12 Molecular Identification of Nematodes Using Polymerase Chain Reaction (PCR)

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12.1 Introduction

Molecular diagnostics are a vital component of the management of economically important pests, including plant-parasitic nematodes. Conventional methods for nematode identification rely on time-consuming morphological and morphometric analysis of several specimens of the target nematode. The accuracy and reliability of such identification depends largely on the experience and skill of the person making the diagnosis, and the number of such qualified and experienced nematode taxonomists is small and currently declining. Molecular methods of nematode identification provide accurate and alternative diagnostic approaches. Molecular diagnostics is a term used more specifically for the characterization of an organism based on information of its DNA or RNA structure.

Compared with biochemical approaches, molecular diagnostics has several advantages. It does not rely on expressed products and is not influenced by environmental conditions or development stage, and any stage (eggs, juveniles, females and males) can be used for diagnosis. It is much more sensitive than any biochemical technique and can be used with nanograms of DNA extracted from one nematode or even part of a nematode's body. It can also be used with various types of samples, such as soil extracts, plant material or formalin-fixed samples.

Various molecular techniques for diagnostics have been introduced to nematology during last decades, but the most popular is Polymerase Chain Reaction (PCR) based. PCR is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules via an enzyme catalyst. Once identified, target nematode DNA generated by PCR amplification can be characterized further by various analyses, including restriction fragment length polymorphism, when variation in sequences in PCR products can be revealed by restriction endonuclease digestion, or sequencing, which is a process of determining the order of

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the nucleotide bases along a DNA strand. Identification of nematodes using PCR requires standard molecular biological equipment: a DNA thermal cycler, set of calibrated pipettes, vortex, centrifuge, horizontal gel electrophoresis box with power supply, freezer, microwave oven and UV light box with camera (Fig. 12.1).

The main DNA regions targeted for diagnostics of nematodes are nuclear ribosomal RNA genes. These include 18S, 28S and especially the Internal Transcribed Spacer 1 (ITS1) and Internal Transcribed Spacer 2 (ITS2), which are situated between 18S and 5.8S, and 5.8S and 28S rRNA genes, respectively. Genes of mitochondrial DNA (mtDNA), with their higher rate of mutations relative to rRNA genes, have great potential for identification of

Fig. 12.1. Laboratory equipment. **(A)** AirClean PCR workstation for preparation of PCR mixture. **(B, C)** Eppendorf PCR thermocycles. **(D)** Gel electrophoresis supplies and equipment. **(E)** Containers for EtBr staining. **(F)** Bio-Rad gel imaging system.

races and populations. Presently, partial cytochrome *c* oxidase subunit I (*COI*) gene of mtDNA is also widely used for nematode diagnostics. The *COI* gene is emerging as the standard barcode for many organisms. The bar-coding technique is based on the idea that a particular nucleotide sequence from a common gene can serve as a **unique identifier** for every species, and a single piece of DNA can identify all life forms on earth.

12.2 Preservation of Nematodes for Molecular Studies

The efficiency of DNA extraction from a sample depends on how the nematodes have been prepared and fixed for molecular analysis. Various fixation methods for molecular study have been proposed and described; however, the best approach is to use live nematodes for diagnostics. If the period between nematode extraction and molecular analysis is several days or weeks, nematodes may be kept at low temperatures (–20°C to +4°C) in a freezer or refrigerator before use. In some cases, quarantine regulations do not allow live nematodes to be kept and transported, so the nematodes should be killed gently using high temperature (75–80°C for 15 min) leaving the DNA undamaged. A sodium chloride (0.1 M NaCl) solution can be used at low or room temperature to store or send dead nematodes for several days. Often during long field sampling trips, it is not possible to keep nematodes at low temperatures and other methods should be used to save nematode DNA.

12.2.1 Dry preservation

Dry preservation is a simple method that has worked successfully with many nematode species, allowing DNA storage for many years. DNA is essentially stable when the sample is properly dried. Live nematodes are placed into a small Eppendorf tube with a drop of distilled water and the tube is kept open at room temperature or gently heated until the water has evaporated. Dead and dried nematodes will be at the bottom of the tube. The tube with nematodes could be stored at room temperature. Before starting DNA extraction, add a few drops of water to the tube and wait for a few minutes for the specimens to rehydrate.

12.2.2 Ethanol (ethyl alcohol) preservation

Ethanol (ethyl alcohol) mixed with distilled water is a good preserving agent. Nematodes preserved in different concentrations (75–95%) of ethanol and stored at ambient temperature can be successfully used in PCR and other molecular analyses. Nematodes fixed in ethanol should always be carefully washed in distilled water before DNA extraction.

12.2.3 DESS preservation

DESS is a solution containing dimethyl sulfoxide, disodium EDTA and saturated NaCl. DESS offers the advantage of preserving both the morphology and DNA with one solution, as opposed to previous sampling methodology that required collection of separate sub-samples in ethanol and formalin for integrative studies. Nematodes picked straight out of DESS can be used successfully for PCR after several months storage (maximum storage time is 7 months). Amplification of DNA fragments from 800 to 1800 bp was 80% successful and sequencing success from these amplicons was greater than 90% for all nematodes preserved in DESS solution between 3 days and 7 months (Yoder *et al.*, 2006). Specimens fixed in DESS solution should be washed in distilled water before DNA extraction.

12.2.3.1 Protocol for preparing DESS solution

After http://www.faculty.ucr.edu/~pdeley/lab/melissa/DESS_protocol_f.doc (See also Eisenback and Hunt, Chapter 5, this volume.)

- For a 250 ml solution of DESS, measure out 23.265 g of disodium EDTA with FW 372.24. (This may vary depending on the FW of your EDTA salt.) Add 50 ml of deionized water to the EDTA salt and stir. Make sure disodium EDTA salt is used, otherwise more NaOH is needed to pH the EDTA.

- Make 1 M NaOH to pH the EDTA. The EDTA should be around pH 3.0 or 4.0 to begin with. It will take approximately 50 ml of 1 M NaOH to bring the EDTA to pH 8.0. The EDTA will then begin to dissolve slowly. Heat to 30°C.
- Once all the EDTA salt is dissolved, bring the volume up to 200 ml with deionized water. Then add the 20% DMSO by volume, which is 50 ml for a 250 ml solution. Return to a beaker and stir for a few minutes.
- Add NaCl until the solution is saturated (i.e. it no longer dissolves); heating will help dissolve the salt. Pour the solution into a bottle leaving most of the salt crystals in the beaker.
- To extract the samples from DESS, pick the nematodes out of the solution and place them in a small Petri dish with distilled water for a few minutes to remove any salt or DESS that might be attached. If the DESS is not completely washed off, the dimethyl sulfoxide and EDTA will inhibit the PCR reaction.

12.3 DNA Extraction

The critical step in molecular identification procedures is the preparation of the template DNA from a nematode sample. Successful molecular identification requires the availability of genomic material of an appropriate quality and concentration. The aim of this procedure is to expose the DNA molecules for further analyses and remove materials that may inhibit subsequent reactions.

Several protocols for the extraction of nucleic acids from nematodes are available (e.g. Curran *et al.*, 1985; Caswell-Chen *et al.*, 1992; Blok *et al.*, 1997). Choosing an appropriate DNA extraction method usually depends on the amount of available nematode material and the method used. Several general conditions should be considered for selection of DNA extraction method: (i) avoid losing DNA and retain as much DNA as possible; (ii) final DNA should be free from inhibiting materials; (iii) the method should be simple with minimal steps and tube changes to avoid laboratory contamination; (iv) the method should involve only limited exposure to toxic chemicals; and (v) the method should be relatively inexpensive and non-labour intensive.

DNA extraction generally follows three basic steps: (i) disruption of nematode cuticle, cell walls and membranes; (ii) separation the DNA from other cell components; and (iii) isolation of the DNA. These three steps or some variation of them can be found in all DNA extraction methods.

