MOLECULAR IDENTIFICATION OF *HETERODERA* SPP., AN OVERVIEW OF FIFTEEN YEARS OF RESEARCH[*]

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SUMMARY

During the last 15 years, researchers have collected and characterised more than 40 species of nematodes from the genus *Heterodera*. The species were identified by sequencing the ITS–rRNA genes and by PCR-RFLP profiles; these tools remain the best available for identifying cyst-forming nematodes. By restricting the ITS amplicons with one or a combination of seven restriction enzymes (*Alu*I, *Ava*I, *Bsh*1236I, *Bsu*RI, *Cfo*I, *Mva*I, and *Rsa*I), researchers can distinguish most of the agriculturally important cyst nematode species from one another and from their sibling species. Species from the Avenae group can be differentiated from one another using the enzymes *Alu*I, *Cfo*I, *Hinf*I, *Ita*I, *Pst*I, *Rsa*I, *Taq*I and *Tru*9I. However, in some cases, it is not possible to use sequences of ITS–rRNA genes and PCR-RFLPs in diagnostic work. In these cases, morphometric characteristics are better for differentiating these species. Intraspecific polymorphism in the ITS sequences can make identification even more difficult; here, more conclusive molecular identification tools are needed to diagnose some species. In the future, end-point PCR and semi-quantitative PCR (SYBR Green I) with species-specific primers (already developed for *Heterodera glycines* and *H. schachtii)* will be the likely choices for fast and reliable detection and quantification of cyst nematodes in samples.

INTRODUCTION

The genus *Heterodera* contains more than 60 species, some of which cause serious yield reduction in crops. Beet crops are intensely affected by *Heterodera schachtii,* and many species of cereal cyst nematodes reduce grain harvests worldwide. The protective cyst stage of these nematodes enables them to withstand desiccation and greatly enhances their dispersal and survival. Rapid and reliable identification of nematodes intercepted by phytosanitary authorities is an important step in monitoring and controlling the movement or introduction of potential pests. Some nematodes are of regulatory concern, and as international trade expands, vigilance and accurate diagnosis become even more important to prevent their dispersion. Application of control measures, especially when growing resistant crops, requires accurate identification of the cyst nematode up to species level.

To date, morphological identification posed particular problems, with many isolates not reliably identifiable to species level. In the last 15 years, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) profiles and sequences of the internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal RNA genes of more than 40 different *Heterodera* spp. have been developed to assess interspecific variability. More recently, emphasis was placed on the creation of species-specific primers and DNA probes to be used in end-point and semi-quantitative/quantitative PCR (real-time PCR). These techniques make diagnostic procedures more effective and accessible, even to scientists not specialised in taxonomy. Several protein coding genes (actin, aldolase, beta-tubulin and hsp90) were recently used for molecular characterisation in cyst nematode diagnostics.

HISTORY

Ferris *et al*. (1993, 1994) were the first to sequence the internal transcribed spacers of ribosomal RNA genes (ITS-rRNA) from several isolates of cyst nematodes belonging to the genus *Heterodera,* and to compare the sequences with those published from *Caenorhabditis elegans*. Universal primers used for PCR allowed amplification of the complete ITS1, 5.8S gene and ITS2 of the rDNA array, including parts of the 18S and 28S genes adjacent to the spacer regions. The ITS sequence data of the cyst nematodes were highly dissimilar to those of *C. elegans*. The sequences for five geographic isolates of *Heterodera glycines* were very similar to one another, but showed nearly as many differences as between this species and either *H. schachtii* or *Heterodera trifolii*. More differences were observed between *H. glycines* and *Heterodera carotae* or *Heterodera avenae*. Despite some mistakes in these newly obtained sequences, these findings confirmed the usefulness of gene and spacer regions of rRNA genes when looking for systematic inference among species and genera of cyst nematodes, and for identification as well. A few years later, Szalanski *et al*. (1997) examined the ITS1, using nucleotide sequencing and PCR-RFLP to assess intraspecific variation between and/or within European, Asian and North American isolates of five heteroderid species. The PCR-RFLP patterns of *Heterodera goettingiana* from Northern Ireland were identical to patterns from the state of Washington. However, sequencing demonstrated that ITS1 heterogeneity existed within individuals and between isolates, but did not result in different restriction patterns. Sequencing of three Indian and two American *Heterodera zeae* isolates revealed variation among ITS1 clones from the same individual, between

