GENETIC VARIABILITY AND PHYLOGENETIC ANALYSES OF *NACOBBUS ABERRANS SENSU LATO* POPULATIONS BY MOLECULAR MARKERS

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ABSTRACT

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Genetic variability of Mexican *Nacobbus aberrans* populations was detected by ISSR markers and some genes used in taxonomic studies. The populations of *N. aberrans* were isolated from different hosts in Guanajuato and Michoacan (Mexico). Partial sequences analysis of the 18S, Internal Transcribed Spacer (ITS) and Cytochrome Oxidase I (COI) regions was used in order to identify and estimate the variability in nematode populations studied. Several inter-simple sequence repeat (ISSR) markers were developed with four anchored primers for the comparative study of genetic variation. According to the ISSR analysis, 88.12% out of 150 bands were polymorphic. This technique grouped populations into two main clusters revealing a high level of genetic variability separating the Romita population from the others, with genetic similarity indices ranging from 0.44 to 0.82. Analysis based on partial sequences of the 18S and ITS regions indicated that our populations of nematodes corresponded to *N. aberrans*. However, the mitochondrial gene revealed significant levels of variation among the sequences analyzed showing 16 variable sites. These results suggested that, in the sampled areas, there are at least two different biotypes of *N. aberrans*.

Key words: Cytochrome c oxidase subunit I, ISSR, ITS, *Nacobbus aberrans*, Sequence analysis.

RESUMEN

Cabrera-Hidalgo, A. J., N. Marbán-Mendoza, G. Valdovinos-Ponce, y E. Valadez-Moctezuma. 2015. Variabilidad genética y análisis filogenético de poblaciones de *Nacobbus aberrans sensu lato* usando marcadores moleculares. Nematropica 45:263-278.

La variabilidad genética de poblaciones mexicanas de *Nacobbus aberrans* fue detectada usando marcadores ISSR y genes utilizados en estudios de taxonomía. Las poblaciones de *N. aberrans* fueron recolectadas de diferentes hospedantes en Guanajuato y Michoacán (México). La identificación y estimación de la variabilidad en estas poblaciones se hizo mediante el análisis de las secuencias parciales de las regiones 18S, ITS y COI. Se desarrollaron varios marcadores inter-simple sequence repeat, ISSR, con cuatro primers anclados para el estudio comparativo de la variación genética. En el análisis ISSR, se consideraron 150 bandas de las cuales el 88.12% fueron polimórficas. Esta técnica agrupó las poblaciones en dos grupos principales revelando altos niveles de variabilidad genética, y separó la población de Romita de las otras poblaciones con índices de similaridad genética de 0.44 a 0.82. El análisis basado en las secuencias parciales de 18S e ITS indicó que las poblaciones en estudio corresponden a *N. aberrans*. Sin embargo, el gen mitocondrial (COI) reveló niveles significativos de variación entre las secuencias analizadas mostrando 16 sitios variables. Estos resultados sugieren que en las áreas muestreadas hay al menos dos biotipos diferentes de *N. aberrans*.

Palabras claves: Análisis de secuencias, Citocromo c oxidasa subunidad I, ISSR, ITS, *N. aberrans*.

INTRODUCTION

The genus *Nacobbus*, distributed mainly in North and South America, are plant endoparasitic nematodes whose taxonomy is controversial. Before Sher's revision of the genus in 1970, there were four species and one subspecies described as *Nacobbus aberrans* (Thorne, 1935) Thorne & Allen, 1944, *N. dorsalis* Thorne & Allen, 1944, *N. batatiformis* Thorne & Schuster, 1956, *N. serendipiticus* Franklin, 1959, and *N. serendipiticus bolivianus* Lordello *et al.* (1961). However, when Sher (1970) revised the genus, he validated only two species: *N. dorsalis* (the type species) and *N. aberrans*. *Nacobbus aberrans* is an important plant-parasitic nematode with a high morphological heterogeneity and a broad host range, which is widely distributed in tropical and temperate regions of the Americas, parasitizing 84 cultivated and non-cultivated plant species classified into 18 families (Manzanilla-López *et al*., 2002; Atkins *et al*., 2005). *Nacobbus dorsalis* has less economic importance because of its limited geographic distribution (Monterey County, California, USA) (Manzanilla-López *et al*., 2002). The heterogeneity found within *N. aberrans* populations suggests the possibility that the genus *Nacobbus* is represented by a species complex with a series of biotypes or physiological races (Reid *et al.*, 2003). On the other hand, the morphological studies have not provided convincing data to establish their taxonomic limits so the application of other tools that might provide a suitable basis to define the classification of this species (Jatala and Golden, 1977; Baldwin and Cap, 1992; Ibrahim *et al*., 1997).

There are several reports about the high intraspecific genetic and physiological variability of *N. aberrans* populations from different geographic areas (Reid *et al*., 2003; Anthoine and Mugniéry, 2005a; Vovlas *et al*., 2007). These support the existence of a species complex in the genus (Reid *et al*., 2003; Lax *et al.*, 2007; Vovlas *et al.*, 2007); or of distinct physiological races, pathotypes or new species (Manzanilla-López, 2010; Ramírez-Suárez, 2011; Lax *et al.*, 2013).

Nacobbus aberrans causes severe yield losses in plants with economic and food value (Inserra *et al.*, 1984; Santa Cruz and Marbán Mendoza, 1986; Cristóbal-Alejo *et al*., 2000; Manzanilla-López *et al.*, 2002). It represents an agricultural threat in Mexico and other countries due to a combination of its wide distribution, broad host range, and peculiar dormant parasitic stages. 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 ten nematodes in plant pathology in a recent survey and review (Jones *et al*., 2013).

Several molecular techniques have been used to study the genetic differences of *N. aberrans sensu lato* (Reid *et al*., 2003) and other species of plant-parasitic nematodes. Inter-simple sequence repeat (ISSR) markers have been widely used in the analysis of genetic variation at the subspecies level, particularly in studies of population structure and differentiation (Huang *et al*., 2012). These molecular markers have great potential for studies of natural populations and have been useful in the study of the population structure of many plant and animal species (Wolfe *et al*., 1998; Vijayan *et al.*, 2004). Unlike ISSRs, the 18S rDNA and COI-mtDNA regions have been often used for nematode molecular systematics. The 18S-rDNA region is highly conserved among species and according to Dixon and Hillis (1993) the intra-specific variation in this region is influenced by many copies of 18S rDNA per genome, and its stability through evolution. The COI-mtDNA is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom (Avise, 2004). Its popularity has increased even more since it appears that 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).

