Development and validation of real-time PCR assays based on novel molecular markers for the simultaneous detection and identification of Globodera pallida, G. rostochiensis and Heterodera schachtii

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Abstract

Considering the growing trades of seed potato, reliable diagnostic protocols are required for the detection of regulated nematode species. In this study, a specific and sensitive multiplex Taqman based real time PCR method was developed in order to detect and identify *G. pallida, G. rostochiensis* and *H. schachtii*. The newly designed primers and probes allowed for the detection of all the target populations tested and with no cross reaction for closely related non-target species (55 populations tested). The limit of detection (LOD) was 1 isolated juvenile for *G. rostochiensis* and *G. pallida*, respectively and 5 juveniles for *H. schachtii*. For monitoring potato cyst nematodes, this analytical tool would extend the number of cyst investigated as 5 juveniles can be detected among 50 cysts in a sample. Furthermore, this multiplex assay detects DNA of the three targeted species in template DNA obtained directly from float material after nematode extraction from soil.

Keywords:

Potato cyst nematodes, beet cyst nematode, diagnostic method, soil extract, TaqMan probes, microsatellite

Expanded international trade is requiring increased awareness of nematodes and there is a core of nematode species that are targeted by legislation around the world. Not all species of plant parasitic nematodes appear on every country's list of regulated pests because they may be endemic in some countries or regions and exotic in others. Among these regulated species are cyst nematodes. Cyst nematodes are notorious not only for their damage they cause to major crop productions, but also because their high dispersion potential has been demonstrated (Plantard & Porte, 2003; Plantard *et al.*, 2008; Alenda *et al.*, 2014). Their ability to withstand desiccation in the protective cyst stage greatly enhanced their dispersal capabilities. Therefore several countries regulate cyst nematodes species such as *G. rostochiensis* (106 countries), *G. pallida* (55 countries), *H. glycines* (52 countries) and *H. schachtii* (22 countries) which are the four main cyst nematode species considering the number of countries regulating them (Hockland *et al.*, 2006). Potato cyst nematodes are subject to quarantine regulations, including in Europe (Council directive 2000/29/EC of 8 May 2000). *H. glycines* is absent from Europe except in Italy (Manachini, 2000). *H. schachtii* is widely distributed in Europe and of main interest for seed potato trade, as it is regulated in several countries in South America, Asia and North Africa. In Europe, the potato cyst nematodes, *G. pallida* and *G. rostochiensis* and the beet cyst nematode, *H. schachtii,* are major pests in potato and sugar beet production areas, and therefore are present within the same field (van Riel & Mulder, 1998; Muller, 1999).

Potato is the fourth most important food crop in the world after corn, rice and wheat. The main potato markets are export and French fries. In Europe the production areas of seed potatoes increase each year and totaled 112 540 ha in 2015. The export of (seed) potatoes and potato products from the EU to other parts of the world plays an important role unlike the case of many other agricultural crops. In 2014, the EU exported 1.44 million tons of potatoes (mainly seed potatoes) for a value of nearly €522 million. Over two thirds of these seed potatoes (67.3 % in value and 69.5 % in volume terms) were exported to southern and eastern Mediterranean countries

(source EuroStat 2016, http://ec.europa.eu/eurostat/statistics-explained/index.php/The_EU_potato_sector_-_statistics_on_production,_prices_and_trade).

There is consequently an increasing demand, particularly for seed potatoes to control and identify cyst nematode species present in Europe such as *G. pallida, G. rostochiensis* and *H. schachtii*. Furthermore, guidelines from the EU Directive 2007/33/EC describe harmonized method for soil sampling which in consequence lead to a significant increase in testing soil samples. In order to address these quarantine and regulation problems, speed, sensitivity, accuracy, specificity, and broad applicability will be required features of any nematode identification protocol and as such, molecular diagnostic tools will provide a convenient approach. PCR and PCR-RFLP tools were already published for the molecular identification of *H. schachtii* (Amiri *et al.* 2002; Madani *et al.* 2005). However, tests carried out in 2009 within the Nematology unit of the French Plant Health Laboratory (Anses) highlighted a lack of specificity or sensitivity of these tools (internal document LSV, 2010). Currently, the only solution is a morphological analysis of cysts after soil extraction. Beyond the skills required for the morphological recognition, this technique has a strong limit for *Heterodera* since it often allows only the identification of a group and not a particular species within this group. Regarding the molecular identification of *G. pallida* and *G. rostochiensis,* existing conventional PCR tools (Fullaondo *et al*. 1999; Bulman & Marshall 1997; Mulholland *et al*. 1996; Skantar *et al*. 2007; Shields *et al*. 1996; Thiery & Mugniéry 1996) often show cross-reactivity with closely related species (*G. tabacum* and *G. mexicana*) and an absence of detection in some South American populations (internal document LSV, 2010), which requires first ever to perform a morphological analysis to process detection of these two species. Some real time PCR tools are described in the literature but they have not been tested against other *Globodera* species such as *G. mexicana, G. ellingtonae* or the subspecies of *G. tabacum*, (Nakhla *et al*. 2010, Reid *et al*. 2010, 2015, Madani *et al*. 2011, Papayiannis *et al.* 2013).

Among the genomic regions that have been extensively characterized among nematode taxa are the ribosomal RNA array and the mitochondrial genome (Powers 2004; Blok 2005). Hence it is not surprising that nearly all the molecular tools used till now for routine nematode diagnostic are mainly based on a single molecular target, the ribosomal RNA array. Extending the range of molecular targets for nematode diagnostic would therefore provide obvious benefits, as different genomic regions could then be investigated and compared for an accurate diagnostic. Next Generation Sequencing (NGS) has extended the number of genomic loci that can now be investigated. In particular, NGS has opened up new opportunities for easier microsatellite isolation (Malausa *et al*., 2011). Microsatellites are simple sequence repeats of 2 to 10 bp that are among the most frequently used DNA markers in many areas of research (Sunnucks 2000). In recent years, these types of markers have been developed successfully from NGS data in several cyst nematode species including *G. rostochiensis, G. pallida, H. schachtii,* and *H. glycines* (Boucher *et al.* 2013; Montarry *et al.* 2015; Kim *et al*. 2016; Wang *et al*. 2015). In all cases, several hundred amplifiable sequences containing a microsatellite motif were obtained. However, very few of them are used in population genetic or phylogeographic studies because all the monomorphic loci and all the loci that contain few repeats of the microsatellite motif are discarded.

This study aims to demonstrate that thanks to the massive data available through NGS, it is feasible to identify microsatellite loci with high potential as novel nuclear species-specific markers. To develop novel diagnostic markers, we screened hundreds of microsatellite primer combinations in the three cyst nematode species *G. pallida, G. rostochiensis* and *H. schachtii* and combined the most interesting ones into a single real time PCR assay. Assays were also performed to define the LOD, the sensitivity and the reproducibility of this molecular tool. Furthermore, this detection method was also able to detect the DNA of *G. pallida, G. rostochiensis* and *H. schachtii* simultaneously, even in mixtures, in a total DNA solution obtained directly from a soil extract. The presented data is

intended to demonstrate these novel and reliable diagnostic markers to scientists and users which, on a practical level, will provide useful tools for the phytosanitary control of exported seed potatoes.

Materials and methods

Nematode populations

A total of 36 populations of *Globodera spp*. and 19 populations of *Heteredera spp*. were used in this study (Table 1). This collection contained 14 populations of *G. pallida*, 13 of *G. rostochiensis*, 7 of *G. tabacum* (*solanacearum, tabacum and virginiae*), one of *G. ellingtonae* and one of *G. mexicana* mainly from Europe and South America. For *Heterodera* species, 8 populations of *H. schachtii* were tested and also 7 other populations belonging to the *Schachtii* group (such as *H. betae*, *H. ciceri*, *H. daverty*, *H. glycines* and *H. trifolii*). Populations of *H. avenae*, *H. carotae*, *H. cruciferae* and *H. goettingiana* were also included in this study. Most of these populations were reared in quarantine glasshouses. The identity of these species was established or confirmed using morphology and morphometrics criteria or molecular assays as for some populations only template DNA was available.

