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Molecular characterization and distribution of the needle nematode *Longidorus laevicapitatus* Williams, 1959 (Nematoda: Longidoridae) in Costa Rica

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Abstract Correct identification of *Longidorus* species in Costa Rica is essential to establish appropriate control strategies for preventing the spread of these nematodes. Nematode surveys conducted in the rainy seasons from 2013 to 2015 in areas arbitrarily chosen and widely distributed in the whole territory of Costa Rica, resulted in an overall prevalence of *Longidorus* spp. infesting soils both cultivated, ornamental and wild plants of 40.26 %. Integrative morphometric and molecular data for *Longidorus* populations were obtained using D2-D3 expansion segments of 28S rRNA, ITS1-rRNA, and the partial 18S-rRNA, identifying a solely species identified as *Longidorus laevicapitatus*. Morphology and morphometrical traits analysis of these populations of *L. laevicapitatus* were in agreement with those of the original and posterior descriptions of the species, except for some minor differences, which may be a result of intraspecific variability. The phylogenetic relationships of this species with other representatives of *Longidorus* spp. using D2-D3 expansion segments and the partial 18S indicated that *L. laevicapitatus* clustered clearly separately in a basal position in both phylogenetic trees.

Keywords Bayesian inference · D2-D3 · ITS1 · 18S · Longidorids · Phylogeny · rDNA · Taxonomy

Needle nematodes of the genus *Longidorus* Micoletzky 1922 includes a wide and diverse group of migratory ectoparasitic nematode species, comprising a number of long to very long body specimens (2–12 mm) with moderate to very long odontostyle (40–200 μm) (Gutiérrez-Gutiérrez et al. 2013; Archidona-Yuste et al. 2016). They are polyphagous species of many plants including various agricultural crops, being able to cause damage both by direct feeding on root cells as well as by transmitting nepoviruses (Taylor and Brown 1997, Decraemer and Robbins 2007). The genus *Longidorus* is considered cosmopolitan, being Europe where it has been found most frequently, followed by India, North America, South Africa and China (Decraemer and Robbins 2007). In South America, only two species have been reported so far, viz. *L. edmundsi* Hunt & Siddiqi 1977 and *L. laevicapitatus* Williams 1959 (Coomans 1996; Doucet et al. 1998; Crozzoli et al. 2000; Decraemer and Coomans 2007). In Costa Rica, *L. laevicapitatus* was the only identified species by Tarjan (1967) in citrus and sugar-cane, as well as several reports of *Longidorus* sp. (Lainer Gonzalez 1978; Lopez and Azofeifa 1985). However, no information exists on morphology and morphometrical traits of *L. laevicapitatus* or other *Longidorus* spp. in Costa Rica, and no molecular data on these species/

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populations were available. Therefore, correct identification of *Longidorus* species in Costa Rica is essential to establish appropriate control strategies for preventing the spread of these nematodes. Molecular approaches using ribosomal DNA (rDNA) sequences including ITS region, the D2 - D3 expansion segments of 28S and 18S rRNA region, have been shown to be useful diagnostic markers in disclosing phylogenetic relationships within Longidoridae, especially in the cases where morphological characters may lead to ambiguous interpretation (De Luca et al. 2004; He et al. 2005; Gutiérrez-Gutiérrez et al. 2013; Subbotin et al. 2014; Archidona-Yuste et al. 2016).

Therefore, the objectives of the present study were: (i) to provide an accurate identification of *Longidorus* species detected in Costa Rica by an integrative approach of morphological and molecular characterization by using the D2-D3 expansion segments of 28S rRNA, 18S rRNA and ITS1 rRNA gene sequences; and (ii) to explore the phylogenetic relationships of detected needle nematodes within *Longidorus* spp.

