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Characterization of Isolates of *Meloidogyne* from Rice-Wheat Production Fields in Nepal

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Abstract: Thirty-three isolates of root-knot nematode were recovered from soil samples from rice-wheat fields in Nepal and maintained on rice cv. BR 11. The isolates were characterized using morphology, host range and DNA sequence analyses in order to ascertain their identity. Results indicated phenotypic similarity (juvenile measurements, perennial pattern, host range and gall shape) of the Nepalese isolates with *Meloidogyne graminicola*, with minor variations. The rice varieties LA 110 and Labelle were susceptible to all of the Nepalese isolates, but differences in the aggressiveness of the isolates were observed. Phylogenetic analyses based on the sequences of partial internal transcribed spacer (ITS) of the rRNA genes indicated that all Nepalese isolates formed a distinct clade with known isolates of *M. graminicola* with high bootstrap support. Furthermore, two groups were identified within the *M. graminicola* clade. No correlation between ITS haplotype and aggressiveness or host range was found among the tested isolates.

Key words: *Meloidogyne graminicola*, morphology, nucleotide polymorphism, *Oryza sativa*, phylogeny, root-knot nematode, systematics, virulence.

The average yield of rice (*Oryza sativa* L.) in Nepal is 1.9 ton/ha, which is below the average yield obtained in neighboring countries including China (3.5 ton/ha), India (2.4 ton/ha) and Pakistan (2.4 ton/ha). Similarly, the average yield of wheat (*Triticum aestivum* L.) in Nepal is 1.6 ton/ha, which is also below the yield average of South Asian countries (2.5 ton/ha) (Kataki, 2001). Several factors are responsible for the low productivity of rice and wheat in Nepal. Of these, availability of irrigation water, soil nutrient status and outbreaks of insect pests and diseases are major constraints to higher productivity (Kataki, 2001). Despite the increasing trend in the use of production inputs and the adoption of new technologies, productivity of rice and wheat is not responding in terms of yield growth as it should be. In fact, the per unit productivity growth of rice and wheat is declining, often resulting in food deficits (Kataki, 2001; Duxbury, 2002). Blast, bacterial blight and sheath blight are among the important diseases of rice; whereas rust, leaf blight and loose smut are prevalent on wheat (Dahal et al., 1992). Plant-parasitic nematodes are also possible causal candidates contributing to the observed yield decline. However, they are often neglected due to lack of conspicuous aboveground symptoms.

More than 200 species of plant-parasitic nematodes have been reported to be associated with rice worldwide (Prot et al., 1994). Among these, the root-knot nematodes (*Meloidogyne* spp.) are considered to be the major problem in rain-fed upland and lowland rice-producing regions, whereas rice root nematode (*Hirschmanniella* spp.) is a problem on lowland rice in South and Southeast Asia (Prot et al., 1994). Though several *Meloidogyne* species are known to attack cereals worldwide, *M. graminicola* is the major species known to be adapted to flooded rice soils and has often been reported from South East Asia, including Nepal (Bridge et al., 1990; Pokharel, 2007). This nematode can cause economic losses to rice in upland, lowland and deep-water production conditions as well as in rice nurseries (Bridge et al., 1990) and was shown to cause up to 70% yield loss on rice in the Philippines (Prot et al., 1994). Information regarding plant-parasitic nematode damage on rice and other crops in Nepal is limited. Recently, research efforts of the Collaborative Research Support Project (CRSP) conducted by scientists at Cornell University and National Institutions in Nepal, Bangladesh and India identified the root-knot nematode as an important problem in rice production, in addition to other crops grown in rice-based crop rotations. Rice yield was increased by 30% or higher in farmers' fields when this nematode was controlled by soil solarization or the application of the nematicide carbofuran (Duxbury, 2002; Padgham, 2003).

The species of root-knot nematode found on rice has been generally considered to be *M. graminicola*, based largely on the symptoms (hook-like galls produced on rice roots) and the ability of this species to cause infection in lowland rice. However, detailed information on identity and variability of observed root-knot nematodes is still lacking. Accurate identification of nematodes is important and is central to understanding the host-parasite relationships and implementing appropriate management options. Traditional methods of nematode identification for root-knot nematode are based on morphology (Chitwood, 1949; Eisenback et al., 1981; van der Beek et al., 1998) and a differential host range test (Sasser and Triantaphyllou, 1977). However, these methods are not completely reliable and can be time consuming (Eshenshade and Trintaphyllou, 1990). Esterase phenotype is considered to be a useful taxonomic character, but requires adult females at a specific developmental stage for accurate diagnosis (Eshenshade and Trintaphyllou, 1990). DNA sequences of the internal transcribed spacer (ITS) region of rRNA genes have been used successfully to identify

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species of nematodes (Zijlstra et al., 1995, 1997; Hugall et al., 1999; Powers, 2004). While PCR-RFLP approaches are useful for nematode diagnostics (Zijlstra et al., 1997), direct comparison of ITS rDNA spacer sequences yield more detailed information about variation within and among nematode species. This project was conducted to characterize the root-knot nematode species occurring in rice fields in Nepal and to study their variability using traditional and molecular tools. A summary of this investigation has been previously published (Pokharel et al., 2005).

Materials and Methods

Sample collection and maintenance: Composite soil samples were collected from rice-wheat fields in Nepal, and root-knot nematodes (*Meloidogyne* spp.) were trapped out on rice cv. BR 11. A total of 57 soil samples was collected either from stunted and symptomatic rice plants or at random, from multiple fields and geographic locations within Nepal. The nematode collection sites were of different soil types (heavy or light texture), cropping patterns (rice-wheat vs. rice-wheat and other crops), sampling criteria (random vs. from around symptomatic plants) and altitudes (mid and high mountain [high altitude] vs. Terai [low altitude]). Soil samples were collected before rice planting or early in the rice season. One composite sample was collected per field (0.1–1 ha area) and consisted of 10 subsamples collected following a "W" sampling pattern. These sub-samples were mixed together, and a representative 200 cc soil sample was placed in a labeled plastic container and kept in a cool room for 3–4 wk before shipping to Cornell University (PPQ 526 permit #54142). At Cornell University, samples were mixed with sterile sand, placed in 15-cm-diam. clay pots, planted to rice cv. BR 11 and maintained in a greenhouse at 25°C for up to 60 d. Plants were then uprooted, and roots washed free from soil and rated for root-galling severity caused by root-knot nematodes.