Moreover, the ITS of ribosomal DNA is a region with high rate of evolution because of frequent mutational events and has been demonstrated to be highly informative as a taxonomic marker at the species level within nematodes (Powers *et al*., 1997). According to Reid *et al.* (2003), *N. aberrans s.l.* (*sensu lato*) is a species complex comprising at least three distinct genotypes, and the populations of *Nacobbus* from Mexico are conspecific with *N. aberrans s.s.* (*sensu stricto*) from the USA. In the absence of conflicting data, they refer to the *N. aberrans s.l.* populations from North America (*i.e*., those from the USA and Mexico) as *N. aberrans s.s*.

Considering this information and the increasing dissemination of this nematode in the region known as Central Bajio (Guanajuato and Michoacan states) -- one of Mexico's most important agricultural areas for vegetable crops, which shares 4.44% of the national income (SIAP, 2010) -- and the high phenotypic heterogeneity (data not included) detected in our populations; the aim of this research was to study the genetic variability of *N. aberrans* populations from different host crops growing in this area, using DNA sequence data.

MATERIALS AND METHODS

Nematode populations

Twelve populations of nematodes were collected from the rhizosphere of tomato (*Solanum lycopersicon* L.), carrot (*Daucus carota* L.), courgette (*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 a greenhouse on tomato (*Solanum lycopersicon* L.) cv. Rio Grande at 28 ± 3°C, 13-hr day length and 40% 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 their morphology, according to the descriptions by Sher (1970).

DNA extraction

Five females from each sample were handpicked from roots, placed in a drop of sterile distilled water on a glass slide, and crushed with a sterile insulin needle. The ground samples were transferred into an Eppendorf tube, mixed with 10 µL of sterile distilled water and crushed with a rounded-tip glass rod. The DNA obtained was dissolved in 40 uL of TE buffer (pH 8) and quantified with a NanoDrop spectrophotometer ND 1000® (Accesolab S.A. de C.V., MEX.). DNA was isolated using the modified DNAzol® protocol of Life Technologies (Molecular Research Center, Inc., USA).

ISSR-PCR amplification and data analysis

Initially, eight anchored ISSR primers were tested; however, only those that produced clear and reproducible polymorphic bands were selected for the analysis (Table 2). The mixture volume for the ISSR-PCR reaction was 25 µL, containing 5 uL of DNA (10 ng.µL-1), 2.5 µL of 10X reaction buffer, 0.75 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.8 pM of primer, and 0.08 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 thermocycling program consisted of 94°C for 1 min, followed by 38 cycles [94°C for 30 sec; 30 sec at 50-54.5°C, depending on the primer (Table 2); and 72°C for 2 min] and a final extension of 10 min at 72°C. Amplification products were separated on 8% acrylamide gel (29:1) in 1X TBE buffer and stained with 0.2% silver nitrate. For reference, two molecular markers of 100 bp and 1-kb (Promega®, Madison, WI, USA) were included. All gel images were recorded digitally with a digital Sony Cyber-shot camera (Carl Zeiss®, USA).

Polymorphic DNA bands were scored "1" for the presence of a band and "0" for its absence, in order to make a binary matrix. Then, data were processed with the FreeTree software (Version 0.9.1.5) to produce a genetic distance matrix using Dice coefficient (Dice, 1945), also known as the Nei and Li similarity coefficient (1979). The resulting matrix was computed with the unweighted pair group method with arithmetic mean algorithm (UPGMA) in order to construct dendrograms with 1,000 bootstrap replicates. The Tree View 1.6.6 software was used to display the tree obtained (Page, 1996).

The ability of the primers to differentiate between populations was assessed by estimating their polymorphic information content (*PIC*), marker index (*MI*), and resolving power (*Rp*) according to the methodology used by Valadez-Moctezuma *et al.* (2014). The *PIC* was calculated using the formula described by Roldán-Ruiz *et al.* (2000): $PICi = 2fi(1\text{-}fi)$, where *PICi* is the polymorphic information content of the primer *i*, *fi* the frequency of the present bands, and 1-*fi* the frequency of the absent bands. The maximum *PIC* value for dominant markers is 0.5 (De Riek *et al*., 2001).

The *MI* was calculated as *MI = PIC*Number of polymorphic bands* and the *Rp* with the formula by Gilbert *et al.* (1999). This function has been found to correlate strongly with the ability to distinguish between taxa and is given by the formula: $Rp=\sum=I_b$, where band informativeness is calculated with the formula $I_b= 1- [2* (|0.5 - p|)]$, and *p* is the proportion of populations containing band (*fi*). The percentage of polymorphic bands (*PPB*) was also determined.

18S, ITS, and COI markers amplification

The 18S rDNA region was amplified using two universal primers: 988F-1912R (Holterman *et al*., 2006); ITS region with the primers ITSF TW81-ITSR AB28 (Phan *et al.*, 2003) and for subunit 1 of the cytochrome oxidase (COI) mtDNA gene, with the primers COIF-JB3/COIR-JB5 (Derycke *et al*., 2005). The following PCR program was used for the 18S region: 94°C for 5 min, 5 cycles [94°C, 30 sec; 45°C, 30 sec; 72°C, 70 sec], followed by 35 cycles [94°C, 30 sec; 54°C, 30 sec; 72°C, 70 sec], and a final extension of 5 min at 72°C. The thermal cycler program for ITS-PCR consisted of 94°C for 4 min, followed by 35 cycles [94°C, 30 sec; 55°C, 60 sec; 72°C, 1.5 min], and 72°C for 10 min. The PCR program used for COI include 94°C for 5

Table 1. Origin of *Nacobbus aberrans sensu lato* populations in the central region of Central Bajio, Mexico. 2010-2011.

