4 Methods for Extraction, Processing and Detection of Plant and Soil Nematodes*

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Diagnosis of nematode damage requires methods for their extraction, handling and detection. The methods take advantage of the size, density and motility of the nematodes to separate them from plant tissue and soil particles by means of sieving, centrifugation and filtration. Different methods allow different applications, such as for diagnosis, determination of infestation levels, monitoring nematode densities and statutory testing for the presence of quarantine nematodes. Besides morphology and morphometrics, molecular techniques are increasingly used for the rapid and accurate identification of nematodes. This chapter provides details on the most common methods, while various modifications to these techniques are mostly determined by local supplies, availability of equipment and operating conditions. Further guidance, with excellent sections on methodology according to different situations, include: Thorne (1961), Ayoub (1980), Zuckerman *et al.* (1985), Southey (1986), Dropkin (1989), Hunt and De Ley (1996), Shurtleff and Averre (2000), Machado *et al.* (2010), EPPO (2013) and Coyne *et al.* (2014).

Sampling

Plants that are heavily stunted and damaged may have too small a root system to support many nematodes, and samples from nearby, less affected plants may yield more specimens. Most migratory plant parasitic nematodes are found around plant roots, and so soil samples from the rhizosphere are preferable. Usually, few nematodes occur in the top 5 cm of soil, which can be discarded from samples. Soil samples are generally taken to a depth of 15–20 cm, but 60 cm may be appropriate for nematodes affecting deep-rooted perennial and tree crops. Nematodes are not distributed uniformly in soil. Areas of nematode damage may be circular to oval or rectangular in outline; patches of poor growth may follow the rows. Sampling for stem and foliar nematodes should be from symptomatic plants. Soil samples and plant material to be examined for nematodes should be kept moist. Polythene bags are excellent containers for samples; soil and/or roots keep well in them, but whole plants are best kept separate from soil. Leaf and stems usually decompose faster than roots and should be stored in separate bags. Warm storage above

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20°C adversely affects nematodes from plants and soil, so samples should be kept cool, at around 5°C in temperate regions, 10–16°C in warmer regions of the middle latitudes and 16–18°C in the tropics and subtropics. Although it is common practice to store samples in refrigerators, low temperature (~5°C) can adversely affect the recovery of some nematodes from tropical soils (Whyte and Gowen, 1974). For more information on sampling procedures, especially sample size and sampling intensity for different crops, see Shurtleff and Averre (2000).

Fixation of Plant Tissue and Soil

In most cases, plant tissue and soil samples should be processed for nematodes within a few days after sampling. However, fixation of plant tissue and soil can be useful in preventing population changes during extended storage and avoiding quarantine restrictions applicable to live material. Roots and shoot tissue can be fixed for storage, subsequent examination or staining by adding to them preferably hot (60–70°C) formal acetic acid (FA, 4:1) or 5% formalin (2% formaldehyde solution). Alternatively, fresh material can be put directly into hot lactoglycerol; this softens tissues and is particularly helpful in the recovery of *Meloidogyne* females from roots. For soil samples, Elmiligy and De Grisse (1970) mixed hot fixative (100 ml of 40% formaldehyde + 10 ml of glycerol + 890 ml of distilled water at ~80°C) with soil samples. Nematodes in soils treated by fixation are extracted using centrifugal flotation.

Materials for Nematode Extraction

Extraction and handling of plant parasitic nematodes require mainly basic materials, which can be bought at the local market (e.g. sieves, dishes, flasks, filters, funnels and tubing) or made individually (e.g. nematode transfer pick, counting dishes, sieves and racks). Plastic or stainless steel is preferable for nematode extraction rather than brass/bronze gauze, rings or pans because metallic ions, especially copper, released into small volumes of static water can be toxic to nematodes, especially dorylaims (Pitcher and Flegg, 1968). However, brief contact with metal sieves, as in the sieving technique, does not appear to be harmful. Stainless steel sieves are available from suppliers, but alternatives can be made using nylon gauze fixed to vinyl rings cut from plastic drainpipe of 15–20 cm in diameter.

Several methods rely on nematode mobility and their ability to pass through a filter, thus separating them from plant debris and soil particles. Cotton wool milk filters, wet-strength paper handkerchiefs and towels are suitable, as are various types of cotton cloth or muslin. Tissues containing odour or toxic substances should be strictly avoided. It is necessary to select a filter that retains as much debris as possible but with sufficiently large pores for the nematodes to migrate through. For large nematodes, such as *Longidorus* spp., a nylon gauze of about 90 µm aperture, secured to a supporting ring, will often give a clean enough extract. Various grades of lingerie material, nylon or terylene, are also suitable. Supports to hold the sample above water level can be made easily by fixing wet-strength viscose or wire mesh between two vinyl rings cut from a drainpipe. A detailed analysis of the cost–benefit ratio of extraction methods, including the advantages and limitations of each method, is given in the EPPO standard PM 7/119 nematode extraction (EPPO, 2013).

Direct Examination of Plant Material

Nematodes can usually be observed by examining small amounts of rinsed plant tissue, such as roots, leaves, stems or seeds, with a stereoscopic microscope at magnifications from 15 to 50× using transmitted and/or incident light. Examine the plant tissue in water in an open Petri dish or large watch glass, and tease it apart with strong mounted needles. Nematodes released from the tissues will float out and can be collected with a handling needle or fine pipette. Migratory endoparasites (e.g. *Aphelenchoides*, *Ditylenchus*, *Hirschmanniella*, *Pratylenchus*, *Radopholus* and *Bursaphelenchus*) emerge quickly and can be found moving about on the bottom of the dish. Sedentary endoparasitic nematodes (e.g. *Globodera*, *Heterodera*, *Meloidogyne* and *Nacobbus*) may be seen attached to the surface of roots or in dissected tissue. Semi-endoparasites

(e.g. *Rotylenchulus* and *Tylenchulus*) and firmly attached ectoparasites can be seen attached to the surface of the roots. Since nematodes tend to migrate from damaged tissue, it is often worthwhile to re-examine the sample after a few hours.

To recover females of root knot nematodes (*Meloidogyne* spp.) from roots, carefully tease away the tissue with forceps and a fine needle to release the head and neck; avoid puncturing the body. Dissection and storage in 0.9% NaCl helps to avoid the osmotic effect of water, which tends to cause females to burst.

Staining of nematodes in plant tissue

Since nematodes are translucent and difficult to see in plant tissues, staining helps to visualize them. Plant material needs to be rinsed free of soil, and thick material sliced thinly before staining. Detection of *Meloidogyne* females can be facilitated by staining the roots in 0.4% cochenilla red food stain for 15–20 min, rinsing and examining them in water; the gelatinous matrix of the egg sac is stained red (Thies *et al.*, 2002).

When staining specimens within leaves, stems and roots, the plant tissue needs first to be cleared in diluted sodium hypochlorite bleach (5.25% NaOCl or Clorox) for about 4 min. Prior assessment is needed to determine a suitable concentration and incubation time for the target tissue, e.g. thin, soft tomato roots clear quickly, but tough, woody coffee roots are difficult to clear. Thoroughly rinse the roots on a 100 µm aperture sieve to remove all traces of the bleach, which inhibits staining by acid fuchsin. Transfer the plant material into a glass vial and cover it with the acid fuchsin solution (3.5 g acid fuchsin, 250 ml acetic acid, 750 ml water, diluted 1:40 with water before use). Boil the solution for a few seconds in the case of seedlings and for up to 30 s for mature tissue in a microwave oven or on a hot plate in a ventilated area, to avoid the vapour of acetic acid. Permit plant tissue to cool in the stain before transferring to a sieve (100 µm aperture) to rinse off excess stain under running tap water. In case of nematode quantification, be aware that the boiling procedure may release nematode stages from the root tissue that appear in the staining solution. If destaining with tap water proves insufficient, transfer the tissue to a solution of glycerol and distilled water, in equal volumes, acidified with a few drops of acetic acid. Depending on the thickness of the material, differentiation may take from several hours to 2–3 days, but the stained nematodes should be seen eventually in largely unstained tissue. Alternatively, plant tissue can be stained in acidified lactoglycerol plus 0.05% acid fuchsin or 0.05% methyl blue stain for a few minutes (Bridge *et al.*, 1982), or in 12.5% (v/v) McCormick Schilling red food colour for 20 min (Thies *et al.*, 2002).

Extraction from Plant Material

The most commonly used methods for the separation of nematodes from plant material rely on nematode activity (e.g. modified Baermann funnel technique), which are therefore not suitable for extracting slow-moving (e.g. *Criconemoides*, *Hemicycliophora* and *Xiphinema*) or sedentary nematodes (e.g. *Globodera*, *Heterodera*, *Meloidogyne*, *Rotylenchulus* and *Tylenchulus*), although juveniles and males of such forms will usually be recovered. For the latter, maceration–filtration or the mistifier technique are more suitable. Comparing the efficiency of these three techniques to extract *Pratylenchus zeae* and *Hirschmaniella oryzae* from rice roots, Prot *et al.* (1993), found the maceration–filtration or mistifier techniques most efficient. Other, less often used methods include the incubation technique (Young, 1954; West, 1957). Nematode extraction from bulky plant substrates, such as bulbs, corms or enlarged storage roots, can present difficulties. In such cases, the plant tissue can be peeled and used for nematode extraction to provide reliable data (McSorley *et al.*, 1999).

Baermann funnel technique

The Baermann funnel technique in its original form should no longer be used, as nematode recovery is less than 20% of that of other methods (Oostenbrink, 1970), mainly because of anaerobic conditions due to bacterial decay of the submerged organic matter and lack of oxygen at the base of the funnel stem. However, this technique has been modified in several ways to become a standard method for extraction of nematodes from plant tissue and soil.

Modifications of the Baermann funnel are used widely to extract active adult and juvenile nematodes (e.g. *Anguina*, *Aphelenchoides*, *Ditylenchus*, *Hirschmaniella*, *Pratylenchus* and *Radopholus*). Examples of modified Baermann techniques are illustrated in Fig. 4.1a–e. The funnel technique uses a supporting mesh (see the section on materials for nematode extraction) to hold the plant tissue partly submerged in water, to avoid anaerobic decomposition (Fig. 4.1b). A milk filter or paper tissue is placed on the support and the chopped plant material placed upon it. Fill the funnel with tap water and set the sieve in the funnel to submerge the filter partly but not completely. After 24–48 h, collect the nematode suspension as described above.

Using a shallow tray, dish or bowl (Whitehead and Hemming, 1965; Rodríguez-Kábana and Pope, 1981) instead of a funnel further improves oxygenation and reduces the number of nematodes remaining on the funnel wall (Fig. 4.1c and d). As above, a milk filter or paper tissue is placed on a support and the chopped plant material placed on it. A circle of muslin or paper tissue placed on top of the material will keep it moist and prevent it from floating. The support, with the sample material, is placed in a tray filled with tap water. Glass rods or small feet attached to the sieve ring provide a space of about 5 mm between the base of the sieve and the collecting tray. The material should be almost submerged. When adding water, do not pour water over the sample to avoid washing debris through the filter. Avoid too large sample sizes; split the sample or use larger trays of 20–30 cm in diameter instead (Fig. 4.1e). After 24–48 h, gently remove the support with the sample and transfer the suspension to a beaker. The sample can be re-immersed in fresh tap water for further extraction of nematodes. Oxygenation, hence nematode extraction, can be improved by wetting the roots with tap water containing 1–3% H_2O_2 (Tarjan, 1967). H_2O_2 helps in extracting migratory endoparasites from fleshy roots (e.g. banana), especially where high temperatures reduce oxygenation.

Mistifier technique (Seinhorst, 1950)

Nematodes recovered using this method are more active than by the previous methods because oxygenation is better, and sap and decomposition products from the material, which inactivate nematodes, are washed away. A fine mist of water is sprayed over the plant material using about 4.5 l/h. Most systems use an intermittent spray of ~1 min every 10 min. Oil burner nozzles or gas jets can sometimes be adapted, and a water pressure of ~2.8 kg/cm² is usually required to give a suitable mist. Plant material is chopped finely to ~3–4 mm long and placed on a milk filter or tissue supported on a mesh set in a funnel or dish for the modified Baermann technique (Fig. 4.2). Optimum sample size depends on sieve diameter and water flow rate; increasing sample size can decrease the efficacy of extraction (De Waele *et al.*, 1987). Nematodes collected in the funnel tube can be released into a beaker. Compared with the modified Baermann techniques, plant tissue will decompose much more slowly, thus allowing prolonged extraction times of up to 3 weeks (e.g. freshly hatched *Meloidogyne* juveniles). Several funnels can be arranged simultaneously on a rack, with one or two nozzles supplying them all. The whole apparatus can be established on the bench if enclosed with a polythene cover and left to stand on a drainage tray. For a more elaborate apparatus using collection trays instead of beakers, see Southey (1986).

