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How to become a parasite – lessons from the genomes of nematodes

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The phylum Nematoda is biologically diverse; it includes parasites of plants and animals in addition to free-living taxa. To date, the genomes of six nematodes have been sequenced. Comparative analyses of these ecologically diverse nematodes are beginning to reveal the mechanisms by which parasites arise and how they evolve. Here, we discuss some emerging principles for the mechanisms and evolution of parasitism. First, horizontal gene transfer represents a common theme in nematode parasites. Second, the human parasite *Brugia malayi* lost otherwise essential genes most probably owing to the mutualistic relationship with a bacterial endosymbiont. Finally, some parasitic features evolved under free-living conditions. A recent study revealed a conserved endocrine mechanism controlling the formation of dauer and infective larvae in nematodes.

Nematology – from species surveys to genome analysis

The nematodes, or roundworms, represent an animal phylum that is best characterized by species richness, numerical abundance and ecological omnipresence. Modern species estimates suggest that there are between 1 and 10 million nematode species on earth, although only ~25 000 of them have been described in the literature [1]. These estimates are based on the enormous diversity of nematodes found in marine environments and in terrestrial, often animal-associated, locations. Some studies indicate that, in soil samples, nematodes can exceed 1 million individuals per meter squared [2]. Nematodes are found in diverse habitats, residing not only in marine, fresh-water and terrestrial habitats, but also in extreme environments, such as polar ice. They occupy a wide range of ecological niches, from free-living microbivores and predators to parasites.

Caenorhabditis elegans, one of the best-studied laboratory model organisms, belongs to the nematode phylum [3]. One group of nematodes that might profit substantially from *C. elegans*, both with respect to methods development and biological insight, is the parasitic nematodes. Parasites are metabolically dependent on other organisms (the hosts) and usually cause their hosts a certain degree of harm. In most cases, however, nematodes are parasites only for a part of their lives because there are some stages inside the host, and other stages outside the host species. Nematode parasites can live on plants, vertebrates and many invertebrate taxa and some of them cause severe agricultural damage or major health problems in humans and livestock (Box 1). Parasites that live on humans present a particular challenge because laboratory models are not available for most of them.

The past decade has seen substantial advances in our understanding of the origin of parasitism in nematodes as a result of application of molecular phylogenetic methods. In 1998, Blaxter *et al.* [4] showed that parasitism evolved at least seven times in nematodes (Figure 1). Four groups of animal parasites and three groups of plant parasites are interspersed with free-living aquatic and terrestrial species in the phylogenetic tree, indicating that, in these groups, parasitism evolved independently from one another. More recently, detailed sampling surveys found that each of the three groups of plant parasitic nematodes – the Tylenchida, Triplonchida and Dorylaimida – have fungal-feeding sister taxa, thereby suggesting that plant parasitism has evolved from fungal associations [5,6].

The ability of nematodes to adapt to nearly all ecosystems and the repeated independent evolution of parasitism is thought to require genomic signatures. Large-scale genomic, transcriptomic and proteomic studies can provide a unique entry point into interdisciplinary investigations of nematode parasitism. The past two years have seen the completion of several important genome-sequencing projects. In 2007, the draft genome of the human parasite *Brugia malayi* was released [7]. In 2008, two plant parasitic nematodes, *Meloidogyne incognita* and *Meloidogyne hapla*, and the beetle-associated *Pristionchus pacificus* were sequenced [8–10] (Table 1).

Here, we discuss the progress made on a variety of nematode genome projects with a focus on what the specific genomic adaptations are revealing about the mechanisms of nematode parasitism and its evolution. We argue that horizontal gene transfer and gene loss are some emerging principles of parasites and speculate on the importance of pre-adaptations for the evolution of parasitism.

Animal parasites have complex life cycles

Approximately 30 genera of nematode parasites cause severe damage to the health of humans and other mammals, including livestock (Box 1). Human parasites include the intestinal parasite *Ascaris lumbricoides*, the filarial nematodes *Onchocerca volvulus* and *Wucheria bancrofti,* which causes elephantiasis (a disorder of the lymphatic system), and the hookworms *Ancylostoma* ssp. and *Necator* ssp. Because none of these human parasites has an animal model, another filarial nematode, *B. malayi*, was chosen for a genome-sequencing project [7]. *B. malayi* lives in southeast Asia and Indonesia and is the only major human filarial parasite that has a laboratory model. Nematode

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Box 1. The diversity of nematode life cycles

Nematode life cycles are very diverse, in particular those of the parasitic nematodes. Here, the life cycles of *M. incognita*, *B. malayi* and *P. pacificus* are shown as being representative of plant parasitic, animal parasitic and necromenic nematodes, respectively, to illustrate some of those species that have recently had their genomes sequenced (Figure I).

