ORIGINAL ARTICLE

Cryptic intraspecific variation in sex determination in *Caenorhabditis elegans* revealed by mutations

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Sex determination mechanisms (SDMs) show striking diversity and appear to evolve rapidly. Although interspecific comparisons and studies of ongoing major transitions in sex determination (such as the establishment of new sex chromosomes) have shed light on how SDMs evolve, comparatively little attention has been paid to intraspecific variation with less drastic effects. In this study, I used mutant strains carrying a temperature-sensitive sex determination mutation, along with a second null mutation, in different wild genetic backgrounds to uncover hidden variation in the SDM of the model nematode *Caenorhabditis elegans*. I then used quantitative trait locus (QTL) mapping to begin to investigate its genetic basis.

I identified several QTLs, and although this variation apparently involved genotype-by-temperature interactions, QTL effects were generally consistent across temperatures. These QTLs collectively and individually explained a relatively large fraction of the variance in tail morphology (a sexually dimorphic trait), and two QTLs contained no genes known to be involved in somatic sex determination. These results show the existence of within-species variation in sex determination in this species, and underscore the potential for microevolutionary change in this important developmental pathway.

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Introduction

Although reproduction with two distinct sexes is taxonomically widespread, the mechanisms that determine an individual's sex are remarkably diverse across species. At the highest level, sex determination mechanisms (SDMs) can be divided into two major categories: those in which the organism's sex is determined by its genotype (genotypic sex determination), and those in which sex is determined by some environmental input during development (environmental sex determination). There is great variation within these categories in the most upstream determiner of sex. There are genotypic sex determination systems, for instance, relying on different types of sex chromosomes as in mammals and birds (Haag and Doty, 2005), systems in which sex determination seems to be influenced by many genes (Vandeputte *et al.*, 2007), and systems in which one sex is haploid and the other diploid (Cook and Crozier, 1995). Similarly, environmental sex determination systems use an array of different environmental variables, including population density and host crowding (Blackmore and Charnov, 1989), light availability (Korpelainen, 1998), and perhaps most commonly, temperature (temperature-dependent sex determination; Janzen and Phillips, 2006).

Within these types of SDMs lies even more diversity, in the genetic pathways that translate the upstream

sex-determining factor into a signal triggering male or female development. Many dipterans, for example, possess an XX/XY sex chromosome system (Sanchez, 2008), but although the most downstream gene in this pathway, *doublesex*, is conserved, there are important differences among species elsewhere in the pathway. For example, sex-specific splicing of *tra* is regulated by *Sxl* in *Drosophila* (Cline and Meyer, 1996) but is directed by *tra* itself in an auto-regulatory feedback loop in *Ceratitis* (Pane *et al.*, 2002) and *Musca* (Hediger *et al.*, 2010), with *Sxl* lacking a sex-specific function in both of these species (Meise *et al.*, 1998; Saccone *et al.*, 1998).

From these types of interspecific pathway comparisons as well as phylogenetic and theoretical studies, we gained some key insights into the evolution of SDMs. First, changes in SDMs may occur commonly and easily, at least in some clades. For example, different genotypic sex determination and temperature-dependent sex determination mechanisms are phylogenetically scattered in reptiles (Janzen and Phillips, 2006) and teleost fishes (Mank *et al.*, 2006), indicating frequent and independent transitions. Second, genes involved in sex determination evolve rapidly and often coevolve with one another. This conclusion is supported by huge sequence divergence among related species in these genes (Whitfield *et al.*, 1993; de Bono and Hodgkin, 1996) and functional analyses (Haag *et al.*, 2002). Finally, changes to SDMs typically occur in the upstream portions of the pathway (Wilkins, 1995; Pomiankowski *et al.*, 2004), as these show more divergence among species, whereas downstream components are more conserved (Raymond *et al.*, 1998).

However, to appreciate more fully how SDMs change over time, an understanding of both between-species divergence and within-species variation is necessary.

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Although earlier work on intraspecific variation and the microevolution of SDMs (Bull and Charnov, 1977) was largely theoretical and abstract because of a lack of molecular understanding of most SDMs (Pomiankowski *et al.*, 2004), great strides are rapidly being made in this area. For example, Hediger *et al.* (2010) recently showed that gain-of-function alleles of the *Musca domestica tra* ortholog correspond to the dominant feminizing factors segregating in some populations. Similarly, various XX/XY and ZZ/ZW SDMs involving LG (linkage group)5 and LG7 occur in different species of Lake Malawi cichlids, which have diversified only in the last million years; in some cases, sex is even determined by an epistatic interaction between LG5 and LG7 (Ser *et al.*, 2010). The maintenance or fixation of these types of sex-determining variants is typically attributed to various types of selection (Kozielska *et al.*, 2009; Roberts *et al.*, 2009).

