Reported populations of *Meloidogyne ethiopica* in Europe identified as *Meloidogyne luci*

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Abstract

The tropical group of root-knot nematodes (RKN) including *Meloidogyne ethiopica* and *M. luci* is a highly polyphagous and damaging group of agricultural pests. *M. ethiopica* has been detected in several European countries (Slovenia, Italy, Greece) and also in Turkey. However, a description of a new sister species *M. luci* calls for reclassification of all European and Turkish *M. ethiopica* populations reported up to date as *M. luci*. Accurate identification can be accomplished through analysis of the esterase isozyme pattern, which is the most distinguishing character between the two otherwise very similar species. Both species display a three banded esterase pattern where the upper band is slightly shifted between the two

species. In addition, molecular characterization of *M. ethiopica* and *M. luci* populations revealed that the ITS, SSU, and LSU of the rDNA regions are not appropriate markers for studying relationships among the tropical group of RKNs. However, the COII/IRNA region on mtDNA proved to be very useful for analyzing the phylogenetic relationship of these very closely related species/populations. Mitochondrial sequences with low levels of heteroplasmy allowed clustering of all *M. luci* populations in a monophyletic clade with a clear separation of this recently described species from *M. ethiopica*. At the same time, a very close relationship between *M. ethiopica* and *M. luci* was confirmed again.

Root knot nematodes (RKNs) of the genus *Meloidogyne* represent one of the most damaging groups of agricultural pests and have been involved in substantial economic loss. The genus consists of approximately 100 nominal species that are obligatory parasites of almost every vascular plant including important agricultural crops. The most damage is caused by the group of four “major” species, *M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*, which are the most distributed worldwide. Additionally, several other RKNs may become emerging pests in the future of agriculture because of intensive global trade, changes in production technologies involving reduced pesticide usage, and climate changes. The emerging species mostly belong to the tropical RKNs group, which reproduce parthenogenetically. They exhibit a wide host range and great damage potential but have limited distribution. One such pest is *M. ethiopica*, which was listed among emerging global agricultural pests (Hunt and Handoo 2009) and added to the EPPO Alert list of harmful organisms in 2011. *M. ethiopica* was described by Whitehead in 1968 from the Mlalo region of Tanzania. It is currently distributed in Africa, South America, Asia, and southern Europe. It has been reported to have been found in Kenya, Ethiopia, Mozambique, Zimbabwe, Tanzania, South Africa (Whitehead 1968, 1969), Chile, Brazil, Peru (Carneiro et al. 2003, 2004), Slovenia (Širca et al. 2004), Greece (Conceição et al. 2012), Turkey (Aydinli et al. 2013), and Italy (Maleita et al. 2012). *M. ethiopica* can parasitize several crops including monocotyledons, dicotyledons, and herbaceous and woody plants (Carneiro et al. 2003, 2004; Lima et al. 2009; O’Bannon 1975; Strajnar et al. 2009; Whitehead 1968). It is widely distributed in vineyards in Chile (Aballay et al. 2009). *M. ethiopica* has been reported to reduce yield quality and quantity in kiwi (*Actinidia deliciosa*) in Brazil (Carneiro et al. 2003) and in grapevine (*Vitis vinifera*) in Chile (Carneiro et al. 2007). Medina et al. (2014) reported *M. ethiopica* on potato plants from Parana State, Brazil, showing root systems with multiple galls and tubers with protuberances.

