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Meloidogyne luci, a new root-knot nematode parasitizing potato in Portugal

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In 2013, during a field survey conducted in Portugal on potato, *Solanum tuberosum*, an unusual esterase (EST) phenotype was detected in a root-knot nematode (RKN) from potato roots collected in Coimbra. This Portuguese isolate was purified and maintained on tomato, *S. lycopersicum*, and morphological, biochemical and molecular characteristics were studied. Perineal pattern morphology was highly variable, similar to *Meloidogyne ethiopica* and not useful for identification. The EST phenotype, from young egg-laying females, displayed three bands similar to the Brazilian *M. luci* (L3) and distinct from *M. ethiopica* (E3). Phylogenetic analyses of mitochondrial cytochrome oxidase subunit I and the mitochondrial DNA region between *COII* and 16S rRNA genes revealed that the Portuguese isolate grouped with *M. luci* isolates close to *M. ethiopica* isolates. However, considering the ITS1-5.8S-ITS2 region, the Portuguese isolate grouped with isolates of *M. luci*, *M. ethiopica* and *M. hispanica*, which limits the confidence of this region for *M. luci* diagnosis, and its differentiation from other species with morphological similarities. The *M. luci* pathogenicity to potato was also assessed in 16 commercial cultivars and compared with *M. chitwoodi*, considered to be a quarantine RKN species by EPPO. All potato cultivars were susceptible to both *Meloidogyne* species with gall indices of 5 and higher reproduction factor values ranging from 12.5 to 122.3, which suggests that *M. luci* may constitute a potential threat to potato production. In the present study, *M. luci* is reported for the first time attacking potato in Portugal.

Keywords: esterase phenotype, mtDNA region, perineal pattern, root-knot nematodes, taxonomy

Introduction

World production of potato, *Solanum tuberosum*, is estimated to be around 382 × 10^6 tonnes annually. China is the world's largest producer (96 × 10^6 tonnes) while Europe produces 125 × 10^6 tonnes, with the Russian Federation being the largest European producer (32 × 10^6 tonnes). In Portugal, an average of 534 × 10^3 tonnes of potato were produced in 2014 with an average potato yield of around 20 tonnes ha^{-1}, compared to 22 tonnes ha^{-1} in Europe (FAO, 2017).

The most damaging nematode pests of potato crops are the potato-cyst nematodes; however these are not the only plant-parasitic nematodes (PPN) responsible for low potato yields. Infestations of root-knot nematodes (RKN), *Meloidogyne* spp., root-lesion nematodes, *Pratylenchus* spp., the false root-knot nematode, *Naccobus aberrans*, stubby-root nematodes, *Paratrichodorus* spp. and *Trichodorus* spp., the potato rot nematode, *Ditylenchus destructor*, and the yam nematode, *Scutellonema bradys*, have also been detected on this crop (Palomares-Rius *et al.*, 2014).

The genus *Meloidogyne* includes nearly 100 described species; however, the majority of research has focused on *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* (Elling, 2013). In turn, several other *Meloidogyne* species, such as *M. chitwoodi*, *M. fallax*, *M. hispanica* and *M. minor*, are perceived as emerging species with potential to cause significant damage to potato crops (Maleita *et al.*, 2012a; Elling, 2013). To prevent further distribution within Europe, *M. chitwoodi* and *M. fallax* are included in the European list of quarantine organisms (Hunt & Handoo, 2009). Infection of potato tubers/fields by *M. arenaria*, *M. chitwoodi*, *M. ethiopica*, *M. fallax*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. minor* has been reported previously in several regions of the world (Jatala & Bridge, 1990; Molendijk & Mulder, 1996; Conceição *et al.*, 2009; Wesemael *et al.*, 2011; Medina *et al.*, 2017). In Portugal these RKN species, with the exception of *M. fallax*, *M. ethiopica* and *M. minor*, have been found parasitizing tubers or infesting potato fields (Conceição *et al.*, 2009).

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In 2013 an unusual esterase (EST) phenotype (L3) was detected in the Portuguese RKN isolate PtL3 from potato roots collected in the Coimbra region. *Meloidogyne* sp. females with similar EST phenotype have been recorded since 1985 in South America (Argentina, Bolivia, Brazil, Chile, Ecuador and Guatemala), Iran and Europe (Turkey and Slovenia) (reviewed in Carneiro *et al.*, 2014; Bellé *et al.*, 2016; Janssen *et al.*, 2016). The description of this RKN species, designated as *M. luci*, was only made in 2014 from isolates from lavender (*Lavandula spica*) collected in Rio Grande do Sul, Brazil (Carneiro *et al.*, 2014). Recently, several populations of *M. ethiopica* in Europe were reclassified, using biochemical and molecular analyses, and identified as *M. luci* (Stare *et al.*, 2017). *Meloidogyne luci* has been associated with several important plant species, such as bean (*Phaseolus vulgaris*), broccoli (*Brassica oleracea*), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), grapevine (*Vitis vinifera*), kiwifruit (*Actinidia deliciosa*), lavender (*Lavandula angustifolia*), lettuce (*Lactuca sativa*), okra (*Abelmoschus esculentus*), rose (*Rosa* sp.), snapdragon (*Antirrhinum majus*), sedum (*Hylotelephium spectabile*), soybean (*Glycine max*), tomato (*S. lycopersicum*) and yakon (*Polymnia sonchifolia*) (Carneiro *et al.*, 2014; Bellé *et al.*, 2016; Janssen *et al.*, 2016).

Current approaches to control RKN include the use of nematicides, but many of these are being phased out due to their harmful effects on human health and the environment (Wesemael *et al.*, 2011). Thus, cultural techniques and resistant cultivars are the only effective way to reduce *Meloidogyne* species infections. Knowledge about *M. luci* pathogenicity on a large number of potato cultivars may also be important for quarantine purposes and to implement effective integrated management programmes to avoid the dissemination of this species through the potato-growing areas.