Maceration techniques

Maceration is used for extracting active nematodes as well as immobile stages of sedentary nematodes from bulbs, cloves, corms, storage roots, crowns, leaves and small plants. The plant material is chopped into ~1 cm lengths and then macerated in about 100 ml of water in an electric blender. The maceration time required depends on the type of mixer used and on the type and thickness of plant material. Maceration needs to be sufficient to enable easy egress of nematodes from the tissues but not render them immobile. For the extraction of eggs (e.g. *Meloidogyne* spp.) from root tissue, the sodium hypochlorite (NaOCl) technique described by Hussey and Barker (1973) is recommended. Maceration methods in general are often quicker and more efficient than those described previously. However, the maceration action may release toxic substances from the plant material that can kill or immobilize nematodes. Toxic substances can

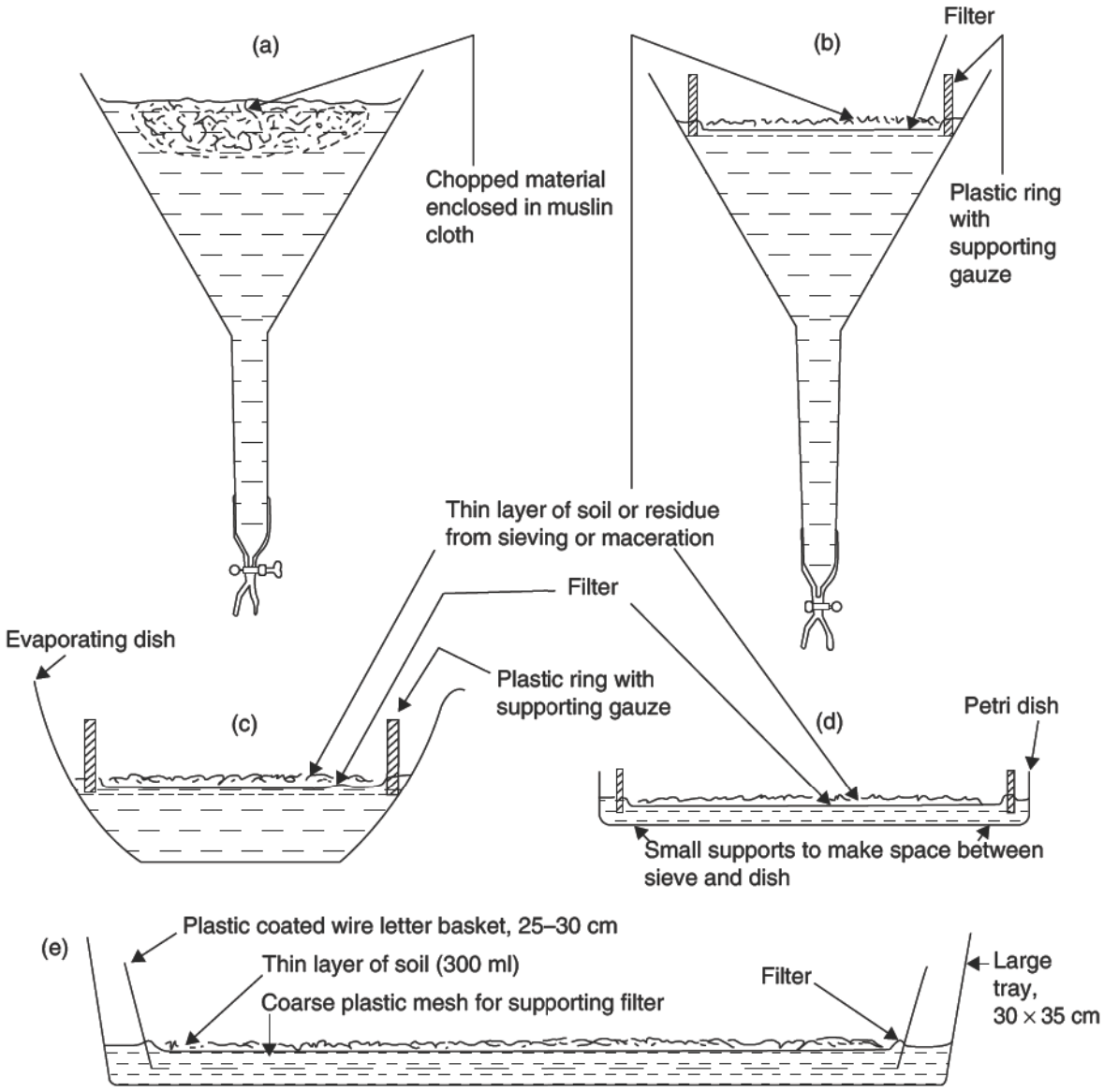

Fig. 4.1. Baermann funnel and modifications for extraction of active nematodes from plant material. (a) Original Baermann funnel technique with plant sample submerged in water. (b) Modification of placing the chopped plant material on a supporting mesh placed in a funnel. (c) Modification of using a bowl instead of a funnel. (d) Modification of using a dish instead of a funnel. (e) Modification of using a tray for large sample sizes. The filter is a cotton wool milk filter, wet-strength facial tissue, coarse cotton cloth, or fine woven nylon or terylene cloth. Plastic rings are cut from perspex, polythene or vinyl tubes. The supporting gauze is a muslin or nylon cloth held with an elastic band, or a coarse plastic mesh stuck or fused to the edge of the ring.

be partially removed and extraction efficacy improved by pouring the macerated debris and water through the filter on the Baermann dish, removing the water in the dish and refilling the dish with tap water. Plant debris hindering nematode observation can be cleaned by the modified Baermann technique (see above) or centrifugal flotation.

For centrifugal flotation (Coolen and D'Herde, 1972; Coolen, 1979), the macerated plant sample is poured on to a 1200 μm aperture sieve resting in a funnel standing in a 500 ml centrifuge tube. The residue on the sieve is washed carefully with a spray before it is discarded. A 5 ml aliquot of kaolin powder is added to the extract in the centrifuge tube and the contents thoroughly

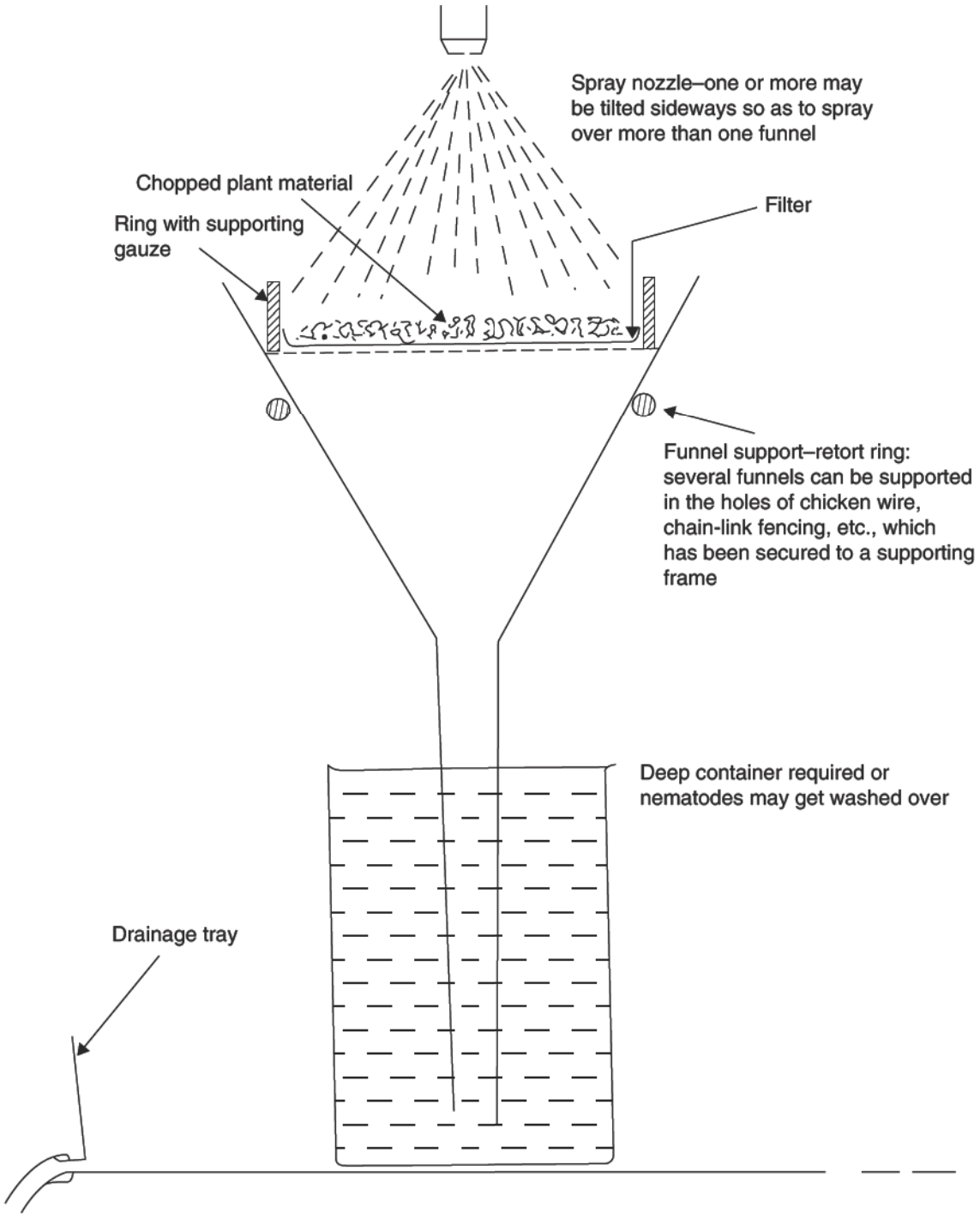

Fig. 4.2. Mist extraction of active nematodes from chopped plant material. The apparatus may be covered with plastic sheeting to prevent spread of the spray.

mixed with a Vibromixer. Tubes are balanced and centrifuged for 4 min at 1500 g; the supernatant is poured off and the residue resuspended in sucrose, $ZnSO_4$ or $MgSO_4$ solution of specific gravity 1.18 and mixed with a Vibromixer or manually for at least 30 s. Tubes are balanced with the appropriate solution and centrifuged for 4 min at 1500 g. The supernatant is then poured through a 20 μm aperture sieve, and the nematodes and eggs collected in a beaker. De Waele *et al.* (1987) found that the efficiency of extraction of *Pratylenchus* from maize roots decreased with an increase in sample size, and so the root mass treated should be standardized for comparative studies.

Extraction of *Bursaphelenchus* from stem tissue

For stem tissue, chop and macerate in a blender for 2 min and then transfer contents to a 2 l conical flask filled with water and allow to stand for 30 min to permit the nematodes to emerge; shake the flask and invert with the neck in a vessel of water and allow the suspension to settle for 30 min. The contents of the lower vessel are discarded and the flask contents are sieved four times through a 63 µm aperture sieve; the residue is washed off each time and collected in a beaker (after Fenwick, 1963). A comprehensive discussion of this method can be found in Ayoub (1980).

Extraction from Soil

Before starting nematode extraction, pass the soil through a coarse sieve of ~1–2 mm to break up clumps and remove stones, roots and plant debris. Then, mix the soil thoroughly and remove a subsample using a beaker of known volume. A 100 ml soil volume is commonly used. Nematode extraction from soil requires techniques different from plant tissue, except for the modified Baermann technique. However, this technique is inefficient in recovering large, slow-moving nematodes (e.g. *Longidorus* and *Xiphinema*) or nematodes with cuticular appendages (e.g. Criconematids). These are best extracted using sieving or elutriation techniques. Sieving or sieving plus filtering are quick methods for assessing all types of active, inactive and dead nematodes in soil, but they are not very quantitative as they are subject to much operator error. Elutriation techniques are very versatile methods capable of extracting wet cysts and vermiform nematodes from soil or root knot females from root debris, providing the appropriate sized sieves and the correct flow rate of water are used. Flotation techniques give the most efficient and quickest extraction of active and sedentary nematodes from soil. Ideally, large centrifuge tubes (300–1000 ml) are preferable, but smaller tubes can be used, especially when used in conjunction with a sieving technique. Other, less frequently used techniques include the Seinhorst two-flask technique, which is a simple method giving a more efficient and cleaner extract than direct sieving (Seinhorst, 1955). A combination of techniques can improve accuracy of the assessment, as noted by Demeure and Netscher (1973) for *Meloidogyne* in a sandy clay soil.

