Russian Journal of Nematology, 2018, 26 (2), 97 – 107

# Simultaneous detection of three plant-parasitic nematode species from the genus *Ditylenchus* using a real-time qPCR matrix-based technology

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Accepted for publication 9 October 2018

**Summary.** Plant-parasitic nematodes of the genus *Ditylenchus* infect a wide range of plants causing serious economic losses in the temperate climatic zones. Among them, two species, *Ditylenchus destructor* and *D. dipsaci*, able to infect potato, are the most destructive and are included in the lists of quarantine and regulated pests in many countries. A proper diagnostics of these species is very relevant for timely and reliable evaluation of the quality of seed potato and the phytosanitary state of potato fields. Based on ITS1-5.8S-ITS2-28S rDNA sequences, test systems for detection of these two species and also for *D. weischeri*, a confounding species, were developed suitable for the use in complex qPCR micromatrices (microarrays) under a standardised real-time PCR protocol. The test systems demonstrated high specificity and reproducibility and satisfactory analytical sensitivity (5 juveniles for *D. destructor* and *D. dipsaci* and 50 juveniles for *D. weischeri*) suitable for practical use. The detection limit was 8-10 pg of DNA for *D. destructor* and *D. dipsaci* and 77 pg of DNA for *D. weischeri*. Working efficiency of the developed test systems after their freeze-drying on preserved silicon micromatrices was not significantly reduced. Due to the simplicity of the use of qPCR micromatrices, flexibility of their structure, and reduced time of analysis, the developed test systems may be recommended for the use in field laboratories including possible cases of combined infection, when the presence of harmful *Ditylenchus* species is masked by symptoms caused by other pathogens and may be overlooked.

**Key words:** *Ditylenchus destructor*, *Ditylenchus dipsaci*, *Ditylenchus weischeri*, qPCR micromatrices, potato pathogens, plant disease diagnostics.

According to the FAO data, potato (*Solanum tuberosum* L.) is the fifth staple food crop in a global context after sugar cane, maize, rice and wheat (FAO Statistical Pocketbook, 2015). This crop may be infected by multiple pathogens resulting in significant yield losses. Tuber rot caused by different pathogens or parasites, such as fungi, bacteria and nematodes, often have similar symptoms. In some cases, primary infection facilitates the subsequent infestation by secondary pathogens or even nonpathogenic microorganisms. For example, among various nematode species, which may inhabit rotten potato tubers, only 3-4% are plant-parasitic nematodes that cause real damage to these tubers (Zhilina, 2004). This group includes two economically important species, potato tuber nematode *Ditylenchus destructor* Thorne, 1945 and stem nematode *D. dipsaci* (Kühn, 1857) Filipjev, 1936. These two polyphagous species cause serious economic losses in agriculture in temperate climate zones and are regulated as quarantine pests in many countries. The host range of both nematodes includes more than 100 and 1200 plant species for *D. destructor* and *D. dipsaci*, respectively (EPPO, 2017).

*Ditylenchus destructor* attacks underground parts of potato plants (tubers, stolons and roots). It was reported to be present in the majority of European countries with registered outbreaks on ware and seed potatoes in Lithuania, Belarus and Estonia (EPPO, 2005; Ilyashenka & Ivaniuk, 2008; Švilponis *et al.*,



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2008; Kruus, 2012). In Russia *D. destructor* is observed in the western and central regions of European Russia and also in the Caucasus and Siberia regions (Shesteperov & Butenko, 2010). This nematode may cause significant potato yield losses reaching up to 30-50% (Zhilina, 2004; Ilyashenka & Ivaniuk, 2008); in addition, storage losses of infected tubers may reach 80% (Zhilina, 2004).

*Ditylenchus dipsaci* parasitises mainly the aerial plant organs (stems and leaves), though is also able to attack tubers and rhizomes, especially in the presence of a fungus *Phoma exigua* (Mugniéry & Phillips, 2007). This nematode is also widespread in Europe and other regions, and may cause up to 60-80% yield losses of different agricultural crops including potato (Goodey, 1956; Sturhan & Brzeski, 1991). Several biological pathotypes or races have been described for *D. dipsaci sensu lato*. Further analysis revealed that it represents a species complex (Subbotin *et al.*, 2005). In 2010, a new species, *D. weischeri*, infesting *Cirsium arvense* and earlier reported as a race of *D. dipsaci*, was described in the Moscow region, Russia (Chizhov *et al.*, 2010); later it was also found in Canada (Tenuta *et al.*, 2014). Though this species is not a parasite of potato, its differentiation from *D. dipsaci* is of phytosanitary importance because of potential misidentification with *D. dipsaci*.

Traditional diagnostics of stem nematodes is based on the examination of morphological features which is a relatively easy task for well-qualified nematologists, but is a serious problem for other plant pathologists and agronomists. In the case of *Ditylenchus* including more than 80 species, only a few species are parasites of higher plants (EPPO, 2017). Morphological traits used for differentiation of these species may vary depending on the developmental stage, temperature and culture medium (Barraclough & Blackith, 1962). In addition, low-level tuber infection with *Ditylenchus* may not have any visible manifestations, yet it is able to cause significant yield and storage losses. Therefore, a sensitive and reliable method is needed to detect and differentiate *Ditylenchus* species, since correct identification to a species level is an absolute prerequisite for the implementation of successful control strategies.

