. var. Gemmifera (cabbage) |
|              | *Brassica pekinensis* (Lour.) Rupr. (celery cabbage) |
| Rosaceae     | *Malus sylvestris* Mill. "M9" (apple) |
|              | *Rosa hybrida* L. (rose)               |
| Fabaceae     | *Trifolium repens* L. (white clover)   |
| Solanaceae   | *Solanum lycopersicum* L. (tomato)     |
| Ulmaceae     | *Ulmus glabra* Huds. (Wych elm)        |

**ISOZYME ANALYSIS**

Esterase and malate dehydrogenase isozymes were analysed for *M. ulmi* sampled at “Mierenbos”, following the method described by Karssen *et al.* (1995). In summary, young females were isolated from roots into an isotonic (0.9% salt) solution. This was followed by a desalting step which involved transfer of the females from the NaCl solution to a reagent–grade water on ice for few minutes. Females were then singly transferred into sample wells.
containing 0.6 µl extraction buffer. With the aid of a small glass rod, the females in the wells were crushed; and the macerated females were then loaded into sample applicators (0.3 µl per well). All twelve wells, with the exception of 6 and 7, were loaded with our test samples of *M. ulmi*. *M. javanica* was used as reference in wells 6 and 7.

Electrophoresis was run using the PhastSystem (Pharmacia Ltd, Uppsala, Sweden) and the gels were subsequently stained in a Petri dish and placed in an incubator at 37 °C. Staining for non–specific esterase (Est; EC 3.1.1.1) was allowed to stand for 60 minutes whiles that for malate dehydrogenase (Mdh; EC 1.1.1.37) stayed for 5 minutes.

Following staining, the gels were rinsed with distilled water and fixed for 5 minutes in a 10% acetic acid / 10% glycerol / 80% distilled water solution. Pictures of the gels were taken by placing them on a glass surface illuminated from below.

**Molecular Analysis**

Already published sequences of both *M. mali* and *M. ulmi* (Holterman et al., 2009) together with our own sequence of the latter were included in this analysis.

**DNA Extraction**

Nucleic acids were isolated from single male or second–stage juveniles of *M. ulmi* populations taken from “Mierenbos” and type populations kept in culture at the Dutch Plant Protection Service on an elm tree (*Ulmus x hollandica* Mill “Wredei”). Genomic DNAs were isolated from these samples using High Pure PCR Template Preparation Kit (www.roche–applied–science.com, Cat. No. 11796828001, Version 16.0) protocol for isolation of nucleic acids from Mammalian Tissue with slight modification in the first step to suit nematode DNA isolation (150 µl tissue lysis buffer added to 50 µl sterile water containing nematodes, minimum protease incubation time of 16 hrs and elution volume of 50 µl).

**PCR and Sequencing**

Amplification of 1000 base pairs (bp) of the large ribosomal subunit (LSU) (28S) was performed using primer set 28–81for (forward) 5’–TTAAGCATATCATTAGCGGAGGA–3’ and 28–1006rev (reverse) 5’–GTTTGATTAGTCTTTGCCCCT–3’ described by Holterman et al. (2008).
To amplify the nearly full length sequence of the small ribosomal subunit (SSU) (18S), two partially overlapping fragments were generated using three universal primers and one nematode–specific primer (1912R) described by Holterman et al. (2006). The latter’s inclusion was to avoid amplification of non–target eukaryotic SSU rDNA, for example from fungal spores attached to the nematode cuticle. The primers 988F (forward) (5’–CTCAAAAGATTAAGCCATGC–3’) and 1912R (reverse) (5’–TTTACGGTCAGAATAGGG–3’) were used to amplify the first fragment. The second fragment was amplified with primers 1813F (forward) (5’–CTCGGTGAGAGGTGAAAT–3’) and 2646R (reverse) (5’–GCTACCTTGTTACGACTTTT–3’). Each PCR reaction mixture contained Molecular Grade Water (MGW) – DNase RNase free water (Sigma–Aldrich, Saint Louis, USA), 1x PCR buffer (incl. 2.0 mM MgCl₂, Roche), dNTPs (0.2 mM each), 0.24 µM of each primer, FastStart Taq DNA polymerase (1 U, Roche) and 1 µl of the template DNA. The final reaction volume was 25 µl. PCR was performed in C1000 touch thermal cycler (Bio–Rad) with the following amplification condition: 15 min at 95°C; 5 cycles of 30 sec at 94°C, 30 sec at 45°C and 30 sec at 72°C; 35 cycles of 30 sec at 94°C, 30 sec at 54°C and 30 sec at 72°C; final extension for 5 min at 72°C. To test for amplification and the quality of PCR products, 5 µl of the PCR products mixed with 1 µl 6x Bromophenol Blue Loading solution (Promega, Madison, USA) were subjected to electrophoresis and SYBR safe (Invitrogen, Carlsbad, USA) staining on a 1.5 % agarose gel by standard methods (Sambrook et al., 1989) along with a 1kb–plus DNA ladder (Invitrogen, Carlsbad, USA) to size fragments. PCR products are imaged under UV light using a GeneGenious gel imaging system (Syngene, Cambridge, United Kingdom).

PCR products were purified after amplification using QIAquick PCR Purification Kit (Qiagen), and the genomic DNA concentration measured using a ND1000 spectrophotometer (NanoDrop). This was followed by a cycle sequencing reaction in a final volume of 20 µl (molecular grade water (Sigma–Aldrich, Saint Louis, USA), BigDye Terminator v1.1, 1x sequencing buffer, purified PCR product and 0.5 µM template–specific forward or reverse primers). Cycling reactions were carried out separately for each of forward and reverse primers. The reaction programme was set for 1 min at 96 °C, 25x (10 sec 96 °C, 5 sec 50 °C, 2.5 min 60 °C), 1 min 20 °C. The cycle sequence products were cleaned up using DyeEx 2.0 Spin Kit (Qiagen) and run on a multi–capillary 3500 Genetic Analyzer DNA sequencer (Applied Biosystems, Carlsbad, USA).
SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

Trace files of D2–D3 expansion segments of 28S and 18S–rRNA genes were assembled into contigs and amplification primer sequences trimmed using Geneious 6.1.6 (Biomatters New Zealand). Additional trimming was performed when needed, to obtain high quality consensus sequence data. Conflicts in the consensus sequence were assessed visually and corrected where possible. The sequences were aligned with selected sequences of other species from GeneBank using MAFFT alignment (Katoh et al., 2002) within the programme Geneious 6.1.6 (Biomatters New Zealand) for both 28S and 18S–rRNA. Alignments were improved manually. Analysis of phylogeny of the sequence data set was performed with Bayesian inference (BI) using MrBayes 3.2.1 (Huelsenback & Ronquist, 2001). The optimal model for nucleotide substitution was obtained using JModelTest ver. 2.1.3 (Darriba et al., 2012) with AIC, AICc, BIC and DT defaulted in JModelTest. For SSU sequence, analysis of Bayesian inference was performed with a random starting tree and four Markov chains for 1 x 10^6 under the model SYM I + G. Trees were sampled at interval of 100 generations. Two independent runs were performed for each analysis. The first 100,000 generations were discarded as burn–ins, and the remaining trees combined to generate 50% majority rule consensus tree which represent posterior probabilities. The same parameter settings were used for LSU phylogenetic analysis, but under the model GTR + G.

Results

MORPHOLOGY

The following are the observations made on selected features considered to be the most differential for species discrimination among members of the genus *Meloidogyne* (Jepson, 1987; Karssen, 2002). Table 3 shows a comparison of our observations of the most important characters with the ones mentioned in the original descriptions.

Female

Perineal pattern

The general shape of the perineal pattern in both species studied ranged from low rounded to oval. The dorsal arch of *M. mali* and *M. ulmi* was mostly low rounded with very few instances where some specimens showed relatively high square patterns. Lateral field was
marked by change in direction or breaks in striae resulting in what would appear as weak lateral lines. The double lateral lines mentioned in the description of \textit{M. mali} were not observed in the studied specimens. The interphasmidial distance in both species was about the same as their corresponding vulva slit lengths. As mentioned in the description, the phasmids were distinct but did not appear large when observed at the correct focus. However, attempting to observe them at the same (relatively deeper) focus as the vulva slit makes them look larger and even farther apart than they really are, due to the diagonally sloping phasmid canals.