Microsatellite markers

All the microsatellite loci used in this study came from previous investigations. The *G. pallida* microsatellite loci were identified by mining the genome of this species as described in Eoche-Bosy *et al.* 2016. The *G. rostochiensis* and *H. schachtii* microsatellite loci were developed using next-generation sequencing of microsatellite enriched libraries as described in Montarry *et al.* (2015) and

Boucher *et al.* (2013). In all cases, the QDD software (Meglécz *et al*. 2010) was used to select sequences and design primer pairs for sequences containing microsatellite motifs.

DNA extraction of isolated nematodes

Concentrated DNA solutions were prepared using QIAamp DNA Mini kit (Qiagen®, ref. 51306). One or more cysts of nematodes were crushed into the lysis buffer solution of the extraction kit using microtube pestles. The DNA extraction protocol used is described in the QIAamp handbook (protocol for tissue). The DNA was eluted in 100 µL of elution buffer and the concentration adjusted to between 2 and 5 ng/µL after measuring the concentration with a spectrophotometer (BioPhotometer Plus, Eppendorf).

A range of DNA solutions from target species (*G. pallida*, *G. rostochiensis* and *H. schachtii*) was also prepared using one, two, five, ten and fifty juveniles (J2), which were transferred into a microtube containing 100 µL of lysis buffer (Tris 10 mM pH = 8, EDTA 1 mM, Nonidet P40 1%, proteinase K 100 µg/mL, Ibrahim *et al.* (1994). Nematodes were crushed by shaking the microtubes containing glass beads (one 3-mm diameter and about fifty of 1-mm diameter per tube, Sigma) using a Tissulyser II (Qiagen®) at 30 beats per second for 40 seconds. Microtubes were then incubated at 55°C for about one hour and at 95°C for 10 minutes. The DNA solutions obtained were stored in a freezer until analysis.

Total DNA extraction from soil extract

Two field soil samples from the “Bourgogne” and “Vendée” regions (France) that were free of cyst nematode were used to produce total DNA extracts free of cyst nematode DNA. First, 300 mL of each soil were extracted using a soil sample extractor (Meku) which works on the Seinhorst elutriation principle (PM7/119 (1), OEPP/EPPO 2013). The soil extracts were then concentrated on

a 250 µm sieve and dried at room temperature for 4 days. The total DNA was extracted from this soil extract using QIAamp DNA Blood Maxi kit (Qiagen®, ref. 51194) following the protocol described in the QIAamp DNA Blood Midi/Maxi handbook.

PCR amplification

The PCR protocols used in this study are described in Table 2. A total of 69 loci for *G. pallida*, 61 for *G. rostochiensis* and 190 for *H. schachtii* were tested by conventional PCR using the primer sequences designed by the QDD software as described above. First, PCR reactions were conducted using an annealing temperature of 55°C. Loci showing promising results were then also tested at higher annealing temperatures to test the specificity. Real time PCR reactions were performed on loci which were first selected by conventional PCR. All real time PCR assays were carried out on a LightCycler® 480 instrument (Roche) using master mix supplied by Roche Diagnostics. SYBR Green master mix (ref. 03515885001) and probe master mix (ref. 04707494001) were used in this study. The automatic analysis option in the software that drives the thermocycler ("Abs Quant/2nd derivative Max") was used to determine the cycle threshold (Ct).

For each assay, DNA was tested in duplicate reactions. The real time PCR amplification parameters are presented in Table 2.

Primers and probes for real time PCR were designed from raw sequence data using Geneious software (version 7.0.6 created by Biomatters, http://www.geneious.com). Sequences of the primers selected in this study for the detection of *G. pallida*, *G. rostochiensis* and *H. schachtii* were as follows: µsatGP-F (5'-AAGGAGTTGTGGTCCAGACG-3'), µsatGP-R (5'-GAAGGCAATCTGTGTTCGGG-3') for *G. pallida*, µsatGR-F (5'-TGACGAGGAACAGTACAAAG-3'), µsatGR-R (5'-GTGTCTCTAATTTGCCATT-3') for *G. rostochiensis*, µsatHS-F (5'-CCCGGACAGCCAAATTGT-3') and µsatHS-R (5'-GGGAAGTGAGTGGGCAGTTT-3') for *H. schachtii*. The Taqman probe sequences were

µsatGP-P for *G. pallida* (5'-JOE-CGCTCGTCGGCCTCCTCCTC-BHQ1-3'), µsatGR-P for *G. rostochiensis* (5'-Cy5-AGGCATTGCTTGAGCGAACGGA-BHQ2-3') and µsatHS-P for *H. schachtii* (5'-FAM-ATGGGAAGGACGGGTGGCGG-BHQ1-3').

Data analysis

The diagnostic specificity, sensitivity and the relative accuracy criteria were assessed by calculating the number of positive and negative agreements and deviations compared to the status of the sample as described in the EPPO standard protocol for test validation (PM7/98 (2), OEPP/EPPO 2010). The diagnostic specificity and the diagnostic sensitivity assess the ability of the test to not detect non target species and to detect target species respectively. Therefore, target and non-target populations covering the genetic diversity and various geographical origins of the species tested were used to evaluate these criteria. The LOD was determined by testing a range of target individuals. The LOD was considered as samples giving positive results (Ct values <35) in all replicates.

Results

Selection of microsatellite loci by conventional PCR

The initial number of amplifiable microsatellite loci was 202 for *G. pallida* (Eoche-Bosy *et al.* 2016), 481 (138 di, 337 tri and 6 tetra) for *G. rostochiensis* and 354 (190 di, 161 tri and 3 tetra) for *H. schachtii* after sequencing of the two microsatellite enriched libraries. Only a subset of these loci were selected in each species based on results previously obtained during the development of these loci as genotyping markers and on additional criteria: loci showing a PCR product <95 pb were discarded, loci with pairs of primers showing melting temperature around 60°C were preferred, loci

showing ePCR (Rotmistrovsky *et al.* 2004) products on both the genomes of *G. pallida* and *G. rostochiensis* were discarded. Based on these criteria, 69 loci for *G. pallida*, 61 loci for *G. rostochiensis* and 190 for *H. schachtii* were tested in conventional PCR against the DNA of target and non-target species. Microsatellite loci were selected both when all the target populations were detected and when no amplification (cross-hybridization) was observed with non-target populations. Finally, 12 loci for *G. pallida* (loci GP143, GP144, GP145, GP150, GP182, GP224, GP228, GP238, GP258, GP272, GP279 and GP286), 6 for *G. rostochiensis* (loci GR63, GR71, GR86, GR90, GR105 and GR121) and 9 for *H. schachtii* (loci HS33, HS43, HS45, HS91, HS95, HS96, HS126, HS127 and HS171) (data/results not shown) fulfilled these criteria. Further testing of these loci was done by testing 14 *G. pallida*, 11 *G. rostochiensis* populations including European and South American populations, and 8 populations of *H. schachtii*. Conventional PCR results obtained for *G. pallida*, *G. rostochiensis* and *H. schachtii* loci are presented in supplementary data. For *G. pallida,* the loci GP145, GP238, GP258, GP272, GP279 and GP286 were removed from the set because of the cross reaction with the *G. mexicana* population. Although amplification is observed for the locus GP182 with the *G. mexicana* population, this locus was still retained at this stage as the signal observed was quite weak. The loci GP143, GP224 and GP228 were removed because they did not detect all the target populations tested. Only the loci GP144, GP150 and GP182 were retained at the end of the conventional PCR screen (Table s1). For *G. rostochiensis*, the loci GR86, GR94 and GR120 were excluded because they either weakly detected the target species or they did not detect all the target populations. Furthermore, the loci GR71, GR83 and GR90 showed amplifications at unexpected sizes and were discarded. The loci GR63, GR105 and GR121 were selected for further development of the assays (Table s2). However, GR105 and GR121 were preferred because they generated fragment sizes of 167 bp and 108 bp, respectively, compared to 215 bp for GR63 loci. For *H. schachtii,* the HS171 locus was the only one retained at the end of the conventional PCR screen (Table s3).

