Diagnostics
Diagnostic

Nacobbus aberrans sensu lato

Specific scope

This standard describes a diagnostic protocol for *Nacobbus aberrans sensu lato*[1].

Specific approval and amendment

Approved in 2000–09. Revision approved in 2009–09.

Introduction

The genus *Nacobbus* includes the two species *N. dorsalis* and *N. aberrans*, but the existence of distinct genotypes (Reid *et al.*, 2003; Vovlas *et al.*, 2007) and observations on reproductive isolation (Anthoine & Mugniéry, 2006) suggests the existence of additional species. While *N. dorsalis* is rare, *N. aberrans* is an important agricultural pest of sugar beet in North America (Mexico and Western USA) and of potatoes in South America. *Nacobbus aberrans* has a very wide host range including members of many plant families, but not *Poaceae*. The nematode has three physiological races, i.e. sugar beet-, potato-, and bean race (Manzanilla-López *et al.*, 2002). It is an endoparasite of roots in which the female swells to become sack-shaped. Obligatory amphimixis has been demonstrated in several South American populations (Anthoine & Mugniéry, 2005b). *Nacobbus* spp. induce similar galls to those caused by root-knot nematodes (*Meloidogyne* spp.) and are, therefore, commonly named the 'false root-knot nematode'.

It infests plants of the families Amaranthaceae, Apiaceae, Asteraceae, Basellaceae, Brassicaceae, Cactaceae, Caryophyllaceae, Chenopodiaceae, Convolvulaceae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Labiatae, Malvaceae, Nyctaginaceae, Oxalidaceae, Plantaginaceae, Polygonaceae, Portulacaceae, Solanaceae, Tropaeolaceae and Zygophyllaceae (Manzanilla-López *et al.*, 2002; and others). It is found on important food crops, such as cabbage, carrot, cucumber, lettuce, mustard, pea, potato, sugar beet and tomato (Canto, 1992). As a soil-borne pest, it can also be spread by means of the soil adhering to agricultural machinery and other commodities.

The taxonomy of *N. aberrans* is not agreed among experts (Sher, 1970; Baldwin & Cap, 1992) and the pest covered in this protocol is *N. aberrans sensu lato,* which can include *Nacobbus batatiformis* from North America and *Nacobbus serendipiticus* as well as *N. aberrans sensu stricto* from South America (see list of synonyms below).

For more general information about *N. aberrans* as a quarantine pest, see EPPO/CABI (1997).

Identity

Name: *Nacobbus aberrans* (Thorne, 1935) Thorne & Allen, 1944 (*sensu lato*)
Synonyms: *Anguillulina aberrans* Thorne, 1935
Pratylenchus aberrans (Thorne, 1935) Filipjev, 1936
Nacobbus batatiformis Thorne & Schuster, 1959
Nacobbus serendipiticus Franklin, 1959
Nacobbus serendipiticus bolivianus Lordello, Zamith & Boock, 1961
Taxonomic position: Nematoda: Tylenchida[2]: Pratylenchidae
EPPO code: NACOBA
Phytosanitary categorization: EPPO A1 list no. 144; EU Annex designation I/A1.

Detection

Symptoms

Nacobbus aberrans can be found in the roots and adhering soil of a wide range of imported plant material. In particular, it can be found within potato tubers, which may or may not show external symptoms.

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

Above-ground symptoms of heavily infested plants include stunting and yellowing, while below ground, galling is typical. The galls are similar to those caused by some *Meloidogyne* species but often occur along the root as discrete and rounded swellings, like beads on a string.

Extraction

In order to identify any nematodes that might be present on an imported commodity, it is necessary to extract specimens from the roots, potato tubers or from soil or growing medium. Mature females can be observed within the roots by means of a dissecting microscope using transmitted light. They can be extracted by dissecting apart the tissues but then should be stored in a 0.9% solution of NaCl in order to avoid possible osmotic disruption in plain water. Other stages of the species can be obtained from plant tissues or soil by suitable extraction techniques (Appendix 1).

Identification

Identification is based on morphological characteristics; molecular methods may be used to confirm *Nacobbus aberrans* identification (Appendix 2).