\textbf{Stylet}

Same variations in stylet knobs shape as described in \textit{M. mali} were observed for \textit{M. mali} paratypes i.e. slightly backwardly sloping to anteriorly concave, with the former being the more frequent. Such variations, however, cannot be mentioned about the \textit{M. ulmi} paratype since there was only a single anterior part of the female on the slides we obtained. We therefore supplemented it with specimens taken from samples from the “Mierenbos”, where the type host originated from. This population showed similar variation as described for \textit{M. mali}, but not reported in table 3. Our observation of the shape of the stylet itself was typical of the genus, i.e. straight shaft with a slightly dorsally curved cone.

\textbf{Secretory–excretory pore}

S–E pore position measured from the anterior end showed quite some variations. Nevertheless, all measurements taken for both species fell within the range described for \textit{M. ulmi}. This character in \textit{M. mali} description was measured on the basis of the number of annuli counted from the anterior end to the one bearing the S–E pore.
On the species status of Meloidogyne ulmi

Fig. 1. LM photograph of perineal pattern and anterior parts of female Meloidogyne mali (A,C,E,G) and Meloidogyne ulmi (B,D,F), bar = 10 µm.
Fig. 2. LM photographs of males anterior part and second stage juvenile tails of *Meloidogyne mali* (A, C, E) and *Meloidogyne ulmi* (B, D, F), bar = 10 µm.
**Table 3.** Observations of the differential characteristics of female, male and second–stage juveniles of *Meloidogyne mali* and *M. ulmi* types in comparison with their interpretation in the original description

| Character | *M. mali* | *M. ulmi* |
|-----------|-----------|-----------|
| **Female** |           |           |
| Stylet knobs | Well developed knobs that tend to slope backward or forward in the ratio of 16 to 8 | Rounded to pear–shaped knobs, set off and slightly anteriorly concave to backwardly sloping | Knobs rounded to transversely ovoid, slightly concave anteriorly | Rounded knobs that are slightly anteriorly concave and offset |
| Perineal pattern | Oval, made up of smooth striae, finely spaced, dorsal arch low and flat. Phasmids large, lateral field clearly marked with single or double incisures | Oval, dorsal arch low to slightly high, rounded to square shaped. Phasmids distinct. Lateral field marked by breaks in the striae or showing indistinct lateral lines. | Oval, dorsal arch flattened to medium high, rounded or somewhat square, phasmids conspicuous, lateral field indistinct or marked by folds, sometimes by lateral lines on one or both sides. | Oval, dorsal arch low to slightly high, rounded to square shaped. Phasmids distinct. Lateral field marked by breaks in the striae or showing indistinct lateral lines. |
| **Male** |           |           |
| Head shape | Head weakly offset, head cap low and slightly narrower than the postlabial region. Postlabial incisures absent. | Head slightly set off, labial cap shallowly rounded, one–fifth to one–fourth as high as postlabial region. | Head weakly offset, head cap low and slightly narrower than the postlabial region. Postlabial incisures absent. |
| Stylet knobs | Knobs rounded | Backwardly sloping with rounded to pear shaped knobs | Knobs rounded to pear shaped more or less backwardly sloping | Backwardly sloping with rounded to pear shaped knobs |
| **Second–stage juvenile** |           |           |
| Stylet knobs | Knobs backwardly sloped | Small rounded knobs, slightly backwardly sloping | knobs rounded and set off from shaft | Small rounded knobs, backwardly sloping |
| Tail shape | Short | Conical with a broad to finely pointed tip | Conical, tapering to a finely rounded almost pointed terminus or broader and rounded at the tip | Conical and tapers to a broadly or finely pointed tip |
| Hyaline tail part | Constrictions present along hyaline part, length short or long. Anterior part clearly delimited | Cuticular constrictions present along hyaline tail terminus, variable in length | Constrictions present along hyaline part, Anterior part clearly delimited. |
Table 4. Morphometrics of *M. mali* and *M. ulmi* females in comparison with the original descriptions. All measurements are in µm and in the form: mean ± sd. (range).

| Species                  | Character | Meloidogyne mali | Meloidogyne ulmi |
|--------------------------|-----------|------------------|------------------|
|                          | Described | Observed         | Described        | Observed         |
| N                        | 25        | 17               | 30               | 2                |
| Body length              | 847       | 762 ± 115        | 771 ± 140        |                 |
|                          | (684–1044)| (608–890)        | (568–1043)       |                 |
| Body width               | 660       | 570±122          | 618±152          |                 |
|                          | (540–864)| (372–700)        | (357–1007)       |                 |
| Neck length              | 166 ± 43.7| 165 ± 62        | 165 ± 67         | 205              |
|                          | (90–252) | (60–265)         | (58–382)         |                 |
| Neck diameter            | –         | 100 ± 34.3       | –                | 152              |
|                          |           | (48–160)         |                 |                 |
| Stylet length            | 15        | 11.9±1.8         | 14.2 ± 1.0       | 13.4             |
|                          | (13–17)  | (7.7–15.4)       | (12.0–15.7)      |                 |
| Stylet knob height       | –         | 1.6 ± 0.3        | 1.8 ± 0.6        | 1.6              |
|                          |           | (1–2.2)          | (1.1–3.9)        |                 |
| Stylet knob width        | –         | 3.2 ± 0.4        | 3.5 ± 0.7        | 3.2              |
|                          |           | (2.6–3.8)        | (2.6–5.2)        |                 |
| DGO                      | 5.5       | 4.3 ± 1.5        | 4.6 ± 0.8        | 3.9              |
|                          | (4–7)     | (2.2–6.7)        | (3.3–6.5)        |                 |
| S–E pore                 | –         | 32.8±5.5         | 32.3 ± 7.8       | 36.5             |
|                          |           | (25–43.5)        | (15.7–45.1)      |                 |
| Metacorpus               | 110       | 103±7.9          | –                | 92.8             |
|                          | (90–147) | (90–117)         |                 |                 |
| Metacorpus length        | 39        | 40.4 ± 4.5       | 42.6 ± 6.5       | 39.7             |
|                          | (32–44)  | (32–50)          | (32.7–58.8)      |                 |
| Metacorpus diameter      | 49        | 39.7 ± 6.5       | 40.9 ± 7.0       | 36.5             |
|                          | (40–73)  | (29–47)          | (31.3–59.0)      |                 |
| Metacorpus valve length  | 12        | 13.0 ± 1.1       | 12.4 ± 1.0       | 17.9             |
|                          | (11–13)  | (11.5–15.4)      | (11.1–14.4)      |                 |
| Metacorpus valve width   | 10        | 9.5 ± 1.3        | 9.7 ± 1.4        | 10.2             |
|                          | (9–11)   | (7–11.2)         | (7.2–12.4)       |                 |
| Vulva – anus distance    | 17 ± 1.8  | 19.1 ± 2.6       | 19.0 ± 1.9       | 19.2 ± 0.8      |
|                          | (14–22)  | (12.8–22.4)      | (15.0–22.2)      | (18.8–19.8)     |
| Interphasmidial distance| 22 ± 3.5  | 24.8 ± 4.7       | 19.2 ± 3.8       | 22.4 ± 4.5      |
|                          | (17–29)  | (17.6–35.2)      | (13.7–28.9)      | (19.2–25.6)     |
| Level of phasmids to     | 25 ± 2.4  | 27.4 ± 2.8       | 25.1 ± 4.2       | 27.6 ± 0.9      |
| vulva                    | (19–31)  | (24–33.9)        | (15.7–39.2)      | (26.9–28.2)     |
| Level of phasmids to     | –         | 8.1 ± 2.5        | 6.9 ± 2.2        | 8.7 ± 1.3       |
| anus                     |           | (5.1–12.8)       | (2.6–15.7)       | (7.7–9.6)       |
| Vulva slit length        | 18 ± 2.5  | 24.4 ± 3.3       | 22.0 ± 2.9       | 24.5 ± 1.1      |
|                          | (12–24)  | (16–28.2)        | (17–28.7)        | (23.7–25.3)     |

*Two perineal pattern and a single anterior part

**Male**

**Head region**

Under light microscope, both species have the same head outline. This was already illustrated in the descriptions of the two species (Itoh *et al.*, 1969; Palmissano & Ambrogioni, 2000). The head cap in both species is low. The presence of lip annuli mentioned in *M. ulmi* was observed on some of the paratypes studied as well as in some of the additional specimens.
included later from “Mierenbos”. The post–labial cephalic region slightly set off from the remainder of the body. SEM observation of the en face view of the lip region was not part of this study. Nevertheless, this will be discussed further on in this work based on previous study conducted by Yaegashi and Okamoto (1981) as well as the original description of *M. ulmi*.

