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Validation report

Ditylenchus dipsaci & *Ditylenchus destructor*

- Diagnostic qPCR assays for identification and detection -

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Validation performed by:

Company	ClearDetections BV
Address	P.O. box 170, NL-6700 AD, Wageningen, The Netherlands
Contact	Dr. R. Landeweert
Phone	+ 31 (0) 6 52561829
E-mail	info@cleardetections.com
Website	www.cleardetections.com

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Abstract

This report describes the in-house validation* of two real-time PCR (qPCR) assays for the identification and detection of the plant pathogenic nematodes *Ditylenchus dipsaci* and *D. destructor*.

Performance characteristics as determined for both qPCR tests:

	D. dipsaci qPCR test	*D. destructor* qPCR test
Accuracy	100%	100%
Diagnostic sensitivity	100%	100%
Diagnostic specificity	100%	100%
Dynamic range	between 10-100 and 0.1 billion copies of target rDNA	between 10-100 and 0.1 billion copies of target rDNA
Analytical sensitivity	100%	100%
Analytical specificity	100%	100%
Selectivity	100%	100%
Robustness	OK	OK
Reproducibility	100%	100%
Repeatability	100%	100%

Conclusion:

The validated diagnostic qPCR assays for identification and detection of D. *dipsaci* and *D. destructor* are suitable for their intended use:

- ✓ species-specific qualitative detection of DNA from *D. dipsaci* and/or *D. destructor* in DNA extracts originating from nematode suspensions isolated from 100 ml soil samples (= highly complex DNA background).
- ✓ species-specific identification of DNA from *D. dipsaci* and/or *D. destructor* originating from individual nematodes.

*The documents 'National guideline for validation of detection and identification methods for plant pathogens and pest organisms' (Dutch National Reference Laboratory, March 2010) and the EPPO document ‘Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity’ (EPPO, 2010) were used as guidelines throughout this validation.

Introduction

Generally spoken, plant-parasitic nematodes are a fairly small minority living in a nematode community that consists for the better part of so-called free-living (= bacterivorous and fungivorous) nematodes. Microscopic identification of plant-parasitic nematodes is laborious and it is often based on only a few subtle morphological characteristics that should be recognized (by specialists) within a huge background of non-target nematodes. Molecular (DNA-based) identification is based on an overwhelming quantity of putative informative characters, and depending on the desired identification level – order, family, genus, species or race – appropriate stretches of DNA can be identified that are suitable for nematode identification at the desired taxonomic level.

The ribosomal DNA cistron is extensively used for the identification of organisms and for the establishment of phylogenetic relationships. It consists of conserved and more variable regions. This gene includes three coding regions: the Small Subunit gene (SSU, 1700 bp), the 5.8S gene (150 bp) and the Large Subunit gene (LSU, 3400 bp). In between there are two non-coding regions called internal transcribed spacer ITS-1 and ITS-2. ITS regions are relatively variable as they are noncoding and among nematodes the intra-specific variation can be considerable. Hence, for detection purposes thorough insight in ITS sequence variation among populations is a prerequisite. For the identification of nematodes (in fact any organism) at any taxonomic level, a low intra-taxon variability should be combined with a relatively high inter-taxon variation. Therefore, tests for the detection of plant-parasitic nematode species are increasingly based on coding regions within the nuclear or mitochondrial genome. For *Ditylenchus* species detection, the SSU rDNA region was chosen, as this gene harbours sufficient informative nucleotide positions for qPCR detection.

Annex 1 of this validation report provides more extensive scientific background information on ClearDetections' nematode diagnostic qPCR assay developments.

Nematode species *Ditylenchus dipsaci* and *D. destructor*

The common name of the stem nematode *Ditylenchus dipsaci* is derived from teasel (*Dipsacus* spp.) – the plant species from which the first specimen of this nematode was described. A typical characteristic of *D. dipsaci* is its low damage threshold density and its desiccation tolerance. The latter characteristic allows for survival in the field for years in absence of a host plant. The common name of the potato rot nematode *Ditylenchus destructor* is slightly misleading as this species has been reported from over 70 crops (including potato) and weeds. A peculiarity of *D. destructor* is its interaction with (soil) fungi; 'rot' local softening of plant tissue is for the better part the result of secondary infections by soil fungi and bacteria.

D. dipsaci and *D. destructor* are categorised as EPPO A2 organisms (EU Annex designation II/A2) (2008 PM7/87(1) OEPP/EPPO Bulletin 38, 363-373).

