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REVIEW / *REVISÃO*

Morphological and molecular diagnostics for plant-parasitic nematodes: working together to get the identification done

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OUTLINE

Nematodes are considered one of the most difficult organisms to identify due to their microscopic size, morphological similarity, limited number of distinguishable taxonomic characters and overlapping morphometric measurements. However, there are few trained nematode taxonomists remaining, primarily due to retirement without replacement and/or young scientists' lack of interest in classical taxonomy. Due to the continuing decline in classical taxonomic expertise of many taxa including Nematoda, there is an increasing reliance on developing molecular-based diagnostic protocols to identify pests and pathogens. To improve the resolution and reliability of nematode phylogenetic and diagnostic studies, they should ideally combine morphological with molecular data. However, few of the published studies have compared phylogenetic trees derived from morphological/morphometric characters with those derived from molecular data. Furthermore, nematode sequences deposited in public databases should have an identification based on morphological and morphometrical characters ascertained by a trained taxonomist. Nevertheless, molecular diagnostics do offer new opportunities in Nematology, such as improving the capacity to deal with large numbers of samples or with species mixtures which have previously been impractical. In conclusion, a balanced molecular and morphological taxonomic approach is required as proposed by the integrative taxonomy concept. Classical taxonomy and molecular diagnostics should be considered as complementary.
Key words: *Nematoda,* diagnostic tools, PCR, plant pathogens, quarantine measure, phylogenetic systematics, taxonomy.

Introduction

The Phylum Nematoda is highly diverse in terms of species richness and one of the most abundant metazoan groups on earth. It is estimated that nematodes comprise nearly 90% of all multicellular organisms. Furthermore, Lambshead (1993) predicted the number of nematode species in marine habitats to be as high as one hundred million, although only 26,646 species have been described thus far from all habitats (Hugot et al., 2001). Nematodes are essentially aquatic organisms and most are microscopic in size (0.3-3.0 mm), living in a range of habitats, from oceans to the microscopic film of water surrounding soil particles. They occur as free-living foragers and as obligate ecto- and endoparasites. Based on their different feeding habits, terrestrial and marine nematodes can be divided into different functional (trophic) groups (Yeates et al., 1993). However, nematodes are considered one of the most difficult organisms to identify due their microscopic size, morphological similarity, limited number of distinguishable taxonomic characters and overlapping morphometric measurements. In a global context there is increasing demand for nematode taxonomists to assess community structure in relation to soil function (Wardle et al., 2004), to develop new tools for agronomic management and to address quarantine regulations (Powers, 2004). However, there are few trained nematode taxonomists remaining, primarily due to retirement without replacement and/or young scientists' lack of interest in classical taxonomy (Ferris, 1994; Coomans, 2002). While there is a continuing decline in classical taxonomic expertise of many taxa including Nematoda (Andre et al., 2001; Coomans, 2002), access to new technologies provides some new opportunities for nematode identification and quantification and there is an increasing reliance on developing molecular-based diagnostic protocols to identify pests and pathogens.

Plant-parasitic nematodes continue to be amongst the most damaging and difficult-to-manage agronomic pests, and pressure to reduce the use of highly toxic nematicides and develop alternative management strategies is growing. The development of molecular diagnostics that can quantify specific plant-parasitic nematode species when combined with epidemiological data provides new tools to assist in management and risk assessment for these pests. Molecular diagnostics being developed for free living nematodes in

soil communities are being used in monitoring soil health and environmental impact assessment.

Molecular diagnostics

Many DNA-based molecular diagnostics have been developed for application in Nematology (see reviews by Jones et al., 1997; Powers et al., 1997; Powers, 2004; Blok, 2005) which are routine, rapid and robust. Polymerase chain reaction (PCR) has revolutionized studies of plant-parasitic nematodes, and in the past decade a steady flow of publications have demonstrated its use in a wide range of applications including phylogenetic studies, molecular breeding for resistance, the discovery of novel pathogenicity genes acquired by horizontal gene transfer and the development of molecular diagnostics.

