RESEARCH/INVESTIGACIÓN

PREVALENCE AND CHARACTERIZATION OF PLANT-PARASITIC NEMATODES IN LOWLAND AND UPLAND RICE AGRO-ECOSYSTEMS IN LUZON, PHILIPPINES

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ABSTRACT

Pascual, M. L. D., W. Decraemer, I. Tandingan De Ley, A. Vierstraete, H. Steel, and W. Bert. 2014. Prevalence and characterization of plant-parasitic nematodes in lowland and upland rice agro-ecosystems in Luzon, Philippines. Nematropica 44:166-180.

The prevalence of plant-parasitic nematodes in lowland and upland rice ecosystems was compared based on soil and root samples from 30 rice fields in five provinces (Bataan, Batangas, Bulacan, Pampanga, and Pangasinan) of Luzon, Philippines. Five nematode genera (*Meloidogyne*, *Hirschmanniella*, *Pratylenchus*, *Tylenchorhynchus*, and *Helicotylenchus*) from lowland and nine genera (*Rotylenchulus*, *Aphelenchoides*, *Criconematidae*, *Rotylenchus*, and those found in lowland) from upland were identified. *Meloidogyne graminicola* was the most prevalent and abundant plant-parasitic nematode in both ecosystems. *Hirschmanniella* was the second most prevalent genus in lowland rice, but its density was low in upland rice. *Pratylenchu*s was present in high densities, but was not widely distributed in either upland or lowland rice fields. The three most prevalent nematode species, *M. graminicola*, *H. oryzae*, and *H. mucronata*, were morphologically and molecularly characterized. Molecular analyses based on D2D3, ITS1-5.8-ITS2, and 18S rDNA regions generally showed an overall congruence, low intraspecific variation and agreement with classical morphological and morphometrical identifications.

Key words: D2D3, 18S, *Hirschmanniella mucronata*, *Hirschmanniella oryzae*, ITS, *Meloidogyne graminicola*, molecular phylogeny, morphology, morphometrics, rDNA.

RESUMEN

Pascual, M. L. D., W. Decraemer, I. Tandingan De Ley, A. Vierstraete, H. Steel, and W. Bert. 2014. Prevalencia y caracterización de nematodos parásitos de plantas en agro-ecosistemas de arroz en tierras bajas y altas en Luzon, Filipinas. Nematropica 44:166-180.

Se comparó la prevalencia de nematodos parásitos de plantas en ecosistemas de arrozales en tierras bajas y altas, basados en muestras de suelo y raíces procedentes de 30 campos de arroz en cinco provincias (Bataan, Batangas, Bulacan, Pampanga, y Pangasinan) de Luzon, Filipinas. Se identificaron cinco géneros de nematodos (*Meloidogyne*, *Hirschmanniella*, *Pratylenchus*, *Tylenchorhynchus*, y *Helicotylenchus*) procedentes de arrozales en tierras bajas y nueve géneros procedentes de arrozales en tierras altas (*Rotylenchulus*, *Aphelenchoides*, *Criconematidae*, *Rotylenchus*, y aquellos encontrados en las tierras bajas). *Meloidogyne graminicola* fue el nematodo parásito de plantas más prevalente y abundante en ambos ecosistemas. *Hirschmanniella* fue el segundo género más prevalente en arrozales de tierras bajas, aunque sus densidades fueron bajas en tierras altas. Se encontraron *Pratylenchus* en altas densidades, pero no estaban ampliamente distribuidos ni en arrozales de tierras bajas ni de altas. Se caracterizaron morfológica y molecularmente las tres especies más prevalentes de nematodos parásitos de plantas, *M. graminicola*, *H. oryzae*, y *H. mucronata*. Análisis moleculares basados en las regiones D2D3, ITS1-5.8-ITS2, y 18S del rDNA mostraron congruencia, baja variación intraespecífica y estuvieron en concordancia con las clásicas identificaciones morfológicas y morfométricas.

Palabras clave: D2D3, 18S, *Hirschmanniella mucronata*, *Hirschmanniella oryzae*, ITS, *Meloidogyne graminicola*, filogenia molecular, morfología, morfométrica, rDNA.

INTRODUCTION

Rice (*Oryza sativa* L.) is the number one agricultural crop in the Philippines, grown in approximately 4.5 million ha with an annual production of 16.3 million metric tons (BAS, 2010). Rice is cultivated in lowland (irrigated or rainfed) and upland (rainfed) agro-ecosystems. Of the total hectares in rice production, 67% are irrigated, and the remaining areas are rainfed (BAS, 2010). A majority of the country's irrigated rice is found on Luzon, the largest island in the Philippines, which accounts for 60% of the Philippines total irrigated rice and about 39% of the rainfed rice, which is about 41% of the national rainfed rice production (BAS, 2010).

In the Philippines, modern high-yielding varieties account for the majority of rice production, with less than 10% of production coming from traditional varieties. However, the average yield is only 3.6 t/ha, which is below the average yield of neighboring countries like China (6.6 t/ha), Vietnam (5.2 t/ha), and Indonesia (5.0 t/ha) (FAOSTAT, 2011). This low productivity may be attributed to various factors including plant-parasitic nematodes, which have been reported to cause 10% yield loss in rice (IRRI, 2004). Plant nematodes can cause extensive damage and substantial yield losses (Sasser and Freckman, 1987). Problems due to plant-parasitic nematodes may be aggravated by lack of awareness among farmers of their existence, the microscopic nature of nematodes, atypical plant symptoms, and the occurrence of multispecies nematode populations and their association with other pathogens (De Waele and Elsen, 2007).

Fig. 1. Sampling areas, indicated on the map of North and Central Philippines.

More than 35 genera and 130 species of plant-parasitic nematodes are reportedly associated with rice (Gerber *et al*., 1987), but only about 29 species are known or suspected to cause yield loss (Bridge *et al*., 2005). Plant-parasitic nematodes occur in all rice environments. Their diversity and distribution vary with different ecosystems. For example a high diversity may occur in rainfed lowland rice ecosystems, while low diversity is common in irrigated rice (Prot and Rahman, 1994). In the Philippines, 11 genera of plant nematodes (*Criconemoides* Taylor, 1936 (*apud* Geraert, 2010); *Helicotylenchus* Steiner, 1945; *Hemicriconemoides* Chitwood & Birchfield, 1957; *Hemicycliophora* de Man, 1921; *Hoplolaimus* Daday, 1905; *Meloidogyne* Goeldi, 1892; *Pratylenchus* Filipjev, 1936; *Rotylenchulus* Linford & Oliveira, 1940; *Rotylenchus* Filipjev, 1936; *Tylenchorhynchus* Cobb, 1913 and *Xiphinema* Cobb, 1913) have been reported in association with upland rice (Villanueva *et al*., 1992) and only six genera (*Criconemella*, *Helicotylenchus*, *Hemicriconemoides*, *Hirschmanniella* Luc & Goodey, 1964, *Meloidogyne*; and *Tylenchorhynchus*) were detected from irrigated rice (Prot *et al*., 1994).

Although a few surveys of rice nematodes have been conducted in the Philippines, changes in land use, intensification of agricultural production, and the introduction of new technologies could contribute to shifts in the occurrence and density of nematode species or emerging nematode species under different rice agro-ecosystems. Moreover, combined molecular and morphological characterization of plant nematodes associated with the different rice agro-ecosystems is lacking. Such information is valuable to gain insight into changes in rice-nematode distribution and frequency between agro-ecosystems and their potential contribution to crop yield loss.

