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ORIGINAL ARTICLE

Bioinformatic analysis of P granule-related proteins: insights into germ granule evolution in nematodes

Luis A. Bezares-Calderón · Arturo Becerra · Laura S. Salinas · Ernesto Maldonado · Rosa E. Navarro

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Abstract Germ cells in many animals possess a specialized cytoplasm in the form of granules that contain RNA and protein complexes essential for the function and preservation of the germline. The mechanism for the formation of these granules is still poorly understood; however, the lack of conservation in their components across different species suggests evolutionary convergence in the assembly process. Germ granules are assumed to be present in all nematodes with a preformed germline. However, few studies have clearly identified these structures in species other than *Caenorhabditis elegans* and even less have carried functional analysis to provide a broader panorama of the granules composition in the phylum. We adopted a bioinformatics approach to investigate the extension of conservation in nematodes of some known *C. elegans* germ granule components, as a proxy to understand germ granules evolution in this phylum. Unexpectedly, we found that, in nematodes, the DEAD box RNA helicase Vasa, a conserved protein among different phyla, shows a complex history of clade-specific duplications and sequence divergence. Our analyses suggest that, in nematodes, Vasa's function might be shared among proteins like LAF-1, VBH-1, and GLH-1/-2/-3 and GLH-4. Key components of P granules assembly in *C. elegans*, like the PGL protein family, are only preserved in *Caenorhabditis* species. Our analysis suggests that germ granules assembly may not be conserved in nematodes. Studies on these species could bring insight into the basic components required for this pathway.

Keywords Nematodes' evolution · Germ granules · RNA helicases · *C. elegans*

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L. A. Bezares-Calderón · L. S. Salinas · E. Maldonado · R. E. Navarro (✉)
Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-600, México 04510, México
e-mail: rnavarro@ifc.unam.mx

A. Becerra
Facultad de Ciencias, Universidad Nacional Autónoma de México, Apartado Postal 70-407, México 04510, México

Introduction

Animals have two main modes to segregate and preserve the germline. In the epigenetic or inductive mode, germ cells arise later in development through a series of signals from surrounding tissues (Extavour and Akam 2003). In the preformation mode, germline is formed through the localization and maintenance of maternally provided determinants before, or immediately after, fertilization (Extavour and Akam 2003). The majority of animals have the former mode of specification, with only a non-monophyletic minority having the latter. Therefore, it has been hypothesized that preformation has arisen independently many times during evolution (Extavour and Akam 2003).

In animals with preformed germline, electrondense granules are present before fertilization and are successively restricted to germ cells. In animals with an induced germline (e.g., mammals), these granules appear in germ cells later in development (Chuma et al. 2009). These aggregates lack a membrane and contain RNAs and proteins essential for germline preservation and function,

and they display a perinuclear localization in many species (see review by Seydoux and Braun 2006). These particles are commonly known as germ granules, although they have been called by a variety of names that reflect their particular morphology at different developmental stages in different organisms (Seydoux and Braun 2006). Germ granules share some components like Vasa, a DEAD box RNA helicase, or the RNA-binding proteins Nanos that help identify germ cells in most animals, although each organism has its own specific components (Seydoux and Braun 2006). The ubiquity of germ granules has led to suggestions that they serve a role in determination, identification, and differentiation of germ cells (Strome and Wood 1982).

Nematodes are one of the phyla that have a preformed germline, but the detailed mechanisms underlying germ granule assembly and preservation are beginning to be understood only in *Caenorhabditis elegans* (Strome 2005). In *C. elegans*, germ granules are known as P granules because they remain associated with the P-cell lineage during early embryogenesis, which is an asymmetrically formed cell lineage that eventually gives rise to the germ cell precursors, Z2 and Z3. P granules are present constitutively throughout the *C. elegans* life cycle and are dynamic structures that change in size and distribution (Strome and Wood 1982). Their cellular function is still unknown, but it is generally accepted that they store RNA and proteins, thereby regulating their expression. Several pieces of evidence suggest that P granules are involved in mRNA trafficking, translation, stability, and RNA interference (RNAi)-related processes (Schisa et al. 2001; Seydoux and Braun 2006; Spike et al. 2008a, b). Recently, it has been shown that P granules share proteins with a couple of ribonucleoprotein complexes known as processing bodies (P-bodies) and stress granules, which are involved, among other processes, in mRNA degradation and in transient storage or protection from stress respectively, suggesting that they might have similar roles (Anderson and Kedersha 2008).

The association of the various P granule components is temporally restricted; only a few of the components are present at almost all developmental stages (Seydoux and Fire 1994; Pitt et al. 2000; Schisa et al. 2001). Only six specific mRNAs have been detected to date in P granules (Subramaniam and Seydoux 1999; Schisa et al. 2001) while more than 20 proteins have been found to associate with these structures; interestingly, all of them have RNA-binding motifs (Strome 2005). It is not clear how the assembly of P granules is achieved. The elimination of some P granules components alters their appearance and distribution; however, they do not disappear completely (Kawasaki et al. 1998; Spike et al. 2008b). This suggests that the mechanism that assembles P granules is redundant. Among the proteins important for P granule assembly are the DEAD box RNA helicases GLH-1/-2/-3 and GLH-4, which all are putative homologs of Vasa, and RNA-binding proteins PGL-1/-2/ and PGL-3, a novel protein family with no similarity to any other protein (Spike et al. 2008a). Epistatic studies in *C. elegans* have shown that GLH-1/-2/-3 and GLH-4 are upstream of PGL-1/-2/ and PGL-3 in the P granule assembly pathway (Spike et al. 2008a). Lack of *glh-1* and *glh-4* causes PGL-1 to dissociate from P granules and to become localized to the cytoplasm (Kawasaki et al. 1998). However *pgl-1*; *pgl-2*; *pgl-3* triple mutants display an apparently normal concentration of GLH-1 in P granules (Kawasaki et al. 2004). This evidence suggests that GLH-1 and GLH-4 may participate directly or indirectly in the recruitment or retention of PGL proteins on P granules.

