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OUTBREEDING DEPRESSION WITH LOW GENETIC VARIATION IN SELFING *CAENORHABDITIS* NEMATODES

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Theory and empirical study produce clear links between mating system evolution and inbreeding depression. The connections between mating systems and outbreeding depression, whereby fitness is reduced in crosses of less related individuals, however, are less well defined. Here we investigate inbreeding and outbreeding depression in self-fertile androdioecious nematodes, focusing on *Caenorhabditis* sp. 11. We quantify nucleotide polymorphism for nine nuclear loci for strains throughout its tropical range, and find some evidence of genetic differentiation despite the lowest sequence diversity observed in this genus. Controlled crosses between strains from geographically separated regions show strong outbreeding depression, with reproductive output of F1s reduced by 36% on average. Outbreeding depression is therefore common in self-fertilizing *Caenorhabditis* species, each of which evolved androdioecious selfing hermaphroditism independently, but appears strongest in *C.* sp. 11. Moreover, the poor mating efficiency of androdioecious males extends to *C.* sp. 11. We propose that self-fertilization is a key driver of outbreeding depression, but that it need not evolve as a direct result of local adaptation per se. Our verbal model of this process highlights the need for formal theory, and *C.* sp. 11 provides a convenient system for testing the genetic mechanisms that cause outbreeding depression, negative epistasis, and incipient speciation.

KEY WORDS: **Androdioecy, *C.* sp. 11, *C. briggsae*, *C. elegans*, genetic diversity, genetic incompatibilities.**

Intrinsic reproductive incompatibilities can derive from negative epistatic interactions of alternative alleles at different loci, and form the crux of genetic models of speciation (Coyne and Orr 2004). Within a species, allelic variation contributing to such strong negative epistasis is thought to occur at only low levels within populations (Phillips and Johnson 1998), although it can manifest as reduced fitness in crosses between subpopulations, known as outbreeding depression (Templeton 1986; Edmands 2002; Escobar et al. 2008). Populations that reveal outbreeding depression lie in a murky genetic space between a simple definition of a single interbreeding species and a simple definition of distinct biological species (Dudash and Fenster 2000), making them intriguing models for understanding the genetic architecture of epistasis, adaptation, and reproductive isolation. Any factor that drives strong genetic structure between subpopulations, including local adaptation, local inbreeding, and reduced dispersal, could exacerbate both extrinsic (environment-dependent; G × E) and intrinsic (environment-independent; G × G) outbreeding depression effects (Templeton 1986; Frankham et al. 2011). This can also lead to heterogeneity in the degree of outbreeding depression

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observed across different pairs of subpopulations analyzed (Waser et al. 2000; Bailey and McCauley 2006; Leimu and Fischer 2010), owing to distinct derived alleles occurring in each of them. In an incipient speciation context, this can be viewed as a source of heritable variation in reproductive isolation (Cutter 2012). Although much previous work on outbreeding depression has emphasized the role of extrinsic, genotype-by-environment factors (Edmands 2002), we will underscore intrinsic genetic effects in generating outbreeding depression.

Here we focus on negative effects of interpopulation crosses on the hybrid progeny. However, it is important to recognize that in many organisms hybrid vigor (heterosis) is seen in the F1 generation, potentially reflecting a masking of deleterious recessive alleles that have accumulated within the subpopulations (or overdominance). But this seeming beneficial effect of outbreeding can break down in F2 and later generations (Lynch 1991; Fenster and Galloway 2000; Edmands 2002). For example, in the copepod *Tigriopus californicus*, beneficial masking of single locus detrimental effects became overwhelmed by more severe negative epistatic interactions with increasing genetic distance of populations (Edmands 1999). In this and other organisms, the improved fitness of F1s belies outbreeding depression that only gets revealed in the F2 (Edmands 2002). Note that underdominance at individual loci, in the absence of epistasis, also could contribute to outbreeding depression (Schierup and Christiansen 1996; Kirkpatrick and Barton 2006).

Plant systems, for which self-fertilization is a common mode of reproduction, have been the subject of intense research on inbreeding depression (Jarne and Charlesworth 1993; Husband and Schemske 1996; Crnokrak and Roff 1999; Charlesworth 2003; Charlesworth and Willis 2009). But is mating system, and self-fertilization in particular, expected to be an important factor in observing outbreeding depression in a given species? Outbreeding depression certainly occurs in selfing species, such as a number of plants, for example, *Amphicarpaea bracteata* (Parker 1992), *Anchusa crispa* (Quilichini et al. 2001), *Silene vulgaris* (Bailey and McCauley 2006), the partially selfing freshwater snail *Physa acuta* (Escobar et al. 2008), and in the selfing nematodes *Pristionchus pacificus* (Click et al. 2009) and *Caenorhabditis elegans* (Dolgin et al. 2007). The strongly inbreeding haplodiploid beetle *Xylosandrus germanus* also shows outbreeding depression (Peer and Taborsky 2005). The expression of outbreeding depression is often tied to the genetic consequences of local adaption of subpopulations, and increased selfing is thought to promote local adaptation (Schmitt and Ehrhardt 1990; Charlesworth 1992; Dudash and Fenster 2000)—although other arguments emphasize a diminished propensity for adaptation by selfing species (Takebayashi and Morrell 2001). Indirect selection from outbreeding depression can favor more extreme selfing as a kind of assortative mating that reinforces local adaptation (Epinat and Lenormand 2009). An important feature of selfing is that it reduces genetically effective recombination, increasing linkage disequilibrium in populations and exacerbating epistatic interactions across the genome. Consequently, we may expect local adaptation in selfing species to yield linked combinations of alleles at different loci that work well together. Such, so-called coadapted gene complexes may be more pronounced in selfing populations (Templeton 1986; Dudash and Fenster 2000).

Local adaptation can evolve in an explicitly coevolutionary way involving multilocus selection favoring certain epistatically interacting combinations of pleiotropic alleles. These multilocus genotypes can become strongly linked by virtue of the restricted recombination afforded by selfing, as is typically implied by discussions of well-integrated genotypes comprising coadapted gene complexes (Dudash and Fenster 2000). Alternatively, epistatic interactions within selfing populations could arise as a by-product of local adaptation or by genetic drift, as through conditional neutrality, whereby a given allele confers a fitness benefit only under some conditions but is otherwise selectively neutral (Waser 1993; Mitchell-Olds et al. 2007; Anderson et al. 2013). Selfing species provide prime examples of adaptation involving conditional neutrality, and this mode of adaptation is hypothesized to predominate in highly structured populations as is often the case for selfing organisms (Anderson et al. 2013). In a similar way, accumulation of conditionally detrimental mutations by drift in small selfing populations could generate linked alleles that interact epistatically to retain population mean fitness within a given genomic context, but that yield reduced fitness when combined with a different genetic background. Selective sweeps could drive fixation of many such linked loci through genetic hitchhiking, as selfing renders large blocks of the genome susceptible to hitchhiking effects (Charlesworth and Wright 2001; Andersen et al. 2012; Cutter and Payseur 2013). Selfing imposes restricted recombination, analogous to the effects of inversions, so individually adapted allele fixation is a more likely scenario than fixation of simultaneously coadapted alleles (Kirkpatrick and Barton 2006). The distribution of dominance coefficients for alleles fixed by selection will include more recessive effects in selfing populations because such populations are less subject to "Haldane's sieve" (Charlesworth 1992; Ronfort and Glemin 2013). Upon fixation and subsequent crosses between populations, such alleles could contribute to either underdominant (strongest in F1s) or epistatic (strongest in F2s and later) causes of outbreeding depression. Strong linkage induced by selfing could generate greater abundance of associative over- (or under-) dominance effects due to gametic phase disequilibrium than in outcrossing species (Ronfort and Glemin 2013). In sum, these effects imply that selfing could foster outbreeding depression in interpopulation crosses. And yet, a simulation study indicated that an intermediate rate of self-fertilization would actually mitigate the magnitude of outbreeding depression relative to

pure outcrossing (Edmands and Timmerman 2003). Thus, there appears to be a disconnect between the predictions of verbal and simulation models, suggesting that additional empirical data will help motivate formal theory about the role of mating system in outbreeding depression.

