RESEARCH/INVESTIGACIÓN

PHYLOGENETIC RELATIONSHIPS AMONG MEXICAN POPULATIONS OF *NACOBBUS ABERRANS* (NEMATODA, PRATYLENCHIDAE) REVEAL THE EXISTENCE OF CRYPTIC (COMPLEX) SPECIES

A. J. Cabrera-Hidalgo[1], N. Marban-Mendoza[1], and E. Valadez-Moctezuma[2*]

[1]Laboratorio de nematodos fitopatógenos, Posgrado en Protección Vegetal, Universidad Autónoma Chapingo, Carretera México-Texcoco km 38.5, Chapingo, Edo. de México, C.P. 56230; [2]Laboratorio de Biología Molecular, Departamento de Fitotecnia, Universidad Autónoma Chapingo, Carr. México-Texcoco km 38.5, Chapingo, Edo. México, C.P. 56230; *Corresponding author: evaladez@chapingo.mx

ABSTRACT

Cabrera-Hidalgo, A. J., N. Marban-Mendoza, and E. Valadez-Moctezuma. 2019. Phylogenetic relationships among Mexican populations of *Nacobbus aberrans* (Nematoda, Pratylenchidae) reveal the existence of cryptic (complex) species. Nematropica 49:1-11.

The plant-parasitic nematode *Nacobbus aberrans* is an agricultural pest of quarantine importance. The taxonomic status of the nematode has been the subject of controversy due to wide degree of variation exhibited by the species. This variability has led to the suggestion that *N. aberrans* is actually a species complex rather than a single species. Nevertheless, all previous studies revealed a limited genetic variability in Mexican populations. The objective of this study was to analyze, for the first time, the Cytochrome c Oxidase Subunit 1 (COI) gene to investigate its variability in 15 Mexican populations of *N. aberrans* and to analyze the phylogenetic relationships of *N. aberrans* with other plant-parasitic nematodes. COI sequences revealed significant intraspecific variation, and the phylogenetic inference showed the formation of two separate groups with different levels of intragroup variability. These results indicate the possibility of the existence of more than one group of *N. aberrans* species in Mexico. COI phylogenetic analyses of *N. aberrans* with related plant-parasitic nematodes confirms its proximity to both cyst and root-knot nematodes.

Key words: Cytochrome c oxidase subunit I, genetic variability, *Nacobbus aberrans*

RESUMEN

Cabrera-Hidalgo, A. J., N. Marbán-Mendoza, y E. Valadez-Moctezuma. 2019. Las relaciones filogenéticas entre poblaciones Mexicanas de *Nacobbus aberrans* (Nematoda, Pratylenchidae) revelan la existencia de especies crípticas (complejas). Nematropica 49:1-11.

El fitonematodo *Nacobbus aberrans* es una plaga agrícola de importancia cuarentenaria. La taxonomía del nematodo es objeto de controversia debido al amplio grado de variación exhibido por la especie. Esta variabilidad sugiere que *N. aberrans* es en realidad, un complejo de especies en lugar de una sola especie. Sin embargo, todos los estudios previos revelan una variabilidad genética limitada en las poblaciones Mexicanas. El objetivo de este estudio fue analizar, por primera vez, el gen Citocromo c Oxidasa subunidad 1 (COI) para investigar su variabilidad en 15 poblaciones Mexicanas de *N. aberrans* y analizar las

relaciones filogenéticas de *N. aberrans* con otros fitonematodos. Las secuencias COI revelan una variación intraespecífica significativa y la inferencia filogenética mostró la formación de dos grupos separados con diferentes niveles de variabilidad intragrupo. Estos resultados indican la posibilidad de la existencia de más de un grupo de especies de *N. aberrans* en México. El análisis filogenético del gen COI de *N. aberrans* con fitonematodos relacionados confirman su proximidad con los nematodos enquistados, así como con los nematodos agalladores.

