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Morphology and DNA sequence data reveal the presence of *Globodera ellingtonae* in the Andean region

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Abstract

Potato cyst nematodes, *G. rostochiensis* and *G. pallida*, are the most economically important nematode pests of potatoes worldwide and are subject to strict quarantine regulations in many countries. *Globodera ellingtonae* was recently described from Oregon (USA), with its host-plant in the field being still unknown. Roots of Andean potatoes from the North of Argentina have been found attacked by this nematode, providing further evidence that this is a potato cyst nematode species, along with *G. pallida* and *G. rostochiensis*. New information about morphological, biological and molecular aspects of *G. ellingtonae* is provided for diagnostic purposes. The Argentine population showed morphological differences from specimens from Oregon; therefore, new diagnostic characters were defined to differentiate *G. ellingtonae* from its closest species. The Hsp90 gene was shown to be a good diagnostic marker for discriminating the three PCN species. The importance of the detection of *G. ellingtonae* on potatoes in the Andean region is not restricted to a regional level, since the nematode is also present in USA. This species can pose a serious problem to potato crop, especially when infected tubers are used as seeds. The distribution in the South American Andes is likely to extend the currently known distribution areas because cysts are passively transported. There is a need to evaluate the possible damage it may cause to potato crops. Morphological and molecular diagnoses conducted in this work provide fundamental information for the protection of potato crops not only in those countries in the Americas where the species has already been detected, but also worldwide.

Content

Introduction

The potato cyst nematodes (PCN) *Globodera rostochiensis* (Wollenweber, 1923) Behrens, 1975 and *G. pallida* (Stone, 1973) Behrens, 1975 are serious pests of potatoes (*Solanum tuberosum* Linnaeus, 1753) worldwide. They cause significant yield losses to the crops and are considered quarantine pests of seed potatoes in many countries. In the European Community, PCN are responsible for losses in potato production of millions of euros every year (Reid, 2009). Economic losses caused by both nematode species in Bolivia and Peru were estimated at 13 and 128 millions of dollars, respectively (Franco-Ponce and González-Verástegui, 2011). Human activities have caused the spread of PCN from South America to many regions of the world (Plantard *et al*., 2008).

In the Americas, the presence of several *Globodera* species and subspecies can generate confusion in the identification of PCN: *G. mexicana* (Campos-Vela, 1967) Subbotin, Mundo-Ocampo and Baldwin, 2010 and one species with three subspecies of tobacco cyst nematodes (TCN): *G. tabacum tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975, *G. tabacum solanacearum* (Miller and Gray, 1972) Behrens, 1975

and *G. tabacum virginiae* (Miller and Gray, 1968) Behrens, 1975. Recently, a new species, *G. ellingtonae* Handoo, Carta, Skantar and Chitwood, 2012, was described from Oregon, USA (Handoo *et al*., 2012). This nematode was detected in soil samples, but the field host-plant is still unknown. Preliminary greenhouse experiments have demonstrated that this nematode can reproduce on potatoes (Handoo *et al*., 2012).

The identification of *Globodera* species largely depends on small differences in measurements, and the high intraspecific variation and overlap between species makes this a difficult task (Stone, 1983). The main DNA regions targeted for the diagnosis of cyst nematodes are nuclear ribosomal RNA genes. The internal transcribed spacer (ITS) region has been a useful marker for identification of *Globodera* species (Subbotin *et al*., 2011).

In the Andean region, tuber crops are a staple food of humans, and represent a valuable source of genetic resources. The production of several varieties of Andean potatoes also has great nutritional importance for rural populations. In 2005, during an inspection of a potato-growing area in the north-western Argentina, cysts belonging to the genus *Globodera* were detected on Andean potato roots in a field in the province of Salta (Lax *et al*., 2005). The population was identified as *G. pallida* based on analyses of diagnostic morphological and morphometric characters indicated by EPPO (2004, 2009). The analysis of ITS region grouped this population with known sequences of *G. pallida*; some RFLP patterns were shared even with *G. pallida* and *G. rostochiensis*. Recently, a high degree of genetic similarity in the ITS region (99%) was observed among the Argentine population from Salta, an unidentified *Globodera* species from Chile and *G. ellingtonae* from USA (Subbotin *et al*., 2011; Handoo *et al*., 2012). The high degree of molecular similarity between these isolates suggests that they belong to the same species.

As the field host of *G. ellingtonae* in USA is still unknown, our finding of the nematode in Argentina naturally parasitizing Andean potatoes shows that the species is another PCN, along with *G. pallida* and *G. rostochiensis*. Here we report morphological, morphometric, biological and molecular data that support the hypothesis that the nematodes found in Salta (Argentina) belong to *G. ellingtonae* and herewith we document the presence of a new PCN in South America.

Material and methods

Nematode sampling

Soil samples were taken from a plot cultivated with Andean potatoes ('Colorada' variety) in the locality of Campo Carreras (2730 m a.s.l.), department of Iruya, province of Salta, Argentina. Potato plants of the same variety were planted in pots containing PCN-infested soil and maintained under laboratory conditions to obtain adults of *G. ellingtonae* for morphological and morphometric analyses. The plants were carefully removed and washed free of soil after 45 days. Females were extracted from plant tissue using dissecting needles under a stereoscopic microscope. Second-stage juveniles (J2), males and cysts were extracted from the soil using a combination of centrifugal-flotation and Fenwick techniques (Doucet *et al*., 2001). Cysts of *G. tabacum* (Province of Jujuy, Argentina), *G. rostochiensis* (Bolivia) and *G. pallida* (Peru) were included in the analysis for comparison of the Hsp90 gene.

Morphological data

Characters of taxonomic value for *Globodera* species identification were evaluated, with an emphasis on those that have been indicated as valid to identify closely related species within this genus (Stone, 1973a, c; EPPO, 2009; Subbotin *et al*., 2010). Males, females and J2 were fixed, dehydrated and mounted in anhydrous glycerine between slides (Seinhorst, 1962). The cysts were temporarily mounted between slides to measure their length and diameter, following the methodology proposed for cysts of *Heterodera glycines* Ichinohe, 1952 (Faghihi *et al*., 1986). Then they were subsequently sectioned at midbody with a scalpel, cleaned internally with a thin needle, and mounted in lactophenol between slides for observation of the vulval region. Morphometric characters were measured using a light microscope with a micrometric ocular scale or *camera lucida*. The mean, standard deviation and range were calculated. In the present study, the ratio b and b' were calculated considering Subbotin *et al*. (2010).

The shape of basal knobs of the stylet in J2 (with the character states rounded, flattened and forward projection) was evaluated based on a classification previously defined for *G. pallida* and *G. rostochiensis* (Behrens, 1972; Manduric *et al*., 2004). The frequency of appearance of each shape within the population studied was recorded.

Between 15 and 20 specimens of each stage were fixed and dehydrated in a graded series of alcohol solutions and critical-point dried using CO_2 for observation with scanning electron microscope (SEM). Individuals were mounted on aluminium stubs with double adhesive tape, coated with a 300 Å layer of gold-palladium and observed under SEM at an accelerating voltage of 15 KV. Cysts were processed following modified techniques (Lax and Doucet, 2002).