PCR-based molecular diagnostics demonstrate the progress made in this area, offering sensitivity, accuracy and confidence when used to complement "conventional" descriptive information. The demonstration that a PCR-based method could amplify polymorphic regions that distinguished closely related species of nematodes was a major achievement (Powers & Harris, 1993), and many subsequent reports have followed that have validated this principle using sibling species. The sensitivity of detection of a specific species within a mixture with other nematodes is also an important advance initially demonstrated using controlled mixtures of purified DNA and subsequently validated with the detection of single individuals within a complex mixture of nematodes from a soil community (Subbotin et al., 2001; Hübschen et al., 2004a). Now several quantitative PCR (qPCR) assays have been developed which can be used to quantify different species in mixtures. Amplification from low- and single- copy genes is also within the sensitivity of PCR, given the multicellular nature of nematodes. Although this level of sensitivity is technically challenging it offers the potential to differentiate isolates with biological differences, such as virulence or avirulence to a specific resistance gene. Other technical improvements in equipment, better and less expensive reagents, new qPCR technologies and robotics will continue to transform the role of molecular diagnostics in Nematology in the future.

Due to their global economic importance, most of the initial molecular-based diagnostics focused primarily on *Meloidogyne*, *Heterodera*, *Pratylenchus* and *Globodera* (for example, Zijlstra et al., 1995; Shields et al., 1996; Uehara et al., 1998; Subbotin et al., 1999, 2000; Siqueira et al., 2009), and also a few were targeted at longidorid species (Hübschen et al., 2004b; Oliveira et al., 2005). Many of these diagnostics involve two stages; a PCR reaction followed by restriction enzyme digestion to produce restriction fragment length polymorphisms (RFLPs). Hübschen et al. (2004a) reiterated that a single step PCR test is preferable, particularly for use in phytosanitary/quarantine laboratories where efficient use of both consumables and human resources is necessary.

Technological advances have improved the sensitivity, accuracy and sample throughput of molecular diagnostics; however, appropriate methods must be used for the particular practical requirements of the diagnosis. Different diagnostic methods have specific practical difficulties, for example those used by the quarantine services. This might include screening imports of tubers for the presence of *Meloidogyne chitwoodi* or *M. fallax,* which involves examination of tubers for symptoms and isolation of individual nematodes from tuber flesh, or distinguishing the pine wood nematode *Bursaphelenchus xylophilus* from the related non-pathogenic but morphologically similar *B. mucronatus* in wood samples. Such tasks raise different challenges with regard to sampling, sensitivity and quantification from, for example, a pre-plant soil test for virus-vectoring nematodes.

Amplification of multicopy targets such as rDNA from single juvenile nematodes and multiple amplifications from a single juvenile are now routine processes in many laboratories. High quality DNA sufficient for up to 80 PCR reactions from (0.5 µL DNA/ 25 µL reaction) can be obtained from a single nematode (Oliveira, 2004). DNA extraction previously required the manual lysis of nematodes e.g. cutting nematodes with a micro-scalpel, which was time-consuming (Stanton et al., 1998). However, DNA can also be extracted from single nematodes without manual disruption of specimens by chemical lysis with sodium hydroxide (Stanton et al., 1998). In addition, DNA extractions can be retained as a voucher for further studies.

The basic reagents and equipment required for molecular diagnostics are now widely available and many laboratories around the world are developing the expertise for undertaking molecular studies of plant-parasitic nematodes. The generic bases of many PCR diagnostic methods mean that the equipment and skills required can be utilized in a wide range of applications in plant pathology (for example: Eiras et al., 2010; Jiménez & Montano, 2010; Rommel et al., 2010). Diagnostic methods for use with individual nematodes, cysts or egg masses are routinely used, and techniques are available that are sufficiently sensitive to detect specific species present in mixtures (see Zijlstra, 2000; Wishart et al., 2002; Subbotin et al., 2001; Hübschen et al., 2004a), host tissue (for example *Aphelenchoides fragariae* found in leaves of diverse ornamental plant species - McCuiston et al., 2007) and total soil extracts (Holeva, 2004; Hübschen et al., 2004a; Machado et al., 2007) and are able to between distinguish closely related species such as *Pratylenchus neglectus* and *P. thornei* in DNA extracts from soil (Yan et al., 2008).

