

RESEARCH/INVESTIGACIÓN

MOLECULAR CHARACTERIZATION AND DISTRIBUTION OF *GLOBODERA PALLIDA* IN THE MAIN POTATO PRODUCTION AREA OF COSTA RICA

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ABSTRACT

Sandoval-Ruiz, R., L. Flores-Chaves, and D. A. Humphreys-Pereira. 2020. Molecular characterization and distribution of *Globodera pallida* in the main potato production area of Costa Rica. *Nematropica* 50:218-228.

Since the first detection of *Globodera pallida* in Costa Rica in 2005, no further research has been done to study the presence and distribution of potato cyst nematodes in the country. The objectives of this study were to identify with molecular approaches the *Globodera* species and their distribution in Cartago, Costa Rica, and to determine the nematode intraspecific variability. Fourteen populations were collected at three elevations in the north of Cartago. In addition, one *Globodera* population collected from Alajuela (the second largest potato area in the country) was included in the molecular analysis. Fifteen populations were characterized with a segment of the ribosomal DNA, ITS1-5.8S-ITS2 (ITS), and the partial mitochondrial DNA gene, cytochrome b (*cytb*). A unique *G. pallida* haplotype from each marker was identified from 202 *cytb* sequences and 188 ITS sequences, suggesting a single introduction into the country and then spread. The phylogenetic relationships based on the *cytb* marker showed that the *G. pallida* populations from Costa Rica have a unique origin, possibly from the northern region of Peru. A pathotype identification study is suggested for the development of nematode-resistant potato varieties in Costa Rica.

Key words: *Globodera pallida*, potato, haplotype, cytochrome b, internal transcribed spacer, phylogeny

RESUMEN

Sandoval-Ruiz, R., L. Flores-Chaves, and D. A. Humphreys-Pereira. 2020. Caracterización molecular y distribución de *Globodera pallida* en la principal área productora de papa de Costa Rica. *Nematropica* 50:218-228.

Desde la primera detección de *Globodera pallida* en Costa Rica en el 2005, no se han realizado investigaciones para estudiar la presencia y distribución de nematodos del quiste de la papa en el país. Los objetivos de este estudio fueron identificar mediante técnicas moleculares las especies de *Globodera* y su distribución en Cartago, y determinar la variabilidad intraespecífica. Catorce poblaciones fueron muestreadas en tres estratos altitudinales en el norte de Cartago. Adicionalmente, una población de *Globodera* colectada en Alajuela (la segunda principal área productora de papa en el país) fue incluida en el análisis molecular. Las 15 poblaciones se caracterizaron con un segmento del ADN ribosomal, ITS1-5.8S-ITS2 (ITS) y el gen parcial de ADN mitocondrial, citocromo b (*cytb*). Un único haplotipo de cada

marcador fue identificado de 202 secuencias del *cytb* y 188 secuencias del ITS, lo que sugiere un único sitio de entrada y de ahí se distribuyó al resto del área. Las relaciones filogenéticas basadas en el marcador *cytb* mostraron que las poblaciones de *G. pallida* de Costa Rica poseen un único origen, y posiblemente de la región norte de Perú. Se sugiere un estudio de identificación de patotipos para el desarrollo de variedades de papa resistentes al nematodo en el país.

Palabras clave: *Globodera pallida*, papa, haplotipo, citocromo b, espaciador interno transcrito, filogenia

INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are native to South America and are ranked fourth in terms of consumption worldwide (Hijmans *et al.*, 2002; Veilleux and De Jong, 2006; Zaheer and Akhtar, 2016). In 2015, potato was the second most consumed vegetable in Costa Rica, with 18.4 kg per capita, and the most consumed in public schools representing 12.2% of all vegetables, followed by tomato with 10.1% (PIMA, 2016). The government health authorities recommend that 25% of the total carbohydrates in a healthy diet can be supplied with potatoes (PIMA, 2016). In 2019, the largest potato-producing area in Costa Rica was the province of Cartago with 63.5% (2,113 ha) of the total production area (3,328 ha), followed by Alajuela (mostly Zarcero) with 32.2% (1,070 ha) and to a minor extent San José with 3.4% (SEPSA, 2020).

