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Molecular characterisation of the potato cyst nematode, *Globodera ellingtonae* Handoo *et al.*, 2012 (Tylenchida: Heteroderidae) from Bolivia

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Summary – In 2022, during potato cyst nematode surveys in several departments of Bolivia, the potato cyst nematode *Globodera ellingtonae* Handoo *et al.*, 2012 was detected for the first time in Bolivia from potato fields located in the Department of Tarija. Cysts and second-stage juveniles of the Bolivian populations were morphologically and morphometrically similar to those of the USA (Oregon and Idaho) and Argentina. Nine new ITS rRNA, 15 *COI*, 13 *cytb* gene sequences of *G. ellingtonae* from ten Bolivian populations were obtained in this study. The phylogenetic analyses of the ITS rRNA and *cytb* genes showed that all Bolivian sequences clustered together and formed a major clade with other *G. ellingtonae* sequences from Argentina, Chile and the USA. Molecular results confirmed the hypothesis that the mountain region in southern Bolivia, northwest Argentina and northern Chile could be considered as an ancient centre of origin of *G. ellingtonae*. The present molecular results suggested that *G. ellingtonae* was likely introduced into the USA from Chile, rather than from Bolivia and Argentina.

Keywords – *COI*, *cytb*, ITS rRNA, phylogeography, potato, Tarija.

In spring 2022, during potato cyst nematode surveys in several departments of Bolivia, the potato cyst nematode (PCN) *Globodera ellingtonae* Handoo *et al.*, 2012 was found from several potato fields in the Department of Tarija located in south-eastern Bolivia. This is the first detection of the nematode in Bolivia. Two other PCN species, *G. rostochiensis* and *G. pallida*, have been reported previously in the country and the former has been considered as the predominant one (Maín *et al.*, 1994; Ramos *et al.*, 1998; Franco *et al.*, 1999; Franco & González, 2011; Silvestre *et al.*, 2021). In previous studies, molecular characterisation of some Bolivian *G. rostochiensis* and *G. pallida* populations was conducted using sequencing of the ITS rRNA gene (Grenier *et al.*, 2010; Subbotin *et al.*, 2020) and genes responsible for parasitism (Geric Stare *et al.*, 2012). However, as PCN species from the Department of Tarija were previously identified using a morphological approach and never with molecular tools, the species diversity of PCN in potato fields of this region of Bolivia remained undetermined. The present study is the first attempt to characterise molecularly PCN parasitising potatoes in this region of the country.

Globodera ellingtonae was first described from samples collected in the USA, in Oregon and Idaho by Handoo *et al.* (2012), and was later reported in Argentina and Chile (Lax *et al.*, 2014; Hesse *et al.*, 2021). *Globodera ellingtonae* can reproduce on potato and tomato (Zasada *et al.*, 2013; Lax *et al.*, 2014); however, it was not considered to be a strong potato pest in experiments conducted in Oregon (Zasada *et al.*, 2019). The molecular analysis showed that several gene markers clearly differentiated this species from all other known *Globodera* and that *G. ellingtonae* was more closely related to *G. rostochiensis* and *G. tabacum* rather than *G. pallida* (Skantar *et al.*,

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2011; Subbotin *et al.*, 2011, 2020; Lax *et al.*, 2014; Zasada *et al.*, 2015; Hesse *et al.*, 2021).

The main goals of this study were: *i*) to provide a short morphological description of Bolivian *G. ellingtonae*; *ii*) to characterise molecularly several Bolivian populations of *G. ellingtonae* using ITS rRNA, *COI* and *cytb* gene sequences; and *iii*) to study phylogenetic relationships among *G. ellingtonae* populations using rRNA and mtDNA gene sequences.

Materials and methods

Nematode populations

In February to May 2022 nematode surveys for PCN were conducted in several regions of Bolivia, with 112 soil samples collected from potato fields in six departments (Cochabamba, La Paz, Oruro, Potosí, Chuquisaca and Tarija). Cysts were extracted from soil samples using standard flotation and sieving techniques in the lab. Cysts with eggs and juveniles were fixed in 70% ethanol, then dried and sent to the CDFA Nematology laboratory for study.

