10
Parasitism Genes of the Pine Wood Nematode

Taisei Kikuchi

10.1 Introduction

Although the life history and some behavioral ecological aspects of *Bursaphelenchus xylophilus*, the pine wood nematode (PWN) are now well documented, little is known about the molecular basis of the nematodes' biology and host-parasite interaction.

Molecular biology has allowed for the details of many aspects of nematode biology and host-parasite interactions to be worked out at a far deeper level than would once have been thought possible. One of the most useful applications of these techniques has been the development of a detailed and accurate phylogeny for the Phylum Nematoda. A lack of clearly homologous characteristics and the absence of a fossil record that would allow the evolution of nematodes to be studied prevent interpretation of the deeper phylogenetic relationships within the Phylum. On the basis of comparisons of the small subunit ribosomal DNA sequences of nematodes, a detailed phylogeny of the Phylum has been drawn up and the interrelationships of the major nematode groups have been established (Blaxter et al. 1998; Dorris et al. 1999). Nematodes can be divided into five major clades, all of which include parasitic species, indicating that parasitism of both animals and plants has arisen multiple times during evolution (Blaxter et al. 1998; Dorris et al. 1999).

In contrast, another technique—analysis of expressed sequence tags (ESTs)—has been of paramount importance in developing an understanding of the proteins produced by nematodes that allow them to parasitize plants. To date, more than 700,000 EST sequences from nematodes, including free-living species as well as animal and plant parasites, are available in databases (Parkinson et al. 2003, 2004). Many parasitism genes have been identified and detailed analysis of such genes present in the EST dataset has been undertaken. One of the most remarkable

Forest Pathology Laboratory Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba 305-8687, Japan

Tel.: +81-29-873-3211, Fax: +81-29-873-1543, e-mail: kikuchit@affrc.go.jp

findings of the EST analysis was the identification of genes, which encode proteins that are likely to be important for plant parasitism (parasitism genes).

Previous studies on the parasitism genes have been largely restricted to economically important Tylenchid, cyst and root-knot nematodes. There have been few studies on plant parasitism genes in other nematodes that are phylogenetically or ecologically distinct from cyst and root-knot nematodes. *Bursaphelenchus xylophilus* is part of the same clade (IVb) as cyst/root-knot nematodes (Blaxter et al. 1998; Meldal et al. 2007); however, *Bursaphelenchus* spp. are not directly related to these nematodes but form a distinct grouping with other mycophagus nematodes including *Aphelenchoides* spp. (Meldal et al. 2007). Studies on the parasitism genes of *B. xylophilus* will undoubtedly provide clues to understanding the mechanisms underlying parasitism in *B. xylophilus*, and will also further our understanding of the evolution of plant parasitism in nematodes.

In this chapter, I present an overview of EST analysis of *B. xylophilus* and detailed characteristics of some parasitism genes in *B. xylophilus* identified during this EST project.

10.2 EST Analysis

The *Caenorhabditis elegans* genome sequence was completed several years ago (The-Caenorhabditis-elegans-Sequencing-Consortium 1998) and since then, substantial annotation of the sequence has taken place. In addition, whole-genome scale gene expression and RNA interference (RNAi) studies have allowed detailed functional analysis of the biological role of many genes (Maeda et al. 2001). Although genome sequencing projects are well underway for several other nematode species, including *C. briggsae*, *Haemonchus contortus* and *Brugia malayi*, such resources are not available for many other nematodes. Consequently, many parasitic nematode genomes are being explored using ESTs. Analysis of ESTs by single-pass random sequencing of cDNA libraries is a powerful tool for rapid and cost-effective gene discovery. High-throughput projects involving more than 30 nematode species have generated nearly 500,000 ESTs from parasitic nematodes, including datasets from animal parasites and plant parasites (Table II.2) (Parkinson et al. 2003, 2004). Including the sequences from *C. elegans* and *C. briggsae*, there are currently over 700,000 nematode ESTs in the publicly accessible dbEST database. EST analysis has been a powerful tool for the identification of plant parasitic nematode genes, which have a possible role in parasitism.

Most of this analysis has been performed on the economically important Tylenchid nematodes. Almost all plant parasitic nematodes in Table II.2 are Tylenchid, with the exception of several groups of ectoparasitic nematodes such as species of *Xiphinema*, *Trichodorus* and *Longidorus*, which are found in clades I and II of the Phylum. By contrast, previous characterization of the *Bursaphelenchus* species genome has been limited to a very few sequences, which were used only for phylogenetic analysis and diagnostic purposes.