Validation of qPCR tests detecting *D. dipsaci* and *D. destructor*

Primer design and assay development

An alignment of 60 SSU rDNA sequences from 7 different known *Ditylenchus* species (*D. dipsaci*, *D. destructor*, *D. angustus*, *D. adasi*, *D. ferepolitor*, *D. gigas* and *D. drepanocercus*) and 9 SSU rDNA sequences of unknown *Ditylenchus* species ('*Ditylenchus* sp.') was used to identify species-specific sequence motives for *D. dipsaci* and *D. destructor*. Subsequently, species-specific SSU rDNA-based primers were designed to identify and detect *D. dipsaci* and *D. destructor*. Primer combinations were tested using cloned SSU rDNA fragments. Clones of nematode species (voucher specimens) are kept at the Laboratory of Nematology (Wageningen University, Wageningen, The Netherlands). Bacterial clones harbouring a TOPO TA vector with a SSU rDNA fragment of interest were grown in 2ml of LB medium (+100 µg/ml of ampicillin) at 37°C. Plasmid extraction was performed using the Wizard Plus Minipreps DNA Purification System (Promega, WI, USA). DNA concentrations were measured with a NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA) and adjusted to 10 ng/µl. For qPCR application 5 µl of plasmid template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Test specifications / scope

The two diagnostic qPCR assays for *D. dipsaci* and *D. destructor* are species-specific, simplex qPCR tests, based on SYBR Green dye detection, with equal amplification protocols (see Annex 2, figure 1 and 2, for qPCR output graphs). The two qPCR tests can be combined in one single 96-well plate in one individual PCR run. The target region of both tests is the 18S (SSU) rDNA gene.

Intended use of the two qPCR tests is:

- ✓ species-specific qualitative detection of DNA from *D. dipsaci* and/or *D. destructor* in DNA extracts originating from nematode suspensions isolated from 100 ml soil samples (= highly complex DNA background).

✓ species-specific identification of DNA from *D. dipsaci* and/or *D. destructor* originating from individual nematodes.

Validation of the DNA extraction method for DNA extraction from individual nematodes or nematode suspensions is not included in this validation report.

Materials

Performance characteristics were determined for two primer combinations:

- *D. dipsaci:* 'Ddip forward' (CD30) and 'Ddip reverse' (CD31/CD32 combination)
- *D. destructor:* 'Ddes forward' (CD60) and 'Ddes reverse' (CD61/CD62 combination)

The two simplex qPCR primer sets are part of an all-inclusive kit for the identification and detection of *D. dipsaci* and/or *D. destructor*. This kit includes primers, positive DNA controls, DNase/RNase free DNA dilution buffer, SYBR Green mix, PCR enhancer and a bench side protocol. For information, please see www.cleardetections.com or contact ClearDetections at info@cleardetections.com.

Experimental validation

This validation report describes the validation of two diagnostic qPCR assays (='methods') for the identification and detection of *D. dipsaci* and *D. destructor*. The documents 'National guideline for validation of detection and identification methods for plant pathogens and pest organisms' (NRL, March 2010) and the EPPO document 'Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity' (EPPO, 2010) were used as guidelines.

The following performance characteristics were determined: Accuracy, diagnostic sensitivity, diagnostic specificity, dynamic range, analytical sensitivity (= detection limit), analytical specificity, selectivity, robustness, reproducibility and repeatability.

Microscopic identification and detection of *D. dipsaci* and *D. destructor* was regarded as the reference method.

Performance characteristics

Accuracy

'The ability of a method to do what it claims to do. In other words: the capacity of the method to detect the target organism within a certain matrix.'

Method:

The ability of the method to accurately detect DNA from *D. dipsaci* and *D. destructor* in DNA extracts from nematode suspensions (obtained from soil) was evaluated.

Ditylenchus-free nematode suspensions obtained from 16 soil samples (100 ml, isolated with Oostenbrink elutriator) were spiked with variable numbers of *D. dipsaci* or *D. destructor* nematodes (Annex 2, table 1). Target nematode species were identified microscopically and originated from The Netherlands and Germany respectively. Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). DNA extracts were split in three separate samples and analysed separately.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Results:

Original Cq values are presented in Annex 2, table 1. Final analysis results are presented in table 1 below.

Table 1: Analysis results of 3 x 16 DNA extracts from spiked nematode suspensions. qPCR tests were carried out to detect *D. dipsaci* and *D. destructor* respectively.

Ditylenchus species	Primer combination	No. of samples correctly diagnosed	No. of samples incorrectly diagnosed	% of samples correctly diagnosed
D. dipsaci	CD30 & CD31/CD32	48	0	100%
D. destructor	CD60 & CD61/CD62	48	0	100%

Conclusion:

Correct detection of DNA originating from *D. dipsaci* and *D. destructor* in a complex DNA background is demonstrated in 3 x 16 DNA extracts from spiked nematode suspensions. The accuracy of the qPCR tests to detect DNA from *D. dipsaci* as well as *D. destructor* (within DNA from a 'natural' soil nematode suspension) is found to be 100%.

Diagnostic sensitivity

'Proportion of infected/infested samples testing positive compared to results from alternative test (or combination of tests). Diagnostic sensitivity = true positives/(true positives + false negatives).'

Method:

Ditylenchus-free nematode suspensions obtained from 2 x 12 soil samples (100 ml, isolated with Oostenbrink elutriator) were spiked with variable numbers of *D. dipsaci* or *D. destructor* nematodes (Annex 2, table 1; samples 1 - 8 and 13 - 20). For high numbers of target nematodes relevant field samples were selected (Annex 2, table 1; samples 9 – 12 and 20 – 24). Target nematode species were identified microscopically and originated from The Netherlands and Germany respectively. Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). DNA extracts were split in three separate samples and analysed separately. The number of false negative signals of both tests was recorded.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Results:

Original Cq values are presented in Annex 2, table 1. Final analysis results are presented in table 2 below. The number of false negative signals of both tests was recorded.