Morphology

As a plant-parasitic nematode belonging to the order Tylenchida, *N. aberrans* has the following characteristics of that order: annulated cuticle; small, pocket-like amphids opening on the lip region; hollow, axial stylet in the stoma which can be protruded for feeding, excretory system with a single lateral canal; pharynx divided into anterior procorpus, metacorpus with metacorpus plates, isthmus surrounded by nerve ring and a glandular basal portion; dorsal pharyngeal gland open into the lumen of the pharynx just behind the base of the stylet.

Nacobbus is the only member of the family *Pratylenchidae* with swollen females. Otherwise, the genus has the characteristics of the family: lip region low with distinct sclerotization; strong stylet with distinct stylet knobs; pharyngeal glands in a long lobe overlapping the intestine; phasmids post-anal near centre of tail; male bursa terminal or sub-terminal.

Description of Nacobbus aberrans (Stone & Burrows, 1985)

See Fig. 1 for position of important diagnostic features. The code letters used in the description are explained in the glossary of nematological terms available on the EPPO website (see http://archives.eppo.org/EPPOStandards/diagnostics.htm).

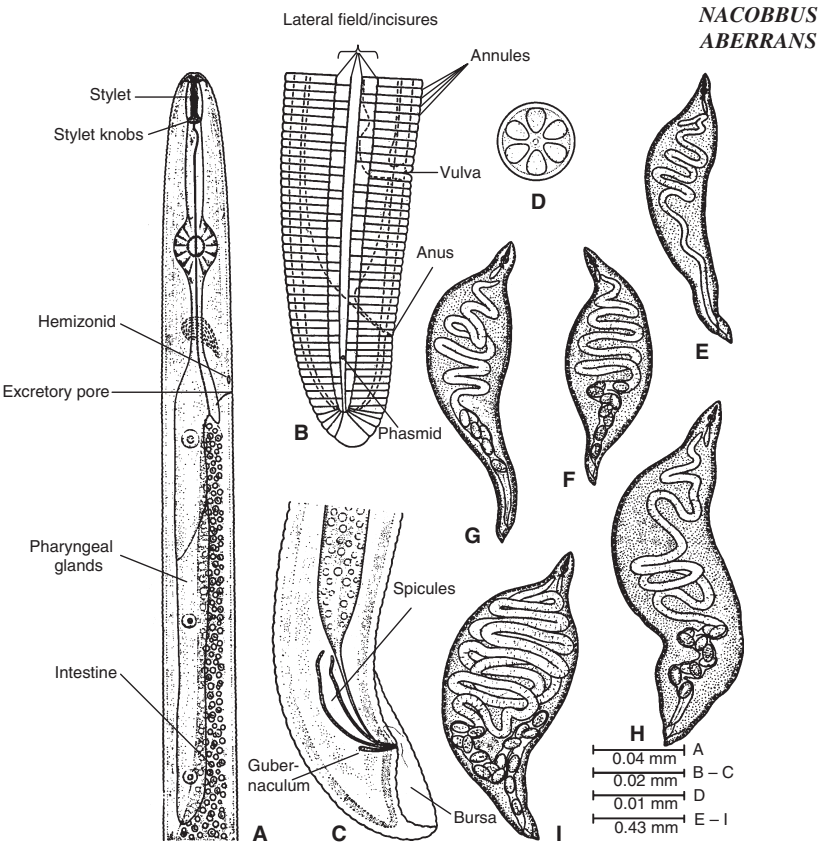

Fig. 1 *Nacobbus aberrans* (A, B): immature female, anterior and posterior regions. (C, D) Male, tail and *en face* view. (E) Mature female, early stage. (F–I) Mature female, successively later development stages (adapted from Sher, 1970, Courtesy *Journal of Nematology*).

Mature female: L = 0.7–1.9 mm; width = 0.3–0.6 mm; stylet = 20–24 µm. Body oval, swollen and white-cream, tapering anteriorly and posteriorly. Lip region annules 3–4. Stylet slender with small rounded knobs. Metacorpus and pharynx well developed, pharyngeal glands overlapping intestine dorsally. A single coiled ovary is present. Vulva and anus located posteriorly sub-terminal. Eggs are deposited in a gelatinous matrix extruded by the female.