Host-plant relationship

In North and Central America, TCNs parasitize tobacco (*Nicotiana tabacum* Linnaeus, 1753) and other solanaceous plants but not potato (EPPO, 2004, 2009). In order to distinguish PCN from *G. tabacum* we evaluated the reproductive capacity of the study population on Andean potato and tobacco plants following the method of Fleming and Powers (1998), as follows. Tobacco seeds of the Virginia cultivar K 326, with known susceptibility to *G. tabacum*, were put to germinate in sterile soil. When four leaves had emerged, seedlings were planted in plastic pots containing 250 g sterile soil. The nematodes were inoculated around the roots (initial density of 250 eggs and/or J2). The 'Colorada' variety of Andean potato which is susceptible to the newly detected nematode was used. Seed tubers were placed in plastic pots containing sterile soil. When the tubers began to sprout the nematodes were inoculated near the roots, as above. Six replicates per host plant species were performed. The plants were grown for 45 days at 25°C. After this period, they were carefully removed and *Globodera* females and/or cysts present in roots were counted. Nematode density in the soil was also evaluated by extracting the individuals using the combination of the flotation-centrifugation-Fenwick techniques mentioned above. The number of eggs and/or J2 inside the cysts was counted. The overall population density was estimated by summing up the number of females and/or cysts on roots, the number of nematodes in the soil and the number of eggs present within cysts. The Reproduction Factor (RF) of the population was calculated for each plant using the final density/initial density relationship.

Molecular analysis

DNA was extracted from single J2 nematodes following Lax *et al*. (2007) and from single filled cysts, using the phenol-chloroform standard extraction technique (Sambrook *et al*., 1989). Different sets of primers were used to amplify three genes (ITS region, D2-D3 expansion segments and Hsp90) under the conditions mentioned in Table 1. The amplification products were separated by electrophoresis on 1% agarose gel in 0.5X TBE buffer. A molecular weight marker of 100 bp DNA Ladder (Promega) was used. Gels were stained with ethidium bromide and photographed with a Kodak-DC digital camera under a UV transilluminator. PCR amplifications from two or three different samples of each species were purified and sequenced in both directions by Macrogen USA Inc. using the primers mentioned in Table 1. DNA fragments were aligned with ClustalX 2.0 (Larkin *et al*., 2007) under default parameters and were manually edited with BioEdit (Hall, 1999). The fragments were aligned with sequences of *G. ellingtonae* and other *Globodera* species from the GenBank (see online supplementary information). The newly obtained sequences of *G. ellingtonae* were submitted to the GenBank database (accession numbers are listed in Table 1).

The phylogenetic analyses were performed with Maximum Likelihood (ML) based on the Tamura-Nei model (Tamura *et al*., 2004) with MEGA 5 software

Table 1. Primers used in this study and accession numbers of *Globodera ellingtonae* from Argentina.

Amplified region	Primer name	5´ to 3´ Sequence	Reference	GenBank accession
ITS1-5.8s-ITS2 rRNA	18S 26S	TTGATTACGTCCCTGCCCTTT GGAATCATTGCCGCTCACTTT	Vrain *et al*., 1992	KF834976
D2-D3 of 28S rRNA	D2A D3B	ACAAGTACCGTGAGGGAAAGTTG TCGGAAGGAACCAGCTACTA	Al-Banna *et al*., 1997	KF834977-KF834979
Heat-shock Hsp90 gene	Hsp90-R1 Hsp90-R2	GGCATTCTTGCTCTTCTTGTTCT TTGACTGCCAAATGGTCTTCC	Madani *et al*., 2011	KF834980-KF834983

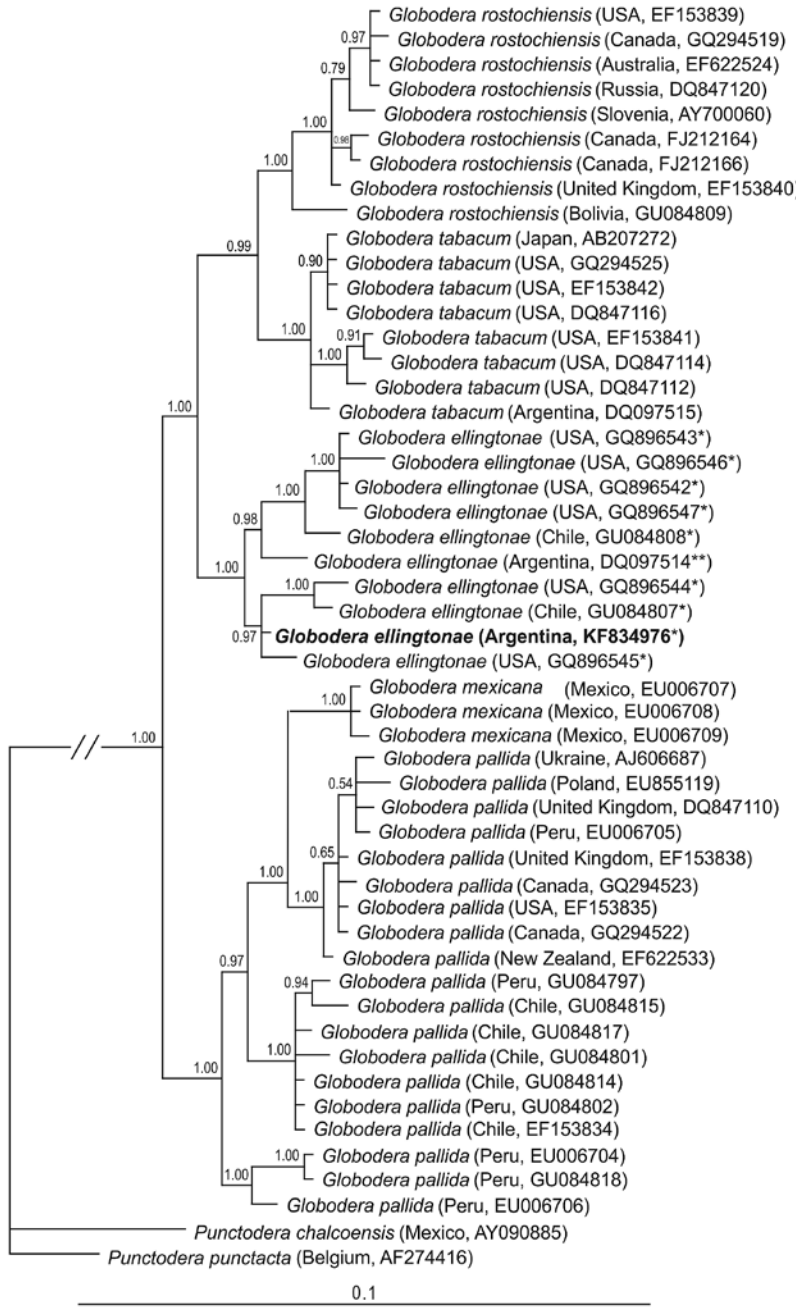

Fig 1. Phylogenetic relationships among *Globodera* species. The majority consensus Bayesian tree obtained from the ITS-rRNA gene sequences after two million generations under the GTR+G model. The Bayesian posterior probabilities are given in the nodes. The newly sequenced sample is indicated in bold. Sequence uploaded as: *Globodera* sp.*, *G. pallida***.