One commonality in these and other cases of intraspecific variation in SDMs (Kallman, 1968; Ogata *et al.*, 2008) is that they involve variants with major effects, such as novel sex-determining genes or chromosomes, or taxa in which related species show very different SDMs (for example, medakas; Takehana *et al.*, 2008). Thus, there is evidence that SDM evolution is often characterized by major and rapid changes. However, what about species not currently experiencing such extreme changes; are they in complete stasis or do SDMs change gradually as well? Intraspecific SDM variation of smaller effect has received comparatively little attention, perhaps because of an ascertainment bias toward variants with drastic effects. However, gradual or cumulative change should not be dismissed completely just yet. For example, heritable quantitative variation has been shown to occur in sex ratio reaction norms in several species with environmental sex determination (Blackmore and Charnov, 1989; Rhen and Lang, 1998), although its genetic basis is unknown and effective heritability in the wild may be low. In addition, minor-effect alleles may be masked in genotypic sex determination species if SDMs show some degree of genetic robustness, allowing the underlying pathway to evolve (True and Haag, 2001). In fact, other developmental signaling pathways have been shown to evolve even without any change in the final phenotypic output, such as the signaling network underlying vulval development in nematodes (Félix, 2007; Zauner and Sommer, 2007; Milloz *et al.*, 2008). Moreover, strong alleles may not necessarily be the result of a single mutation, but rather assembled cumulatively by standing variation and successive minor-effect mutations (Rebeiz *et al.*, 2009). Finally, certain patterns in SDM evolution may not be fully explained by this model of swift and significant changes in master sex determiners. For example, coevolution among interacting sex-determining genes, even intermediate and downstream genes, occurs without changes in the roles of the genes, their interaction or in the master sex-determining signal itself (Haag *et al.*, 2002).

In this study, I investigated variation in sex determination in *Caenorhabditis elegans*, a model nematode 'worm'. This is an ideal study species because its sex determination cascade has been well characterized by years of study (reviewed in Wolff and Zarkower, 2008). In wild-type worms, sex is determined by sex chromosomes; males are XO, whereas hermaphrodites, which are

essentially females that produce a limited supply of sperm early in development, are XX. By examining the joint quantitative effects of specific mutations in two key sex determination genes (Chandler *et al.*, 2009) in different genetic backgrounds, I uncovered a cryptic intraspecific variation in the sex determination pathway. I then used quantitative trait locus (QTL) mapping to begin to investigate its underlying genetic basis and microevolutionary potential, with the goal of addressing the following questions: (1) How many loci are involved, and what are the magnitudes of their effects? (2) Do any QTLs lack candidate genes with known roles in sex determination? and (3) Do QTLs influence overall pathway activity, with effects consistent across environments (in this case, temperatures), or are they more complex, with genotype-by-environment interactions? I conclude by discussing the implications of these findings for our understanding of how SDMs evolve.

Materials and methods

Strains and strain construction

Strain CB5362 *tra-2(ar221ts)II*; *xol-1(y9)X* (Hodgkin, 2002; Chandler *et al.*, 2009) consists of XX worms, which are primarily hermaphrodites at permissive temperatures (< ~16 °C) and males at warmer temperatures (> ~20 °C). At intermediate temperatures, tail phenotypes are variably intersexed (Figure 1). This strain was originally constructed with the N2 genetic background (Brenner, 1974). I introgressed these two mutations into the genetic backgrounds of four additional wild isolates, chosen for their molecular, phenotypic and geographic diversity (Milloz *et al.*, 2008): CB4856 from Hawaii, MY2 from Germany, AB1 from Australia and JU258 from Madeira (Hodgkin and Doniach, 1997; Barriere and Félix, 2005; Haber *et al.*, 2005).

I backcrossed the *tra-2(ar221)* and *xol-1(y9)* alleles into each genetic background separately, selecting for each mutation with a combination of molecular and phenotypic markers, for 10 generations with CB4856, and for 4 generations with MY2, AB1 and JU258. After backcrossing, I crossed the *tra-2* and *xol-1* introgression lines, selfed the offspring and selected double mutants with each new genetic background.