Correct species identification is needed for appropriate pest management. RKN species identification is based on a combination of morphometrical, biochemical, and molecular methods. *M. ethiopica* species identification using classical morphology requires a detailed analysis of morphological features of the female, male, and second-stage juveniles. However, the species is very difficult to distinguish from the sibling species *M. inornata* and the recently described *M. luci* (Carneiro et al. 2014) because of morphometrical similarity and intraspecific variation in the group. Besides, the perineal patterns of *M. ethiopica* are highly variable and similar to *M. luci* (Carneiro et al. 2014) and were characterized as varying from the *M. arenaria* type to the “*acrita*” type of *M. incognita* (Golden 1992). *M. luci* has been reported to have been found in Brazil, Chile, Iran (Carneiro et al. 2014), Guatemala (Janssen et al. 2016), and Portugal (Maleita et al. 2016). Analyzing phenotypes of malate dehydrogenase (MDH) and esterase (EST) isozymes has proved to be a useful approach in RKN species identification (Esbenshade and Triantaphyllou 1985, 1990). Besides morphometrical and biochemical analyses, molecular tools are also used for nematode species determination. Several molecular markers have been used for phylogenetic studies of nematodes from the order Tylenchida and genus *Meloidogyne*. Holterman et al. (2009) reported that the small subunit ribosomal DNA (SSU rDNA) was a good phylogenetic marker for the phylum Nematoda as well as a good species identification marker for Tylenchidae. However, that does not hold for most of the tropical group RKN species that include *M. ethiopica* (Holterman et al. 2006, 2009; Kiewnick et al. 2014) because of high intraspecific variation.

In this study, we have re-examined *M. ethiopica* populations from Slovenia, Greece, Turkey, Brazil, and South Africa and compared them with a population of *M. luci* from Iran to assess whether the closely related *M. luci* and *M. ethiopica* populations can be unambiguously distinguished by differential host, isozyme pattern, and/or molecular marker regions of rDNA and mtDNA widely used in the phylum Nematoda. The present study provides information on the biological, biochemical, and molecular features of *M. luci* and *M. ethiopica* populations from the different continents: South America, Africa, Europe, and Asia.

Materials and Methods

Nematode populations. Nematode populations were collected from different countries and continents where *M. ethiopica* and *M. luci* occur (Supplementary Table S1). Populations from Slovenia, Greece, and Turkey previously identified as *M. ethiopica* (Aydinli et al.

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables and three supplementary figures are published online.

2013; Conceição et al. 2012; Strajnar et al. 2009; Širca et al. 2004) are reclassified in this study to *M. luci*. All populations were maintained on tomato cultures cv. Cuore di Bue planted in sterile sand and kept in a greenhouse at 20 to 25°C. Nematodes used for analyses were isolated from the fresh cultures. Using a dissecting microscope, females and egg masses were isolated from infested roots with a scalpel and a nematological needle. Males were isolated from the sand by the decanting method (Hrzič 1973) followed by Baerman's funnel extraction.

Differential host status. Differential host test was based on the Taylor and Sasser (1978) method. The experiment was set up in the greenhouse at an average temperature of 22°C. Two populations of *M. ethiopica* from Brazil and Africa, and one population of *M. luci* from Slovenia, were chosen in order to represent the esterase phenotypes, E3, E2, and L3, respectively. They were tested for reproduction on watermelon cv. Charleston Gray. Seven-week-old plants were inoculated with an aqueous suspension containing 3,000 RKN eggs obtained from the cultures as described by Hussey and Barker (1973). The experiment was terminated 60 days after inoculation and the final nematode population was determined. Five test plants of each tested RKN population were evaluated for the nematode reproduction rate.

Host suitability was assessed by determining the reproduction factor $R = Pf/Pi$ (final population of nematode eggs and juveniles divided by the number of inoculated nematode eggs). R value defined four plant categories: $R > 10$ = excellent host; $10 > R > 1$ = good host; R close to 1 = maintenance host, and $R < 1$ = poor host to non-host (Ferris et al. 1993).