(Tamura *et al*., 2011). The support for each node was estimated using the bootstrap analysis with 1000 replicates. ITS and Hsp90 sequences were also analysed using the Bayesian inference (BI) with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The most appropriate model of nucleotide substitution was determined with jModelTest 0.1.1 (Posada, 2008) under the Akaike Information Criterion. The GTR+G model was selected for both genes. Two independent runs were performed simultaneously on the data set, each one using one cold and three heated chains. After two million (ITS sequences) and one million (Hsp90 sequences) generations, the average standard deviation of split frequencies between the two independent runs were 0.0067 and 0.0038, respectively. After discarding 25% of burn-in samples and evaluating con-

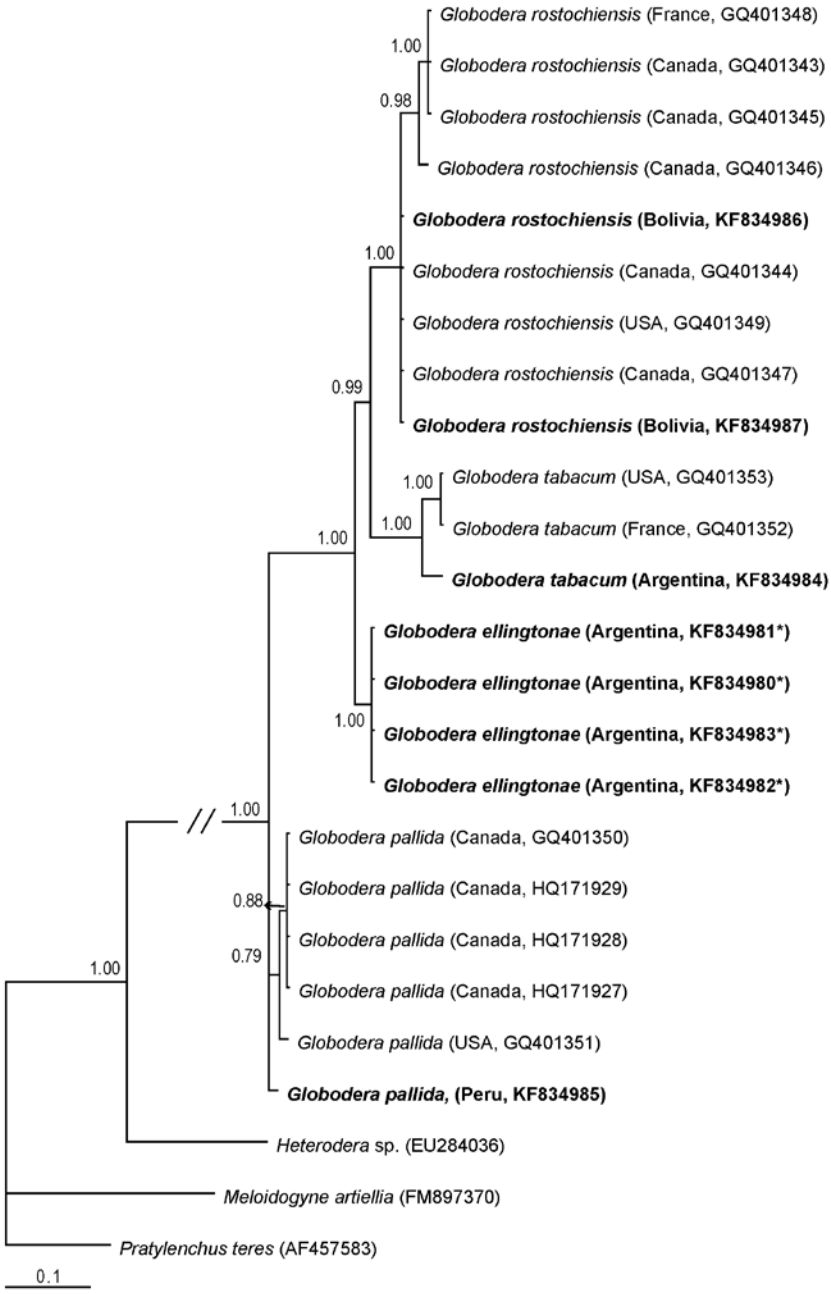

Fig 2. Phylogenetic relationships among *Globodera* species. The majority consensus Bayesian tree obtained from the Hsp90 gene sequences after one million generations under the GTR+G model. The Bayesian posterior probabilities are given in nodes. The newly sequenced samples are indicated in bold. Sequence uploaded as: *Globodera* sp.*

vergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on the appropriate nodes. The trees were visualised with TreeView (Page, 1996). Sequences of D2-D3 expansion segments were not analysed using BI due the unresolved trees obtained.

Results

Morphological data

The morphology of the Argentine population of *G. ellingtonae* is described in Appendix I (Figs 3-7; Tables 2-4). Due to the differences observed from the original population from Oregon (USA), new differential

diagnoses comparing *G. ellingtonae* with other *Globodera* species are presented in Appendix II (Table 5).

Host-plant relationship

The population of *G. ellingtonae* reproduced on Andean potatoes 'Colorada' variety (mean values: 25 cysts; 4190 eggs and RF=16.8); no reproduction was observed on tobacco plants (RF=0.0).

Molecular analysis

Relationships among the ITS rDNA gene sequences were obtained from ML analysis (data not shown) and BI (Fig. 1). The BI and ML tree topologies were congruent. The Argentine population fit in a clade that grouped sequences from Antofagasta (Chile), Oregon and Idaho (USA) (BI bootstrap support=100%; ML=77%). The phylogenetic analyses with the D2-D3 expansion segments of 28S showed one clade comprising the sequences obtained in this study, and one sequence of *G. ellingtonae* from USA (JN712217) and of *G. tabacum* (GQ294489) from Canada; the remaining *G. rostochiensis* and *G. pallida* sequences showed unresolved positions (data not shown). The ML and BI analyses considering the Hsp90 gene showed that Argentine sequences grouped into a highly supported clade (BI=100%; ML=96%) with respect to *G. tabacum* and the other PCN species, *G. rostochiensis* and *G. pallida* (Fig. 2).

Discussion

The diagnostic use of a combination of cyst and J2 characters is recommended for a reliable discrimination between PCN species and morphologically close species (Fleming and Powers, 1998; EPPO, 2009; Subbotin *et al*., 2010). According to Subbotin *et al*. (2010), many diagnostically useful morphometric characters have relatively stable means for *Globodera* species, although in some populations the range may be extended by few individuals and this fact should be taken into account. However, these differences are part of the variability present within each species. Evans and Franco (1977) reported the effects of the environment (temperature, nematode density and day length) on PCN morphometry. As new populations of these species are further characterized, the ranges (of means as well as minimum and maximum values) are widened, and therefore the limits for most of these characters are no longer valid (Appendix II). This was observed for the Argentine *G. ellingtonae* population, which showed certain morphological and morphometric differences from the original description (Handoo *et al*., 2012), therefore extending the limits for the species. Hence, certain characters that those authors considered diagnostic to distinguish *G. ellingtonae* from the closest species are no longer valid (J2: the distinctive tail, the number of refractive bodies in the hyaline tail terminus, the mean stylet length; males: the tip and length of spicules; cysts: the pattern of cuticular ridges between the anus and the vulval basin). For this reason, a reappraisal of the diagnostic characters and a comparison with the closest *Globodera* species were made (Appendix II).

The shape of stylet knob in J2 is one of the useful characters for identification of *Globodera* species (Subbotin *et al*., 2010). Intra-population variability was observed in the anterior side of basal knobs of the Argentine population. In most of the individuals (65%), the anterior surface appeared flattened, whereas in a lower proportion, it was rounded (7.5%) or with a forward projection (27.5%). The latter two shapes agree with descriptions for the specimens from Oregon and Idaho (Handoo *et al*., 2012). Intra-population variability was also observed in *G. pallida* and *G. rostochiensis* populations from Sweden (Manduric *et al*., 2004), in which the three different shapes observed in the Argentine nematodes were detected. Given the variability cited and the overlap of shapes of anterior side of basal knobs among species, this character does not appear to be useful as a diagnostic element to discriminate closely related species of the genus *Globodera*. This character was also found to be variable in males from Argentina (forward projection or backward sloping) and in those from Oregon (rounded to posteriorly directed) (Handoo *et al*., 2012).

