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Morphological and molecular identification of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* in Portuguese potato fields

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Summary – The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* pose one of the greatest threats to potato crops worldwide and are subject to strict quarantine regulations in many countries. The identification of these *Globodera* species based on morphology may be ambiguous due to the variability of the main morphological features and the overlapping of the standard parameters in these two species; thus, confirmation *via* molecular methods is recommended. Multiplex PCR with species-specific primers (ITS5/PITSp4 + PITSr3) allows both species to be distinguished. However, despite the development of molecular identification methods, the morphological approach remains useful as a complementary diagnostic technique. In this work, we report results of morphological and molecular analyses that were carried out in two *Globodera* species from Portuguese potato fields. The average morphometric values of 40 cysts and 40 second-stage juveniles were generally within the expected ranges for *G. pallida* and *G. rostochiensis* with some variations noted. Molecular analysis with multiplex PCR confirmed the morphometric identification. The present results confirmed the occurrence of two potato cyst nematode species, *G. rostochiensis* and *G. pallida*. Surprisingly, the analysis of soils from Portuguese potato fields detected a greater number of samples infested with *G. pallida*, which is contrary to expectation as *G. rostochiensis* has been considered the most widespread species in Portugal. The distinction between the two species is therefore essential in order to detect their presence in the country with a view to re-evaluating the control measures implemented so far and adopting more effective practices.

Keywords – Heteroderidae, morphology, multiplex PCR, *Solanum tuberosum*.

Cyst nematodes (*Heterodera* and *Globodera*) are an economically important group of plant-parasitic nematodes, present throughout the world, affecting all major horticultural crops (Marks & Brodie, 1998). In particular, potato cyst nematodes (PCN) can be devastating to potato fields if they are not controlled in a timely manner, leading to the abandonment of vast areas of production (Van de Vossenberg *et al*., 2014).

Species of PCN include the golden potato cyst nematode, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959, and the pale potato cyst nematode, *G. pallida* Stone, 1973, and are considered harmful quarantine organisms, described in European Union Directives 2000/29/EC and 2009/7/EC and are also part of EPPO A2 List (quarantine species already present in the EPPO region, A2/125 and A2/124, respectively) (EPPO, 2015). These species are regulated by the European Directive 2007/33/EC on the control of potato cyst nematodes and are subject to stringent regulatory measures when detected singly or in combination (EPPO, 2013, 2015).

Globodera rostochiensis and *G. pallida*, originating from the Andes in southern Peru, have spread worldwide

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(Grenier *et al*., 2010). They are thought to have been introduced into Europe in the 16-17th century with potato tubers that would have carried infested soil. In Portugal, *G. rostochiensis* was first reported in 1956 in a field of seed potatoes near Bragança (Trás-os-Montes district) (Macara, 1963) and is currently present in all potato-producing regions of the country (Cunha *et al*., 2004; DGAV, 2015). *Globodera pallida* was first identified in the country in 1988, also in Trás-os-Montes (Santos & Fernandes, 1988).

In Portugal, the potato has a great social and economic importance, since it is grown throughout the country, including on the islands of Madeira and Açores. The most representative production areas are Bragança, Chaves, Aveiro, Viseu, Oeste Region and Montijo. As potato cyst nematodes pose a risk to potato production in Portugal, a national potato nematode survey was implemented in 2010, establishing a new framework for phytosanitary protection measures for these harmful organisms, with a view to avoid dispersion in national and community territories and to ensure potato production of a guaranteed quality for consumers.

Due to their huge economic and trade impacts, it is crucial to distinguish these species accurately using diagnostic tools. Morphological identification, based on a few characters of the second-stage juvenile (J2) and of the perineal area of the cyst, has been quite successful but always carries some uncertainty (Bačić *et al*., 2013; Seesao *et al*., 2016; Tirchi *et al*., 2016). Therefore, due to the variability of the main morphological features and the overlapping of standard diagnostic parameters in these two species, a confirmation through molecular methods is recommended. Molecular methods such as multiplex PCR with species-specific primers (ITS5/PITSp4 + PITSr3) (Bulman & Marshall, 1997) have been successfully applied to differentiate *G. rostochiensis* from *G. pallida*.