Immature female: L = 0.6–0.9 mm; a = 23–40; c = 24–40; V = 91–94; VA = 15–24; stylet = 20–24 µm. Body vermiform with bluntly rounded tail 10–17 annules in length. Lateral field with 4 incisures. Lip region not set off, 3–4 annules present. Stylet robust with rounded knobs. Pharyngeal glands overlapping intestine dorsally. Vulva located about 35 annules anterior to the tail tip. Phasmids posterior to the anus.

Male: except for sexual dimorphism, similar to immature female. Body vermiform with short arcuate tail, equal in length to anal body width, enveloped by a bursa arising close behind position of the anterior spicule; bursa terminal. Lip region with 4 annules. Stylet with robust knobs, projecting anteriorly. Spicules typically tylenchoid, gubernaculum simple.

Second-stage juveniles: L = 300–380 µm; a = 18–26; c = 10–14; width = 12–15 µm; stylet = 11–13 µm. Body vermiform with regularly annulated cuticle and four incisures in lateral field. Lip region set off, 3 annules present. Stylet robust with rounded knobs. Hemizonid anterior to excretory pore. Phasmids small, posterior to the anus, tail bluntly rounded.

If galls are found on roots, all stages of the nematode may be obtained, particularly mature swollen females, immature females, males and 2nd stage juveniles. These should have the characters described above for *N. aberrans* and, especially, the characters to distinguish them from *N. dorsalis* and *Meloidogyne* spp. (see below). If galls are not found but motile nematode stages are obtained from plant tissues (roots or tubers), these should be distinguished from *N. dorsalis* and other endoparasitic nematodes. If galls are not found but motile nematode stages are obtained from soil, these should be distinguished from all other soil-inhabiting nematodes. For motile stages of *N. aberrans*, the key diagnostic features are:

- tylenchid stylet with well developed basal knobs
- lip region shape of males and immature females not off-set
- pharyngeal gland overlapping the intestine dorsally
- lateral field with four incisures
- single ovary
- vulva close to the anus, sub-terminal
- male with bursa
- all measurements within the ranges given above.

Comparison with similar species and genera

The motile stages of *Nacobbus* spp. may be confused with other nematodes of the *Pratylenchidae*, especially endoparasitic species. They can be distinguished from other genera by the combination of the following features: long dorsal overlap of the pharyngeal glands, single ovary, terminal male bursa, and by the short tail length (Luc, 1987).

Although *Nacobbus* spp. induce similar galls to those of *Meloidogyne* spp., the genus *Nacobbus* is relatively easy to separate from *Meloidogyne*. The two genera differ in the pharyngeal gland overlap (dorsal for *Nacobbus* and ventral for *Meloidogyne*), the number of female ovaries (one compared to two, respectively), the immature female shape (vermiform compared to rounded, respectively), the immature female feeding behaviour (migratory versus sedentary, respectively), the presence of a male bursa in *Nacobbus*, and a rounded tail in the second-stage juvenile (compared to tapering in *Meloidogyne*).

Nacobbus dorsalis differs from *N. aberrans* in the number of annules between vulva and anus (VA = 8–14 in *N. dorsalis* compared to VA = 15–24 in *N. aberrans*) and a more posterior vulval position in the immature females (94–97% compared to 91–94%, respectively). Mature *N. dorsalis* females are rounder compared to the spindle-shaped *N. aberrans* females. For a description of *N. dorsalis*, see Thorne & Allen (1944).

Molecular methods

Molecular methods can be used to confirm *N. aberrans* detection and identification, especially when operators are not familiar with this nematode. Two approaches were recently developed. The one described by Atkins *et al.* (2005) is used for specific detection of *N. aberrans* by direct DNA extraction from soil or from tuber combined to a species-specific PCR. This method applies to quite high level of contamination (3000 2nd stage juveniles in 100 g of soil; 5 2nd stage juveniles in 0.1 g of tuber).

The other method developed by Anthoine & Mugniéry (2005a) follows the extraction of nematodes from soils or roots and the observation of morphological and morphometrical features. DNA extracted from isolated nematodes, even a single one, is subjected to species-specific PCR. This method applies to migratory stages.

These two methods are based on detection of minor differences in the ITS-rRNA sequences of *N. aberrans*.

Particular attention must be given to results of molecular methods, as these methods are not fully validated regarding the geographical diversity of nematodes and thus possible variability of DNA (see also Lax *et al.*, 2007).