This study aimed to determine the genera composition, frequency, and abundance of plant-parasitic nematodes in lowland and upland rice agro-ecosystems in Luzon, Philippines; and to characterize the morphology as well as the D2-D3 domains of the large subunit (LSU), internal transcribed spacer (ITS1-5.8-ITS2) regions and 18S or small subunit (SSU) of the rRNA.

MATERIALS AND METHODS

Prevalence of plant-parasitic nematodes in rice agro-ecosystems

Soil and root samples were collected for nematode analysis from 30 rice fields (15 lowland and 15 upland) in Luzon, Philippines. The fields were located in five provinces: Bataan (L1 and U1), Batangas (L2 and U2), Bulacan (L3 and U3), Pampanga (L4 and U4), and Pangasinan (L5 and U5) where lowland and upland rice production were both present (Fig. 1). Three

fields from each rice ecosystem per province were selected for sampling when plants were at tillering and booting stage, except in Pampanga, where fields were sampled when rice was in the seedling stage. In each rice field, five plants were selected randomly and roots and rhizosphere soil were collected up to a depth of 15 cm. Root and soil samples were stored at 4°C until processed.

Nematodes were extracted from roots following the method of Seinhorst (1959) where roots were cut into 1-cm pieces and incubated for 7 d in a mist chamber. Soil samples were processed from a representative 300 cm^3 sample using a combination of sieving and decanting followed by a modified Baermann funnel for 48 hr. Nematodes extracted from roots and soil were preserved in 4% formalin for the estimation of densities and morphological studies and DESS (DMSO-EDTA salt-saturated solution) (Yoder *et al*., 2006) for molecular analysis after morphological identification. The preserved nematode specimens were stored in plastic tubes at 4°C prior to their transport to Ghent University, Belgium.

Plant-parasitic nematodes from root and soil samples were identified to genus, and densities were expressed as nematodes per 100 cm^3 soil or per gram of fresh root weight. The most prevalent nematodes from each agro-ecosystem were identified to species. PRIMER v5.0 software package (Clarke and Gorley, 2001) was used for multivariate analysis. A similarity matrix was constructed using the Bray-Curtis measure of similarity on square-root transformed data of genus densities followed by an ordination analysis using group average sorting with multidimensional scaling ordination (MDS) (Clarke and Warwick, 2001). An analysis of similarities (ANOSIM) (Clarke and Warwick, 2001) was performed to assess significant differences in the nematode genus composition between lowland and upland ecosystems.

Frequency and abundance of each nematode genus were assessed based on the limits established by Fortuner and Merny (1973). The frequency was computed by dividing the numbers of samples in which the nematode was observed by the total number of samples and expressed as a percentage. Abundance was calculated as the sum of the nematodes per 1,000 cm^3 of soil or per gram of fresh root for all samples containing that genus divided by the number of positive samples for that genus and expressed as a decimal logarithm. A nematode was regarded as abundant if abundance value ≥ 1.3 (= 20 individuals/g of roots) or if value ≥ 2.3 (= 200 individuals/1,000 cm^3 of soil). A nematode was regarded as frequent in the soil or the roots when it was observed in at least 30% of the samples.

Morphological characterization

Formalin-preserved specimens were processed to anhydrous glycerol following the glycerol-ethanol method (Seinhorst, 1959 as modified by De Grisse, 1969) and mounted on glass slides with cover glass supported by a wax ring. Measurements were made from pencil line drawings made through a light microscope (Olympus BX 50, Japan) with a drawing tube. Digital images were obtained via an Olympus BX51 microscope with differential interference contrast (DIC) and equipped with Olympus camera.

Molecular characterization

Molecular characterization and analyses were performed on the small subunit (SSU), 18S ribosomal RNA gene, and the large subunit (LSU), 28S D2-D3 expansion segment of the rRNA gene for both *Meloidogyne* (lowland and upland populations) and *Hirschmanniella* (lowland populations). For the latter also the internal transcribed spacer (ITS) of the rRNA gene was analysed. After morphological identification, DESS-preserved nematodes were rinsed in distilled water for about 30 min, individually cut using aseptic techniques, transferred to a 1.5 ml eppendorf tube with 25 µl of worm lysis buffer (WLB, Williams *et al*., 1992: 50 mM KCl; 10 mM Tris-Cl pH 8.3; 2.5 mM MgCl2; 0.45% NP 40 (Tergitol Sigma-Aldrich, St. Louis, MO, USA); and 0.45% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated for at least 10 min at -80°C. To each tube was added 1 µl of proteinase K (60 µg ml^{-1}) prior to incubation at 65°C for 1 hr followed by enzyme deactivation at 95°C for 10 min. To amplify the target genes, 2.5 µl of gDNA suspension was used as template in a 25-µl PCR reaction mix (TopTaq Qiagen, Germany) following the manufacturer's protocol. The primers used were: G18S4 and 4R; 4F and 18P for 18s region (Blaxter *et al*., 1998; Tandingan De Ley *et al*., 2002); D2A and D3B for the D2-D3 region (De Ley *et al*., 1999); and Vrain2F and Vrain2R for the ITS region (Vrain *et al*., 1992; modified by Elbadri *et al*., 1999). Amplification cycles were conducted in the Mastercycler (Eppendorf, Rotselaar, Belgium) programmed for 94°C for 4 min; 40 cycles at 94°C for 30 sec; 54°C (18S and D2D3) or 60°C (ITS) for 30 sec; and 72°C for 1 min. Aliquots of 5 µl of the PCR products were sized with low DNA mass ladder and separated by electrophoresis in 1% agarose gel stained with ethidium bromide and observed using a UV Transilluminator (BioDoc-It Imaging System, UVP, Upland, CA, USA).

The PCR products (5 µl) were enzyme-purified using 1 µl of Exonuclease I + FastAP Thermosensitive Alkaline Phosphatase (ThermoScientific Erembodegem-Aalst, B-9320 Belgium). Purification was done by incubating the mixture for 15 min at 37°C followed by 15 min at 85°C to inactivate enzymes. Cleaned PCR products were then used for cycle sequencing using the ABI Prism BigDye V3.1 Terminator Cycle Sequencing kit (Life technologies, Ghent, Belgium) following the manufacturer's protocol.

Sequencing primers were the same as those used for the PCR of D2D3 and ITS while 9R, 9FX, 26R, 23F, 13R and 2FX were used for 18S (Tandingan De Ley *et al.*, 2002). Sequencing was performed using an Applied Biosystems ABI 3130XL Genetic Analyser. Sequences were edited and assembled with Seqman 7.0 (DNASTAR Lasergene).

For phylogenetic analysis, DNA sequences were aligned with related sequences from GenBank, using MUSCLE (Edgar, 2004). These alignments were also used to check manually sequence differences between taxa. Bayesian phylogenetic inference (BI) was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). A general time-reversible model with a proportion of invariable sites (GTR+I; ITS) or with a gamma distribution for the remaining sites (GTR + I + G; D2D3 and 18S) was used as selected by AIC in MrModeltest 2.3 (Nylander, 2004). Analyses were run for 3×10^6 generations, and trees were generated using the last 1,000,000 generations well beyond the burn-in value.