Of the 57 Nepalese soil samples tested, galls typical of root-knot nematode infection were observed on roots of rice cv. BR 11 growing in the presence of all but four samples (three from Dhading and one from Gorkha). The observed root-galling severity (RGS) was determined on a 1–9 scale (where 1 = no galls and 9 = >76% roots with galls) for all 53 samples and ranged from 2 to 7. Only 33 of the recovered root-knot isolates consistently produced a large number of galls (3–7 RGS ratings) on rice in repeated tests; thus they were maintained and used in this study (Table 1).

To purify the populations, eggs were extracted from infected roots by a modification of the sodium-hypochlorite method (Barker, 1985). The eggs were then used to infest pasteurized (60°C for 30 min) soil placed in 15-cm-diam. clay pots, planted to rice cv. BR 11 and maintained for 10 wk. Since genetic variation in virulence within a single field population is expected, uniform separation of individuals from within each population was essential for further study. Roots of infected plants were washed, female(s) from a single gall of a population teased out and the eggs separated by a forceps and used to inoculate rice cv. Masuli growing in a new pot with pasteurized soil. Five pots were generated (five replications) from each original field population. Thus, five sub-populations (isolates) were established and maintained in the greenhouse for further studies and were designated accordingly.

Morphometric measurements: For second-stage juvenile (J2) measurements, eggs were obtained by blending galled root segments in 1% sodium hypochlorite solution for 3 min and rinsing several times with tap water; they were allowed to hatch in tap water for 48 hr. Juveniles were picked in mass randomly, placed in a drop of water on a glass slide, killed by gentle heat and covered with a glass cover slip. Thirty-five J2 were selected per isolate and measured. Measurements included body length and width, stylet length, tail length and length from the anterior end to the end of the esophagus. The A value (body length/maximum body width), B value (body length/esophagus length) and C value (body length/tail length) were calculated for each individual. Isolate data were compared using Proc GLM (SAS Institute) among the samples used in this study and with those available in the literature.

Perineal pattern study: Mature females were picked from galled roots of rice cv. Mansuli and placed on a glass slide in a drop of water. The posterior end was cut with a sharp razor blade and cleaned. The glass slide with the female end (cut side down) was covered with a cover-slip and sealed. Ten female perineal patterns were analyzed per isolate. Each was examined under a compound microscope at x500 and x640, and selected representative patterns were photographed using Kodak TMAX 100 film.

Host range study: Symptoms produced by the different isolates on rice and barnyard grass (*Echinocloa crusgali* (L.) Beauv., a common weed in rice fields) were recorded. One isolate (NP 29) was randomly selected as a representative isolate sample and used to inoculate host plants of known susceptibility to *M. graminicola*. Reproduction of this nematode isolate was determined on barnyard grass; jute cultivars Tosa and Deshi; cabbage (*Brassica oleracea* var. *capitata* DC.) cv. Dawirth Green; wheat (*Triticum aestivum* L.; different varieties); tomato (*Lycopersicon esculentum* Mill.) cultivars Rutgers, Money Maker and Cherry Large Red; oat (*Avena sativa* L.); barley (*Hordeum vulgare* L.); corn (*Zea mays* L.); and rye (*Secale cereale* L.). These plants were also inoculated with a known isolate of *M. graminis* for comparison. The North Carolina host range test [tobacco (*Nicotiana tabacum* L.) cv. NC 95, watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai.) cv. Charlestone Grey, cotton (*Gossypium hirsutum* L.) cv. Deltapine 61, pepper

TABLE 1. Characteristics of sample collection sites of the isolates of root-knot nematodes used in the study.

Isolate no.	Sample location	District	Soil type	Previous crop	Sample type
NP 1	Bhairahawa	Rupandehi (T)	Clay loam	Wheat	Symptomatic
NP 2	Maghe	Illam (H)	Sandy loam	Fallow-corn	Random
NP 3	Bhairahawa	Rupandehi (T)	Loam	Wheat	Symptomatic
NP 8	Besishar	Lamjung (H)	Loam	Fallow	Random
NP 10	Bhairahawa	Rupandehi (T)	Clay loam	Wheat	Symptomatic
NP 12	Pakribas	Dhankuta (H)	Sandy loam	Fallow	Random
NP 16	Pawanipur	Bara (T)	Sandy loam	Wheat	Symptomatic
NP 17	Pakribas	Dhankuta (H)	Sandy loam	Fallow-rice	Random
NP 19	Jhurkia	Morang (T)	Sandy	Wheat	Random
NP 20	Pakribas	Dhankuta (H)	Sandy loam	Fallow	Random
NP 22	Krishanapur	Chitwan (T)	Sandy clay	Fallow-corn	Random
NP 24	Biratnagar 3	Morang (T)	Clay loam	Wheat	Random
NP 25	Jagatpur	Chitwan (T)	Sandy loam	Wheat	Random
NP 28	Budhabare	Jhapa (T)	Clay loam	Fallow	Random
NP 29	Nayatole	Bara (T)	Clay loam	Wheat	Symptomatic
NP 30	Mulghat	Dhankuta (RB)	Sandy	Fallow-rice	Random
NP 31	Chanauli	Chitwan (T)	Sandy loam	Wheat	Random
NP 33	Archaltar	Chitwan (H)	Sandy loam	Fallow	Random
NP 35	Gitanagar	Chitwan (T)	Sandy	Wheat	Random
NP 36	Prembasti	Chitwan (T)	Sandy loam	Fallow-corn	Random
NP 37	Saranpur	Chitwan (T)	Sandy	Wheat	Random
NP 38	Madi	Chitwan (T)	Sandy	Wheat	Random
NP 39	Bargachi	Morang (T)	Silty clay	Fallow	Random
NP 40	Aitebare	Illan (H)	Clay loam	Fallow	Random
NP 41	Jhurkia	Morang (T)	Sandy loam	Fallow	Random
NP 43	Paudi	Lamjung (RB)	Sandy	Fallow	Random
NP 44	Sisauli	Morang (RB)	Sandy	Fallow	Random
NP 45	Parwanipur	Parsa (T)	Sandy loam	Wheat	Symptomatic
NP 46	Mangalapur	Chitwan (T)	Sandy	Wheat	Random
NP 49	Bardiyah	Bara (T)	Clay loam	Wheat	Random
NP 50	Belwa	Parsa (T)	Sandy	Wheat	Symptomatic
NP 53	Parwanipur	Parsa (T)	Clay loam	Wheat	Symptomatic
NP 55	Mangalapur	Rupandih	Clay loam	Wheat	Random

[a] Closest town to sampling site.
[b] The districts sampled were in the topographic regions designated as Terai (T), Hills (H) or River Basin (RB).
[c] Samples collected from rice fields either randomly or from around symptomatic rice.