Isoenzymes. Isolated egg-laying females of *M. luci* and *M. ethiopica* populations were rinsed with reagent-grade water and transferred to an 8-sample-well stamp placed in an ice bath. A single female was placed in a separate sample well containing 1.3 µl of extraction buffer (20% sucrose, 2% Triton X-100, 0.01% bromophenol blue) (Esbenshade and Triantaphyllou 1985) and macerated with a needle to release the body contents. Samples were loaded on 8/0.5 sample applicators, which were placed into the applicator arms of the Phast-System equipment (GE Healthcare Life Sciences; Pharmacia). Freshly isolated *M. javanica* females (a culture obtained from the Plant Protection Service, Wageningen, The Netherlands) were used as a reference. PhastGel gradient gel (8 to 25) with a buffer system was used according to manufacturer's instructions. The conditions of electrophoresis and gel staining for MDH (EC 1.1.1.37) and EST (EC 3.1.1.1) activity were as described by Karssen et al. (1995). Enzymatic reaction was stopped by rinsing the gel with distilled water and fixed for 5 min in a solution of 10% acetic acid, 10% glycerol, and 80% distilled water. Relative migration rates (Rm) of EST bands were calculated as a ratio of migration distance of EST bands to the upper *M. javanica* EST band.

DNA extraction. DNA was extracted from a single egg mass of each population. A Promega Genomic DNA Wizard purification kit (Madison, WI) was used according to manufacturer's instructions. Extracted DNA was diluted in 10 µl of DNA rehydration solution (10 mM Tris-HCl, 1 mM EDTA).

PCR amplification. SSU rDNA fragments were amplified with two set of primers, 1A (5'-GGCGATCGAAAAGATTAAGCC-3') and 3B (5'-GGCGATCGATTGGCAAATGCTTTCGC-3') described by Baldwin et al. (1997), and Melf (5'-TACGGACTGAGATAATGGT-3') and MelR (5'-GGTTCAAGCCACTGCGA-3') described by Tigano et al. (2005). Both primers sets gave overlapping sequences and resulted in an SSU rDNA gene sequence of approximately 1,700 bp. PCR reactions contained 1 µl of isolated DNA, 10 mM Tris-HCl pH 8.3, 25 mM MgCl₂, 2.5 mM of each of the dNTPs, 1 µM of each primer, 1U GoTaq DNA Polymerase (Promega), and distilled water up to 25 µl. The amplification was carried out in a thermal cycler Veriti (Applied Biosystems) using the following program: initial denaturation at 94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 45 s, and elongation at 72°C for 2 min; followed by a final extension at 72°C for 7 min.

The region of mtDNA, located in the 3' portion of the gene that codes for cytochrome oxidase subunit II (*COII*) through a portion

of the 16S rRNA (*IRNA*) gene, was amplified with primers C2F3 5'-GGTCAATGTTCAGAAATTTGTGG-3' and 1108 5'-TACCTTTGACCAATCACGCT-3' (Powers and Harris 1993). PCR reactions contained 1 µl of isolated DNA (see above), 10 mM Tris-HCl pH 8.3, 33 mM MgCl₂, 2.5 mM of each of the dNTPs, 1 µM of each of the primers, 1U GoTaq DNA Polymerase (Promega), and distilled water up to 25 µl. The amplification was carried out in a thermal cycler Veriti (Applied Biosystems) using the following program: initial denaturation at 93°C for 3 min; 33 cycles of denaturation at 93°C for 4 s, combined annealing and elongation at 48°C for 8 min; followed by a final extension at 48°C for 10 min.

A D2-D3 fragment of LSU rRNA gene was amplified using D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGG AAGGAACCAGCTACTA-3') primers (Rubtsova et al. 2001) while the ITS1-5.8S-ITS2 region of rRNA was amplified using forward 5'-TTGATTACGTCCCTGCCCTTT-3' (Vrain et al. 1992) and reverse 5'-TCCTCCGCTAAATGATATG-3' (Ferris et al. 1993) primers. The PCR conditions were the same as for the amplification of SSU rDNA fragments. Electrophoresis of all amplified DNA products was performed on a 1% TBE agarose gel.

