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PCR-RFLP diagnostic method for identifying *Globodera* species in Slovenia

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Summary. Species identification within the genus *Globodera* is based on the morphological and morphometrical characters of the cysts and second stage juveniles, and these are included in the majority of identification keys. Morphometrical methods are fast and can be applied to most of samples but they demand a trained and experienced specialist. Furthermore, some morphometrical characters may overlap between populations and between species, leading to inaccurate identification. To confirm and complement the morphometrical identification of *Globodera* species molecular methods have been developed. Sequences of the internal transcribed spacer regions ITS1 and ITS2 of the rDNA gene cluster proved to be useful for identifying nematode species identification. A PCR-RFLP molecular method was used to identify *Globodera rostochiensis, G. pallida*, *G. tabacum* and *G. achilleae*. *Globodera rostochiensis, G. pallida*, *G. tabacum* and *G. achilleae* can be distinguished with PCR-RFLP analysis of the rDNA ITS fragment using five restriction enzymes. The RFLP patterns of *G. rostochiensis, G. tabacum* and *G. achilleae* were species-specific, while those of *G. pallida* varied. South American populations of *G. pallida* differed from other populations as their RFLP patterns were demonstrated to be distinct by *in silico* restriction of the ITS sequences deposited at NCBI.

Key words: *Globodera*, potato cyst nematodes, rDNA, ITS, restriction enzymes.

Introduction

Globodera rostochiensis (Woll.) Behrens, the yellow or golden potato cyst nematode, and *G. pallida* (Stone) Behrens, the white or pale potato cyst nematode, belong to the *Globodera* genus, which consists of about twelve species, of which many are unimportant because their distribution is narrow or they do not affect hosts of economic importance. Nevertheless, the genus attracts considerable attention because of the great economic losses that are caused by the potato cyst nematodes (PCN) worldwide (Trudgill *et al.,* 1975; Turner and Evans, 1998). Both PCN species, *G. rostochiensis* and *G. pallida*, are listed as A2 quarantine pests in Europe and the EPPO region countries. In Slovenia, PCN surveys have been carried out by the Agricultural Institute of Slovenia and Phytosanitary Inspection Service since 1963. The first PCN report dates back to 1971 when a single cyst of *G. rostochiensis* was detected in a soil sample from Dobrova close to the Slovene-Austrian border (Hržič, 1971). Afterwards, no PCN was encountered in spite of intensive inspection of fields until 1999, when a relatively severe infestation was discovered at Libeliče, near the Slovene-Austrian border (Urek and Lapajne, 2001). After that, *G. rostochiensis* was detected several times, mainly in the central and western part of Slovenia. *G. pallida* has not yet been detected in cultivated soils in Slovenia. Therefore, the authorities declared the whole of Slovenia a protected zone for *G. pallida* in 2003. *G. pallida* was, however, intercepted in import consignments on several occasions. Besides *G. rostochiensis*, the yarrow cyst nematode, *G. achilleae* (Golden and Klindić) Behrens, has been repeatedly found in cultivated land, mainly in central and eastern Slovenia (Širca and Urek, 2004).

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The three *Globodera* species which can cause confusion in PCN identification in Europe are *G. achilleae*, *G. artemisiae* (Eroshenko and Kazachenko) Behrens and the *G. tabacum* complex. The first two of these species are not parasitic on potato but they are recorded on the weeds *Achillea millefolium* L. and *Artemisia vulgaris* L. respectively. In North and Central America, the *G. tabacum* species complex (*G. tabacum tabacum* [Lownsbery and Lownsbery,] Behrens, *G. tabacum solanacearum* [Miller and Gray] Behrens and *G. tabacum virginiae* [Miller and Gray] Behrens) parasitizes tobacco, potato and solanaceous weeds. *G. tabacum* is also present in southern Europe (EPPO, 2004). The diagnosis of *Globodera* is of great importance because of possible confusion with economically unimportant species and the differences in virulence of *G. rostochiensis* and *G. pallida*.

