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Comparison of three conventional PCR test (Bulman & Marshall, 1997) versions for the molecular identification of *Globodera pallida* and *G. rostochiensis* cysts and juveniles

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Three different versions of the conventional PCR described by Bulman & Marshall (1997) for the identification of cysts and juveniles of *Globodera pallida* and *G. rostochiensis* were compared: the original Bulman & Marshall, Bulman & Marshall as described in EPPO PM 7/40 (2) and an in-house modified version of the Dutch National Plant Protection Organization (NPPO-NL). The versions differ from each other in thermocycler conditions and primer sequences. Two different polymerases (Invitrogen and Roche) were assessed using the different test versions, and performance criteria analytical sensitivity and analytical specificity were determined. Roche-based reaction mixes had the highest amplicon yield and were used for further comparison of the different test versions. The different test versions performed equally well in terms of analytical specificity. No false positive or false negative results were observed. The test version NPPO-NL proved to be the most sensitive test version with a limit of detection of 1 juvenile for both *G. pallida* and *G. rostochiensis*.

Introduction

Cyst nematodes are an economically important group of harmful plant-parasitic nematodes which are found worldwide. In particular, the potato cyst nematodes (PCN) *Globodera pallida* (Stone, 1973) Behrens, 1975 and *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 are a threat for potato growers and subject to strict phytosanitary regulations. A correct PCN species identification is therefore essential, however morphological identification of these sibling species alone is not recommended for routine identification. Therefore a range of DNA based identification methods have been developed for the two PCN.

In 1997, Bulman and Marshall published a conventional PCR for the identification of *G. pallida* and *G. rostochiensis* cysts. This conventional PCR makes use of a generic forward primer ITS5 targeting the small ribosomal subunit (SSU) 18S rRNA gene described by White *et al.* (1990) and two specific reverse primers PITSp4 (*G. pallida)* and PITSr3 (*G. rostochiensis*) targeting the internal transcribed spacer region 1 (ITS1). The test described by Bulman and Marshall is adopted by EPPO as part of EPPO Standard PM 7/40 (2)[1] '*Globodera rostochiensis* and *Globodera pallida*' Appendix 3 (EPPO, 2009). This version of the test is subsequently referred to as PM 7/40 (2) and has a lower annealing temperature (Ta) than the original publication (55°C instead of 60°C). The lower Ta has now been recognised as an editorial error which has been corrected (EPPO, 2013).

A modified version of the conventional PCR described by Bulman & Marshall (1997) was designed by NPPO-NL. Primer sequences of the generic forward primer and the *G. pallida* specific reverse primer were optimised to better fit *Globodera* target sequences and to have a more harmonised melting temperature among the different primers (Fig. 1). Gradient PCR reactions were performed to determine the optimal annealing temperature. Table 1 shows the differences between the test versions.

In this study, the three different PCR versions were compared by determining the performance criteria analytical sensitivity and analytical specificity according to EPPO Standard PM 7/98 (EPPO, 2010). Robustness was also evaluated.

Material and methods

Nematode collection material

All populations used in this study (Table 2) were maintained in a greenhouse on their specific host under quarantine conditions at NPPO-NL. Cysts and second stage juveniles were isolated from live populations, identified morphologically and stored dry (cysts) or in 50 μL molecular grade water (MGW) (juveniles) at 4°C until use.

DNA extraction

The Mammalian Tissue protocol of the High Pure PCR Template Preparation kit (Roche) was used for nematode

[1]This test is also described with the same conditions in version 3 of this EPPO Standard which was published in 2012 (EPPO, 2012).

