Molecular and morphological characterisation of *Meloidogyne hapla* populations from Ethiopia

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Summary. *Meloidogyne hapla*, considered mainly a temperate species, was recently detected parasitising rose plants in glasshouses in Ethiopia. Roses are grown in more than 80% of the existing cut-flower producing farms in Ethiopia. Nevertheless, its production is increasingly facing serious nematode problems. Consequently, soil samples were collected from 12 randomly selected farms distributed in six districts around Addis Ababa. Nine of these farms were positive for *Meloidogyne* spp. and six were positive for *M. hapla*. Pure isolates of 125 *Meloidogyne* spp. from the latter six farms were established from single egg masses on tomato cv. Moneymaker. Based on molecular and morphological data, eighty-two of the isolates were identified as *M. hapla*. Morphological characters based on light and scanning electron microscope images together with morphometric measurements of females, males and second-stage juveniles (J2) were compared with populations of *M. hapla* from different countries. In addition, molecular characterisation was performed based on the 28S D2-D3 expansion segments within the ribosomal DNA and the region located between cytochrome oxidase unit II and the 16S rRNA gene of the mitochondria (mtDNA). Morphological characters of females, males and J2 were in line with descriptions of other *M. hapla* populations but there were a few exceptions in morphometric measurements. The female perennial pattern of the Ethiopian populations fitted the original description but was smaller than that of the population described by Jepson. The J2 body size was larger compared to previous descriptions from Hawaii and East Africa and the *a* ratio value was much greater than for the East African population but similar to the Hawaiian population. Phylogenetic relationships of the Ethiopian *M. hapla* population with other related *Meloidogyne* species on the bases of both mtDNA and D2-D3 expansion segment sequence analysis revealed highly supported clades containing the Ethiopian isolates as well as other published isolates from different countries.

Key words: cut-flowers, glasshouse, molecular diagnostics, mtDNA, phylogeny, rDNA, roses.

Root-knot nematodes (*Meloidogyne* spp.) are endoparasites that are spread worldwide causing severe damage on many economically important crops. They are a problem particularly on glasshouse cultivated crops including ornamentals (Nagesh & Reddy, 2005). Currently, the genus comprises more than 90 species (Hunt *et al*., 2005). Of these, the three tropical species *Meloidogyne javanica*, *M. arenaria* and *M. incognita* have been previously reported from open fields in Ethiopia (O'Bannon, 1975). These species are responsible for most nematode damage on most agricultural crops in the tropics (De Waele & Elsen, 2007). Information regarding nematode problems in glasshouses in Ethiopia is almost non-existent. Nevertheless, cut-flower growers in Ethiopia repeatedly reported plant damages most likely caused by plant-parasitic nematodes, but this was never followed-up. A survey conducted in cut-flower producing glasshouses in 2011 and 2012 revealed the occurrence and wide distribution of several major plant-parasitic nematodes (Meressa *et al*., 2011). This survey indicated among others increased densities of root-knot nematodes with *M.*

hapla usually being the prominent species. Regardless of management strategies, accurate species identification is crucial before any control measures can be started. Hence, the use of both morphological and molecular analysis is important for reliable diagnostics of the species (Orui, 1988; Zijlstra *et al*., 2000; Blok *et al*., 2002; De Luca *et al*., 2011; Birithia *et al*., 2012; Onkendi & Moleleki, 2013). Furthermore, since we detected *M. hapla* only recently in Ethiopia, a comparison of the Ethiopian *M. hapla* population with *M. hapla* populations from other regions was done to see if the general characteristics of this species were met or if there were differences. This should facilitate future diagnostics and control programmes.

Consequently, the objectives of this study were: *i*) to characterise the Ethiopian *M. hapla* population based on morphological and molecular data; *ii*) to compare the Ethiopian isolates of different localities and with populations from other countries based on morphology and morphometry; and *iii*) to reconstruct phylogenetic relationships with *M. hapla* populations of different geographical origin and other *Meloidogyne* species.

MATERIALS AND METHODS

Nematode isolates and pure culturing. During a survey in 2011 soil samples were collected from glasshouses randomly selected from 12 farms distributed in six districts around Addis Ababa, Ethiopia. *Meloidogyne* spp. were detected on nine farms and *M. hapla* on six farms. For the latter a list of the farm code and locality is given in Table 1. Pure nematode cultures of *Meloidogyne* spp. were established for further analysis at Julius Kuehn Institute, Germany. In this case, nematodes were first propagated on roses (*Rosa corymbifera* cv. Laxa) as their original host and then on tomato cv. Moneymaker. All propagation was done in the glasshouse at 20 ± 3°C and 14 h photoperiod.

For each population (hereafter each population represents one farm) 20-60 egg masses were randomly handpicked. Individual egg masses were then placed in 1.5 ml distilled water into wells of a 24 well cell culture plate (Greiner Cellstar®, Frickenhausen, Germany) and kept at room temperature to stimulate hatch of second-stage juveniles (J2). Egg masses from which more than 50 J2 hatched within 3 days received a farm code followed by consecutive isolate number. Hence, each of the six farms was represented by 7 to 51 isolates making a total of 125 isolates. J2 suspensions from each well were then individually pipetted with a micropipette (Eppendorf GmbH, Hamburg, Germany) and injected into holes made around the stem of 2 week-old tomato seedlings grown in 100 ml pots filled with a mix of steam sterilised field soil and silver sand (1:1, v:v).

Seventy-five days later, six egg masses per isolate were handpicked with forceps from each tomato root system under a stereomicroscope and placed in 1.5 ml double deionised water into wells of a 24 well cell culture plate to stimulate J2 hatch. Hatched J2 were then pipetted into 1.5 ml Eppendorf tubes and stored at 4°C until needed for DNA extraction.

Morphometric and morphological analysis. Nine randomly selected *M. hapla* isolates *viz*. STRB6, FYRB60, FYRB62, ETDB47, FLMB18, GUNB26, HRBB82, HRBB100 and HRBB107 representing the different farms (populations) and regions were randomly selected for morphological and morphometrical studies. Males and J2 were killed with gentile heat, fixed in a solution containing 7 ml formalin (40% formaldehyde), 2 ml triethanolamine and 91 ml distilled water (Courtney *et al*., 1955) and processed to anhydrous glycerol over a period of 12 days through slow evaporation technique at 38-40°C (Hooper, 1970). Permanent slide mounts were then made after transferring the nematodes into anhydrous glycerol following the method described by Hooper *et al*. (2005).

For females, perineal patterns were prepared from eight individuals per isolate. Each female was handpicked from galled roots under a stereomicroscope and placed in a drop of water sitting on a microscope slide. The posterior end was cut off with a surgical blade and transferred to 45% lactic acid for 25 min before the inner surface was gently cleaned using a curved fine needle. The cuticle with the perineal pattern was then carefully trimmed. For each isolate, four perineal patterns were transferred to a drop of glycerin for permanent slide mounts. In addition, intact females were prepared for morphometric measurements. Therefore, galled roots were fixed in 3% formaldehyde for 48 h. At least eight females per isolate were handpicked from the root galls and transferred to a drop of water to make temporary slide mounts.

For light microscopy, all specimens were examined under a Leitz Diaplan compound microscope (Leitz, Wetzlar, Germany) at 630-1000× magnification. Morphometric measurements were recorded from digital images taken with Leica DC 180 camera equipped to the microscope and analysed with Leica IM500 image measurement software (Leica Microsystems AG, Wetzlar, Germany). Light microscopic images were taken

with the same camera. All measurements were in micrometers (µm) unless otherwise specified. All morphometric data were subjected to analysis of variance (ANOVA) using SPSS version 20 (SPSS, Inc., Chicago IL, USA). Tukey's HSD (Honest Significant Difference) test was used to compare the means at the 0.05 level.

Nematodes of isolate HRBB100 were processed for scanning electron microscopy (SEM). Specimens were fixed in Trump's fixative and dehydrated in a graded ethanol series, critical point dried (Balzers Union CPD 020, Bal-Tec AG, Balzers, Liechtenstein), mounted on stubs, sputter-coated with gold (Balzers Union SCD 040, Bal-Tec AG, Balzers, Liechtenstein) and examined with a Jeol JSM-840 SEM (Jeol Ltd., Tokyo, Japan).

Molecular analysis. DNA extraction and purification. Total DNA was extracted from the 125 isolates of root-knot nematode in which each farm was represented by varying numbers of isolates *viz*. STR = 7, FYR = 18, ETD = 13, FLM = 18, GUN = 18 and HRB = 51. Nematode suspensions of 100 µl were pipetted onto a cover slip under a stereomicroscope. A minimum of 25 J2 were then handpicked into 10 µl double deionized water in 0.2 ml Eppendorf microtubes. The tubes were then centrifuged for 5 s to get nematodes fully covered with water. For DNA extraction, tubes were left open overnight to let the water evaporate. Next, 10 µl worm lysis buffer (500 mM KCl, 100 mM Tris–HCl pH 8.2, 15 mM $MgCl_2.6H_2O$, 10 mM dithiothreitol (DTT); 4.5% Tween-20) and 2 µl 20 mg ml^{-1} Proteinase K was added. Sterilised tooth sticks were used to crush the nematodes and mix the lysate gently. The lysate was then frozen at –80°C for 20 min and incubated at 63°C for 1 h followed by 95°C for 15 min to stop the activity of Proteinase K.

Total DNA was precipitated from the final aqueous phase by addition of 40 µl isopropanol that was pre-cooled to –20°C, followed by centrifugation at 13,200 *g* for 30 min at 4°C. The DNA was then washed by centrifugation at 13,200 *g* for 15 min with 30 µl of 70% ethanol, air-dried and re-suspended in 35 µl of 1× TE buffer (10 mM Tris and 1 mM EDTA at pH 8).