Table II.2 Nematode expressed sequence tag (EST) projects

Nematode species	Description	Submitted ESTs[a]	Clades[b]	Major EST sources[c]
Bursaphelenchus xylophilus	Pine wood nematode	13,340	IVb	1
Bursaphelenchus mucronatus	Pine wood nematode	3,193	IVb	1
Globodera pallida	Potato cyst nematode	4,378	IVb	2, 3, 4
Globodera rostochiensis	Potato cyst nematode	11,851	IVb	2, 3, 4
Heterodera glycines	Soy bean cyst	24,444	IVb	2
Heterodera schachtii	Sugar beat cyst nematode	2,818	IVb	2
Meloidogyne arenaria	Root-knot nematode	5,018	IVb	2
Meloidogyne chitwoodi	Root-knot nematode	12,218	IVb	2
Meloidogyne hapla	Root-knot nematode	24,452	IVb	2
Maloidogyne incognita	Root-knot nematode	20,334	IVb	2, 4
Meloidogyne javanica	Root-knot nematode	7,587	IVb	2
Meloidogyne paranaensis	Root-knot nematode	3,710	IVb	2
Pratylenchus penetrans	Cobb's lesion nematode	1,928	IVb	2
Pratylenchus vulnus	Plant lesion nematode	5,812	IVb	2
Radopholus similis	Plant migratory endoparasite	1,154	IVb	2
Xiphinema index	California dagger nematode	9,351	I	2, 3
Ancylostoma caninum	Dog hookworm	9,618	V	2
Ancylostoma ceylanicum	Human hookworm	10,651	V	2
Angiostrongylus cantonensis	Rat lung worm	1,279	V	10
Ascaris suum	Swine gut parasite	40,771	III	2, 5
Ascaris lumbricoides	Human gut parasite	1,822	III	2
Anisakis simplex	Marine mammal parasite	475	III	11
Brugia malayi	Human lymphatic parasite	26,215	III	2, 9
Dirofilaria immitis	Canine heart worm	4,005	III	2
Haemonchus contortus	Sheep gut parasite	21,967	V	2, 5
Litomosoides sigmodontis	Mouse filarial worm	2,699	III	5
Necator americanus	Human hookworm	5,032	V	5, 7
Nippostrongylus brasiliensis	Rat gastrointestinal parasite	8,238	V	2, 5
Onchocerca volvulus	Human filarial parasite	14,974	III	9
Ostertagia ostertagi	Cattle gut parasite	7,006	V	2
Parastrongyloides trichosuri	Possum gut parasite	7,963	IVa	2
Strongyloides ratti	Rodent gut parasite	14,761	IVa	2
Strongyloides stercoralis	Human gut parasite	11,392	IVa	2

(continued)

Table II.2 Continued

Nematode species	Description	Submitted ESTs[a]	Clades[b]	Major EST sources[c]
Teladorsagia circumcincta	Sheep gut parasite	4,313	V	5, 7
Toxocara canis	Canine gut parasite	4,889	III	2, 5
Trichinella spiralis	Human muscle parasite	11,568	I	2
Trichuris muris	Mouse threadworm	2,714	I	5, 7
Trichuris vulpis	Dog whipworm	3,063	I	2
Wuchereria bancrofti	Human bancroftian filariasis	4,847	III	2
Caenorhabditis briggsae	Free living	2,424	V	2
Caenorhabditis elegans	Free living	346,046	V	2, 6, 7, 8
Caenorhabditis remanei	Free living	20,292	V	2
Heterorhabditis bacteriophora	Insect associated bacteriovore	7,514	V	2
Pristionchus pacificus	Free living	14,663	V	2
Zeldia punctata	Free living	391	IVb	2
Total		746,647		

[a] ESTs deposited as at 10 October 2004

[b] The phylum Nematoda has previously been defined into five clades (Dorris et al. 1999)

[c] 1, Forestry and Forest Products Research Institute, Japan; 2, Genome Sequencing Center, Washington University, USA; 3, Scottish Crop Research Institute, UK; 4, Wageningen University, the Netherlands; 5, University of Edinburgh, UK; 6, National Institute of Genetics, Japan; 7, The Wellcome Trust Sanger Institute, UK; 8, The Institute for Genome Research, USA; 9, The Filarial Genome Network, UK; 10, Chang Gung University, Taiwan; 11, Pusan University, Korea

The EST project for the PWN was done at the Forestry and Forest Products Research Institute, Japan from 2004 to 2006. In this project, over 13,000 ESTs from *B. xylophilus* and, by way of contrast, over 3,000 ESTs from a closely related species that does not as readily parasitize plants; *B. mucronatus*, were produced (Kikuchi et al. 2007). Four libraries from *B. xylophilus* and one library from *B. mucronatus* were constructed and used to generate ESTs. Sixty-nine percent of the total *B. xylophilus* ESTs were from a mixed-stage library derived from nematodes feeding on fungi, 11% were from a library made from nematodes feeding on plant material and 20% were from two dauer-like larvae libraries (Table II.3). A variety of proteins potentially important in the parasitic process of *B. xylophilus* and *B. mucronatus*, including proteins important in fungal feeding as well as proteins that break down various components of the plant cell wall, were identified in the libraries. As well, several gene candidates potentially involved in dauer entry or maintenance were also identified in the EST dataset.