The aim of this study was to identify *G. rostochiensis* is and *G. pallida* from Portuguese potato fields using morphological and PCR-based methods. The rapid identification of the two species is essential to detect their presence in the nation in order to re-evaluate the control measures implemented so far and adopt more effective practices.

Materials and methods

Nematode sample reference material (cysts of *G. pallida* Pa3 (E400) and *G. rostochiensis* Ro 2/3) was provided by NVWA – the Netherlands Food and Consumer Product Safety Authority, Wageningen, The Netherlands. The Portuguese biological material was extracted from samples of infested soils collected from the main producing potato regions in 2015 and 2016 in the northern (Viana do Castelo, Chaves and Esposende), central (Vagos, Mira and Cantanhede) and southern cultivation areas (Salvaterra de Magos). Those samples were provided by the Laboratory of Nematology of INIAV – the National Institute for Agrarian and Veterinary Research, Oeiras, Portugal. Cysts were extracted from the soil samples using the Fenwick can method (Fenwick, 1940) and the J2 were recovered from cysts. Eight isolates of *Globodera* spp. from 2015 and 32 isolates of *Globodera* spp. from 2016 were used for morphological and molecular analysis.

Morphological characterisation

Vulval cones were cut from cysts with an ophthalmic scalpel and mounted in sterilised tap water. Juveniles were heat-killed and examined using an Olympus BX-41 bright field light microscope. Pictures were taken with an Olympus DP10 digital camera. The observed characters of cysts and J2 were compared with those from the reference materials and descriptions in the literature (Marks & Brodie, 1998; EPPO, 2013). Measurements were made using a ProgResSpeed XT core 5 – Jenoptik image software and dimensions are expressed in μm. Morphological identification of PCN was based on a combination of J2 and cyst characters: body length, stylet length, tail and hyaline region, fenestra diam., distance fenestra to anus, Granek's ratio (the distance from anus to the nearest edge of vulval basin divided by vulval basin diam.), and number of cuticular ridges between vulva and anus.

Nematode DNA

Half cysts containing eggs and J2 (originating from cysts used for the previous morphological characterisation) were used for DNA extraction. Each half cyst was transferred to an Eppendorf tube with 10 μl of distilled sterilised water, frozen in liquid nitrogen and homogenised with a micropestle (Eppendorf). The homogenate was incubated overnight at 56°C in lysis buffer (ATL) and 100 μg ml^{-1} proteinase K. After incubation, total genomic DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions. DNA samples were stored at −20°C.

Multiplex PCR by species-specific primers

The internal transcribed spacer region (ITS) of the ribosomal DNA repeat unit was amplified by multiplex PCR. Multiplex PCR reactions were performed in a 25 μl final volume of using the Promega GoTaq Flexi DNA Polymerase Kit (Promega), containing 1 μl template DNA, 5 μl GoTaq Flexi PCR buffer, 1.5 mM $MgCl_2$, 0.20 mM each dNTPs, 1.25 U GoTaq Flexi DNA Polymerase (Promega) and 0.4 μM of each primer in a Biometra TGradient thermocycler (Biometra). The set of primers for *G. rostochiensis* were: ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and PITSr3 (5′-AGC GCA GAC ATG CCG CAA-3′) and for *G. pallida*: ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and PITSp4 (5′-ACA ACA GCA ATC GTC GAG-3′) (Bulman & Marshall, 1997).

The amplification profile for ITS-rRNA consisted of an initial denaturation of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension of 72°C for 7 min (EPPO, 2013). The amplified products were loaded onto a 1.5% agarose gel containing 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide and 0.5×Tris-borate-EDTA (TBE) running buffer and electrophoresed at 5 V/cm. Amplifications were visualised using the VersaDoc Gel Imaging System (Bio-Rad). The expected length of the PCR products was 265 bp for *G. pallida* and 434 bp for *G. rostochiensis*. Possible contamination was checked by including negative controls (no DNA) in all amplifications.

