J. Agr. Sci. Tech. (2018) Vol. 20: 1293-1305

Prevalence, Identification, and Molecular Variability of Potato Cyst Nematodes in Algeria

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ABSTRACT

The aim of the present study was to evaluate the geographical distribution, infestation degree, and diversity of Potato Cysts Nematode (PCN) in Algeria, including the southern regions. Accurate identification of PCN is essential to determine the appropriate control methods to be used in an Integrated Pest Management program. PCNs were found in forty percent (12 out of thirty) of localities sampled. The average population density of PCN was much higher in the southern regions, compared to the northern regions (9.8 cysts per 100 cm^3 vs.4.6 cysts per 100 cm^3). The southern potato production areas were more infested with PCN than those of the north (7 from 25 fields in North vs. 5 from 5 fields in the South). *Globodera pallida* occurred predominantly in the northern region of Algeria, whereas *G. rostochiensis* occurred predominantly in the southern regions. No mixtures of these species were found in any of the positive studied localities. These species were confirmed by the molecular analysis based on PCR with species-specific primers, ITS-rDNA, and *cytochrome b* of mtDNA. The low molecular diversity and their phylogenetic association with the European populations of PCN suggest that Algerian populations were probably introduced from Europe, probably by infested seed-potato.

Keywords: *Globodera pallida*, *G. rostochiensis*, Molecular markers, PCN distribution, PCN population density.

INTRODUCTION

Production of potatoes (*Solanum tuberosum* L.) occupies an important place in the economy of Algeria. It is cultivated on about 156,176 ha, with a production of 4,673,516 tons, and a yearly yield of 29,920 kg ha^{-1} on average (Algerian Ministry of Agriculture, 2014).There are 3 major potato production zones in Algeria: i) the littoral and sub littoral, ii) the Tellien Atlas and High Plains, and iii) South region. These areas produce 61, 17, and 8.2% of the national potato production, respectively (Technical Institute for Vegetable and Industrial Crops, 2013). However, this crop is affected by several diseases and pests (Mohammadi *et al*., 2001; Mansoori and Smith, 2005; Ashouri, 2007; Yardimci *et al*., 2015), in particular, the potato cyst nematodes (PCNs) *Globodera pallida* (Stone, 1973) Behrens, 1975 and *G. rostochiensis* (Wollenweber, 1923) Behrens 1975. These constitute the most important nematode pathogens, because they can cause considerable damage on potato crop yield, essentially in temperate areas (Winslow and Willis, 1972; Mugnièry, 1984). Worldwide, agricultural losses due to these nematodes could be more than 12% of potato yield (Bates *et al*., 2002).

PCNs were first introduced in Algeria with infested British potato seeds around 1953, and it was established into many regions of Algeria (Frezal, 1954; Scotto La Massesse,

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[DOR: 20.1001.1.16807073.2018.20.6.17.9]

1961). Since that period, PCNs have spread quickly to become a major limiting factor to potato production in several important potato production localities including Aïn Defla, Chlef, Mascara, Sétif, and Tipaza (National Institute of Plant Protection, 2009; Tirchi *et al*., 2016). Recently, a study conducted in the Ain Defla region (Midwest of Algeria) revealed that PCNs, and cereal cyst nematodes are widely distributed in this region and *G. pallida* or *G. rostochiensis* occurred in separate or in mixed populations (Trichi *et al*., 2016). In Algeria, nematode management is usually based on chemical control that threats the human health, and the environment (Cayrol *et al*., 1992). In addition, they are not economically justified in some cases (Janssen *et al*., 1998; Ijani *et al*., 2000). Thus, rapid and accurate identification of the PCN species is critical for planning control measures, and implementing an appropriate integrated management of these nematodes, such as selecting resistant potato varieties. However, identification by morphological characters is time consuming, and becoming more difficult because of increasing number of species groups (Rivoal *et al*., 2003, Donn *et al*., 2008). For these reasons, several molecular techniques have been developed for PCN identification. The majority of them are based on ribosomal DNA (rDNA), including the Internal Transcribed Spacer (ITS) region (Vrain *et al*., 1992; Wendt *et al*., 1993; Zijlstra *et al*., 1995), and on mitochondrial DNA (mtDNA), including the *cytochrome b* gene (cytb). ITS-rDNA sequences can be used to distinguish between many nematode taxa and are used as barcoding regions for many species of cyst nematodes, and to clarify phylogenetic relationships between them (Subbotin *et al*., 2001). Amplification of the ITS regions of rDNA and restriction enzyme digestion of this PCR product (PCR-RFLP) has frequently been used for the identification of cyst nematodes (Thiéry and Mugniéry, 1996; Subbotin *et al*., 2000; Rivoal *et al*., 2003; Madani *et al*., 2004; Abidou *et al*., 2005). Bulman and Marshall (1997) reported on the successful use of species-specific primers in a multiplex PCR to amplify diagnostic ITS-rDNA sequences for potato cyst nematodes. Other methods include the use of Random Amplified Polymorphic DNA (RAPD) fragments (Fullaondo *et al*., 1999) and real-time approaches for identification and quantification (Reid *et al*., 2015).

