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Chapter 3

Molecular Diagnostic Tools for Nematodes

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Abstract

The phylum of Nematoda is a species-rich taxonomic group in abundant numbers across a wide range of habitats, including plant and animal pathogens, as well as good environmental health indicators. Morphological observations are of low throughput and more importantly have problems with their discriminatory capacity, particularly at the species level. For these reasons, diagnostic tools are of paramount importance for all fields of human, animal and plant nematology as well as for environmental studies in water and soil. Accurate, fast and low-cost methodologies are required in order to identify and quantify the population of nematodes in samples from various sources. Scientists in basic research as well as in routine application fields need to have tools for resolving these identification obstacles. Their decisions can be human-, animal- or plant-health related, while many times legally committing. As a result, applicable and accredited methods are required and should be readily available in a common routine lab or in the field of battle or at border control agencies. This chapter aims to inform with the most current information on the available tools for nematode diagnostics, their positives and negatives and hints about the trends in the field and suggestions for those who would like to pursue further this field of biotechnology as researchers or simple users.

Keywords: nematode, detection, quantification, diagnostics, PCR, qPCR, DNA barcoding, Sequencing, NGS, MALDI-TOF

1. Introduction

Nematode identification is crucial for nematologists, diagnosticians and policy-makers. Due to the nematodes small size, life cycle and different habitats, scientists have been struggling to find morphological differences among species that would differentiate them. Nematode identification and differentiation can provide accurate decisions for the control of parasitic nematodes and the

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conservation of non-parasitic nematodes. Many misidentifications due to morphological errors resulted in huge economic impacts around the world. In the 1970s, plant parasitic nematode (PPN) control was based on the use of soil fumigants which made the species identification unnecessary whereas nowadays, the prohibition of those chemicals necessitates the accurate identification of species for the effective implementation of non-chemical management strategies [1].

A huge step towards nematode identification has been the use of biochemical and molecular diagnostic tools such as the enzyme-linked immunosorbent assay (ELISA) [2], isoelectric focusing (IEF) [3] and the polymerase chain reaction (PCR) [4]. The first two biochemical methods, ELISA and IEF, have received limited use as diagnostic tools due to appearance of the most effective, precise and fast PCR-based methods with the use of DNA which has provided solutions in several identification problems. The internal transcribed spacer (ITS) has proven to be a useful DNA region from which universal or species-specific primers are used in PCR reactions. The ITS regions are considered to be the most widely used for identification purposes by nematologists [5]. The use of PCR technology enables nematologists to diagnose nematode diseases rapidly and accurately. Furthermore, the use of PCR is adopted by the European and Mediterranean Plant Protection Organization (EPPO) and used in standardized protocols [6].

2. DNA extraction methods

Plenty of DNA extraction methods have been reported for DNA extraction, from single juveniles to a large number of juveniles, eggs or cysts. DNA extraction methods include commercial kits such as silica columns to bind DNA and switchable magnetic-based surface technology, Chelex® resin, phenol/chloroform and a worm lysis buffer (WLB). From all the methods mentioned above, the silica columns provide the highest quality DNA even from soil samples [7] and thus are widely used by many laboratories despite them being more than three times expensive than the others [8]. Phenol/chloroform is a method widely used before the emergence of commercial kits and although it is still a satisfactory method which provides pure and good-quality DNA template, it is avoided by researchers due toxic effects. Chelex® 100 is a chelating resin that uses ion exchange to bind transition metal ions. The resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions, which act a chelator for polyvalent metal ions. During the extraction process, the alkalinity of the solution and the act of boiling the solution break down the cells and allow the chelating groups to bind to the cellular components, thus protecting the DNA from degradation [9]. Chelex® resin and WLB are two inexpensive methods, rapid and easy to apply and will be presented in this chapter.

2.1. Chelex® resin protocol

1. Prepare 5–10% Chelex® resin solution using deionized water.
2. Boil the nematode (juveniles or cysts) at 95°C for 5 min in the 10% Chelex® resin solution.
3. Centrifuge at 20,000× for 1 min to separate the resin and cellular debris from the supernatant which contains the DNA template.