Morphology has traditionally been used to identify species of cyst-forming nematodes. However, some diagnostic characters, such as characteristics of the cyst perineal region and the stylet of J2, may overlap between populations and between species, leading time-consuming and potentially inaccurate procedures to identify the species (Fleming and Powers, 1998). Several studies have found that the ribosomal DNA (rDNA) gene cluster is very useful for the identification of *Globodera* species (Ferris *et al*., 1993; Mulholland *et al*., 1996; Bulman and Marshall, 1997; Subbotin *et al*., 2000; Širca and Urek, 2004). It is all the more popular because it is both rapid and reliable. The internal transcribed spacer (ITS) region has been used to detect *G. rostochiensis* and *G. pallida* in mixed populations (Mulholland *et al*., 1996; Bulman and Marshall, 1997; Fullaondo *et al*., 1999; Madani *et al*., 2008), while the PCR-RFLP approach has been used to distinguish between *G. rostochiensis, G. pallida* and *G. tabacum* (Thiéry and Mugniéry, 1996; Fleming *et al.,* 2000; Skantar *et al.,* 2007) and to separate *G. rostochiensis* from *G. achilleae* (Širca and Urek, 2004). rDNA-RFLP analysis clearly separates *G. rostochiensis* from *G. pallida* as well as from other *Globodera* species despite a certain sequence diversity within the rDNA haplotypes of these species (Subbotin *et al*., 2000).

In this study, a sample of *G. pallida* that was intercepted at the Slovene-Italian border in a consumption potato import consignment was morphometrically analysed. The main goal of the study was to develop a molecular method to distinguish between *G. rostochiensis, G. pallida*, *G. tabacum* and *G. achilleae*. The PCR-RFLP technique successfully differentiated all the analysed species. The RFLP patterns of *G. rostochiensis, G. tabacum* and *G. achilleae* were species specific while two RFLP patterns were seen for *G. pallida*. To our knowledge, the PCR-RFLP approach described here is the first to differentiate all four of these species.

Materials and methods

Nematodes

The population samples of *G. rostochiensis* and *G. achilleae* from Libeliče and Zadraga respectively, had been morphometrically and molecularly characterised before (Širca and Urek, 2004) since cysts of these populations were collected from the same locations in 2008 and used for PCR-RFLP analysis. The *G. pallida* population sample was intercepted at the Slovene-Italian border in a consumption potato import consignment during a national survey of potato cyst nematodes in 2003. The consignment came from an export company in Padova, Italy, and the precise location of the potato cultivation area was unknown. Cysts of *G. pallida* were identified morphometrically at the time and multiplied in a pot culture of potato cv. Désirée in 2008. The *G. pallida* population sample from this culture was examined morphometrically and subjected to molecular analysis. The population sample of *G. tabacum* was obtained from the Plant Protection Service, Wageningen, The Netherlands. In addition to live nematodes, sequences of *G. rostochiensis, G. pallida, G. tabacum* and *G. achilleae* (Table 1) from NCBI GenBank were used for *in silico* RFLP analysis.

Morphometrical analysis of *G. pallida* population sample

Ten cysts containing eggs and juveniles of the cultured *G. pallida* population sample were analysed. Cyst vulval cones and hatched juveniles fixed in triethanolamine-formalin (TAF) were morphometrically characterized using an

Optiphot-2 light microscope (Nikon, Japan) connected to a DXM 1200F digital camera (Nikon). Photomicrographs of the specimens were analysed with Lucia image analyser software (Nikon) and the diagnostic characters of the species were measured. Specimens were morphometrically identified on the basis of taxonomic keys (Golden, 1986; Brzeski, 1998; Fleming and Powers, 1998).

DNA extraction and PCR amplification

Cyst containing eggs and juveniles of each species of *G. rostochiensis, G. pallida, G. tabacum* and *G. achilleae* were transferred to a 1.5 mL tubes, one cyst per tube. DNA was extracted from each cyst using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted DNA was diluted in 10 μL of TE buffer. A fragment containing the partial 18S gene, complete ITS1, 5.8S and ITS2, and the partial 28S rRNA gene was amplified using forward (CGTAACAAGGTAGCTGTAG) and reverse (TCCTCCGCTAAATGATAT) primers (Ferris *et al*., 1993). Each PCR contained 1 μL of diluted DNA, 10 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$ (Promega), 10 mM of each of the dNTPs (Promega), 1 μM of forward and reverse primers, 1 U *Taq* Polymerase (Promega) and distilled water to make up 25 μL. The amplification was carried out in an AMP PCR system 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following program: 94°C for 2.5 min, 35 cycles

Table 1. List of nematode species, population samples and rDNA sequences used for PCR-RFLP and *in situ* RFLP analyses.

