The spread of a transposon insertion in *Rec8* is associated with obligate asexuality in *Daphnia*

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Although transitions from sexual to asexual reproduction are thought to have important evolutionary consequences, little is known about the mechanistic underpinnings of these changes. The cyclical parthenogen *Daphnia pulex* is a powerful model in which to address these issues because female-limited meiosis suppression can be transmitted to sexual individuals via males, providing the opportunity for genetic dissection of the trait. A previous study identified genomic regions differentiating obligately asexual females from their sexual counterparts, and a candidate gene within one such region, encoding the meiotic cohesin Rec8, is the subject of this investigation. The *D. pulex* genome contains three Rec8 loci, all of which are quite polymorphic. However, at one of the loci, all obligately asexual clones carry an allele containing an identical upstream insertion of a transposable element as well as a frameshift mutation, both of which are completely absent from sexual lineages. The low level of variation within the insertion allele across all asexual lineages suggests that this element may be in the process of spreading through the species, and abrogation or modification of Rec8 function is possibly responsible for converting meiotically reproducing lineages into obligate asexuals.

parthenogenesis | recombination | phylogenetics | cytogenetics

Transitions between sexual and asexual reproduction are believed to have important evolutionary and ecological consequences (1, 2). However, the molecular, genetic, and cytological underpinnings of such transitions remain largely uncharacterized. Most asexual metazoan taxa are obligate parthenogens (3), making formal genetic analysis of these transitions impossible. In contrast, for the cyclical parthenogen *Daphnia pulex*, there are numerous lineages in which the females are obligately asexual, but still retain the ability to produce males capable of haploid sperm production via meiosis. Such males provide a vehicle for fertilizing the eggs of sexual females, thereby transmitting the genetic determinants of female-limited meiosis suppression (4, 5). Such sex-limited meiosis suppression provides a powerful mechanism for the recurrent transformation of sexual lineages into novel asexually produced genotypes by repeated crossings between male progeny of obligate asexuals and sexual females (5–8). Although several genetic types of obligate asexuality may exist within the *Daphnia* species complex (9), our current focus is on a system involving "contagious" obligate asexuality (5, 7, 8), which has resulted in a wide phylogenetic and geographic distribution of obligately parthenogenetic (OP) lineages in the eastern and midcontinental portions of North America.

In cyclically parthenogenetic (CP) *D. pulex*, resting eggs are products of meiosis and fertilization, whereas, in OP females, they are produced by a pseudomeiotic process in which a germline cell undergoes an equational separation (10). Data from laboratory crosses initially suggested that a single dominant allele confers OP (7). However, a recent marker association study of a collection of OP and CP genotypes from across North America revealed single almost perfectly associated regions on chromosomes V, VIII, and X, and an entire copy of chromosome IX significantly associated with this breeding system (8). The discriminatory power of these marker groups is almost perfect, whereas all other chromosomal regions exhibit essentially no subdivision between CP and OP lineages (8). The associated regions on chromosomes V, VIII, and X are on the order of at most 250 to 500 kb, presumably because periodic recombination and segregation via backcrossing OP males reduces linkage disequilibrium (LD) in chromosomal regions with no causal connection to OP. Approximately a dozen genes found in these associated regions have characterized roles in meiosis, including the subject of this study, the genes encoding the meiotic cohesin protein Rec8. Cohesin is a four-protein ring structure that keeps sister chromatids associated during mitosis and meiosis by encircling or clamping them together until regulated cleavage allows separation in the appropriate manner (11). Through its association with other proteins, the meiosis-specific cohesin component Rec8 replaces the mitotic paralogue Rad21/Scc1 to effect a step-wise separation of sister chromatids (12). In all cases examined, *Rec8* mutants have defects in meiotic chromosome segregation and structure (13, 14).

Because mutations that abrogate meiotic cohesin function can cause a meiotic cell to undergo a single equational division [e.g., *spo13* mutations in *Saccharomyces cerevisiae* (15, 16)], because an equational division requires the loss of sister-centromere cohesion, and because Rec8 is essential for meiotic sister-centromere cohesion, the association between OP and the region containing *Rec8* (8) raises the intriguing possibility of a causal link to the origin of obligate parthenogenesis. Here we report the results of our investigations of *Rec8* in *D. pulex* and the discovery of a debilitated allele uniquely carried by all OP lineages.

Results

Genomic Architecture of *Rec8* and Insertion in Obligately Asexual *D. pulex*. As noted earlier, results of our previous association-mapping study (8) strongly indicate that several unlinked loci are associated with OP, including a region of chromosome VIII containing *Rec8*. However, conflicting assemblies of the sequence data from the *Daphnia* genome project involving a CP genotype indicated that there is either one (http://wfleabase.org; 2005 assembly) or two (http://genome.jgi-psf.org/Dappu1/Dappu1.home.html) nearly identical copies of *Rec8* in this region. In addition, it is possible that chromosomal rearrangements in OP *D. pulex* have brought together the region containing *Rec8* on VIII with other diagnostic markers residing on chromosomes V, X, and/or IX in CP *D. pulex*, potentially obscuring the location of the candidate locus. To test the possibility of a chromosomal rearrangement of associated regions in OP and to establish the copy number of *Rec8* genes, we used FISH.