Simplex real time PCR assay development and performances

The second part of the study focused on the development of real time PCR assays from the loci selected by conventional PCR. Several primers and probes were designed for the selected loci in order to have a fragment size of about 150 bp suitable for TaqMan assays while including the microsatellite motif. Primer pairs were tested by simplex real time PCR with SYBR Green mastermix for each target species by using a range of target and non-target DNA. To develop Taqman assays, primers were selected based on their specificity, sensitivity and accuracy.

TaqMan assays were initially tested individually (simplex reaction) by testing the same target and non-target species. It was observed that the different performance criteria (diagnostic specificity, diagnostic sensitivity and relative accuracy) reached 100% for each target species.

To evaluate the analytical sensitivity of the three simplex real time PCR assays, DNA from different numbers of juveniles or from cysts were tested for each target species. DNA from a single juvenile of *G. pallida* and *G. rostochiensis* resulted in a Ct value of 32.8. For the EU regulated species one juvenile was the LOD, whereas 5 juveniles were the LOD for *H. schachtii* with an average Ct value of 33.4 (Table 3).

Multiplex real time PCR performance results

As the performance of the three real time PCR assays tested were promising, triplex real time PCR assays were conducted. The results for the analytical sensitivity are presented in Table 4A. This table also presents results obtained in reproducibility conditions (i.e. two different laboratories, two different operators, different thermocyclers, different days of experiment and different batches of mastermix). As for simplex reactions, the minimum number of nematodes that was detected was one juvenile of *G. pallida* or *G. rostochiensis* which represents the smallest quantity of nematode

that could be encountered in a sample. It was observed that the Ct value obtained for one juvenile of *G. pallida* and *G. rostochiensis* may differ depending on experimental conditions. Indeed, the Ct value obtained when analyzing the same DNA solution composed of one juvenile of *G. pallida* may vary from 32.80 to 34.62. This difference shows that this contamination level is the LOD of the assay as the Ct value can differ depending on the replicates. However, the target species are always detected with a Ct value below 35. Two juveniles of *H. schachtii* could be detected in a sample according to the results presented in Table 4A. However, the results obtained in simplex reaction previously showed a limit of five juveniles. Five juveniles is therefore the LOD of the assay for the detection of *H. schachtii* as this amount of target is detected in all replicates (simplex and triplex). Moreover, it appears that the three target species were still detected in mixtures, even when one of them was in a weaker concentration (10 fold diluted) (Table 4B). For triplex real-time PCR assays, 5 J2 of *G. pallida* were combined with 10, 20, 40 and 50 cysts of *G. rostochiensis* (on average 130 larvae/cyst) and the DNA extracted as described for concentrated DNA solutions. Both target species were detected in mixture by the triplex reaction: the Ct values obtained for *G. pallida* were between 31 and 32 and for *G. rostochiensis* between 22 and 25.

Using our range of target and non-target species, we checked that the target species were detected in the triplex real time PCR assay (no negative deviation) for all populations and that no cross reaction resulted in positive deviations. The performance criteria were still of 100% for the triplex real time PCR assay in terms of diagnostic specificity, diagnostic sensitivity and relative accuracy (supplementary data Table s4). Experiments were also carried out using target DNA added to template DNA from two different soil extracts. Two different soils were chosen for their contrasted physical and chemical properties (data not shown), one from the “Bourgogne” region, the other from the “Vendée” region. The results showed no amplification using the triplex PCR assay by testing the template DNA obtained from soils free of cyst nematode (but containing other commonly found

plant-parasitic and non parasitic species of nematodes). Detection was observed for each target species even when DNA was spiked with template DNA solutions from soil extracts, irrespective of the relative quantities of the target DNA added to the mixture (Table 5). Results were confirmed when carried out in reproducibility conditions (data not shown).

Discussion

In this study we screened hundreds of microsatellite loci to investigate their potential as new species-specific markers for the diagnostic of important or regulated nematodes in seed potato production or trade. Our results demonstrate that these molecular regions can be of value for the development of novel diagnostic as we successfully developed a specific and sensitive real time PCR method for detecting the three species investigated, *G. pallida*, *G. rostochiensis* and *H. schachtii*. The originality of this method is first to allow the detection of different species of interest in a single reaction both on isolated cysts and on float material obtained after cyst nematode extraction from soil, while targeting different genomic regions than the ones used till now in nematode diagnostics. Microsatellites are among the most popular and versatile neutral markers for geneticists. It should be emphasized that the traditional use of microsatellites is the exploitation of the mutation rate of loci (i.e. the number of repeats of the short DNA motifs). In the present study we ignored this variation in size of the amplified products but instead investigated the specificity in the flanking regions of the microsatellites, which explain why these markers do not pass easily the species barrier where they were developed (Fredholm & Wintero, 1995; Selkoe & Toonen, 2006; Alenda *et al*., 2014). Moreover, it was also previously shown that there is a phylogenetic signal in the flanking regions of the microsatellite motif where the primers anneal (Delmotte *et al*., 2003). So clearly while

the variability remains in the microsatellite motif itself, the specificity is in the flanking regions on one or both sides of the motif.

Microsatellite loci were already isolated from several plant parasitic nematode species including *G. pallida* (Thiery & Mugnièry 2000; Montarry *et al.* 2015), *G. rostochiensis* (Boucher *et al.* 2013), *H. schachtii* (Kim *et al*. 2016; Plantard & Porte 2003), *H. glycines* (Wang *et al*. 2015), *H. carotae* (unpublished data), *M. incognita* (Mulet *et al.*, 2011), *B. xylophilus* (Mallez *et al*. 2013) and *Xiphinema index* (Villate *et al.* 2010). Moreover, genome sequencing has revealed that these kinds of loci are present in large numbers ranging from 2,842 in the *M. hapla* genome (Castagnone *et al.* 2010) to 20,015 and 22,627 for the two potato cyst nematode species (Eves van den Akker *et al*. 2016). The increasing availability of genome data for a wide range of nematodes, together with methodological developments of in silico mining of microsatellites, allow for the easier characterization and use of these markers. In this study, the selection process started with 69 loci for *G. pallida*, 60 for *G. rostochiensis* and 190 for *H. schachtii* and resulted in 1 marker for *H. schachtii* and 3 for the two *Globodera* species. Although the success rate varies greatly (0.5% for *H. schachtii*, 4-5% for *G. pallida* and *G. rostochiensis*), it can be assumed that finding species specific markers should be also possible for other species due to the large number of loci present in nematode genomes.