Little is known about the prevalence and distribution of PCNs on the major potato production zones in all Algeria, in particular in the southern region of the country. For this reason, the objectives of this study were: (i) To conduct an extensive nematode survey on different regions of Algeria and to determine the prevalence, distribution, and infestation degree in each area, and (ii) To determine the molecular variability among the PCN populations in Algeria using ITS-rDNA and *cytb* gene of mtDNA sequences.

MATERIALS AND METHODS

Localities Surveyed and Sampling Procedures

Thirty samples were collected using a zig-zag pattern from different potato production areas of Algeria from 2013 to 2016 (Figure 1). Sampling was carried out in the littoral, sub-littoral, Telien Atlas, and south of Algeria. The sites were located at Algiers, Blida, Tipaza, Boumerdés, Chelef, Ain defla, Skikda, Tlemcen, Bouira, Bechar, and El Oued. Each soil sample was obtained using a shovel at a depth of 10-20 cm. The collected samples were placed in a plastic bag, mixed, dried, and divided into 12 sub-samples of 500 g, and kept in a refrigerator at 4°C until nematode extraction.

Nematode Extraction, Counting and Data Analysis

Cysts were extracted from soil using a Fenwick can (Fenwick, 1940). After extraction, cysts were counted and separated from soil debris and other organic materials

[DOR: 20.1001.1.16807073.2018.20.6.17.9]