In the present study, morphological, biochemical and molecular characters were used to identify the PtL3 RKN isolate as *M. luci*. The relationship with other RKN was studied based on analysis of sequences from the mitochondrial DNA (cytochrome oxidase subunit I, *COI*, and between cytochrome oxidase subunits II and the 16S rRNA genes, *COII*/16S rRNA) and the internal transcribed spacer 1, 2 and 5.8S (ITS1-5.8S-ITS2) rDNA region. Furthermore, the host–parasite relationships were evaluated in a pot test using commercial potato cultivars and compared with *M. chitwoodi*, the potato RKN also found in Portugal (Conceição *et al.*, 2009).

Materials and methods

Nematode isolate

The PtL3 RKN isolate was cultured by inoculation of a single egg mass on tomato cv. Coração de Boi, grown in autoclaved sandy loam soil and sand (1:1) in a growth chamber at 25 ± 2 °C, with a 12 h photoperiod. Females were handpicked from infected tomato roots, and second-stage juveniles (J2) hatched from egg masses in moist chambers. Morphological, biochemical and molecular characterization was made from tomato culture. For biochemical studies, one isolate of *M. ethiopica* (BrE3) and one of *M. luci* (BrL3), used on the original species description (Carneiro *et al.*, 2014), both from Brazil, were included for comparison. For the molecular studies, *M. luci* isolates from Greece, Italy and Turkey, previously misidentified as *M. ethiopica* (Stare *et al.*, 2017), were used for mtDNA *COI* region sequencing. Similarly, for the assessment of the pathogenicity of *M. luci* on potato cultivars, a Portuguese isolate of *M. chitwoodi* was included. *Meloidogyne ethiopica*, *M. luci* and *M. chitwoodi* isolates were maintained on tomato cv. Coração de Boi as described above.

Morphological characterization

Perineal patterns of adult females were cut from live specimens in 45% lactic acid and mounted in glycerine. Photographs of females, males and J2 were taken with a Leitz Dialux 20 bright-field light microscope (LM).

Biochemical characterization

For isozyme analysis, five young egg-laying females were handpicked from infected tomato roots and transferred to micro-haematocrit tubes with 5 µL of extraction buffer (20% w/v sucrose with 1% v/v Triton X-100). The females were macerated with a pestle, frozen and stored at −20 °C. Electrophoresis was performed according to Pais *et al.* (1986). Proteins were separated by electrophoresis on thin-slab polyacrylamide gels and the gels stained for EST activity with the substrate α-naphthyl acetate. Protein extracts from females of *M. ethiopica* and *M. luci* were included in each gel for comparison and a protein extract of an isolate of *M. javanica* was used as a reference (Pais *et al.*, 1986).

Molecular characterization

The mtDNA *COI*, mtDNA *COII*/16S rRNA and ITS1-5.8S-ITS2 rDNA genomic regions were selected for molecular characterization of the Portuguese *M. luci* isolate. Total DNA was extracted from J2 using the DNeasy Blood & Tissue kit (QIAGEN). The mtDNA *COI* amplification was performed using primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and 2R5 (5′-YTRWYCTTAAATCTAAATKMGTAT-3′) (Kiewnick *et al.*, 2014), while the mtDNA COII/16S rRNA region was amplified using primers C2F3 (5′-GGTCAATGTTCAGAA ATTTGTGG-3′) and MRH106 (5′-AATTTCTAAAGACT TTTCTTAGT-3′) (Powers & Harris, 1993; Stanton *et al.*, 1997). ITS1-5.8S-ITS2 rDNA amplification was performed using the forward primer 5′-TTGATTACGTCCCTGCC CTTT-3′ and the reverse primer 5′-TCCTCCGCTAAAT GATATG-3′ (Schmitz *et al.*, 1998). For mtDNA *COI*, the PCR was performed in a mixture containing 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 2.5 U *Taq* DNA polymerase (Bioline) and 2 µL DNA with the thermal cycling programme described by Kiewnick *et al.* (2014). The PCRs and the thermal cycling programmes for mtDNA *COII*/16S rRNA were performed as described in Maleita *et al.* (2012b). PCR amplification of ITS1-5.8S-ITS2 rDNA was achieved in a mixture containing 5 µL DNA and 0.8 U *Taq* DNA polymerase (Bioline), in 1× buffer, 1.8 mM $MgCl_2$, 200 µM dNTPs, and 1.5 µM of each primer, and following the thermal cycling programme described by Subbotin *et al.* (2000).

The PCR products were analysed on 1% agarose gel in 1× TBE buffer stained with GreenSafe, purified from the gel with the MiniElute Gel Extraction kit (QIAGEN) and quantified using the NanoDrop 2000C spectrophotometer (Thermo Scientific) following the manufacturer's instructions. Amplified DNA was sequenced in both directions with the same primers used for amplification, by standard procedures at Macrogen, Inc. (Seoul, Korea). Because multiple peaks in the sequencing chromatograms of mtDNA *COII*/16S rRNA and ITS1-5.8S-ITS2 rDNA were observed, suggesting the presence of more than one sequence for each DNA region studied, amplified DNA was cloned by standard procedures at Macrogen, Inc. and three clones were subsequently sequenced. Obtained sequences were aligned and compared with the equivalent sequences of *Meloidogyne* species available in GenBank and ENA databases. Sequences were aligned using CLUSTALW multiple alignment in BIOEDIT. Alignment was manually adjusted when necessary. The length of mtDNA *COII*/16S rRNA sequences of *Meloidogyne* spp. was set to 940 bp by removing several nucleotides to obtain a common start and end point. Phylogenetic relationships were constructed using neighbour-joining (NJ) and maximum likelihood (ML) methods in MEGA 5. The NJ analyses were carried out using the Jukes–Cantor model with complete or pairwise deletion for mtDNA *COI* and ITS1-5.8S-ITS2 rDNA regions, and mtDNA *COII*/16S rRNA region, respectively. One thousand bootstrap replicates were performed to test the support of each node on the trees. The ML analyses were based on the Jukes–Cantor model. All positions containing gaps and missing data were eliminated.

Pathogenicity of *M. luci* and *M. chitwoodi* on potato

The pathogenicity of Portuguese *M. luci* and *M. chitwoodi* isolates was evaluated in 16 potato commercial cultivars. Tomato cv. Coração de Boi was used as a control. Inocula were prepared by extraction of eggs from infected tomato roots using 0.5% NaOCl (Hussey & Barker, 1973).