Species	Location	Material	Accession No.	References
Globodera rostochiensis	Slovenia, Libeliče	Cysts		This study
	Slovenia, Libeliče	Sequence	AY700060	Širca and Urek, 2004
	USA, New York	Sequence	EF153839	Skantar *et al.*, 2007
	Canada, Newfoundland	Sequence	FJ212163	Madani *et al.*, 2008
	Russia, Moscow region	Sequence	DQ847119	Subbotin *et al.*, 2000
	Canada, Quebec	Sequence	FJ212166	Madani *et al.*, 2008
	UK, Scarcliffe	Sequence	DQ847118	Subbotin *et al.*, 2000
G. pallida	Italy, unknown	Cysts		This study
	UK, York	Sequence	EF153838	Skantar *et al.*, 2007
	USA, Idaho	Sequence	EF153836	Skantar *et al.*, 2007
	Canada, Newfoundland	Sequence	FJ212165	Madani *et al.*, 2008
	Poland, unknown	Sequence	EU855119	Nowaczyk *et al.*, unpublished
	Argentina, unknown	Sequence	DQ097514	Lax *et al.*, unpublished
	Peru, Cusco	Sequence	EU006706	Picard *et al.*, unpublished
G. tabacum	USA	Cysts		This study
	USA	Sequence	FJ667946	Geric *et al.*, unpublished
G. achilleae	Slovenia, Trbonje	Cysts		This study
	Slovenia, Trbonje	Sequence	AY599498	Širca and Urek, 2004

of denaturation at 94°C for 1 min, annealing at 56°C for 45 s, elongation at 72°C for 1 min, and a final extension cycle at 72°C for 2 min.

PCR-RFLP and sequence analysis

Four μL of PCR products were digested with 5 U of the restriction endonucleases. The reactions were carried out with five restriction enzymes: *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and *Rsa*I (Fermentas GMBH, St. Leon-Rot, Germany) at 37°C for three hours with 1× restriction enzyme buffer in a 15 μL reaction volume. Restriction fragments were separated on 2% agarose gel, stained with ethidium bromide and photographed using UV illumination. Homologous ITS rDNA sequences (Table 1) of *G. rostochienis*, *G. pallida G. tabacum* and *G. achilleae* population samples from different locations were obtained from the NCBI GenBank database and analysed using BioEdit (Hall, 1999) software. Sequences were aligned, cut at both ends to gain approximately 880 bp long sequences and further cleaved *in silico* with *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and *Rsa*I to generate restriction fragments.

Results

Morphometrics of *G. pallida* population sample

Measurements of second-stage juveniles and cysts of the *G. pallida* population sample from Italy are listed in Table 2. Both cyst and J2 morphometrics fitted well within the ranges of previous *G. pallida* reports (Golden, 1986; Brzeski, 1998; Fleming and Powers, 1998). The distance between anus and vulval basin of the cysts was 50.0 μm (33.3–78.5) slightly greater, than the distance given in the taxonomic keys (22–67 μm), which simply serves to demonstrate a certain intra-specific variation. However,

Table 2. Morphometrical characters of second-stage juveniles and cysts of a *Globodera pallida* sample from Italy. All measurements are in μm, presented as mean ± standard deviation, with the range in parentheses.

Character	Second-stage juveniles (n = 15)	Cysts (n = 10)
Body length	462.4 ± 35.6 (400.0–511.4)	–
Body width	20.2 ± 1.7 (18.4–24.3)	–
Stylet length	24.7 ± 0.9 (23.5–26.6)	–
Body width at stylet knobs	16.7 ± 1.0 (14.5–18.1)	–
Tail length	45.3 ± 2.5 (42.4–49)	–
Tail width	11.5 ± 0.8 (10.1–12.7)	–
Hyaline part of tail	28.0 ± 2.3 (24.1–32.1)	–
Distance from centre of median bulb valve to end of head	66.4 ± 4.6 (57.5–76.6)	–
a	23.1 ± 2.7 (17.1–26.9)	–
c	10.2 ± 0.9 (8.2–11.6)	–
c’	4.0 ± 0.2 (3.3–4.3)	–
Vulval basin diameter	–	21.1 ± 2.2 (17.1–24.2)
Distance - vulval basin to anus	–	50.0 ± 13.1 (33.3–78.5)
No. of cuticular ridges	–	11.0 ± 2.4 (8.0–16.0)
Granek’s ratio	–	2.4 ± 0.6 (1.8–3.5)

distinct morphometrical characters, such as the juvenile stylet length and the stylet knob forward projection (Figure 1), the number of the cuticular ridges of the cyst and Granek's ratio were the same, and indicated that the specimens were *G. pallida* (Stone) Behrens.