Using different extraction methods and commercial kits, nematode DNA can be obtained directly from soil samples (Waite *et al.*, 2003; Yan *et al.*, 2008, 2013; Goto *et al.*, 2009; Sato *et al.*, 2010; Baidoo *et al.*, 2017). Furthermore, extraction of DNA from formalin-fixed material or nematodes embedded in glycerin on slides provides a new opportunity for molecular examination of reference materials (Thomas *et al.*, 1997; Bhadury *et al.*, 2005; Rubtsova *et al.*, 2005).

12.3.1 Protocols for DNA extraction from nematodes

Protocol 1: DNA extraction using proteinase K with Worm Lysis Buffer (WLB) (Waeyenberge *et al.*, 2000)

- Pick a single or several nematodes and place in a 10 µl drop of double distilled water on a glass slide under a dissecting microscope.
- Cut nematodes into three or four pieces with a needle or scalpel.
- Transfer worm pieces with water to a sterile 0.2 ml Eppendorf tube containing 8 µl of WLB (50 mM KCl, 10 mM Tris, pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP40 (Fisher Scientific), 0.45% Tween 20 (Merck) and 0.01% gelatine) and 2 µl of proteinase K (600 µg ml^{-1}).
- Freeze at –80°C for 10 min.
- Incubate at 65°C for 1 h and then heat at 95°C for 15 min.
- Centrifuge for 1 min at maximum speed to remove debris.
- Use 1–4 µl of the supernatant for PCR.

Protocol 2: DNA extraction using proteinase K with 10× PCR buffer (Subbotin *et al.*, 2018)

This is modification of the method of Waeyenberge *et al.* (2000), where WLB is replaced by 10x PCR buffer without losing DNA extraction efficiency.

- Pick a single or several nematodes and place in a 20 µl drop of double distilled water on a glass slide under a dissecting microscope.
- Cut nematodes into three or four pieces with a needle or scalpel. Nematodes in water can also be crushed under a cover slip with careful pressure on the slip.
- Transfer worm pieces with water to a sterile 0.2 ml Eppendorf tube and add 2 µl of 10× PCR buffer and 3 µl of proteinase K (600 µg ml^{-1}).
- Incubate at 65°C for 1 h and then heat at 95°C for 15 min.
- Centrifuge for 1 min at maximum speed to remove debris.
- Use 1–4 µl of the supernatant for PCR.

Protocol 3: DNA extraction using NaOH (Floyd *et al.*, 2002)

- Pick individual nematodes directly into 20 µl of 0.25 M NaOH in a 0.2 ml Eppendorf tube and keep at room temperature from several minutes to several hours.
- Heat the lysate for 3 min at 95°C.
- Add 4 µl of HCl and 10 µl of 0.5 M Tris-HCl buffered at pH 8.0 to neutralize the base.
- Add 5 µl of 2% Triton X-100.
- Heat the lysate for 3 min at 95°C.
- Use 0.5–2.0 µl of lysate for PCR.

Protocol 4: DNA extracted from archived nematodes using an extended hot lysis protocol with Qiagen DNeasy Tissue Kit (Chase *et al.*, 1998; Bhadury *et al.*, 2007)

- Carefully take nematodes off the microscope slides with a sterilized scalpel and place into 0.5 ml PCR tubes containing 200 µl of animal tissue lysis buffer (also known as ATL) from the Qiagen DNeasy Tissue Kit.
- Incubate tubes at 56°C for 24 h.
- Add 5 µl of proteinase K (50 mg ml^{-1}) and an additional 80 µl of the ATL buffer to each tube and incubate for another 72 h at 55°C.
- Complete the extraction procedure according to the DNeasy kit following the manufacturer's instructions. Finally, elute the DNA in 80 µl of MilliQ water.
- Immediately store templates at –20°C until further use.
- Use 5 µl aliquots of the extracted DNA for the PCR.

Effective DNA extraction can be achieved by using commercial kits developed by Qiagen, Promega and other companies.

12.4 PCR Technique

The PCR technique has become one of the most widely used techniques for studying the genetic diversity of nematodes and their identification. PCR is a rapid, inexpensive and simple means of producing large numbers of copies of DNA molecules. Any DNA fragment can be amplified and detected by PCR. The PCR method requires a DNA template (starting material) containing the region to be amplified, two oligonucleotide primers flanking this target region, DNA polymerase and four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) mixed in a buffer containing magnesium ions ($MgCl_2$).

A primer is a short oligonucleotide, containing usually about two dozen nucleotides, that is complementary to the 3′ end of each strand of the fragment that should be amplified. Primers anneal to the denatured DNA template and provide an initiation site for the elongation of the new DNA molecule. Universal primers are those complementary to a particular set of DNA for a wide range of organisms (Table 12.1); primers matching only to certain species are called species-specific primers (Table 12.2). When sequences of the flanking regions of the amplified fragment are unknown, PCR with degenerate primers (i.e. primers containing a number of options at several positions in the sequence that allows annealing and amplification of a variety of related sequences) can be applied. The universal primers for rRNA genes that are currently used for diagnostics of plant-parasitic nematodes are given in Table 12.1. The primers for amplification of some mtDNA genes are given in Table 14.1 (Humphreys-Pereira *et al.*, Chapter 14, this volume).

PCR is performed in a tube in a thermocycler with programmed heating and cooling. The procedure consists of a succession of three steps determined by temperature conditions: template denaturation (95°C for 3–4 min), primer annealing (55–60°C for 30 s to 2 min), and extension of the DNA chain (72°C for 30 s to 2 min). PCR is carried out for 30–40 cycles. As the result of PCR, a single target molecule of DNA is amplified into more than a billion copies. The amplified products are electrophoretically separated according to their size on agarose or polyacrylamide gels and visualized using ethidium bromide (EtBr) or other DNA-staining dyes, which interact with double-stranded DNA and causes it to fluoresce under UV radiation. Once identified, the target nematode DNA generated by PCR amplification can be characterized further by various analyses.

Table 12.1. Some universal primer combinations used for amplification of ribosomal RNA genes of nematodes.