RESULTS

Prevalence of plant-parasitic nematodes in rice ecosystems

Five plant-parasitic nematode genera (*Helicotylenchus*, *Hirschmanniella*, *Meloidogyne,* *Pratylenchus*, and *Tylenchorhynchus*) were associated with lowland rice ecosystem. Nine genera (those found in lowland rice plus *Aphelenchoides*, *Criconematidae*, *Rotylenchus*, and *Rotylenchulus*) were identified from the upland rice ecosystem. Of these genera, *Meloidogyne*, *Pratylenchus*, and *Hirschmanniella* were found in both root and soil samples while the others were only present in the soil samples.

The latter three genera of plant-parasitic nematodes occurred not only in both soil and roots but also in both agro-ecosystems with variable population densities (Fig. 2A and 2B). In roots, *Meloidogyne* had the highest population densities in both lowland and upland rice fields followed by *Pratylenchus* and *Hirschmanniella* (Fig. 2A). Densities of *Meloidogyne* (147 ± 52 & 172 ± 56/1 g roots) did not vary significantly in both ecosystems while the mean densities of *Pratylenchus* were significantly higher in upland (55 ± 16) than in lowland roots (20 ± 11). *Hirschmanniella* occurred at lower densities that ranged only from 1 ± 0 to 4 ± 1 in upland or lowland. In soil (Fig. 2B), generic composition was different. *Pratylenchus*, *Helicotylenchus*, *Tylenchorhynchus*, and *Meloidogyne* were common in both lowland and upland soil; *Hirschmanniella* was only observed in lowland soil and *Rotylenchulus*, the family *Criconematidae*, *Aphelenchoides*, and *Rotylenchus* only occurred in upland soil. *Hirschmanniella* and *Rotylenchulus* had the highest population densities among the nematode genera found in lowland and upland soil, respectively; all other genera were present at lower densities (Fig. 2B).

Differences in generic composition were also illustrated in the non-metric MDS analysis between lowland and upland soil samples, but not with upland and lowland roots (Fig. 2C). Analysis of similarities (ANOSIM) indicated significant differences between upland and lowland soil ($P = 0.01$) as well as upland soil and upland roots ($P = 0.01$). Lowland soil and lowland roots also differed ($P = 0.01$), but no significant differences were noted between lowland and upland roots.

The frequency and abundance of plant-parasitic nematode genera differed in lowland and upland

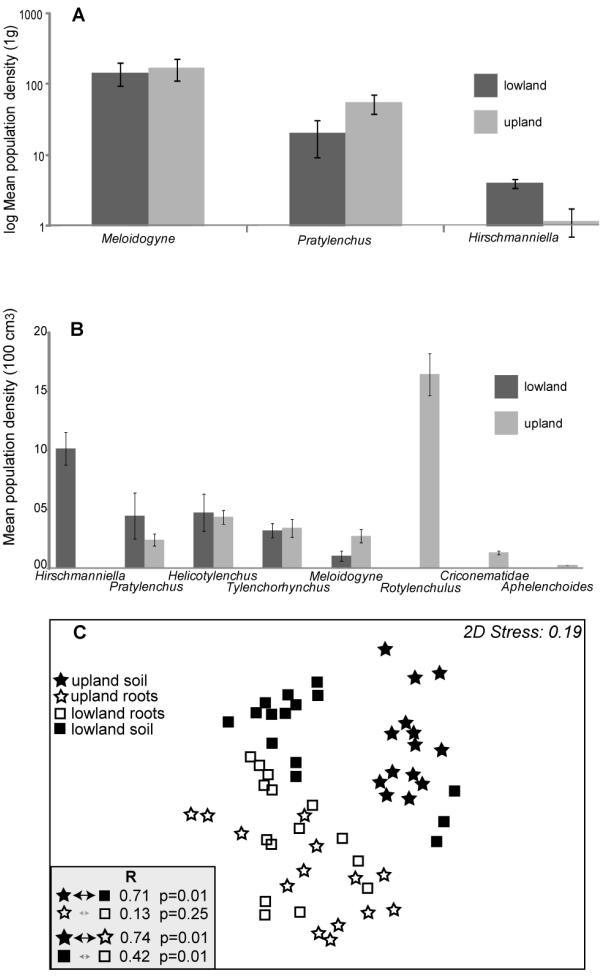

Fig. 2. Mean population densities in roots (A) and in soil (B) and an output of non-metric Multi-Dimensional Scaling (MDS) on square-root transformed genera densities (C) associated with lowland and upland rice ecosystems. The mean is the average of densities of each genus in the 15 fields in each rice ecosystems and standard error bars represent the SE.

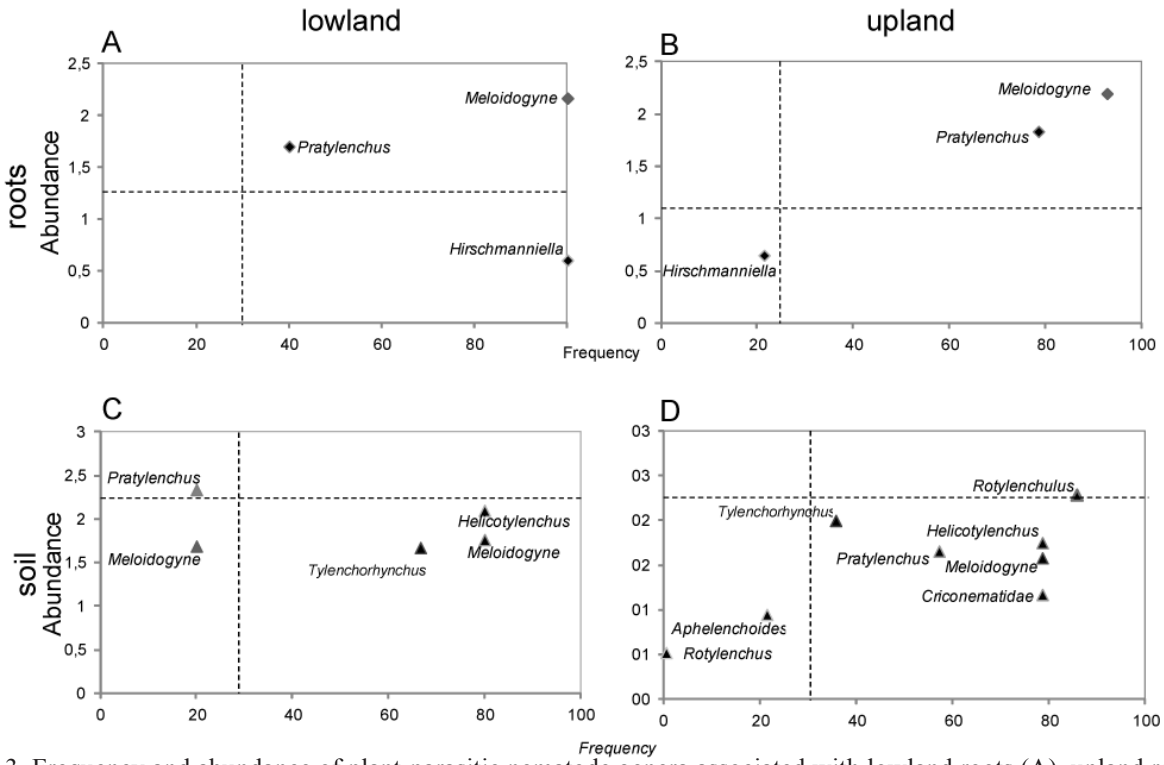

Fig. 3. Frequency and abundance of plant-parasitic nematode genera associated with lowland roots (A), upland roots (B), lowland soil (C) and upland soil (D). Dotted vertical lines represent nematode frequency limit (30%), and the dotted horizontal lines represent the abundance threshold (1.3 for roots & 2.3 for soil) according to Fortuner and Merny (1973).