Code	Locality	Original host	North latitude	West longitude
SJI	S J. Iturbide[y]	Tomato	21°01.517	100°25.087
Ro	Romita[y]	Chilli	20°52.417	101°32.702
SD	Celaya, Santo Domingo[y]	Carrot	20°36.830	100°50.468
SE	Celaya, Santa Elena[y]	Husk tomato	20°29.503	101°12.694
T42	Tanhuato 42[z]	Courgette	20°16.273	102°24.060
T52	Tanhuato 52[z]	Tomato	20°18.695	102°18.409
T81	Tanhuato 81[z]	Tomato	20°16.936	102°17.606
T85	Tanhuato 85[z]	Tomato	20°18.806	102°20.672
T86	Tanhuato 86[z]	Tomato	20°18.731	102°20.559
T87	Tanhuato 87[z]	Tomato	20°18.586	102°20.476
Yu	Yurecuaro[z]	Tomato	20°18.729	102°15.799
Pa	Pajacuaran[z]	Cucumber	20°10.224	102°37.996

[y]Populations collected from Guanajuato.
[z]Populations collected from Michoacan fields.

Table 2. Sequence of ISSR primers, annealing temperature, and number of polymorphic bands scored in the analysis of 12 populations of *Nacobbus aberrans*.

Primer[v]	Sequence (5'-3')	Annealing temperature (°C)	Total bands	Polymorphic bands	*PPB*[w]	*Rp*[x]	*PIC*[y]	*MI*[z]
1	(GA)8YC	50	17	8	47.1	5.50	0.205	1.64
2	(AGAC)4GC	53	56	56	100	32.67	0.383	21.4
3	AC(GACA)4	53	52	52	100	31.83	0.391	20.32
4	(ACGA)4CA	54.5	25	16	64	8.33	0.223	3.57
Average			37.5	33	77.8	19.58	0.300	11.74
Total			150	132		78.33	1.202	46.98

[v]Y substitutes C (Cytosine) residue and T (Thymine),Sigma-Aldrich, St. Louis, MO.
[w]*PPB* = Percentage of polymorphic bands.
[x]*Rp* = Resolving power.
[y]*PIC* = Polymorphic information content.
[z]*MI* = Marker index.

Table 3. List of species of nematodes used by sequence analyses of 18S, ITS, and COI genes obtained from GenBank and used in comparisons.

Nematode species	Region used	Accession number	Country of origin	Locality
N. aberrans	18S	KC875388.1	Netherlands	Unknown
N. aberrans	18S	AF442190	USA	Unknown
N. aberrans	18S	AJ966494.1	Belgium	Unknown
N. aberrans	18S	AY919215.1	USA	Unknown
N. aberrans	ITS	DQ318714.1	Argentina	Unknown
N. aberrans	ITS	DQ318725.1	Argentina	Unknown
N. aberrans	ITS	KF254329.1	Ecuador	Unknown
M. incognita	ITS	KF053038.1	Brazil	Sao Paulo
M. incognita	ITS	KF053037.1	Brazil	Sao Paulo
M. incognita	ITS	LC030367.1	Japan	Nagano
M. javanica	ITS	KC953091.1	China	Unknown
P. penetrans	18S	KC533815.1	Netherlands	Unknown
P. penetrans	18S	KC533828.1	Netherlands	Unknown
M. enterolobii	COI	JX683717.1	China	Hainan
M. enterolobii	COI	JX683716.1	China	Hainan
M. javanica	COI	JX683711.1	China	Yunnan
P. oleae	COI	KJ510866.1	Spain	Almeria

min, followed by 40 cycles [95°C, 60 sec; 41°C, 90 sec; 72°C, 2 min], and 72°C for 10 min. The PCR products were visualized by ethidium bromide staining on a 1% agarose gel in 1X TBE buffer. The amplified products were stored at 4°C for further analysis.

Sequences analysis of genes 18S, COI, and ITS

The amplicons corresponding to the 18S rDNA, ITS rDNA and COI mtDNA region were sequenced using a 3130 Genetic Analyzer sequencer (AB® Hitachi, USA), and after were also aligned with accessions of the Genbank database (Table 3). Nucleotide sequences were aligned with CLUSTALW program (Thompson *et al.*, 1997) using BioEdit software (Hall, 1999) and confirmed by visual inspection. Recently generated ITS-rDNA sequences were deposited at GenBank (accession numbers: KT321114, KT321115, KT321116, KT321117, and KT321118 for populations of Romita, Santo Domingo, Tanhuato 87, San José Iturbide and Tetela, respectively). The 18S sequences of *N. aberrans* reported here have been deposited in GenBank: KT591479, KT591480, KT591481, KT591482, and KT591483.

Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The genetic distances and phylogenetic analyses were conducted using MEGA6.05 software. Intra-specific and inter-specific genetic distances of *N. aberrans* were calculated using complete deletion in NJ algorithm and Kimura 2-parameter (K2-p) and Tamura 3-parameter (T92) model for 18S, and ITS and COI sequences, respectively. Sequences of *N. aberrans* were used in the phylogenetic analyses. These sequences were selected from GenBank database and their access numbers are plotted in phylogenetic tree. *Meloidogyne incognita*, *M. javanica, M. enterolobii*, *Pratylenchus penetrans*, and *P. oleae* genera were used as outgroup taxa (Table 3).

Models with the lowest BIC scores (Bayesian Information Criterion) were considered to describe the best substitution pattern.

RESULTS

ISSR-PCR analysis

High levels of genetic variation were observed using the ISSR markers (Table 2) in *N. aberrans s.l.* populations studied. One hundred fifty bands were scored, among which 88.16% were polymorphic, considering only those bands with sizes of 200-2500 bp. The number of ISSR markers scored per primer ranged between 17 and 56 (Table 2). Eighteen monomorphic bands were obtained in all populations with primers ISSR 1 and ISSR 4 (Fig. 1). The polymorphism percentage ranged between 47% (primer 1) and 100% (primer 2 and 3), with an average of 77.8%.

Resolving power values ranged between 5.5 (primer 1) and 32.67 (primer 2) with an average of 19.58 (Table 2). The ISSR 2 and ISSR 3 primers were the most efficient to evaluate the genetic diversity of populations of *N. aberrans s.l.*, presenting a higher rate of *Rp*. The PIC fluctuated from 0.223 (primer 4) to 0.391 (primer 3) with an average of 0.30, and the marker index rate ranged between 1.64 (initiator 1) and 21.4 (initiator 2) with an average of 11.74. Among the ISSR primers used, Primers 2 and 3 were the most informative to differentiate between populations of *N. aberrans* in this study.

The genetic distance matrix showed an average distance that fluctuated between 0.444 and 0.828, indicating that these isolates are highly divergent at the DNA level. The lowest distance value (0.444) was observed between the populations of Romita and Tanhuato 86 with low genetic similarities; and the highest value (0.828) between Pajacuaran and Tanhuato 85, indicating a high genetic similarity among these last populations. All remaining populations displayed different intermediate levels of similarity.