Comparing the different techniques, Yen *et al.* (1998) found higher recovery rates of *Meloidogyne incognita*, *Pratylenchus coffeae*, *Aphelenchoides besseyi* and free-living nematodes when using the centrifugal flotation method and flotation–sieving technique than the modified Baermann funnel method. Comparing the modified Baermann technique with flotation–sieving, Rodríguez-Kábana and Pope (1981) extracted higher numbers of *Pratylenchus*, *Meloidogyne* and *Heterodera* with the modified Baermann method, but *Helicotylenchus* and *Hoplolaimus* were higher for the flotation–sieving method. Nematode recovery, especially of endoparasitic specimens (e.g. *Meloidogyne* and *Pratylenchus*), can be improved by incubating the soil sample at room temperature for 3–4 weeks prior to extraction. Further information on the advantages and disadvantages of the various techniques is given in the EPPO standard PM7/119 on nematode extraction (EPPO, 2013).

Modified Baermann technique (Whitehead and Hemming, 1965)

The modified Baermann technique requires little labour and uses simple equipment. For soil samples up to 100 ml, flowerpot dishes or plastic bowls of 10 cm in diameter can be used. For handling larger samples, the Baermann tray or dish technique is generally preferred over the Baermann funnel technique. A support to hold the soil above water level is made from a plastic sieve or wire basket. Cotton wool milk filter or paper tissue is laid on the support. The support is held in a collecting tray (e.g. plastic dish or bowl, greenhouse tray). Up to 100 ml soil is spread thinly over the filter in the basket, which should not exceed 5 mm as extraction efficacy will decline rapidly with increasing thickness of the soil layer. Water should be added carefully down the inside edge of the collecting tray until the soil becomes wet (Fig. 4.1e). To obtain a clean extract, it is important not to move the tray once the water has been added. Space can be saved by making a simple rack to hold the trays, and evaporation can be lessened by covering with polythene sheeting. Most nematodes will have collected on the bottom of the tray after 24–48 h,

but root knot juveniles from egg masses, or some endoparasites from root fragments, may take several days to emerge. The support with the soil is then removed slowly and carefully, and the nematode suspension from the tray beneath can be concentrated by pouring into a 100 ml measuring cylinder and leaving to settle for 4 h or more, when the supernatant water can be syphoned off. Alternatively, the suspension can be concentrated quickly by passing it through a 20 µm sieve, washing the nematodes off the sieve and collecting them in a small tube/vial.

Sieving technique (Cobb, 1918)

The sieving technique is also known as the 'bucket-sieving' method. Although crude, it is widely used as it enables the extraction of large numbers of both active and inactive nematodes in a relatively short time. Equipment required includes two plastic buckets (5 l), sieves of 15–20 cm diameter made with wire mesh (preferably stainless steel) of an aperture size of 2 mm, 710, 250, 125, 90, 63, 45 and 25 µm, respectively, and tall 100 ml measuring cylinders for the residue from the sieves.

Usually, only three or four of the set of sieves will be used for a particular sample, with the sieves selected to match the size of nematode it is hoped to extract, and to suit the type of soil involved. In general, sieve openings should be no greater than one-tenth of the nematode length. Most adults of large nematodes (e.g. *Anguina*, *Belonolaimus*, *Hirschmanniella*, *Longidorus* and *Xiphinema*) are caught on a 250 µm aperture sieve, adults of average-sized nematodes (e.g. *Aphelenchoides*, *Ditylenchus* and *Hemicycliophora*) on a 90 µm aperture sieve, and many juveniles and small adults (e.g. *Criconemoides*, *Paratrichodorus*, *Paratylenchus*, *Pratylenchus* and *Radopholus*) on a 63 µm aperture. A 45 µm, or even 25 µm, aperture sieve is used to recover small juveniles (e.g. *Meloidogyne*, *Heterodera* and most others). Use sieves singly, never stack them and never attempt to work a sample through them all simultaneously, as this may reduce the efficiency of recovery. Fine sieves are easily clogged, but this can partially be avoided by pouring the suspension on a sieve inclined at an angle of about 30° to the horizontal; however, the number of nematodes caught on the sieve will also be reduced (Araya *et al.*, 1998). Sonicate sieves for cleaning. The method is as follows:

1. Place a known volume of soil (100–500 ml) in bucket I and fill with about 1–4 l of water. Dry soils should be soaked for a few hours. The mixture is stirred to free nematodes from the soil and suspend them in the water. Flocculating agents, such as Separan NP10 (12.5 µg/ml), can be used to help to break up soil aggregates in heavy clay soils.

2. Allow the mixture to settle for 30–60 s and decant over a 2 mm aperture sieve into bucket II. Avoid pouring the sediment. Add less water to the sediment in bucket I and repeat this step 2–3 times to increase nematode recovery. Any sediment left in bucket I is then discarded and bucket I washed out. The sieve is rinsed over bucket II. The residue on this sieve may contain very large nematodes, but usually it can be discarded safely.

3. The contents of bucket II are stirred, allowed to settle for about 10 s and then poured through a 710 µm aperture sieve into the clean bucket I, leaving behind heavy soil particles to which more water is added and the process repeated, if desired. The sieve over bucket I is rinsed. The residue on this sieve may contain only a few large nematodes, but this often depends on how much debris is present. To collect the residue, hold the sieve over bucket I at a steep angle (35–45°) and direct a gentle stream of water on to its upper side to wash the nematodes to the bottom edge of the sieve. Small nematodes and eggs will be washed through the sieve into bucket I and recovered later. Transfer the nematodes on the sieve into a 250 ml beaker using a gentle stream of water, leaving behind any heavy particles.

4. Bucket II is cleaned and the process repeated using 250, 125 and 90 µm aperture sieves and collecting the residues, as described above. The residues of each sieve can be pooled in one 100 ml measuring cylinder, or kept separate in different measuring cylinders. The contents of the collecting measuring cylinders are allowed to settle for 3–4 h and the supernatant liquid decanted carefully or syphoned off, leaving about 20 ml in the bottom. The suspension can be transferred to a viewing dish and examined.

Some shorten the procedure by transferring the soil suspension directly through a 1–2 mm aperture sieve to remove very coarse material,

followed by a 45 µm aperture sieve to collect the nematodes. This procedure is less suitable for larger sample sizes (>250 ml) and heavy soil, due to clogging of the fine sieve. Although this technique is less laborious, nematode losses may be higher. If the suspension still contains a significant amount of debris, further processing by centrifugal flotation or modified Baermann techniques can result in an almost clean nematode suspension. However, sluggish and inactive nematodes can be lost (e.g. *Longidorus*/*Xiphinema*).

Elutriation techniques

Elutriation techniques extract nematodes from soil samples of 100–1000 ml by using an upcurrent of water to separate them from soil particles and hold them in suspension. They give a cleaner extraction than that obtained by direct sieving; however, further cleaning by the modified Baermann technique or centrifugal flotation might be required. Flow rates can be adjusted readily to suit soil type and the size of nematode to be extracted. Of the models that have been developed (Seinhorst, 1956; Tarjan *et al.*, 1956; Oostenbrink, 1960), the No III model of Oostenbrink is often used because it is robust and easily operated and cleaned. Oostenbrink (1960), Southey (1986) or EPPO (2013) should be consulted for details. Winfield *et al.* (1987) described a column elutriator for extracting nematodes and other small invertebrates, referred to as a Wye Washer. This equipment was shown to achieve extraction rates equal to or better than existing techniques; however, the water use and price are high. Another alternative is the fluidizing column (Trudgill *et al.*, 1973), representing a simple, robust and versatile elutriator.

Centrifugal flotation techniques

Nematodes can be extracted from soil and organic debris by floating them out in a solution of specific gravity greater than their own. As the method does not rely on the mobility of nematodes, it is extremely useful for extracting sluggish forms, such as criconematids, as well as dead, moulting or fixed nematodes and eggs. Centrifugal flotation is generally a more efficient nematode extraction method than the Baermann, sieving or elutriation techniques. Flotation is often used to clean extracts obtained by sieving or elutriation, but can also be applied directly to soil samples. Solutions of sucrose, $MgSO_4$ or $ZnSO_4$ can be used. Sugar is the most used solute because it is cheap; however, Rodríguez-Kábana and King (1975) found that blackstrap molasses was even cheaper and, because of higher viscosity, more effective than sucrose for extracting nematodes. $MgSO_4$ does not have the stickiness of sugar but can be reused, and $ZnSO_4$ has fewer osmotic effects but is more acid and toxic. Other manufactured solutes (Ludox, Ficoll and Percol) have advantages over $MgSO_4$ and $ZnSO_4$ but are more expensive (Viglierchio and Yamashita, 1983; Bloemers and Hodda, 1995). To reduce the osmotic stress by the solutes, nematodes should be rinsed with water as soon as possible to aid their recovery. A solution with a specific gravity of about 1.18 (673 g of sugar dissolved in water and made up to 1 l) is suitable for most nematodes; however, a more dense solution of specific gravity 1.25 (1210 g of sugar dissolved in water and made up to 1 l) is required for very long nematodes, such as *Longidorus* and *Xiphinema*. The specific gravity of a solution should be checked just prior to its use, as changes in temperature and microbial activity can cause a considerable decrease in concentration. The suspensions recovered are caught on a sieve of 20 µm aperture and used for direct counting.

For centrifugal flotation, a soil sample of 100–250 ml is placed in a 800–1000 ml centrifuge tube and water added up to 2 cm from the tube brim. Kermarrec and Bergé (1971) recommend the addition of a tablespoon of kaolin to aid sedimentation and to give a more compact surface to the sediment pellet. The contents are mixed thoroughly using a Vibromixer or mechanical device. The tubes are balanced by adding water and centrifuged at about 1800 g for 4 min. The supernatant containing organic debris is discarded and the tube almost filled with the suspending solution (specific gravity 1.18) and stirred mechanically or with a Vibromixer to resuspend the pellet containing the nematodes. Tubes are balanced by adding more solution and centrifuged again at 1800 g for 4 min. The supernatant is poured through a sieve of 53 µm aperture or less (to avoid loss of smaller nematodes), rinsed quickly with tap water and collected in a

beaker or counting dish. The relative centrifugal force represents the force on particles due to gravity: g = 0.00001118 × radius of centrifuge arm to tip of tube in cm × (speed in r.p.m.)^2. Flocculating agents, such as Separan NP10 (12.5 µg/ml), might be used to help break up soil aggregates in heavy clay soils. Large soil samples of 500–2000 ml can be processed by first applying the sieving technique followed by centrifugal flotation.

Extraction of heteroderid cysts from dry soils

The saccate dead females, 'cysts', containing eggs of heteroderid nematodes differ from other nematode stages in size, shape and weight. Different methods have been developed for extracting cysts from dry soil (e.g. Fenwick can, Schuiling centrifuge) and for extracting from wet or dry soil (e.g. Seinhorst elutriator, centrifugal flotation, Wye Washer). Cysts from dried soil contain air bubbles and float in water. To extract those cysts, a 100–1000 ml sample of the dried soil is placed in a plastic bucket, made up to about 2–5 l with water and thoroughly stirred with a strong stream of water or manually. Allow the coarse material to sediment for 1–3 min. Any cysts present will float to the surface with other organic debris. Decant through a 2 mm aperture sieve over a 250 µm aperture sieve (a 100 µm aperture sieve may be needed to catch small cyst nematodes, such as *Heterodera trifolii*). Repeat the process 2–3 times if necessary. Discard the residue on the 2 mm aperture sieve and collect the cysts on the 250 µm aperture sieve for further examination. Alternatively, the float can be poured on to a filter paper in a funnel, the water drained off and the paper examined for cysts, most of which will occur along the 'tidemark' left at the upper water level (Shepherd, 1986). Methods for extracting cysts from moist soil rely on elutriation that keeps the cysts afloat in the suspension, or on centrifugal flotation using a solution with a higher density then their own (e.g. 1.25). Based on Riggs *et al.* (1997), sieving was more efficient than elutriation for extracting cysts. If cysts are to be used further as inoculum in biotests, note that the contents of *Globodera*, but not *Heterodera*, cysts will survive desiccation. See EPPO (2013) for further details on these methods and their advantages and disadvantages.

Storage

Many nematodes remain in good condition for several days when stored in shallow, fresh tap water at about 5–10°C. Contaminating bacteria can be suppressed by adding three drops of 5% streptomycin sulfate solution per 5 ml of suspension. Tropical nematodes needed for live cultures or for experimental use should be stored at room temperature and aerated with an aquarium pump. For long-term storage (e.g. germplasm collection, maintenance of genetic lines, reference material or inoculum), nematodes can be stored in liquid nitrogen. Cryopreservation has been shown to work for several nematodes (Irdani *et al.*, 2011). For *Pratylenchus thornei*, the survival rate was 76% when nematodes were pre-treated in 14–17% glycerol for 5 days before storage in liquid nitrogen (Galway and Curran, 1995). Thawed nematodes were able to reproduce and infect carrot disc cultures. Similar survival rates were achieved by van der Beek *et al.* (1996) for *Meloidogyne hapla* and *Meloidogyne chitwoodi* in liquid nitrogen after pre-treatment in 10% ethanediol for 2 h at room temperature and 40% ethanediol for 45 min on ice. Cysts of *Heterodera avenae* have been stored successfully at –18°C (Ireholm, 1996).