2.2. Worm lysis buffer: single-worm DNA extraction

1. Prepare the WLB solution by mixing 50-mM KCl, 10-mM Tris, pH 8.2, 2.5-mM $MgCl_2$, 60-μg/ml proteinase K, 0.45% NP40 (Fisher Scientific), 0.45% Tween 20 (Sigma) and 0.01% gelatine [10].
2. Place the juvenile in 10 μL of WLB on a glass microscope slide.
3. Cut the nematode into two pieces using a sterile fine scalpel blade under a stereomicroscope.
4. Transfer the cut nematode using a pipette into a 0.2-ml centrifuge tube containing another 10 μL of WLB.
5. Centrifuge at 13,500 rp.m. for 2 min and then place at −80°C for 15 min.
6. Incubate the nematodes at 60°C for 1 h, followed by 95°C for 10 min to inactivate the proteinase K.
7. Freeze the DNA samples at −20 °C until ready for use.

2.3. Phenol/chloroform extraction/cleanup of genomic DNA

2.3.1. Digestion

1. Drop tissue pieces into liquid nitrogen to freeze.
2. Using a mortar and pestle, crush and grind the frozen tissue to make a powder.
3. Transfer to 50-ml tube and briefly spin to pack tissues to the bottom of tube.
4. Add TNES buffer (50-mM Tris-HCl, pH 7.5, 400-mM NaCl, 100-mM EDTA, pH 8.0 and 0.5% SDS-containing proteinase K (20 mg/ml) to bring volume to around 10 ml).
5. Incubate overnight at 50°C.
6. Adjust to 1.5-M NaCl (add an equal volume of 2.6 M NaCl).
7. Shake vigorously for 30 s.
8. Centrifuge 12,000× g for 5 min.
9. Decant the supernatant to a fresh tube and add an equal volume of ethanol.
10. Spool out the DNA and dissolve in 500 μl of water.

2.3.2. Extraction

1. Add an equal volume of phenol/chloroform/isoamyl alcohol to the DNA solution.
2. Vortex vigorously for 10 s and microcentrifuge for 15 s at room temperature.

3. Carefully remove the top (aqueous) phase containing the DNA and transfer to a new tube. If a white precipitate is present at the aqueous/organic interface, re-extract the organic phase and pool the aqueous phases.
4. Add 1/10 volume of 3-M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly.
5. Add 2–2.5 vol of ice cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 min or longer.
6. Spin 20 min at 13,000× g in the microcentrifuge and remove the supernatant.
7. Add 1 ml of room temperature 70% ethanol. Invert the tube several times and microcentrifuge for 5 min at 13,000× g.
8. Remove the supernatant. Allow to air dry for 15 min.
9. Re-suspend DNA pellet in 100 μl of ultra-pure water or TE buffer.

3. PCR-based methods

PCR-based methods involve the extraction of DNA from single or numerous juveniles, nematocysts or complex soil samples. The PCR-based molecular diagnostic tools used for nematode identification and quantification are restriction fragment length polymorphisms (RFLPs), ribosomal DNA (rDNA) PCR, mitochondrial DNA amplification, microsatellite DNA fragment analysis, real-time PCR, microarrays, sequence-characterized amplified regions (SCARs) and next-generation sequencing (NGS).

3.1. Restriction fragment length polymorphisms

In Ref. [11], Curran et al. differentiated the Meloidogyne population on the level of race and strains by using total genome analysis from washed eggs. The egg DNA was purified and digested with *Eco*RI and electrophoresed in an agarose gel and visualized [11]. Due to the large number of specimens and thus the high amount of DNA needed for RFLPs analysis, the technique was improved in the early 1990s with the use DNA hybridization [12, 13] and finally PCR [4]. The combination of amplification and digestion (PCR-RFLP) of a single DNA strand has been found useful for DNA comparisons among individual nematodes [5]. Various PCR products during restriction endonuclease digestion lead to differences in fragment length within the restriction site yielding different RFLP profiles. To obtain a desirable result, different digestive enzymes participate. Nonetheless, the digestive enzymes used in RFLP do not separate all species within a genus, an issue that will be overcome with the use of species-specific primers. The specificity of RFLP could be used for the examination of a broad range of isolates from different sites around the world and thus confirm the general applicability of the RFLP method [14]. Nevertheless, as a diagnostic tool, PCR-RFLP could eliminate much of the ambiguity involved in morphological identification of nematode specimens since

differences in RFLP can be presented as the existence of differences in restriction sites in the ITS sequence (**Figure 1**) [15, 16]. Nowadays, PCR-RFLP is still used when species-specific primers are absent.