PCR assay. The first primer set used here was as described by Powers & Harris (1993). Primers C2F3 (5'-GGTCAATGTTCAGAAATTTGTGG-3') and 1108 (5'-TACCTTTGACCAATCACGCT-3') amplify the region between the mitochondrial cytochrome oxidase subunit II gene and the large (16S) ribosomal gene. The other set of primers D2A (5'-ACAAGTACCGTGAGGGAAAGTT-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') used was to amplify the D2-D3 expansion region of the 28S rRNA gene (Baldwin *et al*., 1997). All primers were supplied by Eurofins Genomics, Ebersberg, Germany. The overall PCR mix (25 µl) contained 3 µl DNA template, 0.2 mM dNTPs, 1 µl 10 µM each primer, 2.5 µl 10× Dream Taq™ buffer and 1 U of Dream Taq™ DNA polymerase (Fisher Scientific Inc., Schwerte, Germany). The COII-16S PCR reaction was set for heating at 94°C for 3 min followed by 35 cycles of amplification at 94°C for 2 min, 54°C for 90 s, and 72°C for 1 min with a final incubation for 5 min at 72°C. For the D2-D3 region, all conditions were the same as above except that the annealing temperature was 56°C and the timing for the extension phase was extended to 2 min. All PCR reactions were run in Applied Biosystem® 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA). The amplified products were separated on 1.0% agarose gels in 1× TBE buffer at 5 V cm^{-1} for 1 h, stained with 0.0001% ethidium bromide, and visualised at UV-light (Bio-Rad Laboratories GmbH, Munich, Germany).

Cloning and sequencing. The PCR products of both genes amplified by using COII-16S and D2-D3 primer sets were sequenced directly and one isolate per farm was sequenced from cloned products for confirmation. Prior to sequencing or cloning, PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction for PCR-product purification. For direct sequencing, 5 µl of the PCR products and 5 µl of 10 pmole $µl^{-1}$ of the respective forward primers were mixed. PCR products were cloned into pGEM®-T Easy vector (Promega GmbH, Mannheim, Germany). JM109 high efficiency competent *Escherichia coli* cells were used for transformation of the ligation product. Plasmid DNA from the bacteria culture was purified using PureYield™ Plasmid Miniprep System (Promega GmbH, Mannheim, Germany). At least two representative isolates from each rose farm were randomly selected for sequencing in one direction. Sequencing was performed at the Macrogene sequencing facility service (Amsterdam, The Netherlands).

Phylogenetic analysis. Newly obtained sequences of both mtDNA (COII-16S) and D2-D3 expansion segment of 28S rRNA genes plus related published sequences from GenBank were used to reconstruct the phylogenetic trees. *Bursaphlenchus xylophilus* (JQ423203) for mtDNA (COII-16S) and *B. africanus* (HM623784) for D2-D3 was chosen as outgroup taxa. Raw sequences obtained were first proofread in Chromas Lite version 2.1 (2012) (Technelysium Pty Ltd., South Brisbane, Queensland, Australia) to remove ambiguous base

sequences before subjected to BLAST engine (Altschul *et al*., 1990) for sequence similarity search in GenBank NCBI database. All sequences from the GenBank (Table 2) and the new sequences obtained were aligned using ClustalX version 2.0 (Larkin *et al*., 2007) with default parameters. All sequences were trimmed to equal length using alignment editor in MEGA5 (Tamura *et al*., 2011).

Pairwise distance between *M. hapla* isolates from Ethiopia and isolates from The Netherlands (PPSC3093; DQ145641), Germany (JKI-de) and sequences in GenBank from China (JN005873; GQ130139) and Moldova (DQ328685) of D2-D3 sequences was analysed by MEGA5 (Tamura *et al*., 2011).

The variability in nucleotide position was compared. Sequences of six selected isolates were aligned using ClustalX version 2.1. The alignment was then manually edited by GeneDoc multiple sequence alignment editor (Nicholas *et al*., 1997) in which the sequences were trimmed to equal length.

The phylogenetic analysis was carried out with Maximum Likelihood (ML) and Maximum Parsimony (MP). ML analysis was performed using heuristics searches with Nearest-Neighbour-Interchange (NNI) branch swapping filter. The MP analysis was performed using heuristics searches with Tree-Bisection-Reconnection (TBR) with random addition of 10 sequence replicates. The support for each branch was estimated using the bootstrap (bs) method with heuristics search and 1000 replicates in both ML and MP analysis in MEGA5 (Tamura *et al*., 2011). Values of the relative base frequencies, gamma distribution shape parameter, and base substitution rates were also estimated in MEGA5.

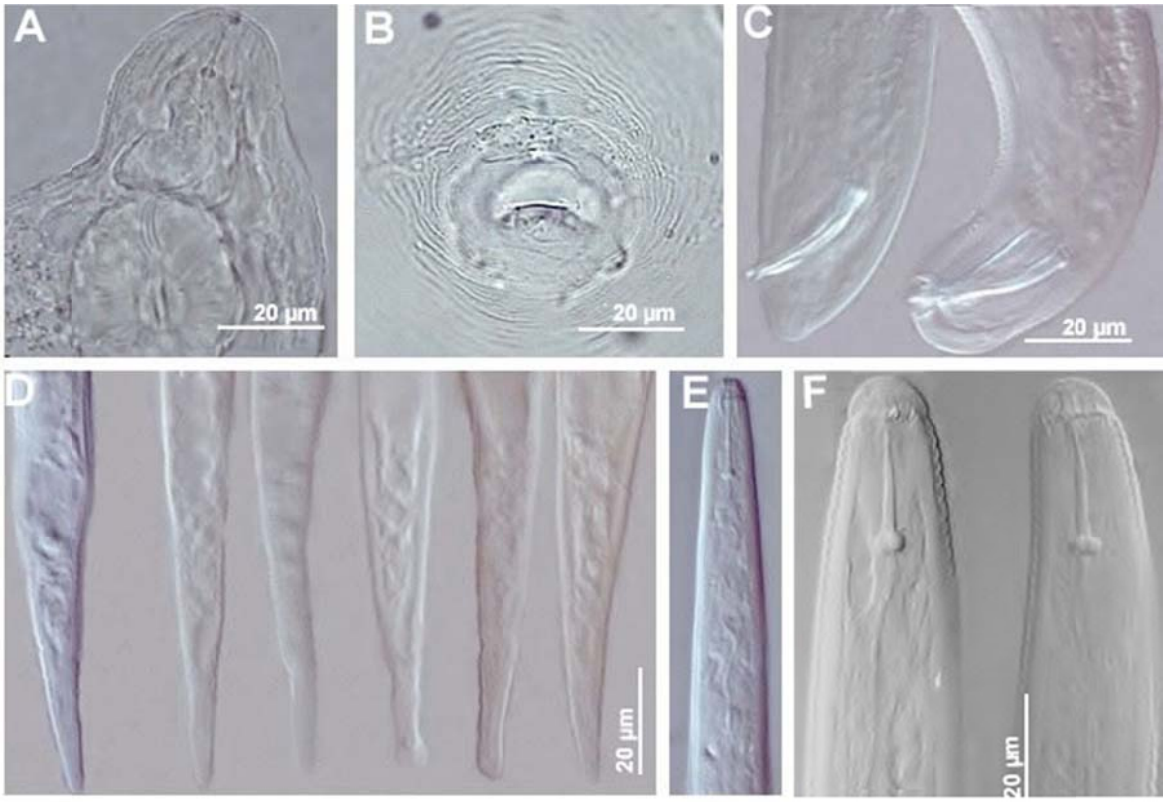

Fig. 1. Light micrographs of *Meloidogyne hapla* isolates. A: female anterior body part; B: perineal pattern; C: male tail and spicule; D: second-stage juvenile (J2) tail types; E & F: anterior part of J2 and male to isolate HRBB100.

RESULTS

Morphology and morphometric analysis. Comparisons between Ethiopian isolates. *Meloidogyne hapla* from Ethiopia was determined by morphological and morphometrical analysis of females, males and juveniles (Figs 1 & 2; Tables 3 & 4). The perineal pattern had generally an ovoidal shape with fine striae forming a wavy pattern on one side. Subcuticular punctuation round the tail terminus appeared from more dispersed to well concentrate manner (Figs 1B, 2G & H). The male had apparent setoff head region and setoff rounded

Table 1. *Meloidogyne hapla* isolates used for molecular analysis in this study

Locality	Altitude m a.s.l	Farm code	Isolate code	Accession	
				mtDNA (COII)	28S D2-D3
Holleta	2300	STR	STRB4	KP681271	KP640618
			STRB6	KP681272	KP410843
		FYR	FYRB60	KP681257	KP410850
			FYRB66	KP681259	KP410851
			FYRB67	KP681260	KP410840
		ETD	ETDB44	KP681252	KP410854
			ETDB47	KP681253	KP410836
			ETDN54	KP681254	KP410845
Menagesha	2300	FLM	FLMB18	KP681255	KP410841
			FLMB24	KP681256	KP410848
Sebeta	2100	GUN	GUNB26	KP681261	KP410838
			GUNB42	KP681258	KP410853
Ziway	1600	HRB	HRBB82	KP681263	KP410842
			HRBB95	KP681264	KP410844
			HRBB98	KP681265	KP410849
			HRBB100	KP681266	KP410852
			HRBB103	KP681267	KP410855
			HRBB107	KP681262	KP410846
			HRBB112	KP681268	KP410837
			HRBB115	KP681269	KP410835
Germany	unknown	JKI	JKI-de	KP681270	KP410839
The Netherlands	unknown	PPS	PPSC3093-nl	–	KP410847

but with varying size of stylet knob (Figs 1F, 2A & B) and tail shape (Figs 2D & E). In J2, most qualitative characters were similar to previous descriptions (Jepson, 1987) (Figs 1E & 2I-M) with various tail shapes. In both male and J2 lateral lines were four in number (Figs 1D & 2K).