Primer combination and code (direction)[a]	Primer sequence (5′-3′)	Amplified region	References
G18SU (f)	GCT TGC CTC AAA GAT TAA GCC	18S rRNA	Blaxter *et al.* (1998)
R18Tyl1 (r)	GGT CCA AGA ATT TCA CCT CTC		Chizhov *et al.* (2006)
F18Tyl2 (f)	CAG CCG CGG TAA TTC CAG C	18S rRNA	Chizhov *et al.* (2006)
R18Tyl2 (r)	CGG TGT GTA CAA AGG GCA GG		
988F (f)	CTC AAA GAT TAA GCC ATG C	18S rRNA	Holterman *et al.* (2006)
1912R (r)	TTT ACG GTC AGA ACT AGG G		
1096F (f)	GGT AAT TCT GGA GCT AAT AC	18S rRNA	Holterman *et al.* (2006)
1912R (r)	TTT ACG GTC AGA ACT AGG G		
1813F (f)	CTG CGT GAG AGG TGA AAT	18S rRNA	Holterman *et al.* (2006)
2646R (r)	GCT ACC TTG TTA CGA CTT TT		
SSU_F_04	GCT TGT CTC AAA GAT TAA GCC	18S rRNA	Blaxter *et al.* (1998)
SSU_R_09	AGC TGG AAT TAC CGC GGC TG		
SSU_F_22	TCC AAG GAA GGC AGC AGG C	18S rRNA	Blaxter *et al.* (1998)
SSU_R_13,	GGG CAT CAC AGA CCT GTT A		
SSU_F_23	ATT CCG ATA ACG AGC GAG A	18S rRNA	Blaxter *et al.* (1998)
SSU_R_81	TGA TCC WKC YGC AGG TTC AC		
designated Nem_18S_F	CGC GAA TRG CTC ATT ACA ACA GC	18S rRNA	Floyd *et al*. (2005)
Nem_18S_R	GGG CGG TAT CTG ATC GCC		
18S-CL-F3	CTT GTC TCA AAG ATT AAG CCA TGC AT	18S rRNA + ITS1-5.8S-ITS2 rRNA + 28S rRNA	Carta and Li (2018, 2019)
28S-CL-R	CAG CTA CTA GAT GGT TCG ATT AGT C		
18S (f)	TTG ATT ACG TCC CTG CCC TTT	ITS1-rRNA	Vrain *et al.* (1992)
rDNA1.58S (r)	ACG AGC CGA GTG ATC CAC CG		Szalanski *et al.* (1997)
TW81 (f)	GTT TCC GTA GGT GAA CCT GC	ITS1-rRNA	Curran *et al.* (1994)
5.8SM5 (r)	GGC GCA ATG TGC ATT CGA		Zheng *et al.* (2000)
18S (f)	TTG ATT ACG TCC CTG CCC TTT	ITS1-5.8S-ITS2 rRNA	Vrain *et al.* (1992)
26S (r)	TTT CAC TCG CCG TTA CTA AGG		
F194 (f)	CGT AAC AAG GTA GCT GTA G	ITS1-5.8S-ITS2 rRNA	Ferris *et al.* (1993)
F195 (r)	TCC TCC GCT AAA TGA TAT G		
TW81 (f)	GTT TCC GTA GGT GAA CCT GC	ITS1-5.8S-ITS2 rRNA	Curran *et al.* (1994)
AB21 (r)	ATA TGC TTA AGT TCA GCG GGT		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of 28S rRNA	Nunn (1992)
D3B (r)	TCG GAA GGA ACC AGC TAC TA		
D2Tyl (f)	GAG AGA GTT AAA NAG BAC GTG A	D2-D3 of 28S rRNA	Chizhov *et al.* (2012)
D3B (r)	TCG GAA GGA ACC AGC TAC TA		Nunn (1992)
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2 of 28S rRNA	Nunn (1992)
D2B (r)	GAC CCG TCT TGA AAC ACG GA		

[a]f, forward; r, reverse.

