New occurrence of *Meloidogyne graminicola* (Nematoda: Meloidogyninae) from rice fields in Italy: Variability and phylogenetic relationships

Elena Fanelli | Francesca Gaffuri | Alberto Troccoli | Stefano Sacchi | Francesca De Luca

1Istituto per la Protezione Sostenibile delle Piante, Bari, Consiglio Nazionale delle Ricerche, Bari, Italy
2Laboratorio Fitosanitario Regione Lombardia presso Fondazione Minoprio, Vertemate con Minoprio, Italy

**Abstract**

Since the first detection of *Meloidogyne graminicola* in Piedmont, North Italy, in 2016, further inspections for the presence of the rice root-knot nematode were carried out in rice fields of neighboring regions, in accordance with the Italian NPPO (National Plant Protection Organization) to support the official phytosanitary measures, to enable the early detection of the rice pest, and to prevent its spread within the national territory. In 2018, surveys of rice fields in Lombardy region revealed a new occurrence of *M. graminicola*. In the present study, we confirmed the identification of the rice nematode in Lombardy using the ribosomal ITS region and the mitochondrial COI and COII genes. The sequences and phylogenetic analyses revealed that Lombardy *M. graminicola* population grouped in all trees in the main cluster containing *Meloidogyne* species belonging to *graminis* group, but always in a different subgroup compared to the Piedmont population of *M. graminicola*. These results clearly suggest that the two Italian populations have been recently and independently introduced and confirm that the geographic origin is not the main factor leading to *M. graminicola* population variability.

**KEYWORDS**
cytochrome c oxidase subunit I (COI), cytochrome c oxidase subunit II (COII), maximum likelihood, ribosomal DNA, rice, root-knot nematode

**TAXONOMY CLASSIFICATION**
Agroecology

1 | INTRODUCTION

Root-knot nematodes are widely distributed causing tremendous economic losses estimated about 80 billion dollars annually (Nicol et al., 2011; Jones et al., 2013). Among *Meloidogyne* species, the root-knot nematode *Meloidogyne graminicola* Golden and Birchfield (1965) is the most important rice pest worldwide causing yield losses up to 87% (Dutta et al., 2012). *Meloidogyne graminicola* is a nematode species well adapted to attack different rice agrosystems, from upland to lowland, and irrigated to deep-water fields, along with more than 98 host plants including cereals and grasses (Pokharel et al., 2010). Khan (2015) reported that rice can be...
attacked by more than 35 plant parasitic nematodes, but *M. graminicola* together with few other *Meloidogyne* spp. is one of the most adapted species to the flooded rice systems, worldwide. Recently, during a survey in Brazil, *M. graminicola* was detected in rice fields along with other *Meloidogyne* spp. belonging to the *graminis* group (Leite et al., 2020; Mattos et al., 2018). It is present in America, Africa, especially in Asia and recently in South Europe (EPPO, 2016, 2018; Fanelli et al., 2017; Mantelin et al., 2017; Rusinque et al., 2021; Sacchi et al., 2021). In Europe, *M. graminicola* was detected for the first time in upland and lowland rice fields in the Piedmont region (Northern Italy) in 2016 and immediately added to the EPPO Alert List. In just one year (2016–2017, EPPO Global Database, 2019), the total infected area increased by fivefold. Italy is the main rice-growing country in Europe, with 217,195 ha of rice (Ente Nazionale Risi, 2018). The most important rice-growing area is the section of the Po River Valley straddling the regions of Lombardy and Piedmont with more than 202,000 hectares representing 93% of the Italian rice surface (Ente Nazionale Risi, 2018; Fanelli et al., 2017; Sacchi et al., 2020, 2021).

In Lombardy region, rice growing is highly specialized and is concentrated above all in the area enclosed between Pavia, Milano, and Lodi provinces. Rice fields constitute a typical element of this territory characterized by a large inter-annual variability related to the meteorological conditions (Zampieri et al., 2019). The Lombardy’s rice fields have sandy soils thus draining faster than in Piedmont during summer, the water table is lower, and larger withdrawals from rivers and channels are needed for rice paddy fields irrigation. Therefore, different water management evolution was observed from rivers and channels are needed for rice paddy fields irrigation. Rice fields have sandy soils thus draining faster than in Piedmont (Torrini et al., 2020; Zampieri et al., 2019). Conditions such as higher increase (+28%) in Piedmont, of rice cultivation in dry paddy fields (Torrini et al., 2020; Zampieri et al., 2019). Conditions such as those described (dry paddy fields and sandy soils) can potentially be scarce in Lombardy (Lombardy).

Soil and root samples from the rice-cultivated area at Cascina Scalina farm in Garlasco (Pavia province) were collected (GPS coordinates: 45.19568654643237, 8.893816095789944) and processed. Second-stage juveniles, females and males were collected from rice galed roots by direct dissection (EPPO PM 7/119 [1] 2013) under LEICA MZ12 stereomicroscope with Canon PowerShot G3 camera. Morphological identification was made on freshly mounted specimens (second-stage juveniles or J2 and adults) and female perineal patterns, to a compound microscope ZEISS AXIOSKOP 40 with TrueChrome HD II digital camera and TCapture software (TUCSEN®) for image capture and analysis. Ethanol-preserved second-stage juveniles were sent to IPSP-Bari Institute for molecular identification.