Nematode surveys were conducted in the rainy seasons from 2013 to 2015 in cultivated, ornamental and wild plants in areas widely distributed in the whole territory of Costa Rica (Table 1). Each soil sample was a composite of 20–25 soil cores arbitrarily chosen from the same field to a depth of 25–40 cm with an Oakfield tube of 2.5-cm diameter. Samples were placed in labelled plastic bags, sealed and brought back to the nematology laboratory where they were stored at 4 °C until processed for nematode extraction. Nematodes were extracted from 500 cm^3 of soil by centrifugal flotation (Coolen 1979), and a modification of Cobb's decanting and sieving method (Flegg 1967). Soil samples were homogenated, washed, and then sieved through a 250-μm pore sieve over a 5-μm pore sieve. Nematodes and root debris retained on the 5-μm pore sieve were separated by centrifuging at 1100×g for 5 min in a magnesium sulphate solution of 1.16 specific gravity (Coolen 1979). Females were processed and mounted in glycerine for diagnostic studies. Specimens for study using light microscopy were killed by gentle heat, fixed in a solution of 4 % formaldehyde +2 % glycerol and processed to pure glycerine using Seinhorst's method (1966). Measurements were carried out using a drawing tube attached to a light microscope and expressed in micrometers (μm). All other abbreviations used are as defined in Jairajpuri and Ahmad (1992).

DNA extraction and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rRNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (De Ley et al. 1999). The ITS1 region was amplified using forward primer 18S (5'TTGATTACG TCCCTGCCCTTT-3') (Vrain et al. 1992) and reverse primer rDNA1 (5'-ACGAGCCGAGTGATCCACCG-3') (Cherry et al. 1997). Partial 18S-rRNA was amplified using primers 988F (5'-CTCAAAGATTAA GCCATGC-3'), 1912R (5'TTTACGGTCAGAACT AGGG-3'), 1813F (5'- CTGCGTGAGAGGTGAAAT-3') and 2646R (5'-GCTACCTTGTTACGACTTTT-3') (Holterman et al. 2006). PCR products were purified, quantified and used for direct sequencing as described by Tzortzakakis et al. (2014). The newly obtained sequences were submitted to the GenBank database under accession numbers KX136865-KX136875 (Table 1).

D2-D3 and 18S rDNA and sequences of different *Longidorus* and *Paralongidorus* spp. from GenBank were used for phylogenetic reconstruction. In addition, sequences from *Xiphinema index* (HM921404), *Xiphinema rivesi* (HM921344), *Xiphidorus minor* (AY604181) and *Tylencholaimus mirabilis* (EF207253) were used as outgroup taxa. The newly obtained and published sequences for each gene were aligned using MAFFT (Katoh and Standley 2013) with default parameters. Sequence alignments were visualized using BioEdit (Hall 1999) and edited by Gblocks v0.91b (Castresana 2000) in Castresana Lab server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (Minimum number of sequences for a conserved or a flanking position: 50 % of the number of sequences +1; maximum number of contiguous nonconserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Phylogenetic analyses of the sequence data sets were performed as described by Tzortzakakis et al. (2014). BI analysis for D2-D3 and 18S rDNA regions under the GTR + I + G model, were initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 2 × 10^6 generations. The MCMC were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50 % majority rule

Table 1 *Longidorus laevicapitatus* Williams 1959 sampled and sequenced in this study from Costa Rica

Host-plant	Locality, province	Sample code	GenBank accession		
			D2-D3	ITS1	18S
1. Sugar-cane	La Virgen de Sarapiquí, Heredia	ACC01	KX136865	KX136869	KX136873
2. Grasses	Carit. Santiago de Puriscal, San José	ACC05	KX136866	KX136870	KX136874
3. Beans	Pueblo Nuevo de Pilas, Buenos Aires, Puntarenas	ACC55	KX136867	KX136871	KX136875
4. Grasses	San Rafael de Paraíso, Sixaola, Limón	ACC70	KX136868	KX136872	-
5. Grapevine	Chirraca. San Ignacio, Acosta, San José	ACC04	*	-	-
6. Grasses	Carit. Santiago de Puriscal, San José	ACC05	*	-	-
7. Sugar-cane	San Antonio de Escazú, San José	ACC06	*	-	-
8. Pineapple	Chilamate, Sarapiquí, Heredia	ACC07	*	-	-
9. Banana	Pueblo Nuevo de Guácimo, Pococí, Limón	ACC09	*	-	-
10. Sugar-cane	San Juan Norte de Poás, Alajuela	ACC12	*	-	-
11. Corn	Pacayas, Oreamuno, Cartago	ACC14	*	-	-
12. Avocado	San Jerónimo de Esparza, Puntarenas	ACC17	*	-	-
13. Pineapple	Pococí, Limón	ACC18	*	-	-
14. Grasses	San Vicente, Ciudad Quesada, Alajuela	ACC20	*	-	-
15. Sugar-cane	Cañas, Guanacaste	ACC24	*	-	-
16. Carrot	Pacayas, Oreamuno, Cartago	ACC25	*	-	-
17. Mirlitons	Río Regado, Ujarrás, Paraíso. Cartago	ACC29	*	-	-
18. Avocado	San José de la Montaña, Barva, Heredia	ACC30	*	-	-
19. Grasses	Grecia, Alajuela	ACC34	*	-	-
20. African oil palm	Paso Canoas, Corredores, Puntarenas	ACC40	*	-	-
21. Grasses	Sucre, Ciudad Quesada, Alajuela	ACC43	*	-	-
22. Robust star-grass	Sucre, Ciudad Quesada, Alajuela	ACC47	*	-	-
23. Forest	Jaris de Mora. San José	ACC48	*	-	-
24. Forest	Estación Biológica Las Cruces, San Vito, Coto Brus, Puntarenas	ACC50	*	-	-
25. Forest	Santa Elena, MonteVerde, Puntarenas	ACC51	*	-	-
26. Sugar-cane	Pacayitas, La Suiza de Turrialba, Cartago	ACC61	*	-	-
27. Grasses	Pacayitas, La Suiza de Turrialba, Cartago	ACC62	*	-	-
28. Stone-fruits	Pacayitas, La Suiza de Turrialba, Cartago	ACC66	*	-	-
29. Fallow	Rancho Redondo, Goicoechea, San José	ACC68	*	-	-
30. Mexican coriander	La Palma, Paso Canoas, Corredores. Puntarenas	ACC72	*	-	-
31. Sugar-cane	Río Cuarto de Grecia, Alajuela	ACC76	*	-	-