I performed QTL mapping using the temperature-sensitive lines with N2 and the CB4856 genetic backgrounds, as they display many single-nucleotide polymorphisms and are commonly used for mapping (Gutteling *et al.*, 2007; Seidel *et al.*, 2008). To construct recombinant inbred lines (RILs), which are available from the author upon request, for QTL mapping, I first performed reciprocal crosses between these strains. I picked F1 offspring at 13 °C, allowed them to self-fertilize and oviposit and used a PCR marker flanking an indel polymorphism on chromosome III to verify heterozygosity of cross-progeny. I picked F2 offspring to individual plates, subsequently transferring 1–4 offspring to new plates for a total of 10 generations. At the F10 generation, RILs were grown to large population sizes to obtain working cultures and frozen stocks.

Phenotypic assays

I scored each temperature-sensitive strain as well as wild-type male and hermaphrodite N2 worms as

Figure 1 Scoring system for tail morphology. 1 = wild-type hermaphrodite; 2 = tail whip is present but shortened or masculinized; 3 = tail whip is absent but tail retains overall hermaphrodite shape; 4 = tail has blunted, male-like shape but lacks fan and rays; 5 = fan or rays present but reduced in size or number; 6 = wild-type male. A full color version of this figure is available at the *Heredity* Journal online.

controls, for both gonadal sex ratio and tail phenotype at four rearing temperatures (24, 18, 16 and 13 °C), using standard bleaching protocols to obtain age-synchronized populations (with approximately 150–200 worms each) for assays. At adulthood, I mounted worms onto glass slides (typically containing 20–50 worms per slide) with 25 mM NaN₃ for Nomarski microscopy, using standard methods (Shaham, 2006). I photographed all worms using a Zeiss Axioplan II (Zeiss, Thornwood, NY, USA) with differential interference contrast optics within a few hours of mounting.

I cropped individual worms from digital images and scored them for gonadal sex and tail phenotypes blindly with regard to strain and rearing temperature. Gonadal sex was scored as a binary trait (0 = hermaphrodite, 1 = male). Hermaphrodites were identified by their distinctive two-armed gonad morphology and/or the presence of oocytes within the adult worm, whereas males were recognized by their one-armed gonads and lack of oocytes. Worms with gonads that were abnormal, unclear or ambiguous (~5% of the total) were excluded from sex ratio analyses. Tail morphology was scored on a semi-quantitative scale from 1 to 6 (Figure 1). A score of 1 was given to worms indistinguishable from wild-type hermaphrodites, with a fully developed tail spike. A score of 2 was assigned to worms with an abnormal but still hermaphrodite-like tail, indicated by a truncated (but still present) tail spike and/or slight swelling at the base of the tail. Worms with more intersexed tail morphology, lacking a tail spike altogether but also lacking a flattened male shape, were given a score of 3. A score of 4 was assigned to individuals with a more male-like tail shape, but without the fan and rays characteristic of normal male tails. A score of 5 was given to worms having a male-shaped tail with at least some of the male characteristics such as a fan or rays, but in which these features were reduced in number or size. A score of 6 was reserved for worms with tails indistinguishable from those of wild-type males, complete with full fan, rays and spicules. To confirm the repeatability of the rating scale, I blindly rescored a subset of worms and calculated

Cohen's κ with quadratic weightings, a statistic commonly used to measure repeatability in ordinal ratings (Banerjee *et al.*, 1999).

Genotyping

I washed worms off stock cultures in deionized water into 1.5 ml tubes, centrifuged them, removed the supernatant and froze the pelleted samples at –80 °C. I extracted DNA from thawed tissue samples using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's directions (Qiagen, Valencia, CA, USA). DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and samples were diluted to 10 ng μ l⁻¹. RIL genotyping at 90 single-nucleotide polymorphisms (Supplementary Table S1) was performed using the Sequenom MassARRAY system (Sequenom, San Diego, CA, USA) at the Iowa State University Genomic Technologies Facility.

Analyses

To test the effects of rearing temperature and genetic background on gonadal sex ratio in CB5362 and the introgression lines, I compared nested models using hierarchical likelihood ratio tests. For sex ratio, I evaluated a series of logistic models to test the effects of temperature, strain and strain-by-temperature interaction. For tail morphology, I used ordered logistic models, an extension of logistic models allowing for more than two categories in the response variable, to test the effects of temperature, strain and two different strain-by-temperature interactions. Each ordered logistic model was composed of a series of five parallel logistic models (because there are six possible tail scores), with each logistic model j expressing the probability that an individual's tail score is greater than j . Maximum likelihood values and parameter estimates for each model were obtained using the 'optim' function in R v2.10.0 (R Development Core Team) using the BFGS (Broyden–Fletcher–Goldfarb–Shanno) search algorithm.

