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Ecological speciation in a cyclic parthenogen: Sexual capability of experimental hybrids between *Daphnia pulex* and *Daphnia pulicaria*

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Abstract

Daphnia pulex and *Daphnia pulicaria* are cyclically parthenogenetic crustaceans that are distributed widely in North American freshwaters. Hybrids of *D. pulex* and *D. pulicaria* are common in the wild, where population genetic analyses have indicated that they are invariably obligately asexual. In this study, we characterize three sexual aspects (sexual egg production, male production, and sexual offspring production) of life history in experimentally bred hybrids to determine the degree of reproductive isolation. In striking contrast to natural hybrids, out of the 53 F_1 hybrids we bred, none were obligately asexual. Neither male production nor sexual egg production was limited by hybridization. Our experimental crosses showed that there was little difficulty in generating or backcrossing the F_1 generation. In addition, we were able to intercross F_1s successfully to produce F_2 progeny. We found no evidence that these taxa are reproductively incompatible, despite other reports of apparent adaptive genetic divergence. We conclude that reproductive isolation between *D. pulex* and *D. pulicaria* is due to ecological separation, not genetic incompatibility. In light of our results and published data on the biogeography and breeding system of these taxa, neither sterility due to hybridization nor direct inheritance of a sex-limited meiosis suppressor appears to provide a satisfactory explanation for the asexuality of wild hybrids. We propose an alternate hypothesis in which one parental taxon invades a population of the other, the resulting F_1 hybrids cross back to a carrier of a meiosis suppressor, and out of the resulting progeny, a high-fitness asexual clone eventually displaces the rest of the population.

Speciation is a multigenerational process during which diverging lineages experience a transformation in their level of reproductive compatibility. Lineages eventually cross a threshold such that they can no longer interbreed, and thereafter they follow independent evolutionary trajectories (Coyne and Orr 2004). One classic complication with this concept arises in organisms that alternate reproduction between sexual and asexual processes. The potential for variation in breeding system means that the threshold may be broad and traversed slowly. By observing such taxa when they are in the midst of speciation, we can gain insight into the interplay of breeding system, ecological heterogeneity, and genetic differentiation. Motivated by the frequent occurrence of asexual natural hybrids in the *Daphnia pulex* species complex, we sought to test the hypothesis that postzygotic reproductive isolation leads to sexual breakdown following hybridization of closely related animals with a sexual–asexual breeding system. To accomplish this we bred experimental hybrids and then characterized the sexual capability of these hybrids by determining whether they could produce sexual females and males and whether successful advanced generations could be bred.

The genus *Daphnia* is a widespread freshwater crustacean whose normal life cycle is cyclic parthenogenesis (Brooks 1957; Zaffagnini 1987; Benzie 2005). Reproduction is typically parthenogenetic, and females produce ameiotic diploid eggs that develop immediately into daughters. Under certain environmental conditions, the diploid eggs develop instead into males, and females also produce haploid eggs. Unlike the diploid parthenogenetic eggs, the haploid eggs are extruded into a durable structure called an ephippium, and, once fertilized, the zygote can remain dormant for days or decades. In *Daphnia*, there are several complexes of young species known to hybridize naturally (Taylor and Hebert 1992; Spaak 1997; Weider et al. 1999). Even infertile hybrids have the potential to reproduce and expand to large numbers as a result of the asexual phase of the life cycle. Hybrids therefore can be demographically persistent and ecologically important, and zooplankton

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biologists have made considerable progress in delineating the ecological ramifications of interspecific hybridization in some of these complexes (Spaak and Hoekstra 1997; Duffy et al. 2004; Wolinska et al. 2004).

D. pulex and *Daphnia pulicaria*

North American *D. pulex* and *D. pulicaria* have long been a model system in ecology, particularly since they often constitute the "big *Daphnia*" that are important in structuring aquatic food webs (Steiner 2001; Tessier et al. 2001; Winder and Schindler 2004). Although these species are currently recognized as both ecologically and genetically distinct (Pfrender et al. 2000; Hebert and Finston 2001; Dudycha 2004), morphological discrimination is unreliable, and *D. pulicaria* was once considered a subspecific variant of *D. pulex* (Brooks 1957; Benzie 2005). Most investigators now view these two species as very closely related lineages within the *pulex* group in the *daphnia s.s.* subgenus (Crease and Lynch 1991; Colbourne and Hebert 1996; Pfrender et al. 2000). This species complex of *Daphnia* has also emerged as an important model system for applying genomic technology to ecological and evolutionary questions, with the complete genome sequenced and numerous full-length complementary DNA libraries constructed. Therefore, investigating reproductive isolation associated with hybridization in this group will shed light not only on how far speciation has progressed but also on how best to apply the new evolutionary genomic tools.

Since morphological discrimination is difficult, in practice most workers distinguish these species ecologically or on the basis of diagnostic alleles of the LDH enzyme (Hebert 1995). Populations occurring in temporary ponds are classified as *D. pulex*; populations coexisting with fish in permanent lakes are classified as *D. pulicaria* (Crease et al. 1997; Pfrender et al. 2000; Hebert and Finston 2001). Alternatively, those *Daphnia* that possess two slow alleles (*SS*) are classified *D. pulex*, while those homozygous for the fast allele (*FF*) are *D. pulicaria* (Hebert et al. 1989; Pfrender et al. 2000). Extensive biogeographic analyses (*see* below), however, have muddied the waters somewhat, as slough populations in the Canadian prairies, and a scattered few elsewhere, can have the *FF* genotype. *SS* genotypes have not been reported from stratified lakes.