Six potato plants per cultivar, grown from single sprouts in pots with 1 L of sterile sandy soil (80–90% sand, 5–10% clay, 5–10% organic matter, pH 6–7), were inoculated with 5000 eggs (initial nematode population density, Pi). Pots were arranged in a completely randomized design in a growth chamber, at 19.0–24.5 °C, with a 12 h photoperiod and plants watered daily. Sixty days after inoculation (DAI), roots were washed free of soil and galls per plant root system rated, and an index of 0–5 was assigned, where 0 = no galls, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, 5 >100 galls (Taylor & Sasser, 1978). Eggs and J2 were extracted from the entire root systems with a 0.5% NaOCl solution and counted to determine the final nematode population density (Pf). The pathogenicity was assessed on the basis of root gall index (GI) and reproduction factor (Rf = Pf/Pi) (Sasser *et al.*, 1984). Plants with GI > 2 and Rf > 1 were considered susceptible, and with GI ≤ 2 and Rf ≤ 1 as resistant (Sasser *et al.*, 1984).

Data (Pf) were checked for evidence of a normal distribution using the Kolmogorov–Smirnov test, and for variance homogeneity using Levene's test, and these two assumptions of analysis of variance (ANOVA) were only assumed after data transformation [$\sqrt{(x + 0.5)}$]. Fisher LSD test was therefore done to test differences among the plant species and cultivars. Statistical analysis of the Pf data was performed using STATISTICA v. 7 for Windows (Statsoft).

Results

Morphological characterization

The morphology of the anterior end of females and anterior/posterior end of males and J2 of the Portuguese *M. luci* isolate were observed at LM (Fig. 1).

Adult females generally showed a prominent neck. The body cuticle was annulated, but in anterior region was less evident. The head region was slightly set off from the body. The stylet was robust, the stylet cone wider near the shaft, and the shaft gradually wider near the junction with stylet knobs. Stylet knobs were well developed and distinct (Fig. 1a). The morphology of female perineal patterns was found highly variable (Fig. 1b–e). Shape was ovoid to squarish; dorsal arch low and rounded to moderately high and some with a higher squarish arch. Dorsal striae were smooth to wavy, sometimes forming rounded shoulders. Ventral striae were usually fine and smooth and lateral lines weakly demarcated. Phasmids ducts were distinct. Some patterns had one or two wings. The perivulval region was not striated (Fig. 1b–e).

Males had an anterior end narrowing and posteriorly were rounded with a short tail (Fig. 1f–h). Body cuticle was annulated, with large annuli. In front and lateral view, the head cap was high and rounded; the head region not set off from the body. The stylet was robust. The stylet cone was larger than the shaft, and increased in width near the junction with the shaft. The shaft was cylindrical. Stylet knobs were rounded and distinct (Fig. 1f, g). Lateral field with four incisures (Fig. 1i).

The J2 had an anterior end truncate; the head region was slightly set off from the body. The stylet was delicate, cone straight, narrow, sharply pointed; shaft slightly wider. Stylet knobs were small and oval shaped (Fig. 1j). The tail was conoid with constrictions near the terminal part which ended in a rounded tip; hyaline tail terminus (Fig. 1k).

Biochemical characterization

Three bands of EST activity were detected in PtL3 (relative mobility, Rm: 2.15, 2.45 and 2.7), similar to the specific phenotype L3 (Fig. 2) exhibited by the Brazilian *M. luci* isolate (BrL3). The *M. ethiopica* isolate (BrE3), had three EST activity bands (Rm: 1.95, 2.4 and 2.65), corresponding to the phenotype E3 (Fig. 2). The three EST bands observed in the *M. javanica* isolate (J3, Rm: 2.15, 2.55 and 2.75) were used as a reference isolate to determine the relative position of *M. luci* and *M. ethiopica* EST bands (Fig. 2). The identification of *M. luci* isolates from Greece, Italy and Turkey were also confirmed by isozyme analysis (data not shown).

Molecular characterization

The PCR amplification of mtDNA *COI*, mtDNA *COII*/16S rRNA and ITS1-5.8S-ITS2 genomic regions of PtL3