**Stylet**

The stylet moderately slender. Conus with bluntly pointed tip. The shaft width the same along its entire length, although in some specimens it appeared to be broader close to the junction with the knobs. Individual knobs rounded to pear shaped. Knobs backwardly sloping in both species.

**Lateral field**

The lateral field marked by four incisures. In most of the specimens studied, the outer lines appeared areolated along most part of the body. No difference in the number of lateral incisures was observed along the body, except at the anterior part where it reduces to two and gradually fades out further anterior.

**Hemizonid position relative to S–E pore**

Although not considered to be of any diagnostic significance, this character remained fairly consistent in all specimens studied. The hemizonid always occurred anterior to the S–E pore, at slightly varying distances.

**Second–stage juveniles**

Examination of the second–stage juvenile characters was based on six *M. mali* type specimens and two of *M. ulmi*.

**Head region**

Head slightly set off from the rest of the body, with a low lip. Post–labial region lacking any annule.

**Stylet**

Stylet somewhat slender, with conus terminating in a fine tip, in both species. Stylet knobs small and rounded; slightly backwardly sloping.

**Hemizonid position relative to S–E pore**

Contrary to the condition in males, hemizonid always located behind the S–E pore in second–stage juveniles. However, the exact position is variable.
Tail
Tail mostly straight, ranging from short to medium; with a fine to bluntly rounded tip. Hyaline tail terminus with varying length, anterior part distinctly delimitated.

MORPHOMETRICS

Females

It may be statistically incorrect to make any meaningful comparisons between our observed morphometrics and those in the original descriptions since our number of measured specimens differ greatly from those measured in the original descriptions, nevertheless almost all our average measurements were within the range of those in the original descriptions (Table 4 to 6). In the case of *M. ulmi*, measured values of the female anterior part are based only on a single paratype specimen. Useful differential characters like the stylet length, stylet knob widths and stylet knob heights showed great similarities. From the perineal patterns, measurements of all the known important features also gave comparable values with those in the descriptions. Interphasmidial distance and the vulva slit were in most cases similar, rarely significantly different. In *M. mali*, these two measurements were almost identical. There was however, a slight difference in these two measurements from *M. ulmi* types (Table 4), probably because only two perineal patterns were studied.

Males

Three male paratypes of *M. mali* and two of *M. ulmi* were measured. Some of the studied characters were only visible enough for measurement on single specimens, and therefore for such characters absolute values were taken rather than their averages. The stylet knobs widths and heights were examples of characters for which measurements were not taken on either species (Table 5) due to the fact that they appeared slightly degenerated on all slides, and so may give false measurements. Nevertheless there were still some outstanding similarities in the stylet length, spicule length and DGO between the observed and the described values.

Second–stage juveniles

Similar to the observations made in the females and the males, the second–stage juvenile morphometrics was very comparable in many features between the two species studied. There was, however an unaccountable difference between stylet length as described for *M. mali* (14 (12 –15)) and that which was measured (12.1 ± 1.5 (10.9 – 13.8)). Values of body width at anus level between the two descriptions were very similar. Some measurements
taken from *M. ulmi*, likewise were quite similar to those in the original descriptions, particularly, the Demanian ratios $a$ and $c'$, while others such as stylet lengths showed slight differences (Table 6).

**Fig. 3.** *Meloidogyne ulmi* type population maintained on *Ulmus x hollandica* cultivar “Wredei” A: Female anterior region; B: Male anterior region; C: Male stylet variation; D: J2 anterior region; E: Male posterior region; F–H: J2 tail regions.
Table 5. Morphometrics of *M. mali* and *M. ulmi* males in comparison with the original descriptions. All measurements are in µm and in the form: mean ± sd. (range).

| Species          | Meloidogyne mali | Meloidogyne ulmi |
|------------------|------------------|------------------|
| Character        | Described        | Observed         | Described        | Observed         |
| N                | 25               | 3                | 30               | 2                |
| Body length      | 1447             | 1428 ± 41.0      | 1462 ± 190       | 1455 ± 64        |
|                  | (1270–1630)      | (1380 – 1452)    | (1053 – 1776)    | (1410 – 15.0)    |
| Body width       | 38               | 34.8 ± 9.6       | 36.9 ± 4.3       | 40.5 ± 0.7       |
|                  | (30–47)          | (28.0 – 41.8)    | (26.6 – 48.4)    | (40.0 – 41.0)    |
| Body width at stylet knobs | 15.7 ± 0.4   | 16.7 ± 1.3       | 17.6 ± 1.1       | 16.8 – 18.4     |
| Body width at S.E pore | 24.5 ± 6.1  | 27.6 ± 2.6       | 28.4 ± 0.6       | 28.0 – 28.8     |
| Stylet length    | 20               | 19.9 ± 1.8       | 19.4 ± 1.2       | 19.9 ± 0.9      |
|                  | 18–22            | (18.6 – 21.1)    | (17.5 – 22.9)    | (19.2 – 20.5)   |
| Stylet knob height | _               | _                | 2.4 ± 0.1        | _                |
|                  |                  |                  | (2.0 – 3.0)      |                  |
| Stylet knob width | _               | _                | 3.9 ± 0.4        | _                |
|                  |                  |                  | (3.0 – 4.8)      |                  |
| DGO              | 8                | 9                | 6.3 ± 0.8        | 7.4 ± 0.5       |
|                  | (6–13)           |                  | (4.8 – 8.5)      | (7.0 – 7.7)     |
| S–E pore*        | _                | 135.2            | 147 ± 18.8       | 139 ± 7.1       |
|                  |                  |                  | (97 – 187)       | 134 – 144       |
| Metacorpus**     | _                | 98 ± 21.5        | 99 ± 9.8         | 83 ± 12.7       |
|                  |                  | (83 – 114)       | (76 – 119)       | (134 – 144)     |
| Spicule          | 32               | 28.1 ± 10.3      | 33.8 ± 1.9       | 29.8            |
|                  | (28–35)          | (20.8–35.3)      | (30.0±37.5)      |                  |
| Gubernaculum     | 8.5              | 10.1             | 9.0 ± 0.9        | 8.4 ± 0.4       |
|                  | (7–10)           |                  | (7.3 – 9.8)      | (8.1 – 8.7)     |
| Testis length    | 788              | 803 ± 125.9      | 716 ± 167        | 752 ± 79        |
|                  | (540 – 970)      | (714 – 892)      | (324 – 977)      | (696 – 808)     |
| T                | 55               | 55.3 ± 8.6       | 48.7 ± 9.7       | 52 ± 3.2        |
|                  | (34 – 65)        | (49.2 – 61.4)    | (27.9 – 71)      | (49 – 54)       |

*distance from anterior end to S–E pore  **distance from anterior end to valve plate of median bulb
Table 6. Morphometrics of *M. mali* and *M. ulmi* second–stage juveniles in comparison with the original descriptions. All measurements are in µm and in the form: mean ± sd. (range).

