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Conspecific and Interspecific Interactions Between the FEM-2 and the FEM-3 Sex-Determining Proteins Despite Rapid Sequence Divergence

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Abstract. Using degenerate oligonucleotide primers, we isolated the *Caenorhabditis remanei* orthologue of the *C. elegans* sex-determining phosphatase gene *fem-2* as well as two other protein phosphatase homologues. Despite the significant sequence divergence between *C. elegans* and *C. remanei* FEM-2, we used RNAi-mediated gene knockdown to demonstrate that at least some aspects of male development require FEM-2 function in *C. remanei*. Consistent with this functional conservation, the conspecific interaction between the FEM-2 and the FEM-3 proteins observed in *C. elegans* also occurs in *C. remanei*. To further explore whether the rapid evolution of FEM-2 and FEM-3 affects their molecular interactions, we tested for cross-species interactions between the proteins from *C. elegans*, *C. briggsae*, and *C. remanei*. Although all FEM-2/FEM-3 pairs from a single species interact, only two out of six interspecific pairs bind each other, showing that FEM-2 and FEM-3 are coevolving. Both interspecific interactions involved *C. briggsae* FEM-3. We constructed chimeric versions of FEM-2 consisting of various combinations of the *C. elegans* and *C. remanei* proteins. *C. briggsae* FEM-3 interacted with all the chimeras, even those that did not interact with either *C. elegans* or *C. remanei* FEM-3. We hypothesize that the promiscuity of *C. briggsae* FEM-3 reflects an increased reliance on evolutionarily constrained regions of FEM-2 for binding. If so, our data support the notion that the coevolution of two interacting proteins sometimes involves a shift in the domains that contribute to binding.

Key words: Sex determination — FEM-2 — FEM-3 — Conspecific protein interactions — *Caenorhabditis* — Protein evolution

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Introduction

Many of the pathways that control development in metazoans are well conserved. Examples include the Wnt, Hh, Ras/MAPK, and TGF-β pathways, which have been described in worms, flies, and mammals (Pires-daSilva and Sommer 2003). This conservation suggests that these pathways arose before the radiation of existing metazoan phyla. In contrast, sex-determination pathways show virtually no conservation between worms, flies, and mammals: each group relies on a distinct set of proteins to regulate sexual fate. Within worms, flies, and mammals the sex-determination pathways are largely conserved, although their constituent proteins show signs of rapid evolution. Both of these features of sex-determination evolution (the presence of distinct pathways in different phyla and rapid component divergence) may be consequences of genome conflict (Haag et al. 2002; Stothard and Pilgrim 2003). Even without a good understanding of what forces are responsible for pathway differences, interspecies comparisons of sex-determination may provide special insight into how

regulatory networks can be assembled and modified. Indeed, several studies of sex determination in a variety of fly species suggest that gene products tend to be lost and gained from the upstream regions of pathways (Graham et al. 2003; Pomiankowski et al. 2004).

A detailed understanding of how sexual fate is regulated in *Caenorhabditis elegans* has emerged through numerous genetic and molecular studies (reviewed in Cline and Meyer 1996; Hansen and Pilgrim 1999; Goodwin and Ellis 2002). We and others have begun to compare the *C. elegans* sex-determination pathway with the pathways that regulate sex in other nematode species such as *C. briggsae* and *C. remanei* (Stothard and Pilgrim 2003; Nayak et al. 2005; Cutter and Ward 2005). Recent phylogenies for the various *Caenorhabditis* species have concluded that *C. briggsae* and *C. elegans*, which are both hermaphrodite/male species, diverged independently from gonochoristic (male/female) ancestors, and most of the closely related species, such as *C. remanei*, have retained that sex determining mechanism (Kiontke et al. 2004; Cho et al. 2004). This implies that the changes that led to hermaphroditism also evolved independently in *C. elegans* and *C. briggsae*, and may involve different regulatory controls. Another popular species for use in comparative studies of nematode development is *Pristionchus pacificus* (Gutierrez et al. 2003; Pires-daSilva and Sommer 2004). It is a much more distant relative of *C. elegans*, and thus can be used as an outgroup to explore pathway change over a broader time scale.

In this study we examine the evolution of FEM-2, one of the factors known to regulate sex in *C. elegans* (Pilgrim et al. 1995; Chin-Sang and Spence 1996). It is required for the male fate to be established in the soma and germline of XO animals, and for the brief period of spermatogenesis that occurs in hermaphrodite (XX) animals. Sequence analysis and biochemical assays indicate that FEM-2 is a protein phosphatase type 2C (PP2C) (Chin-Sang and Spence 1996; Hansen and Pilgrim 1998). It is thought to act in a complex with two other proteins, FEM-1 and FEM-3, which are also necessary for male development and hermaphrodite spermatogenesis. FEM-2′s substrate and the direct downstream targets of the FEM proteins are not known. We previously found that FEM-2 has diverged to a much greater extent than two other PP2Cs between *C. elegans* and the gonochoristic nematode CB5161 (Stothard et al. 2002). This sequence divergence raised the possibility that functional divergence might also have occurred—perhaps FEM-2 is not tethered to the pathway that regulates sex in CB5161 and instead represents a component difference between these two species. Unfortunately CB5161 has not been widely used in comparative studies, and thus the sequences of other sex-determining genes have not been isolated. Here we describe the isolation and characterization of the *fem-2* gene from *C. remanei*. We find that *fem-2* has evolved rapidly in the *C. remanei* lineage, relative to two other PP2Cs. However, despite this sequence divergence the protein-protein interaction between FEM-2 and FEM-3 observed in *C. elegans* also occurs in *C. remanei*, and RNAi indicates that *C. remanei fem-2* is required for normal male development. Thus the rapid evolution of FEM-2 does not necessarily represent sex-determining pathway divergence. We also provide evidence that *C. briggsae* FEM-3 (Cb-FEM-3) is able to interact with the FEM-2 proteins from *C. elegans*, *C. briggsae*, and *C. remanei*. In contrast, *C. elegans* FEM-3 and *C. remanei* FEM-3 appear to interact exclusively with their conspecific partners. We suggest that Cb-FEM-3 may have broader specificity because it interacts with portions of FEM-2 required for other functions, such as phosphatase activity. We also discuss how coevolution might sometimes lead to interaction domain movement and to parallel forms of regulation in pathways.

