56 Let's be inclusive – the time of looking at individual plant parasitic nematodes is over, and new technologies allow for it

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Introduction

Parasitism is a popular life style among members of the phylum Nematoda. Around 46% of the 27,000 described nematode species use either a plant or an animal as a primary food source. A couple of years ago a paper written by John Jones and Roland Perry entitled 'Top 10 plant parasitic nematodes in molecular plant pathology' was published (Jones *et al.,* 2013), and yes, it is true that plant parasitic nematodes cause tremendous crop yield losses. However, it should be kept in mind that probably 95% of these losses are caused by about two dozen nematode taxa. We realize this statement requires some more detailing in order to avoid raising of eyebrows. Just like in the Top 10 paper, we consider root-knot nematodes, cyst nematodes or lesion nematodes (etc.) as a single taxon (harbouring one or, at most, two genera). So, two dozen nematode genera are responsible for the by far major part of the damage inflicted by this group of plant pathogens.

Over the last two decades, molecular phylogenetics has aided in deciphering patterns of evolution and diversification among plant parasitic nematodes. Alignments comprising over 5000 nearly full-length small subunit (SSU) ribosomal (r) DNA sequences (each approximately 1700 bp) with a fairly good coverage of all extant nematode families allowed us to pinpoint patterns with regard to the appearance of plant parasitism. It is justified to label the Trichodoridae (clade 1, for clade delineation see Holterman *et al.*, 2006) as the most basal plant parasite family. Trichodorids have an unusual stylet-like device, an onchiostyle, and one of the peculiarities of this onchiostyle is that it does not have a molecular weight cut off. Trichodorids are unique in that they can ingest relatively large particles such as whole plastids and mitochondria. Outside this lineage, no other plant parasitic nematode is able to do this. The next major branch in which plant parasitism arose is clade 2. The family Longidoridae arose and diversified within the order Dorylaimida. This family is mostly known as a vector of plant viruses (genus *Nepovirus*). It is noted that these two lineages are the only plant parasite harbouring

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branches among the former class Adenophorea. In terms of plant parasitic nematode diversification, clades 10 and 12 are more successful. Clade 10 plant parasites arose relatively recently, and it gave rise to a number of very destructive parasites. The pine wilt nematode *Bursaphelenchus xylophilus* (position 8 in the plant parasitic nematode Top 10) and the red ring nematode *B. cocophilus* are tree parasites vectored by insects that kill their host. The primitive nature of this interaction is illustrated by the fact that no nematode-induced re-differentiation of plant cells takes place. By far most plant parasitic nematodes can be found in clade 12 (mainly order Tylenchida). Within this clade we see a gradual evolution from facultative plant parasites (they also feed on fungi) to sedentary endoparasites. It is worth mentioning that plant parasitic nematodes with a sophisticated and durable interaction with their host are among the most successful ones in terms of proliferation and abundance. The Top 3 of the plant parasitic nematodes according to Jones *et al.* (2013) – the root-knot (*Meloidogyne*), the cyst (*Globodera* and *Heterodera*) and the lesion (*Pratylenchus*) nematodes – all reside in the most distal parts of clade 12 (e.g. Smant *et al.,* 2018). Hence, from the enormous economic and social impact of plant parasitic nematodes worldwide, we should not conclude that we would wish to control nematodes, or even plant parasitic nematodes. We rather should strive to manage specifically a very small subset of plant parasitic nematodes – actually approximately 1% of the total plant parasitic nematode biodiversity. But sure, this is more easily said than done.

State of the art

A large part of the biological diversification patterns described above stem from molecular data. Also here we would like to emphasize there is no principle difference between 'classical' morphological and morphometric data on the one hand, and molecular data on the other. We could summarize this with a very short statement: 'characters are characters'. In fact, it is all about numbers. With molecular data it is pretty straightforward to generate 1000 characters from a single individual nematode. On the other hand, it is more difficult, and maybe even impossible, to generate 1000 morphological characters from a single worm. Another advantage of the use of molecular data is the time efficiency. It is easy to amplify one of multiple fragments within half a day and send them out for DNA sequencing. A more fundamental advantage of the use of molecular data is that one can avoid the effects of convergent evolution. Convergent or parallel evolution has obscured our view on nematode systematics and evolution dramatically. It is hard to find a single morphological characteristic that did not arise at least twice in evolutionary history (Holterman *et al.*, 2017). We think it is fair to state that extensive convergent evolution within the phylum Nematoda is the very reason why nematode systematics has been unstable for decades.