H. schachtii belongs to the *H. schachtii sensu stricto* group, which also contains *H. betae*, *H. ciceri*, *H. daverti*, *H. glycines*, *H. medicaginis*, and *H. trifolii* (Subbotin *et al.* 2000). There are some minor morphological and morphometrical differences which distinguish all of these species from each other but require a high level of morphology skills. In this group only *H. schachtii*, *H. betae* and *H. trifolii* are of economic importance for Western European agriculture. Diagnostic tools for the identification of *H. schachtii* are rare. The currently available are based on polymorphism of the

Internal Transcribed Spacer (ITS) region and are either based on a PCR-RFLP (Amiri *et al.* 2002) or on a real time PCR approach (Madani *et al*. 2005). ITS heterogeneity was reported for several species from the *Schachtii* group (Szalanski *et al.* 1997; Subbotin *et al.* 2000) and such diversity of paralogous ITS-rDNA is known to complicate species diagnostics especially when based on RFLPs (Buckler *et al.* 1997; Fenton *et al.* 1998). Consequently, there is a great benefit to add to the tool box a novel test based on the polymorphism of another genomic region of *H. schachtii.* The *H. schachtii* assay developed in this study proved to be highly specific without cross reaction when testing 19 populations representing 11 non target species of *Heterodera*. It should be noted that in this study we included closely-related species such as *H. betae*, *H. trifolii*, *H. ciceri*, *H. daverty* and *H. glycines* which all belong to the *Schachtii* group of *Heterodera* (Subbotin *et al.* 2000). Hence, slightly more than half of the species diversity known to date in the *Schachtii* group has already been investigated in this study. But it will be of interest to confirm the absence of cross-reaction of this new diagnostic tool with the remaining species of the *Schachtii* group. In terms of sensitivity, the LOD for the *H. schachtii* assay was five juveniles with a Ct value of 33.4. Further improvement of the PCR protocol may help improve this sensitivity. However, Madani *et al.* (2005) also concluded that the sensitivity of their real time PCR diagnostic tool for *H. schachtii* detection was lower than 100% for samples containing fewer than 5 juveniles. Even if the sensitivity of this previous diagnostic tool seems better (5 juveniles were always detected below a Ct value of 30), these authors indicated that a single juvenile and three juveniles were detected in only 20 and 60% of the samples, respectively.

G. pallida and *G. rostochiensis* are major potato pests and are considered as quarantine pests for seed potato production. According to present European Union legislation, the production of seed potatoes must cease for at least 10 years if one single cyst of the above two species is detected in a field. Both species can be found together in potato fields. Therefore it is necessary to identify

unambiguously the nematode species present in contaminated soils. At present the official French method for species differentiation is based on both morphological and biomolecular identification. Thus in order to identify all species belonging to the *Globodera* genus in a field, the analytical process should be repeated for each cyst.

The tools presented in this study were extensively tested for their specificity and proved to be highly specific without any cross reaction against all the *Globodera* species known to date developing on *Solanaceae*. We included in the study several closely related species such as *G. mexicana*, which is closely related to *G. pallida* (Subbotin *et al*. 2011), and the three subspecies of *G. tabacum*, which are closely related to *G. rostochiensis* (Subbotin *et al*. 2011). A total of 36 populations of *Globodera* were used in this study, including several South American populations (5 for *G. rostochiensis* and 7 for *G. pallida*) from different countries. This *Globodera* collection represents one of the largest genetic diversity ever tested against a potato cyst nematode diagnostic tool. While there are numerous conventional PCR diagnostic tests for the specific identification of PCN (Bulman & Marshall, 1997; Fullaondo *et al*. 1999; Mulholland *et al*. 1996; Pylypenko *et al*. 2005; Vejl *et al*. 2002), these have limitations for a screening strategy. Species identification by real-time PCR diagnostics offers much greater potential for high-throughput screening. Several real time PCR assays for the detection of either *G. rostochiensis* or *G. pallida* were developed but data are lacking for the evaluation of their performance especially in terms of sensitivity and specificity against a large range of populations (Reid *et al.* 2010; Nakhla *et al.* 2010; Madani *et al*. 2011). So far, only a commercially available test developed by Blgg is recommended in the EPPO standard protocol *for G. pallida* and *G. rostochiensis* (PM7/40 (3), OEPP/EPPO 2013). This test is a real time SYBR Green test built from rDNA (LSU) sequences, so it cannot be multiplexed to detect *Globodera* potato cyst nematodes simultaneously in a single experiment. Moreover, this commercially available test is not recommended for testing samples from South and Central America (PM7/40 (3), OEPP/EPPO 2013).

Compared to these tools, the added values of the new diagnostic tools presented in this study are that they (i) extend the diagnostics tool box for PCN based on the polymorphism of a new genomic region, (ii) consider for cross reaction particular species such as *G. mexicana* and the newly described PCN species *G. ellingtonae* (Handoo *et al.* 2012) that previously were never considered together, and (iii) cover a larger set of populations from the native area of these nematodes than previously tested (i.e. no South American populations were used in Madani *et al*. (2011) and Papayiannis *et al.* 2013, 2 in Nakhla *et al*. (2010) and no data were published in Reid *et al.* (2010 and 2015)). The perfect accordance in terms both of positive and negative agreement of the tools here described make them tools of interest not only in Europe but also in the Americas (including South American countries) where species such as *G. ellingtonae* or *G. mexicana* can co-occur with *G. pallida* and *G. rostochiensis*. The positive detection obtained for all the native PCN populations tested also ensures an efficient control that should prevent Europe from any new introduction from a third country and the native area in particular. Indeed, the Bulman & Marshall 1997 test does not detect all the native *G. rostochiensis* populations of Bolivia (Anses-LSV internal data), while this test can. The *G. pallida* and *G. rostochiensis* diagnostic markers proved to be very sensitive, as a single juvenile was detected in our tests. This sensitivity is similar to that reported for the previously described multiplex real time PCR assays published to date.

As *G. pallida* and *G. rostochiensis* occur often together, a multiplexed assay saves time and resources. Moreover, associating *H. schachtii* to this analytical process is the best way to take into account the export regulation for seed potato trades. Thus, the study focused on the development of a multiplex diagnostic tool which can detect and identify these three species in a single reaction. Taqman technology with specific probes per species used in multiplex seemed the best option. The main advantages of the multiplexing approach are the reduced cost, implementation time and time

spent analysing the results. However, the development of such multiplex PCR proved to be complex (primer interactions, annealing temperatures, etc) (Dong *et al*. 2001; Blok *et al*. 2005). In our case the multiplex approach was considered for the design of the primers and probes but was facilitated by the fact that different genomic regions were targeted for each species hence, minimizing primer competition problems. All the different multiplex assays showed the same performances as the simplex reaction in terms of specificity, sensitivity and reproducibility.

The detection of the three target species was also investigated directly from float material obtained by the extraction of cyst nematodes from soil. The direct detection approach aims to increase the analytical capacity of routine laboratories performing high volumes of analysis of soil samples, because no morphological analysis is necessary here. The extraction of DNA directly from soil and subsequent detection of plant pathogens is possible as described in Volossiouk *et al*. (1995) and in the international standard ISO 11063:2012. However, DNA is directly extracted from a 0.25 g soil sample, while the minimum quantity of soil to be analyzed for official PCN control is 1500 mL soil (Directive 2007/33/EC). The PCN extraction step from soil using Schuilling, Seinhorst or other elutriation principles remains essential and cannot be overlooked when working on larger volumes of soil. Some studies already presented such direct detection approaches for *G. pallida* and *G. rostochiensis* (Adams *et al.* 2009; Reid *et al.* 2010 and 2015). Adams *et al*. (2009) did not perform sample elutriation to extract PCN but the protocol proposed by these authors is quite fastidious and not automatized. Reid *et al*. (2010 and 2015) did not present any performance results. The assay developed in this study was able to detect target DNA spiked with template DNA extracted from float material. However, despite the fact that this assay could be suitable for direct detection of target DNA in a crude extract, some improvements must be made. The extraction method of

nematodes from soil and the DNA extraction protocol need to be improved to reduce debris recovered from the soil sample, to improve the cyst crushing and reduce potential PCR inhibitors.