Results of the cluster analysis among populations, based on their Nei and Li/Dice similarity values, are shown in Fig. 2. The populations of *N. aberrans s.l.* were grouped according to their genetic divergence using ISSR markers, forming two main groups (1-2) at the Nei and Li/Dice diversity level of 0.75 (Fig. 2). Group 1, formed by the Romita population, was significantly divergent with respect to all remaining populations, and it was separated into an independent group; this suggested that these individuals were the most different genetically. The remaining populations share certain genetic characteristics according to the UPGMA analysis. Grouping of the populations of *N. aberrans s.l.* was independent of the geographic region where they were collected, as well as of the type of host. The ISSR-PCR technique revealed that the Romita population has a high genetic variability and it was able to distinguish high levels of polymorphism DNA fragments between the populations that were subjects of the study.

18S gene analysis

The 18S amplification product was of 900 bp using 988F-1912R primer set. According to electrophoresis of the PCR products, the size of the amplicon was similar for all *N. aberrans* populations studied. Their alignment and comparison of sequences with reference sequences obtained from the GenBank confirmed that the specimens collected in Guanajuato and Michoacan correspond molecularly to *N. aberrans s.l.* (Reid *et al*., 2003). The analysis based on partial sequences of the 18S

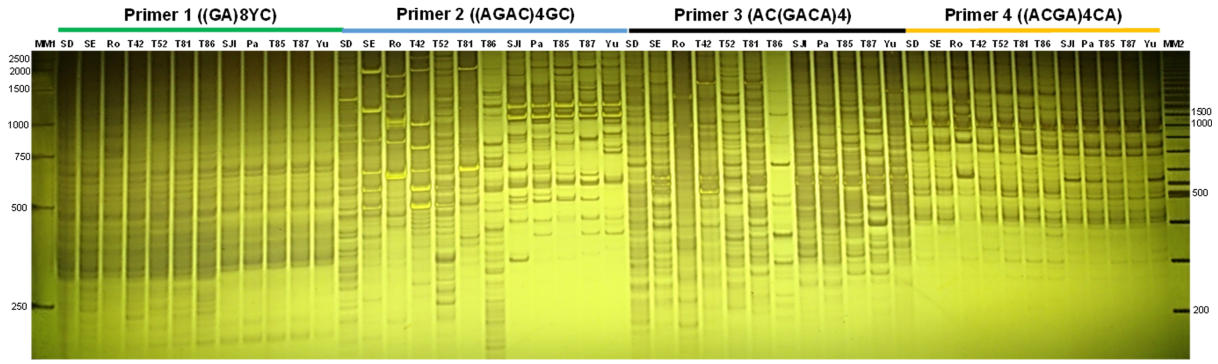

Fig. 1. Inter-simple sequence repeat (ISSR) profiles of 12 populations of *Nacobbus aberrans* from Guanajuato and Michoacan separated by electrophoresis on 8% acrylamide gel. Labels SD to Yu correspond to the *N. aberrans s.l.* populations listed in Table 1. MM1= Molecular marker of 100 bp and MM2= Marker of 1bk.

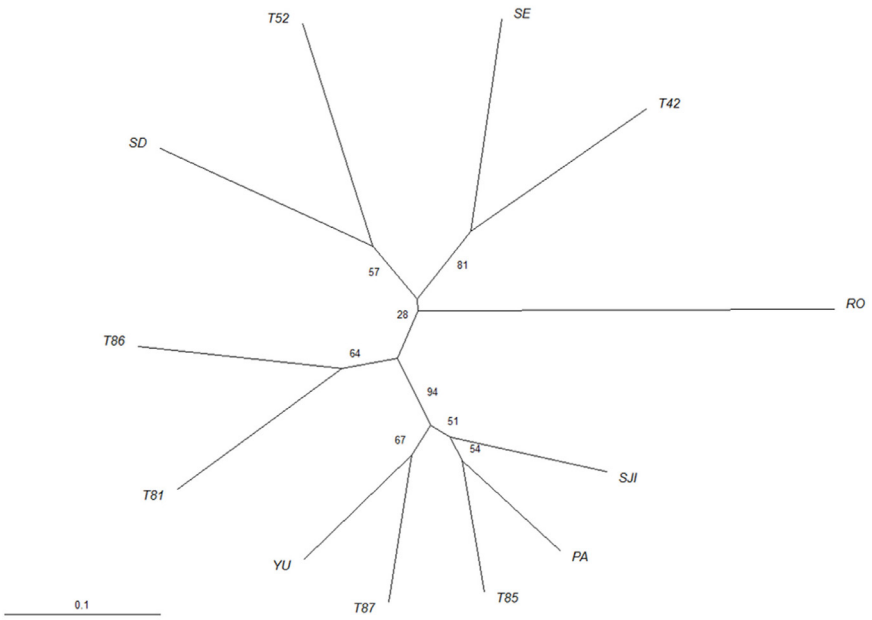

Fig. 2. Phylogenetic relationships between *Nacobbus aberrans* populations based on their Nei and Li/Dice similarity values using Inter-simple sequence repeat (ISSR) markers. UPGMA clustering was constructed using the Free Tree program. Bootstrap values are shown at the nodes. Bar = Genetic distance.

gene only showed one substitution of nucleotide in the population of Tanhuato 86.

The 18S-rDNA data generated after performing multiple sequences alignment showed 928 characters. The sites within the aligned sequences were mainly conserved (99.78%) and only one was variable. This region showed no parsimony-informative sites. All sequences exhibited average nucleotide composition of 25%, 21.7%, 28%, and 25.4 % for thymine (T), cytosine (C), adenine (A), and guanine (G), respectively.

COI gene analysis

The COI amplicon, which is 400 bp long, was similar in all populations of *N. aberrans*. The BLAST (Basic Local Alignment Search Tool) search in the GenBank did not reveal the presence of COI sequences for *N. aberrans* in the database, so it was not possible to make a comparison to corroborate the identity of the species under study with this gene, as was performed with the 18S region. The COI gene revealed 16 variable sites in 203-274 bp long sequence, and 17 sites were found to be parsimony-informative, representing 6.5% of total sites. The average nucleotide composition across all the populations was T = 23.3, C = 24, A = 43.9, and G = 8.9 (Table 4).