Nematode identification

Currently, we see two kinds of molecular approaches for the identification and (quantitative) monitoring of plant parasitic nematodes. There are focused approaches such as real time (RT) PCR. Using large and taxonomically diverse alignments as a starting point, it is most of the time possible to define species-specific DNA motifs. It is technically not overly demanding to design species-specific PCR primers, even for groups of nematodes that are notoriously hard to distinguish such as plant parasitic *Aphelenchoides* species (see e.g. Rybarczyk-Mydłowska *et al.*, 2012). It is noted that for each species a calibration curve that establishes the relationship between C_t value and the number of nematodes needs to be generated. But once this is done, RT or quantitative PCR is a powerful and affordable technique to identify and monitor plant parasitic nematodes. However, qPCR-based detection technologies are by definition focused. One will never see things that one is not looking for. To see the unexpected, another more open approach should be chosen. Lysates from nematode suspensions can be used as a substrate for the amplification of gene fragments of nematodes in general (or even Metazoa). Most of these approaches focus on variable regions within the SSU rDNA, and the V5–V7 regions are quite popular (Capra *et al.*, 2016). Such a meta-barcoding

approach is completely open and allows, as such, for the discovery of unexpected nematodes. However, this comes at a price: the molecular signal present in these V5–V7 regions allow for identification at genus level at best. Moreover, the results of such a nematode community analysis are in essence semi-quantitative.

The soil biome

As already implied by the title, the latest tendency in applied soil ecology is to take a more holistic approach. We no longer focus on a single bad guy – the pathogen threatening our crops – but we rather try to map the biotic environment of the pathogen. In other words, we no longer concentrate on a harmful plant parasitic nematode species, but consider the nematode community as a whole, and even include the bacterial, fungal and protist community. Nowadays it is technically possible to map and monitor the complete soil biome. This approach allows us to map the nematode-suppressive potential of a soil, and experimentally verify whether this potential can be boosted and maximized.

Methodology

Nematode identification in soil

If we really want to assess plant parasitic nematodes in their biotic environment we have to change gears in relation to our methodologies. First of all, the extraction. Currently, techniques to isolate nematodes from the soil matrix differ from the protocols used to extract microbial DNA and RNA from soil. Nematodes will be extracted by Baermann or Oostenbrink elutriation, or any other technique, from >100 g, while microbial DNA/RNA is extracted directly from <2 g of a homogenized soil sample that represents a certain area in a field. This will remain the standard procedure because the size and consequently population density of nematodes determines the soil volume to get a representative sample of the community. The characterization of the nematode community is the part most of us are familiar with. This can be done microscopically, but researchers generally now prefer RT PCR-based methods or meta-barcoding as these methods are easily scalable, time-efficient and do not require as much nematological expertise (see e.g. Quist *et al.*, 2019). With ever decreasing sequencing and data processing costs, PCR-free high-throughput sequencing will replace the at most semi-quantitative nature of meta-barcoding and give access to eco-functionally more relevant transcripts of soil biota.

Microbial community identification in soil

Microbial communities are mostly analysed from rhizosphere or bulk soil. An exciting other niche that should not be overlooked is the microbe community attached to the cuticle of plant parasitic nematodes. These often-non-pathogenic bacteria and/or fungal spores were recently shown to activate the plant innate immunity system (Topalovic *et al.*, 2020), and in that sense contributing to the self-defence of the plant *against* root-invading nematodes. With regard to the soil itself, it is important to know that soils act as a microbial seedbank; only a small part of the soil microbiome is active and the largest fraction is present but inactive ('dormant'). This is especially relevant for bulk soils, where typically 80% of the cells, and 50% of the operational taxonomic units are inactive (Lennon and Jones, 2011). Hence, it is crucial to discriminate between the active microbial fraction of soil – the fraction that potentially interacts with the nematode community – and the resident community that comprises all biodiversity (active and inactive). However, it should be kept in mind that the 'dormant' part of the community partially gets activated by encountering signals from roots or nematodes. Moreover, it is noted that some 'dormant' stages like endospores contain substantial amounts of ribosomal RNA to speed up this activation. Another advantage of targeting ribosomal RNA instead of DNA is the high copy number of ribosomes in active nematodes that allows for their detection within RNA extracts from roots (Topalovic *et al.*, 2020).