Table 12.2. Species-specific primers for conventional PCR used for diagnostics of some plant-parasitic nematodes.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Aphelenchoides besseyi	AbF5 – ATG TGT AAG TAG AGC GTT ATA AbR5 – ATT CGC CGT TTT TAA GGC G-	18S rRNA	~ 340 bp	Devran *et al.* (2017)
Aphelenchoides fragariae	AFragF1 – GCA AGT GCT ATG CGA TCT TCT AfragR1 – GCC ACA TCG GGT CAT TAT TT	ITS rRNA	~ 169 bp	McCuiston *et al.* (2007)
Aphelenchoides ritzemabosi	BSF – TCG ATG AAG AAC GCA GTG AAT T ArtR – CTC CAC ACG CCG ACC GA	ITS rRNA	~ 208 bp	Cui *et al.* (2010)
Bursaphelenchus cocophilus	BC1F – AAC TAC CGT CTT CCG CTG TCG BC1R - TTG AGC ACC AAC ACG CCG TCA	ITS rRNA	~ 528 bp	Silva *et al.* (2016)
Bursaphelenchus fraudulentus	FF – GTG ATG GGT TTG CGG GCG GCG FR – CAA CCA ATG CAC ACC AAC CAA	ITS rRNA	~ 617 bp	Filipiak *et al.* (2010)
Bursaphelenchus mucronatus	MF - TCCGGCCATATCTCTACGAC MR - GTTTCAACCAATTCCGAACC	ITS rRNA	~ 210 bp	Matsunaga and Togashi (2004)
Bursaphelenchus xylophilus	XF - ACGATGATGCGATTGGTGAC XR - TATTGGTCGCGGAACAAACC	ITS rRNA	~ 557 bp	Matsunaga and Togashi (2004)
Bursaphelenchus mucronatus	Y01F - AGT CCG TGC CTT TGC TCT AGC Y01R - CCG AAG TGT CTC CAG CGA AAT	SCAR	~ 609 bp	Chen *et al.* (2011)
Bursaphelenchus xylophilus	BZ2 – TCA CGA TGA TGC GAT TGG TG BF3 – AGA AGA TCT TGG TCG CGG AA	ITS rRNA	~ 580 bp	Jiang *et al.* (2005)
Ditylenchus destructor	D2 – TGG ATC ACT CGG CGG CTC GTA GA D1 – ACT GCT CTG CGT TTG GCT TCA	D2-D3 of 28S rRNA	~ 346 bp	Liu *et al.* (2007)
Ditylenchus dipsaci	DitNF1 – TTA TGA CAA ATT CAT GGC GG rDNA2 - TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 263 bp	Subbotin *et al.* (2005)
Ditylenchus dipsaci	U831 - AAY AAR ACM AAG CCN TYT GGA C Dipsaci-hsp90R - GWG TTA WAT AAC TTG GTC RGC	Hsp90	~ 182 bp	Madani *et al.* (2015)
Ditylenchus dipsaci	H05 - TCA AGG TAA TCT TTT TCC CCA CT H06 - CAACTG CTA ATG CGT GCT CT	SCAR	~ 242 bp	Esquibet *et al.* (2003)
Ditylenchus dipsaci	DdpS1 - TGG CTG CGT TGA AGA GAA CT rDNA2 - TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 517 bp	Kerkoud *et al.* (2007)
Ditylenchus dipsaci	DITuniF – CTG TAG GTG AAC CTG C DITdipR – GAC ATC ACC AGT GAG CAT CG	ITS rRNA	~ 148 bp	Jeszke *et al.* (2015)
Ditylenchus gigas	D09 - CAA AGT GTT TGA TCG ACT GGA D10 - CAT CCC AAA ACA AAG AAA GG	SCAR	~ 198 bp	Esquibet *et al.* (2003)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Ditylenchus gigas	DITuniF – CTG TAG GTG AAC CTG C DITgigR – GAC CAC CTG TCG ATT C	ITS rRNA	~ 270 bp	Jeszke *et al.* (2015)
Globodera rostochiensis	PITSr3 – AGC GCA GAC ATG CCG CAA ITS5 – GGA AGT AAA AGT CGT AAC AAG G	ITS rRNA	~ 434 bp	Bulman and Marshall (1997)
Globodera rostochiensis	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	ITS rRNA	~ 391 bp	Mulholland *et al.* (1996)
Globodera pallida	PITSp4 – ACA ACA GCA ATC GTC GAG ITS5 – GGA AGT AAA AGT CGT AAC AAG G	ITS rRNA	~ 265 bp	Bulman and Marshall (1997)
Globodera pallida	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	ITS rRNA	~ 238 bp	Mulholland *et al.* (1996)
Heterodera avenae	AVEN-COIF - GGG TTT TCG GTT ATT TGG AVEN-COIR - CGC CTA TCT AAA TCT ATA CCA	COI	~ 109 bp	Toumi *et al.* (2013a)
Heterodera filipjevi	FILI-COIF - GTA GGA ATA GAT TTA GAT AGT C FILI-COIR - TGA GCA ACA ACA TAA TAA G	COI	~ 245 bp	Toumi *et al.* (2013a)
Heterodera filipjevi	HfF1 – CAG GAC GAA ACT CAT TCA ACC AA HfR1 – AGG GCG AAC AGG AGA AGA TTA GA	SCAR	~ 646 bp	Peng *et al.* (2013)
Heterodera latipons	Hlat-actF - ATG CCA TCA TTA TTC CTT Hlat-actR - ACA GAG AGT CAA ATT GTG	actin	~ 204 bp	Toumi *et al.* (2013b)
Heterodera glycines	JBG1 – TGG TTT AGT TAG ATT AAC TAT C JB3R – TCC AAA CTW GCG TTA CTY AG	COI	~ 339 bp	Ko *et al.* (2017)
Heterodera glycines	SCNFI – GGA CCC TGA CCA AAA AGT TTC CGC SCNRI – GGA CCC TGA CGA GTT ATG GGC CCG	SCAR	~ 477 bp	Ou *et al.* (2008)
Heterodera glycines	GlyF1 – TTA CGG ACC GTA ACT CAA 26S – TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 181 bp	Subbotin *et al.* (2001)
Heterodera schachtii	JBS1 – GGA TAA TTT ATG CTA TTA TC JB3R –TCC AAA CTW GCG TTA CTY AG	COI	~ 339 bp	Ko *et al.* (2017)
Heterodera schachtii	SHF6 – GTT CTT ACG TTA CTT CCA AB28 - ATA TGC TTA AGT TCA GCG GGT	ITS rRNA	~ 200 bp	Amiri *et al.* (2002)
Hoplolaimus columbus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HC-1r – TCA GCA CAC AAT GGT ACC TTT	ITS1 rRNA	~ 580 bp	Bae *et al.* (2009)
Hoplolaimus galeatus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HG-2r – TCC TCG TTC ACA CAT TGA CA	ITS1 rRNA	~ 120 bp	Bae *et al.* (2009)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Hoplolaimus magnistylus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HM-3r – AGA CTG GAC GGC CAA AGT T	ITS1 rRNA	~ 340 bp	Bae *et al.* (2009)
Longidorus attenuatus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Latten3 - TTC CCT TTT CCC TGA TTA TAA TTT TCT ATC	ITS1 rRNA	~ 419 bp	Hübschen *et al.* (2004)
Longidorus elongatus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lelong1 - TTA TCG TAC GTA TTC CCA GTT CT	ITS1 rRNA	~ 847 bp	Hübschen *et al.* (2004)
Longidorus macrosoma	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lmacro2 - GTT CCC GAC GAT TAT TTT TGT	ITS1 rRNA	~ 705 bp	Hübschen *et al.* (2004)
Longidorus helveticus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lhel1 - CCG CAT CTC TTT ATT TCC GAC CAT CAA CC	ITS1 rRNA	~ 369 bp	Hübschen *et al.* (2004)
Longidorus profundorum	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lprof2 - TTA TTA TTT TTC AGG CTC TAC CTT TCG C	ITS1 rRNA	~ 1071bp	Hübschen *et al.* (2004)
Longidorus sturhani	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lstur - TTT TCC CCA CTA ATA CTC CCT CGT T	ITS1 rRNA	~ 667 bp	Hübschen *et al.* (2004)
Meloidogyne arenaria	Far – TCG GCG ATA GAG GTA AAT GAC Rar – TCG GCG ATA GAC ACT ACA AACT	SCAR	~ 420 bp	Zijlstra *et al.* (2000)
Meloidogyne chitwoodi	Fc - TGG AGA GCA GCA GGA GAA AGA- Rc - GGT CTG AGT GAG GAC AAG AGT A	SCAR	~ 800 bp	Zijlstra (2000)
Meloidogyne enterolobii	Me-F - AACTTTTGTGAAAGTGCCGCTG Me-R - TCAGTTCAGGCAGGATCAACC	IGS rRNA	~ 200 bp	Long *et al.* (2006)
Meloidogyne exigua	Ex-D15-F – CAT CCG TGC TGT AGC TGC GAG Ex-D15-R – CTC CGT GGG AAG AAA GAC TG	SCAR	562 bp	Randig *et al.* (2002)
Meloidogyne fallax	Ff - CCA AAC TAT CGT AAT GCA TTA TT Rf -GGA CAC AGT AAT TCA TGA GCT AG	SCAR	~ 515 bp	Zijlstra *et al.* (2000)
Meloidogyne hapla	Fh – TGA CGG CGG TGA GTG CGA Rh – TGA CGG CGG TAC CTC ATA G	SCAR	610 bp	Zijlstra (2000)
Meloidogyne incognita	Finc – CTC TGC CCA ATG AGC TGT CC Rinc – CTC TGC CCT CAC ATT AGG	SCAR	~ 1200 bp	Zijlstra *et al.* (2000)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Meloidogyne incognita	MI-F – GTG AGG ATT CAG CTC CCC AG MI-R – ACG AGG AAC ATA CTT CTC CGT CC	SCAR	~ 955 bp	Meng *et al.* (2004)
Meloidogyne incognita	F – TAG GCA GTA GGT TGT CGG G R - CAG ATA TCT CTG CAT TGG TGC	SCAR	~ 1350 bp	Dong *et al.* (2001)
Meloidogyne incognita	Inc-K14-F – GGG ATG TGT AAA TGC TCC TG Inc-K14-R – CCC GCT ACA CCC TCA ACT TC	SCAR	~ 399 bp	Randig *et al.* (2002)
Meloidogyne javanica	Fjav – GGT GCG CGA TTG AAC TGA GC Rjav – CAG GCC CTT CAG TGG AAC TAT AC	SCAR	~ 620 bp	Zijlstra *et al.* (2000)
Meloidogyne naasi	N-ITS – CTC TTT ATG GAG AAT AAT CGTR195 – CCT CCG CTT ACT GAT ATG	ITS rRNA	433 bp	Zijlstra *et al.* (2004)
Nacobbus spp.	NacF - GAT CAT TAC ACG TAC CGT GAT GGT C NacR - CTG CTC AAC CAC GCA TAG ACG	ITS rRNA	141-173 bp	Atkins *et al.* (2005)
Paralongidorus maximus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Pmax1 - TGC ATT TCA CCA CTT CTC ACT C	ITS1 rRNA	~ 649 bp	Hübschen *et al.* (2004)
Paratrichodorus allius	BL18 – CCC GTC GMT ACT ACC GAT T PAR2 - CCG TYC AAA CGC GTA TAT GAT C	ITS rRNA	~ 432 bp	Riga *et al.* (2007)
Paratrichodorus teres	BL18 – CCC GTC GMT ACT ACC GAT T PTR4 – CCT GAC AAG CTT GCA CTAG C	ITS rRNA	~ 677 bp	Riga *et al.* (2007)
Pratylenchus brachyurus	18S - TTG ATT ACG TCC CTG CCC TTT ACM7R – GCW CCA TCC AAA CAA YGA G	ITS1 rRNA	~ 267 bp	Machado *et al.* (2007)
Pratylenchus bolivianus	TW81 - GTT TCC GTA GGT GAA CCT GC P-bolivR1 -ATA GCG CAC TGG CGC AGC ATA	ITS rRNA	~ 295 bp	Troccoli *et al.* (2016)
Pratylenchus crenatus	PCR22 (f) – AAA GCC TGA ATG CCC TGA G PCR22 (r) – AAA TTG AAA GAG GTC GGT CGT	ITS rRNA	~ 610 bp	Mekete *et al.* (2011)
Pratylenchus jaehni	Pj1F – TGG TCA ATG AAT GTT ACG 5818 – ACG ARC CGA GTG ATC CAC	ITS1 rRNA	~ 476 bp	Consoli *et al.* (2012)
Pratylenchus neglectus	PNEG – ATG AAA GTG AAC ATG TCC TC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 290 bp	Al-Banna *et al.* (2004)
Pratylenchus neglectus	PNEG-F1 – CGC AAT GAA AGT GAA CAA TGT C D3B5- AGT TCA CCA TCT TTC GGG TC	D3 of 28S rRNA	~ 144 bp	Yan *et al.* (2008)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Pratylenchus oleae	Poleae-fw1 – GAC AGA TTA GAA TGG AAT CTG TTC G Poleae-rv1 – ATC GCT TTT GGA TTC AAT AAT ATA	ITS rRNA	~ 547 bp	Palomares-Rius *et al.* (2014)
Pratylenchus parazeae	PpzF – CTG CTG CTG GAT CAT TAC ATT PpzR –TCA AAT AGA CAT GCC CCA AT	ITS rRNA	~ 570 bp	Wang *et al.* (2015)
Pratylenchus penetrans	PPEN – TAA AGA ATC CGC AAG GAT AC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 278 bp	Al-Banna *et al.* (2004)
Pratylenchus penetrans	PP5 (f) –ACA TGG TCG ACA CGG TGA TA PP5 (r) - TGT TGC GCA AAT CCT GTT TA	beta-1,4-endoglu-canase	~ 520 bp	Mekete *et al.* (2011)
Pratylenchus penetrans	PpenA – TGA CTA TAT GAC ACA TTT RAA CTT G AB28 -ATA TGC TTA AGT TCA GCG GGT	ITS rRNA	~ 660 bp	Waeyenberge *et al.* (2009)
Pratylenchus penetrans	PP1 – ATG ATG GAA GTG TCC GCC T PP2 – CCC AAC GAC GGT CAA AAG G	ITS rRNA	~ 462 bp	Uehara *et al.* (1998)
Pratylenchus scribneri	PSCR – AAA GTG AAC GTT TCC ATT TC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 286 bp	Al-Banna *et al.* (2004)
Pratylenchus scribneri	PsF7 – AGT GTT GCT ATA ATT CAT GTA AAG TTG C PsR7 –TGG CCA GAT GCG ATT CGA GAG GTG T	ITS rRNA	~ 136 bp	Huang and Yan (2017)
Pratylenchus speijeri	TW81 - GTT TCC GTA GGT GAA CCT GC speijeri–specific – GTG CAC TGA TGT TAT TAT GTA TGG	ITS rRNA	~ 102 bp	De Luca *et al.* (2012)
Pratylenchus thornei	PTHO – GAA AGT GAA GGT ATC CCT CG D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 288 bp	Al-Banna *et al.* (2004)
Pratylenchus thornei	Pthf - TTC GGA AGA CAA TAA ATC Pthr - TCC AAA ATG AAA TAA TAA A	SCAR	~ 1078 bp	Carrasco-Ballesteros *et al.* (2007)
Pratylenchus vulnus	PVUL – GAA AGT GAA CGC ATC CGC AA D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 287 bp	Al-Banna *et al.* (2004)
Pratylenchus zeae	TW81 - GTT TCC GTA GGT GAA CCT GC P-zeaeR1 - TAC GCA TAC RGT TCT GCT CAT	ITS rRNA	~ 560 bp	Troccoli *et al.* (2016)
Radopholus similis	PF – CTA CAA ATG TGA CGC GAA PR – CAA TCT GCA CAA TGA ACA TAC	ITS rRNA	~ 500 bp	Liu *et al.* (2011)
Radopholus similis	RsimF – GAT TCC GTC CTT TGG TGG GCA RsimR – GAA CCA GGC GTG CCA GAG G	ITS rRNA	~ 398 bp	Ravindran *et al.* (2011)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Rotylenchulus reniformis	Ren240F – ACC GGC TTA ATT GCA ATG GT Ren240R – ACA ACT GCT CAA CAA CGC AG	ITS rRNA	~ 240 bp	Sayler *et al.* (2012)
Rotylenchulus reniformis	D2A - ACA AGT ACC GTG AGG GAA AGT TG Rrenif-R1A - GAA AAG GCC TAC CCA ATG TG	D2-D3 of 28S rRNA	~ 142 bp	Van den Berg *et al.* (2016)
Rotylenchus robustus	TW81 - GTT TCC GTA GGT GAA CCT GCR-robustus - GACGTGGACATCATACAGTC	ITS rRNA	~ 438 bp	Cantalapiedra-Navarrete *et al.* (2013)
Scutellonema bradys	TW81 - GTT TCC GTA GGT GAA CCT GC S-bradys – GTG ATG GCT AAA CCA CAT TC	ITS rRNA	~ 250 bp	Van den Berg *et al.* (2013)
Scutellonema brachyurus	TW81 - GTT TCC GTA GGT GAA CCT GC S-brachyurus-type A – GCT GAA GTG ACA GCC CAA CTT	ITS rRNA	~ 185 bp	Van den Berg *et al.* (2013)
Tylenchulus semipenetrans	TW81 - GTT TCC GTA GGT GAA CCT GC Semipenetrans – GGA CTC TGC TCA ACC TGG TAG A	ITS rRNA	~ 113 bp	Tanha Maafi *et al.* (2012)
Xiphinema diversicaudatum	TW81 - GTT TCC GTA GGT GAA CCT GC Xip-diver-ITS - GAA TAA ACA CCT TTC AAC GCT C	ITS rRNA	~ 864 bp	Chizhov *et al.* (2014)
Xiphinema index	I27 – GAG TCG TAA CGT TTC TCG TCT ATC AGG A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 340 bp	Wang *et al.* (2003)
Xiphinema vuittenezi	V18 – GTG GAA CGA AAA GAC CTC A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 591 bp	Wang *et al.* (2003)
Xiphinema italiae	ITA26 – GAA ATA AGA ACC CTG AAA AAG ATA GG A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 414 bp	Wang *et al.* (2003)