I performed QTL mapping using the software package R/qtl v1.14-2 in R (Broman *et al.*, 2003), using gonadal sex ratio and tail scores each at four rearing temperatures. To look for QTLs with effects on reaction norm shape, I also performed QTL analyses on tail reaction norm slopes from 13 to 16 °C, and from 16 to 18 °C. Strain means were used as the phenotypic values. I first checked the genotype data for evidence of segregation distortion and disagreement with the publicly available genetic map (WormBase web site, <http://www.wormbase.org>, release WS208). I performed an initial exploratory QTL analysis by interval mapping. Subsequently, I used the 'stepwiseqtl' function to search for additive QTLs and compare multiple-QTL models. The latter approach compares models using logarithm of odds (LOD) scores, imposing a penalty for the inclusion of additional QTLs, and is advantageous because it allows one to test the effects of multiple QTLs simultaneously, offering increased detection power (Broman and Sen, 2009). Penalties were computed from 1000 permutations of the 'scantwo' function. For each QTL, 1.5-LOD confidence intervals were estimated using the 'lodint' function, and effect estimates and the proportion of variance explained were both estimated using 'fitqtl'. All analyses were conducted using multiple imputation with 64 draws.

Results

Variation among genetic backgrounds

A total of 711 worms from CB5362 and the 4 introgression lines, and 9336 worms from the RIL population were examined (average $n = \sim 35$ for each temperature by strain combination). All control hermaphrodite N2 worms were assigned tail scores of 1, and all male N2 worms were given tail scores of 6. Repeatability of tail scores was very high (weighted $\kappa = 0.97$, $n = 368$).

The 'Temp + Strain' model best explained variation in the gonadal sex ratio (Table 1; Figure 2), indicating that sex ratio reaction norms varied among strains in their horizontal positions, but not shapes. The 'Temp + Strain +

I1 + I2' model was selected as the best to explain variation in tail scores (Table 1; Figure 2), suggesting that tail morphology reaction norms in different strains were not simply horizontally displaced as a whole, but rather that reaction norm shape and the distribution of tail scores differed among strains as well, indicating a temperature-by-strain interaction.

Figure 2 Plots of (a) gonadal sex ratio and (b) mean tail morphology score as a function of rearing temperature for *tra-2(ar221); xol-1(y9)* strains with different genetic backgrounds.

Table 1 Hierarchical likelihood ratio tests comparing models evaluated to explain variation in sex ratio and tail morphology in temperature-sensitive *Caenorhabditis elegans* mutants

Model name	Model	$-\log(L)$	χ^2	d.f.	P-value
Sex ratio					
Null	$P(\text{male}) = a$	266.2	—	—	—
Temp	$P(\text{male}) = 1/(1+\exp(-(a+bT)))$	27.7	477.0	1	9.8×10^{-106}
*Temp+Strain	$P(\text{male}) = 1/(1+\exp(-(a_i+bT)))$	12.9	29.7	4	5.6×10^{-6}
Temp+Strain+I	$P(\text{male}) = 1/(1+\exp(-(a_i+b_iT)))$	9.9	6.0	4	0.2
Tail score					
Null	$P(\text{tail} > j) = a_j$	597.6	—	—	—
Temp	$P(\text{tail} > j) = 1/(1+\exp(-(a_j+bT)))$	219.3	756.6	1	1.4×10^{-166}
Temp+Strain	$P(\text{tail} > j) = 1/(1+\exp(-(a_j+bT+h_i)))$	197.5	43.5	4	8.0×10^{-9}
Temp+Strain+I ₁	$P(\text{tail} > j) = 1/(1+\exp(-(a_{ij}+bT)))$	163.1	68.8	16	1.6×10^{-8}
*Temp+Strain+I ₁ +I ₂	$P(\text{tail} > j) = 1/(1+\exp(-(a_{ij}+b_iT)))$	153.8	18.7	4	9.0×10^{-4}

Abbreviation: d.f., degree of freedom.

Sex ratio models are logistic models expressing the probability of a particular individual of strain i being a male; a , a_i , b and b_i are parameters, and T is the rearing temperature. For tail score, I used ordinal logistic models (essentially, a series of parallel logistic models) to express the probability of a particular worm of strain i having a tail score greater than j (where $j = 1, 2, 3, 4$ or 5 , as the tail score can range from 1 to 6). a_{ij} , a_j , b_i , b and h_i are parameters, and T is temperature. Each model is compared with the one above it; P -values < 0.05 indicate that the model performs significantly better than the preceding one. Asterisks (*) indicate the best model for each trait selected by likelihood ratio tests.