Table 2: Analysis results of 2 x 3 x 12 DNA extracts from spiked nematode suspensions. qPCR tests were carried out to detect *D. dipsaci* and *D. destructor* respectively.

Ditylenchus species	Primer combination	No. of positive samples	No. of false negatives	Diagnostic sensitivity
D. dipsaci	CD30 & CD31/CD32	36	0	100%
D. destructor	CD60 & CD61/CD62	36	0	100%

Conclusion:

No false negatives were recorded. The diagnostic sensitivity of both primer combinations (for *D. dipsaci* and *D. destructor*) is therefore 100%.

Diagnostic specificity

'Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from alternative test (or combination of tests). Diagnostic specificity = true negatives/(true negatives + false positives).'

Method:

Ditylenchus-free nematode suspensions obtained from 2 x 12 soil samples (100 ml, isolated with Oostenbrink elutriator) were spiked with variable numbers of *D. dipsaci* or *D. destructor* nematodes (Annex 2, table 1: samples 1 - 8 and 13 - 20). For high numbers of target nematodes relevant field samples were selected (Annex 2, table 1; samples 9 – 12 and 20 – 24). Target nematode species were identified microscopically and originated from The Netherlands and Germany respectively. Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). DNA extracts were split in three separate samples and analysed separately. The number of false positive signals of both tests was recorded.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Results:

Original Cq values are presented in Annex 2, table 1. Final analysis results are presented in table 3 below. The number of false positive signals of both tests was recorded.

Table 3: Analysis results of 2 x 3 x 12 DNA extracts from spiked nematode suspensions. qPCR tests were carried out to detect *D. dipsaci* and *D. destructor* respectively.

Ditylenchus species	Primer combination	No. of negative samples	No. of false positives	Diagnostic specificity
D. dipsaci	CD30 & CD31/CD32	36	0	100%
D. destructor	CD60 & CD61/CD62	36	0	100%

Conclusion:

No false negatives were recorded. The diagnostic specificity of both primer combinations (for *D. dipsaci* and *D. destructor*) is therefore 100%.

Range

'Area between upper limit and lower detection limit where a reliable analysis is applicable.'

Method:

The dynamic range of the detection tests was established by determining detected copy numbers, using dilution series of target plasmid DNA's (*D. dipsaci*, clone no. 1640; *D. destructor*, clone no. 1907). The tested amount of plasmid DNA ranged from 500 pg/qPCR reaction to 0,05 ag/qPCR reaction (see Annex 2, table 2).

For qPCR application 5 µl plasmid template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

For the calculation of the number of target DNA copies the following formula was used:

$$\text{Gene copy no.} = \frac{\text{(Amount of DNA/qPCR)}}{\text{(Molecular mass of target plasmid DNA)}} \times \text{(Avogadro constant)}$$

Molecular mass of target plasmid DNA = Size of plasmid DNA x Molecular mass of one dsNucleotide

Molecular mass of target plasmid DNA: 5.0kb x 660 g/mol ~ $3.3 * 10^6$ g/mol

Avogadro Constant: Number of molecules/mol = 6.022×10^{23}

Results:

Original Cq values are presented in Annex 2, table 2. Final calculation results are presented in table 4 below.

Table 4: Determination of upper and lower detection limit for detection of *D. dipsaci* and *D. destructor* DNA, using dilution series of target plasmid DNA (ranging from 500 pg/qPCR reaction to 0,05 ag/qPCR reaction).

Ditylenchus species	Primer combination	Upper detection limit (gram)	Lower detection limit (gram)	Upper detection limit (copies)	Lower detection limit (copies)
D. dipsaci	CD30 & CD31/CD32	500 pg/qPCR	10 ag/qPCR	$9.1 * 10^7$	9.1
D. destructor	CD60 & CD61/CD62	500 pg/qPCR	10 ag/qPCR	$9.1 * 10^7$	9.1

Conclusion:

The upper limit of detection for both qPCR tests lies around 0.1 billion copies of target DNA. The lower limit of detection for both qPCR tests lies somewhere between 10 and 100 copies of target DNA. The area between the upper limit and lower detection limit where a reliable analysis is applicable lies between 10-100 and 0.1 billion copies of target DNA (SSU rDNA).

Analytical sensitivity (or detection limit)

'Lowest concentration of a target organism which can be detected reliably in a laboratory sample.'

Method:

To determine the analytical sensitivity of the qPCR tests for detection of *D. dipsaci* and *D. destructor*, dilution series of target (genomic) DNA against complex backgrounds of non-target genomic DNA were tested.

Nematode suspensions obtained from soil (containing no target nematodes) were spiked with 10 individual target nematodes. Target nematode species were identified microscopically and originated from The Netherlands and Germany respectively.

Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). Dilution series were made (20x, 80x, 320x, 1,280x, 5,120x, 20,480x) and qPCR was performed.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 °C for 10 sec, 63 °C for 1 min and 72 °C for 30 sec.

According to the Dutch NRL guideline (literature list) the detection limit is described as:

Detection limit = Mean of lowest detectable concentration + 3x standard deviation

In this experiment however, measurement of absolute DNA concentrations is difficult because the target DNA is mixed with background non-target nematode DNA. For this reason the ‘lowest detectable part of one added organism’ was chosen as detection limit instead of the ‘lowest DNA concentration’.