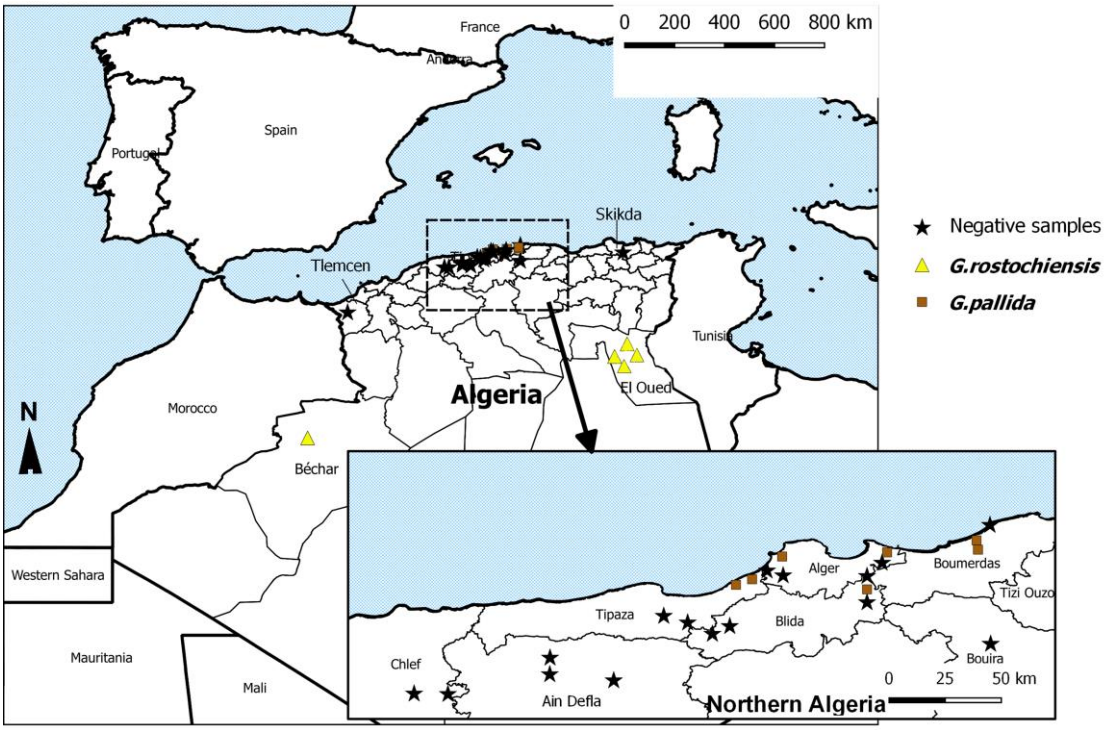

Figure 1. Map of Algeria showing the collection sites of PCN and the identification results using multiplex PCR.

retained on the filter paper using a stereomicroscope. Cysts filled with eggs, and second-stage Juveniles (J2s) were selected and stored dry in tubes at 4°C for molecular identification. The data were analyzed according to Norton (1978) and Wolfgong (1991). The prevalence of nematode populations were estimated based on three factors: (a) The population density, expressed by the average number of cysts in 100 cm^3 of soil; (b) The frequency of nematode species, determined by the relationship between the numbers of samples containing nematodes divided by the total number of collected samples multiplied by 100; and (c) The infestation degree= The number of juveniles-eggs per g of soil calculated as the average number of cysts containing samples in each locality multiplied by the average number of juveniles and eggs/cyst of this locality (an average of ten cysts were used for counting the number of juveniles/cyst) and divided by 500 g.

DNA Extraction

DNA was extracted from individual cysts, and several cysts, at least 5, per sampling point were molecularly identified. Each cyst was crushed and juveniles were cut with a scalpel blade under the stereomicroscope using extraction buffer on glass slide, the cut nematodes from the individual cyst were transferred to a tube containing 20 µL nematode extraction buffer (Thomas *et al.*, 1997). Two µL of proteinase K (600 µg mL^{-1}) were added to each tube and were incubated for 1 hour at 65°C followed by 95°C for 10 minutes.

PCR Reactions and Sequencing

Specific PCR for PCN identification was carried out in a final volume of 25 µL reaction. Two microliters of the DNA

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extract were used for each PCR reaction. PCR reactions were performed as described by Bulman and Marshall (1997). Negative (PCR grade water), and positive controls (*G. pallida* and *G. rostochiensis* kindly provided by Dr. Blok, The James Hutton Institute) were included. All PCR primers used in this study are listed in Table 1. ITS-rDNA fragment amplification was performed using the TW81 and AB28 primers as described by Joyce *et al*. (1994). Two specific primers for *G. pallida cytochrome b* were used to amplify an 872 bp fragment as described by Picard *et al*. (2007). The PCR products were loaded on a 1% agarose gel, separated by electrophoresis, and visualised by ethidium bromide staining and UV illumination. Amplification products were cleaned up and sequenced on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems), at the Stab Vida sequencing facilities (Caparica, Portugal). Sequences were compared to known sequences in the public databases by means of the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table1. Primers used in the present study.

Primer name	Sequence (5'-3')	Amplified region	Size of fragment amplified (bp)	Source
ITS5	*5'-GGAAGTAAAAGTCGTAACAAGG-3'*	18S rRNA gene and ITS1 region		White *et al*. (1990)
PITSp4	*5'-ACAACAGCAATCGTCGAG-3'*	18S rRNA gene and ITS1 region	*G. pallida*-265 *G. rostochiensis*-434	Bulman and Marshall (1997)
PITSr3	*5'-AGCGCAGACATGCCGCAA-3'*	18S rRNA gene and ITS1 region		Bulman and Marshall (1997)
TW8I	*5'-GTTTCCGTAGGTGAACCTGC-3'*	ITS	1.100	Joyce *et al*. (1994)
AB28	*5'-ATATGCTTAAGTTCAGCGGGT-3'*	ITS		Howlett *et al*. (1992)
INRAcytbL	*5'-GGGTGTGGCCTTGTTATTTC-3'*	*cytb* of mtDNA	872	Picard et *al*. (2007)
INRAcytR	*5'-ACCAGCTAAAACCCCATCCT-3'*	*cytb* of mtDNA		Picard et *al*. (2007)

Nematode Molecular Identification, Diversity and Phylogenetic Analysis

ITS-rDNA and *cytb* gene of mtDNA sequences of different cyst nematodes from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to Madani *et al*. (2010). Multiple alignments of the different genes were made using the Q-INS-i algorithm of MAFFT v.7.205 (Katoh and Standley, 2013). Sequence alignments were manually edited using BioEdit (Hall, 1999). Percentage similarity between sequences was calculated using the sequence identity matrix in BioEdit. Phylogenetic analyses of the sequence data sets were performed based on Bayesian Inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003).