Morphological study

Cysts were soaked for 10-20 min in double distilled water. One cyst was placed into 20 μl ddH_2O on a glass slide, then punctured by a needle under a dissecting microscope to release second-stage juveniles (J2) and eggs. Measurements and light micrographs of cysts and J2 were taken with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with Nomarski differential interference contrast.

Molecular study

DNA was extracted from single cysts using a standard protocol with proteinase K as described by Subbotin *et al.* (2020). J2 and eggs suspended in drops of water were cut with a stainless steel dental needle under a stereomicroscope. Fragments of nematodes in 15 μl water were transferred into a 0.2 ml Eppendorf tube containing 3 μl proteinase K (600 μg ml^{-1}) (Promega) and 2 μl 10$\times$ PCR buffer (*Taq* PCR Core Kit, Qiagen). The tubes were incubated at 65°C (1 h) and 95°C (15 min) consecutively. After incubation, the tubes were centrifuged and kept at $-$20°C until use.

PCR and sequencing were performed as described by Subbotin (2020). Several primer sets were used in the present study: forward Het-coxiF (5′-TAG TTG ATC GTA ATT TTA ATG-3′) and reverse Het-coxiR (5′-CCT AAA ACA TAA TGA AAA TGW GC-3′) primers for amplification of the partial *COI* gene, forward Het-cytbF2 (5′-CAR TAT TTR ATR TTT GAR GT-3′) and reverse Het-cytbR3 (5′-ACH ARR AAR TTR ATY TCC TC-3′) primers for amplification of the partial *cytb* gene, forward TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and reverse AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers or forward F194 (5′-CGT ACC AAG GTA GCT GTA G-3′) and reverse 26S (5′-TTT CAC TCG CCG TTA CTA AGG-3′) primers for amplification of the ITS1-5.8S-ITS2 rRNA gene. Amplicons were directly sequenced with forward and reverse primers by Genewiz. New sequences were submitted to GenBank under accession numbers indicated in Table 1, phylogenetic trees and networks. Consensus sequences of *COI* and *cytb* genes for USA and Chilean populations were obtained with Geneious 9.0.5 from the datasets published by Hesse *et al.* (2021).

Alignments with the *COI*, *cytb* and ITS rRNA gene sequences were created using ClustalX 1.83 (Chenna *et al.*, 2003) with default parameters. New sequences were aligned with corresponding published gene sequences (Subbotin *et al.*, 2020; Hesse *et al.*, 2021). Sequence ITS rRNA, *COI* and *cytb* gene alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and with maximum likelihood (ML) using PAUP*4a (Swofford, 2003) as described by Subbotin *et al.* (2020). The alignments for *COI* and *cytb* gene sequences were used to construct phylogenetic network estimation using statistical parsimony (SP) as implemented in POPART software (http://popart.otago.ac.nz) (Bandelt *et al.*, 1999). The haplotypes were identified based on the SP results. Pairwise divergence between sequences was calculated using PAUP*. Species identification of nematodes was made using comparative analysis of several gene sequences obtained from single cysts of Bolivian populations with PCN sequences deposited in the Genbank.

Results

Results of the nematological analyses of the 112 soil samples indicated that *Globodera ellingtonae* was present in ten locations in the Department of Tarija (Table 1). The population levels of *G. ellingtonae* in these samples ranged from two to 100 cysts per 100 g of soil. It is worth mentioning that this species was found in association with

Table 1. Populations of *Globodera ellingtonae* from Bolivia used in this study.