Quantitative PCR

Many nematode molecular diagnostics have relied on size polymorphisms of the PCR products or digestion products when subjected to electrophoresis through an agarose gel. During this latter procedure sample

contamination can occur; moreover, the process is not quantitative and it under-uses the sequence information inherent in the amplification products that could assist in discriminating between samples. More recently specific and sensitive assays using qPCR have enabled the detection and quantification of many plant pathogens (reviewed by Schaad & Frederick, 2002). In the last decade qPCR assays have been designed for many plant-parasitic nematodes (Table 1), though primarily these have been designed for the most economically important genera such as *Bursaphelenchus, Meloidogyne* and *Globodera*. There are technical differences between some of assays; some use the interleaved fluorescent reporter dye SYBR Green whereas others use probes with reported dyes to increase the specificity of the assay. These qPCR assays are used for identification and quantification but also provide new possibilities to deal with large numbers of samples such as from statutory surveys, through automation of extraction and molecular procedures that increase speed and accuracy (Reid et al., 2010). Quantitative PCR also has utility in glasshouse (Gao et al., 2006) and field experiments to determine the effect of different treatments on the reproductive potential of plant-parasitic nematodes or on competition between species, experiments which would have been prohibitive in the past using conventional morphological methods. Other qPCR assays have provided tools for ecological studies quantifying specific species within communities (Holterman et al., 2008; Sato et al., 2007) and host-parasite interaction studies where nematodes are within the host tissue (Francois et al., 2007; Gao et al., 2007; Rahman et al., 2010; Takeuchi & Futai 2009; Zijlstra & Van Hoof, 2006). Quantitative PCR has also been used to determine virus loads in vectoring nematodes (Finetti-Sialer & Ciancio, 2005; Holeva et al., 2006) and the quantity of bacteria (Di Serracapriola et al., 2008) and fungi (Ciancio et al., 2005; Gao et al., 2006) associated with nematodes.

Gene expression in nematodes (Gao et al., 2004; Skantar et al., 2006; Sukno et al., 2007; Dubreuil et al., 2007; De Luca et al., 2009) is also routinely measured with qPCR, particularly genes involved in pathogenicity. In the future the development of assays based on virulence determinants may provide the means for rapid detection of nematodes that can overcome particular resistance genes so that appropriate alternative control methods can be used to prevent the multiplication of resistance-breaking strains.

TABLE 1 - Examples of quantitative PCR assays for plant-parasitic nematodes

Nematode species	PCR target	Real-time assay	Authors
Meloidogyne javanica	rDNA	SYBR Green	Berry et al. (2008)
M. incognita	rDNA	Scorpion probe	Ciancio et al. (2005)
M. chitwoodi and *M fallax.*	rDNA	TaqMan probe	Zijlstra & Van Hoof (2006)
M. incognita	rDNA	SYBR Green	Toyota et al. (2008)
Bursaphelenchus xylophilus	rDNA topoisomerase I	SYBR Green	Huang et al. (2010)
B. xylophilus	rDNA	SYBR Green	Kang et al. (2009)
B. xylophilus	Heat shock protein 70	probe	Leal et al. (2007)
B. xylophilus from *B. mucronatus*	MspI satellite DNA family	TaqMan probe	Francois et al. (2007)
B. xylophilus	rDNA	TaqMan probe	Cao et al. (2005)
B. xylophilus	rDNA	Taqman probe	Wang et al. (2005)
B. xylophilus	rDNA	TaqMan probe	Wang et al. (2006)
Globodera rostochiensis and *G. pallida*	rDNA	SYBR Green	Bates et al. (2002)
G. rostochiensis	rDNA	SYBR Green	Toyota et al. (2008)
G. rostochiensis, G. pallida and *G. tabacum*	rDNA	multiplex	Nakhla et al. (2008; 2010)
G. rostochiensis and *G. artemisiae*	rDNA	TaqMan	Nowaczyk et al. (2008)
G. pallida, Heterodera schachtii	ITS-	SYBR green	Madani et al. (2005; 2008)
G. rostochiensis, G. pallida and *G. tabacum*	ITS-	Taqman	Madani et al. (2008)
G. rostochiensis and *G. pallida*	ITS-	TaqMan	Reid et al. (2010)
Paratrichodorus pachydermus and *Trichodorus similis*	rDNA I	TaqMan	Holeva et al. (2006)
Heterodera glycines	rDNA	SYBR Green	Goto et al. (2008; 2009)
Pratylenchus zeae	rDNA	SYBR Green	Berry et al. (2008)
Xiphinema elongatum	rDNA I	SYBR Green	Berry et al. (2008)
Pratylenchus penetrans	rDNA	SYBR Green	Sato et al. (2007)

Field samples and species mixtures

Detection of nematodes that can cause damage to an annual crop before planting or direct or indirect damage to perennial woody crops is vital for optimizing yields, maintaining plant and soil health particularly with perennial crops such fruit, viticulture and trees. For detection prior to planting, soil samples may be taken when population levels of the target species are low and patchily distributed, exacerbating the difficulties of their detection. Frequently, mixtures of nematode species occur that are morphologically similar, as is common with *Meloidogyne* spp., which are difficult to distinguish using traditional morphological methods.