Results of our cytogenetic analysis showed that there has indeed been a duplication of *Rec8* in the associated region of chromosome VIII (Fig. S1), and further demonstrated the presence of a third copy of this gene in a region of chromosome VIII not previously shown to be associated with OP (8). In addition, dual-color FISH on spermatocyte preparations of OP males

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showed that none of the *Rec8* copies localized to chromosome IX (Fig. S1*C*). Thus, as originally suspected, the asexuality-associated markers in OP are distributed across multiple chromosomes, and have not been translocated to a single driving chromosome in obligate asexuals.

The tight association between the region on chromosome VIII containing two copies of *Rec8* and obligate parthenogenesis (8) raised the possibility of a functional relationship between modified alleles of one or more copies of *Rec8* and meiosis suppression. Therefore, we examined patterns of sequence variation within the three *Rec8* loci by using a broad geographic survey of lineages of CP and OP *D. pulex* (8).

We initially sought to PCR amplify, clone, sequence, and assemble both alleles, from each of the three *Rec8* loci, for eight strains of CP and eight strains of OP genotypes. In the course of characterizing locus B alleles, we discovered a single variant of uniform length carried in heterozygous state in all obligate asexuals, involving the insertion of a 1,239-bp element into a site 604 bp upstream from the annotated transcription start site of *Rec8-B* (Fig. S1*D*). The insertion-site sequence (GAAG/TAAT) is found upstream of all three loci of Rec8, and RT-PCR studies confirmed the site to be within the 5′ UTR of the gene (Fig. S2), thus extending the known transcription-start site for Rec8 to more than 600 bp upstream of the start codon. The insertion, its flanking regions, and most of the associated coding sequence of *Rec8-B* were fully sequenced (directly from PCR products, without cloning) in 31 lines of OP *D. pulex* (Table 1) (17, 18). A further 17 OP lines were screened (but not sequenced) by using insert-specific primers (Table S1), and all were found to contain the insert (strain NDB5 appears to be homozygous for the insertion). Of 44 different sexual strains tested, none showed evidence of the insertion based on PCR primers flanking the insertion or amplifying from inside the insertion to primers anchored in flanking DNA (Table S1).

Thus, insertion alleles for *Rec8-B* are uniquely located within OP lineages, in striking contrast to the vast majority of polymorphisms throughout the genome of *D. pulex*, which exhibit no association with breeding system (8). All the insertion alleles were also monomorphic for a single-base deletion creating a frameshift in exon 1 that would lead to a truncated protein of 31 aa (compared with 640 aa for WT) if the allele's mRNA were to be translated. That is, the "insertion alleles" are all highly similar and contain the same premature termination codon (PTC).

The insertion element has no long ORF or significant homologues in the GenBank nonredundant database, is AT-rich (66% AT), and bounded by terminal nested inverted repeats of 41 bp. When used as a query in BlastN searches of the *D. pulex* reference genome, the element returns 28 loci with sequence identity, several of which have strong similarity to retrotransposons. Given the lack of an ORF, the insertion appears to be nonautonomous, and presumably was transposed by a different *trans*-acting active mobile element into the *Rec8-B* locus of the most recent ancestor of all OP *D. pulex*.

Evolutionary Analysis of Rec8 in *D. pulex*. A phylogeny of *Rec8* sequences revealed several significant patterns (Fig. 1) (19, 20). First, gene sequences from CP and OP genotypes are interspersed throughout the gene genealogy, except for the monophyletic insertion allele of *Rec8-B* found only in obligate asexuals, which appears to be derived. Second, a monophyletic group of *Rec8-C* alleles is sister to the *Rec8-B* insertion-allele grouping, both of which are on longer branches than the remainder of the alleles in the data set. Evidence detailed later suggests that these *Rec8-C* alleles (~15 kb downstream of *Rec8-B*; Fig. 1 and Fig. S1), which we denote the LD alleles because they appear to be in LD with the insertion, are found on the same chromosome as the *Rec8-B* insertion allele in most OP genotypes. However, the fact that not all OP genotypes possess a *Rec8-C* LD allele provides compelling evidence that the causal mutation(s) associated with OP resides upstream of *Rec8-C*. Third, extensive polymorphism within and between the three *Rec8* loci resulted in poor resolution of relationships among most of the remaining alleles, with the exception of those from clones found in far western North America (Table S2) (18, 21).

Table 1. Allelic diversity and length variants at *REC8* loci in *D. pulex* including upstream regions, exons, and introns

Type	Locus	Region	Lineages	Length of locus sequenced	Segregating sites	Haplotypes	Haplotype diversity	Indel events	Indel haplotypes*
OP	*REC8–B*,† insertion allele	Upstream gene‡	31	608	19	6	0.45	3	3
		Intergenic/upstream	31	2,052	3	4	0.21	3	3
		Insertion	31	1,239	1	2	0.08	1	2
		Exons/introns	31	2,388	3	3	0.57	4	3
OP	*REC8–B*, noninsertion allele	Upstream gene	16	618	38	12	0.94	8	6
		Intergenic/upstream	16	2,052	30	12	0.94	24	4
		Exons/introns	16	2,194	166	15	0.99	38	7
CP	*REC8–B*	Upstream gene	18	618	43	5	0.99	24	2
		Intergenic/upstream	18	2,052	72	14	0.98	44	6
		Exons/introns	10	2,388	123	9	0.98	49	4
OP	*REC8–A*§	Exons/introns	8	1,581	158	10	0.99	9	4
CP	*REC8–A*	Exons/introns	8	1,581	174	10	0.99	5	3
OP	*REC8–C*, LD¶	Exons/introns	6	1,581	63	4	0.99	6	3
OP	*REC8–C*, LE¶	Exons/introns	8	1,581	191	15	0.99	11	4
CP	*REC8–C*‖	Exons/introns	8	1,581	235	10	0.99	5	3
	*D. pulex***	13 genes (mean)	—	—	—	21.9	0.91	—	—

Locus and type designations are according to Fig. 1. "Insertion" refers strictly to sequence contained within the insertion element, "intergenic/upstream" to all other sequence upstream of *Rec8-B*.