Phylogenetic analyses based on ITS and Hsp90 sequences showed that *G. ellingtonae* fit in a well-supported clade, more closely related to *G. rostochiensis* and the *G. tabacum* complex than to *G. pallida*. The Hsp90 is one additional gene target for the identification and discrimination of *G. pallida*, *G. rostochiensis* and *G. tabacum* (Madani *et al*., 2011). Those authors considered samples from Canada, USA, France and Belgium. In the present work, we incorporated sequences of populations from South America, confirming the usefulness of this gene to differentiate the three PCN species. It might even be a more powerful marker than the ITS region because it reveals more genetic

differences at the species level (percent genetic similarity of *G. ellingtonae* with *G. tabacum*, *G. rostochiensis* and *G. pallida* for the Hsp90 gene ranged between 86-89%, 82-91% and 76-80%, respectively). Similar analyses incorporating sequences of specimens from Oregon, USA, should be conducted. Contrarily to the results obtained with the ITS and Hsp90 genes, the D2-D3 region of the 28S gene did not contain enough informative sites that allow reconstruction of robust phylogenetic relationships within the genus *Globodera*. This finding is consistent with previous results (Douda *et al*., 2010; Madani *et al*., 2010; Handoo *et al*., 2012).

The Andean region has been a key factor for the evolution and specialisation of at least four *Globodera* species parasitizing solanaceous plants. Since the ecosystems present in that region are variable, species diversity in the genus would be wider than that described by Grenier *et al*. (2010). The differences observed in virulence among European PCN populations would be a small fraction of that found in the 'centre of origin' of PCN diversity in South America; the introduction of such populations into new areas would pose a threat to the use of resistant cultivars as a major tool to reduce the potential spread and damage caused by these species (Hockland *et al*., 2012).

The detection of *G. ellingtonae* on potatoes reveals a serious problem in the Andean region, especially when infested tubers are used as seeds. In agricultural fairs, local farmers trade their products with growers from neighbouring communities; thus, seed potatoes become an important source of passive dissemination of plant-parasitic nematodes of quarantine importance (Rojas *et al*., 1997; Lax *et al*., 2006). For this reason, this nematode is likely to be widely dispersed in the Andean region of Argentina. In previous studies, *Globodera* sp. cysts were found on the peridermis of different varieties of Andean potatoes from localities of the provinces of Salta and Jujuy (Lax *et al*., 2006, 2008). The presence of *G. tabacum* cysts was detected in soil samples collected from different potato fields of Jujuy (Mondino *et al*., 2006). However, the authors did not provide any morphological or morphometric evidence supporting the species identification. Given the similarity between cysts of *G. tabacum* and *G. ellingtonae*, it is necessary to confirm the identity of those populations. Broader samplings should also be made to evaluate the distribution of this new PCN in the Andes and the virulence of different nematode populations, and to estimate the possible damages it may cause to potato crops.

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On-line Supplementary Information (SI)

S1. Sequences used for genetic analyses. Newly sequenced samples are indicated in bold. Sequence uploaded as: *Globodera pallida**; *Globodera* sp.**

APPENDICES

Appendix I

Description of the Globodera ellingtonae *population from Argentina*
Figs 3-7, Tables 2-4

Second-stage juvenile. Cuticle with transverse striae. Lateral field starting or ending with two incisures; external lines starting at the level of the sixth or eighth body annule, then turning into four equidistant incisures forming three bands of equal width. Occasional areolation in external bands. Cephalic region slightly set off, separated by a constriction, occasionally very marked. Lip region with an oral disc: three or four complete or incomplete cephalic annules. Rectangular prestoma located in the centre of the oral disc, surrounded by a slightly elevated structure. Oral disc oval, surrounded by two lateral lips and a pair of dorsal and ventral submedial lips. Lateral lips small, bearing amphid apertures; outer margin complete or incomplete, shape ranging from rounded to irregular. Pair of submedial lips (dorsal and ventral) separated by a slight or marked incisure. Cephalic framework scletorized. Stylet robust, cone occupying about half of the total stylet length. Basal knobs rounded posteriorly; the anterior surface of three shapes: rounded, forward projection anteriorly or flattened, the latter being the most frequent (Table 4). Orifice of dorsal esophageal gland 5-7 μm behind base of stylet. Median esophageal bulb oval. Nerve ring surrounding isthmus, located behind the median bulb, 73-93 μm from anterior end. Esophagus occupying about 40% of total body length; basal pharyngeal bulb ventrally overlapping the anterior part of the intestine. Excretory pore 94-113 μm from anterior end, about 22% of body length. Gonadal primordium about 60% of the body length from anterior region. Tail tapering uniformly, ending in a rounded tip. Hyaline portion about half of tail length; two to six lipid inclusions were observed near the tail tip in some specimens, whereas in others there were no lipid inclusions. No phasmids were observed.

Male. Cuticle transversally striated. Posterior region twisted about 90º along its longitudinal axis in fixed specimens. Lateral field with external lines starting at the level of the seventh to eighth body annule and ending at the tail, not continuing around tip. At midbody, lateral field delimited by four equidistant lines, forming three bands of similar width; external bands with more or less marked aerolations. Lateral field usually converges at the level of the cloacal opening and the pattern is rounded in lateral view. The end view pattern between the two lateral fields shows irregular cuticular tubercles. Cephalic region rounded, set off from the body by a slight or marked constriction. Transverse striae delimit three to five complete or incomplete cephalic annules; short longitudinal striae in the cephalic region occasionally. Prestoma rectangular, surrounded by an elevation and located at centre of a large oval or rounded labial disc. Prominent labial disc surrounded by two lateral lips and a pair of dorsal and ventral submedial lips. Small rounded or elongate lateral lips, containing amphidial apertures; external edge is not visible on some occasions. Pair of submedial (dorsal and ventral) lips of rounded or irregular contour, separated by an incisure. Cephalic framework sclerotized. Stylet robust; basal knobs rounded posteriorly, with a backward sloping or a forward projection anteriorly. Each shape with different frequency of appearance (Table 4). Orifice of dorsal pharyngeal gland located 3-5 μm posterior to stylet base. Median pharyngeal bulb oval. Nerve ring surrounding isthmus, located posterior to median bulb, about 11% of body length (101-134 μm from the anterior end). Excretory pore located at 135-188 μm from the anterior end (about 15% of body length). Pharynx length extending 17% of body length; basal pharyngeal bulb ventrally overlapping the anterior part of the intestine. Spicules curved ventrally, distal end with a rounded tip; gubernaculum present, slightly curved. Tail short, rounded. Phasmids not observed.

Female. Body of variable size, generally spherical and occasionally oval; protuding conical neck of variable size. When female emerges from the root, the white body turns yellowish and then brown with aging, becoming a cyst. Cephalic region with a labial disc of rectangular contour, with slightly concave sides, and the aperture of prestoma in the centre. Pair of dorsal and ventral submedial lips fused forming a structure of irregular edges, located below the labial disc. Lateral lips of round contour, clearly delineated, and differentiated from the submedial lips. Amphidial apertures not observed. Few transversal annules that become fragmented in some parts, giving a smooth ap-

Table 2. Morphometric characters of second-stage juveniles and males of *Globodera ellingtonae* from Argentina and USA (Handoo *et al*., 2012). Measurements are in μm and as: mean ± SD (range).