2.2 | DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from individual nematodes sent to IPSP-Nematology Lab and directly amplified as described by De Luca et al. (2004). The portion of the ITS1-5.8S-ITS2 regions was amplified using the forward primer 18S (5′-TGATTACGTCCCTGGCCTTT-3′) and the reverse primer 26S (5′-TTTCACTCGCCGTTACTAAGG-3′) (Vrain et al., 1992), the portion of the COI gene was amplified as described by Derycke et al. (2005) using forward primer JB3 (5′-TTTTGGGCATCCTGAGGTTTAT-3′) and reverse primer XiphR1 (5′-ACAACCAGTATACCTCTCATTAC-3′) (Lazarova et al., 2006), and the region of the mitochondrial genome between the cytochrome oxidase subunit II (COII) and the 16S rRNA genes was amplified using the forward primer C2F3 (5′-GTTCAATGTCCAGAAATTTGTTG-3′) and the reverse primer 1108 (5′-TACCTTTGACCAATCCGCT-3′) (Powers & Harris, 1993).

PCR cycling conditions used for amplification of the partial ITS were: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 1 min, and a final step at 72°C for 7 min. For the COI, these conditions were: an initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 30 s, and a final step at 72°C for 7 min. For the COII, PCR conditions were: an initial denaturation at 94°C for 3 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 30 s, and a final step at 72°C for 7 min. The 10 J2s of Piedmont *M. graminicola* from upland rice fields were also processed and amplified using ITS and COII primers to confirm the species occurrence in 2018. PCR products of two specimens for each molecular marker were purified after amplification using NucleoSpin (Macherey-Nagel), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies) and used for cloning in 2 of 14
pGEM-T easy vector (Promega). Eight COI, 5 COII, and 1 ITS clones of *M. graminicola* from Lombardy were sent at MWG-Eurofins genomics in Germany for sequencing in both directions. Three ITS and three COII clones from Piedmont upland and lowland rice fields were also sent for sequencing to MWG-Eurofins genomics in the present study.

2.3 | Phylogenetic analysis

A Basic Local Alignment Search Tool (BLAST) search at National Center for Biotechnology Information (NCBI) was performed in order to confirm their nematode origins (Altschul et al., 1997). The newly obtained sequences for ITS containing region and the partial mitochondrial COI and COII were aligned using MAFFT V.7.450 (Katoh et al., 2019). BioEdit program V. 7.2.5 (Hall, 1999) was used for sequence alignments visualization and edited in order to improve the multialignment. Outgroup taxa, *M. incognita*, *M. hapla*, *M. javanica*, and *M. arenaria*, for each dataset were chosen according to the results of previously published data (Fanelli et al., 2017; Mattos et al., 2018; Soares et al., 2020). Phylogenetic trees, obtained for ITS, COI, and COII dataset, were performed with Maximum Likelihood (ML) method using MEGA version 7 software (Kumar et al., 2016). The phylograms were bootstrapped 1000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method. The newly obtained sequences were submitted to GenBank with the following accession numbers: for ITS OM809713-OM809716; for COI OM810293-OM810300; for COII OP024528-OP024535.

2.4 | Estimations of evolutionary divergence between sequences

The pairwise distances within the COI and COII sequences of *M. graminicola* belonging to the graminis-group were done in MEGA7
software package (Kumar et al., 2016). All positions with gaps and missing data were excluded. The COI and COII analyses involved 28 and 25 nucleotide sequences, respectively.

3 | RESULTS

The PCR products of ITS, mitochondrial COI and COII-16rRNA yielded fragments of 876bp, 367bp, 732bp, respectively, by sequencing. BLAST search revealed that M. graminicola sequences from Lombardy were identical to M. graminicola present at NCBI for all genes. ITS region of M. graminicola Lombardy population showed 97–99% similarity (1–24 bp different; 0–13 gaps) with all ITS of M. graminicola present in the database, in particular the highest identity (99%) was with those from China and India. It is interesting to note that ITS sequences of Lombardy population showed 98% similarity (22–24 bp different; 13 gaps) with the ITS sequences of M. graminicola from Piedmont (North Italy). Furthermore, it is noteworthy that M. graminicola population from Lombardy and Piedmont showed 98% similarity with the ITS sequences of M. oryzae populations from Suriname and French Guiana (LS974439, LS974440, and LS974441; 18 nucleotides and 13 gaps) released in Genbank (Besnard et al., 2019). More recently, other two ITS sequences of M. oryzae from Brazil (KY962653, KY962654; Mattos et al., 2018) were released in Genbank and pairwise comparisons showed that both Piedmont and Lombardy M. graminicola populations have a 92 and 93% similarity, respectively.