agro-ecosystems (Fig. 3). *Meloidogyne* was the most prevalent genus in both ecosystems, detected in 100% and 93% of the roots sampled, with abundances of 147 and 157 (2.2, log value), for lowland (Fig. 3A) and upland (Fig. 3B). *Pratylenchus* had an abundance of 50 (1.7, log value) and 68 (1.8, log value) in lowland and upland roots, respectively, but they were only found in 40% of the roots of lowland rice. In contrast, *Hirschmanniella* was found in all lowland roots but its abundance (0.6) was low. In lowland and upland soil, all genera were below the abundance thresholds except *Pratylenchus* in lowland soil (Fig. 3C) and *Rotylenchulus* in upland soil (Fig. 3D). However, most of the genera were found frequently in both rice ecosystems. In lowland soil, *Hirschmanniella* and *Helicotylenchus* occurred most frequently and were present in about 80% of the sampled fields. *Tylenchorhynchus* was also relatively common, occurring in 67% of the samples. In upland soil, *Rotylenchulus* was the most commonly encountered genus, occurring in 86% of the fields while *Aphelenchoides* and *Rotylenchus* were the least prevalent and abundant.

Morphological and molecular characterization of Meloidogyne Goeldi*, 1892 populations*

No morphometric differences were found between lowland and upland populations except for some minor differences in the range of body length, a-value, and body diameter (Fig. 4 and Table 1).

These morphological observations were confirmed by molecular data (new GenBank accession numbers, see Table 3). The lowland and upland populations of *M. graminicola* were 100% similar, for both 18S (new GenBank accession: KF201168) and 28S, D2D3 expansion segments (new GenBank accession: KF201162) of the rRNA gene. The D2-D3 sequence was 100% similar with all *M. graminicola* sequences from GenBank, except for a GenBank sequence of a *M. graminicola* from rice in Taiwan (KF751067). The phylogenetic analysis showed that all *M. graminicola* populations, except for the aberrant KF751067, belonged to the same clade (PP: 88) (Fig. 6). Although our two *M. graminicola* 18 S sequences were identical, they were different from *M. graminicola* AF442196 (GenBank), which had base deletions on six positions. Based on these limited sequence data, the 18S sequence is not species specific for *M. graminicola*. Furthermore, M. graminicola was together with *M. oryzae* and *M. exigua* in the same weakly supported clade (PP: 56).

Morphological and molecular characterization of Hirschmanniella

Two species of *Hirschmanniella*, *H. oryzae* (Van Breda de Haan, 1902) Luc and Goodey, 1964 and

Table 1. Comparative morphometrics of *Meloidogyne graminicola* populations from lowland and upland rice ecosystem in Luzon, Philippines (PR) and other countries. Measurements are in micrometre and in the form: mean (range).

	Males			Second stage juvenile			
				Present study			
Characters	Present study	Mulk (1979) (Louisiana)	Tandingan (1997) (Luzon, PR)	Lowland	Upland	Mulk (1976)	Tandingan (1997) Phil. Pop'n
n	5	20	20	48	33	20	20
L	1313	1222	1333	436	443	441	372
	(1172-1444)	(1020-1428)	(1115-1705)	(389-476)	(412-479)	(415-484)	(345-435)
a	47.5	117.4	-	32.6	33.5	24.8	
	(41.1-50)	(72.8-215.0)	-	(28.9-35.8)	(28.1-38.6)	(22.3-27.3)	
c	125.4	-	-	6.5	6.5	6.2	
	(106.1-139.9)	-	-	(5.9-7.7)	(5.9-7.5)	(5.5-6.7)	
Max. body diameter	27.7	29.8	35.9	13.4	13.3	17.8	15
	(23.6-30.1)	(24.0-34.7)	(30-40.5)	(11.8-14.8)	(11.8-14.8)		(14-16.5)
Stylet length	17.6	16.8	17.8	10.3	10.3	11.4	9.6
	(16.5-18)	(16.2-17.4)	(17-19)	(9.4-11.2)	(9.4-11.2)	(11.2-12.3)	(8.5-11)
Stylet knob height	2.8	-	2.4	1.8	1.9	-	
	(2.4-3)	-	(2.0-3)	(1.2-1.8)	(1.5-2.4)	-	
Stylet knob width	3.9	-	4.1	2	2.1	-	1.8
	(3.5-4.1)	(3.5-4.0)	(4-4.5)	(1.8-2.4)	(1.8-2.4)	-	(1.5-2.5)
DGO	3.4	3.3	3.9	2.5	2.4	2.83	2.3
	(3-4.1)	(2.8-3.9)	(3-4.5)	(1.8-3.5)	(1.8-3.0)	(2.8-3.4)	(1.5-3.0)
Metacorpus diameter	10	-	-	6.9	7.3	-	
	(9.4-10.6)	-	-	(6.5-7.7)	(6.5-8.3)	-	
Excretory pore to	122.5	-	122	71.3	72.9	-	70
anterior end	(109-133)	-	(95-135)	(64.3-77.9)	(64.9-79.7)	-	(60.5-78.5)
Spicule length	27.4	28.1	28.2	-	-	-	
	(26.6-28.3)	(27.4-29.1)	(22.5-32)	-	-	-	
Gubernaculum	7.6	6.1	-	-	-	-	
	(7.1-7.7)	(5.6-6.7)	-	-	-	-	
Tail length	10.5	11.1	10	67.3	68.7	70.9	60
	(9.4-11.2)	(6.2-15.1)	(7-12)	(54.9-75.5)	(59.0-77.9)	(67.0-76.0)	(49-69.5)
Hyaline tail terminus	-	-	-	20.1	20.9	17.9	18
	-	-	-	(17.1-25.4)	(16.5-26.0)	(14.0-21.2)	(14-22)

Table 2. Comparative morphometrics of *Hirschmanniella oryzae* and *H. mucronata* from lowland rice ecosystem in Luzon, Philippines (PR), and other countries. Measurements are in micrometre and in the form: mean (range).