QTL analyses

In total, 157 RILs were generated; a subset of 66 was both phenotyped (Figure 3) and genotyped (Supplementary Table S2); of the 90 original markers, 66 were informative. The range of phenotypes in the RILs exceeded that of the parental lines, except at 24 °C (Figure 3). A predominance of RILs with more feminized phenotypes, particularly at 18 °C, suggests there may have been unintentional selection during RIL construction. Although there were no apparent problems in the genetic map estimated from the genotype data, there were a few regions showing some segregation distortion (Table 2). One region of very strong segregation distortion on chromosome I probably resulted from incompatibility between the N2 and CB4856 alleles at the *zeel-1* and *peel-1* loci (Seidel *et al.*, 2008). Regions of modest segregation distortion on chromosomes III, IV and V may have been the result of inadvertent selection for hermaphrodites during RIL construction, as these regions all contained QTLs, and the overrepresented allele in each case was associated with more feminized phenotypes. Two additional markers on chromosome III and one on chromosome II showing distortion were not closely linked to any QTL but may have been the result of genotyping errors, indicated by the relatively large number of individuals missing genotypes at these markers.

A total of six distinct main QTLs was identified by model selection (Table 3). The only trait for which all of

these QTLs were evident simultaneously was the tail score at 16 °C. However, the QTL on chromosome IV was also evident for the tail phenotype at 13 and 18 °C and for sex ratio at 16 °C, and the QTL on chromosome I and one of the QTLs on chromosome V were also detected for the tail phenotype at 13 °C. No QTLs were detected for the tail score at 24 °C, sex ratio at 13 °C or 24 °C or for either measure of tail reaction norm shape.

The QTLs identified for sex ratio at 18 °C (Table 3) are probably statistical artifacts, for several reasons. The confidence interval for the QTL on chromosome I for this trait is very narrow and is entirely within the region of strong segregation distortion corresponding to the *zeel-1/peel-1* incompatibility (Table 2). Second, the two QTLs on chromosome IV are closely linked, in fact with overlapping confidence intervals, and are in repulsion; such a pattern can often provide a strong but spurious model fit, and therefore it is best not to consider models with more than one QTL between any two adjacent markers, as in this case (Broman and Sen, 2009). Finally, no models considering these three QTLs individually or in pairwise combinations performed better than the null model (all penalized LOD scores <0).

Although not all these QTLs are obvious in LOD profiles from standard interval mapping (Figure 4), this is expected because of the increased power of multiple-QTL models, as they test the effects of each putative QTL while accounting for variation due to other QTLs (similar to composite interval mapping). This scenario is particularly likely for my data, because one QTL (on chromosome IV) had very large effects that would obscure the signal for the QTLs of modest effect during interval mapping. Importantly, composite interval mapping (Wang *et al.*, 2007) produced similar results and did not affect overall conclusions, suggesting that my findings are robust to the particular choice of statistical models for QTL mapping (for a discussion of the differences between these two methods, see Broman and Sen, 2009).

Discussion

To understand how SDMs evolve, we need to investigate variation in these pathways within species, in addition to divergence among species. Most previous studies on SDM microevolution have focused on individual or closely related groups of species segregating alleles with extremely large effects, such as loss-of-function alleles or dominant male- or female-determining factors, and which may be undergoing major transitions to new SDMs (Hediger *et al.*, 2010; Ser *et al.*, 2010). In contrast, in this study, I characterized variation in the SDM of *C. elegans*, an important model organism which is unlikely to be currently experiencing such a radical transition.

My results show that the joint effects of the *tra-2(ar221)* and *xol-1(y9)* alleles on gonadal sex ratio and sexually dimorphic tail phenotypes vary subtly but significantly with the wild genetic background in which they occur. Moreover, the discovery of at least one QTL influencing both gonadal sex and tail morphology (Table 3; Figure 4) supports the idea that at least some of this variation is related to sex determination itself and not, say, genes influencing the differentiation of sex-specific tail phenotypes after sex has been determined. Interestingly, many

Figure 3 Plots of (a) gonadal sex ratio and (b) mean tail morphology score as a function of rearing temperature for recombinant inbred lines. The parental lines are shown in bold.