Sixty-nine sequences, including M. graminicola sequences from Lombardy and Piedmont (North Italy), are obtained in this study, and the corresponding sequences from Genbank, along with those of Meloidogyne spp. belonging to the graminis group and other Meloidogyne spp. from Genbank were aligned. Phylogenetic tree based on the ITS region revealed that M. graminicola from Italy clustered with all other M. graminicola and Meloidogyne spp. belonging to the graminis group (Figure 2). It is interesting to note that M. graminicola from Piedmont grouped in a subclade (83% support) with populations of M. graminicola from Brazil, Meloidogyne sp. 2, Meloidogyne sp. 3 from Brazil, M. trifoliiophila and M. oryzae from Suriname, while M. graminicola from Lombardy with the rest of M. graminicola populations, M. ottersoni from Brazil, and M. oryzae from French Guiana. Closely related to M. graminicola group clustered M. aegraciperi (100% support), while M. naasi, belonging to the graminis group, clustered at a basal position of the M. graminicola and M. aegraciperi cluster. The two populations of M. oryzae from Brazil (KY962653-KY962654; Mattos et al., 2018) grouped with mitotic parthenogenetic species (Figure 1; 98% support).

Two individual COI amplicons of M. graminicola from Lombardy were cloned and eight clones were sequenced. BLASTn analysis at NCBI revealed a 98–99% similarity with all M. graminicola sequences and 97–98% similarity with M. oryzae. Pairwise distances within the graminis-group are shown in Table 1. Most of M. graminicola isolates differ by 0–7 bp each other, while with M. graminicola (MH128475; P169011 isolate) by 7–15 bp. All M. oryzae isolates differ by 5–16 bp from all M. graminicola isolates. COI sequences of M. oryzae (LR215847 and MK507908) were obtained from the complete and mitochondrial genomes, respectively (Besnard et al., 2014, 2019). Nucleotide sequences were also converted into amino-acid sequences and no stop codons or frame shift mutations were observed. The amino-acid analysis revealed that intraspecific amino-acid sequence variation for M. graminicola isolates was only 1–2 amino acids, as that observed with M. oryzae isolates.

Phylogenetic analysis based on mitochondrial COI sequences of M. graminicola from Lombardy and the corresponding available sequences of graminis-group and other Meloidogyne spp. revealed three main groupings (Figure 3). The largest, Group I, contained all M. graminicola and M. oryzae haplotypes; Group II: M. minor, M. ichinoheii, M. exigua, and M. naasi haplotypes; Group III: M. hapla, M. incognita, and M. arenaria haplotypes. Group I showed two subgroups: one containing all M. graminicola haplotypes including Lombardy haplotypes, the second subgroup containing M. oryzae isolates (MO-M1, South East Asia, MH128473 and MH128474 isolates P129052, P129054, respectively), and M. graminicola (MH128475) isolate P169011 which could be misidentified.

The newly obtained COII sequences from Lombardy showed a low intraspecific variability, 1 to 2 bp out of 531 bp, while with the corresponding sequences of M. graminicola present in the database they showed 97–99% similarity (2 to 12 different nucleotides). Pairwise distances within the graminis-group, using 396 sites, revealed that M. graminicola from Lombardy showed the highest variability, namely 8–10 different nucleotides with M. graminicola from Piedmont and Meloidogyne sp. 2 and sp. 3 (MN585713 and MN585714), while with M. oryzae (MNS585712) from Brazil, French Guinea, and Southeast Asia (LR215847 and MK507948; mitochondrial complete genomes) 6–8 different nucleotides (Table 2).

For the COII-16S-rRNA, 68 sequences were aligned, 5 of which were from Lombardy population and 3 from upland rice field from Piedmont. Phylogenetic analysis is based on mitochondrial COII sequences of M. graminicola from Lombardy, and the corresponding available sequences of graminis group and other Meloidogyne spp. revealed five main groups (Figure 4). Group I showed two subgroups (99% support): subgroup A containing haplotypes of M. graminicola from Piedmont and Brazil, including M. salasi, M. ottersoni, Meloidogyne sp. 2 and sp. 3, haplotypes of M. oryzae from Brazil (MN585712), from French Guinea and Southeast Asia (MK507908 and LR215847, respectively); subgroup B containing all haplotypes of M. graminicola from Lombardy and Asian haplotypes (McCure et al., 2012). Group II contained M. aegraciperi at basal position of Meloidogyne spp. belonging to graminis group. Group III contained all haplotypes of M. minor and M. naasi. Group IV contained M. chitwoodi. Group V contained M. hapla, M. arenaria, and M. incognita.

