Nucleotide Polymorphism and Within-Gene Recombination in *Daphnia magna* and *D. pulex*, Two Cyclical Parthenogens

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ABSTRACT

Theory predicts that partially asexual organisms may make the "best of both worlds": for the most part, they avoid the costs of sexual reproduction, while still benefiting from an enhanced efficiency of selection compared to obligately asexual organisms. There is, however, little empirical data on partially asexual organisms to test this prediction. Here we examine patterns of nucleotide diversity at eight nuclear loci in continentwide samples of two species of cyclically parthenogenetic Daphnia to assess the effect of partial asexual reproduction on effective population size and amount of recombination. Both species have high nucleotide diversities and show abundant evidence for recombination, yielding large estimates of effective population sizes (300,000–600,000). This suggests that selection will act efficiently even on mutations with small selection coefficients. Divergence between the two species is less than one-tenth of previous estimates, which were derived using a mitochondrial molecular clock. As the two species investigated are among the most distantly related species of the genus, this suggests that the genus Daphnia may be considerably younger than previously thought. Daphnia has recently received increased attention because it is being developed as a model organism for ecological and evolutionary genomics. Our results confirm the attractiveness of Daphnia as a model organism, because the high nucleotide diversity and low linkage disequilibrium suggest that fine-scale mapping of genes affecting phenotypes through association studies should be feasible.

THE efficacy of natural selection may be severely reduced in asexual compared to sexual organisms due to the absence of recombination and segregation (Fisher 1930; Barton and Charlesworth 1998; Otto and Lenormand 2002; Agrawal 2006). Consequently, asexual populations may adapt more slowly to changing environments (Peck 1994; Orr 2000; Roze and Barton 2006) and suffer from an increased genetic load (Muller 1964; Crow and Kimura 1970; Pamilo *et al.* 1987; Kondrashov 1988; Charlesworth 1994). Both of these factors may contribute to the rarity of obligate asexuality in eukaryotes (Bell 1982), despite its immediate advantages over sexual reproduction (Maynard Smith 1978). The main reason for the decreased efficiency of selection in asexual organisms is that due to the complete linkage of their genomes, selection cannot operate on different mutations independently (the Hill–Robertson effect, Hill and Robertson 1966). Thus, deleterious mutations anywhere in the genome reduce the effective population size (N_e) experienced by other loci (Hill and Robertson 1966; Felsenstein 1974; Charlesworth 1994; Keightley and Otto 2006), resulting in a predicted reduction in neutral genetic variation and accumulation of slightly deleterious alleles, due to inefficacy of selection on mutations with small selection coefficients.

However, even a small amount of sexual reproduction is predicted to greatly alleviate the disadvantages of pure asexual reproduction while conserving most of its advantages (Lynch and Gabriel 1983; Pamilo *et al.* 1987; Charlesworth *et al.* 1993; Green and Noakes 1995; Hurst and Peck 1996). Thus, partially asexual organisms should have N_e and neutral genetic diversity similar to that of obligately sexual organisms of similar body size, abundance, and geographic range. The predominance of obligate sexual life cycles is thus surprising and remains unexplained (Hadany and Beker 2007). Partially asexual life cycles, with only occasional rounds of sexual reproduction, occur in many invertebrates and fungi and may provide insights into the fitness and genomic consequences of occasional sex (Bell 1982). However, few studies of neutral genetic diversity exist for partially asexual organisms, and thus their N_e and levels of recombination are

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TABLE 1

List of the 19 collection sites of the samples used in this study from Europe and North America

Species	Strain[a]	Locality	Latitude	Longitude
D. magna	BE1	Leuven, Belgium	50°52′N	04°41′E
D. magna	CA1	Churchill, MB, Canada	58°46′N	94°11′W
D. magna	CZ1	Sedlec, Czech Republic	48°46.52′N	16°43.41′E
D. magna	FI1	Tvärminne, Finland	59°49.43′N	23°15.15′E
D. magna	FI2	Aland, Finland	60°01.30′N	19°54.15′E
D. magna	GE1	Kniphagen, Germany	54°10.45′N	10°47.3′E
D. magna	GE2	Ismaning, Germany	48°12.2′N	11°41′E
D. magna	HU1	Jaraspuszta, Hungary	46°48′N	19°08′E
D. magna	UK1	Cumnor, UK	51°43.9′N	01°20.4′W
D. magna	UK2	Leitholm, UK	55°42.15′N	02°20.43′W
D. pulex	BW102	Busey Woods, Urbana, IL	40°07′N	88°12′W
D. pulex	CC1	Creswell Court, OR	43°55.5′N	123°01′W
D. pulex	DISP14	Disputed Road, LaSalle, ON, Canada	42°13′N	83°02′W
D. pulex	EB1	Eloise Butler, Minneapolis	44°58.5′N	93°19.5′W
D. pulex	FAT	Fatties, St-Alexis-des-Monts, QC, Canada	46°25.40′N	73°13.44′W
D. pulex	PA32	Portland Arch, Fountain, IN	40°13′N	87°20′W
D. pulex	LP8	Long Point, ON, Canada	42°34′N	80°15′W
D. pulex	MAR	Marion Road, Saline, MI	42°08.5′N	83°48.5′W
D. parvula	A60	Acton Lake, OH	39°57.23′N	84°74.78′W