3.2. Ribosomal DNA polymerase chain reaction (rDNA-PCR)

PCR brought the evolution in molecular diagnostics of nematodes since the early 1990s. Primers were designed to produce large DNA products from which species-specific primers were then designed for producing unique products of each species. By the late 1990s, species-specific primers were designed for quarantine species such as *Globodera pallida* and *Globodera rostochiensis* [17, 18]. Nematode PCR products were derived from the 18S, 28S, 5,8S coding genes and the ITS regions. The ITS region is considered a variable area of DNA that has been repeatedly examined for molecular differences among species. In 1996, Mulholland et al. [19] presented a multiplex PCR technique based on the use of species-specific primers, able to identify potato cyst nematodes (PCN) at the species level and without the use of restriction endonuclease digestion [19].

The PCR method requires DNA extracted from specimens, two pairs of 12–24-bp oligonucleotides named primers, which are complimentary to the 3′end of each strand in a specific binding site of the DNA region that will be amplified, a DNA polymerase (*Taq* DNA polymerase), four deoxynucleotides (dATP, dCTP, dGTP and dTTP) and a buffer-containing $MgCl_2$. The steps of the PCR method contain the activation of the Taq DNA polymerase (usually above 90°C), the denaturation of the DNA chain into two separated strands (usually above 90°C), the annealing of the primers (between 45 and 65°C) and the extension of the new strands, which involves the attachment of the Taq enzyme on the primers 3′end and the moving of the enzyme downstream along the DNA template, incorporating the free dNTPs on the new strand. The extension process is usually done at 72°C. PCR method usually uses around 35–40

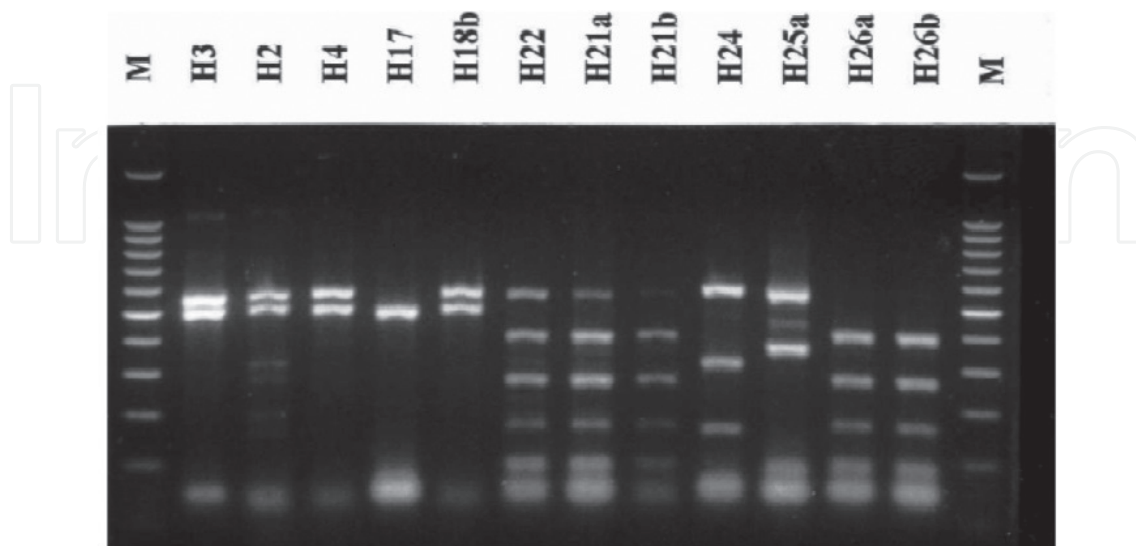

Figure 1. Restriction fragments of amplified ITS regions of cyst-forming nematodes digested by *Tru*9I. M: 100 bp ladder and H: Heterodera species [15].

cycles in a PCR thermocycler. The PCR product is mixed with a fluorescent dye and then transferred into separated wells of agarose gel, with the first well having a DNA ladder used as molecular weight marker. The loaded agarose gel is placed in a tray with buffer (the same buffer with which the gel was prepared) and plugged with electrodes (− electrode in the wells side and + electrode in the other site of the tray) at 100 volts. The higher the voltage, the faster the DNA moves but the heat increases and thus decreases resolution. Agarose gel is then visualized in UV light and photographed. Fleming *et al.* [20], used the PCR method for diagnosing and estimating population levels of PCN. They demonstrated a correlation between the number of viable juveniles hatched from a cyst with the amount of DNA that could be extracted from them in a quantitative manner [20]. A multiplex PCR was presented by Bulman and Marshal, (**Figure 2**), when species-specific primers were used and combined with mixed populations of PCNs [17]. A few years later, the PCR method was named conventional PCR (CoPCR) due the appearance of quantitative real-time PCR (qPCR) [21].

