Hybridization and the Origin of Contagious Asexuality in Daphnia pulex

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Abstract

Hybridization plays a potentially important role in the origin of obligate parthenogenesis (OP) in many organisms. However, it remains controversial whether hybridization directly triggers the transition from sexual reproduction to obligate asexuality or a hybrid genetic background enables asexual species to persist. Furthermore, we know little about the specific genetic elements from the divergent, yet still hybridizing lineages responsible for this transition and how these elements are further spread to create other OP lineages. In this study, we address these questions in Daphnia pulex, where cyclically parthenogenetic (CP) and OP lineages coexist. Ancestry estimates and whole-genome association mapping using 32 OP isolates suggest that a complex hybridization history between the parental species D. pulex and D. pulicaria is responsible for the introgression of a set of 647 D. pulicaria single nucleotide polymorphism alleles that show perfect association with OP. Crossing experiments using males of OP lineages and females of CP lineages strongly support a polygenic basis for OP. Single-sperm analyses show that although normal meiotic recombination occurs in the production of haploid sperm by males of OP lineages, a significant proportion of such sperm are polyploid, suggesting that the spread of asexual elements through these males (i.e., contagious asexuality) is much less efficient than previously envisioned. Although the current Daphnia genome annotation does not provide mechanistic insight into the nature of the asexuality-associated alleles, these alleles should be considered as candidates for future investigations on the genetic underpinnings of OP.

Key words: obligate parthenogenesis, Daphnia, sperm, hybridization, introgression.

Introduction

Obligate asexuals are rare in eukaryotes (approximately 1 in 10,000 species) and exhibit a patchy phylogenetic distribution (Bell 1982), indicating their lack of evolutionary potential due to various evolutionary limitations (Maynard Smith 1978; Lynch et al. 1993; Howard and Lively 1994; Park and Krug 2013). Yet the transition from ancestral sexual reproduction to asexuality has occurred independently in many phylogenetic groups through several different routes, such as mutation, microorganism infection, the spread of contagious asexuality elements, and interspecific hybridization (Lynch 1984; Simon et al. 2003; Neiman et al. 2014).

Among these possible mechanisms of origin, hybridization is involved in probably the largest number of obligate asexuals, as evidenced by the hybrid genomic background in many obligate parthenogens, for example, almost all unisexual vertebrates and an expanding pool of invertebrates such as aphids and grasshoppers (Simon et al. 2003; Neiman et al. 2014). However, whether hybridization plays a causal role in the origin of obligate asexuality remains controversial. On the one hand, direct evidence is scarce that asexuals are generated simply by crossing their putative parental species (Schultz 1973; White et al. 1977; Hotz et al. 1985; Choleva et al. 2012). On the other hand, it remains unclear whether the observed association between hybrid genetic background and asexuality is due to unknown evolutionary advantages conferred by hybridization (rather than triggering asexuality) that allow the newly established asexual lineages to persist. Furthermore, few studies (e.g., Tucker et al. 2013; Xu et al. 2013) have examined whether asexuality is caused by the introgressed genetic elements from diverging, yet still hybridizing lineages.

It is usually difficult to precisely pinpoint the specific genetic elements underlying the origin of obligate asexuality, because classical genetic mapping approaches cannot be applied to obligate asexuals incapable of interbreeding with related sexual species. Nonetheless, in situations where interbreeding is possible, previous studies have identified the genomic loci associated with obligate asexuality in plants including Hieracium (Catanach et al. 2006), Erigeron annuus (Noyes et al. 2007) and Taraxacum (Van Dijk et al. 2009), and animals such as the parasitoid wasp Lysiphlebus fabarum (Sandrock and Vorburger 2011), the honeybee Apis mellifera capensis (Lattorff et al. 2005), and the pea aphid
Acyrthosiphon pisum (Jaquiéry et al. 2014). Although these studies have identified one or multiple genetic markers associated with obligate asexuality, we still need to understand what specific genes and/or alleles confer asexuality, the evolutionary origin of these alleles (e.g., whether these alleles are introgressed from a related sexual species), how these elements are transmitted, and ultimately the genetic mechanisms by which introgressed alleles can induce obligate asexuality.

In this study, we address these questions by examining the genetic underpinning of obligate parthenogenesis (OP) in the North American freshwater microcrustacean, Daphnia pulex, a complex of a few morphologically highly similar species. The nominal species D. pulex mainly inhabits ephemeral woodland ponds throughout the temperate zone. As in all other Daphnia species (e.g., D. longispina and D. magna), D. pulex typically reproduces by cyclical parthenogenesis (CP). Under favorable environmental conditions, females produce directly developing embryos through parthenogenesis, generating genetically identical daughters. However, in unfavorable conditions (e.g., food shortage), males are produced through environmental sex determination, and females switch to producing haploid eggs, engaging in sexual reproduction to produce diapausing, fertilized embryos deposited in a protective case (i.e., ephippium). However, most D. pulex populations in the northeast of the continent reproduce by OP, that is, by engaging in parthenogenesis in favorable conditions, but also producing ephippial resting eggs by parthenogenesis in deteriorating conditions (Hebert et al. 1993). Intriguingly, the inability to engage in sex is limited to females, as some D. pulex OP lineages can still produce functional males (Innes and Hebert 1988). Asexuality elements in D. pulex can then spread through males of OP lineages (i.e., contagious asexuality) when they mate with CP females. It has been postulated that when mating with sexual females, these males can transmit the asexuality-conferring elements to approximately 50% offspring (Innes and Hebert 1988).