Detection limit = Mean of lowest ‘detectable part of one *Ditylenchus*’ + 3x standard deviation

Calculation of ‘detectable part of one *Ditylenchus’*:

$$= \frac{\text{(Volume DNA added to qPCR reaction)}}{\text{(Lysis volume)} \times \text{(Dilution during DNA purification)} \times \text{(Dilution after DNA purification)}}$$

Calculation example (for *Ditylenchus* samples):

$$= \frac{\text{(Volume DNA added to qPCR reaction: 5 µl)}}{\text{(Lysis volume: 205 µl)} \times \text{(Dilution during DNA purification: 200 µl/45 µl)} \times \text{(Dilution after DNA purification: 5x)}}$$

Follows: ‘detectable part of one *Ditylenchus’ =* $5*10/ (205* 4.44 * 5) = 1.10 * 10^{-3}$

Results:

Original Cq values are presented in Annex 2, table 1. Final detection results are presented in table 5 below.

Table 5. Determination of detection limits for *D. dipsaci* and *D. destructor* qPCR tests. Samples 1-6 were analysed with primer combination CD30 & CD31/CD32; Samples 13-18 were analysed with primer combination CD60 & CD61/CD62.

Dilution:		20x	80x	320x	1,280x	5,120x	20,480x	Detection limit: 'part of one target nematode'
Part of one target nematode:		$2.74*10^{-3}$	$6.86*10^{-4}$	$1.71*10^{-4}$	$4.29*10^{-5}$	$1.07*10^{-5}$	$2.68*10^{-6}$	
1	*D. dipsaci 1*	+	+	+	+	+	-	$5.7 * 10^{-5}$
2	*D. dipsaci 2*	+	+	+	+	+	+	
3	*D. dipsaci 3*	+	+	+	+	-	+	
4	*D. dipsaci 4*	+	+	+	+	+	-	
5	*D. dipsaci 5*	+	+	+	+	+	-	
6	*D. dipsaci 6*	+	+	+	+	+	-	
13	*D. destructor 1*	+	+	+	-	-	-	$2.9 * 10^{-4}$
14	*D. destructor 2*	+	+	+	+	-	-	
15	*D. destructor 3*	+	+	+	+	+	-	
16	*D. destructor 4*	+	+	+	+	+	-	
17	*D. destructor 5*	+	+	+	-	-	-	
18	*D. destructor 6*	+	+	+	+	-	-	

Conclusion:

The detection limit of primer combination CD30 & CD31/CD32 is $5.7 * 10^{-5}$ 'part of one *D. dipsaci* nematode'. The detection limit of primer combination CD60 & CD61/CD62 is $2.9 * 10^{-4}$ 'part of one *D. destructor* nematode'.

The tested samples contain (genomic) target DNA of 10 individual nematodes. Diluted 50 times, these samples contain the same amount of target DNA as a sample from one individual target at a regular 5 times dilution. This experiment demonstrates that this method detects one individual target nematode (*D. dipsaci* or *D. destructor*) against a DNA background of thousands of non-target nematodes.

Analytical specificity

'The ability of a detection method to distinguish the target organism from (related) other organisms and the degree in which the method can distinguish known variants of the organism.'

Method:

Here specificity is described as ability of the detection test to distinguish *D. dipsaci* or *D. destructor* from other nematode species that may be present in a soil sample.

Specificity of the primer sets was tested with plasmid DNA (SSU rDNA) and genomic DNA of relevant non-target nematodes species. Possible false positives were selected *in silico*, based on sequence identity, and based on phylogenetic analysis (close relatives). Unfortunately, only a few *Ditylenchus* species could be included in this experiment, as we had no DNA available of other *Ditylenchus* species.

For qPCR application 5 µl of 5x diluted (or plasmid) template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 °C for 10 sec, 63 °C for 1 min and 72 °C for 30 sec.

Results:

Results of the specificity test are presented in table 6.

Table 6: Specificity test results for two qPCR tests detecting *D. dipsaci* and *D. destructor* respectively.

species tested	clone no.	origin	qPCR test result*	
			D. dipsaci	*D. destructor*
Ditylenchus dipsaci	2492	The Netherlands	+	-
Ditylenchus dipsaci	genomic	France (ANSES)	+	-
Ditylenchus dipsaci	genomic	United Kingdom (ADAS)	+	-
Ditylenchus dipsaci	1640	Canada	+	-
Ditylenchus dipsaci	943	unknown	+	-
Ditylenchus destructor	1906	The Netherlands	-	+
Ditylenchus destructor	P299	The Netherlands	-	+
Ditylenchus destructor	P301	The Netherlands	-	+
Ditylenchus destructor	genomic	France (ANSES)	-	+
Ditylenchus destructor	genomic	Germany	-	+
Ditylenchus ferepolitor	2052	from wood'	-	-
Ditylenchus adasi	1143	The Netherlands (nVWA)	-	-
Ditylenchus adasi	2050	The Netherlands (PPO)	-	-
Anguina tritici	710		-	N/T
Anguina tritici	2518		-	N/T
Subanguina radicicola	1211		-	N/T
Paraphanolaimus behningi	2150		-	N/T
Psilenchus hilarulus	2318		-	N/T
Eumonhystera sp	2109		-	N/T
Cephalenchus hexalineatus	660		-	N/T
Metateratocephalus crassidens	75		N/T	-
Aulolaimus oxycephalus	161		N/T	-
Clarkus papillatus	267		N/T	-
Monoposthia sp	1361		N/T	-
Mylonchulus sigmaturus	171		N/T	-
Wisonema otophorum	709		N/T	-
Meloidogyne javanica	1108		N/T	-
Seinura tenuicaudata	1718		N/T	-

* Cut-off value at Cq > 35. In all positive cases melting curves were found to be correct (N/T = not tested).