PCR-RFLP

Amplification of the rDNA ITS fragments yielded PCR products of approximately 1000 bp in length. RFLP profiles distinguished between all four species. The lengths of restricted fragments (Table 3) were determined from the gel (Figure 2) and were supported by the *in silico* cleavage values (Table 4) for the restriction sequences to estimate the precise fragment sizes. *Alu*I digestion was species specific for *G. rostochiensis* and *G. achilleae*, discriminating them from each other and from *G. pallida* and *G. tabacum*, but it could not discriminate *G. pallida* from *G. tabacum*. The *Hinf*I pattern was species specific for *G. pallida* (fragment sizes 769, 152 and 81 bp) and *G. achilleae* (no restriction site), discriminating them from *G. rostochiensis* and *G. tabacum*. *Rsa*I like *Hinf*I, distinguished between *G. pallida* and *G. achilleae* and separated them from *G. rostochiensis* and *G. tabacum*. The RFLP pattern of *G. pallida* was also specific when digested with *Mbo*I and *Mse*I. *Mbo*I and *Mse*I did not distinguish between *G. rostochienis*, *G. tabacum* and *G. achilleae*.

***In silico* RFLP analyses of the sequences**

Aligned sequences cut at both ends ranged from 873 to 888 bp in length (Table 4). Sequences were thus approximately 120 bp shorter than the PCR fragments of live material. Some of the sequences in the database were obtained with different primer pairs and were therefore shorter than our PCR product. *In silico* cleavage of the sequences with *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and

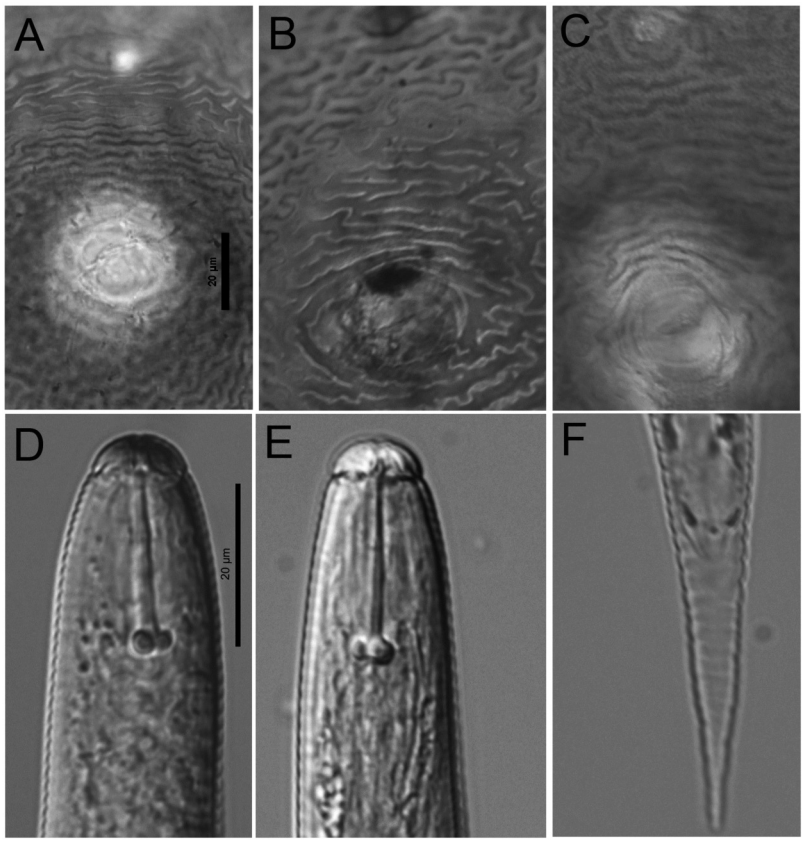

Figure 1. Morphology of a *Globodera pallida* sample from Italy. A, B and C, cyst perineal patterns; D and E, anterior of the second stage juveniles; F, tail of the second stage juvenile. Bar = 20μm.