Amplification PCR products using the same pair of primers were used for one previously confirmed *G. rostochiensis* isolate (Ro 2/3) and one isolate of *G. pallida* (Pa3 (E400)) from NVWA used as positive controls.

Results

Morphology and morphometrics

The morphological characters of the 40 cysts and 40 J2 from Portuguese populations did not always match the descriptions of *G. rostochiensis* and *G. pallida* (Table 1), but the morphometric average values were within the range described in the literature (Marks & Brodie, 1998; EPPO, 2013) (Table 2).

Second-stage juveniles

The J2 of both species follow the general description of these species: a vermiform body, tapering at both extremities, more so posteriorly. The tail reduced, shorter in *G. rostochiensis*. In both species the head is rounded, slightly offset with prominent cephalic sclerotisation. The stylet is well developed with anteriorly flattened to rounded knobs in *G. rostochiensis* and with distinct forwardly projecting knobs in *G. pallida*. However, in some situations, the characterisation of the knob shape may be ambiguous.

Cysts

Cysts were yellow or brown in colour, spherical in shape, with protruding necks and lacking a terminal cone. Vulva fenestrate, anus prominent and visible in almost all specimens, with a V-shaped mark. However, the cyst wall pattern did not always permit species identification.

PCR species-specific primers

All DNA extracts from Portuguese samples produced amplification products of the same size as those obtained from the *G. rostochiensis* and *G. pallida* controls when species-specific primers were used in multiplex PCR reactions.

Nine of the Portuguese samples yielded a single amplification product of *ca* 434 bp, the same size as the one from the known *G. rostochiensis* control and 31 of the Portuguese samples yielded a single PCR product of *ca* 265 bp, the same size as that from the known *G. pallida* control (Fig. 1).

From the molecular data obtained in this study, two potato cyst nematode species (*G. rostochiensis* and *G. pallida*) were identified (Table 3), and the analysis of soils coming from Portuguese potato fields detected a greater number of samples infested with *G. pallida*, which contradicted expectations as *G. rostochiensis* has been considered the most widespread species in Portugal (Conceição *et al*., 2003; Cunha *et al*., 2004).

Discussion

The results in this study clearly demonstrate that the *Globodera* populations present in Portuguese potato fields are morphologically variable, with some features outside the ranges expected for *G. pallida* or *G. rostochiensis*, making it difficult to identify PCN species when cysts and J2 are individually analysed. However, morphometric average values (Table 2) were within the range provided in the literature (Marks & Brodie, 1998; EPPO, 2013).

Table 1. Morphometric characteristics of *Globodera rostochiensis* and *G. pallida* cysts and second-stage juveniles of Portuguese (n = 24 from the northern, n = 15 from the central and n = 1 from the southern cultivation area; total of n = 40 samples) and reference PCN species. All measurements (absolute values) are in μm.