(*Capsicum annuum* L.) cv. California Wonder, tomato cv. Rutgers and peanut (*Arachis hypogaea* L.) cv. Florunner] (Sasser and Triantaphyllou, 1977) was also conducted to assess the reaction of these hosts to the Nepalese isolate (NP 29) and another population of root-knot nematode from Bangladesh (*M. graminicola* BP 3). All tested plants were grown in pots (10-cm-diam.) filled with pasteurized soil infested with one of the isolates (5,000 eggs/pot) and maintained in a greenhouse at 25°C for 75 d. All treatments were replicated five times per experiment, and the experiment was repeated.

Variability in aggressiveness among the isolates: The rice cv. LA 110 and Labelle, previously reported as resistant and susceptible, respectively, to Louisiana populations of *M. graminicola* (Yik and Birchfield, 1979), were used to differentiate aggressiveness of the root-knot nematode isolates from Nepal. Plants were inoculated with the 33 Nepalese isolates and maintained in a greenhouse at 25°C for 75 d. Plants were then removed from pots, roots were washed free of soil and root-galling severity determined for each isolate on each host. Additionally, nematode eggs were extracted from roots by blending (3 min intermittently) in 1% sodium hypochlorite solution. The contents from the blender were emptied into a #100 sieve nested on top of a #500 mesh, washed for 3 min with tap water, and eggs collected into a beaker. The volume was adjusted, and eggs in a 10 ml aliquot were counted with a dissecting microscope. A reproductive factor (RF = total number of eggs and juveniles extracted from the roots divided by 5,000 [the number of eggs used to infest the soil in the pot]) was calculated for each nematode isolate and rice variety.

Gene sequencing and phylogenetic analyses: As ITS sequence data for *M. graminicola* was not available in GenBank, two known *M. graminicola* isolates were also included in the study. One was from Bangladesh (*M. graminicola* BP 3) (Padgham, 2003), and the other (*M. graminicola* US) was from infected nut-sedge in Florida (provided by Dr. Janete A. Brito, Florida Department of Agriculture, Gainesville, FL). Sequence data for *M. graminis* was also unavailable in the GenBank, thus a known *M. graminis* isolate obtained from turf grass near Buffalo, NY (provided by Dr. Nathaniel Mitkowski, University of Rhode Island), was also included in this study. Primers rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-

3′) and rDNA1.58s (5′-ACGAGCCCGAGTGATCCACCG-3′) (Vrain et al., 1992, Powers, 2004) obtained from Sigma Genosys Inc. (St. Louis, MO) were used in this investigation. The 33 isolates described above were utilized for this portion of the study. Eggs were obtained by the sodium hypochlorite method, allowed to hatch for 3 d at room temperature, and 60 J2 were picked. The J2 were then rinsed three times with sterile distilled water, crushed in 60 µl sterile distilled water on a sterile glass slide and transferred to a microfuge tube to be used as DNA template. Each DNA template was distributed into five tubes containing 12 µl of template/tube and frozen until PCR was performed. Each reaction was performed as previously described (Mitkowski et al., 2003) and contained 16.25 µl water, 1.5 µl $MgCl_2$ (25 mM), 2.5 µl magnesium-free buffer (Promega, Madison, WI), 0.75 µl dNTP mix (200 µM each dA, dC, dG, dT) and 1.5 µl each primer (10 mM). Twelve microliters of DNA template was then added, and a drop of light mineral oil was placed in each tube to cover the reaction mixture. The reaction tubes were placed into an MJ PTC-100 thermal cycler (Waltham, MA) at 94°C, and 0.2 unit of Taq DNA polymerase (Promega) was added to each tube through mineral oil. The PCR cycle consisted of an initial step at 94°C for 2 min followed by 25 cycles of 94°C for 1 min, 47°C for 1 min and 72°C for 1 min and a final extension for 5 min at 72°C. The PCR products were purified with the Promega DNA cleaning kit and sequenced in both directions at the Cornell University Biotechnology Resource Center. The ITS sequences of the other *Meloidogyne* species included in the phylogenetic analyses were obtained from the GenBank [*M. chitwoodi* Golden, O'Bannon, Santo and Finley, AY593889; *M. javanica* (Treub) Chitwood, 26892; *M. minor* Karrsen et al., AY53899; *M. incognita* (Kafoid and White) Chitwood, AY438556; *M. arenaria* (Neal) Chitwood, AF 387092; *M. hapla* Chitwood, AF576722; *M. naasi* Franklin, AY59301; and *M. trifoliophila* Bernard and Eisenback, AF077091]. The computer program NTI 6.0 (Infomax, Inc., Bethesda, MD) was used for sequence alignment. For amplification products that could not be sequenced directly, PCR products were cloned using the TA cloning kit (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions. Up to four clones from each isolate were sequenced. Phylogenetic analyses, including maximum parsimony and neighbor-joining, were performed using PAUP*4.0b10 (Swofford, 2002) based on the partial ITS sequence alignment. Maximum parsimony analyses were performed via heuristic search options in PAUP*4.0b10 using the branch swapping algorithm (tree-bisection-reconnection). Neighbor-joining analysis was calculated with uncorrected "p" distance. Based on previous studies, *M. incognita* and *M. arenaria* were chosen as outgroup taxa (Castillo et al., 2003). Bootstrap analyses of 1,000 replicates were conducted to assess the degree of support for each node on the tree.

Results

Morphometric measurements of J2: Average body length, stylet length and A, B and C values of all isolates examined were 450.9 (425–477) µm, 11.37 (9.6–15.9) µm, 25.8 (22.3–30.2) µm, 3.8 (3.5–4.2) µm and 6.4 (5.2–8.1) µm, respectively. The average measurements and range of values recorded for the 33 isolates from Nepal, the *M. graminicola* isolate (BP 3) from Bangladesh and the *M. graminicola* isolate from the US are given in Table 2. Although these measurements varied among the Nepalese isolates, they were within the range of measurements described for *M. graminicola* (Mulk, 1976). However, juvenile measurements of the Nepalese isolates were significantly different in body length from the isolates from Bangladesh (BP 3) and the US. The Bangladesh isolate was significantly longer, whereas the US population was significantly shorter than the Nepalese isolates (Table 2). Minor variability was also observed among the Nepalese isolates (Table 2). A significant correlation ($P = 0.0025$) was observed only between stylet length and body length. In addition, the A, B and C values did not correlate with each other or with the body length.