*Haplotypes defined by the presence of indels, introns excluded.

†Locus identifier: JGI 308284.

‡Locus identifier: JGI 11272.

§Locus identifier: JGI 284385.

¶Alleles in LD (or linkage equilibrium) with the insertion at *Rec8–B*.

‖Locus identifier: JGI 312910 and JGI 312911 (one locus incorrectly split into two gene models by automated genome annotation at JGI, proven inaccurate by cDNA).

**Data are from two published studies (17, 18).

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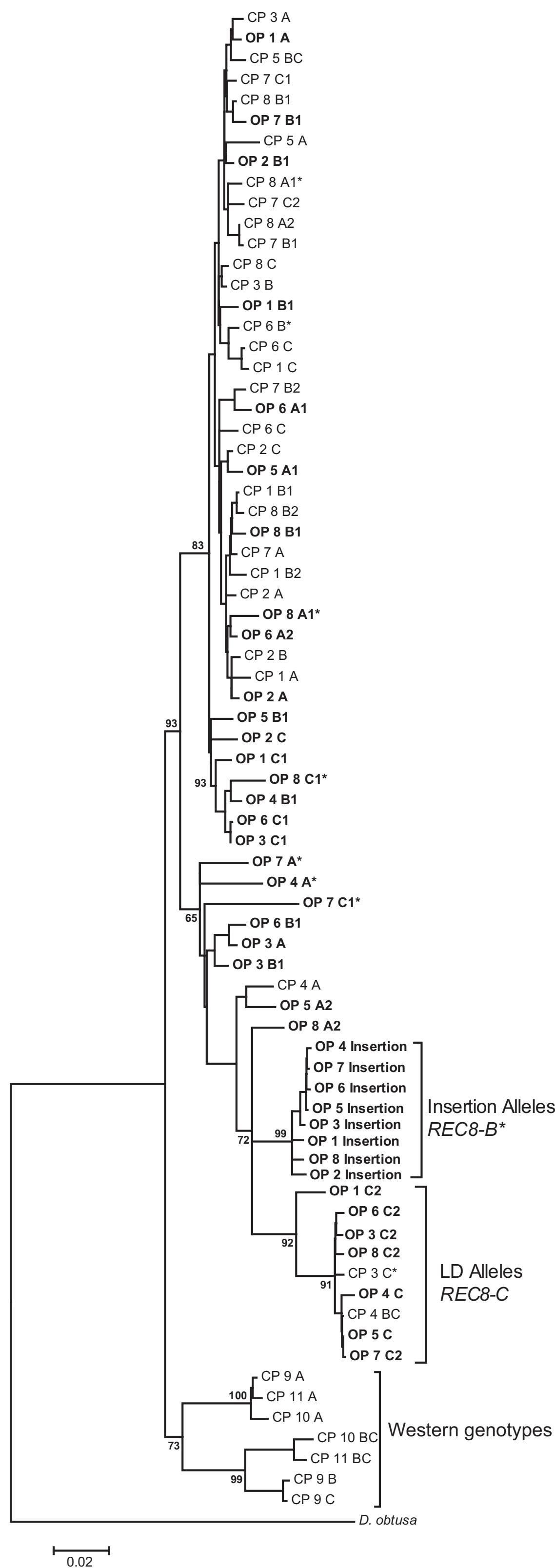

Fig. 1. Phylogenetic analysis of *Rec8* genes in *D. pulex*. The phylogeny was constructed by using the Tamura three-parameter estimation (19) plus gamma plus invariant sites using neighbor-joining (20). Bootstrap values from 1,000 iterations are shown at selected nodes. OP clones are numbered in bold, and sexual genotypes (i.e., CP) are numbered in roman, with loci denoted A, B, or C, and alleles within an individual arbitrarily numbered (1 or 2; homozygotes are not numbered); genotypes are detailed in Table S2. Alleles with a premature stop codon are noted with asterisks. Groupings include insertion alleles at *Rec8-B*, *Rec8-C* alleles putatively in LD with the insertion, and Western clones from Oregon, including the reference genome. Note that the "LD" alleles are not found in complete LD with the insertion element at *Rec8-B*, as one OP genotype lacks this type of allele. However, the LD designation is convenient for purposes of illustration and explanation. Scale is in substitutions per nucleotide site.

We sought to determine whether ectopic gene conversion is responsible for eliminating divergence among paralogues and distributing alleles across multiple loci, using the method of Betrán et al. (22) to detect gene conversion between loci. Six conversion events were detected between loci A and B, eight between loci A and C, and 11 between B and C, with the average conversion-tract length being 78 bp (±34 bp). One gene conversion tract was found between *Rec8-A* in western *D. pulex* and the *Rec8-C* LD alleles in OP *D. pulex*, indicating possible allelic exchange between the groups.