It has also been predicted that larger populations (containing more genetic variation) will be more likely to give rise to outbreeding depression (Edmands and Timmerman 2003; Frankham et al. 2011), although others proposed that outbreeding depression will be more pronounced in small populations when within-population genetic variation is low (Templeton 1986; Escobar et al. 2008; Leimu and Fischer 2010). This is in addition to the more intuitive effect that genetic divergence (and geographic distance) between subpopulations should generally correlate positively with the strength of outbreeding depression (although an intermediate "optimal" outbreeding distance can occur under some circumstances; Lynch 1991; Edmands 2002). Species with high rates of self-fertilization tend to have lower genetic variation than their outbreeding relatives (Hamrick and Godt 1996; Glemin et al. 2006), so it is important to reconcile the independent expectations of mating system and genetic diversity for outbreeding depression.

To explore these outstanding issues surrounding outbreeding depression in the context of mating systems and genetic diversity, here we investigate *Caenorhabditis* nematodes. Most species of *Caenorhabditis* nematodes reproduce by obligatory outcrossing of males and females, but androdioecy, characterized by high inbreeding through self-fertilizing hermaphrodites, has evolved independently at least three times in the genus (Kiontke et al. 2011). The naturally outcrossing species, which retain exceptionally high population genetic variation (Cutter et al. 2013), suffer severe inbreeding depression when inbred under laboratory conditions (Dolgin et al. 2007; Barriere et al. 2009). By contrast, the selfing *C. elegans* and *Caenorhabditis briggsae* show either only weak inbreeding or outbreeding depression, depending on the particular genetic backgrounds used, with a general tendency toward outbreeding depression when genotypes from geographically separated regions are used in crosses (Barrière and Félix 2007; Dolgin et al. 2007, 2008; Seidel et al. 2008; Ross et al. 2011; Baird and Stonesifer 2012; Teotonio et al. 2012). Curiously, this outbreeding depression occurs despite the low species-wide genetic diversity of *C. elegans* and *C. briggsae* (Cutter et al. 2009; Andersen et al. 2012; Félix et al. 2013). Is this phenomenon universal so that it includes the recently discovered selfing species *C.* sp. 11? By analyzing *C.* sp. 11, here we test the hypotheses that all species of *Caenorhabditis* that evolved selfing hermaphroditism (i) have low species-wide genetic variability and (ii) exhibit outbreeding depression, in stark contrast to their congeners that retain the ancestral outbreeding mode of reproduction. We find that, indeed, outbreeding depression despite low genetic variation is a general feature of selfing species of *Caenorhabditis*, in conflict with some aspects of current theory (Edmands and Timmerman 2003; Frankham et al. 2011).

Methods

STRAIN ISOLATION AND MAINTENANCE

A complete list of examined *C.* sp. 11 strains (isolates), their origin, and sampling details are provided in Table S1. All strains except NIC203 (Guadeloupe) have previously been described in Kiontke et al. (2011) and Félix et al. (2013). Species identity was established by ITS2 sequencing and/or mating tests using known *C.* sp. 11 strains (Kiontke et al. 2011). All strains were founded by a single fourth-larval stage (L4) hermaphrodite and were amplified through selfing; strains can therefore be considered to be highly isogenic. Strains were maintained using standard protocols for *C. elegans* (Brenner 1974; Wood 1988) on 2.5% nematode growth medium (NGM) agar plates at 25°C, an optimal growth temperature for *C.* sp. 11 (our own unpublished observations). Dissecting stereomicroscopes at 40–100× magnifications were used to count larvae and dead embryos. All strains have been cryogenically preserved and can be requested from the authors.

SEQUENCE VARIABILITY: STRAINS, AMPLIFICATION, AND SEQUENCING

We collected polymorphism information at nine nuclear loci (Table S2) for 47–54 strains of *C.* sp. 11 from seven tropical localities, with most from the Nouragues Reserve, French Guiana (Table S1). An interactive map of *C.* sp. 11 collection localities and strain information is available online: http://labs.eeb.utoronto.ca/cutter/map. Four loci (p11, p12, p14, and p16) are orthologs of markers previously used in analysis of nucleotide diversity in *C. elegans* and *C. briggsae* (Cutter et al. 2006b; Félix et al. 2013). Orthologs were identified in the *C.* sp. 11 genome assembly (Genome Sequencing Center, Washington University, St Louis, unpubl. data) using the program TBlastN (Altschul et al. 1990). Specific primers were designed for the four orthologs and regions of five additional genes using the *C.* sp. 11 genome sequence (Table S2).

DNA was isolated from large populations of worms of each strain using the DNeasy Blood and Tissue kit (Qiagen, Venlo, The Netherlands). Amplifications were processed in 30 μL reaction volumes with 1.5 μL DMSO, 3 μL dNTPs (6.6 mM), 3 μL 10× Buffer (Fermentas, Waltham, MA), 2.4 μL $MgCl_2$, 0.36 μL of each primer (50 μM), 0.18 μL of Taq polymerase (New England Biolabs, Ipswitch, MA), and 2 μL of genomic DNA. Cycling conditions were: 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 55°C or 58°C for 1 min, and 72°C for 1 min. Amplifications were sequenced at the University of Arizona UAGC sequencing facility. All markers were sequenced on both strands and

all polymorphisms were visually verified using sequencing chromatograms. Primer sequences were manually deleted from each sequence before analysis. Sequences were deposited in GenBank with accession numbers KC794034–KC794497.

SEQUENCE ANALYSIS

Multiple sequence alignments were manually generated for each gene using BioEdit (Hall 1999). We inferred the relationships among *C*. sp. 11 strains with concatenated sequences from the five polymorphic loci in using unrooted neighbor-networks generated with a Jukes–Cantor distance in the program SplitsTree 4.10 (Huson and Bryant 2006). Neighbor-networks are useful for representing the relationships among individuals of the same species for which recombination events may be nonnegligible (Huson and Scornavacca 2011).

We measured nucleotide polymorphism (Nei 1987) at each locus taken separately using DnaSP version 5 (Librado and Rozas 2009) and took the average across loci as a point estimate of nucleotide polymorphism for *C*. sp. 11. We compiled polymorphism data from the literature for *C*. *brenneri* (Dey et al. 2013), *C*. *elegans* (Andersen et al. 2012), *C*. *remanei* (Jovelin et al. 2003; Cutter et al. 2006a; Cutter 2008; Jovelin 2009; Jovelin et al. 2009; Dey et al. 2012), and *C*. sp. 5 (Wang et al. 2010; Cutter et al. 2012) to compare the level of nucleotide diversity between obligately outcrossing and self-fertilizing species within the genus *Caenorhabditis*.