	H. oryzae					*H. mucronata*			
	Present study	Present study	Indonesia (topotypes) Sher, 1966	India Sher, 1966	Taiwan (Yuanli) Chen *et al*., 2006	Present study	India (topotypes) Sher, 1966	PR Sher, 1966	Taiwan (Dounan) Chen *et al*., 2006
Character	Male	Female	Female	Female	Female	Female	Female	Female	Female
n	3	21	17	20	16	1	5	15	12
L	1066	1110	1440	1300	1490	2136	1890	2000	2150
	(989-1166)	(780-1345)	(1140-1630)	(1030-1550)	(1.3-1.76)		(1720-2220)	(1670-2210)	(1940-2490)
a	53.6	53.7	60.0	57.0	64.5	69.6	55.6	62.0	67.3
	(52.1-56.5)	(37.8-61.5)	(50-67)	(53-65)	(52.4-74.8)		(50.8-62.2)	(54.70)	(55.4-82.4)
b									23.2
									(20.9-25.3)
b’	4.9	5.3	5.7	4.9	-		4.9	6.8	5.7
	(49.5-5)	(4.2-7.3)	(4.5-7.2)	(4.3-5.5)	-		(4.6-5.2)	(5.-7.6)	(5.5-6.1)
c	16	14.7	17.0	18.0	16.1	28.3	18.7	22.9	22.4
	(14.4-17.3)	(13.2-15.9)	(15-19)	(16-20)	(15.2-18.9)		(18-20)	()19-26	(19.5-26.8)
c’	4.8	5.3	4.6	4.5	5.8	3.5	4.4	4.0	4.0
	(4.4-4.5)	(4.6-6.0)	(4.3-5.5)	(3.9-5.0)	(4.3-6.5)		(4.1-5.1)	(3.0-4.8)	(3.5-5.1)
V	-	52.5	52.0	54.0	53.4	50.3	51	52.0	51.9
	-	(49.6-70.2)	(50-55)	(51-55)	(51-55.4)		(49-53)	(49-55)	(50.5-54.1)
Stylet length	16.5	17.3	17.0	18.0	17.2	25.4	27	26.0	25.0
	(15.9-17.1)	(14.8-18.9)	(16-19)	(17-20)	(15.0-18.3)		(26-29)	(24-28)	(24.0-26.3)
Excretory pore from	84.2	80.6	-	-	120.0	121.0	-	-	133.0
anterior end	(79.1-89.1)	(53.1-96.2)	-	-	(98-143)		-	-	(117-153)
Metacorpus valve from	61	61.4	-	-	-	86.7	-	-	
anterior end	(59-64.9)	(50.7-70.1)	-	-	-		-	-	
Maximum body diameter	19.9	20.7	-	-	16.0	30.7	-	-	24.0
	(18.9-20.7)	(17.1-22.4)	-	-	(15-18)		-	-	(22-31)
Spicules	28.5	-	-	-	-		-	-	
	(23.6-27.7)	-	-	-	-		-	-	
Guberbacium	7.7	-	-	-	-		-	-	
	(7.1-8.39)	-	-	-	-		-	-	
Tail	66.9	75.5	-	-	93.0	75.5	-	-	98.0
	(64.9-68.4)	(59-88.5)	-	-	(78-111)		-	-	(88-128)

Table 3. List of new GenBank accession numbers.

Species	Sequenced region	GenBank assession number
Meloidogyne graminicola Bataan upland	D2D3	KF201162
M. graminicola Bataan upland	18S	KF201168
Hirschmanniella oryzae Bataan lowland	D2D3	KF201169
H. oryzae Bulacan lowland	D2D3	KF201161
H. oryzae Batangas lowland	D2D3	KF201165
H. oryzae Batangas lowland	18S	KF366907
H. oryzae Bataan lowland	18S	KF366906
H. oryzae Bulacan lowland	ITS1-5.8-ITS2	KF201164
H. oryzae Batangas lowland	ITS1-5.8-ITS2	KF201163
H. mucronata Bataan lowland	D2D3	KF201167
H. mucronata Bataan lowland	ITS1-5.8-ITS2	KF201166

H. mucronata (Das, 1960) Luc and Goodey, 1963, were identified morphologically from the lowland rice ecosystem. The different lowland populations of *H. oryzae* were morphometrically similar and corresponded very well with previous *H. oryzae* descriptions (Sher, 1968; Loof, 1991; Chen *et al.,* 2006) (Table 2, Fig. 5). The nematodes in the present study showed distinct similarities with *H. oryzae* in stylet length (range from 16.5 – 17.3 µm); tail with one mucro and without a subterminal notch; c' value of 4.3 – 5.3 (Table 2). *Hirschmanniella mucronata* was identified by having a long stylet (25.4 µm), c value of 21.8, areolated lateral field near the tail region, and longer body (L = 2.1 mm), having a short mucro, which was sometimes absent in juvenile specimens.

Molecular analysis confirmed morphological identifications. Both *Hirschmanniella* species were placed with maximum support (PP 100) among previously sequenced species and for all available sequenced regions (Fig. 7). From the four *H. oryzae* D2-D3 sequences obtained, three populations (Pampanga, Bataan, and Bulacan) were completely identical (new GenBank accession KF201169) and had only one nucleotide difference from the Batangas (new GenBank accession KF201165) and IRRI populations as well as from the Vietnamese population. For the ITS1-5.8-ITS2 region, the *H. oryzae* sequences in this study were all identical (new GenBank accession KF201164) except the Batangas population that had 11 nucleotide differences. The other two GenBank sequences, *H. oryzae* DQ5095889 and EU722286, had 9 and 16 nucleotide differences from the present study, respectively. The 18S sequence of Bataan was identical to the IRRI population, but had 2 nucleotides different from the Batangas population.

For *H. mucronata*, the ITS1-5.8-ITS2 sequence of the Bataan population (new GenBank accession KF201165) only differed for one nucleotide with the *H. mucronata* DQ309589 from GenBank. Our study revealed the first D2D3 sequences for *H. mucronata* (new GenBank accession KF201167), and the populations from Bataan and Bulacan were identical. The phylogenetic position of *H. mucronata* was similar for both the D2D3 and ITS1-5.8-ITS2 based trees, namely sister to the clade comprising *H. loofi* and *H. kwazuna*. An 18S sequence was not successfully obtained for *H. mucronata*.

DISCUSSION

Prevalence of plant-parasitic nematodes

With nine genera detected from upland and only five genera from lowland, present observations agree with previous reports (Prot and Rahman, 1994) that upland rice ecosystems have higher nematode diversity than lowland rice ecosystems. Greater diversity in an upland rice ecosystem can be attributed to soil type and different cropping practices. In this study, genera found in each agro-ecosystem were generally similar to those observed from earlier surveys by Pokharel (1991), Villanueva *et al*. (1992) and Prot *et al.* (1994) with the exception of *Criconemoides* and *Scutellonema* in lowland and *Xiphinema*, *Hemicriconemoides*, *Hemicycliophora*, and *Hoplolaimus* in upland, which were not found in the present study. It is important to note, however, that the previous studies collectively surveyed the whole country while our study only surveyed the Luzon area.

The frequency and abundance of the nematode genera particularly *Meloidogyne*, *Pratylenchus*, and *Hirschmanniella*, differed from former studies (Pokharel, 1991; Villanueva *et al*., 1992; Prot *et al*., 1994). This difference was notable in both rice ecosystems where the frequency and abundance of *M. graminicola* was considerably higher in this study for both upland and lowland (93% and 100% vs. 35% and 25% frequency in upland and lowland, respectively). The noticeable increase in the prevalence and densities of *M. graminicola* in both rice ecosystems could be due to changes in agricultural practices, particularly in water regimes, and with the intensification of rice cropping. Farmers are shifting from permanent flooding to intermittent irrigation due to the increasing