Examination of Nematode Suspensions

Direct examination

Extracted nematodes can be examined directly under a microscope to the genus level using open counting dishes or fixed capacity, usually 1 ml, covered counting slides (Fig. 4.3). A good stereoscopic microscope with a range of magnifications 10× to 100×, a fairly flat field and good resolution are essential. All or part of the extracted suspension, according to nematode density, is placed in a counting dish/slide and examined under the microscope. When samples are taken with a pipette, it should have a wide outlet to prevent debris or large nematodes

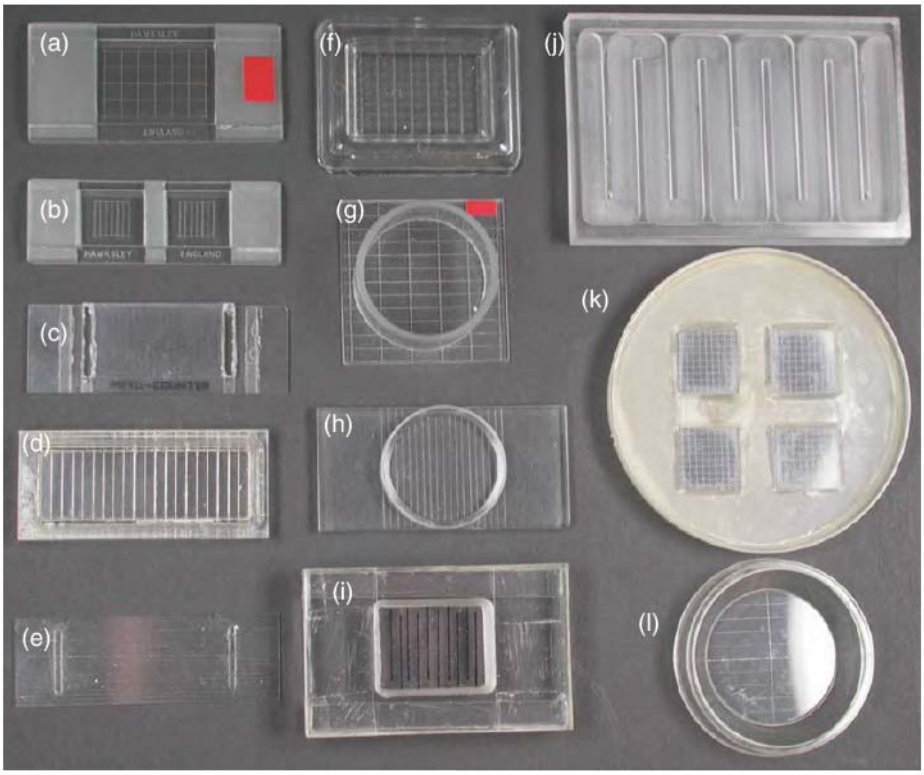

Fig. 4.3. Examples of counting slides/dishes: (a) Peter's 1 ml counting slide in glass, as made by Hawksley, UK; (b) multichamber counting slide in glass, as made by Hawksley; (c) 1 ml counting slide made by MEKU, Germany; (d) 2 ml counting slide in plastic (made at JKI Münster, Germany); (e) microscope slide with ridges to hold a large cover slide, 1 ml volume (made by Sikora, Bonn University, Germany); (f) moulded plastic dish, 5 ml, with sloping sides and ridged grid (made at Rothamsted Experimental Station, UK); (g) glass ring, 38 mm, glued on a glass plate for counting cysts (made at JKI Münster); (h) 2 ml counting slide with sloping sides consisting of a 2 mm high plastic ring glued on a plastic plate of 75 × 37 mm (made by Sikora, Bonn University, Germany); (i) 2 ml counting slide in plastic with a coverglass of 78 × 48 mm, as the bottom to allow examination with an inverse microscope (made at JKI Münster); (j) 10 ml winding-track counting tray in plastic, as made by Nordmeyer and Sikora (at Bonn University, Germany); (k) multichamber counting slide with sloping sides made in paraffin within a 90 mm diameter plastic Petri dish (made at JKI Münster); (l) 50 mm diameter plastic tissue culture Petri dish marked for examination at 20–40×, base lines are cut with a plastic or glass writing knife into the lid. (Photograph courtesy of JKI Münster.)

clogging it. Petri dishes or flat-bottomed Syracuse watch glasses (Shurtleff and Averre, 2000) are often used, and a grid is etched, or scratched with a marking diamond, on the inside of the base to act as a guide when counting. Small disposable tissue culture plastic Petri dishes (5 cm in diameter) that have sloping sides can be used on which a grid is scratched easily with a needle (Fig. 4.3). To be sure of searching over the whole area of the dish, the space between the grid lines should be a little less than the field width of the microscope at the magnification being used. Thus, a dish with an extract containing average size nematodes would be examined at about 50× and have lines about 3 mm apart. Some workers prefer to examine extracts in a dish with a thin base (e.g. a disposable plastic Petri dish) using the low/medium power objectives of an inverted compound microscope when nematodes can be seen in more detail. Covered counting slide chambers are useful for routine counts when immediate access to nematodes within the suspension is not required. Examples are shown in Fig. 4.3. Counting slides and dishes are in many cases custom made (Doncaster *et al.*, 1967; Southey, 1986); others are commercially available, such as 1 ml covered counting slides from Chalex LLC (www.vetslides.com, accessed

2 November 2017) or 10 ml open counting dishes from Wageningen University (https://www.wur.nl/en/show/Nematode-counting-dishes-2.htm, accessed 2 November 2017). A hand tally counter or a multiple bank of counters is an essential aid for counting different genera. For nematode identification to the species level, temporary or permanent slides need to be prepared, which includes handling of the nematodes.

Handling nematodes

There are various methods for handling nematodes. Small batches of nematodes can be selected and transferred from a suspension by using a fine pipette. The modified Hesling's device (Alam, 1990) or the suction device described by Sehgal and Gaur (1988) even allow the selection of individual specimens. However, in most cases, a handling needle is preferred, which is a dissecting needle handle to the end of which is attached with glue a nylon toothbrush bristle, sharpened bamboo splinter, eyebrow hair, fine wire or small wire loop. To 'fish' nematodes, the specimens should be in shallow water, near the centre of the dish, and the lowest convenient microscope magnification used to give the greatest possible depth of focus and working distance. While viewed with the stereoscopic microscope, the handling needle is used to lift the nematode to the surface of the water; the bristle is then held immediately underneath the nematode and flicked up quickly so that the nematode is pulled out through the meniscus. The surface tension can be removed by adding a small drop of detergent. Picking up fixed nematodes from glycerine is generally easier, due to its higher viscosity.

Killing and fixing nematodes

For identification to the species level and permanent storage, nematodes must first be killed, fixed and properly mounted. The following method is recommended for killing and fixing nematodes in one step (Seinhorst, 1966): specimens are concentrated in ~3 ml of water in a 10 ml glass vial, either by centrifuging or by letting them settle and siphoning off the supernatant. The vial is shaken to disperse the nematodes. Fixatives that can be used are TAF (2 ml of triethanolamine, 7 ml formaldehyde 40%, 91 ml distilled water) or 4:1 FA containing 10 ml formalin (40% formaldehyde), 1 ml glacial acetic acid and distilled water up to 100 ml. If equal amounts of fixative are added to the nematode suspension, the fixative needs to be double strength. This can be made up using half the amount of water indicated above. The fixative is heated to 70–75°C in a small tube held in a water bath of the required temperature for a few minutes, preferably monitored with a thermometer, and added to the nematode suspension. This method gives a very good fixation of glands and gonads. Nuclei tend to expand and are seen more easily. Although specimens appear rather dark as soon as they are fixed, processing to glycerol will eventually clear them. However, fixatives usually cause some shrinkage and/or distortion of the specimen (Grewal *et al.*, 1990). The addition of 2% glycerol to the above means that nematodes can be brought directly from fixative to glycerol by slow evaporation (see below). Also, as noted by Hooper (1987), nematodes stored in vials will eventually end up in glycerol should the fixative evaporate. Nematodes will be spoiled if placed alive into cold fixative. Alcoholic fixatives should be avoided as they usually shrink nematodes. Well-fixed specimens have a smooth outline. Nematodes can be stored in formalin indefinitely. However, due to toxic fumes, all work with formaldehyde must be conducted under the exhaust hood.

Comparing the different methods, Grewal *et al.* (1990) found that killing and fixing with the addition of hot (95°C) TAF produced the least affected specimens compared with FA 4:1. Chakrabarti and Saha (2001) arrived at a similar conclusion using TAF at 50°C. The most lifelike specimens were produced when fixed in TAF and processed to glycerol by the slow method (outlined below) (Grewal *et al.*, 1990; Siddiqi, 2000).

Processing and Mounting Nematodes

In fixed nematodes, much of the internal body contents, especially gonad structure, may be obscured by the granular appearance of the intestine. Specimens can be cleared by processing with lactoglycerol or glycerol, which are also suitable mountants. Lactoglycerol is a solution of equal amounts of lactic acid, glycerol and

distilled water, to which can be added 0.05% acid fuchsin or 0.05% methyl blue to stain the specimen, if required. However, glycerine mounts are preferred. Several techniques exist that allow processing of the specimens through alcohol to glycerine with minimum time and effort (Hooper, 1987). Mounted specimens can deteriorate, and the storage of some representatives in glycerol in vials is recommended.

Glycerol method

Most nematodes are best preserved in anhydrous glycerol. Transfer from the fixative to glycerol can follow a slow or rapid method. The former usually gives better preservation and is therefore recommended if time is not a limiting factor.

Slow method

Remove most of the fixative from preserved specimens in a small dish or deep glass block with a fine pipette, but take care not to draw nematodes inadvertently. Add 3–4 ml of the following solution: anhydrous glycerol, 2 ml; 96% ethanol, 1 ml; distilled water, 90 ml.

Cover the dish loosely and let the sample stand at room temperature for 2–3 weeks or until water and ethanol have all evaporated. The process can be speeded up in an oven at 30–40°C, but the container needs to be well covered to ensure that the evaporation takes several days. If evaporation is too rapid, the nematodes shrink and become distorted. Golden (in Hooper, 1970) recommends the addition of a few drops of picric acid, which helps to prevent clearing and fading of nematode stylets and the growth of moulds. If completed, the nematodes are in pure glycerol and can be stored indefinitely or used for preparing permanent microscope slides. Note that nematodes processed to glycerol are very soft and should be handled carefully, preferably using a mounted eyebrow hair or similar soft bristle.

Rapid method (Seinhorst, 1962)

Fixed specimens are transferred to a small, concave glass dish of 2–4 ml capacity containing about 0.5 ml of the following solution: 96% ethanol, 20 ml; glycerol, 1 ml; distilled water, 79 ml.

The dish with nematodes is placed into a closed glass vessel containing an excess (e.g. 1/10 volume of the vessel) of 96% ethanol. The dish is supported above the ethanol on a platform or grid. After a minimum of 12 h in an oven at 40°C, the specimens will be in a mixture of mainly ethanol, with some glycerol. Remove the dish from the vessel; excess ethanol can be withdrawn using a pipette, and add a solution of five parts glycerol and 95 parts of 96% ethanol. Then place the dish in a partly closed Petri dish in an oven at 40°C until the ethanol has evaporated. This should take at least 3 h; the nematodes are then in pure glycerol and should be mounted immediately in anhydrous glycerol.

Mounting nematodes

The nematodes are best mounted on thin microscope glass slides (25 × 76 mm) using 19 mm diameter round cover slips. Supports (e.g. stainless-steel wire, tungsten filaments of calibrated diameter, glass fibre or beads) with similar thickness as the nematodes are required to prevent deformation of the specimens from the weight of the cover glass.