Previous genome-wide association studies contrasting diploid CP and diploid OP D. pulex from woodland ponds revealed that all asexuals share the same haplotype in at least four genomic regions, including nearly the entirety of chromosomes 8 and 9 (Lynch et al. 2008; Tucker et al. 2013; Xu et al. 2013). The asexual-specific haplotype occurring on a chromosomal scale seems to suggest that these two chromosomes are nonrecombining when transmitted through male meiosis. The ultimate source of the asexual-specific haplotype is introgression from another species in this complex, D. pulicaria, raising the hypothesis that OP originated through an historical introgression event (Tucker et al. 2013; Xu et al. 2013). Daphnia pulicaria mostly inhabits permanent stratified lakes in North America and reproduces by CP. To date, direct experimental tests have failed to reveal any D. pulicaria lineage to be obligately parthenogenetic (Heier and Dudycha 2009).

Interestingly, OP lineages in the D. pulex complex occupy several other temperate-zone habitats that differ drastically from ordinary woodland ponds. For example, triploid OP lineages are widespread in ephemeral ponds in the lower Canadian Arctic (Weider et al. 1987). Moreover, some OP lineages carry diagnostic D. pulicaria alleles (e.g., Ldh Fast allele) in addition to the chromosomes 8 and 9 where asexuality-conferring elements appear to reside (Hebert and Crease 1983; Xu et al. 2013). This latter class of OP lineages was previously called the “urban-clone” group (Hebert and Crease 1983) because they are often found in recently deforested areas. These urban clones exhibit significant ecological tolerance and can inhabit some extremely inhospitable water bodies contaminated by heavy metals (Shaw et al. 2007). They are also notoriously invasive, achieving dominance in many African lakes in just a couple of decades (Mergeay et al. 2006).

However, because the triploid and urban OP lineages were not examined in previous whole-genome association studies, their genetic basis remains unclear.

In this study, we performed association mapping using the whole-genome sequences of a wide geographic collection of CP and OP isolates, extensive crossing experiments, and genetic analysis of single sperm. Our results elucidate the role of hybridization in the origin of OP in Daphnia, the polygenic architecture of OP, and the great inefficiency of transmission of asexuality-conferring elements through males. Moreover, we identified a set of 206 protein-coding candidate genes where introgressed alleles may jointly confer OP, which will greatly facilitate future investigations on the mechanistic basis of the origin of obligate asexuality.

Results

Ancestry of Obligately Parthenogenetic (OP) Lineages

We sequenced the full genomes of 32 OP isolates, including 14 urban clones (10 from metal contaminated habitats) and 3 triploid isolates, 11 CP D. pulex, and 14 D. pulicaria isolates across North America (supplementary fig. S1 and table S1, Supplementary Material online). The phylogeny based on the full mitochondrial genome reveals that all diploid OPs are robustly grouped within the D. pulex clade (fig. 1A), indicating their D. pulex maternal ancestry, whereas the triploid OP isolates can have either D. pulex or D. pulicaria mitochondrial ancestry (one in the pulex clade, and two in the pulicaria clade). A principle component analysis (PCA) using 639,225 bi-allelic nuclear single nucleotide polymorphisms (SNPs) excluding chromosomes 8 and 9 (because the D. pulicaria alleles on these two chromosomes in OP lineages would bias the genome-wide estimate) shows that CP D. pulex and D. pulicaria form two distinct clusters, whereas most OP isolates are scattered in between (fig. 1B). OP isolates 23–32 from heavy metal contaminated habitats in Sudbury, Ontario form a separate cluster mainly because these isolates are genetically highly similar. In line with a previous hypothesis (Xu et al. 2013), the ancestry estimates for OP isolates (fig. 1C) derived from Bayesian- and introgression-based methods using 10,000 randomly selected SNPs consistently demonstrate extensive variation in both the diploid and triploid lineages, suggesting that these asexuals originated from pulex–pulicaria hybrid lineages with variable introgression from D. pulicaria. In extreme situations, they can carry a nearly pure D. pulex or D. pulicaria background, and very
often they are characterized by a higher *D. pulex* ancestry, indicating that their ancestral lineages are advanced back-crosses with *D. pulex*.

**Association Mapping**

To localize the asexual-specific genetic elements, we used a total of 927,855 SNPs to perform association mapping. We found that 647 sites (from 13 scaffolds on chromosomes 5, 6, 8, and 9) contain specific SNPs present in all diploid OP isolates (allowing a maximum of one missing isolate) but not found in any CP lineages (supplementary table S2, Supplementary Material online). In more than 90% of the OP isolates, these sites are heterozygous for the asexual-specific SNP and the SNP found in all CP *D. pulex*.

Because these asexual-specific SNPs are introgressed from *D. pulicaria*, we could estimate their approximate frequency in *D. pulicaria*. In total, 598 of the asexual-specific SNPs are fixed and 49 SNPs represent segregating alleles in our sample of *D. pulicaria* (mean allele frequency 0.59). We note that none of the sequenced *D. pulicaria* isolates possesses the complete suite of asexual-specific SNPs, implying that the OP lineages are most likely not F1s between *D. pulex* and *D. pulicaria*.