P granules are assumed to be present in those nematodes with a preformed germline. Despite this, few studies have clearly identified these structures in species other than *C. elegans*, and they have revealed variation in their localization and in their mode of segregation. More importantly, no functional analysis on P granules in other nematodes has been carried out to date that helps generalize (or restrict) the findings in *C. elegans* (Skiba and Schierenberg 1992; Goldstein et al. 1998). Here, we adopted a bioinformatics approach to investigate the extent to which known *C. elegans* P granule components are conserved in other nematode species, as a proxy to understand germ granule evolution in this clade. We have also the aim to advance our understanding of the plasticity of germline specification mechanisms, which are hypothesized to be of different origins in different phyla (Extavour and Akam 2003). We took advantage of the genome sequences currently available for ten nematode species: five species of *Caenorhabditis* (*C. elegans*, *Caenorhabditis briggsae*, *Caenorhabditis remanei*, *Caenorhabditis brenneri*, and *Caenorhabditis japonica*) and five more of distant nematodes (*Pristionchus pacificus*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Brugia malayi*, and *Trichinella spiralis*; consortium TCes 1998; Stein et al. 2003; Ghedin et al. 2007; Abad et al. 2008; Dieterich et al. 2008; Opperman et al. 2008). This allows us to discern possible gene origins, duplication, and loss across this phylum. Our results showed that GLH family homologs are very divergent beyond *Caenorhabditis* species, and more distant homologs resemble *Drosophila* Vasa instead. However, we did detect other important DEAD box RNA helicases like CGH-1 and LAF-1, which are well conserved in all nematodes analyzed. We found poor conservation for components without recognizable domains, including PGL-1 family, MEG-1/-2, and DEPS-1, which are presumably involved in P granule assembly and/or in germ cell development in *C. elegans* (Spike et al. 2008a). Our results suggest that P granules assembly and regulation pathways may not be conserved in nematodes.

Materials and methods

Proteome and genome datasets acquirements

Proteome datasets and genome assemblies of all five *Caenorhabditis* species and *P. pacificus* were downloaded from the WormBase FTP site (http://www.wormbase.org release WS200, date 02 Mar 2009 for *Caenorhabditis* genus and *P. pacificus* proteome; WS198 date 13 Jan 2009 for *P. pacificus* genome assembly). *B. malayi* contigs (version WS195 08 Oct 2008) were also downloaded from the same website, but its protein homologs were searched online with NCBI BLAST (http://blast.ncbi.nlm.nih.gov). *M. hapla* genome assembly was obtained from http://www.hapla.org. *M. incognita* contigs were from GenBank (sequences CABB01000001–CABB01009538) and *T. spiralis* contigs and supercontigs from http://genome.wustl.edu.

Homologs recovery

Data sets were repackaged in databases to which we submitted *C. elegans* P granule protein sequences to search for homologs using TBLASTN and BLASTP programs for DNA and Protein datasets, respectively. For the former case, the best-hit contigs were further submitted to two gene prediction servers: Eukaryotic GeneMark.hmm (using the models of *Caenorhabditis* species) and AUGUSTUS (using only the *B. malayi* or the *C. elegans* model). Putative homologs were blasted back against *C. elegans* protein database to support homology. The longest and highest quality (i.e., the least truncated important domains) prediction was considered for subsequent phylogenetic analyses; however, if an equivalent version of the protein was already present in protein datasets (with the same or better quality) of version WS200 (if applicable), we chose this instead (see Supplementary Table S1 for a list of Wormpep numbers of proteins recovered). Moreover, we better elected predicted proteins without big fused domains (see Supplementary Table S3 for predicted gene products included in phylogenetic analyses).

Although some nematodes have proteome datasets available at WormBase, we nonetheless carried out TBLASTN searches on their genome contigs to minimize the possibility that we missed the presence of some not previously predicted homologs. Additionally, we iterated the searching process using the successively recovered homologs for more divergent (i.e., non-*Caenorhabditis*) nematodes to account for too much divergence in the sequence of *C. elegans* proteins that might have prevented us from finding homologs in other species.

We extended the search for homologs to nematode ESTs contigs and genes available at Nematode.net (http://www.nematode.net/) through its NemaBLAST tool. We however, used this strategy only to investigate the presence of the proteins of interest, but not for recovery of sequences for later analyses. This is because the predicted translations from ESTs (even from clustered ones) are generally too short.

Orthology/paralogy detection

To look for paralogs, we decided to blast homologs recovered for a particular species against its own Proteome and Genome datasets. We carefully analyzed significant hits (i.e., those with low *e*-values and alignments of reasonable length) to obtain putative paralog sequences. In cases in which multiple copies of the *C. elegans* protein homologs were found, we tried to determine which one was the ortholog copy to that present in the model nematode. For that, we compared gene contexts, using the UCSC Genome Browser website (http://genome.ucsc.edu/) and its BLAT tool (genome assemblies: *C. brenneri* Feb 2008, *C. briggsa*e Jan 2007, *C. japoni*ca Mar 2008, *C. elegan*s May 2008, *C. remanei* May 2007, and *P. pacificus* Feb 2007). We also performed phylogenetic analyses to discern the homology relationships between considered proteins (see below).

It has been recently reported that the shotgun genome assemblies of the three gonochoristic *Caenorhabditis* species*: C. brenneri*, *C. japonica*, and *C. remanei* considered in this study are highly heterozygous. Therefore, we took a conservative criterion to define paralogs in these species. We first compared putative paralogs in a given species to determine the percent identity between proteins; higher than 90% identity values made proteins candidates for being different alleles instead of paralogs. Then, we compared the context of genes encoding the pair of proteins analyzed: Identical (or very similar) contexts were a strong indication of heterozygosity at that locus. If both of the mentioned situations were met, we rejected one of the two putative alleles for subsequent analyses. For all nematodes, we discarded all low-quality versions of predicted paralogs (see Supplementary Table S2 for discarded WormBase predictions).

Protein alignment and model selection

Collected homolog proteins of each family here studied were aligned using MUSCLE software. For DEAD box helicases, each subfamily was separately aligned and then all alignments were merged into one using the profile option of MUSCLE. The resulting alignment was trimmed to preserve only the truly homologous region that would be utilized for phylogenetic analyses (e.g., DEAD box and helicase C-terminal domains). The protein model that best fitted this alignment was LG + I + G (AICw was 1), according to the AIC calculated by Prottest; for computational restric-

tions, tree topology was fixed using a Bayesian precalculated tree. PGL and DEPS family members were aligned employing the same algorithm; PGL alignment was trimmed from the N-terminal region until the first homologous methionine. DEPS-1 alignment suffered no further refinement.