3.3. Real-time PCR

While conventional PCR was used worldwide for identification purposes, there was a need for more rapid, sensitive and cost-efficient method for identifying nematodes. As the genome analysis was heading deeper and deeper, more and more sequence data became available which made nematode identification and species discrimination more rapid and accurate [22]. Real-time PCR provides simultaneous amplification of the DNA target sequence and direct analysis of the PCR products by incorporating fluorescent probes or dyes into the reaction mix and thus the need for gel electrophoresis is avoided [23]. In real-time PCR, the fluorescent molecule (probe or dye) reports the amount of DNA as it is multiplied in each cycle as the fluorescent signal increases proportionally. The two types of fluorescent molecules used in real-time PCR bind on DNA as DNA-binding dyes or fluorescently labelled specific primers

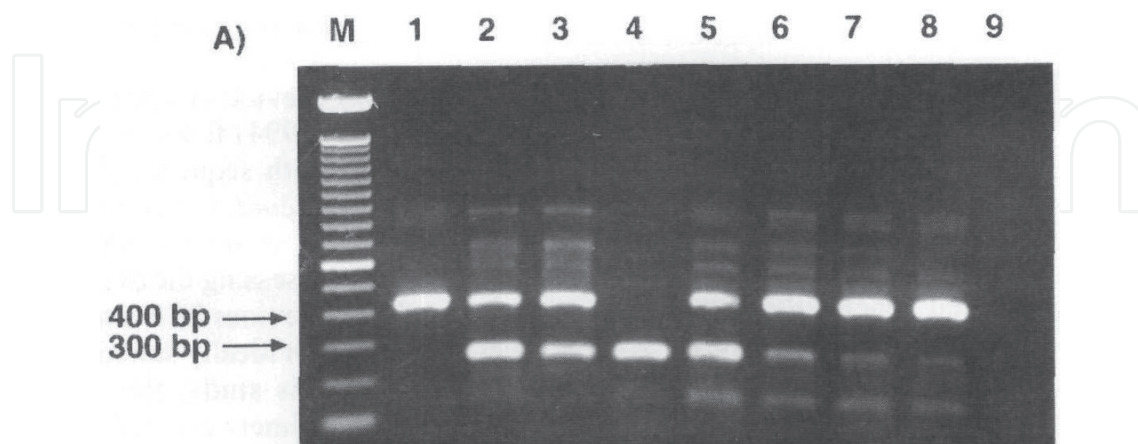

Figure 2. Polymerase chain reaction (PCR) differentiation of the potato cyst nematode (PCN) species, *Globodera rostochiensis* and *Globodera pallida*, with various concentrations of DNA. A: multiplex PCR with primers Plp4, Plr3 and ITS% upon DNA from Ro1 Lincoln and Pa2/3 Lincoln. M: ladder, Lane 1, Ro1 1:20 H_2O, Lane 2, Ro1 1:20 Pa2/3, Lane 3, Ro1 1:1 Pa2/3 (1:20 H_2O each), Lane 4, Pa2/3 1:20 H_2O, Lane 5, Ro1 10:1 Pa2/3, Lane 6, Ro1 20:1 Pa2/3, Lane 7, Ro1 50:1 Pa2/3, Lane 8, Ro1 100:1 Pa2/3, Lane 9, no DNA control [17].

or probes and specialized thermal cyclers detect, monitor and measure the fluorescence which reflects the amount of the amplified products in each cycle, in real time.

Quantitative real-time PCR is used for the detection and quantification of DNA present in a sample which is reflected by the number of nematodes present in the sample. For the quantification of nematodes using qPCR, a standard curve is needed (**Figure 3**) [24]. Standard curves are constructed by plotting the Ct values against the logarithm of the DNA amount isolated from different amounts of nematode eggs and juveniles. The amplification efficiency (E) is calculated from the slope of the standard curve using the following formula $E = 10^{[-1/\text{slope}]} - 1$ [25]. qPCR is used for the quantitative detection, species identification and discrimination in plant and in veterinary parasitic nematodes [8, 23, 24, 26–30].