Materials and Methods

Nematode Strains. The Caenorhabditis Genetics Center provided the following nematode strains: *Caenorhabditis elegans* strain N2, *Caenorhabditis briggsae* strain AF16, *Caenorhabditis remanei* strain SB146, *Caenorhabditis* sp. strain PS1010, and *Pristionchus pacificus* strain PS312.

Isolation of PP2C Genes by PCR. RNA was isolated from nematodes and converted to cDNA as described (Stothard et al. 2002). Degenerate oligonucleotides BJM1 (5′-GSIGTITWYGA YGGICAYGSIGG-3′) and BJM2 (5′-ACRTCCCADAIICCR TCRCAIGC-3′) were then used to amplify cDNAs related to known PP2Cs. PCR was performed in a custom buffer (60 m*M* Tris−SO_4, pH 9.1; 18 m*M* $[NH_4]_2SO_4$; 2.6 m*M* $MgSO_4$) using the following program: 94°C for 2 min, 50°C for 2 min, 72°C for 2 min, one cycle; 94°C for 1 min, 50°C for 2 min, 72°C for 2 min, 35 cycles. The resulting products were cloned into pGEM-T (Promega) and sequenced. The PCR and cloning procedures were repeated using the primers F25FI (5′-GTCCACGATTGCTTCGCTATCT-3′) and F25RO (5′-ACACATGCTTGATGTCACGTTG-3′), which are designed to anneal to the predicted *C. elegans* PP2C gene F25D1.1. Approximately 30 clones were sequenced for each nematode species. BLAST searches and phylogenetic analysis were used to classify the clones, and a representative sequence for each PP2C-related gene was submitted to GenBank (Table 1). To obtain a full-length *C. remanei fem-2* cDNA suitable for use in the yeast two-hybrid assay, additional primers were designed based on the sequence of a partial *C. remanei fem-2* clone isolated using the above procedure. These primers were used in 3′RACE and SL1-PCR reactions as described (Stothard et al. 2002).

RNA Interference. Double-stranded RNA was synthesized from a *C. remanei fem-2* template using the MEGAscript *in vitro* transcription kit (Ambion). The RNA was injected at a concentration of approximately 3 mg/ml into the gonad or gut of adult *C. remanei* females. At 20 h after injection, the worms were transferred to new plates. The ratio of males to females was determined for the brood arising from each animal, to test whether

Table 1. Comparison of partial PP2C domain sequences with their *C. elegans* orthologues: Only the sequence regions between the BJM1 and the BJM2 primer annealing sites were used in the alignment and % identity calculations

Putative orthologue	Species (strain)	GenBank accession No.	% identity[a]	Reference
F25D1.1	*C. briggsae* (AF16)	AF507022	90	This work
F25D1.1	*C. remanei* (SB146)	AF507021	92	This work
F25D1.1	*C.* species (CB5161)	AF268069	90	Stothard et al. (2002)
F25D1.1	*P. pacificus* (PS312)	AF507024	53	This work
fem-2	*C. briggsae* (AF16)	AF054982	76	Hansen and Pilgrim (1998)
fem-2	*C. remanei* (SB146)	AF507019	77	This work
fem-2	*C.* species (CB5161)	AF177870	68	Stothard et al. (2002)
T23F11.1	*C. briggsae* (AF16)	AF507023	96	This work
T23F11.1	*C. remanei* (SB146)	AF507020	93	This work
T23F11.1	*C.* species (CB5161)	AF177866	95	Stothard et al. (2002)
T23F11.1	*C.* species (PS1010)	AF507027, AF507026	79	This work
T23F11.1	*P. pacificus* (PS312)	AF507025	63	This work

[a]Amino acid sequence identity when aligned with the *C. elegans* orthologue.

the RNAi was causing sexual transformation. Worms were then examined for signs of partial sexual transformation using differential interference contrast microscopy. Several adult progeny were also dissected and stained with 4′,6-diamidino-2-phenylindole (DAPI) to assess the effects of the RNAi on gonad morphology and germ cells. The fertility of some *fem-2(RNAi)* worms was assayed by crossing them with unmated wild-type females or wild-type males.

Yeast Two-Hybrid Analysis. PCR was used to add restriction sites to the ends of the complete *fem-2* cDNAs from *C. elegans*, *C. briggsae*, and *C. remanei*, so that they could be ligated to the GAL4 activation domain plasmid pGAD-C3 (James et al. 1996). Several fusions containing truncated versions of *C. elegans* and *C. remanei fem-2* were generated in a similar fashion using different primers. Chimeric *fem-2* constructs, consisting of portions from both the *C. elegans* and *C. remanei* genes, were prepared by swapping conserved restriction fragments, and by using mismatched PCR primers to convert sequences. All constructs were sequenced, and versions containing mutations were discarded. L40 yeast cells, which contain His and LacZ reporters regulated by the LexA operator, were transformed with the *fem-2* constructs and with plasmids expressing the *fem-3* genes from the same three species, fused with the LexA DNA binding domain (Haag et al. 2002). Reporter gene expression was assayed by growth on −Leu−Trp−His plates at 30°C and by X-gal staining after overnight growth at 25°C on nylon filters. For some strains a quantitative X-gal assay was performed using a commercial kit (Pierce).

Sequence Analysis. Multiple sequence alignments were prepared using CLUSTAL X with the default settings (Thompson et al. 1997), and pairwise sequence alignments and identity scores were determined using The Sequence Manipulation Suite 2 (Stothard 2000). A phylogenetic tree was generated from aligned PP2C domains using CLUSTAL X and the neighbor-joining method (Saitou and Nei 1987). Portions of the alignment containing gaps were included in the analysis, and the correction for multiple substitutions was not applied. The results were analyzed using the bootstrap method (1000 replicates) to provide confidence levels for the tree topology.