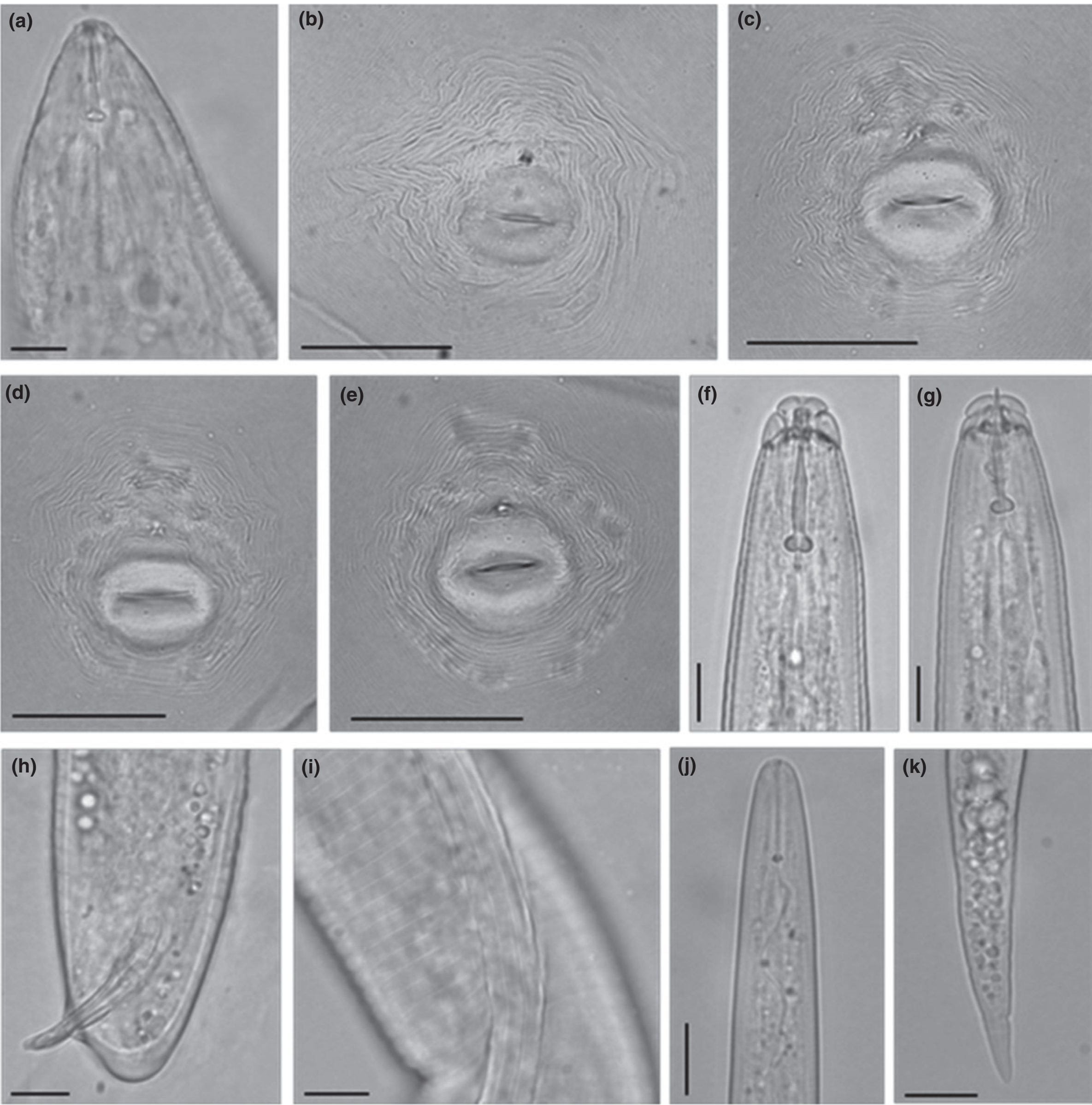

Figure 1 *Meloidogyne luci* light microscope photographs. Female: anterior end (a); and representative perineal patterns (b–e). Male: anterior region in front and lateral view (f and g, respectively); posterior region in lateral view (h); and lateral field with four lateral lines (i). Second-stage juveniles: anterior region (j); and tail region (k). Scale bars: 10 μm (a, f–k), and 50 μm (b–e).

yielded single fragments of around 800, 1800 and 800 bp, respectively (data not shown). Sequences were submitted to GenBank database under the accession numbers KY563093 for mtDNA *COI*, KM042847 and KM042848 for mtDNA *COII*/16S rRNA clones and KY554194, KY554195 and KY554196 for ITS1-5.8S-ITS2 clones. mtDNA *COI* sequences of Greek, Italian and Turkish isolates were also submitted to GenBank under accession numbers MF280974, MF280975, MF280976 and MF280973.

Phylogenetic analysis from the sequence alignment of PtL3 mtDNA *COI* sequence with the here determined mtDNA *COI* sequences of *M. luci* isolates and the other corresponding available sequences of *Meloidogyne* species revealed that PtL3 formed a single cluster with all *M. luci* isolates (63% bootstrap; Fig. 3). Additionally, NJ analysis from the sequence alignment of mtDNA *COII*/16S rRNA sequences of PtL3 with the corresponding fragments with similar length from the closely related *Meloidogyne* species available, revealed that PtL3 formed a single cluster with the *M. luci* isolates, confirming that the Portuguese isolate belongs to *M. luci* (Fig. 4). However, in this mtDNA *COII*/16S rRNA analysis, *M. ethiopica* appeared as sister taxa to *M. luci* with only

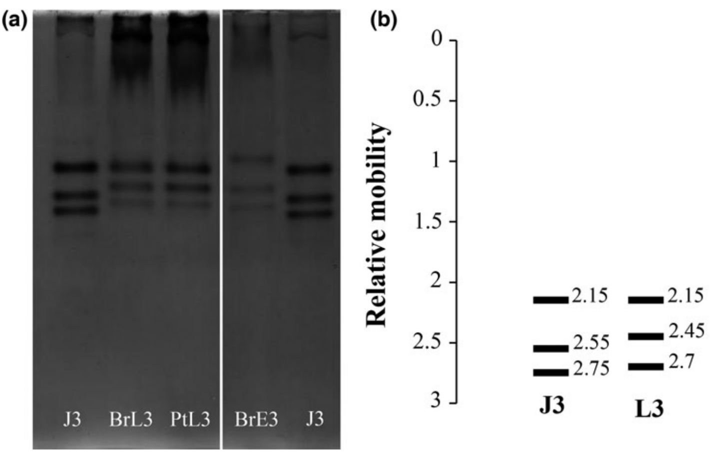

Figure 2 Esterase phenotypes of protein homogenates (a) from five egg-laying females of *Meloidogyne* species isolates, and respective relative mobility (b). BrL3 and PtL3, *M. luci* isolates from Brazil (Br) and Portugal (Pt), respectively; BrE3, *M. ethiopica* isolate from Brazil; and J3, *M. javanica* (reference isolate).

30% bootstrap and in ML analysis *M. luci* and *M. ethiopica* formed a single cluster with 86% bootstrap (data not shown). *Meloidogyne hispanica* was the most divergent RKN species (Fig. 4).

Considering the ITS1-5.8S-ITS2 region, the sequences of the three PtL3 clones differed by 1 to 6 nucleotide positions (data not shown). The phylogenetic tree from NJ analysis of ITS1-5.8S-ITS2 showed that the three clones of PtL3 formed one clade with *M. luci* isolates from Greece, Slovenia and Turkey (LN626962.1, LN626963.1 and LN626964.1), *M. ethiopica* isolates from Brazil and Chile (KF482366.1, LN626965.1 and KF482367.1) and two isolates of *M. hispanica* (EU443613.1 and EU443614.1), with a bootstrap support of 61%. Other *M. luci* isolates (Brazil, Chile and Iran, accessions KF482363.1, KF482364.1 and KF482365.1, respectively) appeared together in a well-separated clade with a bootstrap support of 99% (Fig. 5).

Both NJ and ML trees obtained for the mtDNA *COI* and ITS1-5.8S-ITS2 genomic regions analysis were similar; therefore, only the NJ trees are shown.