Although quantification of nematodes was a step forward for estimating population levels of parasitic nematodes in a sample, the stability of DNA from dead specimens in samples especially those extracted from cysts appears to be an obstacle [26]. In the case of PCN, it is very common for dead juveniles to be present within a cyst (in-egg mortality) [31] and their DNA intact, while in soil, dead juveniles' DNA can be degraded in a short time. The DNA of *Phasmarhabditis hermaphrodita* was degraded in unpasteurized soil within 6 days as the dead juveniles were in direct contact to soil microflora [7]. Christoforou et al. [24] reported the detection and amplification of nematodes DNA in a 34-year-old cyst stored at room temperature using PCR (**Figure 4**) and qPCR with Taqman probes.

Although the use of DNA appears to be the best approach for live/dead specimen differentiation, its stability outside cell membranes allows the amplification of outbound DNA from dead cells as well, thus introducing inaccuracies in live nematode quantification. Recently, a

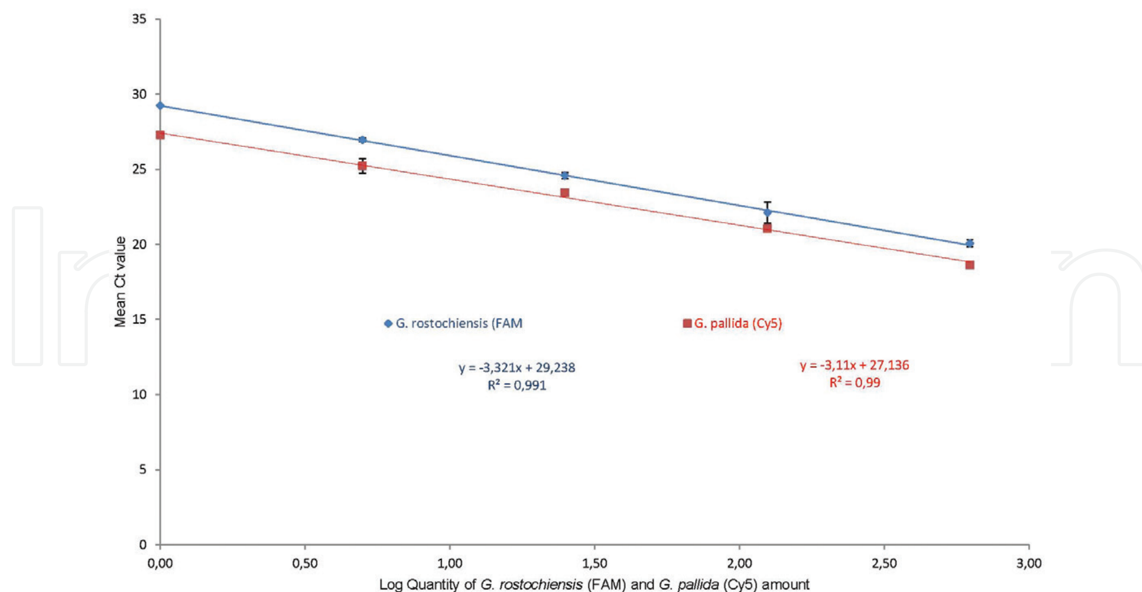

Figure 3. PMA-qPCR method for the detection and quantification of viable potato cyst nematodes. A: standard curves generated by duplex real-time PCR using DNA isolated from standard PCN solutions containing 1, 5, 25, 125 and 625 live eggs or juveniles (J2), respectively. The mean Ct values corresponding to the PCR cycle number are plotted against the logarithmic quantity of nematodes DNA used in triplicate as standards. The error bars represent standard deviations of three samples [24].