Department, municipality, community	GPS coordinates: latitude, longitude, altitude	Sample codes	GenBank accession number		
			ITS rRNA	*COI*	*cytb*
Tarija, El Puente, Chilcayo Sud	−21.50637, −64.96164, 3477	CD3815, TJ7	OQ339228	OQ337994, OQ337995	OQ352154, OQ352155
Tarija, El Puente, Papa Chacra	−21.52620, −64.94526, 3578	CD3902, TJ8	OQ339230	OQ338006, OQ338008	–
Tarija, El Puente, Chorcoya Mendez	−21.56038, −65.00891, 3629	CD3887a, TJ9	–	OQ337997	OQ352165
Tarija, El Puente, Carolina	−21.54247, −64.99975, 3567	CD3888, TJ10	OQ339229	OQ337999	OQ352164
Tarija, El Puente, Campanario	−21.52511, −64.98993, 3521	CD3897, TJ11	OQ339231	OQ338007	–
Tarija, El Puente, San Roque	−21.47291, −64.96775, 3420	CD3871, TJ13	OQ339224	OQ337998, OQ338000	OQ352156, OQ352157
Tarija, Padcaya, Rejara	−22.01479, −64.99151, 3192	CD3872, TJ16	–	OQ338001	–
Tarija, Padcaya, Rejara	−21.99824, −64.98560, 3006	CD3889, TJ18	OQ339226	OQ337996	OQ352158
Tarija, San Lorenzo, Cerro Redondo	−21.17892, −64.71314, 2549	CD3890, TJ19	OQ339225	OQ338002, OQ338003	OQ352160, OQ352162, OQ352163
Tarija, San Lorenzo, Leon Cancha	−21.17727, −64.71221, 2716	CD3873, TJ24	OQ339223, OQ339227	OQ338004, OQ338005	OQ352159, OQ352161, OQ352166

G. rostochiensis in two samples collected in Padcaya, Rejara (CD3872, TJ16) and El Puente, Chorcoya Mendez (CD3887a, TJ9) (Subbotin *et al.*, unpubl.).

Morphological characterisation of *Globodera ellingtonae*

For morphological identification, several cysts and second-stage juveniles were photographed (Fig. 1) and measured from the sample collected in Chilcayo Sud (CD3815).

Cysts (n = 8)

L = 606.3 ± 103.5 (420-760) μm; W = 585.0 ± 83.2 (450-700) μm; L/W = 1.0 ± 0.1 (0.8-1.2); vulval plates (n = 3): fenestral diam. = 20.0 ± 2.5 (17.5-22.5) μm; anus-fenestra basin distance = 55.8 ± 8.0 (50.0-65.0) μm; Granek's ratio = 2.8 ± 0.5 (2.2-3.3). Cyst brown, spherical. Circumfenestra rounded, anus located in a smooth depression.

Second-stage juveniles (n = 10)

L = 497.0 ± 24.8 (445.0-532.5) μm; a = 24.8 ± 1.4 (21.7-26.6); c = 9.0 ± 0.7 (7.7-9.9); stylet length = 22.5 ± 1.1 (21.3-25.0) μm; tail length = 55.4 ± 2.8 (50.0-58.8) μm; hyaline part of tail length = 29.5 ± 2.6 (25.0-32.5) μm. Cephalic region slightly set-off, stylet robust with rounded basal knobs. Tail tapering uniformly with rounded terminus. Hyaline portion almost about half of tail length.

Molecular characterisation of *Globodera ellingtonae*

Nine new ITS rRNA, 15 *COI* and 13 *cytb* gene sequences of *G. ellingtonae* were obtained in this study from ten populations collected in the Department of Tarija. Amplification or sequencing of ITS rRNA, *COI* or *cytb* genes failed in four samples studied. BLAST search of the sequences in the GenBank database revealed that Bolivian sequences were similar to those of *G. ellingtonae* from the USA and South America in more than 98% for the ITS rRNA gene and in more than 89% for *COI* and *cytb* genes.

ITS rRNA gene

Nine new sequences of *G. ellingtonae* from Bolivia were obtained from eight populations in this study. Sequence alignment contained 28 *Globodera* sequences and was 871 bp in length. Phylogenetic relationships of Bolivian *G. ellingtonae* species with other populations of this species and some *Globodera* species parasitising solanaceous plants as inferred from analysis of the