Pathogenicity of *M. luci* and *M. chitwoodi* on potato

Tomato cv. Coração de Boi, included as positive control, was highly infected with GI = 5 and Rf values of 72.90 and 60.8 for *M. luci* and *M. chitwoodi*, respectively (Table 1). Both *Meloidogyne* species reproduced in all potato cultivars, with high numbers of eggs and J2 recovered from roots at 60 DAI, so that all cultivars were classified as susceptible (GI = 5 and Rf > 11.1; Table 1). However, Pf varied between potato cultivars and RKN species. The Pf across potato cultivars indicated that the most susceptible to *M. luci* were cv. Carolus > cv. Raja > cv. Camberra (decreasing order of susceptibility); and to *M. chitwoodi* were cv. Carolus > cv. Camberra > cv. Monalisa. *Meloidogyne chitwoodi* tended to be more aggressive than *M. luci*: potato cultivars Agria, Camberra, Carolus, Désirée, Kondor, Monalisa, Romana and Stemster showed significantly greater Pf levels when inoculated with *M. chitwoodi* than with *M. luci* ($P < 0.05$; Table 1). *Meloidogyne chitwoodi* Rf values varied between 40.4 (cv. Stemster) and 122.3 (cv. Carolus); whilst for *M. luci* they varied between 12.5 (cv. Stemster) and 63.6 (cv. Carolus). The egg population recovered from roots was significantly greater for *M. luci* than *M. chitwoodi* only in potato cv. Evolution ($P > 0.05$). All the other potato cultivars outside these two groups did not differ statistically between the two RKN species ($P > 0.05$; Table 1).

Discussion

In the past, RKN were identified by laborious microscopic examination of morphological and biometric characters that rely on measurements and comparison of morphological structures. *Meloidogyne* species identification based on these characters is a challenge because morphological differences between RKN species are, in most cases, very subtle and measurements of individual specimens, in general, overlap. *Meloidogyne luci* can be distinguished by the combination of the perineal pattern and stylet morphology of the females, stylet, head and tail morphology in the males and head and tail morphology in the J2 (Carneiro *et al.*, 2014). The Portuguese isolate had the morphological characteristics described for *M. luci*. However, the presence of intraspecific variability hinders its identification, because some of the characters, such as morphology of the perineal patterns, are highly variable and can be found in isolates from other species: *M. ethiopica*, for example, exhibits a mix of *M. arenaria* and *M. incognita* patterns, and *M. inornata*, is closer to *M. incognita* (Carneiro *et al.*, 2014).

Several biochemical studies have demonstrated that the analysis of nonspecific EST phenotypes can be very useful for *Meloidogyne* species differentiation, remaining, in many laboratories and countries of the world, the first step in the RKN species identification process (Blok & Powers, 2009). The esterase phenotype detected in the Portuguese isolate PtL3 was similar to the Brazilian isolate BrL3. The first band (Rm 2.15) of *M. luci* was

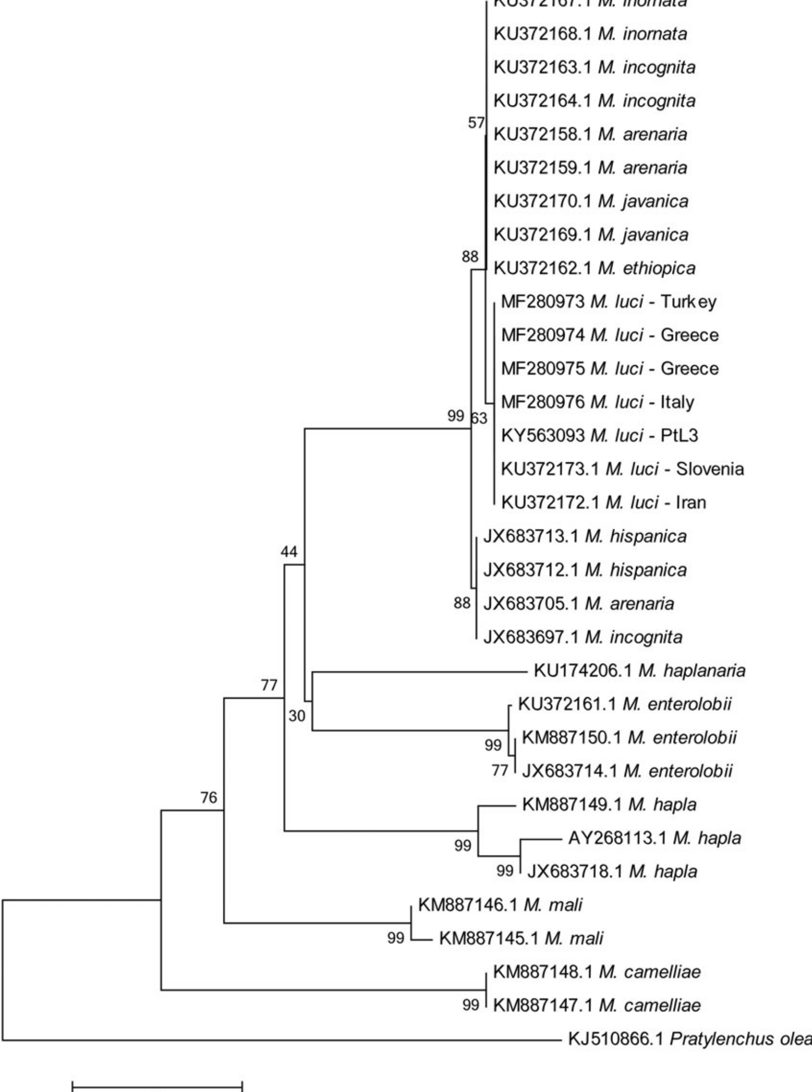

Figure 3 Neighbour-joining tree based on analysis of alignment of available *Meloidogyne* species mtDNA *COI* sequences. PtL3, Portuguese *M. luci* isolate. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Jukes–Cantor model and all positions containing gaps and missing data were eliminated.

located at the same level of the first band of the reference isolate, *M. javanica*. Considering the Brazilian *M. ethiopica* phenotype (E3), included for comparison, the Rm of the first band (Rm 1.95) is clearly different and located above. More than one phenotype for EST has been described for several *Meloidogyne* species, such as *M. arenaria* (A2, A3), *M. incognita* (I1, I2) and *M. exigua* (E1, E2, E3) (Carneiro *et al.*, 1996; Oliveira *et al.*, 2005; Muniz *et al.*, 2008). Nevertheless, to the best of the authors' knowledge, those variations occurred only in the presence/absence of minor and fainter bands and not in terms of relative mobility. The main band, used to characterize the RKN species, is always present in all phenotypes and more bands may or may not be detected, depending on the EST activity (Carneiro *et al.*, 1996). Thus, despite the similarity with *M. ethiopica*, the *M. luci* pattern is unique and useful to differentiate this species from the other RKN species found associated with potato plants.