The asexual-specific haplotype was reconstructed by assigning the asexual-specific SNPs into the same haplotype in OP isolates and *D. pulicaria* and the other allele into the background haplotype (see Materials and Methods). Consistent with previous analyses (Tucker et al. 2013), our neighbor-joining tree clearly demonstrates the origin of asexual-specific haplotypes from *D. pulicaria*, forming a robust clade with *D. pulicaria* haplotypes, whereas most of the background haplotypes in asexuals are placed in the *D. pulex* clade (fig. 2). The background haplotypes of three asexual isolates (op18–20) formed a sister clade to *D. pulicaria* largely because these haplotypes contain the asexual-specific SNPs at a large fraction of sites (i.e., many sites in these asexuals are homozygous for asexual-specific SNPs). Gene conversion may be responsible for the frequent occurrence of asexual-specific SNPs in these background haplotypes as *Daphnia* experience a high rate of gene conversions (Omlilan et al. 2006; Xu et al. 2011; Tucker et al. 2013). However, it remains plausible that these alleles are accumulated through backcrossing with *D. pulicaria*.

**FIG. 1.** (A) Neighbor-joining tree based on full mitochondrial genome sequence for all sequenced cyclical parthenogenetic *Daphnia pulex* (px), obligately parthenogenetic *D. pulex* (op), and *D. pulicaria* (pa) isolates. Asterisks indicate triploid OP isolates. The unit for the scale bar is the number of substitutions per site. (B) PCA plot using 639,225 nuclear SNPs (excluding chromosomes 8 and 9) for all *Daphnia* isolates. (C) Bayesian ancestry estimates for all sequenced isolates based on the same set of SNPs, with CP *D. pulex* and *D. pulicaria* designated as the ancestors. Each bar represents an individual, with blue color corresponding to the ancestry of *D. pulex* and red the ancestry of *D. pulicaria*. Open oval shape represents values of transformed hybrid index ($1 - H$) for OP isolates, which can be considered as the amount of *D. pulex* ancestry.
Annotation of Asexual-Specific SNP Alleles

In total, 489 of the 647 SNPs affect protein-coding genes (e.g., exons, introns, and untranslated regions), whereas 158 are located in intergenic regions. Based on the current *Daphnia* genome annotation, we examined the functions of the affected 206 protein-coding genes and classified them into different KOGs (Eukaryotic Ortholog Groups) to determine whether any category is significantly enriched. Although no KOG shows significant enrichment, we identified 22 KOGs that affect many biological functions (table 1), including meiosis (e.g., chromatin structure and dynamics, replication, recombination, and repair, and cell cycle control, cell division, chromosome partitioning), signal transduction, transcription, and posttranslational modification, and so forth. The $d_{ns}/d_s$ (ratio between nonsynonymous and synonymous divergence) values for these 206 genes in *D. pulex* and *D. pulicaria* are all significantly less than 1, indicating the absence of positive selection at the whole-gene level during the divergence of these two species.

Single-Sperm Analysis

We performed single-sperm analyses to examine whether males from OP lineages are an efficient vector for the spread of asexuality-conferring elements. Utilizing fluorescence-activated cell sorting, we isolated single sperm from clonally produced males for four asexual (op5, op7, op9, and op32) and two sexual isolates (px2 and px5). In contrast to the large amount of haploid sperm from sexual isolates, the total amount of sperm from males of asexuals is much lower (supplementary fig. S2, Supplementary Material online) except for op9. More strikingly, three OP isolates produce a significant proportion of diploid and even tetraploid sperm, indicating low fertility, whereas in one OP isolate (op9) only haploid sperm occurs (supplementary fig. S2, Supplementary Material online).

The transmission of the whole suite of asexuality-conferring elements to offspring (i.e., when males of OP lineages cross with CP females) rests critically on whether meiotic recombination occurs on chromosomes 8 and 9.
constructed their haplotypes by genotyping microsatellite loci. Furthermore, the test between the marker d007 (chr 8) and d118 (chr 9) supports the hypothesis that these two chromosomes can freely segregate. Under the assumption that the co-occurrence of asexual-specific alleles on chromosomes 8 and 9 is essential to asexuality, the occurrence of recombination during OP male spermatogenesis suggests that only a small fraction of haploid sperm may serve as an efficient vector for the spread of asexuality elements, although in principle some fraction of sperm will still transmit the full chromosome 8/9 asexual-specific haplotype.

This is because the asexual-specific haplotype spans a large portion of both chromosomes 8 and 9, which can be broken apart by recombination and segregation. To detect meiotic recombination on these chromosomes, we whole-genome amplified 88 single haploid sperm from op9, and reconstructed their haplotypes by genotyping microsatellite loci. A four-gamete test for recombination within chromosome 8 strongly suggests the occurrence of crossover events (table 2), although chromosome 9 remains inconclusive due to low amplification success and small number of available microsatellite loci. Furthermore, the test between the marker d007 (chr 8) and d118 (chr 9) supports the hypothesis that these two chromosomes can freely segregate. Under the assumption that the co-occurrence of asexual-specific alleles on chromosomes 8 and 9 is essential to asexuality, the occurrence of recombination during OP male spermatogenesis suggests that only a small fraction of haploid sperm may serve as an efficient vector for the spread of asexuality elements, although in principle some fraction of sperm will still transmit the full chromosome 8/9 asexual-specific haplotype.