(-) Not obtained

(*) Sequenced population but not deposited in GenBank database because of their similarity (see discussion section)

consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 100 replicates and fast step search.

Overall prevalence of needle nematodes of the genus *Longidorus* infesting soils from a wide range of cultivated, ornamental and wild plants in Costa Rica was of 40.26 %, being this nematode genus in 31 out of 77 sampling sites widely distributed all over the country (Table 1, Fig. 1). Needle nematode population density ranged from 1 to 6 specimens/500 cm^3 of soil. Preliminary morphological observations indicated that all populations of *Longidorus* spp. found appeared to be identified as the same morphotype. Detailed observations using light microscopy and several diagnostic

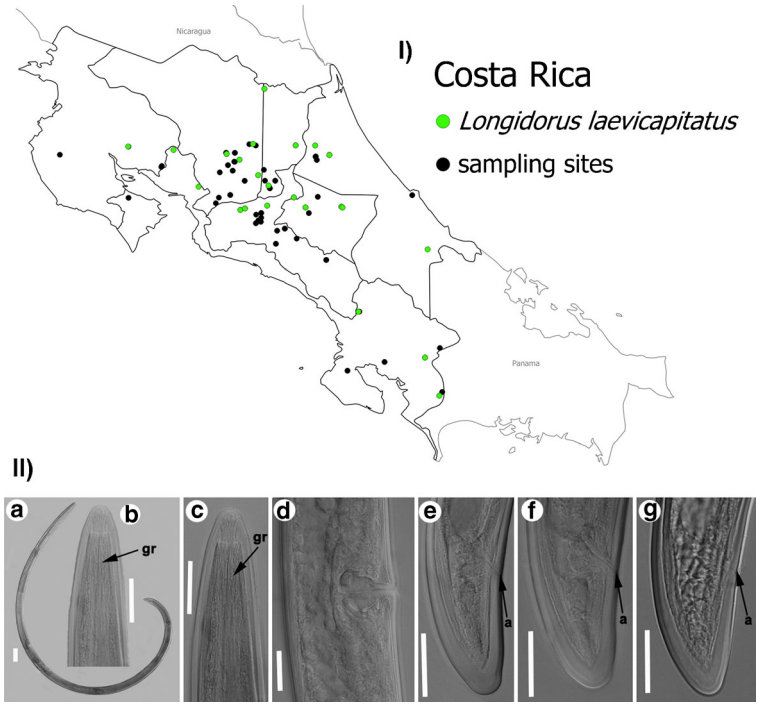

Fig. 1 *I* Map of Costa Rica showing sampling sites and detection of *Longidorus laevicapitatus* Williams 1959. *II*) Light micrographs of *Longidorus laevicapitatus* Williams 1959. **a** Female whole body. **b**, **c** Female anterior regions. **d** Detail of vagina. **e**–**g** Female tail regions. Abbreviations: a = anus; gr = guiding ring. (Scale bars: **a** = 100 μm; **b**–**g** = 20 μm)

morphological characters as well as morphometric studies indicated that the parthenogenetic populations resembled fairly well with the original description of *Longidorus laevicapitatus* (Williams 1959).