Conclusion:

Several target and non-target species (from different origins) were tested and no cross reactions were noted for the *D. dipsaci* qPCR test as well as the *D. destructor* qPCR test.

Selectivity

'The ability of a detection method to distinguish the target organism from other components in a sample.'

Method:

Here selectivity is described as ability of the detection tests to distinguish *D. dipsaci* and *D. destructor* from thousands of other nematodes (consisting of many different nematode species) present in a soil sample.

Ditylenchus-free nematode suspensions obtained from 6 soil samples (100 ml, isolated with Oostenbrink elutriator) were spiked with 2, 2 and 5 individuals of *D. dipsaci* and 2, 3 and 4 individuals of *D. destructor* (Annex 2, table 1). Target nematode species were identified microscopically and originated from The Netherlands and Germany respectively. Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). DNA extracts were split in two separate samples and analysed separately.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Results:

Original Cq values are presented in Annex 2, table 1. Final selectivity results are presented in table 7 below.

Table 7: Results of analysing 3 x 2 different DNA extracts for determining the selectivity of the molecular detection of *D. dipsaci* and *D. destructor.*

Ditylenchus species	Primer combination	No. of positive samples	No. of false negatives	Selectivity
D. dipsaci	CD30 & CD31/CD32	6	0	100%
D. destructor	CD60 & CD61/CD62	6	0	100%

Conclusion:

No false negatives were recorded. The selectivity of both primer combinations (for *D. dipsaci* and *D. destructor*) is therefore 100%. This experiment demonstrates that DNA from small numbers of *D. dipsaci* or *D. destructor* nematodes is detected against a complex DNA background (from thousands of soil-derived non-target nematodes).

Robustness

'The degree of insensitivity of the measurement result for deviations in implementation, circumstances and quality of materials, which can occur in practice.'

Method:

The two qPCR tests for identification of *D. dipsaci* and *D. destructor* are provided to end-users as all-inclusive identification and detection kit, including the SYBR Green mix to be used with the primer sets and a bench-side protocol describing the laboratory procedure. In practice, only the PCR equipment may vary, depending on the end-user.

Like any other primer set, the robustness of the designed primer sets for identification and detection of *D. dipsaci* and *D. destructor* depends on their sensitivity to possible variations in annealing temperature (Ta). Manufacturers of PCR equipment usually assume a possible temperature variation around the set Ta of + or – 0.5 ºC. This variation in Ta may possibly (negative) influence the performance of the primer set. In order to test the sensitivity of the *D. dipsaci* and *D. destructor* primer pairs for (unintended) variations in Ta, the primers were exposed (in the presence of the correct target DNA: *D. dipsaci*, clone no. 1640; *D. destructor*, clone no. 1907) to a temperature gradient around the optimal Ta (at least 1 degree higher and 1 degree lower than Ta, here 63 ^{o}C).

For qPCR application 5 µl plasmid template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 ^{o}C for 10 sec, 61,4 – 65,5 ^{o}C for 1 min and 72 ^{o}C for 30 sec.

Results:

Original Cq values of a temperature gradient for both primer pairs are presented in table 8.

Table 8: qPCR test results (Cq values) of temperature gradient for two qPCR tests detecting *D. dipsaci* and *D. destructor* respectively.

Gradient Ta (ºC)	Cq values of CD30 & CD31/32 on *D.dipsaci* (clone 1640)	Cq values of CD60 & CD61/62 on *D.destructor* (clone 1907)
65,5	22.07	19,40
65,3	21.31	18,48
64,8	20.08	17,32
64,0	19.73	16,27
63,0	**19.35**	**15,78**
62,2	19.36	15,51
61,7	19.39	15,62
61,4	19.64	15,94

Conclusion:

No qPCR failure is observed when primer combinations CD30 & CD31/CD32 and CD60 & CD61/CD62 are exposed to a temperature gradient. With a deviation in Ta of (plus or minus) 1.0 ^{o}C from the normal Ta (63 ^{o}C), all ΔCt values remain < 1. The qPCR tests for the detection of *D. dipsaci* and *D. destructor* are robust.