DNA-based technologies are highly sensitive and independent of phenotypic variations, so their use makes it possible to overcome limitations of a morphological identification. In relation to nematodes, such methods can be used in the case of low infestation levels and are able successfully to identify damaged or atypical adults and even juveniles, for which morphological identification to species is not possible. Several conventional PCR techniques have been developed for identification of *Ditylenchus* species. Wendt *et al.* (1993) proposed the use of the ITS rDNA region in the RFLP-PCR test for distinguishing *D. destructor* and *D. dipsaci*. The ITS region was also used to develop a RFLP-PCR test to identify *D. dipsaci* and *D. weischeri* (Sturhan & Brzeski, 1991; Chizhov *et al.*, 2010; Tenuta *et al.*, 2014). SCAR markers derived from AFLP-PCR data were developed to identify *D. dipsaci* and *D. gigas* (Esquibet *et al.*, 2003). In addition, some other standard PCR assays based on 18S and ITS1 (Subbotin *et al.*, 2005), 5.8S rDNA (Marek *et al.*, 2005), or rDNA-ITS regions (Kerkoud *et al.*, 2007) were developed for *D. dipsaci* detection and identification. Species-specific primers have also been developed on the basis of the *hsp90* gene for distinguishing *D. dipsaci* and *D. weischeri* (Madani *et al.*, 2015).

The first multiplex conventional PCR assay was developed for simultaneous identification of *D. destructor*, *D. dipsaci sensu stricto* and *D. gigas* (Marek *et al.*, 2010). Though succeeding in distinguishing *D. destructor* from the other two species, authors were unable to reveal *D. gigas* in samples, co-infested with D. *dipsaci sensu stricto*. Another example of a multiplex approach is described by Jeszke *et al.* (2015). Using ITS1 region of rDNA, authors developed species-specific primers for diagnostics of *D. destructor*, *D. dipsaci* and *D. gigas*. The developed test systems provided high sensitivity (detection limit of 16 pg of DNA) and specificity and were suitable for singleplex or multiplex conventional PCR, as well as for singleplex real-time PCR. Two other real-time PCR approaches were proposed by Cheng *et al.* (2015) and the ClearDetections company (Wageningen, The Netherlands; www.cleardetections.com). In the first case, authors developed ITS-based species-specific primers for *D. destructor* detection in soil and plant samples with the detection limit of about 4 nematodes per sample. In the second case, the company developed commercial real-time PCR kits for detection of *D. destructor*, *D. dipsaci* and also a range of other plant-parasitic nematodes. The announced analytical sensitivity of these tests is about 3 cells of a target nematode (EPPO, 2017). The company does not disclose primer sequences; however, it is known they target the 18S (SSU) rDNA gene, which, together with the 28S (LSU) rDNA and mitochondrial COI genes, is used for DNA barcoding of selected regulated nematode species (EPPO, 2016).

**Table 1.** Nematode populations used in the study.

| Species | Sample code | Host | Source or sampling region |
|---|---|---|---|
| *Ditylenchus destructor* | N2 | *Solanum tuberosum* | Kaluga Region, Russia |
| *Ditylenchus destructor* | N3 | | Sverdlovsk Region, Russia |
| *Ditylenchus destructor* | N10 | | Bryansk Region, Russia |
| *Ditylenchus destructor* | N13 | | Moscow Region, Russia |
| *Ditylenchus dipsaci* | N8 | *Allium sativum* | Stavropol Region, Russia |
| *Ditylenchus dipsaci* | N9 | *Phlox paniculata* | Moscow Region, Russia |
| *Ditylenchus weischeri* | N11 | *Cirsium arvense* | Moscow Region, Russia |
| *Heterodera schachtii* M100266 | Hsch | *Beta vulgaris* | SCPPM ARRIP, Russia |
| *Heterodera medicaginis* M1300IP | Hmed | *Medicago sativa* | SCPPM ARRIP, Russia |
| *Globodera rostochiensis* Ro1 M100206 | Gros | *Solanum tuberosum* | SCPPM ARRIP, Russia |
| *Globodera pallida* Pa2 M100113 | Gpal | | Bari Unit ISPP, Italy |
| *Meloidogyne hapla* | N12 | *Daucus carota* subsp. *sativus* | Moscow Region, Russia |
| *Meloidogyne incognita* | N14 | *Apium graveolens* | Moscow Region, Russia |
| *Meloidogyne arenaria* | N15 | *Apium graveolens* | Moscow Region, Russia |
| *Caenorhabditis elegans* Bristol N2 wild-type | Cel | – | BIMLFS, Germany |
| **Field samples** | | | |
| *Ditylenchus destructor* | P11 | *Solanum tuberosum* | Ivanovo Region, Russia |
| *Ditylenchus destructor* | P16 | | Kaluga Region, Russia |
| *Ditylenchus destructor* | P17 | | Sverdlovsk Region, Russia |

Compared to morphological and protein-based methods, conventional PCR techniques have some advantages: they do not depend on life stage or environment of nematodes, are relatively rapid and reliable, provide good specificity and sensitivity, and sometimes represent the only tool of discriminating between confounding species. At the same time, some of these assays are not sensitive enough to detect low amounts of target DNA, require expensive techniques and skilled personnel, or are not suitable for multiplexing, or do not provide the possibility for routine high-throughput analysis (Braun-Kiewnick & Kiewnick, 2018).