The *L. laevicapitatus* populations found in Costa Rica were characterized by a moderately short body length assuming a spiral body habitus, lip region continuous with the body contour, amphidial fovea pouch like (not bilobed), and tail elongate-conoid, ending in a rounded terminus (Fig. 1). Morphology and morphometrical traits agree fairly well with the original and other descriptions of the species (Table 2), with minor morphometric differences, which may be a result of the small number of specimens studied in the original description or intraspecific variability (Williams 1959; Merny 1966; Hooper 1985; Azpilicueta and Chaves 2013).

Longidorus laevicapitatus is widespread in tropical/subtropical regions on various crops, and has been reported several times from Latin America, from the rhizosphere of banana, citrus, and grasses in Dominica and Santa Lucia (Hunt 1977); sugar-cane in Martinique, and sugar-cane, citrus, and sweet potato in Guadeloupe (Scotto la Massese 1969); from sugar-cane in Antilles (Dalmasso 1967); and from a mussel bed in Argentina (Azpilicueta and Chaves 2013). Also has been reported in Africa, viz. Congo (Merny 1966), Ethiopia (Meressa et al. 2015), Kenya (Kaiser et al. 1978), South Africa (Jacobs and Heyns 1982), Sao Tomé (Lamberti et al. 1987), and Egypt (Lamberti et al. 1996).

The amplification of D2-D3 expansion segments of 28S rRNA, ITS1, and the partial 18S regions yielded a single fragment of approximately 900 bp,1100 bp, and 1800 bp, respectively, based on gel electrophoresis. D2-D3 expansion segments of 28S rRNA of *L. laevicapitatus* (KX136865-KX136868), ITS1 (KX136869-KX136872) and 18S rRNA (KX136873-KX136875) were obtained for the first time in this study. D2-D3 sequences from *L. laevicapitatus* showed similarity values of 83 % with several *Longidorus* and *Paralongidorus* spp. such as, *Longidorus vineacola* (KT308865), *Longidorus wicuolea* (KT308865), *Longidorus pseudoelongatus* (KJ802873), and *Paralongidorus maximus* (AF480083). The new ITS1 sequences obtained from *L. laevicapitatus* (KX136869-KX136872) showed scare similarity with *Longidorus* spp. deposited in GenBank, and no accession with coverage values above 50 % were found. Finally, 18S rRNA sequences (KX136873-KX136875) matched closely with 98 % similarity with *Paralongidorus bikanerensis* (JN032586), *Longidorus ferrisi* (AY283163) and *P. maximus* (AJ875152). Intraspecific

Table 2 Morphometrics of *Longidorus laevicapitatus* Williams 1959 from the rhizosphere of several crops and wild plants from Costa Rica. All measurements in μm and in the format: mean ± s.d. (range)