Results

The genome sequence of *C. remanei* has not yet been completed, so to obtain a partial *fem-2* coding sequence from *C. remanei* we used RT-PCR with degenerate oligonucleotide primers. The annealing sites of these primers correspond to portions of PP2C motifs two and eight, of the 11 conserved motifs detected in a detailed sequence analysis of the PP2C superfamily (Bork et al. 1996). In an attempt to identify more distant PP2C orthologues, RNA from *P. pacificus* and *Caenorhabditis* species PS1010 was processed in the same manner, and *C. briggsae* RNA was used as a control to test the efficacy of the primers and methods used. Apparent orthologues of the *C. elegans* PP2C gene T23F11.1 were isolated from all the species examined (Table 1, Fig. 1). Two types of T23F11.1 cDNAs were isolated from PS1010 multiple times. They differ at five nucleotide positions, and the changes all involve synonymous C/T transitions, suggesting the presence of two alleles, two genes, or RNA editing. Putative orthologues of the predicted PP2C F25D1.1 were isolated from *C. briggsae*, *C. remanei*, and *P. pacificus*. A previously characterized *fem-2* orthologue was obtained from *C. briggsae*, and a new *fem-2* orthologue was isolated from *C. remanei*. Approximately 40 additional RT-PCR clones were sequenced for both PS1010 and *P. pacificus*, but no *fem-2* orthologues were obtained for these species.

The 5′ end of the *C. remanei fem-2* cDNA was amplified using a primer that anneals to the SL1 spliced leader sequence, and the remaining portions were obtained using 3′ RACE and RT-PCR. New primers were designed from the hypothetical sequence assembled from the cDNA fragments, and these were used to isolate a complete cDNA. The predicted product of the cDNA, which we refer to as Cr-FEM-2, is shown in Fig. 2. Cr-FEM-2 contains the seven metal binding and phosphate binding residues that are typically found in PP2Cs. The residues that are mutated in two temperature-sensitive alleles of *C. elegans fem-2* are also conserved. Cr-FEM-2 contains a long amino terminal domain and an acidic carboxy terminus, both of which are found in the

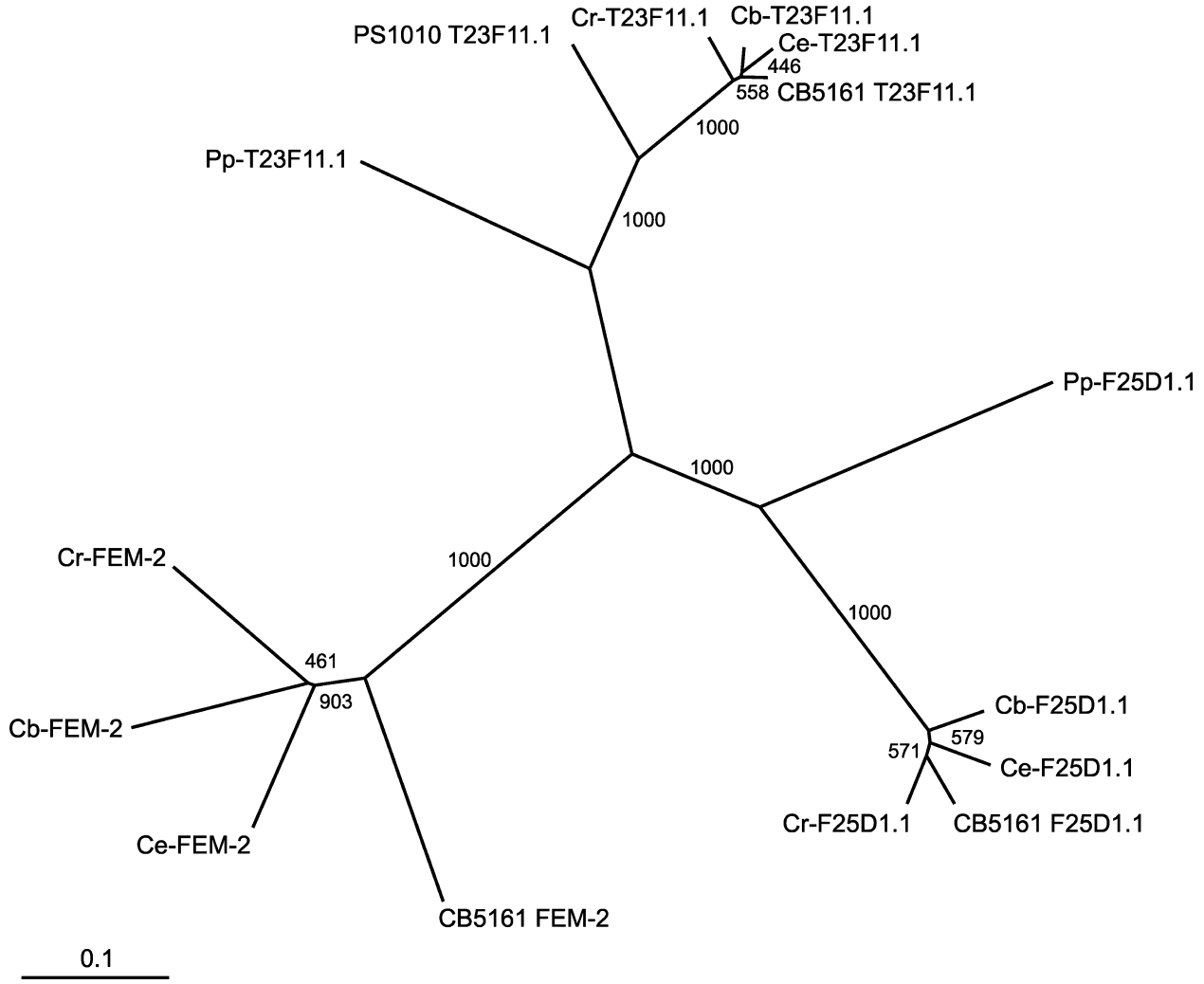
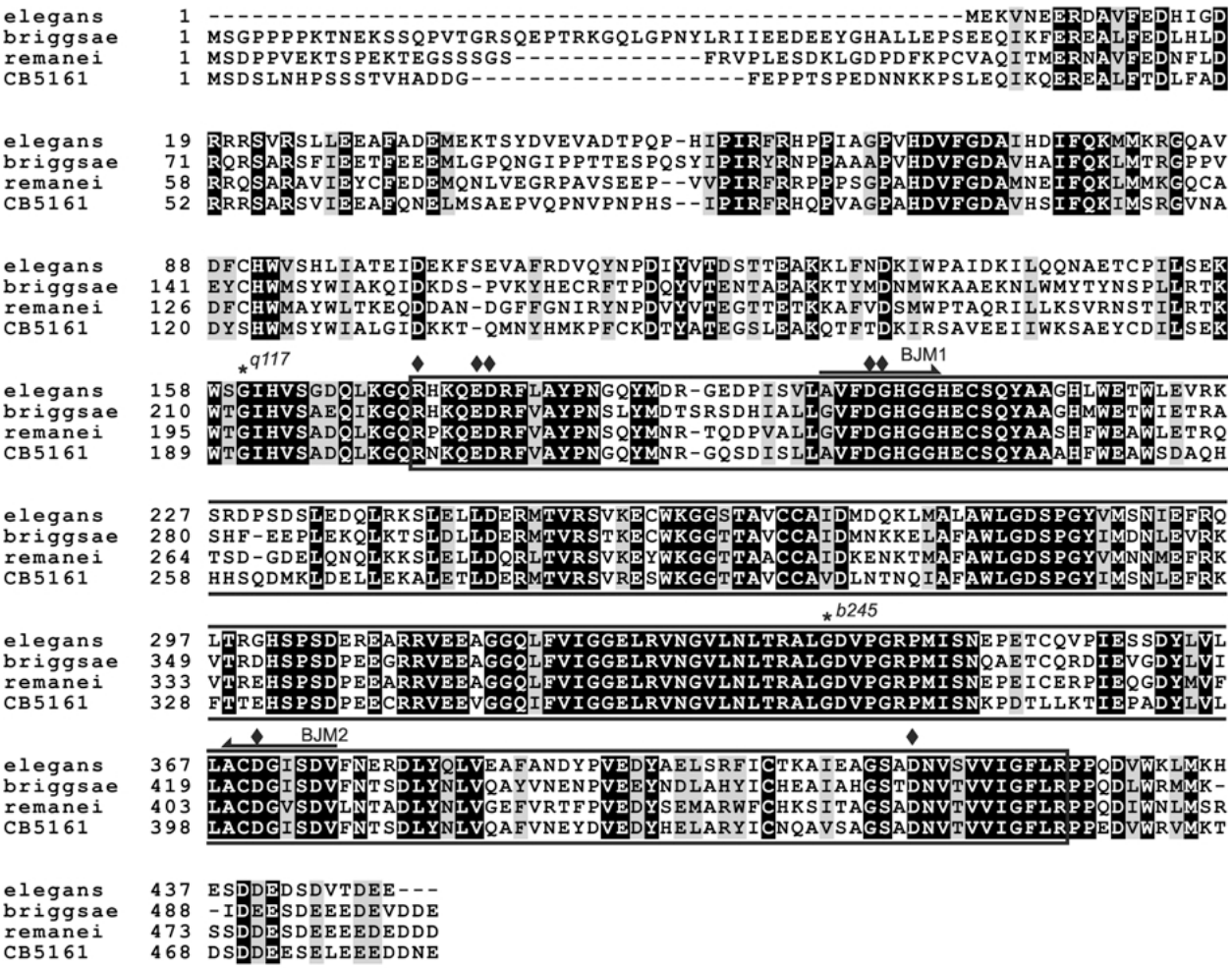