### Table 1. KOG Classification for the Number of Protein-Coding Genes Associated with Asexual-Specific Alleles.

<table>
<thead>
<tr>
<th>KOG</th>
<th>No.</th>
<th>Genome Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>General function prediction only</td>
<td>39</td>
<td>2,859</td>
</tr>
<tr>
<td>Signal transduction mechanisms</td>
<td>22</td>
<td>1,846</td>
</tr>
<tr>
<td>Function unknown</td>
<td>13</td>
<td>1,052</td>
</tr>
<tr>
<td>Transcription</td>
<td>13</td>
<td>1,112</td>
</tr>
<tr>
<td>Posttranslational modification,</td>
<td>10</td>
<td>1,209</td>
</tr>
<tr>
<td>protein turnover, chaperones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translation, ribosomal structure,</td>
<td>10</td>
<td>466</td>
</tr>
<tr>
<td>and biogenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid transport and</td>
<td>9</td>
<td>643</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate transport and</td>
<td>9</td>
<td>633</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy production and conversion</td>
<td>9</td>
<td>256</td>
</tr>
<tr>
<td>Intracellular trafficking, secretion, and vesicular transport</td>
<td>9</td>
<td>633</td>
</tr>
<tr>
<td>Inorganic ion transport and</td>
<td>8</td>
<td>554</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA processing and modification</td>
<td>8</td>
<td>664</td>
</tr>
<tr>
<td>Secondary metabolites biosynthesis, transport, and catabolism</td>
<td>8</td>
<td>210</td>
</tr>
<tr>
<td>Chromatin structure and dynamics</td>
<td>7</td>
<td>393</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>7</td>
<td>603</td>
</tr>
<tr>
<td>Extracellular structures</td>
<td>6</td>
<td>494</td>
</tr>
<tr>
<td>Lipid transport and metabolism</td>
<td>6</td>
<td>473</td>
</tr>
<tr>
<td>Replication, recombination, and repair</td>
<td>6</td>
<td>422</td>
</tr>
<tr>
<td>Cell cycle control, cell division, chromosome partitioning</td>
<td>3</td>
<td>342</td>
</tr>
<tr>
<td>Defense mechanisms</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>Coenzyme transport and metabolism</td>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>Nucleotide transport and</td>
<td>1</td>
<td>114</td>
</tr>
<tr>
<td>metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td><strong>206</strong></td>
<td><strong>15,144</strong></td>
</tr>
</tbody>
</table>

Note.—Genome total designates the total number of known genes in the genome.

Crossing Experiments

Our extensive laboratory crosses confirm the inefficiency of contagious asexuality in *D. pulex*. In accord with the sperm data, crosses using males of op9 (producing only haploid sperm) with females of multiple CP isolates had one of the highest *F*₁ hatching rates (36%), whereas crosses involving males of op5 that make diploid sperm had a hatching rate of only 1% (table 3). A total of 122 *F*₁ hybrids were established. To determine the reproduction mode of these *F*₁s, we used the rule that in the absence of males, OP females should be able to deposit resting eggs into ephippia, whereas sexual females cannot do so and produce empty ephippia. However, the reproductive mode of most hatched *F*₁s is difficult to determine because there is a great amount of variation in ephippial production among clones (e.g., many of them do not produce ephippia), presumably due to some reproductive deficiency, although they can produce parthenogenetic offspring through directly developing embryos. Moreover, some *F*₁s occasionally produce both developing eggs and resting eggs inside of ephippia (table 4). Only 7 of the total established 122 *F*₁s can be assigned a reproductive mode (three possible OPs; table 4), which still remains quite uncertain because some of their resting embryos did not successfully hatch.

### Discussion

Hybridization and the Origin of OP

The current data and previous analyses (Tucker et al. 2013; Xu et al. 2013) unambiguously show that the asexual-specific alleles in OP *D. pulex* originated in *D. pulicaria*, strongly suggesting that *D. pulex* constitutes another example of an obligate parthenogen with a hybrid ancestry. The association between obligate asexuality and hybrid ancestry can be interpreted as evidence for meiotic abnormalities derived from hybridization causing asexual reproduction. Our observation that all OP isolates carry the same suite of asexual-specific SNPs introgressed from *D. pulicaria* clearly supports this interpretation. Below we consider two different, but closely related roles of hybridization in creating the asexuality-conferring haplotype in the first place and in the subsequent spread of this haplotype to create new lineages.

Despite the large number of obligate asexuals with hybrid ancestry (Bell 1982; Lynch 1984), only a few crossing experiments have directly generated asexual *F*₁s using the identified parental species, for example, marsh frog *Rana ridibunda* (Hotz et al. 1985), the fish *Poeciliopsis* (Schultz 1973), Cobitis loaches (Choleva et al. 2012), and the grasshopper Warramaba (White et al. 1977). Previous crossing experiments using *D. pulex* females and *D. pulicaria* males (because all diploid OP isolates have *D. pulex* maternal/mitochondrial ancestry; fig. 1A) from multiple locations have only generated CP *F*₁s (Heier and Dudycha 2009), suggesting that hybridization need not directly create asexual lineages. Indeed, given the substantially variable amount of *D. pulex* and *D. pulicaria* ancestry in different OP isolates (fig. 1C), we can exclude the idea that continuous direct hybridization between an unknown lineage of *D. pulicaria* and CP *D. pulex* directly
duced eggs through modified meiosis and the spontaneous lineage giving rise to asexual lineages. In purely sexually reproducing species, two genetic modifications are essential for switching to asexuality: a haplotype that can produce unreduced eggs in a parthenogenetic fashion, and a haplotype that can negate meiotic recombination in the progenitor lineages. The presence of four haplotypes for d077/d118 strongly suggests that chromosomes 8 and 9 can segregate in male meiosis. The total number of haplotypes for each pair of loci is different because sperm cells did not show consistent amplification across all loci. The recombinant frequency is calculated as the proportion of the two least common haplotypes among the four possible haplotypes.