Locality (sample code) Character	La Virgen de Sarapiquí, Heredia (ACC01)	Carit. Santiago de Puriscal, San José (ACC05)	Chilamate, Sarapiquí, Heredia (ACC07)	Pacayas, Oreamuno, Cartago (ACC14)	San Vicente, Ciudad Quesada, Alajuela (ACC20)	Paso Canoas, Corredores, Puntarenas (ACC40)	San Rafael de Paraíso, Sixaola, Talamanca (ACC70)
n	10	3	4	5	2	3	2
L	22310 ± 258 (1938–2690)	2534 ± 363 (2296–2952)	22401 ± 213 (2151–2639)	2346 ± 144 (2108–2499)	2290 ± 147 (2186–2393)	2107 ± 222 (1874–2316)	2398 ± 18 (2386–2411)
a	53.8 ± 4.5 (48.8–60.5)	44.4 ± 1.9 (43.1–46.6)	52.7 ± 8.4 (44.1–63.9)	48.2 ± 2.2 (45.0–50.3)	54.1 ± 3.4 (51.7–56.5)	46.3 ± 9.9 (36.7–56.4)	43.4 ± 3.2 (41.2–45.7)
b	8.2 ± 1.0 (6.7–9.7)	9.9 ± 0.5 (9.3–10.2)	8.8 ± 1.3 (7.4–10.1)	8.3 ± 0.5 (7.8–8.9)	10.4 ± 1.4 (9.4–11.3)	8.6 ± 0.1 (8.5–8.7)	12.4 ± 0.3 (12.2–12.6)
c	60.7 ± 7.6 (49.7–78.5)	62.9 ± 4.7 (59.9–68.3)	66.6 ± 10.1 (57.0–77.7)	68.7 ± 5.4 (62.5–76.9)	66.7 ± 7.7 (61.3–72.1)	56.1 ± 7.0 (50.6–64.0)	54.7 ± 0.1 (54.6–54.7)
c’	1.3 ± 0.1 (1.2–1.6)	1.3 ± 0.0 (1.3–1.3)	1.3 ± 0.1 (1.2–1.4)	1.2 ± 0.1 (1.0–1.3)	1.2 ± 0.1 (1.1–1.2)	1.2 ± 0.1 (1.1–1.4)	1.4 ± 0.2 (1.2–1.5)
V	52.1 ± 2.6 (46.0–55.0)	46.4 ± 1.1 (45.4–47.5)	45.6 ± 1.5 (44.0–47.0)	45.8 ± 1.5 (44.0–48.0)	46.7 ± 0.8 (46.0–47.0)	47.4 ± 2.4 (45.0–50.0)	45.5 ± 0.3 (45.0–46.0)
Odontostyle length	60.5 ± 2.5 (57.0–64.5)	62.1 ± 3.2 (59.0–65.0)	59.7 ± 1.1 (59.0–61.0)	62.4 ± 1.7 (60.0–65.0)	70.3 ± 13.9 (60.5–80.0)	63.5 ± 1.8 (62.0–65.5)	59.3 ± 0.4 (59.0–59.5)
Odontophore length	50.8 ± 6.2 (46.0–64.0)	42.0 ± 2.6 (40.0–45.0)	41.6 ± 2.5 (39.0–45.0)	43.9 ± 1.1 (42.5–45.5)	49.5 ± 7.8 (44.0–55.0)	50.3 ± 7.6 (42.0–57.5)	50.2 ± 13.9 (40.0–60.0)
Lip region width	8.7 ± 0.7 (7.0–10.0)	9.8 ± 1.1 (9.0–11.0)	10.0 ± 0.6 (10.0–10.5)	10.1 ± 0.6 (9.2–10.8)	10.1 ± 0.8 (9.5–10.5)	9.1 ± 0.5 (8.5–9.5)	9.0 ± 1.4 (8.0–10.0)
Oral aperture-guiding ring	23.6 ± 2.7 (20.0–30.0)	24.0 ± 1.1 (23.0–25.0)	26.4 ± 6.7 (22.0–36.5)	24.1 ± 0.6 (23.5–25.0)	24.9 ± 2.0 (24.0–26.0)	26.5 ± 9.3 (19.5–37.0)	33.0 ± 5.7 (29.0–37.0)
Tail length	38.4 ± 5.0 (32.5–46.5)	40.2 ± 2.7 (38.0–43.0)	36.3 ± 2.5 (34.0–39.0)	34.2 ± 2.5 (31.0–37.4)	34.5 ± 1.8 (33.0–36.0)	38.3 ± 8.4 (29.0–46.0)	44.3 ± 0.4 (44.0–44.5)
J	8.3 ± 1.3 (7.0–10.0)	8.2 ± 0.6 (8.0–9.0)	8.1 ± 0.4 (8.0–8.5)	9.3 ± 0.5 (8.5–10.0)	7.8 ± 0.4 (7.5–8.0)	7.6 ± 1.8 (5.5–9.0)	6.8 ± 1.1 (6.0–7.5)

Abbreviations are defined in Jairajpuri and Ahmad (1992)