In brief, we reported here a new real time PCR tool based on microsatellite markers for the specific (simplex) and simultaneous (triplex) detection of the three major cyst nematodes which are often investigated for seed potato trade, i.e. *G. pallida*, *G. rostochiensis* and *H. schachtii*. It could be used both for the identification of isolated cysts (one to 50 cysts analyzed in the same tube) and direct detection on float material after nematode extraction from soil. For the direct detection of the two EU quarantine PCNs, *H. schachtii* DNA could be used as internal positive control to rule out failure of amplification in cases where the target sequence is not detected.

Over two thirds of seed potatoes produced in Europe are exported to Southern and Eastern Mediterranean countries. There is therefore an increasing demand in the seed potato export market regarding the control and identification of not only the quarantine potato cyst nematodes but also other cyst nematode species such as the beet cyst nematode, *H. schachtii* which is regulated in several countries to which seed potatoes are exported. The diagnostic tools presented in this article were developed to respond to this increasing demand and to improve and facilitate sanitary controls for the export of seed potatoes. We can assume that with the amount of novel sequencing data that will be available in different nematode species and because of the routine multiplexing of microsatellites, the current diagnostic assay could easily evolve and adapt to future demands in order for example to include another cyst nematode species of interest or to develop a similar assay for a subset of regulated or emergent root knot nematode species.

Acknowledgements

We are grateful to the different people quoted in the paper for providing us with some useful non target species. We thank all the colleagues from the Anses and INRA nematology teams who have contributed to this project. We thank Sebastian Kiewnick for his useful comments on the manuscript. This study was funded by Anses and INRA Plant Health and Environment department.

References

Adams I., Woodhall, J., Glover, R., Shah, F., Hockland, S., Boonham, N., and Marshall, J. (2009). Validation of quantitative DNA detection systems for PCN. Potato Council, Kenilworth, UK, pp.1-36.

Alenda, C., Montarry, J., and Grenier, E. (2014). Human influence on the dispersal and genetic structure of French *Globodera tabacum* populations. *Infection, Genetics and Evolution* **27**, 309-317. DOI: 10.1016/j.meegid.2014.07.027

Amiri, S., Subbotin, S. A., and Moens, M. (2002). Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* **108**, 497-506. DOI: 10.1023/A:1019974101225

Anonymous (2000). Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official J. Eur. Communities* L169(43):1-112.

Anonymous (2007). Council Directive 2007/33/EC of 11 June 2007 on the control of potato cyst nematodes and repealing Directive 69/465/EEC. *Official Journal of the European Union* L156/12.

Anonymous (2010). Preliminary tests for the identification of different species of *Heterodera*. Anses-LSV-Nematology unit internal document. 10/02, 1-11.

Anonymous (2010). Comparative evaluation of different molecular tools for the identification of *Globodera pallida* and *Globodera rostochiensis*. Anses-LSV-Nematology unit internal document. 10/03, 1-18.

Blok, V. C. (2005). Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal of Plant Pathology* **27**, 176-185. DOI: 10.1080/0706660509507214

Boucher, A. C., Mimee, B., Montarry, J., Bardou-Valette, S., Bélair, G., Moffett, P., and Grenier, E. (2013). Genetic diversity of the golden potato cyst nematode *Globodera rostochiensis* and determination of the origin of populations in Quebec, Canada. *Molecular Phylogenetics and Evolution* **69**, 75-82. DOI: 10.1016/j.ympev.2013.05.020

Buckler, E. S., Ippolito, A., and Holtsford, T. P. (1997). The evolution of ribosomal DNA divergent paralogues and phylogenetic implications. *Genetics* **145**, 821-832.

Bulman, S., and Marshall, J. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* **25**, 123-129.

Castagnone-Sereno, P., Danchin, E. G. J., Deleury, E., Guillemaud, T., Malausa, T., and Abad, P. (2010). Genome-wide survey and analysis of microsatellites in nematodes, with a focus on the plant-parasitic species *Meloidogyne incognita*. *BMC Genomics* **11**. DOI: 10.1186/1471-2164-11-598

Delmotte, F., Sabater-Muñoz, B., Prunier-Leterme, N., Latorre, A., Sunnucks, P., Rispe, C., Simon, J.-C., and Crespi, B. (2003). Phylogenetic evidence for hybrid origins of asexual lineages in an aphid species. *Evolution* **57**, 1291-1303. DOI: 10.1554/02-557

Dong, K., Dean, R. A., Fortnum, B. A., and Lewis, S. A. (2001). Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. *Nematropica* **31**, 271-280.

Eoche-Bosy, D., Gauthier, J., Juhel, A. S., Esquibet, M., Fournet, S., Grenier, E., and Montarry, J. (2016). Experimentally evolved populations of the potato cyst nematode *Globodera pallida* allow the targeting of genomic footprints of selection due to host adaptation. *Plant Pathology*, n/a-n/a. DOI: 10.1111/ppa.12646

Eves-van den Akker, S., Laetsch, D. R., Thorpe, P., Lilley, C. J., Danchin, E. G. J., Da Rocha, M., Rancurel, C., Holroyd, N. E., Cotton, J. A., Szitenberg, A., *et al.* (2016). The genome of the yellow potato cyst nematode, *Globodera rostochiensis*, reveals insights into the basis of parasitism and virulence. *Genome Biology* **17**. DOI: 10.1186/s13059-016-0985-1

Fenton, B., Malloch, G., and Germa, F. (1998). A study of variation in rDNA ITS regions shows that two haplotypes coexist within a single aphid genome. *Genome* **41**, 337-345.

Fredholm, M., and Winterø, A. K. (1995). Variation of short tandem repeats within and between species belonging to the *Canidae* family. *Mammalian Genome* **6**, 11-18.

Fullaondo, A., Barrena, E., Viribay, M., Barrena, I., Salazar, A., and Ritter, E. (1999). Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology* **1**, 157-163.

Handoo, Z. A., Carta, L. K., Skantar, A. M., and Chitwood, D. J. (2012). Description of *Globodera ellingtonae* n. sp. (Nematoda: Heteroderidae) from Oregon. *Journal of Nematol* **44**, 40-57.

Hockland, S., Inserra, R., Millar, L., Lehman, P., Perry, R., and Moens, M. (2006). International plant health-putting legislation into practice. *Plant nematology*, 327-345.

Ibrahim, S. K., Perry, R. N., Burrows, P. R., and Hooper, D. J. (1994). Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. *Journal of Nematology* **26**, 412-421.

Kim, J., Kim, T., Lee, Y. C., Chun, J. Y., Kern, E. M. A., Jung, J., and Park, J. K. (2016). Characterization of 15 microsatellite loci and genetic analysis of *Heterodera schachtii* (Nematoda: Heteroderidae) in South Korea. *Biochemical Systematics and Ecology* **64**, 97-104. DOI: 10.1016/j.bse.2015.11.013

Madani, M., Subbotin, S. A., and Moens, M. (2005). Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. *Molecular and Cellular Probes* **19**, 81-86. DOI: 10.1016/j.mcp.2004.09.006

Madani, M., Ward, L. J., and De Boer, S. H. (2011). Hsp90 gene, an additional target for discrimination between the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, and the related species, *G. tabacum tabacum*. *European Journal of Plant Pathology* **130**, 271-285. DOI: 10.1007/s10658-011-9752-z

Malausa, T., Gilles, A., Meglécz, E., Blanquart, H., Duthoy, S., Costedoat, C., Dubut, V., Pech, N., Castagnone-Sereno, P., Délye, C., *et al.* (2011). High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* **11**, 638-644. DOI: 10.1111/j.1755-0998.2011.02992.x

Mallez, S., Castagnone, C., Espada, M., Vieira, P., Eisenback, J. D., Mota, M., Guillemaud, T., and Castagnone-Sereno, P. (2013). First Insights into the Genetic Diversity of the Pinewood Nematode in Its Native Area Using New Polymorphic Microsatellite Loci. *PLoS ONE* **8**. DOI: 10.1371/journal.pone.0059165

Manachini, B. (2000). First report of *Heterodera glycines* Ichinohe on soybean in Italy. *Bolletino di Zoologia Agraria e di Bachicoltura Serie II* **32**, 261-267.