The observation of similarity between species, intraspecific variability, and discovery of new EST patterns make the use of additional molecular information of these nematodes necessary (Muniz *et al.*, 2008; Blok & Powers, 2009; Maleita *et al.*, 2012b). Phylogenetic studies conducted by Carneiro *et al.* (2014) using the ITS1-5.8S-ITS2 region of rDNA found that *M. luci* isolates belonged to a single cluster, supporting the differentiation of this species from other RKN. Considering this genomic region, the Portuguese isolate grouped not only with *M. luci* isolates from Greece, Slovenia and Turkey but also with *M. ethiopica* isolates from Brazil and Chile and two isolates of *M. hispanica*. Only *M. luci* isolates from Brazil (KF482363.1), Chile (KF482364.1) and Iran (KF482365.1) formed a well-supported clade with 99% bootstrap. Considering the main clades, the results here agree with those obtained by Stare *et al.* (2017) for the ITS rDNA region. On the other hand, this analysis revealed that the ITS divergence was not significant to

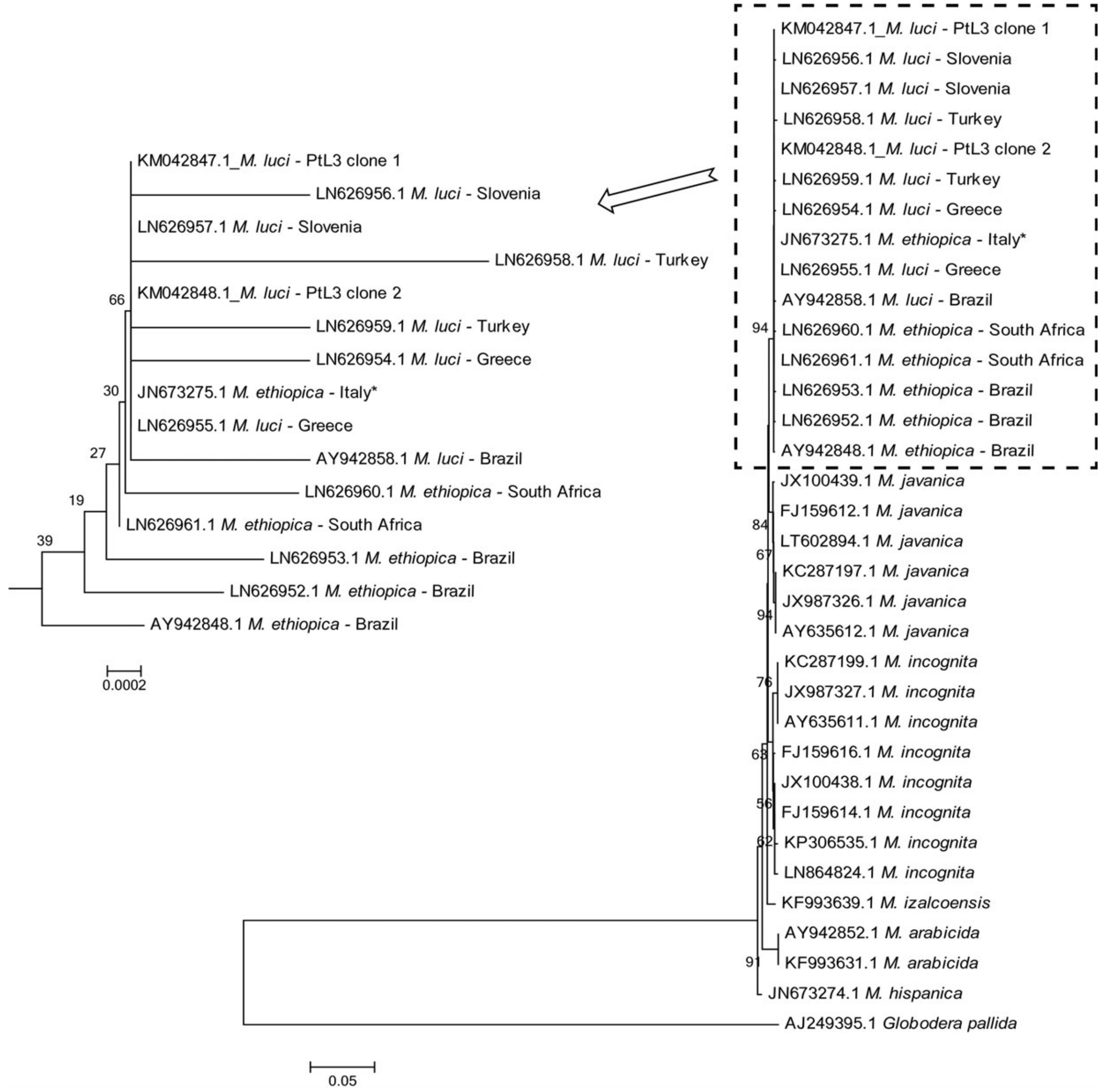

Figure 4 Neighbour-joining tree based on mtDNA *COII*/16S rRNA sequences of *Meloidogyne* species with approximate amplification product sizes. PtL3, Portuguese *M. luci* isolate. The percentage of replicate trees in which the associated *Meloidogyne* species clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Jukes–Cantor model and all ambiguous positions were removed for each sequence pair. *Recently, reclassified as *M. luci*, according to Stare *et al.* (2017).

differentiate *M. arenaria*, *M. incognita* and *M. javanica* sister species, as previously described (Blok *et al.*, 1997; Carneiro *et al.*, 2014; Stare *et al.*, 2017). Relationships between isolates of these species were not clarified using the ITS1-5.8S-ITS2 sequences, which also limits the confidence of this DNA region for *M. luci* diagnosis, and its differentiation from other species with morphological similarities, such as *M. ethiopica*. Additionally, the ITS region analysis of the Portuguese and other *M. luci* isolates revealed an intraspecific genetic diversity that has been previously reported within species and individuals from a range of nematodes, including *Meloidogyne* species (Hugall *et al.*, 1999).