QUANTIFICATION OF OUTBREEDING DEPRESSION

We examined offspring production of F1 animals derived from interstrain crosses between different *C*. sp. 11 wild strains, analogous to experiments performed in *C*. *elegans* and *C*. *briggsae* (Dolgin et al. 2007, 2008; Fig. 1A). Using 11 distinct parental strains, a total of 23 crosses were performed across five experimental blocks, representing 19 unique crosses (Table 1). These 19 crosses comprised five crosses for intralocality comparison, that is crosses between strains from the same locality, and 14 for interlocality comparisons (Table 1). Localities refer to distinct geographically separated regions, that is different regions of mainland South America and different tropical islands (Table 1 and Fig. S1). Intralocality crosses were performed between independently derived strains from different sampling spots within the same locality (French Guiana, Guadeloupe, Cape Verde) or from the same sampling spot, yet isolated at different time points (La Réunion, Puerto Rico). Strains from the same locality can therefore be considered to represent distinct genotypes.

A given interstrain cross (designated by "Cross ID") includes the four possible cross combinations between parental strains, divided into two distinct breeding classes (Fig. 1A). The breeding class "pure" consists of the two crosses between males and sperm-depleted hermaphrodites for each of the two parental strains (resulting in F1 pure strains); the breeding class "hybrid" consists of the two reciprocal crosses for the strain pair (resulting in F1 hybrid strains). Reciprocal hybrid crosses were obtained for all intralocality crosses; however, for seven of the 14 interlocality crosses, only one of the two possible reciprocal hybrid crosses could be obtained (Table 1), which was likely due to the low mating ability of parental males and the advanced age of sperm-depleted hermaphrodites, although premating isolation cannot be completely ruled out. Note that parental crosses did not produce any defective or abnormal F1 individuals and embryonic mortality

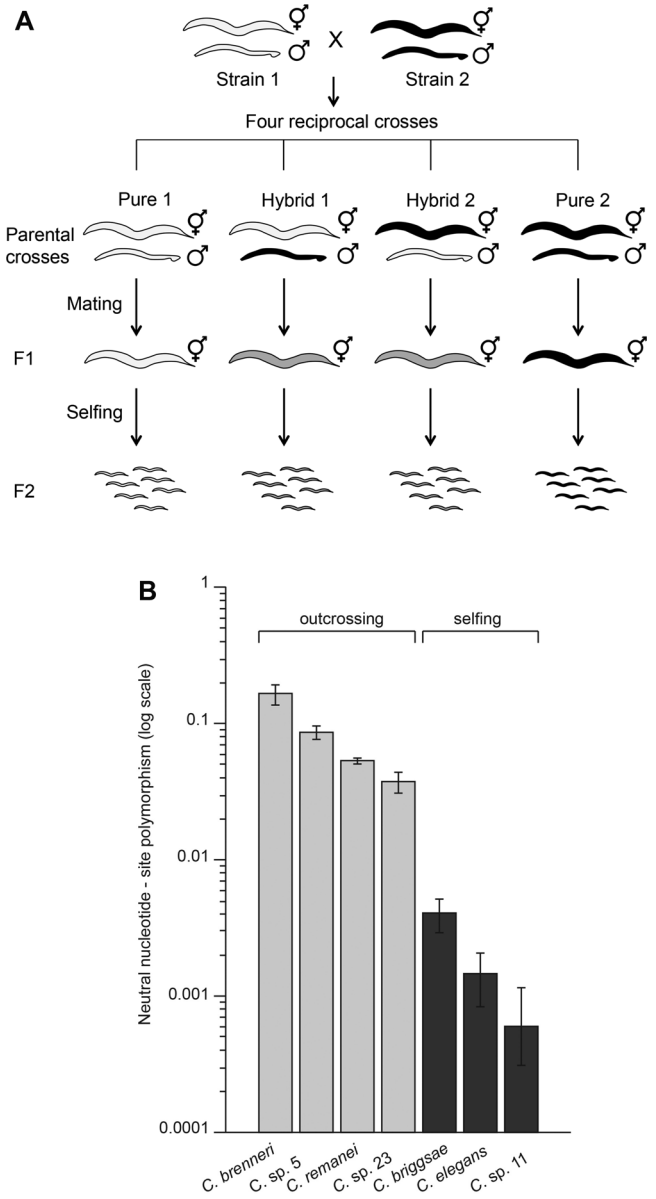

Figure 1. (A) Schematic view of reciprocal interstrain crosses performed for a given pair of strains (for details, see Methods section). (B) Outcrossing *Caenorhabditis* species (light gray) exhibit on average more nucleotide diversity than hermaphroditic species (dark gray). Error bars indicate ± 1 SE.

Table 1. Overview of inter-strain crosses.

	Strains crossed	Strain origin	Block
Intra-locality			
	NIC58×JU1818	French Guiana×French Guiana [FG×FG]	2,3
	EG6180×EG5889	Puerto Rico×Puerto Rico [PR×PR]	3
	JU1630×JU1639	Cape Verde×Cape Verde [CV×CV]	4
	NIC122×NIC203	Guadeloupe×Guadeloupe [G×G]	4
	JU1374×JU1800	La Réunion×La Réunion [R×R]	5
Inter-locality			
	JU1818×EG6180	French Guiana×Puerto Rico	1
	JU1818×QG131	French Guiana×Hawaii [FG×H]	1,2
	NIC58×JU1639[1]	French Guiana×Cape Verde	4
	EG6180×QG131	Puerto Rico×Hawaii	1
	EG6180×JU1800[1]	Puerto Rico×La Réunion	3
	EG6180×NIC58	Puerto Rico×French Guiana	3
	JU1639×EG6180	Cape Verde×Puerto Rico	4
	JU1639×NIC203[1]	Cape Verde×Guadeloupe	4
	NIC203×EG6180	Guadeloupe×Puerto Rico	1,3,4
	JU1800×NIC203[1]	La Réunion×Guadeloupe	4
	JU1800×NIC58	La Réunion×French Guiana	5
	QG131×NIC58[1]	Hawaii×French Guiana	2
	QG131×JU1800[1]	Hawaii×La Réunion	3
	QG131×NIC203[1]	Hawaii×Guadeloupe	1

[1]Crosses for which only one of the two hybrid crosses was obtained.

was not observed in F1 progeny. Crosses for which only one of the pure F1 individuals could be obtained were excluded from analysis.

The experimental procedure for the above-described F1 animals was as follows: for each cross, approximately 10 hermaphrodites per strain were isolated at the L4 stage, transferred to fresh plates every 24 h over 3–4 days to ensure self-sperm depletion before mating with males (Baird et al. 1992). Crosses were initiated 24 h after cessation of hermaphrodite egg laying by establishing multiple mating plates, each containing an isolated hermaphrodite and three young adult males (i.e., males selected in the L4 stage and allowed to mature overnight). After 48 h, F1 hermaphrodite progeny at the mid-L4 stage were isolated from mating plates in which the proportion of males was >40%, indicating exclusive cross fertilization.

F1 hermaphrodites were isolated (15–30 replicates for each of the four strain combinations) and then transferred daily to fresh plates during the reproductive period (3 days) and F2 offspring were counted as the number of larvae present 24 h after egg laying; F2 embryonic mortality was quantified as the number of unhatched embryos 24 h after egg laying. During the course of the experiments, we noticed the presence of morphologically abnormal and developmentally arrested F2 larvae in some of the crosses, and we quantified the proportion of such larvae in subsequent experimental blocks (three of five blocks); these abnormal larvae are included in all analyses of larval offspring number.