Reproducibility and repeatability

<u>Reproducibility</u>: *'The degree of agreement between the results of two consecutive measurements on same specimens conducted under the same measuring conditions. To determine the repeatability, measurements should be carried out to a laboratory sample, by the same performer with the same measuring equipment and within the smallest possible time interval.'*
<u>Repeatability</u>: *'The degree of agreement between the results of measurements of the same specimens conducted under different measuring conditions. Measuring conditions are: time, equipment, instruments, and performer. For determining the reproducibility analysis will be performed on multiple time moments and preferably on multiple devices and by several performers.'*

Method:

The performance of both qPCR test was tested under repeatability and reproducibility conditions. For this experiment, 16 *Ditylenchus*-free nematode suspensions were spiked with *D. dipsaci* or *D. destructor* nematodes and 8 extra actual field samples were chosen to represent samples with higher number of nematodes (Annex 2, table 1). Subsequently, nematode DNA was extracted (Nematode DNA extraction and purification kit, ClearDetections). DNA extracts were split into one repeatability sample and two reproducibility samples.

For qPCR application 5 µl of 5x diluted template was mixed with 2 µl species-specific primers (end concentrations for both primers 250 nM), 3 µl PCR enhancer (ClearDetections), and 10 µl qPCR SYBR Green mix (ClearDetections) in a total reaction volume of 20 µl. Thermal cycling was performed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA) and consisted of 95°C for 3 min; followed by 40 cycles of 95 °C for 10 sec, 63 °C for 1 min and 72 °C for 30 sec.

The positive signals of the qPCR analyses performed were collected to calculate the statistical characteristics using the guidelines from the document 'National guideline for validation of detection and identification methods for plant pathogens and pest organisms' (NRL, March 2010). The tests were carried out as shown in Annex 2, table 3 (analysis scheme).

Results:

Original Cq values are presented in Annex 2, table 1. Final reproducibility and repeatability results are presented in table 9 and 10 respectively.

Table 9: Final reproducibility results after analysis of 3 x 12 different DNA extracts for the detection of *D. dipsaci* and *D. destructor.*

Ditylenchus species	Primer combination	Total no. of samples	No. of false negatives	Reproducibility
D. dipsaci	CD30 & CD31/CD32	36	0	100%
D. destructor	CD60 & CD61/CD62	36	0	100%

Table 10: Final repeatability results after analysis of 2 x 12 different DNA extracts for the detection of *D. dipsaci* and *D. destructor.*

Ditylenchus species	Primer combination	Total no. of samples	No. of false negatives	Repeatability
D. dipsaci	CD30 & CD31/CD32	24	0	100%
D. destructor	CD60 & CD61/CD62	24	0	100%

Conclusion:

Reproducibility is 100% for both primer combinations (detecting *D. dipsaci* and *D. destructor*).

Repeatability is 100% for both primer combinations (detecting *D. dipsaci* and *D. destructor*).

Literature

- 'National guideline for validation of detection and identification methods for plant pathogens and pest organisms' (Dutch National Reference Laboratory, March 2010)
- EPPO document PM7/98 Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity, 2010 OEPP/EPPO Bulletin 40, 5-22
- Diagnostic Standard PM7/87(1) *Ditylenchus destructor* and *Ditylenchus dipsaci*, 2008 OEPP/EPPO Bulletin 38, 363-373
- Rybarczyk-Mydłowska, K. *et al.* (2012) SSU rDNA-based phylogenetic analysis of foliar nematodes (*Aphelenchoides* spp.) and their quantitative detection in complex DNA backgrounds. Phytopathology 102 (12): 1153-1160.
- Van Megen, H. *et al.* (2009) A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. Nematology 11: 927-950.
- Holterman, M. *et al.* (2008) A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats Mol Ecol Resour. 8 (1): 23-34.
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Annex 1: Scientific background information

Build-up of nematode rDNA sequence database

The Laboratory of Nematology (Wageningen University, The Netherlands) constructed a rDNA sequence database of over 2.800 nematode species (Holterman *et al*., 2006, 2008; Van Megen et al. 2009). Nematodes were collected from various habitats throughout The Netherlands or obtained from the Dutch Plant Protection Service (Prof. dr. Gerrit Karssen). Nematodes were extracted from soil using standard techniques and identified under a light microscope. Prior to DNA extraction, pictures were taken using a CCD camera (CoolSnap, RS Photometrics, Tucson AZ). SSU and LSU rDNA sequences from collected species were supplemented with sequences taken from GenBank. Clones of each species (voucher specimens) are kept at the Laboratory of Nematology (Wageningen University, Wageningen, The Netherlands). Information can be obtained from Dr. Hans Helder (e-mail: hans.helder@wur.nl).

rDNA sequencing method (as described in Holterman *et al*., 2008)

Single nematodes were transferred to 0.2 ml PCR tube containing 25 µl sterile water. An equal volume of lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% (v/v) β-mercaptoethanol and 800 µg/ml proteinase K was added. Lysis took place in a Thermomixer (Eppendorf, Hamburg, Germany) at 65° C and 750 r.p.m. for 2 hours followed by a 5 minute incubation at 100° C. Lysate was immediately used or stored at -20° C. SSU rDNA was amplified as two partially overlapping fragments using three universal and one nematode-specific primer (1912R). For the first SSU fragment, either the primer 988F (5'-ctcaaagattaagccatgc-3') or the primer 1096F (5'-ggtaattctggagctaatac-3') was used in combination with the primer 1912R (5'-tttacggtcagaactaggg-3'). The second SSU fragment was amplified with primers 1813F (5'-ctgcgtgagaggtgaaat-3') and 2646R (5'-gctaccttgttacgacttttt-3'). LSU rDNA (5'end includingD—D3 region (≈ 1,000 bp)) was amplified as two partially overlapping fragments using either primer 28–61for or 28–81for (28–61for, 5'-gtcgtgattacccgctgaactta-3'; 28–81for, 5'-ttaagcatatcatttagcggaggaa-3') in combination with either primer 28–1006rev or 28–1032rev (28–1006rev, 5'-gttcgattagtctttcgcccct-3'; 28–1032rev, 5'-tcggaaggaaccagctacta-3'). PCR was performed in a final volume of 25 µl containing 3 µl of 100x diluted crude DNA extract, 0.1 µM of each PCR primer and a 'Ready-to-go' PCR bead (GE Healthcare, Little Chalfont, UK). The