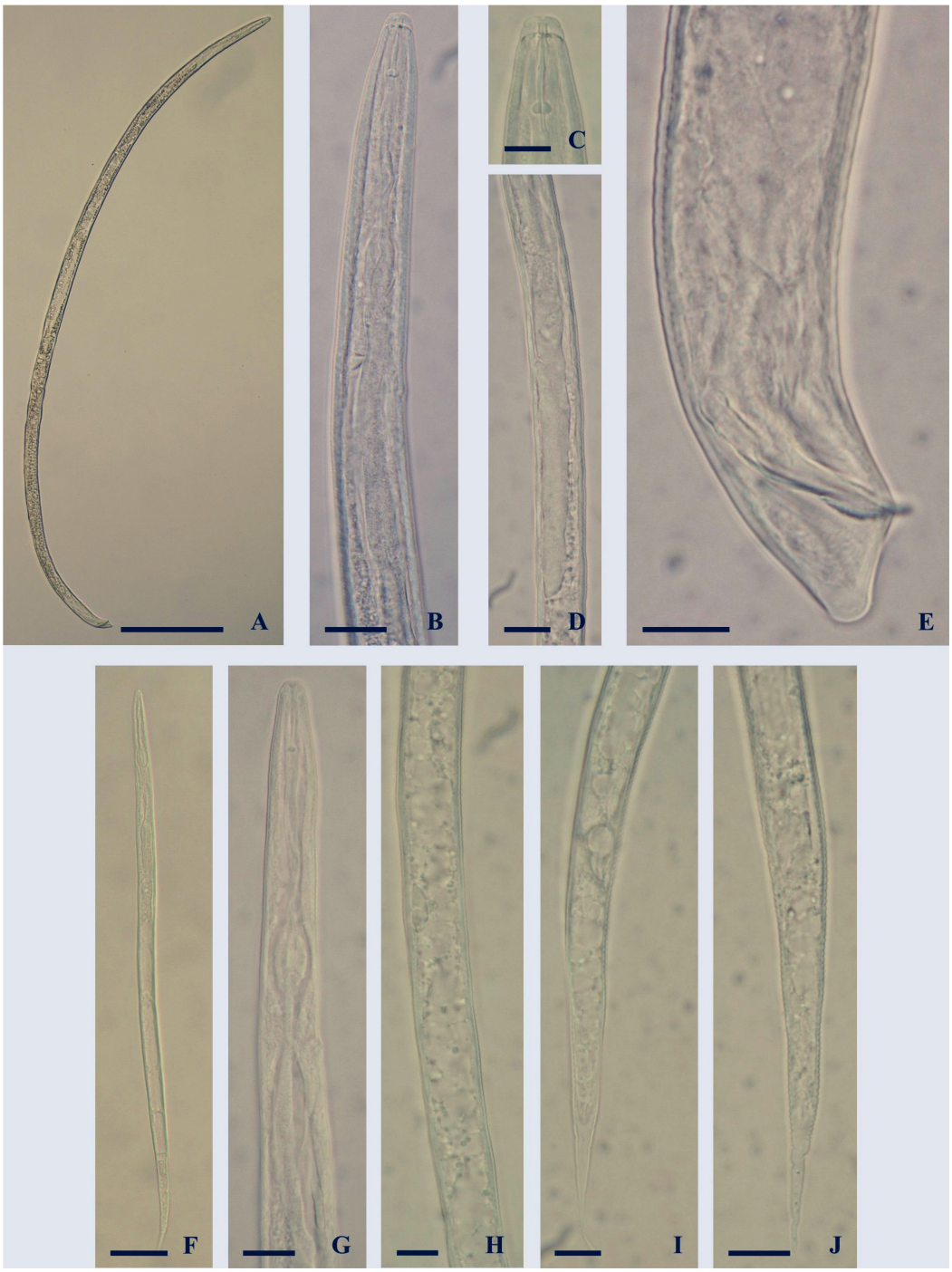

Fig. 4. *Meloidogyne graminicola*. LM of male and second stage juvenile (J2). A: Entire male; B: Anterior region of male; C: Head end of male; D: Pharyngeal gland of male; E: Tail region of male; F: Entire J2; G: Anterior region of J2; H: Genital primordium; I,J: Tail regions of J2. (Scale bars: C-E, G-J = 10 µm; B = 20 µm; A = 150 µm)

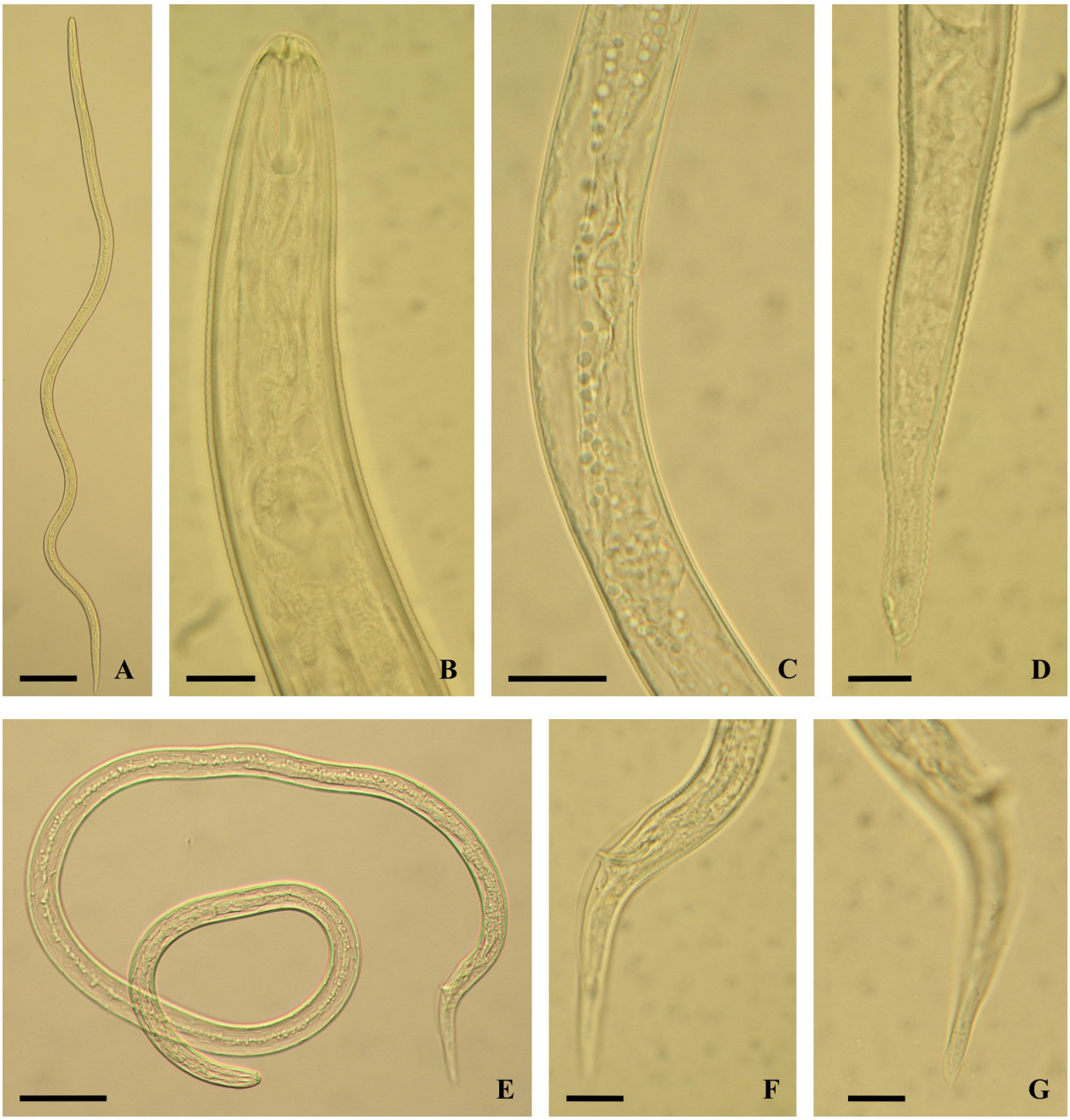

Fig. 5. *Hirschmanniella oryzae*. LM of female and male. A: Entire female; B: anterior region of female; C: Vulval region and spermatheca; D: Female tail; E: Entire male; F: Tail region of male; G: Tail tip of male. (Scale bars: B,D = 10 µm; C,F,G = 20 µm; E=50 µm; A=100 µm).