Table 2 Markers showing evidence of segregation distortion

Marker ID	Chromosome	Position (cM)	No. with missing genotype	No. with N2 allele	No. with CB4856 allele	P-value
CE1-159	I	-17.9	1	48	17	1.21×10^{-4}
CE1-242	I	-9.5	0	64	2	2.32×10^{-14}
pas2341	I	-3.7	0	47	19	5.68×10^{-4}
CE2-161	II	0.6	13	40	13	2.08×10^{-4}
CE3-104	III	-26.8	2	22	42	1.24×10^{-2}
CE3-109	III	-22.5	0	23	43	1.38×10^{-2}
CE3-122	III	-11.2	0	24	42	2.67×10^{-2}
CE3-198	III	13.2	30	31	5	1.47×10^{-5}
CE3-205	III	18.1	28	33	5	5.57×10^{-6}
uCE4-670	IV	-7.3	0	22	44	6.77×10^{-3}
CE4-120	IV	-3.8	1	20	45	1.93×10^{-3}
CE4-224	IV	-1.2	2	19	45	1.15×10^{-3}
uCE4-905	IV	1.4	2	16	48	6.33×10^{-5}
uCE4-1020	IV	3.6	1	14	51	4.45×10^{-6}
CE4-1	IV	5.1	2	18	46	4.65×10^{-4}
CE4-194	IV	8.6	2	18	46	4.65×10^{-4}
uCE4-1337	IV	11.9	7	18	41	2.75×10^{-3}
CE4-213	IV	14.8	1	18	47	3.22×10^{-4}
CE4-221	IV	16.4	2	20	44	2.70×10^{-3}
CE5-102	V	-20.0	0	46	20	1.37×10^{-3}
CE5-128	V	-12.8	2	44	20	2.70×10^{-3}
CE5-134	V	-8.0	13	46	7	8.46×10^{-8}
CE5-139	V	-5.0	0	49	17	8.18×10^{-5}
CE5-147	V	-1.9	4	46	16	1.39×10^{-4}
CE5-228	V	8.3	0	44	22	6.77×10^{-3}

Table 3 Positions and 1.5-LOD confidence intervals, LOD scores, effect estimates, percentage of variance explained and potential candidate sex-determining genes of loci identified in QTL mapping

Trait/temperature	Chromosome	Position (cM)	CI—Min (cM)	CI—Max (cM)	Max LOD	Effect	% Variance explained	SD candidates
Tail/13 °C	I	2.7	-1.9	7.1	4.9	-0.3	12.2	<i>gld-1*</i> <i>fog-3*</i> <i>rpn-10*</i>
	IV	12.7	9.7	14.7	12.5	-0.65	12.5	<i>tra-3</i>
	V	10.0	6.0	12.0	7.0	0.43	7.0	<i>sd-3</i>
Tail, 16 °C	I	5.0	0.1	9.1	6.0	-0.32	11.6	<i>gld-1*</i> <i>fog-3*</i> <i>rpn-10*</i>
	II	-8.6	-11.6	1.4	4.2	-0.22	7.7	<i>sea-1</i> <i>fkh-6</i> <i>mog-5*</i> <i>fbf-1*</i> <i>fbf-2*</i> <i>tra-2</i> <i>nos-2*</i> <i>gld-3*</i>
								<i>fem-2</i>
								<i>tra-3</i>
								—
								<i>oma-2*</i>
								<i>her-1</i>
								<i>sd-3</i>
								<i>sel-10</i>
								<i>fem-1</i>
								<i>oma-1*</i>
								<i>fem-3</i>
								<i>tra-3</i>
	III	-26.8	-26.8	-19.8	3.8	-0.25	6.8	—
	IV	10.7	9.7	14.7	16.4	-0.74	48.3	—
	V	-20.0	-20.0	-13.0	6.1	0.35	11.9	—
	V	15.0	-4.0	22.4	3.2	0.21	5.6	—
Tail, 18 °C	IV	11.9	-9.0	16.4	3.2	-0.67	20.0	<i>oma-2*</i> <i>her-1</i> <i>sd-3</i> <i>sel-10</i> <i>fem-1</i> <i>oma-1*</i> <i>fem-3</i> <i>tra-3</i>
Sex ratio, 16 °C	IV	9.7	-17.3	16.4	2.6	-0.018	16.8	<i>fem-1</i> <i>oma-1*</i> <i>fem-3</i> <i>tra-3</i>
Sex ratio, 18 °C	I	-9.5	-9.9	-8.9	5.5	0.28	28.4	—
	IV	8.7	6.7	10.7	3.7	0.23	17.7	—
	IV	10.7	9.7	11.7	4.9	-0.27	24.7	—

Abbreviations: CI, confidence interval; LOD, logarithm of odds; QTL, quantitative trait locus; SD, sex determining.