	Second-stage juveniles				Males			
Character	n	Argentina	n	USA	n	Argentina	n	USA
Lip region height	37	4 ± 0.2 (3-4)	-	-	25	5 ± 0.6 (4-6)	-	-
Lip region width	37	9 ± 0.3 (9-10)	-	-	24	11 ± 0.7 (10-12)	-	-
L (body length)	37	458 ± 23 (418-526)	106	450 ± 28 (365-515)	24	1076 ± 138 (717-1368)	20	981 ± 97 (787-1150)
a (body length/maximum body width)	37	25.9 ± 1.4 (23.3-30.2)	46	22.9 ± 1.38 (18.25-25.56)	24	32.9 ± 4.1 (25.3-44.5)	20	26.3 ± 1.32 (23.75-28.72)
b (L/distance from anterior end to junction of pharynx and intestine)	37	4.7 ± 0.3 (4-5.4)	47	2.98 ± 0.22 (2.35-3.42)	20	8.2 ± 0.6 (7.1-9)	20	6.81 ± 0.92 (5.25-8.63)
b' (L/pharynx length)	37	2.5 ± 0.2 (2.2-2.8)		-	18	5.9 ± 0.6 (4.7-6.8)		-
c (body length/tail length)	37	9.2 ± 0.4 (8.5-10.3)	90	9.69 ± 0.74 (8.06-12.25)	23	200.1 ± 67 (112.6-335.3)	19	278.3 ± 77.6 (164-432)
c' (tail length/anal body width)	37	4.3 ± 0.3 (3.7-5)	38	3.68 ± 0.19 (3.31-4)	24	0.4 ± 0.1 (0.2-0.6)	-	-
Maximum body width	37	18 ± 0.6 (17-19)	46	20 ± 0.58 (18-21)	25	33 ± 4 (26-39)	20	37.3 ± 2.75 (32-43)
Distance from anterior end to junction of pharynx and intestine	37	97 ± 4 (88-109)	-	-	21	132 ± 11 (101-152)	-	-
Distance from anterior end to valve of median bulb	37	69 ± 3 (61-76)	-	-	25	93 ± 9 (68-106)	-	-
Distance from anterior end to excretory pore	36	101 ± 4 (94-113)	-	-	24	160 ± 13 (135-188)	-	-
Distance from anterior end to nerve ring	36	86 ± 3 (79-93)	-	-	13	120 ± 8 (101-134)	-	-
Pharynx length	37	184 ± 11 (167-220)	47	153.8 ± 8.93 (138-180)	18	184 ± 18 (149-225)	20	145.2 ± 12.6 (120-160)
Stylet length	41	23 ± 0.6 (22-24)	108	20.9 ± 0.85 (19-22.5)	25	26 ± 0.9 (25-27)	20	22.9 ± 1.13 (21-25)
Conus length	41	11 ± 0.5 (10-12)	-	-	25	13 ± 0.8 (12-15)	-	-
Shaft length	41	12 ± 0.4 (11-12)	-	-	25	13 ± 0.6 (12-14)	-	-
Tail length	37	50 ± 3 (43-56)	93	46.7 ± 3.45 (39-55)	24	6 ± 2 (3-9)	19	3.74 ± 0.86 (2.5-5)
Tail hyaline portion length	37	25 ± 3 (19-31)	106	24.3 ± 2.66 (20-32.5)	-	-	-	-
Anal body width	37	12 ± 0.4 (10-12)	38	12.3 ± 0.82 (10-13.5)	-	15 ± 2 (13-19)	-	-
Spicule length	-	-	-	-	22	39 ± 2 (36-44)	20	33.5 ± 1.94 (30-37)
Gubernaculum length	-	-	-	-	20	11 ± 1 (9-13)	16	11.5 ± 1.70 (8-15)

pearance, especially near the labial region. Posteriorly, the presence of protuberances of variable arrangement, shape and sizes is observed; in some specimens the protuberances form transverse rows, giving a ring-like appearance, whereas in other specimens, protuberances are irregularly arranged. Near the neck base, those protuberances are closer to one another; they become fused occasionally, exhibiting a much more compact pattern. In some individuals, the neck shows few longitudinal lines that extend up to the middle of the body. Stylet slightly curved in its anterior region and widened towards posterior region; basal knobs

Table 3. Morphometric characters of females and cysts of *Globodera ellingtonae* from Argentina and USA (Handoo *et al*., 2012). Measurements are in µm and as: mean ± SD (range). [1] Measurement indicated as Fenestra length.

	Females				Cysts			
Character	n	Argentina	n	USA	n	Argentina	n	USA
L (excluding neck)	24	600 ± 87 (406-736)	10	480 ± 91 (312-618)	50	583 ± 138 (370-860)	20	599 ± 95 (470-815)
Maximum body width	24	423 ± 97 (179-585)	10	409 ± 140 (178-585)	50	544 ± 174 (320-890)	20	568 ± 112 (415-792)
L/maximum body width	24	1.5 ± 0.2 (1.2-2.3)	10	1.25 ± 0.27 (0.96-1.75)	50	1.1 ± 0.1 (0.9-1.4)	20	1.07 ± 0.11 (0.9-1.22)
Stylet length	10	25 ± 2 (22-27)	8	21.3 ± 1.07 (20-22.5)	-	-	-	-
Number of cuticular ridges between anus and vulval basin	-	-	-	-	42	13 ± 4 (8-25)	23	13 ± 2.31 (10-18)
Distance from anus to vulval basin	-	-	-	-	42	52 ± 12 (32-84)	23	64.5 ± 10.3 (50-85)
Diameter of vulval basin	-	-	-	-	42	23 ± 5 (12-36)	22	27.4 ± 5.44[1] (20-42.5)
Granek's ratio (distance from anus to vulval basin/diameter of vulval basin)	-	-	-	-	42	2.6 ± 0.9 (0.9-5.9)	22	2.37 ± 0.37 (1.69-3)

rounded with slight backward slope. Excretory pore at the base of the neck within a smooth depression, surrounded by the tubercles. At midbody, the cuticle pattern turns into a zigzag pattern. Vulval region opposite the neck. Vulva a transverse slit, located in a rounded or oval depression (vulval basin), flanked by two vulval crescents made up of small perineal tubercles that cover half of the basin. Anus a small slit located in a smooth depression (anal basin), of irregular contour. Lines between the vulva and anus (perineal lines) parallel, some anastomosed and having some cross connections. Lines can be more or less separated, in the latter case showing a more compact pattern. Lines change into a more reticulate pattern around vulval and anal regions, with a high number of connections.

Cyst. Body brown, generally spherical, occasionally oval (especially small cysts). Protuberant neck, generally broken; it appears complete in a few cases. Neck cuticle pattern similar to that of females. Zigzag-like pattern at midbody; transverse striae at this level rarely present. At the terminal region, cuticle lines of circular pattern, surrounded by vulval and anal regions. In some specimens, vulval region may remain intact, maintaining perineal tubercles. In other cysts, circumfenestra, generally rounded, is observed, which may occupy part or the entire vulval basin. Number and morphology of lines between the vulva and the anus more variable than in females; generally arranged in parallel, perpendicular to the vulva-anus axis, with few or several connections among them. In some specimens, the lines are closer to one another, showing a more compact pattern, whereas in other individuals they are more separated, with presence of wide grooves. Some individuals exhibit ridges highly anastomosed among one another, showing a very irregular pattern. Anus a small slit located in a smooth depression (anal basin), of variable size and shape (oval, rectangular or irregular); anal basin delimited by anastomosed perineal and preanal lines surrounding it or

Table 4. Stylet shape of the anterior surface of basal knobs in second-stage juveniles and males of *Globodera ellingtonae* from Argentina. Data are expressed as percentages.