Plant-parasitic nematode infestation can decrease tuber quality and reduced potato yield by more than 25% (Youssef, 2013). The most important plant-parasitic nematode genera associated with potato production are *Meloidogyne*, *Pratylenchus*, and *Globodera* (Brodie, 1999). Potato cyst nematodes (PCN; *Globodera*) are a major problem for the potato industry (Fatemy, 2018; Koirala *et al.*, 2020) and primarily consist of two main species, the white potato cyst nematode, *G. pallida*, and the golden potato cyst nematode, *G. rostochiensis* (Manduric and Andersson, 2003; Boucher *et al.*, 2013). Both species are global quarantine pests (EPPO, 2015) for several reasons: their ability to survive in the soil for long periods (more than 20 years) without a host (Perry *et al.*, 2013); yield losses that can reach 100% (Banks *et al.*, 2012); the presence of pathotypes that reproduce on resistant potato varieties (Schouten and Beniers, 1997; Mwangi *et al.*, 2019) and, their ability to have high reproductive potential (Evans and Stone, 1977).

The first report of a PCN in Costa Rica was in October 1972, in Cartago, with the identification of *G. rostochiensis* (Ramírez and Bianchini, 1973). Later, between 1972 and 1974, 18 potato fields were found positive for *G. rostochiensis*, but pathogenicity assays demonstrated that juveniles did not develop, and there was no infection on susceptible varieties (Atzimba and Red Pontiac) (Ramírez, 1979). It was concluded that local conditions were not conducive for the development of the nematode or the identification of *G. rostochiensis* was questionable (Brodie, 1998). In a survey between 1975 and 1977, *G. rostochiensis* was not found in the same area (Ramírez, 1979). Hence, an international panel of experts certified the country to be free of *G. rostochiensis* (Brodie, 1998). Consequently, the European and Mediterranean Organization for Plant Protection (EPPO) declared the country free of *G. rostochiensis* in 1999 (EPPO, 1999). In 2005, samples from one potato field near the Irazú volcano, Cartago, were processed in the Laboratory of Nematology at the University of Costa Rica (San Jose, Costa Rica), and *Globodera* cysts were found. The cysts were morphologically identified as *G. pallida* (Salazar *et al.*, 2005). However, the identity of this species in the country was not confirmed with molecular tools until 2009 (García *et al.*, 2009).

The potato seed production area in Costa Rica is located at altitudes over 2,500 m.a.s.l. (meters above sea level), mostly to avoid the incidence of diseases (Vásquez *et al.*, 2006). It is in this area where *G. pallida* was found for the first time in the country (García *et al.*, 2009). There are no studies on the distribution of *G. pallida*, its intraspecific variability, and the origin of Costa Rican *G. pallida* populations. Knowing the intraspecific variability of the nematode will allow for a better understanding of the ecology and the evolutionary biology of the species (Cianciaruso *et al.*, 2009). Moreover, practical implications can be obtained through the study of the nematode population

origins because the genetic basis of virulence depend on the site of origin (Blok *et al.*, 1998). It has been suggested that the presence of cryptic species within *G. pallida* exist due to the high genetic diversity found in South Peru, which is attributed to the geographic distances, climate and soil characteristics (Thevenoux *et al.*, 2019). Therefore, it is necessary to determine the genetic diversity of *Globodera* in Costa Rica that could result in different management and quarantine measurements. The objectives of this study were to: i) identify the *Globodera* species in the most important potato production area of Costa Rica, and ii) study the intraspecific variability of the species.

