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Molecular identification of entomopathogenic nematodes-an overview

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Abstract
Entomopathogenic nematodes (EPNs) are one of the important biocontrol agent in IPM system and in sustainable agriculture. Identification of EPNs is important for proper classification, biodiversity assessment, habitat specificity and their potential use in pest management programs. By using overlapping morphological characters, very closely related species are difficult to connect phylogenetically. Therefore, application of molecular methods advances our understanding of nematodes inter-relationships and evolution. Various molecular techniques have been developed which are capable of identification of species of EPNs. This review attempts an overview of some of the molecular methods that are helpful for taxonomy, biodiversity and biogeography studies of EPNs.

Keywords: Entomopathogenic nematodes (EPNs), taxonomy, phylogeny, molecular methods of identification, insect pest, biocontrol agent

Introduction
Entomopathogenic nematodes (EPNs), *Steinernema* spp. and *Heterorhabditis* spp. are natural enemies of insect pests of agricultural importance. EPNs have long been studied for their distinct virulence against a wide range of insects belonging to orders Lepidoptera, Coleoptera, Diptera, Thysanoptera, and Orthoptera. Their utilization has formed part of a more integrative approach in reducing crop losses (Shapiro-Ilan *et al*., 2017) [19]. EPNs have been considered non-toxic alternative to chemical pesticides, in cases where resistance to insecticides has developed (Ehlers, 1998) [6], enabling producers to use an additional biological resource to control pests in an environmentally friendly manner (Platt *et al*., 2020) [17].

Mode of action of Entomopathogenic nematodes (EPNs)
Their parasitic life cycle is initiated by the infective juveniles (IJ), either cruisers or ambushers for their insect host. They enter the host through body openings or by penetrating directly the cuticle to reach the hemocoel. After entering in the hemocoel, they release their bacterial endosymbionts (*Xenorhabdus* and *Photorhabdus* for *Steinernema* and *Heterorhabditis*, respectively) that multiply very rapidly in the hemolymph resulting to host death within 24 to 72 hr. The nematodes then feed on the bacteria and complete their life cycle inside the insect cadavers. They eqmerge from the failing cadaver, carrying the bacterial symbiont in the anterior part of their intestine to begin another infection cycle (Burnell and Stock, 2000) [5]. EPNs are naturally found in both agriculturally disturbed and undisturbed soil environments with reports of occurrences from many temperate and tropical countries. To date, there are at least 90 *Steinernema* and 20 *Heterorhabditis* species reported (Shapiro-Ilan *et al*., 2017) [19].

Importance of identification of EPNs
Research into the biocontrol potential of EPNs is gaining more importance and has opened up opportunities to explore the rich diversity of nematode species that are beneficial to human. In Europe and many other developed countries, EPNs have been commercialized and successfully used for pest management (Malan and Ferreira, 2017) [12]. EPNs can control a variety of soil-dwelling insect pests (Hiltpold, 2015) [7] as well as aboveground herbivorous insects (Shapiro-Ilan *et al*., 2017) [19]. The selection of an EPN for the control of a particular insect pest is determined by factors such as the nematode's host range, host finding strategy, soil pH, texture, aeration, temperature, atmospheric CO_2, application methods, tolerance of environmental factors and their effects on survival and efficacy. More surveys need to be conducted across the globe in order to identify and isolate new species that are more virulent

and locally adapted to the environmental conditions of the region. Identification of EPNs is a prerequisite for proper classification, biodiversity studies, habitat specificity and their potential use in pest management programs (Nasmith *et al*., 1996) [13]. Nematodes are characterized on the basis of morphologic and morphometric traits (Nguyen and Smart, 1996) [14]. As the overlapping morphological characters are common among species within each genus, molecular tools are needed to identify and distinguish species. Moreover, by using morphological characters very closely related species are difficult to connect phylogenetically. Subbotin and Moens (2006) [23] suggested that delimitation of species should be based either mainly in an amalgamation of the phylogenetic species concept and evolutionary species concept. Molecular techniques in the field of biology have helped us to get the accurate identification of nematode species and to detect the smallest variations within species and even within individual strains. The application of molecular systematic advances our understanding of nematodes inter-relationships and evolution. Hominick *et al*., (1996) [8] observed that differentiation among species of *Heterorhabditis* is difficult due to the extreme morphological conservation and difficulties in performing cross breeding tests.