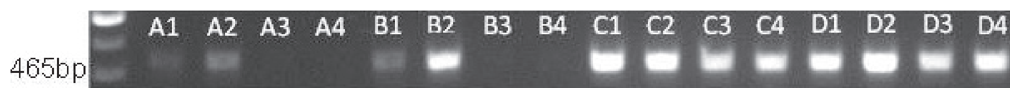

Figure 4. PCR-amplified products at 465 bp of genomic DNA from non-PMA and PMA-treated cysts (A1–A4: 1976, A1 and A2 non-PMA and A3 and A4 PMA; B1–B4: 1990, B1 and B2 non-PMA and B3 and B4 PMA; C1–C4: 2007, C1 and C2 non-PMA and C3 and C4 PMA; D1–D4: 2010, D1 and D2 non-PMA and D3 and D4 PMA) [24].

new chemical dye propidium monoazide (PMA) has been used for selective detection of viable bacteria, fungi and nematodes, in combination with qPCR [24, 32–34]. PMA is a photoreactive DNA-intercalating dye which renders exposed DNA of dead cells, is unable to amplify and thus, only DNA from viable/intact cells is PCR amplified and detected. Christoforou *et. al.* [24], presented a qualitative estimation of viable PCN inocula using species-specific primers and Taqman probes designed by Papayiannis *et al.* [8], in a PMA-qPCR method which was developed for the two PCN species. The PMA-qPCR method successfully discriminates dead from living specimens in heat-treated samples as also the eggs from old and newly formed cysts.

qPCR method proves to be very useful for routine identification and discrimination of nematode species from field samples. The optimization of the qPCR and DNA extraction methods is essential for the specificity, sensitivity and accuracy of the procedure. Madani *et al.* [26], described a real-time PCR method using SYBR green-I dye with melting curve analysis for the detection and quantification of PCN species and mentioned the dependence of nematode quantification on the efficacy of DNA-extraction methods. Papayiannis *et al.* [8], evaluated five DNA extraction methods (silica columns, magnetic-based surface, Chelex resin, chloroform-based and disruption in TE) and compared them for their preparation time, cost and technical difficulty as well as the limit of detection between PCR and qPCR assays for all extraction methods. Another important factor for an accurate qPCR assay is the primers' specificity and the limitations in detecting nematodes when species are mixed in a sample. When three plant parasitic nematodes (PPNs), *Meloidogyne javanica*, *Pratylenchus zeae* and *Xiphinema elongatum*, were tested for identification and quantification in a mixture of species and primers, competition between the DNA of *M. javanica* with *P. zeae* and *X. elongatum* was found [27].

4. Microarrays

Microarrays show high potential for discriminating nematodes in multi-complex samples since many targets can be identified simultaneously due to the specificity of the microarray method to detect unique sequences for each target species [35, 36]. Microarrays are composed of complementary DNAs (cDNAs) that can be detected due to a fluorescence bind on the cDNA, on microscope slides or silicon chips, which contain specific synthesized known DNA after hybridization of the cDNA. Ahmed et al. [36] mentioned the potential of using the microarrays to identify gastrointestinal nematodes. Besides the high prospective of microarrays as diagnostic tools for identifying nematodes, it still has not been achievable. The high cost, the amplification of unknown sequences in mixed samples and the better hybridization of mismatched targets rather than the perfectly matched targets lead to the limited use of the microarray method as a diagnostic tool for nematodes [35].

5. DNA sequencing

DNA sequencing or DNA barcoding is referred to many nematode-related publications and has been the main driving force in studies, and as availability of instrumentation increases while cost is constantly reduced, it is apparent that it will be the dominating approach. The Sanger method or NGS approaches accumulate a substantial amount of genetic data with sufficient, if not to say overwhelming, information on sequence divergence, which may be often characterized as erroneous due to sample or analysis limitations.

For diagnostic purposes, most studies have targeted two main genomic regions for sequence divergence. These regions are the nuclear ribosomal RNA genes and their transcribed and untranscribed spacers and the mitochondrial cytochrome oxidase I (COI) gene. These regions are highly conserved but sufficiently divergent and occur in multiple copies in the genome, thus made easily amplifiable by PCR. A key element of this approach is the use of standardized markers and a relatively standardized experimental approach not introducing significant subjectiveness. On the other hand, this methodology builds taxonomic reference libraries where all submitted sequences from different organisms can be compared. As a result, unidentified organisms can be determined according to the level of DNA homology [37]. Results can be acquired in as fast as 8–12 h, making the method competent to be used in control of pest movement within trade activities and border control [38]. rDNA genes are preferred over COI gene in most studies due to the availability of sequences and the level of conservation in order to design universal primers even though COI is capable of discriminating between species at a better level. Porazinska et al. [39] had shown that the use of SSU and LSU genes together improves resolution.