| Species Character | Meloidogyne mali | Meloidogyne ulmi |
|-------------------|------------------|-----------------|
|                   | Described        | Observed        | Described        | Observed        |
| N                 | 25               | 5               | 30               | 3               |
| Body length       | 418 (390 – 450)  | 420 ± 21.7      | 413 ± 20.6       | 384 ± 9.5       |
|                   | (14 – 16)        | 14.5 ± 1.1      | (373 – 460)      | (374 – 394)     |
|                   | (7 – 9)          | 8.5 ± 1.1       | 9.4 ± 1.8        | 8.4 ± 1.0       |
| Body width        | 14.5 (12.2 – 15.2)| 14.0 ± 1.1      | 14.2 ± 1.8       | 12.7 ± 3.8      |
|                   | (7 – 9)          | 8.5 ± 1.1       | 9.4 ± 1.8        | 8.4 ± 1.0       |
| Body diameter at anus | 8.5       | 9.4 ± 1.8       | 8.4 ± 1.0        | 6.5 ± 0.7       |
| Stylet length     | 14 (12 – 15)     | 12.1 ± 1.5      | 10.0 ± 0.8       | 11.1 ± 0.6      |
| Tail length       | 31 (24.3 – 33.9) | 30.2 ± 4.3      | 31.3 ± 3.1       | 24.2 ± 0.8      |
| Tail terminus length | –             | 7.0 ± 2.1       | 8.2 ± 1.8        | 5.7 ± 1.1      |
| Anus–primordium   | –                | 139 ± 11.4      | –                | 126 ± 21.8      |
| a                 | 28.5 (27 – 31)   | 30.2 ± 3.2      | 29.5 ± 3.4       | 32.5 ± 10.9     |
| c                 | 13.3 (12 – 15)   | 14.4 ± 2.3      | 13.3 ± 1.2       | 16.3 ± 0.7      |
| c’                | 3.7 (3 – 5)      | 3.3 ± 0.7       | 3.7 ± 0.5        | 3.7 ± 0.5       |

HOST TEST

The ability of *M. ulmi* to reproduce on various plant species was examined under greenhouse conditions. Table 7 presents host status of the various plants used in the greenhouse test. *M. ulmi* population from “Mierenbos” used as inoculum was able to induce galls and reproduce on both *Ulmus glabra* and *U. hollandica ‘belgica’*. The apple ‘M9’ also had galls which contained egg–laying females. Although galls were induced by *M. ulmi* on *Brassica oleracea* var. Gemmifera, most of these galls contained small non–gravid females whose development seemed to have ceased at some point. Therefore, it is herein not considered as a host. There were no galls on *Rosa hybrida* and the other cabbage species, *B. pekinensis*.

Additionally, samples collected during 2011 and 2012 revealed that *M. ulmi* is able to parasitize one or more species of *Acer* (Aceraceae), *Impatiens* (Balsaminaceae), *Taraxacum* (Compositae), *Dryopteris* (Dryopteridaceae), *Fagus* (Fagaceae), *Quercus* (Fagaceae), *Geranium* (Geranium), *Geum* (Rosaceae), *Rubus* (Rosaceae), *Sorbus* (Rosaceae), *Taxus* (Taxaceae), *Urtica* (Urticaceae), as shown in Table 7.
Table 7. Plants species identified as host of *M. ulmi* from the green house experiments and field survey at “Mierenbos.”

| Family          | Genus + species                                      |
|-----------------|------------------------------------------------------|
| **Greenhouse test** |                                                      |
| Ulmaceae        | *Ulmus glabra* Huds.                                  |
|                 | *Ulmus hollandica* ‘belgica’                          |
| Rosaceae        | *Malus sylvestris* Mill. ‘M9’                         |
| Solanaceae      | *Solanum lycopersicum* L.                             |
| **Field hosts** |                                                      |
| Sapindaceae     | *Acer pseudoplatanus* L.                              |
| Balsaminaceae   | *Impatiens parviflora* DC.                            |
| Boraginaceae    | *Pulmonaria officinalis* L.                           |
| Asteraceae      | *Taraxacum officinale* F.H. Wigg.                    |
| Dyopteridaceae  | *Dryopteris filix–mas* (L.) Schott                   |
|                 | *Dryopteris cartusiana* (Vill.) H.P. Fuchs            |
| Fagaceae        | *Fagus sylvatica* L.                                   |
|                 | *Quercus robus* L.                                    |
| Geraniaceae     | *Geranium robertianum* L.                             |
| Rosaceae        | *Geum coccineum* Lindl.                               |
|                 | *Rubus idaeus* L.                                      |
|                 | *Sorbus aucuparia* L.                                 |
| Taxaceae        | *Taxus baccata* L.                                     |
| Ulmaceae        | *Ulmus davidiana var. japonica* Rehder                |
| Urticaceae      | *Urtica dioica* L.                                     |

**Isozyme Analysis**

Samples taken from the trial field “Mierenbos” all gave the same type of esterase isozyme pattern of weak single bands, corresponding to the VS1 type (Esbenshade and Triantaphyllou, 1985). When analysed for MDH, some individuals gave single–banded patterns of the H1 type (Esbenshade and Triantaphyllou, 1985), while others revealed a three–banded pattern, herein designated H3. Usually, the H1 type had two additional weaker bands at the same
level as the upper two H3 bands. There was also an additional observation in the types of single bands some of the specimens produced (Fig 4). These single bands were positioned at the same level as the upper H3 band, which herein are given the name H1a.

**PHYLOGENETIC RELATIONSHIP BETWEEN MELOIDOGYNE MALI AND M. ULMI**

The obtained SSU rDNA and LSU rDNA sequence lengths for *Meloidogyne ulmi* were 810bp (including gaps) and 669bp (including gaps) respectively. In addition to our four SSU rDNA sequences of *M. ulmi* (1-B J2 PPS, 2-A male PPS, 3-B J2 PPS and 4-A male GW), 74 accessions belonging to other species of *Meloidogyne* from GeneBank were included in the local alignment (781 aligned positions, including gaps). For LSU rDNA, we had only one sequence of *M. ulmi* (1-B J2 PPS_28) due to poor data, resulting in lack of consensus sequence. Therefore the local alignment included this sequence and 70 other GeneBank accessions from other species of *Meloidogyne*. *Pratylenchus vulnus* was selected as outgroup for constructing gene trees using Bayesian inference from both SSU rDNA and LSU rDNA sequences. SSU rDNA–based phylogenetic analysis put all sequences of *M. ulmi* obtained together with those of *M. mali* and *M. ulmi* from GeneBank in one strongly supported polytomous branch. Despite the relatively short sequence length of SSU rDNA, the tree was able to resolve relationship between certain species in a way comparable with that of Holterman *et al.*, (2009). Clades I, II and III as defined by Tandingan De Ley *et al.* 2002 were also found in our BI of the partial SSU rDNA, indicated herein in colours (Fig. 5). LSU rDNA–based Bayesian analysis revealed higher resolution within the clade containing our sequence and sequences of *M. mali* and *M. ulmi* from GeneBank. Our sequence of *M. ulmi* was positioned in a branch that contained three sequences of *M. mali* from GeneBank forming a sister group to another branch composed also of two sequences of *M. ulmi*. As would be expected, there was higher resolution in the overall topology of LSU rDNA–based tree than that of SSU rDNA.
Fig. 4. Isozyme phenotypes from ten individual females of *Meloidogyne ulmi* from “Mierenbos”. A: Esterase; B: Malate dehydrogenase. *M. ulmi* (1–5 and 8–12); *M. javanica* (6 and 7) as reference marker.
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Fig. 5. Bayesian tree inferred from part of 18S rRNA using SYM I+G model. Sequences were aligned with MAFFT alignment. Numbers near the nodes indicate posterior probabilities. NCBI accession numbers are listed with the species names.
Fig. 6. Bayesian tree inferred from part of D2 D3 of 28S rRNA using GTR+I+G model. Sequences were aligned with MAFFT alignment. Numbers near the nodes indicate posterior probabilities. NCBI accession numbers are listed with the species names.
Discussion

Type specimens representing holotypes and paratypes of the two *Meloidogyne* species were analysed in order to demonstrate the morphological similarities that existed between them. Most of the slides we received were in good conditions except for some few specimens that showed some signs of deterioration due to long period of storage. This impaired the quality of some of the slides and made it difficult to examine and measure certain characters.