The purpose of this study, representing a part of a complex project (Nikitin *et al.*, 2017, 2018), was the development of reliable test systems for simultaneous detection and identification of three species, *D. destructor*, *D. dipsaci* and *D. weischeri*, suitable for qPCR microarrays (micromatrices).

## MATERIAL AND METHODS

**Nematode populations.** Nematode species used in the study are listed in Table 1. Target species included four populations of *D. destructor* isolated from *Solanum tuberosum*, two populations of *D. dipsaci* isolated from *Allium sativum* and *Phlox paniculata*, and one population of *D. weischeri* isolated from *Cirsium arvense*. All populations were isolated from fresh plant material (stems or tubers) collected in various regions of Russia and identified by microscopical examination. *Meloidogyne hapla* population was collected from roots of a carrot growing on a commercial field in the Moscow Region. *Meloidogyne incognita* and *M. arenaria* populations were collected from roots of celery growing in a glasshouse (Moscow Region). Other nematode species used for the specificity assay included pure cultures of *Globodera rostochiensis*, *G. pallida*, *Caenorhabditis elegans*, *Heterodera schachtii* and *H. medicaginis* provided by the Bari Unit of the Institute for Sustainable Plant Protection (ISPP, Italy), Buchmann Institute for Molecular Life Sciences (BIMLS, Germany), and State Collection of Plant Pathogenic Microorganisms of the All-Russian Research Institute of Phytopathology (SCPPM ARRIP, Russia).

The efficiency of the developed test systems was tested using three field samples of potato tubers (‘Innovator’) with visible manifestations of *Ditylenchus*-like infection; samples were collected in Ivanovo (P11), Kaluga (P16) and Sverdlovsk (P17) regions.

All *Ditylenchus* species were isolated from infected tissues of the aforementioned plants using a modified Baermann technique (Hooper, 1986). Extracted adult

and juvenile specimens were mounted on microscope slides in water for visual identification. Morphological observations and metrics were performed using an AxioImager Z2 light microscope (Zeiss) with Nomarski differential contrast.

**DNA extraction.** DNA was extracted from nematode samples using an AmpliSens® DNA-sorb B DNA extraction kit (Russian Central Institute of Epidemiology, Russia) in accordance with the manufacturer's recommendations. Total isolated DNA concentration was determined at 260 nm using a SmartSpec Plus spectrophotometer (BioRad, USA). The obtained DNA samples were stored at –20°C until use.

**Primer design.** For each target species, species-specific real-time PCR primers and fluorescent probes based on the available ITS1-5.8S-ITS2-28S sequences of these species from GenBank, were designed using Oligo Primer Analysis v. 6.0 software (Molecular Biology Insights, Inc., USA). DNA sequences were aligned using a Clustal W software (http:// workbench. sdsc.edu) (Edgar, 2004). Since the amplification of all three test systems composed on the same micromatrix should occur simultaneously, one of the key factors was the possibility to apply similar amplification parameters to provide efficient use of all primer pairs. The preferable annealing temperature ($T_a$) was chosen to be 60°C. The designed test systems were additionally checked by the Primer Blast program (http:// www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of the developed test systems was tested using the BLAST homology search option of the NCBI database.

**PCR analysis.** A PCR mastermix of total 16 µl containing 2.5 µl of dNTP mix (0.25 µM), 2 µl of the oligonucleotide mix (0.4 µM of each primer and 0.2 µM of the fluorescent probe); 2.5 µl of a 10× PCR buffer (Sibenzyme, Russia), 1.25 µl of 50 mM $MgCl_2$, 0.5 µl of Taq DNA polymerase (0.1 U $\mu l^{-1}$, Sibenzyme, Russia), and 7.25 µl of deionized water was prepared in 0.2-ml tubes.

For preliminary evaluation of the test system efficiency in a tube format, 9 µl of template DNA (~50 $\mu g\ ml^{-1}$) or deionized water (negative control) was added to the PCR mastermix, and the resulted PCR mix was analysed using a DTLite 4 amplifier (DNK-Tekhnologiya, Russia).

At the second stage of the study (sensitivity, specificity and reproducibility tests), PCR analysis was carried out using empty 30-well aluminum micromatrices (Lumex Marketing LLC, Russia). After installation of a micromatrix into a holder cartridge, the whole reaction zone was accurately covered with a sealing layer of mineral oil (620 µl). The above-mentioned PCR mix containing template DNA or deionized water (negative control) was added into reaction wells (1.2 µl each) under the oil layer, and the subsequent analysis was carried out.