With the development of NGS approaches, similarly to metagenomics, a term that has been used solely for microorganisms, DNA metabarcoding, is rapidly evolving. Bulk DNA deriving from environmental samples (water, soil) but same approach can be applied elsewhere (i.e. infected plant tissues, animal gut, blood samples), can uncover the entire hidden microcosm [37]. This approach can be used both for ecology studies, including soil quality and health, and for plant/animal diagnostics.

Limitations of high-throughput DNA barcoding still exist and are mainly the following: (1) Efficiency of DNA recovery is an issue but experimentation and protocol development studies will soon address this, (2) identification of a suitable marker to provide good taxonomic coverage and species resolution and (3) formation of chimeras (artefacts of PCR when an incompletely extended DNA fragment from one cycle anneals to a template of an unrelated taxon and gets copied to completion in subsequent cycles). Bioinformatic tools are trained to identify and discard such sequences.

The second limitation referred to above can be indirectly resolved by taking advantage of the high throughput of NGS technology, where multiple genes can be simultaneously sequenced and analysed with relatively low cost. In our times, genetic information will be easily acquired and our main technical obstacle is the vastness of information in genetic repositories of sequences, that storage and computing capacities require constant upgrade to convey biological and taxonomic meanings to scientists.

It is worth referring the most recent achievement of DNA sequencing, using third-generation sequencing technology and providing whole genome analysis that has used the portable device MinION. Tyson et al. [40] have reported performing the whole genome and assembly of a *Caenorhabditis elegans* genome with complex genomic arrangements. Two astonishing elements of this study are the USB type and sized instrument of MinION and the long reads that the technology offers. This second attribute improves immensely the NGS technology for de novo sequencing of complex genomes, in part due to repeat regions that nematodes as metazoans have in common. The flowcell of MinION is currently able to provide 5–10 Gb of sequence, which is a sufficient performance for a 100-Mb genome of a nematode with long reads for an unambiguous assembly of the chromosome.

6. Other methods

A variety of biochemical methods have been used in the past for nematode identification. They relied mainly on protein analysis using isoelectric focusing, two-dimensional electrophoresis and serological techniques using monoclonal or polyclonal antibodies. None of these techniques reached an application level, and research has been seized. Recently, the use of analytical instrumentation for protein analysis has acquired the attention of the research and application scientific community. MALDI-TOF mass spectrometry is a method that can be used for microorganism's identification [41] and has been reported by Pepera et al. [42] for nematodes as well with very good results. The authors discriminated up to a race level for *Ditylenchus dipsaci*. Although in microorganisms the ribosomal proteins seem to be the prominent identification/fingerprinting molecules, in this report [42], an array of other proteins of housekeeping importance were analysed and sequenced (LC MS/MS). The discriminatory differences found on proteomic approaches such as the aforementioned can more easily contribute and lead to the identification of pathogenicity factors important for development of new disease management strategies, through resistant plant cultivars. Conclusively, MALDI-TOF technology beside the instrumentation cost (in 2017, prices are about 150–200 K euros) is a robust technique, with very low cost per sample preparation and analysis (1-h for sample preparation and 3 min for analysis). Similar to microbial proteomic instrumentation, commercially available databases (Bruker MALDI Biotyper, Biomerieux VITEK MS) can be developed for nematode identification.

7. Conclusions

Molecular diagnostics are used as tools for the identification of parasitic and free-living nematodes since the early 1990s. Currently, most of the veterinary and plant protection laboratories use molecular tools for the identification, discrimination and quantification of important parasitic nematodes for common everyday diagnostic activities. From all the molecular tools and methods mentioned in the literature and in this review, only few are used in routine protocols. These selected ones are highly correlated with the reliability, the time and cost effectiveness as well as the expertise necessary for applying the methods.

From the methods reviewed in this chapter, real-time PCR is currently the fastest, most-sensitive and accurate method. Taqman PCR assay could detect, identify and quantify nematodes, reaching 100% accuracy. Real-time PCR methodologies can be of use in field applications with the use of a mobile qPCR instrument that is able to operate in field conditions along with easy-to-perform kits like DNA extraction and PCR reaction chemistries. For more analytical protocols and methodologies, DNA barcoding is fast progressing as DNA sequencing tools develop. However, we need to inform our readers that DNA barcoding based on NGS technologies and proteomic analysis based on mass spectrometry will soon dominate the market and offer low-budget, kit-type applications even for mobile diagnostic laboratories.

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