Palabras clave: Citocromo c oxidasa subunidad 1, *Nacobbus aberrans*, variabilidad genética

INTRODUCTION

The genus *Nacobbus*, the false root-knot nematode, initially included four species: *N. dorsalis* Thorne & Allen, 1944, *N. aberrans* (=*Anguillulila aberrans* Thorne, 1935), *N. batatiformis* Thorne & Schuster, 1956, and *N. serendipiticus* Franklin, 1959 (Lax *et al*., 2014). Sher (1970) reported no significant morphological differences between these nominal species and proposed two species in the genus, *viz*., *N. dorsalis* and *N. aberrans*. These two species were separated, among other characters, by the number of annuli between vulva and anus, the more posterior position of the vulva and the presence or absence of fully embryonated J2 within the female. *N. dorsalis* has been recorded only in the USA, its variation and biology being scarcely known. By contrast, *N. aberrans* has a wide-ranging distribution, being found in North and South America. Variations in morphology and morphometrics, physiology, and host-range provide an increasing body of evidence that *N. aberrans* embraces substantially more variation than could be easily limited within a single species (Jatala and Golden, 1977; Manzanilla-López *et al*., 2002). Several *N. aberrans* populations can have shorter or longer embryogenesis and generation times, the duration of the life cycle being affected by temperature, diapause, and host (Manzanilla-López *et al*., 2002; Anthoine and Mugniéry, 2005a). Biochemical and molecular studies of populations attributed to *N. aberrans* have uncovered differences that in other taxa could be attributed to the species or even genus level (Ibrahim *et al*., 1997; Reid *et al*., 2003). Within the scheme proposed by Reid *et al*. (2003), *N. aberrans sensu* Sher was divided into two species, *viz*., *N. aberrans sensu* Reid *et al*. and *N. bolivianus* Lordello, Zamith & Boock, 1961. Others studies (Anthoine and Mugniéry, 2005b; Lax *et al*., 2007; Vovlas *et al*., 2007) have provided molecular evidence to support the existence of at least two major clades within South American populations of *Nacobbus*. Evidence of cryptic species within the *N. aberrans* were reported by Lax *et al*. (2014) based on sequences of ITS and D2-D3 expansion segments of 28S rDNA from 10 Argentine populations.

Nacobbus aberrans sensu lato (EPPO, 2009) has a broad host range encompassing 84 known plant species across 18 families. This host range includes crops such as potato, tomato, beans, and sugar beet, and many common weeds (Manzanilla-López, 2010). *Nacobbus aberrans* causes yield losses up to 65% for potato in Andean Latin America and 10-20% for sugarbeet in the USA (Manzanilla-López *et al*., 2002). Due to a combination of its wide distribution, broad host range, potential yield losses, and peculiar dormant parasitic stage, *N. aberrans* is recognized as an EPPO A1-rated quarantine pest and also has quarantine status in the United States (Manzanilla-López, 2010). At least 40 countries have implemented quarantine measures to prevent the introduction of this nematode. Consequently, *N. aberrans* has been listed as one of the top 10 nematodes in plant pathology (Jones *et al*., 2013).

In Mexico, *N. aberrans* was first reported as *N. serendipiticus* in 1967, from Chile pepper in Chapingo, Mexico State (Brunner de Magar, 1967). At present, it is distributed in several states *viz*. Mexico State, Coahuila, Federal District, Guanajuato, Hidalgo, Michoacán, Morelos, Puebla, Oaxaca, San Luis Potosí, Tlaxcala, and Zacatecas. This nematode affects several crops of great agronomic importance causing considerable losses in chili, tomato, potato, lettuce, beet, cucumber, zucchini, and beans (Brunner de Magar, 1967; Cid del Prado, 1993; Toledo *et al*., 1993; Manzanilla-Lopez *et al*., 2002; Cristobal *et al*., 2006; Cabrera-Hidalgo *et al*., 2014). Yield losses are estimated at 36% for bean (Manzanilla-López *et al*., 2002) and 12-83% for tomato in Mexico

(Cristóbal *et al*., 2006). For most crops, yield loss statistics are not available.