At the third stage, each assay was tested using preserved 30-well silicon (Si) micromatrices (Fig. 1) containing preliminary stabilised and lyophilised components of the three species-specific amplification mixtures. Preparation of preserved micromatrices was carried out by the GenBit LLC specialists using a special technology developed independently of this study. Template DNA samples were mixed with 10× PCR buffer (Sibenzim, Russia) at a 1:9 ratio; the resulting DNA concentration was 1 $\mu g\ ml^{-1}$. After installation of a micromatrix into a holder cartridge and covering of the reaction zone with 620 µl of mineral oil, 1.2 µl of a sample DNA or deionized water (negative control) was added into each well under the sealing oil layer according to the particular matrix topology.

Real-time qPCR assays on micromatrices were performed using an AriaDNA® microarray amplifier (Lumex Marketing LLC, Russia). Thermal cycling conditions for DNA amplification included an initial denaturation step at 94°C for 120 s followed by 45 cycles at 94°C for 5 s and 56-60°C (see Results section) for 25 s. The total amplification time was 30 min. Data acquisition occurred on the FAM (*D. destructor*, *D. dipsaci*, *D. weischeri*) and ROX (internal control) channels at the end of each cycle. The baseline was set in an automatic mode. Signal recording, calculation of critical threshold cycles ($C_t$), and analysis of the results were carried out automatically using an AriaDNA® software package (Lumex-Marketing LLC, Russia).

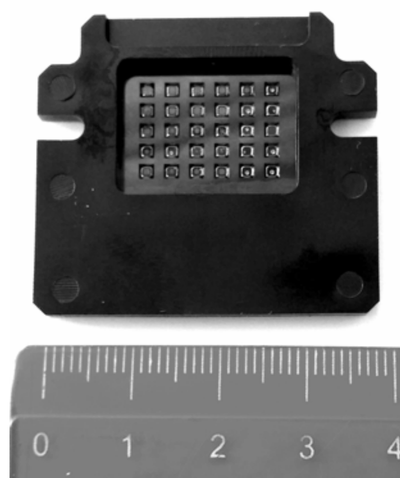


**Fig. 1.** Micromatrix with 30 reaction wells (the numbers on the ruler represent centimeters).

**Detection limit determination.** To determine the detection limit of the developed test systems, five serial dilutions of DNA of the target species were tested. The concentration range included: 8.6, 0.86, 0.086, 0.0086 and 0.0008 μg $ml^{-1}$ for *D. destructor* N3; 10, 1, 0.1, 0.01 and 0.001 μg $ml^{-1}$ for *D. dipsaci* N8; and 14.8, 1.48, 0.148, 0.0148 and 0.0014 μg $ml^{-1}$ for *D. weischeri* N11. DNA of each species was tested in three repetitions for all dilutions; the dilutions were arranged in the same micromatrix. An additional assay included the testing of DNA samples obtained from different number of nematode juveniles (1, 5 and 50) also performed in three replications. According to the earlier obtained empiric data (Nikitin *et al.*, 2018), we considered the $C_t$ level threshold to be equal to 35. In the case of $C_t$ slightly exceeding this value, one cannot be sure whether it is false positive result or just very low DNA presence.

**Specificity assessment.** To evaluate specificity of the developed primer pairs, each of them was tested with DNA of other target species as well as with reference nematode species included into the study. All DNA used were adjusted to a final concentration of 1 μg $ml^{-1}$.

**Reproducibility assessment.** A reproducibility of the approach was examined by the analysis of the same sample of each target pathogen (N3, N8 and N11) in nine repetitions arranged on the same Si micromatrix. DNA concentrations used in this assay were adjusted to 1 μg $ml^{-1}$.

## RESULTS

**Primer design.** Since the main task of this study was to develop test systems able to be freely combined on the same micromatrix with the test systems for other DNA-based potato pathogens (that would provide a possibility of simultaneous diagnostics of samples for a range of pathogens), one of the main criteria for selection of primers and probes was melting temperature close to 60°C. Additionally, a possible dimer or hairpin formation was evaluated for each set of oligonucleotides. After the alignment of the DNA sequences of the ITS1-5.8S-ITS2-28S regions, the best primer set for each nematode species was selected according to these criteria (Table 2). The length of the resulted amplicons corresponded to the calculated values.

A preliminary testing of the developed test systems in a tube format showed their sufficient working capacity; all three systems successfully detected the target nematode species (data not shown). Then the developed test systems were examined under the same amplification conditions, but at four different annealing temperatures ($T_a$) to evaluate their efficiency at the desired $T_a$ (60°C). According to the obtained results (Table 3), $T_a$ = 60°C provided satisfactory results for all three primer sets, *i.e.*, the test systems can efficiently work under 'standard' amplification conditions.

**Specificity assay.** All test-systems were subjected to specificity examination at both development stage (using NCBI nucleotide-BLAST tool) and through laboratory testing. In the latter case, each test-system was checked for possible cross-reactions with genomic DNA of a range of nematode species. For all three test systems, only DNA samples of a target pathogen provided sufficient signal intensity, while signals from other nematode species did not exceed a threshold level (Table 4). Thus, no false positive results for related species were observed.