ITS analysis

The ITS amplification product varied between 700-900 bp using the ITSF TW81-ITSR AB28 primer set. ITS-rDNA data generated after performing multiple sequences alignment had 678 sites. Sites within the aligned sequences were conserved, and parsimony-informative sites, represented 666 (98.37%) and 11 (1.63%) sites, respectively. These regions showed single-base change, two or more base changes, and a combination of singletons and parsimony-informative sites, respectively. All sequences exhibited average nucleotide frequencies of 29.3 %, 21.7 %, 24.4 %, and 24.7 % for thymine (T), cytosine (C), adenine (A), and guanine (G) (Table 5). The alignment of sequences with the accessions DQ318714.1, DQ318725.1 and KF254329.1 of *N. aberrans* isolates showed 11 polymorphic sites.

18S, COI, and ITS: Phylogenetic analysis

In several independent runs with nearly identical results, maximum likelihood (ML) analysis based on the 18S rDNA gene generated a phylogenetic tree strongly supported (*PP* = 0.99) that grouped all populations of *N. aberrans s.l.* in a monophyletic clade together with accessions of this species obtained from GenBank (Fig. 3). Evolutionary statistics were conducted to find the best model for distances and phylogeny. The best DNA model for estimating evolutionary distances was determined to be the number of nucleotide substitutions occurring between them in a nucleotide-by-nucleotide comparison (Nei and Kumar, 2000). This analysis showed that the Kimura 2-parameter method was the

Table 4. Parsimony-informative sites and nucleotide composition within the COI mt-RNA regions of female of *Nacobbus aberrans* isolates from vegetable crops of Mexico.

Isolate	Variations at specific base locations in COI mt-RNA[z]																																																		
	38	39	40	41	47	48	49	50	51	60	61	62	63	64	65	66	67	68	69	71	72	73	74	75	76	77	82	83	84	85	86	87	88	89	94	95	96	97	98	99	100	101	110	111	112	113	115	116	117	118	119	120
SD	-	-	-	-	-	-	-	-	-	T	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
T42	A	A	C	T	A	G	G	G	A	T	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
T52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
T81	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
YU	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
PA	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
RO	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T85	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T86	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
SE	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
SJI	A	T	C	T	A	A	G	G	A	T	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T87	A	A	C	T	A	G	G	G	A	T	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A

Isolate	Variations at specific base locations in COI mt-RNA[z]																												Nucleotide				
	142	144	145	146	147	148	149	150	177	178	179	180	181	182	183	248	249	250	251	252	253	254	258	259	260	261	262	263	T(U)	C	A	G	Total
SD	T	A	T	A	C	G	C	A	A	T	A	T	A	T	A	A	A	C	A	C	C	A	G	C	T	A	A	T	23.9	25.2	41.3	9.6	218
T42	T	A	T	A	C	G	C	A	A	T	A	T	A	T	A	A	A	C	A	C	C	A	G	C	T	A	A	T	22.6	24.5	43.8	9.1	274
T52	T	A	T	A	C	G	C	A	A	T	A	T	A	T	A	A	A	C	A	C	C	A	G	C	T	A	A	T	23.2	24.6	42.4	9.9	203
T81	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.5	23.5	44.1	8.8	272
YU	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.2	23.9	44.5	8.5	272
PA	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.2	23.9	44.5	8.5	272
RO	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.2	23.9	44.5	8.5	272
T85	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.5	23.5	44.1	8.8	272
T86	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	-	23.5	23.5	44.2	8.8	260
SE	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.5	23.5	44.1	8.8	272
SJI	T	A	T	A	A	G	C	A	A	T	A	C	A	T	A	A	A	C	G	C	C	A	G	C	C	A	A	T	23.5	23.5	44.1	8.8	272
T87	T	A	T	A	C	G	C	A	A	T	A	T	A	T	A	A	A	C	A	C	C	A	G	C	T	A	A	T	22.6	24.5	43.8	9.1	274
Average																													23.3	24.0	43.9	8.9	261.1

[z] Highlighted regions in yellow show parsimony-informative sites and positions with gaps are 241 and 242 (Not shown).

Table 5. Parsimony-informative sites and nucleotide composition within the ITS rRNA regions of females of *Nacobbus aberrans* isolates from vegetable crops of Mexico.

Isolate	Variations at specific base locations in ITS rRNA[z]																																									
	61	62	63	64	65	66	67	68	69	71	72	73	74	75	76	77	82	83	84	85	86	87	88	89	94	95	96	97	98	99	100	101	110	111	112	113	115	116	117	118	119	120
N aberrans DQ318725.1	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
N aberrans DQ318714.1	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
Nacobbus sp. KF254329.1	-	-	-	-	-	-	-	-	-	-	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A
RO	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
PA	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
SD	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T87	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	C	C	A	A	A	A	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
SJI	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T86	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
TETELA	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
T81	A	T	T	A	C	A	A	C	C	C	G	T	A	C	A	A	A	T	T	A	A	T	C	A	A	A	G	A	C	T	T	T	T	A	G	G	A	C	A	G	C	A
SE	A	T	A	A	C	G	A	C	C	C	A	T	A	T	A	A	A	T	C	A	G	C	C	A	A	A	A	A	C	C	T	T	T	G	G	G	A	C	C	G	C	A

Isolate	Variations at specific base locations in ITS rRNA[z]																			Nucleotide				
	190	191	192	193	194	195	196	197	663	664	665	666	667	668	669	670	671	672		T(U)	C	A	G	Total
N aberrans DQ318725.1	G	T	T	G	C	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.1	22.1	24.0	24.8	678.0
N aberrans DQ318714.1	G	T	T	G	C	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.0	21.7	24.5	24.8	677.0
Nacobbus sp. KF254329.1	G	T	T	G	C	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.2	21.9	24.5	24.4	677.0
RO	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.4	21.6	24.4	24.7	677.0
PA	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.4	21.6	24.4	24.7	677.0
SD	G	T	T	G	G	T	C	A	G	A	C	C	C	C	C	A	A	T	T	29.0	22.0	24.4	24.7	677.0
T87	G	T	T	G	G	T	C	A	G	A	C	C	C	C	C	A	A	T	T	29.0	22.0	24.4	24.7	677.0
SJI	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.5	21.3	24.3	24.9	675.0
T86	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.5	21.4	24.2	24.8	677.0
TETELA	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.5	21.4	24.4	24.7	677.0
T81	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.4	21.4	24.4	24.7	676.0
SE	G	T	T	G	G	T	C	A	G	A	C	C	T	T	T	A	A	T	T	29.4	21.4	24.4	24.7	676.0
																	Average			29.3	21.7	24.4	24.7	676.8

[z]Highlighted regions in yellow show parsimony-informative sites and positions with gaps are 51, 52, and 607 (Not shown).