Statistical tests were carried out to test for differences in reproductive traits of F1 hybrid versus F1 pure strains (number of larval offspring, embryonic mortality, proportion of abnormal larvae) both for intra- and interlocality crosses. This was done using mixed-effects REML models (JMP 9.0), testing for the fixed effects of "Breeding Class" (pure vs. hybrid), "Cross ID" (a given strain combination), and the interaction between "Breeding Class" and "Cross ID"; the effect "Block" was included as a random effect nested within breeding class and Cross ID. To compare hybrid performance between intra- and interlocality crosses, we tested for differences in F1 hybrid performance, measured as the differential between individual F1 hybrid values and mean midparent values of corresponding F1 pure strains. Positive deviations of F1s from the midparent value provides the standard metric of heterosis, with negative values indicating outbreeding depression (Falconer and Mackay 1996); note that the genetic architecture to heterosis and outbreeding depression differ (Escobar et al. 2008). Specifically, we used a mixed-effects REML model (JMP 9.0), testing for the fixed effects of "Locality" (intralocality vs. interlocality) and "Cross ID" (nested in "Locality"), and the effect "Block" was included as a random effect nested within breeding class and Cross ID. In addition, for each of the 23 crosses, we carried out separate analyses of variance (ANOVAs) testing for the

fixed effects of "Breeding Class" and "Cross Direction" (nested in "Breeding Class").

All statistical tests were performed using the software programs JMP 9.0 or SPSS 21.0 for Macintosh. Data were transformed where necessary to meet the assumptions of ANOVA procedures (homogeneity of variances and normal distributions of residuals; Sokal and Rohlf 1995). For post hoc comparisons, Tukey's honestly significant difference (HSD) procedure was used (Sokal and Rohlf 1995).

MALE MATING EFFICACY

We used mating assays to assess the capacity of males to successfully mate with and fertilize hermaphrodites for each of five *C*. sp. 11 wild strains (JU1373, JU1630, JU1818, NIC58, EG6180) and for the reference strains of *C. elegans* (N2) and *C. briggsae* (AF16). For each strain, L4 males were isolated and allowed to mature to adulthood overnight. The next day, three young adult males were placed together with a single mid-L4 (i.e., unmated) hermaphrodite on a 55-mm-diameter NGM agar plate at 25°C (10–15 replicates per strain). After 24 h, hermaphrodites were transferred to fresh plates for the next 48 h. The resulting progeny were counted and sexed as they reached L4 and adult stages; the proportion of male progeny was used as indicator of male mating efficacy. In parallel, we scored spontaneous male production of self-fertilizing hermaphrodites by scoring the progeny of isolated L4 hermaphrodites in the same fashion as described earlier.

Results

PATTERNS OF NUCLEOTIDE DIVERSITY

We investigated nucleotide diversity in the recently discovered species *C*. sp. 11 using single nucleotide polymorphism information collected from partial gene sequences of nine nuclear loci. Overall, we found very little nucleotide diversity, with only nine single nucleotide polymorphisms (SNPs) in 5.9 Kb of sequence from a collection of 47–54 strains representing seven localities (Tables 2, S1). Low nucleotide diversity is fully consistent with the pattern expected for organisms with a highly selfing mode of reproduction (Liu et al. 1999; Sweigart and Willis 2003; Ness et al. 2010). Moreover, these data for *C*. sp. 11 agree qualitatively with previously reported disparities between selfing and outcrossing species of *Caenorhabditis* (Graustein et al. 2002; Jovelin et al. 2003; Cutter et al. 2009; Fig. 1B). However, the difference in mating system alone cannot explain the much greater than 2-fold disparity in polymorphism observed between selfing and outcrossing *Caenorhabditis* species, and likely additionally reflects the combined influence of linked selection and extinction–recolonization dynamics (Charlesworth and Wright 2001; Graustein et al. 2002; Sivasundar and Hey 2005; Cutter et al. 2009).

Despite the broad similarity in levels of diversity among self-fertilizing *Caenorhabditis*, we document a nearly 7-fold lower point estimate of polymorphism at silent sites in *C*. sp. 11 relative to *C. briggsae* (Fig. 1B; Félix et al. 2013). A caveat is that strains from French Guiana dominate our sample for *C*. sp. 11 (55%), which could lead to underestimation of global diversity for this species. However, sampling to date suggests a narrower, tropical geographic range for *C*. sp. 11 than for the more cosmopolitan *C. elegans* and *C. briggsae* (Kiontke et al. 2011). Importantly, *C. briggsae* strains from French Guiana are still 1.5-fold more diverse than the full sample of *C*. sp. 11 strains (*C. briggsae*: $\pi_{si} = 0.089\%$; *C*. sp. 11: $\pi_{si} = 0.060\%$) and 32 times more diverse than *C*. sp. 11 strains from French Guiana (*C. briggsae*: $\pi_{si} = 0.089\%$; *C*. sp. 11: $\pi_{si} = 2.78 \times 10^{-5}$; Table 2; Félix et al. 2013), which argues for a real disparity between these species.

Because five of the seven sampling localities are islands, we next investigated the possibility that *C*. sp. 11 strains were genetically differentiated according to their geographical origin. The multilocus analysis shows that strains from La Réunion and Cape Verde each form separate multilocus haplotype groups that differentiate from a third group that includes strains from French Guiana, Guadeloupe, Brazil, Puerto Rico, and Hawaii (Fig. 2 and Table S3). This result indicates some level of global population differentiation within *C*. sp. 11, despite its overall low nucleotide polymorphism, and warrants further investigation into the extent of population structure within this species.

QUANTIFICATION OF OUTBREEDING DEPRESSION

Intralocality crosses

Five crosses were generated between each of two strains from the same locality (French Guiana, Guadeloupe, Cape Verde and Puerto Rico; Table 1). Breeding class and Cross ID had no or only weak effects on larval offspring production of F1 hybrid strains (Fig. 3 and Table 3). In separate analysis of individual crosses, significant outbreeding depression was detected for two of the five intralocality crosses of Puerto Rico and La Réunion (Figs. 3, 4A, S2 and Table S4).

Interlocality crosses

A total of 14 different crosses were generated between strains from geographically separated localities (Table 1). F1 hybrids showed greatly reduced numbers of larval offspring, as indicated by significant negative heterosis values for 13 of 14 crosses (Figs. 3, 4A, S2 and Table 3). The magnitude of outbreeding depression, however, depended strongly on the strain combination, ranging from a 10% to >50% reduction of larval offspring number relative to pure F1 strains (Fig. 3 and Table 3).

Table 2. Summary statistics of the per locus nucleotide diversity within *Caenorhabditis* sp. 11.