4 | DISCUSSION

In the present study, we report on the second occurrence of M. graminicola population in rice fields in Lombardy (North Italy), after the first occurrence in lowland and upland rice fields in Piedmont in
**FIGURE 2** Phylogenetic tree of ITS containing region describing the evolutionary relationships among different geographical populations using Maximum Likelihood (ML) method. Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method with the invariant site plus gamma options. Numbers at nodes indicate bootstrap values. *Meloidogyne incognita*, *M. hapla*, *M. javanica*, and *M. arenaria* were used as outgroups. Newly obtained sequences are in bold. GenBank accession numbers are along with the species names.
|   | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    | 15    | 16    | 17    | 18    | 19    | 20    | 21    | 22    | 23    | 24    | 25    | 26    | 27    | 28    |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | OM810293 | Meloidogyne graminicola isolate Lombardy | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 2 | OM810294 | Meloidogyne graminicola isolate Lombardy | 0     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 3 | OM810295 | Meloidogyne graminicola isolate Lombardy | 0     | 0     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 4 | OM810296 | Meloidogyne graminicola isolate Lombardy | 0     | 0     | 0     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 5 | OM810297 | Meloidogyne graminicola isolate Lombardy | 1     | 1     | 1     | 1     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 6 | OM810298 | Meloidogyne graminicola isolate Lombardy | 1     | 1     | 1     | 1     | 2     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 7 | OM810299 | Meloidogyne graminicola isolate Lombardy | 2     | 2     | 2     | 2     | 3     | 3     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 8 | OM810300 | Meloidogyne graminicola isolate Lombardy | 0     | 0     | 0     | 0     | 1     | 0     | 1     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 9 | LR215858 | Meloidogyne graminicola | 5     | 5     | 5     | 5     | 6     | 4     | 5     | 3     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 10| LR215853 | Meloidogyne graminicola | 5     | 5     | 5     | 5     | 6     | 4     | 5     | 3     | 0     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 11| LR215854 | Meloidogyne graminicola | 5     | 5     | 5     | 5     | 6     | 4     | 5     | 3     | 0     | 0     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 12| MH332672 | Meloidogyne graminicola | 4     | 4     | 4     | 5     | 5     | 6     | 4     | 3     | 3     | 3     | 0     |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  | 25  | 26  | 27  | 28  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 13| KY250093 Meloidogyne graminicola | 4   | 4   | 4   | 5   | 5   | 6   | 4   | 3   | 3   | 3   | 0   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 14| LR215857 Meloidogyne graminicola | 6   | 6   | 6   | 7   | 5   | 6   | 3   | 0   | 0   | 0   | 3   | 3   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 15| LR215848 Meloidogyne graminicola | 6   | 6   | 6   | 7   | 5   | 6   | 3   | 0   | 0   | 0   | 3   | 3   | 0   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 16| MW411965 Meloidogyne graminicola | 6   | 6   | 6   | 6   | 7   | 5   | 6   | 3   | 0   | 0   | 0   | 3   | 3   | 0   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 17| MN017128 Meloidogyne graminicola | 5   | 5   | 5   | 6   | 4   | 7   | 3   | 2   | 2   | 2   | 3   | 2   | 2   | 2   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 18| MG917045 Meloidogyne graminicola | 3   | 3   | 3   | 3   | 4   | 3   | 4   | 3   | 0   | 0   | 1   | 1   | 0   | 0   | 0   | 0   |     |     |     |     |     |     |     |     |     |     |     |     |
| 19| MG917043 Meloidogyne graminicola | 0   | 0   | 0   | 0   | 1   | 0   | 1   | 0   | 0   | 0   | 1   | 1   | 0   | 0   | 0   | 0   | 0   |     |     |     |     |     |     |     |     |     |     |
| 20| NC056772 Meloidogyne graminicola | 6   | 6   | 6   | 6   | 7   | 5   | 6   | 3   | 0   | 0   | 0   | 3   | 3   | 0   | 0   | 0   | 2   | 0   | 0   | 0   |     |     |     |     |     |     |     |
| 21| HGS29223 Meloidogyne graminicola | 7   | 7   | 7   | 7   | 8   | 6   | 7   | 4   | 1   | 1   | 1   | 4   | 1   | 1   | 1   | 3   | 1   | 1   | 1   | 0   |     |     |     |     |     |     |     |
| 22| LR215855 Meloidogyne graminicola | 3   | 3   | 3   | 3   | 4   | 2   | 2   | 0   | 0   | 0   | 0   | 3   | 3   | 0   | 0   | 0   | 2   | 0   | 0   | 0   | 1   | 0   |     |     |     |     |
| 23| LR215851 Meloidogyne graminicola | 6   | 6   | 6   | 6   | 7   | 5   | 6   | 3   | 0   | 0   | 0   | 3   | 3   | 0   | 0   | 0   | 2   | 0   | 0   | 0   | 1   | 0   |     |     |     |     |
| 24| MH128473 Meloidogyne oryzae | 11  | 11  | 11  | 11  | 12  | 10  | 11  | 8   | 7   | 7   | 7   | 10  | 7   | 7   | 9   | 7   | 5   | 7   | 8   | 5   | 7   | 0   |     |     |     |     |
| 25| MH128474 Meloidogyne oryzae | 11  | 11  | 11  | 11  | 12  | 10  | 11  | 8   | 7   | 7   | 7   | 10  | 7   | 7   | 9   | 7   | 5   | 7   | 8   | 5   | 7   | 0   | 0   |     |     |     |
| 26| MH128475 Meloidogyne graminicola | 13  | 13  | 13  | 13  | 14  | 14  | 15  | 11  | 14  | 14  | 13  | 13  | 14  | 14  | 14  | 12  | 11  | 7   | 14  | 15  | 9   | 14  | 15  | 15  | 0   |     |
| 27| LR215847 Meloidogyne oryzae isolate Mo-M1 | 14  | 14  | 14  | 14  | 15  | 13  | 15  | 12  | 10  | 10  | 9   | 12  | 12  | 10  | 10  | 10  | 11  | 9   | 7   | 10  | 11  | 7   | 10  | 12  | 12  | 14  | 0   |
| 28| MK507908 Meloidogyne oryzae isolate Southeast Asia | 15  | 15  | 15  | 15  | 16  | 14  | 16  | 12  | 10  | 10  | 9   | 12  | 12  | 10  | 10  | 10  | 11  | 9   | 7   | 10  | 11  | 7   | 10  | 12  | 12  | 14  | 0   | 0   |
2016 (Fanelli et al., 2017). Piedmont and Lombardy regions are the main rice-growing areas (more than 202,000 hectares, 93% of the Italian rice surface). In Piedmont region, most of the rice fields are under controlled field flooding, while in Lombardy region most of the rice fields are upland as the soil structure is characterized by a low water retention capacity (Sacchi et al., 2020) and water shortage as a result of local climate change (Zampieri et al., 2019). Recently, several species of *Meloidogyne* associated with rice causing severe damages were described (Kyndt et al., 2014; Mattos et al., 2018; Soares et al., 2020). In 2018, many rice fields in Lombardy showed the typical symptoms caused by *M. graminicola*: poor growth and stunting, chlorosis, and heavily diseased roots with differently shaped and sized galls (Figure 1). The average measurements and the perineal patterns of females were in the range reported for *M. graminicola* in literature. Thus, the population of *M. graminicola* from Cascina Scalina field was characterized by sequencing the ITS, mitochondrial COI and COII to confirm the identification and assess its phylogenetic relationships with Piedmont and other *M. graminicola*. The ITS-RFLP of Lombardy isolate produced restriction profiles identical to upland and lowland populations from Piedmont. Phylogenetic relationships based on ITS sequences using ML method confirmed that Lombardy population grouped with all *M. graminicola* sequences available in Genbank, but in a different subgroup compared with Piedmont *M. graminicola* isolates from upland and lowland rice fields (Figure 2). This finding confirms that *M. graminicola* populations do not group according to the geographical origin due to the high level of intraspecific variability. Thus, Lombardy population of *M. graminicola* could have been introduced independently from the Piedmont population confirming a recent expansion of this species in Italy and all over the world (Fanelli et al., 2017; Salalia et al., 2017; Soares et al., 2020). It is also noteworthy that in our phylogenetic analysis, *M. oryzae* isolates from French Guinea and Suriname, *M. trifoliiophila* and *M. salasi* grouped within *M. graminicola* cluster confirming the occurrence of cryptic species and races of *M. graminicola* (Besnard et al., 2019; Mattos et al., 2018).
TABLE 2  Pairwise distances of mitochondrial COII of *Meloidogyne graminicola* populations in bold the sequences obtained in this study.