Stage	n	Rounded	Flattened	Forward projection	Backward sloping
Second-stage juveniles	40	7.5%	65%	27.5%	-
Males	25	-	-	20%	80%

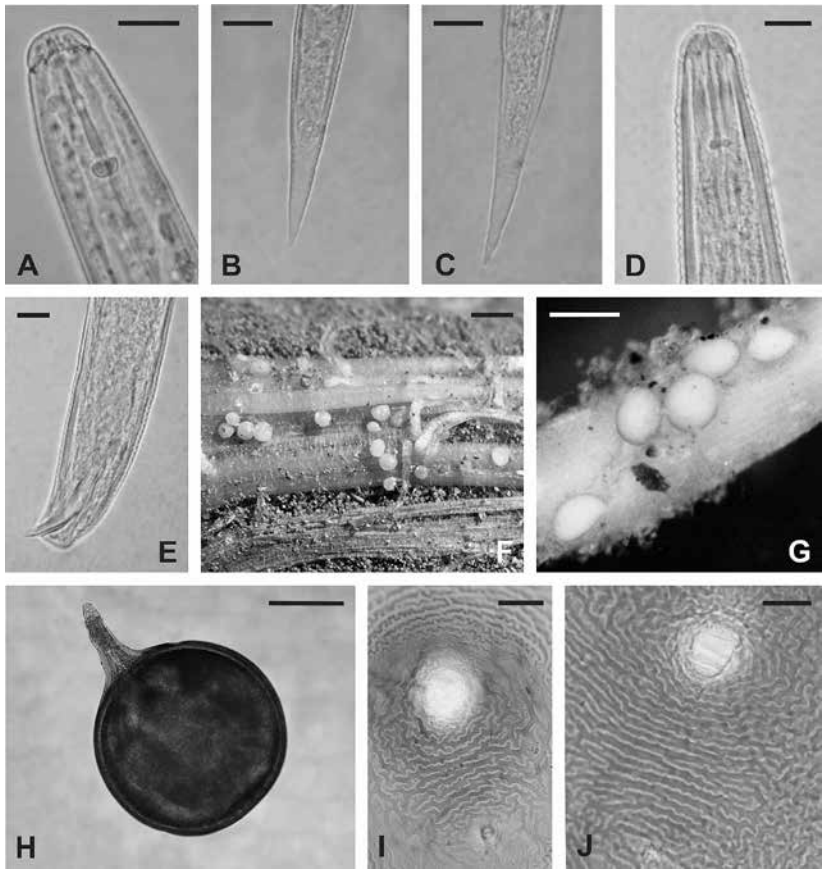

Fig 3. *Globodera ellingtonae* from Argentina. Second-stage juvenile: A) anterior region; B, C) tail region. Male: D) anterior region; E) posterior region. Females: F, G) roots of Andean potatoes 'Colorada' variety with white females. Cyst: H) whole cyst; I, J) vulval region. Scale bars: A, B, C, D and E - 10 μm; F - 1 mm; G - 500 μm; H - 200 μm; I and J - 20 μm.

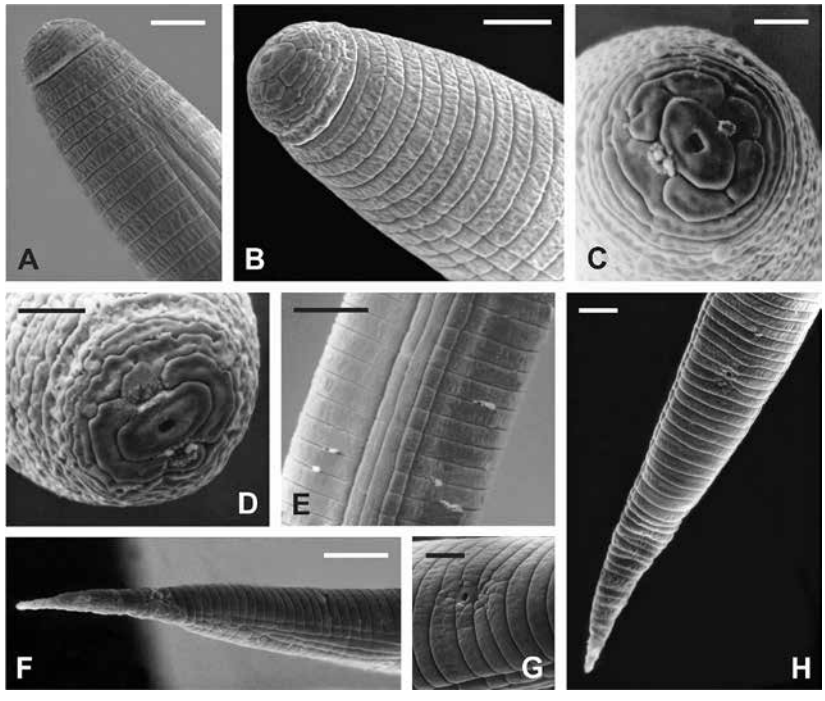

Fig 4. Scanning electron microscope image of the second-stage juveniles of *Globodera ellingtonae* from Argentina. A) anterior region, lateral view; B) anterior region, ventral-lateral view; C) anterior region, apical view; D) anterior region, lateral-apical view; E) lateral field; F) tail region, lateral view; G) detail of anus; tail region, ventral view. Scale bars: A, B, E and H - 5 μm; C, D and G - 2.5 μm and F - 10 μm.

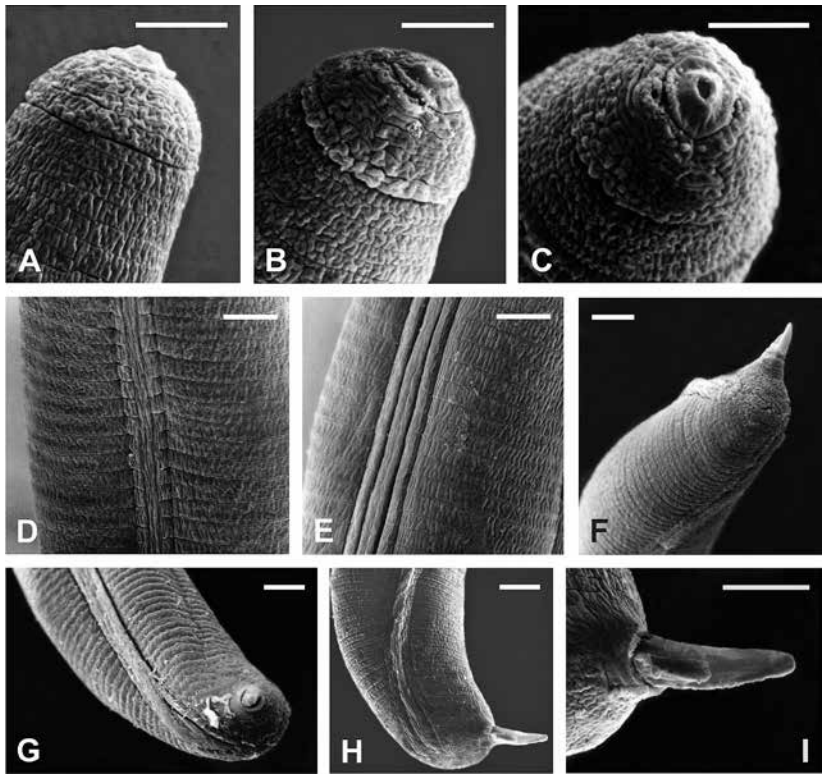

Fig 5. Scanning electron microscope image of the males of *Globodera ellingtonae* from Argentina. A, B) anterior region, lateral view; C) anterior region, ventral-apical view; D, E) lateral field, at midbody; F) posterior region, dorsal view; G) posterior region, ventral view; H) posterior region, lateral view; i) detail of spicule tip. Scale bars: 5 μm each.

interrupting there. In some individuals, the anal basin is very small or very wide. Inside the cyst, a ring of thick cuticle formed by the joining of bullae is below the anal basin. At anus level the V-shaped mark in the cuticle was not observed inside the cyst. Preanal lines parallel or showing different degrees of anastomosis. Few or no small vulval bodies or bullae; they were abundant and larger only in one specimen. Small tubercles surrounding the anal basin. Fine sub-cuticular punctations on the body.