Activity of microbiome

RNA from the soil or nematode cuticle can be used to map the active microbiome, whereas

DNA is used to provide an overview of the resident community (Ofek *et al.*, 2014; Harkes *et al.*, 2019). Subsequently, fragments of the 16*S* or 18*S* ribosomal DNA are amplified, and the resulting complex amplicons are labelled. Currently, paired end 2 × 300 bp MiSeq sequencing-based analysis is frequently used. In a single run about 22 million reads from nearly 100 samples can be generated to characterize a taxonomic fraction of the soil biota. Curated databases with taxonomic information or sequence similarities within the dataset are used to translate these rather large data sets into amplicon sequence variant (ASV) tables (a matrix that gives the number of reads per sample per ASV). Such an ASV table can be used to check for the presence and activity of known nematophagous fungi and/or nemato-toxic microorganisms. The overall aim of this approach is to have a methodological framework to map the actual and the potential nematode suppressiveness, as well as a tool to verify the validity of any tool of management practice that is suggested to boost this very wanted soil characteristic.

Pros and cons

We are convinced that the combined use of host plant resistances, smart soil management practices (including pathogen-informed (cover-) crop rotation schemes), and an optimal exploration of the soil nematode-suppressive potential is key to future-proof plant parasitic nematode management. In this scientific brief, we paid most attention to endogenous soil suppressiveness as it is the least well-characterized of the main control options. That is no wonder: the soil microbiome is highly complex, and only in recent years have the DNA sequencing costs been dropped to a level that we can use and explore for agroecological purposes. Nevertheless, we currently are able to handle and analyse the literally tens of millions of DNA reads that are typically produced by microbiome monitoring studies. Moreover, we are able to pinpoint the effects of various soil management systems on the active and resident microbiome in association with increased levels of soil suppressiveness against plant parasitic nematodes.

What are currently the major cons? It is work in progress, it is quite complex, and we are only just starting to understand the underlying mechanisms. The following can be said about this complexity: currently we are reasonably well able to map soil biodiversity and monitor management-induced changes. On several occasions we were able to link desired traits to specific bacteria or fungi. A logical next step is to search for ecological characteristics of these organisms. The crux is in this last step: not always but regularly it appears that literally close-to-nothing is known about the ecological functions of these soil inhabitants. So ecological characterization of soil inhabitants is lagging behind our dramatically increased capability to map soil life. Soil is no longer a black box, but the functional understanding of interesting and probably relevant community shifts is currently the limiting factor in our understanding of the soil biome.

Outlook: a vision of the future

One thing is for sure – future durable plant parasitic nematode management is much more knowledge intensive than it was in previous times. To illustrate this: in 'soil fumigant times' the nature of the nematode problem did not matter at all. In the end it even did not need to be a nematode. With the application of, for instance, systemic acetyl cholinesterase inhibitors, it became a bit more subtle, but not too much. After all, most nematodes use acetyl choline as a neurotransmitter. The current approaches – the combined use of host plant resistances, smart soil management techniques and soil suppressiveness (in combination with biocontrol) – require in-depth knowledge about the biological system. Fortunately, this is happening as we speak: reference genomes have been or are currently generated for the most important plant parasitic nematode species, and molecular pathotyping will allow for a much more effective use of resistance genes. Moreover, we will soon be able to pinpoint the actual and the potential endogenous nematode-suppressive potential of soil in agroecosystems, and help applied science explore and boost this potential and breed crops with a high capacity for the induction of defence by associated microbiomes to achieve tolerance to plant parasitic nematodes in managed soil systems.

An aspect of concern is the accessibility of these knowledge-intensive approaches. It is of utmost importance that we use our host plant resistances in a more durable way as prolonged and uninformed use will unavoidably lead to the appearance of virulent plant parasitic nematode populations. It is also clear that exploring and boosting the soil suppressive potential will be a major additional tool in the foreseeable future. Let us be clear: these approaches are under development and there are no practical applications yet. However, this will not take long and it would be a shame if only farmers that happen to live in countries with an excellent knowledge infrastructure could benefit from it.

Last, but not least, we would plea to value the biodiversity and the functioning soil living community as an intrinsic asset of that soil. So, soils would not only be valued by their physical and biochemical characteristics but also by the condition of the soil biota. This would imply that farmers that invest in soil biological functioning will see a return on their investments in terms of better market values of their acreage. We know this sounds far-fetched, but we are convinced we should strive for this in order to create a healthy economic basis for soil management that includes the durable exploration of soil biodiversity.

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