Meglécz, E., Costedoat, C., Dubut, V., Gilles, A., Malausa, T., Pech, N., and Martin, J.-F. (2010). QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* **26**, 403-404. DOI: 10.1093/bioinformatics/btp670

Montarry, J., Jan, P. L., Gracianne, C., Overall, A. D. J., Bardou-Valette, S., Olivier, E., Fournet, S., Grenier, E., and Petit, E. J. (2015). Heterozygote deficits in cyst plant-parasitic nematodes: possible causes and consequences. *Molecular Ecology* **24**, 1654-1677. DOI: 10.1111/mec.13142

Mulet, K., Fargette, M., Richaud, M., Genson, G., and Castagnone-Sereno, P. (2011). Isolation of microsatellites from an enriched genomic library of the plant-parasitic nematode *Meloidogyne incognita* and their detection in other root-knot nematode species. *European Journal of Plant Pathology* **129**, 501-505. DOI: 10.1007/s10658-010-9721-y

Mulholland, V., Carde, L., and O'Donnell, K. (1996). Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. *MONOGRAPHS-BRITISH CROP PROTECTION COUNCIL*, 247-252.

Muller, J. (1999). The economic importance of *Heterodera schachtii* in Europe. *Helminthologia* **36**, 205-213.

Nakhla, M. K., Owens, K. J., Li, W. B., Wei, G., Skantar, A. M., and Levy, L. (2010). Multiplex real-time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Disease* **94**, 959-965. DOI: 10.1094/PDIS-94-8-0959

OEPP/EPPO (2013). EPPO Standard PM 7/40 (3). *Globodera rostochiensis* and *Globodera pallida*. *Bulletin OEPP/EPPO, Bulletin 43 (1),* 119-138. DOI: 10.1111/epp.12025

OEPP/EPPO. (2014). EPPO standard PM 7/98 (2). Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *Bulletin OEPP/EPPO, Bulletin, 44 (2)*, 117–147. DOI: 10.1111/epp.12118

OEPP/EPPO (2013). EPPO Standard PM 7/119 (1). Nematode extraction. Bulletin OEPP/EPPO, Bulletin 43 (3), 471-495. DOI: 10.1111/epp.12077

Papayiannis, L. C., Christoforou, M., Markou, Y. M., and Tsaltas, D. (2013). Molecular typing of cyst-forming nematodes *Globodera pallida* and *G. rostochiensis*, using real-time PCR and evaluation of five methods for template preparation. *Journal of Phytopathology* **161**, 459-469. DOI: 10.1111/jph.12091

Plantard, O., Picard, D., Valette, S., Scurrah, M., Grenier, E., and Mugni,ry, D. (2008). Origin and genetic diversity of Western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* **17**, 2208-2218. DOI: 10.1111/j.1365-294X.2008.03718.x

Plantard, O., and Porte, C. (2003). Isolation and characterization of microsatellite loci in the sugar beet cyst nematode *Heterodera schachtii Molecular Ecology Notes* **3**, 139-141. DOI: 10.1046/j.1471-8286.2003.00383.x

Powers, T. (2004). Nematode Molecular Diagnostics: From bands to barcodes. *In* "Annual Review of Phytopathology", Vol. 42, pp. 367-383. DOI: 10.1146/annurev.phyto.42.040803.140348

Pylypenko, L., Uehara, T., Phillips, M., Sigareva, D., and Blok, V. (2005). Identification of *Globodera rostochiensis* and *G. pallida* in the Ukraine by PCR. *European Journal of Plant Pathology* **111**, 39-46. DOI: 10.1007/s10658-004-2732-9

Reid, A., Evans, F., Mulholland, V., Cole, Y., and Pickup, J. (2015). High-Throughput Diagnosis of Potato Cyst Nematodes in Soil Samples. *In* "Plant Pathology: Techniques and Protocols" (C.

Lacomme, ed.), pp. 137-148. Springer New York, New York, NY. DOI: 10.1007/978-1-4939-2620-6_11

Reid, A., Kenyon, D. M., Evans, F. F., Mulholland, V., Pickup, J., Blok, V. C., Paterson, A., and Phillips, M. S. (2010). Development of a high-throughput method for the detection and species determination of PCN. *Aspects of Applied Biology*, 13-16.

Rotmistrovsky K., Jang W., Schuler G.D. (2004). A web server for performing electronic PCR. *Nucleic Acids Research* **32**, W108-12. DOI: 10.1093/nar/gkh450

Selkoe, K. A., and Toonen, R. J. (2006). Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**, 615-629. DOI: 10.1111/j.1461-0248.2006.00889.x

Shields, R., Fleming, C. C., and Stratford, R. (1996). Identification of potato cyst nematodes using the polymerase chain reaction. *Fundamental and Applied Nematology* **19**, 167-173.

Skantar, A. M., Handoo, Z. A., Carta, L. K., and Chitwood, D. J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* **39**, 133-144.

Subbotin, S. A., Prado Vera, I. C. d., Mundo-Ocampo, M., and Baldwin, J. G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology* **13**, 805-824. DOI: 10.1163/138855410X552661

Subbotin, S. A., Waeyenberge, L., and Moens, M. (2000). Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology* **2**, 153-164. DOI: 10.1163/156854100509042

Sunnucks, P. (2000). Efficient genetic markers for population biology. *Trends in Ecology and Evolution* **15**, 199-203.

Szalanski, A. L., Sui, D. D., Harris, T. S., and Powers, T. O. (1997). Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology* **29**, 255-267.

Thiery, M., and Mugniery, D. (1996). Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. *Fundamental and Applied Nematology* **19**, 471-479.

Thiery, M., and Mugniery, D. (2000). Microsatellite loci in the phytoparasitic nematode *Globodera*. *Genome* **43**, 160-165.

Van Riel, H., and Mulder, A. (1998). Potato cyst nematodes (*Globodera* species) in Western Europe. *Potato Cyst Nematodes, Biology, Distribution and Control, CAB international, Wallingford, UK*, 271-298.

Vejl, P., Skupinova, S., Sedlak, P., and Domkarova, J. (2002). Identification of PCN species (*Globodera rostochiensis*, *G. pallida*) by using of ITS-1 region's polymorphism. *Rostlinna Vyroba* **48**, 486-489.

Villate, L., Esmenjaud, D., Van Helden, M., Stoeckel, S., and Plantard, O. (2010). Genetic signature of amphimixis allows for the detection and fine scale localization of sexual reproduction events in a mainly parthenogenetic nematode. *Molecular Ecology* **19**, 856-873.

Volossiouk, T., Robb, E. J., and Nazar, R. N. (1995). Direct DNA extraction for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology* **61**, 3972-3976.

Wang, H.-M., Zhao, H.-h., and Chu, D. (2015). Genetic structure analysis of populations of the soybean cyst nematode, *Heterodera glycines*, from north China. *Nematology* **17**, 591-600.