For permanent mounts, a very small drop of anhydrous glycerol (heated for 4 h at 40°C in an oven) is placed in the centre of a clean microscope slide and nematodes of about equal diameter are transferred to it, using a handling needle, and arranged in the centre of the drop so that they are touching the slide surface, not floating. Three cover glass supports should be arranged around the nematodes. Paraffin wax of melting point 60–65°C is used as seal, but also provides additional support. A wax ring is prepared using a copper tube (15 mm in diameter, heatproof handle) heated in a flame, dipped in paraffin wax and applied to the centre of the slide surrounding the mountant. A clean cover glass (19 mm diameter circle No 1) held with fine forceps is lowered on to the drop. A mounted needle held in the other hand can be used to help prevent the cover glass from sliding sideways when it is applied. It helps to prevent air bubbles from being trapped if the drop is kept as hemispherical as possible before applying the cover glass. The slide is placed on a hotplate at 65°C for a few seconds. As soon as the wax melts, press lightly with a mounted needle on the cover glass

to make sure it has settled far enough; thick mounts prevent oil immersion objectives being used. The wax will set rapidly when the slide is placed on a cool surface. A secondary seal is desirable to prevent drying out and to prevent immersion oil dissolving the wax, such as Permount (Thermo Fisher Scientific, USA), Corseal (Sabir, 1997) or Glyceel (Bates, 1997), which are excellent, or nail varnish. Seal the cover glass using a small soft brush, with a thick but fairly narrow band of the sealant, making sure there is sufficient on the cover glass as well as on the slide. Repeat the process when the first ring has dried, to give a good seal.

Instead of a wax ring, Siddiqi (2000) recommends the use of three small lumps of wax, each about the size of the mounting drop, arranged around the drop, and the cover glass is placed on the lumps and the slide then heated. The wax melts, allowing the cover glass to settle down, and confines the glycerol to the centre of the mount. It is important to retain a hemispherical drop of mountant before applying the cover glass, or the wax may swamp the specimens. Supports, however, remain useful to prevent deformation of the nematodes.

Posterior cuticular patterns of *Meloidogyne* spp.

The cuticular markings surrounding the vulva and anus (posterior cuticular pattern, or 'perineal' pattern) of females of *Meloidogyne* spp. are used in their identification (Taylor *et al.*, 1955; Franklin, 1962). Fresh or fixed galled roots are stained in cotton-blue lactophenol or lactoglycerol. Females stained in fresh root material are preferable, because their body contents are removed more easily (Franklin, 1962). About 20 females are dissected out and transferred, using fine-pointed forceps, to 45% lactic acid on a transparent perspex slide or plastic Petri dish cover. Working at a magnification of at least 32×, preferably more, the swollen female is speared at the neck end with a very sharp, fine needle and held so that the posterior end can be cut off with an oculist's scalpel or sharp Borradaile needle. A hypodermic needle mounted on a handle also serves as a useful cutting tool. The inner tissue is removed carefully by brushing lightly with a flexible bristle. The cuticle is transferred to a drop of glycerol, where it is trimmed to a size slightly greater than the pattern, which is then transferred to a drop of glycerol on a clean glass slide. The posterior patterns, outside uppermost, are arranged in one or two neat rows, and a cover glass is applied and sealed. Supports are optional. At least ten specimens from a population should be examined. The patterns can usually be seen satisfactorily at a magnification of about 500×, but for species having small or indistinct patterns, an oil immersion objective and higher magnification may be needed.

As noted by Taylor (1987), the lip region shape and the position of the excretory pore in mature females are an aid to the identification of *Meloidogyne* spp. Gerber and Taylor (1988) give details of preparation and mounting so as to show the anterior end and perineal pattern on one specimen. The preparation is similar to that described above for perineal patterns only, but the mature female is pierced once or twice in the mid-body region and the body contents squeezed out carefully. The female is then orientated with the perineal pattern to one side and, using a fine scalpel or hypodermic needle, the posterior quarter of the body, without the pattern, is cut away, taking care not to damage the pattern. The prepared specimens are then mounted in glycerol, with the cut opening underneath and the perineal pattern uppermost. For additional information on preparation methods for culturing and identification of *Meloidogyne* spp., see Barker *et al.* (1985) or Jepson (1987).

Vulval cones of cyst nematodes

The structure of the vulva, fenestra and associated internal structures as well as the general shape of cysts are used for identifying cyst nematodes (e.g. *Globodera* and *Heterodera*) (Hesling, 1978). A detailed protocol for the preparation of vulval cones of cyst nematodes is given by Subbotin *et al.* (2010). Dry cysts should be soaked in water for up to 24 h before dissection. Place a moist cyst on a perspex slide on the stage of a stereomicroscope and cut the posterior end off so that the fenestral area is in the centre of the cut piece. Trim the cut end so that it is no more than 5–10 times the fenestral area. Using fine forceps and a flexible probe (e.g. eyebrow or fine

toothbrush bristle), clean away any adhering body contents, e.g. eggs, taking particular care not to damage the structures associated with the vulva. Thick-walled and heavily pigmented species, bleached for a few minutes in H_2O_2, often have more visible structures. Avoid overbleaching. Wash the cleaned vulval cones in distilled water and then pass through 70, 95 and 100% ethanol to clove oil. After clearing in clove oil, mount in Canada balsam. Support the cover glass with pieces of glass rod or broken cover glass to prevent distortion of the specimen. Vulval cones may also be mounted in 'Euparal', after passage through 70% ethanol and isobutanol, or directly in glycerine and sealed.

A simpler method for the examination of the vulval cone of mature *Heterodera* cysts is described by Esser (1988). Place a block of 1.7% water agar (15 mm × 15 mm × 2 mm high) on a slide and make a small 1 mm deep cavity slightly less than the diameter of the cyst with a fine needle. Gently press the cyst into the cavity with the anterior end down until the vulva region of the cyst is at the same level as the agar surface. Add a small drop of water to a 15 mm cover slip, which is inverted and dropped over the embedded cyst, which can then be viewed under the microscope. Correia and Abrantes (1997) describe an improved technique for mounting *Heterodera* cysts in glycerine agar.

Computerized systems

Image analysis systems can assist with the examination of nematode samples by counting nematodes in a suspension (Been *et al.*, 1996) or with automatic recognition of nematodes (Fernandez-Valdivia *et al.*, 1989). Furthermore, computerized keys can help with the identification of species (Viscardi and Brzeski, 1993, 1995). An example of a computerized key is freely accessible on the website of the University of Nebraska, Lincoln (http://nematode.unl.edu/key/nemakey.htm, accessed 2 November 2017).

Molecular Diagnostics

Most methods of nematode diagnostics have their limitations. Species identification based on morphological and morphometrical characters requires much skill, but can often be inconclusive for individual nematodes. Isozyme or total protein analyses are relatively fast ways to identify root knot or cyst-forming nematode species. Although differences in isozyme or protein patterns show significant consistency and are useful for species identification, reliable results can only be obtained with nematodes of specific developmental stage. DNA-based diagnostics do not rely on the express products of the genome, and are independent of environmental influence or developmental stage. Recent progress in nematode diagnostics has been achieved due to introducing the polymerase chain reaction (PCR), a powerful method with widespread application in many biological fields (Fig. 4.4). A single nematode, egg, or even a part of the nematode body, can be identified using this technology. The majority of PCR-based techniques developed for nematode diagnostics indicate differences of the ribosomal RNA (rRNA) or mitochondrial DNA (mtDNA) gene sequences.

rRNA and mtDNA genes

The rRNA genes are arranged as tandem repeats, with several hundred copies per genome. Each repeat includes the small subunit (SSU) gene, or 18S gene, the 5.8S gene and the large subunit (LSU) gene, or 28S gene, the spacer region between the subunit and 5.8S gene, called the internal transcribed spacers (ITS1 and ITS2), and between the gene cluster, called the intergenic spacer (IGS). In the root knot nematodes, the 5S gene is found in the IGS. The 18S gene evolves relatively slowly and is useful for comparison of distantly related groups, whereas ITS and IGS are considerably more variable and can be used to distinguish species or subspecies. Some regions of the 28S gene are also useful for species differentiation.

MtDNA is a circular, double-stranded, closed, small structure that is present in large copy numbers in the cell. Rapid evolution rates of specific genes in the mtDNA, which evolve ten times faster and more than nuclear genes, result in accumulated sequence polymorphism. This allows this molecule to be used as a useful marker for differentiation of nematode populations and of closely related species. For example, sequences

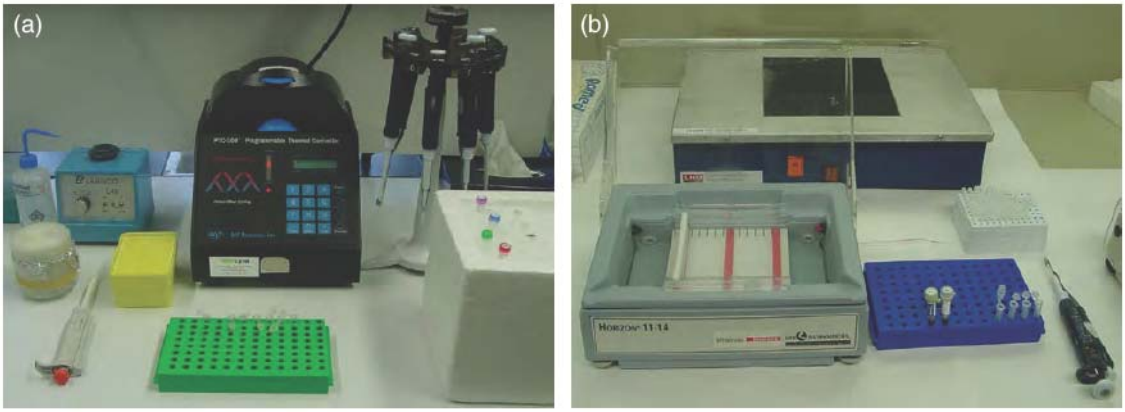

Fig. 4.4. Equipment required for PCR (a), electrophoresis and visualization of the PCR product on agarose gel (b).

of intergenic spacer, large subunit of the rRNA, mitochondrial cytochrome c oxidase 1 (COI) and NADH dehydrogenase subunit 5 (NAD5) genes can be used successfully for differentiation of root knot nematodes from the *M. incognita* group (Powers and Harris, 1993; Pagan *et al.*, 2015; Janssen *et al.*, 2016).

DNA extraction

The first step in molecular diagnostic procedures is the preparation of the template DNA (see Examples 1 and 2 below). Several protocols for the extraction of nucleic acids from nematodes are available (Curran *et al.*, 1985; Caswell-Chen *et al.*, 1992; Blok *et al.*, 1997). Some of these allow the isolation of microgram quantities of pure genomic DNA. However, because only small quantities of starting DNA are required for PCR amplification, simplified and rapid procedures can generally be used (Harris *et al.*, 1990; Subbotin *et al.*, 2000; Waeyenberge *et al.*, 2000; Floyd *et al.*, 2002). Using different extraction methods and commercial kits, nematode DNA can be obtained directly from soil samples (Nazar *et al.*, 1995; Waite *et al.*, 2003). Furthermore, extraction of DNA from formalin-fixed materials or nematodes embedded in glycerine on slides provides a new opportunity for molecular examination of reference materials (Thomas *et al.*, 1997; Rubtsova *et al.*, 2005).

Example 1: protocol for DNA extraction using proteinase K with worm lysis buffer (WLB) (Waeyenberge *et al.*, 2000).

1. Select a single or several nematodes and place in a 10 µl drop of double-distilled water on a glass slide under the dissecting microscope.
2. Cut nematodes into three or four pieces with a needle or scalpel.
3. Transfer worm bits with water to a sterile 0.2 ml Eppendorf tube containing 8 µl of WLB (500 mM KCl, 100 mM Tris–HCl pH 8.3, 15 mM $MgCl_2$, 10 mM dithiothreitol (DTT); 4.5% Tween-20) and 2 µl of proteinase K (600 µg/ml).
4. Freeze at –80°C for 10 min.
5. Incubate at 65°C for 1 h and then heat at 95°C for 15 min.
6. Centrifuge for 1 min at maximum speed to remove debris. Use 1–4 µl of the supernatant in the PCR.

Example 2: protocol for DNA extraction using NaOH (Floyd *et al.*, 2002).

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

PCR

This enzymatic reaction allows *in vitro* amplification of target DNA fragments by up to a billion-fold from complex DNA samples within a

test tube. Any nucleic acid sequence can be detected by PCR amplification. The method requires a DNA template containing the region to be amplified, two oligonucleotide primers flanking this target region (Table 4.1), DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) mixed in buffer containing magnesium ions ($MgCl_2$) (Example 3). The PCR is performed in tubes, with final volumes of 20–100 µl. The PCR procedure consists of a succession of three steps, which are determined by temperature condition: template denaturation at 95°C for 3–4 min, primer annealing at 55–60°C for 1–2 min and extension at 72°C for 1–2 min. The PCR is carried out for 30–40 cycles in a thermocycler with programmed heating and cooling. Finally, PCR products are separated electrophoretically, according to their size, on agarose gels and visualized by ethidium bromide under ultraviolet (UV) light. Once identified, nematode target DNA generated by PCR amplification can be characterized further by various analyses: restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism or sequencing.

Example 3: PCR protocol.