Table 2. Four Gamete Test: Occurrences of the Four Possible Haplotypes for Four Pairs of Microsatellite Loci on Chromosomes 8 and 9.

<table>
<thead>
<tr>
<th>Locus/Locus</th>
<th>Haplotype1</th>
<th>Haplotype2</th>
<th>Haplotype3</th>
<th>Haplotype4</th>
<th>Recombinant Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>d077/d117</td>
<td>7</td>
<td>3</td>
<td>15</td>
<td>11</td>
<td>0.39</td>
</tr>
<tr>
<td>d117/d150</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0.27</td>
</tr>
<tr>
<td>d150/d192</td>
<td>1</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>0.18</td>
</tr>
<tr>
<td>d077/d118</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Note.—The markers d077, d117, d150, and d192 are on chromosome 8, whereas d118 is on chromosome 9. The haplotype data for d077/d117, d117/d150, and d150/d192 support the presence of meiotic recombination within chromosome 8. The presence of four haplotypes for d077/d118 strongly suggests that chromosomes 8 and 9 can segregate in male meiosis. The total number of haplotypes for each pair of loci is different because sperm cells did not show consistent amplification across all loci. The recombinant frequency is calculated as the proportion of the two least common haplotypes among the four possible haplotypes.

Table 3. Summary of the Numbers of Ephippia, Resting Eggs, Hatched Eggs, and Hatching Rate of Resting Eggs in Crossing Experiments between Males of CP and OP Isolates and Females of Multiple CP Isolates.

<table>
<thead>
<tr>
<th>Males</th>
<th>Ephippia</th>
<th>Resting Eggs</th>
<th>Hatched Eggs</th>
<th>Hatching Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP pulex</td>
<td>9</td>
<td>11</td>
<td>10</td>
<td>91</td>
</tr>
<tr>
<td>op5</td>
<td>44</td>
<td>76</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>op6</td>
<td>36</td>
<td>40</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>op8</td>
<td>14</td>
<td>15</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>op9</td>
<td>173</td>
<td>229</td>
<td>83</td>
<td>36</td>
</tr>
<tr>
<td>op10</td>
<td>20</td>
<td>29</td>
<td>5</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 4. Results of Reproductive Mode Test for Seven Cases of F1s between CP Females and Males of OP Isolates, Showing the Number of Ephippia with No Resting Eggs, Number of Ephippia with Resting Eggs, Number of Hatched Resting Eggs, and Possible Reproduction Mode.

<table>
<thead>
<tr>
<th>Parents</th>
<th>Eggs</th>
<th>Eggs</th>
<th>Hatched Eggs</th>
<th>Reproduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>px2/</td>
<td>op10</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>px4/</td>
<td>op9</td>
<td>20</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>px4/</td>
<td>op9</td>
<td>8</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>px4/</td>
<td>op9</td>
<td>31</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>px4/</td>
<td>op9</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>px6/</td>
<td>op9</td>
<td>40</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>px5/</td>
<td>op6</td>
<td>49</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Note.—Question marks indicate resting egg production in the absence of males does not strictly match expectation of our sexuality test because the F1s did not consistently produce resting eggs (although still capable of parthenogenesis).

Cases where developing eggs were deposited into ephippia.

gave rise to all the OP lineages, which, in contrast to our observation, predicts all asexual lineages to have similar ancestry profiles. We therefore hypothesize that the creation of the asexuality-conferring haplotype involved one original cross between D. pulex and D. pulicaria followed by one or multiple backcrosses events with other D. pulicaria lineages. However, it remains unknown whether the asexuality-conferring haplotype originated once or multiple times through hybridization in the evolution of Daphnia.

Under the scenario of contagious asexuality (Innes and Hebert 1988), males of OP lineages can spread an asexuality-conferring haplotype to some offspring through crossing with females of CP lineages (see below). Given the variable amount of D. pulicaria ancestry across the four loci that we examined, we suggest that they most likely originated from CP progenitors that carry different amounts of ancestry of D. pulicaria through multiple hybridization events before capturing the asexuality-conferring haplotype and becoming asexual. Nonetheless, we cannot determine whether the loss of sexual reproduction is due to the capture of asexuality-conferring elements in the progenitor or whether these progenitor lineages became asexual through hybridization/backcross that independently recreates the asexuality-conferring elements.

Genetic Basis of OP

The fact that Daphnia is typically a cyclical parthenogen distinguishes itself from other cases of obligately sexual ancestors giving rise to asexual lineages. In purely sexually reproducing species, two genetic modifications are essential for switching to asexuality, that is, the capacity to produce unreduced eggs through modified meiosis and the spontaneous development of eggs without fertilization by sperm. Because CP D. pulex already can produce directly developing embryos through a modified meiosis in its parthenogenetic cycle of life history (Hirata et al. 2010), the most parsimonious way to become obligately asexual is to simply replace the sexual cycle with existing capability of producing unreduced eggs in a parthenogenetic fashion, with the provision that such eggs be diapausing rather than directly developing.