No agreement exists on the taxonomy of *N. aberrans* due to variability in morphometric characters and morphological traits of populations (Manzanilla-López *et al*., 1999; Lax *et al*., 2007). Mexican populations represent a less heterogeneous group than other American groups (Anthoine and Mugniéry, 2005a; Reid *et al*., 2003; Lax *et al*., 2007; 2014). Also, variability in host range (Manzanilla-López *et al*., 2010), reproductive potential of populations on different plants (Lax *et al*., 2011), variation in number of chromosomes (Anthoine and Mugniéry, 2005a), isoenzyme patterns (Ibrahim *et al*., 1997; Doucet *et al*., 2002), RAPD and ISSR molecular marker variability (Lax *et al*., 2007: Cabrera-Hidalgo *et al*., 2015) has generated further controversy on the taxonomy of *N. aberrans*.

The COI-mtDNA is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom (Avise, 2004). The M1-M6 partition of the COI gene is an efficient identification tool for Metazoan species, turning it into the core fragment for DNA barcoding (Hebert *et al*., 2003). No information about the COI gene of *N. aberrans* is available in the literature. Consequently, the objectives of this research were to investigate the variability of the COI gene in 15 populations of *N. aberrans s.l.* from Mexico and to analyze the phylogenetic relationships of *N. aberrans s.l.* with others plant-parasitic nematodes based on COI sequences.

MATERIALS AND METHODS

Nematode populations

Fifteen populations of nematodes were collected from the rhizosphere of tomato (*Solanum lycopersicon* L.), carrot (*Daucus carota* L.), zucchini (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), husk tomato (*Physalis ixocarpa* Brot.), and pepper (*Capsicum annuum* L.) plants from Central Bajio, Mexico (Table 1). The isolates were increased and maintained in greenhouse on tomato cv. Rio Grande at 28 ± 3°C, 13-hr day length and 40% of relative humidity. The populations were maintained in the original field soil and cultivated in isolation from each other to avoid cross-contamination. All populations were identified as *N. aberrans* based on morphological identification according to Sher (1970). The 18S region of ribosomal DNA region was sequenced to confirm the identification (Cabrera-Hidalgo *et al*., 2015).

DNA extraction

From each population, total genomic DNA was obtained from manually selected nematodes. Five females were transferred to 1.5 mL microcentrifuge tubes containing 500 µL of lysis buffer (0.1 M Tris, pH 8.0; 10 mM EDTA; 2% SDS; 0.2 mg/L proteinase K), crushed with a microhomogenizer, and incubated at 38°C for 30 min. After incubation, 30 µL of 10% CTAB and 70 µL of 5M NaCl were added to the tubes and the samples were incubated at 60°C for 10 min. After the 10 min, 50 µL of 5 M potassium acetate was added and the tube incubated on ice for 5 min. After ice incubation, 700 µL of chloroform-isoamyl alcohol (24:1) was added, and the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new sterile tube and 640 µL of cold isopropanol and 60 µL of 3M sodium acetate pH 5.8 were added. The tube was mixed thoroughly. The sample was incubated for 10 min at -20°C and subsequently centrifuged at 13,000 rpm for 10 min. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol and centrifuged at 13,000 rpm for 5 min. The DNA obtained was resuspended in 40 µL of TE buffer (pH 8.0). The DNA quantification was estimated by spectrophotometry (ND-1000, Thermo Scientific, USA), and the DNA quality was determined in 1% agarose gels.

COI gene amplification and sequencing

The Cytochrome c oxidase subunit 1 (COI) mtDNA gene was amplified using the primers JB3 5'- TTTTTTGGGCATCCTGAGGTTTAT-3' (Bowles *et al*., 1992) and JB5 5'-AGCACCTAAACTTAAAACATAATGAAAAT G-3' (Derycke *et al*., 2005). The PCR reactions were performed in total volumes of 25 µL, containing 5 muL of DNA (10 ng/µL), 2.5 µL of 10X reaction buffer, 3 µL of 25 mM $MgCl_2$, 5 µL of 1 mM dNTPs, 1 µL of 10 pM primers, and 1 U of Taq DNA polymerase (Integrated DNA Technologies, IDT®, San Diego, CA). The PCR amplification was carried out in a Thermocycler MaxyGene (Applied Biosystem, USA). The PCR

Table 1. Samples of *Nacobbus aberrans* populations from central Mexico.