Transformation, isolation of plasmid DNA, and sequencing. SSU rDNA and mtDNA PCR products, and PCR products of selected populations for ITS and LSU rDNA regions, were transformed using a pGEM-T Easy Vector System II (Promega) according to the manufacturer's instructions. Plasmid DNA was isolated by JetQuick Plasmid Miniprep (Genomed) following the manufacturer's instructions. Macrogen Inc. (Korea) provided sequencing of plasmids with universal primers SP6 and T7, while the amplicons of LSU rDNA and ITS regions (except for the populations that were cloned) were sequenced directly with the primers used in amplification.

Sequence analysis. DNA sequences were assembled using the BioEdit v. 7.0.5.2 software (T. A. Hall). An alignment was prepared with EMBL-EBI Multiple Sequence Alignment Tools MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and edited manually if necessary. To assess relationships between populations and related species, DNA sequences determined in this study and sequences from the NCBI GenBank public database were included in the analyses. The neighbor joining (NJ) method conducted in MEGA v. 6.06 (K. Tamura et al.), maximum likelihood (ML) method in PhyML 3.0 (S. Guindon et al.; <http://www.atgc-montpellier.fr/>), and Bayesian inference method in MrBayes v. 3.2.2. (F. Ronquist and J. P. Huelsenbeck) were all used for phylogenetic and molecular evolution analyses. Bayesian phylogeny inferences in MrBayes and the ML analysis in PhyML implemented a substitution model selected according to the Akaike information criterion calculated with the software jModelTest v. 2.1.4. (D. Darriba et al.). Model parameters used for the NJ method were determined with MEGA 6. An analysis of five million generations based on four Markov chains sampled every 100 generations was performed with MrBayes. Calculation of the 50% majority rule consensus tree ignored the first 0.5% of trees. A burn-in of 25% was used when the 50% majority rule consensus tree was calculated to receive posterior probabilities.

Results

Differential host status. Watermelon cv. Charleston Gray was a poor host for populations of *M. ethiopica* and *M. luci*. All three RKN populations of *M. ethiopica* and *M. luci* had a reproduction factor under 1. The Brazilian and African populations of *M. ethiopica* and the Slovenian population of *M. luci* had Rf of 0.3, 0.7, and 0.6, respectively.

Isoenzymes. MDH phenotype N1 was determined in all the *M. ethiopica* and *M. luci* populations that were examined. The EST phenotype E3 was determined in the Brazilian *M. ethiopica* population, while the phenotype E2 was exhibited in the South African *M. ethiopica* population. The two banded phenotype E2 differs from E3 by the absence of the middle band of E3. All *M. luci* populations, including populations from Slovenia, Greece, and Turkey, previously identified and reported as *M. ethiopica* with E3 EST pattern, displayed EST phenotype L3. Phenotypes E3 and L3 differed in the Rm of the upper EST band of 0.96 and 1.00, respectively (Fig. 1, Table 1).

Sequence variability and comparison. All sequences obtained in this study were deposited at the European Nucleotide Archive, EMBL Nucleotide Sequence Database with the accession numbers LN626920–LN626967 and LN713293–LN713302. Phylogenetic relationships were evaluated between *M. ethiopica* and *M. luci* populations from different regions and compared with related *Meloidogyne* species using four molecular markers: SSU rDNA, LSU rDNA, ITS rDNA, and COII/IRNA mtDNA.

Low variation with 99.6% sequence identity was observed in the mtDNA region COII/IRNA among *M. luci* sequences from this study and other sequences available in the database. A slightly higher level of variation (99.3% sequence identity) was observed in a combined set of *M. ethiopica* and *M. luci* sequences (Supplementary Table S2). Considerably higher variation was observed in the three rDNA markers. Sequence identities for *M. luci* sequences from this study and other available sequences in the database are 97.8, 94.8, and 89.1% for SSU, LSU, and ITS, respectively (data not shown).