Brugia malayi

B. malayi switches between a mosquito vector and the final human host. In infected humans, adult female nematodes are ovoviviparous. The fully developed active juveniles, so-called microfilariae, are found in the blood or skin. Upon ingestion by the mosquito vector during a blood meal, the microfilariae initiate further development and form so-called 'infective third-stage larvae' after molting twice. The nematodes are transferred back to humans when the mosquito takes the next meal of human blood. Adult nematodes can live in humans for more than a decade and can cause tremendous health problems. Over this long time period, the environment of the parasite is completely controlled by its human host.

Meloidogyne incognita

Meloidogyne females deposit eggs on the surface of the root system of the host plant. Embryogenesis and the first molt take place in the egg, generating second-stage juveniles (J2). Those stages represent the exophytic phase of the life cycle, because they are free-living. The J2 animals penetrate the plant root and migrate between cells. The rest of the life cycle represents the endophytic phase. Nematodes induce the growth of enlarged multi-nuclear cells, which feed the nematode after the plant metabolism has been redirected. The nematode becomes sedentary and goes through three molts to finally develop adult females. Males are only observed occasionally and it is believed that they have no role in reproduction in *M. incognita*.

Pristionchus pacificus

P. pacificus has a hermaphroditic mode of reproduction. If food is presented and the animals are not under stress conditions, they go through a direct life cycle: the first stage juveniles develop in the egg and the J2 stage juveniles are the hatching stage. After three molts, animals become adult and complete the life cycle. Under laboratory conditions, this is achieved in 3–4 days at 20 °C. Under stress conditions (high temperature, low food, high density of conspecifics), the animals go through an indirect life cycle and form arrested dauer juveniles, which only develop further under favorable conditions.

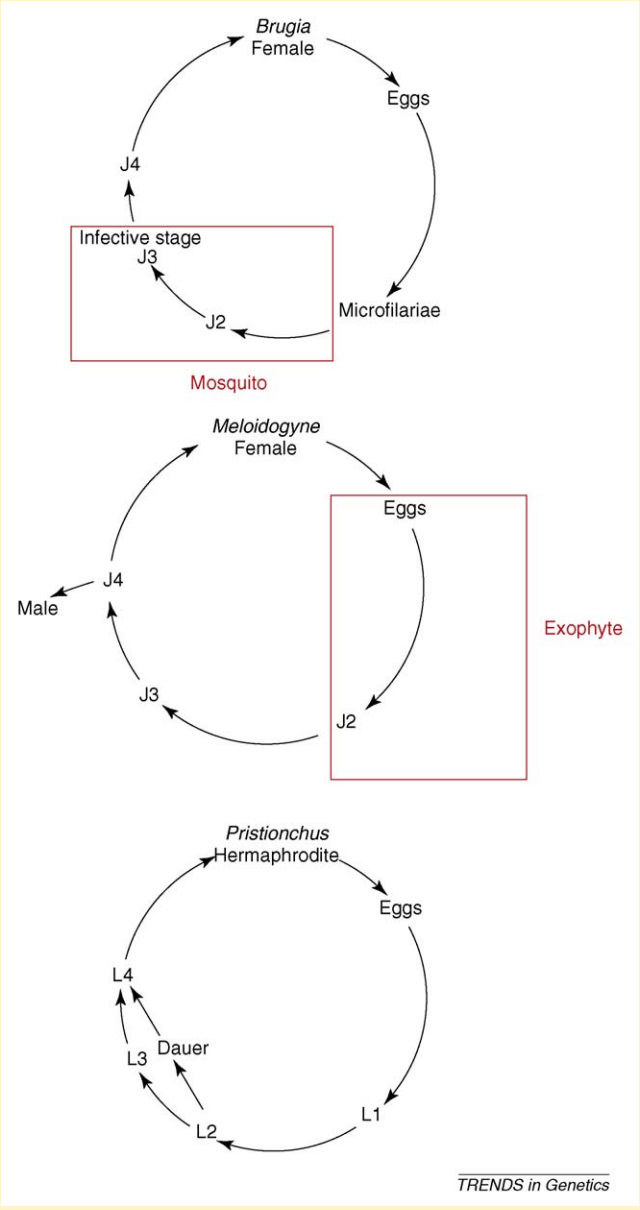

Figure I. The life cycles of *M. incognita*, *B. malayi* and *P. pacificus* are shown here as being representative of plant parasitic, animal parasitic and necromenic nematodes, respectively.