Phylogenetic analyses

From aligned DEAD box helicase homologs of interest (i.e., CGH-1, GLH-1/-2/-3, VBH-1, and LAF-1; Dm_EIF4A and Ce_INF-1 translation initiation factors as outgroups), a Bayesian estimation of the most likely phylogeny was carried out using Mr.Bayes 3.1.2. A fixed WAG+ I + G unpartitioned model was used, as an LG model (see above) could not be implemented in this version of Mr.Bayes, and WAG was the second best general model considering likelihood (AICw was 0). To consider I and G, we implemented the covarion model; thus, we do not assume all helicases are varying in the same sites (in fact, some sites are invariable in a subfamily but variable in other). Covarion model also allow us to take into account differences in substitutions rates across nematode phylogeny. Posterior probabilities were based on two independent MCMC runs, each consisting of four chains; a sample frequency of 200 generations for a total of two million generations was chosen. The 20% fraction of each run was eliminated as burn-in. Convergence status was based on the average standard deviation of split frequencies for the cold chain likelihoods between the two independent MCMC runs. A 50% majority-rule consensus tree was constructed from the resulting trees.

Aligned PGL family members were used to build a maximum likelihood (ML) tree using PhyML 3.0 software; it was generated under a LG substitution model, and the program estimated the proportion of invariable sites and the Gamma shape parameter. Tree improvement was done using the Subtree Pruning and Regrafting search strategy using five random initial trees to evaluate the abundance of local optima. The statistical confidence was evaluated both with 500 bootstrapped samples and with the newly developed approximate likelihood ratio test (aLRT) statistical criterion (SH-like test); the former values were mapped on the resulting phylogeny of the latter analysis. The same strategy was followed to infer phylogenetic relationships between DEPS-1 family members, but an LG + G + F model was used.

Results and discussion

We made a phylogenetic study of those proteins that have been implicated in structuring or localizing P granules and are important for germline function in *C. elegans*. The analysis includes GLH-1/-2/-3/ and GLH-4, VBH-1, LAF-1, CGH-1, PGL-1/-2/ and PGL-3, DEPS-1, and MEG-1/-2 proteins. We BLAST-searched these proteins against genome and/or protein databases of genome-sequenced nematodes; we found a whole spectrum of conservation, possibly reflecting the heterogeneity of functions that this protein set has.

GLH family of proteins and VASA homologs in nematodes are divergent in sequence and domain architecture

DEAD box RNA helicases are involved in many aspects of mRNA metabolism and processing including transcription, splicing, ribosome biogenesis, nuclear export, mRNA turnover, ribonucleoprotein remodeling, and RNAi (reviewed by Linder 2006). RNA helicases belong to a conserved group of proteins, which unwind RNA and may facilitate RNA–protein interactions in an ATP-dependent manner (Hay et al. 1988; Andersen et al. 2006; Linder 2006). Among these proteins, Vasa is the best-known germline RNA helicase; it is widely conserved across organisms (Hay et al. 1988; Lasko and Ashburner 1988; Tanaka et al. 2000). This protein was first discovered in *Drosophila* where it has an essential role for germline specification and function (Schupbach and Wieschaus 1989; Liang et al. 1994).

The first Vasa homologs found in *C. elegans* were proteins GLH-1/-2/-3 and GLH-4, which turned out to be constitutive P granule components; we will refer to them as the GLH family or GLH proteins as a group (Roussell and Bennett 1993; Gruidl et al. 1996; Kuznicki et al. 2000). GLH-1 is critical for fertility in animals grown at elevated temperature (26°C). Animals lacking GLH-2, GLH-3, or GLH-4 are generally fertile at all temperatures. Loss of both GLH-1 and GLH-4 function causes the same effect as lack of GLH-1 but at any temperature (Spike et al. 2008b).

As expected, we found putative members of the GLH/Vasa family homologs in all nematode genomes studied but in variable number of copies (Table 1). We built a Bayesian phylogenetic tree to discern orthology/paralogy relationships between them. Homologs of other DEAD box RNA helicases important for germline development that will be discussed later were also included (Fig. 1). We could not group all putative Vasa/GLH under one clade. Instead, we recovered three well-supported groups with members of this family (Fig. 1). One group contained all GLH-4 and GLH-1/-2/-3 homologs of *Caenorhabditis* species and *P. pacificus* (posterior probability (PP) of 0.9), a second that grouped *B. malayi* and *Meloidogyne* homologs (the Vasa-like group, PP = 1). *T. spiralis* homolog was closest to the non-nematode Vasa clade forming the third group (built from mouse and fly homologs). Due to the low posterior probabilities associated to these clades (PP of 0.54 and 0.63), it is hard to suggest the relationship between these three clades.

Table 1 Phylogenetic distribution of P granules proteins across sequenced nematodes

Protein	Recognizable domains	*C. briggsae*	*C. remanei*	*C. brenneri*	*C. japonica*	*P. pacificus*	*M. hapla*	*M. incognita*	*B. malayi*	*T. spiralis*
GLH-1/-2/-3	DEAD box helicase domains, Zn CCHC fingers	+	+	++	+	++++[a]	+++[b]	++++[b]	+[b]	+/−[b]
GLH-4	DEAD box helicase domains, Zn CCHC fingers	+	+	+	+	−	−	−	−	−
LAF-1	DEAD box helicase domains	+	+	+	+	+	+	+	+	+
VBH-1	DEAD box helicase domains	+	+	−	+	+	−	−	−	−
CGH-1	DEAD box helicase domains	+	+	+	+	+	+	+	+	+
PGL-1/-3	RGG box	+	+	+	+	−	−	−	−	−
PGL-2	Without any recognizable domains	+	+	+	−	−	−	−	−	−
DEPS-1	Without any recognizable domains	++	+	+	+	+	−	−	+/−[c]	−
MEG-1/-2	Without any recognizable domains	−	−	−	−	−	−	−	−	−

Amount of + symbols indicate the number of copies of a particular homolog found in the considered organism

[a] Only one of the four homologs conserves Zn CCHC fingers

[b] Although homologs to GLH/VASA proteins, these proteins do not conserve the Zn CCHC finger that characterize the ones in *C. elegans*