Descriptions of protocols for the PCR with species specific primers methods are given in details in the Appendix 2.

Reporting and documentation

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

Further information

Further information on this organism can be obtained from:

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Acknowledgements

This protocol was originally drafted for EPPO by: G Karssen & L den Nijs, Plant Protection Service, National Reference Laboratory, Wageningen (NL). It has been revised by G Anthoine (French National Laboratory, Nematology section, Le Rheu, FR), G Karssen (see above) and C Magnusson (Norwegian Institute for Agricultural and Environmental Research, Aas, NO).

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Appendix 1 – Extraction of motile stages of *Nacobbus aberrans* from tubers, roots and soil

Examples are given here of some methods that are known to be effective for obtaining specimens for identification.

Extraction from potato tubers

Juveniles and immature females and males overwinter in tubers and can be found in the superficial (0.5 mm) tuber tissue. Extraction from tubers is important in inspection work, and can be made by incubating thin (2 mm) peels of the tubers on paper filter in the Baermann funnel (Fig. 2) for 24–72 h (Manzanilla-López *et al.*, 2002). After the extraction the migratory stages can be obtained by releasing a small amount of water from the base of the funnel into a glass dish. Any nematodes recovered are examined under a dissecting microscope at ×25 magnification. Direct dissection of the superficial tuber tissue is also possible.

Alternatively all stages of the nematode can be extracted from tubers by maceration and filtration. Here tuber peels (0.5 mm thick) are macerated in a blender. The resulting suspension is

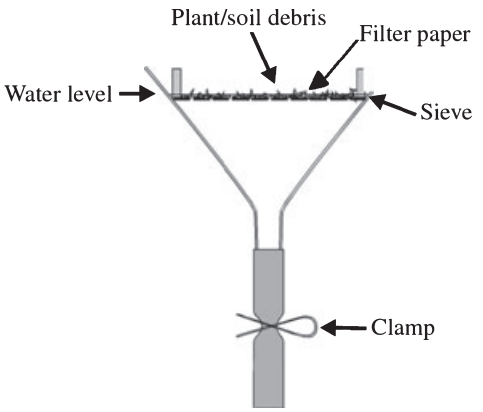

Fig. 2 Baermann funnel for extracting nematodes from roots or soil.

passed through a bank of sieves with decreasing apertures. Debris is collected on a 250 or 150 μm-aperture sieve and the nematodes will collect on a 45-μm aperture sieve. Starch grains will complicate the examination of the suspension from the 45 μm-sieve unless it is heated. Nematodes can be made more visible by staining with 15 pPM Phloxine B in the proportion 1 part suspension to 4 parts staining solution before heating (Manzanilla-López *et al.*, 2002). Recovered nematodes are examined under the microscope at ×25 magnification.

Extraction from roots

All stages of the nematode, apart from the mature, swollen females, can be extracted from the roots of suspect plants by the method of Tarjan (1972). Roots are washed free from adhering soil and cut into short lengths (5–10 cm). These are moistened and placed in a polythene bag, which is then closed and left for approximately 7 days. During this period, most of the nematodes will leave the root tissues and can be recovered by washing the roots and the inside of the bag with a small amount of water. The efficiency of extraction can be improved by using a 1–3% solution of H_2O_2 to moisten the roots. The washing water is poured into a glass dish and examined under a dissecting microscope at ×25 magnification.

A quicker extraction method is the maceration/centrifugal/flotation method of Coolen (1979). The roots are homogenized in an electric macerator at about 12 600 rev min^{-1} for 30 s and the resulting suspension is poured onto a sieve of 1200 μm pore size and washed. The washing water that passes through the sieve is centrifuged with 1% kaolin powder at 1500 ***g*** for 4 min. The sediment is re-suspended in sucrose, $MgSO_4$ or $ZnSO_4$ solution (sucrose 484 g L^{-1}; 1.18 specific gravity, others 1.16 s.g.) and centrifuged at 1500 ***g*** for 4 min. The supernatant is poured through a sieve of maximum 20 μm pore size. Nematodes can be washed off the surface of the sieve and collected in a glass dish for examination under a microscope at ×25 magnification.