PCR with specific primers enables the detection of species in a nematode mixture by a single PCR test. Oligonucleotide primers for this PCR are designed to bind to regions of the gene that are conserved over the particular taxon or a group of taxa, so that it may be species-specific or it may target a group of species that differ from other such groups. Detection of a specific size amplicon in a gel indicates the presence of a certain species within a sample (Fig. 12.2). This PCR type constitutes a major development in DNA diagnostics. It enables the detection not only of a single species but also of several species in a nematode mixture by a single PCR test (multiplex PCR), provided several sets of specific primers for different species are mixed.

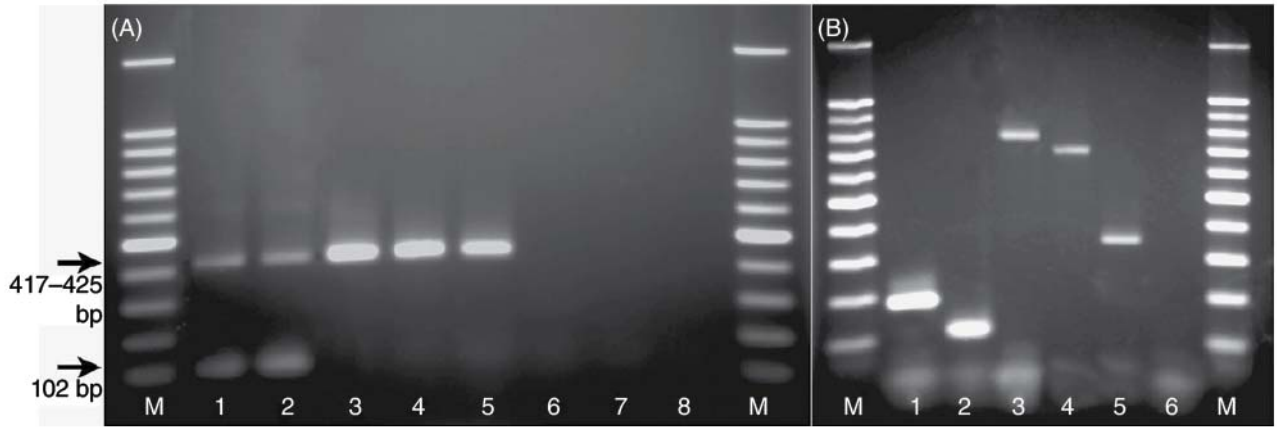

Fig. 12.2. **(A)** Duplex PCR with the *Pratylenchus coffeae* group-specific and *P. speijeri* species-specific primers. Lanes: M, 100 bp DNA marker (Promega); 1–4, *Pratylenchus* sp. C1; 5, *P. coffeae*; 6, *P. neglectus*; 7, *P. brachyurus*; 8, control without DNA. Arrows indicate a group-specific band for *P. coffeae* species complex (417–425 bp) and a species-specific band for *P. speijeri* (102 bp). (After De Luca *et al.*, 2012.) **(B)** PCR with the *Tylenchulus* species-specific primers. Lanes: M, 100 bp DNA marker (Promega); 1, *Tylenchulus musicola*; 2, *T. semipenetrans*; 3, *T. graminis*; 4, *T. furcus*; 5, *T. palustris*; 6, control without DNA. (After Tanha Maafi *et al.*, 2012.)