Effect indicates the effect of the CB4856 allele relative to the N2 allele. Asterisks (*) denote genes currently known to be involved in germline sex determination only.

Figure 4 LOD plots from exploratory interval mapping (IM) analysis of tail morphology and sex ratio at four rearing temperatures and of tail reaction norm slope in two temperature intervals, showing overall qualitative similarity for sex ratio and tail phenotypes across temperatures. Horizontal dashed lines indicate significance thresholds for IM computed by permutation. Sex ratio at 13 °C is omitted because there was no variation in sex ratio among RILs at that temperature. QTL significance (Table 3) was then assessed using a more powerful multiple-QTL model selection approach.

RILs showed more extreme phenotypes than either parental line, similar to the results of Zauner and Sommer (2007). This pattern of transgressive segregation is not uncommon (Rieseberg *et al.*, 1999, 2003) and can be explained by, among other mechanisms, the co-occurrence of antagonistic QTL alleles within each parental line, which then recombine to produce more extreme phenotypes in the F₂ and later generations. In this case, the transgressive phenotypes were biased in one direction (Figure 3), specifically toward hermaphrodite-skewed sex ratios and feminized tail morphology. This bias may reflect an unintentional selection for less masculinized hermaphrodites during RIL construction, consistent with patterns of segregation distortion at markers linked to QTL (Tables 2 and 3).

The presence of a genotype-by-environment ($G \times E$; in this case, the environmental variable is temperature) interaction for tail phenotype (Table 1; Figure 2) suggests that this variation is not merely due to differences in overall pathway activity, but rather due to variation in how sex-determining genes are regulated. In addition, although variation in transcription levels of sex-determining genes has been documented in *Drosophila melanogaster* (Tarone *et al.*, 2005), translational and posttranslational regulation both have crucial roles in sex determination in *C. elegans* (Clifford *et al.*, 2000; Sokol and Kuwabara, 2000) and therefore may be important components of this variation. QTL mapping showed a small handful of loci underlying this variation, individually explaining moderate to large proportions (up to ~50%) of the among-strain variance in tail morphology and sex ratio at any particular temperature (Table 3). Thus, although this variation in *C. elegans* is less

obvious compared with other species with known SDM polymorphisms, as it is only revealed in the context of a mutant genotype, its genetic basis is probably not too complex to dissect further with high-resolution mapping and candidate gene studies.

Some QTLs had apparent effects at multiple temperatures. Although finer mapping is necessary to completely exclude the possibility that the seemingly common QTLs at different temperatures are distinct but closely linked, this observation suggests that these QTLs have effects that are generally consistent across environments, particularly the QTL with the largest effects, and therefore may reflect differences in overall pathway activity. Other QTLs had effects that were temperature-specific, suggesting possible regulatory differences among these genetic backgrounds. However, qualitative similarity of LOD profiles across temperatures for both tail morphology and sex ratio (Figure 4) is also suggestive of common effects that were simply too weak to be detected in some cases. Moreover, despite the presence of $G \times E$ interactions among the five genetic backgrounds (Table 1) and apparently substantial variation for reaction norm slope between 16 and 18 °C (Figure 3) among the RILs, no QTLs influencing the reaction norm slope were detected. This finding implies that the genetic architecture of the reaction norm shape may differ from that of the reaction norm intercept (and hence overall pathway activity or expression), perhaps distributed across more loci with effects too small to be identified in this study. Whether this conclusion applies to other reaction norms (such as thermal sex ratio reaction norms in reptiles with temperature-dependent sex determination) remains to be tested. In contrast, however, several QTL mapping

studies of phenotypic plasticity and $G \times E$ interactions in both morphological and life history traits have detected QTLs influencing reaction norm slopes and with environment-specific effects (Gurganus *et al.*, 1998; Vieira *et al.*, 2000; Gutteling *et al.* 2007).