The relative sequence homogeneity among copies of the *Rec8-B* insertion alleles and among *Rec8-C* LD alleles contrasts with the situation observed for the remaining alleles characterized. Excluding the insertion and LD alleles, all three *Rec8* loci exhibit substantial amounts of sequence variation (Table S3), pronounced differences in the size of exon 11 associated with variable numbers of a tandem repeat sequence, and numerous insertion/deletions (Table 1).

Although no clades consisting only of alleles containing PTCs have been revealed beyond the *Rec8-B* insertion-allele clade, such alleles are common. Across all three loci, eight segregating alleles containing unique PTCs were found in both CP and OP clones. Phylogenetic analysis of *Rec8* from other *Daphnia* species suggests that duplications of this locus are restricted to the *D. pulex* species complex, as no more than two sequences were recovered (by using universal primers; Table S1) from isolates of outgroup species (Fig. S3). Thus, the extra copies of *Rec8* in *D. pulex* may allow the Rec8 proteins in this species to evolve under a relaxed selection regime, with little or no short-term cost associated with the loss of function in one or more alleles. The relatively high ratio of nonsynonymous to synonymous substitutions in *Rec8* alleles provides further support for this contention (Table S4) (23).

Taken together, these observations are consistent with three different processes influencing the evolution of *Rec8* loci in *D. pulex*: (*i*) a homogenizing mechanism such as ectopic gene conversion acting to reduce interlocus diversity; (*ii*) a relaxation of purifying selection on most *Rec8* alleles at all three loci; and (*iii*) a spread of the insertion-containing allele (and the likely partial hitchhiking of an associated allele at locus C, as discussed later) across all OP lineages of *D. pulex*. The finding that the transposon insertion in *Rec8-B* is always present in OP genomes and never in CP genomes leads to the hypothesis that the insertion is tightly linked to (or is possibly itself) the genetic element that suppresses meiosis in OP.

Evidence That Insertion Allele Induces Obligate Parthenogenesis. To further test the hypothesis that the insertion-containing *Rec8-B* allele in obligate asexuals has been creating novel OP lineages by spreading into CP genetic backgrounds, we constructed a phylogeny for the upstream region of *Rec8-B*, by using a larger collection of 23 OP and 10 CP genotypes (beyond the 16 in our initial study). If the proposed model of causality is correct, copies of the insertion allele are expected to be monophyletic, whereas the other (i.e., noninsert) *Rec8-B* alleles in OP should be interspersed with copies from CP genotypes. That is, the meiosis suppressor and DNA linked to it are expected to be essentially the same in each OP genotype, as they will have been transmitted via backcrossing through an OP male, whereas allelic regions inherited from CP mothers are expected to be derived from random, sexual genetic backgrounds.

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Results of our phylogenetic analysis of the region upstream of *Rec8-B* (with the insertion removed) demonstrate that the alleles containing the insertion in OP genotypes are monophyletic, whereas alleles without the insertion from these lineages are more closely related to alleles from CP genotypes (Fig. 2). This phylogenetic pattern is reflected in disparate levels of sequence variation found among these alleles. For example, as shown in Table S3, the *REC-B* noninsertion alleles from OP actually show higher levels of nucleotide diversity (Watterson estimator of the population mutation rate $4N_e\mu$, $\theta_W = 0.0283$; average number of total nucleotide differences per site, $\pi_T = 0.0227$) than *Rec8-B* from CP ($\theta_W = 0.0196$, $\pi_T = 0.\ 0156$). In contrast, the *Rec8-B* insertion alleles show approximately 100-fold less nucleotide diversity than the noninsertion alleles ($\theta_W = 0.0003$, $\pi_T = 0.0007$). In addition, careful analysis has shown an abrupt breakdown of LD of OP-associated markers approximately 40 kb upstream of the insertion (*SI Materials and Methods*). The markers are found specifically on the insertion chromosome in all 11 OP clones tested, and not in any of 12 CP clones tested, thus delimiting the region associated with OP to, at most, approximately 60 kb.

We formally tested for LD by comparing alleles at *Rec8-B* and *C* separately in OP and CP clones, measuring associations between parsimony-informative sites (227 informative sites in OP; 206 informative sites in CP) by using the summary statistics Z_{nS} [average of r^2 (24)], Z_a (average r^2 between adjacent polymorphic sites), and ZZ [Z_a minus Z_{nS} (25)]. Significance of the pairwise associations between polymorphic sites, or LD, was tested by using Bonferroni correction of the χ^2 distribution, which is quite conservative (25). When testing for nonrandom associations between all pairwise parsimony-informative polymorphic sites, results showed 771 significant pairwise comparisons (3%) between *Rec8-B* and *Rec8-C* in OP (after Bonferroni correction), but none in CP (Table S5). An example of nonrandom association is the presence of a splice-site polymorphism shared among the LD and insertion alleles, in which all other alleles except those from Oregon have a derived site. Significant LD is also shown by the small value of ZZ in the OP haplotypes at *B* and *C* (Table S5), which indicates lower intragenic recombination in these haplotypes. Taken as a whole, these data strongly support grouping the *Rec8-B* insertion alleles and the *Rec8-C* LD alleles into an incompletely linked haplotype.