Locus	*Caenorhabditis elegans* ortholog	Chr	n	L	N_c	N_{si}	S	π_t (%)	π_a (%)	π_{si} (%)	θ_{si} (%)
p11	Y25C1A.5	II	52	696	215.17	442.833	3	0.094	0	0.14	0.15
p12	E01G4.6	II	53	599	393.17	203.833	0	0	0	0	0
p13	D1005.1	X	52	804	572	226	1	0.014	0.019	0	0
p14	R160.7	X	54	586	30.83	512.167	1	0.072	0	0.076	0.043
p16	T24D11.1	X	52	628	0	600	2	0.019	NA	0.019	0.074
p17	E01A2.5	I	49	758	373.83	382.167	0	0	0	0	0
p18	ZK858.8	I	52	842	343.50	466.501	0	0	0	0	0
p19	F47G6.1	I	53	374	254.50	118.5	2	0.191	0.14	0.301	0.186
p21	ZK686.5	III	47	605	0	599	0	0	NA	0	0
Average			51.56	654.67	242.55	394.56	1	0.043	0.023	0.060	0.050

Chr = chromosome in *C. elegans*, n = number of strains, L = length, N_c = number of coding sites, N_{si} = number of silent sites (synonymous and intronic), S = number of segregating sites, π_t = total polymorphism, π_a = average number of nucleotide differences per amino acid replacement site, π_{si} = average number of nucleotide differences per silent site, θ_{si} = nucleotide diversity per silent site.

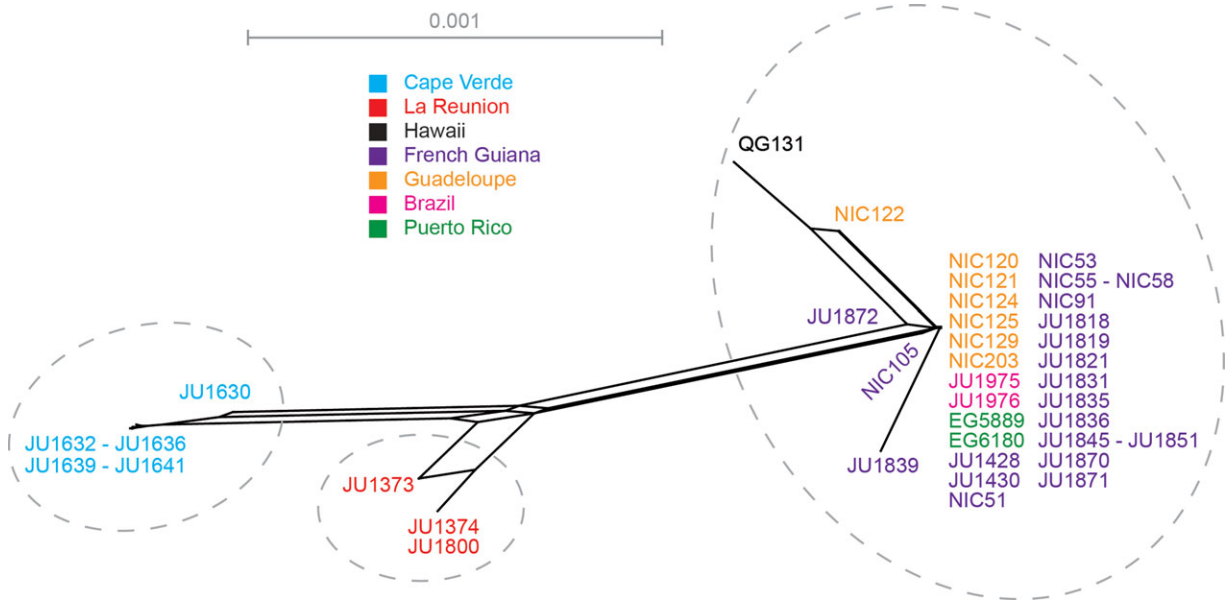

Figure 2. Neighbor-Joining network based on the concatenated sequences of five polymorphic nuclear loci depicting the relationships among 51 *Caenorhabditis* sp. 11 strains. Strains are partitioned into three groups according to their geographical origin.

Differences in outbreeding depression between intralocality versus interlocality crosses

Outbreeding depression in reproductive performance (number of larval offspring) was significantly higher among hybrid strains derived from interlocality crosses compared to intralocality crosses (Fig. 4A and Table 4). Mean heterosis values were -0.11 ± 0.04 for intralocality hybrids ($N = 5$) and -0.36 ± 0.05 for interlocality hybrids ($N = 14$). Note that heterosis and mid-parent values only provide a limited summary of information on mean hybrid performance, and complete information on all reciprocal crosses performed is therefore shown in Figure S2. However, for the large majority of crosses, for which significant negative heterosis was observed, the two reciprocal hybrid strains showed significantly lower reproductive performance than either of the pure strains (Fig. S2). The only clear-cut exception occurred in the interlocality cross between strains EG6180 × NIC58 (Fig. S2O), where the reproductive performance of the two hybrid strains was the same as in the pure NIC58 strain, yet significantly reduced relative to the pure EG6180 strain. In this case, the significant negative heterosis reported for this cross using mid-parent values (Fig. 3) should be interpreted with caution.

Increased mortality of F2 hybrid embryos

Embryonic lethality of F2 progeny was low for all pure F1 strains (<2%) but reached up to 20% in progeny of F1 hybrid strains from

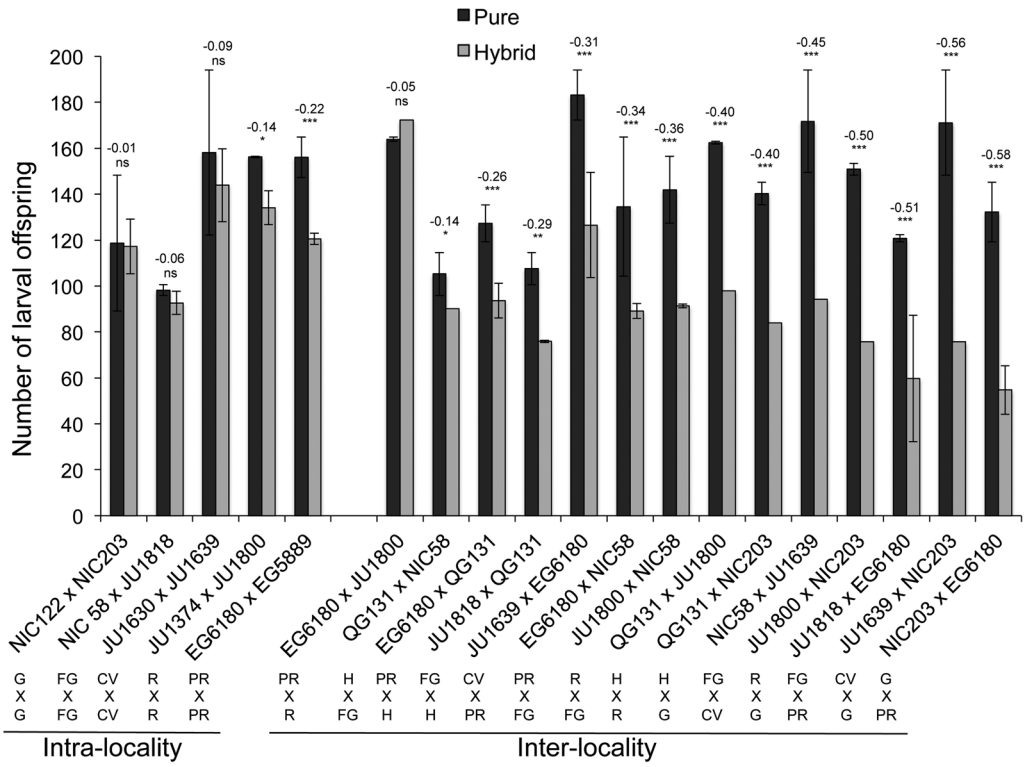

Figure 3. **Outbreeding depression in *Caenorhabditis* sp. 11 wild strains: larval offspring number. Mean numbers of larval offspring of F1 hermaphrodites derived from pure (black bars) and hybrid (gray bars) crosses. Bars indicate the mean midparent value for pure and hybrid strains derived from reciprocal crosses for a given pair of parental strains. Numbers above the bars show heterosis values (indicating the relative increase of reproductive performance of F1 hybrids), which were calculated as previously described (Falconer and Mackay 1996; Dolgin et al. 2007). Asterisks indicate significant differences between means of pure vs. hybrid strains using one-way analyses of variance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Error bars indicate ± 1 SE. Locality abbreviations are as indicated in Table 1. (Note that for crosses that were assayed multiple times in different experimental blocks, the results for a single, arbitrarily chosen block are shown.)**