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1 | OP024530 *Meloidogyne graminicola* isolate Lombardy | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 2 | OP024532 *Meloidogyne graminicola* isolate Lombardy | 0  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 3 | OP024529 *Meloidogyne graminicola* isolate Lombardy | 1  | 1  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 4 | OP024528 *Meloidogyne graminicola* isolate Lombardy | 1  | 1  | 2  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 5 | OP024531 *Meloidogyne graminicola* isolate Lombardy | 0  | 0  | 1  | 1  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 6 | OP024535 *Meloidogyne graminicola* isolate Piedmont | 8  | 8  | 9  | 9  | 8  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 7 | OP024534 *Meloidogyne graminicola* isolate Piedmont | 8  | 8  | 9  | 9  | 8  | 0  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8 | LT669811 *Meloidogyne graminicola* isolate Piedmont | 9  | 9  | 10 | 10 | 9  | 1  | 1  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 9 | OP024533 *Meloidogyne graminicola* isolate Piedmont | 8  | 8  | 9  | 9  | 8  | 0  | 0  | 1  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 10| LT669812 *Meloidogyne graminicola* isolate Piedmont | 8  | 8  | 9  | 9  | 8  | 0  | 0  | 1  | 0  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 11| MH332687 *Meloidogyne graminicola* Vietnam | 0  | 0  | 1  | 1  | 0  | 8  | 8  | 9  | 8  | 8  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 12| KY020413 *Meloidogyne graminicola* China | 0  | 0  | 1  | 1  | 0  | 8  | 8  | 9  | 8  | 8  | 0  | 0  |    |    |    |    |    |    |    |    |    |    |    |    |
| 13| MG356944 *Meloidogyne graminicola* China | 0  | 0  | 1  | 1  | 0  | 8  | 8  | 9  | 8  | 8  | 0  | 0  | 0  |    |    |    |    |    |    |    |    |    |    |    |
| 14| JN241929 *Meloidogyne graminicola* USA | 0  | 0  | 1  | 1  | 0  | 8  | 8  | 9  | 8  | 8  | 0  | 0  | 0  | 0  |    |    |    |    |    |    |    |    |    |    |