Municipality	Population	State	Crop
Tanhuato 42	T42	Michoacán	Zucchini
Tanhuato 52	T52	Michoacán	Tomato
Tanhuato 53	T53	Michoacán	Tomato
Tanhuato 81	T81	Michoacán	Tomato
Tanhuato 85	T85	Michoacán	Tomato
Tanhuato 86	T86	Michoacán	Tomato
Yurécuaro	YU	Michoacán	Tomato
Romita	RO	Guanajuato	Pepper
Celaya, Santo Domingo	SD	Guanajuato	Carrot
Celaya, Santa Elena	SE	Guanajuato	Husk tomato
Silao	SILAO	Guanajuato	Cucumber
San Carlos, Libres	LIBRES	Puebla	Tomato
Tétela	TE	Puebla	Tomato
Calpulalpan	TLAX	Tlaxcala	Tomato
Chapingo	CHA	Mexico State	Pepper

program included 94°C for 5 min, followed by 40 cycles of 95°C for 60 sec; 41°C for 90 sec; and 72°C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were visualized with ethidium bromide staining on 1% agarose gel. PCR amplifications of each population were purified and sequenced in both directions using the same primers mentioned above by Macrogen Korea Inc. The obtained sequences were submitted as *Nacobbus aberrans* to the GenBank database under accession numbers as indicated on the phylogenetic trees.

Phylogenetic analyses

A quality check of the sequences was performed (Song *et al*., 2008). The sequence chromatograms were investigated for the presence of double peaks without indication of additional products on agarose gel and the translated sequences were checked for the presence of frame-shift mutations or stop codons. Nucleotide and amino acid composition were calculated in MEGA6 (Tamura *et al*., 2013). The nucleotide sequences were edited using BioEdit 7.2.3 (Hall, 1999), and the assembly of both strands of DNA was conducted by applying CAP contig assembly software. The consensus sequences were submitted to GenBank database (accession numbers indicated in Fig. 1). The nematode origin of sequences was first investigated by a blastx algorithm search against the non-redundant nucleotide database in Genbank. In view of the low number of nematode COI sequences available and the high sequence

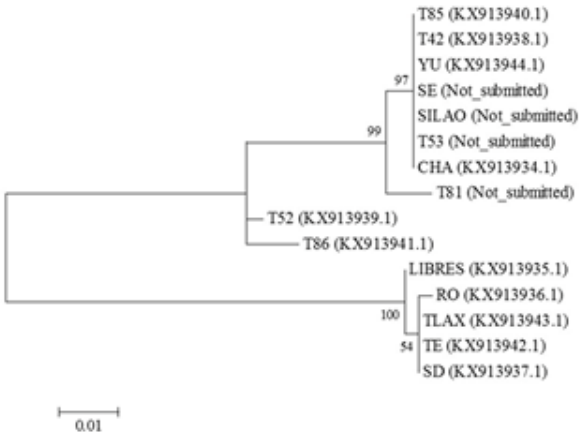

Figure 1. Maximum likelihood phylogenetic tree based on COI gene with HKY+G model of *Nacobbus* populations from Mexico. Text in parenthesis indicates number of accessions assigned in Genbank database. Bootstrap values from 500 replicates are given in nodes.

divergence between distantly related nematode species, identity matches with nematodes or with any other organism were generally lower than 70%. Consequently, COI sequences were validated by constructing a phylogenetic tree. Nucleotide alignments were produced using ClustalX implemented in MEGA6 with default parameters. Phylogenetic analyses were performed based on two data sets: (1) 15 COI sequences of *N. aberrans* obtained in the present study and (2) 18 COI sequences from plant-parasite nematodes species including two sequences of *N. aberrans*. Analyses were conducted with maximum likelihood (ML) method based on the HKY+G (Hasegawa *et al*., 1985) and K2P models using MEGA6 software. Phylogenetic reconstruction with Maximum Parsimony and Minimum Evolution methods were also evaluated. The estimation of the support for each node was assessed by bootstrap analysis with 500 replicates.