[a] Strains result from a single individual isolated from the wild population, which is subsequently clonally propagated in the lab.

largely unknown (*e.g.*, Hughes and Verra 2001; Delmotte *et al.* 2002; D'souza and Michiels 2006).

The genus Daphnia belongs to a clade of brachiopod crustaceans, which, on the basis of phylogenetic and paleontological evidence, has a long evolutionary history (>100 MY) of partially asexual reproduction (Taylor *et al.* 1999). Daphnia generally reproduce by cyclical parthenogenesis, with typically one sexual generation and ~5–20 asexual generations per year. The two species investigated here, *Daphnia magna* and *D. pulex*, inhabit small to medium-sized freshwater ponds. Both species are widely distributed and locally abundant throughout the northern hemisphere, which would suggest a very large population size, and, if they were sexual, a large N_e, comparable to other widely distributed invertebrates such as common Drosophila or outcrossing Caenorhabditis species. Here, we assess the consequences of partial asexual reproduction on nucleotide diversity and recombination, and estimate N_e for Daphnia. Ideally, estimates of diversity, recombination, and N_e would be compared between partially asexual and strictly sexual Daphnia species, but this is impossible, because no strictly sexual Daphnia are known.

We analyzed nucleotide diversity and linkage disequilibrium in eight housekeeping genes in *D. magna* and *D. pulex*. Allozyme studies have already shown that Daphnia can have considerable genetic diversity, both within and between populations (*e.g.*, Hebert 1978; Lynch and Spitze 1994; De Meester *et al.* 2006). However, data on nucleotide diversity are needed to estimate silent and synonymous diversity, which are likely to be neutral or weakly selected, so as to estimate N_e. Although diversity at silent sites can be influenced by selection at linked sites, this will generally reduce diversity (Begun and Aquadro 1992), leading to an underestimation of neutral diversity and thus to an underestimation of N_e, unless there is pervasive long-term balancing selection, which is implausible. Hence, a finding of high diversity is likely to be conservative with respect to N_e. In addition, DNA sequence data allow estimates of recombination and linkage disequilibrium (LD) over the small physical distances at which LD is likely to exist. As Daphnia is being developed as a model organism for ecological and evolutionary genomics and is the first crustacean to have its genome sequenced (draft genome accessible on http://genome.jgi-psf.org/Dappu1/Dappu1.home.html), data on nucleotide diversities and LD are useful because several types of analyses would be impeded by low diversity and long-range linkage disequilibria.

MATERIALS AND METHODS

Origin of samples: We investigated 10 strains of *D. magna*, 8 strains of *D. pulex*, and 1 strain of *D. parvula*. Each strain originated from a different population (Table 1, Figure1). The strains were isolated as single females and multiplied by clonal reproduction in the laboratory before DNA/RNA extraction. All the strains originated from cyclically parthenogenetic populations.

Molecular methods: We extracted DNA from *D. magna* strains using the DNeasy Blood and Tissue kit (QIAGEN). Because the *D. pulex* and *D. parvula* strains were used in a different project that required cDNA, we extracted RNA from

