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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/87 (2)

Diagnostics
Diagnostic

PM 7/87 (2) *Ditylenchus destructor* and *Ditylenchus dipsaci*

Specific scope

This Standard describes a diagnostic protocol for *Ditylenchus destructor* and *Ditylenchus dipsaci*.[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology.[2]

Specific approval and amendment

Approved in 2008-09.

This revision was prepared on the basis of the IPPC Diagnostic Protocol adopted in 2015 on *D. dipsaci* and *D. destructor* (Annex 8 to ISPM 27 *Diagnostic protocols for regulated pests*). The EPPO Diagnostic Protocol is consistent with the text of the IPPC Standard for morphological identification for this species. For comparison with other species the IPPC table includes *Ditylenchus africanus* whereas the EPPO table includes *Ditylenchus convallariae* due to the different distribution of the species. The molecular tests for which there is experience in the EPPO region are described in full in the appendices (some of these are additional tests to those in the IPPC protocol). Reference is given to the IPPC protocol for tests for which there is little experience in the EPPO region. DNA barcoding is also included.

Revision approved in 2017-04.

1. Introduction

Among the more than 80 species currently recognized in the genus *Ditylenchus* Filipjev, 1936, only a few are parasites of higher plants, such as *D. destructor*, *D. dipsaci* and *Ditylenchus gigas*, while the majority of species are mycophagous. *Ditylenchus destructor* (potato tuber nematode, potato rot nematode) is recorded from all continents, mainly from temperate regions. The known host range comprises more than 100 species of plants from a wide variety of families. Economically important crops are *Solanum tuberosum*, *Iris* spp., *Tulipa* spp., *Dahlia* spp., *Gladiolus* spp., *Rheum rhabarbarum*, *Trifolium* spp., *Beta vulgaris* and *Daucus carota*. Some weeds (e.g. *Cirsium arvense*, *Mentha arvensis*, *Potentilla anserine*, *Rumex acetosella* and *Stachys palustris* (Andersson, 1971)) can also be hosts and can act as sources of infection to crop plants. *Ditylenchus destructor* is also capable of reproducing on the mycelium of many fungi (Namjou *et al.*, 2013). The nematode attacks subterranean parts of plants (tubers, stolons, bulbs, rhizomes, roots), but may occasionally also invade aboveground parts, mainly the base of the stem. *Ditylenchus destructor* is able to withstand desiccation, but not to the extent that *D. dipsaci* does and unlike this species it does not clump together in the cryptobiotic state to form 'nematode wool' when the plant begins to dry. *Ditylenchus destructor*, however, is capable of surviving low temperatures and overwinters as eggs, which gives this species' eggs some advantage over *D. dipsaci*.

Ditylenchus dipsaci sensu lato (*s.l.*) (stem nematode, stem and bulb nematode; Sturhan & Brzeski, 1991) is among the plant-parasitic nematodes of greatest economic impact worldwide and is widely distributed, mainly in temperate areas. Almost 1200 wild and cultivated plant species are known as hosts for *D. dipsaci* but the different biological races of this nematode each have limited host ranges. On the basis of morphological, biochemical, molecular and karyological analyses a total of 13 nominal species have been synonymized and up to 30 host races of *D. dipsaci s.l.* have been suggested, divided into two groups (Jeszke *et al.*, 2013). The larger group, named *D. dipsaci sensu stricto* (*s.s.*), comprises diploidal populations characterized by their 'normal' size. The second group is polyploidal and currently comprises *Ditylenchus gigas* – previously known

[1]Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

[2]http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf

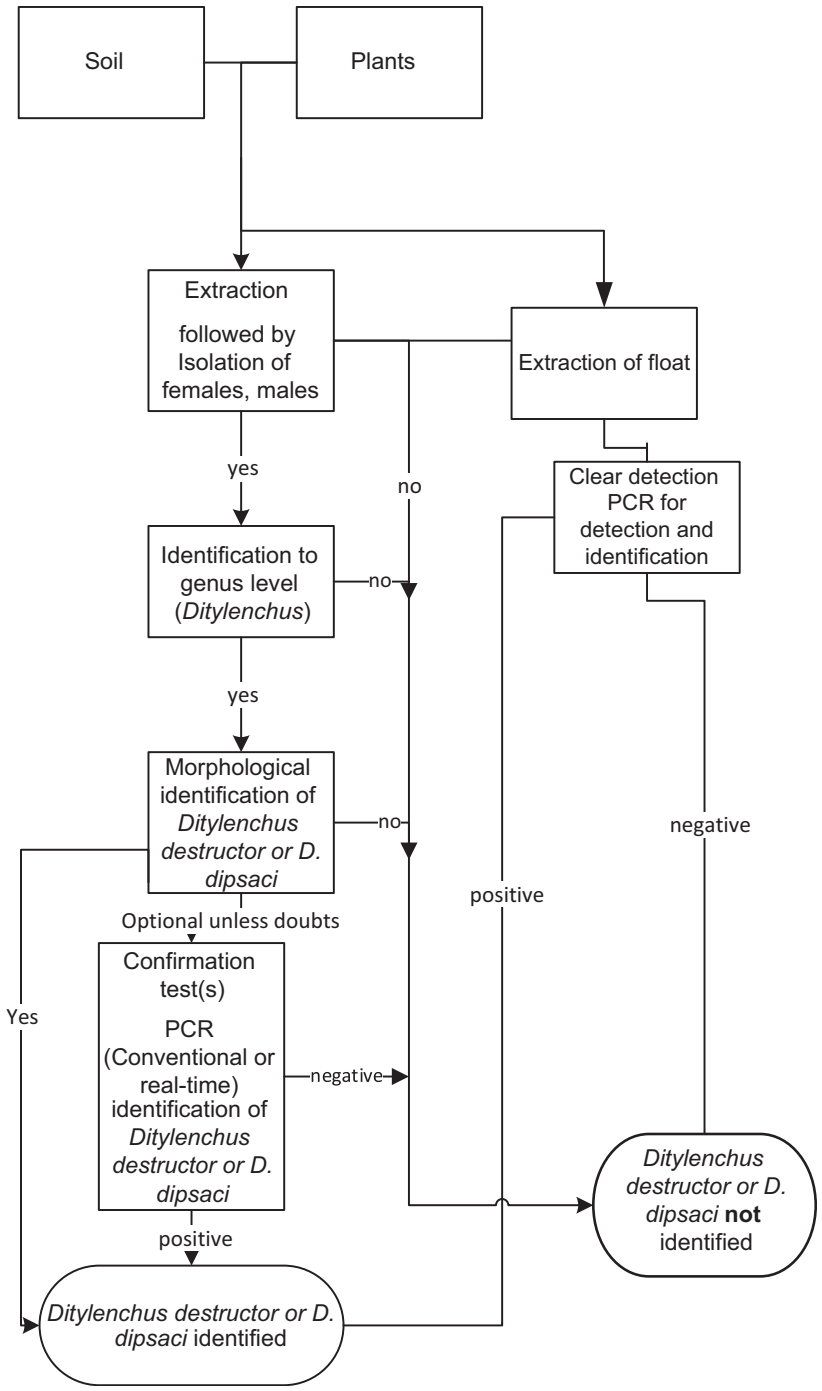

Fig. 1 Flow diagram for the detection and identification of *D. destructor* and *D. dipsaci*.

as the 'giant race' of *D. dipsaci* parasitizing *Vicia faba* (broad bean) (Vovlas *et al.*, 2011), *D. weischeri* parasitizing *Cirsium arvense* (creeping thistle) (Chizhov *et al.*, 2010) and three undescribed *Ditylenchus* spp., which are associated with plant species of the Fabaceae, Asteraceae and Plantaginaceae, respectively (Jeszke *et al.*, 2013). This protocol includes information to distinguish between the most important plant pests within this complex – *D. dipsaci s.s.* and *D. gigas*.

Ditylenchus dipsaci s.s. (hereafter referred to as *D. dipsaci*) lives mostly as an endoparasite in aerial parts of plants (stems, leaves, flowers), but also attacks bulbs, tubers and rhizomes. The nematode is seed-borne in *V. faba*, *Medicago sativa*, *Allium cepa*, *Trifolium* spp., *Dipsacus* spp. and *Cucumis melo* (Sousa *et al.*, 2003; Sikora *et al.*, 2005). *Ditylenchus dipsaci* readily withstands desiccation and can be isolated even from completely dry plant material after moistening (resistant stage = fourth-

stage juveniles). Similar to *D. destructor*, *D. dipsaci* can reproduce on the mycelium of soil fungi (Viglierchio, 1971). It is worth mentioning that although *D. dipsaci* and *D. destructor* have common hosts (e.g. beets, lucerne, clover, potato), the two species rarely occur together in the same plant (Andrássy & Farkas, 1988).