Table 3. **Variation in larval offspring number in intralocality vs. interlocality crosses. Mixed-effects REML model testing for the fixed effects of pure vs. hybrid breeding class, Cross ID, and the interaction breeding class × Cross ID; block was treated as a random effect nested within breeding class and Cross ID.**

	Source	df	dfDen	*F*	*P*
Intra-locality					
	Breeding class	1	2.62	6.68	0.0935
	Cross ID	4	2.56	17.30	0.0315
	Breeding class×Cross ID	4	2.56	1.20	0.4756
Inter-locality					
	Breeding class	1	6.05	46.36	0.0005
	Cross ID	13	6.02	1.77	0.2472
	Breeding class×Cross ID	13	6.02	0.90	0.5937

interlocality crosses (Fig. 5). Embryonic mortality was highly variable among different crosses, but significantly increased in hybrid relative to pure strains, and was higher in interlocality crosses compared to intralocality crosses (Figs. 4B, 5 and Tables S5, S6). For interlocality crosses, F1 hybrid strains with highly significant negative heterosis (for larval offspring number) also showed the highest embryonic mortality, suggesting that the observed decrease of larval offspring in these F1 hybrids was apparently due to an increase in embryonic mortality (Figs. 3, 5).

Production of developmentally abnormal F2 larvae

For several F1 hybrid strains, we observed the production of developmentally abnormal F2 larvae (Figs. S4, S5). Such larvae showed

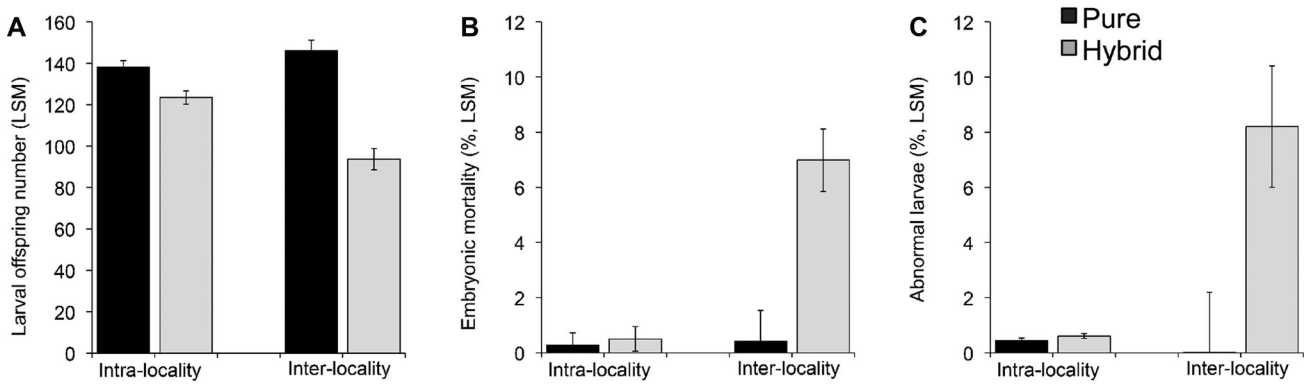

Figure 4. Differences in outbreeding depression between intra- and interlocality crosses of *Caenorhabditis* sp. 11 strains. Least square means (LSM) for (A) larval offspring number (for statistical test, see Table 3), (B) embryonic mortality (for statistical test, see Table S5), and (C) proportion of abnormal larvae (for statistical test, see Table S7). Error bars indicate ± 1 SE.

Table 4. Effect of locality (intralocality vs. interlocality) on F1 hybrid performance, measured as the differential between individual hybrid larval offspring number and mean midparent larval offspring number of corresponding pure-bred strains. Mixed-effects REML model testing for the fixed of locality and Cross ID nested within locality; block was treated as a random effect nested within breeding class and Cross ID.

Source	df	dfDen	*F*	*P*
Locality	1	4.44	233.54	<0.0001
Cross ID (locality)	17	4.06	21.64	0.0041

unusual morphology and extreme slowing of development; and in many cases, larvae were not able to complete moulting in the first larval stage, likely leading to developmental arrest and death (Fig. S6). This suggests that the number of viable larvae reaching reproductive maturity is substantially lower than the larval offspring numbers reported earlier. Abnormal F2 larvae were rare in all pure crosses (<2%), but common (5–15%) in most hybrid strains derived from interlocality crosses (Figs. 4C, S4 and Tables S7, S8). Production of abnormal F2 larvae varied greatly among F1 hybrid strains and was strongly increased in interlocality crosses (Tables S7, S8).

Outbreeding depression extends to F2 hybrids

To confirm the maintenance of outbreeding depression across generations, we selected fertile F2 hermaphrodites of two crosses showing strong outbreeding depression in F1 hybrids: NIC203 (Guadeloupe) × EG6180 (Puerto Rico) (Fig. S2P, heterosis −0.46) and EG6180 (Puerto Rico) × NIC58 (French Guiana; Fig. S2O, heterosis −0.38). We then quantified the production of F3 larval offspring as described earlier. F2 hybrids exhibited very similar levels of outbreeding depression, with heterosis values of −0.38 for NIC203 × EG6180 and heterosis values of −0.34 for EG6180 × NIC58 (Fig. S7).

Male mating efficacy

Male mating efficacy varied considerably among *C*. sp. 11 strains, but was mostly lower than in reference strains of *C. elegans* and *C. briggsae* (Fig. 6). Several *C*. sp. 11 strains (e.g., JU1630 or JU1818) showed male proportions of only 5–10%, and such low male mating efficacy is commonly observed for crosses between males and non sperm-depleted hermaphrodites for many *C*. sp. 11 strains (data not shown).

Discussion

Despite strong inbreeding depression for species of *Caenorhabditis* that outcross obligatorily (Dolgin et al. 2007; Barriere et al. 2009), it is outbreeding depression that appears to be a common feature in selfing species in this group, including for *C*. sp. 11 investigated here. The pattern is also seen in the more distantly related selfing nematode *P. pacificus* (Click et al. 2009). We observe that F1 animals derived from parents of distinct populations produce on average 36% fewer offspring than do "pure" strains under controlled conditions, and even find significant outbreeding depression between some strain pairs collected at the same locality. Although interlocality crosses resulted in a globally higher outbreeding depression than intralocality crosses, there was no obvious relationship between geographic distance and degree of outbreeding depression in interlocality crosses. The magnitude of outbreeding depression, however, varies among crosses as seen in other organisms (Waser et al. 2000; Leimu and Fischer 2010), indicating the presence of heritable differences in its genetic basis. Moreover, the strain combinations with the strongest outbreeding depression tend to have the highest incidence of embryonic mortality, suggesting that postzygotic Dobzhansky–Muller-like incompatibilities could be involved.