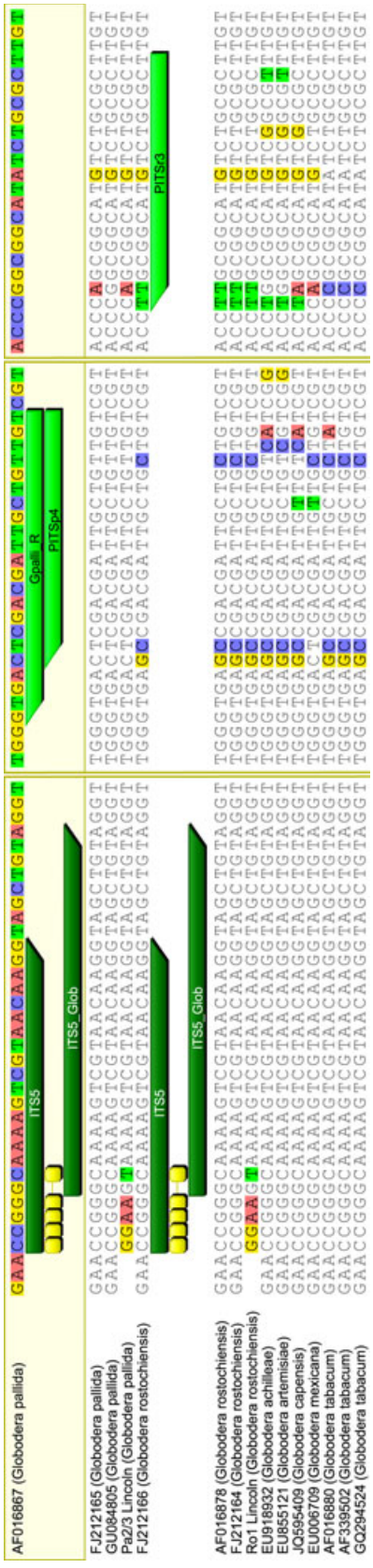

Fig. 1 Detail of primer sites in a SSU 18S rRNA gene (ITS5 and ITS5_Glob) and ITS1 (Gpalli_R, PITSp4 and PITSr3) alignment of 15 *Globodera* specimens. Seven *Globodera* species are shown in the alignment: *G. pallida* (4), *G. rostochiensis* (4), *G. tabacum* (3), *G. achilleae* (1), *G. artemisiae* (1), *G. capensis* (1), *G. mexicana* (1). *G. pallida* and *G. rostochiensis* sequence data from Bulman & Marshall that were not submitted to NCBI GenBank was copied from the original paper. Additional sequence data was selected from the top-100 hit list in NCBI GenBank obtained after a BLAST with specific reverse primers PITSr3 and PITSp4. Sequence data was uploaded in Geneious 6.1 (Drummond *et al.*, 2012) and aligned using the MAFFT alignment tool. *Globodera pallida* (AF016867) was set as reference sequence, and nucleotides that are different from the reference sequences are highlighted. Primer sequences of ITS5, ITS5_Glob, PITSr3, PITSp4 and Gpalli_R were uploaded as separate documents and annotated as primer. One sequence per target organism was tested with the available primers (mismatches allowed = 5). Primer annealing sites and primer orientation are shown as annotated regions (green). Mismatches in primer sequence in regard to the template sequence are shown as separate annotations (yellow). Five mismatches are present in forward primer ITS5 for all *Globodera* specimens, except for Pa2/3 Lincoln and Ro1 Lincoln which are both copied from Bulman & Marshall (1997). When Bulman & Marshall published their paper, sequence data of the ITS5 primer site in *Globodera* spp. was not available and the sequence for *G. pallida* and *G. rostochiensis* of that particular region was copied from the primer sequence. The ITS5 primer sequences in Pa2/3 Lincoln and Ro1 Lincoln are therefore listed as part of the SSU 18S rRNA sequence and should be ignored. The generic in-house optimised primer ITS5_Glob has no mismatches with the *Globodera* specimens included in the alignment. Primers PITSr3, PITSp4 and Gpalli_R are target specific and show almost no mismatches with the target organisms (see *in silico* analytical specificity), or possible annealing with the non-target organisms.