individuals, and between isolates. An additional, variant ITS1 region present in the isolates from the US but not in the Indian isolates, created a composite PCR-RFLP pattern. The authors concluded that heterogeneity might contribute to the complexity of the restriction digestion pattern and can serve as highly specific genetic markers.

From 1997 to 2009, a number of articles contributed to the expansion of ITS-RFLP profiles and sequences of more than 40 different species of cyst nematodes belonging to the genus *Heterodera*. The conclusion drawn from new species descriptions, identification and phylogenetic studies was that ITS-RFLP profiles and sequences are most useful for *Heterodera* species identification. Restriction of the ITS amplicons with one or a combination of seven restriction enzymes (*Alu*I, *Ava*I, *Bsh*1236I, *Bsu*RI, *Cfo*I, *Mva*I, and *Rsa*I) enables discernment of agriculturally important cyst nematode species, both from one another and from their sibling species (Subbotin *et al.* 2000). Species of *Heterodera* from the Avenae group (*H. arenaria*, *H. aucklandica*, *H. australis*, *H. avenae*, *H. hordecalis, H. filipjevi*, *H. mani*, *H. latipons*, *H. pratensis* and *H. ustinovi*) can be differentiated from one another using the enzymes *Alu*I, *Cfo*I, *Hinf*I, *Ita*I, *Pst*I, *Rsa*I, *Taq*I and *Tru*9I (Subbotin *et al.* 2003). Restriction profiles for Chinese populations of cereal cyst nematodes were published recently by Ou *et al.* (2008a). The studies also revealed that heterogeneity is present in several *Heterodera* species, resulting in composite RFLP profiles that depend on the enzymes used. Subbotin *et al.* (1999) distinguished two types of ITS regions within *H. avenae*. Bekal *et al.* (1997) also observed polymorphism between *H. avenae* populations, but those observations differed from those of Subbotin *et al.* (1999). Szalanski *et al.* (1997), Subbotin *et al.* (2000, 2003), Wouts *et al.* (2001) and Zheng *et al.* (2000) reported intraspecific variations within the *Heterodera* species *H. betae*, *H. carotae*, *H. ciceri*, *H. cruciferae*, *H. filipjevi*, *H. glycines*, *H. pratensis*, *H. schachtii, H. trifolii, H. urticae* and *H. zeae*. Madani *et al.* (2004) and Rivoal *et al.* (2003) reported a relatively high level of sequence divergence between populations of *H. hordecalis,* and suggested that two species may be grouped under this taxon. The same level of sequence divergence was observed between *H. latipons* populations (Rivoal *et al.* 2003). This is likely a case of sibling species, as Ferris *et al.* (1999) proposed earlier. The number of genetically different populations within the same species may be even higher than what has been observed so far.