MATERIALS AND METHODS

Sampling and nematode populations

Fourteen potato fields from Cartago and one from Zarcero with the presence of *Globodera* sp. cysts were sampled systematically in a zigzag pattern. A total of five composite samples (five soil subsamples per sample) were collected per potato field with a shovel, at a depth of approximately 0-30 cm. Each composite sample was homogenized and 200 ml of dry soil per composite sample was processed using the Fenwick method (Fenwick, 1940). The 15 populations were located between 1,956 to 3,193 m.a.s.l. (Table 1). In Cartago, seven populations were sampled from the potato seed production area (> 2,500 m.a.s.l.). Each location, with exception of GP-12, was recorded using a Garmin GPSMAP 76CSx (Garmin, Lenexa, KS). A distribution map was created with the location of the populations using ArcMap 10.3 (ESRI, 2015).

DNA extraction

Fifteen to 24 cysts per potato field were randomly picked and placed separately in a Syracuse glass with sterile water. The cysts were opened to release juveniles and one juvenile per cyst was individually transferred to a DNA extraction solution (10 µl water, 10 µl PCR DreamTaq reaction buffer (Thermo Fisher Scientific, Waltham, MA) and 1.5 µl Proteinase K (20 mg/ml; Thermo Fisher Scientific) and were incubated for 16 hr at 57°C and 95°C for 20 min (a modified method from Williamson *et al.*, 1997; D. Humphreys-Pereira, unpublished data).

Analysis of ribosomal DNA and the mitochondrial cytb partial gene

The intraspecific variability of *Globodera* sp. was studied by sequencing a fragment of ribosomal DNA, which includes the ITS1-5.8S-ITS2 region (ITS region), and a partial gene of the mitochondrial genome, cytochrome b (*cytb*). The ribosomal DNA was amplified with primers TW81 (GTTTCCGTAGGTGAACCTGC) (Joyce *et al.*, 1994) and AB28 (ATATGCTTAAGTTCAGCGGGT) (Howlett *et al.*, 1992), whereas the mitochondrial DNA was analyzed with primers INRAcytL (GGGTGTGGCCTTGTTATTTTC) and INRAcytR (ACCAGCTAAAACCCCATCCT) (Picard *et al.*, 2007). The PCR reaction for the amplification of the partial *cytb* gene included 1× iProof HF buffer (Bio-Rad, Hercules, CA), 0.2 mM of dNTP's (Bio-Rad), 0.4 µM of each primer, 1 µl of 25 mg/ml BSA (Thermo Fisher Scientific), 1.25 U of iProof DNA polymerase (Bio-Rad) and 2 µl of the crude DNA extract from a single juvenile in a final volume of 25 µl. The ITS region PCR reaction included 1× Phusion HF Buffer (Thermo Fisher Scientific), 0.2 mM of dNTP's (Thermo Fisher Scientific), 0.4 µM of each primer, 1 µl of BSA (25 mg/ml, Thermo Fisher Scientific), 0.5 mM MgCl₂ (Thermo Fisher Scientific), 1.25 U Phusion DNA polymerase (Thermo Fisher Scientific) and 2 µl of the crude DNA extract from a single juvenile in a final volume of 25 µl. The PCR amplification conditions with primers INRAcytL/INRAcytR were as follows: 98°C for 30 s, followed by 37 cycles at 98°C for 5 s, 54°C for 30 s, 72°C for 40 s and a final extension step at 72°C for 3 min. The amplification conditions with primers TW81/AB28 were 98°C for 30 s, followed by 35 cycles at 98°C for 5 s, 58°C for 30 s, 72°C for 40 s and a final extension step at 72°C for 3 min. All PCR reactions were performed in a Mastercycler® Pro thermal cycler (Eppendorf, Hamburg, Germany). DNA amplification products were visualized by electrophoresis using a 1% agarose gel (1 g of agarose on 100 ml of 0.5X TBE) mixed with DNA Gel Loading Dye 6X (Thermo Fisher Scientific) and GelRed™ (Biotium, San Francisco, CA) following manufacturer's instructions. Electrophoresis was run at 110V for 1 hr and observed in a transilluminator, BioDoc-It2® 315 Imaging System LMS-26 (UVP, Upland, CA). Only strong amplification products checked on gel

Table 1. Geographical location of *Globodera pallida* populations from Costa Rica and number of sequences analyzed per DNA marker.