Morphometrics for females and J2 of nine selected Ethiopian isolates representing six farms are presented in Table 3 and 4. Comparisons of morphometrics revealed intra-population variations in some characters. Female body length ($P = 0.001$), stylet length ($P = 0.04$), distance between DGO and stylet base ($P = 0.01$) varied significantly within the Ethiopian isolates. Isolate ETD47 showed the greatest body length (706.9 µm) of all the isolates but on the other hand had the shortest DGO length from stylet base (4.1 µm). By contrast, isolate HRBB100 had the smallest body length (528.6 µm) but the greatest DGO length from the stylet base (5.2 µm).

Second-stage juveniles showed significant differences in body length ($P < 0.001$), head height ($P = 0.03$), centre of median bulb from anterior end ($P < 0.001$), stylet length ($P = 0.01$), distance of DGO from stylet base ($P = 0.002$), tail length ($P = 0.02$), tail width at hyaline portion ($P = 0.01$), *a* ratio ($P < 0.01$) and caudal ratio A ($P = 0.01$). Isolate STRB6 from the highland region had a lower

average body length, centre of median bulb from anterior end, excretory pore to anterior end, DGO from base of stylet and tail length than the rest of the isolates. By contrast, HRBB107 of the lowland region had the greatest average body length, centre of median bulb from anterior end, excretory pore to anterior end, DGO from base of stylet, tail length and a ratio of all isolates.

Table 2. GenBank accession numbers for reference sequences used for phylogenetic analysis

Nematode species	COII-16S of mtDNA	Reference and origin (country)	28S D2-D3	Reference and origin (country)
M. arenaria	FJ159610	Fargette *et al*., 2010; Australia	KF112873	Zeng & Huang, 2013; China
	–	–	AF435803	De Ley *et al.*, 2001; USA
M. chitwoodi	–	–	JN019321	McClure *et al*., 2011; USA
M. ethiopica	AY942848	Tigano *et al*., 2006; Brazil	KF482372	Carneiro *et al*., 2013; Brazil
	JN673275	Maleita *et al*., 2012; Portugal	–	–
M. enterolobii	–	–	JN005866	Hu *et al.*, 2011; China
			KJ146862	Wang *et al.*, 2014; China
M. graminis	–	–	JN019339, JN157849	McClure *et al*., 2012; USA
M. hapla	AY539839	Handoo *et al*., 2005; Hawaii	DQ145641	Nadler *et al*., 2006; The Netherlands
	FJ159608	Fargette *et al*., 2010; Australia	DQ328685	Subbotin *et al*., 2005; Moldova
	AY757902, AY757903, AY757889, Y757887, AY757901, AY757899	Powers *et al*., 2005; USA	JN005873	Hu *et al*., 2011; China
	L76262	Hugall *et al*., 1997; Australia	GQ130139	Wang *et.al.*, 2009; China
	AY757900	Powers *et al*., 2005; USA	KJ755183	Lio & Zhang, 2004; China
	AY942850	Tigano *et al*., 2006; Brazil	KJ598136	Chen, 2014; China
M. hispanica	JN673274	Maleita *et al*., 2012; Portugal	EU443606	Landa *et al*., 2008; Spain
	–	–	KF501128	Tzortzaka *et al.*, 2013; Greece
M. incognita	FJ159628	Fargette *et al*., 2008; Australia	JX100424	Wu *et al*., 2012; Taiwan
			KF482374	Carneiro *et al.*, 2013; Brazil
M. javanica	JX100439	Wu *et al*., 2012; Taiwan	KC953092	Wang *et al*., 2013; China
			JQ317912	Naz *et al.*,2011; Pakistan
M. marylandi	–	–	JN019363, JN019351	McClure *et al*., 2012; USA
M. partityla	AY672412	Thomas *et al*., 2004; USA	–	–
	AY757908	Powers *et al*., 2005; USA	–	–
M. thailandica	–	–	EU364890	Skantar *et al*., 2007; USA
B. xylophilus	JQ423194	Valadas *et al.*, 2012; Portugal	HM623784	Gu & Wang, 2010; China

The Ethiopian isolates were primarily collected from localities at altitudes ranging from 1600-2500 m a.s.l. having varying mean annual precipitation and temperatures. However, lack of consistency for variation within the isolates from the same farm may suggest that the difference seen between isolates is not strong enough to be due to the influence of geographical factors.

Comparison between Ethiopian and other populations. For comparison of *M. hapla* from Ethiopia with populations from other countries all Ethiopian, *M. hapla* isolates were considered to be of the same origin and therefore were pooled to form the so-called Ethiopian population of *M. hapla*. The Ethiopian population was then compared in terms of morphometric characters with populations described from Hawaii (Handoo *et al*., 2005), East Africa (Whitehead, 1968), and those mentioned in Jepson (1987) and Chitwood (1949) (Tables 5 & 6). In general, most morphometrics of the Ethiopian population of *M. hapla* fitted closely within the range of the beforehand mentioned populations, but some exceptions.

Female body length (577.1 µm *vs* 781 µm) and width (410 µm *vs* 492 µm) of the Ethiopian population was smaller than that of the population described by Jepson (1987). The stylet length was similar for the Ethiopian population (14.6 µm) and the population described by Jepson (14.6 µm) but greater than the East African population (11 µm). The inter phasmids distance of the Ethiopian population (20.2 µm) was similar to the population from Hawaii (20.9 µm) but smaller than that of the original description (24.4 µm) by Chitwood (1949). In terms of length of vulva slit, the Ethiopian population (19 µm) could be distinguished from the Hawaiian population (23.9 µm).

The mean J2 body length and tail length was greater for the Ethiopian population (369.7 µm; 47.9 µm) than for the Hawaiian population (323.1 µm; 42.6 µm) and East African population (337 µm; 43 µm). The population described in Jepson (1987) had the greatest mean tail length (59 µm) of all those populations. Moreover, both the East African and Hawaiian populations showed a lower *a* ratio (31 *vs* 23.9) and a shorter length of hyaline tail terminus (14 µm *vs* 10.9 µm), respectively, than the Ethiopian population.

Molecular analysis. Sequence analysis. The amplification of the mtDNA (COII-16S) and 28S D2-D3 expansion segment of the 28S rDNA region produced single fragments of about 540 bp and 740 bp, respectively. All tested 125 isolates of *Meloidogyne* spp. gave the same fragment size for the D2-D3 region, similar to the two *M. hapla* isolates form The Netherlands and the isolate from Germany. For mtDNA, only 82 of the 125 isolates yielded a PCR product size similar to the control of *M. hapla* from Germany. Out of the 82, twenty isolates were randomly selected and sequenced for both genes. A BLAST search at NCBI for both genes confirmed the species identity of the Ethiopian isolates (data not shown). For the mtDNA, each isolate revealed 99-100% sequence identity with one or more previously published isolates of *M. hapla*. For the D2-D3 region, 93-99% identity was obtained. Moreover, both sequence and phylogenetic analysis confirmed that the Ethiopian isolates represent *M. hapla*. Furthermore, when compared to other *Meloidogyne* species, the highest percentage identity obtained for mtDNA was 86% with *M. partityla*, whereas for D2-D3 region it was 89% with *M. ethiopica*.

Nucleotide characteristic. The final mtDNA sequence alignment contained 511 positions with 162 parsimony informative sites. Within the Ethiopian isolates, there were four groups based on A+T richness: 83%, 87%, 88% and 89%. The Ethiopian isolates shared an overall intra-sequence similarity value of 98.4%. The sequence similarity among all isolates of *M. hapla* sequences aligned in the sequence analysed was 91-100%. For comparison, mtDNA sequence similarity with other *Meloidogyne* species ranged between 23% for *M. partityla* and 29% for *M. javanica* (data not shown).

Similarly, the ClustalX alignments for the D2-D3 region consisted of 762 nucleotide positions with 255 parsimony-informative. When the % AT was compared among the Ethiopian isolates, three types of AT richness were observed: 54%, 55% and 56%. Out of those, HRBB107 showed low % AT in both gene sequences.

Nucleotide position in D2-D3 sequence. The alignment for the 28S D2-D3 region was compared between three Ethiopian isolates (GUNB26, STRB6, FLMB24) and other isolates from Germany (JKI-de), The Netherlands (PSSC3093) and GenBank (GQ130139; China) (Fig. 3). Overall, the alignment consists of six sequences with 683 bp in length (after trimming to equal length). A total of 61 variable positions were found among sequences of those six isolates. The variability is described as 16 transitions (11 G↔A; 5 C↔T); 43 transversions (8 A↔C, 9 G↔T, 12 A↔T and 14 G↔C) and two indels ↔ T/C. The highest nucleotide variation (45 nucleotides) was between The Netherland's isolate PSSC3093 and the Ethiopian isolate GUNB26. The other two Ethiopian isolates varied from PPSC3093 with 6 and 17 nucleotides for FLMB18 and STRB6, respectively. On the other hand, FLMB18 and STRB6 varied from GUNB26 with 39 and 28 nucleotides, respectively. FLMB18 and STRB6 differ from each other by 11 nucleotides and from the German isolate JKI-de with 5 and 6 nucleotides, respectively.

Sequence similarity of the D2-D3 region was compared between Ethiopian isolates, three other isolates from GenBank (DQ328685, JN005873 and GQ130139), one isolates from The Netherlands (PSSC3093) and one isolate from Germany (JKI-de). Results indicated that Ethiopian isolates vary in sequence homology between each other and isolates originating from other countries (Table 7). Isolate HRBB107 shared low similarity (83%) with isolate ETDB47, HRBB95 and JKI-de. The Isolate JKI-de from Germany shared a maximum of 99% similarity with five Ethiopian isolates. Isolates originated from The Netherlands (PPSC3093), Moldova (DQ328685) and China (JN005873, GQ130139) shared 100% similarity with six of Ethiopian isolates.