Interstrain crosses in *C. briggsae* previously uncovered little outbreeding depression (for reproductive output) both within and

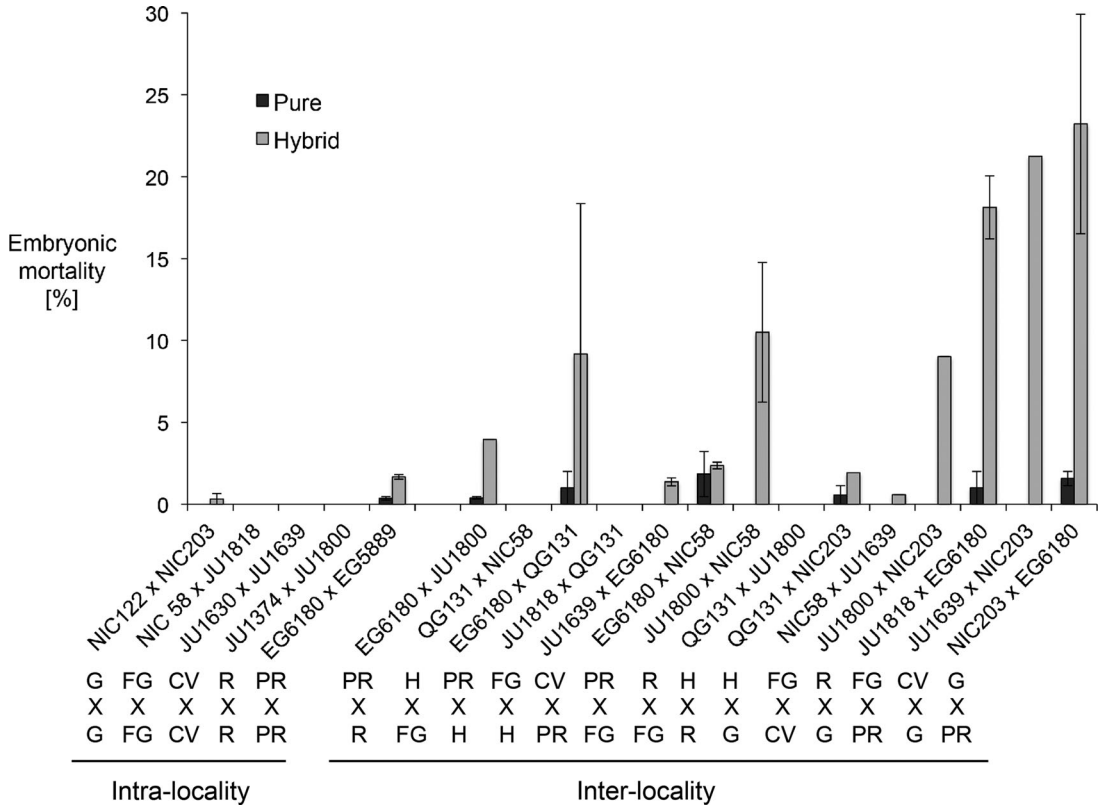

Figure 5. Outbreeding depression in *Caenorhabditis* sp. 11 wild strains: embryonic mortality. Mean proportion of dead embryos produced by F1 hermaphrodites derived from pure (black bars) and hybrid (gray bars) crosses. Bars indicate the mean midparent value for pure and hybrid strains derived from reciprocal crosses for a given pair of parental strains. Error bars indicate ± 1 SE. Locality abbreviations are as indicated in Table 1.

among localities (Dolgin et al. 2008); in particular, F1 strains derived from parents belonging to genetically distinct clades did not show strong outbreeding depression as observed here for *C*. sp. 11 strains. In the same study, some *C*. *briggsae* strain combinations also showed inbreeding depression of F1 hybrids (Dolgin et al. 2008). However, additional analyses of *C*. *briggsae* F1 hybrids revealed significant outbreeding depression, for example the developmental time of F2 hybrids may be significantly longer (Ross et al. 2011; Baird and Stonesifer 2012). In *C*. *elegans*, outbreeding depression has been observed for crosses between strains from the same locality (mainland France), reaching heterosis values of approximately −0.05 to −0.20 (Dolgin et al. 2008). Moreover, widespread outbreeding depression (embryonic lethality) occurs among F1 hybrids of *C*. *elegans* strains both within and among localities, due to incompatibilities of allelic variants at two loci (Seidel et al. 2008, 2011). Outbreeding depression is thus a shared characteristic of selfing *Caenorhabditis* species, but seems most pronounced in *C*. sp. 11, which also shows the lowest genetic diversity of the three species, accompanied by a very low male mating capacity. These combined observations suggest elevated levels of inbreeding of *C*. sp. 11 relative to *C*. *elegans* and *C*. *briggsae*.

The striking outbreeding depression in *C*. sp. 11 is coupled with surprisingly little neutral genetic differentiation among populations inferred from molecular sequence data. More generally, this species has very little nucleotide polymorphism, qualitatively similar to other selfing species (*C*. *elegans* and *C*. *briggsae*) but quantitatively the lowest diversity observed to date for any species of *Caenorhabditis* (Cutter et al. 2009, 2013; Jovelin et al. 2013). Although differences in mutation rates could contribute to differences in population variation, current estimates of single nucleotide mutation rate do not differ significantly between *C*. *elegans* and *C*. *briggsae* (Denver et al. 2012). Similar to the case of *Caenorhabditis*, the island endemic plant *A*. *crispa* also has small populations with low molecular variation, and exhibits outbreeding depression (Quilichini et al. 2001; Coppi et al. 2008). By contrast, crosses between strains of *C*. *brenneri* that differ enormously at the molecular level show no detectable differences in progeny production for inter- and intrapopulation crosses (Dey et al. 2013). Some theory predicts the opposite: that species with large population sizes (and correspondingly high neutral polymorphism) will show stronger outbreeding depression (Edmands and Timmerman 2003; Frankham et al. 2011). With this in mind,