animal parasites have a complex life cycle, which differ between individual parasitic groups and often involve two distinct hosts. For example, *B. malayi* first develops within a mosquito before it is transmitted to the human host (Box 1). In addition to interacting with its insect vector and human host, *B. malayi* carries an α-proteobacterial endosymbiont, *Wolbachia* [11]. Usually, *Wolbachia* are known endosymbionts of several nematode and arthropod species. The genomes of all four organisms have been sequenced, thus providing the opportunity to understand the mechanisms of parasitism in *B. malayi* and to manipulate it [7,11–14].

The *Brugia* genome – multiple genome interactions

The draft genome of *B. malayi* indicates that 20% of the identified gene predictions are *B. malayi*-specific, which has been interpreted as a huge pool of genes that might encode factors involved in nematode defense against the insect and human hosts [7]. Several potential drug targets have been identified in the genome sequence, including genes that encode proteins with a presumed function in molting, neuronal signaling and cell-surface collagens. One of the most striking features of the *B. malayi* genome is the loss of genes encoding for a large amount of enzymes required for *de novo* purine synthesis, heme biosynthesis and all enzymes required for *de novo* riboflavin biosynthesis. One potential source of these vital components is the *Wolbachia* genome, in which all three pathways are intact [7]. Interestingly, this mutualistic relationship between *Brugia* and *Wolbachia* might result in gene loss in both organisms because the *Wolbachia* genome has been shown to have lost several membrane biogenesis genes. Also, *Wolbachia* is unable to synthesize lipid A, a usual

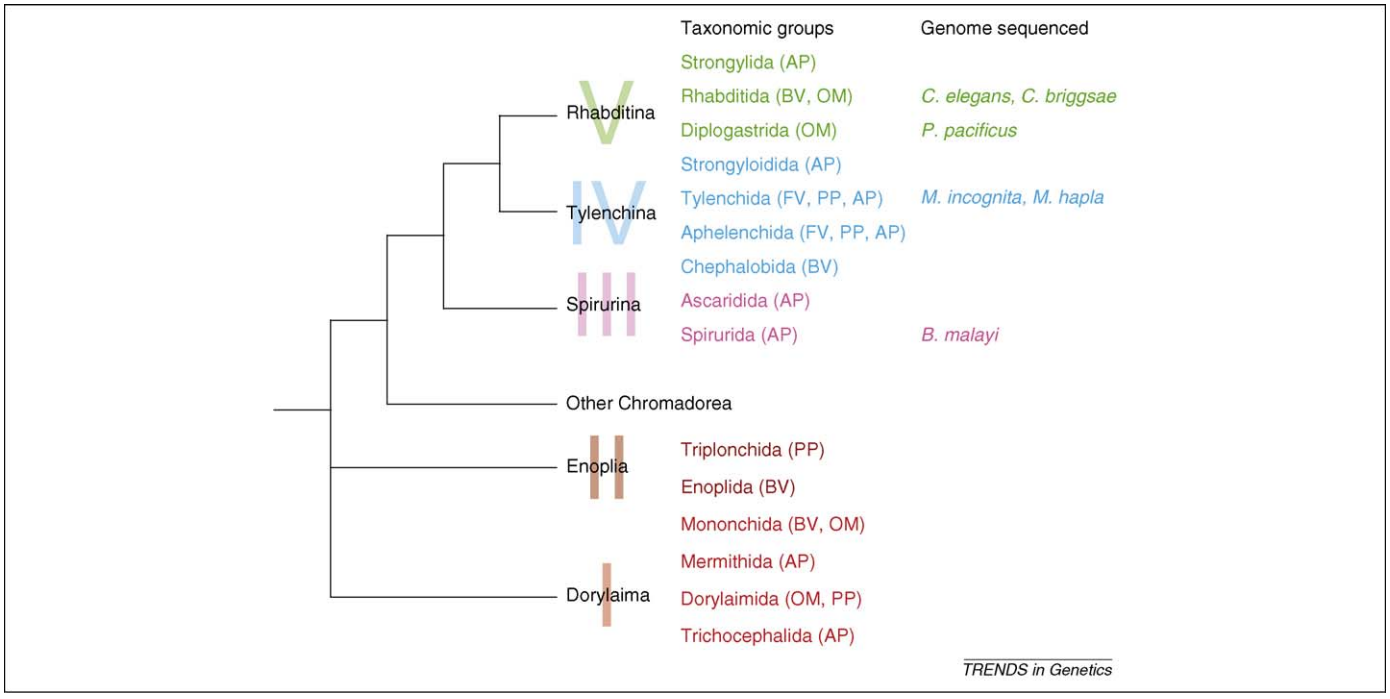

Figure 1. Major taxonomic groups of the phylum Nematoda. Most nematode taxa have been grouped into one of five clades based on molecular studies [4]. The species discussed in this review are in clades V (*C. elegans, P. pacificus*), clade IV (*M. incognita, M. hapla*) and clade III (*B. malayi*). The trophic mode of each taxonomic group is indicated by the following abbreviations: AP, animal parasite; BV, bacteriovore; FV, fungivore; OM, omnivore; PP, plant parasite. All nematode species with a published genome sequence are listed to the right of their taxonomic group.

component of the proteobacterial membrane [7]. The *Wolbachia* genome of *B. malayi* is ~1 Megabase in size. It has now been phylogenetically compared with the Wolbachian genomes of other nematodes and arthropods, indicating the monophyly of nematode and arthropod *Wolbachia* [15,16]. One recent study states widespread occurrence of horizontal gene transfer (HGT) from the *Wolbachia* bacteria to their multicellular insect and nematode hosts [17]. This finding might prove to be important to our understanding of the evolution of parasitism, but this idea is still controversial [18].

The genome of *B. malayi* is also the basis for studying its proteome. Of particular interest is the secretome, which comprises proteins that are secreted by the nematode and that might therefore interfere with the immune system of the host [19,20]. Although proteomic analysis is still in its infancy, the availability of the genome sequence and new proteomic methods are of tremendous medical potential.

Plant parasitism causes severe agricultural damage

Plant parasitic nematodes are of tremendous agricultural importance, but they have almost nothing in common with animal parasites in terms of their parasitic lifestyle (Box 1). In general, plant parasitic nematodes are not as highly specialized to individual species as animal parasites, although host preferences do exist. All plant parasites are characterized by a stylet, which enables the nematode to puncture the plant cell wall. This induces – by an unknown mechanism – the formation of multinucleated plant cells, which provide nutrition for the worm. Among the most devastating plant parasites are the root-knot nematodes (RKNs) of the genus *Meloidogyne*, two species of which have been recently sequenced. *Meloidogyne incog-*

Table 1. Key features of nematode genome assemblies and gene structures[a]

Features	*C. elegans*	*C. briggsae*	*P. pacificus*	*B. malayi*	*M. incognita*	*M. hapla*
General						
Size of assembled sequence (Mb)	100	104	142	88	82	53.5
Number of scaffolds	6	142[b]	2894	8180	2817	1523
Chromosome number	6	6[c]	6	5	Variable[d]	16
GC content (%)	35.4	37.4	42	30.5	31.4	27.4
Protein-coding regions						
Number of protein-coding genes	20 060	19 934	29 201	11 515	19 212	14 420
Mean number of coding exons per transcript	6.38	6	9	7.27	6.62	6
Median exon length (bp)	147	150	85	140	136	145
Median intron length (bp)	68	54	110	219	82	55

[a]Note that the genome features of *P. pacificus, B. malayi, M. incognita* and *M. hapla* are based on incomplete genome drafts and therefore, represent statistical estimates. By contrast, the *C. elegans* and, to a certain extent, *C. briggsae* have fully sequenced genomes that have been robustly annotated.
[b]142 physical map-based contigs plus 463 sequence supercontigs.
[c]89.4 Mb could be placed on the six linkage groups.
[d]Wild isolates of *M. incognita* vary in their number of chromosomes.

Table 2. Genes encoding glycosylhydrolase family members in six sequenced nematodes

Species	Cellulase (GHF5)	Xylanase (GHF5)	Arabinase (GHF43)	Pectinase (GH28)	Pectate lyase (PL3)	Sum
C. elegans	0	0	0	0	0	0
C. briggsae	0	0	0	0	0	0
P. pacificus	6(7)	0[a]	0	0	0	6
B. malayi	0	0	0	0	0	0
M. incognita	21	6	2	2	30	61
M. hapla	6	1[b]	2[c]	4[c]	22	35

[a]No xylanase activity could be detected *in vivo*.
[b]Only one secreted xylanase has been reported in Opperman *et al.* [9].
[c]The copy numbers are estimates based on whole genome sequence similarity searches.

nita is a cosmopolitan parasite with asexual reproduction that attacks cotton, tomatoes, coffee and other agricultural crops [8] (Box 1). *Meloidogyne hapla* reproduces by facultative meiotic parthenogenesis [9]. The occurrence of sex has been used to establish the diploid species *M. hapla* as a genetic model for plant parasitism [21].

Horizontal gene transfer in Meloidogyne as a source for novelty

One basic requirement that RKNs have to meet is the ability to invade the root of the host plant. The genome sequences of the two *Meloidogyne* species now provide clues as to how this is achieved. The genomes of *M. incognita* and *M. hapla* encode numerous cell-wall-degrading enzymes that have no counterpart in most other animals. More specifically, the complete genome sequences of *M. incognita* and *M. hapla* now indicate the presence of 61 and 35 cell-wall-degrading carbohydrate-active enzymes (CAZymes), respectively [8,9,22]. These sets of enzymes include cellulases, xylanases and other members of the glycosylhydrolase family, and pectate lyases (Table 2). As first pointed out by Smant *et al.* [22], the cell-wall-degrading CAZymes of plant parasitic nematodes might have been acquired by HGT because similar genes are absent from most free-living nematodes and they are most similar to those of unicellular organisms and bacteria. Thus, one of the key adaptations towards plant parasitism was most probably achieved by HGT of cell-wall-degrading enzymes [8,9,22]. Although the mechanism of HGT remains largely elusive, it has been speculated that HGT from a rhizobial bacterium, followed by strong selection pressure for the retention of parasitism-enhancing genes, represent important initial steps facilitating the invasion of plants by nematodes [23].

Host specificity is an important aspect of nematode plant parasitism. *M. incognita* is a tropical species and has an unusually wide range of hosts, as indicated earlier [8]. It is now thought that the asexual mode of reproduction by mitotic parthenogenesis is crucial for the evolution of this extraordinary host range. In the absence of males and recombination, alleles can evolve independently, resulting in enormous sequence diversity. Indeed, the 82-Mb genome of *M. incognita* with 19 200 predicted genes (Table 1) contains many highly diverged segment pairs, which might represent ancient alleles [8]. By contrast, the genome of the diploid *M. hapla* is only 54 Mb in size, contains 14 400 gene predictions and lacks diverged alleles [9].

Based on the available genome sequence, transcriptome and proteome analyses can now be used to study the invasion of the plant by the nematode in detail. As is the case for many nematode parasites, the life cycle of *Meloidogyne* consists of two distinct phases, the exophytic and endophytic phase, respectively (Box 1). A comparison of the *M. incognita* transcriptome of the pre-parasitic exophytic stage with that of the parasitic endophytic third-stage juvenile indicated the upregulation of genes encoding detoxification and protein degradation enzymes [24]. Also recently, mass spectrometry has been used to identify the *M. incognita* secretome [25]. This study revealed 486 secreted proteins, several of which might function in host-cell reprogramming during the invasion of the plant by *Meloidogyne* ssp. Proteomic approaches represent a powerful tool to study the mechanisms of parasitism and will provide important new insight in the near future.

Evolution of parasitism

Parasitism has evolved independently several times in nematodes, but little is known about the initial steps towards it. How does the transition from the free-living to the parasitic lifestyle occur? How do the physiological and morphological adaptations evolve in the first place? What initiates the evolution from a direct free-living life cycle to a complex one with host switching? Unfortunately, we are not close to answering these questions. The key problem is that multiple independent changes are

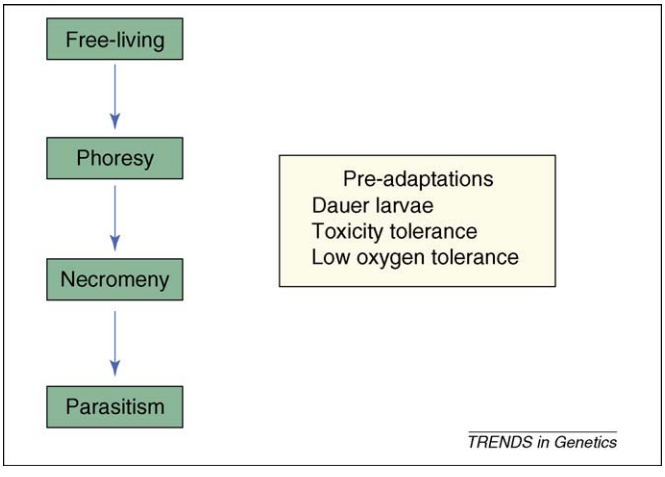

Figure 2. Evolutionary trends towards parasitism in nematodes. Although many of the non-parasitic nematode species are free-living in marine, fresh-water and terrestrial habitats, some species show typical associations with arthropods, other invertebrates and even vertebrates. Phoretic species associate with a host for transportation in an often, unspecific manner. Necromenic nematode species associate with a host, wait for its death and then feed on the developing microbes on the carcass of the host. This type of association is mostly species-specific. Several studies have argued that phoretic and necromenic associations provide important pre-adaptations for the evolution of parasitism [26–31].

necessary for a free-living organism to transform into a parasite, most of which do not have strong phenotypic consequences. Therefore, their successive fixation by natural selection is difficult to explain. Are there trends towards parasitism and intermediate stages? Are there adaptive forces that select for genetic and genomic alterations in a different environmental context before an organism evolves into a parasite?

Several authors have claimed, on theoretical grounds, that pre-adaptations are crucial for the evolution of parasitism [26–28] (Figure 2, Box 2). This argument goes back to Osche [29], who suggested that the phoretic and the necromenic associations of nematodes with insects and other invertebrates could be considered as pre-adaptations towards parasitism. It is usually in the dauer stage of the nematode, a developmentally arrested, non-feeding stage specialized for survival and dispersal, that these associations are found. In the case of phoresy, the dauer juveniles use insects or other invertebrates for transportation between habitats without being specific about their choice. *C. elegans* is usually considered to fall into this category [30]. In the case of necromeny, the dauer stage associates with their insect host in a mostly species-specific manner. They then wait for the death of the host species to feed on the developing microbes (e.g. bacteria and fungi and other nematodes growing on the carcass) [30].

Morphologically, the dauer stage of free-living nematodes resembles the infective larvae of many nematode parasites. It has, therefore, long been argued that the dauer stage in general and its phoretic and necromenic associations in particular represent pre-adaptations towards parasitism [29]. However, molecular similarities in the regulation of dauer and infective larvae formation have not been identified until recently. Ogawa *et al.* [31] have now shown that a conserved endocrine signaling mechanism involving the hormone dafachronic acid and the nuclear hormone receptor DAF-12 controls the formation of dauer and infective larvae in nematodes. This study presents the first link between dauer and infective juvenile formation and is consistent with the idea that the dauer stage is a pre-adaptation for parasitism.

Potential pre-adaptations for parasitism in the *P. pacificus* genome

Phoretic and necromenic associations provide an ecological context for intermediate phenotypes and can be considered as a step towards parasitism [26–30] (Figure 2, Box 2). One of the recently sequenced nematodes, the beetle-associated *P. pacificus*, supports this idea [10,32]. *P. pacificus* has been developed as a model system in evolutionary biology with genetic and transgenic tools [33]. Ecologically, *P. pacificus* and its relatives are often found in a necromenic association with scarab beetles [34–36], representing an intermediate type of association between the phoretic *C. elegans* and true parasites.

Several features of the *P. pacificus* genome might support the pre-adaptation hypothesis. For example, there is a drastic increase in the number of detoxification enzymes and, in comparison to *C. elegans*, *P. pacificus* has many more cytochrome P450 enzymes, glycosyltranferases, sulfotranferases and ATP-binding cassette (ABC) transporters [10].

Box 2. Pre-adaptations

The term pre-adaptation has been coined by Osche [29] and others to explain the evolution of complex traits, such as those associated with changes in the ecology of organisms. Changes in niche composition from marine to land or from free-living to parasitism requires several independent physiological and morphological alterations. The concept of pre-adaptation argues that such transitions are, in part, facilitated by the current environment of an organisms and adaptations associated with it. Pre-adaptations are adaptations to the current environment of the organism and its life style. In the future, such adaptations might be co-opted to a new function and facilitate the transition to a new environment. Therefore, pre-adaptations might be helpful for an organism in the acquisition of a new niche. For parasitism, the known types of nematode associations with insects and other invertebrates could be considered as pre-adaptations towards parasitism. Physiologically, nematodes might acquire adaptations to low oxygen concentrations and tolerance to host enzyme toxicity. Morphologically, the ability to form dauer larvae has been considered as an important pre-adaptation [31]. However, phoretic and necromenic associations are not yet parasitic, a strict metabolic dependence on a host must evolve later as the result of functional changes of the pre-adapted characters. Most recently, Poulin [28] has followed this argument in his monograph on the evolutionary ecology of parasites. It should be stressed that the concept of pre-adaptation remains largely hypothetical. Because one cannot foresee evolution, there is no proof that any extant non-parasite is a 'pre-parasite' of tomorrow. Similarly, the pre-parasites of present-day parasites are no longer around. Furthermore, there are no phylogenetic groups that harbor free-living species, parasites and species in transition, so a phylogenetic argument cannot be made. Thus, the evolution of parasitism remains as much hypothetical as the evolution of other complex traits, such as the eye, behavior or sociality.

Although the exact functions of individual paralogous genes are challenging to study (e.g. there are 198 cytochrome P450 enzymes predicted in the *P. pacificus* genome), many of them could be part of the detoxification machinery. A second example is the unexpected presence of glycosylhydrolase-encoding genes in the *P. pacificus* genome. At the sequence level, these genes are most similar to cellulases and cellulase activity has been observed in supernatant of *P. pacificus* mixed-stage cultures [10] (Figure 3). Although the exact functions of the products of these *P. pacificus* genes are still under investigation (L.N. Schuster and R.J.S., unpublished), this case represents the first example of glycosylhydrolases or cellulases found in a non-parasitic nematode [10]. This example indicates that a feature commonly regarded as typically parasitic has evolved in a necromenic ancestor under free-living conditions. Both the detoxification and cellulase genes in the *P. pacificus* genome are part of the versatile and complex life cycle of this organism. They are important adaptations for the survival in the beetle ecosystem but, in addition, might also represent examples for molecular pre-adaptations for the evolution of parasitism (Box 2).

It should be noted, however, that not all nematodes are excellent candidates for reaching parasitism and no one species can be a model for all types of nematode parasites. For example, *P. pacificus* is not a good model for RKN. In this case, the phylogenetically related genus *Bursaphelenchus*, which is a plant parasite that is transmitted by insects, can be considered a good model [37]. Also for *Bursaphelenchus*, the role of the dauer stage in this parasitic life cycle has also been discussed [38].

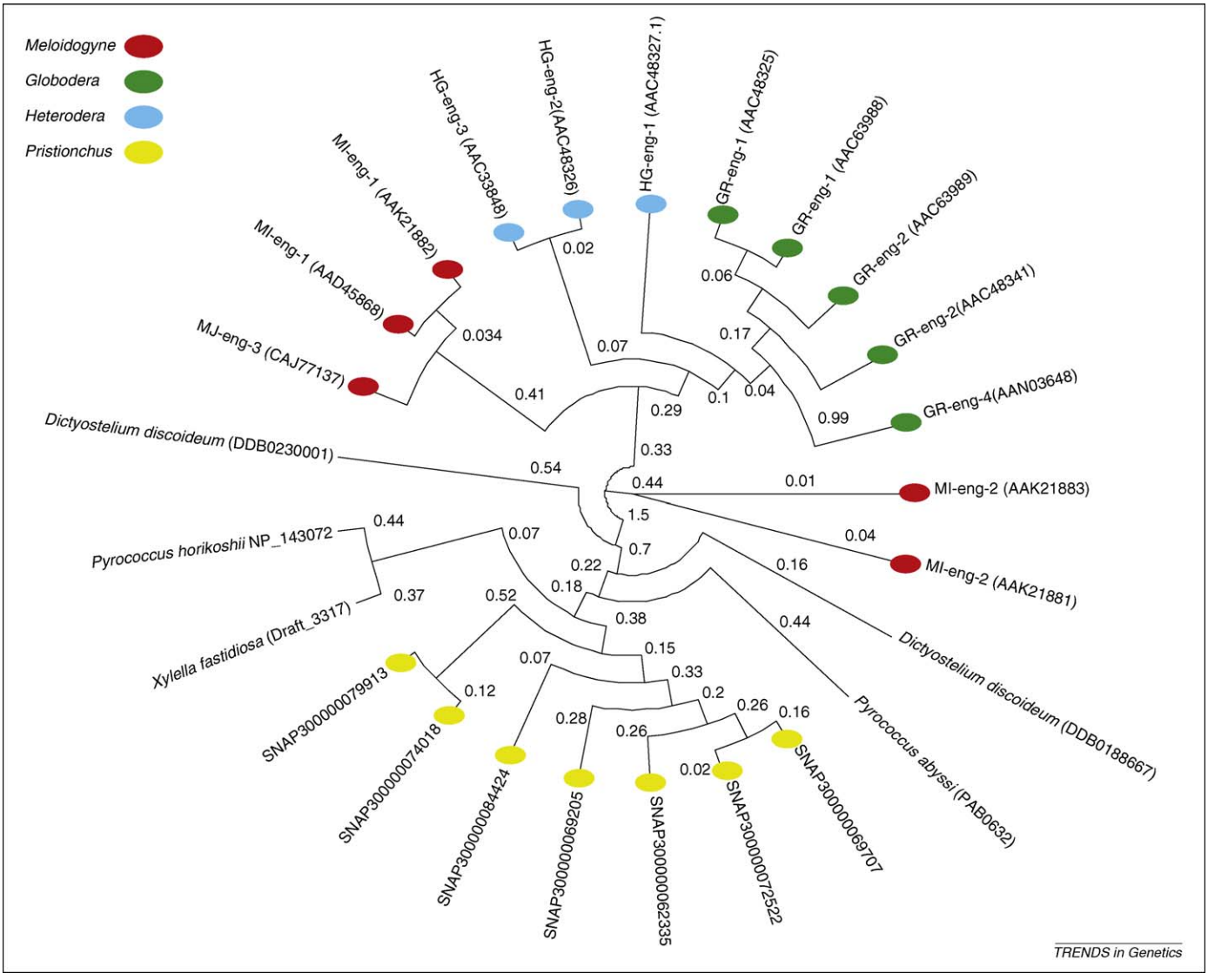

Figure 3. Phylogeny of cellulase genes from *P. pacificus* and plant parasitic nematodes. The phylogeny of cellulases indicates that plant parasitic nematodes (green) and *P. pacificus* (red) have acquired cellulases independently by horizontal gene transfer. The rectangular phylogram and branch length estimates (expected number of amino acid substitutions) are based on a multiple alignment of the conserved region (~70 amino acids) around the C-terminal active site. Protein alignments were generated by MUSCLE program [39]. The phylogeny was built by using a maximum likelihood approach (fproml with default settings; http://emboss.sourceforge.net) [40]. Only nematode cellulases with >10% sequence divergence are shown. Additionally, best non-nematode protein matches to *P. pacificus* cellulases are shown.

Concluding remarks

The availability of six genomes of ecologically diverse nematodes provides a unique starting point to analyse genomic features involved in becoming a plant or animal parasite. We have described HGT as seen in the plant parasitic nematodes of the genus *Meloidogyne* and the gene loss observed in the human parasite *B. malayi*. We also speculated on the importance of the concept of pre-adaptation for the evolution of complex traits, such as parasitism. Given the huge diversity of nematode parasites, the available genome sequences can currently only present scattered evidence of the genomic signatures involved in the mechanisms and evolution of parasitism. Only by sequencing many more phylogenetically related and unrelated nematode parasites can we begin to reveal robust evolutionary patterns that might indicate how and why parasitism evolved independently so often in the nematode phylum. The advances in sequencing technology make such projects feasible, and nematodes are perhaps one of the most rewarding animal phyla to be investigated in greater detail by comparative genomics.

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