Phylogenetic analysis. The ML analysis from a total of 44 sequences for mtDNA and 38 sequences for D2-D3 were able to infer the phylogenetic relationship among the root-knot nematodes. Based on default parameters, the best sequence evolution model for mtDNA was found to be T92+G representing the model Tamura 3-parameter (Nei & Kumar, 2000). The base frequency varied in that

Table 3. Morphometrics comparison of females of Ethiopian isolates of Meloidogyne hapla (n = 45)

Morphological character[1]	*M. hapla* isolates								
	HRBB100	HRBB107	FYRB60	FYRB62	HRBB82	ETDB47	FLMB18	GUNB26	STRB6
Body length	528.6 ± 42.4^{c} (486–574)	549.8 ± 74.8^{bc} (442–640)	551 ± 40.8^{bc} (512–605)	596 ± 83.2^{bc} (463.8–685.8)	638.6 ± 54.1^{ab} (582.5–704)	706.9 ± 101.3^{a} (606–877.6)	579.8 ± 99.2^{bc} (482.8–718)	520.2 ± 53^{c} (468–587)	523.1 ± 31.7^{c} (481.6–551)
Stylet length	14.7 ± 0.9^{ab} (13.8–16)	16.1 ± 1.5^{a} (14–17.6)	14.7 ± 1.7^{ab} (13–17.4)	15.1 ± 2.3^{ab} (12.4–17.7)	13.4 ± 0.5^{b} (12.8–14)	14.3 ± 1.2^{ab} (13.2–16.2)	13.5 ± 0.6^{b} (12.9–14.2)	14.7 ± 1^{ab} (13.8–16.2)	13.6 ± 1^{b} (12.8–15)
Stylet knob width	3.5 ± 0.5^{a} (3–4)	3 ± 0.1^{c} (2.8–3.2)	3.2 ± 0.3^{abc} (3–3.6)	3.4 ± 0.4^{ab} (3–4.1)	3 ± 0.1^{bc} (3–3.2)	3 ± 0.1^{bc} (3–3.2)	3 ± 0.1^{bc} (3–3.2)	3.1 ± 0.3^{bc} (2.9–3.6)	2.9 ± 0.3^{c} (2.5–3.2)
DGO from stylet base	5.2 ± 0.4^{a} (5–6)	5.1 ± 0.2^{a} (4.8–5.4)	5.4 ± 0.6^{a} (5–6.2)	5.1 ± 0.2^{a} (5–5.4)	4.9 ± 0.2^{ab} (4.6–5)	4.1 ± 0.6^{c} (3.4–5)	4.8 ± 0.4^{ab} (4.1–5)	4.4 ± 0.5^{bc} (4–5)	5.4 ± 0.5^{a} (5–6)
Excretory pore from anterior end	33.4 ± 5.8) (28–41)	31.4 ± 4.6 (26–37)	29.4 ± 5.2 (23–35)	34.2 ± 3 (31.4–38.4)	30.4 ± 4.4 (25–36)	34 ± 4.4 (29.7–41.3)	33.5 ± 5.8 (27–42)	30.8 ± 5.2 (26.3–39.4)	30.1 ± 4.9 (23–36.4)
Distance between phasmids	21.3 ± 1.8 (20–24)	20.7 ± 3.7 (14.5–23.2)	18.7 ± 1.2 (17.4–20)	21.8 ± 2.2 (19.8–24.4)	20.6 ± 3.1 (18–26)	19.3 ± 2.5 (16.4–23)	18.6 ± 1.8 (16.8–21.6)	20.6 ± 1.3 (18.6–21.8)	20 ± 1.2 (18.6–21.6)
Vulva slit length	19 ± 2 (17–22)	18.2 ± 1.6 (16–20)	18.9 ± 0.4 (18.4–19.2)	20.2± 2.4 (17.6–24)	18.4 ± 2.1 (16.3–21)	18 ± 3.7 (13–21)	19.9 ± 2.5 (16.8–23.7)	19.2 ± 0.6 (18.2–20)	18.8 ± 0.4 (18.4–19.2)
Vulva-anus distance	16.6 ± 1.3 (15–18.4)	15.6 ± 1.9 (14–18.4)	16.5 ± 1 (15.8–18.2)	17.6 ± 3 (14.8–22.4)	15.8 ± 0.6 (15–16.6)	16.4 ± 2.1 (14–19)	18.6 ± 3.4 (14.4–23.6)	16.1 ± 1.5 (13.6–17.6)	17.1 ± 1.3 (15–18.4)
Distance from anus to tail terminus	18.9 ± 3.3 (14.9–22.8)	16.5 ± 2 (13.3–18.6)	15.3 ± 2 (12.8–18.4)	17 ± 1.5 (14.6–18.4)	16.5 ± 1 (14.9–17.4)	16.5 ± 2.7 (13.8–20.8)	17 ± 1.4 (15.2–18.4)	16.8 ± 1.1 (15.2–18.4)	16 ± 1.9 (13.8–18.4)
‘a’ ratio	1.6 ± 0.1 (1.4–1.7)	1.4 ± 0.3 (1.1–1.7)	1.4 ± 0.1 (1.2–1.6)	1.4 ± 0.2 (1.2–1.6)	1.5 ± 0.3 (1.2–1.9)	1.6 ± 0.2 (1.4–1.8)	1.4 ± 0.1 (1.3 – 1.5)	1.3 ± 0.1 (1.2–1.4)	1.3 ± 0.1 (1.1–1.4)
Excretory pore/stylet length	2.3 ± 0.3 (1.9–2.6)	2 ± 0.3 (1.6–2.3)	2 ± 0.3 (1.6–2.4)	2.3 ± 0.5 (1.8–2.7)	2.3 ± 0.4 (1.9–2.8)	2.4 ± 0.4 (1.8–2.9)	2.5 ± 0.4 (2.1–3.2)	2.1± 0.5 (1.6–2.9)	2.2± 0.4 (1.5–2.6)

[1] Means in the same row followed by the same letter are not significantly different at $P = 0.05$, according to Tukey HSD Multiple comparison test.

Table 4. Morphometrics comparison of second stage juvenile of Ethiopian isolates of Meloidogyne hapla (n = 45). All measurements are in µm as mean ± s.d. (range)

Morphological character[1]	*M. hapla* isolates								
	STRB6	FYRB62	FLMB18	HRBB82	HRBB100	FYRB60	HRBB107	ETDB47	GUNB26
Body length	335.8 ± 16.5^{d} (315–351)	373.24 ± 11.1^{bc} (355–385)	380.2 ± 18.8^{b} (350–402)	361.8 ± 17.3^{bc} (340–380)	360.7 ± 14.7^{bc} (345–375.4)	364.8 ± 9b^{b} (352.7–378.1)	407.4 ± 6.8^{a} (399–417)	368.1 ± 8.2^{b} (354–373)	374.7 ± 8.4^{c} (362.5–384.8)
Head height	2.1 ± 0.1^{abc} (2–2.2)	1.96 ± 0.3^{bc} (1.4–2.2)	2.3 ± 0.4^{bc} (2–3)	2.1 ± 0.1^{abc} (2.0–2.3)	2.5 ± 0.2^{abc} (2.3–2.8)	1.76 ± 0.3^{ab} (1.4–2)	2.1 ± 0.3^{ab} (1.6–2.3)	2.1 ± 0.1^{c} (2–2.2)	2.2 ± 0.5^{a} (1.8–3)
Centre of median bulb from anterior end	43.7 ± 4.7^{d} (40–51)	49.64 ± 2.6^{c} (45.6–52)	52.3 ± 3.5^{a} (47.6–55.7)	49.8 ± 2.2^{bc} (46.4–52)	54.3 ± 2.1^{abc} (51.3–56.2)	53.1 ± 1.8^{abc} (51.2–55)	55 ± 2^{ab} (52–57)	52.3 ± 3^{abc} (48.8–57)	53.8 ± 3.1^{a} (48.8–56.4)
Excretory pore to anterior end	63.2 ± 3.5^{d} (60–68.8)	70.76 ± 1.8^{c} (68.8–73)	76.88 ± 2.5^{ab} (74.8–79.8)	71.6 ± 1.1^{ab} (70.4–72.8)	71.1 ± 2.1^{bc} (68.8–74)	75.3 ± 1.7^{ab} (73.6–78)	78.6 ± 3.7^{a} (76–84)	73.7 ± 3.9^{ab} (68.4–79.1)	75.5 ± 2.2^{c} (73.2–79)
Stylet length	12.8 ± 0.3^{c} (12.4–13)	13.48 ± 0.7^{abc} (12.8–14.4)	13.4 ± 0.9^{ab} (12–14)	13.3 ± 0.5^{abc} (12.8–14)	12.9 ± 0.4^{c} (12.2–13.3)	14.2 ± 0.4^{abc} (14–15)	14.2 ± 0.4^{a} (14–15)	12.7 ± 1.7^{c} (10.4–14)	13.9 ± 0.2^{bc} (13.6–14)
DGO from stylet base	2.3 ± 0.3^{c} (1.9–2.5)	2.34 ± 0.2^{bc} (2.1–2.5)	2.64 ± 0.1^{abc} (2.5–2.7)	2.6 ± 0.1^{abc} (2.4–2.7)	2.5 ± 0.2^{a} (2.2–2.7)	2.5 ± 0^{ab} (2.4–2.5)	2.6 ± 0.4^{a} (2.1–3.2)	2.84 ± 0.2^{bc} (2.5–3.1)	2.8 ± 0.2^{bc} (2.5–3.1)
Tail length	45.8 ± 2.5^{cd} (42–48.8)	47.28 ± 3^{abcd} (44–51)	46.92 ± 2.1^{abc} (44.4–49.6)	47.4 ± 1.7^{abcd} (44.8–49.6)	47.4 ± 3.1^{d} (42.4–50.4)	50.4 ± 5.8^{bcd} (44.8–59.2)	51.5 ± 0.5^{a} (51–52)	44.8 ± 1.3^{ab} (43.2–46.4)	49.9 ± 3.9^{abcd} (45.2–55)
Tail width at hyaline portion	3.9 ± 0.7^{ab} (3.2–5)	3.92 ± 0.2^{ab} (3.6–4)	3.44 ± 0.5^{a} (3–4)	3.9 ± 0.3^{ab} (3.6–4.3)	4 ± 0.1^{ab} (4–4.2)	3.6 ± 0.5^{ab} (3–4)	3.1 ± 0^{b} (3–3.1)	3.4 ± 0.5^{ab} (3–4)	4 ± 0.7^{a} (3–5)
a ratio	29 ± 2^{c} (26.3–31.8)	29.7 ± 1.9^{c} (26.9–31.6)	29.2 ± 2.3^{ab} (25.7–32.2)	30.6 ± 1.8^{bc} (28.3–33.3)	30.7 ± 1.1^{ab} (29.3–31.9)	29.2 ± 3^{abc} (24.3–32.1)	35 ± 2.7^{a} (32.1–38.9)	32.3 ± 1.8^{c} (30.8–34.7)	33.3 ± 4.3^{bc} (27.7–37.8)
Head width/height	2.1 ± 0.3^{ab} (1.8–2.5)	2.1 ± 0.5^{ab} (1.7–2.9)	2.2 ± 0.7^{ab} (1.7–3.3)	1.9 ± 0.1^{ab} (1.8–2)	1.7 ± 0.2^{ab} (1.5–1.9)	2.6 ± 0.6^{ab} (2–3.6)	2.1 ± 0.3^{ab} (1.7–2.5)	1.8 ± 0.2^{b} (1.5–2)	2.2 ± 0.4^{a} (1.7–2.5)
Caudal ratio A	3.4 ± 0.7^{c} (2.6–4.3)	3.3 ± 0.4^{c} (2.8–3.8)	4.2 ± 1^{ab} (3–5.1)	3.5 ± 0.2^{bc} (3.2–3.8)	3.6 ± 0.6^{abc} (3.2–4.5)	4.6 ± 1.1^{abc} (3.6–6.4)	4.4 ± 0.1^{c} (4.2–4.5)	4.2 ± 0.5^{a} (3.6–4.8)	3.3 ± 0.3^{bc} (2.9–3.7)