[c] It is a very divergent protein without any similarity to any GenBank protein. It has significant similarity (6e−10) with Pp_DEPS-1 but not with the other DEPS-1 found. It was not included in the ML analysis

The GLH-1/-2/-3 and GLH-4 clade is divided into GLH-4 and GLH-1/-2/-3 homologs (Fig. 1). Only proteins within these two subtrees conserve the characteristic FGG repeats and zinc CCHC fingers observed in *C. elegans* versions (Fig. 2). Within the GLH-1/-2/-3 clade, *C. briggsae*, *C. remanei*, and *C. japonica* only possess one homologous copy in contrast to the three members found in *C. elegans*, while in *C. brenneri*, we found two copies close to each other in the genome. According to functional analyses, it has been hypothesized that GLH-1 gave rise to GLH-2 and GLH-3 in *C. elegans* after the divergence with the other sequenced species of its genus (Spike et al. 2008a). In agreement with this, GLH-1/-2/-3 were grouped with each other (Fig. 1) evidencing duplications after speciation. In addition, we found that all of the GLH homologs in *Caenorhabditis* species have FGG repeats and three or four CCHC zinc fingers at the N terminus portion, as does GLH-1 (Fig. 2). However, the gene neighborhood (i.e., synteny) is not conserved between different putative *Caenorhabditis* GLH-1 homologs to further support this hypothesis (data not shown).

Three GLH-1/-2/-3 putative homologs were found in *P. pacificus*, and they group with each other in the phylogenetic tree, forming a sister clade with the *Caenorhabditis* homologs (Fig. 1). This phylogenetic pattern suggests that *P. pacificus* GLH-1/-2/-3 homologs arose by duplication only after the *Caenorhabditis* and *Pristionchus* clades diverged. We considered Pp_GLH-1A as the potential ortholog of GLH-1 from the topology of the tree and for the following important traits: Firstly, Pp_GLH-1A is the only copy that conserves the characteristic CCHC zinc fingers and the FGG repeats of GLH family (Fig. 2). Secondly, in the *C. elegans* genome next to *glh-1*, there is a gene that encodes for the putative RNA helicase H27M1.19; this synteny is also conserved in the *P. pacificus* genome (data not shown). Interestingly, however, Pp_GLH-1A zinc CCHC finger domain architecture resembles more the one seen in GLH-4 homologs (Fig. 2). This could be due either to architectural constraints, in the sense that the zinc CCHC finger number and position is needed in a GLH-4-like conformation, or to confounded orthology relationships. Whatever the case, these observations suggest that Pp_GLH-1A is the best candidate to be involved in processes related to germline formation and function.

P. pacificus possesses two other GLH family homologs, Pp_GLH-1B and Pp_GLH-1C, which only conserve the DEAD box RNA helicase domain and have acquired other domains (Fig. 2). Pp_GLH-1B has two RNA recognition domains, and Pp_GLH-1C possesses one glycosyl hydrolase domain (Fig. 2), an abundant domain in *P. pacificus* genome (Dieterich et al. 2008). These proteins might function redundantly with Pp_GLH-1A or might have a novel function. Another putative homolog to GLH-1, Pp_GLH-1D, is severely truncated due to sequencing gaps, and therefore, it was not included in the phylogenetic analysis (Supplementary Table S2). The natural architecture and function of these proteins remains to be determined.

GLH-4 was only found within the genus *Caenorhabditis*; these proteins group with the GLH-1/-2/-3 homologs (Fig. 1). All conserve the zinc fingers and the FGG repeats,

albeit in different numbers and positions; they also preserve the long extension seen on *C. elegans* GLH-4, but at the N terminus portion, the homology is difficult to discern (Fig. 2). The presence of a GLH-1 and a GLH-4 homolog in each *Caenorhabditis* species confirms the importance of these two proteins in *C. elegans* and suggests they play fundamental roles in P granule function. Whether the functions associated to these two proteins can be carried out solely by Pp_GLH-1A in *P. pacificus* or by duplicated GLH/Vasa homologs in other nematodes (see below) is an interesting open question.

As mentioned above, putative GLH/Vasa family homologs in *Meloidogyne* species and *B. malayi* located together in the tree (Fig. 1). We assigned these proteins to a GLH-1/Vasa-like independent category, as we did not find other proteins in the genome of these organisms that could fit better this assignation (Table 1; Fig. 1). *B. malayi* only has one member, whereas *M. hapla* and *M. incognita* have three and four conserved homologs, respectively (Fig. 1), although one of the copies in *M. incognita* is probably only an allelic form. None of the proteins in this clade have zinc fingers or FGG repeats at their N-termini, although interestingly Mi_GLH-1/Vasa-likeB and Mh_GLH-1/Vasa-likeB show an enrichment in RGG repeats (data not shown), as does *Drosophila* Vasa (Hay et al. 1988; Lasko and Ashburner 1988).

In *T. spiralis*, we could not recover an unambiguous GLH/Vasa homolog, although two possible candidates were included in the Bayesian phylogenetic inference (Fig. 1). According to the resulting tree, one of the members (Ts_Belle/LAF-1) is more related to the Belle family, whereas the other (Ts_GLH-1/Vasa-like) is outside Vasa/GLH/Belle clade, though it also has small N-terminal region of RGG repeats.