Cyst No.	Second-stage juvenile				Cyst			
	Body length	Tail length	Hyaline region	Stylet length	Fenestra to anus	Fenestra diam.	Granek's ratio	No. cuticular ridges
Portuguese samples								
1	385.0	(*)	26.4	*28.9*	*42.6*	*21.5*	*2.0*	(*)
2	*499.0*	29.5	19.5	20.8	*34.7*	19.1	*1.8*	13
3	395.0	(*)	(*)	*23.7*	55.6	14.7	3.8	>14
4	*477.9*	*52.7*	(*)	22.1	51.1	*25.3*	*2.0*	>14
5	*481.9*	*52.1*	26.9	*23.9*	76.8	*22.3*	3.5	>14
6	*534.0*	50.2	*28.8*	*26.3*	*40.0*	*29.3*	*1.4*	*11*
7	414.7	(*)	(*)	21.4	92.6	*22.6*	4.1	>14
8	375.4	42.2	23.0	*24.8*	*42.4*	*20.6*	*2.1*	*12*
9	406.1	49.3	23.4	*28.7*	109.1	*36.1*	3.0	*>14*
10	399.6	42.4	17.5	*27.4*	52.0	*23.5*	*2.2*	14
11	379.0	48.8	19.7	*28.1*	59.9	19.2	3.1	>14
12	422.2	43.8	*28.6*	*27.1*	51.9	*23.8*	*2.2*	(*)
13	404.1	44.2	22.5	21.4	152.3	*31.8*	4.8	(*)
14	(*)	46.3	*28.2*	21.4	93.4	*24.1*	3.9	>14
15	444.9	50.9	*28.0*	*25.0*	58.6	*24.8*	*2.4*	15
16	405.0	50.3	25.7	*25.2*	*30.6*	18.8	*1.6*	(*)
17	405.9	48.5	*27.7*	22.1	87.6	*30.2*	2.9	(*)
18	390.0	(*)	18.6	21.5	71.1	18.8	3.8	>14
19	416.6	50.1	26.3	*25.5*	*44.4*	*22.2*	*2.0*	*10*
20	433.7	49.3	26.7	*25.0*	*46.7*	*22.2*	*2.1*	(*)
21	401.4	50.1	26.3	*23.2*	92.7	*24.0*	3.9	>14
22	*476.8*	*51.6*	*28.6*	*24.5*	63.7	13.3	4.8	*12*
23	*511.2*	*51.3*	23.7	*26.4*	*32.9*	10.6	3.1	14
24	436.9	46.0	28.3	22.2	58.1	17.6	3.3	17
25	437.9	46.9	19.1	*23.8*	61.7	*22.4*	2.8	20
26	405.7	46.5	21.5	*24.5*	*30.1*	13.0	*2.3*	*7*
27	487.3	45.3	*32.2*	22.8	55.7	16.7	3.3	>14
28	458.7	(*)	*43.5*	20.7	*48.3*	*23.6*	*2.0*	*12*
29	381.9	42.5	22.8	*23.8*	*45.7*	19.4	*2.4*	>14
30	*483.0*	47.8	*29.4*	22.2	*21.2*	19.6	*1.1*	*10*
31	*477.1*	49.4	25.6	22.7	65.3	*28.8*	*2.3*	16
32	393.3	45.6	19.3	22.0	93.0	*26.1*	3.6	>14
33	386.7	34.6	19.2	21.9	*47.2*	16.3	2.9	12
34	425.9	49.6	25.9	*25.6*	*42.0*	*24.5*	*1.7*	*8*
35	390.8	41.5	19.9	*24.8*	*32.0*	*27.3*	*1.2*	*9*
36	375.8	31.7	17.3	*25.3*	63.0	18.8	3.4	16
37	420.9	44.4	21.8	22.2	66.6	17.9	3.7	(*)
38	463.3	46.0	31.2	23.1	52.6	*26.9*	*2.0*	(*)
39	446.9	50.3	23.9	22.1	54.7	*21.0*	2.6	(*)
40	380.2	42.5	22.8	*23.2*	*36.8*	19.0	*1.9*	*10*

Table 1. (Continued.)

Cyst No.	Second-stage juvenile				Cyst			
	Body length	Tail length	Hyaline region	Stylet length	Fenestra to anus	Fenestra diam.	Granek's ratio	No. cuticular ridges
Reference samples								
P3	(*)	50.7	17.0	21.2	63.7	20.3	3.1	14
P4	(*)	*76.0*	*34.3*	*25.8*	52.0	*35.0*	*1.5*	11
P5	(*)	*61.4*	*28.0*	*25.1*	52.2	*30.4*	*1.7*	(*)
P6	(*)	48.4	23.6	*25.8*	*47.0*	*21.7*	*2.2*	*9*
R4	396.20	*65.3*	*29.1*	*23.5*	*49.6*	*31.8*	*1.6*	(*)
R5	(*)	*52.5*	*30.4*	*23.4*	*31.3*	*21.0*	*1.5*	(*)
R11	(*)	48.0	20.3	22.6	59.7	18.2	3.3	(*)
R12	441.90	47.2	25.5	22.2	76.6	*33.7*	*2.3*	(*)
Reference measurements								
Marks & Brodie (1998)	(*) *(*)*	(*) *(*)*	(*) *(*)*	21-23 (22) *21-26 (>23)*	37-77 (>55) *22-67 (<50)*	8-20 (<19) *18-21 (>19)*	1.3-9.5 (>3) *1.2-3.5 (<3)*	12-31 (>14) *8-20 (<14)*
PM 7_70 (3)	*452-486*	*50-53*	*26-27*	*23-24*	*48-54*	*(*)*	*2.1-2.5*	*12*
PM 7_70 (3)	392-468	44-51	20-27	20-22	51-70	(*)	3.0-4.5	17-20
PM 7_70 (3)	452-468	50-51	26-27	22-23	51-54	18-20	2.5-3.0	12-17