Population code	Altitude (m.a.s.l.)	Locality	<i>cytb</i>	ITS
GP-01	2833	Santa Rosa, Oreamuno	15	12
GP-02	2886	Santa Rosa, Oreamuno	12	15
GP-03	2619	Santa Rosa, Oreamuno	15	11
GP-04	2631	Near Irazú volcano, Santa Rosa, Oreamuno	11	12
GP-05	2918	La Pastora, Santa Rosa, Oreamuno	16	11
GP-06	2083	Charcalillos, Pacayas	18	21
GP-07	2184	Tierra Blanca, Cartago	24	10
GP-08	2482	Bajo Rojas, Pacayas, Alvarado	15	18
GP-09	2489	Bajo Rojas, Pacayas, Alvarado	12	12
GP-10	2819	Capellades, Alvarado	12	15
GP-11	1956	Buenos Aires, Pacayas, Alvarado	10	17
GP-12	2080	Tierra Blanca, Cartago	15	12
GP-13	3193	Near Irazú volcano, Santa Rosa, Oreamuno	12	-
GP-14	2424	Near Irazú volcano, Santa Rosa, Oreamuno	12	12
GP-15	2108	Zarcelero, Alajuela	3	10
Total No. of sequences ^z :			202	188

^zEach sequence represents a single individual (infective juvenile).

with a size of slightly above 1000 pb were sent directly for purification and bidirectional sequencing with the Sanger sequencing method at Elim Biopharmaceuticals, Inc. (Hayward, CA). At least 10 individuals per population were analyzed for each DNA marker, except for population GP-15 (Table 1). A total of 15 populations were studied with the *cytb* marker and 14 populations with the ITS region.

Phylogenetic Analysis

Sequences were edited with the sequence alignment editor BioEdit (Hall, 1999) and compared with other *Globodera* spp. using BLAST Search (National Center for Biotechnology Information, NCBI, USA). A total of 47 ITS sequences and 40 *cytb* sequences of *Globodera* spp. were retrieved from the GenBank. Sequences were aligned with the MUSCLE algorithm (Edgar, 2004) implemented in Mega 6 (Tamura et al., 2013). The positions with gaps and the ends were eliminated. The JmodelTest program (Guindon and Gascuel, 2003; Darriba et al., 2012) was used to determine the evolutionary DNA model that best fit the data. The Bayesian information criterion (BIC) was used to run the phylogenetic analyses with MrBayes 3.2 program (Ronquist and Huelsenbeck, 2003) under the HKY + G model for both markers. The phylogenetic trees were visualized with FigTree 1.4.3 (Rambaut, 2012).

RESULTS

DNA amplification products of the *cytb* and the ITS gene regions from all *Globodera* sp. populations resulted in a size of ~1000 bp. A total of 202 *cytb* and 188 ITS sequences (each sequence represents one individual) were analyzed from 15 and 14 *Globodera* sp. populations, respectively (Table 1). Based on sequencing, all populations were identified as *G. pallida*, and the analyses of both DNA markers indicated that a single haplotype of *G. pallida* was present in the most important potato-producing areas of Costa Rica (GenBank accession numbers MT834971 - MT834972 for ITS and MT872309 - MT872310 for *cytb*). The *cytb* sequence was identical (100% GenBank identity) with *G. pallida* accession number AY851624 from Otuzco, a region in northern Peru. The ITS region sequence had 100% identity with *G. pallida* Genbank accession number HQ670264 also from South America. The locations positive for *G. pallida* in the north of Cartago were Santa Rosa, Faldas del volcán Irazú, La Pastora, Charcalillos, Tierra Blanca, Pacayas, Capellades and Buenos Aires (Fig. 1; Table 1).