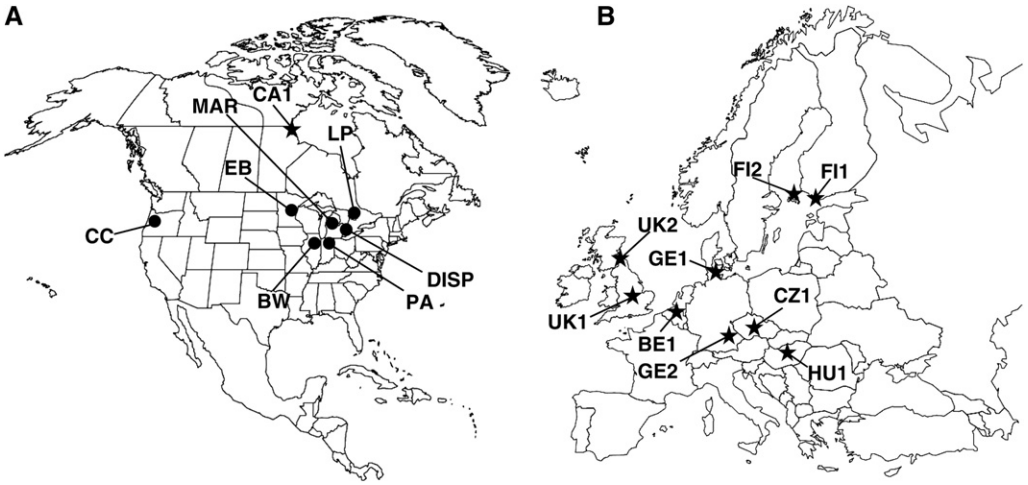

FIGURE 1.—Geographical locations of the Daphnia populations surveyed from (A) North America and (B) Europe. Population locations of *D. magna* are indicated by stars and those of *D. pulex* by circles. For more details, see Table 1. Maps of North America and Europe are not to scale.

these species with the RNeasy kit (QIAGEN). Reverse transcription was carried out using the Reverse Transcription System (Promega).

We studied eight genes (amino acid identity with *Drosophila melanogaster*: 44–85%). They include central metabolic genes, a translation initiation factor, and a nuclear receptor protein (Table 2). Daphnia have no sex chromosomes, so all the genes are autosomal. Primers were designed with Primer3 (ROZEN and SKALETSKY 2000). Primers for *D. magna* were based on sequences available in GenBank or in the *D. magna* EST library (COLBOURNE *et al.* 2005), and for *D. pulex* they were based on the draft genome sequence, which we annotated using blast hits of sequences from *D. magna* (when available) or *D. melanogaster*. The initial sequence of *Mpi* in *D. magna* was obtained using degenerate primers designed with Codehop (ROSE *et al.* 2003). Primers and sizes of amplicons are given in supporting information, Table S1. In a few cases, additional, internal primers (sequences available upon request) were used to verify specific regions or to check for allele-specific amplification in cases of all-homozygous sequences. PCR was carried out using GoTaq polymerase (Promega) and 57° as annealing temperature (in a few instances modified by ±2–4°). Sequences (GenBank accession nos. FJ668030–FJ668168) were obtained by direct sequencing from purified PCR product.

Analysis: Sequences were aligned using Sequencher version 4.8 (Gene Codes) and BioEdit (HALL 1999). All heterozygous sites were confirmed by resequencing from independent PCR reactions, and primer sequences were removed before analysis. In *D. magna*, two short regions within *Pgi* and one within *Got* could not be sequenced in one individual each, due to two or more length variants within the same amplicon. In *D. pulex*, *Usp* had multiple heterozygous length variants and consequently we were unable to obtain high-quality sequences for most individuals. Hence this gene was not included in the *D. pulex* analysis.

From the aligned heterozygous sequences, we obtained pseudohaplotypes using the program PHASE version 2.1 (STEPHENS *et al.* 2001). Since the *D. pulex* sequences were obtained from cDNA, introns were not sequenced and thus genomic distances were not preserved. We therefore determined the location and length of introns from the draft genome sequence, and inserted an appropriate number of "N"s into the sequences. The pseudohaplotypes obtained ($N = 20$ for *D. magna*, $N = 14$ for *D. pulex*) were then used in the analyses.

Neighbor-joining trees were constructed using MEGA 4 (TAMURA *et al.* 2007). In addition to our own data, we included a single haplotype obtained from the *D. pulex* draft genome. The trees indicated that one individual from each species (the *D. magna* from Canada and the *D. pulex* from Oregon) might belong to different subspecies (see RESULTS). Including different subspecies would inflate measures of diversity; hence, these individuals were excluded from the remaining analyses.

We used DnaSP version 4.50.3 (ROZAS *et al.* 2003) to estimate diversity from pairwise differences (π) and from the number of segregating sites (θ) and for recombination and linkage disequilibrium (LD) analyses. Further analyses of recombination and LD were performed with RecMin (MYERS and GRIFFITHS 2003), maxdip (http://genapps.uchicago.

TABLE 2

List of the eight nuclear loci analyzed in this study

Locus	Abbreviation	Characterization
Eukaryotic translation initiation factor 2γ	*Eif2γ*	Translation initiation factor
Enolase	*Eno*	Metabolic enzyme
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	Metabolic enzyme
Glutamine-oxaloacetic transaminase	*Got*	Metabolic enzyme
L-lactate dehydrogenase	*Ldh*	Metabolic enzyme
Mannose-phosphate isomerase	*Mpi*	Metabolic enzyme
Phosphoglucose isomerase	*Pgi*	Metabolic enzyme
Ultraspiracle	*Usp*	Nuclear receptor protein