(*): no data; italics: *G. pallida* data; underlined: *G. rostochiensis* data; roman: both species characteristics.

Table 2. Morphometric characteristics of cysts and second-stage juveniles of Portuguese (Pt) compared to reference (Ref) *Globodera pallida* and *G. rostochiensis*. All measurements are in μm and in the form: mean $\pm$ s.d. (range).

	G. pallida (Pt) (n = 31)	*G. rostochiensis* (Pt) (n = 9)	*G. pallida* (Ref) (n = 4)	*G. rostochiensis* (Ref) (n = 4)
Second-stage juvenile				
L	428 $\pm$ 42.8 (375-534)	429 $\pm$ 43.9 (380-499)	(*)	419 $\pm$ 32.3 (396-442)
Stylet length	24.4 $\pm$ 2.3 (20.7-28.9)	22.4 $\pm$ 0.9 (20.8-23.7)	24.5 $\pm$ 2.2 (21.2-25.8)	23.0 $\pm$ 0.8 (22.2-23.5)
Tail length	47.1 $\pm$ 4.5 (31.7-52.7)	44.7 $\pm$ 5.2 (34.6-50.3)	52.8 $\pm$ 5.9 (48.4-61.4)	49.9 $\pm$ 2.6 (47.2-52.5)
Hyaline region	25.4 $\pm$ 5.3 (17.3-43.5)	22.9 $\pm$ 4.1 (19.2-31.2)	24.4 $\pm$ 2.4 (22.9-28.0)	26.2 $\pm$ 4.3 (20.3-29.8)
Cyst				
Fenestra diam.	22.2 $\pm$ 5.4 (10.6-36.1)	21.1 $\pm$ 5.0 (14.7-28.8)	23.9 $\pm$ 4.5 (20.3-30.4)	21.0 $\pm$ 2.2 (18.2-23.7)
Fenestra to anus	59.7 $\pm$ 27.4 (21.2-152.3)	56.3 $\pm$ 17.6 (34.7-93.0)	53.7 $\pm$ 7.1 (47.0-63.7)	54.3 $\pm$ 18.9 (31.3-76.6)
Granek's ratio	2.8 $\pm$ 1.1 (1.1-5.1)	2.8 $\pm$ 0.8 (2.0-3.8)	2.1 $\pm$ 0.7 (1.5-3.1)	2.2 $\pm$ 0.8 (1.5-3.3)
No. cuticular ridges	12.5 $\pm$ 3.6 (7-20)	12.8 $\pm$ 2.5 (10-16)	11.3 $\pm$ 2.5 (9-14)	>14

(*): no data.