RESULTS

The JB3-JB5 pair primer allowed amplification of a clear fragment of estimated size of 450 bp without generating any nonspecific product in the 15 analyzed populations of *N. aberrans* collected from Mexico. Sequencing of the amplified fragments in both directions permitted obtaining 15 COI sequences varied ca 446-447 bp in length. The JB3 forward primer generated clear sequences varied in length from 355 to 390 nucleotides with an average of 376 bases whereas the JB5 reserve primer generated good length sequences that ranged from 370 to 400 nucleotides with an average of 388 bases. The obtained consensus sequences were deposited in Genbank database with number of accessions from KX913934.1 to KX913944.1. Four sequences corresponding to SE, SILAO, T53, and T81 populations were not deposited in Genbank because no variations in sequence nucleotides were observed compared to the other populations.

From the multiple sequences alignment of the 15 populations obtained with the ClustalW program, a total of 36 informative sites were calculated on a total of 446 nucleotides representing 8.07% of the total. Informative polymorphic sites were found in the interval of 33 to 417 nucleotides. The multiple amino acid alignments obtained from the translation of the COI sequences revealed the presence of 148 amino acids, of which 8 (5%) sites were polymorphic through the 15 sequences. The polymorphic sites were found in the interval of 18 to 104.

Maximum Likelihood analysis with the HKY + G model revealed the formation of two clades comprised of 10 and 5 populations each (Fig. 1). The topology of the tree was similar when analyzing the data with the K2P model. The tree was also comparable when applying the Minimum Evolution method and the Maximum Parsimony analysis. The grouping of the populations followed neither the geographical origin nor the crop from which the nematode was isolated. Average evolutionary divergence over sequence pairs within groups (Kimura, 1980) was greater in the first clade (0.011 ± 0.003) than in the second clade (0.002 ± 0.001). Proportion of interpopulation diversity (Coefficient of Evolutionary Differentiation) was $d=0.837 \pm 0.034$.

Within clade 1 (10 populations), the number of polymorphic nucleotides varied from 1 to 3 in a total of 15 information sites in the multiple alignment. Within clade 2 (5 populations), only two mutation sites were detected, each one with a single base. The multiple alignment of the amino acids obtained from the translation of the COI sequences revealed that four amino acids (2.5%) were shown to be polymorphic through the 10 sequences of clade 1 of *N. aberrans*. The polymorphic sites in this clade were found in the interval of 19 and 104 amino acids. In clade 2, the multiple amino acids alignment of the 5 sequences showed that all these populations have the same amino acid composition.

COI phylogenetic of N. aberrans

We were able to construct phylogenetic relationships between 17 species representing 7 genera of the most economically important plant-parasitic nematodes related to *N. aberrans*, including a representative of each clade obtained in the tree described above. Using all species, with nucleotide alignment eliminating all gaps, we constructed a robust phylogeny representing the likely relationships between the major plant-parasitic nematode species. The topology obtained is in agreement with phylogenetic trees based on a single SSU rDNA sequence (Holterman *et al*., 2009; van Megen *et al*., 2009), and with phylogenetic trees based on 65 putatively single copy core eukaryotic genes (Eves-van den Akker *et al*., 2014). In this study, phylogeny reconstruction

analysis with Maximum Likelihood method based on GTR + G substitution model and Maximum Parsimony analysis placed *N. aberrans* at the closest outgroup position relative to the cyst nematodes, *Hoplolaimus,* and *Rotylenchulus* (Fig. 2). However, phylogeny reconstruction analysis with Maximum Likelihood method based on K2P substitution model placed *N. aberrans* at the closest outgroup position relative to the root-knot nematodes and *Pratylenchus* species (Fig. 3). *N. aberrans* has different positions at the base of either the cyst nematode clade or the root-knot/*Pratylenchus* nematode clade. The uncertainty in the positioning of *N. aberrans* may reflect its proximity to the bifurcation of these different genera.