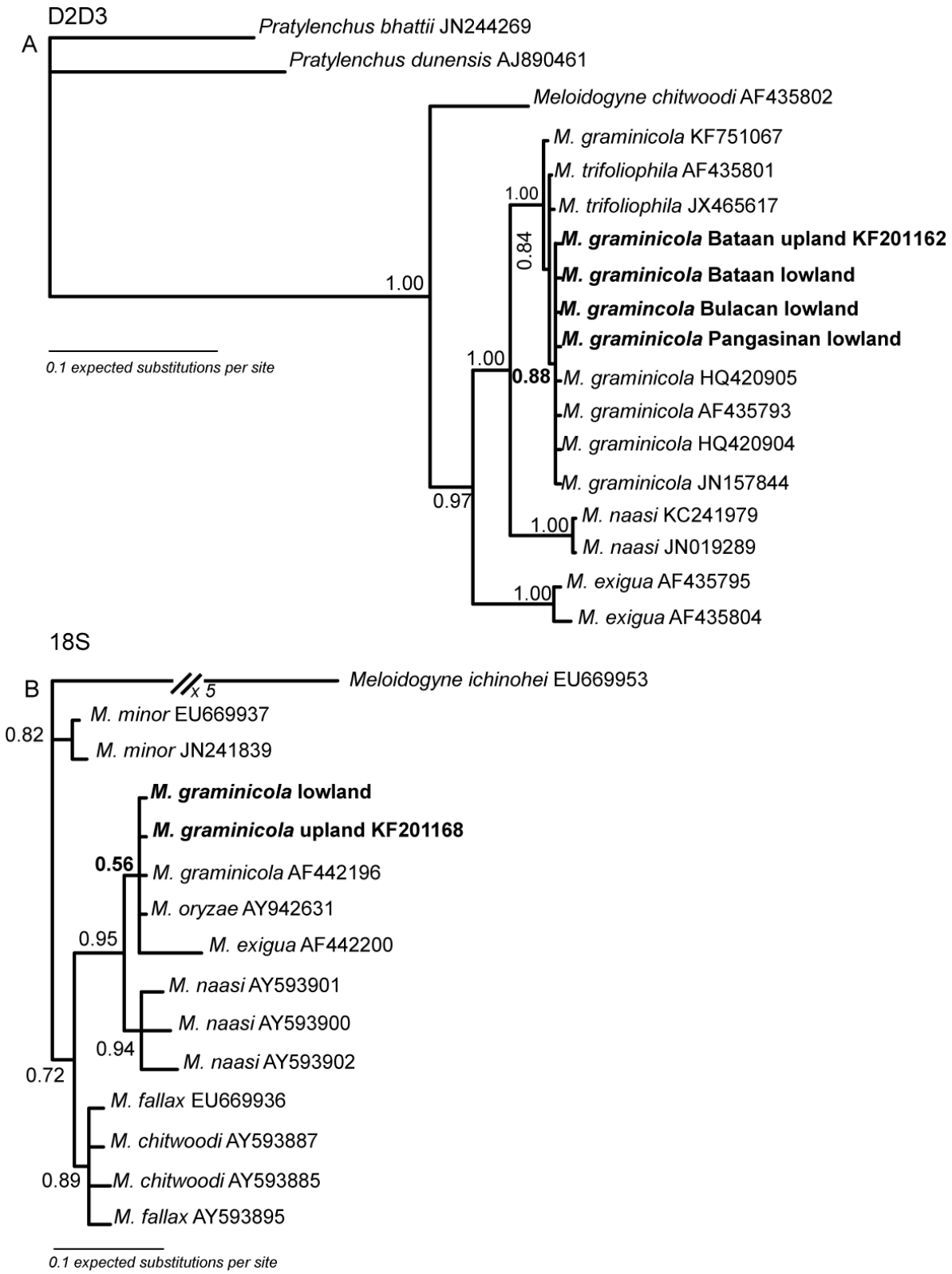

Fig. 6. Phylogenetic tree of *Meloidogyne* species close to *Meloidogyne graminicola* based on D2D3 LSU rDNA data (A) and 18S rDNA data (B) obtained from Bayesian inference (BI) performed with MrBayes v3.1.2 using a GTR + I + G model. Branch support is indicated with PP: high (≥95% PP)/ medium (>95% to ≥ 50% PP)/ low (<50% PP). Out group: *Pratylenchus bhattii* JN244269 and *P. dunensis* AJ890461 for D2D3 analysis, and *Meloidogyne ichinohei* EU669953 for the 18S analysis.