On the morphology, an important character like the occurrence of double lateral lines mentioned in the description and by Jepson (1987) was not observed in all specimens. In fact this area was marked by breaks in the striae on some of the specimens studied. Morphometric values of some characters for the two species fell within the range of values reported in the original description of *M. mali* (Itoh et al., 1969), for example position of DGO in females, as well as in males, vulva–anus distance, level of phasmids to vulva, vulva slit length, male stylet length, testis length, spicule length gubernaculum length, J2 body length, and J2 c’ values (Tables 4 – 6). For some characters, however, morphometric values recorded agreed more with those reported for *M. ulmi*. For example, the stylet length measured in females and J2s was significantly lower than the values given for *M. mali*, but comparable with that of *M. ulmi*. A possible explanation might be the fact that stylet in juveniles and sometimes in females appear less visible, causing the anterior end to be mistaken for the tip of the conus. This leads to misleadingly higher values for the stylet length.

Already in the original description of *M. ulmi*, only a few differences could be found to separate it from *M. mali*. And in some cases, the differences emanated from some apparent mistakes on the part of the first authors. An example is the use of male tail length of the two species to draw differences. Tail length in males of *M. mali* was given as ranging between 28 and 44 µm, making it extremely longer than that of *M. ulmi* 10.9 µm. However, it is important to mention that tail length values as long as the range of between 28 and 44 µm never exists among males of species of the genus *Meloidogyne*. Such differences, therefore, cannot be considered as correct.

The DGO position in males with reference to the stylet knobs according to Jepson (1987) bears some broad interspecific variation, making it very useful for species discrimination. *M. mali* is by far the species with the most farther DGO position (6–13 µm), within the genus *Meloidogyne*. The observation of similar values for both species studied here therefore
separates the two from all other species that have relatively shorter DGO position. Additional *M. ulmi* specimens studied also gave DGO position values averaging 8 µm (data not shown).

Eisenback and Hirschmann (1981) highlighted the significance of SEM studies of male head shapes in *Meloidogyne* taxonomy, outlining the role SEM has played in raising the value of males for use in comparison of species. Head and stylet shape morphologies of males and juveniles are the most useful supplemental taxonomic characters that SEM studies have given new insights into (Eisenback & Hirschmann, 1979; 1981). It is not surprising that a number of variability in these characters were outlined to separate *M. ulmi* from *M. mali* by Palmissano and Ambriogioni (2000). It was mentioned that *M. ulmi* differed from *M. mali* in the former having no or vestigial lateral lips, which is apparent in the latter. Interestingly, this contradicted the comparison made by Toida and Yaegashi (1984) when they attempted to point out the differentiating characters between *M. suginamiensis* and *M. mali*. In their comparison, the *en face* view of the lip region of *M. mali* was mentioned as having no or obscure lateral lips to separate it from *M. suginamiensis* in which lateral lips were described as clear. One would not expect such contradicting accounts especially with the possibility that both works referred to the same publication (Okamoto et al., 1983). Referring to a separate work (Yeagashi & Okamoto, 1981), the account given by Toida and Yaegashi (1984) seems to us more probable, the first reason being that they published the same work which is being referred to. Therefore they understand the details of their results more. And the second reason is that our observations of SEM images of (Yeagashi & Okamoto, 1981) agree more with the account that lateral lips were vestigial and not apparent in *M. mali*.

Both apple and elm trees supported *M. ulmi* reproduction. This does not only provide a strong support for the synonymization of *M. ulmi* with *M. mali*, but represents the first and only test involving the former on an apple plant. In principle, however, the first actual report was the description of *M. mali* on apple in Japan (Itoh et al., 1969). Contrary to the finding in the original description, the status of white clover as host to *M. mali* could not be confirmed with *M. ulmi*. Again, although representatives of the family Rosaceae form the larger part of the plants *M. mali* parasitizes (Itoh et al., 1969; Toida, 1979), rose (*Rosa hybrida*) could not support the reproduction of *M. ulmi*. This contradicts earlier finding, Itoh et al., 1979, who identified rose as host. *M. ulmi* was also able to induce galls on *Prunus yodoensis* grown in the field, confirming the earlier report of the latter’s status as a host for *M. mali* by Toida (1979). Results of the sampling has also revealed new natural host for *Meloidogyne ulmi* like
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Acer pseudoplatanus L., Fagus sylvatica L., Quercus robus L., Rubus idaeus L., Sorbus aucuparia L., Taxus baccata L., Dryopteris filix-mas (L.) Schott, Dryopteris cartusiana (Vill.) Fuchs, Geranium robertianum L., Urtica dioica Rehder., Impatiens parviflora DC., Taraxacum officinale L., and Geum coccineum Lindl. The strongest evidence deduced from this host plant test is the ability of *M. ulmi* to reproduce on apple.

It is interesting to mention that the observed variability of the MDH isozyme phenotypes among the different specimens was similar to the findings of Sakai and Mizukubo (2009) when they studied two populations of *M. mali* from Hokkaido on apple and Saitama on cherry in Japan. The populations from Hokkaido gave phenotypes with single MDH bands whereas those from Saitama on cherry produced variable patterns with single and triple bands. PCR–RFLP of D2/D3 expansion segment of 28S rDNA and mtDNA intergenic region with *Alu* I was able to confirm that population from Saitama were all identical, despite their expression of variable MDH isozyme phenotypes. Similar observations of intraspecific phenotype variations were made by Dalmasso and Bergé (1979) among a certain *M. arenaria* population where there were three MDH bands instead of two. Such type of variable isozyme patterns were also observed within one population of the sexually reproducing species *M. microtyla* (Karssen, unpublished). This indicates that *M. mali* could also be a sexually reproducing species, a claim which is further supported by the frequency at which males are encountered in galled root samples – at least one male per female in a gall. Meanwhile, the esterase phenotypes were rather stable across all studied specimens and were characterised by weak indistinct single bands.

Trimming the SSU and LSU datasets to high quality sequence data may have caused a loss in phylogenetic signal. For SSU rDNA, over half of the target sequence length was trimmed out because of the poor quality of the dataset obtained. Although not ideal for reconstruction of phylogeny, it was still sufficient to resolve the taxa on a species level. Moreover, it has to be emphasized that the purpose here is not to reconstruct any formal phylogeny of *Meloidogyne*, a subject which is well covered already in previous studies (Tandingan De Ley *et al.*, 2002; Holterman *et al.*, 2009), but only to demonstrate that *M. mali* and *M. ulmi* are highly similar at the molecular level and belong to the same clade. Two previous phylogenetic analyses involving these two species have already pointed to the fact that the two can hardly be separated based on their SSU rDNA sequences (Holterman *et al.*, 2009; Rybarczyk–Mydlowska *et al.*, 2013). The SSU rDNA sequence once again gave resolution
till the species level, confirming previous proposition that SSU rDNA sequence signatures can be defined at species level for a wide range of parasitic and non–parasitic nematodes (Holterman et al., 2006). As was expected, LSU rDNA–based analysis gave even higher resolution and more clearly defined the relationship between M. mali and M. ulmi. On the SSU rDNA based tree, it is unquestionable that our sequence of M. ulmi with all the other sequences of M. mali and M. ulmi are the same (Fig. 5). The clustering of our sequence of LSU rDNA for M. ulmi with those for M. mali may be an indication that the branching could only be due to some intraspecific sequence variation. This branching cannot be attributed to species variation but rather to inter population variation since our M. ulmi sequence would have otherwise, clustered with other M. ulmi sequences instead of M. mali as shown in Fig. 6.

In conclusion, the evidence from morphological and morphometrical studies of holo– and paratype materials of Meloidogyne mali and M. ulmi as well as host plant studies, isozyme analysis and DNA analysis all confirm the status of M. ulmi as a junior synonym of M. mali.

NOTES ON THE LIFE CYCLE AND BIOLOGY OF MELOIDOGYNE MALI ON ULMUS SPP.

The life cycle Meloidogyne mali is in many respect typical of the genus. Meloidogyne mali requires 18–22 weeks to complete one full generation on apple and does so only once in a year (Inagaki, 1978). The study also reported that adult males and females first were observed after twelve and continued to increase till the 20th week, when egg masses began to appear. There was also some reports on the distribution of M. mali in the field, both vertically and horizontally. However, nothing is known so far about its survival on apple or any other plant during frost conditions of winter. Regarding this, a very interesting observation was made during early spring of 2013 at the trial field “Mierenbos”. Egg–laying females were already found in most galls that were examined, a rare phenomenon known to occur only in M. ardenensis (Stephan & Trudgill, 1982). The only plausible explanation to why egg–laying females can be observed so early in the year is that, like reported for M. ardenensis, the nematodes overwintered in the roots. Additional observations need to be made to find out exactly what stage in the development overwinters in the root.