Ditylenchus gigas represents the formerly known 'giant race' of *D. dipsaci*. It is a serious pest on *V. faba* in Mediterranean countries including Algeria, Morocco, Portugal, Italy and France, but has also been reported in temperate countries such as the United Kingdom and Germany (Sturhan & Brzeski, 1991; Sikora *et al.*, 2005). Besides infecting *V.faba*, its host range seems to be limited to wild plants such as *Ranunculus arvensis*, *Convolvulus arvensis*, *Lamium* spp. and *Avena sterilis* (Augustin & Sikora, 1989; Vovlas *et al.*, 2011). *Ditylenchus gigas* is generally more damaging to *V. faba* and produces more infested seed than *D. dipsaci*.

A flow diagram for the detection and identification of *D. destructor and D. dipsaci* is given in Fig. 1.

2. Identity

Name: *Ditylenchus destructor* Thorne, 1945
Synonyms: About 30 synonyms but none used in recent years (Sturhan & Brzeski, 1991)
Taxonomic position: Nematoda: Tylenchida[3]: Anguinidae
EPPO Code: DITYDE
Phytosanitary categorization: EU Annex designation: II/A2

Name: *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936
Synonyms: About 30 synonyms but none used in recent years (Siddiqi, 2000)
Taxonomic position: Nematoda: Tylenchida[3]: Anguinidae
EPPO Code: DITYDI
Phytosanitary categorization: *Ditylenchus dipsaci*: EPPO A2 List no. 174, EU Annex designation: II/A2

3. Detection

3.1. Symptoms

This section describes some of the most common symptoms of *Ditylenchus* species on crop plants. A more detailed description is given in the IPPC protocol on *D. dipsaci* and *D. destructor*.

3.1.1. Symptoms caused by D. destructor

Common symptoms of infestation of *D. destructor* are discoloration and rotting of the plant tissue of underground parts of plants (tubers, stolons, bulbs, rhizomes, roots). A few examples of symptoms are given below. For a more comprehensive description of symptoms on a broader spectrum of plants consult the IPPC diagnostic protocol (IPPC, 2016).

Potatoes. The first symptoms are white spots under the tuber skin. Badly affected tubers have slightly sunken areas with cracked and papery skin (Figs 2 and 3); the tissue below the skin is darkened and may have a mealy or spongy appearance. Symptoms may be more visible after storage. There is in general a secondary invasion of fungi, bacteria and free-living nematodes.

Above-ground symptoms may occur rarely and include dwarfing, thickening and branching of the stem and dwarfing, curling and discoloration of the leaves (Sturhan & Brzeski, 1991).

Fig. 2 *Ditylenchus destructor* damage on potatoes. Crown copyright, reproduced courtesy of Fera, York, GB.

Fig. 3 *Ditylenchus destructor* damage on potatoes (LNPV Nematology, Rennes, FR).

Flower bulbs and corms. Infestations usually begin at the base of the bulb and extend up to the fleshy scales with yellow to dark brown lesions. Secondary rotting may occur and the bulbs can be destroyed. Specimens of *D. destructor* are accumulated on the boundary between distinctly diseased parts and healthy sections, but are rarely isolated from completely decayed tissues.

[3]Recent development combining a classification based on morphological data and molecular analysis refer to 'Tylenchomorpha' (De Ley & Blaxter, 2004)

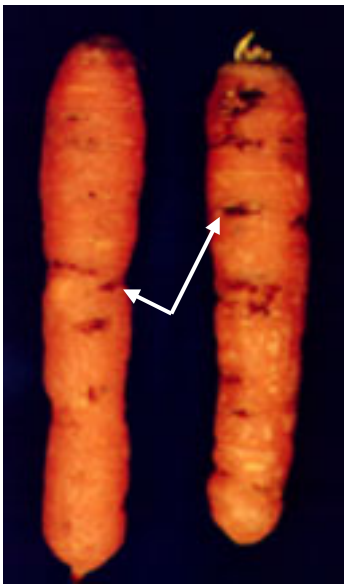

Fig. 4 Carrots showing transverse cracks upon infection by *D. destructor*.

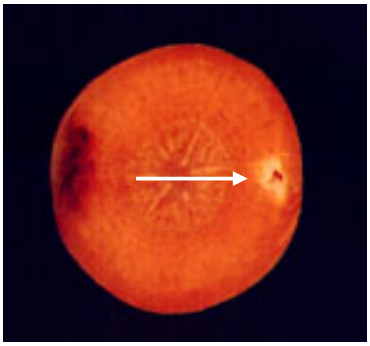

Fig. 5 Carrot disc with white sub-cortical patch caused by *D. destructor.*

Carrots. Damage to carrots appears as transverse cracks in the skin with white patches in the sub-cortical tissue (Fig. 4). The patches are easily seen in a transverse cut (Fig. 5). Infested areas are subject to secondary infections by fungi and bacteria resulting in decay and rot.

Sugar beet. Symptoms comprise dark, necrotic lesions on roots and rhizomes, similar to crown canker (Dallimore & Thorne, 1951). In addition, yield loss and sugar content will be reduced.

3.1.2. Symptoms caused by D. dipsaci

Common symptoms of infestation are swelling, distortion, discoloration and stunting of above-ground plant parts (Fig. 6), necrosis and rotting of bulbs and tubers. During the season various generations of *D. dipsaci* are present in a host. *Ditylenchus dipsaci* usually leaves the plant before it dies due to infection. A few examples of symptoms are given below. For a more comprehensive description of symptoms on a broader spectrum of plants consult the IPPC Diagnostic Protocol (IPPC, 2016).

Fig. 6 *Ditylenchus dipsaci* damage on bean plants. Crown copyright, reproduced courtesy of Fera, York, GB.

Seeds. Small seeds (e.g. *M. sativa*, *A. cepa*, *Trifolium* spp.) generally show no visible symptoms of infestation, but in larger seeds (e.g. *Phaseolus vulgaris* and *V. faba*) the skin may be shrunken and show discolored spots (Figs 7 and 8).

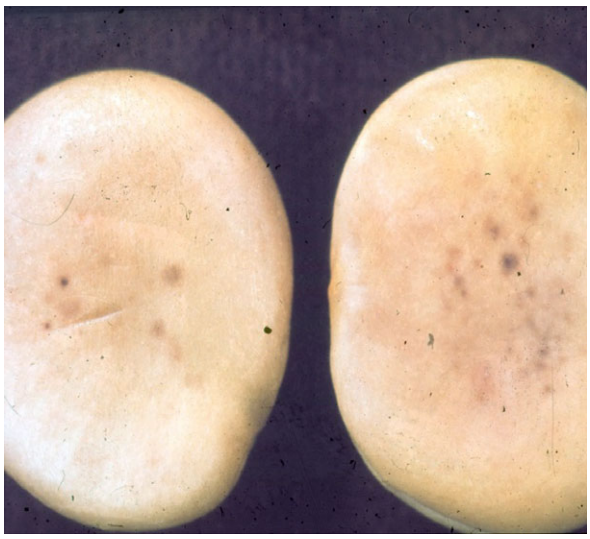

Fig. 7 *Ditylenchus dipsaci* symptoms on *V. faba* seeds (Plant Protection Service, the Netherlands).

Bulbs, tubers. Infested tissue is generally necrotic, and bulbs (Figs 9 and 10) show browning of the scales in concentric circles (seen in transverse sections; see Fig 11); entire bulbs often become soft and occasionally, in the case of garlic, a strong smell may develop.

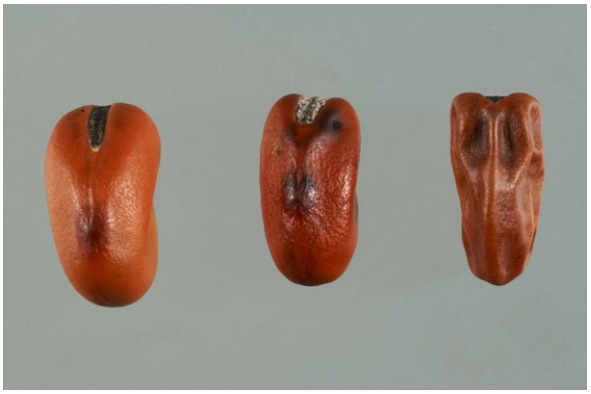

Fig. 8 *Ditylenchus dipsaci* damage on *Phaseolus vulgaris* seeds.