DNA extraction. Slight modifications were made to suit nematode DNA extraction: a minimum Proteinase-K incubation time of 16 h and an elution volume of 50 µL were used. Cyst material was crushed in Tissue Lysis Buffer using a micro-pestle prior to DNA extraction. For the extraction of DNA from juveniles, 150 µL Tissue Lysis buffer was added to 50 µL MGW containing the nematodes. Positive (1 cyst of either *G. pallida* or *G. rostochiensis*) and negative isolation controls were used during each DNA extraction run to monitor possible contamination and the effectiveness of the DNA extraction. DNA extracts were stored at −20°C until use.

Conventional PCR

As the Taq DNA polymerase (LifeTechnologies, Carlsbad, California, United States of America) used in Bulman and Marshall and PM 7/40 (2) was not available at NPPO-NL, a robustness experiment using two different polymerases (Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, United States of America) and FastStart Taq DNA polymerase (Roche, Basel, Switzerland)) was performed first. Per test version (Bulman & Marshall, PM 7/40 (2) and NPPO-NL), two different reaction mix set-ups were tested based on different polymerases. In the description of the different performance criteria (I) or (R) is added to the

Table 1 Different versions of the conventional PCR for *Globodera pallida* and *G. rostochiensis*. Parameters that are different from the PCR test described by Bulman and Marshall are underlined

	Bulman & Marshall (1997)	PM 7/40 (2) appendix 3A	NPPO-NL
Forward primer	ITS5	ITS5	ITS5_glob
Sequence	GGA AGT AAA AGT CGT AAC AAG G	GGA AGT AAA AGT CGT AAC AAG G	GCA AAA GTC GTA ACA AGG TAG CTG TA
Tm (PrimerExpress 3)	51.6	51.6	59.0
Reverse primer *G. pallida*	PITSp4	PITSp4	Gpalli_R
Sequence	ACA ACA GCA ATC GTC GAG	ACA ACA GCA ATC GTC GAG	ACA ACA GCA ATC GTC GAG TCA C
Tm (PrimerExpress 3)	51.4	51.4	58.9
Reverse primer *G. rostochiensis*	PITSr3	PITSr3	PITSr3
Sequence	AGC GCA GAC ATG CCG CAA	AGC GCA GAC ATG CCG CAA	AGC GCA GAC ATG CCG CAA
Tm (PrimerExpress 3)	63.6	63.6	63.6
PCR program	2 min 94°C, 35× (30 s 94°C, 30 s 60°C, 30 s 72°C)	2 min 94°C, 35× (30 s 94°C, 30 s 55°C, 2 min 72°C), 5 min 72°C	2 min 95°C, 35× (15 s 94°C, 30 s 64°C, 45 s 72°C), 5 min 72°C, 1 min 20°C
Amplicon *G. pallida* (bp)	265	265	261
Amplicon *G. rostochiensis* (bp)	434	434	430
Polymerase used	Taq DNA polymerase (Life technologies)	Taq DNA polymerase (Life technologies)	Platinum Taq DNA polymerase (Invitrogen)

Table 2 Materials used in this study

				Performance criteria		
Scientific name	Population	Material	Host	Sensitivity	Specificity	Robustness
Globodera pallida	Pa2	Cysts and juveniles	Potato	X	X	X
G. pallida	Pa3Nl	Cysts and juveniles	Potato	X	X	
G. pallida	Pa3Fr	Cysts and juveniles	Potato	X	X	
Globodera rostochiensis	Ro 1	Cysts and juveniles	Potato	X	X	X
G. rostochiensis	Ro2/3	Cysts and juveniles	Potato	X	X	
G. rostochiensis	Ro4	Cysts and juveniles	Potato	X	X	
Globodera tabacum	C6876	Cysts	Tobacco		X	X
Heterodera schachtii	C9872	Cysts	Beet		X	
Heterodera trifolli	9	Cysts	Clover		X	
Heterodera betae	6B	Cysts	Beet		X	
Heterodera glycines	Not available	Cysts	Soya		X	