[1] Means in the same row followed by the same letter are not significantly different at $P = 0.05$, according to Tukey's HSD Multiple comparison test.

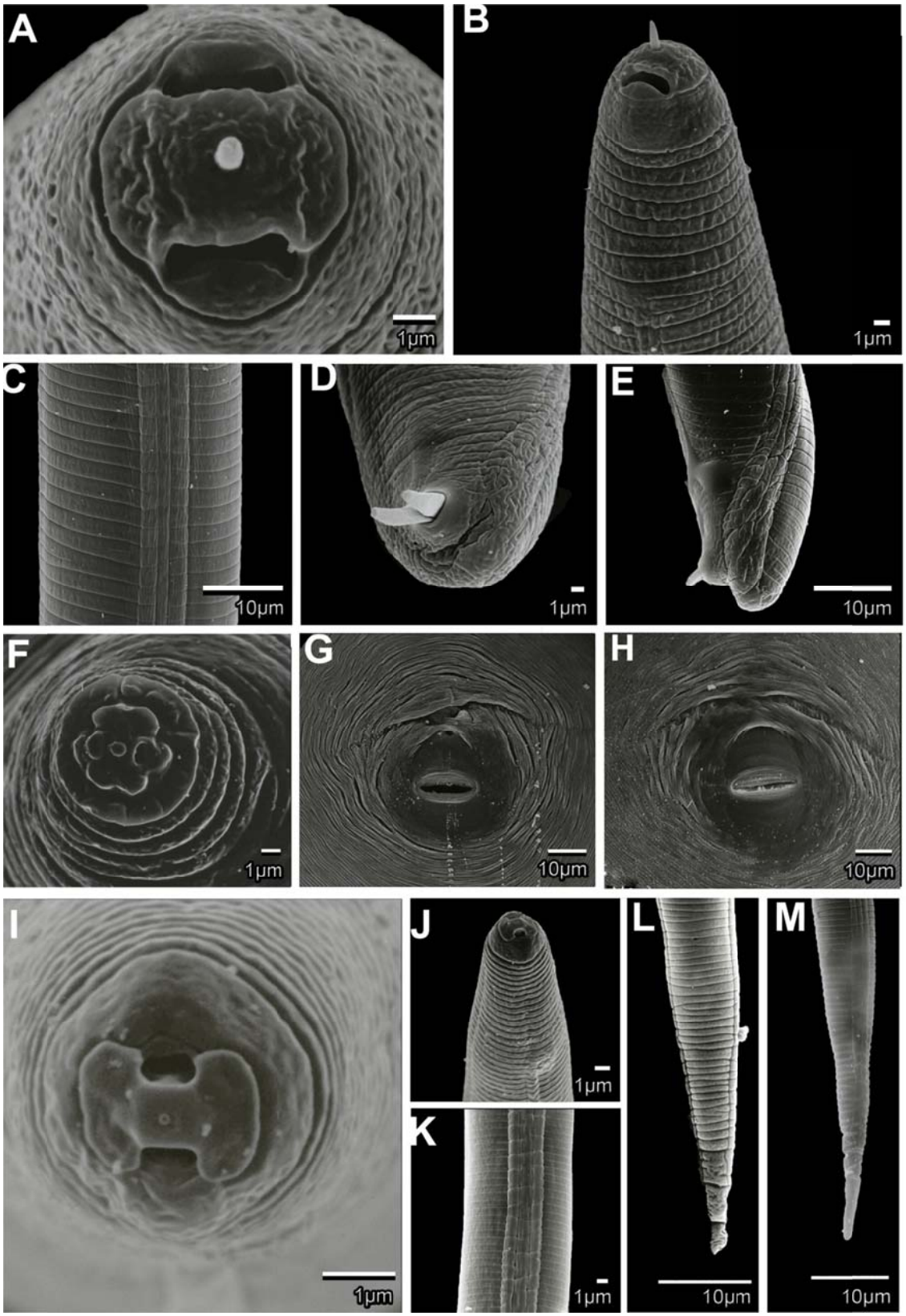

Fig. 2. Scanning electron micrographs of *Meloidogyne hapla* isolate HRBB100. A & B: male face view and head lateral view; C: lateral field and tail; D & E: spicule. F: female face view; G & H: typical perineal pattern. I: second-stage juvenile face view; J: head lateral view; K, L & M: lateral field and tail.

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PPSC3093  : TGAGGAGGACACGGATAGAGTCGGCGTATCTTGCAAGTATTCAATTACTTTATTATTGTGTTGTTATCTCTGAGCT :   76
GQ13013   : .........A.................................................................. :   76
FLMB24    : .........A.................................................................. :   76
JKI-de    : .........A.................................................................. :   76
STRB6     : .........A.................................................................. :   76
GUNB26    : .........A..............................................................G.......... :   76

PPSC3093  : CCAGATTGGGACAGAGGAAAGCAGCATGATTTAATGTGATGCATTTACTTGTCTGGTGTGTGGGGGTATCTTAAGA :  152
GQ13013   : ..............................................................................-.... :  151
FLMB24    : ..............................................................................-.... :  151
JKI-de    : ..............................................................................-.... :  151
STRB6     : ..............................................................................-.... :  151
GUNB26    : ............................TT...........G....C.................-G... :  151

PPSC3093  : TGGATTTGCAACCAATGTTTTGAGGCCAGCTTGCTGGTACCCAAACATTGTTAACATTTTTTATCTTGGATATTCG :  228
GQ13013   : ..................................................................... :  227
FLMB24    : ..........................................G.......................... :  227
JKI-de    : ..................................................................... :  227
STRB6     : ..................................................................... :  227
GUNB26    : .........TG.................................G.....T.....................T :  227

PPSC3093  : AGTACGGCTTACGTGCATTTTTTGTATTGATCTAAGTGCAAGTTACGGTCGCATGCGACACGTGCTTTTCATTTAG :  304
GQ13013   : ..................................................................... :  303
FLMB24    : ................................A.................................A.... :  303
JKI-de    : ..................................................................... :  303
STRB6     : ................................A.........C.........C................... :  303
GUNB26    : .A.........C...........G...C.A.......G.......C.....C...C..C..............C :  303

PPSC3093  : TTCGGTGCAGTTAATGCTCTCGTACTCTCCCCCCATGTAAAAGCCGGTCATCTATTTGACCCGTCTTGAAACACGG :  380
GQ13013   : ..................................................................... :  379
FLMB24    : ................................A.................................... :  379
JKI-de    : ..................................................................... :  379
STRB6     : ..............................................................C.C. :  378
GUNB26    : ...C.....................C.........A...........G...T.....C.A.....C......C.C. :  379

PPSC3093  : ACCAAGGAGTTTATCGTGTGCGCAAGTTTTTGGGTGTTAAAAACCTAGAAGCGAAATGAAAGTAAATGATTCTTTA :  456
GQ13013   : ..................................................................... :  455
FLMB24    : ..................................................................... :  455
JKI-de    : ..............................................A...................... :  455
STRB6     : ..................................................C................A..... :  455
GUNB26    : .......G....T..C.............................C....................... :  455

PPSC3093  : GAGTCTGATGTGCAATCTTTTTTCAGAAAAGTGCATCATGGCCCC-ATTCTAACTGTTTACAGTAGGGTGGCGGAA :  531
GQ13013   : ..............................................-...................... :  530
FLMB24    : ..............................................-...................... :  530
JKI-de    : ........................A...........C.......-...................... :  530
STRB6     : ......................TCA...........C.......-...................... :  530
GUNB26    : T......................A.................C.................A......... :  531

PPSC3093  : GAGCGTACGCGGTGAGACCCGAAAGATGGTGAACTATTCCTGAGCAGGACGAAGCCAGAGGAAACTCTGGTGGAAG :  607
GQ13013   : ..................................................................... :  606
FLMB24    : ..................................................................... :  606
JKI-de    : ........................T............................................ :  606
STRB6     : ..................................................A.....C.............. :  606
GUNB26    : ..................................................................... :  607

PPSC3093  : TCCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA :  683
GQ13013   : ..............................................................T....... :  682
FLMB24    : ..................................................................... :  682
JKI-de    : ......C.T.................T.............T.............................. :  682
STRB6     : ..................C.....C.............................................. :  682
GUNB26    : ..........................................ATA.......................... :  683
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Fig. 3. Alignment of D2-D3 28S expansion segment gene of six selected *Meloidogyne hapla* isolates. Isolates GUNB26, STRB6 and FLMB24 are from Ethiopia, whereas isolate PPSC3093 and JKI-de are newly obtained sequences from The Netherlands and Germany, respectively. GQ130139 is from GenBank originally from China. Identical nucleotide positions are indicated with "." Indels are indicated with "**—**".