Fig. 10 *Ditylenchus dipsaci* damage on potatoes.

Fig. 9 *Ditylenchus dipsaci* damage on onions.

Fig. 11 symptoms of *D. dipsaci* on *Narcissus* sp. (LNPV Nematology, Rennes, FR).

Carrots and sugar beet. On carrots early symptoms of *D. dipsaci* attacks are straddled leaves, multi-bud plant crowns and light discolorations of tap-root tops. The portion of the plant most affected by *D. dipsaci* is that 2–4 cm below and above ground. Severe symptoms of attacks by the nematode are leaf death and tap-root rot (Greco *et al.*, 2002). These symptoms are similar to those described on sugar beet roots (Dunning, 1957) (see Fig. 12).

Fig. 12 *Ditylenchus dipsaci* symptoms on *Beta vulgaris.*

3.1.3. Symptoms caused by D. gigas

Plant damage has so far only been reported for *V. faba*. Common symptoms are severe swelling and deformation of stem tissue or lesions which turn reddish-brown then black (Vovlas *et al.*, 2011). Lesions envelope the stem, increase in length and often reach the edge of an internode. These symptoms can be confused with those caused by fungal leaf pathogens. Newly formed pods turn dark brown. Seeds affected by the nematode may appear darker, distorted and smaller in size and with speckle-like spots on the surface.

3.2. Extraction of the nematodes

The choice of the extraction method depends on the substrate to be analysed. *Ditylenchus* spp. are either extracted from plant tissue (roots, tubers, stolons, stems, seeds) or soil (however, it should be noted that these nematodes are rarely found in soil unless the latter has been associated with an infested host). For each application detailed information is given in EPPO Standard PM 7/119 *Nematode extraction* (EPPO, 2013).

4. Identification

The identification of *D. destructor* and *D. dipsaci* should always be based on morphological features of adult specimens first.

Molecular methods have been developed for the identification of *D. destructor* and *D. dipsaci*, and these methods may be used when there is a low level of infestation or when only juvenile stages are present.

4.1. Morphological methods

For identification, individual nematodes or entire nematode suspensions are heated (to approximately 60°C) until the nematodes become immobile. Specimens are then evaluated under the light microscope at a magnification of ×500 to ×1000 (oil immersion lens). It is recommended that this is done in combination with differential interference contrast microscopy.

The body of *D. destructor* killed by gentle heat is almost straight and never C-shaped, spiral or with its posterior end markedly bent to the ventral side. Generally all developmental stages (females, males, 3 juvenile stages, eggs) can be isolated from infested plant tissues. Contamination of the extract by free-living mycophagous and bacteriophagous nematodes is common, in particular in decaying plant material (e.g. potato tubers).

The body of *D. dipsaci* killed by gentle heat is straight or almost so and never C-shaped, spiral or with its posterior end markedly bent to the ventral side. In seed samples, nematodes other than *D. dipsaci* will rarely be found but contamination by other nematodes may occur, particularly with samples containing other plant debris. From seeds, generally only fourth-stage juveniles of *D. dipsaci* can be isolated (no adults or early juvenile stages) and the specimens have a similar appearance. In bulbs, tubers, rhizomes etc. a variety of nematodes which invaded the plant tissues from the soil may be present. In rotting tissue bacteriophagous or mycophagous nematodes are often dominant. *Ditylenchus dipsaci* is mainly found in plant tissues which are still viable. All stages of *D. dipsaci* can be isolated from bulbs etc. (females, males, 3 juvenile stages, eggs), but fourth-stage juveniles often prevail.

The body of *D. gigas* killed by gentle heat is straight or almost so and never C-shaped, spiral or with its posterior end markedly bent to the ventral side. All nematode stages can be extracted from soil and infested plant material, Contamination with free-living mycophagous and bacteriophagous nematodes is common, especially for decaying plant tissue. Extraction from dry seeds generally results in a pure suspension of fourth-stage juveniles of *D. gigas* with

Table 1. Key to distinguish *Ditylenchus* spp. from other tylenchid and aphelenchid genera (modified from Heyns (1971) and Siddiqi (2000))

1	Outlet of dorsal pharyngeal gland near base of stylet; median bulb roundish, ovoid or absent	Tylenchida 2
	Outlet of dorsal pharyngeal gland in median bulb; median bulb a prominent feature, usually oblong	Aphelenchida
2	Anterior part of pharynx (procorpus) and metacorpus not united into single unit; stylet never exceptionally long	3
	Procorpus gradually widened and fused with metacorpus; stylet very long, its base often located in anterior part of metacorpus	Other genera
3	Adult female vermiform	4
	Adult female saccate or pyriform sessile parasite on roots	Other genera
4	Median bulb with metacorpus plates	5
	Median bulb without metacorpus plates*	Other genera
5	Pharyngeal glands contained within basal bulb, not overlapping or slightly overlapping intestine; cephalic framework rarely conspicuous; stylet weak to moderately strong	6
	Pharyngeal glands lobe-like, overlapping intestine; cephalic framework strong; stylet massive	Other genera
6	Single prodelphic ovary; vulva posterior	7
	Ovaries two, amphidelphic; vulva slightly post-equatorial	Other genera
7	Female not swollen; crustaformeria in female in form of quadricollumella with four rows of four cells; bursa in males enveloping one-third or more of tail	*Ditylenchus*
	Female swollen; crustaformeria with more than 20 cells	Other genera

*A few non-plant parasitic species of *Ditylenchus* do not have a median bulb with metacorpus plates.

little contamination of free-living mycophagous and bacteriophagous nematodes.

Microscopic examination has to concentrate on stylet-bearing nematodes (the stylet is delicate) with a slender and (almost) straight body and a conical pointed tail. To distinguish *Ditylenchus* spp. from other tylenchid genera see Table 1. Specimens are then evaluated under the light microscope at a magnification of ×500 to ×1000 (oil immersion lens). It is recommended that this is done in combination with differential interference contrast microscopy.

In plant-parasitic *Ditylenchus* species the stylet is >10 µm long and metacorporeal plates are clearly visible, while in non-plant-parasitic species the stylet is often <10 µm long and metacorporeal plates are weak or even absent.

In non-related nematode genera the tail ranges from filiform to dome-shaped and the body of heat-relaxed specimens from straight to C-shaped and spiral with the posterior end often ventrally curved, a stylet may be absent, the pharynx is distinctly off-set from the intestine, the vulva is located at mid-body, the male tail lacks a bursa, etc. Specimens of *Aphelenchoides* spp. are mostly easily distinguished by their well-demarcated rounded median pharyngeal bulb, long overlap of the pharynx over the intestine, presence of a mucro on the tail tip and lack of a bursa on the male tail.

Discriminating morphological characteristics of *D. destructor, D. dipsaci, D. gigas, D. convallariae* and *D. myceliophagus* adult females are presented in Table 2 (see also Figs 13–17).

Ditylenchus destructor, *D. dipsaci* and *D. gigas* have a stylet with a cone of length about 50% of the total stylet length, whereas most other fungivorus species have stylets with cones shorter than 50% of the total stylet length (mainly about 1/3 of its length). In *D. dipsaci* and most similar species, the lateral fields have four incisures, the basal pharyngeal bulb is distinctly off-set from the intestine and the tail terminus is sharply pointed. *Ditylenchus gigas* is morphologically close to

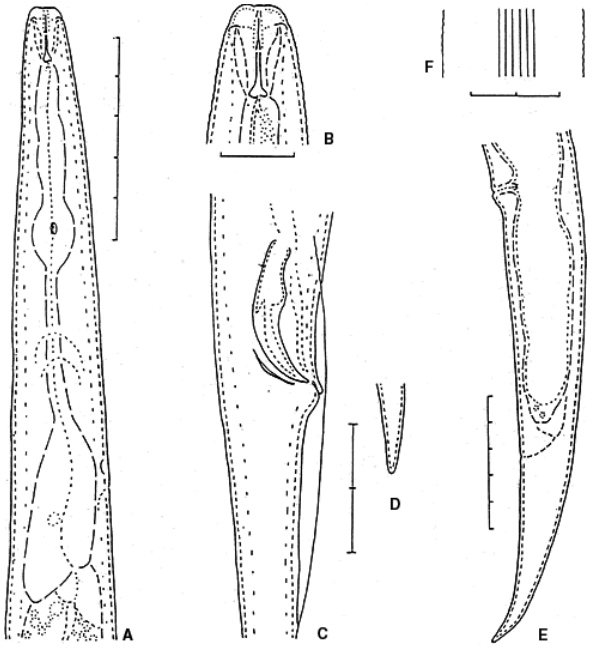

Fig 13 *Ditylenchus destructor*: (A) female, pharyngeal region; (B) head of female; (C) male, spicule region; (D) end of female tail; (E) posterior portion of female; (F) lateral field at midbody. Each unit on bars = 10 µm. After Sturhan & Brzeski (1991).