HOST PLANTS AND DISTRIBUTION IN EUROPE

In addition to the host plant reported already for M. mali, additional hosts identified in this study include the following woody plants: Acer pseudoplatanus L., Fagus sylvatica L.,
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*Quercus robur* L., *Rubus idaeus* L., *Sorbus aucuparia* L., *Taxus baccata* L.; and herbaceous plants: *Dryopteris filix-mas* (L.) Schott, *Dryopteris cartusiana* (Vill.) Fuchs, *Geranium robertianum* L., *Urtica dioica* Rehder., *Impatiens parviflora* DC., *Taraxacum officinale* L., *Geum coccineum* Lindl.

*Meloidogyne mali* induces a similar type of galls as do *M. arenaria* on tomatoes, a type of gall commonly referred to as bead-like galls (Fig. 7). Concerning the current distribution of the nematode in Europe, no study has yet been done to investigate this. However, it would be rational to speculate that *M. mali* may be found in all the ten European countries to which rooted seedlings were sent after the breeding programme. These countries include Belgium, England, France, Ireland, Italy, Spain, Denmark, Germany, Slovakia and Romania (Heybroek, 1993). Elsewhere in Asia, it has also been found in *Acer palmatum* trees from Japan that were intercepted in China (Gu, unpublished). Sequence data from these were also included in the analysis.

**Table 8.** A compilation of all known host plants of *Meloidogyne mali* to date.

| Family   | Genus + species             | Reference     |
|----------|-----------------------------|---------------|
| Rosaceae | *Malus* pumila Mill.        | Itoh et al., 1969 |
|          | *Malus prunifolia* Borkh.   | Itoh et al., 1969 |
|          | *Malus* Sieboldii Rehd.     | Itoh et al., 1969 |
|          | *Malus* “M9”                | Itoh et al., 1969 |
|          | *Prunus* yedoensis Matsum   | Itoh et al., 1969 |
|          | *Rosa* hybrida Hort.        | Itoh et al., 1969 |
|          | *Geum coccineum* Lindl.     | Current work   |
|          | *Vitis vinifera* L.         | Itoh et al., 1969 |
|          | *Rubus* idaeus L.           | Current work   |
|          | *Sorbus aucuparia* L.       | Current work   |
| Moraceae | *Morus bombycis* Koidz.     | Itoh et al., 1969 |
|          | *Ficus* carica L.           | Toida, 1979    |
|          | *Maclura tricuspidata* (Carriere) Bureau | Toida, 1979 |
| Family            | Species                                                   | Reference                  |
|------------------|-----------------------------------------------------------|----------------------------|
| Fagaceae         | *Broussonetia papyrifera* (L.) Vent                      | Toida, 1979                |
|                  | *Broussonetia kazinoki* Seibold.                         | Toida, 1979                |
|                  | *Castanea crenata* Seib. Et Zucc                         | Itoh et al., 1969          |
|                  | *Fagus sylvatica*                                        | Current work               |
|                  | *Quercus robus* L.                                       | Current work               |
| Ulmaceae         | *Ulmus davidiana var. japonica*                          | Toida, 1979                |
|                  | *Ulmus chennouei* Cheng                                  | Palmissano & Ambrogioni, 2000 |
|                  | *Ulmus glabra* Hud.                                      | Palmissano & Ambrogioni, 2000 |
|                  | *Ulmus x hollandica* “belgica”                           | Current work               |
| Sapindaceae      | *Acer palmatum* Thunb                                     | Itoh et al., 1969          |
|                  | *Acer pseudoplatanus* L.                                 | Current work               |
|                  | *Trifolium repens* L.                                    | Itoh et al., 1969          |
| Taxaceae         | *Taxus baccata* L.                                        | Current work               |
| Fabaceae         | *Impatiens parviflora* DC.                               | Itoh et al., 1969          |
| Solanaceae       | *Solanum lycopersicum* L.                                | Toida, 1979                |
|                  | *Solanum melongena* L.                                   | Toida, 1979                |
|                  | *Capsicum annuum* L.                                     | Toida, 1979                |
| Cucurbitaceae    | *Cucumis sativus* L.                                     | Toida, 1979                |
|                  | *Cucurbita spp.*                                          | Toida, 1979                |
|                  | *Citrillus vulgaris* Schrad. ex Eckl. & Zeyh.            | Toida, 1979                |
| Cruciferae       | *Brassica pekinensis* Rupy.                              | Toida, 1979                |
|                  | *Brassica oleracea var. capitata* L.                     | Toida, 1979                |
|                  | *Brassica napus var. *oleifera* L.                       | Toida, 1979                |
| Compositae       | *Arcutium lappa* L.                                       | Toida, 1979                |
| Umbelliferae     | *Daucus carota* var. *sativa* L.                         | Toida, 1979                |
| Leguminaceae     | *Glycine max* (L.) Merr.                                 | Toida, 1979                |
| Urticaceae       | *Urtica dioica* L.                                        | Current work               |
Fig. 7. Root gall symptoms of *Meloidogyne mali* infection on (A, B) *Malus* "M9" (C, D) *Ulmus davidiana* var. *japonica* and (E, F) *Solanum lycopersicum*. 
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References

Blok, V.C. & Powers, T.O. (2009). Biochemical and molecular identification. In: Perry, R. Moens, M. and Starr, J. (Eds). Root–Knot Nematodes. CABI Publishing, Wallingford, UK, pp. 98–118.

Brinkman, H. (1980). Wortelknobbelaaltjes (Meloidogyninae). Verslagen en Mededelingen Plantenziektenkundige Dienst Wageningen (Jaarboek 1979), 56, 47–47.

Carneiro, R.M.D.G., Almeida, M.R.A. & Queneherve, P. (2000). Enzyme phenotypes of Meloidogyne spp. populations. Nematology 2, 645–654.

Dalmasso, A. & Bergé, J. (1978). Molecular polymorphism and phylogenetic relationship in some Meloidogyne spp.: Application to the taxonomy of Meloidogyne. Journal of Nematology 10, 323–332.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). JModel Test 2: more models, new heuristics and parallel computing. Nature Methods, 9, p. 772

Eisenback, J.D. & Hirschmann, H. (1981). Identification of Meloidogyne species on the basis of head shape and stylet morphology of the male. Journal of Nematology 13, 513–521.

Eisenback, J.D. & Hirschmann, H. (1979) Morphological comparison of second–stage juveniles of several Meloidogyne species (root–knot nematodes) by scanning electron microscopy. Scanning Electron microscopy III, 223–229.

Esbenshade, P. & Triantaphyllou, A. (1985). Use of enzyme phenotypes for identification of Meloidogyne species. Journal of Nematology 17, 6–20.

Heybroek, H.M. (1983). Resistant elms for Europe. in Research on Dutch elm disease in Europe, Burdekin, D.A.(ed.). Forestry Commission Bulletin 60, HMSO, UK. pp. 108–113.

Heybroek, H.M. (1993). The Dutch elm breeding program: In Dutch elm disease research: Cellular and molecular approaches, Sticklen, M.B. and J.L. Sherald (eds.). Springer–Verlag, NY. pp. 16–25.

Heybroek, H.M., Goudzwaard, L., & Kaljee, H. (2009). Iep of olm, karakterboom van de Lage Landen (:Elm, a tree with character of the Low Countries). KNNV, Uitgeverij, pp 272.

Holterman, M., Karssen, G., van den Elsen, S., van Megen, H., Bakker, J. & Helder, J. (2009). Small subunit rDNA–based phylogeny of the Tylenchida sheds light on
relationships among some high–impact plant–parasitic nematodes and the evolution of plant feeding. *Phytopathology* 99, 227–235.

Holterman, M., Rybarczyk, K., van den Elsen, S., van Megen, H., Mooymam, P., Santiago, R. P., Bongers, T., Bakker, J., & Helder, J. (2008). A ribosomal DNA–based framework for the detection and quantification of stress–sensitive nematode families in terrestrial habitats. *Molecular Ecology Resources*. 8, 23–34.