Fig. 1. Phylogenetic analysis of partial PP2C domains isolated from various nematode species, in this work and in previous studies. Only the sequence regions between the BJM1 and the BJM2 primer annealing sites were used. Numbers indicate bootstrap support values obtained from 1000 replicates. The GenBank accession numbers for these sequences are given in Table 1. The scale bar represents 0.1 change per site.

other FEM-2 proteins but not in PP2Cs in general. On average, the complete FEM-2 sequences are about 60% identical when aligned with one another (Fig. 2). Compared with the two non-sex-determining PP2C sequences, FEM-2 is evolving rapidly (Table 1, Fig. 1). However, FEM-2 contains a greater proportion of conserved residues than FEM-3 (Haag et al. 2002), which is between 35% and 38% identical among *C. elegans*, *C. briggsae*, and *C. remanei*.

In *C. elegans*, germline and somatic sex determination are regulated by different pathways, although many proteins, such as the FEMs, are components of both. In CB5161, we were unable to confirm whether the Cs-FEM-2 protein has a role in controlling somatic or germline sexual fate in that species, since RNAi against *Cs-fem-2* did not produce any detectable phenotype in either XX or X0 animals (Stothard et al. 2002). The same question arises with Cr-FEM-2, but as with CB5161, no genetic lesions in any of the *C. remanei* sex-determining genes have yet been reported, leaving RNAi as the only tool available at this time to reduce the level of gene function. In *C. remanei*, RNAi against *Cr-fem-3* and *Cr-glp-1* have been successful at producing phenotypes in the soma and early embryo, respectively (Haag et al. 2002; Rudel and Kimble 2001). To explore the biological function of *fem-2* in *C. remanei*, we also used RNA interference. Broods from adult *C. remanei* females injected with *Cr-fem-2* dsRNA were examined for evidence of a sex ratio skewed toward females (which might suggest complete feminization of XO animals) and for the presence of partially feminized males. No significant alteration in the ratios of males to females was observed in the progeny following RNAi (Table 2), and we observed no males with feminization of the tail or germline. However, a highly penetrant gonad development defect, in which two gonad arms were formed instead of one, was present in most of the F1 males

A



B

Pair	% aa identity
Ce-FEM-2 vs Cb-FEM-2	56
Ce-FEM-2 vs Cr-FEM-2	59
Ce-FEM-2 vs CB5161-FEM-2	58
Cb-FEM-2 vs Cr-FEM-2	61
Cb-FEM-2 vs CB5161-FEM-2	60
Cr-FEM-2 vs CB5161-FEM-2	58

Fig. 2. Comparison of *C. elegans* FEM-2 with putative orthologues from *C. briggsae*, CB5161, and *C. remanei*. **A** A multiple sequence alignment of the four FEM-2 sequences. Residues identical among all four sequences are shaded black, while similar residues are shaded gray. The PP2C domain region is outlined, and the residues thought to bind metal or phosphate ions are marked with diamonds (Das et al. 1996). The asterisks mark the positions of altered residues in the proteins produced by two temperature-sensitive loss-of-function alleles of *C. elegans fem-2*. The relative annealing positions of the two degenerate primers used to isolate the partial PP2C domains are shown as arrows. **B** Percentage identity of FEM-2 proteins when aligned individually.

arising from injected mothers (Table 2, Fig. 3). Several males also had a defect in the ventral hypodermis, which we interpret as a partially formed vulva (Fig. 3). These RNAi phenotypes appear to involve the development or attempted development of female structures in an XO animal and, thus, are consistent with *C. remanei fem-2* controlling some aspects of sex determination. As with *Cr-fem-3* RNAi (Haag et al. 2002), no germline feminization was observed with *Cr-fem-2*, although we were able to reproduce the highly penetrant embryonic phenotype with *Cr-glp-1* (Rudel and Kimble 2001) ($>99.9\%$ lethality; $n = 18$ injections), showing the ability of injected RNAi to act efficiently in the germline of *C. remanei*.