The phylogenetic analysis based on *cytb* (Fig. 2) showed two main clades within *G. pallida* sequences from different countries (Posterior probability, PP = 99%). Clade I was formed with *G. pallida* sequences from Peru and the haplotype from Costa Rica (PP = 94). Clade II included

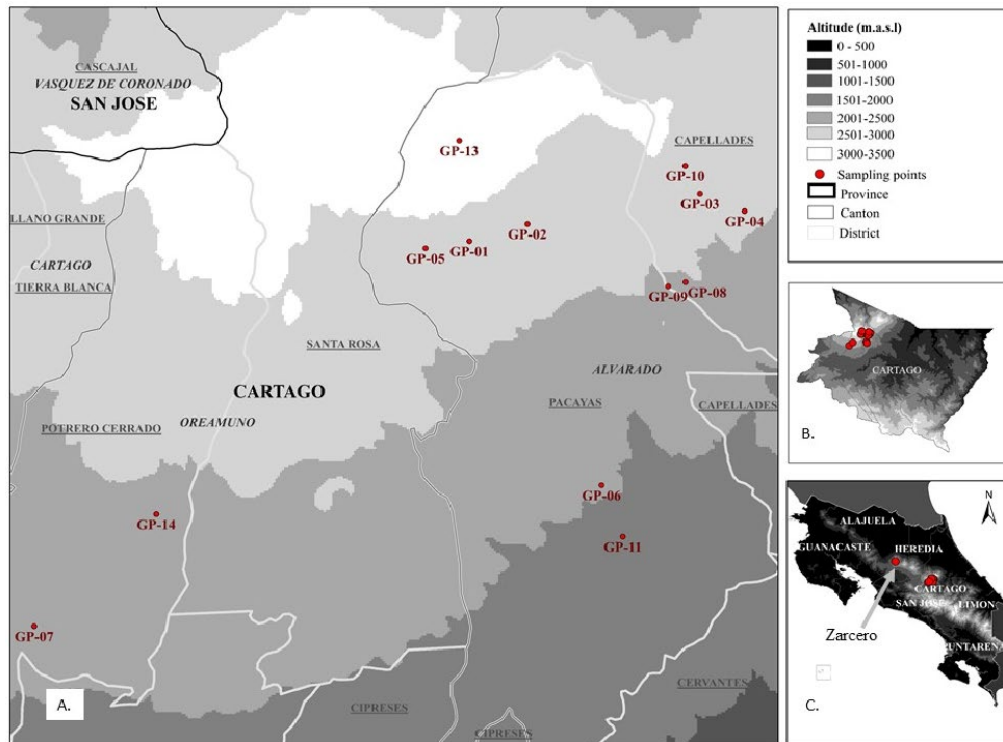


Figure 1. Geographic distribution of *Globodera pallida* populations from Cartago (excepting GP12) and one sample from Zarcerro (Alajuela), Costa Rica. Bolded text represents province, italicized text represents canton, underlined text represents district. Red dots represent sampling locations.

sequences from Europe, North America, and Peru (PP = 100). Clade I was subdivided into three subclades (I.1, I.2 and I.3). Subclade I.1 included the sequence from Costa Rica and from North and South Peru (PP = 100). Subclade I.2 was formed with samples only from North Peru (PP = 99). The third subclade, I.3, corresponded to a sample from South Peru (PP < 80). Clade II was divided into 4 subclades (PP = 100). Subclade II.1 consisted of sequences from Europe, North America, and a sequence from South Peru (PP = 100). Subclade II.2 included the European populations and subclades II.3 and II.4 included samples from the South of Peru.

Phylogenetic relationships based on sequences of the ITS region of *Globodera* spp. showed four main clades (Fig. 3). Clade I (PP = 99%) was formed with *G. pallida* sequences from different potato-producing countries. This clade was subdivided into three subclades: I.1 included populations from the North and South of Peru, an accession from England and the haplotype from Costa Rica (PP = 91%); I.2 was formed with

sequences from Chile, south Peru, Bosnia, and Algeria (PP = 91%); and, clade I.3 consisted of sequences mainly from Europe and North America, also an accession from Japan and one from south Peru (PP = 100%). Clade II consisted of sequences from *G. ellingtonae*, clade III from *G. tabacum*, and clade IV from *G. rostochiensis*.