Appendix II

New differential diagnosis comparing Globodera ellingtonae *with other* Globodera *species*

Information of the diagnostic characters useful for identification of *Globodera* species (Fleming and Powers, 1998; EPPO, 2009; Subbotin *et al*., 2010) was compiled for *G. ellingtonae*, *G. rostochiensis*, *G. pallida*, *G. tabacum* and *G. mexicana* (Table 5). A new cyst nematode species, *G. capensis* Knoetze, Swart and Tiedt, 2013, was also included. This species was described from South Africa; it was found in cleared potato fields and the host plant is currently unknown. According to the characters considered as diagnostic, the Argentine population would be assigned to the *G. pallida* species or the *G. tabacum* complex because the mean values agree with those known for populations of those species. However, if only ranges are considered, a marked overlap with the values of *G. rostochiensis* is also found.

According to Handoo *et al*. (2012), *G. ellingtonae* differs morphologically from the related species (*G. pallida*, *G. rostochiensis*, *G. tabacum complex* and *G. mexicana*) in the distinctive J2 tail. It tapers uniformly but abruptly narrows with a constriction near the posterior third of the hyaline portion and ends with a peg-like, finely rounded to pointed terminus. However, such constriction was not found to be a distinctive character in the Argentine population.

Considering the results obtained in the present work, *Globodera ellingtonae* can be distinguished from the related species of the genus present in the American continent (*G. pallida*, *G. rostochiensis*, *G. mexicana* and *G. tabacum* complex) using, besides the molecular analyses, one or some of the following characteristics: *i)* the cuticle colour transition of females during cyst formation (chromogenesis), *ii)* the stylet length of fe-

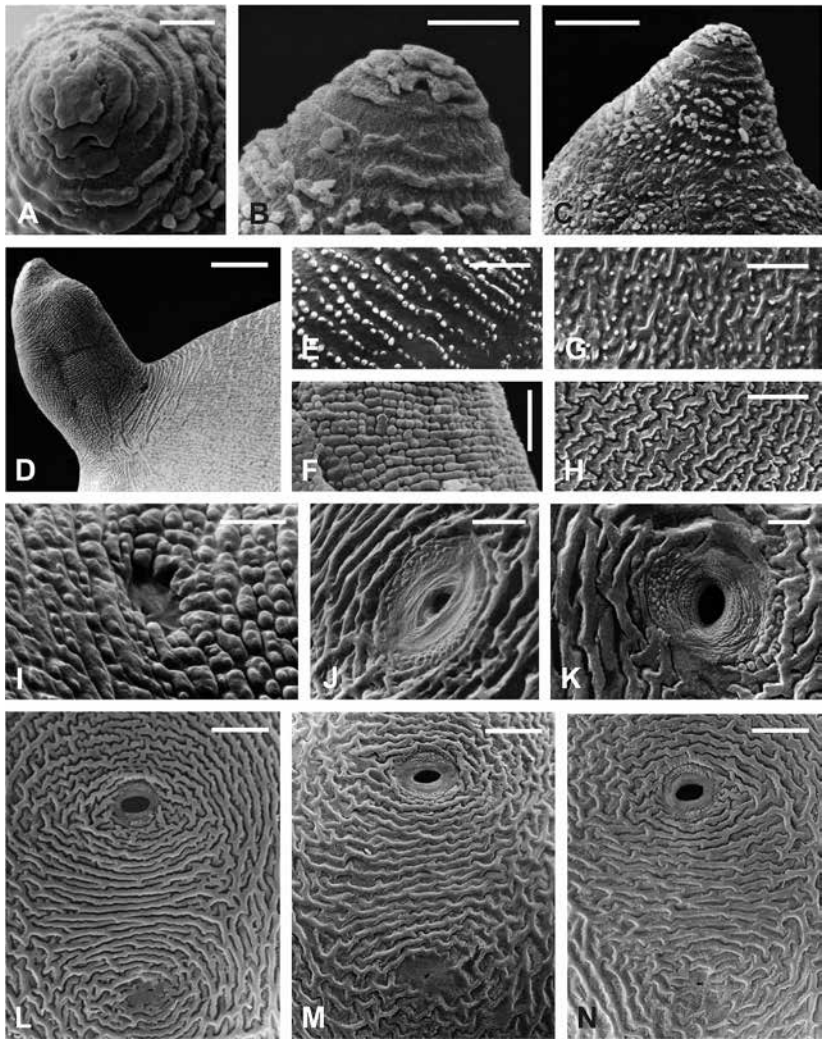

Fig 6. Scanning electron microscope image of the females of *Globodera ellingtonae* from Argentina. A) anterior region, apical view; B, C) anterior region, lateral view; D) anterior region, neck; E) tubercles at neck; F) tubercles at the base of the neck; G-H) detail of cuticle at midbody; I) excretory pore; J-K) detail of terminal area; L-N) terminal area of different females. Scale bars: A - 2.5 μm; B and I - 5 μm; C, D, E, F, G, H, J and K -10 μm; L, M and N - 20 μm.

males and males; *iii)* the morphology of the labial region of J2, males and females, *iv)* the morphology and morphometry of the terminal area of females and cysts; *v)* the gubernaculum length; *vi)* the ability/inability to reproduce on potato or tobacco plants.

Globodera ellingtonae differs from *G. pallida* in several characteristics: *i)* a slightly shorter stylet mean of females and males [21.3-25 µm *vs*. 26-27.4 µm (Stone, 1973a), 22.9-26 µm *vs*. 26.6-27.5 µm (Stone, 1973a), respectively]; *ii)* J2 with oval *vs*. irregular rectangular labial disc (Stone, 1973a), *iii)* males with a pair of submedial (dorsal and ventral) lips separated by an incisure *vs*. fused (Othman *et al*., 1988), *iv)* females with few and small perineal tubercles occupying half of the vulval basin *vs*. small and discrete tubercles (Mulvey and Golden, 1983), occupying most of the vulval basin (Stone, 1973a); *v*) when transforming into a cyst, the white body of the female becomes yellowish and then brown *vs*. a change from white directly to brown (which confers the common name of 'white or pale potato cyst nematode') (EPPO, 2004).

Globodera ellingtonae differs from *G. rostochiensis* in the following characteristics: *i)* adjacent submedial lips separated and clearly delineated lateral lips *vs*. males with adjacent submedial lips fused (partially or completely) with each other or occasionally separated and with lateral lips tending to form a continuous annule (Othman *et al*., 1988); *ii)* lateral lips delineated and differentiated from dorsal and ventral submedial lips *vs*. female labial region with lateral lips not clearly demarcated (Othman *et al*., 1988); *iii)* a white-yellowish-brown transition *vs*. the white female