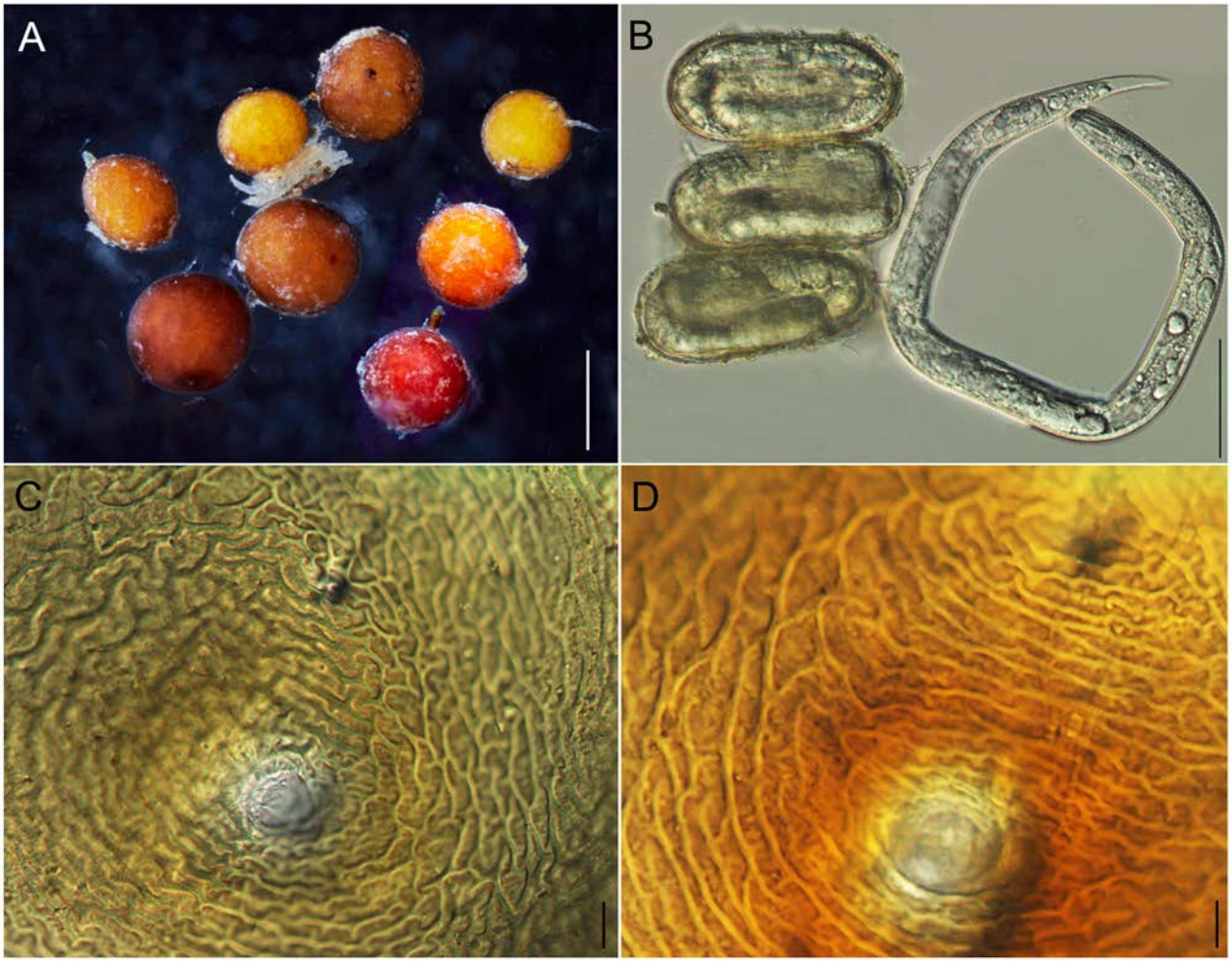

Fig. 1. *Globodera ellingtonae* from Bolivia. A: Cysts; B: Eggs and second-stage juvenile; C, D: Vulval plates. (Scale bars: A = 0.5 mm, B = 50 μm; C, D = 20 μm.)

ITS rRNA gene sequence alignment are given in Figure 2. The ITS rRNA gene sequences clearly differentiated three PCN species from each other and Bolivian *G. ellingtonae* from Argentina, Chile and the USA (Idaho and Oregon) populations. Sequences of *G. ellingtonae* were distributed within five clades: *i*) Bolivian populations; *ii*) Idaho, Oregon and Chilean populations; *iii*) Oregon and Chilean populations; *iv*) Oregon populations; and *v*) Argentina population. Maximal intraspecific ITS rRNA gene sequence diversity for *G. ellingtonae* was 2.4%. The ITS rRNA gene sequences of all Bolivian *G. ellingtonae* samples were identical and they differed from those of other populations in up to 1.8%.