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The best fitted model of DNA evolution was obtained using Jmodel Test v.2.1.7 (Darriba *et al*., 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis under a General Time Reversible of Invariable sites and a Gamma-shaped distribution (GTR+I+G) model for ITS dataset, and a Transition Model of Invariable sites, and a Gamma-shaped distribution (TIM1+I+G) model for the *cytb* gene of mtDNA were run with four chains for 1×10^6 generations, respectively. The Markov chains 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 consensus tree. Bayesian Posterior Probabilities (BPP) were given on appropriate clades. Trees from all analyses were visualized using FigTree software version v.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Nematode Sampling and Occurrence of PCNs

PCNs were found in 12 out of thirty localities sampled in Algeria with a prevalence of 40% (Table 2). In the northern regions (Mefteh 2, Lagata, Mandoura, H'rawa, Bou Ismail, Douaouda and Staouali), PCNs were found at average density of 4.60 cysts per 100 cm^3 (ranging from 3 to 11.5 cysts) and an infestation degree from 10.7 to 43.7 juveniles and eggs per 100 cm^3 of soil. However, in the south of Algeria (Al Abadella, Trifaoui 1, Trifaoui 2, Hassi khelifa 2 and Hassi khelifa 1), the average population density was 9.76 cysts per 100 cm^3 (ranging from 6.4 to 13.4 cysts) and an infestation degree from 67.62 to 98.22 juveniles and eggs per 100 cm^3. There was not a noteworthy variability in infestation degree and cyst number per 100 cm^3 of soil among localities (Table 2).

Nematode Molecular Identification, Diversity, and Phylogenetic Analysis

PCNs were detected and identified in six provinces of Algeria (Algiers, Boumerdés, Blida, Tipaza, Bechar and El Oued) (Figure 1; Table 2), represented by twelve localities. No mixtures of both species were found in any of the positive localities. In Algeria, *Globodera pallida* occurred predominantly in the northern region (Algiers, Blida, Tipaza and Boumerdés), whereas *G. rostochiensis* occurred predominantly in the southern regions (El Oued and Bechar).

Amplification of ITS-rDNA region from *G. pallida* and *G. rostochiensis* yielded a single fragment of approximately 1,100 bp, and the amplification corresponding to *cytb* gene of mtDNA from *G. pallida* yielded a single fragment of approximately 1,000 pb. No intraspecific sequence diversity (uncorrected p-distance) was found among the six ITS sequences from *G. rostochiensis* (from KY513118 to KY513123) obtained in this study. Similarly, intraspecific diversity of ITS-rDNA from *G. pallida* was very low and ranged from 0.0 to 0.2% (from KY513111 to KY513117). BLAST results showed that ITS-rDNA sequences from *G. pallida* and *G. rostochiensis* were matched well with other sequences from these species deposited in GenBank. Intraspecific sequence diversity was from 0.0 to 1.8% and from 0.2 to 0.9%, respectively, for *G. pallida* and *G. rostochiensis*. Eight new *cytb* sequences from *G. pallida* were obtained in the present study (KY513124-KY513131) and no intraspecific sequence diversity was found, although high intraspecific sequence diversity values are common amongst other *G. pallida* sequences deposited in GenBank, where the values range from 0.0 to 11.0%, with the highest values corresponding to South American sequences.

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Table 2. Codes and origin of potato cyst nematode populations used in this study.