Table 2. Effects of RNAi against *fem-2* in *C. remanei*: Each row corresponds to a single experiment

Treatment (individuals)	F_1 males[a]	F_1 females[a]	Phenotypes of males[b]			Phenotypes of females[b]		
			WT	FS	FG	WT	MS	MG
fem-2 RNA (7)	342 (49%)	359 (51%)	14	101[c,d]	0	45	0	0
fem-2 RNA (4)	197 (53%)	176 (47%)	3	110[c]	0	80	0	0
None (5)	352 (49%)	364 (51%)	65	0	0	57	0	0

[a]Worms were classified on the basis of size and tail morphology, as seen under low magnification.
[b]Several worms from each sex were examined using DIC microscopy for signs of a feminized soma (FS), feminized germline (FG), masculinized soma (MS), or masculinized germline (MG). Worms with no defects were classified as wild type (WT).
[c]These worms had two-armed gonads and, sometimes, a partially formed vulva. The tail appeared to be wild-type male.
[d]Fifteen of these worms were moved to separate plates containing several wild-type adult females. The transferred worms attempted to mate with the females, but no progeny were produced.

In *C. elegans*, the FEM-2 and FEM-3 proteins interact and both are required for male development. Although they are evolving rapidly, some conserved motifs are apparent in each protein when the available orthologues are aligned (Fig. 2) (Haag et al. 2002). One possibility is that these conserved motifs are all that are needed for mediating an interaction between the two proteins. Another is that the divergence reflects a loss of the FEM-2/FEM-3 interaction in some species and that the conserved motifs have been maintained for some other reason (catalytic activity, for example). A third possibility is that FEM-2 and FEM-3 are coevolving. In an attempt to differentiate between these possibilities we used the yeast two-hybrid assay to test for interspecific and conspecific interactions between FEM-2 and FEM-3. Conspecific interactions were observed for all three species examined (Fig. 4A). Of the six interspecies combinations assayed, only two interactions were apparent, both involving *C. briggsae* FEM-3 (Fig. 4A). Each interacting pair was further examined using a quantitative reporter assay (Fig. 4B). Similar levels of reporter expression were observed for all the interacting pairs, with the notable exception of the conspecific *C. briggsae* interaction, which yielded significantly more β-galactosidase activity ($\alpha = 0.01$).

The interaction tests indicate that Ce-FEM-3 and Cr-FEM-3 bind specifically to their conspecific partners. In an attempt to isolate regions of the proteins involved in the FEM-3 interaction, additional two-hybrid assays were performed using truncated versions of Ce-FEM-2 and Cr-FEM-2, and interspecies domain swapping experiments were performed to determine which parts of FEM-2 convey species specificity. Only 2 of 14 truncated versions of FEM-2 interacted with FEM-3 (data not shown), and based on their sizes (12 residues missing from the carboxy end of Ce-FEM-2 and 15 residues missing from the carboxy end of Cr-FEM-2), little of Ce-FEM-2 and Cr-FEM-2 can be said to be unnecessary for FEM-3 binding. The absence of interactions with the larger deletions is not informative, since it could simply reflect improper protein folding or expression. In the domain swapping experiments, portions of the *C. elegans* and *C. remanei fem-2* cDNAs were swapped between two-hybrid constructs. The exchanged segments correspond approximately to the amino-terminal domain, PP2C domain, and carboxy-terminal domain of the proteins they encode. Ce-FEM-3 recognized the complete Ce-FEM-2 and Ce-FEM-2 containing the *C. remanei* carboxy terminal domain (Fig. 5). Similarly, Cr-FEM-3 interacted with the complete Cr-FEM-2 and Cr-FEM-2 containing the *C. elegans* carboxy terminal domain. Chimeric proteins containing an interspecies combination of the amino terminal and PP2C domains interacted very weakly or not at all with FEM-3 from either *C. elegans* or *C. remanei*. In contrast, interactions were seen between *C. briggsae* FEM-3 and all of the chimeric proteins (Fig. 5).

Discussion

The FEM-2 protein is evolving rapidly compared to two other PP2Cs (Stothard et al. 2002; this work). However, FEM-2 does not show as much divergence as FEM-3: between *C. remanei* and *C. elegans*, FEM-2 is 59% identical, while FEM-3 is just 31% identical (Haag et al. 2002). Many residues in the phosphatase domain of FEM-2 are conserved among PP2Cs in general (Stothard et al. 2002). Thus selection maintaining the phosphatase activity of FEM-2 likely accounts to some extent for the higher conservation of FEM-2. Indeed, when the PP2C domain is excluded from the pairwise comparison, Cr-FEM-2 and Ce-FEM-2 are 43% identical.