In contrast to the previous hypothesis suggesting that approximately 50% of the offspring of crosses between males of OP lineages and CP females are asexual (Innes and Hebert 1988), our crossing experiments suggest that this mode of transmission is much less efficient. Most hatched F1s from crosses using different combinations of parental lineages produced few ephippial resting eggs, although they could produce offspring parthenogenetically through directly developing embryos. It is possible that our experimental conditions are not optimal for resting egg production. However, a similar observation was made by Innes and Hebert (1988) in the same kind of crossing experiments, where the majority of F1s only produces ephippia containing no resting embryos even in the presence of males, which could be interpreted as incompatibility between parental genomes. This observation seems to be consistent with a polygenic model of OP, as the low frequency of observed OP F1s (3 of 122 F1s) in our data cannot be reconciled with a single-locus model. Because the asexual-specific haplotype spans a large fraction of two
separate chromosomes 8 and 9, the chances appear to be low that the complete asexual-specific haplotype is kept intact during OP male meiosis (i.e., not affected by recombination and independent assortment) and inherited by F₁s. Incomplete asexuality-specific haplotypes resulting from recombination could then create difficulties in producing resting eggs/ephippia in most F₁s, consistent with our observation that the vast majority of F₁s cannot even be assessed for their reproduction mode. Furthermore, the polygenic model is consistent with the results of our association mapping (see below).

The Degenerative Male Function of OP Lineages

Our conventional understanding of OP in Daphnia is that meiosis-suppression is sex-limited in OP lineages, that is, males still can engage in meiosis whereas females cannot. However, our single-sperm analysis suggests that males in many OP isolates are not fully functional. Males in three of the four OP isolates examined produced polyploid sperm and much smaller total amounts of sperm than males of CP isolates. The observation of polyploid sperm is consistent with the finding of triploid isolates from laboratory crosses and in the field (Lynch et al. 2008; Vergilino et al. 2011), which presumably arise through fertilization of haploid eggs by diploid sperm. More specifically, the presence of diploid sperm in males of OP Daphnia lineages may provide an explanation for the origin of triploid OP Daphnia lineages, which could result from a haploid egg fertilized by diploid sperm carrying the asexuality-conferring alleles. This possibility needs to be further investigated in future studies.

Asexuality-Conferring Haplotype

Although we have identified 206 protein-coding genes associated with 647 asexual-specific SNPs, the specific genetic factors underlying OP in Daphnia remain elusive. With 52 of the affected genes having only general function predictions or unknown functions, no particular categories of cellular function show enrichment of genes. Despite some interesting candidate genes affecting meiosis, cell cycle, and DNA replication, we currently are not able to distinguish between genes resulting from spurious association and the causal genes. Future association studies using a larger panel of asexuals of different geographic origins would alleviate this issue to some extent. Although not all marker-associated genes need be involved in OP, the large number of identified genes is qualitatively consistent with the proposed model of the polygenic model of OP. It should be noted that our set of candidate factors does not include the Rec8 gene, which was previously hypothesized to have a causal relationship with OP in Daphnia (Eads et al. 2012). A single genetic modification is not supported by our crossing experiment and seems unlikely to meet the requirements of our proposed model unless the affected genes are pleiotropic.

Future Directions

Based on the data presented in this study, we envision at least two necessary genetic modifications for the origin of OP, that is, the ameiotic production of unreduced eggs using the parthenogenetic reproductive pathway, and genetic modifications to transform unreduced, directly developing eggs into resting eggs. To further test this hypothesis and to pinpoint the genes critical for OP, we suggest the initial step would be to examine genome-wide gene-expression data contrasting CP and OP lineages at the stage immediately before ephippial reproduction. Although it is well known that Daphnia can switch to producing resting eggs under unfavorable environmental conditions, genome-wide gene expression profiles for Daphnia entering the phase of resting egg production in CP and OP lineages are lacking. Such data sets would be complementary to what has been understood at the genome-wide DNA sequence level, and will be invaluable for deciphering the genetic basis for sexual reproduction and genetic mechanisms for the abolition of sex.

Materials and Methods

Sampling, Library Preparation, and Sequencing

A total of 11 cyclically parthenogenetic (CP) D. pulex isolates and 32 obligately parthenogenetic (OP) D. pulex (including three triploid isolates) were collected from ephemeral ponds and metal-contaminated habitats across their known distribution range in the United States and Canada. Additionally, 14 CP D. pulicaria isolates were collected from stratified, permanent lakes across the continent (supplementary fig. S1 and table S1, Supplementary Material online). Furthermore, we sampled one isolate of D. arenata and one isolate of D. obtusa (as outgroups in phylogenetic analysis, see below). The collected isolates were brought to the laboratory, isolated, and maintained in filtered lake water at 20 °C. These isolates were maintained under benign laboratory conditions so that they can reproduce parthenogenetically essentially indefinitely. A clonal line for each isolate was fed ad libitum with a suspension of Scenedesmus obliquus. We performed allozyme assays on the LDH (lactate dehydrogenase) locus for all asexuals. We determined SF heterozygotes as the urban clones, as D. pulex is homozygous for the S (slow) allele and D. pulicaria is homozygous for the fast (F) allele (Hebert et al. 1989).

DNA from approximately 60 individuals of each clonal line was extracted using a cetyltrimethylammonium bromide method (Doyle JJ and Doyle JL 1987) after the animals were starved in clean water overnight. DNA libraries for short-read sequencing were prepared following Illumina’s standard protocol. Paired-end sequencing was performed on the Illumina platform with 100-bp or 150-bp reads.

Reproductive Mode Test

The reproductive mode of each isolate was determined using a sexuality test. Because cyclical parthenogens need sperm fertilization to produce diapausing embryos, the sexuality tests involved examining whether, in the absence of males, diapausing embryos were deposited into ephippia by females. Three to five parthenogenetic offspring descended from each isolate were used for sexuality tests. Consistent results of no ephippial embryos from at least three consecutive rounds of
tests suggest the status of cyclical parthenogen, whereas consistent presence of embryos indicates the status of obligate parthenogen.