Table 2. Discriminating morphological characteristics of *D. destructor, D. dipsaci, D. gigas, D. convallariae* and *D. myceliophagus*

	D. destructor (after Hooper, 1973)	*D. dipsaci* (after Hooper, 1972)	*D. gigas* (after Vovlas *et al.*, 2011)	*D. convallariae* (after Sturhan & Brzeski, 1991)	*D. myceliophagus* (after Hesling, 1974)
c (body length/tail length) of female	14–20	11–20	15.8–27.6	12–15	8.2–17
Female body length (mm)	(0.8–) 1.0 (–1.9)	(1.0–) 1.1 (–1.7)	(1.6–) 1.8 (–2.2)	(0.9–) 1.1 (–1.3)	(0.6–) 0.9 (–1.4)
Stylet length (µm)	10–14	10–12	10.5–13.0	11–13	7–8
Posterior bulb	Short, dorsally overlapping	Not overlapping	Slightly overlapping	Not overlapping	Short, dorsally overlapping
Number of lateral lines	6	4	4	6	6
Vulva–anus length	1¾–2⅓ tail length	1¾–2¼ tail length	(202–) 228 (–266)	2–2¼ tail length	2–2¼ tail length
PUS/vulva–anus length (%)	53–90	40–70	About 50*	25–47	30–69
Form of tail terminus	Rounded	Pointed	Pointed to finely founded	Rounded	Rounded
Spiculum length (µm)	24–27	23–28	23.5–28	20–26	15–20
Length of cone/total stylet length	About 50%	About 50%	>50%	≪50%	≪50%
Host preference†	Higher plants and mycelia of fungi	Higher plants and mycelia of fungi	Higher plants	Higher plants	Mycelia of fungi

PUS, postvulval part of the uterine sac.
*Calculated from species description.
†Helpful in case of confusing morphological criteria.

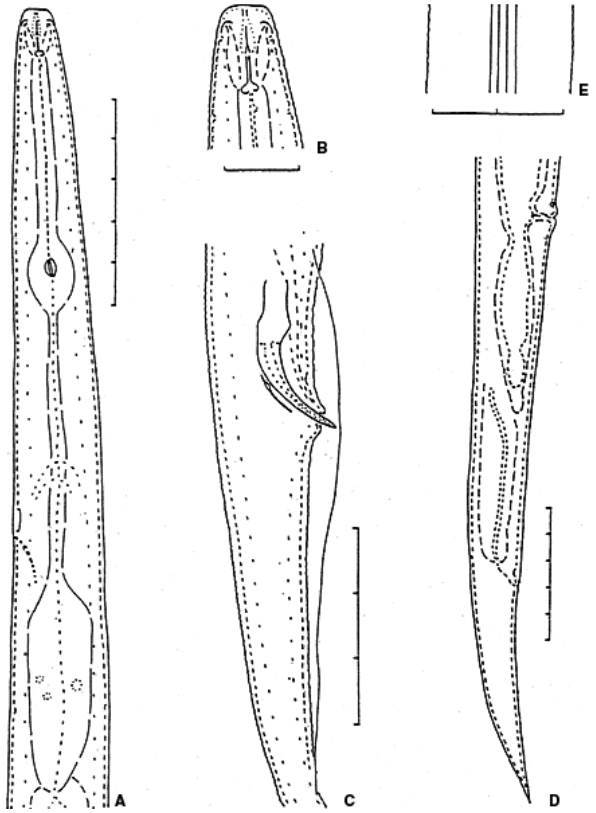

Fig 14 *Ditylenchus dipsaci*: (A) female, pharyngeal region; (B) head of female; (C) male, spicule region; (D) posterior portion of female; (E) lateral field at midbody. Each unit on bars = 10 µm. After Sturhan & Brzeski (1991).

D. dipsaci, from which it differs by its longer body size (1.5–2.2 mm vs. 1.0–1.7) and longer vulva–anus distance (202–266 vs. 132–188 µm). *Ditylenchus gigas* and *D. dipsaci* can be distinguished from *D. destructor* by having 4 lateral lines compared with 6 lateral lines for *D. destructor*. The presence of males can be helpful for identification as the spiculum of *D. destructor* differs from *D. dipsaci* in having a ventral tumulus in the calomus area (Karssen & Willemsen, 2010)

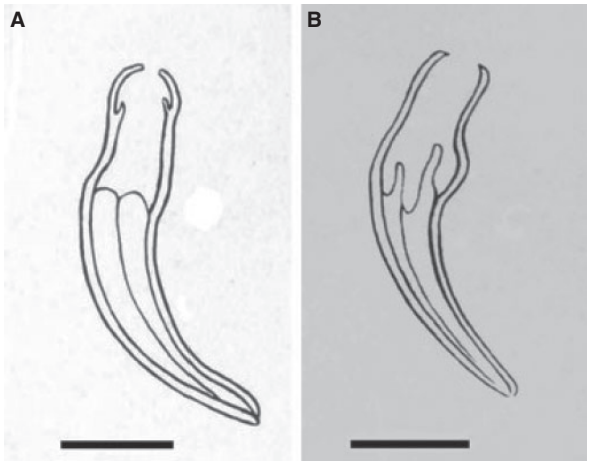

Fig 15 *Ditylenchus* spiculum line drawings: (A) *D. dipsaci*; (B) *D. destructor*. Each bar = 8 µm (after Thorne, 1945).

(Figs 15 and 16). Furthermore, in *D. destructor* cuticle parts within the lamina are anteriorly pointed and less sclerotized than in *D. dipsaci*. A list of *Ditylenchus* species with specific characters has been published by Brzeski (1991, 1998), Sturhan & Brzeski (1991), Wendt *et al.* (1995), Andrássy (2007), Karssen & Willemsen (2010) and Vovlas *et al.* (2011).

4.2. Molecular methods

Several molecular techniques have been developed and are in use now for diagnostics of *Ditylenchus* species and those for which there is experience in the EPPO region are described in full in the Appendices.

A PCR-restriction fragment length polymorphism (RFLP) of the ITS rRNA (Wendt *et al.*, 1993) is described in Appendix 1. A PCR with specific primers for *D. dipsaci* and *D. gigas* and tested in an interlaboratory comparison as part of the Testa project is described in Appendix 2. Marek *et al.* (2010) developed a multiplex PCR for *D. dipsaci s.s.*, *D. gigas* and *D. destructor* (Appendix 3). A real-time PCR test for detection and identification of

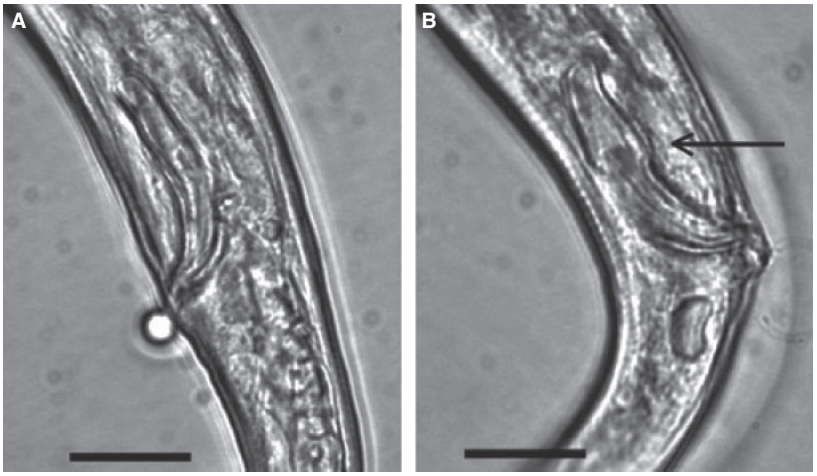

Fig 16 *Ditylenchus* spiculum photographs: (A) *D. dipsaci*; (B) *D. destructor* (arrow = tumulus). Each bar = 12 µm After Karssen & Willemsen (2010).