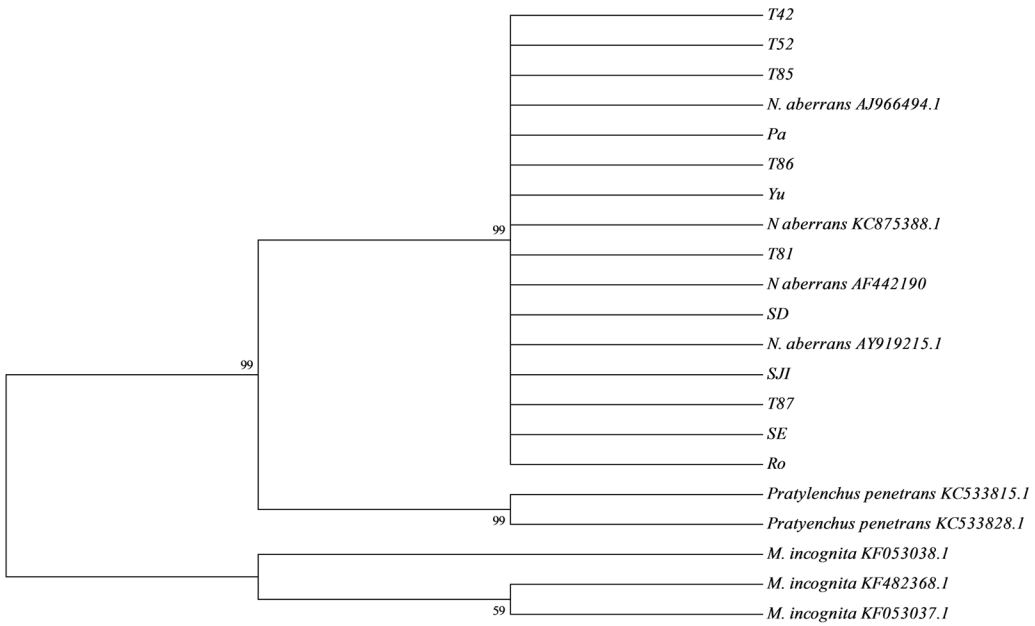

Fig. 3. Maximum-Likelihood tree constructed with 18S rDNA sequences of *Nacobbus aberrans* populations from Guanajuato and Michoacan, Mexico. The Kimura 2-parameter was used as evolutionary model (BIC= 2648.297; AICc= 2384.383; Ln Likelihood= -1151.891). *Pratylenchus penetrans* and *Meloidogyne incognita* were used as outgroup species. Bootstrap values are shown at the nodes.

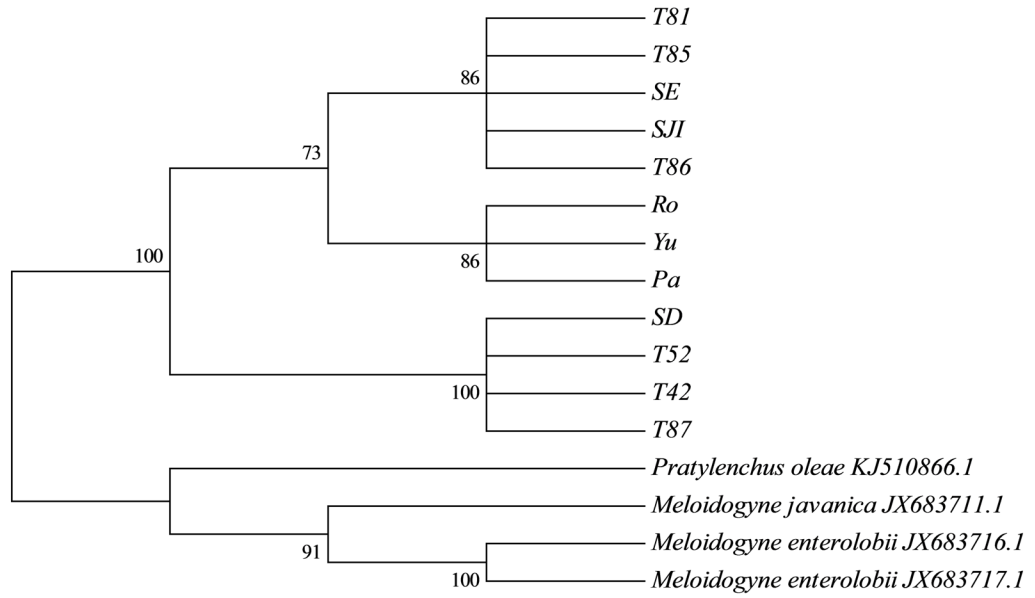

Fig. 4. Maximum-Likelihood tree constructed with COI mtDNA sequences of *Nacobbus aberrans* populations from Guanajuato and Michoacan, Mexico, using Tamura 3-parameter model of DNA sequence evolution (BIC= 1826.37; AICc= 1640.59; Ln Likelihood= -788.965). *Meloidogyne enterolobii*, *M. javanica*, and *Pratylenchus oleae* were used as outgroup species. Bootstrap values are shown at the nodes.