It is expected with the unprecedented movement of plant-derived commodities around the globe, climate change, the continuing growth of the human population and consequent changes in land use and agricultural practices, and new threats to agricultural production from either undescribed or recently distributed species of plant-parasitic nematodes will continue to occur and be an ongoing concern. Paul Lehman compiled a list of the top 15 plant-parasitic nematode species regulated by twenty or more countries in international quarantine legislation in 2000 (http://nematode.unl.edu/regnemas.htm); *Globodera rostochiensis* is at the top of the list with 106 countries, and despite its status as a quarantine pest new incidents continue to be found. There is a need for standardized diagnostic protocols for plant protection agencies that are rapid and accurate to limit the movement of regulated species. Diagnostic protocols are being developed by the European and Mediterranean Plant Protection Organisation with quarantine status in the European Union (http://www.eppo.org) and these protocols provide a resource for the international community of diagnostics that are standardized and comparable between laboratories. Diagnostics need to be subjected to ring-testing via inter-laboratorial assessments as done in virology (for example, Massart et al., 2008).

The feasibility of designing molecular diagnostics to distinguish plant-parasitic nematode species is well established, but there are still practical constraints, such as sampling, which are limiting their general use. Diagnostic primers should, where possible, be tested on target organisms from geographically disparate locations; however, relatively small samples of isolates from restricted geographic regions compared to the total number of individuals of that species present have generally been tested. In an ideal world, validation through routine use of the molecular diagnostics alongside conventional morphological identification is needed to demonstrate reliability in generating a species-specific signature, but this is becoming limited by the reduction in numbers of trained taxonomists available.

Combining morphological with molecular data

Recent phylogenetic studies of nematodes have demonstrated that molecular techniques based on sequence analyses can be used to support and challenge classical taxonomic groupings and reveal new species relationships (for example, van Megen et al., 2009). The concept of a "molecular barcode" to distinguish species based on sequences of 18S rDNA was described by Floyd et al. (2002) using communities of soil nematodes sampled from different research plots at a site in Scotland. The molecular barcode concept extends the phylogenetic groundwork established from classical taxonomic descriptions based on traditional morphological, morphometric and biological information of approximately 25,000 nematode species (Powers, 2004). In fact, as proposed by the integrative taxonomy concept (Valdecasas et al., 2008; Pires & Marinomi, 2010), classical taxonomy and DNA barcoding should be consider as complements of each other. The rapidly accumulating molecular taxonomic data provide a robust context for the development and *in silica* validation of molecular diagnostics. As part of the global tree of life project (http://tolweb.org/tree/phylogeny.html), NemATOL, the nematode-specific Tree of Life project (http://nematol.unh.edu/) was created as a public database dedicated to collecting, archiving and organizing reference materials including morphological information and DNA sequences for the study of the phylogeny, diversity, taxonomy, systematics and ecology of nematodes (Powers, 2004).

It is imperative that the specimens used for sequence generation are correctly identified, preferably by a trained taxonomist using morphological and morphometrical characters, before the sequence data is submitted to public databases. Luc et al. (2010) highlighted the limitations of using DNA barcodes from sequences deposited in the GenBank database that had been incorrectly identified. To avoid incorrect labeling, the DNA barcode must be taken from one of the paratypes or paralectotypes when the nominal species-group taxon has been established on many specimens. If such material does not exist, or cannot be used, specimens should be sought in the type locality, or a practicable place as near to the original type locality as possible; for parasitic species, it should be from the same host species and the identity confirmed by a specialist in the group in question. Alternatively, DNA can be extracted from archived specimens. For example, successful PCR amplification and sequencing of the 18S rRNA fragment was achieved for marine nematodes that had been fixed and stored for up to 20 years using a modified hot lysis protocol (Bhadury et al., 2007). To improve the resolution and reliability of nematode systematics, studies should ideally combine morphological with molecular data (De Ley & Blaxter, 2002). However, phylogenetic trees derived from morphological/morphometric characters (Coomans et al., 2001; Lamberti et al., 2002) have mainly been analyzed separately from those derived from molecular data (Fitch et al., 1995; Liu et al., 1997; Aleshin et al., 1998; Blaxter et al., 1998; Kampfer et al., 1998; Blaxter et al., 2000; De Ley et al., 2002; Kanzaki & Futai, 2002; Rusin et al., 2003; Boutsika et al., 2004; Neilson et al., 2004; van Megen et al., 2009). An exception is a comparative analysis of