Given the proximity of *Rec8-B* and *C* and the extreme reduction in diversity seen for the insertion allele at locus *B*, a similar reduction in diversity is expected of linked alleles at *C*. In accordance with this expectation, we found a marked decrease in genetic variation among the subset of *Rec8-C* alleles that we surmise to be linked to the *Rec8-B* insertion allele (Table 1 and Table S3). Among the Rec8-C LD alleles, π_T is 0.0011, whereas, among the other *Rec8-C* alleles in OP, π_T is 0.0468 (Table S3). Our data thus indicate a distinct difference between the insertion alleles and the alleles putatively in LD at locus *C* on the one hand, and all other alleles in our data set on the other.

Decreased nucleotide variation may result from several types of population-genetic process (reviewed in ref. 26), including hitchhiking as a result of linkage with a beneficial allele (27), or background selection as a result of linkage with deleterious mutations (28). Thus, we used site-frequency tests to determine the most likely explanation for the observed patterns. Specifically, we estimated the Tajima *D* (22) and the Fay and Wu scaled *H* (29) in an effort to distinguish the effects of population growth and recombination from selection (30). The hallmark of a selective sweep is an excess of high-frequency, derived variants, even in regions where normal levels of recombination are occurring (29), and the Fay and Wu *H* is effectively able to distinguish sweeps from demographic effects and background selection by the occurrence of these variants (31). The frequency of the insertion allele is consistent with a sweep, rather than a population bottleneck or background selection, as shown by a very small value for *H* for the OP *Rec8-B* alleles (Table S5).

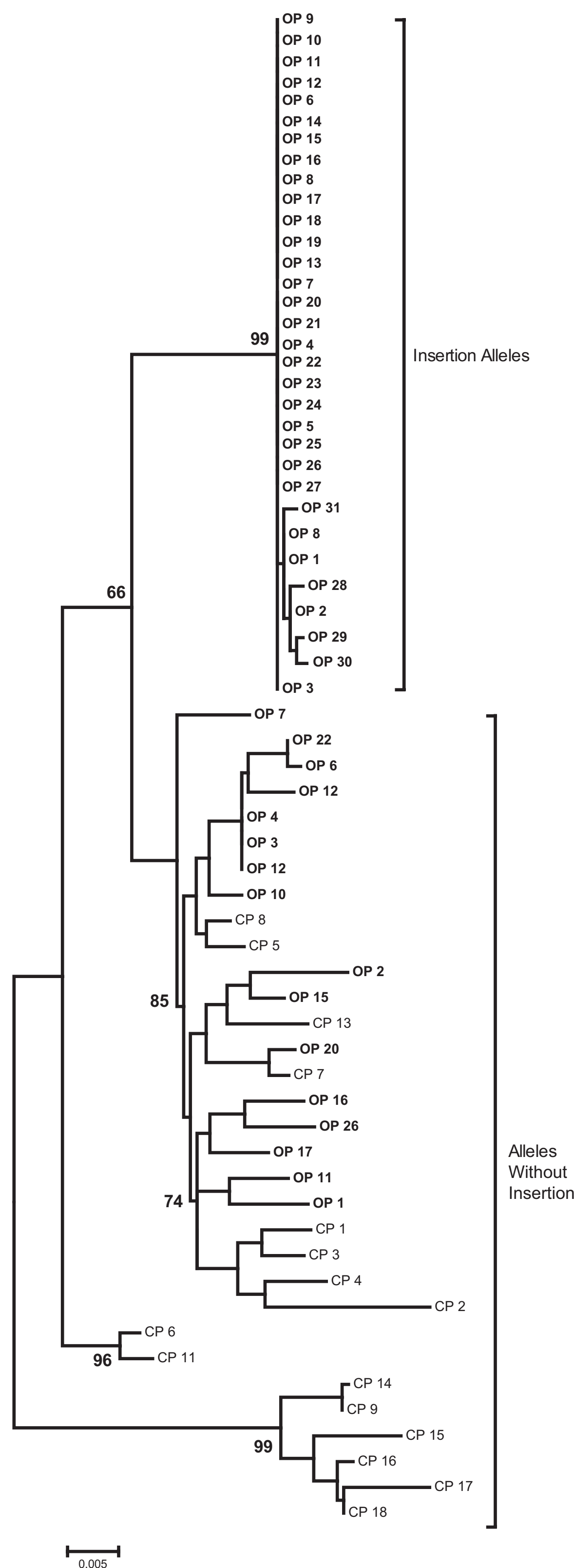

Fig. 2. Phylogenetic analysis of the upstream region of *Rec8-B*. The phylogeny was constructed by using the Tamura three-parameter estimation (19) plus gamma plus invariant sites and the neighbor-joining method (20). The sequences consisted of approximately 1.7 kb of noncoding DNA from the insert and noninsert alleles of OP (bold) and CP; genotypes are detailed in Table S2. Bootstrap values from 1,000 iterations are shown at selected nodes. Although the insertion was removed from this alignment, the insert alleles form a monophyletic group. Scale is in substitutions per nucleotide site.

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Functional Consequences of Insertion. To determine whether there are any systematic differences between sexual and obligately asexual genotypes with respect to *Rec8* gene expression, we measured mRNA levels (Table S6) and compared shotgun-sequenced RT-PCR products in CP and OP genotypes. By using RT-PCR, we tested *Rec8* expression levels in OP or CP females producing resting or direct developing eggs, and in OP and CP males. We also cloned and sequenced a sample of cDNAs created from sexual and obligately asexual females making resting eggs (pooling 15 genotypes each) to assess whether differences exist between these groups with respect to splicing, RNA editing, or other transcriptional features.