COI *gene*

Fifteen new *COI* gene sequences for this species were obtained from ten populations in this study. A total of 21 sequences of this species was analysed. The alignment length was 441 bp. The SP network of haplotypes is given in Figure 3A. Only one haplotype was found in most samples. Seven haplotypes were revealed in this study and six of them came from Bolivia. New Bolivian haplotypes (GeCOI2-GeCOI7) clearly differed from the Oregon and Chilean haplotype (GeCOI1). Maximal intraspecific *COI* gene sequence diversity was 11.7%. The geographical distribution of studied *COI* haplotypes in Bolivia and Chile is presented in Figure 4. Phylogenetic relationships of Bolivian *G. ellingtonae* populations

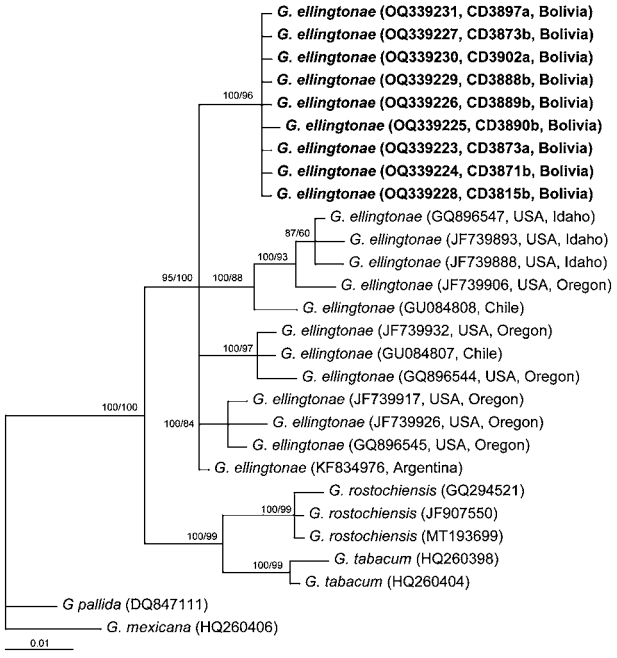

Fig. 2. Phylogenetic relationships of *G. ellingtonae* with some *Globodera* species parasitising solanaceous plants: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities (BI) and bootstrap values (ML) more than 60% are given for appropriate clades. New sequences are indicated by bold letters.

with other populations of this species and some *Globodera* species parasitising solanaceous plants as inferred from analysis of the *COI* gene sequence alignment are given in Figure 5A. Three distinct groups (a, b, c) could be distinguished within *G. ellingtonae* sequences in the phylogenetic SP network and tree. In the phylogenetic tree, the relationships among these groups were not well resolved.

Cytb *gene*

Thirteen new *cytb* gene sequences for this species were obtained from seven populations in this study. A total of 20 sequences of this species was analysed. The alignment length was 463 bp. The haplotype network is given in Figure 3B. Eleven haplotypes were revealed in this study. Strong sequence heterogeneity was observed for some samples. Ten haplotypes were found from Bolivia. These new Bolivian haplotypes (GeCb2-GeCtb11) clearly differed from the Oregon and Chilean haplotypes (GeCb1). Maximal intraspecific *cytb* gene sequence diversity was 8.6%. Phylogenetic relationships of Bolivian *G. ellingtonae* populations with other populations of this species and some *Globodera* species as inferred from analysis of the *cytb* gene sequence alignment are given in Figure 5B. Three groups (a, b, c) could be distinguished within *G. ellingtonae* sequences in phylogenetic SP network and tree. In the phylogenetic tree, all Bolivian *G. ellingtonae* populations (b and c groups) clustered together.