Holterman, M., van der Wurff, A., van Den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J. & Helder, J. (2006). Phylum wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. *Molecular Biology and Evolution* 23, 1792–1800.

Hu, M., Hoglund, J., Chilton, N.B., Zhu, X.Q. & Gasser, R.B., (2002). Mutation scanning analysis of mitochondria cytochrome c oxidase 1 reveals limited gene flow among bovine lungworm subpopulations in Sweden. *Electrophoresis* 23, 3357–3363.

Huelsenback, J.P. & Ronquist, F., (2001). MR–BAYES: Bayesian inference of phylogeny. *Bioinformatics* 17, 754–755.

Inagaki, H. (1978). Apple root–knot nematode *Meloidogyne mali*, its taxonomy, ecology, damage and control. *Second Asian Regional Conference on root–knot nematodes, Thailand Kasetsart Journal*, 12, 25–30.

Itoh, Y., Ohshima, Y. & Ichinohe, M. (1969). A root–knot nematode, *Meloidogyne mali* n. sp. on apple–tree from Japan (Tylenchida: Heteroderidae). *Applied Entomology and Zoology* 4, 194–202.

Jepson, S.B. (1987). *Identification of Root–knot Nematodes* (Meloidogyne species). Commonwealth Agricultural Bureaux, Farnham Royal, UK. 265pp.

Karssen, G. & Moens, M. (2006). Root–knot nematodes. In: Perry, R.N. and Moens, M. (eds) *Plant Nematology*. CAB International, Wallingford, UK, pp. 59–90.

Karssen, G. (2002) *The plant–parasitic nematode genus Meloidogyne Göldi, 1892 (Tylenchida) in Europe*. Brill Academic Publishers, Leiden, The Netherlands, 161pp.

Karssen, G. (2009). Een Nieuwe iepenwortelparasiet. In: Heybroek, H. M., Goudzwaard, L. & Kaljee, H. (eds) *Iep of Olm: Karakterboom van de Lage Landen*. KNNV Uitgeverij, Zeist, 2009, p 132.
On the species status of Meloidogyne ulmi

Karssen, G., van Keulen, I., van Hoenselaar, T. & van Heese, E. (2008). Meloidogyne ulmi: een nieuwe iepen parasiet in Nederland? Boomzorg 1, 62–63.

Karssen, G., van Hoenselaar, T., Verkeberk-Bakker, B. & Janssen, R. (1995). Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females. Electrophoresis 16, 105–109.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Resources 30, 3059–3066.

Okamoto, K., Yaegashi, T. & Toida, Y. (1983). Morphological differences among some populations of Meloidogyne mali from apple and mulberry. Japanese Journal of Nematology 12, 26–32.

Oostenbrink, M. (1961). Enige bijzondere aaltjesaantastingen in 1960. Tijdschrift Over Plantenziekten, 57–58.

Palmisano, A. & Ambrogioni, L. (2000). Meloidogyne ulmi sp. n., a root–knot nematode from elm. Nematologia Mediterranea 28, 279–293.

Rybarczyk–Mydlowska, K., van Megen H., van den Elsen, S., Mooyman P., Karssen, G., Bakker J. & Helder, J. (2013). Both SSU rDNA and RNA polymerase II data recognize that root knot nematodes arose from migratory Pratylenchidae, but probably not from one of the economically high-impact lesion nematodes. Nematology (accepted for publication).

Sakai, H. & Mizukubo, T. (2009). Root-knot nematodes parasitizing the Japanese flowering cherry trees. Japanese Journal of Nematology, 39 (2), 74. Abstract of papers presented at the 17th meeting of the Japanese Nematological Society, Kumamoto, Japan, September 3-5, 2009.

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (US) pp. 2344.

Sasser, J. (1977). Worldwide dissemination and importance of the root–knot nematodes, Meloidogyne spp. Journal of Nematology 9, 26–29.

Sasser, J. N. & Carter, C. C. (1982). Root–knot nematodes (Meloidogyne spp.): Identification, morphological and physiological variation, host range, ecology, and control. In: Riggs, R. D. (Ed). Nematology in the southern region of the United States.
Southern Cooperative Series Bulletin 276, Arkansas Agricultural Experimental Station, Fayetteville, Ark., pp. 21–32

Stephan, Z.A. & Trudgill, D.L. (1982). Population fluctuation, life cycle of root–knot nematode, *Meloidogyne ardenensis* in Cupar, Scotland, and the effect of temperature on its development. *Revue de Nématologie*, 5, 281–284.

Tandingan De Ley, I., De Ley, P., Vierstraete, A., Karssen, G., Moens, M. & Vanfleteren, J. (2002). Phylogenetic analyses of *Meloidogyne* small subunit rDNA. *Journal of Nematology* 34, 319–327.

Toida, Y. & Yaegashi, T. (1984). Description of *Meloidogyne suginamiensis* n. sp. (Nematoda: Meloidoygnidae) from Mulberry in Japan. *Japanese Journal of Nematology* 12, 49–57.

Toida, Y. (1979). Host plants and morphology of the 2nd–stage larvae of *Meloidogyne mali* from mulberry. *Japanese Journal of Nematology* 9, 20–24.

Triantaphyllou, A.C. (1979) Cytogenetics. In: Lamberti, F. and Taylor, C. E. (eds) Root–knot nematodes (*Meloidogyne* species): Systematics, Biology and Control. Academic Press. New York, pp. 85–114.

Yaegashi, T. & Okamoto, K. (1981). Observations of six *Meloidogyne* species by scanning electron microscope 2. *En face* views of males. *Japanese Journal of Nematology* 10, 43–51.
Appendix

Appendix 1: List of Genebank accessions used in phylogenetic analysis of SSU rDNA

| Scientific names            | Accession numbers | Authors                  |
|-----------------------------|-------------------|--------------------------|
| *Meloidogyne incognita*     | HE667742          | Tomalova *et al.*, 2012  |
| *Meloidogyne hispanica*     | HE667741          | Tomalova *et al.*, 2012  |
| *Meloidogyne hispanica*     | EU443610          | Landa *et al.*, 2008     |
| *Meloidogyne hispanica*     | EU443609          | Landa *et al.*, 2008     |
| *Meloidogyne ethiopica*     | AY942630          | Tigano *et al.*, 2006    |
| *Meloidogyne sp. MS3*       | AY942636          | Tigano *et al.*, 2006    |
| *Meloidogyne paranaensis*   | AY942622          | Tigano *et al.*, 2006    |
| *Meloidogyne izalcoensis*   | HE667743          | Tomalova *et al.*, 2012  |
| *Meloidogyne arabicida*     | HE667738          | Tomalova *et al.*, 2012  |
| *Meloidogyne arabicida*     | AY942625          | Tigano *et al.*, 2006    |
| *Meloidogyne morocciensis*  | AY942632          | Tigano *et al.*, 2006    |
| *Meloidogyne javanica*      | JX100422          | Wu *et al.*, 2012        |
| *Meloidogyne javanica*      | AY942626          | Tigano *et al.*, 2006    |
| *Meloidogyne incognita*     | JX100421          | Wu *et al.*, 2012        |
| *Meloidogyne incognita*     | JX100420          | Wu *et al.*, 2012        |
| *Meloidogyne incognita*     | HQ709102          | Sirias *et al.*, unpublished |
| *Meloidogyne incognita*     | AY942624          | Tigano *et al.*, 2006    |
| *Meloidogyne floridensis*   | AY942621          | Tigano *et al.*, 2006    |
| *Meloidogyne ethiopica*     | JQ768373          | Conceicao *et al.*, 2012 |
| *Meloidogyne ethiopica*     | FJ559408          | Strajnar *et al.*, unpublished |
| *Meloidogyne cruciani*      | HE667740          | Tomalova *et al.*, 2012  |
| *Meloidogyne mayaguensis*   | AY942629          | Tigano *et al.*, 2006    |
| Species                  | Accession Number | Reference                      |
|-------------------------|------------------|--------------------------------|
| *Meloidogyne exigua*    | AY942627         | Tigano *et al*., 2006          |
| *Meloidogyne duytsi*    | AF442197         | Tandingan De Ley *et al*., 2001|
| *Meloidogyne minor*     | JN241839         | McClure *et al*., 2012         |
| *Meloidogyne minor*     | EU669937         | Holterman *et al*., 2009       |
| *Meloidogyne fallax*    | EU669936         | Holterman *et al*., 2009       |
| *Meloidogyne fallax*    | EU669935         | Holterman *et al*., 2009       |
| *Meloidogyne fallax*    | AY593895         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | EU669934         | Holterman *et al*., 2009       |
| *Meloidogyne chitwoodi* | EU669933         | Holterman *et al*., 2009       |
| *Meloidogyne chitwoodi* | EU669932         | Holterman *et al*., 2009       |
| *Meloidogyne chitwoodi* | AY593888         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AY593886         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AY593885         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AY593884         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AY593883         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AY593887         | Helder *et al*., 2009          |
| *Meloidogyne chitwoodi* | AF442195         | Tandingan De Ley *et al*., 2001|
| *Meloidogyne naasi*     | AY593902         | Helder *et al*., 2009          |
| *Meloidogyne naasi*     | AY593901         | Helder *et al*., 2009          |
| *Meloidogyne naasi*     | AY593900         | Helder *et al*., 2009          |
| *Meloidogyne oryzae*    | AY942631         | Tigano *et al*., 2006          |
| *Meloidogyne exigua*    | HQ709101         | Sirias *et al*., unpublished    |
| *Meloidogyne silvestris*| EU570215         | Castillo *et al*., 2009        |
| *Meloidogyne maritima*  | EU669945         | Holterman *et al*., 2009       |
On the species status of *Meloidogyne ulmi*