DISCUSSION

Nacobbus aberrans s.l. is widely distributed in Mexico encompassing different agronomical regions. The distribution of this nematode has expanded since its discovery in Chapingo (Brunner de Magar, 1967) and currently is found in more than a third of the country's states affecting crops of economic importance (Cristóbal *et al*., 2006; Manzanilla-Lopez *et al*., 2012; Cabrera-Hidalgo *et al*., 2015). We have published the first 11 COI sequences for *N. aberrans s.l.* The COI gene was used in this study because it is currently one of the most used and accepted in nematology for species identification and due to the fact that it is used as a biological barcode (Hebert *et al*., 2003; Elsasser *et al*., 2009; Ferri *et al*., 2009; Derycke *et al*., 2010). Our COI sequences are highly AT-rich (A: 24.06%, T: 44.72%, C: 9.87%, and G: 21.35%). Derycke *et al*. (2010) reported similar results in marine nematodes for the I3-M11 partition (A: 27.1%, T: 42.8%, C: 11.5%, G: 18.6%). A moderate variability was observed at the nucleotides and amino acid levels, with 33 out 417 nucleotides (8.07%) and 8 out of 148 amino acids (5%) being variable. Both JB3 and JB5 primers used to amplify and sequence the COI gene allowed obtaining good-quality sequences of similar lengths in both directions. Contrary to what was found by Derycke *et al*. (2010), where reverse sequences generally produced more unambiguous chromatograms than forward sequences (73% and 56%, respectively), good-quality sequence consensus of ≈ 450 bp were obtained. This result confirms the usefulness of this pair of primers to analyze Mexican populations of *N. aberrans*. Likewise, the COI gene is able to differentiate most of nematode marine species tested by Derycke *et al*. (2010). Similar results were obtained in a barcoding study on parasitic nematode species (Ferri *et al*., 2009) where taxa-specific primers were used to amplify ca 550 bp (Casiraghi *et al*., 2001). Nevertheless, the extensive utility of JB3 and JB5 primers in *N. aberrans* should be evaluated with populations originating from South America.

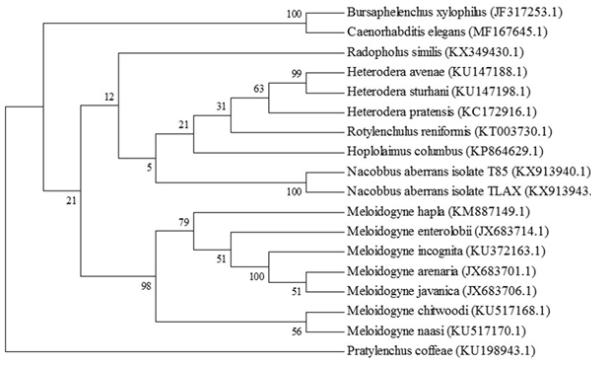

Figure 2. Maximum likelihood phylogenetic tree based on COI gene with HKY+G model of *Nacobbus* populations from Mexico and related plant-parasitic nematodes. The bootstrap values (500 replicates) are shown next to the branches. Text in parenthesis indicates number of accessions assigned in Genbank database.

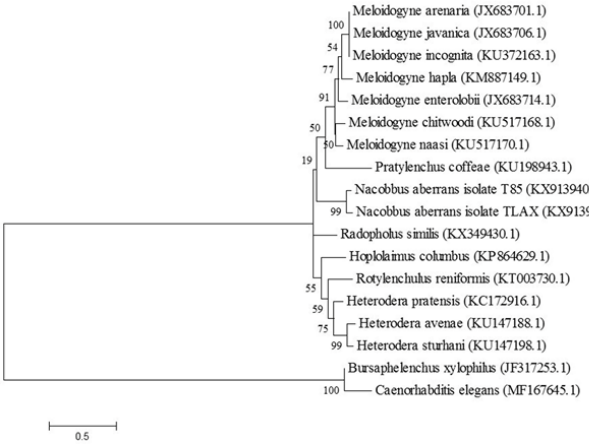

Figure 3. Maximum Likelihood phylogenetic tree based on COI gene with K2P model of *Nacobbus* populations from Mexico and related plant-parasitic nematodes. The bootstrap values (500 replicates) are shown next to the branches. Text in parenthesis indicates number of accessions assigned in Genbank database.