Sampling province	Locality	Infestation Degree (ID) Juveniles g^{-1} of soil	Cysts per 100 cm^3 of soil	Geographical coordinates	Altitude (m)	Nematode species	Population code	Potato cultivar	GenBank accessions[a] ITS	GenBank accessions[a] *cytb*
Algiers	Staouali	43.7	11.5	36°45'5.943''N 2°53'17,446''E	45	*Globodera pallida*	AC150	Spunta	KY513117	KY513131
	H'Rawa	18.19	3.4	36°46'6.297''N 3°18'31.24''E	23	*G.pallida*	AC146	Désirée	KY513113 KY513116	KY513129 KY513130
Boumerdés	Mandoura	18.15	3	36°49'0.001''N 3°40'0.001''E	38	*G.pallida*	AC121	Désirée	KY513112	KY513126
	Lagata	30.81	3	36°46'45,465"N 3°40'20,873''E	91	*G.pallida*	AC132	Désirée	KY513115	KY513128
Blida	Meftah2	12.19	2.4	36°37'11.234"N 3°13'41.513''E	101	*G.pallida*	AC125	Timate	KY513114	KY513127
Tipaza	Bou Ismaîl	19.95	5.8	36°38'17.517"N 2°42'12,087''E	118	*G.pallida*	AC114	Désirée	KY513111	KY513124
	Douaouda	10.70	3.16	36°40'42.889"N 2°47'50.148''E	71	*G.pallida*	AC116	Désirée	*	KY513125
Bechar	Al Abadella	72.83	6.4	31°0'36.825''N 2°44'35.311''W	780	*Globodera rostochiensis*	AC136	Désirée	KY513118	-
El Oued (Oued souf)	Trifaoui1	67.62	8.4	33°25'10.753"N 6°56'8.426''E	71	*G.rostochiensis*	AC167	Spunta	KY513121	-
	Trifaoui 2	93.5	10	33°25'10.753"N 6°56'8.426''E	71	*G.rostochiensis*	AC160	Spunta	KY513122 KY513123	-
	Hassikhelifa1	98.22	13.4	33°33'36.455"N 6°59'26.59''E	51	*G.rostochiensis*	AC140	Bartina	KY513119	-
	HassiKhelifa2	98	10.6	33°33'36.455"N 6°59'26.59''E	51	*G.rostochiensis*	AC157	Spunta	KY513120	-

[a] (-) Not obtained, (*) Sequenced population but not deposited in GenBank database because of their high similarity with others.

The 50% majority rule consensus phylogenetic trees generated from the ITS-rDNA, and the partial *cytb* alignments are presented in Figures 2 and 3. The 50% majority rule BI tree of a multiple alignment including 212 ITS-rDNA sequences and 928 bp long showed two highly supported (BPP= 100) major clades, separating the two species, *G. pallida* and *G. rostochiensis* (Figure 2). Species identified as *G. pallida* occupied a superior position within the tree and showed similar topology to the *cytb* tree (Figure 3). ITS-rDNA sequences from Peru and Chile tend to cluster forming two different sub-clades, one of them not well supported, however, some sequences from the UK and one GenBank accession from Algeria (LT159838) clustered within these sub-clades. On the other hand, five sequences from Peru clustered outside these sub-clades with sequences from around the world. ITS sequences from *G. rostochiensis* occupied a basal position in the tree, forming a unique major clade (PP= 86), any sub-clade were shown but not well supported (Figure 2). Only one GenBank accession for *G. rostochiensis* (GU084809, Bolivia) clustered separately.

The 50% majority rule consensus *cytb* gene BI tree of *G. pallida* based in a multiple edited alignment including 56 sequences and 1,020 total characters showed three clearly separated (PP= 1.00) major clades (Figure 3). Clade I grouped all *cytb* sequences from Algeria, Europe, Canada, USA and 4 GenBank accessions from Peru (AY851639, AY851647, AY851648, and AY851641). Clade II and III were formed by the rest of sequences from Peru (Figure 3).

DISCUSSION

This study provides valuable new information on the distribution and frequency of PCNs in Algeria. However, our investigation indicated the presence of PCNs in samples of six provinces and twelve localities including Staouali, Mandoura, Lagata, H'rawa, Meftah2, Bou Ismail, Douaouda, Al Abadella, Trifaoui1, Trifaoui2, Hassi khelifa1 and Hassi Khelifa 2.