Discussion

From 112 soil samples collected from potato fields in six departments of Bolivia: Cochabamba, La Paz, Oruro, Potosí, Chuquisaca and Tarija, *G. ellingtonae* was found in ten samples from the Department of Tarija. Most cysts collected during this survey in the Bolivian departments belonged to *G. rostochiensis* (Sainz *et al.*, unpubl.; Subbotin *et al.*, unpubl.). The finding of *G. ellingtonae* is the first report of this species in Bolivia.

Tarija is a department in south-eastern Bolivia bordered by Argentina to the south and Paraguay to the east. Only *G. rostochiensis* and *G. pallida* have been previously identified in this department (Franco & González, 2011; Silvestre *et al.*, 2021). *Globodera ellingtonae* was found in three municipalities in the Department of Tarija: El Puente, Padcaya and San Lorenzo, which have ecoregions with different climates, such as the sub-Andean zone, plateaux, high valleys, low valleys, and subtropical zone, with altitudes ranging from 1600 to 2800 m a.s.l., and where agriculture is the main economic activity. One of the most widely used agricultural practices is crop rotation, which consists of intentionally planting different types of crops in different parts of the field and in different seasons in a sequential manner to limit the concentration of pests and diseases. In the study area, a 3-year rotation of potato to broad bean, barley/oats and fallow is the preferred cropping system. *Globodera ellingtonae* was found on two local potato varieties: 'Runa Iscayachi' and 'Imilla Negra' and on the 'Desiree' potato, which is widely cultivated in the country.

Limited distribution of *G. ellingtonae* in the Department of Tarija only and absence of this species in other regions of Bolivia could be explained by the isolation of the Central Valley of the Tarija surrounded by mountain ridges, which could serve as physical barriers for nematode dispersal to northern Bolivia, as well as by the use of local potato varieties, which are only cultivated predominantly in this area and not in the rest of the country.

As cysts were fixed in ethanol and J2 were slightly deformed and with undiscernible internal structures, only a few morphometric characters were measured. Our study