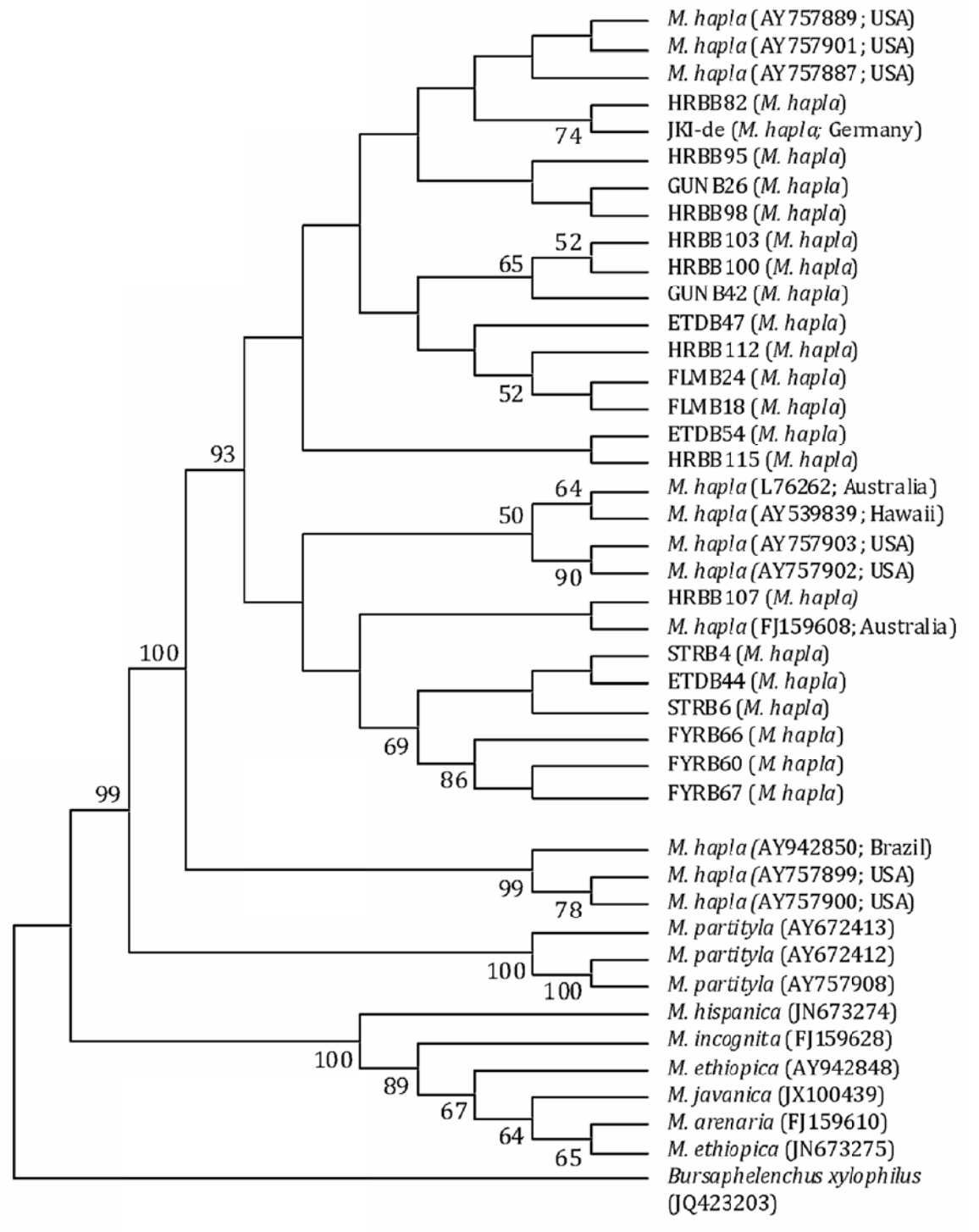

Fig. 4. Maximum Parsimony analysis of mtDNA (COII-16S) of *Meloidogyne hapla* isolates from Ethiopia and other related sequences. Sequences from GenBank are given their corresponding accession number in a parenthesis. Newly obtained sequences for isolates from Germany is in bold. Sequences for Ethiopian isolates are given by farm codes (STR, HRB, FYR, FLM, GUN, and ETD) followed by "B" + isolate number. All ambiguous positions were removed for each sequence pair. The analysis was performed using 1000 bootstrap replicates. The MP tree out of the several parsimonious trees was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm by the random addition of sequences (10 replicates). Corresponding bootstrap values above 50% are shown on each node. *Bursaphelenchus xylophilus* has been used as an outgroup taxon.

both A and T had 0.44 while C and G on the other hand had same 0.06. The estimated gamma distribution shape parameter was 0.67 and the phylogenetic tree had a –ln likelihood of 2433.68. With a similar default search, the best sequence evolution model for the D2-D3 region obtained was K2+G representing the model Kimura 2-parameter (Nei & Kumar, 2000). Each base had equal frequency of 0.25 and the estimated gamma distribution shape parameter was 1.32. The phylogenetic tree obtained had a –ln likelihood of 5737.45.

Identification of *M. hapla* isolates based on sequences of the mtDNA (COII-16S) and rDNA region of the D2-D3 was in agreement with both morphological and morphometrical data. Phylogenetic trees of both genes demonstrated that *M. hapla* isolates from Ethiopia formed highly supported clades with those of published *M. hapla* isolates of different geographical origin (Fig. 4).

Phylogenetic analysis of mtDNA of the Ethiopian *M. hapla* isolates with *M. hapla* isolates and isolates other *Meloidogyne* species obtained from GenBank using Maximum parsimony formed three highly supported clades (Fig. 4): Clade I includes *M. hispanica*, *M. javanica*, *M. arenaria* and *M. ethiopica*; Clade II consists of only *M. partityla*, and Clade III contains *M. hapla* which was separated from the other *Meloidogyne* species with a bootstrap value of 99%. In turn, two *M. hapla* isolates from USA (AY757899, AY757900) and Brazil (AY942850) formed an independent subclade with 100% bootstrap, while other isolates from USA were placed into two highly supported subclades (93% bs). One subclade consists of *M. hapla* isolates from USA (AY539839, AY757903 and AY757902), Australia (L76262, FJ159608) and half of the Ethiopian isolates originally collected from higher altitude regions of Ethiopia except isolate *HRBB107*. The second subclade includes three isolates from USA (AY757889, AY757901, AY757887), one isolate from German (JKI-de) and the remaining isolates from Ethiopia. In summary, the Ethiopian *M. hapla* isolates were placed on the tree sandwiched between isolates originating from USA.

For the D2-D3 expansion segment, phylogenetic trees obtained with both ML and MP revealed consistent topology and thus the ML tree is presented here (Fig. 5). In ML phylogenetic analysis, *Meloidogyne* species formed two highly and moderately distinct clusters supported by bootstrap value 64% and 99%, respectively. The first highly supported cluster comprised all the tropical *Meloidogyne* species (*M. incognita*, *M. javanica*, *M. arenaria*, *M. ethiopica*, *M. enterolobii*, *M. thailandica* and *M. hispanica*) sandwiched between the temperate species (*M. graminis*, *M. marylandi*, *M. chitwoodi*) and population of *M. hapla*. The second clade formed two subclades of *M. hapla* populations including: *i*) one isolate from Germany (JKI-de) separated from other isolates; and *ii*) isolates from China (JN005873, GQ130139, KJ755183, KJ598136), The Netherlands (DQ145641, PPSC3093), Moldova (DQ328685), and all isolates from Ethiopia.

DISCUSSION

In this study, on the basis of comprehensive molecular and morphological analysis, *M. hapla* from Ethiopia is characterised and compared to populations from other countries. In Ethiopia, several plant-parasitic nematode genera were reported to be associated with rose plants cultivated in different regions (Meressa *et al*., 2014a). Of these genera, *Meloidogyne* was frequent as well as abundant. Based on preliminary observation on morphology and morphometry of some characters, we identified *M. hapla*, which was unexpected in the tropical environment of Ethiopia. Thus, we propagated a pure culture and studied details of its morphology, morphometry and molecular characteristics as described in this paper.

Morphological and morphometrical analysis. The identification of *M. hapla* to species level might depend on a specific population that bears varying forms of morphological characters (Eisenback, 1982). Based on sampling locality, our isolates were roughly grouped into those from lowland (≈ 1600 m) or highland (≥ 2300 m) regions. Isolates obtained from Ziway locality represented the lowland regions. There were differences in morphometrics of some characters in female, male and J2 between the Ethiopian isolates. Quite striking was the fact that isolate HRBB107 collected from the lowland revealed considerable variation in some morphometrics from all other isolates. Unfortunately, we have no explanation for such differences because except for HRBB107 all other isolates from the same farm did show high similarity with the isolates collected from the highlands. Thus, the variation observed lacks consistent correlation with sampling localities to infer the type of environment from which they were sampled.