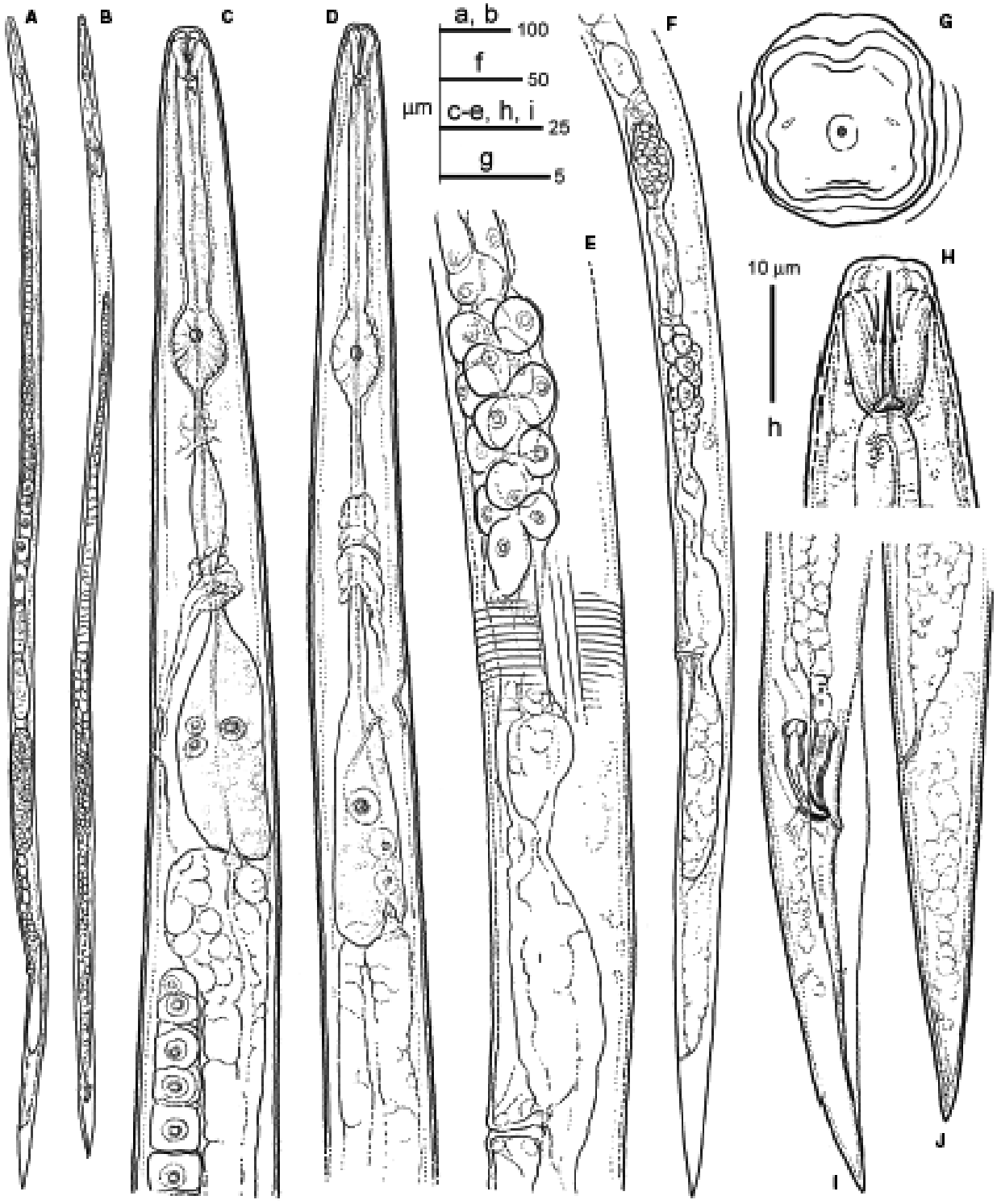

Fig 17 *Ditylenchus gigas*: (A) female; (B) male; (C) female pharyngeal region; (D) male pharyngeal region; (E) quadricolumella and vulval region; (F) female posterior body region; (G) female *en face* view showing quadrangular outline of the first lip annulus (amphidial apertures are ideally reconstructed); (H) female (holotype) lip region; (I) male tail; (J) female tail. After Vovlas *et al.* (2011).

D. dipsaci and *D. destructor*, based on SSU rDNA, has been developed by ClearDetections (Appendix 4). A PCR test with specific primers for *D. dipsaci*, *D. destructor* and *D. gigas* is described by Jeszke *et al.* (2015); this has also been adapted for real-time PCR. The real-time PCR test described in Appendix 5 only covers *D. dipsaci* and

D. gigas because the identification of *D. destructor* is not described in sufficient detail. The original paper also describes a conventional PCR.

Other molecular tests for *Ditylenchus* species exist (Esquibet *et al.*, 2003; Subbotin *et al.*, 2005; Marek *et al.*, 2005; Zouhar *et al.*, 2007) and are described in the IPPC Protocol (IPPC, 2016).

4.2.2. DNA barcoding

A protocol for DNA barcoding based on 18S rDNA and 28S rDNA is described in Appendix 5 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016)*: DNA barcoding of nematodes* and can support the identification of *D. dipsaci* and *D. destructor*. Sequences are available in Q-bank (http://www.q-bank.eu/Nematodes/) and other databases recommended in PM 7/129.

5. Reference material

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Pest and Disease Identification Team, Fera Science Limited, Sand Hutton, York YO41 1LZ (GB).

Plant Protection Service Servizio Fitosanitario Regionale Via di Saliceto, no. 81, 40128 Bologna (IT).

National Plant Protection Organization, National Reference Center PO Box 9102, 6700 HC Wageningen (NL). Reference material can be found through different resources (e.g. Q-bank, http://www.q-bank.eu/Nematodes/; NCE, http://www.nce.nu/).

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77(1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and consultation of this database is recommended as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this organism can be obtained from: Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11/12, 38104 Braunschweig (DE); Plant Protection Service, Diagnostic Centre, PO Box 9102, 6700 HC Wageningen (NL); Istituto per la Protezione delle Piante, Sezione di Bari, C.N.R., Via G. Amendola, 165/A, 70126 Bari (IT); Pest and Disease Identification Team, Fera Science Limited, Sand Hutton, York YO41 1LZ (GB); Department of Plant Protection Biology – Nematology, Swedish University of Agricultural Sciences, Alnarp (SE).

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included in it, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was originally drafted by D. Sturhan (retired), formerly Federal Biological Research Centre for Agriculture and Forestry, now Julius Kühn-Institute (DE). The molecular part has been added by S Subbotin, Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832 (US) and G Anthoine LNPV-Unité de Nematologie, Domaine de la Motte au Viconte BP 35327 Le Rheu (FR). The revision has been prepared by J. Hallmann (Julius Kühn-Institut, DE) and L. Pylypenko (National Academy of Agrarian Sciences of Ukraine). It was reviewed by the Panel on Diagnostics in Nematology.

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Appendix 1 – ITS rDNA PCR-RFLP test for *D. dipsaci* and *D. destructor* (Wendt *et al.*, 1993)

This test is quite old and some of the reagents and equipment may have changed. It is strongly recommended to validate this protocol under the operator's laboratory conditions.

1. General information

1.1 Protocol developed by Wendt *et al.* (1993).

1.2 The test can only be used on nematodes morphologically identified as *Ditylenchus* spp., as the primers are not specific for *Ditylenchus* spp.

1.3 Individuals from *D. dipsaci* (in the paper referred to as a host race), *D. gigas* (in the paper mentioned as *D. dipsaci* giant race), *D. destructor* and *Ditylenchus myceliophagous* are extracted from plant material.

1.4 The targeted region is the ITS region (including ITS1, ITS2 and the 5.8S rDNA gene). They are not specific regions.

1.5 Oligonucleotides: ITS-specific universal primers described by Vrain *et al.* (1992): 18S (5′-TTG ATT ACG TCC CTG CCC TTT-3′) and 26S (5′-TTT CAC TCG CCG TTA CTA AGG-3′). The amplicon is approximately 900 bp for *D. dipsaci, D. gigas* and *D. myceliophagus*, and 1200 bp for *D. destructor.*

1.6 The amplification mix is provided in a kit containing Taq DNA polymerase, nucleotides and reaction buffer (Perkin Elmer).