This research showed a clear separation, in geographical distribution, between *G. pallida* and *G. rostochiensis*, the former being more prevalent in northern areas while the latter was in the southern part of the country. This study increases our knowledge about the prevalence, distribution, and molecular diversity of PCNs in Algeria concerning recent studies that were focused on five northern localities on Ain Defla province only (Tirchi *et al*., 2016). Interestingly, in our case, even identifying more than 5 cysts per locality, only single species population per locality was found, whereas Tirchi *et al*. (2016) detected mixed populations based mainly on morphometric measurements such as Granek's ratio. Two main explanations could be plausible for this distribution: i) the major tolerance of *G. rostochiensis* to higher temperatures developing in southern regions of Algeria, while *G. pallida* could reproduce better at lower temperatures (Kaczmarek *et al*., 2014) in the northern regions; and ii) the repeated use of cultivars resistant to *G. rostochiensis* ('Spunta' and 'Bartina') in the southern region. The distribution and predominance of one of the two species depends on the susceptibility or resistance of the potatoes grown in successive rotations (Jones, 1979). Even using resistant cultivars, the southern regions have shown higher levels of infestation than the north, which could be the result of a selection to resistant nematode pathotypes to these cultivars, probably because of the monoculture of potato in the south. These resistant cultivars, i.e. 'Spunta' and 'Bartina', are susceptible to PCN Ro1 and Pa2 and Pa3, respectively, and to the other pathotypes to a lesser extent (https: //www.hzpc.com).

The low molecular diversity and their association with the European populations of PCNs suggest the close relationship with those populations, probably by infested-seed potato in few introductions. This result is

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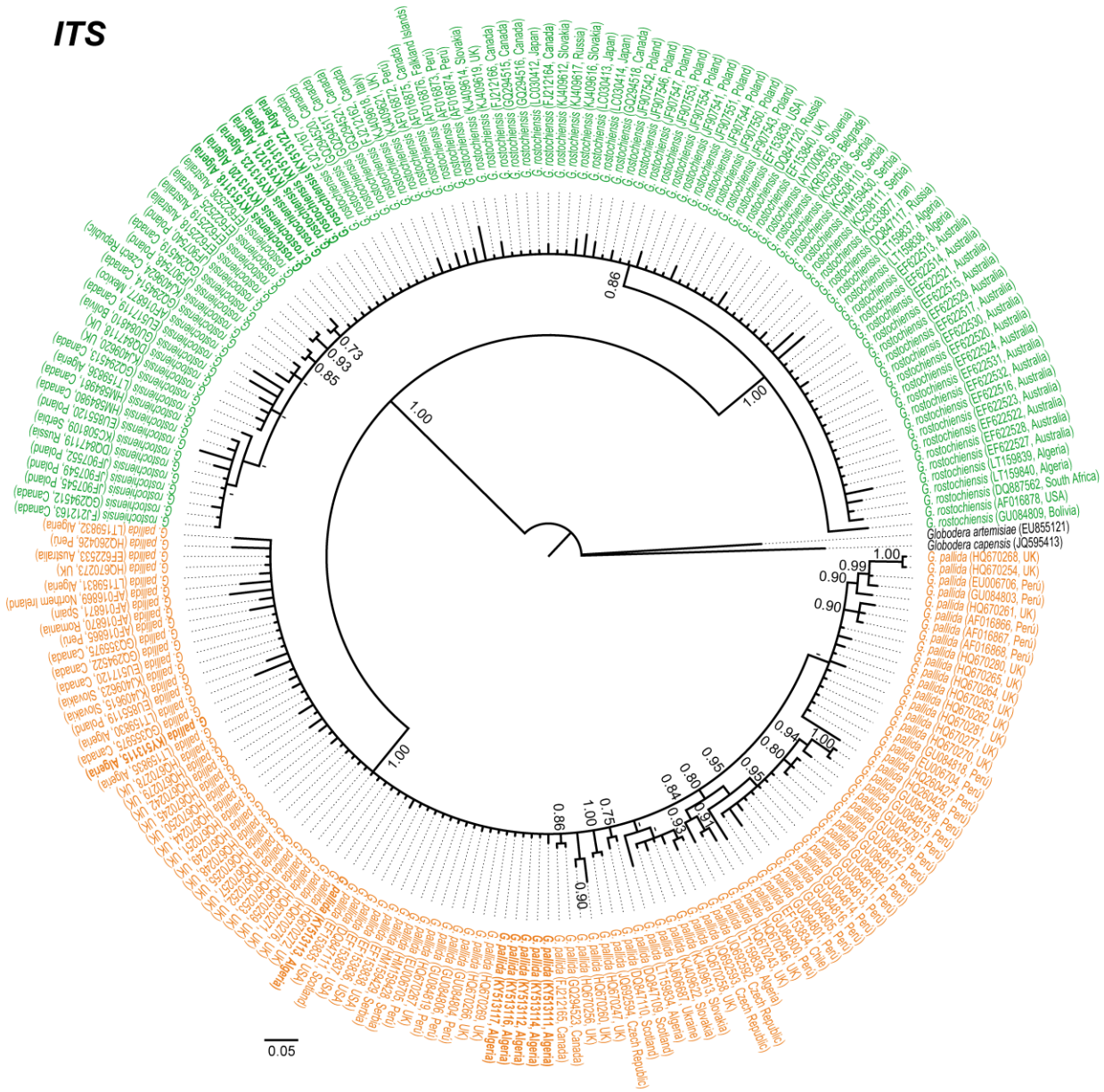