12.4.1 Protocols for PCR

Protocol 1: PCR with Taq PCR Core Kit (Qiagen) for amplification of rRNA and protein-coding genes (Subbotin *et al.*, 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 2.5 µl 10× PCR buffer, 5 µl Q solution, 0.5 µl dNTPs mixture (10 mM each) (*Taq* PCR Core Kit, Qiagen), 0.15 µl of each primer (1.0 µg µl^{-1}), 0.1 µl *Taq* Polymerase, and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 94°C for 4 min, 35 cycles of 1 min at 94°C, 1 min 30 s at 55°C, and 2 min at 72°C, followed by a final step of 10 min at 72°C.

Protocol 2: PCR with DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific) for amplification of mitochondrial gene (Subbotin *et al.*, 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 10 µl DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific), 10 µl water and 0.15 µl of each primer (1.0 µg µl^{-1}), and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min.

Protocol 3: PCR with Apex Taq RED DNA Polymerase Master Mix (2×) (Genesee Scientific) for amplification of ribosomal RNA and mitochondrial gene (Ye *et al.*, 2015)

- Add 1 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 12.5 µl 2× Apex Taq red master mix DNA polymerase (Genesee Scientific), 9.5 µl water, 1 µl each of 10 µM forward and reverse primers.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C, with a final extension at 72°C for 10 min.

Protocol 4: PCR with HOT FIREPol® (Solis BioDyne) for amplification of rRNA and protein-coding genes (Archidona-Yuste *et al.*, 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 5 µl of 5× HOT FIREpol® Blend Master Mix (with 10 mM $MgCl_2$), 0.15 µl of each primer (1.0 µg $µl^{-1}$), and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 15 min, 35 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, followed by a final step of 5 min at 72°C. For some specific A + T rich gene region as the *coxII*-16S used for identification of *Meloidogyne* species: an initial denaturation at 95°C for 15 min, 40 cycles of 1 min at 95°C, 1 min at 54°C, and 2 min 30 s at 66°C, followed by a final step of 7 min at 68°C.

Protocol 5: PCR with TaKaRa Ex Taq (Takara Bio) for amplification of rRNA (Carta and Li, 2018)

- Add 4 µl of extracted DNA to 0.2 ml Eppendorf tube containing 5 µl 10× Ex Taq Buffer, 4 µl dNTP mixture (2.5 mM each), 1 µl of each primer (10 µM), 0.25 µl of TaKaRa Ex Taq (5 units $µl^{-1}$) and 34.75 µl of distilled water.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 3 min, 5 cycles of 30 s at 94 °C, 40 s at 45°C, 2 min at 72°C, 40 cycles of 30 s at 94°C, 40 s at 55°C, 2 min at 72 °C, followed by a final step of 5 min at 72°C.

Protocol 6: PCR with Phusion Taq (BioLabs) for amplification of rRNA (Carta and Li, 2018)

- Add 4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 10 µl of 5× Phusion HF Buffer, 1 µl of 10 mM dNTPs, 2.5 µl of each 10 µM primer, 0.5 µl of Phusion DNA polymerase and distilled water added up to 29.5 µl. It is recommended that all reaction components are assembled on ice and quickly transferred to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA polymerase last in order to prevent any primer degradation.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 98°C for 30 s, 35 cycles of 10 s at 98°C, 30 s at 59°C, 90 s at 72°C followed by a final step of 5 min at 72°C.

12.4.2 Gel electrophoresis

Pour 100 ml of 1x TAE into a clean 250 ml flask. Add 1 g of agarose to make a 1% gel; microwave until the solution is clear. Pour the warm liquid agarose into a gel casting tray. Wait until the gel polymerizes. Run 2–5 µl of PCR product on an agarose gel for 30–60 min at 90–100 V. Put the unstained gel in a container and pour some TAE buffer and EtBr solution. Incubate with mild shaking. DNA-staining dye (GelRed® or GelGreen®) could be added directly in an agarose gel instead of staining in EtBr solution. Use the UV transilluminator to visualize the DNA bands.

12.5 PCR-Restriction Fragment Length Polymorphism

Variation in sequences in PCR products can be revealed by restriction endonuclease digestion. The PCR product obtained from different species or populations can be digested by a restriction enzyme, after which the resulting fragments are separated by electrophoresis. If differences in fragment length occur within restriction sites, the digestion of the PCR products will yield restriction fragment length polymorphism (RFLP), i.e. different RFLP profiles. PCR-RFLP of the ITS region of the rRNA gene is a very reliable method for identification of many plant-parasitic nematode groups including cyst forming, root-knot, lesion, stem, gall forming and longidorids (Fig. 12.3), as well as nematodes from the genera *Bursaphelenchus* and *Aphelenchoides*. Six to nine restriction enzymes enable most of the economical important species of cyst forming nematodes to be distinguished from each other as well as from their sibling species. RFLP of the ITS-rDNA obtained after restriction with several enzyme combinations enables identification of important root-knot nematode species; however, it fails to separate species from the tropical group, including *M. javanica*, *M. incognita* and *M. arenaria*. PCR-RFLP of mtDNA fragments between cytochrome oxidase *COII* gene and 16S has been successfully applied for diagnostics of these nematodes.