This sequencing of mRNAs confirmed intron boundaries of previously annotated cDNA models (http://wfleabase.org and http://genome.jgi-psf.org/Dappu1/Dappu1.home.html), and did not reveal any notable differences in Rec8 transcript sequences between CP and OP females (Table S6). By using RT-PCR, we assayed near–full-length transcripts using primers in conserved regions of the protein-coding sequence (Table S1). We also found evidence of transcription from 655 bp upstream of the start codon into the gene, although the primers used cannot distinguish the originating locus because they are in repetitive regions (Fig. S2), and attempts to use internal locus-specific primers together with upstream primers in repetitive regions were inconclusive. Thus, at least some transcripts are considerably longer than suggested by the available annotation as a result of this extended 5′ UTR. By using a primer anchored in the insertion and another in exon 2, a very faint band of the expected size (with the first intron spliced out) was seen in OP samples, indicating that the insertion allele is transcribed at a very low level.

We also performed quantitative, allele-specific RT-PCR studies of gene expression in a sexual lineage (TEX21) and an OP lineage (NDB5), by using three biological replicates of females producing resting eggs or directly developing embryos, or with empty ovaries (Table S6). Results showed that, for each allele assayed, there was a high degree of variability in the amounts of transcript detected within and among replicates, so no significant differences were found either among loci or between samples (Table S6). In summary, the data do not reveal any large differences between CP and OP clones in levels of transcripts across loci, except for transcription of the insertion allele at *Rec8-B*, which occurred at such low levels that we were unable to reliably detect it. We also did not find systematic differences in transcript sequences, or splice variants. Any functional effect of the insertion on other *Rec8* loci is therefore likely mediated at the posttranscriptional level.

Discussion

In an effort to uncover the mechanism(s) responsible for the origin of obligate asexuality, we have characterized an insertion that is uniquely found in all obligately asexual clones of *D. pulex,* upstream of one copy of a gene encoding the meiotic cohesin Rec8. Our observations are consistent with the idea that this insertion, likely derived from a transposon, has been spreading through sexual populations along with the associated *Rec8-B* allele, and in doing so, conferring the ability to produce resting eggs in the absence of canonical meiosis. A virtually identical copy of the insertion has been found in every OP *D. pulex* tested. Moreover, abrogation of Rec8 function, which could plausibly result from the retroelement insertion, is also known in other species to result in phenotypes similar to those seen in OP *D. pulex* (32–37). The finding that the *Rec8* insertion and portions of three other chromosomes (8) are all strongly associated with OP is concordant with results from other systems, in which mutation of just a small number of loci is required to induce a switch from sexual to asexual reproduction.

In *Arabidopsis thaliana*, meiosis can be engineered to become mitosis by mutations to three different genes, including *rec8*, although the gametes produced by these plants were diploid and not capable of autonomous development (32). Although the causal loci in *D. pulex* remain to be determined, results of our previous association-mapping study (8) indicate the possibility of a comparable functional epistatic interaction, with similarly altered kinetochore orientation and homologous recombination (HR) in OP *D. pulex* being a result of mutation of uncharacterized loci in associated regions of chromosomes V, VIII, IX, or X (8).

Although mutations to multiple loci are required to induce obligate asexuality in *A. thaliana* (32, 33), it is theoretically possible that Rec8 abrogation alone is sufficient to induce pseudomeiosis in *D. pulex*, because loss of Rec8 function affects each of the three processes modified during pseudomeiosis (HR, sister-chromatid orientation, and cohesion). Although we know of no examples of *rec8* mutants that specifically result in successful clonal propagation, mutant or biochemical studies in *Schizosaccharomyces pombe* and other organisms (34–37) are consistent with the idea that loss of Rec8 function could be sufficient to abrogate meiotic sister chromatid cohesion and HR, and to orient sister chromatids to opposite poles. In normal meiosis, the Rec8 protein is specifically required for normal levels of HR and for kinetochore orientation (13, 38–41). For example, in *S. pombe*, when meiotic cohesin Rec8 is replaced by mitotic cohesin Rad21, monopolar attachment of sister kinetochores is not established and aneuploidy is common; in addition, homozygous null mutants for *rec8* exhibit significantly reduced HR (14).

If the loss or disruption of Rec8 protein activity in *Daphnia* is sufficient to induce OP, the remaining regions on chromosomes V, VII, IX, and X associated with OP in *D. pulex* (8) may have no direct mechanistic role in the suppression of meiosis but might instead play roles in processes related to the spread of the suppressor itself, such as male production or activation of parthenogenetically-produced resting eggs.

The perfect association between the presence of the insertion allele and OP raises the question of whether the suppression of meiosis is caused by the insertion per se, the PTC-causing frameshift mutation in the coding sequence downstream, or an unidentified but closely linked genetic element. Although we cannot rule out the last possibility, our data suggest it is unlikely. The region known to be absolutely associated with OP encompasses, at most, a 60-kb stretch flanking *Rec8-B*, from *Rec8-C* (15 kb downstream) to the six genes 40 kb upstream. None of the genes have any characterized roles in meiosis or reproduction. It is also unlikely that the element would be the PTC-debilitated allele itself. Although the PTC may induce haploinsufficiency in OP as a result of lower Rec8 protein titers, similarly defective alleles also exist among the CP lineages we characterized, so they would presumably suffer from the same problem.