DISCUSSION

The main potato production region of Costa Rica, the north of Cartago, also includes the potato-seed producing area. *Globodera pallida* was found for the first time in this region of Costa Rica in 2005 (Salazar *et al.*, 2005; García *et al.*, 2009). Due to the value of the region as potato seed producer, the present study molecularly characterized *G. pallida* populations collected from additional locations in the north of Cartago. *Globodera pallida* was the only PCN species identified in the country based on two DNA fragments (one nuclear and one mitochondrial) sequenced from 14 *G. pallida* populations from Cartago and one from

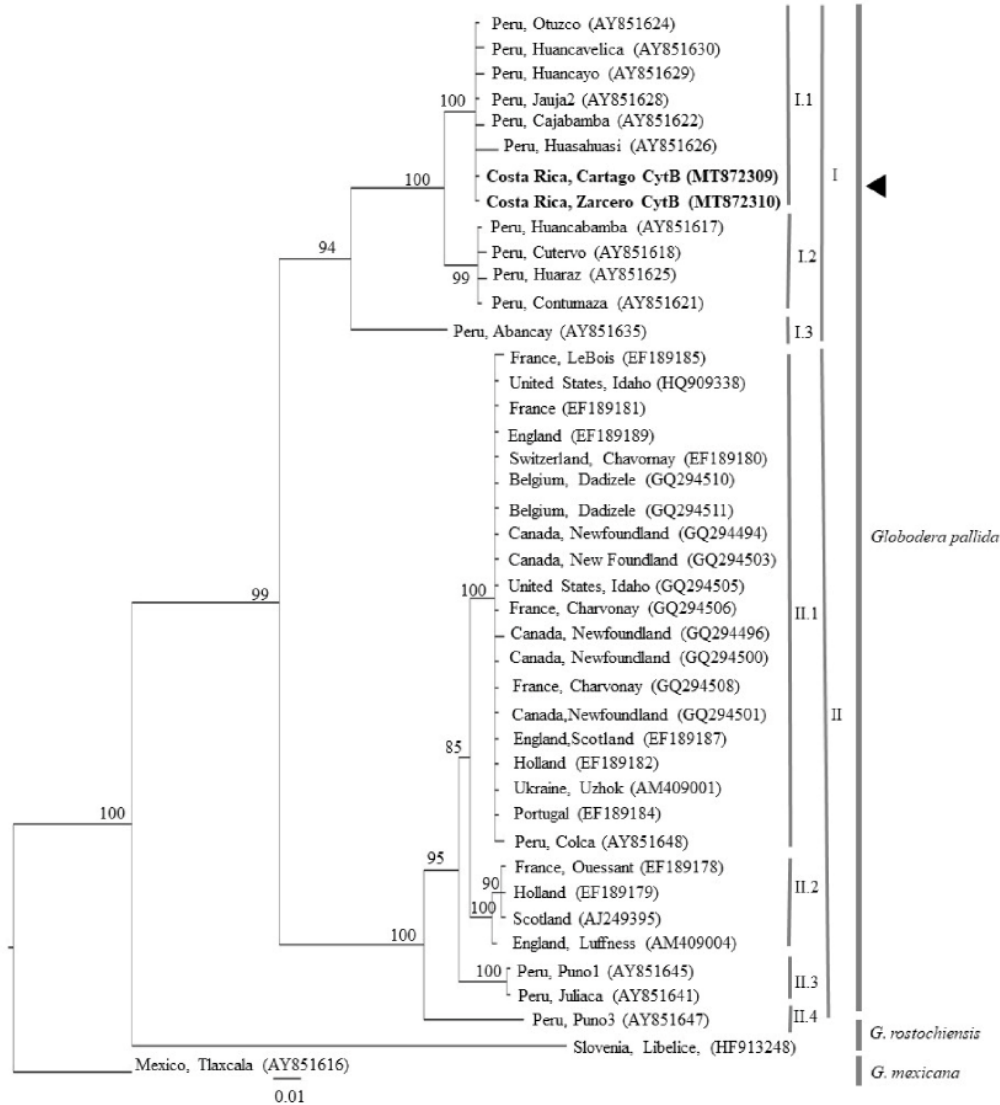


Figure 2. Phylogenetic relationships between *Globodera pallida* from Cartago (GenBank accession number MT872309) and Zarcero (MT872310), Costa Rica and populations from different countries. Bayesian inferred method based on the mitochondrial DNA gene *cytb* gene. The tree was generated with the HKY + G evolutionary model. The posterior probability values are shown in the clades.