Wild hybrids of *D. pulex* and *D. pulicaria*

Populations of hybrids between *D. pulex* and *D. pulicaria* are common in North America (Hebert et al. 1989; Hebert and Finston 1996; Crease et al. 1997). These species may be in the midst of speciation, rather than clearly discrete species in the sense of the biological species concept. Several early reports found that ponds will often contain only LDH heterozygotes (Hebert and Crease 1980, 1983; Innes et al. 1986); such ponds were later recognized as containing entirely hybrid populations (Hebert et al. 1993; Hebert and Finston 2001). Coexistence between hybrids and *D. pulex* has been reported to be uncommon (Hebert et al. 1988, 1993; Hebert and Finston 2001). Coexistence between hybrids and *D. pulicaria*, and coexistence of all three taxonomic classes, is rare, with fewer than 3% of temporary pond populations harboring all three LDH genotypes; and where these populations do harbor all three genotypes, allele frequencies indicate that the genotypes are not freely interbreeding (Hebert and Finston 2001). Populations of LDH heterozygotes can persist independent of one or both parental taxa because of the parthenogenetic phase of the *Daphnia* life cycle. Even if hybridization led to sexual infertility, a successful genotype could persist asexually. Although F_1-generation hybrids are common, multilocus genotypic variation indicates that introgression between the two species is rare or absent in areas where *D. pulex* and *D. pulicaria* coexist (Innes et al. 1986; Hebert et al. 1993; Hebert and Finston 2001). This is somewhat surprising, since similar studies of the *longispina* group, another hybridizing complex, show frequent advanced intercrossing (Taylor and Hebert 1993; Taylor et al. 2005). Geographically broad analyses have shown that hybridization events are widespread and due to recurrent local events rather than long-distance dispersal from rare events (Crease et al. 1989, 1990; Hebert and Finston 2001).

The naturally occurring hybrids tend to be found in different niches from the parents and have different life histories. Hybrids were originally considered to be a variant of *D. pulex*, and Hebert and Crease (1983) observed that in Ontario they tended to occur in disturbed areas, where forests had been cleared. In ponds that were still forested and had a closed canopy, pure *D. pulex* was typical. Thus, these types became known as the "urban" (i.e., hybrids that were LDH heterozygotes) and "forest" (i.e., *D. pulex* that were LDH *SS*) types (Hebert et al. 1988, 1989; Crease et al. 1989). Further work has indicated that hybrids are more likely to dominate permanent fishless waters and other small lakes in a variety of regions (Hebert and Finston 2001). In Michigan's lower peninsula one of us (J. L. Dudycha unpubl.) found hybrids frequently in open-canopy fishless waters, but rarely in closed-canopy fishless waters, repeating the ecological separation in Ontario. Wild hybrids also have distinct life histories that combine elements of *D. pulex* (high fecundity) and *D. pulicaria* (long life span) (Dudycha and Tessier 1999; Dudycha 2001, 2003). There is some indication that wild hybrids exhibit hybrid vigor and have a competitive advantage over parental genotypes (Loaring and Hebert 1981).

Wild hybrids have also subverted the sexual phase of the life cycle and instead produce dormant eggs asexually, a breeding system called obligate parthenogenesis or obligate asexuality (OA) in the *Daphnia* literature. Numerous studies have analyzed the breeding system of wild hybrids. Invariably, these studies show that hybrids reproduce via obligate asexuality (Hebert and Crease 1980, 1983; Innes et al. 1986; Hebert et al. 1988, 1989, 1993; Crease et al. 1990; Crease and Lynch 1991; Hebert and Finston 1996, 2001). Wild hybrids are not restricted to permanent habitats and indeed often become the sole resident of permanent and ephemeral water bodies. This obligate asexuality of wild hybrids—in contrast to the normal cyclic parthenogenesis (CP)—is one of the strongest pieces of evidence indicating that *D. pulex* and *D. pulicaria* are distinct species. In a

number of cases, hybridization of different species of animals is known to produce obligately asexual lineages (Cuellar 1977; Moritz et al. 1992; Radtkey et al. 1995), so it would not be surprising to find it in an organism that already has an asexual reproductive cycle. Thus, one obvious explanation of obligate asexuality in hybrid *Daphnia* is that obligate asexuality may be a result of postzygotic reproductive isolation that blocks normal meiosis.

The commonplace occurrence of *D. pulex–D. pulicaria* hybrids shows that these species can interbreed. However, genetic analyses indicate that wild hybrids have *D. pulex* dams and *D. pulicaria* sires (Crease et al. 1989), contradicting the proposition of hybrid asexuality arising from OA male *D. pulex* crossing with CP female *D. pulicaria* (Hebert et al. 1989). Instead, there may be asymmetrical reproductive isolation (Arnold et al. 1996; Lee 2000), the result being that only crosses in this direction are successful. Since the typical *D. pulex* population must invest in dormant reproduction annually, the potential for sexual females is much higher in *D. pulex*. Indeed, comparisons of the frequency of ephippial reproduction show that sexual females are a far more important component of the demography of *D. pulex* than they are of *D. pulicaria* (Caceres and Tessier 2003, 2004). Thus, a wayward *D. pulicaria* male is more likely to encounter a sexual *D. pulex* female than a wayward *D. pulex* male is to encounter a sexual *D. pulicaria* female. A further possibility is that there are *D. pulicaria* whose mitochondria genetically resemble those of *D. pulex*, and it is only these *D. pulicaria* whose females are capable of mating successfully with *D. pulex* males; such examples of *pulex*-type mitochondria in *D. pulicaria* have been observed (Crease et al. 1989). We do not attempt to distinguish among these explanations in this report, since all three may contribute to the apparent uniformity of *D. pulex* dams for the wild hybrids.