Figure 2. Bayesian 50% majority rule consensus tree of ITS-rDNA containing region describing the evolutionary relationships among different geographical populations of PCN (*Globodera pallida* and *G. rostochiensis*). Alignments under the GTR+I+G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are in bold.

different from the important variability found by Tirchi *et al*. (2016) in the ITS-rDNA region, used for sequencing cloned PCR products. In our case, the depurated sequences were based on direct PCR sequencing, obtaining only the majoritarian haplotypes present in the genomes. Cloning and sequencing of PCR products could show more intra-individual variability because of the intra-genomic variation in the ribosomal repeats, while, in our case, only the majoritarian repetition was sequenced. In fact, it has been documented that this locus is affected by the process of concerted evolution, which is a homogenizing force

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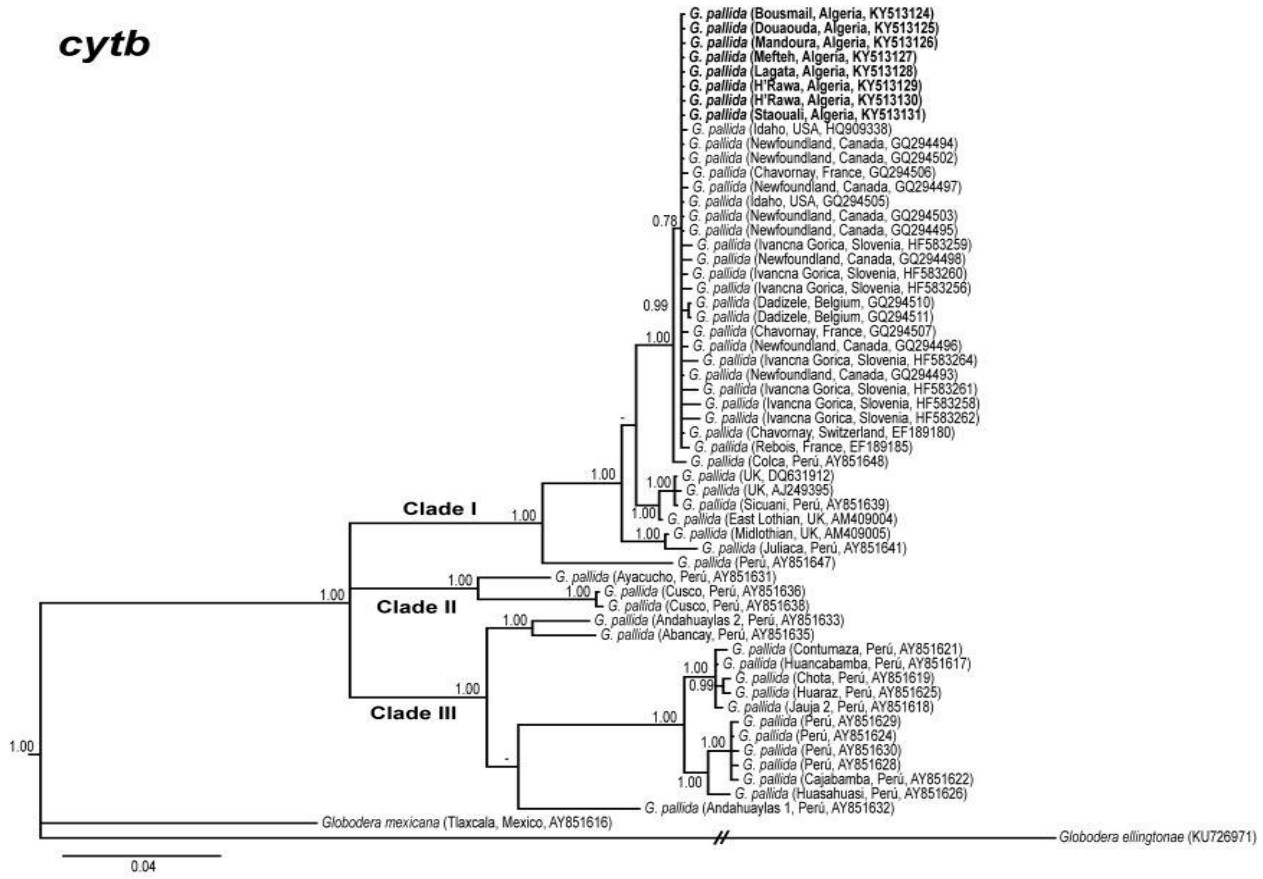