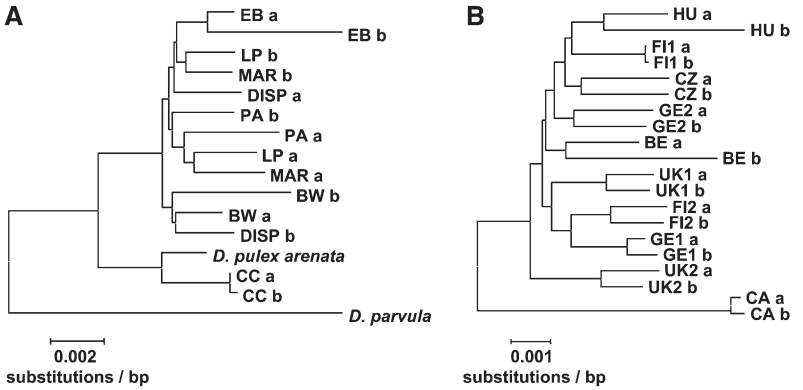

FIGURE 2.—Neighbor-joining trees of (A) *D. pulex* and (B) *D. magna*. Trees were constructed from concatenated sequence data from seven (*D. pulex*) and eight (*D. magna*) genes. Pseudohaplotypes are indicated by the suffix "a" or "b" after the strain name. The *D. pulex arenata* sequences were obtained from the draft genome available at http://wfleabase.org/. Strains CC (*D. pulex*) and CA (*D. magna*) were considered putative subspecies and removed from further analyses.

edu/maxdip/index.html; HUDSON 2001), and LI1AN (HAUBOLD and HUDSON 2000). In the analysis of the *D. magna* data, we distinguished between synonymous sites (indicated by the subscript s), nonsynonymous sites (a), noncoding sites (nc), and silent sites (si, *i.e.*, synonymous and noncoding sites combined). For *D. pulex*, noncoding sites were not sequenced.

In the analysis of between-species divergence, we report the net divergence, which subtracts π (or average π if polymorphism data from both species is available) from the observed divergence (NEI 1987). To estimate divergence times, T (in units of $2N_e$ generations), we use the framework of the HKA test (HUDSON *et al.* 1987). To correct for multiple hits at fourfold degenerate sites, we employed the Tamura–Nei correction (TAMURA and NEI 1993) using MEGA 4 (TAMURA *et al.* 2007).

RESULTS

Stratification of samples: Neighbor-joining trees revealed that the *D. magna* individual from Canada (CA) and the *D. pulex* individual from Oregon (CC) were highly divergent from all other individuals in their respective species clades (Figure 2), supporting an earlier conclusion from mitochondrial haplotypes that European and American *D. magna* are highly divergent (DE GELAS and DE MEESTER 2005). The *D. pulex* from Oregon grouped with the sequence from the draft genome (Figure 2), which belongs to the subspecies *D. p. arenata* (https://dgc.cgb.indiana.edu), which is

TABLE 3

Number of segregating sites, genetic diversity, and number of length variants in eight nuclear loci in *D. magna* and seven nuclear loci in *D. pulex*

Species	Locus	N_{Hap}[a]	Sequence length (nt)		S[b]	Diversity (π)					N length variants[e]
			Coding	Noncoding		π_s	π_{nc}	π_{si}	π_a	π_{all}	
D. magna	*Eif2γ*	18	429	186	14	0.0210	0.0091	0.0130	0.0013	0.0067	0
D. magna	*Eno*	18	438	193	12	0.0092	0.0100	0.0098	0.0016	0.0053	0
D. magna	*Gapdh*	18	369	70	9	0.0164	0.0220	0.0190	0.0000	0.0068	0
D. magna	*Got*	18	1188	348	19	0.0008	0.0130	0.0072	0.0008	0.0034	5
D. magna	*Ldh*	18	999	493	9	0.0004	0.0022	0.0016	0.0004	0.0010	8
D. magna	*Mpi*	18	645	319	14	0.0008	0.0059	0.0043	0.0011	0.0026	3
D. magna	*Pgi*	18	1611	957	112[c]	0.0280	0.0160	0.0190	0.0024	0.0110	13
D. magna	*Usp*	18	333	141	14	0.0440	0.0120	0.0220	0.0000	0.0100	2
D. pulex	*Eif2γ*	12	1233	0	15	0.0130	NA	NA	0	0.0031	0
D. pulex	*Eno*	14	933	0	14	0.0180	NA	NA	0.0002	0.0043	0
D. pulex	*Gapdh*	14	762	0	8	0.0120	NA	NA	0	0.0030	0
D. pulex	*Got*	12	852	0	14	0.0170	NA	NA	0	0.0039	0
D. pulex	*Ldh*	12	672	0	18	0.0260	NA	NA	0.0032	0.0087	0
D. pulex	*Mpi*	12	759	0	6	0.0062	NA	NA	0.0010	0.0022	0
D. pulex	*Pgi*	12	1365	0	78[d]	0.0620	NA	NA	0.0024	0.0164	0
D magna	Average				24.3	0.0150	0.0110	0.0120	0.0009	0.0059	
D. pulex	Average				21.9	0.0220	NA	NA	0.0009	0.0059	

s, synonymous; nc, noncoding; si, silent (synonymous and noncoding combined); a, nonsynonymous, NA, not assessed.
[a] Number of haplotypes.
[b] Number of segregating sites.
[c] Includes four triallelic sites.
[d] Includes one triallelic site.
[e] All length variants are in introns.