Extraction from soil

To extract immature females, males and second-stage juveniles from small amounts of adhering soil, a thin layer of soil (2.5 mm) is placed on a suitable filter, which is laid over a coarse nylon sieve. The sieve is carefully suspended over a Baermann funnel (Fig. 2) filled with water so that the soil is just wetted (Fig. 2). Over 48 h, nematodes will emerge from the soil and sink to the bottom of the funnel. This technique can be modified by sieving techniques prior to Baermann extraction. In this case, the fraction >25 μm and <1000 μm will be used for extraction. Nematode suspensions may be further concentrated by centrifugation steps following extraction.

For larger amounts of soil, standard methods for extracting motile nematodes of lengths from 0.3 to 1.2 mm from soil may be employed (Southey, 1986). For example, the following simple flotation/sieving technique may be used. A 100-g sample of soil is added to a 10-L bucket of water. The soil particles are suspended in the water by stirring vigorously for 10 s, and then allowed to settle for a further 45 s. The supernatant is poured through a bank of 3 sieves of 50-μm pore size. The soil debris collected on the sieves is washed, collected in a beaker, and poured onto a nylon sieve which is suspended on a Baermann funnel (as described above, but without the paper filter). After 24 h, nematodes can be collected by releasing a small amount of water from the base of the funnel into a glass dish for examination under a dissecting microscope at ×25 magnification. This simple method can extract nematodes for identification but fewer nematodes are recovered compared to other more complex methods.

Appendix 2 – Molecular detection of *N. aberrans*

Species-specific PCR protocol (Anthoine & Mugniéry, 2005a)

1. General information

1.1. This method was developed by Anthoine & Mugniéry (2005a,b).

1.2. The test was evaluated with two Peruvian, two Bolivian and two Argentinean *Nacobbus aberrans* populations, either able to develop on potato or not. The test was also evaluated against non target genus and species (*Meloidogyne arenaria*, *M. chitwoodi, M. fallax, M. hapla, M. hispanica, M. incognita, M. javanica, M. mayaguensis* and *M. naasi*, *Pratylenchus scribneri* and *thornei*, *Globodera pallida* and G. *rostochiensis*, *Rotylenchulus reniformis*, *Radopholus similis*, *Hirschmanniella sp*. and *Zygotylenchus guevarai).*

1.3. The target region of the primer set is located in the 18S rDNA region.

1.4. The amplicon's size is approximately 295 bp.

1.5. Primer set:
18SintF: 5′-ACG TCT AAG GAT GGC AGC AG-3′.
18SintR: 5′-AGG AGT TGA GCG GAA AAC C-3′.

1.6. Taq DNA polymerase (MP Biomedicals, exQ-Biogene, Illkirch, FR) used for the amplification.

1.7. Nucleotides are used at a final concentration of 0.2 μM each.

1.8. Taq buffer and nucleotides from MP Biomedicals (ex Q-Biogene) and molecular grade water (MGW) are used to make reaction mixes.

2. Methods

2.1. Nucleic acid extraction

DNA is extracted from handpicked individuals nematodes (approximately 10), which were placed in a drop of sterile water on a glass slide. As the drop dries, the nematodes are crushed between the glass slide and the cover slip by gentle pressure. The extract is recovered with 20 μL of lysis buffer (10 mM Tris pH = 8.8, 1 mM EDTA, 1% Nonidet P40, 100 μg per mL proteinase K) (Ibrahim *et al.*, 1994) and incubated at 60°C for 1 h and at 95°C for 10 min. This crude DNA extract can be directly used or frozen until needed. For DNA extraction of one

individual (whatever the development stage, except female), the same procedure is used but using only 10 µL of lysis buffer.

1.1. Polymerase chain reaction

2.2.1. Master mix (concentration per 25-µL single reaction)
1X Taq DNA Polymerase Buffer (MP Biomedicals, ex Q-Biogene)
1.5 mM $MgCl_2$ (MP Biomedicals, ex Q-Biogene)
0.2 mM each DNTP (MP Biomedicals, ex Q-Biogene)
0.1 µM each primer
0.5 unit Taq DNA Polymerase (MP Biomedicals, ex Q-Biogene)
5 µL crude DNA extract

2.2.2. PCR cycling conditions

Initial denaturation at 94°C for 2 min followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 67°C for 30 s, elongation at 72°C for 2 min, and final elongation at 72°C for 5 min.