following PCR program was used: 94° C for 5 min; 5 x (94° C, 30 s; 45° C, 30s; 72° C, 70s); 35 x (94° C, 30 s; 54° C, 30s; 72° C, 70s); 72° C for 5 min. Gel-purified (Marligen) amplification products (SSU and LSU rDNA fragments) were cloned into a TOPO TA vector (Invitrogen) and sequenced using standard procedures.

Sequence alignment and phylogenetic analysis

The sequences were aligned using the ClustalW algorithm as implemented in the program BioEdit 7.0.1 (Hall, 1999). The alignment was then manually corrected using arthropod secondary structure information in accordance with Ben Ali *et al.* (1999) (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/index.html).

Annex 2: Analysis data

Figure 1: Graphic representation of qPCR output of *D. dipsaci* qPCR test (amplification curve and melting peak curve with Tm = 85,5 °C). Test was performed on a Biorad CFX Connect thermal cycler with protocol and materials from the all-inclusive kit for the identification and detection of *D. dipsaci* and/or *D. destructor* (ClearDetections).

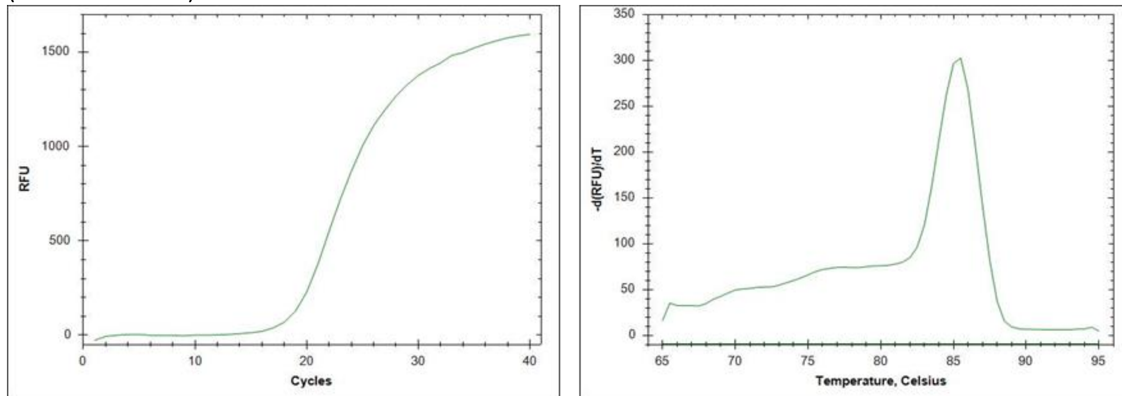

Figure 2: Graphic representation of qPCR output of *D. destructor* qPCR test (amplification curve and melting peak curve with Tm = 86,0 °C). Test was performed on a Biorad CFX Connect thermal cycler with protocol and materials from the all-inclusive kit for the identification and detection of *D. dipsaci* and/or *D. destructor* (ClearDetections).

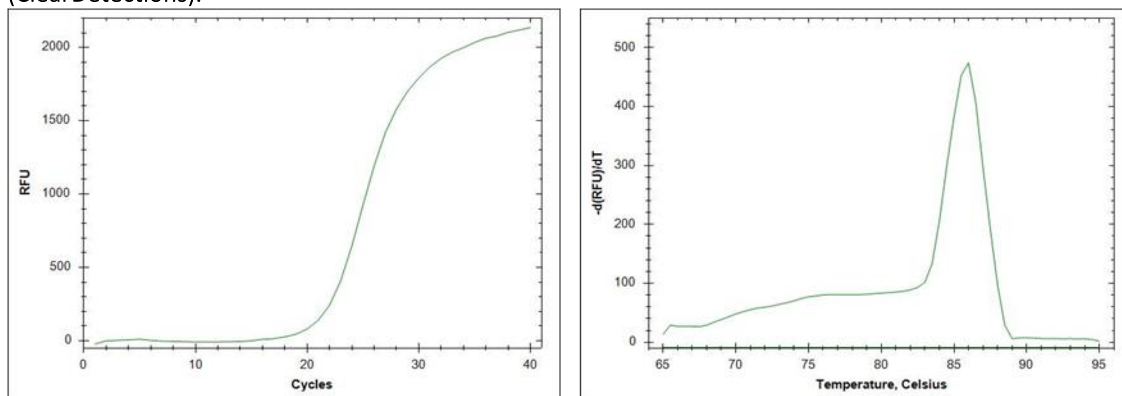

Table 1. Molecular analyses (Cq values) of 3 x 2 x 12 different DNA extracts of spiked (soil) nematode suspensions. Analyses results were used to determine accuracy, diagnostic sensitivity, diagnostic specificity, reproducibility, repeatability and selectivity of two simplex qPCR tests for identification and detection of resp. *D. dipsaci* and *D. destructor*.