The 13,327 *B. xylophilus* ESTs grouped into 6,487 clusters and the 3,193 *B. mucronatus* ESTs formed 2,219 clusters. Assuming 19–20,000 total genes as in *C. elegans*, these clusters are likely to represent 30% of all *B. xylophilus* genes and 11% of *B. mucronatus* genes (Kikuchi et al. 2007).

Table II.3 ESTs generated from *Bursaphelenchus* cDNA libraries

Species	Library name	Strain	Stage	Description	ESTs	Average length (bp)
B. xylophilus	K1	Ka-4	Mixed stage	Vigorously growing on fungi	9,194	578
	KP	Ka-4	Mixed stage	Growing on plants	1,476	550
	KDw	Ka-4	Dauer larvae (JIV)[a]	Separated from wood	658	442
	KDi	Ka-4	Dauer larvae (JIV)[a]	Separated from insect	1,999	455
					13,327	550
B. mucronatus	U1	Un1	Mixed stage	Vigorously growing on fungi	3,193	564

[a] JIV: dispersal fourth stage juvenile

The availability of these sequences and further bioinformatic analysis, including functional categorization and detailed comparative analysis of the ESTs, will provide useful information to investigate the biology, pathogenicity and evolutionary history of *B. xylophilus*.

10.3 Parasitism Genes

Plant parasitic nematodes are mainly biotrophic root parasites, and can be sedentary or migratory, and ectoparasites or endoparasites (Gheysen and Jones 2006). Migratory nematodes feed on plant cells, frequently causing cell death, and then move to another cell to repeat the feeding. Sedentary endoparasites, including cyst and root-knot nematodes, feed from a single cell or a group of cells for a prolonged period of time. For this sustaining feeding, sedentary parasites have the ability to dramatically modify root cells into elaborate feeding cells.

All plant parasitic nematodes have evolved a hollow, called a stylet, and use it to penetrate the wall of a plant cell, to remove plant cell contents during feeding and to introduce nematode secretion into plant tissue.

Secretions from the stylet, which are produced in esophageal gland cells, play important roles in plant parasitism of nematodes. Plant parasitic nematodes have two sets of these gland cells, dorsal and sub-ventral, and these gland cells enlarged considerably as nematodes evolved from bacterial-feeding nematodes to parasites of higher plants. Tylenchid nematodes have two sub-ventral gland cells and one dorsal gland cell, and the products of the gland cells are developmentally regulated (Gheysen and Jones 2006).

Bursaphelenchus xylophilus have a stylet and the same number of esophageal gland cells as other plant parasitic nematodes although it is difficult to distinguish

each cell because the three-esophageal gland cells of *Bursaphelenchus* dorsally overlap and all connect to similar positions in the large median esophageal bulb.

However, *B. xylophilus* is unique compared to the major plant parasitic nematodes as it is a parasite of aboveground parts of trees, and does not enter the soil but migrates through plant tissues. Furthermore, *B. xylophilus* is basically a fungal feeder and uses an insect as a transmitting vector. This implies that *B. xylophilus* should have a set of parasitism genes distinct from the major plant parasitic nematodes.

10.3.1 Cell Wall-Degrading Enzymes

The plant cell wall is the primary barrier faced by most plant pathogens and the production of enzymes able to degrade this cell wall is of critical importance for plant pathogens including plant parasitic nematodes. The plant cell wall is a complex but highly organized composite of polysaccharides and protein (Fig. II.11). Plant cell wall-degrading enzymes had previously only been shown to be produced by plants, bacteria and fungi, with no clear reports of their production by animals. Although the ability of animal to hydrolyze cellulose had been the subject of various studies, it is difficult to establish whether the enzyme is synthesized by an animal or by associated microorganisms without isolating the corresponding gene. The first animal cellulase genes were described in plant parasitic cyst nematodes in 1998. Since then many plant cell wall-degrading enzymes have been identified