Table 3. Length of rDNA amplified fragments and fragment length after digestion with five restriction enzymes of four *Globodera* species (*Globodera rostochiensis, G. pallida, G. tabacum* and *G. achilleae*).

Species	Fragment size	*Alu*I	*Hinf*I	*Mbo*I	*Mse*I	*Rsa*I
G. rostochiensis	1002	381, 359, 148, 100, 14	921, 81	537, 226, 165, 35, 16, 14	502, 500	603, 221, 169, 9
G. pallida	1002	520, 382, 100	769, 152, 81	433, 226, 165, 140, 35, 16, 14	502, 442, 58	603, 399
G. tabacum	1000	519, 380, 100	920, 80	537, 224, 165, 35, 16, 14	502, 498	603, 221, 169, 7
G. achilleae	1012	888, 14	1012	560, 236, 165, 35, 16	512, 500	608, 283, 112, 5, 4

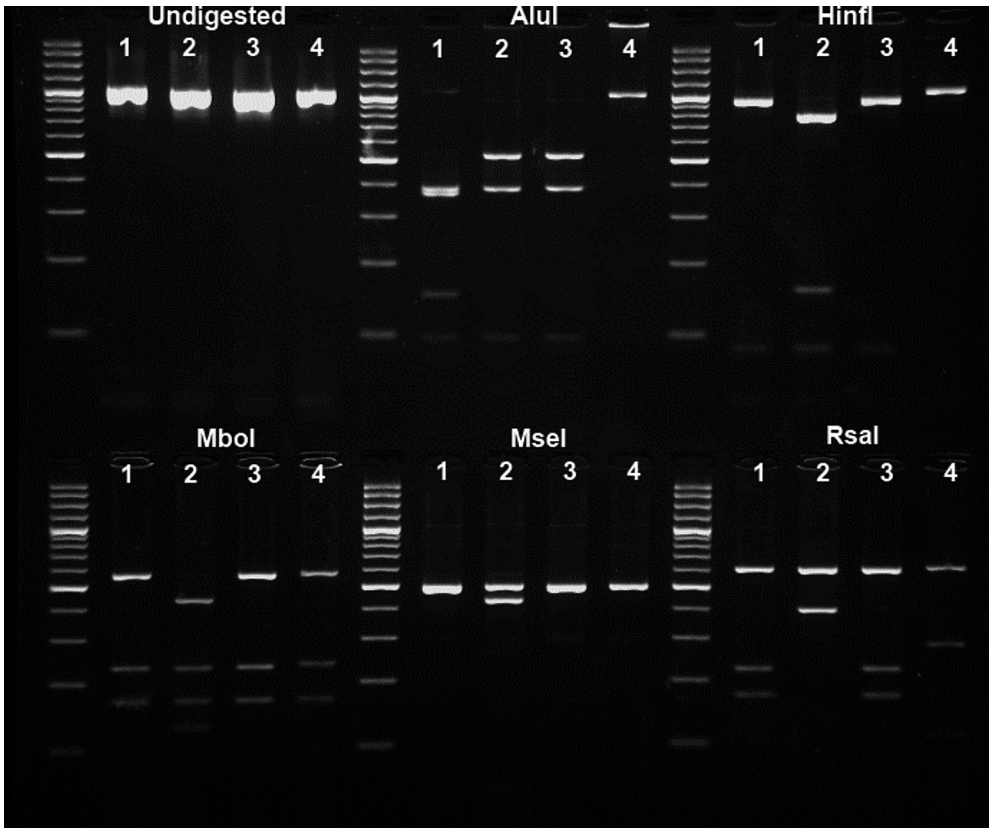

Figure 2. Agarose gel with PCR-RFLP patterns of *Globodera rostochiensis* (1), *G. pallida* (2), *G. tabacum* (3) and *G. achilleae* (4). DNA ladder 100 bp Plus (Fermentas GMBH, Germany), restriction enzymes used: *Alu* I, *Hinf*I, *Mbo*I, *Mse*I, and *Rsa*I.

Table 4. *In silico* cleavage and fragment sizes of *Globodera rostochiensis, G. pallida, G. tabacum* and *G. achilleae* rDNA-ITS sequences with *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and *Rsa*I.