**Table 2.** Species-specific primers and probes designed for the qPCR-based diagnostics of three *Ditylenchus* species.

| Nematode species | Primers and probe sequences (5'-3') | Melting temperature $T_m$, °C | Calculated working $T_m$ for the whole system, °C | Amplicon size, bp |
|---|---|---|---|---|
| *Ditylenchus destructor* | F: TGTTTGGTGACATTGCTGT | 58 | 58 | 133 |
| | R: AAGCATATCAGTAAGCGGA | 58 | | |
| | Probe: TTATGGACGTAAGGCTTTGAA | 63 | | |
| *Ditylenchus dipsaci* | F: ACTTGCCTACCGGATGAT | 59 | 59.4 | 92 |
| | R: TCTGGATGTACGTTGGCA | 59 | | |
| | Probe: AATAGCCAGTCGATTCCGTCT | 65 | | |
| *Ditylenchus weischeri* | F: GATTGCCTACCGGATGAT | 59 | 59.4 | 96 |
| | R: GGAC**R**ATATTAGGCATCGAT | 59 | | |
| | Probe: ACGGAATCAG**Y**GGCTGTTTCA | 67 | | |

**Note.** Degenerate nucleotides are indicated in bold.

**Table 3.** Effect of different annealing temperatures on the efficiency of test systems developed for detection of *Ditylenchus destructor* N3, *D. dipsaci* N8 and *D. weischeri* N11.

| Annealing temperature $T_a$, °C | Maximum fluorescence level, $F_m$ | Threshold fluorescence level, $F_t$ | Threshold cycle, $C_t$ |
|---|---|---|---|
| *Ditylenchus destructor* (sample H10) | | | |
| 62 | 800 | 213.3 | 32.2 |
| 60 | 1200 | 177.5 | 27.6 |
| 58 | 3000 | 197.5 | 25.27 |
| 56 | 3700 | 153.0 | 24.08 |
| *D. dipsaci* (sample H9) | | | |
| 62 | 11500 | 108.2 | 18.78 |
| 60 | 11000 | 221.4 | 19.70 |
| 58 | 9500 | 33.3 | 17.59 |
| 56 | 14000 | 123.7 | 19.36 |
| *D. weischeri* (sample H11) | | | |
| 62 | 7600 | 82.1 | 30.53 |
| 60 | 7600 | 133.8 | 29.32 |
| 58 | 9000 | 41.8 | 26.28 |
| 56 | 10000 | 30.9 | 25.80 |

**Detection range determination and regression curves.** In the case of *D. destructor* and *D. dipsaci*, only the lowest tested DNA concentrations (less than 0.86 and 1 ng $ml^{-1}$, respectively) provided a signal intensity that did not exceed the threshold level ($C_t$ = 35); all other dilutions were reliably detected. For *D. weischeri*, the lowest DNA concentration providing $C_t < 35$ was 77 ng $ml^{-1}$. Therefore, the detection limit of the developed diagnostic systems was determined to be about 8-10 pg of DNA for *D. destructor* and *D. dipsaci* and 77 pg for *D. weischeri*.

Standard regression curves were generated using the range of DNA concentrations of target species from 8.6 to 0.0086 µg $ml^{-1}$ for *D. destructor*, from 10 to 0.01 µg $ml^{-1}$ for *D. dipsaci*, and from 7.7 to 0.0077 µg $ml^{-1}$ for *D. weischeri* (Fig. 2). A linear dynamic range of amplification was exhibited for the concentration ranges used, and a highly significant negative correlation between the $C_t$ value and the DNA concentration over the range studied ($R^2$ = –0.999, –0.999, and –0.995 for *D. destructor*, *D. dipsaci* and *D. weischeri*, respectively) was found.

An additional detection limit assay in relation to a number of juveniles detected by the developed test systems was carried out (Table 5). To do this, total DNA was isolated from samples containing 1, 5 or 50 juveniles, and then 1.2 µl of each DNA sample was added into a separate well of the same micromatrix. According to the obtained results *D. destructor* and *D. dipsaci* were successfully detected at the level of 5 juveniles or more, whereas *D. weischeri* was revealed only at the level of 50 juveniles. These results correspond to those obtained for serial DNA dilutions.

**Table 4.** Specificity test for the developed test systems for three *Ditylenchus* species.

| Species | Code | $C_t$ values* | | |
|---|---|---|---|---|
| | | ***D. destructor*** | ***D. dipsaci*** | ***D. weischeri*** |
| *Ditylenchus destructor* | **N2** | 24.9±1.10 | –** | – |
| *Ditylenchus destructor* | **N3** | 23.9±0.09 | – | – |
| *Ditylenchus destructor* | **N10** | 29.5±3.07 | – | – |
| *Ditylenchus destructor* | **N13** | 26.4±1.14 | – | – |
| *Ditylenchus dipsaci* | **N8** | – | 27.5±1.73 | – |
| *Ditylenchus dipsaci* | **N9** | – | 20.3±0.01 | – |
| *Ditylenchus weischeri* | **N11** | – | – | 25.8±0.58 |
| *Globodera rostochiensis* | **Grost** | – | – | – |
| *Globodera pallida* | **Gpal** | – | – | – |
| *Heterodera medicaginis* | **Hmed** | – | – | – |
| *Heterodera schachtii* | **Hcsh** | – | – | – |
| *Caenorhabditis elegans* | **Cel** | – | – | – |
| *Meloidogyne hapla* | **N12** | – | – | – |
| *Meloidogyne incognita* | **N14** | – | – | – |
| *Meloidogyne arenaria* | **N15** | – | – | – |

*Averaged $C_t$ values for three replications. For all samples, DNA concentration was adjusted to ~1 µg $ml^{-1}$.