(Continues)
|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  | 25  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 15| JN241927 | Meloidogyne graminicola | Bangladesh | 0 | 0 | 1 | 1 | 0 | 8 | 8 | 9 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16| LR215858 | Meloidogyne graminicola | Vietnam | 0 | 0 | 1 | 1 | 0 | 8 | 8 | 9 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17| JN241939 | Meloidogyne graminicola | India | 0 | 0 | 1 | 1 | 0 | 8 | 8 | 9 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18| KJ576894 | Meloidogyne graminicola | Taiwan | 0 | 0 | 1 | 1 | 0 | 8 | 8 | 9 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19| MN585713 | Meloidogyne sp. 2 | 8 | 8 | 9 | 9 | 8 | 4 | 4 | 5 | 4 | 4 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 0 | 0 | 0 | 0 |
| 20| MN585715 | Meloidogyne sp. 3 | 9 | 9 | 10 | 10 | 9 | 7 | 7 | 8 | 7 | 7 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 6 | 0 | 0 | 0 | 0 |
| 21| MN585714 | Meloidogyne sp. 3 | 9 | 9 | 10 | 10 | 9 | 7 | 7 | 8 | 7 | 7 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 6 | 0 | 0 | 0 |
| 22| MN585720 | Meloidogyne salasi | Brazil | 3 | 3 | 4 | 4 | 3 | 7 | 7 | 8 | 7 | 7 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 7 | 8 | 8 | 0 |
| 23| MN585712 | Meloidogyne oryzae | 7 | 7 | 8 | 8 | 7 | 3 | 3 | 4 | 3 | 3 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 3 | 6 | 6 | 6 | 0 |
| 24| LR215847 | Meloidogyne oryzae isolate | French Guiana | 6 | 6 | 7 | 7 | 6 | 10 | 10 | 11 | 10 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 11 | 11 | 7 | 9 | 0 |
| 25| MK507908 | Meloidogyne oryzae isolate | Southeast Asia | 6 | 6 | 7 | 7 | 6 | 10 | 10 | 11 | 10 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 9 | 11 | 11 | 7 | 9 | 0 | 0 | 0 | 0 |

**TABLE 2 (Continued)**
FIGURE 4 Phylogenetic tree of partial mitochondrial COII sequences describing the evolutionary relationships among different geographical populations using Maximum Likelihood (ML) method. Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method with the invariant site plus gamma options. Numbers at nodes indicate bootstrap values. *Meloidogyne incognita*, *M. hapla*, *M. javanica*, and *M. arenaria* were used as outgroups. Newly obtained sequences are in bold. GenBank accession numbers are along with the species names.

NC056772 Meloidogyne graminicola China
HG529223 Meloidogyne graminicola Philippines
LR215851 Meloidogyne graminicola
LR215855 Meloidogyne graminicola
LR215857 Meloidogyne graminicola
LR215853 Meloidogyne graminicola Laos
LR215848 Meloidogyne graminicola Brazil
KR234081 Meloidogyne graminicola China
KM111533 Meloidogyne graminicola China
KM236559 Meloidogyne graminicola China
OK245418 Meloidogyne graminicola

**I**

**a**

**II**

**III**

**IV**

**V**

**Meloidogyne incognita**, **M. hapla**, **M. javanica**, and **M. arenaria** were used as outgroups. Newly obtained sequences are in bold. GenBank accession numbers are along with the species names.
The isolates of *M. oryzae* from Brazil clustered with mitotic parthenogenetic species showed 51–55 chromosomes, while *M. oryzae* isolates from French Guinea and Suriname showed 18 chromosomes (Mattos et al., 2018) corroborating the suspicions of Negretti et al. (2017) that these isolates (French Guinea and Suriname) could be misidentified (Carneiro et al., 2000) because the authors used only esterase phenotype diagnosis and thus they have to be considered as *M. graminicola* isolates (Alvarez-Ortega et al., 2019; Mattos et al., 2018; Negretti et al., 2017). Recently, genome analyses of *M. graminicola* and *M. oryzae* reported by Besnard et al. (2019) revealed the presence of two divergent copies of ribosomal DNA in *M. graminicola* and two or three divergent copies in *M. oryzae*. One type of ribosomal sequence was shared by *M. graminicola* and *M. oryzae* suggesting that it could arise by hybridization or duplication (Alvarez-Ortega et al., 2019; Besnard et al., 2019; Hugall et al., 1994; Phan et al., 2020; Szitenberg et al., 2017). These findings overall suggest a closely related ancestor of *M. graminicola* and *M. oryzae*. In the present study, the mitochondrial COI and COII-16SrRNA genes were also used to investigate the genetic diversity among Italian and other isolates of *M. graminicola* and the geographical origin of Italian populations. Mitochondrial DNA shows high mutation rate and thus is a powerful marker to detect the intraspecific and interspecific variability in order to distinguish closely related and cryptic species (Blok & Powers, 2009; Powers et al., 2018). COI barcode, as a protein coding gene, is a powerful region to identify and to recognize groupings at species level. The phylogenetic tree based on COI sequences revealed that all haplotypes from Lombardy population formed a supported subgroup within *M. graminicola* group, whereas all *M. oryzae* isolates were positioned at basal position of the *M. graminicola* group (Figure 3). To further evaluate the genetic diversity and phylogenetic relationships between *M. graminicola* and *M. oryzae*, the ML COXII-16SrRNA genes tree revealed that Lombardy haplotypes grouped in a subgroup with Asian *M. graminicola* haplotypes sharing common COII haplotypes with geographical distant populations, while Piedmont haplotypes formed a separated subgroup with *M. graminicola* haplotypes from Brazil, *M. oryzae*, *M. salasi*, *M. ottersoni*, *Meloidogyne* sp. 2 and *Meloidogyne* sp. 3 (Figure 4). Furthermore, COII Piedmont haplotypes belonged to type A for the presence of one *Dra* I and four *Ssp* I restriction sites, while those from Lombardy belonged to type B with two *Dra* I and four *Ssp* I restriction sites. These findings suggest that geographical distance is not the main factor leading to *M. graminicola* population differentiation. As the Italian isolates always grouped in two different subgroups with geographical distant isolates sharing the same haplotypes, this observation seems to confirm that the two Italian *M. graminicola* populations may have been recently and independently introduced. Furthermore, in the current study, some *M. oryzae* isolates grouped within *M. graminicola* cluster confirming their close relationships due to recent evolution or hybrid origin of these species well adapted to irrigated rice (Besnard et al., 2019).