While protein interactions assayed using the yeast two-hybrid assay must be understood to be subject to the caveats of the assay method, the FEM-2/FEM-3 interaction originally described in *C. elegans* also appears to occur in *C. briggsae* and *C. remanei* (Fig. 2). The FEM-2/FEM-3 binding shows partial species specificity, as two of the six cross-species

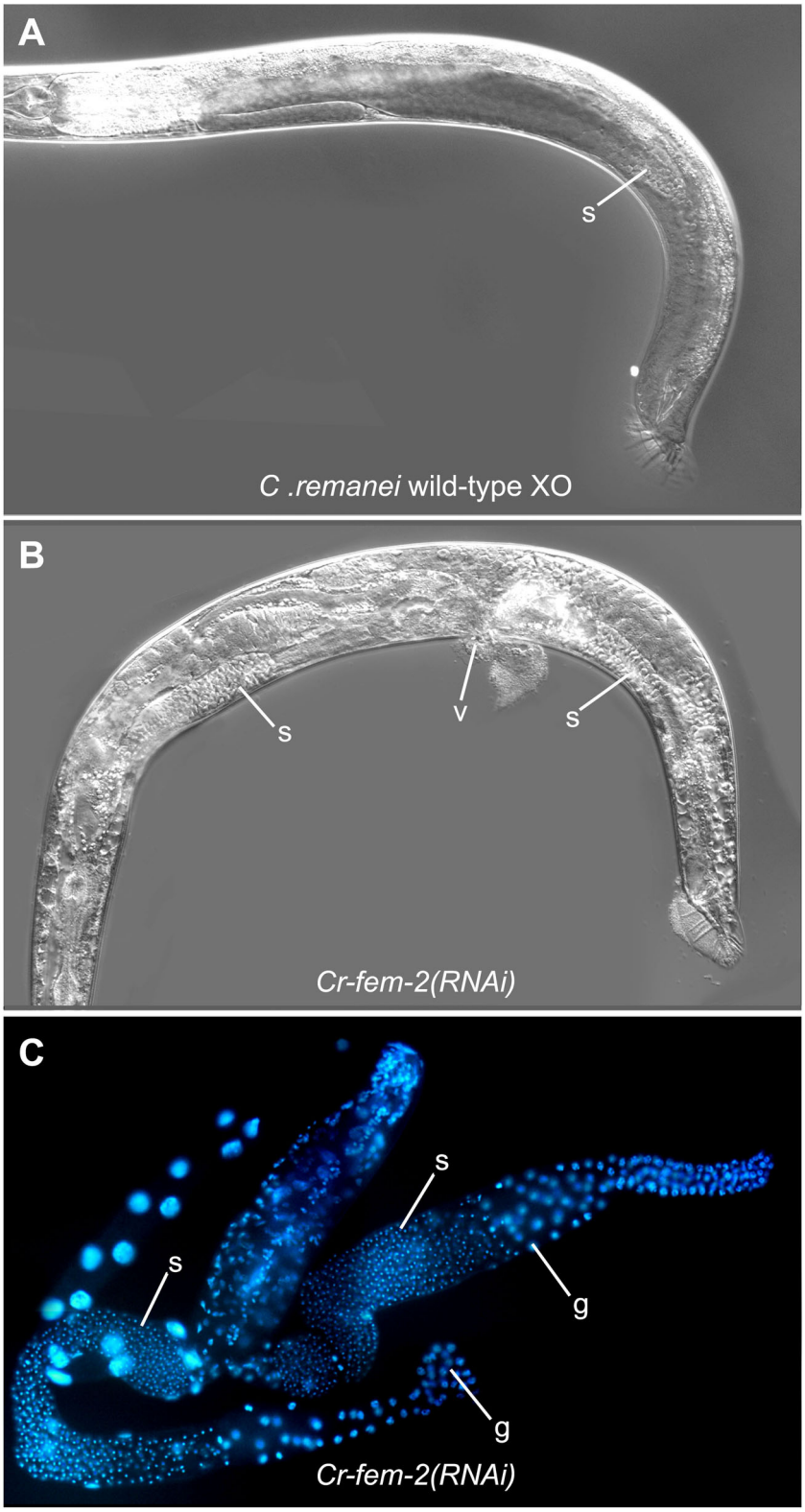


Fig. 3. Apparent feminization of the soma of *C. remanei* animals following RNAi against *fem-2*. **A** Wild-type *C. remanei* male with single-armed gonad. s, sperm. **B** Presumptive XO progeny of a female injected with *fem-2* dsRNA. Gonad development is abnormal, as there are two gonad arms containing sperm. The rupture of the ventral hypodermis is at a position correlating roughly with the position of the vulva in a wild-type XX animal. The tail fan and rays are apparently normal. s, sperm; v, vulva. **C** A worm similar to the one shown in A, but dissected and stained with DAPI. The two gonad arms are clearly visible, as are the sperm nuclei. s, sperm; g, gonad arm.

combinations of these proteins led to reporter expression in yeast two-hybrid assays. This apparent mixture of promiscuous and specific binding differs from what has been observed for other nematode sex-determining proteins. The TRA-1 and TRA-2 proteins interact in *C. elegans* and in *C. briggsae*, but not between species (Lum et al. 2000; Wang and Kimble 2001). Cross-species binding of FEM-3 to TRA-2 also does not occur, despite the maintenance of the interaction in *C. elegans*, *C. briggsae*, and *C. remanei* (Haag et al. 2002). From these other studies it is clear that compensatory coevolution of the binding part-