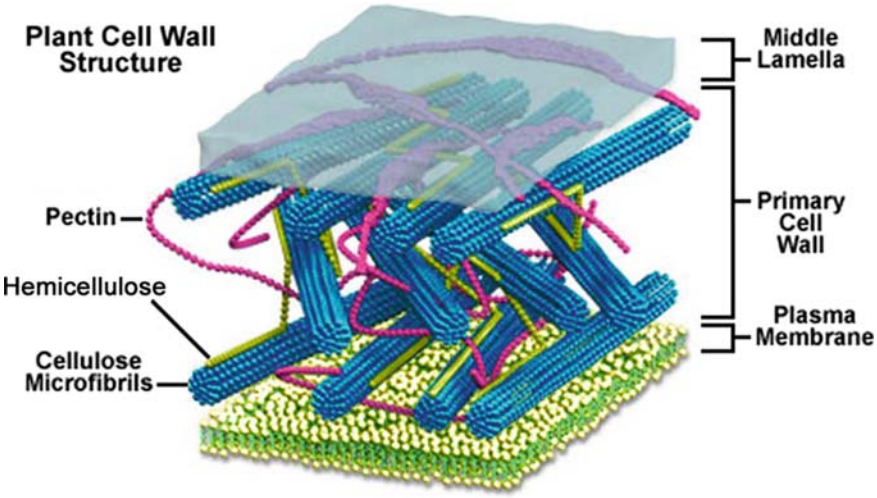

Fig. II.11 Structure of primary plant cell wall, from http://micro.magnet.fsu.edu/ (see Color Plates)

in Tylenchid nematodes, including endo-β-1,4-glucanases (cellulases) (Rosso et al. 1999; Smant et al. 1998), pectate lyase (Doyle and Lambert 2002; Popeijus et al. 2000), polygalacturonase (Jaubert et al. 2002) and xylanase (Dautova et al. 2001), as well as proteins that disrupt non-covalent bonds in plant cell walls (Qin et al. 2004).

1 Cellulase

Cellulose is a major component of plant cell walls (Fig. II.11) and consequently, cellulases (endo-β-1,4-glucanases) are produced by many plant pathogens including bacteria and fungi (Barras et al. 1994; Walton 1994). Endogenous cellulase genes have been identified from plant parasitic nematodes, including *Heterodera*, *Globodera* (cyst nematode) and *Meloidogyne* (root-knot nematode) species (Smant et al. 1998; Rosso et al. 1999; Goellner et al. 2000). These cellulases are produced within the esophageal gland cells of these nematodes and secreted through the nematode stylet into plant tissues (de Boer et al. 1999). They are therefore likely to facilitate the penetration and migration of nematodes into root tissues during parasitism. In addition, their removal using RNAi prevents successful invasion of plant roots (Chen et al. 2005). The proteins encoded by these genes belong to the glycosyl hydrolase family (GHF) 5 and are far more similar to bacterial than to eukaryotic cellulases. It has therefore been suggested that these genes have been acquired via horizontal gene transfer from bacteria (Yan et al. 1998). GHF5 cellulase genes have also been found in one migratory endoparasitic nematode *Pratylenchus penetrans* (Uehara et al. 2001) that is related to cyst and root-knot nematodes.

Cellulase genes of the PWN were identified by screening the EST dataset. Surprisingly, the cellulases showed high similarity with GHF45 cellulases from fungi (Fig. II.12). The catalytic domain of *B. xylophilus* cellulases shows 62–66% overall amino acid identity with cellulases from two fungi, *Scopulariopsis brevicaulis* and *Rhizopus oryzae*. This extremely high similarity between *B. xylophilus* cellulases and fungal cellulases, together with the absence of sequences resembling GHF45 cellulases from other nematodes, including *C. elegans* and *C. briggsae* for which full genome sequences are available, suggests that *B. xylophilus* cellulases might have been acquired via horizontal gene transfer from fungi.

GHF45 cellulase genes were shown to exist as a multiple gene family in the *B. xylophilus* genome. The enzymatic activity of the protein was confirmed by heterologous expression in *Escherichia coli* and the endogenous nature of the genes was confirmed by Southern blotting. The presence of predicted signal peptide sequences at the N-termini of the proteins encoded by these genes coupled with the specific localization of the transcripts imply that these cellulases could be secreted into plant tissues to help nematodes feed and migrate into plants (Fig. II.13). Localization of the protein by immunofluorescence confirmed this (Fig. II.13).

The biochemical properties were examined using purified recombinant proteins expressed in *Pichia*. These analyses suggested that *B. xylophilus* cellulases act not only on cellulose but also on hemicellulose. Among celluloses, these enzymes act