1. Add a DNA suspension to the Eppendorf tube containing a PCR mixture with 5 µl of 10× PCR buffer, 10 µl of Q-solution, 1 µml of dNTP mixture (10 mM each) (*Taq* PCR Core Kit, Qiagen), 0.5 µl of each primer, 1 U of *Taq* polymerase, and double-distilled water to a final volume of 50 µl.
2. Place the tube in the PCR machine with an initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, 55°C for 1.5 min, 72°C for 2 min and a final elongation step at 72°C for 10 min.
3. Run 2–5 µl of PCR product on a 0.8–1% agarose gel for 30–60 min at 90–100 V.

PCR-RFLP

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 and the resulting fragment is separated by electrophoresis (Example 4). If there is some difference in sequences situated within the restriction site of the enzyme, the digestion of the PCR products will lead to different electrophoretic profiles. It has been shown that the comparison of restriction patterns derived from amplified ITS regions is a very useful approach to distinguish species and populations. PCR-RFLP protocols are available for all relevant genera, often even with several protocols for one genus. Just a few examples are provided here, such as for *Aphelenchoides* (Ibrahim *et al.*, 1994), cyst-forming nematodes (Thiéry and Mugniéry, 1996; Szalanski *et al.*, 1997; Subbotin *et al.*, 2000) (Fig. 4.5), *Ditylenchus* (Ibrahim *et al.*, 1994), *Hemicycliophora* (Subbotin *et al.*, 2014), *Longidorus* (Subbotin *et al.*, 2013), *Nacobbus* (Reid *et al.*, 2003), *Pratylenchus* (Waeyenberge *et al.*, 2000), *Radopholus* (Fallas *et al.*, 1996), root knot nematodes (Zijlstra *et al.*, 1995; Schmitz *et al.*, 1998), *Trichodorus* (Kumari and Subbotin, 2012), *Tylenchulus* (Tanha Maafi *et al.*, 2012) and *Xiphinema* (Vrain *et al.*, 1992). Comparison of RFLP profiles from newly obtained samples with those from known species provide a quick tool for nematode identification. PCR-RFLPs are especially suited to identify nematodes of monospecific probes; this strategy does not allow mixed species populations to be identified.

Example 4: RFLP protocol.

1. Add 2–8 µl of PCR product to an Eppendorf tube containing 1.0 µl of 10× restriction enzyme buffer, 1 µl of restriction enzyme and double-distilled water to a final volume of 10 µl.
2. Place the tube in a water bath at 37°C (or other temperature required for digestion) for 1–12 h.
3. Centrifuge the tube for 30 s at maximum speed.
4. Run the reaction mixture on a 1.5% agarose gel in 1× TBE for 60–90 min at 90–100 V.

The restriction enzymes recommended for species identification are *Alu*I, *Ava*I, *Bsh*1236I, *Bsu*RI, *Cfo*I, *Hin*fI, *Mva*I, *Rsa*I and *Pst*I for cyst-forming nematodes (Fig. 4.5), and *Alu*I, *Dra*I, *Hin*fI, *Msp*I, *Pvu*II and *Rsa*I for root knot nematodes.

Sequencing

Direct sequencing of PCR products or sequencing of cloned PCR fragments provides full characterization of amplified target DNA. One of the first applications of PCR in plant nematology was presented by Ferris *et al.* (1993), who used the ITS rDNA sequences to establish the taxonomic and phylogenetic relationships of cyst-forming nematodes. The sequences of the ITS regions, fragments of 18S and 28S of rRNA genes, have been examined for a wide range of plant parasitic nematodes.

Table 4.1. Universal primers frequently used for nematode diagnostics.

Code	Primer (5′–3′)								Amplified region	Reference
C2F3	GGT	CAA	TGT	TCA	GAA	ATT	TGT	GG	3′ of COII to 16S mitochondrial genes	Powers and Harris (1993)
1108	TAC	CTT	TGA	CCA	ATC	ACG	CT			
18S	TTG	ATT	ACG	TCC	CTG	CCC	TTT		ITS1 region of rDNA	Szalanski *et al*. (1997)
rDNA1.58S	GCC	ACC	TAG	TGA	GCC	GCG	CA			
18S	TTG	ATT	ACG	TCC	CTG	CCC	TTT		ITS1–5.8S–ITS2 region of rDNA	Vrain *et al*. (1992)
26S	TTT	CAC	TCG	CCG	TTA	CTA	AGG			
F194	CGT	AAC	AAG	GTA	GCT	GTA	G		ITS1–5.8S–ITS2 region of rDNA	Ferris *et al*. (1993)
F195	TCC	TCC	GCT	AAA	TGA	TAT	G			
SSU18A	AAA	GAT	TAA	GCC	ATG	CAT	G		18S gene of rDNA	Blaxter *et al*. (1998)
SSU26R	CAT	TCT	TGG	CAA	ATG	CTT	TCG			
D2A	ACA	AGT	ACC	GTG	AGG	GAA	AGT	TG	D2–D3 expansion segments of 28S gene of rDNA	De Ley *et al*. (1999)
D3B	TCG	GAA	GGA	ACC	AGC	TAC	TA			
TW81	GTT	TCC	GTA	GGT	GAA	CCT	GC			
AB28	ATA	TGC	TTA	AGT	TCA	GCG	GGT		ITS1–5.8S–ITS2 region of rDNA	Joyce *et al*. (1994)

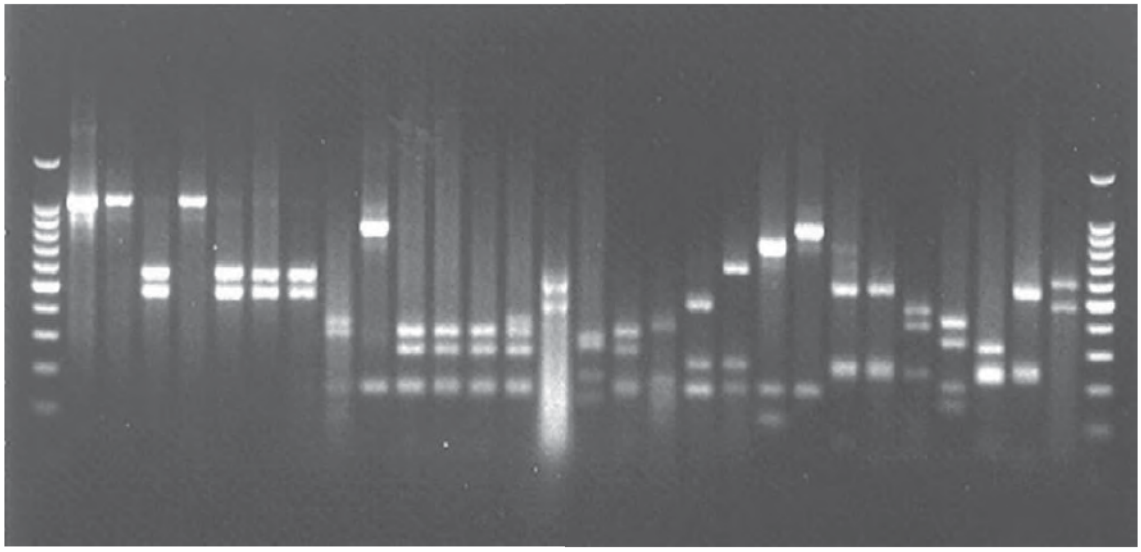

Fig. 4.5. RFLP patterns obtained after *Alu*I digestion of the amplified PCR product of the ITS-rDNA for cyst-forming nematodes. L, 100 bp DNA ladder; U, unrestricted PCR product; 1, 2, *Heterodera avenae*; 3, *Heterodera arenaria*; 4, *Heterodera filipjevi*; 5, *Heterodera aucklandica*; 6, *Heterodera ustinovi*; 7, *Heterodera latipons*; 8, *Heterodera hordecalis*; 9, *Heterodera schachtii*; 10, *Heterodera trifolii*; 11, *Heterodera medicaginis*; 12, *Heterodera ciceri*; 13, *Heterodera salixophila*; 14, *Heterodera oryzicola*; 15, *Heterodera glycines*; 16, *Heterodera cajani*; 17, *Heterodera humuli*; 18, *Heterodera ripae*; 19, *Heterodera fici*; 20, *Heterodera litoralis*; 21, *Heterodera carotae*; 22, *Heterodera cruciferae*; 23, *Heterodera* sp.; 24, *Heterodera cyperi*; 25, *Heterodera goettingiana*; 26, *Heterodera urticae*; 27, *Meloidodera alni*. (From Subbotin *et al*., 2000.)

The comparison of newly obtained sequences from samples with those published or deposited in the GenBank (www.ncbi.nlm.nih.gov/genbank/) is a most reliable approach for molecular identification. Increasing numbers of deposited nematode rDNA sequences, as well as decreasing costs for sequence analyses, will allow wider application of this still rather expensive procedure for routine nematode diagnostics in the future.

PCR with species-specific primers

PCR with specific primer combinations or multiplex PCR constitute a major development in DNA diagnostics and allow the detection of one or several species in a nematode mixture by a single PCR test, thus decreasing diagnostic time and costs. Species-specific primers are designed based on the broad knowledge of sequence divergence of the target DNA region in many populations of the same species and in closely related species. This knowledge allows the detection of populations with small differences in sequences, and avoids the amplification of an identical specific fragment in other species. The principle of this method is the alignment of the sequences from target and non-target organisms and the selection of primer mismatches to non-target organisms, but it shows sufficient homology for efficient priming and amplification of the target organism. This diagnostic tool has been developed for the identification of many agriculturally important plant nematodes (Fig. 4.6; Table 4.2). The multiplex PCR with specific primers for the identification of several nematode targets in one assay is limited by the number of primer pairs that can be used in a single reaction and the number of bands that can be identified clearly without giving false-positive results. This technique requires precise optimization of the reaction conditions for the primer sets used simultaneously in the test.

Reverse dot-blot hybridization

This technique involves the use of PCR for simultaneous amplification and labelling of target DNA to generate digoxigenin-dUTP-labelled amplicons, which are hybridized to specific immobilized

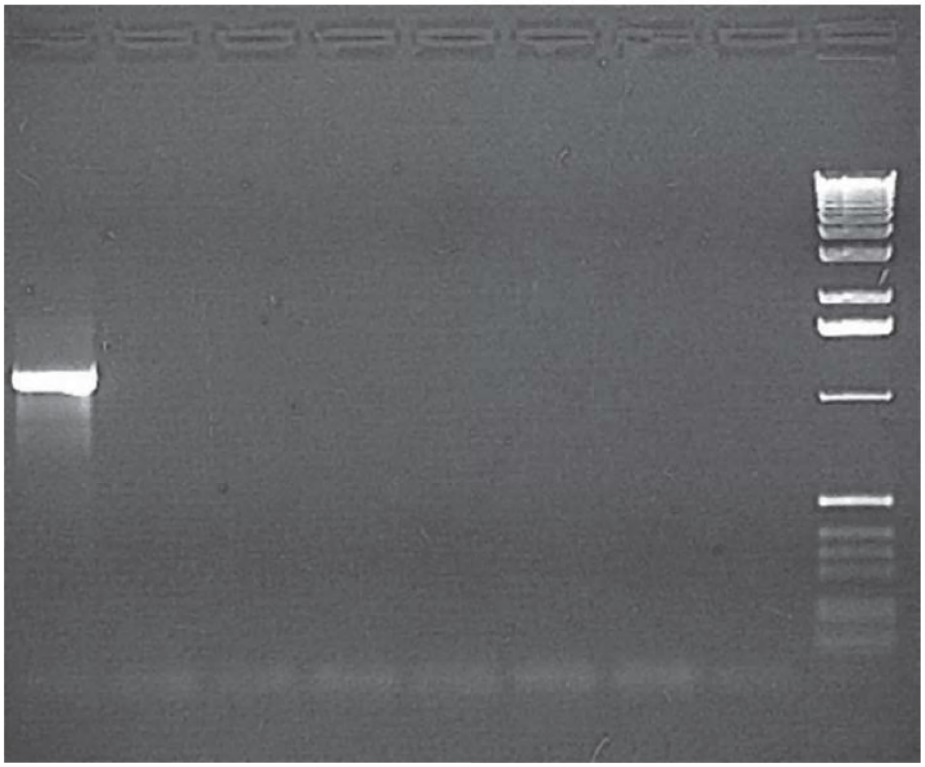

Fig. 4.6 Amplification product of PCR with species-specific primer Finc/Rinc for *Meloidogyne incognita*. I, *Meloidogyne incognita*; J, *Meloidogyne javanica*; A, *Meloidogyne arenaria*; M, *Meloidogyne mayaguensis*; H, *Meloidogyne hapla*; C, *Meloidogyne chitwoodi*; F, *Meloidogyne fallax*; W, no template DNA control; S, size marker. (From Zijlstra *et al.*, 2000.)

oligonucleotide probes on a membrane. This approach can be used for the simultaneous identification of many different nematodes from a single sample. Uehara *et al.* (1999) demonstrated that this technology could be used for the identification of *Pratylenchus* species (Fig. 4.7).