Zarcero, the second largest potato production area in the country. The presence of *G. pallida* across the potato seed production area (> 2,500 m.a.s.l.) might explain the wide distribution of the nematode in production fields in the north of Cartago (samples collected between 1,956 and 3,193 m.a.s.l.). Moreover, in the Zarcero region, seed potato production is not common, therefore, it is possible that contaminated material came from the potato-seed producing area in Cartago. A unique *cytb* and ITS *G. pallida* haplotype was found in the country, suggesting a single

introduction of this nematode in Costa Rica and spread to other areas. Therefore, new areas must be explored to produce *G. pallida*-free planting material.

The phylogenetic analysis based on the mitochondrial DNA gene *cytb* showed the *G. pallida* populations from Costa Rica are similar to populations from the North of Peru, and potentially has its origin from this area or from a transiting country near to Costa Rica. The introduction of *G. pallida* to Costa Rica may have occurred independently from the North American, European

and Japanese *G. pallida* introductions that had their origin from southern Peru (Picard *et al.*, 2007; Plantard *et al.*, 2008; Madani *et al.*, 2010; Ohki *et al.*, 2018). The high similarity of the *G. pallida* populations from Costa Rica with populations from northern Peru, could have negative implications because it has been reported that populations from northern Peru reproduce on resistant potato materials (Hockland *et al.* 2012; Mugniery *et al.* 2007). Therefore, strategies for risk assessment

must be implemented constantly at the borders to avoid the introduction of virulent populations.

The presence of a single haplotype based on the *cytb* or the ITS DNA markers suggested that the *G. pallida* populations from Costa Rica are less diverse than the European populations, which displayed several haplotypes based on the *cytb* gene. Plantard *et al.* (2008) found four *cytb* haplotypes in populations from Western Europe, whereas Hockland *et al.* (2012) showed an increase