Some morphometrical measurements were found to distinguish the Ethiopian population from others. The Ethiopian female population was different from the Hawaiian in possessing wider body diameter (410 µm *vs* 342.3 µm) and narrower vulva slit (19 µm *vs* 23.9 µm). J2 were comparably larger in body

Table 5. Morphometrical comparison of *Meloidogyne hapla* female populations from Ethiopia, Hawaii, Jepson (1968) and East Africa. All measurements are in µm as mean ± s.d. (value range) except for the last column given as mean (value range)

Morphological character[1]	Ethiopia (this study)	Hawaii (Handoo *et al*., 2005)	Jepson (1987)	East Africa (Whitehead, 1968)
Body length	577.1 ± 86.1 (442-877.6) [a]	–	781 ± 76 (643-952) [b]	612 ± 118 (419-845) [a]
Body width	410 ± 55 (284-510.2) [ab]	342.3 ± 65.2 (225-495) [a]	492 ± 40 (426-559) [b]	430 ± 81 (311-561) [a]
Stylet length	14.4 ± 1.4 (12.4-17.7) [b]	–	14.6 ± 0.53 (12-15) [b]	11 (10-13) [a]
Stylet knob height	2.6 ± 0.4 (2-3.2)	2.5 ± 0.1 (2.5-3)	–	–
DGO from stylet base	4.9 ± 0.6 (3.4-6.2)	5.2 ± 0.5 (5-6.5)	–	5 (4-6)
Inter-phasmids distance	20.2 ± 2.3 (14.5-26) [a]	20.9 ± 4.6 (12.5-30) [a]	–	24.4 (17-38) [b]*
Vulva slit length	19 ± 2 (13-24) [a]	23.9 ± 2.7 (20-30) [b]	–	19 (13-24.5) [a]*
Vulva slit to anus distance	16.7 ± 2 (13.6-23.6)	17.6 ± 2.7 (14-22.5)	–	14.6 (9.5-24.5) *
Distance from anus to tail terminus	16.7 ± 2 (12.8-22.8) [b]	15.4 ± 3.3 (12.5-25) [b]	–	9.3 (5.5-13) [a]*

* Measurements are from the original description of the species by Chitwood (1949).
[1] Means in the same row followed by the same letter are not significantly different at $P = 0.05$, according to Tukey's HSD Multiple comparison test.

Table 6. Morphometrical comparison of *Meloidogyne hapla* second-stage juvenile populations from Ethiopia, Hawaii, Jepson (1968) and East Africa. All measurements are in µm as mean ± s.d. (value range) except for the last column given as mean (value range)

Morphological character[1]	Ethiopia (this study)	Hawaii (Handoo *et al*., 2005)	Jepson (1987)	East Africa (Whitehead, 1968)
Body length	369.7 ± 21.6 (315-417) [b]	323.1 ± 18.4 (284-355) [a]	–	337±11.4(312-355) [a]
Body width	12 ± 1 (9.6-15)	10.8 ± 0.9 (10-12)	–	–
Excretory pore to anterior end	73 ± 5 (60-84)	66.9 ± 7.6 (60-88)	–	–
Stylet length	13.4 ± 0.9 (10.4-15)	–	–	9.7 ± 0.9 (7.9-10.9)
DGO from stylet base	2.5 ± 0.3 (1.9-3.2)	2.5 ± 0 (2.5-2.5)	–	–
Tail length	47.9 ± 3.5 (42-59.2) [b]	42.6 ± 4.2 (30-47.5) [a]	59 ± 5.3 (48.2-69.8) [c]	43 ± 4 (33-48) [a]
Length of hyaline tail terminus	14 ± 2.1 (10-19.2) [b]	10.9 ± 2.1 (5-15) [a]	15.7 ± 1.7 (11.7-18.9) [b]	–
Tail width at hyaline portion	3.7 ± 0.5 (3-5)	3.7 ± 0.5 (3-4.5)	–	–
Ratios				–
a	31 ± 3 (24.3-38.9)[b]	30.2 ± 2.6 (24.1-35.5) [b]	–	23.9±1.7(20.1-26.6)[a]
c	7.7 ± 0.6 (6.4-9.1)	7.7 ± 0.6 (6.8-9.4)	–	7.9 ± 0.2 (7.3-10.2)
Caudal ratio A	3.8 ± 0.7 (2.6-6.4)	3 ± 0.7 (1.7-5)	–	–

[1] Means in the same row followed by the same letter are not significantly different at $P = 0.05$, according to Tukey's HSD Multiple comparison test.

Table 7. Sequence similarity (%) of D2-D3 expansion segments of 28S rDNA of Meloidogyne hapla isolates from Ethiopia, The Netherlands (nl), Germany (de) , China (cn) and Moldova (md). PPSC3093 and JKI-de are newly obtained sequences. Additional sequences from GenBank database are shown with theirs corresponding accession number

Isolates		*M. hapla* populations																									
		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
01.	HRBB115	–																									
02.	DQ328685 (md)	100	–																								
03.	ETDB54	100	100	–																							
04.	HRBB98	100	100	100	–																						
05.	JN005873 (cn)	100	100	100	100	–																					
06.	STRB4	96	96	96	96	96	–																				
07.	ETDB47	94	94	94	94	94	93	–																			
08.	GUNB26	92	92	92	92	92	92	90	–																		
09.	HRBB107	84	84	84	84	84	84	83	86	–																	
10.	GUNB42	92	92	92	92	92	91	89	93	84	–																
11.	HRBB95	98	99	99	99	99	96	93	92	83	92	–															
12.	FYRB60	99	99	99	99	99	97	94	93	85	93	97	–														
13.	FYRB66	99	99	99	99	99	96	94	92	85	92	98	100	–													
14.	STRB6	98	98	98	98	98	97	94	93	84	92	97	99	99	–												
15.	JKI-de (de)	99	99	99	99	99	95	93	92	83	91	98	98	98	97	–											
16.	PPSC3093 (nl)	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	–										
17.	FLMB24	99	99	99	99	99	97	95	92	84	92	98	98	98	98	98	99	–									
18.	HRBB100	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	100	99	–								
19.	HRBB82	100	100	100	100	100	96	94	93	85	92	99	99	99	98	99	100	99	100	–							
20.	HRBB103	98	99	99	99	99	97	94	93	84	92	98	98	98	97	98	99	99	99	99	–						
21.	FYRB67	99	100	100	100	100	97	94	92	84	92	99	98	99	98	99	100	100	100	100	99	–					
22.	ETDB44	97	98	98	98	98	95	93	91	84	91	97	96	97	97	97	98	98	98	98	97	98	–				
23.	HRBB112	98	99	99	99	99	96	93	93	85	92	98	98	98	98	98	99	99	99	99	98	99	97	–			
24.	FLMB18	99	99	99	99	99	96	94	93	85	92	98	98	99	98	98	99	99	99	100	99	100	98	99	–		
25.	GQ130139(cn)	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	100	99	100	100	99	100	98	99	99	–	
26.	DQ145641 (nl)	100	100	100	100	100	96	94	93	85	92	99	99	99	98	99	100	100	100	100	99	100	98	99	100	100	–

size (369.7 µm *vs* 323.1 µm) and had longer tail length (48 µm *vs* 42.6 µm). One population from The Netherlands was previously reported to have an average vulva slit length of 18.9 µm, which is almost identical to the Ethiopian population (Handoo *et al*., 2005).

Our population differed from a population described from East Africa (Tanzania) in some morphometrics of female and J2 (Whitehead, 1968). The Ethiopian female had relatively shorter (577 µm *vs* 612 µm) and narrower (410 µm *vs* 430 µm) body compared to the East African population. There was also a considerable difference in female stylet length (11 µm *vs* 14.4 µm). On the other hand, the Ethiopian J2 had greater body size than the East African (369.7 µm *vs* 337 µm). The tail length of the Ethiopian population (48 µm) falls between the East African (43 µm) and the population described by Jepson (1987) (59 µm). The smaller *a* ratio (23.6) of the East African J2 than the Ethiopian (31) J2 indicates that the Ethiopian population possess a thinner body.

Previously, based on J2 body length, the possible geographical origin of the Hawaiian population was suggested to be associated with the origin of *Coffea arabica* in Ethiopia. Although, a warmer environment might correlate with smaller J2 body size (Handoo *et al*., 2005), the Ethiopian J2 populations were apparently larger in size than both the East African and Hawaiian populations. Hence, it is unlikely that the Ethiopian population shares a similar origin with these two populations.

Molecular analysis. DNA based molecular diagnostics avoids ambiguous diagnosis in root-knot nematodes (Hyman, 1990). Amplification of mtDNA (COII-16S) produced a fragment size of approximately 540 bp in agreement with the reports by Orui (1998) and Powers & Harris (1993). Using the same primer pairs used here, the three tropical root-knot nematode species are consistently known to produce a typical mtDNA PCR fragment size of 1.7 kb for *M. incognita* and *M. javanica* and 1.1 kb for *M. arenaria* (Powers & Harris, 1993). Hence, our PCR product size gave the first indication that *M. hapla* could be discriminated at least from those known tropical root-knot nematode species. The presence of unusual PCR size polymorphism, even within isolates of same species as reported for *M. incognita* (Blok *et al*., 2002), shows that mere use of PCR fragment size is not sufficient for the correct identification of this species.

The PCR fragment size (740 bp) for D2-D3 expansion segment of 28S rDNA was not species-specific because *M. incognita* and *M. chitwoodi* included as a control also yielded the same fragment size (data not shown). Nevertheless, sequence analysis of the PCR products of both genes confirmed the identity of our specimens as *M. hapla*.

Phylogenetic analysis. In our study, the MP analysis of the mtDNA gene of *M. hapla* isolates formed a highly supported clade together with other isolates retrieved from the GenBank. Nevertheless, considerable nucleotide variation has been observed within the Ethiopian isolates. For example, the overall sequence variability with in isolates was 11.5%. On the other hand, the sequence variability among those *M. hapla* isolates retrieved from the GenBank was 12%. Therefore, the sequence disparity observed between the Ethiopian isolates can be considered realistic. The rapid evolutionary nature of mtDNA (Powers *et al*., 1986) combined with the reproduction behaviour of *M. hapla* (Hugall *et al*., 1994) could explain the sequence variation observed between isolates.