RAPD-PCR

In contrast to the above-mentioned classical PCR method, the random amplified polymorphic DNA PCR (RAPD-PCR) or PCR with arbitrary primer (AP-PCR) does not require any information on the primer design. This PCR technology uses a single random primer of about ten nucleotides long, approximately 50% GC rich and lacking any internal inverted repeats. By lowering the annealing temperature during the amplification cycle, the primer anneals at random in the genome, allowing the synthesis of highly polymorphic amplification products. RAPD-PCR distinguishes nematode species and subspecies for root knot nematodes (Cenis, 1993; Blok *et al.*, 1997; Cofcewicz *et al.*, 2005) and cyst-forming nematodes (Caswell-Chen *et al.*, 1992; Thiéry *et al.*, 1997) (Fig. 4.8). However, the reproducibility of the results is the most critical point for application of this technique for diagnostic purposes. Specific sequences for certain species or races, called SCARs (sequence characterized amplified regions), can be derived from RAPD fragments and further used to design species-specific primers.

AFLP

The amplified fragment length polymorphism (AFLP) technique was developed by Vos *et al.* (1995) and was based on the selective amplification of genomic restriction fragments. AFLP involves three steps: (i) digestion of DNA with two restriction enzymes and ligation of specific adapters to the restriction fragments; (ii) PCR amplification of a subset of the restriction/adapter

Table 4.2. Species-specific primers developed for identification of some nematodes.

Species	Primer set (5′–3′)	Amplicon length	Reference
Ditylenchus destructor	D2 TGG ATC ACT CGG CGG CTC GTA GA D1 ACT GCT CTG CGT TTG GCT TCA	346 bp	Liu *et al*. (2007)
Heterodera latipons	Hlat-actF ATG CCA TCA TTA TTC CTT Hlat-actR ACA GAG AGT CAA ATT GTG	204 bp	Toumi *et al*. (2013)
Heterodera filipjevi	HfITS-F1 CCC GTC TGC TGT TGA GA HfITS-R1 ACC TCA GGC TTT TAT TAT CAC	170 bp	Yan and Smiley (2010)
Heterodera avenae	HaITS-F6 ATG CCC CCG TCT GCT GA HaITS-R4 GAG CGT GCT CGT CCA AC	242 bp	Yan and Smiley (2010)
Globodera pallida	PITSp4 ACA ACA GCA ATC GTC GAG ITS5 GGA AGT AAA AGT CGT AAC AAG G	265 bp	Bulman and Marshall (1997)
Meloidogyne arenaria	Far TCG GCG ATA GAG GTA AAT GAC Rar TCG GCG ATA GAC ACT ACA ACT	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	800 bp	Zijlstra (2000)
Meloidogyne exigua	Ex-D15-F CAT CCG TGC TGT AGC TGC GAG Ex-D15-R CTC CGT GGG AAG AAA GAC TG	562 bp	Randing *et al*. (2002)
Meloidogyne hapla	Fh TGA CGG CGG TGA GTG CGA Rh TGA CGG CGG TAC CTC ATA G	610 bp	Zijlstra (2000)
Meloidogyne incognita	Finc CTC TGC CCA ATG AGC TGT CC Rinc CTC TGC CCT CAC ATT AGG	1200 bp	Zijlstra *et al*. (2000)
Meloidogyne paranaensis	Par-C09-F GCC CGA CTC CAT TTG ACG GA Par-C09-R CCG TCC AGA TCC ATC GAA GTC	208 bp	Randing *et al*. (2002)
Meloidogyne arabicida	ar-A12F TCG GCG ATA GTA CGT ATT TAG CG ar-A12R TAG TGA TTT CGG CGA TAG GC	300 bp	Correa *et al*. (2013)
Meloidogyne ethiopica	meth-F ATG CAG CCG CAG GGA ACG TAG TTG meth-R TGT TGT TTC ATG TGC TTC GGC ATC	350 bp	Correa *et al*. (2014)
Meloidogyne enterolobii	MK7-F GAT CAG AGG CGG GCG CAT TGC GA MK7-R CGA ACT CGC TCG AAC TCG AC	520 bp	Tigano *et al*. (2010)

Continued

Table 4.2. Continued.

Species	Primer set (5′–3′)	Amplicon length	Reference
Meloidogyne naasi	N-ITS CTC TTT ATG GAG AAT AAT CGT R195 CCT CCG CTT ACT GAT ATG	433 bp	Zijlstra *et al*. (2004)
Pratylenchus penetrans	PNEG ATG AAA GTG AAC ATG TCC TC D3B TCG GAA GGA ACC AGC TAC TA	278 bp	Al-Banna *et al*. (2004)
Pratylenchus vulnus	PVUL GAA AGT GAA CGC ATC CGC AA D3B TCG GAA GGA ACC AGC TAC TA	287 bp	Al-Banna *et al*. (2004)
Rotylenchus robustus	TW81 GTT TCC GTA GGT GAA CCT GC R_rob GAC GTG GAC ATC ATA CAG TC	438 bp	Cantalapiedra-Navarrete *et al*. (2013)
Tylenchulus semipenetrans	TW81 GTT TCC GTA GGT GAA CCT GC Sem_spec GGA CTC TGC TCA ACC TGG TAG A	113 bp	Tanha Maafi *et al*. (2012)
Xiphinema index	I27 GAG TCG TAA CGT TTC TCG TCT ATC AGG A-ITS1 GAA TAG CCA CCT AGT GAG CCG AGCA	340 bp	Wang *et al*. (2003)

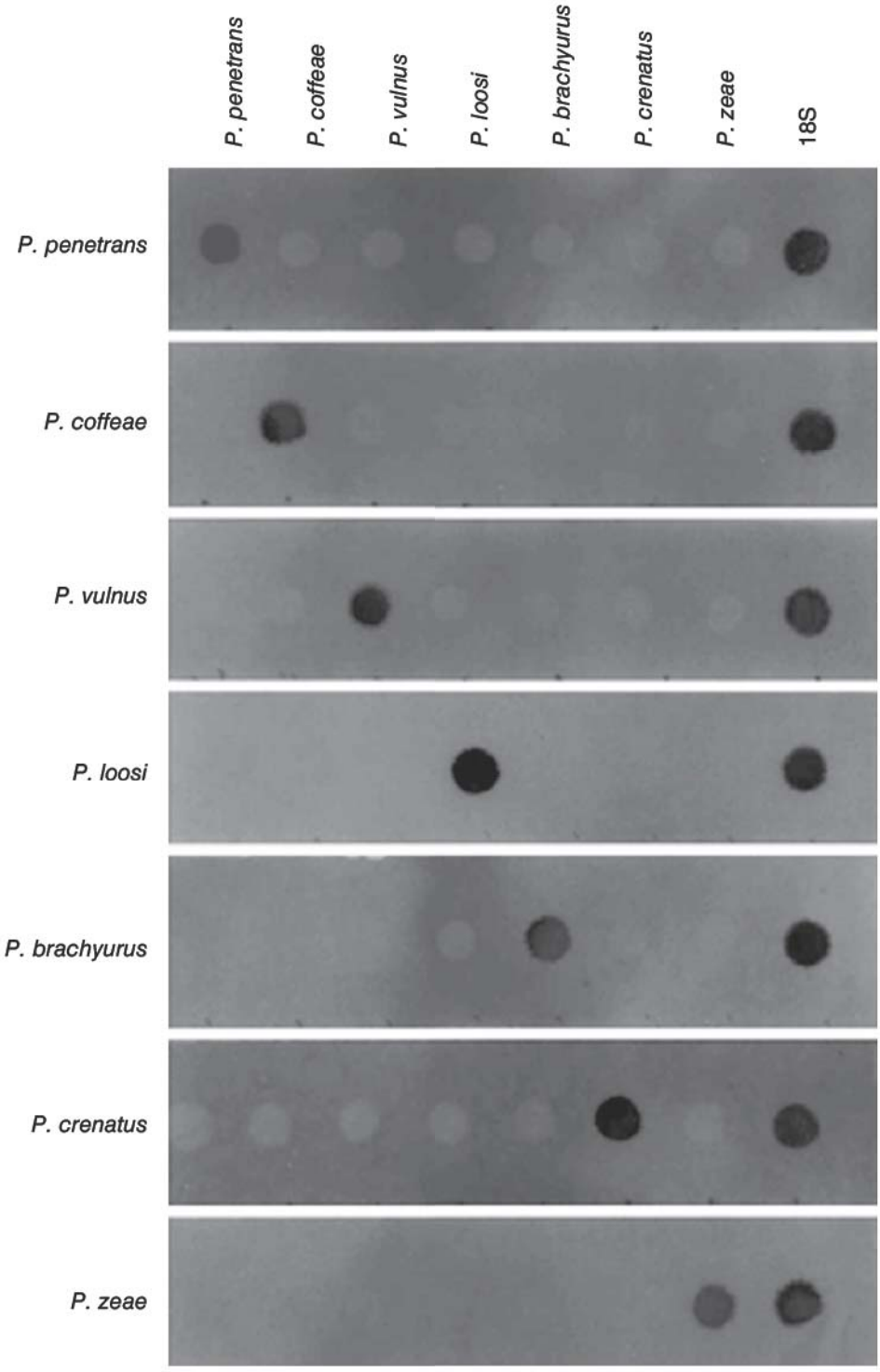

Fig. 4.7. Reverse dot-blot hybridization with immobilized specific oligonucleotides. The *Pratylenchus* species listed on the left were used for each hybridization. (From Uehara *et al*., 1999.)

fragments under stringent conditions; (iii) gel electrophoresis analysis of the amplified restriction fragments. The AFLP technique has several advantages over RAPD in that it produces results that are highly reproducible and has higher resolutions generating many more amplified fragments. AFLP fingerprinting has been applied successfully for the evaluation of inter- and

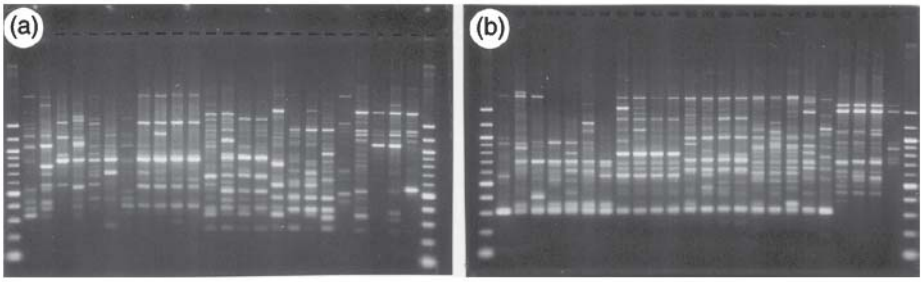

Fig. 4.8. RAPD patterns of 26 populations of the *Heterodera avenae* complex. Primers: (a) A-16; (b) A-18. Populations: 1, *H. avenae* (Taaken, Germany); 2, *H. avenae* (Santa Olalla, Spain); 3, *H. avenae* (Çukurova Ebene, Turkey); 4, *H. avenae* (Saudi Arabia); 5, *H. avenae* (Ha-hoola, Israel); 6, *H. avenae* (Israel); 7, *H. avenae* (near Delhi, India); 8, *Heterodera australis* (South Australia, sample 3); 9, *H. australis* (Beulah, Australia); 10, *H. australis* (Victoria, Australia); 11, *H. australis* (Yorke Peninsular, Australia); 12, *Heterodera mani* (Bayern, Germany); 13, *H. mani* (Heinsberg, Germany); 14, *H. mani* (Andernach, Germany); 15, *H. mani* (Germany); 16, *Heterodera pratensis* (Missunde, Germany); 17, *H. pratensis* (Östergaard, Germany); 18, *H. pratensis* (Lindhöft, Germany); 19, *H. pratensis* (Lenggries, Germany); 20, *Heterodera aucklandica* (One Tree Hill, New Zealand); 21, *Heterodera filipjevi* (Saratov, Russia); 22, *H. filipjevi* (Akenham, England); 23, *H. filipjevi* (Torralba de Calatrava, Spain); 24, *H. filipjevi* (Selçuklu, Turkey). M, 100 bp DNA ladder (Biolab). (From Subbotin *et al.*, 2003.)

intraspecific genetic variation of cyst-forming nematodes (Folkertsma *et al.*, 1996; Marché *et al.*, 2001), root knot nematodes (Semblat *et al.*, 1998) and stem nematodes (Esquibet *et al.*, 2003).