What conclusions can we draw from these findings? First, even if a species is not currently experiencing a radical transition between SDMs, such as the fixation of a novel male-determining factor or the conversion of an autosome to a sex chromosome, it may still harbor functional variation in its SDM. This observation suggests that in addition to major transitions in upstream sex-determining genes, SDMs may also be subjected to more gradual change in other parts of the pathway, for example, in the relative importance of redundant gene interactions. In *C. elegans*, for instance, *tra-2* regulates *tra-1* by two distinct manners: directly and through the *fem* genes; both are required for hermaphrodite spermatogenesis (Lum *et al.*, 2000). In *Caenorhabditis briggsae*, although, which evolved self-fertility independently, indirect regulation through *fem* intermediates is dispensable for spermatogenesis in hermaphrodites (Hill *et al.*, 2006). Moreover, both *fem-3* and *fem-2* are epistatic to *tra-1* in the *C. elegans* germ line, but only *fem-3* is in *C. briggsae* (Hill and Haag, 2009). Thus, these species have taken different paths to the same trait. Such observations have led to the proposal that the differential loss and strengthening of ancestral redundant gene interactions in separate lineages (driven by either drift or, probably in the case of this example, selection) caused some of the interspecific differences in SDMs that we see today (Ellis, 2006). Sex determination in *C. elegans* must involve such previously unknown, and probably minor or redundant, factors, and at least some of these are variable within this species. For example, two of the QTLs (on chromosomes I and V) influencing somatic sexual phenotypes contained no candidate genes with a known role in somatic sex determination (Table 3). It will be interesting to see whether these minor components are shared by other *Caenorhabditis* species; it is worth noting that qualitative similarities have been discovered between intraspecific and interspecific variation in the signaling pathways underlying *Caenorhabditis* vulva patterning (Félix, 2007; Milloz *et al.*, 2008) and in the basis of sexually dimorphic abdominal coloration in *Drosophila* (Kopp *et al.*, 2003).

In addition, the presence of this variation may help explain the coevolutionary change seen in interacting sex-determining genes. Coevolution is expected if amino-acid substitutions altering the function of one gene help promote the fixation of compensatory mutations in its interaction partners (Haag *et al.*, 2002). Although polymorphism in the sex-determining genes that have been examined in *C. elegans* is low (Graustein *et al.*, 2002; Haag and Ackerman, 2005), to illustrate this possibility, there is one nonsynonymous single-nucleotide polymorphism in *tra-3*, the function of which in sex determination is to activate TRA-2A protein by cleavage (Sokol and Kuwabara, 2000), and which is an excellent candidate for the strongest QTL identified in this study (Table 3). If this polymorphism affects the affinity of TRA-3 for TRA-2A and is responsible for selectable phenotypic variation, it could favor compensatory changes in TRA-2A structure. Interestingly, this single-nucleotide polymorphism mediates the influence

of temperature on body size in *C. elegans* (Kammenga *et al.*, 2007), suggesting that the initial changes to sex-determining genes might have pleiotropic effects that could promote their fixation.

Along those lines, the selective forces influencing the maintenance or fixation of these variants in SDMs are an important consideration. Previous theoretical and empirical work has suggested that sex ratio selection (Bulmer and Bull, 1982; Conover and Van Voorhees, 1990; Wilkins, 1995), sexual selection and sexual conflict (Pomiankowski *et al.*, 2004; van Doorn and Kirkpatrick, 2007), as well as parent-offspring conflict (Werren *et al.*, 2002) can drive the evolution of SDMs. This and other recent work examining the 'cryptic' evolution of developmental signaling pathways may offer additional hypotheses. Milloz *et al.* (2008) hypothesized that cryptic variation among strains of *C. elegans* in cell signaling related to vulva patterning (1) could be neutral, (2) might not be cryptic at all, but phenotypically exposed to selection in different environments or (3) may be a correlated response to selection on other traits affected by the same signaling pathways. Zauner and Sommer (2007) proposed that variation in cell-cell interactions involved in vulval patterning within and among *Pristionchus* species might be due to selection for robustness to developmental noise in different environments, similar to the second hypothesis mentioned above. These possibilities likely apply to this SDM variation as well. For example, many 'core' sex-determining genes have secondary roles in dosage compensation, meiosis, etc. (for example, *gld-1*; Francis *et al.*, 1995; Clifford *et al.*, 2000). Moreover, this type of cryptic variation may have evolutionary consequences, for example, on evolvability (de Visser *et al.*, 2003), and SDM and sex chromosome evolution in particular may contribute to post-zygotic reproductive isolation (Wade *et al.*, 1997; Baird, 2002).

This study illustrates how laboratory-generated mutations can be exploited to expose hidden variation in, and thus explore the microevolution of, sex determination, even in species that appear to lack such variation at first glance. I have shown that even taxa not currently undergoing major transitions in SDMs may harbor variation in sex determination, and that such hidden variation is thought to lead eventually to pathway divergence. Further investigation of the QTL identified in this study, such as fine mapping to pinpoint the causative genes and more detailed cellular analyses of phenotypes, should provide a better view of their molecular bases. Examining how these variants interact with one another, and whether they have any phenotypic effects in nonmutant genotypes, will also illuminate what selective forces, if any, influence their evolution.

Conflict of interest

The author declares no conflict of interest.

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