Characterization of perineal patterns: The perineal patterns of the Nepalese isolates were dorso-ventral, oval to almost circular in shape, moderate in height of arc, and no lateral incisures or gaps were observed. Tail tip was marked with prominent, coarse, fairly well separated striae that sometimes formed an irregular tail whorl. These perineal patterns were similar to the pattern described for *M. graminicola*, with some minor variations and overlap with those of *M. oryzae* Maas, Sanders and Dede and *M. trifoliophila* (Fig. 1).

Infection, symptoms and host range studies: All 33 Nepalese isolates examined caused swelling and root galls of different shape and size throughout the root system. In addition to hook-shaped root tips, infected plants produced numerous small fibrous and by-forked roots (Fig. 2). However, symptoms observed were not correlated with particular root-knot nematode isolates, but appeared to be a characteristic of the rice germplasm, as the susceptible varieties exhibited a larger number of bigger galls. The initial white root galls became dark brown and necrotic at plant maturity. However, infected roots of older plants always yielded larger numbers of eggs and J2, regardless of the number and size of galls observed. Eggs were laid within the root cortex in an egg sac that was not easy to observe. Higher severity of infection and large, white, spongy root galls were observed on *E. crusgali*, which also had higher RF values than those of the susceptible rice cultivar Masuli (Pokharel, 2007). The symptoms produced by these isolates were similar to the symptoms observed in the field and also described for *M. graminicola* on rice (Fig. 2).

TABLE 2. Averages and ranges of the morphometric measurements (μm) of second-stage juveniles of the Nepalese isolates on rice cv. BR 11 in the greenhouse.

Isolate no.	Body length	Stylet length	A value[a]	B value[b]	C value[c]
NP 1	438.3 (400–470)	10.1 (5.5–15.5)	25.5 (17.7–9.6)	3.7 (2.7–3.8)	6.2 (5.5–7.6)
NP 2	431.6 (390–465)	12.9 (9.2–17.6)	22.9 (17.2–1.9)	3.9 (2.5–2.8)	7.3 (5.7–9.2)
NP 3	448.8 (410–510)	11.4 (7.3–16.5)	22.4 (16.2–27.4)	3.9 (2.4–2.9)	6.2 (4.4–12.1)
NP 8	463.2 (425–515)	9.8 (5.5–14.1)	27.5 (20.2–37.4)	4.3 (2.8–3.1)	6.6 (5.8–10.6)
NP 10	477.0 (445–510)	12.0 (9.2–15.5)	25.9 (15.7–35.6)	3.9 (32.5–4.3)	6.9 (5.8–8.1)
NP 12	456.5 (400–575)	11.9 (8.5–13.5)	26.4 (18.3–32.2)	4.2 (2.2–5.4)	8.2 (6.0–10.2)
NP 16	429.8 (385–460)	11.2 (8.5–14.1)	24.4 (15.7–36.1)	3.8 (2.8–4.5)	6.9 (5.9–8.0)
NP 17	457.6 (400–675)	11.5 (9.2–14.1)	26.3 (19.6–39.2)	3.3 (2.4–6.1)	6.7 (6.0–8.0)
NP 19	448.5 (410–485)	11.9 (9.2–13.3)	23.3 (18.5–25.8)	3.7 (2.7–4.3)	6.8 (6.2–7.4)
NP 20	449.2 (425–475)	11.5 (9.2–12.6)	25.4 (21.6–30.8)	3.6 (2.6–4.6)	7.3 (6.0–8.9)
NP 22	456.6 (405–500)	11.5 (9.2–14.1)	26.2 (18.1–36.3)	3.2 (2.4–3.8)	6.6 (6.1–7.6)
NP 24	437.4 (390–495)	11.5 (8.2–14.1)	23.8 (17.2–31.2)	3.1 (2.5–4.3)	6.4 (6.0–8.2)
NP 25	462.7 (425–505)	12.0 (9.2–14.1)	25.0 (18.6–29.5)	3.0 (2.4–3.7)	6.2 (5.2–7.6)
NP 28	446.4 (400–485)	11.4 (9.2–13.3)	25.9 (23.0–33.0)	3.3 (2.5–3.9)	6.9 (6.2–8.1)
NP 29	453.8 (410–490)	11.4 (9.2–14.1)	25.0 (20.0–34.5)	3.1 (2.3–3.8)	6.3 (6.2–8.1)
NP 30	444.4 (375–510)	10.3 (5.5–15.5)	27.1 (16.8–40.0)	2.8 (1.8–3.8)	5.7 (4.6–7.2)
NP 31	435.0 (405–460)	9.6 (5.5–14.1)	26.5 (16.4–39.2)	2.6 (2.1–3.3)	7.4 (6.0–9.3)
NP 33	469.0 (425–525)	11.6 (9.2–14.1)	25.5 (18.5–32.7)	3.9 (2.5–4.0)	7.0 (6.0–8.6)
NP 35	443.6 (400–500)	11.3 (9.2–13.3)	27.0 (17.9–39.2)	3.0 (2.1–3.7)	6.2 (5.5–8.3)
NP 36	361.2 (425–500)	11.6 (9.2–13.9)	27.6 (21.9–38.7)	3.7 (2.8–4.8)	6.9 (6.0–7.7)
NP 37	473.6 (445–515)	11.5 (9.2–13.4)	25.9 (19.6–35.6)	3.9 (2.8–4.3)	7.1 (6.3–7.8)
NP 38	453.4 (425–480)	11.2 (7.3–14.1)	26.2 (21.3–33.0)	3.6 (2.5–4.0)	6.7 (5.6–7.9)
NP 39	425.2 (390–475)	11.0 (9.2–13.3)	28.5 (20.7–37.9)	3.1 (2.8–4.3)	6.4 (5.7–8.0)
NP 40	454.2 (425–490)	11.3 (9.2–13.4)	29.9 (24.0–34.4)	3.1 (2.4–4.8)	7.2 (6.3–8.1)
NP 41	442.0 (410–470)	11.2 (8.2–13.4)	24.8 (20.9–28.6)	3.2 (2.4–5.2)	6.6 (5.8–7.3)
NP 43	430.0 (390–455)	11.3 (9.2–12.7)	23.6 (18.5–27.7)	3.7 (2.3–4.3)	6.6 (6.0–7.6)
NP 44	469.2 (440–500)	10.2 (8.5–14.1)	30.3 (24.6–40.9)	3.0 (2.1–4.0)	7.1 (6.5–7.9)
NP 45	467.2 (425–510)	11.5 (7.3–14.1)	26.9 (20.5–38.8)	3.3 (2.4–4.4)	7.1 (6.3–8.3)
NP 46	438.2 (400–490)	11.5 (9.2–13.4)	23.8 (16.3–31.6)	3.1 (2.3–4.1)	6.5 (4.5–8.0)
NP 49	449.3 (410–475)	11.3 (9.2–13.4)	25.6 (19.5–33.4)	3.2 (2.3–4.1)	7.1 (6.1–8.1)
NP 50	466.0 (410–495)	11.5 (9.2–14.1)	26.4 (20.7–35.6)	3.1 (2.5–3.8)	7.1 (5.8–8.4)
NP 53	449.0 (400–505)	11.3 (7.3–14.1)	27.1 (22.8–31.6)	3.7 (2.1–4.1)	6.6 (5.9–7.8)
NP 55	460.6 (415–480)	11.7 (9.2–14.1)	26.2 (19.7–34.5)	3.8 (2.3–4.1)	7.0 (6.1–7.8)
BP3	509.2 (425–585)	11.2 (8.2–13.4)	25.0 (18.5–29.9)	3.1 (2.1–4.3)	7.4 (6.0–9.3)
US 1	403.6 (375–470)	10.3 (9.2–11.0)	30.0 (20.4–37.9)	2.9 (2.2–3.9)	6.3 (5.3–8.1)
LSD	1.01	2.58	15.6	7.77	0.17
(log values)	(0.0100)	(0.9470)	(1.1928)	(0.8904)	(–0.7696)