Breeding system variation in the *D. pulex* complex

In addition to the wild hybrids, there are some populations of the parental *Daphnia* species that are known to be obligately asexual (Hebert and Crease 1983; Hebert et al. 1988, 1989). Circumstantial data strongly indicate that in *D. pulex*, at least, obligate asexuality is caused by a dominant, sex-limited meiosis suppressor (Innes and Hebert 1988; Hebert et al. 1989; Innes et al. 2000). No meiosis suppressor gene has yet been found, nor has the mechanism through which the suppression operates been determined, though there is evidence that obligate asexuality can spread through its inheritance from sexually capable males that are the clonal sons of sexually incompetent females (Innes and Hebert 1988; Innes et al. 2000; Paland et al. 2005). In fact, most populations of *D. pulex* in eastern Canada and the northeast United States are obligately asexual, with populations of mixed breeding system occurring in the southern Great Lakes region (Hebert et al. 1993; Hebert and Finston 2001; Paland et al. 2005). Much of the work identifying obligately asexual populations is based solely on analysis of genotypic diversity, though this approach has been validated with direct tests of breeding system (Hebert and Crease 1983; Innes et al. 1986, 2000). For temporary pond populations, analysis of genotypic diversity is not complicated by the potential for long-term clonal selection, though this is a serious problem for interpreting data from sloughs and lake populations. Populations of *D. pulicaria* have therefore rarely been identified as obligately asexual (Hebert et al. 1993; Hebert and Finston 2001), and to our knowledge, no suggestion of obligate asexuality in *D. pulicaria* has been verified with a direct test of breeding system.

Another polymorphism in the *Daphnia* breeding system is the presence or absence of the ability to produce males (Innes and Dunbrack 1993; Innes 1997). There is no known sex chromosome in *Daphnia*; sex determination is instead hormonally induced between 48 and 72 h into the ovarian egg development (Olmstead and LeBlanc 2002). This enables females to produce males asexually in response to environmental cues. However, there is evidence of a genetic component to the production of male offspring (Innes and Dunbrack 1993). Naturally occurring non–male producing (NMP) *Daphnia* are known as well and may possess an advantage over male producers (MP) as a result of the cost of male production. Thus, if a hybrid *Daphnia* genotype cannot produce males, this may be a result of hybrid incompatibility, or it may simply be due to inheritance of the putative NMP locus.

Objectives of our study

Our main objective was to generate experimental hybrids and determine whether they are sexually competent. If obligate asexuality is a consequence of reproductive isolation between *D. pulex* and *D. pulicaria*, our experimental hybrids should be obligately asexual regardless of parental breeding system. The second component of evaluating sexual competence was to determine whether the hybrids could produce males. If NMP hybrids were the offspring of MP females, then NMP status could be a consequence of reproductive isolation. If, however, NMP status was seen only in offspring of NMP females, this could be explained by the hypothesis that NMP status is a consequence of a simple polymorphism. Finally, we sought to breed backcross and F_2 genotypes. If there was no reproductive isolation between *D. pulex* and *D. pulicaria*, advanced intercrosses should be no more difficult to produce than intraspecific crosses. If speciation has progressed to the point at which the lineages have substantial genetic incompatibilities, advanced intercrosses should not be possible.

Methods

Parents, culturing, and crosses—We obtained parental individuals from clonal lineages originally isolated for other projects from lakes and ponds in the Midwestern United States and Oregon (*see* Table 1), choosing clones known to produce males for sires and clones known to produce ephippia sexually for dams. Clones were classified as *D. pulex* or *D. pulicaria* on the basis of LDH genotype via

Table 1. Parentage, geographic origins, and reproductive capabilities of specific experimental clones, based on monitoring stock cultures. Stock cultures of F_1-020, F_1-031, and F_1-045 went extinct prior to reproductive assessments. In most cases, only a single clone from a population was used as a sire or dam, but several clones from the Warner population were used as sires, and two clones each from the Busey and RW populations were used as dams.