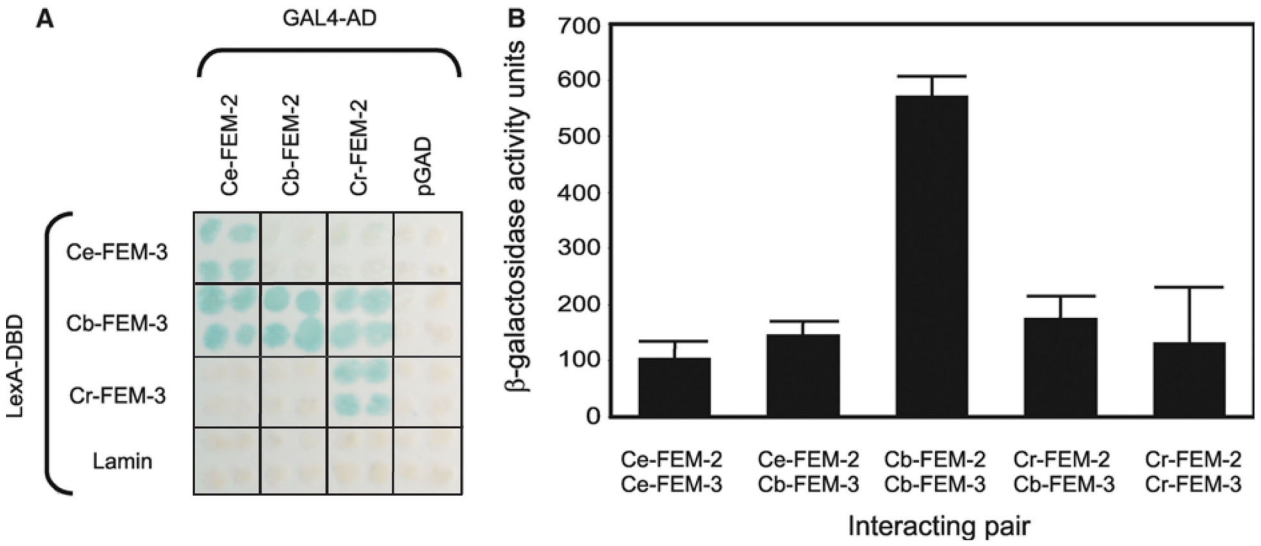


Fig. 4. Analysis of conspecific and interspecific interactions between FEM-2 and FEM-3 using the yeast two-hybrid system. **A** Yeast containing plasmids encoding FEM-2 fused to the GAL4 activation domain and FEM-3 fused to the LexA DNA-binding domain were tested for reporter gene expression using a β-galactosidase filter-lift assay. Plasmids encoding just the GAL4 activation domain or a Lamin-LexA DNA-binding domain fusion were used to test for nonspecific interactions. Four independent transformants were examined for each construct pair. **B** Quantitative β-galactosidase assays were performed for pairs showing a positive response in the qualitative assay. The mean value of four measurements is shown for each pair, with bars indicating standard deviation.

ners has occurred. Has coevolution shaped the FEM-2/FEM-3 interaction? In the absence of coevolution we would expect there to be complete interspecies compatibility between FEM-2 and FEM-3. Given that interspecies interactions do not occur in the majority of cases, and that conspecific interactions always occur, we believe that FEM-2 and FEM-3 are coevolving.

If we assume that FEM-2 and FEM-3 are coevolving, why are any interspecies interactions observed between these proteins? First, it is important to note that protein coevolution does not necessarily occur because there is selection for changes that prevent interspecies protein interactions. Sex-determination proteins that bind specifically to their conspecific partners are thought to contribute to hybrid sterility in nematodes (Baird 2002), and this hybrid sterility could serve as a source of reproductive isolation and speciation. However, as discussed by Haag et al. (2002), the parents of the sterile hybrids gain no fitness advantage, as their gametes are effectively wasted. Thus it is difficult to see hybrid sterility as a plausible selective force for sex-determination protein divergence. Instead, the rapid protein evolution may be occurring because of genomic conflict (Haag et al. 2002), with hybrid sterility arising as a consequence of independent sex-determining protein adaptations occurring in different lineages. Given that there may be no selection favoring species specificity of sex-determination proteins, interspecies interactions may still occur after extensive coevolution. In the case of FEM-2 and FEM-3, FEM-2 has several conserved motifs, many of which are likely required for phosphatase activity, and several of these appear to be located on the surface of the protein (Das et al. 1996). We hypothesize that during the coevolution of FEM-2 and FEM-3 in *C. briggsae*, Cb-FEM-3 began to interact with regions of Cb-FEM-2 that are evolutionarily constrained. This involvement of constrained regions of FEM-2 can explain why Cb-FEM-3 is able to interact with all of three natural FEM-2 proteins and all six of the FEM-2 chimeras (Fig. 5). In contrast, our results suggest that the FEM-3 proteins in *C. elegans* and *C. remanei* rely on portions of FEM-2 that are not well conserved, as both Cr-FEM-3 and Ce-FEM-3 bind only to FEM-2 proteins containing conspecific amino-terminal and PP2C domains (Figs. 1 and 5). Our results do not indicate that Cb-FEM-3 binds only to the conserved PP2C domain, since Cb-FEM-3 interacts more strongly with Cb-FEM-2 than with either Ce-FEM-3 or Cr-FEM-3 (Fig. 5). Another explanation for the observed results is that Ce-FEM-2 and Cr-FEM-2 are promiscuous. However, Ce-FEM-2 and Cr-FEM-2 do show some specificity, as they do not interact with all three of the FEM-3 proteins. Also, domain swapping experiments show that the conspecific *C. elegans* and *C. remanei* interactions each involve unique structural versions of FEM-2—pollute either FEM-2 with too much sequence from the other species and the interactions are abolished. Chimeras that no longer interact with Ce-FEM-3 or Cr-FEM-3 still interact with Cb-FEM-3. If we accept that Ce-FEM-2 and Cr-FEM-2 are promiscuous, then we would also