TABLE 4

Summary statistics of recombination and linkage disequilibrium estimates in *D. magna* and *D. pulex*

Species	Locus	R_m [a]	R_{min} [b]	ρ	I_{AS} [c]	Z_{ns} [d]	Z_a [e]	ZZ [f]	*P*(ZZ)
D. magna	*Eif2γ*	1	2	0.016	0.27****	0.26	0.44	0.18	0.01
D. magna	*Eno*	3	2	0.025	0.27****	0.15	0.21	0.06	0.19
D. magna	*Gapdh*	0	0	0	0.32****	0.30	0.26	−0.03	0.65
D. magna	*Got*	0	0	0.002	0.21****	0.31	0.37	0.06	0.18
D. magna	*Ldh*	0	0	0.001	0.39****	0.36	0.50	0.15	0.055
D. magna	*Mpi*	2	2	0.440	0.11****	0.16	0.03	−0.13	0.98
D. magna	*Pgi*	20	31	0.017	0.02****	0.10	0.17	0.07	0.006
D. magna	*Usp*	3	4	0.015	0.17****	0.25	0.33	0.08	0.10
D. pulex	*Eif2γ*	3	4	0.040	0.07****	0.15	0.20	0.05	0.26
D. pulex	*Eol*	5	5	0.289	0.03****	0.13	0.24	0.11	0.10
D. pulex	*Gapdh*	1	1	0.011	0.12****	0.10	0.05	−0.05	0.73
D. pulex	*Got*	0	0	0.008	0.10****	0.20	0.33	0.13	0.10
D. pulex	*Ldh*	3	4	0.013	0.08****	0.28	0.30	0.02	0.37
D. pulex	*Mpi*	1	1	0.039	−0.03	0.08	0.17	0.09	0.17
D. pulex	*Pgi*	19	25	0.207	0.03****	0.14	0.18	0.05	0.13
D. magna	Average	3.6	5.1	0.064	0.22	0.23	0.29	0.05	
D. pulex	Average	4.6	5.7	0.087	0.06	0.15	0.21	0.06	

****$P < 0.0001$.

[a] R_m, minimum number of recombination events (Hudson and Kaplan, using pseudohaplotypes).

[b] R_{min}, minimum number of recombination events (Recmin, using pseudohaplotypes).

[c] I_{AS}, LD summary statistics (LIAN).

[d] Z_{ns}, average r^2.

[e] Z_a, average r^2 between adjacent polymorphic sites.

[f] $ZZ = Z_a - Z_{ns}$, tested with coalescence simulations in DnaSP.

restricted to a small area in Oregon (*e.g.*, Colbourne *et al.* 1998; Lynch *et al.* 1999), whereas *D. pulex* from other populations included in this study have previously been identified as *D. pulex* s. str. (Crease *et al.* 1997). We concluded that these two specimens (CA and CC) potentially belong to different subspecies and thus excluded them from further analysis.

Nucleotide polymorphism: Across loci, we obtained 8719 bp of sequence for *D. magna* and 6576 bp for *D. pulex*, and found high average silent or synonymous site diversity in both species, although synonymous site diversity in *D. pulex* is almost twice as high as in *D. magna* (Table 3). All loci had low diversity at nonsynonymous sites, as expected for highly conserved sequences (Table 3).

Tajima's *D* (Tajima 1989) was significantly negative in *D. pulex* (one sample *t*-test for *D* on the basis of synonymous sites, mean across loci = −0.62, $t = -4.76$, d.f. = 6, $P = 0.0031$). In *D. magna*, Tajima's *D* also tended to be negative, but was not significant (one sample *t*-test for *D* based on synonymous sites, mean = −0.48, $t = -1.51$, d.f. = 7, $P = 0.17$, one sample *t*-test based on all silent sites: mean = −0.35, $t = -1.38$, d.f. = 7, $P = 0.21$).