Analysis on log transformed data by GLM (General Linear Models).
[a] Refers to the ratio of body length/maximum body width.
[b] Refers to the ratio of total body length/neck length.
[c] Refers to the ratio of total body length/tail length.

The Nepalese isolate (NP 29) and the Bangladesh isolate (BP 3) did not reproduce on any of the hosts included in the North Carolina host-range test in repeated experiments. Similarly, these same isolates did not increase on tomato cv. Rutgers, Money Maker or Red Cherry or jute cv. Tosa. They did, however, reproduce on cabbage cv. Dawirth Green and jute cv. Deshi. In addition, they parasitized all varieties of rice, wheat, oat and barley tested, but not on rye or corn. The reproductive factor of the Nepalese isolate was lower in cabbage and oat as compared to rice, wheat and *E. crusgali*. *Meloidogyne graminis* reproduced well on *E. crusgali*, but poorly on tomato cv. Rutgers and rice cv. Masuli.

Variability in aggressiveness of the isolates: Rice varieties Labelle and LA 110 were both found to be susceptible to all the Nepalese isolates of *M. graminicola* tested in this study. However, root-galling severity and RF values varied among the Nepalese root-knot isolates on both varieties (Table 3). Isolates NP 37 and NP 36 were found to be the most aggressive, and isolate NP 39 was the least aggressive. The isolates could be classified into three categories based on the results of aggressiveness tests: the most aggressive, the intermediate and the least aggressive. Four isolates (NP 37, NP 12, NP 36 and NP 50) were in the most aggressive category, and four (NP 49, NP 40, NP 19 and NP 29) were categorized as the least aggressive. The remaining isolates were categorized as intermediate types in their aggressiveness on these two rice varieties. There was no correlation between the aggressiveness of the isolate and the geographic area of collection, soil types or crop rotation (Table 3). In addition, no correlation between root-galling severity (RGS) and reproductive factor was observed (Table 3). Similar results were observed when the experiment was repeated.

Gene sequencing and phylogenetic analyses: An approximately 450 bp DNA region, which included a portion of

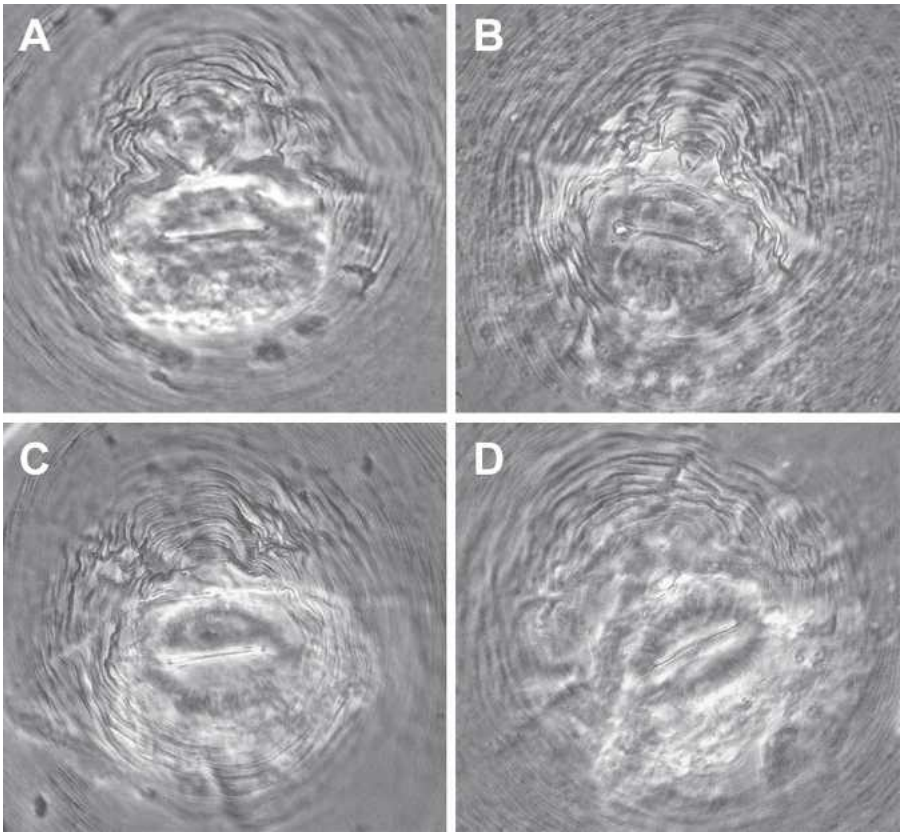

FIG. 1. Minor variants identified in the perineal pattern observed among the Nepalese isolates of *Meloidogyne graminicola*.

the 18S rRNA gene, complete ITS 1 and part of the 5.8S rRNA gene, was amplified and sequenced. For most isolates in this study, clones from the same isolate had either identical ITS sequence or grouped together in the phylogeny. In these cases, one clone was chosen to represent the isolate for further phylogenetic analyses. For isolate 37, however, clones were placed in two groups following phylogenetic analysis, and therefore two representatives were included (Fig. 3). The maximum number of nucleotide differences between clones within the same isolates was three. All sequences, including those that were not selected for the analysis, were deposited in the Genbank (Table 4). Differences observed in the ITS sequences between clones of the same isolate could be due to variation among copies of the ITS within an individual, or due to errors introduced during PCR or sequencing.