Read Mapping and SNP Identification

Raw reads for each sequenced isolate were mapped to the *D. pulex* reference nuclear genome (Colbourne et al. 2011) and mitochondrial reference genome (GenBank accession number AF117817.1) using the software Novoalign (Novocraft Technologies) and the default mapping parameters, as previously done in Tucker et al. (2013). The raw fastq files have been deposited at NCBI Short Read Archive under study accession number SRP062107 and accession numbers SAMN02252729–SAMN02252752 (Tucker et al. 2013). To eliminate misidentification of SNPs in the downstream analysis, reads that were mapped to multiple locations (e.g., duplicate genes, repetitive regions, mobile elements) were removed (-r none option in Novoalign).

To identify the genomic regions associated with OP, we generated a set of 927,855 SNPs using the sequenced CP, OP *D. pulex* and *D. plicaria* genomes. To avoid problems associated with SNP loci where three alleles could exist in triploid OP isolates, we restricted our analysis to only bi-allelic loci. An initial set of SNPs was generated using the mpileup function and bcftools in Samtools 0.1.18 (Li et al. 2009). False SNP calls often result from sequencing errors, PCR artifacts in library construction, mismapped reads, and misalignment caused by indels. To filter out the false-positive SNPs, we employed the following set of stringent criteria (see Auton et al. 2012): Phred-scaled quality score greater than 30 (i.e., <0.1% chance of a wrong base call), no signs of strand bias (i.e., covered by both forward and reverse reads), and no signs of tail distance bias (i.e., not enriched at the ends of reads that are prone to sequencing errors). Further filtering was performed to exclude indels, sites with minor-allele frequency less than 0.05 and genotype call quality less than 30 (using sequence coverage information), and sites where genotypes cannot be determined for more than 10% of the samples.

Mitochondrial Phylogenetic Analysis

In the mitochondrial genome analysis, the consensus sequence of the full mitochondrial genome sequence was generated for each isolate using Samtools 0.1.18 (Li et al. 2009). Furthermore, we added to the data set the full mitochondrial genome sequences of an isolate of *D. obtusa* and an isolate of *D. arenata*. We used the software Muscle 3.8 (Edgar 2004) to generate the multiple sequence alignment for the mitochondrial genomes. To examine the relationship among the mitochondrial genomes of the sequenced isolates, we constructed a neighbor-joining tree using the full sequence. Bootstrap test with 1,000 replicates was done to test the robustness of major nodes on the phylogeny.

Principle Component Analysis

PCA aims to reduce the dimensionality of data while retaining most of the variation in the data. With the identified principle components, it is possible to represent the data in a much smaller number of components and to identify the grouping and similarities of samples. Because the OP isolates share alleles on chromosomes 8 and 9 with *D. pulicaria*, we removed SNPs on the scaffolds belonging to these chromosomes to provide an unbiased view of the grouping of the sequenced *Daphnia* isolates. PCA was performed using 639,225 SNPs from the rest of the genome for all sequenced CP and OP *D. pulex* and *D. pulicaria* isolates in the R software package adegenet (Jombart 2008; Jombart and Ahmed 2011).

Ancestry Estimates

We used the software Structure 2.3.4 (Pritchard et al. 2000) to estimate the ancestry of the sequenced OP isolates, using CP *D. pulex* and *D. pulicaria* as the putative ancestral species (i.e., K = 2). All OP isolates were analyzed as unknowns under an admixture ancestry model. Because Structure cannot efficiently handle the large number of loci (approximately 0.6 million) for this data set, we randomly selected 10,000 loci for each analysis and repeated it ten times. Burn-in and run lengths were set to 200,000 and 1,000,000, respectively. As the results from each analysis were nearly identical, we present the average estimates across the runs. We also used the R software package INTROGRESS that does not assume Hardy–Weinberg equilibrium to estimate the hybrid index of the OP individuals (Gompert and Buerkle 2010). INTROGRESS is a regression-based method and provides maximum-likelihood estimates of hybrid index using multilocus genotype data. With CP *D. pulex* and *D. pulicaria* as fixed parental sources, a hybrid index (H index) of 0 represents pure *D. pulex* genomic background, whereas an H index of 1 represents pure *D. pulicaria* background. It should be noted that a low H index value corresponds to a low *D. pulicaria* and high *D. pulex* Bayesian ancestry estimate.

Asexual-Specific SNPs

We performed genome-wide association analysis to detect the asexual-specific SNPs. A previous study (Tucker et al. 2013) identified a set of 27,760 SNPs that are perfectly associated with OP with a smaller set of OP isolates. With a much wider geographic sampling, we detected asexual-specific alleles using 927,855 high-quality SNPs across the genome. An asexual-specific allele is called when found in all diploid OP isolates with a maximum of one missing isolate, whereas not detected in any CP *D. pulex* (with at most one CP isolate missing). For all detected asexual-specific alleles, we examined the frequency of asexual-specific alleles in the pool of 14 *D. pulicaria* isolates excluding all the missing data at these sites.

In total, we found asexual-specific SNPs at 647 sites (supplementary file S1, Supplementary Material online). These loci are homozygous for the nonasexual-specific allele in CP *D. pulex*, whereas in diploid OP *D. pulex* they are heterozygous or homozygous for the asexual-specific allele greater than 90% of the time. Based on this feature of the data, these genotypes were phased into two haplotypes using the following method. For CP *D. pulex*, the two identical alleles were randomly...
placed into each of the haplotypes, whereas in asexual D. pulex and D. pulicaria the asexual-specific allele is always put into the first haplotype (asexual-specific haplotype) with the other allele into the second haplotype (background haplotype). After phasing, these sites were concatenated to form the asexual-specific haplotype and background haplotype. Using D. arenata as an outgroup, we constructed a neighbor-joining tree including all phased haplotypes. A bootstrap of 1,000 replicates was done to generate statistical support for major clades.