In conclusion, the present study clearly demonstrates that Italian *M. graminicola* populations show the same genetic profiles of those from Asia and America suggesting that this species prefers asexual reproduction and is well adapted to different rice fields. In this context, it can be understood that appropriate control measures are needed to manage this pest. Rice field flooding seems to be an efficient control technique for *M. graminicola* in Piedmont but not in Lombardy due to the soil structure. Thus, in Lombardy, researchers are testing several trap cropping strategies to maintain a low nematode population in infested fields and also balancing water shortage due to climate change and dry-seeding practices. Regarding the entry of *M. graminicola* in Italy, it is not well understood yet. It seems through the movement of infested host plants, soil, waterbodies, aquatic plants or weeds acting as reservoirs for this nematode.

**AUTHOR CONTRIBUTIONS**

Elena Fanelli: Conceptualization (equal); formal analysis (lead); investigation (equal); validation (equal); writing – original draft (equal).

Francesca Gaffuri: Formal analysis (equal); methodology (equal); writing – original draft (equal).

Francesca De Luca: Conceptualization (lead); formal analysis (equal); funding acquisition (lead); project administration (lead); supervision (lead); writing – original draft (lead); writing – review and editing (lead).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

DNA sequences obtained in the present study are available at Genbank accession numbers: for ITS OM809713–OM809716; for COI OM810293–OM810300; for COII OP024528–OP024535.

**ORCID**

Francesca De Luca [https://orcid.org/0000-0002-6646-8066](https://orcid.org/0000-0002-6646-8066)

**REFERENCES**

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389–3402.

Alvarez-Ortega, S., Brito, J. A., & Subbotin, S. A. (2019). Multigene phylogeny of root-knot nematodes and molecular characterization of *Meloidogyne nataliei* Golden, *Rose & Bird*, 1981 (Nematoda: *Tylenchida*). *Scientific Reports*, 9, 11788–11798. [https://doi.org/10.1038/s41598-019-48195-0](https://doi.org/10.1038/s41598-019-48195-0)

Besnard, G., Juhling, F., Chapuis, E., Zedane, L., Lhuillier, E., Mateille, T., & Bellafiore, S. (2014). Fast assembly of the mitochondrial genome of a plant parasitic nematode (*Meloidogyne graminicola*) using next generation sequencing. *Comptes Rendus Biologies*, 337, 295–301. [https://doi.org/10.1016/j.crvi.2014.03.003](https://doi.org/10.1016/j.crvi.2014.03.003)

Besnard, G., Thi-Phan, N., Ho-Bich, H., Dereeper, A., Nguyen, H. T., Quénehervé, P., Aribi, J., & Bellafiore, S. (2019). On the close relatedness of two rice-parasitic root-knot nematode species and the recent expansion of *Meloidogyne graminicola* in South east Asia. *Genes*, 10, 175–190. [https://doi.org/10.3390/genes10020175](https://doi.org/10.3390/genes10020175)
Derycke, S., Remerie, T. M., Vierstraete, A., Backeljau, T., Vanfleteren, J., Dutta, T. K., Ganguly, A. K., & Gaur, H. S. (2012). Global status of rice. EPPO. (2016). Reporting Service (2016/211); First Report of Meloidogyne graminicola in Italy. EPPO. (2019). EPPO Global Database (available online). https://gd.eppo.int

Leite, R. R., Mattos, V. S., Gomes, A. C. M. M., Grimaldi Py, L., Souza, D. A., Castagnone-Sereno, P., Carneiro, R. M. D. G. (2020). Integrative taxonomy of Meloidogyne otersoni (Thorne, 1969) Franklin, 1971 (Nematoda: Meloidogynidae) parasitizing flooded rice in Brazil. European Journal of Plant Pathology, 157, 943–959. https://doi.org/10.1007/s10658-020-02049-y

Mantelin, S., Bellafiore, S., & Kyndt, T. (2017). Meloidogyne graminicola: A major threat to rice agriculture. Molecular Plant Pathology, 18, 3–15. https://doi.org/10.1111/mpp.12394

Mattos, V. S., Caraes, J. E., Gomes, C. B., Gomes, A. C. M. M., Monteiro, J. D. M. S., Gomez, G. M., Castagnone-Sereno, P., & Carneiro, R. M. D. G. (2018). Integrative taxonomy of Meloidogyne oryzae (Nematoda: Meloidogyninae) parasitizing rice crops in Southier Brazil. European Journal of Plant Pathology, 151, 649–662. https://doi.org/10.1007/s10658-020-02049-y