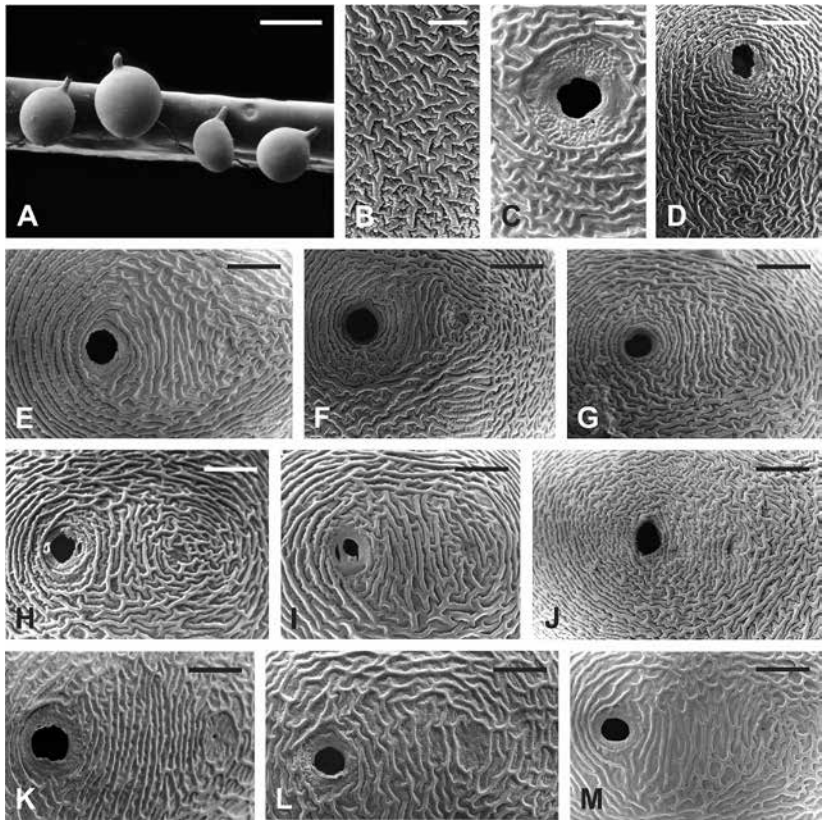

Fig 7. Scanning electron microscope image of the cysts of *Globodera ellingtonae* from Argentina. A) cysts of different sizes; B) detail of cuticle at midbody; C) detail of terminal area; D-M) terminal area, showing different patterns of ridges between vulval basin and anus. Scale bars: A - 500 μm; B and C - 10 μm; D, E, F, G, H, I, J, K, L and M - 20 μm.

goes through a golden yellow phase before turning into a brown cyst ('golden nematode') (Stone, 1973c); *iv)* vulval crescent area with small perineal tubercles, covering half of the basin *vs*. females with large and coalesced perineal tubercles (Mulvey and Golden, 1983), occupying most of the vulval basin (Stone, 1973c); *v)* a lower number of perineal lines between the anus-vulva with mean values ranging between 13-13.2 *vs*. 16-21.6 (Stone, 1973c; Manduric *et al*., 2004); *vi)* a higher mean value of diameter of vulval basin: 23-27.4 µm *vs*. 14.1-20.7 µm (Evans and Franco, 1977; Subbotin *et al*., 2010); *vii)* a lower mean Granek's ratio: 2.37-2.6 *vs*. 2.7-4.5 (Manduric *et al*., 2004; Subbotin *et al*., 2010).

Globodera ellingtonae differs from the *G. tabacum* complex in the following aspects: *i)* cysts with a slightly higher mean number of cuticular ridges between anus-vulval basin: 13-13.2 *vs*. 7.2-12 (Mota and Eisenback, 1993a); *ii)* female labial region with delineated lateral lips, and differentiated from dorsal and ventral submedial lips *vs*. lateral and submedial lips typically fused (Mota and Eisenback, 1993b); *iii)* inability *vs*. ability to reproduce on tobacco plants (Subbotin *et al*., 2010).

Globodera ellingtonae differs from *G. mexicana* (Subbotin *et al*., 2010) in the following characteristics: *i)* males with a longer mean gubernaculum length: 11-11.5 μm *vs*. 9.2 μm; *ii)* cyst with a longer mean diameter of vulval basin: 23-27.4 μm *vs*. 20.85; *iii)* ability *vs*. inability to reproduce on potatoes.

Globodera ellingtonae can be distinguished from *G. capensis* (Knoetze *et al*., 2013) mainly by a lower mean stylet length of J2 (20.9-23 μm *vs*. 24.1-26.3 μm), a smaller mean length of cysts (583-599 μm *vs*. 447-457 μm), and a greater mean diameter of vulval basin (23-27.4 μm *vs*. 19.7-20.6 μm), distance from anus to vulval basin (52-64.5 μm *vs*. 23.8-37.6 μm), and Granek's ratio (2.37-2.6 *vs*. 1.3-2).

Table 5. Update of the principal diagnostic characters (following Fleming and Powers, 1998; EPPO, 2009; Subbotin *et al*., 2010) for the potato cyst nematodes (*G. ellingtonae*, *G. rostochiensis*, *G. pallida*), *G. capensis*, *G. mexicana* and the *G. tabacum* complex (*Globodera tabacum tabacum*, *G. t. virginiae* and *G. t. solanacearum*). Measurements are given in μm: range of means (minimum and maximum value). [1] Measurement indicated as Fenestra length; [2] including neck; [3] measurement indicated for females.

	Second-stage juveniles					Cysts					
Species	Body length	Stylet shape of anterior surface of basal knobs	Stylet length	Tail length	Hyaline portion length	Length	Number of ridges between anus-vulval basin	Diameter of vulval basin	Distance from anus to vulval basin	Granek's ratio	References
G. ellingtonae	428-458 (365-526)	Flattened, rounded, forward projection	20.9-23 (19-24)	46.7-50 (39-56)	24.3-25 (19-32.5)	583-599 (370-860)	13-13.2 (8-25)	23[1]-27.4 (12-42.5)	52-64.5 (32-84)	2.37-2.6 (0.9-5.9)	Skantar *et al*., 2011; Handoo *et al*., 2012; present study
G. capensis	452-495 (430-528)	Rounded to flattened	24.1-26.3 (23-28)	47.1-54.6 (44-73)	26-33.1 (24-41.5)	447-457 (365-590)	8.2-12.6 (7-20)	19.7-20.6 (16-28.5)	23.8-37.6 (19-60)	1.3-2 (0.7-4)	Knoetze *et al*., 2013
G. mexicana	468.5 (333-587)	Forward projection	23.3-23.5 (20-27)	54 (44.2-74.2)	24.5 (19.8-28.8)	815[2] (671-999)	-	20.85 (15.2-28.5)	58.6 (34-110)	2.8 (1.7-5.3)	Subbotin *et al*., 2010
G. pallida	441.1-547.3 (380-533)	Flattened, rounded, forward projection	21.8-24.9 (20.6-25.5)	47.9-63.3 (40-57)	23-31 (20-31)	510-675 (400-748)	12-15.2 (7-26)	20.7-26[1] (17.5-45)	48-59.2 (28-88)	2.1-2.6 (1.2-3.6)	Stone, 1973a, b; Øydvin, 1973; Evans and Franco, 1977; Manduric *et al*., 2004; Skantar *et al*., 2011; Subbotin *et al*., 2010
G. rostochiensis	392-506 (366-505)	Flattened, rounded, forward projection	19.9-22.5 (18.9-24)	43.5-52.2 (36.4-57)	19.9-26.5 (18-30)	445-680 (450-990)	16-21.6[3] (12-31)	14.1-20.7[1] (8-20.7)	50.7-75.8 (29-149)	2.7-4.5 (1.2-9.5)	Stone, 1973c; Øydvin, 1973; Evans and Franco, 1977; Manduric *et al*., 2004; EPPO, 2009; Subbotin *et al*., 2010
G. tabacum complex	433-576.1 (384-661.2)	Pointed, concave, rounded	21.2-24.9 (18.9-28)	49.8-57.6 (34-71.1)	20.9-29.8 (16.2-37.8)	460.5-767.3 (348-937)	7.2-12 (4.1-26)	15[1]-27.6[1] (12.6-37.8)	35-59.2 (17.5-92.4)	1.6-2.8 (1-4.9)	Chaves, 1987; Mota and Eisenback, 1993a; Abelli, 2003; EPPO, 2004; 2009; Subbotin *et al*., 2010