1.7 Amplification is performed in a thermocycler (e.g. Twin Block System EC Cycler, Ericomp).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Nematodes that had migrated to the lid of a Petri dish are rinsed off with 0.05 M NaCl, and concentrated by centrifugation at 2000 rpm for 2 min at room temperature. The NaCl solution is discarded and the nematodes resuspended in seven volumes of Proteinase K buffer (0.1 M Tris pH = 8.0, 0.05 M EDTA, 0.2 M NaCl and 1% SDS) containing 1.0 mg mL^{-1} proteinase K. The solution containing the nematodes is frozen in liquid nitrogen, transferred to a mortar and ground into a fine powder. After thawing the solution is transferred to a 50 mL Falcon tube and extracted three times with TE (10 mM Tris pH = 8.0 and 1.0 mM EDTA)-saturated phenol pH 8.0 and twice with 24:1 chloroform, iso-amyl alcohol. The clean DNA is precipitated by two volumes of 95% ethanol, pelleted, dried and redissolved in TE with 10 μg mL^{-1} RNAse A (Webster *et al.*, 1990).

2.1.2 Store overnight at 4°C or at –20°C for longer periods.

2.2 Polymerase chain reaction

2.2.1 Master mix

Reagent*	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water†		60.8	
Taq DNA polymerase buffer	10×	10	1×
$MgCl_2$ (if not included in the Taq DNA buffer)	25 mM	8	2 mM
dNTPs	10 mM each	1	100 μM each
Forward primer 18S	10 μM	5	0.5 μM
Reverse primer 26S	10 μM	5	0.5 μM
Taq DNA Polymerase (MP Biomedicals, ex Appligene Oncor)	5 U/μL	0.2	0.5 U
Subtotal		90	
Genomic DNA		10	20 ng
Total		100	

*Quantities in the table are based on laboratory practice.
†Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.

2.2.2 PCR cycling parameters

1 cycle of 1.5 min at 96°C, 30 s at 50°C, 4 min at 72°C; 40 cycles of 45 s at 96°C, 30 s at 50°C, 4 min at 72°C; 1 cycle of 45 s at 96°C, 30 s at 50°C, and a final extension of 10 min at 72°C.

2.3 Restriction of PCR amplicon

2.3.1 Reaction mix

Reagent*	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water†	N.A.	12.8	N.A.
Restriction enzyme buffer	10×	2.0	1×
Restriction enzyme (s) (*Alu*I‡)	10 U μL^{-1}	0.2	2 U
Subtotal		15	
(Purified) PCR product		5	
Total		20	

*Quantities in the table are based on laboratory practice.
†Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.
‡The same protocol for each individual restriction enzyme: *Acc*I, *Bam*HI, *Dde*I, *Dra*I, *Hae*III, *Hinc*II, *Hinf*I, *Hpa*II, *Nsi*I, *Pst*I, *Rsa*I, *Sau*3A, *Taq*I.

Table 3. Restriction patterns of *D. destructor, D. myceliophagus, D. dipsaci* and *D. gigas* with different restriction enzymes (– indicates data not available). The length of restriction bands is given in bp) (Wendt *et al.*, 1993)

	D. destructor	*D. myceliophagus*	*D. dipsaci*[*]	*D. gigas*†
Unrestricted PCR product	1200	900	900	900
*Hae*III	450, 170	450, 200	900	800, 200
*Hpa*II	1000	900	320, 200, 180	600, 200
*Hinf*I	780, 180‡	630, 310	440, 350, 150	350, 150
*Rsa*I	600, 250, 170	900	450, 250, 140	490, 450
*Acc*I	1200	900	900	–
*Alu*I	370, 290	900	900	–
*Bam*HI	1000	900	340, 220, 180	–
*Dde*I	670, 570	300, 250, 130‡	310, 290, 200	310, 290, 200
*Dra*I	1200	900	340, 250	–
*Hinc*II	900, 250	900	800	800
*Nsi*I	1200	900	900	–
*Pst*I	850, 400	620, 400	650, 400	650, 400
*Sau*3A	540, 400, 180	440, 100	340, 260, 200, 110, 100	–
*Taq*I	640, 200, 150	320, 260, 160	340, 230, 130	–

[*]Referred to as '*D. dipsaci* host race' in Wendt *et al.* (1993).
†Referred to as '*D. dipsaci* giant race' in Wendt *et al.* (1993).
‡More restriction bands is shown in Wendt *et al.* (1995).

2.3.2 Incubation time/temperature for digestion: 2 h for 37°C

3. Essential procedural information

3.1 Analysis of DNA fragments

DNA fragments are separated by electrophoresis on agarose gel and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

3.2 Identification of speciesRestriction patterns for different species are presented in Table 3.

3.3 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:[4]

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism or other nematode species (as primers used are universal), whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid using conserved primers that amplify a conserved non-target nucleic acid that is also present in the sample (e.g. eukaryotic 18S rDNA), amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls), or amplification of a duplicate sample spiked with the target nucleic acid.

3.4 Interpretation of results

Verification of the controls:

- All samples should produce an amplicon
- NIC and NAC should not produce amplicons
- PAC should produce restricted fragment lengths as given in Table 3
- When relevant the IPC should produce the expected amplicon

When these conditions are met:

- A sample will be considered positive if it produces the restriction fragment lengths as given in Table 3
- A sample will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria from Wendt *et al.*, 1993, 1995

This test was evaluated against *D. dipsaci* normal race (7 populations from different hosts and locations), *D. gigas* (2 populations isolated from *V. faba* from different locations and referred as *D. dipsaci* giant race in Wendt *et al.*, 1993), *D. destructor* (2 populations isolated from *S. tuberosum* from different locations), *D. africanus* (one population isolated from peanuts) and *D. myceliophagus* (one population).

[4]As the primers are universal the positive isolation control is not needed.

Appendix 2 – 5.8S rDNA and ITS-specific PCR test for *D. dipsaci* for separation from *D. gigas* Kerkoud *et al.* (2007)

1. General information

1.1 Protocol developed by Kerkoud *et al.* (2007).

1.2 Targets are individuals of *D. dipsaci* and *D. gigas* (in the paper mentioned as *Ditylenchus* sp. B).

1.3 The targeted regions are the 5.8S rDNA gene and flanking ITS spacer regions using specific primers for *D. dipsaci*.

1.4 Oligonucleotides: two different primers sets are available, one for the identification of *D. dipsaci* alone and one for the identification of *D. dipsaci* and *D. gigas*. The use of both primer sets allows separation of *D. dipsaci* from *D. gigas*.

The first set is composed of DdpS1 primer (5′-TGG CTG CGT TGA AGA GAA CT-3′) and rDNA2 (5′-TTT CAC TCG CCG TTA CTA AGG-3′) (Vrain *et al.*, 1992). The amplicon with this primer set is approximately 517 bp for *D. dipsaci.*

The second primer set is composed of DdpS2 primer (5′-CGA TCA ACC AAA ACA CTA GGA ATT-3′) and rDNA2 (5′-TTT CAC TCG CCG TTA CTA AGG-3′) (Vrain *et al.*, 1992). The amplicon with this primer set is approximately 707 bp for *D. dipsaci* and *D. gigas*.

The use of both primer sets allows separation of *D. gigas* from *D. dipsaci*.

1.5 Taq DNA polymerase 5 U μL^{-1} (MP Biomedicals) is used for PCR amplification.

1.6 Molecular-grade water (MGW) is used to make up reaction mixes; this should be purified (deionized or distilled), sterilized (autoclaved or 0.45 µm filtered) and nuclease free.

1.7 Amplification is performed in a thermocycler (e.g. GeneAmp PCR System 9600; applied Perkin-Elmer Inc. Foster City, CA).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Stock genomic DNA was extracted according to the phenol–chloroform procedure (Sambrook *et al.*, 1989). Following ethanol precipitation, DNA was resuspended in TE buffer (0.01 M Tris, pH 8.0; 0.001 M EDTA) and stored at –20°C.

2.1.2 A fast method was developed to obtain DNA from single individuals. Single nematodes were hand-picked, transferred onto a glass slide under a dissection microscope and crushed when dry by gentle pressure with the tip of a micropipette. The crushed nematode was recovered carefully with 13.7 µL of sterile distilled water and transferred into a 0.2 mL Eppendorf tube. The tube was immediately placed in ice. The crushed suspension was stored at –20°C and used for further study.