The parsimony analysis based on 46 informative characters resulted in eight equally most parsimonious trees, differing slightly in the branching pattern. The lengths of the most parsimonious trees are 83 steps, with consistency index of 0.77, retention index of 0.88 and rescaled consistency index of 0.68 (Fig. 3). All Nepalese isolates formed a distinct clade together with *M. graminicola* isolates BP 3 and US, and also *M. trifoliophila*, with a 96% bootstrap value (*M. graminicola* clade) (Fig. 3). The neighbor-joining analysis also resulted in a tree with the same distinct clade, with a 100% bootstrap support. The parsimonious tree showed that the *M. graminicola* clade formed two major groups, Group I and Group II, with a 54% bootstrap support for Group I (Fig. 3). Group II had seven polymorphic nucleotide positions in ITS sequences among the 18 isolates. Group I contained eight isolates with only two polymorphic nucleotide positions in the ITS sequences. A fixed sequence difference was found between the two groups. ITS sequences of isolates in Group I had CATA at positions 277 to 280 bp, whereas the ITS sequences of Group II isolates had TATT in the same positions.

No correlation of nucleotide substitution patterns and identified characteristics of isolate collection sites including cropping history (rice-wheat vs. rice-other rotations), soil texture types (heavy vs. light) and sampling designs (random vs. symptomatic plants) was observed. Similarly, ITS sequences did not correlate with any of the morphometric measurements made (juvenile measurements and perennial patterns) or the pathological differences detected among the isolates.

DISCUSSION

Results of this investigation suggest that only *M. graminicola* occurs in the rice-wheat production fields in Nepal. This finding was confirmed by morphometric measurements of J2, perennial pattern of females, host

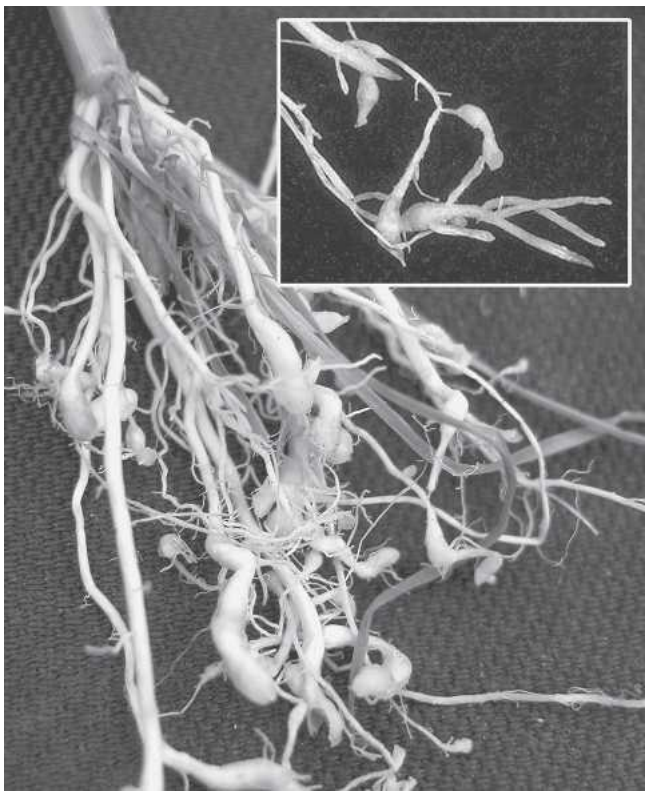

FIG. 2. Different shape and size of root-galls, extensive branching and root tip thickenings caused by infections of *Meloidogyne graminicola* in rice.

range tests and amplification and sequencing of the ITS regions. The results also documented the existence of some variability among the Nepalese isolates of *M. graminicola* in juvenile size, host range, aggressiveness on rice germplasm and ITS sequences.

Several *Meloidogyne* species such as *M. artiellia* Franklin, *M. chitwoodi*, *M. naasi*, *M. microtyla* Mulvey, Townshend and Potter and *M. ottersoni* Thorne (Sikora, 1988), *M. graminicola*, *M. graminis*, *M. kikuyensis* de Grisse and *M. spartinae* (Rau and Fassuliotis) Whitehead (Taylor and Sasser, 1978) and *M. incognita*, *M. javanica* and *M. arenaria* (Swarup and Sosa-Moss, 1990) were reported to attack wheat and other cereal crops worldwide. However, only *M. graminicola*, *M. tritocoryzae*, *M. oryzae*, *M. incognita*, *M. javanica* and *M. arenaria* were reported attacking rice. Furthermore, *M. graminicola* has been predominantly reported on rice from lowland production conditions (with short-term flooding and water not more than 10-cm deep), which is common in the majority of the rice fields in the Terai region of Nepal.

The average values for juvenile measurements of individuals among the Nepalese isolates falling beyond the range described in literature for *M. graminicola* (Mulk, 1976) may be due to the high variability of this nematode and the diversity in geographic locations, soil types and management practices at the collection sites. However, there was no correlation between the variability in juvenile measurements with that of geographic

TABLE 3. Root-galling severity (RGS) ratings and reproductive factor (RF) resulting from inoculating roots of rice cv. Labelle and cv. LA 110 with *M. graminicola* isolates from Nepal.

	cv. LaBelle[c]		cv. LA 110[c]	
Isolate no.	RGS[a]	RF[b]	RGS[a]	RF[b]
NP 49	4.2	10.5	5.2	12.7
NP 40	4.6	12.6	5.2	12.6
NP 19	4.6	65.3	6.4	21.7
NP 29	4.8	14.8	5.0	10.6
NP 46	5.0	13.5	6.4	22.2
NP 44	5.2	17.2	5.0‘	12.7
NP 55	5.4	23.5	5.0	22.2
NP 25	5.6	26.5	5.4	22.1
NP 41	6.0	21.7	6.4	18.5
NP 31	6.0	22.1	5.6	16.6
NP 1	6.0	26.1	6.2	20.8
NP 28	6.0	29.1	5.2	23.9
NP 3	6.2	18.7	5.6	19.4
NP 8	6.2	19.0	6.2	18.0
NP 33	6.2	26.7	6.2	20.8
NP 2	6.4	21.9	6.0	21.3
NP 22	6.4	27.2	6.8	31.3
NP 24	6.4	30.0	6.2	26.6
NP 35	6.4	30.8	5.6	21.7
NP 30	6.4	44.7	6.0	20.8
NP 43	6.6	19.5	4.8	11.0
NP 10	6.6	30.9	5.4	15.4
NP 39	6.6	31.7	6.4	21.8
NP 17	6.6	34.0	6.6	22.7
NP 16	6.6	39.4	6.4	23.7
NP 45	6.6	55.9	6.4	34.6
NP 38	6.8	19.5	4.4	9.4
NP 53	6.8	46.9	5.6	40.0
NP 20	6.8	47.2	6.2	28.9
NP 50	7.2	123.9	7.0	79.5
NP 36	7.2	127.7	7.4	160.0
NP 12	7.8	131.1	7.0	73.3
NP 37	7.8	199.4	7.2	111.1
LSD	1.41	29.45	0.12	20.29
(log values)	(0.1492)	(1.4691)	(–0.9208)	(1.3073)

Analysis on log transformed data by GLM (General Linear Models).