Piwi-interacting RNA (piRNA) has roles in gene silencing consistent with characteristics of the meiosis suppressor as well as with patterns of *Rec8* gene expression. In the germline, transposons are silenced by piRNA via translational inhibition and other mechanisms (42–44). Robust transcription of *Rec8* by OP and barely detectable transcription of the insertion allele are consistent with regulated cleavage and accumulation of antisense insertion allele fragments, which could target other alleles via sequence homology to inhibit *Rec8* translation [e.g., by hybridization, which precludes ribosome binding (42–44)]. This hypothesis explains why long insertion-allele transcripts are vanishingly rare, why transcripts of other alleles are not, and how paralogous loci could in principle be silenced by an allele showing very low mRNA levels. Evidence that piRNA can inhibit translation of mRNA from paralogous loci comes from *Drosophila* oogenesis, in which piRNA silences multiple loci as a result of the activity of a single transposon (44). The involvement of piRNA in pseudomeiosis could be tested by transcriptional analysis of germline RNAs, including short RNAs, because silencing RNAs, microRNAs, and piRNAs can be differentiated by their distinctive lengths, sequence motifs, and biochemical modifications (43).

A basic premise of the contagious spread of obligate asexuality in *D. pulex* is that the phenotype is limited to females (5–7), implying that Rec8 activity should be disrupted in OP females but not males. However, there is good evidence of meiotic problems in OP males based on genetic mapping of controlled crosses (8),

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showing that a high proportion of mature sperm have some degree of aneuploidy. Twenty of 28 progeny from our laboratory cross were triploid at some loci coming from the OP male (8). An earlier report that laboratory crosses resulted in a 1:1 segregation of the OP phenotype used seven genetic markers among 10 progeny to conclude that conversion to OP occurs via haploid sperm from OP males (7). However, given the limitations of that study and our observations, the rate of haploid-sperm production among OP males may be quite variable, raising questions about whether all OP clones are equally proficient at faithfully transmitting the genetic determinants of meiosis suppression.

Materials and Methods

The work reported here is based on the same *D. pulex* populations from a North American phylogeographic survey (Table S2) used in earlier association studies (8, 45), which are all lactate dehydrogenase SS genotypes and not hybrids with the subspecies *Daphnia pulicaria*, which appear to have a different genetic basis for obligate asexuality (9).

DNA and RNA Extraction and Analysis. Genomic DNA from clonal isolates was extracted using a modified cetyl trimethyl ammonium bromide method as described previously (45) and quantified by spectrophotometry. Details about PCR amplifications, cloning, and sequencing are provided in *SI Materials and Methods*. Total RNA from whole animals was isolated from clonal females as described (46). RNA from dissected ovaries or individual oocyte clusters of four cells was obtained by hand-dissection in PBS solution followed by extraction with a PicoPure RNA isolation kit (MDS). Details regarding quantitative and standard RT-PCR are provided in *SI Materials and Methods*.

Phylogenetic and Sequence Analysis. Sequence chromatograms were quality-trimmed and, in some cases, assembled by using DNAstar (LaserGene) and aligned initially by using ClustalW, version 1.8 (47), and manually refined in MEGA3 (48). Phylogenetic reconstruction was performed by using jModelTest (49) to select the best-fit models for our data sets, which was the Tamura three-parameter plus gamma plus invariant sites (19), and by using neighbor-joining (20). Tree topology was tested by bootstrap resampling 1,000 times. Population-genetic parameters, including nucleotide diversity estimates, allele-frequency tests, and estimates of LD and gene conversion were performed by using DnaSP, version 4.50 (25).