phylogenetic tree topologies derived from a combination of morphometric and morphological characters with a molecular-based tree which demonstrated congruence in the relationships between *Xiphinema* and *Xiphidorus* species from Brazil. Species belonging to the *X. americanum*-group formed a single group that was clearly separate from the other *Xiphinema* and *Xiphidorus* species. Therefore it was possible to reclassify the *X. americanum*-group as a sub-genus as proposed by Cohn & Sher (1972) or even a distinct genus, within the Longidoridae. These unique and novel analyses, in a nematological context, further demonstrate the validity of the three taxonomic extant clades (Oliveira et al., 2004a). Additionally, maximum likelihood phylogenetic trees derived from both 18S rDNA and ITS-1 sequences discriminated six *Xiphidorus* species (Oliveira et al., 2004b). Sequence divergence was noted between *X. parthenus* and *X. yepesara* and together with morphometric data it was possible to conclude that these nematodes were distinct taxonomic species contrary to their previous subspecies status and synonymization (Chaves et al., 1999). These studies demonstrate that combining morphological/morphometric and molecular approaches is effective in untangling taxonomic controversies.

The future of nematode taxonomy in a molecular era will be challenging because of an ever decreasing classical taxonomy skill-base. The delineation of a "species", based solely on sequence data will continue to be debated and will inform future decisions that consider intra- and inter-population variability. Although recognized in classical taxonomy, molecular studies will need to deal with this issue. However, it is imperative that studies that aim to make definitive comment about Nematoda at the species level, e.g. NemaTol and diagnostics, address natural variation.

Case study

Xiphinema krugi is a pseudomonodelphic species originally described from the rhizosphere of natural vegetation in Piracicaba, SP, Brazil. It is widespread throughout Brazil and considered pantropical in distribution. As a result of interpopulational morphological and morphometric heterogeneity, the taxonomic status of *X. krugi* has been questioned and for 50 years, *X. krugi* was assumed by many taxonomists to be a variable species. For example, an examination of more than 300 *X. krugi* females from populations collected in eight Brazilian states (Ferraz, 1980) noted considerable variability in tail shape. The majority (approximately 90%) of the specimens had a characteristic ventral tail peg, whilst the rest were hemispheroidal. The reported variability in *X. krugi* strongly suggested the possibility of a species complex.

Therefore, the study of 14 *X. krugi* populations reported by Oliveira et al. (2006), encapsulating both molecular and classical taxonomic data, has demonstrated the possibility that in fact *X. krugi* is a species complex comprised of four distinct genotypes and/or cryptic species that have a morphological basis, albeit defined by minor morphometric differences. The 14 morphologically putative populations of *X. krugi* were clearly separated into four different profiles by RFLP analysis (*Alu*I and *Hinf*I), sequencing of the ITS-1 region, and subsequent Maximum Likelihood phylogenetic analyses. These four profiles were further supported by a principal component analysis of morphometric characters that yielded four taxonomic clusters matching those produced by the molecular data. Sequence homology was greater amongst populations that represented the same RFLP profile than between profiles and similar both between representative populations of the RFLP profiles and putative closely related *Xiphinema* species. As a consequence, a thorough re-examination of the taxonomic status of *X. krugi* is clearly required. The contemporary techniques of molecular biology used in this study have demonstrated their potential utility in nematode taxonomy to assist with the resolution of taxonomic controversies such as *X. krugi* and putative evolutionary studies. Similarly, in a combination of classical and molecular techniques to resolve controversial taxonomic issues related to *Xiphinema cf. pyrenaicum* from Spain, Gutierrez-Gutierrez et al. (2010) provided unequivocal proof that there is genomic variability amongst various putative *X. pyrenaicum*-group populations.

Concluding remarks

The requirement for taxonomists with specialist skills that are expert in dealing with the conservative morphology of plant parasitic nematodes is not a problem unique to Nematology. Many areas of plant pathology are using molecular diagnostics to assist with identifications of difficult taxonomic groups or to take advantages of the sensitivity and through-put possible with new technologies. Paradoxically, Lee (2004) asserts that the application of molecular techniques can be useful in species taxonomy, including nematodes, only when combined with taxonomic skills which, as already noted, are declining. Thus, molecular approaches should not be considered in isolation as proposed by Tautz et al. (2003), but a balanced molecular and morphological taxonomic approach is required.

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