DNA extracts of spiked (soil) nematode suspensions used for:			Accuracy, diag. sens., diag spec., reproducibility, selectivity				Accuracy, diag. sens., diag spec., reproducibility, selectivity			
					Repeatability				Repeatability	
	Target species	No. spiked individuals	*D. dipsaci* (Cq values* CD30 & CD31/CD32)				*D. destructor* (Cq values* CD60 & CD61/CD62)			
			1	2	3	4	1	2	3	4
1	*D.dipsaci*	2	28.09	28,39	28,69	28,14	N/D	N/D	N/D	
2	*D.dipsaci*	2	28.25	27,57	27,95	28,00	N/D	N/D	N/D	
3	*D.dipsaci*	5	26.47	26,45	25,86	26,19	N/D	N/D	N/D	
4	*D.dipsaci*	5	26.25	26,58	26,59	26,60	N/D	N/D	N/D	
5	*D.dipsaci*	10	25.80	25,64	25,59	25,50	N/D	N/D	N/D	
6	*D.dipsaci*	10	25.28	25,60	25,80	25,49	N/D	N/D	N/D	
7	*D.dipsaci*	20	24.44	24,95	24,92	24,67	N/D	N/D	N/D	
8	*D.dipsaci*	40	23.32	23,38	23,34	23,25	N/D	N/D	N/D	
9	*D.dipsaci*	~80	24.19	24,18	24,20	24,19	N/D	N/D	N/D	
10	*D.dipsaci*	~110	22.54	22,88	22,79	22,82	N/D	N/D	N/D	
11	*D.dipsaci*	~250	22.13	22,27	22,34	22,32	N/D	N/D	N/D	
12	*D.dipsaci*	~430	22.03	22,26	22,21	22,18	N/D	N/D	N/D	
13	*D.destructor*	2	N/D	N/D	N/D		31,17	31,04	30,27	30,02
14	*D.destructor*	3	N/D	N/D	N/D		29,52	30,42	29,52	29,35
15	*D.destructor*	4	N/D	N/D	N/D		31,98	33,77	31,14	32,19
16	*D.destructor*	5	N/D	N/D	N/D		28,62	28,29	28,30	28,30
17	*D.destructor*	8	N/D	N/D	N/D		25,70	25,85	25,89	25,60
18	*D.destructor*	10	N/D	N/D	N/D		26,93	26,84	26,82	26,50
19	*D.destructor*	20	N/D	N/D	N/D		24,60	24,71	24,71	24,50
20	*D.destructor*	50	N/D	N/D	N/D		23,31	23,57	24,02	23,83
21	*D.destructor*	~60	N/D	N/D	N/D		23,84	23,72	23,91	23,84
22	*D.destructor*	~100	N/D	N/D	N/D		24,15	23,86	24,03	24,09
23	*D.destructor*	~200	N/D	N/D	N/D		22,38	22,19	22,19	22,37
24	*D.destructor*	~300	N/D	N/D	N/D		22,49	22,10	22,14	22,27

* Cut-off value at Cq > 35. In all positive cases melting curves were found to be correct (N/D = not detected).

Table 2: Determination of upper and lower detection limit (range) for detection of *D. dipsaci* and *D. destructor* DNA, using dilution series of plasmid DNA.

	Plasmid DNA concentration	Cq values of CD30 & CD31/32 on *D. dipsaci* (clone 1640)	Cq values of CD60 & CD61/62 on *D. destructor* (clone 1907)
1	100 pg/µl	13.36	11.87
2	10 pg/µl	16.50	15.04
3	1 pg/µl	19.63	18.20
4	100 fg/µl	23.06	21.69
5	10 fg/µl	26.64	22.25
6	1 fg/µl	29.50	26.23
7	100 ag/µl	30.71	30.32
8	10 ag/µl	35.13	35.17
9	1 ag/µl	N/D	N/D
10	0.1 ag/µl	N/D	N/D
11	water	N/D	N/D
		N/D = not detected	

Table 3: Analysis scheme of qPCR tests carried out for the underlying validation report.

Samples	qPCR test	Performance characteristics	Technician 1				Technician 2			
			Test event				Test event			
			1	2	3	4	1	2	3	4
24 Ditylenchus samples	*D.dip., D.des.*	Accur., diag. sens., diag. spec., reprod., select.	x							x
12 positive Ditylenchus samples	*D.dip., D.des.*	Repeatability			xx					
Plasmid DNA dilution series	*D.dip., D.des.*	Range	x							
6-fold Nematode DNA dilution series	*D.dip., D.des.*	Analytical sensitivity		x						
Target + Non target Plasmid DNA	*D.dip., D.des.*	Specificity			x					
qPCR gradient	*D.dip., D.des.*	Robustness				x				

ClearDetections BV
Binnenhaven 5
6709PD Wageningen
The Netherlands
info@cleardetections.com
www.cleardetections.com