DNA bar coding

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. DNA bar coding first came to the attention of the scientific community when 'Biological identifications through DNA barcodes' was published, in which the authors proposed a new system of species identification and discovery using a 648-bp region of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene as a standard bar code in the animal kingdom (Hebert *et al.*, 2003). There are considerable debates among taxonomists about DNA bar-code application. Floyd *et al.* (2002) were the first to develop a 'molecular operation taxonomic unit' approach when they applied a molecular bar code, derived from single-specimen PCR and sequencing of the 5′ segment of the 18S rRNA gene, to estimate nematode diversity in Scottish grassland. Further studies showed that in some cases the 18S rRNA gene did not contain sufficient resolution for nematode identification to species level. Moreover, a single bar-code region may be insufficient for the identification of the majority of nematodes, and presently several markers (18S rRNA, D2–D3 of 28S rRNA, ITS rRNA, COI and other genes) are proposed and used for nematode bar coding. The markers should fit three criteria: (i) show significant species-level genetic variability and divergence; (ii) be an appropriately short sequence length so as to facilitate DNA extraction and amplification; (iii) contain conserved flanking sites for developing universal primers. It is important to note that DNA bar coding is only as good as the reference database, and it can only be used to identify species already catalogued. DNA bar coding will be also most reliable for the identification of putative new species, but only for species groups whose genetic diversity has been well surveyed.

Presently, the results of many nematode DNA bar-coding projects are compiled in a central integrative bioinformatics platform – BOLD (Barcode of Life Data Systems, 2009) – that supports all phases of the analytical pathway, from specimen collection to tightly validated bar-code library, and can also accommodate externally produced sequences, either through direct submission or regular incorporation of GenBank sequences (Ratnasingham and Hebert, 2007, 2013).

Real-time PCR

A real-time polymerase chain reaction is a laboratory technique that monitors the amplification of a targeted DNA molecule using sequence-specific primers, fluorescent probes or fluorescent DNA-binding dyes. Real-time PCR is able to quantify the amount of DNA in a sample. This technique indirectly measures the nematode number by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes (Fig. 4.9). Many real-time fluorescent PCR chemistries exist, but the most widely used are SYBR Green I dye-based

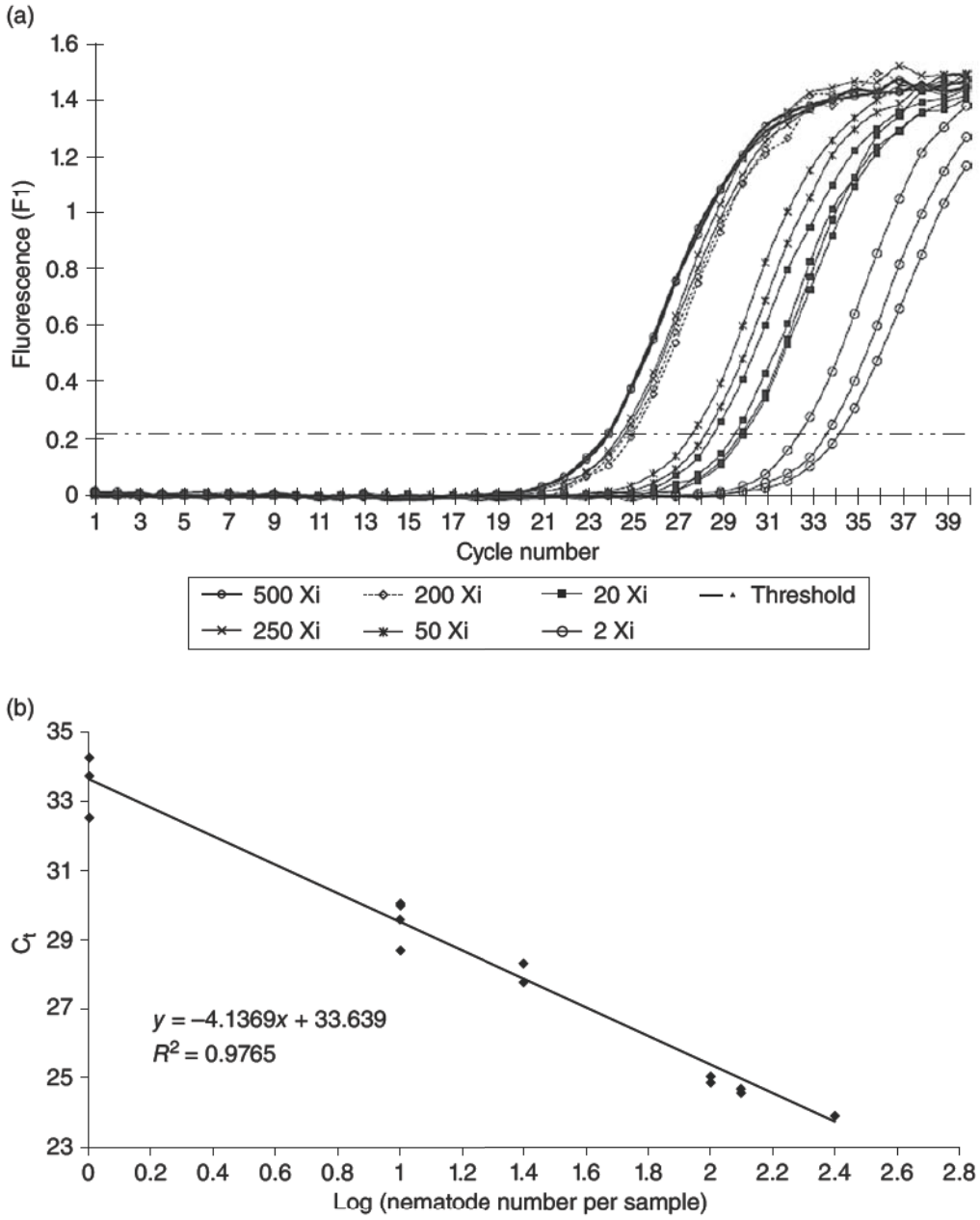

Fig. 4.9. Relationship between nematode density and the threshold cycle number (C_t) using the real-time PCR method for *Xiphinema index*. (a) Amplification curves for pure samples. From left to right, the curves correspond respectively to 500 (two replicates), 250 (two replicates), 200 (two replicates), 50 (two replicates), 20 (four replicates) and two (three replicates) individuals in a 2 µl total volume of extraction buffer. (b) Standard linear curve of Ct plotted against the log-transformed *X. index* numbers per sample. R^2: linear correlation coefficient. (From Van Ghelder *et al*., 2015.)

and TaqMan assays. SYBR Green I 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, the dye binds also to any non-specific product, including primer dimers, and to overcome this problem, the melting curve analysis can be employed. Increasing the temperature of the sample melts the PCR products. The non-specific product tends to melt at a much lower temperature than the longer specific product. Bates *et al.* (2002) were the first to use real-time PCR with SYBR Green I for plant parasitic nematodes, to detect *Globodera* species.

The disadvantage of using a fluorescent dye is that it binds to any double-stranded DNA and cannot be used for quantification of individual 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 needed. In the TaqMan assay, a DNA probe consisting of approximately 25–30 nucleotides in length and labelled with a fluorescent reporter permits detection only after hybridization of this probe with its complementary sequence. Cao *et al.* (2005) developed a method for detecting the pinewood nematode, *Bursaphelenchus xylophilus*, using TaqMan probes. The PCR assay detected DNA template concentrations as low as 0.01 ng. The Ct values were correlated with the DNA template concentration ($R^2 = 0.996$), indicating the validity of the assay and its potential for quantification of target DNA. The real-time PCR assay also detected DNA from single specimens of *B. xylophilus*.

Presently, real-time PCR methods have been developed for species of *Bursaphelenchus* (Kang *et al.*, 2009), *Ditylenchus* (Subbotin *et al.*, 2005), *Heterodera* (Madani *et al.*, 2005; Ye, 2012); *Globodera* (Madani *et al.*, 2005, 2008; Nowaczyk *et al.*, 2008; Nakhla *et al.*, 2010; Papayiannis *et al.*, 2013), *Meloidogyne* (Berry *et al.*, 2008; Agudelo *et al.*, 2011), *Paratrichodorus* (Holeva *et al.*, 2006), *Pratylenchus* (Sato *et al.*, 2007; Berry *et al.*, 2008; Yan *et al.*, 2012; Mokrini *et al.*, 2013), *Xiphinema* (Berry *et al.*, 2008; Van Ghelder *et al.*, 2015) (Fig. 4.9) and others.

The real-time PCR method is straightforward, sensitive and reproducible and, compared with conventional PCR methods, has several advantages. The technique allows a simultaneous faster detection and quantification of target DNA, and the automated system overcomes the laborious process of estimating the quality of PCR product after electrophoresis.

Loop-mediated isothermal amplification (LAMP)

The LAMP technique is a simple, rapid, specific, sensitive and cost-effective nucleic acid amplification technology developed by Notomi *et al.* (2000). Amplification is completed by incubating the mixture of DNA template, a set of 4–6 specially designed primers based on six or eight distinct regions of the target DNA and a strand displacement DNA polymerase in a single tube at an isothermal temperature of 60–65°C. It provides high amplification efficiency, with replication of the original template copy, occurring 10^{9-10} times during a 15–60 min reaction. Detection of the amplification product is determined by intercalating dyes such as SYBR Green I (Fig. 4.10) or ethidium bromide, or measuring the turbidity caused by the formation of magnesium pyrophosphate. Presently, LAMP methods have been developed for *B. xylophilus* (Kikuchi *et al.*, 2009; Kanetani *et al.*, 2011), *Meloidogyne* spp. (Niu *et al.*, 2011, 2012; He *et al.*, 2013) (Fig. 4.10), *Radopholus similis* (Peng *et al.*, 2012) and *Tylenchulus semipenetrans* (Lin *et al.*, 2016). In order to identify living organisms specifically, the LAMP technique was adapted into a reverse transcriptase assay (RT-LAMP), specifically targeting RNA by isolating RNA instead of DNA and using an additional reverse transcription step before or during amplification. In order to detect living *B. xylophilus* in wood, the RT-LAMP assay was developed by Leal *et al.* (2015), detecting the presence of mRNA encoding an expansin gene. The result indicated that the RT-LAMP assay was able to detect the target expansin mRNA 2 days after the nematodes were killed, but not 4 days after their deaths. On the contrary, DNA can still be probed from nematodes even 3 months after their death.

Compared with PCR methods, the LAMP is simple to operate and does not require specialized equipment, even for the nematode extraction step, which allows for application under field conditions.

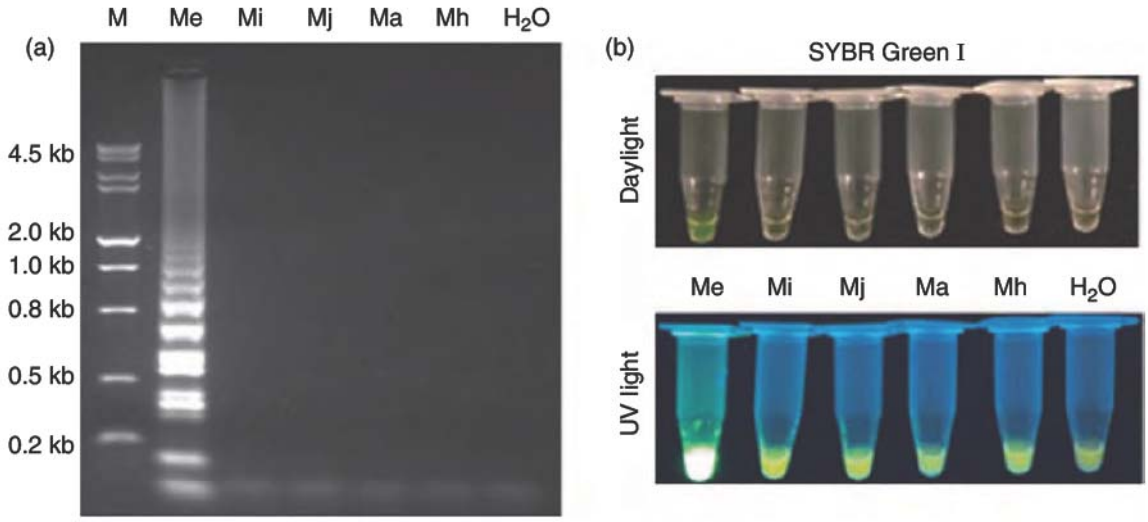

Fig. 4.10. Specificity of *Meloidogyne enterolobii* LAMP detection and product confirmation. (a) LAMP product on a gel; (b) specificity of the LAMP assay products visualized by adding SYBR Green I. Top row: direct visualization by the naked eye. Bottom row: observation under UV transillumination. M = molecular marker; Me = *M. enterolobii*; Mi = *Meloidogyne incognita*; Mj = *Meloidogyne javanica*, Ma = *Meloidogyne arenaria* and Mh = *Meloidogyne hapla*. The H_2O tube was used as a negative control without DNA template. (From Niu *et al*., 2012.)

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