Therefore, mtDNA *COI* and mtDNA *COII*/16S rRNA genes were selected to complement the molecular characterization of the Portuguese *M. luci* isolate. The mtDNA evolves, creating sufficient nucleotide variation for species-level diagnosis (García & Sánchez-Puerta, 2015). Mitochondrial DNA *COI* was proposed as a molecular marker for DNA barcoding and is capable of discriminating between RKN species more than the rRNA genes (Ahmed *et al.*, 2016). Moreover, the mtDNA *COII*/16S rRNA region has been used in diagnosis and large-scale regional RKN surveys through PCR and RFLP (Tigano *et al.*, 2005; Humphreys-Pereira *et al.*, 2014; Baidoo *et al.*, 2016; Karuri *et al.*, 2017). *Meloidogyne lopezi*,

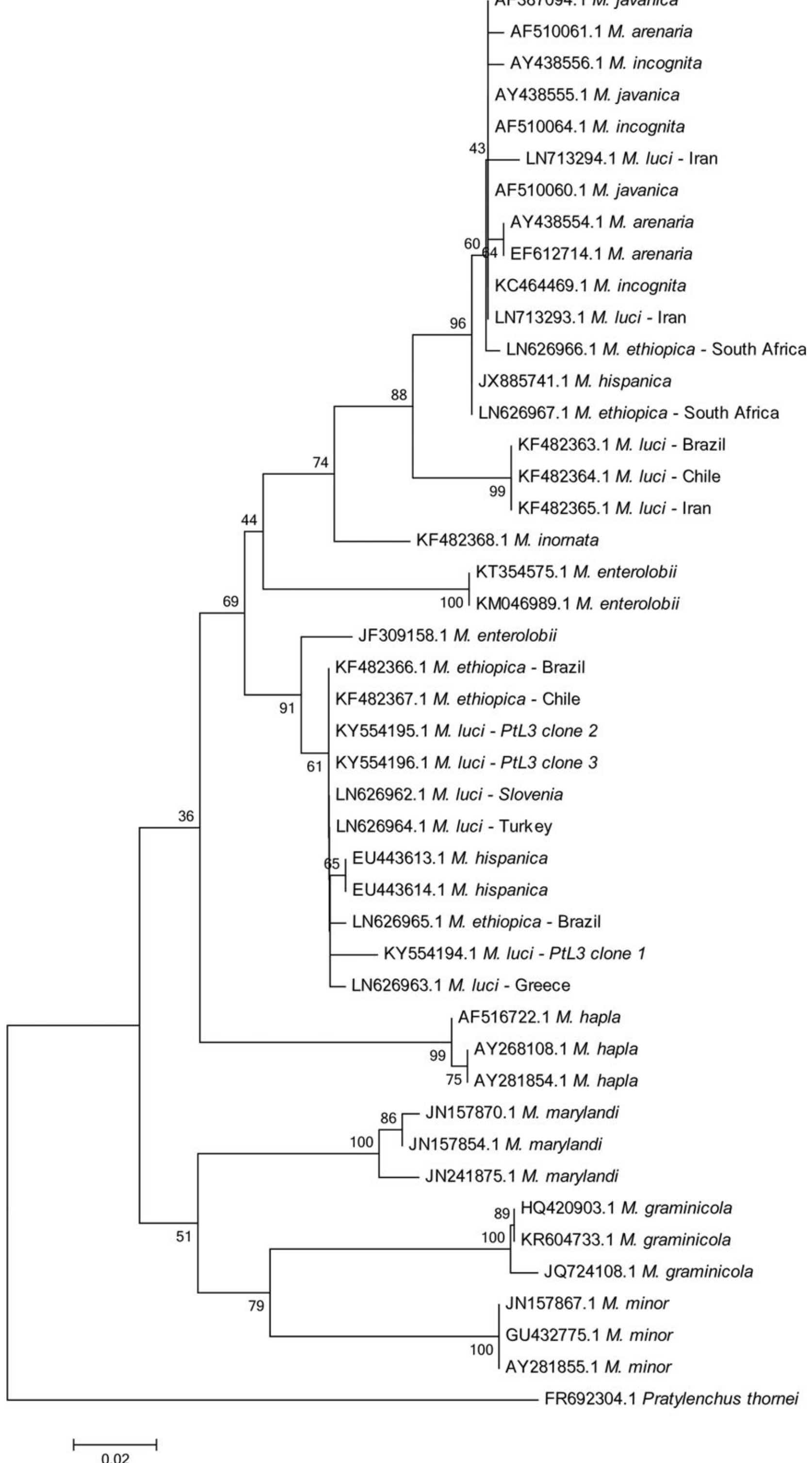

Figure 5 Neighbour-joining tree based on analysis of alignment of available *Meloidogyne* species ITS1-5.8S-ITS2 sequences. PtL3, Portuguese *M. luci* isolate. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Jukes–Cantor model and all positions containing gaps and missing data were eliminated.

closely related to other tropical *Meloidogyne* species that infect coffee, especially *M. arabicida*, *M. izalcoensis* and *M. paranaensis*, could be differentiated from these species by PCR-RFLP of the mtDNA *COII*/16S rRNA (Humphreys-Pereira *et al.*, 2014). A nematode survey of sweet potato fields and cut foliage crops was conducted in Kenya and Florida, respectively, and the mtDNA *COII*/16S rRNA region was used to determine the distribution and prevalence of *Meloidogyne* species (Baidoo *et al.*, 2016; Karuri *et al.*, 2017). Additionally, mtDNA