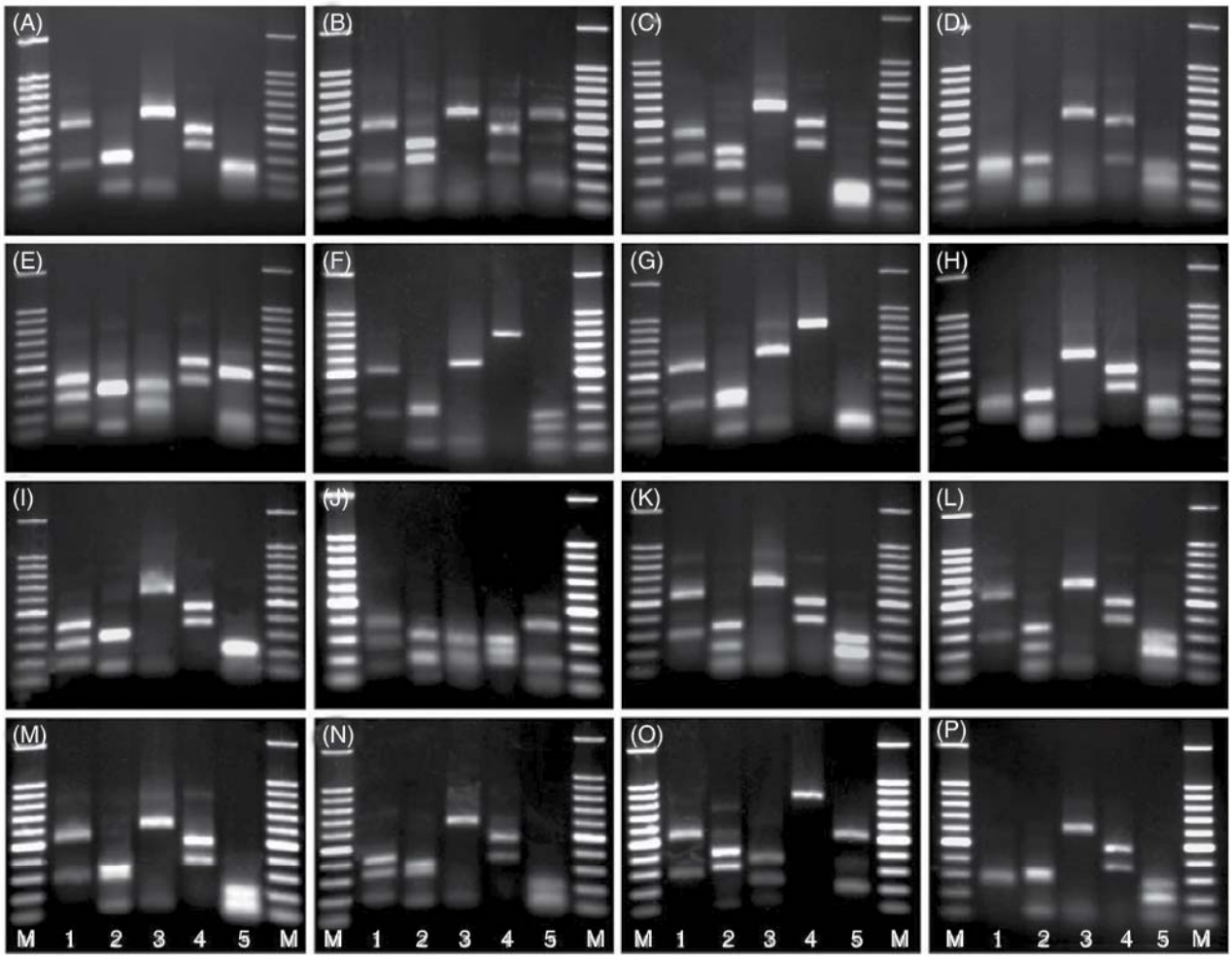

Fig. 12.3. PCR-D2-D3-28S-RFLP diagnostic profiles for some *Longidorus* species. **(A)** *L. aetnaeus*. **(B)** *L. africanus*. **(C)** *L. americanus*. **(D)** *L. artemisiae*. **(E)** *L. caespiticola* type B. **(F)** *L. elongatus*. **(G)** *L. euonymus*. **(H)** *L. intermedius*. **(I)** *L. leptocephalus*. **(J)** *L. lignosus*. **(K)** *Longidorus* sp. 1. **(L)** *Longidorus* sp. 2. **(M)** *Longidorus* sp. 3. **(N)** *Longidorus* sp. 4. **(O)** *Longidorus* sp. 5. **(P)** *Longidorus* sp. 6. Lanes: M, 100 bp DNA marker (Promega); 1, *Alu*I; 2, *Hinf*I; 3, *Bsp*143I; 4, *Tru*1I; 5, *Rsa*I. (After Subbotin *et al.*, 2013.)

12.6 Real-time PCR

DNA technology also provides several methods for quantification of nematodes in samples. Real-time PCR requires an instrumentation platform that consists of a thermal cycler, optics for fluorescence excitation and emission collection, and computerized data acquisition and analysis software (Fig. 12.4A,B). Real-time PCR is the continuous collection of fluorescent signals from one or more polymerase chain reactions over a range of cycles. The real-time technique allows monitoring of the sample during PCR using hybridization probes (TaqMan, Molecular Beacons, and Scorpions) or double-stranded dyes, such as SYBR Green, resulting in an increase in fluorescence signal. The amplification of any template is defined by four phases: (i) baseline; (ii) exponential; (iii) linear; and (iv) plateau. Quantitative PCR requires the measurement to be taken before the plateau phase, so the relationship between the number of cycles and molecules is relatively linear. The length of exponential phase depends on the template concentration and the quality of the real-time assay. Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample. Real-time PCR instruments use for calculations the cycle threshold (C_t) or the point when the level of fluorescence exceeds some arbitrary threshold. A plot of cycle number

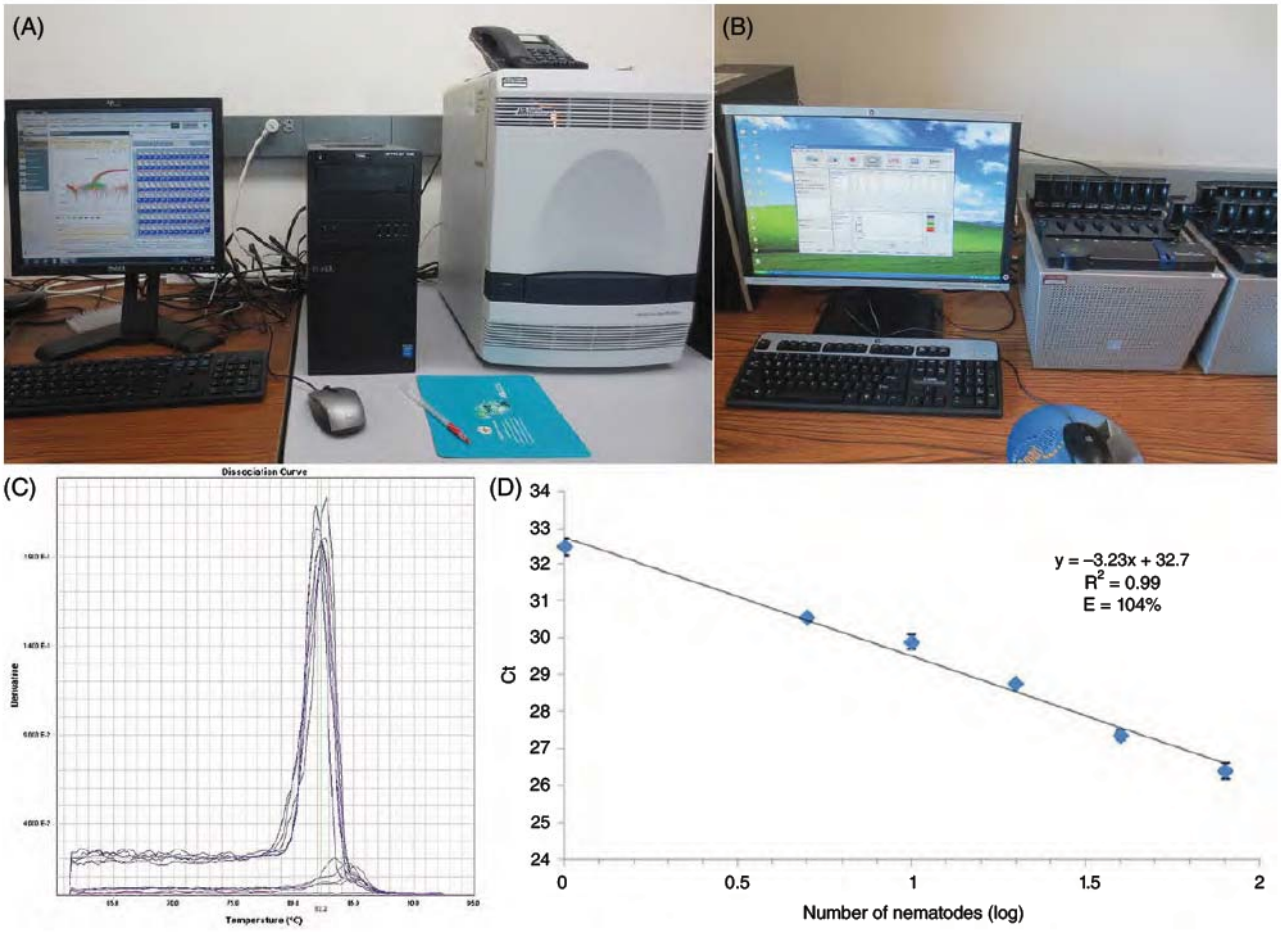

Fig. 12.4. **(A)** Applied Biosystems 7500 Real-Time PCR System. **(B)** SmartCycler Thermal Cycler Automated Real-Time PCR System. **(C)** Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 62°C showing high peaks at ± 82.2°C of two *Pratylenchus penetrans* populations (n = 3) and minor peaks for a population of *P. thornei* (n = 3) and NTC (n = 2). **(D)** Standard curve of the qPCR assay (SensiFAST Probe Hi-ROX) for *P. penetrans*: threshold cycle number (Ct) plotted against the log of the number of individuals of *P. penetrans* (1, 5, 10, 20, 40 and 80). (After Mokrini *et al.*, 2013.)

versus a log scale of the DNA concentration should result in a linear relationship during the exponential phase of PCR amplification.