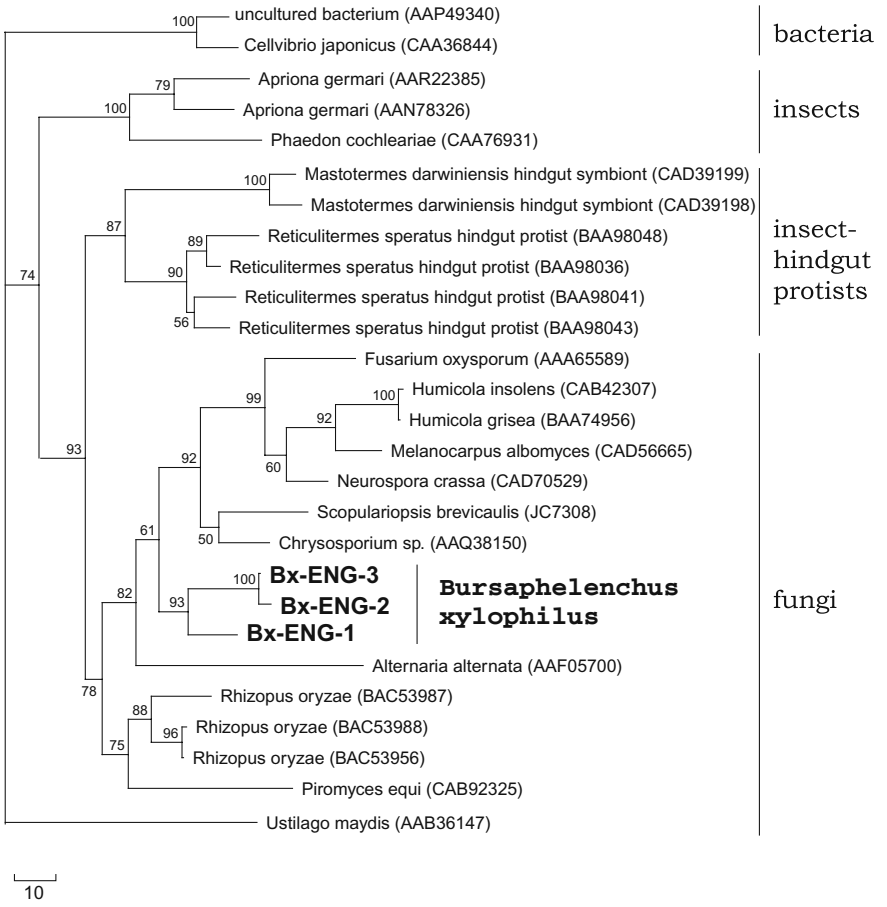

Fig. II.12 Unrooted, phylogenetic tree of GHF45 cellulases. *Scale bar* represents 10 substitutions per 100 amino acid positions

largely on amorphous cellulose but hardly hydrolyze crystalline cellulose, indicating that the nematode does not use cellulases for complete digestion of the cell walls. Once a nematode invades a pine tree, it migrates primarily through the resin canals in the tree and feeds on parenchyma cells surrounding the canals. The cell wall of the parenchyma cell consists of a primary wall, without a thick and rigid secondary wall. In the primary walls, cellulose exists as elementary fibrils that form a complex with hemicellulose.

Before the identification of the cellulase genes, cellulase enzymatic activity was reported in homogenates and secretions of *B. xylophilus* (Odani et al. 1985; Yamamoto et al. 1986). Moreover, close observations of pine tissues infected with *B. xylophilus* suggested that the destruction of pine cells might be a result of cell