Table 1 Host status of potato (*Solanum tuberosum*) cultivars for *Meloidogyne luci* and *M. chitwoodi*, measured 60 days after inoculation with 5000 eggs (initial population density) per plant

	M. luci				*M. chitwoodi*			
Cultivar	*n*[a]	GI[b]	Pf[c]	Rf[d]	*n*	GI	Pf	Rf
Agria	6	5	125 777.8 ± 22 916.5 h–o	25.2	6	5	355 022.2 ± 40 598.3 b–e	71.0
Berber	4	5	128 866.7 ± 50 219.3 h–p	25.8	6	–[e]	–	–
Camberra	6	5	242 111.1 ± 71 257.2 c–j	48.4	6	5	535 555.6 ± 97 328.5 a	107.1
Carolus	6	5	317 777.8 ± 70 818.3 b–g	63.6	6	5	611 733.3 ± 121 741.9 a	122.3
Colomba	6	–[e]	–	–	5	5	110 788.9 ± 88 501.4 i–q	22.2
Désirée	6	5	178 555.6 ± 59 309.2 f–m	35.7	6	5	367 444.4 ± 124 578.2 b–e	73.5
Evolution	5	5	100 933.3 ± 35 958.6 j–q	20.2	5	5	55 386.7 ± 18 821.5 m–q	11.1
Fortus	6	5	92 600.0 ± 32 757.6 k–q	18.5	5	5	141 333.3 ± 46 009.7 h–o	28.3
Kennebec	4	5	172 533.3 ± 71 469.7 f–n	34.5	5	5	235 200.0 ± 73 316.8 c–j	47.0
Kondor	6	5	231 288.9 ± 52 199.9 d–j	46.3	6	5	363 400.0 ± 122 500.2 b–e	72.7
Lusa	6	5	127 466.7 ± 63 399.6 h–o	25.5	4	5	304 800.0 ± 137 415.4 b–h	61.0
Monalisa	6	5	102 555.6 ± 30 855.1 j–q	20.5	6	5	529 400.0 ± 116 406.2 a	105.9
Picasso	6	5	140 088.9 ± 41 892.5 h–o	28.0	6	5	242 533.3 ± 86 680.0 c–j	48.5
Raja	5	5	242 453.3 ± 98 511.3 c–j	48.5	6	5	300 900.0 ± 131 449.0 b–h	60.2
Romana	5	5	108 411.1 ± 56 849.2 j–q	21.7	6	5	308 777.8 ± 72 152.0 b–g	61.8
Stemster	6	5	62 627.8 ± 36 414.5 m–q	12.5	5	5	201 760.0 ± 67 772.3 e–l	40.4
Volumia	5	5	72 433.3 ± 29 512.3 l–q	14.5	4	5	106 750.0 ± 63 157.7 j–q	21.4
Coração de Boi[f]	5	5	364 320.0 ± 56 165.6 b–e	72.9	5	5	304 000.0 ± 144 406.8 b–f	60.8

[a]No. of replicates per cultivar.
[b]GI, gall index: 0 = no galls, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, 5 = >100 galls.
[c]Pf, final population density (J2 + eggs). Data are means of replicates ± standard deviation. Means in this column followed by the same combination of letters do not differ significantly at $P > 0.05$, according to the Fisher LSD test.
[d]Rf, reproduction factor = Pf/initial population density.
[e]No data available.
[f]Tomato used as a control.

COII/16S rRNA data improved the discrimination sensitivity of RKN species and atypical, non-identified isolates (Tigano *et al*., 2005). Recently, Stare *et al*. (2017) have reclassified *M. ethiopica* isolates from Europe and Turkey as *M. luci*, and have considered that the mtDNA *COII*/16S rRNA region is useful for analysing the phylogenetic relationship of these closely related species. However, in the present study, phylogenetic analysis of this region was not very robust differentiating *M. luci* from *M. ethiopica* but, on the other hand, the mtDNA *COI* region was revealed as a useful region to differentiate *M. luci* from other species with morphological similarities, namely *M. ethiopica*. Nevertheless, mtDNA *COI* region analysis was not useful to differentiate *M. arenaria*, *M. ethiopica*, *M. incognita*, *M. inornata* and *M. javanica* species. *Meloidogyne luci* clearly differs from other RKN species by isozyme analysis and molecular characters. The application of mtDNA molecular markers integrated with biochemical studies supported the diagnosis and characterization of *M. luci*. However, a careful selection of molecular markers is needed and further studies should be performed to analyse/characterize a broad range of *M. luci* isolates to validate the mtDNA *COI* results and strengthen their potential applicability in diagnosis.

The pathogenicity assays revealed that all of the 16 potato cultivars were susceptible (GI = 5 and Rf > 1) to *M. luci* and *M. chitwoodi*. Nonetheless, for eight of the 16 potato cultivars, the Rf values for *M. chitwoodi* were higher than for *M. luci*. Therefore, greater potato losses can be expected from *M. chitwoodi* than from *M. luci*. *Meloidogyne hispanica*, a RKN species found associated with potato roots in Portugal, also reproduced on potato cvs Agria (Rf = 232.08) and Monalisa (Rf = 117.27), being classified as susceptible (Maleita *et al*., 2012a). Although these RKN species are known to infect potato and *M. chitwoodi* cause significant losses, reducing tuber quality and potato production, their exact impact is unknown. Consequently, preventive measures to limit the spread of *M. luci* and *M. chitwoodi* from infested to non-infested fields are recommended.

Taking into account the published data, *M. luci* can be considered a potential polyphagous species with a wide host range, including plants from several botanical families (EPPO, 2016). Further studies should be conducted to evaluate the host status of a large number of economically important crops to *M. luci* and to evaluate the ability of this species to reproduce on plants with genetic resistance against RKN. Genetic resistance against RKN has been investigated in several crops and a broad availability of effective resistance genes was identified in a wide range of host plant taxa (Williamson & Roberts, 2009).

This research reports for the first time *M. luci* associated with potato roots in Portugal. Moreover, to the best of the authors' knowledge, this is also the first report of

this RKN species parasitizing potato worldwide. The high reproduction factors of the *M. luci* isolate in potato make it a potential species of emerging significance for this economically important crop, thus further research should also be developed to increase the knowledge about its biology and ecology.

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