** no amplification.

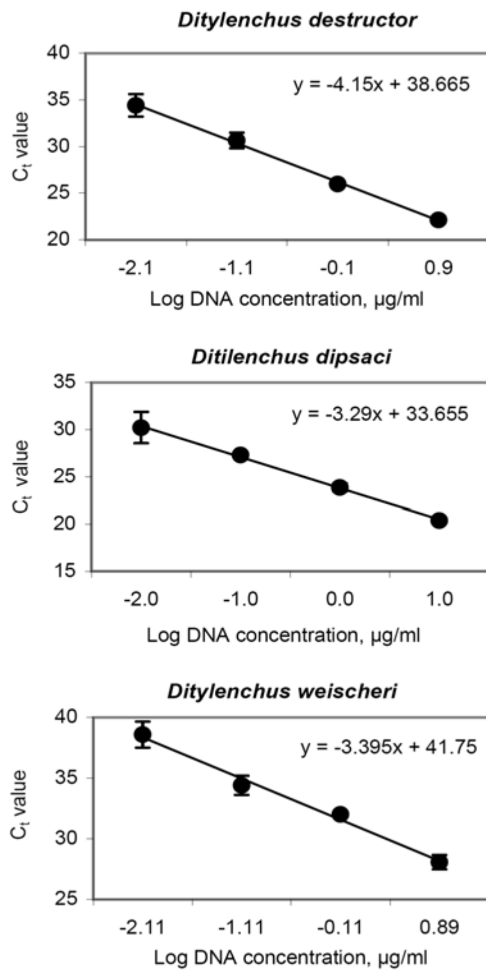


**Fig. 2.** Standard regression curves of a serial dilution of DNA from three *Ditylenchus* species.

**Table 5.** Variations in the critical threshold ($C_t$) values for different number of *Ditylenchus* juveniles per a sample.

| Number of juveniles per sample | $C_t$ value (M±SD) | | |
|---|---|---|---|
| | *D. destructor* | *D. dipsaci* | *D. weischeri* |
| 1 | –* | – | – |
| 5 | 30.51±0.56 | 27.6±3.63 | – |
| 50 | 27.26±0.13 | 26.1±0.23 | 25.8±0.13 |

* Fluorescence signal intensity did not exceed the threshold value.

**Reproducibility assay**. Results of the reproducibility assay for the developed test systems are shown in Fig. 3. All test systems showed high reproducibility. Mean $C_t$ values for *D. destructor*, *D. dipsaci* and *D. weischeri* obtained for nine repetitions were 23.97 ± 0.37, 29.60 ± 0.20 and 27.77 ± 0.15, respectively; for all of them, standard deviation of $C_t$ value did not exceed 1.55%.

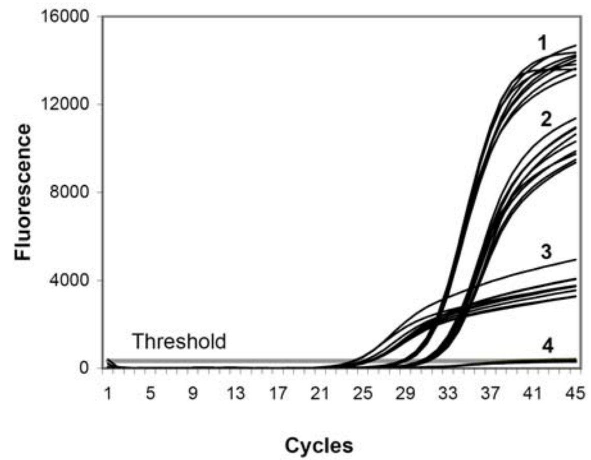


**Fig. 3.** Reproducibility of the test systems for detection of three *Ditylenchus* species. 1 – *D. weischeri*; 2 – *D. dipsaci*; 3 – *D. destructor*; 4 – negative control. Detection threshold is indicated with grey line. Each species was analysed in nine replications.

**Evaluation of the efficiency of lyophilised test systems.** After a completion of the specificity, sensitivity and reproducibility tests, all three test systems were examined in the format of a ready-to-use silicon micromatrix. After the application of the required PCR components on the surface of reaction wells and their lyophilisation, detection efficiency of the resulting micromatrix was tested using several DNA samples of target nematode species. Results of the examination are shown in Fig. 4. All developed test systems worked properly and provided reliable detection of the corresponding *Ditylenchus* species with no cross-reactions with non-target species.