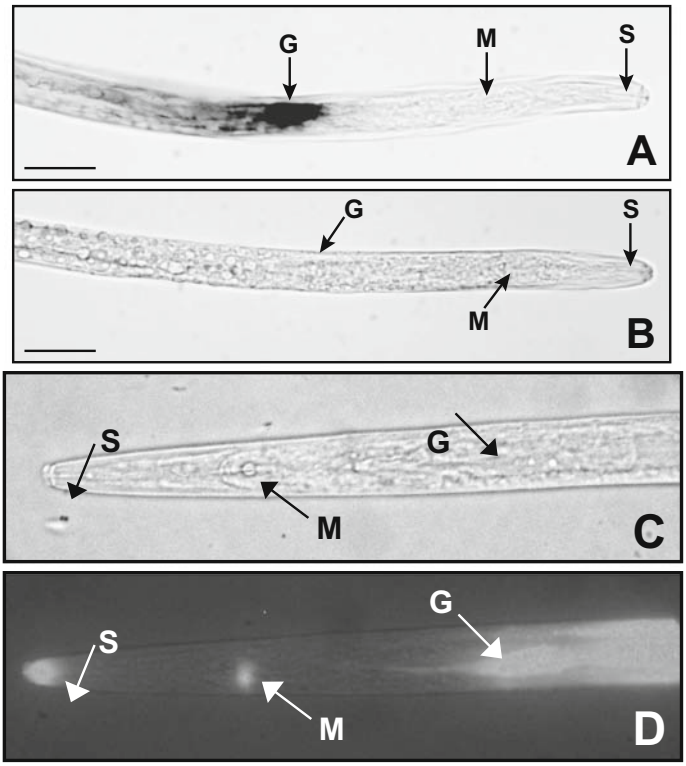

Fig. II.13 Localization of the *Bursaphelenchus xylophilus* cellulase 1 (Bx-ENG-1) transcript and protein. **A**, **B** localization by in situ hybridization of Bx-eng-1 transcripts in oesophageal gland cells of *B. xylophilus* adult female with antisense (**A**) and sense (**B**) Bx-eng-1 digoxigenin-labelled cDNA probes. Expression is restricted to oesophageal gland cells (*bar*, 20 μm); **C**, **D** immunofluorescence localization with antiserum against recombinant Bx-ENG-1, showing that protein is present in the oesophageal gland cells of the nematode and on the exterior of the nematode's head; **C** illustrates the bright-field image, whereas **D** illustrates the same specimen viewed under fluorescence optics; *G* oesophageal glands; *S* stylet, *M* metacarpus

wall-degrading enzymes such as cellulase (Ishida et al. 1997; Ichihara et al. 2000a). Thus, these results taken together suggest that *B. xylophilus* secretes cellulases to act on the cellulose–hemicellulose complex, resulting in weakening of the mechanical strength of the cell wall.

It was shown that *B. xylophilus* cellulases are secreted through the nematode's stylet and, like the cellulases of other plant parasitic nematodes, may soften the plant cell wall to facilitate their feeding and migration; however, the *B. xylophilus* cellulases showed most similarity to fungal cellulases and were classified into the glycosyl hydrolase family (GHF) 45, while cellulases of cyst/root-knot nematodes belong to GHF5 and are most similar to bacterial cellulases. It was therefore proposed that cyst/root-knot nematodes and *Bursaphelenchus* spp. might have evolved

both the ability to digest cellulose and the ability to parasitize plants independently (Kikuchi et al. 2004).

2 Pectate Lyases

Pectin is a major structural component of the plant cell wall along with cellulose and hemicellulose (Fig. II.11). Pectin is located mainly in the middle lamella and primary cell wall, and functions as a matrix anchoring cellulose and hemicellulose fibers (Carpita and Gibeaut 1993). The breakdown of pectin consequently leads to the maceration of plant tissues, a characteristic symptom of soft-rot diseases (Lietzke et al. 1994). Pectin degradation requires the combined action of several enzymes. These can be divided into two groups, namely pectin esterases, which remove the methoxyl groups from pectin and depolymerases (hydrolases and lyases) that cleave the backbone chain (Tamaru and Doi 2001). Pectate lyase (pectate transeliminase, EC 4.2.2.2), which catalyzes the cleavage of internal α-1,4-linkages of unesterified polygalacturonate (pectate) by beta-elimination, is known to play a critical role in pectin degradation (Barras et al. 1994).

Pectate lyases are widely distributed among bacterial and fungal plant pathogens and have been the focus of several studies that have aimed to ascertain their function as virulence factors (Barras et al. 1994). They are used by plant pathogens to degrade host cell walls in order to allow penetration and colonization. Plant parasitic cyst nematodes and root-knot nematodes are known to secrete pectate lyases. Genes encoding pectate lyases have been cloned from several species of plant parasitic nematodes including *Heterodera*, *Globodera* (cyst nematode) and *Meloidogyne* (root-knot nematode) species (Popeijus et al. 2000; de Boer et al. 2002; Doyle and Lambert 2002; Huang G et al. 2005). These pectate lyases are produced in esophageal gland cells and are secreted from the stylet of the nematode. They are thought to play an important role in the infection and parasitism of plants.

Two pectate lyase genes have been cloned from *B. xylophilus*. Like cellulases, the pectate lyases were shown to be secreted from the stylet of the nematode (Kikuchi et al. 2006). *Bursaphelenchus xylophilus* is required to migrate within plant tissue during its lifecycle. Once the nematode invades the pine tree, it migrates primarily through resin canals of the tree and feeds from parenchyma cells surrounding the canals (Mamiya 1983). Although pectin substrates are scarce in the xylem of woody plants, they are present in the primary cell wall of the cambium and parenchyma cells of woody plants, including pine trees (Hafren et al. 2000; Westermark et al. 1986). As such it is likely that pectate lyases are secreted from the nematode stylet and help the nematode to migrate and feed within the tree.