Sequence similarity trees based on mtDNA and rDNA sequences were constructed using NJ, ML, and Bayesian inference methods. Tree topologies were fairly consistent irrespective of the algorithm and are represented by the ML trees (Fig. 2, Supplementary Figs. S1, S2, and S3). Evolutionary relationships of RKN sequences inferred using the mtDNA region of 40 RKN species and populations clustered all *M. luci* sequences in a monophyletic clade (Fig. 2). On the other hand, trees based on SSU, LSU, and ITS representing 64, 28, and 58 sequences of RKN species and populations do not cluster *M. luci* sequences in monophyletic clades.

Our study comprehensively analyzed rDNA and mtDNA sequences of *M. ethiopica* and *M. luci* populations from different countries including sequences of other *Meloidogyne* spp. from GenBank. The study revealed that the SSU, LSU, and ITS regions of the rDNA are not appropriate markers for studying relationships among *M. ethiopica* and *M. luci* populations as well as other species from the RKN tropical group. The sequences of the tropical RKN species *M. ethiopica*, *M. luci*, *M. arenaria*, *M. javanica*, and *M. incognita* did not form monophyletic groups in any of the rDNA studied markers. Furthermore, genetic variability was greater within the species than between species even on the relatively conserved region SSU.

On the contrary, the COII/IRNA region of mtDNA proved to be very useful for analyzing the phylogenetic relationship of the these very closely related RKN species and populations. Low levels of heteroplasmy in this region allowed the formation of a monophyletic clade for *M. luci* with a clear separation of this recently described

species. At the same time, a very close relationship between *M. ethiopica* and *M. luci* and also *M. inornata* was observed.

Discussion

The characteristics of the newly described species *M. luci* (Carneiro et al. 2014) and our results indicate a very close relationship between the sister species of *M. luci*, *M. inornata*, and *M. ethiopica*. Morphologically *M. luci* can be distinguished from other RKNs through several characteristics such as perineal pattern, morphology of the male or female stylet, and male and second stage juvenile head and tail morphology (Carneiro et al. 2014). However, on the basis of morphological features alone, the sister species *M. luci* and *M. ethiopica* are very difficult to distinguish. For example, the feature of the *M. luci* perineal pattern, which is often used as a diagnostic characteristic, is very similar to *M. ethiopica* (mix of *M. arenaria* and *M. incognita*). Using scanning electron microscopy Carneiro et al. (2014) reported the following morphological differences between *M. luci* and *M. ethiopica*: the reniform stylet knobs, the cephalic region of males, and the shape of male stylet. For example, in *M. luci* the stylet knobs are small and rounded to a heart shape as compared with the reniform shape of *M. ethiopica*. In fact, all the above mentioned differences are quite difficult to observe using the most widely used light microscopy and even then, highly trained and experienced diagnostic specialists are needed. In addition, most of the morphometrical characteristics of juveniles, females, and males overlap between sister species, thus making morphological identification unreliable.

Host status. Watermelon cv. Charleston Gray was one of the hosts of the North Carolina differential host test, and it was used to determine the host status of the South African and Brazilian populations of *M. ethiopica* and Slovenian populations of *M. luci*. All populations in our study had a reproduction factor under 1 and therefore watermelon cv. Charleston Gray was determined to be in the range of a poor to non-host. Our results contradict the finding of Carneiro et al. (2003, 2007) who reported that watermelon cv. Charleston Gray was a good host for *M. ethiopica* (although the exact Rf was not reported). On the other hand, watermelon cv. Charleston Gray was reported as a non-host for the sister species *M. luci* (Carneiro et al. 2014). The reported Rf = 0.3 in *M. luci* is not different from the Rf = 0.3 of the Brazilian *M. ethiopica* population from this study. Although previously reported data suggests that watermelon cv. Charleston Gray could be used as a differential host between *M. ethiopica* (Carneiro et al. 2007) and *M. luci* (Carneiro et al. 2014), our results do not support this.