Species (sequence accession No., source)	Fragment size	*Alu*I	*Hinf*I	*Mbo*I	*Mse*I	*Rsa*I
G. rostochiensis						
AY700060 Slovenia: Libeliče	874					
EF153839 USA: New York	874	381, 310,	856, 18	522, 170,	449, 425	538, 221,
FJ212163 Canada: Newfoundland	874	148, 35		165, 16		106, 9
DQ847119 Russia: Moscow region	874					
FJ212166 Canada: Quebec	874					
DQ847118 UK: Scarcliffe	873					
G. pallida						
EF153838 UK: York	876	459, 382,	706, 152,	431, 171,	426, 392,	539, 328,
EF153836 USA: Idaho	876	35	19	165, 93, 16	58	9
FJ212165 Canada: Newfoundland	877					
EU855119 Poland	876					
G. pallida						
DQ097514 Argentina	874	459, 382,	706, 152,	524, 170,	450,426	539, 222,
EU006706 Peru	876	35	19	165, 16		106, 9
G. tabacum		458, 383,	860, 17	524, 172,	452, 425	541, 222,
FJ667946	877	36		165, 16		105, 9
G. achilleae		888	888	530, 177,	460, 428	549, 218,
AY599498 Slovenia: Zadraga	888			165, 16		112, 5, 4

*Rsa*I yielded species specific RFLP profiles with the exception of *G. pallida*, where diverse RFLP profiles were seen. Sequences of *G. pallida* populations from UK, USA, Canada and Poland differed from the sequences of populations from Argentina and Peru in their *Mbo*I, *Mse*I and *Rsa*I RFLP profiles (Table 4). As was the case with PCR-RFLP, *Alu*I cleavage distinguished *G. rostochiensis* from *G. achilleae*, *G. pallida* and *G. tabacum* but not from any *G. pallida* populations or from *G. tabacum*. *Hinf*I was species specific for *G. pallida* (fragment sizes 706, 152 and 19 bp) and *G. achilleae* (no restriction site), discriminating them from *G. rostochiensis* and *G. tabacum*. *Mbo*I and *Mse*I distinguished *G. pallida* populations from UK, USA, Canada and Poland from populations of *G. rostochiensis, G. tabacum, G. achilleae* and *G. pallida* from Argentina and Peru. *Rsa*I fragments of *G. achilleae* were species specific. *Rsa*I also discriminated between *G. pallida* (UK, USA, Canada and Poland) and *G. rostochiensis, G. pallida* (Argentina and Peru) and *G. tabacum*. *In silico* RFLP patterns agreed with the PCR-RFLP analyses except for sequences of *G. pallida* populations from South America.

Discussion

The correct and rapid identification of a pest is fundamental for it's control. Species of PCN, can be distinguished by morphometrical differences and by their capacity to reproduce on various hosts, but this is time-consuming. Some of the diagnostic characters, such as cyst cone and J2 stylet characteristics, may overlap between various populations of the different species (Baldwin and Mundo-Ocampo, 1991). Besides morphometrical examination, molecular methods are gaining in importance in identifying plant-parasitic nematodes. The ITS region many times shown itself to be a useful marker for the diagnosis of *Globodera* species, but intra-specific variation also needs to be considered. In addition non-homogenized paralogues of rDNA at different loci in a genome can present difficulties when the ITS

region is used in making a diagnosis. Subbotin *et al*. (2000) found several haplotypes within the genome of *G. rostochiensis* while Blok *et al*. (1998) found one *G. pallida* population with different RFLP profiles, showing that haplotypes could occur. However, selecting appropriate restriction enzymes may overcome this problem, and enable the RFLP profiles to be species specific. The PCR-RFLP of the rDNA ITS region developed in this study rapidly identified *Globodera* species and distinguished between potato, tobacco and yarrow cyst nematodes. Several restriction enzymes (not shown) were used for *in silico* restriction of *Globodera* sequences from different locations in this study but only five enzymes were effective. A combination of *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and *Rsa*I restriction yielded species-specific RFLP profiles from species except *G. pallida*. The sequences of *G. pallida* populations from South America had an additional *Rsa*I restriction site and lacked one *Mbo*I and one *Mse*I restriction site, and thus generated RFLP profiles distinct from the profiles of *G. pallida* populations from other parts of the world. These findings reflect variations in the rDNA that may be useful to discriminate South American from other *G. pallida* populations. To our knowledge, the PCR-RFLP approach described here is the first molecular method that differentiates *G. rostochienis*, *G. pallida, G. tabacum* and *G. achilleae*.

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