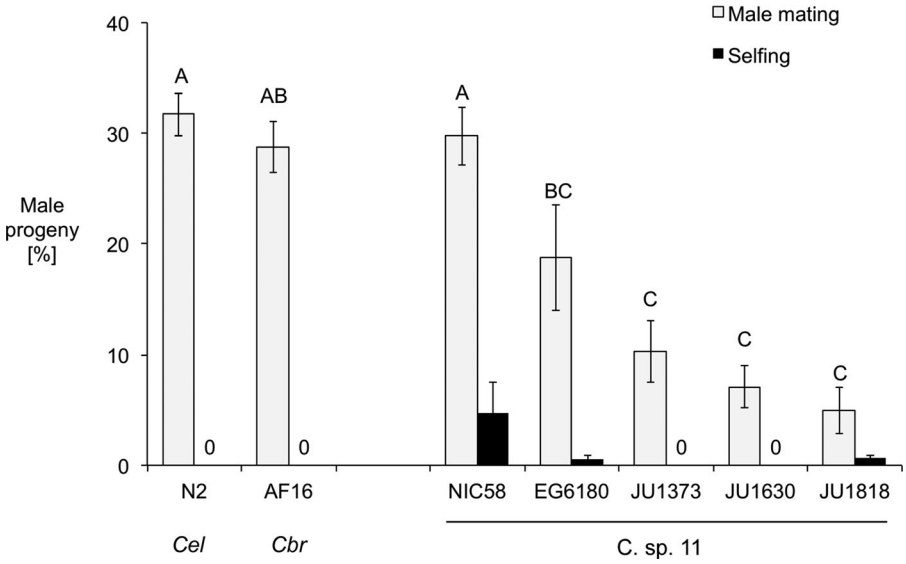

Figure 6. **Male mating efficacy of *Caenorhabditis* sp. 11 wild strains and the reference strains of *Caenorhabditis elegans* (N2) and *C. briggsae* (AF16) at 25°C. For each strain, the percentage of males was measured in the offspring of 10–15 single hermaphrodites kept in isolation (selfing) or single hermaphrodites kept together with three young adult males (male mating). Examined strains differed significantly in the proportion of male progeny (ANOVA, $F_{6,97} = 15.97$, df = 6, $P < 0.0001$). Values for male mating treatments with different letters are significantly different from each other (Tukey's honestly significant difference). Error bars indicate ± 1 SE.**

how can it be that interpopulation hybrids of *C*. sp. 11 show such pronounced outbreeding depression?

We propose that the highly selfing mode of reproduction in this species is the key driver of outbreeding depression in *C*. sp. 11, in line with some previous predictions (Templeton 1986; Dudash and Fenster 2000; Leimu and Fischer 2010). We find that despite ostensibly being androdioecious, males in this species are extremely ineffectual at mating (Fig. 6), being even less efficient than *C. elegans* males that have notoriously poor mating ability (Garcia et al. 2007). This ought to lead to nearly complete self-fertilizing reproduction in nature, which, in turn, will promote independent evolutionary trajectories of lineages in different places with the entire genome being propagated as a linked unit within each lineage. These conditions will facilitate intragenomic coadaptation (i.e., "co-adapted gene complexes"), in the sense that conditionally deleterious mutations, compensatory mutations, and adaptive mutations can all accumulate in a given genomic background, quickly becoming homozygous with little reassortment by recombination, and therefore contributing to divergence between lineages. Much like models of restricted recombination in inversions, alleles that get fixed by selection in a given selfing population need not involve epistatic selection, but more plausibly could arise from fixation of "individually adapted" alleles and still contribute to outbreeding depression in crosses (Kirkpatrick and Barton 2006). We note that allele combinations involved in this process need not explicitly be associated with extrinsic adaptation to local ecological circumstances, although clearly local adaption will hasten divergence owing to both focal selected mutations and linked mutations that hitchhike along with them (Hartfield and Otto 2011; Cutter and Payseur 2013). With small effective population size, as implied by the low neutral polymorphism of *C*. sp. 11, even slightly deleterious mutations could accrue in genomes. Such independent evolution of fitness-affecting mutations provides the raw material for negative epistatic associations when distinct lineages are brought together in crosses. In the diction of speciation geneticists, such loci are the stuff of Dobzhansky–Muller incompatibilities (Coyne and Orr 2004).

Negative epistatic incompatibilities manifesting in crosses between populations need not be the only cause of outbreeding depression, especially in highly selfing species. Models of adaptation in the context of inversions, which restrict recombination analogously to selfing, suggest that underdominant alleles could contribute an important source of reproductive incompatibilities (Kirkpatrick and Barton 2006). In *Caenorhabditis*, however, inversions appear relatively uncommon in interspecies genome comparisons (Wolfe and Shields 1997; Hillier et al. 2007), although little is known about population variation for inversions. Perhaps adaptive alleles favoring the evolution of inversions per se might be less favored in selfers, because selfing itself does the job of restricting recombination, thus reducing selection for stronger linkage induced by inversions (Kirkpatrick and Barton 2006), although underdominant rearrangements of all sorts can accumulate more quickly in selfing than outcrossing lineages (Charlesworth 1992). For *C*. sp. 11, we find intrinsic F2 outbreeding depression in a controlled environment; we also did not observe an

obvious difference between pure and hybrid F1 viability, where underdominance effects should be particularly strong (Schierup and Christiansen 1996). This suggests that direct effects of dominance might play a less prominent role compared to epistatic gene interactions in interpopulation crosses.

At the molecular level, there are many potential causes of genetically intrinsic outbreeding depression. The simplest to conceive are standard point mutational changes to protein-coding genes or their regulatory regions that contribute to negatively epistatic interactions (or, potentially, underdominance effects). More drastic changes like rearrangements, however, are predicted to accrue more rapidly in independently evolving selfing lineages (Charlesworth 1992). Such rearrangements could easily lead to outbreeding depression in F2 and later generations of crosses between populations with high selfing rates. The "divergent resolution" of gene duplicates in different lineages also is proposed as an important mechanism of reproductive incompatibility (Lynch and Force 2000), which could similarly manifest more rapidly in distinct selfing populations. Under some conditions, selfing populations can experience a proliferation of transposable elements in their genomes (Wright and Schoen 1999; Morgan 2001). If expansions of transposable elements occur independently in distinct populations, then hybridization could lead to accelerated element-induced dysgenesis (i.e., not necessarily requiring negative epistasis) or reduced fitness owing to ectopic recombination (Langley et al. 1988; Petrov et al. 1995; Gray 2000). None of these genetic causes are precluded from causing outbreeding depression in non selfing taxa, but selfing could modulate their relative contributions. The larger types of mutational event, which will not be reflected simply in patterns of single nucleotide polymorphism, provide intriguing possible proximate genetic mechanisms for the outbreeding depression observed in *C*. sp. 11 and other highly selfing species. Population genomic sequencing could help test their plausibility in nature.

The genetic causes of outbreeding depression can be thought of in the same way as reproductive incompatibilities between species, as we have outlined earlier. What distinguishes outbreeding depression within a species from hybrid breakdown between species? Should the different populations of *C*. sp. 11 be considered new species or subspecies? The answers to these questions, of course, are somewhat subjective, but in this case we are comfortable considering *C*. sp. 11 to be a cohesive species that simply has some degree of population genetic differentiation that may or may not correspond to locally adaptive differences among parts of its range. However, the strong outbreeding depression does motivate application of this species to understand the genetic basis to the evolution of reproductive incompatibilities, incipient speciation, and epistasis generally. This species provides a unique position within *Caenorhabditis* for studying divergence, intermediate between the isolation of distinct "good species" (e.g., *C. remanei*—*C*. sp. 23, *C. briggsae*—*C*. sp. 9) and differentiated populations (Woodruff et al. 2010; Baird and Stonesifer 2012; Dey et al. 2012; Kozlowska et al. 2012). We anticipate that the experimental tractability of *C*. sp. 11 will contribute substantively to emerging studies of speciation in this genus.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. List of isolated *Caenorhabditis* sp. 11 strains up to date.
Table S2. List of primers used to investigate nucleotide polymorphism in *Caenorhabditis* sp. 11.
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Figure S2. Individual representation of larval offspring production of each of the 23 interstrain crosses performed.
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Figure S5. Individual representation of proportions of abnormal larvae produced in of each of the 23 interstrain crosses performed.
Figure S6. Outbreeding depression in *Caenorhabditis* sp. 11 strains: production of abnormal larvae.
Figure S7. Outbreeding depression is maintained in F2 hybrids.