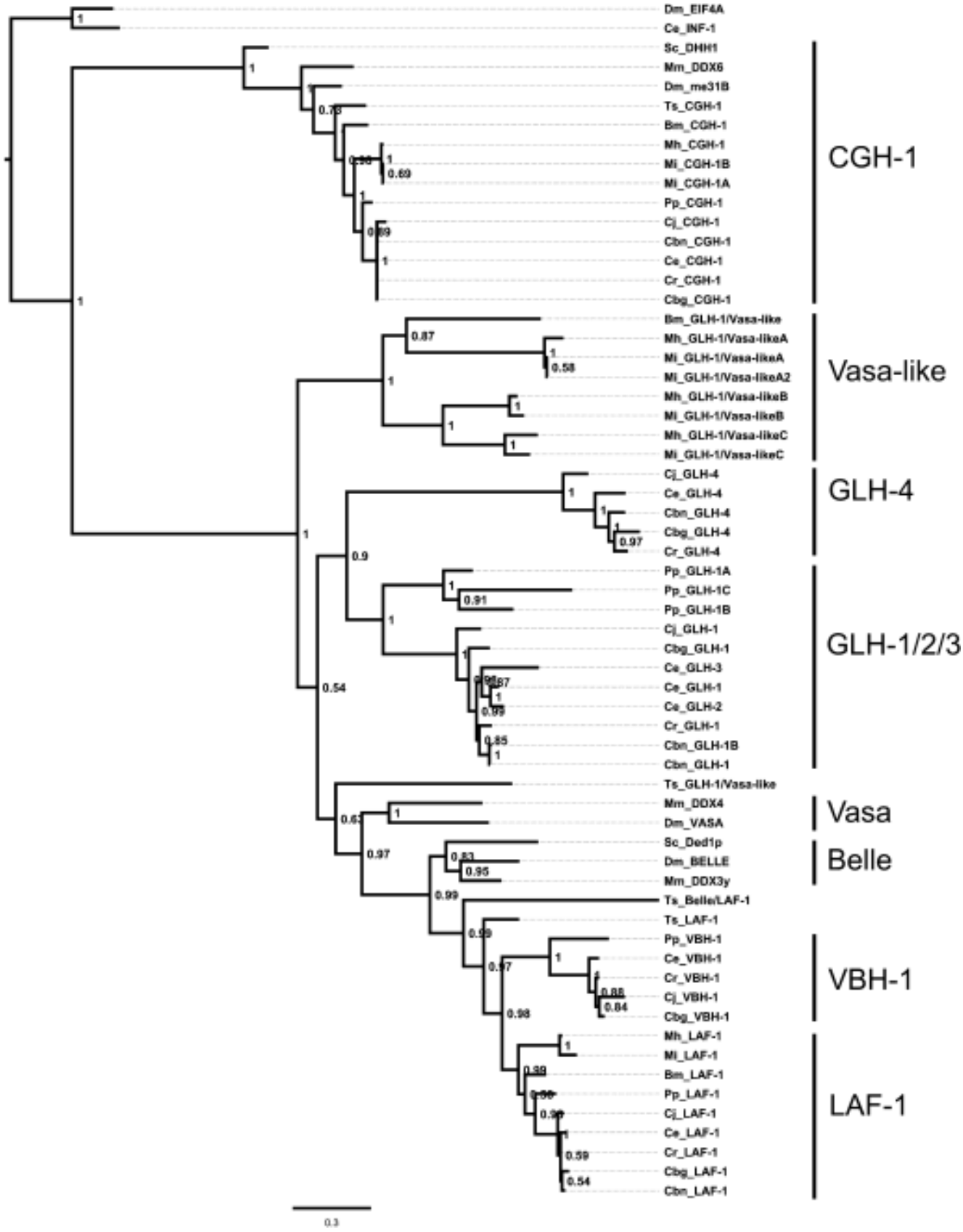
Altogether, our analysis suggests that the GLH family has suffered multiple changes across nematode phylogeny. In some species, e.g., *C. elegans*, *P. pacificus*, *M. hapla*, and *M. incognita*, members of this family of proteins are present in multiple copies. It is also clear that the addition of CCHC zinc fingers and FGG repeats at the N-terminal portion occurred probably only at clade 9 of nematodes (i.e., where *Caenorhabditis* species and *P. pacificus* are classified) (Holterman et al. 2006), while the more distant homologs have a more similar architecture to that found in D*rosophila*. More sequence and functional sampling is definitely needed in other nematodes to propose a more general view of GLH/Vasa family functions in this clade.

LAF-1 is an ancestral protein that might have given rise to VBH-1

Vasa probably originated from the ancestral Belle/Pl10 DEAD box RNA helicase family (Mochizuki et al. 2001), which has homologs in yeast, plants, and animals. Members of this family have been implicated in both promoting and repressing translation (Chuang et al. 1997; Johnstone et al. 2005), and they also have functional implications in male and female fertility (Johnstone et al. 2005). In *C. elegans*, the most similar proteins to this family are LAF-1 and VBH-1 (Salinas et al. 2007; Hubert and Anderson 2009). VBH-1 is important for embryogenesis, spermatogenesis, and oocyte function (Salinas et al. 2007). LAF-1 has a somatic role, is also important for embryogenesis, and, together with VBH-1, regulates the sperm/oocyte switch (Hubert and Anderson 2009). Additionally, LAF-1 is an mRNA regulator of *tra-2*, a gene implicated in the sex determination pathway (Hubert and Anderson 2009). VBH-1 associates constitutively to P granules (Salinas et al. 2007; A Franco-Cea and RE Navarro unpublished results), whereas LAF-1 has only been shown to localize to these structures in two-cell embryos (Hubert and Anderson 2009).

We traced the conservation of these proteins in sequenced nematodes, and LAF-1 turned out to be more ancestral than VBH-1. The former is present in all the species analyzed (Table 1). In contrast, VBH-1 is restricted to sampled species from clade 9 (except in *C. brenneri* where it may have been lost). The Bayesian tree presented in Fig. 1 shows a putative *T. spiralis* LAF-1 homolog outside LAF-1/VBH-1 clade. Nonetheless, in distance matrix analyses, this protein is grouped with the other LAF-1 homologs with confident bootstrap values (data not shown). As Bayesian inference is more sensitive to small differences in sequence, Ts_LAF-1 may be an ancestral version of LAF-1 and VBH-1. Interestingly, *T. spiralis* has another putative LAF-1 homolog that falls outside the LAF-1/VBH-1 clade. This could represent a duplication of an ancestral LAF-1, independent of the event that gave origin to the VBH-1 version. The later origin of VBH-1 is consistent with a more germline-specific function and with less pleiotropic effects observed when is silenced (Salinas et al. 2007). In contrast, LAF-1 is