As the new millennium began, another approach to *Heterodera* species identification was being developed. In 2001, Amiri *et al.* designed a primer, using the available ITS-rDNA sequence information, that is specific for species from the *H. schachtii sensu stricto* group. The primer was evaluated with 30 populations and species within this *Heterodera* group, as well as several other parasitic nematode species. Subsequent digestion of amplified PCR product by *Mva*I and *Pvu*II allowed separation of the morphologically poorly distinguishable *H. schachtii*, *H. betae* and *H. trifolii* from one another. This method of identification is highly sensitive: amplification was obtained even when a single second-stage juvenile or a single cyst was mixed with other nematode species. In the same year, Subbotin *et al.* (2001) described a method to rapidly identify juveniles and cysts of the soybean cyst nematode, based on PCR with species-specific primers (Figure 1). The PCR assay was tested on 53 populations originating from China, Russia, the US, and Brazil. This method could detect a single cyst or second-stage juvenile of *H. glycines,* alone or in a mixture with other soil-inhabiting nematodes. In 2002, Amiri *et al.* supplemented their research with a species-specific primer to detect only *H.*
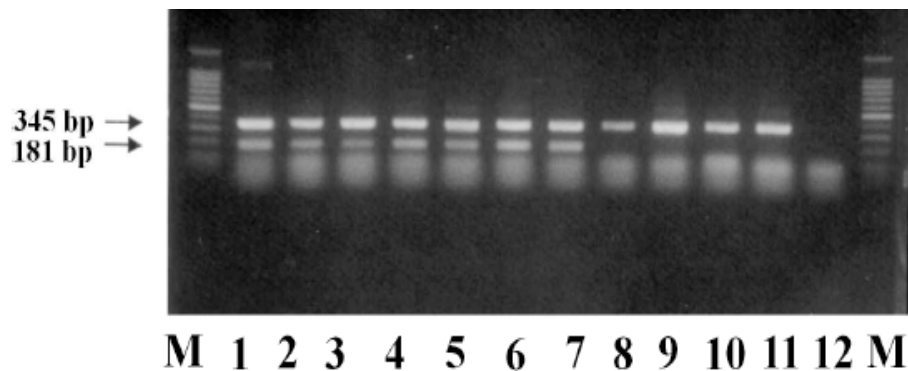
Figure 1. Duplex PCR with *H. glycines* specific primer resulting in the *H. glycines* specific 181 bp and in the universal 345 bp PCR product. M: 100 bp DNA ladder; 1-7: *H. glycines*, 8: *H. schachtii*, 9: *H. ciceri*, 10: *H. medicaginis*, 11: *H. cajani*, 12: negative control (Subbotin *et al.* 2001).

schachtii. Recently, a PCR test with species-specific SCAR primers for *H. glycines* was developed by Ou *et al.* (2008b). In 2005, Madani *et al.* (2005) used the *H. schachtii* specific primer in combination with SYBR green I dye to detect and quantify *H. schachtii* nematodes in samples using real-time PCR. Real-time PCR is faster than end-point PCR, especially since it eliminates the time-consuming post-PCR agarose gel electrophoresis.

PRESENT STATUS

Currently, most of the agriculturally important cyst-forming nematodes of the genus *Heterodera* are identified by using PCR-ITS-RFLP and sequencing of the ITS-rRNA genes, and PCR using species-specific primers developed for *H. schachtii* and *H. glycines*. However, PCR-ITS-RFLP diagnostics profiles have only been generated for 40 species, half of the valid *Heterodera* species, whereas another 40 known species have not been molecularly characterised. For several *Heterodera* species (*H. avenae, H. carotae, H. filipjevi, H. hordecalis, H. latipons* and *H. salixophila*), interspecific RFLP polymorphism with one or several restriction enzymes has been reported. This should be taken in account when diagnosing these species. Several studies have also revealed that, in some cases, identical ITS sequences can be found in morphologically clearly distinct *Heterodera* species such as *H. avenae* and *H. arenaria, H. carotae* and *H. cruciferae* (Subbotin *et al.* 2000), and *H. trifolii* and *Heterodera daverti* (S. A. Subbotin, unpublished data). Consequently, identification of these species should be based on IEF profiles of proteins and/or morphometric characteristics, until reliable markers for differentiation of these species have been published.

PROSPECTS AND RECOMMENDATIONS

It is clear from the work over the last 15 years that molecular techniques are powerful tools for nematode diagnosis. Using these tools, scientists have solved a number of problems, but an even larger number remain unsolved. The promising

and encouraging results have increased demand for better-performing techniques and applications in new fields. People now expect faster diagnostic results, preferably with on-the-spot sample examination. Direct diagnosis from soil samples, without first extracting the nematodes, would make diagnosis even faster.

Mobile molecular equipment may be particularly useful in quarantine applications, where the detection of single individuals is of paramount importance. Techniques must also be able to distinguish between dead and living individuals. Molecular nematode diagnostic techniques are evolving toward nanodiagnostics, which is still primarily in the research stage.