The difficulties in partitioning morphological and morphometrical variation within *N. aberrans s.l.* have been attributed to cryptic or sibling species whose existence is more likely to be demonstrated by employing molecular methodologies than by relying upon classical methods (Blouin, 2002; Manzanilla-López, 2010). The phylogenetic reconstruction of the 15 populations of *N. aberrans s.l.* studied here showed the formation of two constant clades of 10 and 5 populations with a coefficient of evolutionary differentiation of 0.837. The genetic variability was different in each group so we assume that it is necessary to study more populations of nematodes to estimate the overall variation in Mexican populations. Similar results were found using other molecular markers. ISSR analysis of 12 Mexican populations of *Nacobbus* from different hosts and of different origin revealed a high genetic variability (Cabrera-Hidalgo *et al*., 2015).

One of the major challenges in the taxonomy of *Nacobbus* has always been the robust separation and identification of its species. Reid *et al*. (2003), using RFLP and sequencing of the ITS rDNA region, designated the existence of more than two species within *N. aberrans sensu* Sher, a grouping which was considered to represent a nascent complex of species along its range of distribution from North to South America. Their results were supported by studies on morphometrics and other characters and divided *Nacobbus* into several population groups *viz.* the North American (Mexican and Argentinean), South American (Bolivian and Peruvian) and Argentinean groups (Ibrahim *et al*., 1997; Manzanilla-López *et al*., 1999; Reid *et al*., 2003; Anthoine and Mugniéry 2005a; Lax *et al*., 2007; Vovlas *et al*., 2007; Lax *et al*., 2014). All the previous studies revealed limited genetic variability in Mexican populations, since they were grouped together or with some populations from Argentina, probably due to the analysis of few representative samples from Mexico. This contradicts the present work. Similar results were reported by Ramirez-Suarez (2011) based on molecular markers. The latter author reported that Mexican populations can be divided into at least two groups *viz*. Mexican North and Mexican Central. In the present study, the COI analysis showed that in Mexico there may also be more than one group of *N. aberrans* species or cryptic species as was suggested by Lax *et al*. (2014). Considering the COI available sequences, and in the hope of expanding the database, we suggest using standard BLAST search tool with default parameters for the location/identification of a new population in a group of *N. aberrans* considering a threshold of 5% of the identity percentage.

The grouping of the populations was neither according to the geographical origin nor to host. Similar results were reported by Cabrera-Hidalgo *et al*. (2015) based on ISSR molecular markers and by Doucet *et al*. (2002) based on biochemical markers. For their part, Anthoine and Mugniéry (2005b) reported that in six South American populations, the chromosome number was not linked to race group, or even to geographical origin. ITS sequences analysis resulted in similar conclusions (Vovlas *et al*., 2007). This could be due to multiple introductions of nematodes from different geographical origins (Plantard *et al*., 2008). Also, the rental of agricultural machinery and the exchange of propagative plant material are common activities in these areas although it is difficult to elucidate the contribution made by agricultural interchange to the *N. aberrans* genetic structure (Manzanilla-López, 2010). Furthermore, nematodes can move only short distances in the soil and have no natural media for long-distance movement, so gene flow among populations may be generated by passive transport of nematodes across fields by human activities, by water, or by wind (Plantard and Porte, 2004).

Using the information generated here, a local phylogenetic history of the "false root-knot" nematodes is proposed. We present a comprehensive phylogeny of 16 species representing seven genera of the most economically important plant-parasitic nematodes, including representatives from the two major groups obtained from COI gene analysis. The topology described here is in agreement with phylogenetic trees based on a single SSU rDNA sequence (Holterman *et al*., 2009; van Megen *et al*., 2009), and with phylogenetic tree based on 65 putatively single copy core eukaryotic genes (Eves-van den Akker *et al*., 2014). However, it is important to note that despite this apparent congruence, the phylogenetic position of *N. aberrans* fluctuates as a function of methods used to phylogenetic analysis. *N. aberrans* has different positions at the base of either the cyst/*Hoplolaimus*/*Rotylenchulus* nematode clade or the *Meloidogyne*/*Pratylenchus* nematode clade.