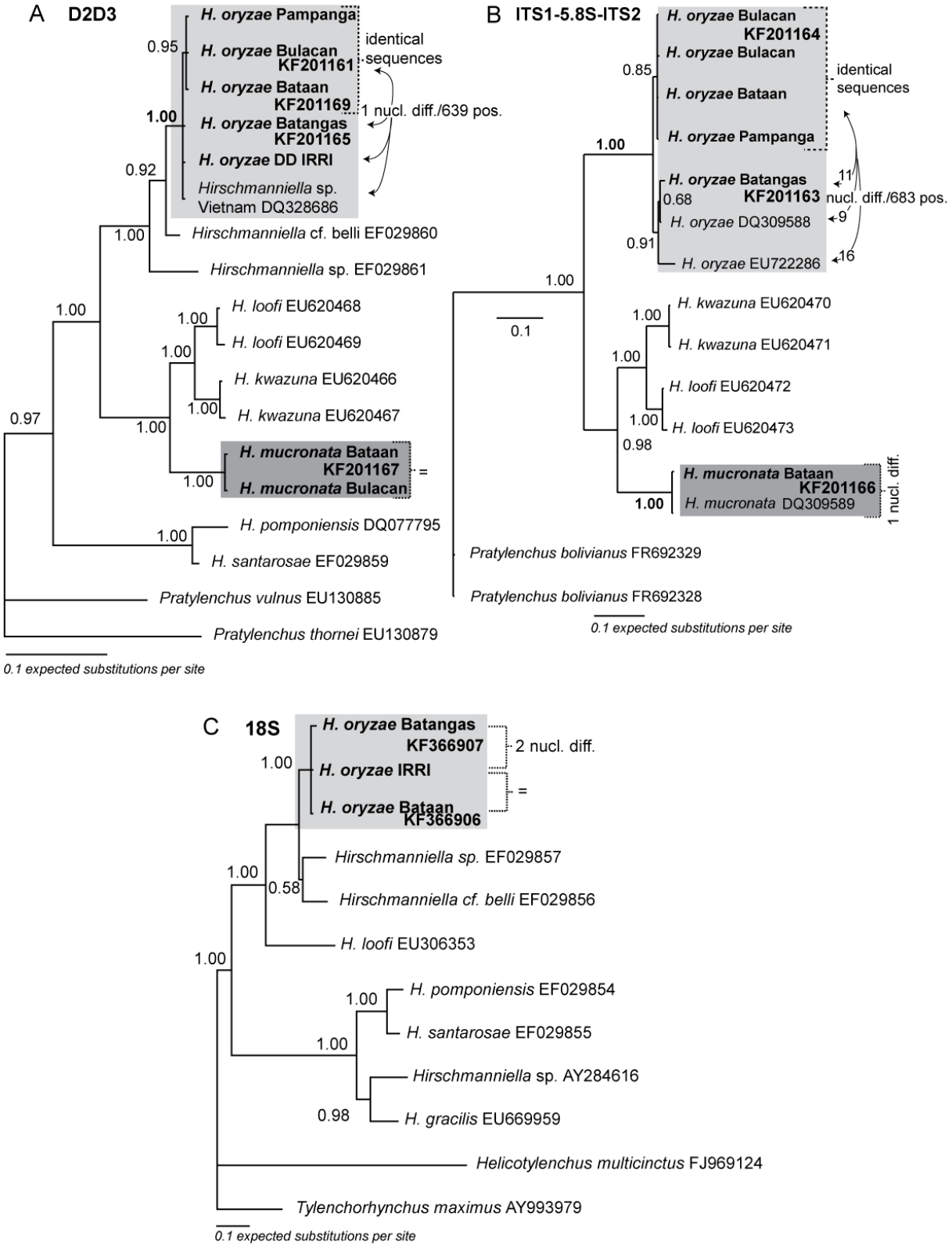

Fig. 7. Phylogenetic tree of *Hirschmanniella* species based on D2D3 LSU rDNA data (A), ITS1-5.8-ITS2 rDNA data (B) and 18S rDNA data (C) obtained from Bayesian inference (BI) performed with MrBayes v3.1.2 using a GTR + I + G model. Branch support is indicated with PP: high (≥95% PP)/ medium (>95% to ≥ 50% PP)/ low (<50% PP). Out group: *Pratylenchus vulvus* EU130885 and *Pratylenchus thornei* EU130879 for D2D3 analysis; *Pratylenchus bolivianus* FR692329 and *Belonolaimus longicaudatus* DQ672372 for ITS1-5.8-ITS2 analysis; and *Helicotylenchus multicinctus* FJ969124 and *Tylenchorhynchus maximus* AY993979 for 18S region.

scarcity and cost of water. Such change could possibly favour the population dynamics of *M. graminicola*. Although *M. graminicola* is generally known to be well adapted to flooded conditions (Bridge *et al*., 2005), Netscher and Erlan (1993) reported that, under experimental conditions, roots of rice plants grown under continuous flooding were free of *M. graminicola*, and Win *et al.* (2013) observed that *M. graminicola* population levels did not build up during the wet monsoon season. Other reports also indicated that *M. graminicola* has become one of the constraints in rice production associated with the intensification of rice cropping and the increasing scarcity of water (Bridge *et al*., 2005; Soriano and Reversat, 2003). In Myanmar, higher frequency of occurrence and population density of *M. graminicola* was observed in lowland compared with upland rice ecosystems (Win and de Waele, 2011).

In the study by Villanueva *et al.* (1992), *Pratylenchus* was identified as the most prevalent (100%) genus in upland rice, and *P. zeae* as the most prevalent species. Although *Pratylenchus* was not the most prevalent species in this survey, it was found in 79% of samples collected from the upland ecosystems. Only in the Pangasinan (L5) region was this genus found at higher population density than *Meloidogyne.*

In the lowland rice, *Hirschmanniella* was prevalent in all irrigated fields although at densities lower than previously reported (Prot *et al*., 1994). *Hirschmanniella oryzae* was reported to be the most common plant-parasitic nematode on irrigated rice especially in areas with a long history of irrigated rice cultivation where the plants were constantly flooded (Bridge *et al*., 2005). In previous studies, *H. oryzae* was recorded in 94% of the irrigated rice fields in the Philippines (Prot *et al*., 1994), in 99% of the rice fields in the Mekong Delta in Vietnam (Cuc and Prot, 1992), and in 90% of rainfed lowland (monsoon) fields in Myanmar (Maung *et al.*, 2010).

The same nematode genera (*Meloidogyne*, *Pratylenchus*, and *Hirschmanniella*) were observed in both lowland and upland roots implying that they were root parasites of rice as was indicated in previous reports (Bridge *et al*., 2005). Other potentially important nematode taxa (*Tylenchorhynchus*, *Helicotylenchus, Criconematidae*, *Rotylenchulus*, *Rotylenchus*) were only found in soil samples, usually at relatively low densities, and their association with rice has not been established. These taxa were not included in the list of nematodes known or suspected to cause yield loss in rice (Bridge *et al*., 2005).

Morphological and molecular characterization of Meloidogyne graminicola *and* Hirschmanniella

The morphological and morphometric data of the second-stage juveniles (J2) and one male from the two ecosystems largely agree with *M. graminicola* descriptions (Golden and Birchfield, 1965; Esser *et al*., 1976; Jepson, 1987; Pokharel *et al*., 2007). The truncate male head morphology along with the stylet length (16.5-18 µm), DGO (3-4.1 µm), and stylet knob shape (smooth, rounded, backwardly sloping) observed in this study were all similar to *M. graminicola* as described by Jepson (1987). The tail shape, tail length and hyaline tail length of J2 also matched with previously reported *M. graminicola*. Results of our morphological and molecular analyses are congruent. All analyzed *Meloidogyne* populations were identified as *M. graminicola*, the most important root-knot pest of rice. The important diagnostic characters of males (head shape, stylet length, stylet knob shape, and DGO) and second stage juveniles (tail shape, tail length and hyaline tail length) by Jepson (1983, 1987) and Esser *et al*. (1976) were found useful in species identification. Nevertheless, only the combined use of these diagnostic characters was species informative because morphometric and morphological data overlapped among closely related *Meloidogyne* species; this is in agreement with Pokharel *et al*. (2007). Corroboration of molecular analysis/data is then important. The obtained 18S SSU and 28S LSU D2D3 sequences of lowland and upland *M. graminicola* were 100% similar. The D2D3 sequences of the Luzon specimens were also identical with those of GenBank, while our obtained identical 18S sequences were different from *M. graminicola* AF442196 from GenBank. Since all differences were ascribed to indels, analysis of more *M. graminicola* populations needs to be done to check if the 18S region is either not species specific or if differences can be attributed to sequence errors.

Based on morphological identification, two *Hirschmanniella* species (*H. oryzae* and *H. mucronata*) were identified in the lowland rice ecosystem. The morphological characters and measurements of *H. oryzae* slightly extend the lower limits of other *H. oryzae* populations described from other countries, particularly with respect to body length; a and c values, distance from SE-pore to anterior end; and tail length. While for *H. mucronata*, the morphometric data are within the range of the original descriptions (Sher, 1968), and with those of other countries except for the lower tail length (Loof, 1991; Chen *et al.*, 2006). Current analyses on D2D3 and ITS1-5.8-ITS2 region from the different lowland populations of *Hirschmanniella* confirms the presence of both *H. oryzae* and *H. mucronata*, in Luzon, Philippines, as revealed by the phylogenetic analysis and the direct sequence comparisons. Based on the sequences of ribosomal DNA (including complete 5.8S gene, partial 18S and 28S gene, and complete internal transcribed spacer ITS-1, ITS-2), Chen *et al.* (2006) also reported *H. oryzae* and *H. mucronata* from several rice fields in Taiwan. Data in this study support the usefulness of 18S, D2D3 and ITS rRNA to identify species within the genus *Hirschmanniella*. The D2D3, sequences of *H. mucronata* and *H. oryzae*, as well as the 18S SSU

of *H. oryzae*, are new sequences obtained from these species.

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