Recent progress in sequencing nematode genomes, including the recent sequencing of the *H. glycines* genome, supports the search for more reliable markers for use in diagnostics. DNA sequencing costs have decreased more than 100-fold over the past decade, fuelled in large part by tools, technology and process improvements developed as part of the successful effort to sequence the human genome. New technology opens the door to the next generation of sequencing methods, which include, pyrosequencing, sequencing-by-synthesis and sequencing using nanopores. There are many opportunities to reduce the cost and increase the throughput of DNA sequencing, which are likely to lead to very different and novel approaches to diagnostics (Perry *et al.* 2007).

ACKNOWLEDGMENT

REFERENCES

Amiri S, Subbotin SA, Moens M (2001) An efficient method for identification of the *Heterodera schachtii sensu stricto* group using PCR with specific primers. *Nematologia Mediterránea* 29, 241-246.

Amiri S, Subbotin SA, Moens M (2002) Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108, 497-506.

Bekal S, Gauthier JP, Rivoal R (1997) Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. *Genome* 40, 479-486.

Ferris VR, Ferris JM, Faghihi J (1993) Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177-184.

Ferris VR, Ferris JM, Faghihi J, Ireholm A (1994) Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *Journal of Nematology* 26, 144-151.

Ferris VR, Subbotin SA, Ireholm A, Spiegel Y, Faghihi J, Ferris JM (1999) Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. *Russian Journal of Nematology* 7, 121-125.

Madani M, Subbotin SA, Moens M (2005) Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*,

using real-time PCR with SYBR green I dye. *Molecular and Cellular Probes* 19, 81-86.

Madani M, Vovlas N, Castillo P, Subbotin SA, Moens M (2004) Molecular characterisation of cyst nematode species (*Heterodera* spp.) from the Mediterranean basin using RFLPs and sequences of ITS-rDNA. *Journal of Phytopathology* 152, 229-234.

Ou S, Peng D, Li Y, Wang YJ (2008a) Restriction fragment length polymorphism and sequences analysis of rDNA-ITS region of cereal cyst nematode (*Heterodera avenae*) on wheat from Zhengzhou. *Acta Phytopathologica Sinica* 38 407-413.

Ou S, Peng D, Liu X, Moens M (2008b) Identification of *Heterodera glycines* using PCR with sequence characterised amplified region (SCAR) primers. *Nematology* 10, 397-403.

Perry RN, Subbotin SA, Moens M (2007) Molecular diagnostics of plant-parasitic nematodes. In 'Biotechnology and plant disease management.' (Eds ZK Punja, S De Boer, HI Sanfacon) pp. 195-226. (CAB International: Wallingford, UK)

Rivoal R, Valette S, Bekal S, Gauthier J-P, Yahyaoui A (2003) Genetic and phenotypic diversity in the graminaceous cyst nematode complex, inferred from PCR-RFLP of ribosomal DNA and morphometric analysis. *European Journal of Plant Pathology* 109, 227-241.

Szalanski AL, Sui DD, Harris TS, Powers TO (1997) Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology* 29, 255-267.

Subbotin SA, Deliang P, Moens M (2001) A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology* 3, 365-371.

Subbotin SA, Sturhan D, Rumpenhorst HJ, Moens M (2003) Molecular and morphological characterisation of the *Heterodera avenae* species complex (Tylenchida: Heteroderidae). *Nematology* 5, 515-538.

Subbotin SA, Waeyenberge L, Moens M (2000) Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology* 2, 153-164.

Subbotin SA, Waeyenberge L, Molokanova IA, Moens M (1999) Identification of *Heterodera avenae* group species by mophometrics and rDNA-RFLPs. *Nematology* 1, 195-207.

Wouts WM, Rumpenhorst HJ, Sturhan D (2001) *Heterodera betae* sp. n., the yellow beet cyst nematode (Nematoda: Heteroderidae). *Russian Journal of Nematology* 9, 33-42.

Zheng J, Subbotin SA, Waeyenberge L, Moens M (2000) Molecular characterization of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. *Russian Journal of Nematology* 8, 109-113.