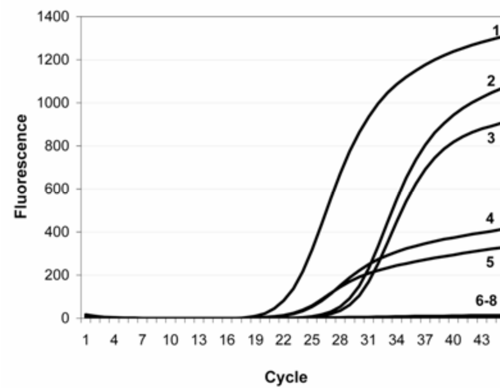


**Fig. 4.** Detection efficiency of the lyophilised test systems applied on a preserved silicon micromatrix. 1 – *Ditylenchus dipsaci* N9; 2 – *D. dipsaci* N8; 3 – *D. weischeri* N11; 4 – *D. destructor* N10; 5 – *D. destructor* N3; 6-8 – negative controls for the three *Ditylenchus* species.

**Examination of field samples.** Field samples (P11, P16, P17), for which infection with stem nematodes was suspected, were collected on potato fields of three different regions and examined using test systems for all three target species arranged in the same micromatrix with a parallel microscopic examination to verify the obtained results. Results of microscopic examination and PCR analysis are showed in Table 6.

Morphological identification of field populations of *Ditylenchus* species extracted from potato tubers was based on morphological characteristics and morphometric measurements of females and males of each population. According to the obtained results, a conclusion was made that all three examined populations represent *D. destructor*. The same results were obtained by a PCR identification: for all samples, only test systems specific for *D. destructor* provided a positive result, whereas zero fluorescence was observed in micromatrix wells corresponding to two other test systems (*D. dipsaci* and *D. weischeri*). Thus, primers developed for *D. destructor* confirmed their efficiency for the field sample analysis. The similar examination of two other test systems requires field samples with the corresponding nematode species and will be probably performed next season.

**Table 6.** Morphological characteristics and morphometric measurements of nematode females and males isolated from the field samples of potato.

| **Parameter** | **Field sample** | | |
|---|---|---|---|
| | **P11 (Ivanovo Region, Russia)** | **P16 (Kaluga Region, Russia)** | **P17 (Sverdlovsk Region, Russia)** |
| Number of nematodes analysed | 35 | 35 | 30 |
| Female body length, mm | 1.0-1.4 (1.2±0.2) | 0.9-1.5 (1.2±0.3) | 0.8-1.5 (1.1±0.3) |
| Female stylet length, µm | 9-13 (11.0±1.5) | 8-12 (10.5±1.1) | 8-12 (10.3±1.4) |
| Posterior bulb | short, dorsally overlapping | short, dorsally overlapping | short, dorsally overlapping |
| Number of lateral lines | 6 | 6 | 6 |
| Vulva position, % | 80-83 | 80-82 | 78-81 |
| Vulva-anus distance | 170-200 (185±12) | 172-190 (183±11) | 170-190 (179±9) |
| Tail tip | finely rounded | finely rounded | finely rounded |
| Male spiculum length, µm | 24-26 (25±1.1) | 24-27 (25±1.1) | 23-25 (24±0.9) |
| **PCR identification systems** | | | |
| *Ditylenchus destructor* | + | + | + |
| *D. dipsaci* | – | – | – |
| *D. weischeri* | – | – | – |

## DISCUSSION

Both *D. destructor* and *D. dipsaci* are listed quarantine pests. Due to their polyphagous nature, they are able to survive on the infected fields for a long time even in the absence of potato crops (Švilponis *et al.*, 2008). Therefore, timely and precise diagnostics is very important for adequate assessment of the state of both seed potato and potato fields.

Traditionally, identification of *D. dipsaci* and *D. destructor* is based on morphological methods. However, such identification may be inefficient in the case of atypical adults, or juveniles only, or occurrence of confounding species, such as *D. gigas*, *D. weischeri*, *etc*. In addition, low infestation levels may easily be overlooked. Morphological identification is also constrained by the need for highly trained taxonomists, whose numbers have declined over the past years. The efficiency of protein-based technologies depends on the particular life stage of analysed nematodes, environmental conditions, and occasional cross-reactivity (Ahmed *et al.*, 2016). Thus, training of scientists, quarantine experts, and technicians involved in the diagnostics of plant-parasitic nematodes is now focused on the modern DNA-based identification methods.

To date, existing PCR assays are intended mainly for static laboratories, since they either require highly qualified personnel and complex equipment, or are sensitive to possible contamination and environmental conditions. At the same time, the role of field diagnostics, especially in the case of regulated pests, is very important, since it provides rapid on-site results that may be crucial for timely

control of pathogens and pests. Diagnostic test systems suitable for field laboratories should be not only sensitive and specific, but also simple in use, storage, and transportation; their use and interpretation of results should be available even for inexperienced users.

The development of test systems described in this study was intended to meet the above-listed requirements. A qPCR micromatrix technology provides a simultaneous identification of a range of pathogens within a single run *via* parallel real-time PCR reactions occurring in micromatrix wells. Due to a small reaction volume (1.2 µl) and the use of a special technology for stabilisation of PCR mix components within reaction wells providing a long-term storage of preserved micromatrices at room temperature, the number of required steps in the procedure is significantly reduced, as well as the total time of analysis (~1.5 h). Taking into account the above features and also the automatic mode of data interpretation, the proposed method of analysis is suitable for use by unskilled persons under conditions of field laboratories.