							No. of ephippia collected with			
Clone No. F_1-	Sire clone	Sire from	Dam clone	Dam from	Males?	MP dam?*	No eggs, males present	No eggs, males not seen	Eggs, males not seen	Eggs, males present
001	Gull 10	Michigan	RW 1	Michigan	No	No	0	157	0	0
002	Gull 10	Michigan	RW 1	Michigan	No	No	0	247	0	0
003	Gull 10	Michigan	RW 1	Michigan	Yes	No	3	11	43	0
004	Gull 10	Michigan	RW 1	Michigan	Yes	No	34	24	23	17
005	Gull 10	Michigan	RW 1	Michigan	No	No	0	130	0	14
006	Warner 14	Michigan	Busey 4	Illinois	Yes	Yes	10	0	32	0
007	Warner 14	Michigan	Busey 4	Illinois	Yes	Yes	42	74	30	0
008	Gull 10	Michigan	RW 1	Michigan	No	No	0	200	0	0
009	Warner 14	Michigan	Busey 4	Illinois	Yes	Yes	72	0	34	0
010	Warner 14	Michigan	Busey 4	Illinois	Yes	Yes	29	0	134	0
011	Warner 5	Michigan	West 2	Illinois	Yes	Yes	25	0	28	0
012	Warner 5	Michigan	West 2	Illinois	Yes	Yes	6	0	21	0
013	Warner 5	Michigan	LL3 110	Iowa	No	No	0	60	0	0
014	Lost Creek	Oregon	Busey 16	Illinois	Yes	Yes	21	0	22	0
015	Lost Creek	Oregon	Busey 16	Illinois	Yes	Yes	69	0	46	0
016	Lost Creek	Oregon	Busey 16	Illinois	Yes	Yes	55	0	75	0
017	Lost Creek	Oregon	Busey 16	Illinois	Yes	Yes	63	3	9	0
018	Warner 2	Michigan	POVI 4	Michigan	No	No	0	85	0	0
019	Warner 2	Michigan	POVI 4	Michigan	No	No	0	436	0	0
021	Warner 2	Michigan	POVI 4	Michigan	No	No	0	211	0	0
022	Warner 2	Michigan	POVI 4	Michigan	No	No	0	198	0	0
023	Gull 10	Michigan	RW 1	Michigan	Yes	No	7	0	0	0
024	Gull 10	Michigan	RW 1	Michigan	No	No	0	100	0	0
025	Little Cultis	Oregon	West 2	Illinois	Yes	Yes	47	0	55	0
026	Warner 17	Michigan	RW 2	Michigan	No	No	0	135	0	0
027	Warner 14	Michigan	Busey 4	Illinois	Yes	Yes	8	10	32	12
028	Warner 5	Michigan	West 2	Illinois	Yes	Yes	3	18	31	32
029	Gull 10	Michigan	RW 1	Michigan	Yes	No	20	0	38	0
030	Gull 10	Michigan	RW 1	Michigan	Yes	No	38	0	29	0
032	Warner 15	Michigan	RW 2	Michigan	Yes	No	18	9	21	7
033	Warner 15	Michigan	RW 2	Michigan	Yes	No	28	0	0	0
034	Warner 9	Michigan	LL3 110	Iowa	No	No	0	91	0	0
035	Warner 5	Michigan	LL3 110	Iowa	No	No	0	62	0	0
036	Warner 5	Michigan	LL3 110	Iowa	No	No	0	145	0	0
037	Warner 5	Michigan	LL3 110	Iowa	No	No	0	56	0	0
038	Warner 5	Michigan	LL3 110	Iowa	No	No	0	84	0	0
039	Warner 5	Michigan	LL3 110	Iowa	No	No	0	96	0	0
040	Warner 2	Michigan	Busey 16	Illinois	Yes	Yes	13	22	46	28
041	Warner 2	Michigan	Busey 16	Illinois	Yes	Yes	13	26	23	28
042	Warner 2	Michigan	Busey 16	Illinois	Yes	Yes	4	26	22	0
043	Warner 2	Michigan	POVI 4	Michigan	Yes	No	26	88	5	23
044	Warner 2	Michigan	POVI 4	Michigan	Yes	No	40	9	5	0
046	Fish Lake	Oregon	LL3 110	Iowa	Yes	No	4	93	0	32
047	Fish Lake	Oregon	LL3 110	Iowa	No	No	0	82	0	0
048	Fish Lake	Oregon	LL3 110	Iowa	Yes	No	4	15	0	0
049	Fish Lake	Oregon	LL3 110	Iowa	No	No	0	83	0	1
050	Pine 1	Michigan	POVI 4	Michigan	No	No	0	126	0	0
051	Fish Lake	Oregon	West 5	Illinois	Yes	Yes	0	0	0	0
052	Fish Lake	Oregon	RW 2	Michigan	Yes	No	5	0	84	0
053	Fish Lake	Oregon	RW 2	Michigan	Yes	No	8	0	51	0
054	Warner 5	Michigan	LL3 110	Iowa	Yes	No	45	0	16	0
055	Warner 5	Michigan	LL3 110	Iowa	No	No	0	178	0	0
056	Warner 5	Michigan	LL3 110	Iowa	No	No	0	138	0	0

* MP, male producing.

cellulose acetate electrophoresis (Hebert and Beaton 1993). In all cases, specific status indicated by LDH coincided with habitat, with *SS* clones from temporary ponds and *FF* clones from stratified lakes. Parental and descendant clones were maintained throughout the experiment in stock cultures via standard methods, with duplicate copies of each genotype kept at 20°C and 10°C on a 12 : 12–h photoperiod. Cultures were kept in filtered lakewater and were fed a vitamin-enriched strain of *Scenedesmus obliquus*. Feeding occurred daily at 20°C and weekly at 10°C at a level sufficient to maintain high lipid stores and clutch sizes.

Since sex in *Daphnia* is induced primarily by environmental factors, the conditions to which experimental individuals were exposed were similar in all three of our tests of sexual function. Test *Daphnia* were exposed to a long-day photoperiod (16 : 8 h light : dark [LD]) at 20°C. Food levels were cycled to expose the test *Daphnia* to periods of abundance followed by periods of scarcity. This boom–bust food cycle served to provide sufficient resources for reproductive activity, but sufficient unpredictability to encourage sex.

Mitochondrial DNA evidence indicates that wild hybrids have *D. pulex* dams and *D. pulicaria* sires (Crease et al. 1989), so we attempted to produce experimental hybrids by crossing *D. pulicaria* sires to *D. pulex* dams. *D. pulicaria* clones rarely or never produced ephippia in our lab cultures, so reciprocal crosses would not have been possible. We focused our effort on an initial set of 53 experimental F_1s, although we eventually generated >400 hybrid genotypes.

For each cross, 250-mL beakers were set with three adult male individuals and 10–12 adult females of the parental genotypes. Some variation occurred as a result of the differential availability of mature individuals of the needed sex. Prior to a cross, maternal genotypes were kept at low density (three to four adult females per 250-mL beaker, with offspring removed on alternate days) for at least two generations. Individuals used as dams were removed from their natal beaker as neonates and transferred to a new beaker to ensure they had never been in the presence of a male until we introduced the sires. To prevent production of inbred ephippia (via male progeny of the dam individuals), crosses were transferred every 3–4 d. Crosses were done in filtered (1 μm) lakewater that had been preconditioned with a very high density (>500 adults L^{-1}) of *Daphnia* to stimulate sexual reproduction. To further encourage sex, food was restricted to approximately half the level in our standard cultures.

Ephippia were collected from crosses every few days, stored in 12-well cell culture plates, and frozen in a conventional freezer for 2–4 weeks before hatching. Hatching was induced in the plates by replacing the water with fresh, oxygen-saturated, filtered lakewater. Plates of ephippia were then placed in a controlled-environment chamber at 10°C under intense light on a 16 : 8 LD photoperiod. After several days, plates were examined daily for hatchlings. Hatchlings were removed to individual beakers with filtered lakewater and allowed to continue growing at 10°C until they reproduced. At this point, newly established lines were split into duplicates and kept at normal stock culture conditions.