test version to indicate the use of the Invitrogen or the Roche enzyme respectively. Reaction mix set-up is provided as supportive data [http://archives.eppo.int/files/blackwell/44_1/vandeVossenberg.doc]. Bulman and Marshall (1997) described a final primer concentration of 250 μM. This final concentration was also used in EPPO Standard PM 7/40 (2) and Skantar *et al.* (2007). Personal communication with S.R. Bulman and S.A. Skantar revealed that the actual final concentration was a 1000-fold lower: 250 nM. In this study, a final concentration of 250 nM was used for both the Bulman & Marshall and PM 7/40 (2) and PM 7/40 (3) test versions.[2]

To determine the amplify-ability of extracted DNA that produced no amplicons in the *G. pallida* and *G. rostochiensis* specific tests, a 833 bp fragment of the SSU 18S rRNA gene was amplified using primers 1813F and 2646R (Holterman *et al.*, 2006). Positive (DNA extract of 1 cyst of *G. pallida*) and negative (MGW used for reaction mix set-up) amplification controls were used in each run to monitor possible contamination and the effectiveness of the PCR run. PCR reactions were performed in C1000 and S1000 Thermal Cyclers (Bio-Rad, Hercules, California, United States of America). To test for amplification and the quality of PCR products, electrophoresis of 5 μL of PCR product and 1 μL 6× Bromophenol Blue Loading solution (Promega, Fitchburg, Wisconsin, United States of America),

[2]An erratum has now been published in the *EPPO Bulletin* to correct this (EPPO, 2013).

was performed in a 1.5% RESult LE General Purpose Agarose (BIOzymTC, Landgraaf, the Netherlands) gel with SYBR safe (Life Technologies, Carlsbad, California, United States of America) staining. Bands were visualized and images were captured using a GeneGenious gel imaging system (Syngene, Cambridge, United Kingdom). A 1Kb Plus DNA ladder (Promega, Fitchburg, Wisconsin, United States of America) was used to estimate amplicon size.

Robustness

Single cysts of sample material indicated in Table 2 were used to determine the robustness (i.e. optimal polymerase) of the test versions Bulman & Marshall (I) and (R), PM 7/40 (2) (I) and (R) and NPPO-NL (I) and (R). Amplicon yield and occurrence of false positive or false negative results were used to assess the optimal polymerase for each test version.

Analytical sensitivity

The analytical sensitivity gives a measure of the amount of target that can be reliably identified: the limit of the detection (LOD). In this study, the LOD is expressed in biological units (juveniles and cysts). Per test version (Bulman & Marshall (R), PM 7/40 (2) (R) and NPPO-NL (R)), the following series were analysed for the populations mentioned in Table 2: 2 cysts, 1 cyst, 20 juveniles, 10 juveniles, 5 juveniles, 2 juveniles and 1 juvenile.

The LOD was calculated per test version with a 99.7% (3x standard deviation) confidence level using:

$$\mathrm{LOD} = \frac{x_{\mathrm{series1}} + x_{\mathrm{series2}} + x_{\mathrm{series}} \cdots}{n_{\mathrm{series}}} + 3\sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

In which: x, lowest number of cysts or juveniles that could be identified; $\bar{x}$, average; n, number.

Analytical specificity

The analytical specificity was determined per test version (Bulman & Marshall (R), PM 7/40 (2) (R) and NPPO-NL (R)) by using single cysts of different populations of the target organism, and selected populations of closely related or otherwise associated non-target organisms (Table 2). The analytical specificity was calculated according to EPPO Standard PM 7/98 (1). In addition, the analytical specificity of the primers was determined *in silico* to get an impression of their usability.

Other performance criteria

The performance criteria selectivity, repeatability and reproducibility were not determined in this study. The PCR test developed by Bulman & Marshall (1997) was found to be widely used in the EPPO region in a survey carried out in 2013 (EPPO Secretariat, pers. comm.). Widely used tests from EPPO Standards are considered as giving appropriate confidence regarding selectivity, repeatability and reproducibility (Petter & Suffert, 2010).