Recombination and linkage disequilibrium: At least one recombination event was detected in most genes, even with the conservative four-gamete test (Table 4) (Hudson and Kaplan 1985). The per-site recombination parameter ρ estimated from unphased genotypic data (using the program maxdip) varied greatly among loci (Table 4). Average ρ is 0.064 in *D. magna* and 0.087 in *D. pulex* (Table 4), but median values are lower (0.015 and 0.039, respectively). When estimated from phased pseudohaplotypes (using DnaSP), the values were somewhat lower in *D. magna* (mean = 0.019, median = 0.007), and similar in *D. pulex* (mean = 0.091, median = 0.041).

LD (assessed with the program LIAN, Haubold and Hudson 2000) was significant but weak at all loci except *Mpi* in *D. pulex* (Table 4). LD decreased significantly with distance for some loci (Table 4, evaluated with a ZZ test, Rozas *et al.* 2001). LD was assessed using phased pseudohaplotypes, rather than from genotypic data, because the latter methods assume Hardy–Weinberg equilibrium, whereas PHASE infers haplotypes very accurately even when this assumption is not met, as is likely in our samples (Smith and Fearnhead 2005).

Effective population size: Assuming that a population is at equilibrium, N_e can be estimated from either nucleotide polymorphism $\theta = 4N_e\mu$ or the recombination parameter $\rho = 4N_e r$, where μ is the mutation rate, and *r* is the recombination rate per nucleotide; we refer to these estimates as $N_e(\theta)$ and $N_e(\rho)$, respectively. The mutation rate in Daphnia is not yet known, but, at least for microsatellites, it appears to be similar to that of *Caenorhabditis elegans* and *D. melanogaster* (Seyfert *et al.* 2008). We thus used the average genomic mutation rate

TABLE 5

Divergence between species and presumed subspecies

	D. magna to *D. pulex*		European *D. magna* to Canadian *D. magna*:	*D. pulex* to *D. parvula*:	*D. pulex* s. str. to *D. p. arenata*:
Locus	K_s(net)[c]	K_s(corr)[d]	K_{si}(net)[c]	K_s(net)[c]	K_s(net)[c]
Eif2γ	0.437	0.631	0.003	0.022	0.017
Eno	0.482	1.141	0.022	0.059	0.016
Gapdh	0.426	62.123	0.002	0.048	0.028
Got	0.542	3.744	0.026	0.115	0.038
Ldh	0.620	0.968	0.022	0.093	0.019
Mpi	0.338	0.413	0	0.055	0.008
Pgi	0.541	1.792	0.003	0.035	0.007
Usp[a]	0.563	1.138	0.010		
Average	0.494	1.982[b]	0.011	0.061	0.019

s, synonymous; si, silent (synonymous and noncoding combined).
[a] Estimate for *Usp* corrects for polymorphism in *D. magna* only, compared to the genome sequence of *D. pulex*.
[b] Geometric mean.
[c] Net divergence, corrected for within-species polymorphism.
[d] Divergence at fourfold degenerate sites corrected for multiple hits (Tamura–Nei correction).

of *D. melanogaster* and *C. elegans*, $\mu \approx 10^{-8}$ per generation (Denver *et al.* 2004; Haag-Liautard *et al.* 2007). For the average recombination rate, we use an estimate based on the *D. pulex* genetic map (Cristescu *et al.* 2006) of $r \approx 7.5 \times 10^{-6}$ cM/bp per sexual generation, or $r \approx 7.5 \times 10^{-7}$ per generation (sexual and asexual generations combined, assuming one sexual generation per 10 asexual ones). Using these estimates, and the average θ_{si} and ρ values from our data, we obtain estimates of N_e for *D. magna* $N_e(\theta) = 311{,}000$ and $N_e(\rho) = 2{,}148{,}000$, and for *D. pulex* $N_e(\theta) = 642{,}000$ and $N_e(\rho) = 2{,}890{,}000$. Using the more conservative median values for ρ, $N_e(\rho)$ is 503,000 for *D. magna*, and 1,286,000 for *D. pulex*. Although these estimates are highly uncertain, because local mutation and recombination rates may deviate substantially from genomewide averages, the N_e of European *D. magna* is clearly of the order of 300,000–500,000 and that of North American *D. pulex* at least twice as high. Using diversity at fourfold degenerate sites, the *D. pulex* N_e estimate is 3.3 times that of *D. magna*.

Between-species divergence: As expected from sequences of mitochondrial genes (*e.g.*, Lehman *et al.* 1995; Colbourne and Hebert 1996), the net synonymous site divergence between *D. magna* and *D. pulex* is high, averaging $K_s = 0.494$ across loci (Table 5). Multiple mutations at the same site are thus likely, and indeed the Tamura–Nei corrected divergence estimates for fourfold degenerate sites (Table 5) indicate a substantial but (with exception of the *Gapdh* locus) not extreme underestimation of divergence. Using the geometric mean rather than the arithmetic mean, to reduce any undue influence of *Gapdh*, we obtained an average divergence of 1.98 substitutions per site (Table 5).