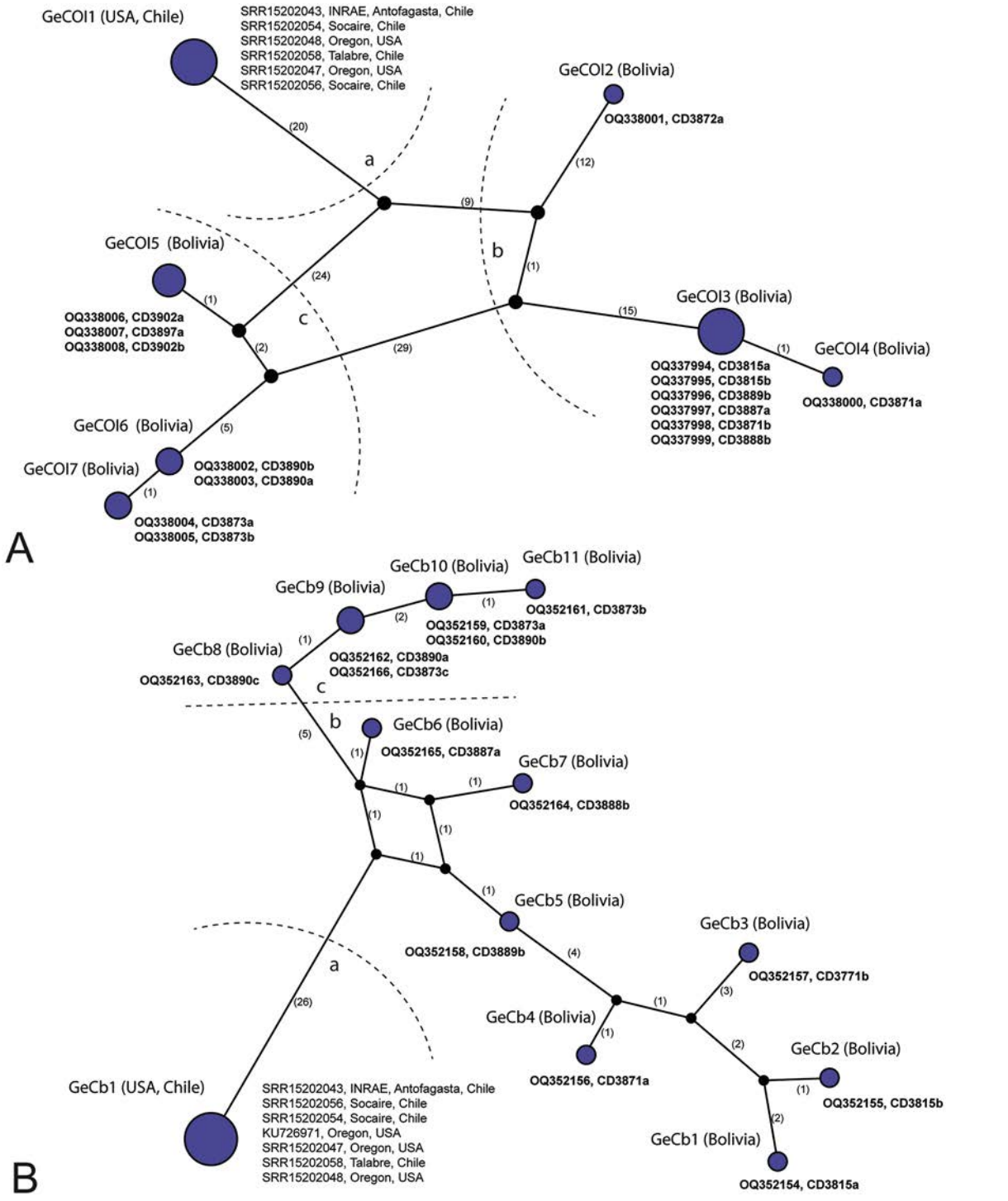

Fig. 3. Statistical parsimony networks showing the phylogenetic relationships between the *COI* (A) and *cytb* (B) gene haplotypes of *Globodera ellingtonae*. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype. Nucleotide changes between haplotypes are given for appropriate branches. New sequences are indicated by bold letters.

Fig. 4. Map of the Andes with distribution of seven *COI* haplotypes for *Globodera ellingtonae* obtained from original samples from Bolivia and the sequence datasets from Chile published by Hesse *et al.* (2021). Location with *G. ellingtonae* reported by Lax *et al.* (2014) in Argentina is also marked on the map.

showed that cysts and J2 of the Bolivian population were morphologically and morphometrically similar to those of Argentina and the USA (Handoo *et al.*, 2012; Lax *et al.*, 2014).

The phylogenetic analyses of the ITS rRNA and *cytb* genes showed that all Bolivian sequences clustered together and formed a major clade with other *G. ellingtonae* sequences from Chile, Argentina and the (Oregon and Idaho) USA. In the ITS rRNA gene tree, Bolivian populations formed a separate lineage and did not show the same high sequence diversity that was observed for USA and Chilean populations. Conversely, high diversity was revealed for *COI* and *cytb* gene sequences of Bolivian samples as compared to uniform USA and Chilean isolates. Two groups of sequences among Bolivian populations and a distinct group for Chilean-USA sequences were found for both mtDNA genes, and this finding suggests a unique population structure for this species that warrants further study with more samples.

Brücher (1959) proposed that PCN might have their origin in north-west Argentina. Franco (1977) agreed with this hypothesis and suggested that PCN species evolved in that region in scattered populations isolated during the Pleistocene glaciations and then migrated north (Stone, 1979). The phylogenetic analysis of mtDNA sequences made by Subbotin *et al.* (2020) suggested that the South Andes, including north-west Argentina, might indeed be considered as an ancient centre of origin for *G. rostochiensis*, *G. ellingtonae* and *G. tabacum*. Our results revealed a high diversity of *COI* and *cytb* gene sequences from samples collected in Bolivia, comparing with those from Chile and the USA published by Hesse *et al.* (2021). The phylogenetic analysis of the current dataset suggested that the mountain region located in southern