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1. Engelstädter J (2008) Constraints on the evolution of asexual reproduction. *Bioessays* 30:1138–1150.
2. Maynard Smith J (1978) *The Evolution of Sex* (Cambridge Univ Press, Cambridge, UK).
3. Suomalainen E, Saura A, Lokki J (1987) *Cytology and Evolution in Parthenogenesis* (CRC, Boca Raton, FL).
4. Eads BD, Andrews J, Colbourne JK (2008) Ecological genomics in *Daphnia*: Stress responses and environmental sex determination. *Heredity* 100:184–190.
5. Paland S, Colbourne JK, Lynch M (2005) Evolutionary history of contagious asexuality in *Daphnia pulex*. *Evolution* 59:800–813.
6. Hebert PD, Beaton MJ, Schwartz SS, Stanton DJ (1989) Polyphyletic origins of asexuality in *Daphnia pulex*. I. Breeding-system variation and levels of clonal diversity. *Evolution* 43:1004–1015.
7. Innes DJ, Hebert PD (1988) The origin and genetic basis of obligate parthenogenesis in *Daphnia pulex*. *Evolution* 42:1024–1035.
8. Lynch M, Seyfert A, Eads B, Williams E (2008) Localization of the genetic determinants of meiosis suppression in *Daphnia pulex*. *Genetics* 180:317–327.
9. Decaestecker E, De Meester L, Mergeay J (2009) Cyclical parthenogenesis in *Daphnia*: Sexual versus asexual reproduction. *Lost Sex: The Evolutionary Biology of Parthenogenesis*, eds Schon I, Martens K, van Dyke P (Springer, Dordrecht, The Netherlands), pp 295–316.
10. Zaffagnini F (1987) Reproduction in *Daphnia*. *Memoirie Instituto Italiano Idrobiologia* 45:245–284.
11. Nasmyth K (2001) Disseminating the genome: Joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu Rev Genet* 35:673–745.
12. Kitajima TS, Kawashima SA, Watanabe Y (2004) The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. *Nature* 427:510–517.
13. Nasmyth K, Haering CH (2009) Cohesin: Its roles and mechanisms. *Annu Rev Genet* 43: 525–558.
14. Watanabe Y, Nurse P (1999) Cohesin Rec8 is required for reductional chromosome segregation at meiosis. *Nature* 400:461–464.
15. Klapholz S, Esposito RE (1980) Isolation of *SPO12-1* and *SPO13-1* from a natural variant of yeast that undergoes a single meiotic division. *Genetics* 96:567–588.
16. Lee BH, Kiburz BM, Amon A (2004) Spo13 maintains centromeric cohesion and kinetochore coorientation during meiosis I. *Curr Biol* 14:2168–2182.
17. Haag CR, McTaggart SJ, Didier A, Little TJ, Charlesworth D (2009) Nucleotide polymorphism and within-gene recombination in *Daphnia magna* and *D. pulex*, two cyclical parthenogens. *Genetics* 182:313–323.
18. Omilian AR, Scofield DG, Lynch M (2008) Intron presence-absence polymorphisms in *Daphnia*. *Mol Biol Evol* 25:2129–2139.
19. Tamura K (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol Biol Evol* 9:678–687.
20. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.
21. Colbourne JK, Hebert PD (1996) The systematics of North American *Daphnia* (Crustacea: Anomopoda): A molecular phylogenetic approach. *Philos Trans R Soc Lond B Biol Sci* 351:349–360.
22. Betrán E, Rozas J, Navarro A, Barbadilla A (1997) The estimation of the number and the length distribution of gene conversion tracts from population DNA sequence data. *Genetics* 146:89–99.
23. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585–595.
24. Kelly JK (1997) A test of neutrality based on interlocus associations. *Genetics* 146: 1197–1206.
25. Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–2497.
26. Andolfatto P (2001) Adaptive hitchhiking effects on genome variability. *Curr Opin Genet Dev* 11:635–641.
27. Smith JM, Haigh J (1974) The hitch-hiking effect of a favourable gene. *Genet Res* 23: 23–35.
28. Charlesworth B, Morgan MT, Charlesworth D (1993) The pattern of neutral molecular variation under the background selection model. *Genetics* 134:1289–13033.
29. Fay JC, Wu CI (2000) Hitchhiking under positive Darwinian selection. *Genetics* 155: 1405–1413.
30. Zeng K, Mano S, Shi S, Wu CI (2007) Comparisons of site- and haplotype-frequency methods for detecting positive selection. *Mol Biol Evol* 24:1562–1574.
31. Zeng K, Shi S, Wu CI (2007) Compound tests for the detection of hitchhiking under positive selection. *Mol Biol Evol* 24:1898–1908.
32. d'Erfurth I, et al. (2009) Turning meiosis into mitosis. *PLoS Biol* 7:e1000124.
33. Marimuthu MP, et al. (2011) Synthetic clonal reproduction through seeds. *Science* 331:876.
34. Ding DQ, et al. (2006) Meiotic cohesins modulate chromosome compaction during meiotic prophase in fission yeast. *J Cell Biol* 174:499–508.
35. Onn I, Heidinger-Pauli JM, Guacci V, Unal E, Koshland DE (2008) Sister chromatid cohesion: a simple concept with a complex reality. *Annu Rev Cell Dev Biol* 24:105–129.
36. Storlazzi A, et al. (2008) Coupling meiotic chromosome axis integrity to recombination. *Genes Dev* 22:796–809.
37. Severson AF, Ling L, van Zuylen V, Meyer BJ (2009) The axial element protein HTP-3 promotes cohesin loading and meiotic axis assembly in *C. elegans* to implement the meiotic program of chromosome segregation. *Genes Dev* 23:1763–1778.
38. Brar GA, Amon A (2008) Emerging roles for centromeres in meiosis I chromosome segregation. *Nat Rev Genet* 9:899–910.
39. Gerton JL, Hawley RS (2005) Homologous chromosome interactions in meiosis: diversity amidst conservation. *Nat Rev Genet* 6:477–487.
40. Watanabe Y (2004) Modifying sister chromatid cohesion for meiosis. *J Cell Sci* 117: 4017–4023.
41. Pasierbek P, et al. (2001) A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes Dev* 15:1349–1360.
42. Jordan I, Miller WJ (2008) Genome defense against transposable elements and the origins of regulatory RNA. *Transposons and the Dynamic Genome*, eds Lankenau D-H, Volff J-N (Springer, Dordrecht, The Netherlands), pp 77–94.
43. Aravin A, Hannon GJ (2008) Small RNA silencing pathways in germ and stem cells. *Cold Spring Harb Symp Quant Biol* 73:1–8.
44. Khurana JS, Theurkauf W (2010) piRNAs, transposon silencing, and *Drosophila* germline development. *J Cell Biol* 191:905–913.
45. Cristescu ME, Colbourne JK, Radivojac J, Lynch M (2006) A microsatellite-based genetic linkage map of the waterflea, *Daphnia pulex*: On the prospect of crustacean genomics. *Genomics* 88:415–430.
46. Schurko AM, Logsdon JM, Jr., Eads BD (2009) Meiosis genes in *Daphnia pulex* and the role of parthenogenesis in genome evolution. *BMC Evol Biol* 9:78.
47. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
48. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5:150–163.
49. Posada D (2008) jModelTest: Phylogenetic model averaging. *Mol Biol Evol* 25: 1253–1256.

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