All three developed test systems are species-specific and do not show any cross-reactions in relation to other target and non-target nematode species. Analytical sensitivity of test systems for *D. destructor* and *D. dipsaci* started from 8-10 pg of DNA or 5 juveniles that is comparable to the sensitivity of primers developed by other authors: 16 pg (Jeszke *et al.*, 2015) and 4 nematodes (Cheng *et al.*, 2015). In the case of *D. weischeri*, the detection limit was only 77 pg of DNA or 50 juveniles, which might be related to two possible reasons. First, according to Sato *et al.* (2007), a $C_t$ value depends on the developmental stages of nematodes (minimal for adult males and maximal for juveniles); for root-lesion nematode *Pratylenchus penetrans*, this difference may reach three cycles. This phenomenon was also confirmed for *D. destructor* (Cheng *et al.*, 2015). Second, Cheng *et al.* (2015) showed that DNA isolated from non-mobile (probably dead) individuals of *D. destructor* provided $C_t$ values five cycles higher (that corresponded to a 32× lower DNA copy number) compared to mobile (living) nematodes; the authors explained this fact by a possible low metabolic activity of non-mobile individuals (or the lack of this activity if these individuals were dead). Thus, relatively low sensitivity of our primers might be caused by a poor state of the nematodes in samples. Some additional studies with fresh *D. weischeri* samples are planned next season to check for these reasons and to improve the result; nevertheless, even at the current stage, this test system is suitable for our purpose, since *D. weischeri* does not have any economic importance for potato and just should be distinguished from *D. dipsaci*.

The developed test systems showed high reproducibility and were successfully tested in both fresh and preserved micromatrices. In spite of reduced fluorescence intensity, lyophilisation of PCR mix components still provided good detection efficiency. Further sensitivity improvement is planned, and the further validation of these test systems may be carried out using a larger number of *Ditylenchus* populations; at the same time, we consider test systems for *D. destructor* and *D. dipsaci* to be ready for use as the components of micromatrices for detection and identification of various potato pathogens. To date, test systems for identification of two potato cyst nematodes (Nikitin *et al.*, 2017) and also eight viral and seven bacterial/oomycetal potato pathogens (Nikitin *et al.*, 2018) have been already developed, and the creation of similar test systems for seven fungal and four phytoplasma potato pathogens is still in process. Since infestation of potato with stem nematodes is often accompanied with fungal and bacterial diseases, their presence in tubers is often masked and can be easily overlooked. Therefore, combining test systems for identification of different pathogens (including regulated species) on the same micromatrix may have some clear benefits for the proper assessment of seed potato quality and the control of phytosanitary state of fields.

## ACKNOWLEDGEMENTS

This study was financially supported by the Russian Science Foundation (project no. 16-16-04109). We thank Renat Khusainov (A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Science) for morphological identification of nematode samples.

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**К.О. Дейч, М.В. Приданников, М.М. Никитин, Н.В. Стацюк, В.Г. Джавахия и А.Г. Голиков.** Одновременное выявление трех видов паразитических нематод растений из рода *Ditylenchus* в режиме реального времени с использованием технологии qPCR матриц.

**Резюме.** Паразитические нематоды растений рода *Ditylenchus* поражают широкий спектр растений и вызывают серьезные экономические потери в различных климатических зонах. Среди них два поражающих картофель вида, *Dytlenchus destructor* и *D. dipsaci*, являются наиболее вредоносными и включены в перечни карантинных и регулируемых вредителей во многих странах. Правильная диагностика этих видов очень важна для корректной и своевременной оценки качества семенного картофеля и фитосанитарного состояния картофельных полей. На основе последовательностей ITS1-5.8S-ITS2-28S регионов были разработаны тест-системы для одновременной детекции этих двух видов, а также близкого к ним вида *D. weischeri* методом ПЦР в реальном времени с использованием qPCR матриц (микрочипов). Тест-системы продемонстрировали высокую специфичность и воспроизводимость, а также удовлетворительную аналитическую чувствительность (5 личинок для *D. destructor* и *D. dipsaci* и 50 личинок для *D. weischeri*). Предел обнаружения составил 8-10 пг ДНК в пробе для *D. destructor* и *D. dipsaci* и 77 пг ДНК для *D. weischeri*. Лиофилизация компонентов ПЦР-смеси на кремниевых микроматрицах длительного хранения не оказала существенного влияния на эффективность их работы. Благодаря простоте использования qPCR микроматриц, гибкости их конфигурации и сокращению времени анализа до 1 ч. (с учетом процедуры выделения ДНК), разработанные тест-системы (отдельно или в сочетании с аналогичными системами, созданными для других патогенов картофеля) могут быть рекомендованы для использования в полевых лабораториях для одновременного обнаружения и идентификации целевых видов нематод, включая возможные случаи комбинированной инфекции, когда наличие паразитических видов *Ditylenchus* замаскировано симптомами, вызванными другими патогенами.

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