The other species or putative subspecies pairs show much less sequence divergence. Between *D. pulex* and *D. parvula*, net divergence at synonymous sites averaged $K_s = 0.061$. Between *D. pulex* s. str. and *D. pulex arenata*, $K_s = 0.019$, and between Canadian and European *D. magna*, silent site divergence $K_{si} = 0.011$ (Table 5). The divergence at nonsynonymous sites was low in all comparisons (data not shown).

Using the polymorphism and divergence data for synonymous sites, and assuming neutrality of synonymous differences, we estimated divergence times T for the different species/putative subspecies pairs in units of $2N_e$ generations (N_{magna} and N_{pulex} denote the N_e of *D. magna* and *D. pulex*, respectively). The estimated divergence times for the closely related species/subspecies pairs were 1.8 for *D. pulex vs. D. parvula* (in units of $2N_{pulex}$), and, in the same units, 0.48 for the divergence between *D. pulex* s. str. and *D. p. arenata*, and 0.74 for the divergence between European and Canadian *D. magna* (in units of $2N_{magna}$, based on silent sites). Note that there are no polymorphism data from the second species/subspecies, so that these estimates are based on assuming equal N_e. Using polymorphism and divergence at fourfold degenerate sites, the divergence time between *D. magna* and *D. pulex* is much greater, estimated as 76.1 in units of $2N_{pulex}$ generations.

DISCUSSION

Abundant diversity and recombination: Both cyclically parthenogenetic, Daphnia species studied here have considerable silent site diversity (1–2%), frequent intragenic recombination, and low levels of linkage disequilibrium. Compared to other abundant and widely distributed invertebrate species, diversity levels are intermediate between outcrossing and self-fertilizing species of Caenorhabditis (Cutter 2006; Cutter *et al.* 2006a,b), and similar to or somewhat lower than in

Drosophila species (*e.g.*, Hamblin and Aquadro 1999; Andolfatto 2001; Dyer and Jaenike 2004; Maside and Charlesworth 2007). Daphnia species are currently being developed as new model organisms for evolutionary and ecological genomics (Colbourne *et al.* 2005). Several approaches proposed for such studies, including fine-mapping by association analyses, demand high diversity and low levels of linkage disequilibrium. The results of our study suggest that these approaches might indeed be feasible in Daphnia.

Genetic differentiation among Daphnia populations can be strong (*e.g.*, Hebert 1978; Lynch and Spitze 1994; De Meester *et al.* 2006). Population subdivision can contribute to high continentwide diversity (Whitlock and Barton 1997; Pannell and Charlesworth 2000; Wakeley 2000), but should also decrease recombination (Nordborg 2000; Wakeley and Aliacar 2001). Under the island model of population subdivision, diversity is increased and recombination decreased by a factor $(1 - F_{ST})$ compared to a panmictic population (Conway *et al.* 1999; Ingvarsson 2004). Our sample included just one individual per population (a "scattered sample"), and hence our diversity estimate are π_T, the continentwide values; this is ideal for estimating LD, as it avoids excess LD that is expected within populations due to recent common ancestry (Wakeley and Aliacar 2001; Song *et al.* 2009). π_T allows us to obtain an estimate of N_e, the "metapopulation effective size," which determines, for instance, fixation times of neutral alleles (Roze and Rousset 2003; Whitlock 2003). However, a scattered sample does not allow us to estimate within-deme diversity π_S. An estimate of π_S can, however, be obtained from $\pi_S = \pi_T\ (1 - F_{ST})$ (Pannell and Charlesworth 2000). Because F_{ST} measures the *proportion* of the total diversity that is found between populations, it is not very sensitive to the type of genetic information used, and thus we can tentatively use earlier allozyme-based estimates of $F_{ST} \approx 0.3$ in *D. magna* and *D. pulex* based on allozymes (Vanoverbeke and De Meester 1997; Lynch *et al.* 1999). This suggests that removing the effect of population subdivision would reduce our estimate of $N_e(\theta)$ by a factor of $\sim$0.7 and increase the estimate of $N_e(\rho)$ by the inverse of this factor. Despite these potentially substantial effects of population subdivision, within-population diversity ($\sim$1%) and $N_e(\theta)$ (>100,000) are still high and recombination abundant.

While it may be questioned whether the above F_{ST}-estimates indeed apply to our data, the evidence for abundant recombination (see also below) speaks against highly subdivided populations. Thus N_e is certainly high, even taking into account the effect of population subdivision, and hence selection is expected to be effective in the Daphnia species studied here, even for mutations with small selection coefficients (mutations with selection coefficients $s > 1/2N_e$ are predominantly influenced by selection as opposed to genetic drift). This situation is contrary to that expected in strictly asexual or highly inbreeding organisms, where selection is expected to be inefficient for mutations with small selection coefficients (Hill and Robertson 1966; Felsenstein 1974; Charlesworth 1994; Cutter and Charlesworth 2006; Keightley and Otto 2006).