Phylogenetic analysis of pectate lyases, including those from bacteria, fungi and nematodes, resulted in a tree in which the nematode sequences were not monophyletic (Fig. II.14). Although it is difficult to determine conclusively from this analysis whether nematode pectate lyase genes have an ancient, common origin, it seems

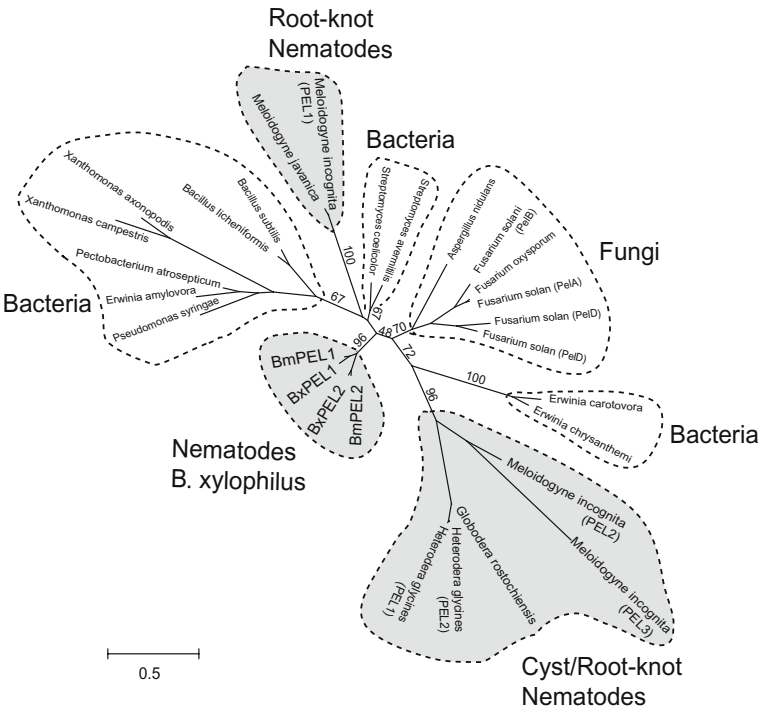

Fig. II.14 Unrooted phylogenetic tree of pectate lyases belonging to polysaccharide lyase family 3. Numbers on the node represent bootstrap support percentages. The *scale bar* represents 50 substitutions per 100 amino acid positions

likely that this is the case. Further support for this idea comes from observation of the conserved position of the intron. Pectate lyases are likely to be widely distributed in plant parasitic nematodes.

3 1,3-Glucanases

β-1,3-Glucanases are widely distributed among bacteria, fungi and higher plants. β-1,3-Glucanases catalyze the hydrolysis of β-1,3-D-glucosidic linkages in β-1,3-D-glucan. This polymer is a major component of fungal cell walls and a major structural and storage polysaccharide (in the form of laminarin) in marine macroalgae (Hong et al. 2002). The physiological functions of β-1,3-glucanases are distinct and depend on their source. Although they have the same hydrolytic activity, bacterial enzymes are classified into glycosyl hydrolase family 16 (GHF16), whereas most plant and fungal enzymes are grouped in GHF17, on the basis of differences in their amino acid sequences (Henrissat and Bairoch 1993).

In the animal kingdom, functionally characterized β-1,3-glucanases are restricted to *B. xylophilus* and marine invertebrates. Genes encoding β-1,3-glucanases have been cloned from the sea urchin *Strongylocentrotus purpuratus* (Bachman and McClay 1996) and the bivalve mollusk *Spisula sachalinensis* (Kozhemyako et al. 2004). They are classified into GHF16 and are thought to be involved in the digestion of algal food. In addition, many β-1,3-glucanase-like proteins have been isolated, and the encoding genes cloned, from insects (Dimopoulos et al. 1997; Kim et al. 2000; Ma and Kanost 2000; Ochiai and Ashida 2000; Zhang et al. 2003) and other invertebrates (Seki et al. 1994; Beschin et al. 1998; Lee et al. 2000; Sritunyalucksana et al. 2002). Although these sequences contain regions that are very similar to the activation region of GHF16 β-1,3-glucanases, they have not been shown to exhibit glucanase activity. These proteins bind specifically to β-1,3-glucan, which is found on the cell surface of microbes but is absent in the host, and they have been shown to play a role in the innate immune system by recognizing foreign material.

Most *Bursaphelenchus* species are solely fungal feeders and all species rely on fungi as a food source at some stage in their life cycle. Many *Bursaphelenchus* species feed on fungi colonizing dead trees. Many *Bursaphelenchus* species, including *B. xylophilus*, therefore have a close association with fungi. Since β-1,3-glucan is the main structural component of fungal cell walls, it seems likely that β-1,3-glucanases play an important role in the life cycle of these nematodes.

The first nematode β-1,3-glucanase genes were identified from *B. xylophilus* during an EST project (Kikuchi et al. 2005). The *B. xylophilus* gene is expressed in oesophageal gland cells and, like the previously characterised cellulases, the enzyme is secreted from the nematode stylet. However, in contrast to cellulases, glucanase is most similar to bacterial enzymes. It is therefore suggested that the *B. xylophilus* β-1,3-glucanase was acquired by horizontal gene transfer from bacteria.