2.2 Polymerase chain reaction

2.2.1 Master mix for simplex PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*		9	
PCR buffer (Thermo SA,France)	10×	2	1×
$MgCl_2$ (if not included in the Taq DNA buffer)	25 mM	1.6	2 mM
dNTPs	20 mM	0.2	200 µM each
Forward primer DdpS1 (or DdpS2)	10 µM	1	0.5 µM
Reverse primer rDNA2	10 µM	1	0.5 µM
Taq DNA polymerase (Thermo SA, France)	5 U μL^{-1}	0.2	1 U
Subtotal		15	
Genomic DNA		5	5 ng
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.2 Master mix for duplex PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*		6	
PCR buffer (Thermo SA, France)	10×	2	1×
$MgCl_2$ (if not included in the Taq DNA buffer)	25 mM	1.6	2 mM
dNTPs	20 mM	0.2	200 µM each
Forward primer DdpS1	10 µM	1	0.5 µM
Forward primer DdpS2	10 µM	2	1.0 µM
Reverse primer rDNA2	10 µM	2	1.0 µM
Taq DNA polymerase (Thermo SA, France)	5 U μL^{-1}	0.2	1 U
Subtotal		15	
Genomic DNA		5	5 ng
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.2 PCR cycling parameters:

1 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C and a final extension of 10 min 72°C.

Table 4. PCR patterns of *D. dipsaci, D. destructor, D. africanus, D. myceliophagus* and *Ditylenchus gigas* (Kerkoud *et al.*, 2007)

	D. dipsaci	*D. destructor*	*D. africanus*	*D. myceliophagus*	*Ditylenchus gigas*[*]
DdpS1-rDNA2	517 bp	–	–	–	–
DdpS2-rDNA2	707 bp	–	–	–	707 bp

[*]Referred to as '*Ditylenchus* sp. B. from *Vicia faba*' in Kerkoud *et al.* (2007).

3. Essential procedural information

3.1 Analysis of DNA fragments

DNA fragments are separated by electrophoresis on agarose gel and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

3.2 Identification of species (Table 4)

3.3 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or a synthetic control (e.g. a cloned PCR product).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify a conserved non-target nucleic acid that is also present in the sample (e.g. eukaryotic 18S rDNA), amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls), or amplification of a duplicate sample spiked with the target nucleic acid.

3.4 Interpretation of results:

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC and PAC should produce restricted fragment lengths as given in Table 4
- When relevant the IPC should produce the expected amplicon

When these conditions are met:

- A sample will be considered positive if it produces the amplicons as given in Table 4
- A sample will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

From Kerkoud *et al.* (2007):

This test was evaluated against *D. dipsaci* (ten populations from different hosts and locations); *D. africanus*, *D. destructor*, *D. myceliophagus*, *Anguina tritici*, *Aphelenchoides ritzemabosi* (one population for each species) and *D. gigas* (according to the paper – *Ditylenchus* sp. isolated from *V. faba* from different locations).

Appendix 3 – Multiplex PCR according to Marek *et al.* (2010)

1. General information

1.1 Protocol developed by Marek *et al.* (2010).

1.2 Nematode individuals are extracted from plant material (especially potato tuber tissue).

1.3 The targeted region is the ITS region (including ITS1, ITS2 and the 5.8S rDNA gene).

1.4 Oligonucleotides: *D. dipsaci s.s.*, *D. gigas* (in the paper mentioned as *Ditylenchus* sp. B).
Forward primer DipU-F (5′-CCC ATT TTT GAA CTT TTT TAC AAG-3′); reverse primer DipU-R (5′-CTA GAT TAG CAA AGA CGT ATA TC-3′). The amplicon with this primer set is approximately 333 bp for *D. dipsaci s.s.* and *D. gigas*.
Ditylenchus dipsaci s.s.
Forward primer DipU-F (5′-CCC ATT TTT GAA CTT TTT TAC AAG-3′); reverse primer Dip1-R (5′-GAA AAG CAC CCA ACC AGT ACC-3′). The amplicon with this primer set is approximately 256 bp for *D. dipsaci s.s*.
Ditylenchus destructor
Forward primer Des2-F (5′-GTG CTT GTA TTT GCG GTT GTG-3′), reverse primer Des1-R (5′-TGC TAG GCC AAA GAG ACA GC-3′). The amplicon with this primer set is approximately 453 bp for *D. gigas*.

1.5 Taq DNA Polymerase 5 U μL^{-1} (Fermentas) is used for PCR amplification.

1.6 Amplification is carried out in an automated thermal cycler (e.g. PTC 200, MJ Research Inc).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 DNA is extracted from nematode-infested plant tissues (especially potato tuber tissue). Approximately 0.5–1.0 g plant tissue naturally or artificially infested with 30 individuals of an appropriate phytonematode species is crushed in liquid nitrogen using a mortar and pestle and homogenized in 300 µL lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 0.4 mg mL^{-1} proteinase K). The mixture is incubated for 1 h at 37°C with shaking and finally denaturated for 5 min at 85°C. The homogenate is mixed 1:1 with phenol (pH 8.0)–chloroform–isoamylalcohol (25:24:1), vortexed for 15 min and centrifuged at 7000*g*. Each lysate (water phase) is transferred to a new tube, an equal volume of chloroform added, and the extraction repeated. DNA is precipitated with an equal volume of isopropanol at −20°C overnight or in liquid nitrogen for 20 min and centrifuged at 10 000*g* for 10 min. The supernatant is removed and the remaining pellets vacuum-dried. The pellets for each sample are resuspended in 50 µL TE (10 mM Tris, 1.0 mM EDTA, pH 8.0) or double-distilled water. DNA is stored at −20°C. The working stock of DNA for PCR amplification is diluted to approximately 100 ng mL^{-1} after quantification using a Helios Gamma spectrophotometer (ThermoSpectronic).

2.2 Polymerase chain reaction

2.2.1 Master mix for multiplex PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		10.45	
PCR buffer (Fermentas)	10×	2.5	1×
$MgCl_2$ (if not included in the Taq DNA buffer)	25 mM	1.5	1.5 mM
dNTPs	20 mM	0.25	200 µM
Forward primers (DipU-F and Des2-F)	100 µM	0.1	0.4 µM
Reverse primer (DipU-R, Dip1-R and Des1-R)	100 µM	0.1	0.4 µM
Taq DNA polymerase (Fermentas)	5 U/µL	0.3	1.5 U
Subtotal		20	
Genomic DNA		5	100 ng
Total		25	

2.2.2 PCR cycling parameters:
2 min at 94°C; 30 cycles of 30 s at 94°C, 20 s at 60°C, 1 min at 72°C and a final extension of 7 min at 72°C.

3. Essential procedural information

3.1 Analysis of DNA fragments:
DNA fragments are separated by electrophoresis on agarose gel (1.2% agarose) and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989). The lengths of the DNA fragments are estimated by comparison with the MassRuler Low Range 100-bp DNA ladder or MassRuler MixDNA ladder (Fermentas).

3.2 Identification of species (Table 5)

Table 5. PCR patterns of *D. dipsaci, D. gigas* and *D. destructor* (Marek *et al.*, 2010)

	D. dipsaci (sensu stricto)	*D. gigas*	*D. destructor*
DipU-F–DipU-R	333	332	–
DipU-F–Dip1-R	256	–	–
Des2-F–Des1-R	–	–	453

3.3 PCR amplification of the host chloroplast gene of ribulose-1.5-(bis)-phosphate-carboxylase is used as an internal PCR control. The nucleotide sequences of these plant-specific primers (Heinze, 2007) are: forward primer, Rbcl-F (5′-AGT CAT GCA TTA CGA TCC GAA-3′); reverse primer, Rbcl-R (5′-CCA GTC TTG ATC GTT ACA AA-5′); the expected length of PCR product is 582 bp.

3.4 Controls
For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or a synthetic control (e.g. a cloned PCR product).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic

acid, using conserved primers that amplify a conserved non-target nucleic acid that is also present in the sample (e.g. eukaryotic 18S rDNA), amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls), or amplification of a duplicate sample spiked with the target nucleic acid.

3.5 Interpretation of results

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC and PAC should produce restricted fragment lengths as given in Table 5
- When relevant the IPC should produce the expected amplicon

When these conditions are met:

- A sample will be considered positive if it produces the amplicons given in Table 5
- A sample will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Data from Marek *et al.* (2010).

This test was evaluated in bioinformatics-based and laboratory tests against *D. dipsaci s.s.* (thirteen European populations from different hosts), *D. gigas* (three European populations from *V. faba*; referred to as *Ditylenchus* sp. B in *Marek et al.,* 2010), *D. destructor* (four European populations from *S. tuberosum*), *Ditylenchus askenasyi* (one European population), *D. myceliophagus* (one European population) as well as non-target species (*Heteroanguina ferulae*, *A. tritici*, *Globodera pallida*, *Globodera rostochiensis*, *Caenorhabditis elegans*, *Rhabditis* spp.); no amplification products were observed for non-target phytonematodes, or for free-living nematodes tested.