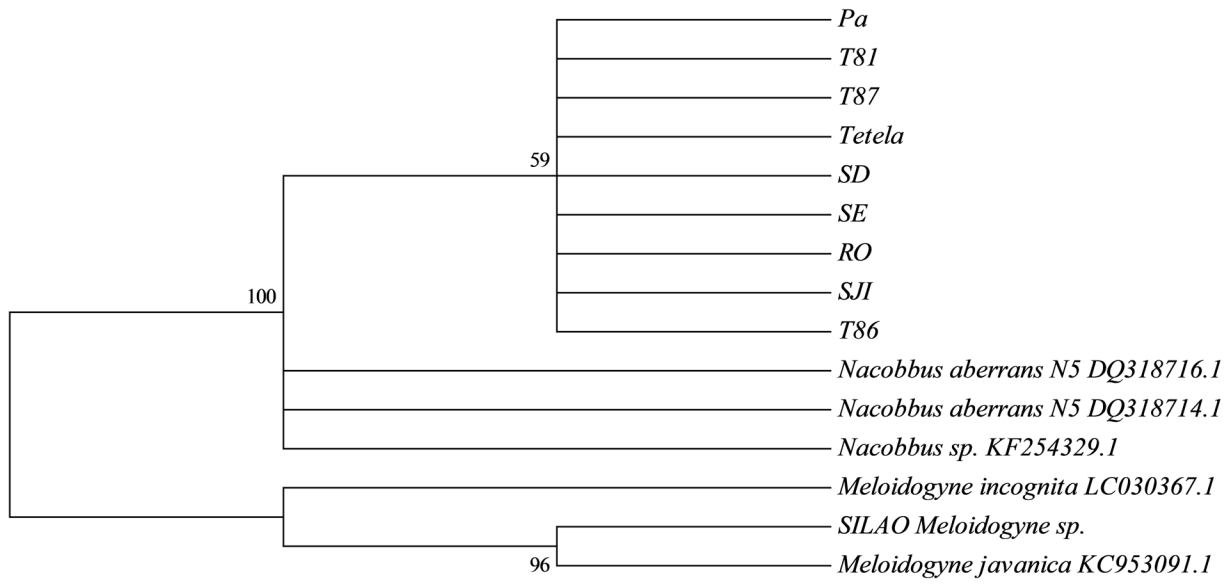

Fig. 5. Maximum-Likelihood tree obtained from analysis of alignments of ITS rDNA sequences of *Nacobbus aberrans* populations from Guanajuato, and Michoacan, Mexico, using Tamura 3-parameter evolutionary model (BIC=2089.57; AICc= 1898.025; Ln Likelihood= -919.854). *Meloidogyne incognita* was used as outgroup species. SILAO *Meloidogyne* sp. is property of authors and actually is not in Genbank. Bootstrap values are shown at the nodes.

best because this had lowest Bayesian information criterion (BIC) (2648.297) score, which was considered to be the best substitution pattern. The rate of nucleotide substitutions ranged from 0.056 (AT, AC, TA, TG, CA, CG, GT, and GC) to 0.138 (AG, TC, CTnm, and GA) (Table 6).

With the COI mt-DNA gene, the ML estimate of substitutions method was also performed in which the best model was Tamura 3-parameter (BIC= 1826.37; AICc= 1640.59). The probability of substitutions from one base to another base was considered along with the rate of nucleotide substitutions that varied between 0.032 (AC, TG, CG, and GC) and 0.205 (GA) (Table 6). Rates of different transitional substitutions were obtained, where the highest value was 29.4518 for substitution A with G, whereas that rate of transversionsal substitutions was highest for A with T and A with C (7.5230 each one).

The COI mt-DNA tree in Fig. 4 revealed an important degree of differentiation in populations, grouping all *N. aberrans* sequences in a monophyletic clade with three subclades and a high bootstrap value (100%). The first subclade was formed with populations of Santa Elena, San José Iturbide, Tanhuato 81, 85, and 86; these were associated with carrot and tomato in farms where farmers usually change cropping patterns, alternating with crops that are not hosts and where gravity and drip irrigation are commonly used. A second subclade, close to the previous one, contained Yurecuaro, Romita, and Pajacuaran populations associated with crops of tomato, pepper, and cucumber in farms where rotation is usually not practiced, but crops that are non-hosts are planted and gravity irrigation is mainly used. A third subclade included the populations of Santo Domingo, Tanhuato 42, 52, and 87, which were related with crops of husk tomato, squash and tomato, with a very similar agronomic management to the previous group.

The maximum likelihood analysis based on the ITS rDNA region generated a phylogenetic tree strongly supported (100% bootstrap value) that grouped all our populations of *N. aberrans s.l.* in a monophyletic clade, separating them of the accessions of this species obtained from GenBank (Fig. 5). Evolutionary statistics were performed to find the best substitution model for distances and phylogeny. This analysis showed that Tamura 3-parameter was the best model with BIC and AICc score of 2089.57 and 1898.025 respectively. The rate of nucleotide substitutions ranged from 0.044 (AC, TG, CG, and GC) to 0.168 (CT and GA) (Table 6). Rates of different transitional substitutions were obtained, where the highest value was 18.6764 for substitution T with C, whereas that rate of transversionsal substitutions was highest for T with A, and T with G (6.0471 each).

Table 6. Maximum likelihood fits of the best nucleotide substitutions model selected for calculating the nucleotide frequency, substitutions, and further evolutionary analyses.

					Nucleotide rates of base substitutions for each nucleotide pair[z]											
Model	BIC	AICc	lnL	R		AC	AG	TA	TC	TG	CA	CT	CG	GA	GT	GC
K2-18S	2648.297	2384.383	-1151.891	1.23	0.056	0.056	0.138	0.056	0.138	0.056	0.056	0.138	0.056	0.138	0.056	0.056
T92-COI	1826.37	1640.59	-788.965	1.34	0.067	0.032	0.098	0.067	0.098	0.032	0.067	0.205	0.032	0.205	0.067	0.032
T92-ITS	2089.57	1898.025	-919.854	1.53	0.054	0.044	0.136	0.054	0.136	0.044	0.054	0.168	0.044	0.168	0.054	0.044

[z]K2 = Kimura 2-parameter; T92 = Tamura 3-parameter; BIC = Bayesian information criterion; AICc = Akaike information criterion corrected value; lnL = Maximum Likelihood value; R = Assumed or estimated value of transition/transversion rate for each model.

DISCUSSION

Twelve populations of *N. aberrans s.l.* isolated from Guanajuato and Michoacan, Mexico, were examined using molecular tools to study their genetic variability. The ISSR test consistently supported the presence of highly divergent specimens at the DNA level and a genetic similarity index, which ranged from 0.44 to 0.82. This analysis revealed a high genetic variability between the populations that were studied, possibly as result of their mode of reproduction. Lax *et al.* (2007) reported similar results within Argentinean populations of *N. aberrans s.l.* with ISSR loci (95% of total polymorphism detected), indicating that one possible source of these levels of polymorphisms might be related to the reproduction mode of the nematode. According to Huang *et al.* (2012), this may be due to one individual *N. aberrans* male that could mate with different females. Occasionally, several males would attempt to mate with the same female at the same time, and these multiple matings would favor offspring with higher genetic diversity. There is controversy in the reproduction behavior of this species. Anthoine and Mugniéry (2005b) stated that amphimixis is the only reproduction mode in *N. aberrans* while Jones *et al.* (2013) indicated that there is uncertainty as to whether the nematode is compulsorily amphimictic or whether facultative parthenogenesis is possible.