Sequence variation in the D2-D3 region within isolates of *M. hapla* ranged from 0% to 17%. Subbotin *et al*. (2011) reported variability of 6% within clones of the same PCR for *Helicotylenchus martini*. Sequence variability in the rDNA within individuals of the same species has also been reported previously (De Ley *et al*., 2002). On the other hand, Blok *et al*. (2002) found identical D2-D3 sequence for three isolates of *M. hispanica* collected from different geographical origins. However, given our isolates were collected from different geographical regions; the level of heterogeneity observed between isolates may still be considered acceptable. Both ML and MP analysis of the two gene regions placed all the *M. hapla* isolates on a strongly supported clade separated from other *Meloidogyne* species.

Biogeography of *Meloidogyne hapla*. Accurate information on the species and distribution of particular plant-parasitic nematodes is important to develop appropriate control strategies. The occurrences of *M. incognita*, *M. javanica*, *M. arenaria* and *M. ethiopica* have been long known from Ethiopia (O'Bannon, 1975), whereas the occurrence of *M. hapla* in Ethiopia was only recently reported (Meressa *et al*., 2014a).

The most common tropical *Meloidogyne* species, *M. incognita*, *M. arenaria* and *M. javanica*, occur in areas with an average temperature up to 36°C (Taylor *et al*., 1982). *Meloidogyne hapla* is generally distributed in cooler parts of the world where it survives mean annual low temperature of –15°C, but also occurs in highland areas of tropical and subtropical countries where the temperature is below 27°C (Taylor *et al.*, 1982). However, its occurrence in the rift valley of Ethiopia

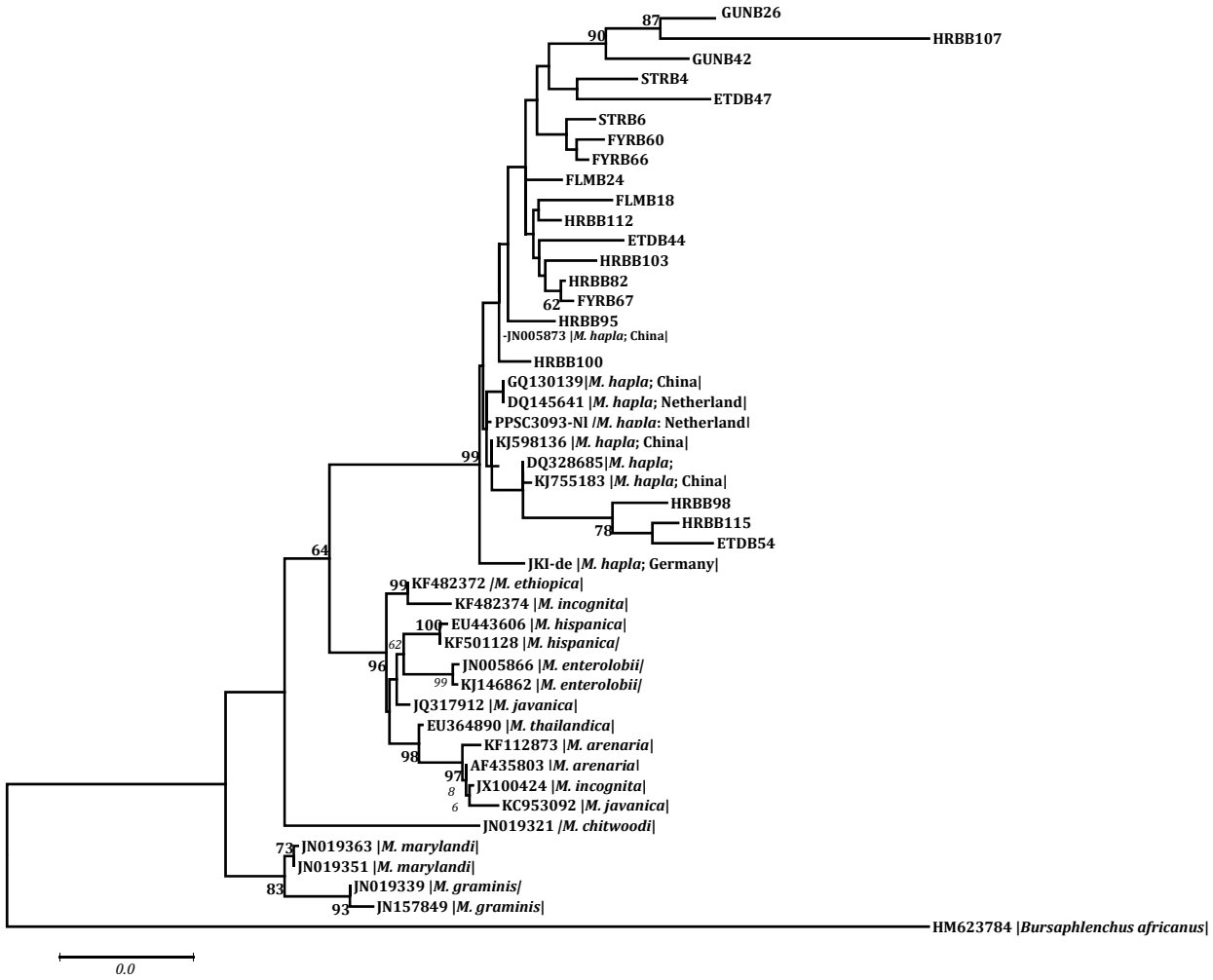

Fig. 5. Maximum Likelihood analysis of the D2-D3 expansion segment of 28S ribosomal DNA of *Meloidogyne hapla* isolates from Ethiopia and other related sequences. Sequences from GenBank are given with their corresponding accession number. Newly obtained sequences for isolates from The Netherlands and Germany are in shown in bold. Whereas sequences for Ethiopian isolates are given by farm codes (STR, HRB, FYR, FLM, GUN and ETD) followed by "B" + isolate number. The analysis was performed using 1000 bootstrap replicates. Corresponding bootstrap support more than 50% are given for each appropriate clade. *Bursaphelenchus africanus* has been used as an outgroup taxon.

(Meressa *et al*., 2014a), South Africa (Onkendi & Moleleki, 2013) and highlands of Tanzania (Whitehead, 1968) might indicate a possible wider climate range of this species than previously expected. The temperature of our sampling localities generally varied throughout the year but can reach up to 35°C, which is already suboptimal temperature conditions for this species, thus reducing its pathogenicity (Wong & Mai, 1973). However, the fact that rose glasshouses used shading and irrigation to reduce temperature peaks might have helped *M. hapla* to establish and cause damage under those otherwise extreme conditions.

Although *M. hapla* was shown under experimental conditions to multiply on cut-flower species other than roses, such as on statice, carnation, gypsophila and freesia (Meressa *et al*., 2014b), *M. hapla* was not detected on these cut-flower species in Ethiopia (Meressa *et al*., 2014a). One reason for this restricted distribution might be that *M. hapla* was introduced to Ethiopia with rooted rose plants, whilst for the other cut flowers seeds were used that are free of *M. hapla*. This hypothesis is supported by the fact that the Ethiopian isolates shared high sequence similarity with *M. hapla* populations from other part of the

world. Consequently, *M. hapla* might have a historical connection with the import of rose rootstocks on growth media from outside Ethiopia.

In conclusion, the association between *M. hapla* and rose cut-flowers will remain a challenge to the growers in Ethiopia. Optimistically, this report will trigger alarm and generate a coordinated search for sustainable management strategies for *M. hapla* on rose cut-flowers.

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B.-H. Meressa, H. Heuer, H.-W. Dehne and J. Hallmann. Молекулярная и морфологическая характеристика популяций *Meloidogyne hapla* из Эфиопии.

Резюме. Вид *Meloidogyne hapla* часто рассматривается как форма, характерная для регионов с умеренным климатом. Недавно этот вид был выявлен в Эфиопии в теплицах с розами. Розы представляют собой основную культуру в более чем 80% хозяйств, экспортирующих срезанные цветы из Эфиопии. В последнее время эти хозяйства сталкиваются с серьезными проблемами из-за поражения нематодами. Образцы почвы из случайно выбранных 12 хозяйств были собраны в 6 административных районах вокруг Аддис-Абебы. На девяти из них были выявлены нематоды рода *Meloidogyne*, а на шести – нематоды вида *M. hapla*. Чистые лабораторные культуры мелойдогин были основаны с использованием яйцевых масс от 125 одиночных особей, происходивших из этих 6 хозяйств. В качестве растения-хозяина использовали томаты сорта 'Moneymaker'. Основываясь на морфологических и молекулярных данных, 82 культуры были идентифицированы как *M. hapla*. Морфологические особенности этих нематод были исследованы с помощью светового и сканирующего электронного микроскопа. Морфологические и морфометрические признаки самок, самцов и личинок 2-й стадии (J2) сравнивали с данными по популяциям *M. hapla* из различных стран. Проведенная молекулярно-таксономическая характеристика эфиопских популяций основывается на последовательностях D2-D3 сегмента большой субъединицы рибосомы и участке митохондриального генома, лежащего между генами COII и 16S мтДНК. Морфологические особенности самок, самцов и J2 соответствовали таковым у других популяций *M. hapla*, с некоторыми различиями в морфометрических параметрах. Вульварные пластинки эфиопских популяций соответствовали первоописанию, но были меньше по размерам, чем у форм, исследованных Джепсоном (Jepson, 1987). Размеры тела у J2 были крупнее, чем у ранее исследованных популяций с Гавайских островов или из Восточной Африки, а индекс *a* был выше, чем у восточноафриканских популяций, и сходным с таковым у гавайских форм. Филогенетический анализ взаимоотношений эфиопских популяций *M. hapla* с другими популяциями этого вида по последовательностям мтДНК и D2-D3 сегмента рДНК выявил высокую статистическую поддержку эволюционной линии, объединяющей популяции из Эфиопии с исследованными популяциями из различных стран мира.