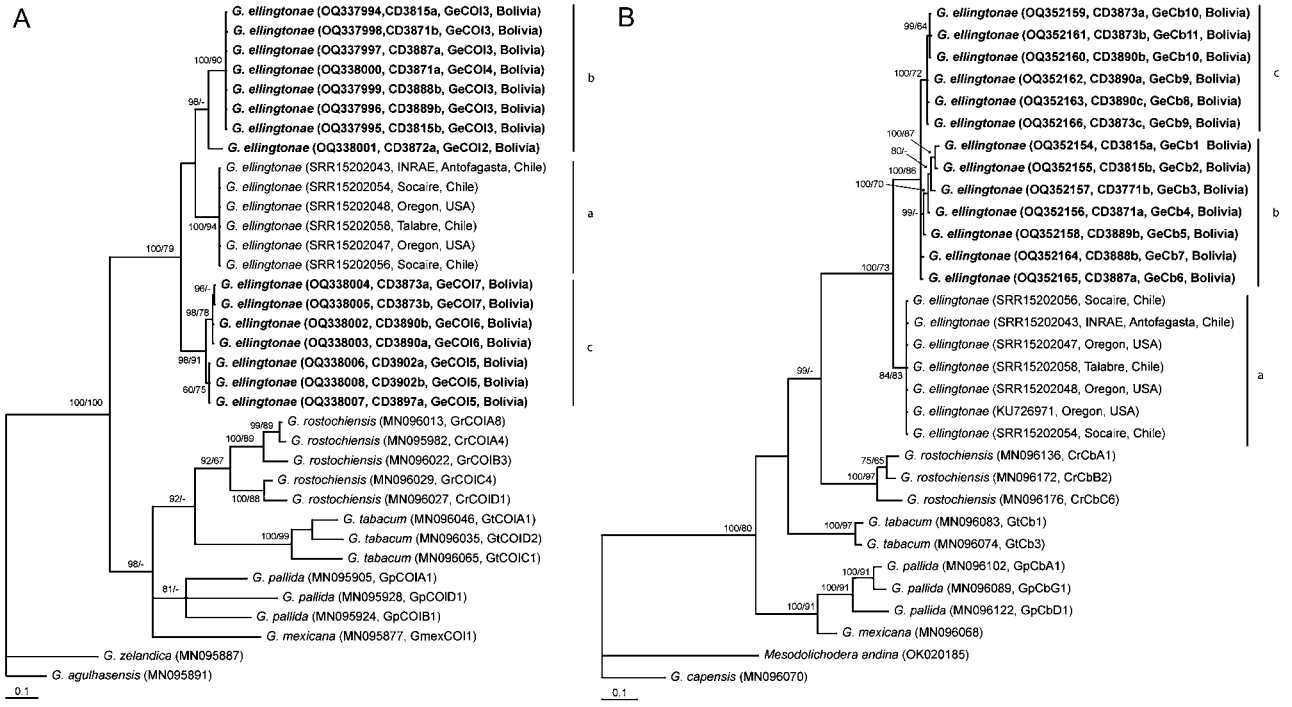

Fig. 5. Phylogenetic relationships of *G. ellingtonae* with some *Globodera* species parasitising solanaceous plants: Bayesian 50% majority rule consensus trees from two runs as inferred from analysis of the *COI* (A) and *cytb* (B) gene sequence alignments under the GTR + I + G model. Posterior probabilities (BI) and bootstrap values (ML) more than 60% are given for appropriate clades. New sequences are indicated by bold letters.

Bolivia, north-west Argentina and northern Chile could be considered as an ancient centre of origin of *G. ellingtonae*. Isolated valleys, in which wild potatoes have been growing, served as refugia for survival of this nematode during the last ice age, and favored limited gene exchange between local *G. ellingtonae* populations and, finally, high genetic diversity of this species in this region.

Although the origin of USA populations found in potato fields in Idaho and Oregon is still unclear, the present molecular results showing high similarity in *COI* and *cytb* gene sequences between Chilean and USA populations indicate that they likely were introduced from Chile, rather than from Bolivia and Argentina.

Additional new nematode surveys in potato fields located at high altitude and native areas in southern Bolivia, northern Argentina and Chile could give more information on genetic diversification of *G. ellingtonae* and other *Globodera* species and provide a more distinct picture of distributions and phylogeographic structures for PCN.

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