Table 1 Nematode populations used for this study

Nematode species	Name	Location	Source
Globodera ellingtonae	Oregon	USA	Oregon State University
Globodera mexicana	Santa Ana	Mexico	INRA laboratory collection[a]
Globodera pallida	Pa2/3 Chavornay	Switzerland	’’
	Pa2/3 Rookmaker	The Netherlands	’’
	Pa2/3 Noirmoutier	France	’’
	Pa2/3 Pukekohe	New Zealand	’’
	Pa2/3 Luffness	Scotland	’’
	Pa2/3 Ouessant	France	’’
	Pa1 Duddingston	Scotland	’’
	309	Peru	’’
	251	Peru	’’
	212	Peru	’’
	224	Peru	’’
	38	Peru	’’
	120803	Chile	’’
	3343	Chile	’’
Globodera rostochiensis	Ro1 Sedan	France	’’
	Ro1 Nîmes	France	’’
	Ro1	Portugal	’’
	Ro1	Scotland	’’
	Ro1 Alsonemedi	Hungary	’’
	Ro4	The Netherlands	’’
	Ro5	The Netherland	’’
	Ro3 CZ0B	The Netherlands	’’
	228	Peru	’’
	267	Peru	’’
	3346	Chile	’’
	B1	Bolivia	’’
	B2	Bolivia	’’
Globodera tabacum tabacum	Agen	France	’’
	Connecticut	USA	’’
	Rosalejo 11093	Spain	’’
	La Réole 33190	France	’’
Globodera tabacum solanacearum	Gs3	USA	’’
	75140	Mexico	’’
Globodera tabacum virginiae	GV2	USA	’’
Heterodera avenae	Ha1	France	LSV laboratory collection[b]
Heterodera betae	Fin C1	Finland	INRA laboratory collection[a]
	Hb2	Finland	Wageningen University
Heterodera carotae	HCA 50.01	France	INRA laboratory collection[a]
Heterodera ciceri	Hci1	Syria	ILVO[c]
Heterodera cruciferae	Hcr35.01	France	LSV laboratory collection[b]
Heterodera daverty	Hd1	The Netherlands	ILVO[c]
Heterodera glycines	Hgl1	Russia	ARRIP[d]
	Hgl2	USA	(1)University of Illinois
Heterodera goettingiana	Hgo1	Italy	ARRIP[d]
Heterodera schachtii	Tur.C4	Turkey	INRA laboratory collection[a]
	Pol.C1	Poland	’’

	It.C2	Italy	’’
	Esp.C1	Spain	’’
	USA.C1	USA	’’
	Mar.C2	Morocco	’’
	Fra C1	France	’’
	Hs1	Belgium	ILVO[c]
Heterodera trifolii	Ht1	The Netherlands	’’

[a] INRA, Le Rheu, France

[b] Anses – Plant Health Laboratory – Nematology unit, Le Rheu, France

[c] ILVO (Flanders Research Institute for Agriculture, Fisheries and Food), Belgium

[d] All-Russian Research Institute of Phytopathology, Russia

(1) Only DNA solution supplied

Table 2 PCR amplification parameters: mix composition (final concentration per reaction) and program details

Conventional PCR amplification parameters used for microsatellite marker selection		
Target organism	***G. pallida and G. rostochiensis***	***H. schachtii***
Taq buffer	1X	1X
$MgCl_2$*	3 mM	1.5 mM
Primer (each)	0.4 µM	0.3 µM
dNTP (each)	0.2 mM	0.2 mM
Taq polymerase	0.6 U	0.5 U
DNA solution	1 µl	5 µl
Final volume	12.5 µl	25 µl
Amplification program	2 min at 95°C, 35 cycles (20 sec at 95°C, 20 sec at 55°C, 30 sec at 72°C), 5 min at 72°C	2 min at 94°C, 30 cycles (30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C), 10 min at 72°C
*already contained in Taq buffer		

Real time PCR amplification parameters used in Taqman and SYBR Green reactions		
	SYBR Green reaction	**Probe reaction (simplex and triplex)**
Target organism	***G. pallida or G. rostochiensis or H.schachtii***	***G. pallida and/or G. rostochiensis and/or H.schachtii***
Master mix	1X	1X
Primer (each)	0.25µM	0.5µM
Probe (each)	/	0.2µM
DNA solution	3 µl	5 µl
Final volume	20 µl	20 µl
Amplification program	10 min at 95°C, 40 cycles (10 sec at 95°C, 10 sec at 60°C; 25sec at 72°C);	10 min at 95°C, 40 cycles (10 sec at 95°C, 50 sec at 60°C)

Table 3: Cycle threshold values obtained by testing a range of juveniles (J2) and a cyst of *G. pallida*, *G. rostochiensis* and *H. schachtii* target species in simplex real time PCR reaction

	G. pallida	***G. rostochiensis***	***H. schachtii***
1 J2	32.8	32.8	ND
2 J2	32.3	31.9	>35
5 J2	31.4	30.3	33.4
10 J2	30.0	29.7	31.9
50 J2*	27.4	27.3	31.8
1 cyst	25.9	26.1	29.3

* 30 J2 for *H. schachtii*

ND: Not Detected

Table 4: Cycle threshold (Ct) values obtained by triplex real time PCR analyzing a range of juveniles of each target species (A) and a mixture of DNA from the three target species (B)

Target organism			***G. pallida***		***G. rostochiensis***		***H. schachtii***	
Reproducibility conditions			**1**	**2**	**1**	**2**	**1**	**2**
A - Range of target species:								
1 J2			34.62	32.80	32.60	31.33	ND	ND
2 J2			32.39	31.77	31.90	30.30	34.13	34.07
5 J2			31.76	31.44	30.47	29.28	32.89	32.38
10 J2			30.39	30.10	29.72	29.33	31.87	31.03
50 J2*			27.76	27.71	27.28	27.00	31.74	31.42
1 cyst			25.95	25.91	26.14	26.02	29.43	29.24
B - Mixture of DNA from the different target species								
Gp	***Gr***	***Hs***						
1	*1*	*1*	26.83	26.79	26.24	26.19	27.69	27.73
1/10	*1*	*1*	30.02	29.91	26.60	26.53	28.08	28.05
1	*1/10*	*1*	26.98	26.98	29.90	29.15	28.19	28.23
1	*1*	*1/10*	26.90	26.83	26.40	26.35	29.94	30.11

GP: *G. pallida*

GR: *G. rostochiensis*

HS: *H. schachtii*

* 30 J2 for *H. schachtii*

ND: Not Detected

1: 3.35 ng/well

1/10: 0.34 ng/well

Table 5: Cycle threshold (Ct) value obtained by triplex real time PCR assay analyzing different quantities (in µL) of target DNA (0.6 ng/µl) spiked with a total DNA extracted from float material obtained by the extraction of cyst nematodes from the soil of two different regions, “Bourgogne” and “Vendée”

Target organism				***G. pallida***	***G. rostochiensis***	***H. schachtii***
Total DNA soil extract +	GP +	GR +	HS			
"Bourgogne"	0	0	0	ND	ND	ND
"Bourgogne" +	3 +	3 +	3	27.60	26.50	27.75
"Bourgogne" +	1 +	1 +	6	28.93	27.75	27.36
"Bourgogne" +	1 +	6 +	1	29.05	25.73	28.50
"Bourgogne" +	6 +	1 +	1	26.37	27.55	28.96
"Vendée"	0	0	0	ND	ND	ND
"Vendée" +	3 +	3 +	3	27.59	26.68	28.00
"Vendée" +	1 +	1 +	6	29.00	27.89	27.59
"Vendée" +	1 +	6 +	1	29.09	26.04	29.03
"Vendée" +	6 +	1 +	1	26.58	27.71	29.10

GP: *G. pallida* GR: *G. rostochiensis* HS: *H. schachtii*
ND: Not Detected

Table s1: Results obtained by conventional PCR for the selection of relevant loci for G. pallida detection by testing a range of target and non-target populations and calculating the diagnostic specificity, the diagnostic sensitivity and the relative accuracy