| Species                        | Accession       | Authors                        |
|-------------------------------|-----------------|-------------------------------|
| *Meloidogyne maritima*        | EU669944        | Holterman et al., 2009        |
| *Meloidogyne maritima*        | AF442199        | Tandingan De Ley et al., 2001 |
| *Meloidogyne spartinae*       | EF189177        | Plantard et al., unpublished  |
| *Meloidogyne ardenesia*       | EU669946        | Holterman et al., 2009        |
| *Meloidogyne ardenesia*       | AY593894        | Helder et al., 2009           |
| *Meloidogyne hapla*           | EU669943        | Holterman et al., 2009        |
| *Meloidogyne hapla*           | EU669943        | Holterman et al., 2009        |
| *Meloidogyne hapla*           | EU669943        | Holterman et al., 2009        |
| *Meloidogyne hapla*           | AY942628        | Tigano et al., 2006           |
| *Meloidogyne hapla*           | AY593893        | Helder et al., 2009           |
| *Meloidogyne hapla*           | AY593892        | Helder et al., 2009           |
| *Meloidogyne ulmi*            | EU669947        | Holterman et al., 2009        |
| *Meloidogyne ulmi* 4 2498+2499|                 | Holterman et al., 2009        |
| *Meloidogyne ulmi* 3 K162+K163|                 | Holterman et al., 2009        |
| *Meloidogyne mali*            | JX978225        | Gu, unpublished               |
| *Meloidogyne mali*            | EU669949        | Holterman et al., 2009        |
| *Meloidogyne mali*            | EU669948        | Holterman et al., 2009        |
| *Meloidogyne sp. MHMH-2008*   | EU669951        | Holterman et al., 2009        |
| *Meloidogyne sp. MHMH-2008*   | EU669952        | Holterman et al., 2009        |
| *Meloidogyne sp. MHMH-2008*   | EU669950        | Holterman et al., 2009        |
| *Meloidogyne coffeicola*      | HE667739        | Tomalova, 2012                |
| *Meloidogyne ichinohei*       | EU669954        | Holterman et al., 2009        |
| *Meloidogyne ichinohei*       | EU669953        | Holterman et al., 2009        |
| *Meloidogyne camelliae*       | JX912884        | Gu, unpublished               |
Appendix 2: List of Genebank accessions used in phylogenetic analysis of LSU rDNA

| Scientific names          | Accession numbers | Authors                  |
|---------------------------|-------------------|--------------------------|
| Meloidogyne naasi         | JN019272          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019291          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019271          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019267          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019266          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019289          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019265          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019299          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019286          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019283          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019304          | McClure et al., 2012    |
| Meloidogyne naasi         | JN019312          | McClure et al., 2012    |
| Meloidogyne graminicola   | JN157844          | McClure et al., 2012    |
| Meloidogyne graminicola   | AF435793          | Tenente et al., unpublished |
| Meloidogyne graminicola   | HQ420905          | Lui et al., unpublished  |
| Meloidogyne graminicola   | HQ420904          | Lui et al., unpublished  |
| Meloidogyne exigua        | AF435804          | Tenente et al., unpublished |
| Meloidogyne exigua        | AF435796          | Tenente et al., unpublished |
| Meloidogyne exigua        | AF435795          | Tenente et al., unpublished |
| Meloidogyne minor         | JN628437          | McClure et al., 2012    |
| Meloidogyne minor         | JN628436          | McClure et al., 2012    |
| Meloidogyne minor         | JN019323          | McClure et al., 2012    |
| Meloidogyne minor         | JN019322          | McClure et al., 2012    |
On the species status of *Meloidogyne ulmi*

| Species                     | Accession Number | Authors, Year       |
|-----------------------------|------------------|---------------------|
| Meloidogyne minor           | JN157846         | McClure et al., 2012|
| Meloidogyne chitwoodi       | AF435802         | Tenente et al., unpublished |
| Meloidogyne chitwoodi       | JN019321         | McClure et al., 2012|
| Meloidogyne fallax          | JN157848         | McClure et al., 2012|
| Meloidogyne incognita       | JQ317917         | Naz et al., unpublished |
| Meloidogyne hispanica       | EU443606         | Landa et al., 2008  |
| Meloidogyne konaensis       | AF435797         | De Ley et al., 2005 |
| Meloidogyne javanica        | JQ317915         | Naz et al., unpublished |
| Meloidogyne hapla           | DQ145641         | Nadler et al., 2006 |
| Meloidogyne hapla           | DQ328685         | Subbotin et al., 2006|
| Meloidogyne silvestris      | EU570214         | Castillo et al., 2009|
| Meloidogyne dunensis        | EF612712         | Palomares-Rius et al., 2007|
| Meloidogyne marylandi       | JN019366         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019340         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019334         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019338         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019355         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019342         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019336         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019343         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019364         | McClure et al., 2012|
| Meloidogyne marylandi       | JN157845         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019352         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019333         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019337         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019350         | McClure et al., 2012|
| Meloidogyne marylandi       | JN019359         | McClure et al., 2012|
| Species                        | Accession Number | Authors                  |
|-------------------------------|------------------|--------------------------|
| *Meloidogyne maryandi*        | JN019363         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019339         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019326         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019331         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019328         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019330         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019329         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN019327         | McClure *et al.*, 2012   |
| *Meloidogyne graminis*        | JN157849         | McClure *et al.*, 2012   |
| *Meloidogyne mali*            | JX978227         | Gu, unpublished          |
| *Meloidogyne mali*            | JX978226         | Gu, unpublished          |
| *Meloidogyne mali* 21390 LSU  |                  |                          |
| *Meloidogyne ulmi* 11201 LSU  |                  |                          |
| *Meloidogyne ulmi* 21838 LSU  |                  |                          |
| *Meloidogyne artiellia*       | AY150369         | Castillo *et al.*, 2003  |
| *Meloidogyne artiellia*       | AF248477         | De Giorgi *et al.*, 2002 |
| *Meloidogyne baetica*         | AY150367         | Castillo *et al.*, 2003  |
| *Meloidogyne camelliae*       | JX912886         | Gu, unpublished          |
| *Meloidogyne ichinohei*       | EF029862         | Tandingan De Ley *et al.*, unpub. |
| *Pratylenchus vulnus*         | JX261945         | Majd Taheri *et al.*, 2013 |
Appendix 3

ALIGNMENT VIEW FOR SSU SEQUENCES WITH SPECIES NAMES AND ACCESSION NUMBERS
Appendix 4
ALIGNMENT VIEW FOR SSU SEQUENCES WITH SPECIES NAMES AND ACCESSION NUMBERS