To understand what genomic features (e.g., exon, intron, and intergenic region) these asexual-specific alleles affect, the genomic context of these alleles was annotated using the software snpEff (Cingolani et al. 2012) and the genomic context of these alleles affect, the genomic context of these alleles was annotated using the software snpEff (Cingolani et al. 2012) and the Daphnia-frozen gene catalog (http://genome.jgi-psf.org/Dappu1/Dappu1.home.html, last accessed September 14, 2015). We classified the affected protein-coding regions based on their KOG terms.

dn/ds Analysis

We calculated the ratio of nonsynonymous divergence and synonymous divergence (dn/ds) for the 206 genes associated asexual-specific SNPs using their sequences in 11 CP D. pulex and 14 D. pulicaria isolates. The consensus sequence for the genes in each isolate was generated using Samtools 0.1.18 (Li et al. 2009). Because the consensus sequence contains heterozygous sites, we used the program Phase version 2.1 (Stephens et al. 2001; Stephens and Scheet 2005) to computationally recreate the two separate haplotypes. The divergence at nonsynonymous sites (dn) and synonymous sites (ds) were computed using custom scripts, assuming no multiple mutation hits per site and corrected weighting for 2-fold degenerate sites. A dn/ds value greater than 1 indicates positive selection, whereas a value less than 1 signifies purifying selection.

Sperm Analysis

We performed sperm ploidy analysis for four OP isolates (op5, op7, op9, and op32) and two CP isolates (px2 and px5). For each isolate, we collected 30 mature males and collected sperm by squeezing the abdominal part of the animal under a cover slip. The presence of sperm was confirmed by morphological examination under microscope. The collected sperm was stained using Hoechst Blue, a dye that binds to double-stranded DNA, and the stained sperm was analyzed for ploidy levels and sorted by an FACS Ariall Flow Cytometer (BD Biosciences) for microsatellite genotyping.

To genotype each sperm and detect recombination using genotype data, a total of 88 isolated single sperm for op9 was whole-genome amplified following the MALBAC (multiple annealing and looping-based amplification cycles) protocol (Zong et al. 2012). We genotyped 17 microsatellite markers spanning the chromosomes 8 and 9 (Cristescu et al. 2006) using an ABI 3730 genetic analyzer (Life Technologies). The allele sizes of the genotyped microsatellite loci were analyzed using GeneMapper software 4.0 (Life Technologies). With the obtained haplotype data, we performed the four-gamete test (Hudson and Kaplan 1985) to determine the presence of recombination (i.e., crossover). In this test, the presence of recombination between two bi-allelic loci is inferred when the four possible genotypes are observed, assuming the possibility of repeated mutation is zero. Despite the high mutation rate for microsatellite loci ($10^{-5}$ event/locus/generation) relative to base-substitution rate ($10^{-9}$) in Daphnia, the chance of repeated mutation at a locus is negligible given the single generation time.

Crossing Experiments

We performed crosses between CP females and males of OP isolates in 40-ml scintillation vials containing filtered, autoclaved lake water. Furthermore, as a positive control to show that the experimental conditions used for hatching F1’s are optimal, we crossed CP females and CP males. The single-celled alga S. obliquus was used as food, with the standard concentration 120,000 cells/ml. Males were obtained from mass culture that were fed irregularly and allowed to reach high density. Examination of the ovaries can be used to distinguish females that will produce ameiotic subitaneous (i.e., directly developing) eggs in their next clutch from those that will produce meiotic ephippial eggs. The former have rather bulbous ovaries that tend toward green in color, whereas the later have reduced, smoother and blacker ovaries. The latter is called pre-ephippial females. Because fertilization is thought to occur very close in time to molting, the use of pre-ephippial females (still in the pre-ephippial instar) assures greater crossing success.

To obtain pre-ephippial females, batches of eight newborn females were placed in vials with the standard food concentration at 20 °C with a 12/12 light cycle. No further food was added. Over the course of the next several days, decreasing food availability occurred as the individuals approached maturity. These conditions were found to maximize the proportion of females that produced meiotic eggs in either the first or second mature instar. Pre-ephippial females could be identified and isolated by examination under a compound dissecting microscope. Multiple pre-ephippial females were put into a fresh vial with standard food along with at least one male. Starting on the third day and thereafter, the contents of each vial were examined for the presence of released ephippia. Each batch of one or more resting eggs decapsulated from ephippia was placed in distilled water in a plastic petri dish, wrapped in aluminum foil, and placed in a refrigerator at 4 °C. After 2 weeks, the covered petri dishes were unwrapped and put under bright 24 h light at room temperature and examined daily. Those showing clear signs of segmentation in developing embryos were transferred into a clean petri dish containing 5 ml of standard food and returned to the bright light. After 2 weeks (assuming no further development of the remaining eggs was observed), the dishes were cycled through additional treatment cycles. Often, several cycles were necessary to get some of the eggs to develop. Each day, any hatchlings were transferred to individual 40-ml vials containing standard food, which was replenished as needed. To determine the reproductive mode
of F1s, we conducted sexuality test following the protocol specified in the section Reproductive Mode Test.

**Supplementary Material**

Supplementary file S1, figures S1 and S2, tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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**References**