Fig. 1 Majority-rule consensus tree that resulted from a Bayesian analysis of the aligned DEAD box helicase C domains of CGH-1, GLH-1/-2/-3, VBH-1, and LAF-1 and their recovered homologs in sequenced nematodes. Node labels are posterior probabilities supporting each subtree topology (see "Materials and methods"). The tree was rooted by the outgroup criterion. Branch lengths are proportional to the amount of change in sequences. Uncharacterized homologs were named according the *C. elegans* (or experimentally characterized) protein, which was used to recover them, unless otherwise indicated in the text. *Meloidogyne* and *B. malayi* GLH-1/Vasa-like proteins were the most similar proteins to both *C. elegans* GLH-1 and Dm_VASA recovered by our method. TS_LAF-1 is assumed to be the corresponding homolog although it is not in the group of LAF-1 proteins. Ts_Belle/LAF-1 is between the Belle and LAF-1 groups. Species codes: *Ce C. elegans*, *Cr C. remanei*, *Cbg C. briggsae*, *Cbn C. brenneri*, *Pp P. pacificus*, *Bm B. malayi*, *Mh M. hapla*, *Mi M. incognita*, *Ts T. spiralis*, *Dm Drosophila melanogaster*, *Mm Mus musculus*, *Sc Saccharomyces cerevisiae*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Ce_INF-1
Sc_DHH1
Mm_DDX6
Dm_me31B
Ts_CGH-1
Bm_CGH-1
Mh_CGH-1
Mi_CGH-1B
Mi_CGH-1A
Pp_CGH-1
Cj_CGH-1
Cbn_CGH-1
Ce_CGH-1
Cr_CGH-1
Cbg_CGH-1
CGH-1
Bm_GLH-1/Vasa-like
Mh_GLH-1/Vasa-likeA
Mi_GLH-1/Vasa-likeA
Mi_GLH-1/Vasa-likeA2
Mh_GLH-1/Vasa-likeB
Mi_GLH-1/Vasa-likeB
Mh_GLH-1/Vasa-likeC
Mi_GLH-1/Vasa-likeC
Vasa-like
Cj_GLH-4
Ce_GLH-4
Cbn_GLH-4
Cbg_GLH-4
Cr_GLH-4
GLH-4
Pp_GLH-1A
Pp_GLH-1C
Pp_GLH-1B
Cj_GLH-1
Cbg_GLH-1
Ce_GLH-3
Ce_GLH-1
Ce_GLH-2
Cr_GLH-1
Cbn_GLH-1B
Cbn_GLH-1
GLH-1/2/3
Ts_GLH-1/Vasa-like
Mm_DDX4
Dm_VASA
Vasa
Sc_Ded1p
Dm_BELLE
Mm_DDX3y
Belle
Ts_Belle/LAF-1
Ts_LAF-1
Pp_VBH-1
Ce_VBH-1
Cr_VBH-1
Cj_VBH-1
Cbg_VBH-1
VBH-1
Mh_LAF-1
Mi_LAF-1
Bm_LAF-1
Pp_LAF-1
Cj_LAF-1
Ce_LAF-1
Cr_LAF-1
Cbg_LAF-1
Cbn_LAF-1
LAF-1
0.3

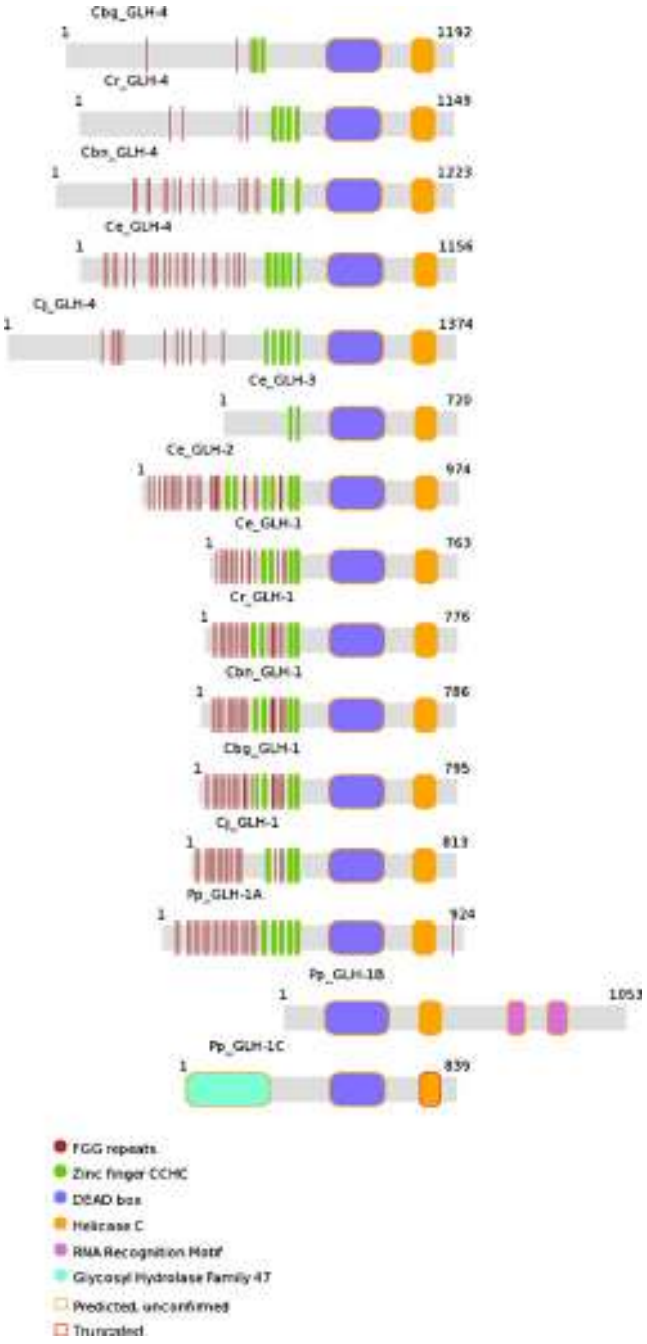

Fig. 2 Protein domain architectures of selected GLH homologs. Domains were predicted using Pfam (http://pfam.janelia.org/) and schematized to scale with DomainDraw (http://domaindraw.imb.uq.edu.au/). FGG repeats were defined as tracks of one Phe and (at least) two Gly residues. In Cj_GLH-1 and Ce_GLH-2, some tracks were interspersed by a Thr or Ser residue, respectively

expressed both in soma and in germline and carries more general functions (Hubert and Anderson 2009).