After putative hybrid genotypes were established as clonal lineages, their hybrid status was confirmed through LDH electrophoresis. One hundred percent of our F_1 hatchlings were heterozygotes, clearly showing that our hatchlings were indeed progeny of interspecific crosses and that none of our dam genotypes were actually obligate asexuals.

Tests for obligate asexuality—Once a genotype was established as a clonal line, we routinely examined cultures for males and production of ephippia. These examinations were conducted for about 11 months. Obligately asexual genotypes produce ephippia more often than sexual genotypes, and they usually deposit eggs into their ephippia. Therefore, a reliable test for obligate asexuality is to determine whether viable ephippial eggs are produced in the absence of males (Innes et al. 1986). Any ephippia that were seen in our routine examinations were collected and dissected to determine whether viable eggs were present. Eggs were determined to be viable by having a characteristic size, ovate shape, and smoothness; rare eggs that appeared shrunken and wrinkled were classified as nonviable. We generally examined 40–150 ephippia from each F_1 clone (mean: 103; range: 7–436; full clone-specific data are given in Table 1) and then used this information to determine which F_1s to test further. All clones that were observed to produce viable resting eggs inside their ephippia when no males were observed in the cultures were classified as potential obligate asexuals. These clones were tested more rigorously to determine whether they were indeed obligate asexuals, since it was possible that we had failed to observe males that were previously or rarely in a culture. Clones that produced ephippia with eggs only in the presence of males were classified as cyclic parthenogens, and some of these, along with clones that never put eggs into ephippia in stock cultures, were then used in further crosses to confirm sexual competence (*see* Advanced intercrosses, below).

For each potential obligately asexual genotype, three to five females that had never been in the presence of a male were placed in 150-mL beakers. As a control, two previously confirmed obligately asexual *D. pulex* clones and three confirmed sexual *D. pulex* clones were run through the same test (confirmation of breeding system was done by analysis of heterozygous microsatellite markers in exephippial hatchlings; J. L. Dudycha unpubl.). These controls served to characterize the ephippial production of sexual and asexual *Daphnia*. Since it takes approximately 4–5 d for *Daphnia* to reach sexual maturity and since it was necessary to ensure the absence of mature males, the test females were transferred to new beakers, with removal of their offspring every 3–4 d. During these transfers, any ephippia present were removed for dissection, and the number of viable eggs was recorded.

Tests for male production—All experimental hybrid clones that had not been observed to produce males under casual observation of stock cultures were brought to

crowded conditions. These crowded cultures were surveyed weekly for the presence of males for at least 1 month. Hybrid genotypes that had not produced males in the crowded cultures and all eight of the *D. pulex* dam genotypes were subjected to hormonal induction. In these tests, female *Daphnia* were placed in filtered lakewater with 400 nmol L^{-1} methyl farnesoate (MF), which triggers the male production pathway in MP genotypes (Olmstead and LeBlanc 2002). Control genotypes known to produce males were used to ensure that the MF did induce male production. Each test included three replicate beakers of each tested genotype, with 8–10 individual females per 250-mL beaker. These individuals were transferred to new lakewater + MF every 2 d. After 2 weeks, fresh females were used to guard against any effects of acclimation to MF. All of our known–male-producing controls produced 100% male offspring in the first two clutches that completed oogenesis in the presence of MF. After 1 month (i.e., 10–12 clutches), any genotype that had not produced males was classified as a non–male producer.

Advanced intercrosses—Finally, we characterized the ability of experimental hybrids to produce viable offspring sexually. Several F_1s and *D. pulex* that produced males were used as sires in these experimental crosses, while dams were chosen from cyclically parthenogenetic genotypes that produced ephippia on a consistent basis. Where possible, clones were chosen in a manner that created a set of crosses that represented geographic diversity and simultaneously provided a maximum amount of data on individual clones through their presence in several different crosses.

Five types of crosses were carried out. In order to avoid confusion with the original F_1s, these crosses will collectively be referred to as the reproductive isolation test crosses (RITC). Individually, the five types of crosses we attempted were coded as follows: XX = *D. pulex* intraspecific crosses, F_1 = *D. pulex*–*D. pulicaria* interspecific crosses, FX = F_1–*D. pulex* maternal backcrosses, CF = *D. pulicaria*–F_1 paternal backcrosses, and FF = experimental F_1–experimental F_1 crosses. Diverse F_1 intercrossing in an attempt to produce an F_2 generation was carried out prior to this work and yielded scattered success (J. L. Dudycha unpubl.); in this experiment, those F_1s that previously produced the most ephippia from attempted sexual crosses were chosen for the F_1–F_1 crosses. The F_1–F_1 crosses in this experiment serve to maximize the probability of producing an F_2 generation, but because they were not a random set of parents, we make no attempt to quantify genetic parameters such as dominance and epistasis. In addition, this restricts the inferences from statistical analyses to the specific crosses we performed, as the focus of this project was whether such crosses could work at all rather than a precise assessment of the relative performance of different cross types.

Crosses were conducted between three to five sires and six to 10 dams, with replacement of *Daphnia* that died, and each cross was run for up to 13 weeks. Every 3–4 d, crosses were transferred to new beakers. At that time, ephippia were removed and placed into cell plates for hatching, as described above. Hatchlings were then monitored for viability and reproduction. Hatchlings that produced a sufficient number of parthenogenetic offspring to create stable stock cultures were classified as successful.

Parentage analysis with microsatellites—To confirm sexual production of offspring, the parents of all successful offspring from the original F_1 crosses and the RITC crosses were verified through microsatellite analysis. This analysis was conducted by using the polymerase chain reaction to amplify 12–18 microsatellite loci for each parent and offspring clone, following the methods and primers described in Colbourne et al. (2004) and the Daphnia Genomics Consortium website (http://daphnia.cgb.indiana.edu). This allowed us to confirm that offspring were produced sexually, rather than via obligate asexuality, and to confirm parentage. We used the following microsatellite loci: P1-O12, P1-O17, P1-P16, P2-B18, R1-34, P3-I9, P2-M10, P3-I21, P3-122, P3-I7, P2-L6, P2-K24, P3-F3, P2-A21, P3-A9, GTT12, P1-L1, P1-F15, P1-C11, Dpl CAA30, Dpl 1/41, P3-I5, and P2-n17. Not all loci were amplified in all clones.

Results

Obligate asexuality tests—Of the first 53 experimental F_1 hybrid clonal lineages that we established, 16 produced ephippia with eggs only when males were present and were classified as cyclic parthenogens. Eleven clones produced ephippia with eggs when no males were observed and were subjected to the formal obligate asexuality tests. However, all 11 of these clones did at some point produce male offspring. The remaining 26 clones never produced ephippia with eggs in stock cultures. Of these, 25 clones produced empty ephippia and never had males. In the formal obligate asexuality tests, 5.69% of the ephippia produced by the OA controls (clones Lin A and OL3) were empty (Table 2). Assuming this fraction is representative of OA genotypes, we conclude that the 25 F_1s that produced only empty ephippia in the absence of males are obligately asexual (of these, the fewest ephippia produced by a clone was seven, leading to a probability that the clone is not OA that is $<2.0 \times 10^{-9}$). Only one clone, F_1-051, never produced any ephippia, though it did produce males. Five of these 26 clones were used as dams in the RITC; all produced ephippia with eggs when mated. Data for each individual genotype can be found in Table 1.

None of the 11 clones identified as possible obligate asexuals in the preliminary survey were found to be OA upon direct testing (Table 2). In clone F_1-040, two out of the 22 ephippia produced did contain eggs. However, they appeared in a beaker that inadvertently contained an adult male whose sex had been misidentified as a neonate. During these tests, the two obligately asexual control clones deposited eggs in 94.9% and 92.0% of the ephippia they produced. The three sexual control clones produced fewer ephippia overall, and none contained eggs.

Male production tests—Males were observed in either standard cultures or the crowded cultures in 31 hybrid genotypes and four *D. pulex* dam genotypes. The remaining

Table 2. Ephippia produced during experimental test for obligate asexuality. Tested clones are experimental hybrids that produced ephippia with viable eggs in stock cultures at a time when males were not observed. Controls are genotypes of *D. pulex* known to be obligately asexual (OA) or cyclically parthenogenetic (CP) based on microsatellite analysis of their ephippial offspring.

Clone	No. of ephippia with		
	No eggs	1 Egg	2 Eggs
Hybrids			
F_1-004	1	0	0
F_1-005	50	0	0
F_1-025	1	0	0
F_1-027	7	0	0
F_1-028	6	0	0
F_1-032	4	0	0
F_1-040	20	1*	1*
F_1-041	9	0	0
F_1-043	26	0	0
F_1-046	51	0	0
F_1-049	34	0	0
Controls			
Lin A (OA)	5	12	81
OL3 (OA)	2	8	15
RW 1 (CP)	9	0	0
POVI 4 (CP)	20	0	0
Busey 16 (CP)	1	0	0

* An adult male was inadvertently present prior to these ephippia being produced.

Table 3. Male production by family. Family size is the number of F_1 hybrids bred from a particular sire–dam pair. ''Non–male producers'' comprises the number of offspring genotypes in a family that cannot produce male offspring. Non–male producing dam genotypes are indicated in bold.

Sire	Dam	Family size (No.)	Non–male producers
Lost Creek	Busey 16	4	0
Warner 14	Busey 4	5	0
Warner 5	West 2	3	0
Little Cultis	West 2	1	0
Warner 2	Busey 16	3	0
Fish Lake	West 5	1	0
Gull 10	**RW1**	10	6
Warner 2*	**POVI 4**	6	4
Warner 5	**LL3 110**	9	8
Fish Lake	**LL3 110**	4	2
Warner 17	**RW 2**	1	1
Warner 15	**RW 2**	2	0
Warner 9	**LL3 110**	1	1
Pine 1	**POVI 4**	1	1
Fish Lake	**RW 2**	2	0

* Microsatellite genotyping later revealed that Warner 2 was not actually the sire of this family; most likely another clone from the Warner population was.

22 hybrid genotypes and four dam genotypes never produced males in standard cultures, crowded cultures, or in the MF tests. In the MF tests, known–male-producing control genotypes did produce males when exposed to MF. All 22 NMP hybrid genotypes were offspring of an NMP dam genotype (Table 3).

Advanced intercrosses (RITC)—Each of the five cross types (XX, CX, FX, CF, and FF) produced ephippia during the course of the experiment, from which both successful and unsuccessful hatchlings were obtained (Table 4). Successful hatchlings were those that reached maturity and reproduced, establishing a clonal line. Unsuccessful hatchlings usually grew but never reproduced. The most successful cross type was CX (*D. pulicaria* × *D. pulex*), which produced new F_1 genotypes (Table 3). Treating each cross as a replicate in a one-way ANOVA with Tukey's test for multiple comparisons, cross type had a significant influence on success (overall model $F_{2,39} = 4.24$, $p = 0.0217$), where in particular the CX crosses performed better than either intraspecific crosses ($p < 0.05$) or the advanced intercrosses ($p < 0.05$). Surprisingly, the least successful cross type, based on number of successful hatchlings produced per unit of crossing effort, were the intraspecific (XX) crosses, although this finding did not represent a statistically significant difference from the advanced intercrosses.

Although our *D. pulex* intraspecific crosses produced more ephippia than did the *D. pulicaria* × *D. pulex* hybrid crosses for the given amount of effort (1.6 ephippia per unit of crossing effort [EPUE], compared to 1.1 EPUE), our hybrid crosses exhibited a higher frequency of success, because hybrid ephippia were more likely to hatch (Fisher's exact test, $p < 0.0001$) and hybrid hatchlings were more likely to survive (Fisher's exact test, $p = 0.0011$). All six of the CX crosses that produced hatchlings produced successful hatchlings, with 76% of the total CX hatchlings reproducing successfully. In the XX crosses, only two out of the six crosses that produced hatchlings yielded successful hatchlings. The superior performance of hybrid ephippia indicates that at the F_1 generation, rather than there being reproductive isolation, there is hybrid vigor. The success of hatchlings produced by the backcrosses was intermediate to the XX or CX success rates discussed above (Fisher's exact test, $p = 0.0036$), with 34% of the backcross hatchlings being successful. There may have been asymmetry between the backcrosses, resulting in a higher rate of ephippia production (1.75 EPUE, as opposed to 0.27 EPUE; *t*-test: $t = 2.69$, df = 16, $p = 0.0161$) by backcrossing F_1s to *D. pulicaria* males than to *D. pulex* females. This may simply be due to a greater propensity of F_1s to produce and provision ephippia relative to *D. pulex*. Because the success rate of F_2 and backcrosses was higher than that of *D. pulex* intraspecific crosses (Fisher's exact test, $p = 0.0161$), there is little evidence that reproductive isolation is limiting intercrossing beyond the F_1 generation.

Parentage verification—Informative data were obtained for at least 10 different microsatellite loci for each of the original F_1s examined. All 53 F_1 genotypes were clearly the sexual offspring in the families indicated. However, it was also apparent that although the six hybrid clones in one family (Warner 2 × POVI 4) were full-sibs, they were not half-sibs of the other families sired by Warner 2 and were

Table 4. Reproductive isolation test crosses (RITC). Crossing effort values were obtained by multiplying the number of beakers within a cross type by the duration of the cross (in weeks). EPUE is the ephippia produced per unit effort of crossing. Total crosses is the number of unique combinations of parental genotypes in that cross type. Success rate is the number of successful hatchlings per unit of crossing effort.

Cross type	Crossing effort	No. of ephippia	EPUE	Total No. of crosses	No. of crosses with			No. of hatchlings	Successful hatchlings	Success rate
					Ephippia	Hatchlings	Successful hatchlings			
XX	143	250	1.7	11	8	6	2	10	2	0.014
CX	130	147	1.1	10	10	6	6	34	26	0.200
FX	104	28	0.3	8	7	2	1	3	2	0.019
CF	130	228	1.8	10	9	7	4	32	10	0.077
FF	39	33	0.8	3	2	1	1	3	2	0.051

not in fact sired by Warner 2. Thus, the sire of this family is unknown, but it appears to be another clone from the Warner *D. pulicaria* population.

Microsatellite analysis of the RITC offspring was conducted for 12 different loci. Informative data were obtained from each of the cross families (not shown); examination of these data confirmed that each of the RITC offspring were indeed sexually produced by the specific parents involved in each particular cross.

Discussion

Obligate asexuality, male suppression, and sterility are not consequences of hybridization between *D. pulex* and *D. pulicaria*, which shows that hybridization for these two taxa is not an evolutionary dead end. This is in stark contrast to population genetic surveys and experimental tests showing that wild hybrids are invariably obligately asexual. Our evidence shows that experimental hybrids suffer no loss of sexual function, and intercross generations beyond the F_1 can be successful. Therefore, these taxa are clearly not discrete species in terms of the biological species concept, though other evidence indicates that they are indeed being driven toward separate evolutionary trajectories by ecological pressures.

The nature of obligate asexuality in wild hybrids—Many population surveys and experimental tests have established that wild hybrid populations are invariably obligate asexuals (Hebert and Crease 1980, 1983; Innes et al. 1986; Hebert et al. 1988, 1989, 1993; Crease et al. 1990; Crease and Lynch 1991; Hebert and Finston 1996, 2001). The most obvious explanation for this—that the sexual incapacity of wild hybrids is a direct consequence of genetic incompatibilities between the parental taxa—is apparently incorrect. The next obvious explanation for obligate asexuality in wild hybrids is also unsatisfactory. It has been proposed that hybrid populations are obligately asexual as a result of simple inheritance of a meiosis-suppressor gene from the paternal genotype (Hebert et al. 1989). This is unsatisfactory for two reasons. First, it implies that carriers of the meiosis-suppressor gene are much more likely to hybridize than are noncarriers. Why would these genotypes be favored for hybridization events? This seems unlikely, as it may require that the meiosis-suppressor gene has several disparate pleiotropic effects, such as increased dispersal and weakened mate recognition. Furthermore, hybridization events appear to occur throughout North America, even in regions in which obligately asexual *D. pulex* genotypes are rare (Crease et al. 1989, 1990; Hebert and Finston 1996, 2001). Second, although our understanding of breeding system in *D. pulicaria* and of the origins of wild hybrids is limited, it appears that wild F_1 hybrids are unlikely to inherit a meiosis-suppression gene from their sire. This is because molecular evidence indicates that *D. pulicaria* is the sire genotype of wild hybrids (Crease et al. 1989; Crease and Lynch 1991). There are no reports of obligate asexuality in lacustrine *D. pulicaria*, and reports of OA *D. pulicaria* in other habitats may be unreliable, since they are based on indirect evidence that has not been experimentally

confirmed. Furthermore, it is difficult to reconcile the commonness of hybrids with the rarity of OA *D. pulicaria*. In contrast, the indirect and experimental evidence is strong that obligate asexuality is common in *D. pulex* (Hebert and Crease 1983; Hebert et al. 1989; Paland et al. 2005). We are left with a substantial puzzle: If experimental hybrids are sexually competent, why are wild hybrids consistently obligate asexuals?

We propose a multistage invasion–conversion–exclusion model that could account for the frequency and fitness of hybrid populations, the apparent unidirectional parentage, and the ubiquity of obligate asexuality in wild hybrid populations. In the first stage, "invasion," a *D. pulicaria* migrates from lake to pond and mates with the native *D. pulex*. In the second stage, "conversion," hybrid F_1s hatch and mate with the native *D. pulex*, some of which may be obligate asexuals (as females, though not necessarily as males), producing a backcross-to-*pulex* generation (BC_x). In the final stage, "exclusion," a high-fitness obligately asexual "hybrid" displaces the remaining genotypes in the population.

We believe that the invasion stage is both plausible and more likely than the reverse of *D. pulex* migrating to and mating in a *D. pulicaria* lake for demographic and ecological reasons. *D. pulex* is much more prone to producing ephippia offspring because its typical habitat requires diapause (Caceres and Tessier 2004). Thus, an immigrant into a *D. pulex* pond is more likely to find a receptive female mate than an immigrant into a *D. pulicaria* lake. In addition, population sizes in small ponds are substantially lower than those in large lakes, so there is a greater probability that an immigrant allele will spread through a *D. pulex* population than through a *D. pulicaria* population. Finally, fish are size-selective predators in lakes, and on average, *D. pulex* is slightly larger than *D. pulicaria* (Dudycha and Tessier 1999), so a *D. pulex* that migrated to a lake has a greater chance of becoming prey. The consequences of the invasion stage are that there will be occasional production of ephippia containing F_1 genotypes that are LDH heterozygotes (and thus identifiable as hybrids), that carry a pulex-type mitochondrion, and that are destined to be fully competent sexually.

In the conversion stage, F_1 hybrid ephippia hatch, multiply through the normal parthenogenetic phase of the *Daphnia* life cycle, and mate with the native *D. pulex*. This is plausible, because hybrids obviously can expand in numbers and may also have hybrid vigor for short-term fitness (Dudycha and Tessier 1999). These F_1 hybrids will be present in the water column in only one season, since they need to reproduce sexually to produce diapausing offspring. These hybrids are more likely to mate with natives than each other simply because after an initial invasion event, hybrids will be overwhelmingly outnumbered by natives. If the native *D. pulex* are a mixture of obligately asexual and cyclically parthenogenetic genotypes (a not uncommon situation; Hebert et al. 1989, 1993; J. L. Dudycha pers. obs.), then native obligately asexual *D. pulex* males could mate with a hybrid female. If the meiosis-suppressor is a paternally inherited dominant locus, such a mating could produce offspring that were either OA or CP and either LDH heterozygotes or *SS* (the *D. pulex* genotype). All would have a *D. pulex* mitochondrion.

Although many different genotypes could be produced in the conversion stage, the diverse condition would be transitory, as eventually a highly fit, obligately asexual genotype would come to dominate the population in the final exclusion stage. This dominance would come about through the short-term fitness advantage of hybrids via hybrid vigor and the long-term demographic advantage of obligate asexuality. Thus, our model predicts two different possible endpoints for hybrid populations. In one case, an obligately asexual LDH heterozygote displaces all other genotypes. Such a population would be identified as hybrid and would carry *D. pulex* mitochondrial DNA, but in reality would be a backcross (rather than an F_1), with 75% of its nuclear genome from *D. pulex* and 25% from *D. pulicaria*. The other case is a similar endpoint, except that it produces a population that is an LDH *SS* homozygote and that would be incorrectly identified as a pure *D. pulex* population. Note that our model does not imply that all OA populations of *SS* homozygotes are of hybrid origin, and those populations that are not may show a relatively high level of homozygosity throughout their genome.

The inheritance of male suppression—Our data on the suppression of male production support prior evidence that it is inherited as a dominant allele (Innes and Dunbrack 1993). We found NMP hybrids were always the offspring of NMP dams. In crosses of NMP dams, approximately one third of the offspring were NMP, which is not in accord with a one- or two-locus model of inheritance. However, our sample sizes were small enough that our data cannot statistically be distinguished from either a one- or two-locus model. We are working to expand our NMP families to more clearly determine the inheritance pattern, but it is evidently not a consequence of hybridization. Furthermore, since MF did not induce male production in the NMP clones, our data confirm that male suppression is likely associated with an MF receptor or some other downstream process in the male production pathway (Rider et al. 2005) and is not associated with sensitivity to environmental cues for male production.

The specific status of D. pulex *and* D. pulicaria—Our hybridization data show that *D. pulex* and *D. pulicaria* are not reproductively isolated by postzygotic factors such as genetic incompatibilities. However, we recommend retaining both names at the species level, since they define useful categories from the perspectives of both ecology and evolution. Field data indicate that these species are ecologically separated or that LDH is subject to differential selection in ponds and lakes, directly or indirectly (Pfrender et al. 2000; Dudycha 2004). In addition, molecular divergence data indicate that these taxa are on different evolutionary trajectories (Pfrender et al. 2000). Together, these lines of evidence indicate a taxon in the process of evolutionary divergence, and fairly early in the process at that. Other hybrid species complexes of *Daphnia* similarly show ecological and genetic divergence in the face of

ongoing hybridization. It is clear that these divergences have been going on for thousands of years, and one may wonder why speciation seems to take so long in *Daphnia*. Perhaps the clonal phase of the life cycle, allowing hybrids to spread without relying on sex, creates more opportunities for introgressive backcrossing and thus acts as a strong adhesive, holding species together.

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