[a] Root-galling severity ratings were recorded on a scale of 1 (healthy roots, no galls observed) to 9 (>76% of roots with galls).

[b] Refers to reproductive factor (RF = Pf/Pi).

[c] The rice cultivars Labelle and LA 110 were reported as most susceptible and resistant, respectively, to isolates of *M. graminicola* in Louisiana (Yik and Birchfield, 1979).

regions. A total of 1,155 individuals were measured in the present study, whereas measurements reported previously were generally based on 20 to 30 individuals. Lower and higher ranges of measurements observed in this study suggested either that the juvenile measurements are not reliable means of identification of this species due to high variability or they may need readjustment to account for the observed differences. Similar variability in the measurements of *Meloidogyne* juveniles was recently described for *M. thailandica*, a new species of root-knot nematode of ginger from Thailand (Handoo et al., 2005), and for *M. graminicola* from Florida (Handoo et al., 2003).

Perineal pattern morphology has been the standard characteristic used for the identification of the most common *Meloidogyne* species since 1949 (Chitwood,

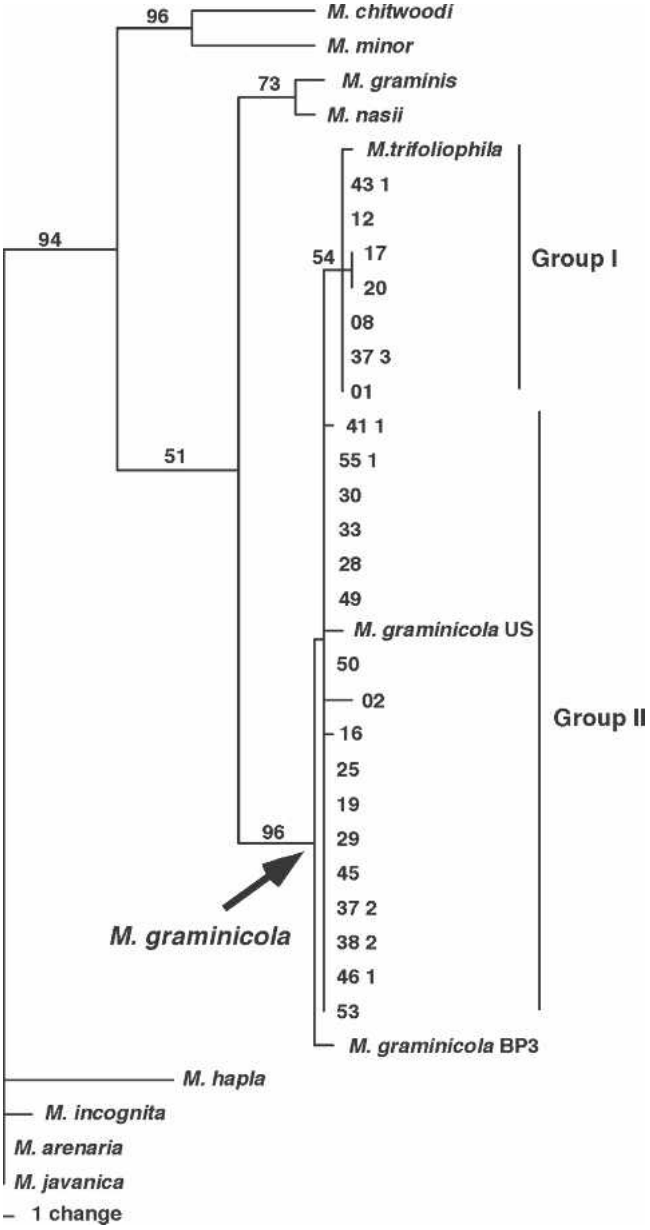

FIG. 3. One of the eight most parsimonious trees showing relationships of Nepalese, Bangladesh and US isolates of root-knot nematodes (*M. graminicola, M. graminis* and other *Meloiodgyne* species) based on partial ITS sequences. Bootstrap values of 50% and higher are labeled on the branches.

1949). The perineal patterns of the Nepalese isolates in this study were similar to published perineal patterns for *M. graminicola*, with minor variability. The perineal patterns of these isolates were distinctly different from those of other species of *Meloidogyne* and even those of the closely related species *M. graminis*, as the lateral incisures diagnostic for the latter species were absent. However, variants of the perineal pattern observed on the collected Nepalese isolates suggested some overlap with those of *M. oryzae* and *M. trifoliophila*. *Meloidogyne oryzae* has been reported on rice only once from Surinam (Maas et al., 1978). *Meloidogyne trifoliophila* has only been isolated from white clover and is not known to attack rice or wheat (Bernard and Eisenback, 1997), but all of the Nepalese isolates attacked both rice and wheat. The minor variation observed in the perineal pattern of the Nepalese isolates could be of natural occurrence within the species, caused primarily by difference in size of females examined or due to variation in genetic make-up of the species. Variations in perineal pattern have also been described within other species of *Meloidogyne* (Maas et al., 1978; Mitkowski et al., 2003; Handoo et al., 2005). In addition, minor variation (markings on perineum) in pattern of *M. graminicola* was noted in the original description of the species (Mulk, 1976). The perineal patterns alone could not be used to confirm the identity of the tested root-knot nematode from Nepal, as the perineal patterns for *M. oryzae, M. graminicola* and *M. trifoliophila* are not distinct enough to allow a definitive diagnosis.