CGH-1 is a highly conserved RNA helicase

CGH-1 is a DEAD box RNA helicase required for embryonic development, germ cell function, and survival in *C. elegans* (Navarro et al. 2001; Navarro and Blackwell 2005). Besides P granules, CGH-1 is also expressed in other RNA granules found in the core of the hermaphrodite gonad, oocytes, and somatic and germ cells during early embryogenesis that might resemble P-bodies (Navarro et al. 2001). CGH-1 protein was found in a single copy in almost all nematode genomes analyzed confirming that this protein is highly conserved. The only exception was *M. incognita*, which has two copies, but this may also reflect heterozygosity rather than duplication (Table 1; Fig. 1). Association of CGH-1 with P-bodies and stress granules is interesting because these particles are also present in organisms without a preformed germline and suggests that P granules might have a similar role (reviewed by Rajyaguru and Parker 2009).

The PGL family of proteins is only present in the *Caenorhabditis* genus

The PGL family of proteins has been only found to date in *C. elegans* and consists of three members: PGL-1/-2/ and PGL-3. PGL-1 and PGL-3 possess an RGG box at the C-termini, a motif known to function as a RNA-binding domain. PGL-1 and PGL-3 are constitutively associated to P granules, whereas PGL-2 localizes to these structures only in postembryonic development (Kawasaki et al. 1998, 2004). Moreover, *pgl-1*; *pgl-3* double mutant has severe germline defects at all temperatures, while *pgl-1*; *pgl-2* mutant shows no greater defects than those seen in *pgl-1* single mutant. As a consequence, PGL-1 and PGL-3 are considered to function redundantly in some aspect of P granule assembly and stability, as well as in germline proliferation (Kawasaki et al. 1998, 2004; Strome 2005).

Due to its apparent importance in germline development, PGL protein family may be inferred to be at least present in nematodes; however, we could not find any traceable homolog in non-*Caenorhabditis* species (Table 1). The ML phylogenetic tree inferred from these sequences revealed that PGL-1 and PGL-3 are paralog proteins formed after *C. elegans* diverged from the other species (Fig. 3a). Only one PGL-1/-3 homolog is present in the other four *Caenorhabditis* species, and all conserve the characteristic RGG box motifs at the C-terminal portion (data not shown). Nonetheless, synteny is not conserved between these proteins' loci to determine whether *C. elegans* PGL-1 or PGL-3 is their ortholog protein (data not shown). More importantly, the redundancy observed for the function carried out by PGL-1 and PGL-3 in *C. elegans* may not be present in the other four nematodes.

Homologs to PGL-2 were only found in three additional *Caenorhabditis* species (Table 1). The inferred tree also indicated that PGL-2 and its homologs are fast-evolving proteins (relative to PGL-1/-3), as judged by their long