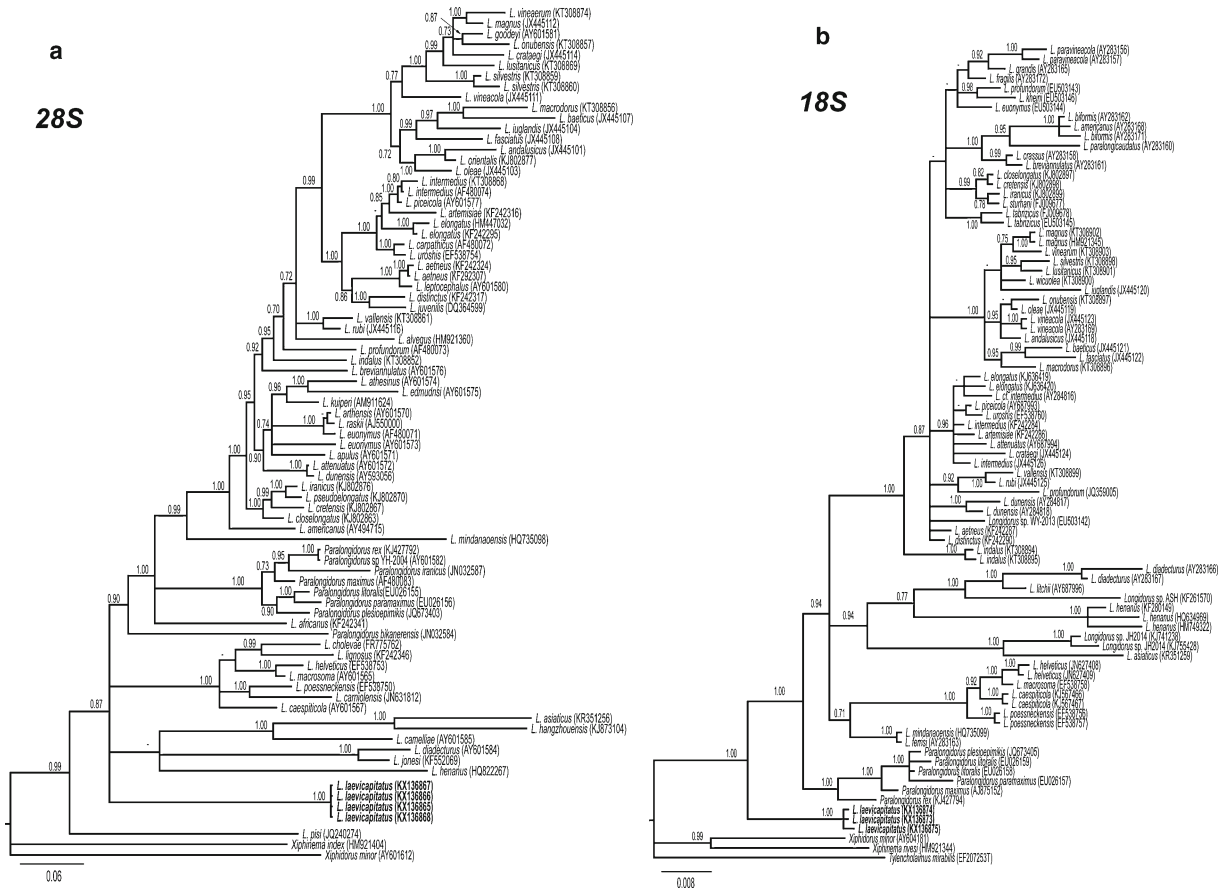

Fig. 2 Phylogenetic relationships *Longidorus laevicapitatus* Williams 1959. Bayesian 50 % majority rule consensus trees as inferred from D2-D3 expansion segments of 28S (**a**) and (**b**) 18S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities more than 0.70 are given for appropriate clades (in bold letters). Newly obtained sequences in this study are in bold

variation for D2-D3, ITS1 and 18S sequences was low, only 1, 3 and 4 nucleotides (0.11 %, 0.27 %, and 0.22 %, respectively) and no indels, respectively.

The phylogenetic trees obtained using the D2-D3 and partial 18S regions were congruent with the results obtained in previous studies. (Gutiérrez-Gutiérrez et al. 2013; Archidona-Yuste et al. 2016 and). *Longidorus laevicapitatus* occupied a basal position in both trees (Fig. 2) and did not cluster with any *Longidorus* spp. used in these analysis. These results suggest that *L. laevicapitatus* is not closely related phylogenetically with any *Longidorus* spp. sequenced to date.

In summary, the present study provides new molecular markers (D2-D3 expansion segments of the 28S rRNA, ITS1 and 18S rRNA) for precise and unequivocal diagnosis of this species and elucidates phylogenetic relationships with other *Longidorus* spp., which may help for effective quarantine inspection and appropriate application of exclusion principles.

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