Molecular methods

Nucleic acid analysis, have enhanced nematode identification and phylogenetic investigations. Nucleic acids are complex organic substance present in all living cells, especially DNA or RNA, whose molecules consist of many nucleotides linked in a long chain. DNA is a biopolymer of two complementary strands of nucleotides. There are four nucleotides in DNA and their order in the genome is the code which determines the identity of the individuals. Segments of genome that vary between taxon and are conserved within taxon are useful for identification. In order to perform analysis on DNA and RNA, it is necessary to first extract these molecules from cells or tissues and then go for analyzing process by using different techniques. The pattern of resolution of the DNA fragments is used for identification and /or phylogenetic analyses of the nematode taxa considered. These may include Restriction Fragment Length Polymorphism (RFLP) (Nasmith *et al*., 1996) [13], Amplified Fragment Length Polymorphism (AFLP), random-amplified polymorphic DNA (RAPD), and the use of species-specific primers, which relies on the presence/absence of a PCR amplification product. Except for RFLP, where PCR may not be needed, all other methods involve PCR followed by electrophoresis. PCR offers several advantages: organisms do not need to be cultured prior to processing by PCR, the technique is rapid and versatile and has sensitivity (Lee *et al*.,1993) [9]. Gel electrophoresis can separate fragments of DNA on the basis of their sizes, base pair and form a useful method to characterize nematode species. By comparing the base sequences of nematode species, one can determine the exact number of mutational variations.

RFLP analyses can be made using fingerprint generated from genomic DNA (gDNA) digested with one or more endonucleases. Genomic DNA-RFLPs likely to be complex, but potentially reveal more polymorphisms owing to the size of the gDNA template. Also, gDNA-RFLPs do not require knowledge of sequence information a priori. Care must be taken to let restriction digestions go to completion since incomplete digestions may lead to non-reproducible fingerprints.

The AFLP technique improves upon gDNA-RFLP by selectively amplifying fewer restriction products and producing less-complex fingerprints. Genomic DNA is digested with two restriction enzymes that produces sticky ends, to which are ligated adaptors. A subset of these adaptor-ligated fragments is then selectively amplified using primer sets that recognize sequences of the adaptors, the sticky ends, and one to three nucleotides inside the restriction sites. As with gDNA-RFLPs, AFLPs do not require prior knowledge of sequence information, and completion of restriction digestions is crucial for reproducible fingerprints.

RAPD involves PCR amplification of gDNA fragments using short (usually 10 bp) primer of arbitrary sequences. The primers bind to several regions on the DNA, and amplification results if two primers bind on opposite strands of the DNA with their 3 ends facing each other at a distance that can be traversed by the polymerase. Consequently, fragments of various sizes may be generated, with sizes of the larger fragments dependent on efficiency of the polymerase used. The use of large, intact gDNA template is important for this reason. Because RADPs are done at lower temperatures, which create lower stringency for primer annealing, reproducibility especially between laboratories also poses a limitation. One advantage of this method is that it does not require prior knowledge of sequence information about the template DNA.

The nucleotide-based methods involve PCR amplification, specific probe hybridizations and sequencing of a region(s) of the DNA. Sequence-based methods may involve analyses of nucleotide sequence information from specific segment(s) of the nuclear DNA(rDNA), mitochondrial DNA (mtDNA), or the whole genome (gene regions and the corresponding primer sets). Nucleotide sequence analysis has proven to be a useful tool not only for diagnostics at different taxonomic levels, but also for providing valuable data for phylogenetic inference or evolutionary interferences about EPNs (Adams *et al*., 2007) [1]. Ribosomal DNA contains internal transcribed spacer (ITS) array, which consists of the entire ITS1, 5.8S rRNA gene, and ITS2 regions of the nuclear rDNA cistron. The rDNA encompasses conserved coding regions (28S, 18S, and 5.8S subunits) and variable non-coding regions (ITS and ETS; the external-transcribed region) organized as tandem repeats, with intergenic spacers separating the repeating units. The bulk of the sequence variability in the rDNA is harbored in the internal transcribed spacer (ITS), which is interrupted by the 5.8S coding region in the rDNA ciston into ITS1 and ITS2, making the ITS useful in molecular systematic of closely related nematode species. The 5.8S gene sequence is highly conserved, whereas the ITS1 and ITS2 sequences are more variable and highly polymorphic, but are more similar within species and more divergent between species. As the 5.8S rRNA region of the ITS is short and highly conserved than the ITS-1and ITS-2 regions (Stock 2009) [22], evolve more rapidly than the 18S and 28S genes. The 28S rRNA varied more rapidly than the 18S rRNA and had fewer positional ambiguities during alignment than ITS (Stock 2009) [22]. However, the 28S rRNA has been attested more informative and suitable for the assessment of phylogenetic relationships, delimitation of terminal taxa, and for diagnostic purposes among *Steinernema* spp. (Stock and Hunt, 2005) [21]. Thus, they are ideal for the EPN taxonomic studies at species (population) levels and for population genetic studies (Stock

2009) [22]. Suggested that ITS-1 region is reportedly sufficient at differentiating species and assessing their evolutionary relationships, particularly among the *Heterorhabditis* spp.