Results and discussion

Robustness

The outcome of the Robustness experiment was used to establish the optimal reaction mix composition for the determination of the performance criteria analytical sensitivity and analytical specificity. Comparing the different PCR test versions with different polymerases, all tests performed equally well in terms of qualitative results (Table 3). *G. pallida* produced *G. pallida*-specific bands, and *G. rostochiensis* produced *G. rostochiensis*-specific bands in all reactions. *G. tabacum* produced no bands at all. In all test versions, the Roche-based reaction mix produced the highest amplicon yield. The Bulman and Marshall version seemed to have a lower amplicon yield (Fig. 2). Roche-based reaction mixes were used for further comparison of the different test versions.

Analytical sensitivity

Sample sets consisting of 2 cysts, 1 cyst, 20 juveniles, 10 juveniles, 5 juveniles, 2 juveniles and 1 juvenile from three populations per target organism were analysed to determine the LOD of the test versions. Table 4 shows the results per test version for each sample set. The LOD was calculated with a 3 standard deviation (SD) 99.7% confidence level per test version: Bulman & Marshall (R): *G. pallida* 9 juveniles and *G. rostochiensis* 9 juveniles, PM 7/40 (2) (R): *G. pallida* 3 juveniles and *G. rostochiensis* 8 juveniles, NPPO-NL (R): *G. pallida* and *G. rostochiensis* 1 juvenile. Alternatively, the LOD is often determined using the amount of individuals that produced the expected results in all cases (SD = 0). This results in a LOD of Bulman & Marshall (R): *G. pallida* and *G. rostochiensis* 5 juveniles, PM 7/40 (2) (R): *G. pallida* 2 juveniles and *G. rostochiensis* 5 juveniles, NPPO-NL (R): *G. pallida* and *G. rostochiensis* 1 juvenile. The authors prefer to include a confidence level to take into account slight variations in the constitution and quality of biological material used for routine testing.

Sample '*G. rostochiensis* Ro2/3' produced a fainter amplicon for 2 juveniles than for 1 juvenile. This was not observed for the other populations and was regarded as a pipetting error. Test version NPPO-NL (R) had the highest amplicon yield resulting in the lowest LOD (using both analysis methods) and therefore was found to be the most sensitive test version.

Following the comparison carried out in this study personal communication with Ms G. Anthoine (Anses, Plant Health Laboratory, Angers, France) also provided information on the test version used in the French laboratory. Single cysts were crushed in 100 µL of lysis buffer (Tris 10 mM

Table 3 Results robustness

	Bulman & Marshall (1997)		PM 7/40 (2)		NPPO-NL	
Material	Invitrogen (I)	Roche (R)	Invitrogen (I)	Roche (R)	Invitrogen (I)	Roche (R)
Globodera rostochiensis	GR	GR	GR	GR	GR	GR
Globodera pallida	GP	GP	GP	GP	GP	GP
Globodera tabacum	–	–	–	–	–	–

GR, *Globodera rostochiensis* specific band; GP, *Globodera pallida* specific band, –, no amplicon.