The high level of genetic variability found in *N. aberrans s.l.* populations might also be due to multiple introductions of nematodes from different geographical origins (Plantard *et al.*, 2008). This is possible if we consider that, the rental of agricultural machinery and the exchange of propagative plant material are common activities in these areas of study although it is difficult to elucidate the contribution made by agricultural commerce 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 could be generated by passive transport of nematodes across fields by human activities or by water (*e.g.*, floods, irrigation, etc.) and wind (Plantard and Porte, 2004).

The Romita population associated with pepper was clearly separated from the rest of the isolates according to ISSR results. This population came from an important agricultural area and it is geographically isolated from other populations in Guanajuato and Michoacan. This geographic barrier may limit inter-population gene flow (Curran *et al*., 1985), thus generating specimens with different genetic features than other populations. However, according with Curran and Webster (1989), the differences in polymorphic profiles cannot be used as the sole criterion for species delimitation.

The 18S rDNA region was highly conserved and alignment allowed the molecular identification of our populations. This region showed very slight or no variation to nucleotide level, as was also reported by Anthoine and Mugniéry (2005a) and Vovlas *et al.* (2007) in South American populations of *N. aberrans*. The nucleotide dissimilarity in *N. aberrans s.l.* 18S sequences, when compared with related sequences of GenBank, ranged from 0 to 5%. Maximum interspecific differences were observed in populations of Romita, Santa Elena, Yurecuaro, Tanhuato 81, 85, and 86 (3-5%); similar data were reported by Ibrahim *et al*. (1997) comparing isolates from Mexico, Peru, and Argentina. The consensus tree obtained with the ML analysis was strongly supported (99% bootstrap value) without showing any intra- or interspecific variation. All *N. aberrans* populations were clustered in a monophyletic clade that allowed confirmation that specimens collected from Guanajuato and Michoacan belong to the same species, being clearly separated from *M. incognita* and *P. penetrans* used as outgroup taxa. The phylogenetic relationships among nematodes using DNA sequence data from the 18S rDNA have been reported (Blaxter *et al.*, 1998).

The ITS region is known to be useful as a target for species identification, and for inferring phylogenetic relationships between nematodes (Chilton *et al*., 2001; Andrade *et al*., 2013). In this study, the sequence analysis of ITS-rDNA revealed relatively low intraspecific variation in rDNA

of *N. aberrans*. This variation might result from differences in the rates of ITS evolution occurring within the nematodes (Nyaku *et al.*, 2013). Other possible explanation for the observed ITS variation might be related to the presence of several major rDNA gene loci in the genome of *N. aberrans* (Huang *et al.*, 2012). Variation within this region also was observed by Tilahun *et al.* (2008) and Nyaku *et al.* (2013) in *Rotylenchulus reniformis*.

Indels and substitutions were observed in our ITS sequences after multiple sequence alignment was performed. According to Dixon and Hillis (1993), mutation rates can occur at higher rates in loops and stems of rRNA, while according with Huang *et al.* (2012), base transition and transversion of rDNA may result from different individuals of the populations or from different operons of the same nematode. These differences allowed separating our populations of *N. aberrans* with the reference sequences obtained from the GenBank of this species. The estimate of evolutionary divergence among the sequences showed more divergence in ITS sequences than 18S sequences mainly in the rate of nucleotide transition/transversion of nucleotide substitutions model (T92).

The cytochrome oxidase I (COI) gene was used in this study because it is currently one of the most used and accepted in nematology (Hugall *et al.*, 1994; Floyd *et al*., 2002; Elsasser *et al*., 2009; Ferri *et al*., 2009; Derycke *et al.*, 2010; Siddall *et al*., 2012) for species identification because of its high rate of change, and the fact that it is used as a biological barcode (Floyd *et al*., 2002; Hebert *et al.*, 2003). Our COI sequences were shown to be highly rich in adenine and contained relatively high levels of nucleotide variation. Derycke *et al.* (2010) reported similar results in marine nematodes. The ML analysis using COI mtDNA sequences was able to differentiate our *N. aberrans* populations in a monophyletic clade with three subclades strongly supported (100% bootstrap value). Its analysis showed that these populations shared a common maternal ancestor quite recently. The grouping obtained shared certain characteristics with the clustering generated with the ISSR analysis, and each one contained different isolates from different geographical areas and hosts. This could be due to a combination of recent microevolutionary changes (Avise *et al.*, 1987) or to a local differentiation through selection among parthenogenetic lines, since the migration of this nematode is a very slow phenomenon and dispersal depends on different mechanical media (Moritz *et al.*, 1992; Whipple *et al.*, 1998).

The alignment of the COI sequences of nematode specimens from Guanajuato and Michoacan revealed more indels than ITS sequences. The genetic structure of these sequences was characterized by relatively high nucleotide variation among the twelve populations of *N. aberrans s.l.* (1-2.2 in the ratio of thymine, adenine, guanine, and cytosine). This was also reported by Ramírez-Suárez (2011) when analyzing the genetic structure of D2-D3 and COII/16S sequences from nematodes in Mexico's central region and the Argentinean lowland region, suggesting that those isolates might be the result of recent expansion from relatively few individuals. The ML tree obtained with the 18S, ITS, and COI sequences showed a similar topology to those generated with MP method (data not included). Both ML and MP trees revealed genus-specific clusters supported by strong bootstrap values (85-100% bootstrap values).

The present study shows that the Kimura 2-parameter (K2p) and Tamura 3-parameter (T92) methods were the best substitution pattern test of homogeneity between sequences to compute evolutionary distances between the populations studied. ISSR analysis on the Romita population showed clearly that it was highly divergent from the rest, but it shares genetic features with Yurecuaro and Pajacuaran populations. However, the sequence analysis of the 18S and ITS rDNA region indicated that it corresponds to the *N. aberrans* complex.

These results suggest that there are at least two different biotypes of *N. aberrans* in the sampled areas, and according to Huang *et al.* (2012), the hybridization, multiple introductions, passive dispersal by anthropogenic activities, and some natural means would probably be responsible for the genetic variation of *N. aberrans*. Our results also show that analysis based on mitochondrial and ribosomal genes can be useful for describing phylogenetic relationships in the genus *Nacobbus*.

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