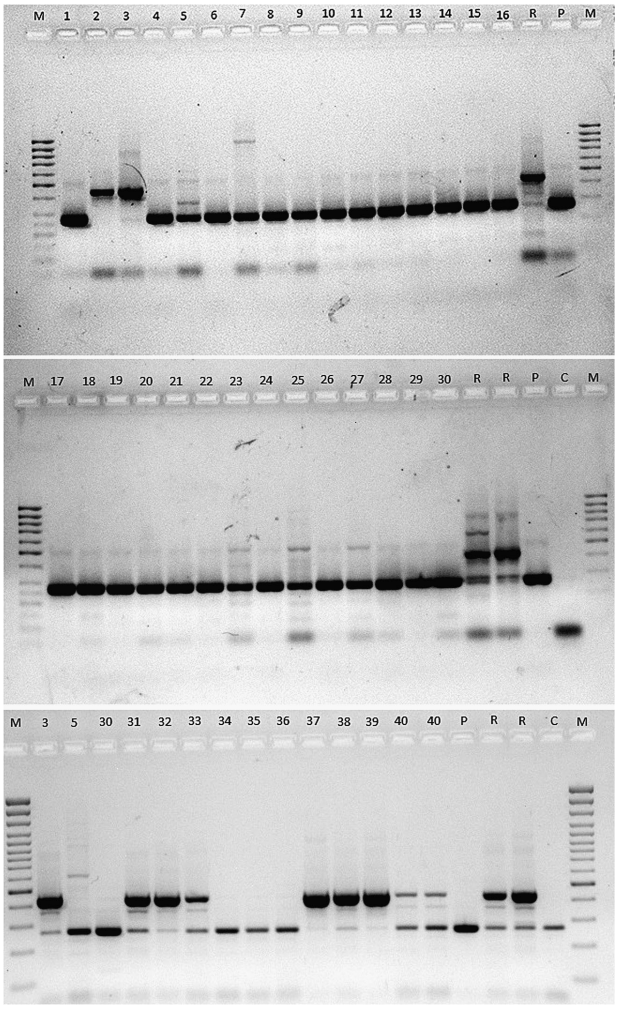

Fig. 1. Molecular identification of Portuguese PCN. Agarose gel (1.5%) of the amplified products obtained with multiplex polymerase chain reaction using primers PITSr3, PITSp4 and the common primer ITS5 M = GeneRuler 100 bp DNA Ladder (Thermo Scientific); 1-40 = Portuguese *Globodera* spp.; R = reference *Globodera rostochiensis*; P = reference *G. pallida*; C = PCR control.

Table 3. Identification of *Globodera* species extracted from soil samples.

Cyst No.	Soil sample	*Multiplex* PCR identification
1	Gb1_2016	*G. pallida*
2	Gb10_2015	*G. rostochiensis*
3		
4	Gb17_2015	*G. pallida*
5		
6		
7		
8		
9	Gb2_2016	*G. pallida*
10		
11		
12		
13		
14	Gb3_2016	*G. pallida*
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31	Gb4_2016	*G. rostochiensis*
32		
33		
34	Gb5_2016	*G. pallida*
35		
36		
37	Gb6_2016	*G. rostochiensis*
38		
39		
40	Gb4_2016	*G. rostochiensis*

According to the obtained morphometric data, morphological characterisation did not always match the molecular identification (as shown by comparing the morphological characteristics typical of *G. rostochiensis* species of samples nos 7, 11, 13, 18, 24, 27 and 36 in Table 1 and their molecular identification as *G. pallida* in Table 3. On the other hand, sample no. 38 presented morphological characteristics of *G. pallida* yet was molecularly identified as *G. rostochiensis*). These species may be variable at morphological level, so it is recommended that sequence-specific multiplex PCR (Bulman & Marshall, 1997) is used to complement the morphological diagnosis of the Portuguese populations.

Also, the analysis of soils from the national potato fields in 2015 and 2016 revealed a greater number of samples infested with *G. pallida*. Five of the nine soil samples analysed were infested with this species, showing

a trend towards an increase of *G. pallida* in Portugal. In addition, it was verified that all the main areas of potato cultivation are already infested with *G. pallida*. This may have resulted from the application of control measures for potato cyst nematodes through the intensive use of *G. rostochiensis*-resistant cultivars, as occurred in the UK (Varypatakis *et al*., 2016). It is crucial to distinguish these species by phytosanitary diagnostic tools since resistant potato cultivars for *G. pallida* are less available, whilst the occurrence of *G. rostochiensis* does not impact potato cultures significantly as most of the potato cultivars used nowadays are resistant or tolerant to this species (Douda *et al*., 2014).

In this context, the National Survey Plan for PCN should cover the largest potato production area possible, with particular attention to the seed potato fields for the exclusion of both species. If feasible, this survey should also be intensified in order to confirm the dispersion trend of *G. pallida* with a view to re-evaluate the control measures so far implemented and for the adoption of more effective practices.

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