pH = 8.8, EDTA 1 mM, Nonidet P40 1%, proteinase K 100 μg mL^{-1}). The crude DNA obtained was stored at –20°C until use. Single nematodes were taken from suspensions and placed in a 1.5 mL tube with 100 μL lysis buffer (Tris 10 mM pH = 8.8, EDTA 1 mM, Nonidet P40 1%, proteinase K 100 μg mL^{-1}). One 3 mm and several 1 mm glass beads (Sigma, Saint Louis, Missouri, United States of America) were added to the tube and shaken with a Tissulyser II (Qiagen, Venlo, the Netherlands) at 30 beats s^{-1} for 40 s. Afterwards, tubes were incubated at 55°C for one hour and 15 min at 95°C, and DNA solutions obtained were frozen at –20°C until use. PCR was performed in a 25 μL reaction mix containing 1× Taq buffer (MP Biomedicals, Santa Ana, California, United States of America), 2 mM $MgCl_2$ (MP Biomedicals, Santa Ana, California, United States of America), 0.25 mM dNTPs (MP Biomedicals, Santa Ana, California, United States of America), 640 nM of each primer as described by Bulman & Marshall, 0.6 Units of Taq Polymerase (MP Biomedicals, Santa Ana, California, United States of America), and 5 μL template. PCR reactions were performed in GeneAmp (Applied Biosystems, Carlsbad, California, United States of America) PCR systems, either 2400, 2700 or 2720 using the following conditions: 2 min 94°C, 35 cycles (30 s 94°C, 30 s 60°C, 30 s 72°C), 7 min 72°C). This version, hereafter referred to as the 'French version' is as sensitive as the test version

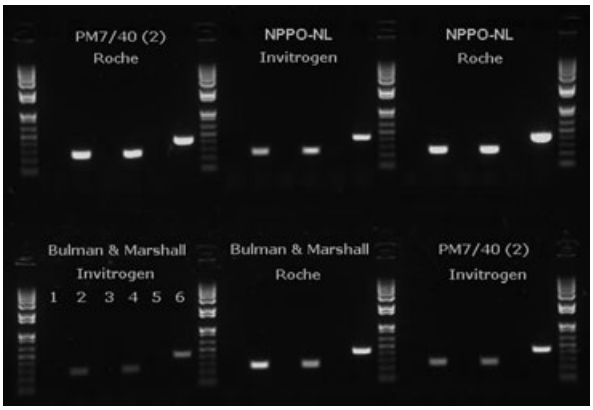

Fig. 2 Results Robustness. Two different polymerases (Platinum Taq DNA polymerase (Invitrogen) and FastStart Taq DNA polymerase (Roche)) were tested for all test versions. The following samples were tested: 1. negative amplification control, 2. positive amplification control *G. pallida,* 3. negative isolation control, 4. *G. pallida* Pa2 1 cyst, 5. *G. tabacum* C6876, and 6. *G. rostochiensis* Ro1 1 cyst.

NPPO-NL, i.e. 1 juvenile for both *G. pallida* and *G. rostochiensis*. The authors recognise that performance criteria of molecular tests may be influenced by reagents and equipment used, and that each laboratory needs to determine if a test is fit for purpose under their conditions through a verification process upon implementation.

Analytical specificity

Analytical specificity was determined by testing the two target organisms and 5 non-target species (*G. tabacum*, *Heterodera schachtii*, *H. trifolli*, *H. betae* and *H. glycines*). Three populations per target organisms and 1 population per non-target organism were tested. The different test versions performed equally well in terms of analytical specificity. All test versions scored 100% for both diagnostic specificity and diagnostic sensitivity (Table 5). No false positive or false negative results were observed for the species included in this study. DNA from specimens that produced no amplicon in the *G. pallida* and *G. rostochiensis* specific tests could be amplified using generic primers 1813F and 2646R.

For *in silico* analysis of *G. pallida* and *G. rostochiensis* specific primers, 93 *G. pallida* and 77 *G. rostochienis* 18S and ITS1 annotated sequences were downloaded from NCBI and aligned with the MAFFT alignment tool in Geneious 6.1. Both *G. pallida* specific primer Gpalli_R and PITSp4 showed a single mismatch in 4 (GU084803, HQ260426, HQ260428, HQ670246) out of 93 *G. pallida* sequences. In a single case (*G. pallida* DQ097514, Argentina), 4 and 3 mismatches were observed compared to primers Gpalli_R and PITSp4 respectively. *G. rostochiensis* specific primer PITSr3 showed two mismatches at the 3′ end in 1 (GU084809) out of 77 *G. rostochiensis* sequences. Amplification of the species specific PCR product could be negatively influenced in six out of 170 specimens included in the *in silico* analysis, in particular *G. pallida* DQ097514 from Argentina. It should be noted that mismatches observed *in silico* are indicative, and do not necessarily result in false negatives; the ITS5 primer used by Bulman & Marshall (5 mismatches) clearly demonstrates this. Specimens with mismatches in the primer sites were not available to us, and therefore not tested in this study.