10.3.2 Other Parasitism Genes

Analysis of the EST datasets revealed that other proteins potentially important for parasitism were present, including chitinase and expansin (Kikuchi et al. 2007). Chitinase was described from the cyst nematode *Heterodera glycines*, where it was found to be expressed in subventral oesophageal gland cells, suggesting a role in parasitism, although the precise nature of its potential role remains uncertain (Gao et al. 2002). While a similar function for chitinase may be possible for *B. xylophilus*, this nematode uses fungi as a food source and a beetle as a transmission vector, whose structures contain chitin, and it is possible that chitinase plays a role in these processes. Expansins have been described from cyst nematodes (Qin et al. 2004) and it is thought that these proteins disrupt non-covalent bonds in the plant cell wall, enhancing the activity of other enzymes such as cellulases.

10.4 Conclusions

In the EST project of the PWN, over 13,000 ESTs from *B. xylophilus* and over 3,000 ESTs from a closely related species, *B. mucronatus*, were generated. This project demonstrated that EST generation is an effective method for the discovery of new genes in plant parasitic nematodes. Previous characterization of the *Bursaphelenchus* species genome has been limited to a very few sequences which were used only for phylogenetic analysis and diagnostic purposes. The EST sequences will provide a solid base for future research to investigate the biology, pathogenicity and evolutionary history of this nematode.

One of the most remarkable findings of the EST analysis was the identification of genes, which encode cell wall-degrading enzymes that are likely to be important for plant parasitism. Distinct types of cell wall-degrading enzyme genes were identified from the EST dataset: cellulase, β-1,3-glucanase, pectate lyase. Molecular characterization of these genes showed that they are endogenous nematode genes and are each present as gene families. In situ hybridization showed that all these enzymes are produced in esophageal glands and are therefore extremely likely to be secreted from the nematode through the stylet to the external environment. Further evidence for this was obtained from experiments in which enzyme activity was detected in stylet secretions collected from nematode samples. It has therefore been suggested that these nematodes use a mixture of enzymes to attack the plant or fungal cell wall. These secreted enzymes have been suggested to help in feeding and migration of the nematode.

Cell wall-degrading enzymes of cyst/root-knot nematode are suggested that they were acquired by horizontal gene transfer (HGT) from bacteria (Jones et al. 2005; Scholl et al. 2003) because they are not found in other nematodes or almost any other animals, and are most similar to bacterial genes. *Bursaphelenchus xylophilus* also showed that at least two independent HGT events, one each from bacteria (Kikuchi et al. 2005) and fungi (Kikuchi et al. 2004), have occurred during the evolution of the *Bursaphelenchus* group and that these events have helped shape the evolution of two feeding strategies (fungal feeding and plant parasitism) within this group.

It seems clear therefore that horizontal gene transfer has played an important role in the evolution of plant parasitism in at least two major groups of plant-parasitic nematodes. ESTs have also been obtained from a representative of a third group, *Xiphinema index* (Table II.2). Analysis of these ESTs suggests that cellulase-like genes may also be present in this nematode and that these are most similar to GHF12 cellulases. Further studies on these genes are required and are currently in progress (J. Jones, personal communication) but if these genes are confirmed as genuine *X. index* genes, this will provide another example of the role of genes acquired by HGT in plant-nematode interactions. Studies on other nematodes, particularly the less intensively studied ectoparasites such as *Trichodorus* and fungal-feeding nematodes belonging to groups different from *Bursaphelenchus* such as *Aphelenchus* and *Tylencholaimus*, would be useful to determine whether

the presence of cellulases or other cell wall-degrading enzymes is a requirement for nematode parasitism of plants and whether HGT has driven the evolution of plant parasitism in other nematode groups.

As more genome sequences are obtained from a wider range of nematodes and as EST datasets are compared and analyzed in more detail, it is possible that other horizontally acquired genes may be identified and the role that this process plays in the evolution of nematodes will be fully appreciated.

RNAi (RNA interference) is a powerful tool for the analysis of gene function that has been used extensively for model organisms such as *C. elegans*. This technique exploits the fact that exposure of an organism to double-stranded RNA (dsRNA) from a gene of interest causes silencing of the endogenous gene and allows the null phenotype to be mimicked (Fire et al. 1998). RNAi has been used for genomic scale studies in *C. elegans* (Maeda et al. 2001). In recent years, many groups have been working to transfer this technology to plant parasitic nematodes. To date, successful application of RNAi has been published for plant parasitic cyst and root-knot nematodes (Urwin et al. 2002; Bakhetia et al. 2005; Chen et al. 2005; Fanelli et al. 2005; Rosso et al. 2005). This technique will surely be helpful in determining the detailed functions not only of the genes encoding cell wall-degrading enzymes but also other interesting genes in *B. xylophilus*.