There is no dependable differential host between *M. ethiopica* and *M. luci* and it seems that these two species are so closely related that there is no biological difference between the two species in terms of parasitizing different plant species. Based on crop damage and economic losses that *M. ethiopica* has been causing on grapevines and kiwi (Carneiro et al. 2003), *M. ethiopica* has been included on the EPPO Alert list of harmful organisms in 2011. *M. ethiopica* is also reported to affect potato production (Medina et al. 2014) and *M. luci* has been shown as a potential threat to the potato production as well (Maleita et al. 2016). Further, *M. luci* has been reported on several vegetables, flowers, and fruits species (Carneiro et al. 2014). It should also be noted that some hosts previously reported for

Fig. 1. Isozymes malate dehydrogenase (MDH) and esterase (EST) phenotype patterns of individual female of *Meloidogyne luci* and *M. ethiopica*. **A**, lanes: 1 - *M. luci* from Turkey, 3 - *M. luci* from Slovenia, 4 - *M. ethiopica* from Brazil, 5 - *M. ethiopica* from South Africa, 7 - *M. luci* from Greece, *M. javanica* (lanes 2, 6) as a reference. **B**, *M. javanica* (lanes 1, 2), *M. luci* from Slovenia (lanes 3, 4), and *M. luci* from Iran (lanes 5, 6, 7). Populations from Slovenia, Greece, and Turkey have been previously identified as *M. ethiopica* (Aydinli et al. 2013; Conceição et al. 2012; Strajnar et al. 2009; Širca et al. 2004), but are reclassified in this study to *M. luci*.

Table 1. The relative migration rates (Rm) of esterase isozymes of studied *Meloidogyne ethiopica* and *M. luci* populations and of reference *M. javanica* population

Species and populations	Rm
<i>M. javanica</i>	1.00, 1.11, 1.16
<i>M. ethiopica</i> Brazil	0.96, 1.05, 1.13
<i>M. ethiopica</i> South Africa	0.96, 1.13
<i>M. luci</i> Slovenia	1.00, 1.05, 1.13
<i>M. luci</i> Greece	1.00, 1.05, 1.13
<i>M. luci</i> Turkey	1.00, 1.05, 1.13
<i>M. luci</i> Iran	1.00, 1.05, 1.13

Fig. 2. Evolutionary relationships of *Meloidogyne* sequences inferred using the mtDNA region between COII and 16S rRNA genes of 40 *Meloidogyne* species and populations and the ML method. Bootstrap support over 50% shown above branches, posterior probabilities from the Bayesian inference above 0.90 shown below branches.

M. ethiopica (Strajnar et al. 2009) are in fact hosts of *M. luci*, as the identity of Slovene *M. ethiopica* population has been reclassified into *M. luci*. In addition, the potential of *M. ethiopica* to survive outdoors under a continental climate even in areas where soil temperatures fall below zero during winter, as well as under a sub-Mediterranean climate (Strajnar et al. 2011) now applies to *M. luci*. This indicates that *M. luci* could establish and spread in the southern and central parts of the EPPO region. Based on the potential threat *M. luci* represents for agriculture in the EPPO region, it is the opinion of the authors that *M. luci* should be added to the national and international lists of harmful organisms of phytosanitary concern.