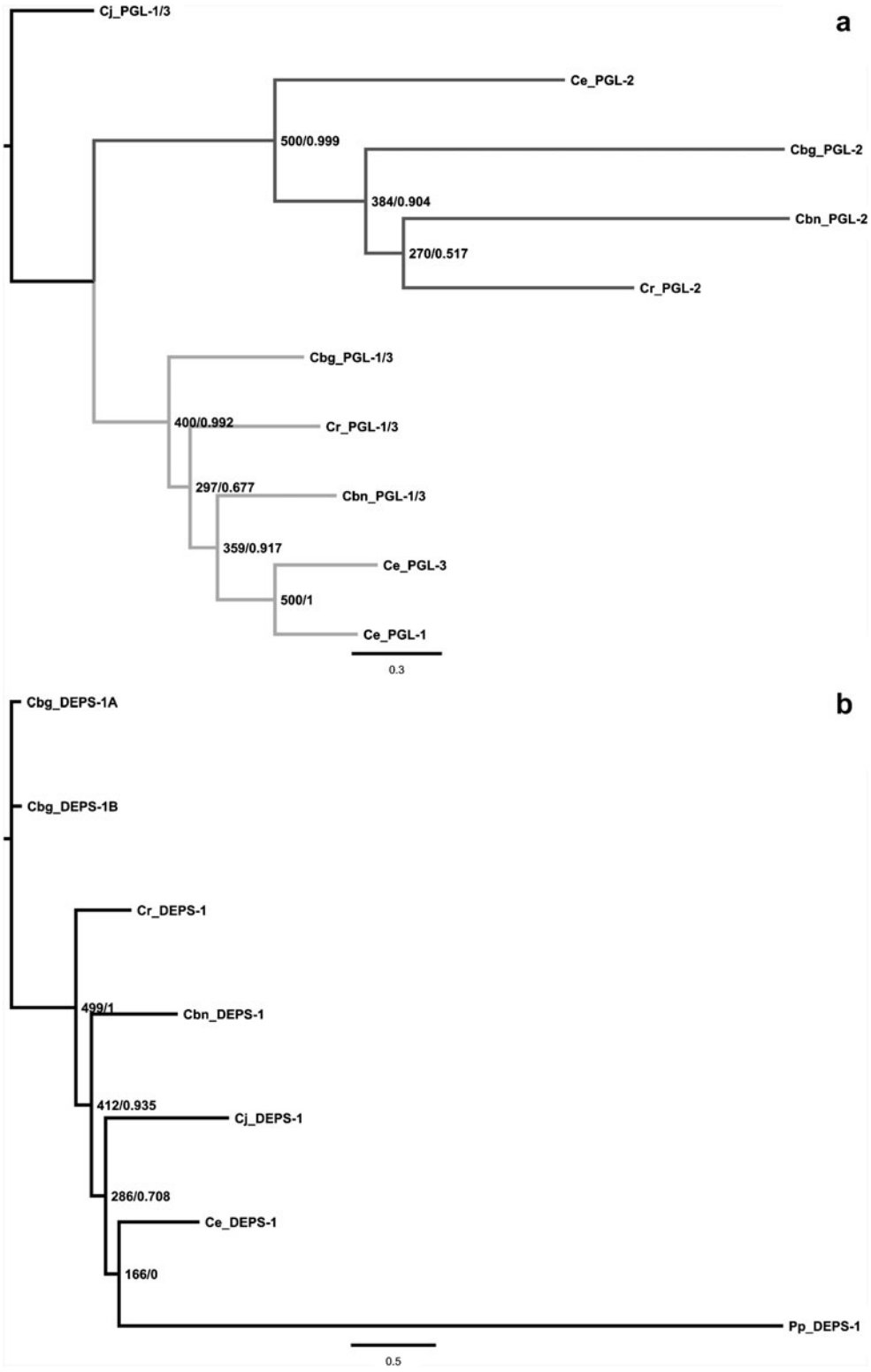

Fig. 3 Inferred maximum likelihood tree from aligned (**a**) PGL and (**b**) DEPS-1 homologs recovered from the search analysis. Branch length mirrors relative amount of substitution rates. **a** For PGL, two groups, a PGL-1/-3 clade and a PGL-2, were defined based on the position in the tree of the three members in *C. elegans*; sequences within these groups were named accordingly. The tree was rooted at the only *C. japonica* PGL family member assuming PGL-2 did never exist in this species. **b** DEPS-1 tree was not rooted. Species codes for both trees are as in Fig. 1. Bootstrap values (500 repetitions) support the branching order calculated from the data; aLRT was also computed for each tree; the resulting probability for each node is thus displayed as bootstrap value/aLRT probability. Trees were edited with FigTree software (http://tree.bio.ed.ac.uk/software/figtree/)

branches (Fig. 3a). Despite this, synteny is conserved among them (data not shown). PGL-2 probably arose after the ancestor of PGL-1/-3 because *C. japonica* lacks a copy of it, a species that appears as an outgroup to the other *Caenorhabditis* nematodes (Kiontke et al. 2007). Thus, as PGL-2 was originated and maintained in various *Caenorhabditis*, it might have an important function that could be elucidated more easily in nematodes with fewer copies of this family. Another result that supports PGL being a phylogenetically restricted family is the fact that

non-*Caenorhabditis* ESTs from the collection available at NematodeNet have no detectable similarity to any of its members (data not shown). The protein alignment revealed few conserved motifs between all PGL family members; however, a [D/E]L[D/E]FD[A/V][F/Y/L]V[F/L] motif was recovered, so it may be important for them to function. PGL family is therefore very recent in evolutionary terms, and whether it realizes novel functions in the *Caenorhabditis* species included in these analyses or it replaced an analogous protein carrying out a similar task in other nematodes remains to be elucidated.

DEPS-1 protein is present in the *Caenorhabditis* species analyzed and in *P. pacificus*

DEPS-1 is another P granule constitutive component possibly involved in structuring P granules and in other functions like RNA interference in *C. elegans* (Spike et al. 2008a). *deps-1* encodes a novel protein with a C-terminal domain of low amino acid complexity rich in S, T, R, and A residues (Spike et al. 2008b). *deps-1* mutant animals deprived of both maternal and zygotic protein are sterile at 24.5°C but fertile at higher temperatures (Spike et al. 2008b). DEPS-1 is important for PGL-1 localization to P granules and for the accumulation of *glh-1* mRNA and protein (Spike et al. 2008b). DEPS-1 was only recovered in *Caenorhabditis* species and *P. pacificus* (Table 1). In *C. briggsae*, this protein was found to be duplicated, and the putative homolog in *P. pacificus* is highly divergent to the other proteins (Fig. 3b) suggesting that it may not have the same function as that reported in *C. elegans*. It would be interesting to elucidate its function in *P. pacificus*, where no PGL homolog is encoded; it may turn out that this protein acquired its germline functions later in evolution, as it seems to regulate other processes as well (Spike et al. 2008b).

MEG-1 and MEG-2 are present exclusively in *C. elegans*

MEG-1 and MEG-2 are proteins of low complexity that associate transiently with P granules during embryogenesis (Leacock and Reinke 2008). Loss of *meg-1* and *meg-2* results in sterile animals that have undersized gonads due to failure of larval germ cell proliferation. Animals that lack these proteins also present ectopic P granules in somatic blastomeres (Leacock and Reinke 2008). Surprisingly, we failed to find homologs in any of the nematode genomes analyzed (Table 1), despite the fact that these proteins function redundantly at different temperatures in P granule segregation and repression (Leacock and Reinke 2008). Why MEG-1/-2 evolved in *C. elegans* and not in the other nematodes (although they may have reached a convergent solution) and how these proteins connected to the germline specification network are obvious questions arising from this finding.

Conclusions

Nematodes comprise one of the most diverse animal phyla, which argues for a consideration of the representativeness of the genomes available to date. Holterman et al. classified nematodes into 12 clades, which by the rate of their SSU rDNA evolution were further divided in slow (1–7)- or fast (8–12)-evolving taxa, the latter being the group "Secernentea" (Holterman et al. 2006). Genomes sequenced to date belong to nematodes mainly clustered in clade 9 (*Caenorhabditis* spp. and *P. pacificus*), but other genomes, all from parasitic nematodes, are classified in clade 8 (*B. malayi*), clade 12 (*Meloidogyne* spp.), or clade 2 (*T. spiralis*; Holterman et al. 2006). Divergence times between *C. elegans* and the other genera range from 280 Ma (with *P. pacificus*; Dieterich et al. 2008) to 700 Ma (with *T. spiralis*; Mitreva and Jasmer 2006). Therefore, the current nematode genome set represents isolated clades located at both extremes and at the center of the phylum, but with a bias toward fast-evolving taxa. In spite of this fact, the distribution was wide enough to reveal varying levels of conservation among P granule proteins, which in turn may suggest diversity in granule assembly mechanisms in the phylum. More information at the sequence and functional level from non-parasitic basal nematodes, whose germline may follow a pattern of specification more similar to other animal phyla, as well as other clades of nematodes not yet represented will be important to obtain a broader view of these structures and of their evolution in the phylum.

From the presented analysis, some important consequences arise. First, germ granule mechanism of assembly and stability may differ greatly across nematodes. This suggestion stems from the fact that poor conservation was observed for important components in *C. elegans*. Alternatively, some as yet to be identified component(s) may be sufficient for giving the most important characteristics to these germinal structures throughout the phylum. Second, germ granule proteins and functions associated with them might be relatively easy to be incorporated during evolution. How this process has been achieved and whether it happened in other nematodes are issues that could be tackled in experimentally amenable species like *C. briggsae*, *C. remanei*, and *P. pacificus*. Finally, the absence and modification of known germ granule components may reflect deeper differences in underlying molecular mechanisms of germline specification and development in nematodes.

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