Mitochondrial DNA (mtDNA) is highly variable, maternally inherited, lacks recombination and seems to be selectively neutral (Liu *et al*., 1998) [2]. High evolution rates of mtDNA genes permit their use to compare both inter and intra specific variation. Among mitochondrial genes, NADH dehydrogenase subunit 4 (nd4) and cytochrome c oxidase subunit 1 (cox1) provide ideal markers for population genetic structure and molecular evolution (Blouin 2002) [4]. The higher level of sequence diversity in the variable region makes cox1 preferable for resolution at lower taxonomic levels such as species and subspecies groups, while the higher level of sequence conservation in the flanking regions, which allows for universal primers to be designed, has made the rDNA more suitable for use in wider taxonomic levels. An added advantage of cox1 and rDNA is that both genes occur in multiple copies in nematode genomes enabling PCR amplifications from small amounts of DNA templates such as that can be obtained from single nematodes.

Currently, the 18S and 28S rRNA genes are among the most common markers used to identify and classify members of the phylum Nematoda, however, these loci often lack species-level taxonomic resolution. To address the potential limitations of single-locus molecular hypotheses, multilocus approaches has been proposed to assess phylogenetic relationships among *Steinernema* taxa (Lee and Stock, 2010) [10]. The combination of rDNA ITS1 and mtDNA 16S-CO II markers provides an informative dataset for future studies on phylogenetic relationships and molecular diagnostics of *Steinernema* at the species level (Szalanski *et al*.,2000) [24]. Similarly, DNA sequencing of the nuclear ribosomal DNA (rDNA) first internal transcribed spacer (ITS1) region and mitochondrial DNA (mtDNA) nd4 gene has proven useful for studying speciation, phylogenetic relationships, and molecular evolution in the Heterorhabditidae. Blouin *et al*., (1998) [3] which reported relatively low mtDNA diversity both at the population and the species level in *H. marelatus*. The rRNA gene sequences can vary in length and nucleotide composition, insertion, and deletion events, sometimes involving blocks of multiple nucleotides occur frequently (ITS sequence length differences of >100 bp can be observed), and can result in rDNA size differences between sequences. These, unlike protein-coding genes, do not compromise the function of the ribosome (Nguyen *et al*., 2001) [15], but can render dubious the homological position of characters (useful for delimitating species) during the phylogenetic reconstruction. Thus, inaccurate tests of homology statements during alignment, compounded by a high number of taxa (which drastically increases the number of possible phylogenetic solutions) can lead to spurious phylogenies.

The use of phenotypic traits, biochemical and biophysical techniques, and molecular techniques for diagnosis and identification of bacterial symbiont, *Xenorhabdus* and *Photorhabdus* species and strains. Molecular methods such as restriction analysis of PCR amplified gene products have been employed to determine diversity among entomopathogenic bacterial species. Sequence data of single and multigene datasets has also been used to identify *Xenorhabdus* and *Photorhabdus* species and /or strains and their evolutionary relationships (Lee and Stock, 2010) [10]. In addition, sequence data has been used to develop coevolutionary hypotheses between these bacteria and their nematode hosts. Comparison of 16S rDNA gene sequences is useful for determining molecular taxonomy and has been used in the description of the genera *Xenorhabdus* and *Photorhabdus* (Rainey *et al*.,1995) [18]. At present, phylogenetic analysis of the genera *Xenorhabdus* and *Photorhabdus* is based on a multigene approach with the recombinase A(recA), DNA polymerase III beta chain (dnaN), glutamyl-tRNA synthetase catalytic subunit (gltX), DNA gyrase subunit B (gyrB) and initiation factor B (infB) genes. Furthermore, DNA-DNA hybridization analysis needs to be done if 16S rDNA similarity is over 97%. For this analysis DNA-DNA relatedness should be below the 70% threshold for description as a new taxon.

Each of these methods has its own advantages and/or disadvantages compared to other nematode identification methods. A great advantage of sequence based methods is that sequence information is stored in publicly available databases such as GenBank (ncbi.nlm.nih.gov) and NEMBASE (nematodes.org).This facilitates identification of nematodes based on sequence information through comparison with that available in these databases (Blaxter *et al*.,1998) [2]. At present, nucleotide differences on the level of 4-5% of compared sequences are sufficient to identify new species (Spiridonov, 2004) [20]. Accuracy of identification, however, depends on the quality of sequences deposited in the databases and the authenticity of the taxa the sequences originated from.

Conclusion

New genome sequences of these mutualistic partners together with new analytical methods will help improve our understanding of their phylogenies, and will contribute to the advancement of the evolutionary history of EPNs, their symbionts, their insect hosts, and their ecological roles. Availability of global DNA sequence databases make molecular methods a great tool for rapid and accurate identification of nematodes. This development is likely to contribute to the global initiatives in promoting agriculture and thereby sustainably resolving the challenges pest attack.

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