Numerous forked roots and swelling of root-tips in addition to hook-like galls (Fig. 2) were observed on rice plants infected with the Nepalese isolates. Root-knot nematode infections on lowland rice resulting in the production of hook-like galls on roots have been attributed to *M. graminicola* or possibly *M. oryzae*, as they are the only known root-knot species that can survive in flooded soil conditions (Bridge et al., 1990). However, *M. oryzae* was reported to survive in shallow-flooded (<10-cm) rice fields for relatively short periods (Bridge et al., 1990). In contrast, *M. graminicola* can survive in soil flooded to a depth of 1 m for at least five months. Many rice production sites in Nepal are under flooding for extended period of time. Thus, the characteristic root-gall symptoms (the hook-like galls) in rice are considered diagnostic for infection caused primarily by *M. graminicola*. Females of the collected Nepalese isolates deposited their eggs in the root cortex in mass and without an easily observable egg sac, which further suggested that the identity of the isolates was *M. graminicola* (Mulk, 1976).

All the Nepalese isolates of root-knot nematode infected and multiplied well in barnyard grass in a similar manner to *M. graminis* (Birchfield, 1965). In addition, all the Nepalese isolates reproduced in rice, in contrast to *M. graminis*. These results indicated that barnyard grass, which is commonly available and easy to grow, can serve as an experimental differentiating host for both species. A number of isolates of root-knot nematode from Nepal reproduced in jute cv. Deshi, but not in jute cv. Tosa. Similar results were observed with an *M. graminicola* isolate from Bangladesh on jute (Padgham, 2003). The Nepalese isolate NP 29 also reproduced on other crops such as cabbage, wheat and barley. The latter crops are known to be good hosts for *M. graminicola*, whereas corn and oat are not. However, oat and corn were previously reported as hosts for *M. graminicola* (MacGowan and Langdon, 1989). This contradicting result may be due to the differences in virulence of isolates of *M. graminicola* and the crop varieties

TABLE 4. GenBank Accession numbers for the ITS sequences of isolates of *M. graminicola* generated in this study. Isolates were originally collected in Nepal (NP), Bangladesh (BP) or Florida (US).

Isolate no.[a]	GenBank accession no.	Isolate no.	GenBank accession no.	Isolate no.	GenBank accession no.
NP 01	DQ909021	NP 30	DQ909032	NP 46.1	DQ909044
NP 02	DQ(09022	NP 33	DQ909034	NP 46.2	DQ909045
NP 08	DQ909025	NP 37.1	DQ909035	NP 49	DQ909046
NP 12	DQ909023	NP 37.2	DQ909036	NP 50	DQ909047
NP 16	DQ909024	NP 38.2	DQ909037	NP 53	DQ909048
NP 17	DQ909027	NP 41.1	DQ909038	NP 55.1	DQ909049
NP 19	DQ909028	NP 41.2	DQ909039	NP 55.2	DQ909050
NP 20	DQ909029	NP 43.1	DQ909040	BP 3	EF432570
NP 25	DQ909030	NP 43.2	DQ909041	US	EF432571
NP 28	DQ909033	NP 43.3	DQ909042		
NP 29	DQ909031	NP 45	DQ909043		

[a] The isolates with multiple copies of ITS sequences are differentiated by the decimal point in isolate number.

used in the previous study compared with those used in the current investigation. In addition, the tested isolates of root-knot nematode from Nepal and Bangladesh did not multiply in tobacco cv. NC 95, watermelon cv. Charlestone Grey, cotton cv. Deltapine 61, pepper cv. California Wonder, tomato cv. Rutgers and peanut cv. Florunner that are used in the North Carolina host range test (Sasser and Triantaphyllou, 1977). The reaction of these host plants to *M. graminicola* was not known. Tomato cv. Rutgers is an experimental host for most of the root-knot species, but not the root-knot species attacking cereals, like *M. graminicola, M. oryzae* and *M. graminis*. However, *M. oryzae* multiplied well in tomato cv. Money Maker (Maas et al., 1978), in contrast to *M. graminicola* that did not multiply in any of the tomato cultivars tested (Manser, 1971). None of the Nepalese isolates with perineal patterns observed to overlap those of *M. oryzae* multiplied in any of the above tomato cultivars. This finding further indicated that these isolates were not *M. oryzae*.

No information was available on the variability in the aggressiveness of isolates of *M. graminicola*. However, significant variation in aggressiveness was observed among the collected isolates of this nematode from Nepal in the present study. This finding is important because the efficiency of host resistance depends largely on the genetic variation in the aggressiveness of the pathogen. A significant interaction of lettuce genotypes and *M. hapla* isolates was previously reported (Mitkowski and Abawi, 2003). However, van der Beek et al. (1998) observed no significant interactions between 10 isolates of *M. chitwoodi* and *M. fallax* Karssen, 1996 on 10 potato cultivars. Similarly, variability of virulence was not observed among the New Zealand isolates of *M. hapla* on potato germplasm (van der Beek et al., 1998).

To our knowledge, this is the first report of ITS sequences for *M. graminis* and *M. graminicola*. The ITS sequences of the *M. graminicola* isolates were very similar to that of *M. trifoliophila* (Birchfield, 1965). Unfortunately, we did not have isolates of *M. trifoliophila* to include in the test. This species has only been reported from white clover and does not infect rice. The ITS sequence data from *M. graminis* and *M. graminicola* were very distinct and easily separable from each other and from other species.

Several studies have demonstrated that ITS sequencing was not only useful as a diagnostic tool for *Globodera, Heterodera, Xiphenema, Longidorus* and *Pratylenchus* species (Powers, 2004), but also for phylogenetic analyses of relationships of a number of species in the genera *Heterodera, Meloidogyne* and *Bursaphelenchus* (Hugall et al., 1999; Powers, 2004). In this study, phylogenetic analyses based on the partial ITS sequences of *Meloidogyne* species and the unknown Nepalese root-knot isolates resulted in a well-supported clade, which includes the Nepalese isolates and known *M. graminicola* isolates. Therefore, we propose the species boundary of *M. graminicola* (Fig. 3). This study provides the basis for future studies on the systematics of *M. graminicola*, particularly in relationship to *M. trifoliophila*. ITS sequences of isolates from other sources and sequences of other genes are needed to test the phylogenetic relationship indicated here (Fig. 3).

Variability in ITS sequences among the *M. graminicola* isolates indicates that the ITS region may be useful for population studies of *M. graminicola*. In addition, sequence polymorphisms within individuals were observed in six of the 33 isolates tested, which may be due to the presence of multiple alleles and/or multiple copies of the sequences, both of which were reported previously in other root-knot nematode species (Powers et al., 1997; Hugall et al., 1999). ITS sequences and the two groups of *M. graminicola* recognized among the Nepalese isolates agree with the observations made by Hugall et al. (1999) with *M. arenaria, M. incognita* and *M. javanica*. Further studies are warranted to understand the observed genetic variability that exists within *M. graminicola*.

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