3. Essential procedural information

3.1. Analysis of DNA fragments: DNA fragments are separated by electrophoresis on agarose gel (1%) and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

3.2. Identification of species: expected amplicons for *N. aberrans*: 295 bp. No amplification was observed for *Meloidogyne arenaria, M. chitwoodi, M. fallax, M. hapla, M. hispanica, M. incognita, M. javanica, M. mayaguensis* and *M. naasi, Pratylenchus scribneri* and *thornei, Globodera pallida* and *G. rostochiensis, Rotylenchulus reniformis, Radopholus similis, Hirschmanniella* sp. and *Zygotylenchus guevarai.*

3.3. A negative control (no DNA target) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen).

Species-specific PCR protocol (Atkins *et al.*, 2005)

1. General information

1.2. This method was developed by Atkins *et al.*, 2005.

1.3. The test was evaluated *in silico* against sequences from *Nacobbus aberrans s.l* (4 populations from Mexico and 1 from Equator) and *Nacobbus 'bolivianus'* (3 populations from Bolivia). The test was also evaluated *in vivo* with one Mexican *N. aberrans s.l.* population, one Bolivian *N. 'bolivianus'* population and one Argentinean *Nacobbus n. sp.* population, and non target species (*Globodera tabacum, Heterodera glycines, Meloidogyne incognita, Pratylenchus goodeyi* and *Radopholus similis*).

1.4. The target region of the primer set is located in ITS rDNA region.

1.5. No information is available on the precise position of the primers.

1.6. The amplicon's size varies from 141 to 173bp depending of the *Nacobbus* population tested. The *N. 'bolivianus'* population gave a 148-bp amplicon.

1.7. Primer set:
NacF: 5′-GAT CAT TAC ACG TAC CGT GAT GGT C-3′
NacR: 5′-CTG CTC AAC CAC GCA TAG ACG-3′

1.8. Taq DNA polymerase (RedTaq Sigma Poole, Dorset, GB) used for the amplification.

1.9. Taq buffer and nucleotides from RedTaq mega mix (Sigma) and molecular grade water (MGW) are used to make reaction mixes.

2. Methods

2.1. Nucleic acid extraction

The DNA from *Nacobbus* was either extracted from individuals in the soil or from potato tubers.

DNA extraction from soil: DNA was extracted from 0.5 g of soil taken from pooled soil cores using a soil DNA extraction kit (Mo-Bio Laboratories Inc., USA). Soil samples were disrupted before DNA extraction for 20 s in a Mo-Bio Vortex Adapter (Fast Prep FP 120). All soil DNA extractions were diluted 1:10 before using in the PCR.

DNA extraction from tubers: the skin was peeled from washed tubers using a metal potato peeler, which was flame-sterilised between each tuber. The skin was frozen using liquid nitrogen and crushed to a fine powder using a pestle and a mortar. A sub-sample (100 mg) was taken and DNA extracted using the Nucleospin® (Abgene, Epsom, GB) tissue kit. Due to insoluble particles being present after lysis, samples are centrifuged for 1 min at 9300 ***g*** and the supernatant added to the filter column following the protocol. The extracted DNA was diluted 1:10 for PCR reaction.

2.2 Polymerase chain reaction

2.2.1 Master mix (concentration per 20-µL single reaction)
10 µL 2X Red Taq maga mix, including Taq DNA polymerase (Sigma)
0.1 µM each primer

For samples extracted from soil, the $MgCl_2$ concentration is increased from 1.5 mM to 2.5 mM.

2.2.2 PCR cycling conditions

95°C followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, with a final incubation at 72°C for 5 min.

For samples extracted from soil, the number of cycles increases to 45.

3. Essential procedural information

3.1. Analysis of DNA fragments: DNA fragments are separated by electrophoresis on agarose gel (2%) and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

3.2. Identification of species: expected amplicons for *N. aberrans*: from 141 to 173 bp and for *N. bolivianus* 148 bp. No amplification was observed for *Globodera tabacum*, *Heterodera glycines, Meloidogyne incognita, Pratylenchus goodeyi* or *Radopholus similis.*

3.3. A negative control (no DNA target) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen).