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7-2-2016

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## *Trichinella spiralis*: Adaptation and parasitism



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### ARTICLE INFO

#### Article history:

Received 5 January 2016

Received in revised form 29 June 2016

Accepted 2 July 2016

#### Keywords:

Adaptation

Genomics

Parasitism

Proteomics

*Brugia*

*Caenorhabditis*

*Meloidogyne*

*Trichinella*

### ABSTRACT

Publication of the genome from the clade I organism, *Trichinella spiralis*, has provided us an avenue to address more holistic problems in parasitology; namely the processes of adaptation and the evolution of parasitism. Parasitism among nematodes has evolved in multiple, independent events. Deciphering processes that drive species diversity and adaptation are keys to understanding parasitism and advancing control strategies. Studies have been put forth on morphological and physiological aspects of parasitism and adaptation in nematodes; however, data is now coming available to investigate adaptation, host switching and parasitism at the genomic level. Herein we compare proteomic data from the clade I parasite, *Trichinella spiralis* with data from *Brugia malayi* (clade III), *Meloidogyne hapla* and *Meloidogyne incognita* (clade IV), and free-living nematodes belonging to the genera *Caenorhabditis* and *Pristionchus* (clade V). We explore changes in protein family birth/death and expansion/reduction over the course of metazoan evolution using *Homo sapiens*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* as out-groups for the phylum Nematoda. We further examine relationships between these changes and the ability and/or result of nematodes adapting to their environments. Data are consistent with gene loss occurring in conjunction with nematode specialization resulting from parasitic worms acclimating to well-defined, environmental niches. We observed evidence for independent, lateral gene transfer events involving conserved genes that may have played a role in the evolution of nematode parasitism. In general, parasitic nematodes gained proteins through duplication and lateral gene transfer, and lost proteins through random mutation and deletions. Data suggest independent acquisition rather than ancestral inheritance among the Nematoda followed by selective gene loss over evolutionary time. Data also show that parasitism and adaptation affected a broad range of proteins, especially those involved in sensory perception, metabolism, and transcription/translation. New protein gains with functions related to regulating transcription and translation, and protein family expansions with functions related to morphology and body development have occurred in association with parasitism. Further gains occurred as a result of lateral gene transfer and in particular, with the cyanase protein family. In contrast, reductions and/or losses have occurred in protein families with functions related to metabolic process and signal transduction. Taking advantage of the independent occurrences of parasitism in nematodes, which enabled us to distinguish changes associated with parasitism from species specific niche adaptation, our study provides valuable insights into nematode parasitism at a proteome level using *T. spiralis* as a benchmark for early adaptation to or acquisition of parasitism.

Published by Elsevier B.V.

### 1. Introduction

Parasitic nematodes comprise the majority of eukaryotic parasites and have a broad host range which includes plants, insects, and vertebrates. In humans, nematode parasitism triggers a vari-

ety of diseases (Liu et al., 1999; de Silva et al., 2003; Bethony et al., 2006), which pose both physical and financial burdens on health-care. Indeed, parasitic nematodes infect over 50% of the world's population (Steppek et al., 2006) and impose substantial strain on the global food supply where economic losses in Australia alone exceed 1 billion US dollars; a number that increases 10 fold when losses are examined worldwide (Roeder et al., 2013).

Today, issues have surfaced on control strategies that rely heavily on anthelmintics, such as rapid re-infection, drug resistance, and

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water and soil contamination. Further, as in the case of parasites in the genus *Trichinella*, sylvatic hosts can act as reservoirs to introduce or maintain the parasite in the local food supply. Because of the escalation of drug-resistance and the inability to generate immune strategies to curb animal parasitism, producers are once again seeking alternative approaches to controlling parasitic nematodes. Inasmuch as new biochemical approaches are both target- and system-specific, they are intimately linked to basic nematode biology, and in particular, the genetic/genomic mechanisms of parasitism.

It is clear that adaptation, exaptation and host switching have had profound effects on parasitism at the genetic, physiological and ecological levels. As example, plant parasites developed stylets to pierce host cell walls for secretion and feeding, and Tylenchids and Aphelenchids have evolved enlarged secretory cells to produce proteins critical for plant parasitism (Hussey, 1989; Davis et al., 2004;). The parasite cuticle allows for the worm to escape the physiologic and immune defenses of the host (Jones et al., 1993). Parasites of the genus *Trichinella* use an oral stylet for mechanical rather than enzymatic host cell penetration. Further, *Trichinella* spp. have adapted to a simple life cycle that is confined to a single host and does not involve a free-living stage; both of which are quite unique among the parasitic nematodes.

Although it is well known that parasitism among the Nematoda evolved multiple times spanning hundreds of millions of years (Dorris et al., 1999), and tropism is not a delineating factor that links parasite clades, questions remain as to whether or not there are conserved, intrinsic genetic factors or external genetic events that link nematode parasites beyond physiological shape and host associations. As example, lateral or horizontal gene transfer appears to have played a role in the evolution of plant parasitism (Kikuchi et al., 2004; Jones et al., 2005; Mitreva et al., 2009). Wang et al. (2010) observed a number of parasitic specific insertions and deletions (indels) in a variety of proteins suggesting that this genetic character could be one mechanism related to the evolution of parasitism. Further, among the sequences currently available, parasite genomes tend to encode fewer genes than those of free-living nematodes suggesting gene deletion and the evolution of a genetic profile more focused on a well-defined and confined life-style are important in parasitism (Mitreva et al., 2011).

Parasitism is a process involving a complex set of genes. Exploring parasitism, therefore, requires data at a genome level that spans the phylum. A recent paper by Zarowiecki and Berriman (2015) showed that few similarities exist among independently evolving parasitic worms; however, they go on to suggest that the process of adaptation is not without some level of conservation or independent convergence on a common outcome, in particular the use of later gene transfer. Fortunately, the number of available draft genomes that cover several major lineages and span both parasitic and free-living nematodes is increasing. In addition, the deduced proteomes of these species have been well characterized based on primary sequence similarity. These data make available a systematic analysis of genetic linkages among parasitic nematodes for determining if specific proteins favor parasitism and for characterizing the parasitic proteins involved. This may also provide critical information for designing alternative treatments to control nematode infections. In this study, we investigated parasitism through protein family dynamics utilizing genomes of species spanning the phylum Nematoda. The genome from *Trichinella spiralis* appeared prominently in our analysis because it represents a unique organism and a member of the most diversified clade in the Nematoda. Our analyses provide new insights into parasitism and it identified potential targets for better parasite control. Clearly, findings and hypotheses presented in this paper will be tested with

more exhaustive comparisons as the nematode genomic database expands.

## 2. Materials and methods

### 2.1. The proteomes

The sequenced and annotated genomes that comprised the analysis included 6 free-living nematodes and 4 parasitic nematodes. The 6 free-living nematode genomes were from *Caenorhabditis elegans* (Consortium, 1998), *Caenorhabditis briggsae* (Stein et al., 2003), *Caenorhabditis remanei*, *Caenorhabditis brenneri*, *Caenorhabditis japonica*, and *Pristionchus pacificus* (Dieterich et al., 2008). The 4 parasitic nematode genomes were *T. spiralis* (Mitreva et al., 2011), *Brugia malayi* (Ghedini et al., 2007), *Meloidogyne incognita* (Abad et al., 2008) and *Meloidogyne hapla* (Opperman et al., 2008). All nematode deduced proteomes were obtained from WormBase [version 204], or the above citations. In addition, the proteomes of *Saccharomyces cerevisiae*, *Homo sapiens*, and *Drosophila melanogaster* were downloaded from BioMart (Smedley et al., 2009) and used as outgroup genomes. Isoforms of the collected proteomes were screened out and only the longest sequences were utilized.

### 2.2. Family reconstruction

Protein families were defined using Tribe-MCL, a robust algorithm for protein family reconstruction at a genome level (Enright et al., 2002; Enright et al., 2003). Non-clustered proteins from each species as well as clusters composed of single protein members were treated as singleton families.

### 2.3. Phylogeny reconstruction

The phylogeny of the 10 nematodes together with the 3 outgroup species was inferred based on 94 universal orthologous protein families generated using ORTHOMCL (Li et al., 2003) each occurring exactly once in each of the 13 species. A phylogenetic tree was reconstructed for each family using PROTDIST and FITCH (PHYLP; Felsenstein, 1989) after having been aligned using MUSCLE (Edgar, 2004). A single consensus tree derived from these family trees was used as the working phylogeny. Branch lengths of the consensus tree were computed using PROML (PHYLP) based on concatenated sequences from the 94 protein families.

### 2.4. Inference of protein duplication and deletion

Family member duplication and deletion was inferred using URec (Gorecki and Tiuryn, 2007), which employs tree reconciliation. A species tree was generated using all protein families having one sequence per species and spanning all 13 species (hereafter, referred to as “single member universal families”); the gene trees in this study were those derived from each of the families. Specifically, family sequences were aligned using MUSCLE (Edgar, 2004), protein distances were computed using PROTDIST (Felsenstein, 1989) and family gene trees were reconstructed using FITCH (Felsenstein, 1989). For the species tree, the aligned sequences from all the single member universal families were merged. Subsequently, protein duplications and deletions within each family were inferred using URec by reconciling the species and gene family trees.

### 2.5. Protein family birth/death inference

Protein family birth and death events were inferred using DOLLOP (Felsenstein, 1989) by treating each family as a character where presence and absence were defined as individual states.

This reconstruction of protein family birth/death events permitted exploration of adaptation. Because of the phylogenetic distribution of our species where all free-living organisms were from the “crown” clade i.e., clade V, and all parasitic nematodes were from “root” clades i.e., clades I, III, and IV (Blaxter et al., 1998), death events uniformly occurring among all parasites would be inferred mistakenly as birth events at the ancestor of the clade V free-living nematodes using DOLLOP. Protein sequence similarities were therefore used to correct this misconception. Specifically, for the families inferred as born at the ancestor of clade V free-living nematodes, pairwise protein distance (i.e. similarities) of all the family member sequences were examined. If there existed any pair of paralogs whose distance was more than twice the length (2X) of the longest distance of orthologous pairs from one of the paralogs, the family was treated as ancestral and assumed to have gone through uniform parasite death. A cutoff of 2X was chosen because the phylum first diverged in the Precambrian approximately 543 million years ago (Mya) whereas those of the crown clades are estimated to have begun diversification 300–380 Mya (Blaxter, 2009). Therefore ancestral paralogs diverged 543 Mya while orthologs among the clade V free-living nematodes diverged no more than 300–380 Mya.

### 2.6. Inferring parasite uniform protein deletions

Tree reconciliation can track duplication and deletion events; however, with respect to multiple copy families, the program is unable to assign gene deletions to a specific copy. Thus, we examined the family protein distance matrix. If a protein within the free-living nematodes exhibited distances with all other homologs that were larger than the average distances of all orthologs between free-living nematodes and *T. spiralis* (the most highly diverged among the group examined), we assumed these orthologs were uniformly deleted among all other parasites examined. Because the branch distances of orthologs follow speciation, all homologs with large distances was interpreted as the deletion of its orthologs.

Insertions and deletions were inferred from sequence alignments using MUSCLE (Edgar, 2004). Indels at the ends of sequences were excluded as were those that were less than 4 residues in length. Indels specific to parasites were further explored through functional analyses.

In order to construct the phylogenetic tree based upon the cyanase protein family, a BLAST search of NCBI sequences was performed using default parameters and based upon a truncated *T. spiralis* protein sequence. The longest sequence from each taxonomic order was then used to build the tree. Protein sequences were aligned with MUSCLE (Edgar, 2004), and phylogenetic tree was reconstructed using PROTML (Felsenstein, 1989).

### 2.7. Functional analysis and gene ontology (GO) enrichment

Putative evolutionary events were detected and assimilated to parasitism and adaptation through functional analyses of the associated protein families and/or proteins. The functional analyses utilized either the GO annotation of *C. elegans* proteins (from WormBase), or the GO annotation derived from InterProScan (Zdobnov and Apweiler, 2001). GO enrichment/depletion probabilities (multiple test corrected) were computed using FUNC (Prufer et al., 2007).

## 3. Results

### 3.1. Reconstruction of protein families and inference of species phylogeny

From the proteomes of the 13 species collected (total of 265,454 proteins) 139,901 protein families were built including 118,174

singleton protein families (Table 1). The 21,727 non-singleton families contained members from *C. elegans*, *C. brenneri*, *C. japonica*, *C. remanei*, *C. briggsae*, *P. pacificus*, *M. hapla*, *M. incognita*, *B. malayi*, *T. spiralis*, *H. sapiens*, *D. melanogaster*, and *S. cerevisiae*, (Supplemental Table 1). Among these 1589 families were universal to all nematodes, and 739 families were universal to all species. In addition, there were 683 families universal and specific to free-living nematodes i.e., present in all free-living nematodes in this study. In contrast, there were only 8 families universal and specific to all non-parasitic species (including outgroups), and only three families were universal and specific to parasites (Supplemental Table 2). One of these three families is a LEM domain containing protein that shares similarity with the human MAN1 gene which regulates the expression of several fundamental genes; the second family is an RNA recognition motif domain containing protein (*T. spiralis* annotation), that shares similarities with a splice factor in *Schistosoma japonicum*; the third family is cyanase, a gene family evolutionarily linked to plants, bacteria and fungi.

To better explore parasitism and adaptation, a reliable phylogeny was needed. Current nematode phylogeny is mainly based on the 18 s rRNA study of Blaxter et al. (Blaxter et al., 1998; Holterman et al., 2006; Meldal et al., 2007). In addition, the relationship between the nematodes and the outgroups included is also not well resolved (Wolf et al., 2004; Philip et al., 2005;). For this purpose, we identified 94 single copy orthologous universal protein families using OrthoMCL (Li et al., 2003) to reconstruct a consensus tree. Branch lengths of the tree were calculated using PROML of PHYLIP (Felsenstein, 1989) based on the concatenated sequences from the 94 single copy universal orthologous families. This tree (Supplemental Fig. 1) differs from that based upon the 18 s rRNA (Blaxter et al., 1998; Meldal et al., 2007) which depicted *B. malayi* as an outgroup of *Meloidogyne* and other nematodes.

It is clear that parasitic nematodes do not have extended branch lengths, suggesting that positive selection on parasites did not lead to increased amino acid substitutions at least among conserved proteins. Indeed, the branch lengths of the free-living nematodes in particular those of the genus *Caenorhabditis*, are longer than those of parasites.

### 3.2. Protein family birth/death

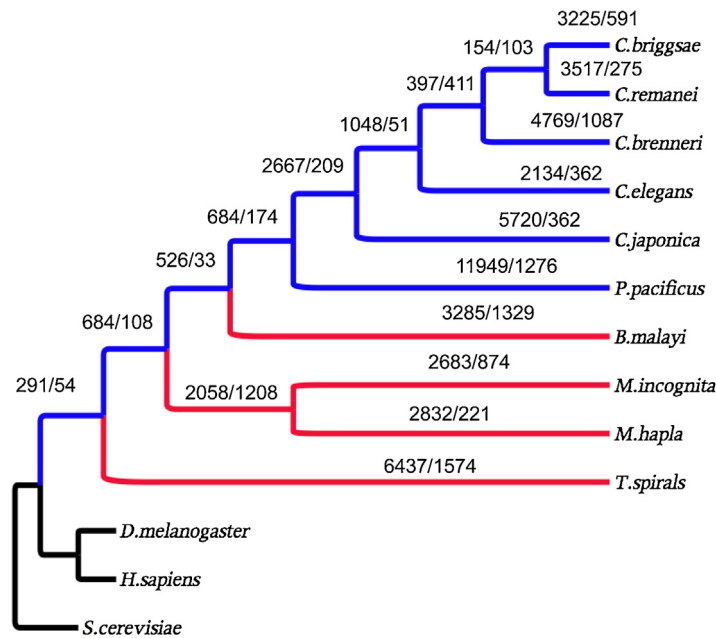
Protein family birth/death events are defined as the emergence and disappearance of protein families, respectively. In the process of nematode evolution, protein encoding genes were very dynamic where thousands of protein births and deaths occurred in conjunction with each speciation event (Fig. 1). Generally and coincident with speciation, organisms tend to experience net gains in the number of protein families they acquire. However, parasitic nematodes exhibited more protein family deaths than those observed in the free-living organisms we examined (except for *P. pacificus* and *C. brenneri*). More than 1000 families disappeared after the splits of *T. spiralis*, *B. malayi*, and the common ancestor of *M. hapla* and *M. incognita* (Fig. 1). Further, parasitic nematodes showed fewer protein family births, though it is not clear if this is related to the higher sampling density of the free-living nematodes. Thus, an increase in deaths is one contributing factor to parasites having fewer protein families (Table 1).

As the number of family deaths increases along each individual parasite lineage, there is also an increase in family deaths at the last common ancestor (LCA) of the free-living nematodes of clade V if considering the branch lengths. The branch length of the LCA of free-living nematodes is 0.13, and that of the LCA of free-living nematodes and *B. malayi* is 0.07; however, the family deaths at these two LCAs are 174 and 33, respectively (Fig. 1). These increased deaths are again related to parasitism and adaptation since 113 of these 174 families are parasite specific.

**Table 1**  
Reconstruction of protein families from the proteomes of 6 free-living nematodes, 4 parasitic nematodes and 3 outgroup species.

	Species	Total proteins	Proteins in OPFs <sup>a</sup>	Clusteredsingletons	OPFs with >1 member	Proteins in OPFs with >1 member
Outgroups	<i>Homo sapiens</i>	24,013	17,807	6206	5463	11,669
	<i>Drosophila melanogaster</i>	14,141	9466	4675	4274	8949
	<i>Saccharomyces cerevisiae</i>	6698	3343	3355	1725	5080
Nematodes	<i>Caenorhabditis elegans</i>	20,173	18,602	1571	9234	10,805
	<i>C. brenneri</i>	30,702	26,949	3753	9650	13,403
	<i>C. japonica</i>	25,870	20,695	5175	8672	13,847
	<i>C. remanei</i>	31,518	28,675	2843	10,171	13,014
	<i>C. briggsae</i>	21,974	19,194	2780	9626	12,406
	<i>Pristionchus pacificus</i>	29,201	18,649	10552	6401	16,953
	<i>Meloidogyne hapla</i>	14,421	11,824	2597	6141	8738
	<i>M. incognita</i>	19,212	17,109	2103	5833	7936
	<i>Brugia malayi</i>	11,407	8376	3031	4686	7717
	<i>Trichinella spiralis</i>	16,124	10,804	5320	4064	9384

<sup>a</sup> OPF, orthologous protein families.



**Fig. 1.** Protein family birth (emergence)/death (disappearance) over the course of evolution. Blue = free-living nematodes; Red = parasitic nematodes; Black = outgroups.

Because of their phylogenetic distribution, protein families born after the split of the nematodes and arthropods, and that subsequently died in all nematode parasites could be inferred as families born at the LCA of the clade V, free-living nematodes. Given that parasitism evolved numerous times within the phylum Nematoda, and that diversification only supports free-living nematodes as the ancestral state, we corrected these inferences by comparing the distances between orthologs and paralogs of these families. Among the 684 families inferred as arising at the ancestor of the free-living nematodes examined herein, 398 have paralogs in some of the free-living nematodes and 225 of them possess old paralogs (more than twice the age of their oldest orthologs, (see Materials and Methods)) indicating their births occurred before the split of the basal groups i.e., *T. spiralis*. We suggest that these families died uniformly in parasites.

### 3.3. Protein family birth/death and their parasitic association

The association between parasitism and the birth and death of protein families can be illustrated by the GO term enrichments of families related to parasites. The above 113 parasite specific families coincided with 28 GO terms in biological process and 44 GO terms in molecular function (Table 2). These biological process GO

terms have functional enrichment in regulation of translational fidelity (GO:0006450), cyanate metabolic process (GO:0009439), and transcription initiation (GO:0006352). And the 225 families that died uniformly in parasites have enrichments in GO terms related to free-living, and depletions in GO terms related to basic cellular functions (Table 3). The most highly enriched biological process GO terms are G-protein coupled receptor protein signaling pathway (GO:0007186), neuropeptide signaling pathway (GO:007218), and activation of MAPKK activity (GO:0000186).

The limited numbers of shared families suggests the independent occurrences or evolution of parasitism and adaptation. However, the functional enrichments of protein families that were born or that died at each parasitic lineage illustrated the commonality of functional adaptation. In spite of the fact that these families appeared or disappeared in distinct lineages, they shared functional enrichments. Protein families born at different parasitic lineages exhibited significant enrichment in molecular function terms relative to biological process terms. These families shared enrichments in zinc ion binding (GO:0008270), chromatin binding (GO:0003682), protein dimerization activity (GO:0046983), and cyclin-dependent protein kinase inhibitor activity (GO:0004861) (Fig. 2, and Supplemental Table 3). In contrast, the protein families that died at different parasitic lineages exhibited greater signifi-

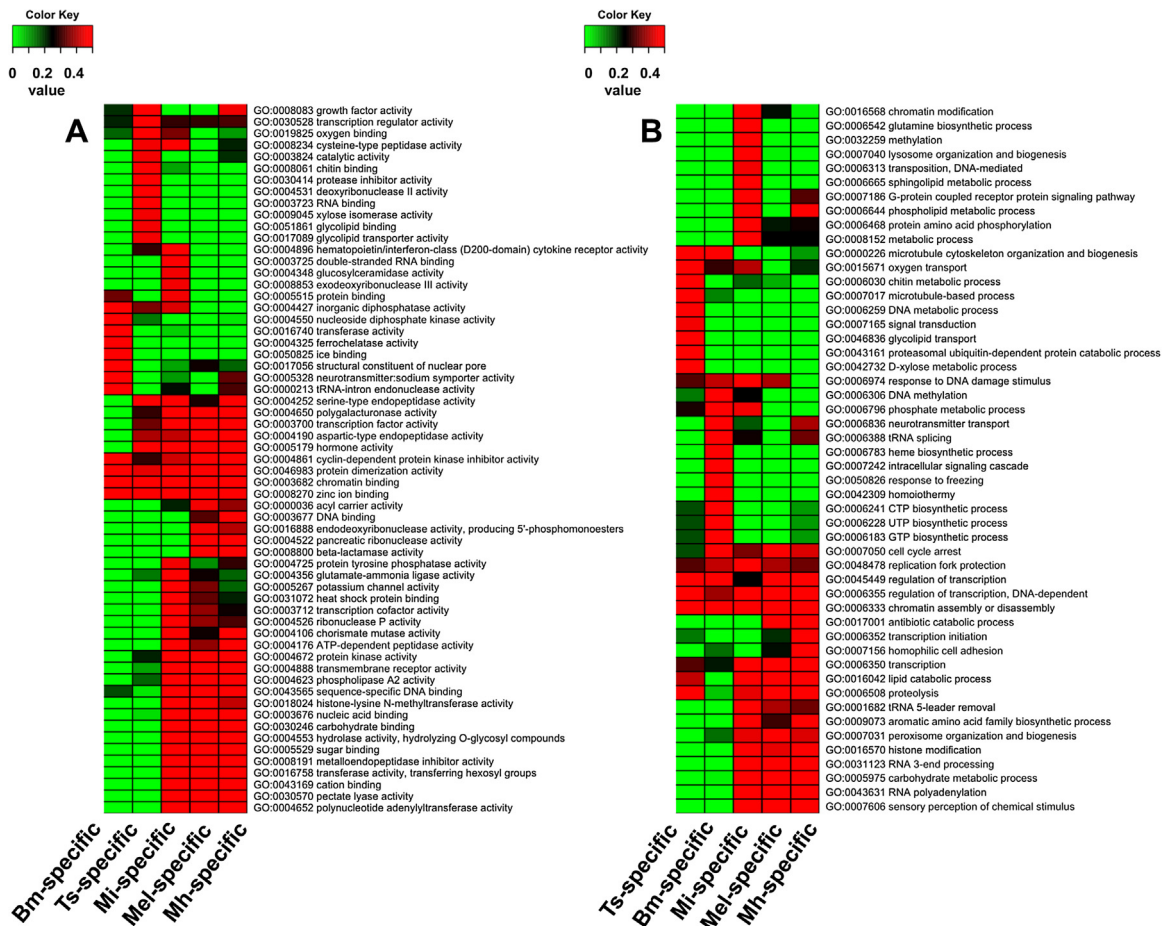
**Table 2**  
GO term enrichment of protein families died at the ancestor of free-living nematodes and not present in the outgroup species.

GO Category	GO term	GO descriptor	Under-representP value	Over-representP value	
Biological processes	GO:0006139	nucleobase, nucleoside, nucleotide and na metabolic process	0.137	1.000	
	GO:0006260	DNA replication	0.977	0.215	
	GO:0006281	DNA repair	0.964	0.265	
	GO:0006351	transcription, DNA-dependent	0.409	0.872	
	GO:0006352	transcription initiation	1.000	0.006	
	GO:0006397	mRNA processing	0.998	0.065	
	GO:0006450	regulation of translational fidelity	1.000	0.002	
	GO:0006461	protein complex assembly	0.996	0.092	
	GO:0006508	proteolysis	0.933	0.249	
	GO:0006511	ubiquitin-dependent protein catabolic process	0.983	0.186	
	GO:0006810	transport	0.249	1.000	
	GO:0006811	ion transport	0.439	1.000	
	GO:0006886	intracellular protein transport	0.967	0.251	
	GO:0007049	cell cycle	0.988	0.154	
	GO:0007154	cell communication	0.292	1.000	
	GO:0007186	G-protein coupled receptor protein signaling pathway	0.900	0.424	
	GO:0008152	metabolic process	0.193	1.000	
	GO:0008154	actin polymerization and/or depolymerization	1.000	0.016	
	GO:0009058	biosynthetic process	0.395	1.000	
	GO:0009439	cyanate metabolic process	1.000	0.004	
	GO:0009987	cellular process	0.131	0.975	
	GO:0016192	vesicle-mediated transport	0.987	0.160	
	GO:0016998	cell wall catabolic process	0.999	0.041	
	GO:0032259	methylation	0.990	0.143	
	GO:0043283	biopolymer metabolic process	0.204	0.953	
	GO:0044238	primary metabolic process	0.326	0.874	
	GO:0044249	cellular biosynthetic process	0.179	1.000	
	GO:0050794	regulation of cellular process	0.096	0.983	
	Molecular function	GO:0000166	nucleotide binding	0.924	0.217
		GO:0003676	nucleic acid binding	0.847	0.262
GO:0003700		transcription factor activity	0.462	0.780	
GO:0003735		structural constituent of ribosome	0.424	1.000	
GO:0003824		catalytic activity	0.137	1.000	
GO:0003896		DNA primase activity	1.000	0.016	
GO:0004175		endopeptidase activity	0.452	1.000	
GO:0004252		serine-type endopeptidase activity	0.941	0.328	
GO:0004298		threonine endopeptidase activity	0.999	0.036	
GO:0004871		signal transducer activity	0.151	1.000	
GO:0005083		small GTPase regulator activity	0.941	0.328	
GO:0005179		hormone activity	0.885	0.444	
GO:0005198		structural molecule activity	0.962	0.169	
GO:0005215		transporter activity	0.384	1.000	
GO:0005509		calcium ion binding	0.407	1.000	
GO:0005515		protein binding	0.996	0.010	
GO:0005524		ATP binding	0.178	0.959	
GO:0008073		ornithine decarboxylase inhibitor activity	1.000	0.020	
GO:0008158		hedgehog receptor activity	0.999	0.048	
GO:0008234		cysteine-type peptidase activity	0.899	0.420	
GO:0008270		zinc ion binding	0.957	0.089	
GO:0008324		cation transmembrane transporter activity	0.321	1.000	
GO:0008824		cyanate hydratase activity	1.000	0.007	
GO:0009055		electron carrier activity	0.450	1.000	
GO:0016301		kinase activity	0.477	0.831	
GO:0016491		oxidoreductase activity	0.061	1.000	
GO:0016740		transferase activity	0.198	0.952	
GO:0016787		hydrolase activity	0.428	0.857	
GO:0016788		hydrolase activity, acting on ester bonds	0.262	1.000	
GO:0016836		hydro-lyase activity	0.996	0.094	
GO:0016874		ligase activity	0.483	1.000	
GO:0016986		transcription initiation factor activity	1.000	0.003	
GO:0017111		nucleoside-triphosphatase activity	0.437	1.000	
GO:0022857		transmembrane transporter activity	0.498	1.000	
GO:0022892		substrate-specific transporter activity	0.414	1.000	
GO:0030234		enzyme regulator activity	0.386	1.000	
GO:0030528		transcription regulator activity	0.312	1.000	
GO:0042578		phosphoric ester hydrolase activity	0.489	1.000	
GO:0043169		cation binding	0.459	1.000	
GO:0043565		sequence-specific DNA binding	0.150	1.000	
GO:0046872	metal ion binding	0.320	1.000		
GO:0046983	protein dimerization activity	0.901	0.416		
GO:0048037	cofactor binding	0.427	1.000		
GO:0051537	2 iron, 2 sulfur cluster binding	1.000	0.032		

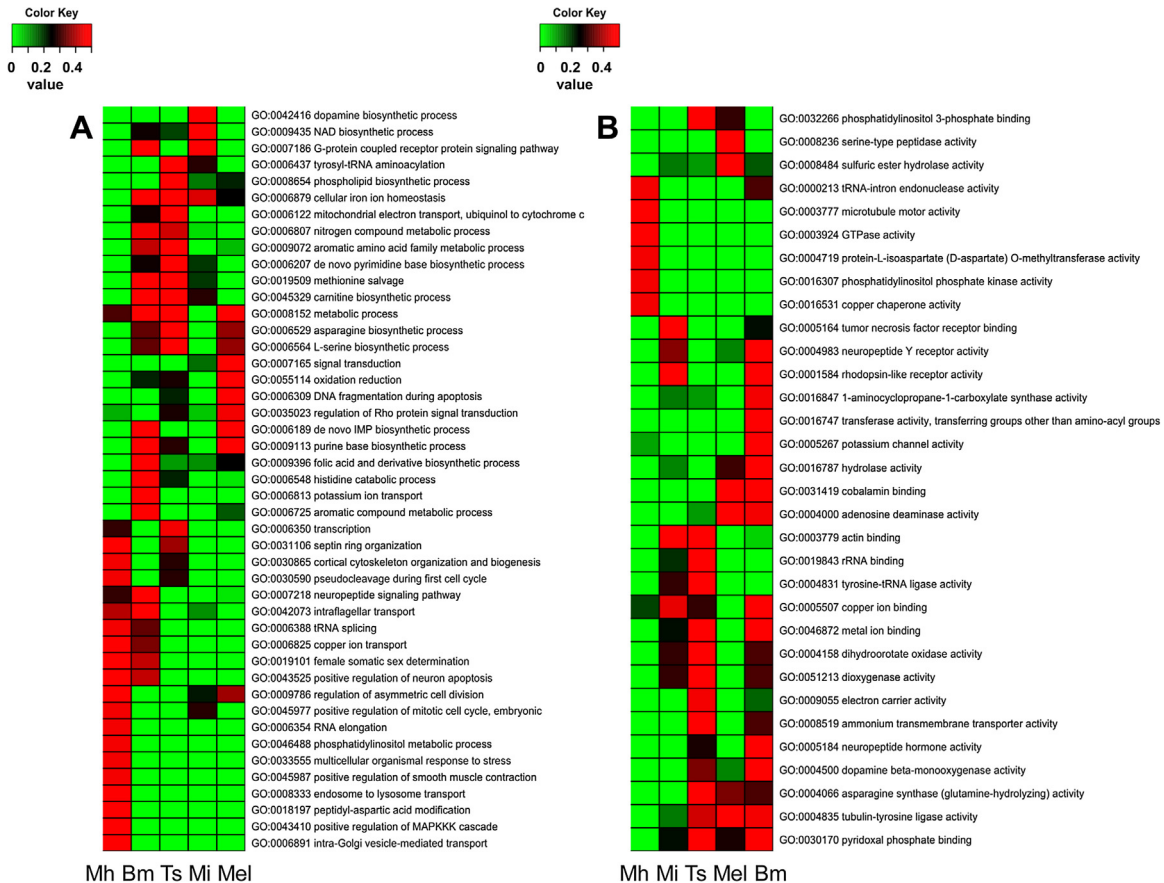
**Table 3**

GO enrichment of protein families disappeared uniformly in all nematodes (free-living nematode specific families having born time before the split of *Trichinella spiralis*).

GO Category	GO terms	GO descriptor	Under-representP value	Over-representP value
Biological processes	GO:0000186	activation of MAPKK activity	1.00E + 00	2.45E – 02
	GO:0002119	nematode larval development	2.90E – 02	9.83E – 01
	GO:0006303	double-strand break repair via non-homologous end joining	9.99E – 01	4.85E – 02
	GO:0006412	translation	1.74E – 02	1.00E + 00
	GO:0007186	G-protein coupled receptor protein signaling pathway	9.99E – 01	4.96E – 03
	GO:0007218	neuropeptide signaling pathway	9.99E – 01	1.92E – 02
	GO:0009792	embryonic development ending in birth or egg hatching	2.28E – 03	9.99E – 01
	GO:0040017	positive regulation of locomotion	3.40E – 02	1.00E + 00
	GO:0048268	clathrin coat assembly	9.99E – 01	4.85E – 02
	Molecular function	GO:0001584	rhodopsin-like receptor activity	9.96E – 01
GO:0003707		steroid hormone receptor activity	1.00E + 00	5.07E – 04
GO:0003735		structural constituent of ribosome	4.83E – 02	1.00E + 00
GO:0003993		acid phosphatase activity	9.99E – 01	1.64E – 02
GO:0004190		aspartic-type endopeptidase activity	1.00E + 00	3.39E – 03
GO:0004497		monooxygenase activity	9.98E – 01	1.79E – 02
GO:0004792		thiosulfate sulfurtransferase activity	9.99E – 01	4.47E – 02
GO:0004930		G-protein coupled receptor activity	9.98E – 01	2.88E – 02
GO:0005158		insulin receptor binding	9.99E – 01	4.47E – 02
GO:0005506		iron ion binding	9.96E – 01	1.79E – 02
GO:0005545		phosphatidylinositol binding	9.99E – 01	4.47E – 02
GO:0008191		metalloendopeptidase inhibitor activity	9.99E – 01	4.47E – 02
GO:0008825		cyclopropane-fatty-acyl-phospholipid synthase activity	1.00E + 00	2.26E – 02
GO:0009055		electron carrier activity	9.99E – 01	3.99E – 03
GO:0020037		heme binding	9.98E – 01	1.16E – 02
GO:0045296		cadherin binding	9.99E – 01	4.47E – 02
GO:0051015		actin filament binding	1.00E + 00	9.87E – 03



**Fig. 2.** Heatmap showing the GO enrichment of protein families born at each parasite lineage A = molecular function, B = biological process. Bm = *B. malayi*; Ts = *T. spiralis*; Mi = *M. incognita*; Mel = all *Meloidogyne*; Mh = *M. hapla*.



**Fig. 3.** Heatmap showing GO enrichment of families that died at the different parasitic lineages. A = Biological process; B = molecular function.

cant enrichment in biological process GO terms than in molecular function GO terms. These families did not show common functional enrichment over all four parasitic lineages, but have three enriched terms shared by *B. malayi*, *T. spiralis*, and *Meloiodogyne* (Fig. 3, and Supplemental Table 4). As expected, the enrichment patterns of *B. malayi* and *T. spiralis* are close, and those of *M. incognita* and *M. hapla* are similar. In addition, those of the families that died at the LCA of *Meloiodogyne* share high commonality with those of *B. malayi* and *T. spiralis*. This suggests that among this group, parasitism occurred at the LCA of *M. hapla* and *M. incognita*, and that *M. hapla* and *M. incognita* acquired host specificity after becoming species.

### 3.4. Protein family expansion and reduction

The dynamic nature of whole protein families is mirrored in the plasticity of individual protein family members. Examining member duplication and deletion within families universal to nematodes revealed that each family underwent many duplication and deletion events (Fig. 4) over evolution. This in turn resulted in changes to the sizes of the families. Unlike changes in protein family births and deaths, greater numbers of deletions occurred relative to duplications in association with each speciation event suggesting a reduction in family sizes. Parasitic and free-living nematodes are somewhat similar in the numbers of deletion events that occurred; however, parasitic nematodes tend to have fewer duplications than free-living nematodes (with the exception of *M. incognita*) which leads to smaller protein families in parasites ( $P < 0.008$ , TTEST) (Table 1).

More than 80% of the families were reduced in size over the course of nematode evolution and parasitism. In fact, more than 50% of the families were reduced in size in all the parasites exam-

ined. In contrast, less than 10% of the protein families expanded in size in *B. malayi*, *T. spiralis* and *M. hapla*. Expansion in *M. incognita* is different where 30% of the families expanded predominantly due to an increase in duplications (1336 duplications in 725 families), no reduced deletions. The top three expanded families are those homologous to *C. elegans* R09B3.4 (ubc-12, ubiquitin-conjugating enzyme; 24 duplications; 4 deletions), K03E6.3 (ncs-3, neuronal calcium sensor, calcium binding; 14 duplications; 1 deletion) and C39E9.6 (scl-8, SCP-like extracellular protein, defense related protein; 20 duplications; 5 deletions).

Consistent with the independence of parasitism and adaptation, only one family has shown gene expansion over all four parasites examined. Meanwhile these independent events also lead to an excessive number of reductions in common families, though parasitic nematodes do not show increased numbers of total protein deletions. Among the 1572 families exhibiting inferences of duplications and deletions, 790 families showed reductions over all four parasites examined. Also, this appeared to be non-random protein deletion wherein the orthologs of 524 *C. elegans* genes were uniformly deleted in all four parasites. These orthologs were distributed only over 93 families.

### 3.5. Protein family expansion and reduction and their parasitic association

We examined the GO term enrichment of families that expanded or reduced over individual parasite lineages and over all parasite lineages investigated. The GO enrichments of the above 524 *C. elegans* orthologs were also examined. The families that expanded at the different parasitic lineages exhibited different functional enrichments (Fig. 5) while the families that were reduced at dif-



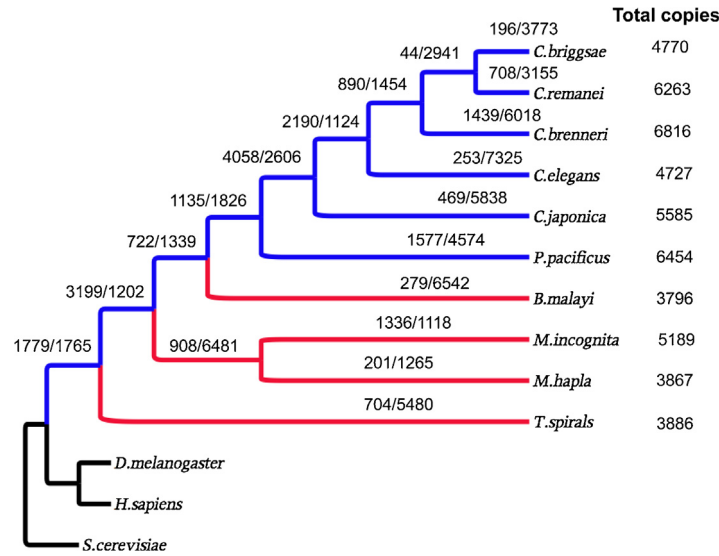


Fig. 4. Duplications/deletions of all universal nematode protein families (1572 families). Blue = Free living nematodes; Red = parasitic nematodes; Black = outgroups.

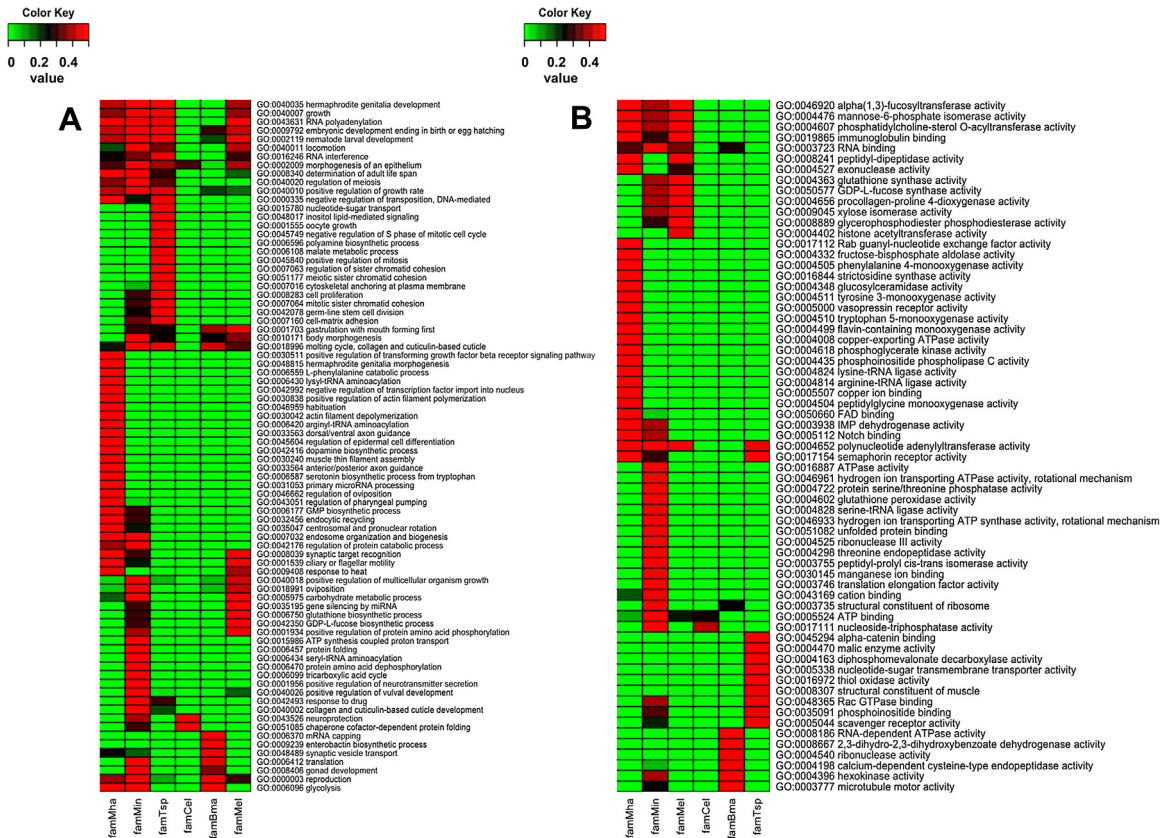
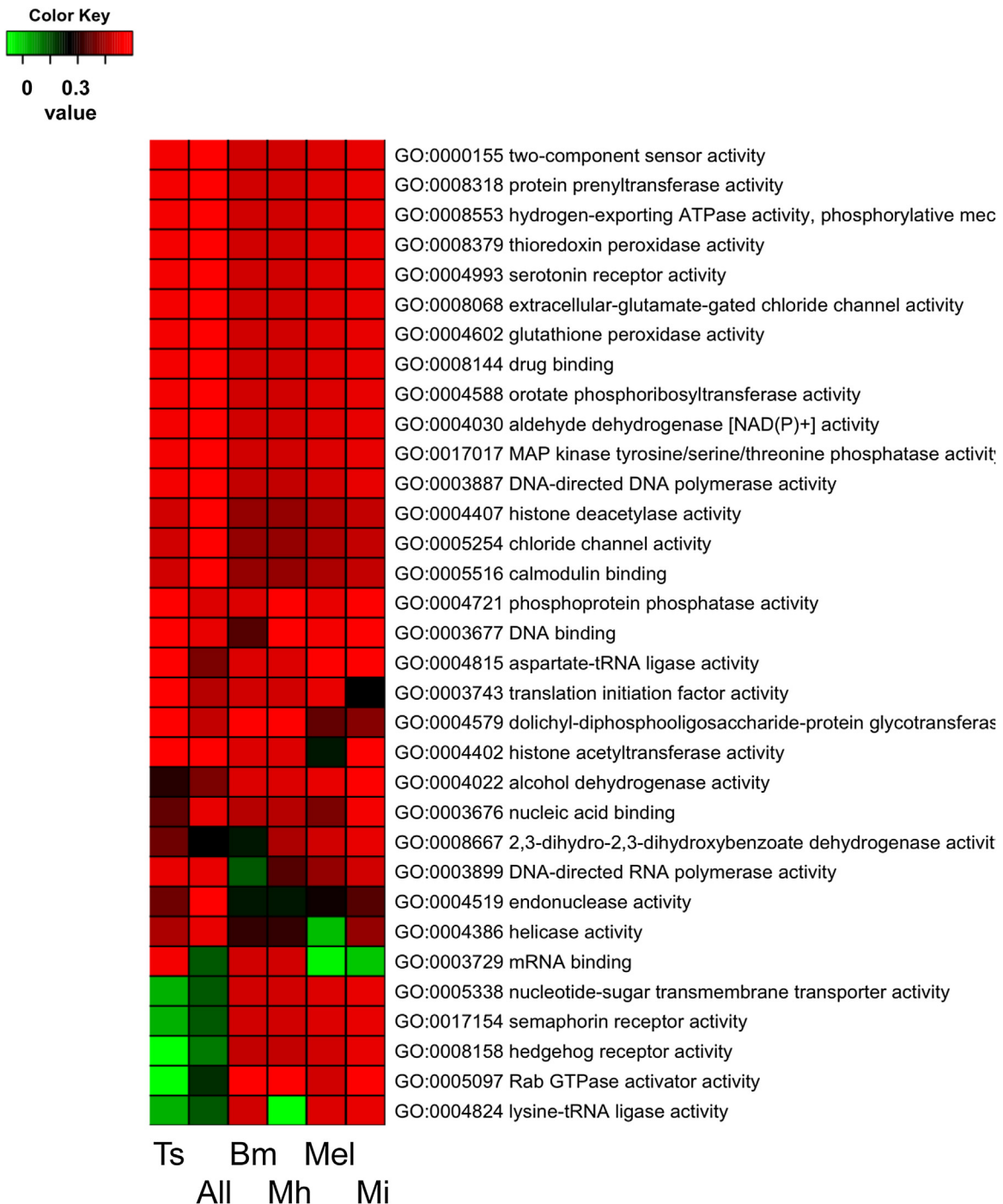


Fig. 5. GO term enrichment pattern of protein families expanded at different parasite lineages. *C. elegans* lineage was added as reference; A = biological process; B = molecular function; Bm = *B. malayi*; Ts = *T. spiralis*; Mi = *M. incognita*; Mel = all *Meloidogyne*; Mh = *M. hapla*; Ce = *C. elegans*.

ferent parasitic lineages showed similar functional enrichments (Fig. 6). In fact, the functional enrichments of reduced families in parasites have a more than 60 percent overlap with those also reduced in *C. elegans*. Fig. 6 displays only the parasite specific enrichments. Many of these parasite specific enrichments are related to signal processing, such as two-component sensor activity (GO:0000155), protein prenyltransferase activity (for receptor localization, GO:0008318), serotonin receptor activity (GO:0004993), drug binding (GO:0008144) and extracellular-

glutamate-gated chloride channel activity (GO:0008068). Despite the different enrichment patterns, the majority of the families found to contain enriched terms in parasites are related to growth and development possibly associated with the unique food sources. Glycolysis was not enriched in the protein families that expanded in *T. spiralis* though it is enriched in those of the other three parasites (Fig. 5A). The 524C. *elegans* genes where their orthologs were uniformly deleted in all parasites were predominantly kinases and involved channel activities such as ATP



**Fig. 6.** GO enrichment pattern (molecular function) of families that were reduced over different parasite lineages. Bm = *B. malayi*; Ts = *T. spiralis*; Mi = *M. incognita*; Mel = all *Meloidogyne*; Mh = *M. hapla*; All = all parasites.

binding (GO:0005524), nicotinic acetylcholine-activated cation-selective channel activity (GO:0004889), protein serine/threonine kinase activity (GO:0004674), protein tyrosine kinase activity (GO:0004713), etc. (Table 4).

### 3.6. Origination of parasitic proteins

Birth of parasite specific protein families can occur through sub-functionalization, lateral gene transfer (LGT) or protein family expansion. As stated above, we found only 3 families that were common to all parasitic nematodes. The majority of the families (94/113) were common among *B. malayi* and *M. incog-*

*nita* or *M. hapla* (77 were common to all three). Consistent with our inferred tree (Supplemental Fig. 1), very limited numbers of families are common among *B. malayi* and *T. spiralis* and even less so with *Meloidogyne*. The limited number of protein families that may be linked to parasitism, and their consistency within the phylogeny suggest that parasitism and adaptation occurred independently over different species, and that parasitism proteins were acquired mainly from speciation through duplication and through sub-functionalization or exaptation.

It should also be noted that LGT played a role in the birth of some of these families. The phylogenetic tree of cyanase clearly illustrates this. Despite the independent acquisition of parasitism,

**Table 4**Molecular function GO enrichment of the 524 *Caenorhabditis elegans* genes whose orthologs were uniformly deleted in all parasitic species.

Molecular functions (12426)	GO Descriptor	Under-represent P value	Over-represent P value
GO:0005524	ATP binding	1.00E+00	9.61E−26
GO:0004889	nicotinic acetylcholine-activated cation-selective channel activity	1.00E+00	1.55E−25
GO:0004674	protein serine/threonine kinase activity	1.00E+00	9.24E−19
GO:0004713	protein tyrosine kinase activity	1.00E+00	2.68E−18
GO:0016787	hydrolase activity	1.00E+00	9.48E−17
GO:0004386	helicase activity	1.00E+00	9.84E−16
GO:0005230	extracellular ligand-gated ion channel activity	1.00E+00	3.68E−12
GO:0005328	neurotransmitter:sodium symporter activity	1.00E+00	1.21E−10
GO:0015464	acetylcholine receptor activity	1.00E+00	5.93E−09
GO:0019001	guanyl nucleotide binding	1.00E+00	8.36E−08
GO:0004222	metalloendopeptidase activity	1.00E+00	2.10E−04
GO:0004725	protein tyrosine phosphatase activity	1.00E+00	4.18E−04
GO:0004879	ligand-dependent nuclear receptor activity	1.00E+00	5.80E−04
GO:0019787	small conjugating protein ligase activity	1.00E+00	7.09E−04
GO:0022848	acetylcholine-gated cation channel activity	1.00E+00	9.93E−04
GO:0005388	calcium-transporting ATPase activity	1.00E+00	1.31E−03
GO:0003677	DNA binding	9.99E−01	1.60E−03
GO:0004016	adenylate cyclase activity	1.00E+00	5.71E−03
GO:0004402	histone acetyltransferase activity	9.99E−01	8.75E−03
GO:0004871	signal transducer activity	9.97E−01	9.42E−03
GO:0016706	oxidoreductase activity, acting on paired donors	9.99E−01	9.63E−03
GO:0017111	nucleoside-triphosphatase activity	9.96E−01	9.69E−03
GO:0005509	calcium ion binding	9.92E−01	1.52E−02
GO:0005330	dopamine:sodium symporter activity	1.00E+00	3.15E−02
GO:0030297	transmembrane receptor protein tyrosine kinase activator activity	1.00E+00	3.15E−02
GO:0035034	histone acetyltransferase regulator activity	1.00E+00	3.15E−02
GO:0016849	phosphorus-oxygen lyase activity	9.91E−01	3.95E−02
GO:0003712	transcription cofactor activity	9.92E−01	4.74E−02
GO:0003723	RNA binding	1.76E−02	9.96E−01
GO:0004888	transmembrane receptor activity	1.63E−02	9.96E−01
GO:0003707	steroid hormone receptor activity	3.01E−03	9.99E−01
GO:0003824	catalytic activity	8.97E−04	1.00E+00
GO:0005215	transporter activity	3.18E−04	1.00E+00
GO:0016491	oxidoreductase activity	1.26E−04	1.00E+00
GO:0005488	binding	2.06E−05	1.00E+00
GO:0003735	structural constituent of ribosome	3.38E−05	1.00E+00
GO:0004497	monooxygenase activity	1.73E−02	1.00E+00
GO:0008270	zinc ion binding	2.40E−09	1.00E+00
GO:0009055	electron carrier activity	2.27E−04	1.00E+00
GO:0016301	kinase activity	2.31E−02	1.00E+00
GO:0016747	transferase activity, transferring non amino-acyl groups	2.03E−02	1.00E+00
GO:0016758	transferase activity, transferring hexosyl groups	1.52E−02	1.00E+00
GO:0016773	phosphotransferase activity, alcohol group as acceptor	2.31E−02	1.00E+00
GO:0020037	heme binding	1.96E−03	1.00E+00
GO:0046872	metal ion binding	2.90E−02	1.00E+00
GO:0050660	FAD binding	3.09E−02	1.00E+00

all the parasites in our studies appear to have acquired cyanase through LGT. Reconstructing the evolution of cyanase showed no less than 4 independent acquisitions of cyanase in nematodes, one for the ancestor of *Trichinella*, one for *B. malayi*, one for the ancestor of *Meloidogyne*, and possibly one additional acquisition for *T. spiralis* (Fig. 7). Cyanase was also found in *Loa loa*, a filarial nematode that was highly similar to that of *B. malayi* suggesting a common origin for these acquisitions (data not shown)

#### 4. Discussion

Studies on nematode parasitism and adaptation usually focus on a specific species, and explore only a single protein or protein family. Consequently, there is a lack of understanding of these events at the holistic level. In this study, we explored at the genome level, similarities and distinctions among a subset of parasitic and free-living nematodes as they relate to parasitism and adaptation. We used a subset of genomes from organisms representing different clades and tropisms among the Nematoda. Our results revealed some genetic mechanisms underlying different modes of adapta-

tion and validated the multiple independent adaptation events that occurred during the evolution of parasitism.

##### 4.1. Independent adaptation among parasitic nematodes

Parasitism independently originated multiple times over the evolution of the nematodes (Dorris et al., 1999; Blaxter, 2003). These independent events are supported by the observation that only three protein families were exclusively shared by all parasitic nematodes, and that only one family expanded in this group of nematodes. One of the families exclusively shared by all parasites examined here is cyanase. The independent origins of these parasitic cyanases, reflected by their phylogenetic relationships with other cyanases, provides direct evidence for the acquisition of the gene via LGT, which was key to the evolution of the parasitic character of these organisms (Jones et al., 2005; Mitreva et al., 2009). The three plant parasitic cyanases appear to have similar origins, meaning that LGT occurred before the split of *M. hapla* and *M. incognita*. It is interesting to note that this gene was either not acquired by the free-living nematodes, or that after early acquisition, it was lost in the process of further diversification of the free-living state.

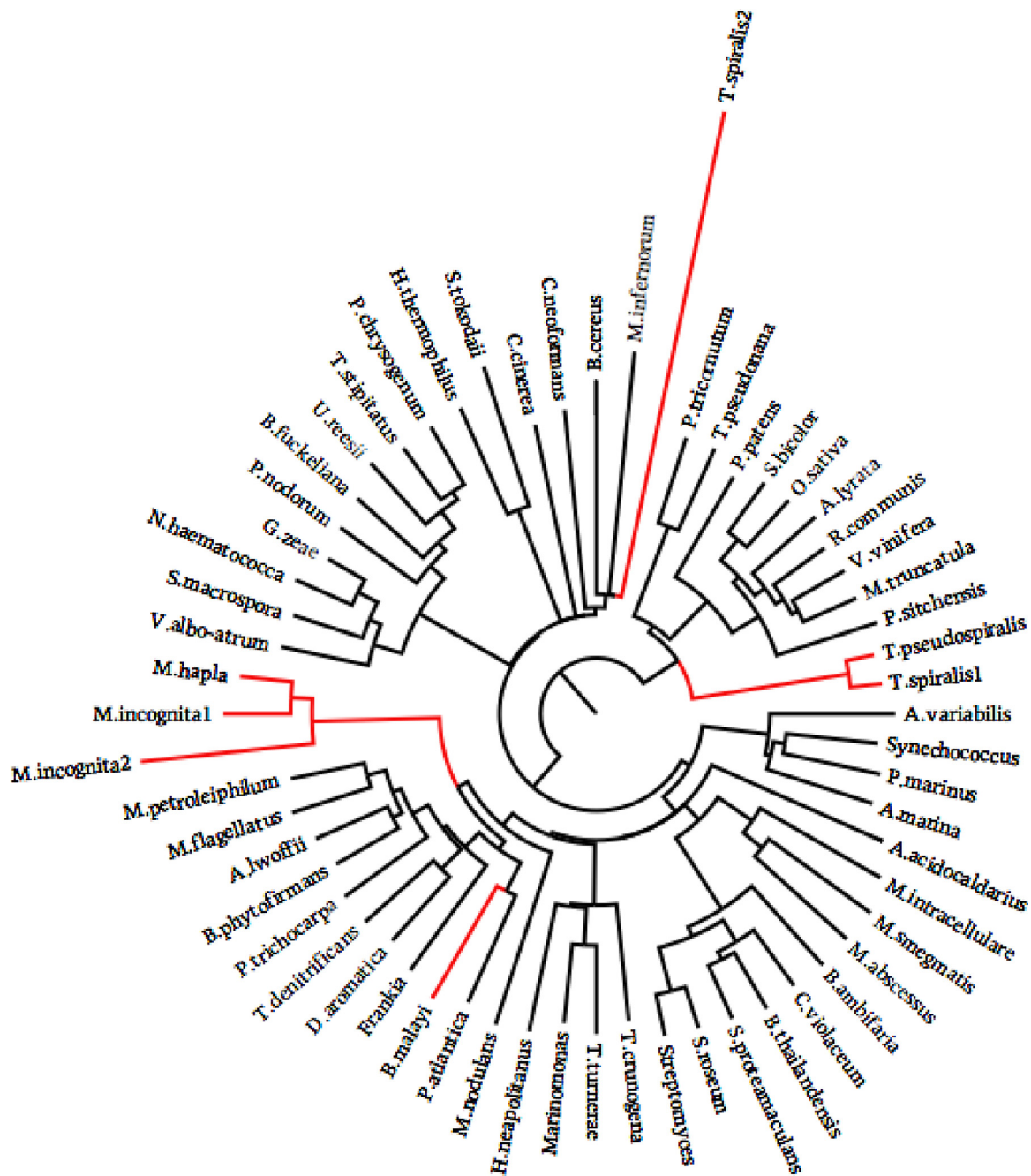


Fig. 7. Evolutionary tree of cyanase showing 4 independent events of lateral gene transfer.

Meanwhile, the free-living nature of nematode ancestor is illustrated by examining the functions of the protein families that emerged before the split of the clade I nematode, *T. spiralis* and that were lost in the evolution of the nematode parasites studied herein. The top enriched biological process GO terms are G-protein coupled receptor protein signaling pathway (GO:0007186), neuropeptide signaling pathway (GO:007218), and activation of MAPKK activity (GO:0000186). One can envision where all three processes are crucial for free-living nematodes by permitting more rapid responses to environmental change e.g., olfactory perception and the localization of food resources (Thomas and Robertson, 2008). Further, MAPKK activity is key to osmolarity sensing and thus important for free-living organisms. These functional enrichments in free-living worms further support the ancestral character of the Nematoda was the free-living state and that adaptation associated with par-

asitism resulted in the independent death or loss of these families in the parasites examined here.

#### 4.2. Parasitic adaptation through gene loss

The proteomes of nematodes have exhibited a high level of plasticity over the course of evolution. Parasite-based adaptations resulted in reductions in the sizes of the proteomes investigated to date (Mitreva et al., 2011), including fewer protein families and a reduction in the number of members of those protein families. More protein families disappeared in the evolution of parasitic nematodes than in the adaptation of free-living nematodes to their local environments. However, the protein families that disappeared at the lineages of *T. spiralis*, *B. malayi* and the common ancestor of *Meloidogyne* seemed to exhibit conservation in function. The

molecular functions of the families that disappeared (relative to free-living nematodes) were significantly enriched in asparagine synthase activity, tubulin-tyrosine ligase activity, and pyridoxal phosphate binding. Among the biological processes examined, the most significant enriched GO term was metabolic processes.

One can envision where adaptation to parasitism can lead to reductions in genes involved in metabolic processes because of a simplification in food and energy resources for parasites as their life cycles become more predictable, confined and less dependent upon the outside environment (Blaxter, 2003). Examining shared molecular functional enrichments provides more enlightenment on this process of adaptation. Asparagine synthase involves the biosynthesis of L-asparagine from L-aspartate in an ATP-dependent reaction. It has been shown that asparagine synthase expression correlates with nutrient stress (Fafournoux et al., 2000) where increasing nutrients decreases gene expression. Thus the disappearance of these proteins in the parasitic nematodes may relate to the abundance of available asparagine in the host and a reduced need for biosynthesis by the parasite. Pyridoxal phosphate binding involves vitamin B6 activity, where vitamin B6 is a cofactor in many biological processes, including antioxidant activity (Denslow et al., 2005). Again, the disappearance of these proteins may reflect reduced oxidative stress of the parasite within the host environment compared to that of free-living nematodes that are exposed to a larger number of more complex metabolic substrates and other metabolizing organisms (Dieterich and Sommer, 2009; Zarowiecki and Berriman, 2015). While parasite-based, adaptation-associated gene loss can consist of functionally related proteins; such loss can also trigger ortholog deletion over all parasitic nematodes. We detected a loss of 524C. *elegans* orthologs within the 93 nematode universal members.

In addition to the individual families that disappeared at each parasitic lineage, there were 250 protein families that emerged prior to the split of *T. spiralis* then disappeared uniformly across all parasites. As stated previously, these families were enriched in functions that probably exhibit redundancy in parasitic nematodes. Besides the disappearances of whole protein families, a large number of the remaining protein families also lost members. Nearly 50% of the families that emerged before the split of *T. spiralis* were reduced in size in all the parasites examined. When checked individually, more than 80% percent of those families were reduced in size in at least one parasite group. This group of protein families possesses functional enrichment patterns biased towards signal processing which uniformly disappeared in all nematode parasites in this study. Interestingly, enrichments in drug binding and extracellular-glutamate-gated chloride channel activity among the reduced families suggest the build-up of drug resistant character. It has been shown that mutations in extracellular-glutamate-gated chloride channel decrease the sensitivity to the anthelmintics (Njue et al., 2004). Further, the enrichment of ATP binding and kinases suggests a deeper involvement of cellular signaling in the process of parasite adaptation. Similar GO term enrichments were found in the nematode intestinal genes (Yin et al., 2008). It is possible that genes expressed in nematode intestines experienced significant change related to adaptation in relation to the changes in food and energy resources during the evolution of parasitism.

#### 4.3. Parasitism and adaptation through gene duplication and LGT

During the evolution of parasitism, gene gain appears to have occurred far less extensively than did gene loss. Proteins exhibited significantly lower numbers of duplications among the parasitic nematodes possibly reflecting less of a need for redundancy in a more predictable host environment when compared to free-living nematodes. Constant environment imposes less selective pressure on protein diversification. Nevertheless, gene gain has played a role

in parasitism and adaptation. This is reflected by functional enrichment in the protein families specific to parasites and in the protein families that expanded in the parasite species. The protein families that expanded in parasites were predominantly related to growth and development. As example, many parasites have very well controlled free-living and parasitic stages where the free-living stages are characterized by developmental arrest prior to transitioning to the parasitic stages. Consistent with this, the parasite-associated protein families showed functional enrichment in transcription and translation related terms in addition to cyanate metabolism, much of which was acquired via LGT. It is not clear yet what role cyanase played in the origins or persistence of parasitism. Clearly this is an evolutionarily conserved protein with foundations in detoxification in early organisms during a period in the earth's development when hydrogen cyanide was a key component of our atmosphere. Cyanide undergoes rapid oxidation to cyanate which in turn is degraded to NH<sub>3</sub> and CO<sub>2</sub>. Bacteria, early plants and fungi subsequently utilized the nitrogen for growth and development. It is possible that the detoxifying components of cyanase were important in early parasites and the protein has since been repurposed (Elleuche and Poggeler, 2008). Surprisingly, to date it has not been identified in any free-living or clade V nematodes.

The majority of gene gains are species specific; however, these have common functional enrichments in zinc ion binding, chromatin binding, protein dimerization activity, and cyclin-dependent protein kinase inhibitor activity. Chromatin binding and cyclin-dependent protein kinase inhibitors are associated with gene expression and thus to a great extent, growth and development. A key function of zinc ion binding involves control of gene transcription through selective DNA binding (Iuchi, 2001). Zinc ion binding is also a GO term that is enriched in the transcripts expressed in the L3 and adult *A. caninum* (Wang et al., 2010) and in excretory-secretory products of microfilariae of *B. malayi* (Moreno and Geary, 2008) suggesting a link to parasitism and/or adaptation.

## 5. Conclusions

It is well known that parasitism evolved independently at least 18 times over the course of evolution of the Nematoda. Given the uniqueness in lifecycles, origins and tropisms, it can be difficult to ask and answer questions related to genetic and proteomic conservation among parasitic nematodes associated with parasitism and adaptation. And yet, one must wonder if in fact certain well-rooted mechanisms or biological processes act prominently to advance host-pathogen associations, disparate thought they may be. Using *T. spiralis* to represent parasites that diversified early in the evolution of the Nematoda (clade I), along with a group of free-living clade V nematodes and a collection of parasites that represent members of clades III and IV, we attempted to look for similarities that might link the parasitic nematodes, and differentiate this group from the most recently evolved free-living nematodes. Although one must temper these data with the proviso that only a small subset of organisms was used in our analysis, we were intrigued to find a few similarities among the parasites. We identified three protein families that were shared by all parasitic nematodes: 1) a LEM domain-containing protein which is an inner nuclear membrane protein that can function as a specific repressor of Tgf- $\beta$ , activin, and BMP signaling through interaction with the R-SMAD proteins, clearly associated with growth and development; 2) an RNA recognition motif domain-containing protein that in *S. japonicum* has been linked to alternative splicing and would certainly advance the parasite directive on adaptation; and 3) a cyanase family which was putatively acquired via LGT. The most intriguing questions to ask and answer are why would such a peculiar gene such as cyanase, which functions in the detoxification of cyanate, be

independently acquired by parasites of such unique tropisms and yet not appear in free-living nematodes or in other metazoa? This finding has spurred a new set of studies to: 1) identify the depth of conservation in this independent event among the Nematoda; 2) determine if the nematode cyanase family is a non-functional relic or a biologically relevant protein in the life of these parasites; 3) better discern why such a gene would remain functional long after the potential need for detoxification of cyanate in the life cycles of nematodes has ceased; and 4) determine if LGT played a larger role than currently believed in adaptation and the parasitic character among the Nematoda.

### Conflict of interest

All authors express no financial, personal or organizational conflicts of interest relative to the content of this manuscript.

### Acknowledgements

This work was supported by the National Institute of Health NIAID (R01 AI081803) and NIGMS (R01 GM097435) to M.M.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetpar.2016.07.003>.

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