Isozyme profile. Carneiro et al. (2004, 2007) reported that isozyme phenotyping is currently the most useful procedure for differentiation of *M. ethiopica* from other species as the esterase phenotype E3 is species-specific for *M. ethiopica*. However, E3 is not the only esterase phenotype found in *M. ethiopica*. Mandefro and Dagne (2000) previously reported two esterase phenotypes, E2 and E3, for *M. ethiopica* but the relative migration rates were not reported. While some *Meloidogyne* species have one species-specific esterase pattern, some species like *M. arenaria* (Esbenshade and Triantaphyllou 1985), *M. incognita* (Carneiro et al. 2000; Esbenshade and Triantaphyllou 1985), and *M. exigua* (Muniz et al. 2008) are known to display several esterase patterns. Some patterns can be found in more than one species; for example, *M. morocciensis* (Rammah and Hirschmann 1990) had the same esterase phenotype as *M. arenaria* (A3). In this study we have shown that Slovene, Greek, and Turkish RKN populations previously identified as *M. ethiopica* and reported to have esterase phenotype E3 (Aydınlı et al. 2013; Conceição et al. 2012; Strajnar et al. 2009) in fact exhibit a three banded esterase pattern where the upper band is slightly lower than in *M. ethiopica* populations from Brazil, Chile, and Peru. This pattern has recently been recognized as L3 and specific for a newly described species *M. luci* (Carneiro et al. 2014). A close look at the published esterase profile of the *M. ethiopica* from Italy reveals that this population also has the L3 esterase phenotype, although it was reported as an E3 esterase phenotype (Maleita et al. 2012). Therefore, all previously reported populations of *M. ethiopica* in Europe and Turkey in fact belong to the recently described *M. luci*. This misidentification is the consequence of the fact that L3 and E3 phenotypes differ in migration rate of one band only, which is hard to spot unless populations with both phenotypes are analyzed on the same gel.

Isozyme MDH pattern does not differentiate between *M. ethiopica* and *M. luci* as phenotype N1 was determined in all tested populations, which is in accordance with previous reports (Carneiro et al. 2007, 2014; Strajnar et al. 2009).

Sequences, phylogeny, and evolution. The majority of tropical RKNs, including *M. ethiopica* and *M. luci*, reproduce by obligate mitotic parthenogenesis (Carneiro et al. 2004, 2014; Castagnone-Sereno and Danchin 2014). High diversity, even within individual nematodes, has previously been reported for the ITS region in *M. arenaria*, *M. javanica*, and *M. incognita* by Hugall et al. (1999). Based on the high variability of ITS sequences in tropical *Meloidogyne* that reproduce with obligate mitotic parthenogenesis, Hugall et al. (1999) hypothesized that these species have hybrid origin and reticulate evolution. Recent comparative genomics studies of three RKN species confirmed hybrid origin of apomictic RKNs (Lunt et al. 2014). Taking into account the hybrid origin of apomictic RKNs, finding high variability of rDNA regions in *M. luci* is not unexpected. However, high genetic variability represents a challenge for the description of a new species in the group of tropical RKNs as well as for the development of identification tools. While mtDNA in apomictic RKNs can be used for species identification (Janssen et al. 2016; Pagan et al. 2015), it cannot be used to study species evolution within the group as the reticulate evolution of these species was very complex (Lunt et al. 2014). While we observed a close relationship between *M. ethiopica*, *M. luci*, and *M. inornata* based on COII/IRNA region of mtDNA, a close relationship between these three species has also been shown on coding mtDNA regions by Janssen et al. (2016).

Since variability of both the rDNA as well as the mtDNA regions has been well documented in this and previous studies (Holterman et al. 2006, 2009, 2012; Kiewnick et al. 2014; Powers et al. 2005;

Tigano et al. 2005), careful selection of molecular markers to study phylogenetic relationships or to confirm the identification of *Meloidogyne* species is needed. Our study suggests that mtDNA is suitable for *M. luci* species identification, while the SSU rRNA gene, ITS region, and a segment of LSU rDNA were revealed as poor identification markers for tropical *Meloidogyne* species identification. Analyzing phenotypes of MDH and EST isozymes in combination with phylogenetic analysis of mtDNA should be used to distinguish between *M. ethiopica* and *M. luci*. In the study by Janssen et al. (2016), *M. luci* from Slovenia and Iran shared the same haplotype, while *M. luci* from Guatemala exhibited another haplotype occurring in a paraphyletic position. It seems that genomic approach will be needed to resolve phylogenetic relationships of closely related populations of *M. ethiopica*, *M. luci*, and *M. inornata*.

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