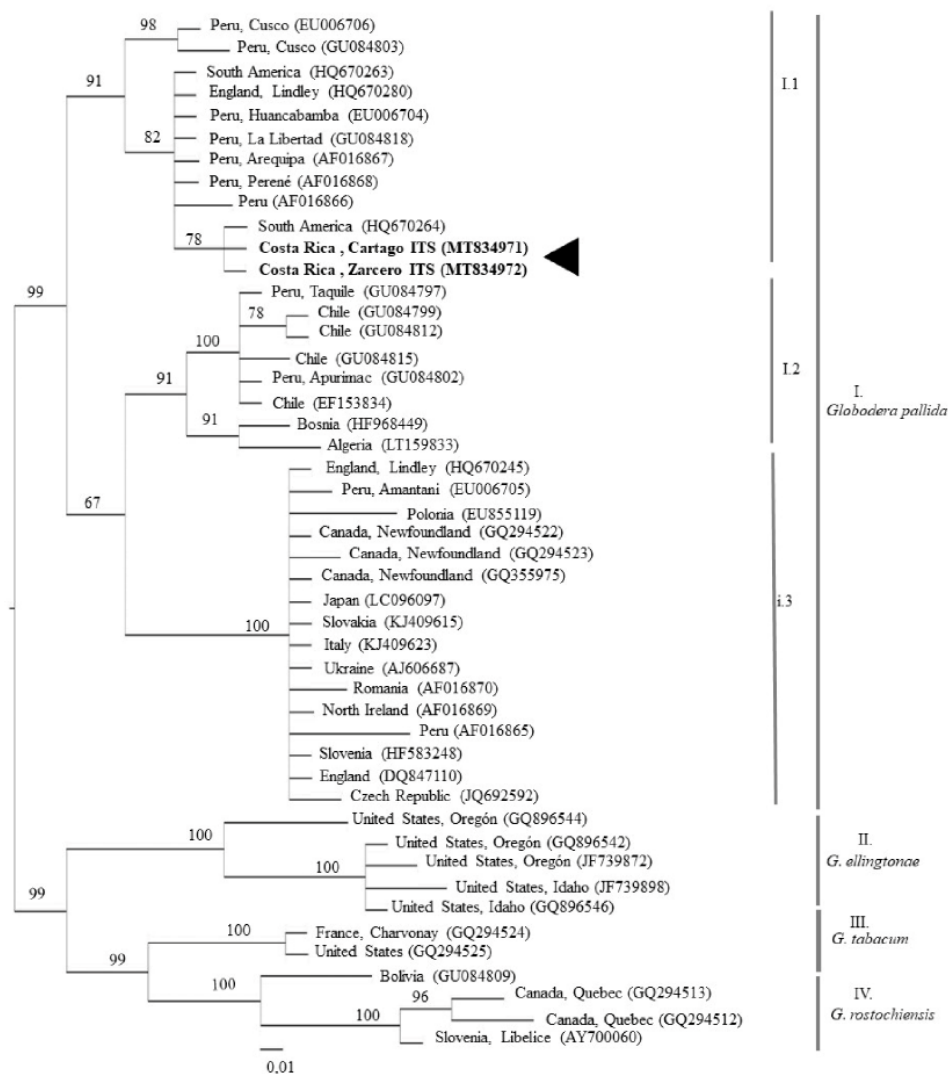


Figure 3. Phylogenetic relationships between *Globodera pallida* from Cartago (GenBank accession number MT834971) and Zarcero (MT834972), Costa Rica and populations from different countries. Bayesian inferred method based on the ITS region of the nuclear DNA. The tree was generated with the HKY + G evolutionary model. The posterior probability values are shown in the clades.

of the haplotype diversity by including more sequences in the analysis. Eves-van den Akker *et al.* (2015) through a large-scale metagenomic approach with DNA samples from 687 potato fields in Scotland identified hundreds of unique *cytb* amplicons that were categorized into three groups or mitotypes. The fact that *G. pallida* from Costa Rica had a low haplotype diversity could indicate limited routes of entry to the country, as reported in Japan with the same nematode (Ohki *et al.*, 2018). Furthermore, the presence of one *cytb* haplotype suggest that the introduction of *G. pallida* into the national territory is very recent, which agrees with the first observation in the field and the description of the nematode in the country (Salazar *et al.*, 2005; García *et al.*, 2009).

The analysis of the ITS region showed a high similarity of the *G. pallida* from Costa Rica with the "Pa5" population from South America that has been reported as more virulent than the European populations (Kort and Jaspers, 1973; Phillips and Blok, 2008), and highly virulent to different potato genotypes (Blok and Phillips, 2012). Populations of *G. pallida* from northern Peru have shown differences in virulence on the potato cultivars Désirée and María Huanca, and the clone identified as 280090.1 (Franco and Gonzalez, 1990). Pathogenicity and virulence studies should be conducted with *G. pallida* populations from Costa Rica and include varieties grown in the country.

Globodera pallida has a restricted distribution status in Costa Rica according to the EPPO A2 list. However, our study reports for the first time *G. pallida* in the second largest potato-producing region in the country, Zarcero. An expanded survey of potato production areas in this region is warranted. *Globodera pallida* is a threat to potato production, and it should be treated as a quarantine species. Hence, to avoid the introduction of genetic variants, and to reduce the spread of *G. pallida* throughout the country, management strategies should be defined, such as the use of certified tubers, the establishment and monitoring of new potato seed-producing areas, the development of resistant varieties and tracking the movement of potato seed from one farm to another. Additionally, while *G. rostochiensis* was not found in this study, measures should be taken to prevent the entry of this species into the country.

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