Species	Name	GP143	GP144	GP145	GP150	GP182	GP224	GP228	GP238	GP258	GP272	GP279	GP286
G. pallida	Pa1 Duddingtston	+	+	+	+	+	-	-	+	+	+	+	+
"	Pa2/3 Chavornay	+	+	+	+	+	+	-	+	+	+	+	+
"	Pa2/3 Luffness	+	+	+	+	+	+	-	+	-	+	+	+
"	Pa2/3 Rookmaker	*nt*	+	*nt*	+	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	Pa2/3 Ouessant	*nt*	+	*nt*	+	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	Pa2/3 Noirmoutier	*nt*	+	*nt*	+	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	Pa2/3 Pukukoe	*nt*	+	*nt*	+	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	3343	*nt*	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	120803	*nt*	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	309	-	+	+	+	+	-	-	+	-	+	+	+
"	251	+	+	+	+	+	-	-	+	+	+	+	+
"	212	-	+	+	+	+	-	-	+	-	+	+	+
"	224	*nt*	+	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*
"	38	+	+	+	+	+	-	-	+	-	-	+	+
G. rostochiensis	Ro1	-	-	-	-	-	-	-	-	-	-	-	-
G. mexicana	Santa Ana	-	-	+	-	(+)	-	-	+	+	+	+	+
G. elingtonae	Oregon	-	-	-	-	-	-	-	-	-	-	-	-
G. tabacum virginiae	Gv2	-	-	-	-	-	-	-	-	-	-	-	-
G. tabacum tabacum	Gtt Agen	-	-	-	-	-	-	-	-	-	-	-	-
G. tabacum solanacearum	Gs3	-	-	-	-	-	-	-	-	-	-	-	-
Negative Agreement *		6	6	5	6	5	6	6	5	5	5	5	5
Positive Agreement *		5	14	7	11	11	2	0	7	3	6	7	7
Positive Deviation *		0	0	1	0	1	0	0	1	1	1	1	1
Negative Deviation *		2	0	0	0	0	5	7	0	4	1	0	0
Diagnostic Specificity *		**100%**	**100%**	**83%**	**100%**	**83%**	**100%**	**100%**	**83%**	**83%**	**83%**	**83%**	**83%**
Diagnostic Sensitivity *		**71%**	**100%**	**100%**	**100%**	**100%**	**29%**	**0%**	**100%**	**43%**	**86%**	**100%**	**100%**
Relative Accuracy *		**85%**	**100%**	**92%**	**100%**	**94%**	**62%**	**46%**	**92%**	**62%**	**85%**	**92%**	**92%**

+ : amplification at the expected size

- : NO amplification at the expected size

(+): weak signal

nt : not tested

* : compared to the status of the sample

Table s2: Results obtained by conventional PCR for the selection of relevant loci for G. rostochiensis detection by testing a range of target and non target populations and calculating the diagnostic specificity, the diagnostic sensitivity and the relative accuracy

Species	Name	GR63	GR71	GR83	GR86	GR90	GR94	GR105	GR120	GR121
G. rostochiensis	Ro1	+	+	+	(+)	+	(+)	+	-	+
"	Ro1 Sedan	+	+	*nt*	(+)	+	*nt*	+	*nt*	+
"	Ro1 Nîmes	+	+	*nt*	(+)	+	*nt*	+	*nt*	+
"	Ro1 Portugal	+	+	*nt*	(+)	+	*nt*	+	*nt*	+
"	Ro1 Alsonemedi	+	+	*nt*	(+)	+	*nt*	+	*nt*	+
"	Ro4	+	+	+	(+)	+	(+)	+	-	+
"	Ro5	+	+	+	(+)	+	(+)	+	-	+
"	228	+	+	+	(+)	+	(+)	+	-	+
"	267	+	+	+	(+)	+	(+)	+	-	+
"	3346	+	+	+	(+)	+	(+)	+	-	+
"	B1	+	+	+	(+)	+	(+)	+	-	+
G. pallida	Pa2/3 Chavornay	-	-	-	-	-	-	-	-	-
G. mexicana	Santa Ana	-	-	-	-	-	-	-	-	-
G. elingtonae	Oregon	-	-	-	-	-	-	-	-	-
G. tabacum virginiae	Gv2	-	-	-	-	-	-	-	-	-
G. tabacum tabacum	Gtt Agen	-	-	-	-	-	-	-	-	-
G. tabacum solanacearum	Gs3	-	-	-	-	-	-	-	-	-
Negative Agreement *		6	6	6	6	6	6	6	6	6
Positive Agreement *		11	11	7	11	11	7	11	0	11
Positive Deviation *		0	0	0	0	0	0	0	0	0
Negative Deviation *		0	0	0	0	0	0	0	7	0
Diagnostic Specificity *		**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
Diagnostic Sensitivity *		**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**0%**	**100%**
Relative Accuracy *		**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**46%**	**100%**

+ : amplification at the expected size

- : NO amplification at the expected size

(+): weak signal

nt : not tested

* : compared to the status of the sample

Table s3: Results obtained by conventional PCR for the selection of relevant loci for H. schachtii detection by testing a range of target and non-target populations and calculating the diagnostic specificity, the diagnostic sensitivity and the relative accuracy

Species	Name	HS33	HS43	HS45	HS91	HS95	HS96	HS126	HS127	HS171
H. schachtii	Tur.C4	+	+	+	+	+	+	+	+	+
	Pol.C1	+	+	+	-	+	+	+	+	+
	HS1	+	+	+	+	+	+	+	+	+
	It.C2	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	+
	Esp.C1	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	+
	USA.C1	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	+
	Mar.C2	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	+
	Fra C1	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	+
G. pallida	Pa2/3 Noirmoutier	-	-	-	-	-	-	-	-	-
G. rostochiensis	Ro1	-	-	-	-	-	-	-	-	-
G. tabacum tabacum	Gtt Agen	-	-	-	-	-	-	-	-	-
H. avenae	Ha1	-	-	-	-	-	-	-	-	-
H. betae	Fin C1	-	+	+	+	-	-	+	+	-
	Hb2	-	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	-
H. carotae	HCA 50.01	-	-	-	-	-	-	-	-	-
H. ciceri	Hci1	-	-	+	-	-	+	+	-	-
H. cruciferae	HCr35.01	-	-	-	-	-	-	-	-	-
H. daverty	Hd1	-	+	(+)	-	-	-	+	(+)	-
H. glycines	Hgl1	-	-	+	-	+	-	+	+	-
	Hgl2	-	-	+	-	-	-	+	+	-
	Hgl3	-	-	+	-	+	-	+	+	-
	Hgl4	-	-	+	-	+	-	+	+	-
	Hgl5	-	-	+	-	-	-	+	+	-
H. goettingiana	Hgo	-	-	-	-	-	-	-	-	-
H. trifolii	Ht1	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	*nt*	-
Negative Agreement *		16	13	7	14	12	14	7	8	17
Positive Agreement *		3	3	3	2	3	3	3	3	8
Positive Deviation *		0	2	8	1	3	1	8	7	1
Negative Deviation *		0	0	0	1	0	0	0	0	0
Diagnostic Specificity *		**100%**	**87%**	**47%**	**93%**	**80%**	**93%**	**47%**	**53%**	**94%**
Diagnostic Sensitivity *		**100%**	**100%**	**100%**	**67%**	**100%**	**100%**	**100%**	**100%**	**100%**
Relative Accuracy *		**100%**	**89%**	**56%**	**89%**	**83%**	**94%**	**56%**	**61%**	**96%**

+ : amplification at the expected size

- : NO amplification at the expected size

(+): weak signal

nt : not tested

* : compared to the status of the sample

Table s4: Results for diagnostic specificity, diagnostic sensitivity and relative accuracy obtained in triplex real time PCR assay

Target organism	***G. pallida***	***G. rostochiensis***	***H. schachtii***
Negative Agreement *	41	42	47
Positive Agreement *	14	13	8
Positive Deviation *	0	0	0
Negative Deviation *	0	0	0
Diagnostic Specificity *	**100%**	**100%**	**100%**
Diagnostic Sensitivity *	**100%**	**100%**	**100%**
Relative Accuracy *	**100%**	**100%**	**100%**

* Compared to the status of the sample