Figure 3. Bayesian 50% majority rule consensus tree of *cyt b* gene of mtDNA describing the evolutionary relationships among different geographical populations of *Globodera pallida*. Alignments under the TIM1+I+G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are in bold.

across rRNA repeats within a genome (Dover, 1982).

In summary, the results of this survey revealed the differential prevalence and distribution of *G. rostochiensis* and *G. pallida* in potato production areas of Algeria, as well as the molecular identification and their phylogenetic relationship with European populations. The high infestation of PCNs in some areas, even using resistant cultivars, makes this species a severe threat to potato production, particularly in the south of the country, which is considered nowadays among the first production areas of potato in Algeria. For this reason, correct identification of the nematode species needs to be emphasized in order to find the most suitable control methods, which should use the safe natural crop production methods such as resistant cultivars, rotations, and biological control using bacterium, fungi, and plant extracts. Additional studies about these nematodes of more variable and informative regions, especially in the Algerian Sahara, are required to allow the construction of a complete picture of the distribution of PCN in Algeria.

ACKNOWLEDGEMENTS

This research was financially supported by the National Upper School of Agronomy (ENSA), El Harrach, Algiers, and the Ministry of Higher Education and Scientific Research Algeria.

[DOR: 20.1001.1.16807073.2018.20.6.17.9]

The first author thanks the Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Spain for the help. Also, all authors thank Mr. Guillermo León for the excellent technical assistance.

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شیوع، شناسایی، و تغییرات ملکولی نماتد کیست سیب زمینی در الجزایر

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چکیده

هدف این پژوهش ارزیابی توزیع جغرافیایی، درجه آلودگی، و تنوع نماتد کیست سیب زمینی (PCN) در الجزیره و منجمله بخش های جنوبی آن بود. شناسایی درست PCN برای تعیین روش مناسب کنترل این آفت در برنامه یکپارچه مدیریت آفات ضروری است. PCN های مزبور در ۴۰٪ مناطق نمونه برداری شده (۱۲ منطقه از ۳۰ تا) حضور داشت. میانگین تراکم جمعیت PCN در مناطق

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جنوبی بسیار بیشتر از مناطق شمالی بود(۹/۸ کیست در cm^3 ۱۰۰ در مقایسه با ۴/۶ کیست در cm^3 ۱۰۰). نواحی جنوبی تولید سیب زمینی آلودگی بیشتری از مناطق شمالی داشت (هفت مزرعه در شمال در مقایسه با ۵ مزرعه در جنوب) . *Globodera pallida* بیشتر در مناطق شمالی الجزیره وجود داشت در حالیکه *G. rostochiensis* بیشتر در مناطق جنوبی بود. هیچ آمیزه ای از این دو گونه در هیچیک از مناطق مطالعه شده مثبت یافت نشد. این گونه ها با تجزیه تحلیل ملکولی بر مبنای PCR با آغازگرهای ویژه-گونه، ITS-rDNA، و *cytochrome b* of mtDNA تایید شدند. تنوع کم ملکولی و همراهی فیلو ژنیک آن ها با جمعیت های اروپایی چنین اشارت دارد که جمعیت های الجزیره ای احتمالا با سیب زمینی های بذری آلوده از اروپا وارد کشور شده اند.

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