SYBR Green binds only to double-stranded DNA and becomes fluorescent only when bound. This dye has the virtue of being easy to use because it has no sequence specificity and it can be used to detect any PCR product. However, this virtue has a drawback, as the dye binds also to any non-specific product, including primer dimers. To overcome this problem, the melting curve analysis can be employed. The products of PCR reaction are melted by increasing the temperature of the sample (Fig. 12.4C). The non-specific product tends to melt at a much lower temperature than the longer specific product. Both the shape and position of the DNA melting curve area are a function of the GC/AT ratio. The length of amplicon can be used to differentiate amplification products separated by less than 2°C in *T*m (the melting temperature).

The disadvantage of using a fluorescent dye is that it binds to any double-stranded DNA and then it cannot be used for quantification of several targets in a multiplex real-time PCR because it cannot distinguish between different sequences. In this case, sequence-specific fluorescent probes, such as TaqMan probes, are employed.

The rise in fluorescence is correlated to the initial DNA template amounts when compared with samples of known DNA concentration. Several DNA samples with known concentrations are used to generate a

standard curve based on their measured C_t values. A sample with an unknown DNA quantity can be compared to this standard curve to calculate its initial DNA template concentration (Fig. 12.4D). The PCR quantification technique measures the number of nematodes indirectly by assuming that the amount of target DNA concentration (copies) in the sample is proportional to the number of targeted nematodes.

Several real-time PCR assays for detection and quantification of different plant-parasitic nematodes have been published and are briefly reviewed by Braun-Kiewnick and Kiewnick (2018). Compared with the traditional PCR method, real-time PCR has several advantages. It allows for faster, simultaneous detection and quantification of target DNA. The automated system overcomes the laborious process of estimating the quantity of the PCR product after gel electrophoresis and results can be seen in real-time. PCR assays that can be easily adapted for high-throughput analyses of many samples at a time – 96 or 384 formats.

Protocol 1: Detection and quantification of *Pratylenchus penetrans* using SensiFAST SYBR Hi-ROX (after Mokrini *et al.*, 2013)

- The reaction tube contains 20 µl reaction mixture with 10 µl of SensiFAST SYBR Hi-ROX (2×), 400 nM of each primer (PpenMFor 3′-CCA ACC TCT GCT ACA CTA-5′ and PpenMRev 3′-CAG TGC CGT ATT CAG TGA-5′), 200 nM of the probe (PpMPb 3′-CAC TAT GCC GC-5′, labelled with 6-FAM) and 3 µl of DNA template.
- The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 63°C for 30 s and 72°C for 1 min.

Protocol 2: Detection and quantification of *Meloidogyne hapla* using Bio Probe Mix LoRox (Sapkota *et al.*, 2016)

- The tube contains a reaction mixture of 13 µl with 0.9 µM of each of primer (Mhaplafwd 5′-TGG TTC AGG GTC ATT TTT CTA TAA AGT-3′ and Mhaplarev 5′-CAA ATC GCT GCG TAC CAA CA-3′), 0.25 µM of probe (Mhapla MGB Probe 5′-FAM-CCA TTG GCA CTA TAA C-MGB-3′) and 7.5 µl of Bio Probe Mix LoRox (PCR Biosystems). Water and 2 µl of DNA template are added to a total volume of 15 µl.
- The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min.

12.7 Problems with PCR-based Methods

The application of conventional PCR with universal and specific primers for diagnostic purposes has revealed several limitations. First, so-called universal primers, which are designed based on existing knowledge of DNA sequences for target species, might not work for some other species that have different sequences in this target DNA region. Second, universal primers presently available for application in diagnostics can also amplify organisms other than nematodes, such as fungi and plants.

Application of PCR with a specific primer could face similar problems. One limitation is the potential for obtaining a false positive reaction. As a specific primer design is always based on analysis of DNA sequences for a target species and closely related nematodes, there is a possibility that similar fragments can be obtained for another, previously non-investigated and tested, nematode species. A second limitation is the possibility of a false negative reaction. Although a region with a conserved sequence should be used for primer design, the possibility cannot be excluded that some mutations might have occurred in this region in some specimens or populations of the target taxon. As a result, such specimens or populations might not be detectable by the PCR test. Another limitation that should be always considered is the possibility of sample contamination, which might occur during sample preparation in a laboratory. This might give a false positive reaction due to the great sensitivity of the PCR method with a specific primer. Following strict rules to prevent contamination during preparation of the PCR mixture and adding a control tube without DNA in the PCR test is imperative for all diagnostics tests.

12.8 PCR Purification

Purification of DNA from a PCR reaction is necessary for sequencing, and facilitates the removal of enzymes, nucleotides, primers and buffer components. Commonly used methods employ spin columns containing a silica membrane, which binds DNA under specific salt concentrations and the remaining sample is washed

out. These are performed during the successive wash steps. DNA elution can then be performed in either Tris buffer or distilled water. The PCR product can be cleaned using QIAquick PCR Purification Kit (Quagen), DNA Clean & Concentrator-5 (Zymo Research) or kits provided by other companies. Another approach is PCR product cleaning with enzyme digestion. ExoSAP-IT™ (Applied Biosystems) PCR Product Cleanup Reagent is used for enzymatic cleanup of the amplified PCR product. It hydrolyses single-stranded DNA, primers and nucleotides in a single step. The reaction setup is complete with one pipetting step, which is followed by two incubations. The first incubation digests excess primer and dephosphorylates nucleotides. The second, high temperature incubation inactivates the enzymes.

12.9 Cloning of PCR Products

In a genome, rRNA and protein-coding genes are present in many copies and, although their sequences are often identical, some copies may have sequence variations. PCR amplification of such gene fragments yields a complex mixture with a product containing copies with different sequences. If some highly abundant copies have insertions/deletions, often the PCR product cannot be directly sequenced and should be cloned. Cloning is a method in which double-stranded DNA fragments amplified by PCR are ligated directly into a vector. The vector transports the DNA fragment into a bacterial host cell. Within the host cell the vector multiplies, producing numerous identical copies of the recombinant DNA. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one copy of the recombinant DNA molecule. PCR of colonies enables an amplicon from a single copy to be obtained and then this product can be submitted for sequencing.

The cloning technique requires a small amount of starting template materials and, thus, poorly amplified PCR product that failed for direct sequencing can be submitted for cloning to get high-quality DNA sequencing results from its clones. The cloning of DNA fragments essentially involves several steps: (i) preparation and purification of target DNA; (ii) preparation of vector DNA; (iii) creation of recombinant DNA; (iv) introduction of recombinant DNA into bacteria; and (v) selection of bacterial clones containing recombinant DNA. Cloning kits are provided by many companies, including Promega (pGEM®-T Easy Vector Systems, pGEM®-T Vector Systems), Qiagen (QIAGEN PCR Cloning Kit) (see: https://www.promega.com/-/media/files/resources/education-and-training/unit-6/sm0060710.pdf).

12.10 DNA Sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. Nucleic acid sequencing methods have undergone tremendous advances over the past decade. The rRNA, mtDNA and other gene sequences have been determined for a large number of nematode species and have been deposited into the GenBank database (http://www.ncbi.nlm.nih.gov/). In general, the comparison of the genes with reference data using sequence and phylogenetic analysis enables the identification of nematode samples.

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