The French version was used to test European and non-European populations of both *G. pallida* (Bulgaria, Switzer-

Table 4 Results analytical sensitivity

	Bulman & Marshall (R)						PM 7/40 (2) (R)						NPPO-NL (R)					
	G. rostochiensis			*G. pallida*			*G. rostochiensis*			*G. pallida*			*G. rostochiensis*			*G. pallida*		
Material	Ro 1	Ro2/3	Ro4	Pa2	Pa3Nl	Pa3Fr	Ro 1	Ro2/3	Ro4	Pa2	Pa3Nl	Pa3Fr	Ro 1	Ro2/3	Ro4	Pa2	Pa3Nl	Pa3Fr
2 cysts	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
1 cyst	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
20 juveniles	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
10 juveniles	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
5 juveniles	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
2 juveniles	–	–	GR	GP	–	–	GR	–	GR	GP	GP	GP	GR	GR	GR	GP	GP	GP
1 juvenile	–	–	–	–	–	–	–	–	–	–	GP	GP	GR	GR	GR	GP	GP	GP

GR, *Globodera rostochiensis* specific band; GP, *Globodera pallida* specific band, –, no amplicon.

Table 5 Results analytical specificity

		Bulman & Marshall (R)		PM 7/40 (2) (R)		NPPO-NL (R)	
Scientific name	Population	*G. rostochiensis*	*G. pallida*	*G. rostochiensis*	*G. pallida*	*G. rostochiensis*	*G. pallida*
Globodera pallida	Pa2	–	+	–	+	–	+
G. pallida	Pa3Nl	–	+	–	+	–	+
G. pallida	Pa3Fr	–	+	–	+	–	+
Globodera rostochiensis	Ro 1	+	–	+	–	+	–
G. rostochiensis	Ro2/3	+	–	+	–	+	–
G. rostochiensis	Ro4	+	–	+	–	+	–
G. tabacum	C6876	–	–	–	–	–	–
Heterodera schachtii	C9872	–	–	–	–	–	–
Heterodera trifolli	9	–	–	–	–	–	–
Heterodera betae	6B	–	–	–	–	–	–
Heterodera glycines	Not available	–	–	–	–	–	–

land, the United Kingdom, Canada, Chile, and Peru) and *G. rostochiensis* (Bulgaria, the Czech Republic, Finland, Latvia, Romania, Slovenia, the United Kingdom, Bolivia, Canada, and Chile). One out of two Bolivian *G. rostochiensis* populations could not be identified as *G. rostochiensis*. Also, one out of three *G. tabacum* populations from the United States of America, and one *G.* '*mexicana*' population from Mexico yielded false positive *G. pallida* results (G. Anthoine, pers. comm.).

The usefulness of PCR tests based on ITS1 sequences in nematology have been debated (Frey & Helder, 2013). It is recognised that the ITS1 region shows more intra-species variation in many nematode species than, for instance, the SSU 18S rRNA gene (e.g. *G. pallida* ITS 5.1% (87 NCBI ITS1 annotated sequences, alignment length 490 nt, sequence lengths 486–489 nt) and SSU 2.8% (44 NCBI 18S annotated sequences, alignment length 1775 nt, sequence lengths 128–1775 nt)). This could render the ITS1 region less suitable for phylogenetic studies, but this is not necessarily true for specific identification tests. The usefulness of any molecular test, regardless of the locus used, is dependent on the test design and the scope of the test (e.g. usable for all populations of a species, or restricted to a subset of populations based on geographical distribution). Method validation is carried out to provide objective experimental evidence that a test is fit for purpose. Since 2005, test version NPPO-NL is used as a verification test for morphologically identified *G. pallida* and *G. rostochiensis* specimens obtained from national surveys at the Dutch NPPO and is found to be fit for purpose under that particular scope. When unclear results are obtained, additional tests are performed for species identification (i.e. PCR sequencing of SSU, LSU and COI). These cases are rare at the NPPO-NL.

Conclusion

The different test versions (Bulman & Marshall, PM 7/40 (2) and NPPO-NL) all managed to correctly identify different populations of the target species *Globodera pallida* and *G. rostochiensis*. None of the test versions produced false positive results with the non-target species *G. tabacum*, *Heterodera schachtii*, *H. trifolli*, *H. betae* and *H. glycines*. This implies using the lower Ta in test version PM 7/40 (2) did not influence the reliability of the test. Using a Roche-based reaction mix (FastStart Taq DNA polymerase, Basel, Switzerland) proved to be beneficial for the ampli-

con yield for all test versions, compared to reaction mixes based on Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, United States of America). The only difference between the test versions was observed in the analytical specificity. Test version NPPO-NL proved to be the most sensitive test version with a LOD of 1 juvenile for both *G. pallida* and *G. rostochiensis* and the authors recommend this test version for routine molecular identification of PCN and for addition to EPPO Standard PM 7/40 when it is next revised.

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Comparaison de trois versions d'un test PCR conventionnel (Bulman & Marshall, 1997) pour l'identification moléculaire des kystes et des juvéniles de *Globodera pallida* et de *G. rostochiensis*

Trois versions différentes d'un test PCR conventionnel (Bulman & Marshall, 1997) pour l'identification moléculaire des kystes et des juvéniles de Globodera pallida et de G. rostochiensis ont été comparées: la version originale de Bulman & Marshall, Bulman & Marshall tel que décrits dans la Norme OEPP PM 7/40 (2) et une version modifiée en interne par l'Organisation Nationale de Protection des Végétaux des Pays-Bas (ONPV-NL). Les versions diffèrent entre elles par les conditions du thermocycleur et les séquences d'amorce. Deux polymérases différentes (Invitrogen et Roche) ont été évaluées en utilisant différentes versions du test, et les critères de performance (sensibilité analytique et spécificité analytique) ont été évalués à chaque fois. Les mélanges réactionnels basés sur les composés Roche avaient le meilleur rendement en amplimère, et furent utilisées pour les comparaisons plus poussées des différentes versions du test. Les différentes versions du test ont abouti à des performances équivalentes en termes de spécificité analytique. Aucun faux positif ni faux négatif ne furent observés. Il a été démontré que la version du test modifiée par l'ONPV-NL a était la plus sensible avec une limite de détection d'un juvénile, tant pour G. pallida que pour G. rostochiensis.

Сравнительный анализ трех традиционных вариантов тестов ПЦР (Bulman & Marshall, 1997) при молекулярной идентификации кист и ювенальных особей *Globodera pallida* и *G. rostochiensis*

Три различных варианта традиционного ПЦР, описанного Bulman & Marshall (1997) сравнивались для идентификации кист и ювенальных особей *Globodera pallida* и *G. rostochiensis*: в оригинале Bulman & Marshall, описанный в стандарте ЕОКЗР PM 7/40 (2), и собственный измененный вариант Национальной организации защиты растений Нидерландов (НОКЗР Нидерландов). Эти варианты отличались по условиям термоциклера и последовательностям праймеров. Две различных полимеразы (Invitrogen и Roche) оценивались с использованием различных версий теста и определялись чувствительностью и специфичностью анализов). Смеси реактивов на базе Roche показали самый высокий выход ампликона и использовались для дальнейшего сравнения различных вариантов этого теста, которые с точки зрения специфичности анализа работали одинаково. Никаких ложных положительных или отрицательных результатов не наблюдалось. Вариант НОКЗР Нидерландов оказался наиболее чувствительным с пределом выявления одной ювенальной особи как *G. pallida*, так и *G. rostochiensis*.

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