Similar observations were reported by Eves-van den Akker *et al*. (2014) where the location of *N. aberras* depended on the set of species included in the analysis. The removal of *P. coffeae* from the analysis caused *N. aberrans* to group with the cyst nematode clade; however, the additional removal of *Radopholus similis* caused *N. aberrans* to group with the *Meloidogyne* clade for protein alignment but with the cyst nematode clade for back-threaded nucleotide alignment (Eves-van den Akker *et al*., 2014). The relative positions of all other nematodes do not change. Only *N. aberrans* has different positions at the base of either the cyst/*Hoplolaimus*/*Rotylenchulus* clade or the *Meloidogyne*/*Pratylenchus* clade.

Nacobbus spp. are thought to be more closely related to the root-knot nematodes than the cyst nematodes (Holterman *et al*., 2009; van Megen *et al*., 2009). The phylogenetic closeness of *N. aberrans* to *Meloidogyne* spp. correlates poorly with the biology of this nematode. The parasitic habit of *Nacobbus* is complex, with migratory, vermiform juveniles and immature adults moving through the root and feeding on cells causing cavities and lesions inside the root tissues, similar to those caused by *Pratylenchus* spp. The juveniles of *Nacobbus* can also repeatedly leave and re-enter the root, causing additional damage (Manzanilla-López *et al*., 2002). By contrast, the mature females are sedentary and induce partial dissolution of the cell walls and the fusion of cell protoplasts, resulting in syncytia (Jones and Payne, 1977). The majority of defining features of the biotrophic interaction, with the exception of the galls, are more similar to those of heteroderids and *R. reniformis* than those of *Meloidogyne* spp. For example, the juveniles of cyst nematodes migrate destructively and intracellularly, whereas the juveniles of root-knot nematodes migrate intercellularly (Atkinson *et al*., 1995). The juveniles of *N. aberrans* migrate destructively and intracellularly as the cyst nematodes (Manzanilla-López *et al*., 2002). The syncytial feeding structure of *Nacobbus* is similar in structure and ontogeny to that of cyst nematodes, and in particular *R. reniformis* (Jones and Payne, 1977; Vovlas *et al*., 2007), and does not resemble the giant cells of *Meloidogyne* spp. Females of *Meloidogyne*, *R. reniformis*, and *Nacobbus* all produce eggs into a gelatinous matrix. In *Meloidogyne*, the matrix originates from rectal glands, whereas in *R. reniformis*, the matrix originates from vaginal glands (Robinson *et al*., 1998). The origin of the gelatinous matrix of *N. aberrans* is not known (Manzanilla-López *et al*., 2002). These observations can be put in parallel with the lateral gene transfer and the effector complement analyses (Eves-van den Akker *et al*., 2014). Classes of glycoside hydrolase GH enzyme present in either *Meloidogyne* or heteroderid nematodes are both present in the *N. aberrans* transcriptome. In addition, heteroderid nematode specific effectors, absent from *Meloidogyne*, are also present in the *N. aberrans* transcriptome. The difficulties in positioning *N. aberrans,* with respect to heteroderid and *Meloidogyne* clades depending on the number of species, number of sequences, type of alignment used (Eves-van den Akker *et al*., 2014), or phylogenetic reconstruction method, may indicate that it is at the base of this bifurcation.

The variation within *Nacobbus* assessed in Mexican populations will now be considered in order to demonstrate the advances in our knowledge of the variation occurring among *Nacobbus* populations and how this evidence may be interpreted regarding species concept within the genus. The need for a practical resolution of the diagnostic difficulties inherent for this species complex must be tackled. Accurate identification is vital, and the increasing number of deposited DNA sequences for *N. aberrans s.l.* will facilitate the design of specific primers for routine identification of these species/groups.

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