McClure, M. A., Nischwitz, C., Skantar, A. M., Schmitt, M. E., & Subbotin, S. A. (2012). Root-knot nematodes in golf course greens of the western United States. Plant Disease, 96, 635–647. https://doi.org/10.1094/PDIS-09-11-0808

Negretti, R. R., Gomes, C. B., Mattos, V. S., Somavilla, L., Manica-Berto, R., Agostinetto, D., Castagnone-Sereno, P., & Carneiro, R. M. D. G. (2017). Characterisation of Meloidogyne species complex parasitising rice in Southern Brazil. Nematology, 19, 403–412. https://doi.org/10.1116/15685411-00003065

Nicol, J. M., Turner, S. J., den Nijs, L., & Hockland, S. (2011). Current nematode threats to world agriculture. In J. T. Jones, G. Gheyseran, & C. Ponnell (Eds.), Genomics and Molecular Genetics of plant-parasitic nematode interactions (pp. 21–43). Springer.

Phan, N., Orjuela, J., Danchin, E. G. J., Klop, C., Perus-Barbecho, L., Kozlowski, D. K., Koutsovoulos, G. D., Lopez-Roques, C., Bouchez, O., Zahn, M., Besnard, G., & Bellafiore, S. (2020). Genome structure and content of the rice root-knot nematode (Meloidogyne graminicola). Ecology and Evolution, 10, 11106–11201. https://doi.org/10.1002/eece.6680

Pokharel, R. R., Abawi, G. S., Duxbury, J. M., Smat, C. D., Wang, X., & Brito, J. A. (2010). Variability and the recognition of two races in Meloidogyne graminicola. Australasian Plant Pathology, 39, 326–333. https://doi.org/10.1071/ap09100

Powers, T. O., & Harris, T. S. (1993). A Polymersens Chain Reaction method for identification of five major Meloidogyne species. Journal of Nematology, 25, 1–6.

Discovery and identification of Meloidogyne species using COI DNA barcoding. Journal of Nematology, 50, 399–412. https://doi.org/10.21307/jofnem-2018-029

Rusinque, L., Maleita, C., Abrantes, J., Palomares-Rius, J. E., & Inácio, M. L. (2021). Meloidogyne graminicola-A threat to rice production: Review update on distribution, biology, identification, and management. Biology, 10, 1163. https://doi.org/10.3390/biology10111163

Sacchi, E., Brenna, S., Fornelli Genot, S., Leoni, A., Sale, V. M., & Setti, M. (2020). Potentially Toxic Elements (PTEs) in cultivated soils from Lombard (Northern Italy): Spatial distribution, origin, and management implications. Minerals, 10, 298.

Sacchi, S., Torrini, G., Marianelli, L., Mazza, G., Fumagalli, A., Cavagna, B., Ciampitti, M., & Roovers, P. F. (2021). Control of Meloidogyne graminicola a root-knot nematode using rice plants as trap crops: Preliminary results. Agriculture, 11, 37.

Salalia, R., Walia, R. K., Somavansi, V. S., Kumar, P., & Kumar, A. (2017). Morphological, morphometric, and molecular characterization of inaspecific variations within Indian populations of Meloidogyne graminicola. Journal of Nematology, 49, 254–267.

Soares, M. R. C., Mattos, V. S., Leite, R. R., Gomes, A. C. M. M., Gomes, C. B., Castagnone-Sereno, F., Dias-Arieta, C. R., & Carneiro, R. M. D. G. (2020). Integrative taxonomy of Meloidogyne graminicola populations with different esterase enzymophores parasitizing rice in Brazil. Nematology, 23, 1–17. https://doi.org/10.1116/15685411-bja10065

Szitzenberg, A., Salazar-Jaramillo, L., Blok, V. C., Laetsch, D. R., Joseph, S., Williamson, V. M., Blaxter, M. L., & Lunt, D. H. (2017). Comparative genomics of apomictic root-knot nematodes: Hybridization, ploidy, and dynamic genome change. Genome Biology and Evolution, 9(10), 2844–2861. https://doi.org/10.1093/gbe/evx201
Torrini, G., Roversi, P. F., Cesaroni, C. F., & Marianelli, L. (2020). Pest risk analysis of rice root-knot nematode (Meloidogyne graminicola) for the Italian territory. Bulletin OEPP/EPPO Bulletin, 50, 1–10.

Vrain, T. C., Wakarchuck, D. A., Lévesque, A. C., & Hamilton, R. I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the Xiphinema americanum group. Fundamental and Applied Nematology, 15, 563–573.

Zampieri, M., Ceglar, A., Manfron, G., Toreti, A., Duveiller, G., Romani, M., Rocca, C., Scoccimarro, E., Podrascanin, Z., & Djurdjevic, V. (2019). Adaptation and sustainability of water management for rice agriculture in temperate regions: The Italian case-study. LDD—Land Degradation Development, 30, 2033–2047. https://doi.org/10.1002/ldr.3402

How to cite this article: Fanelli, E., Gaffuri, F., Troccoli, A., Sacchi, S., & De Luca, F. (2022). New occurrence of Meloidogyne graminicola (Nematoda: Meloidogyninae) from rice fields in Italy: Variability and phylogenetic relationships. Ecology and Evolution, 12, e9326. https://doi.org/10.1002/ece3.9326