Appendix 4 – Diagnostic real-time PCR tests for identification and detection of *D. dipsaci* and *D. destructor*, based on SSU rDNA (provided by ClearDetections)

1. General information

Scope of the tests: identification of *D. dipsaci* and *D. destructor* nematodes by real-time PCR and detection of *D. dipsaci* and *D. destructor* nematodes in both simple (plant material) and complex (soil or substrate) DNA backgrounds by real-time PCR.

1.1 These tests targets the SSU (18S) rDNA gene.

1.2 Amplicon sizes: *D. dipsaci* 87 bp; *D. destructor* 635 bp.

1.3 Oligonucleotide sequences are not disclosed. These tests are available as all-inclusive real-time PCR kit (ClearDetections, the Netherlands; http://www.cleardetections.com)

2. Methods

2.1 Nucleic acid extraction

These real-time PCR tests can be combined with any nematode DNA extraction method delivering target DNA. Validation was performed with the Nematode DNA Extraction and Purification Kit from ClearDetections. When using this test for nematode quantification purposes it is highly recommended to include an (internal or external) DNA standard in the extraction procedure to correct for potential DNA losses during the DNA extraction and purification process.

2.2 Real-time PCR

The real-time PCR kit includes target and general nematode DNA real-time PCR primer sets, positive amplification control(s) (PACs) and PCR mix with fluorescent DNA-binding dye.

2.2.1 PCR cycling conditions:

Enzyme activation: 3 min at 95°C. Amplification: 35 cycles of 10 s at 95°C, 1 min at 63°C, 30 s at 72°C. Melt curve: 0.2–0.5°C steps 72°C → 95°C.

3. Essential procedural information

3.1 **Controls**

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism. Alternatively, the all-inclusive real-time PCR kit contains a separate real-time PCR primer set for the detection of 'nematode DNA', which can be used to check for the presence and quantity of nematode DNA in the nucleic acid sample.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PACs, included in the kit) to monitor the efficiency of the amplification of nucleic acid of the target organism. This can include genomic DNA extracted from the target organism, a cloned PCR product (plasmid DNA) or synthetic DNA.

3.2 Interpretation of results

In order to obtain results from PCR-based tests the following criteria should be met:

Verification of the controls:

- The PAC and PIC amplification curves should be exponential
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve
- A test will be considered negative if it produces no exponential amplification curve or if it produces a curve which is not exponential
- A melt curve analyses is performed and the obtained melting temperature (T_M) value equals the T_M value of the PAC ($\pm$1°C)
- Tests should be repeated if any contradictory or unclear results are obtained
- The real-time PCR primer set included in the kit for the detection of 'nematode DNA' can be used when in doubt about the presence of nematode DNA in a DNA sample (check for possible false negatives)

4. Performance criteria available

These real-time PCR tests are validated in line with PM 7/98.

4.1 Analytical sensitivity: less than one individual nematode (about 3 cells of the target nematode).

4.2 Diagnostic sensitivity: 100%

4.3 Analytical specificity

Specificity value 100%

In silico primer design: 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 motifs for *D. dipsaci* and *D. destructor*.

Specificity of the primer sets was tested with plasmid DNA (SSU rDNA) and genomic DNA of relevant non-target nematode species. Possible false positives were selected *in silico*, based on sequence identity, and on phylogenetic analysis (close relatives). Unfortunately, only a few *Ditylenchus* species were included, as there was no DNA available from other *Ditylenchus* species. Primer sets were tested against: *D. dipsaci*, *D. destructor*, *D. ferepolitor*, *D. adasi*, *Anguina tritici*, *Subanguina radicicola*, *Paraphanolaimus behningi*, *Psilenchus hilarulus*, *Eumonhystera* sp., *Cephalanchus hexalineatus*, *Metateratocephalus crassidens*, *Aulolaimus oxycephalus*, *Clarkus pappilatus*, *Monoposthia* sp., *Mylonchulus sigmaturus*, *Wisonema otophorum*, *Meloidogyne javanica* and *Seinua tenuicaudata.*

Target nematode species were identified microscopically and originated from the Netherlands and Germany respectively.

4.4 Diagnostic specificity: 100%

4.5 Reproducibility: 100%

4.6 Repeatability: 100%

4.7 Accuracy: 100%

4.8 Dynamic range: between 10–100 and 0.1 billion copies of target DNA

4.9 Selectivity: 100%

4.10 Robustness: no real-time PCR failure is observed when the primer combinations are exposed to a temperature gradient. With a deviation in annealing temperature (T_a) of $\pm$1.0°C from the normal T_a (63°C), all ΔCt values remain <1. The real-time PCR tests for the detection of *D. dipsaci* and *D. destructor* are robust.

Appendix 5 – Real-time PCR tests for identification of *D. dipsaci* and *D. gigas* according to Jeszke *et al.* (2015)

1. General information

1.1 Protocol developed by Jeszke *et al.* (2015). The test described here only covers *D. dipsaci* and *D. gigas* because the identification of *D. destructor* is not described in sufficient detail. The approach is based on DNA intercalating dyes (EvaGreen or Sybr-Green). The article also describes a conventional PCR.

1.2 DNA is extracted from a single nematode or a few nematodes.

1.3 The targeted region is ITS1 rDNA.

1.4 Oligonucleotides:

Ditylenchus dipsaci, *D. gigas*

The forward universal primer DITuniF 5′- CTG TAG GTG AAC CTG C -3′.

Ditylenchus dipsaci

The reverse species specific primer DITipR 5′- GAC ATC ACC AGT GAG CAT CG -3′.

Ditylenchus gigas

The reverse species-specific primer DITgigR 5′- AC CAC CTG TCG ATT C-3′.

1.5 Allegro Taq DNA Polymerase (Novazym) is used for PCR amplification.

1.6 The amplification is carried out in an automated thermal cycler (e.g. a Rotor Gene 6000, Corbett Research).

2. Methods

Nucleic acid extraction and purification

2.1 DNA was extracted with DNeasy Blood and Tissue kit reagents (Qiagen) according to the

manufacturer's recommended procedure with a final volume of 50 µL.

2.2 Polymerase chain reaction

2.2.1 Master mix for singleplex real-time PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		6.76	
EvaGreen dye (Biotium)	20×	0.5	1×
Allegro Taq polymerase buffer	10×	1	1×
$MgCl_2$ (if not included in the Taq DNA buffer)	25 mM	0.4	1 mM
dNTPs	20 mM	0.1	200 µM
Forward universal primer (DITuniF)	100 µM	0.1	1 µM
Reverse primer (DITipR, or DITgigR)	100 µM	0.1	1 µM
Allegro Taq polymerase (Novazym)	5 U/µL	0.04	0.2 U
Subtotal		9	
Genomic DNA		1	2 ng
Total		10	

2.2.2 Simplex real-time PCR cycling parameters: 5 min at 95°C; 35 cycles of 20 s at 95°C, 20 s at 63.5°C, 30 s at 72°C at 82°C for 15 s for the DITuniF/DITdipR and DITuniF/DITgigR primer sets.

3. Essential procedural information

3.1 The melting phase begins at 72°C and ends at 95°C, with an increase of 1°C in each step.

3.2 Identification of species (Table 6)

3.3 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. eukaryotic 18S rDNA), amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls), or amplification of a duplicate sample spiked with the target nucleic acid.

3.4 Interpretation of results

Verification of the controls:

- The PAC and PIC amplification curves should be exponential
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve
- A test will be considered negative if it produces no exponential amplification curve or if it produces a curve which is not exponential
- A melt curve analysis is performed and the obtained T_M value is as expected (see Table 6)
- Tests should be repeated if any contradictory or unclear results are obtained

Table 6. PCR product sizes and melting temperatures of *D. dipsaci* and *D. gigas* (Jeszke *et al.*, 2015)

	PCR product size (bp)	PCR product melting temperatures, T_M
D. dipsaci	148	86
D. gigas	270	88

4. Performance criteria available

Data from Jeszke *et al.* (2015).

Analytical sensitivity: one specimen (it is possible to detect 0.016 ng of *Ditylenchus* DNA in one reaction).

Analytical specificity: this test was evaluated in bioinformatics-based and laboratory tests against *D. dipsaci s.s.* (twelve European populations from different hosts), *D. gigas* (seven European populations from *V. faba*), *D. destructor* (six Polish populations from *S. tuberosum*), *D. acutus* (one Polish population from *H. vulgare*), *Ditylenchus* sp.1 (one Polish population from *H. vulgare*), *Ditylenchus* sp.2 (one Polish population from *H. vulgare*); no amplification products were observed for non-target nematodes tested.