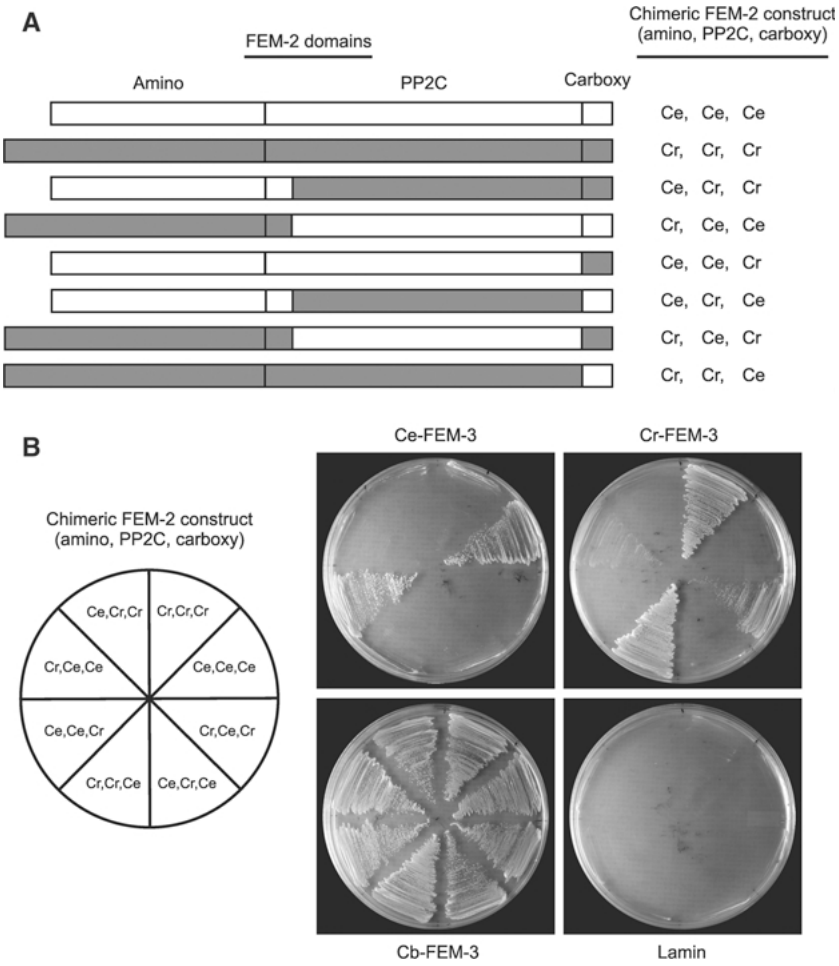


Fig. 5. Interaction of chimeric *C. elegans* and *C. remanei* FEM-2 proteins with FEM-3. **A** Schematic representations of the FEM-2 chimeric proteins that were tested. Protein segments from *C. elegans* are drawn as open rectangles, while protein segments from *C. remanei* are drawn as filled rectangles. Vertical lines divide the amino, PP2C, and carboxy regions of the proteins. **B** Selective medium growth assay for reporter expression. Chimeric *fem-2* constructs were transformed into yeast containing plasmids encoding full-length FEM-3 fused with the LexA DNA-binding domain. The resulting strains were grown on −Leu−Trp−His plates for 3 days. The two strains on the Cr-FEM-3 plate showing weak growth (Cr,Ce,Ce, and Cr,Ce,Cr) produced very faint staining in a β-galactosidase filter assay, while the strains showing more growth produced strong staining (data not shown). The strains showing no growth produced no staining in a β-galactosidase assay (data not shown).

be forced to believe that we succeeded in recombining their parts to make four independent nonpromiscuous FEM-2 proteins, all of which are specific for Cb-FEM-3.

A clearer picture of how Cb-FEM-3 differs from the other FEM-3 proteins in terms of how it binds FEM-2 will require structural comparisons of the proteins involved. Nevertheless, the possibility of rapid coevolution leading to changes in which regions of a protein participate in an interaction raises some intriguing scenarios. For example, if one of the proteins participating in an interaction contains a constrained domain that is part of a protein family, then coevolution might eventually lead to that domain being recognized by the binding partner. The binding partner might then be able to act on the other proteins in the same family to some extent. This type of coevolution could thus serve to promote the entry of proteins into new regulatory pathways. Another interesting possibility involves coevolving protein complexes. Consider a complex consisting of three proteins, A, B, and C, where A and B interact directly with C but not with each other. Changes in which regions interact because of coevolution could yield a complex in which A interacts directly with both B and C. This type of evolution could explain how parallel forms of regulation arise in pathways. In *C. elegans*, for example, FEM-3 and TRA-2 interact (Mehra et al. 1999), and TRA-2 interacts with TRA-1 (Lum et al. 2000; Wang and Kimble 2001), which is one of the presumed targets of the FEMs. A pathway in which TRA-2 bound to FEM-3, and FEM-3 bound to TRA-1, could have been modified as outlined above, such that TRA-2 developed the ability to bind with both proteins.

Several sex-determining genes have been shown to have conserved biological roles among *C. briggsae*, *C. remanei*, and *C. elegans*. RNAi against the *C. briggsae* orthologues of *tra-2* (Kuwabara 1996), *her-1* (Streit et al. 1999), and *fog-3* (Chen et al. 2001) produces phenotypes that closely resemble those observed in mutant *C. elegans* animals. Similarly, *Cr-tra-2(RNAi)* and *Cr-fog-3(RNAi)* animals resemble their *C. elegans* counterparts (Haag and Kimble 2000; Chen et al. 2001). In contrast, the RNAi phenotypes observed for the *fem* genes are not consistent across species. *Ce-fem-2(RNAi)* causes germline feminization in male and hermaphrodite animals, while *Cb-fem-2(RNAi)* feminizes the male germline but not the hermaphrodite germline (Stothard et al. 2002). While it is possible that the efficiency of RNAi differs between

species, or between the soma and germline within a species, there is increasing evidence for different roles between species for proteins that are necessary for sex determination in *C. elegans*. As an extreme example, *C. briggsae* lacks a *fog-2* orthologue, and the GLD-1 protein, which in *C. elegans* interacts with FOG-2 to control *tra-2* translation in the germline, has an RNAi phenotype in *C. briggsae* which is opposite to that seen in *C. elegans* (Nayak et al. 2005). A *C. briggsae fem-2* deletion produces mutant XX animals that develop into normal hermaphrodites, while mutant XO animals are feminized (R. Hill and E. Haag, personal communication). *Cb-fem-3(RNAi)* and *Cb-fem-1(RNAi)* also fail to interfere with hermaphrodite spermatogenesis (reviewed in Stothard and Pilgrim 2003). The *C. remanei fem-2(RNAi)* phenotypes observed in this study resemble those described for *Cr-fem-3(RNAi)* (Haag et al. 2002) in that both lead to two-armed gonads and vulva-like structures but not to germline feminization. Thus although we can conclude that *fem-2* regulates some aspects of *C. remanei* sex determination, these findings raise the possibility that *fem-2* and *fem-3* have a reduced role in the germline. One possibility, which we have discussed previously (Stothard et al. 2002), is that the TRA-2/TRA-1 is used instead of the FEM complex in the germline of *C. briggsae* and *C. remanei*. Since the phylogenetic relationships of these three species suggest that hermaphroditism evolved independently in *C. elegans* and *C. briggsae* (Kiontke et al. 2004), parsimony suggests that a role for the FEM proteins in germline sex determination may be a derived trait in *C. elegans*. Such a modification could be imagined to occur as a consequence of the same selective forces that led to the rapid protein coevolution. However, RNAi phenotypes need to be interpreted with caution, as they may be less severe than those obtained with mutations, and a clearer picture of how the sex-determining pathways differ between species will require genetic analysis. Both forward and reverse genetic screens are being used to obtain *C. briggsae* sex-determining gene mutations, and reverse screens should soon be feasible in *C. remanei*.

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