Has a partially asexual life cycle affected N_e of Daphnia? Although estimates of $N_e(\theta)$ in the Daphnia species we studied are large, they are lower (up to one order of magnitude) than for outcrossing *C. remanei* (Cutter *et al.* 2006a) and most Drosophila species (Wall *et al.* 2002; Yi *et al.* 2003), especially if corrected for population subdivision. It is, however, unclear whether this is a consequence of the partial asexual life cycle. The census population size N_c (*i.e.*, the actual number of individuals) may also be lower. For example, the obligately sexual invertebrate *D. miranda* has low diversity, presumably due to low N_c (Bachtrog 2003; Yi *et al.* 2003).

Most Daphnia, including all the strains we analyzed, reproduce by cyclical parthenogenesis (partial asexuality), while some strains of *D. pulex* (and a few other Daphnia species) have become obligate asexuals (Hebert and Crease 1980). Observations of low numbers of clones in obligate parthenogenetic Daphnia populations (Weider *et al.* 1987; Hebert *et al.* 1989), and a higher rate of accumulation of slightly deleterious mutations than in cyclically parthenogenetic strains (Paland and Lynch 2006), suggest small N_e values in the obligate asexuals, and a comparison of two mitochondrial genes between *D. pulex* populations with differing breeding systems found slightly lower genetic diversity in obligate asexual populations (Paland *et al.* 2005). However, the sample may have included several independently arisen asexual lines, and thus no direct conclusions could be made about the relative N_e of populations of obligate and partial asexuals. We did not include obligate asexual strains in the present study because (in addition to the possible inclusion of independently arisen asexual lines) estimates of N_e in obligate asexuals from nuclear gene diversity may be affected by ancestral polymorphism, and possibly, if they have been asexual for long evolutionary times, by divergence between the alleles within lineages, and thus within individuals (Butlin 2002; Balloux *et al.* 2003).

Recombination: Per-site recombination parameters in both species of Daphnia are similar to those in outcrossing *C. remanei* and most Drosophila species (Andolfatto and Przeworski 2000; Yi *et al.* 2003; Haddrill *et al.* 2005; Cutter *et al.* 2006a). In addition, there is clearly much lower linkage disequilibrium in Daphnia than in self-fertilizing species such as *Arabidopsis thaliana* or *C. elegans* (Nordborg *et al.* 2002; Cutter 2006), consistent sexual reproduction in Daphnia being more common than outcrossing in the latter species.

The N_e estimated from the recombination parameters are somewhat larger than values from diversity, but, given the uncertainties about the underlying assump-

tions, the two estimates of effective population sizes are quite consistent, especially with $N_e(\rho)$ from the median ρ estimates. The consistency between the estimates of effective population size from recombination and from diversity is, however, altered if population structure is taken into account. The ρ/θ ratio is an estimate of r/μ, the relative frequency of recombination compared to mutation. Given the effects of population subdivision, we would expect $\rho/\theta = (r/\mu)(1 - F_{ST})^2$ (Conway *et al.* 1999; Nordborg 2000; Ingvarsson 2004). On the basis of our assumed mutation rate μ, recombination rate r, and frequency of sexual reproduction, we have $r/\mu = 0.75$, and thus (using $F_{ST} = 0.3$, as above), we expect $\rho/\theta = 0.37$. However, the observed values are 1.25 in *D. magna* and 1.75 in *D. pulex*, even using the more conservative median estimates of ρ. Thus, recombination in Daphnia is surprisingly high, particularly considering the fact that demographic history can also affect diversity and recombination (Hudson 1987; Andolfatto and Przeworski 2000; Wall *et al.* 2002; Haddrill *et al.* 2005): Demographic events that produce genomewide negative Tajima's *D* (as observed in *D. pulex*, see also Paland *et al.* 2005), for example, a strong population bottleneck followed by population growth, should increase LD (*e.g.*, Tishkoff *et al.* 1996; Haddrill *et al.* 2005) so that the observed low levels of LD are even more surprising.

The unexpectedly high levels of recombination may be explained if our assumptions about the mutation rate, recombination rate, the frequency of sexual reproduction or the F_{ST} value are incorrect. However, at least two additional effects might increase the effective recombination rate in Daphnia. First, two recent studies provided evidence that, contrary to our assumptions above, some recombination also takes place during the clonal phase in Daphnia (Omilian *et al.* 2006; Mctaggart *et al.* 2007), which would increase the estimate of r per sexual generation. Second, the offspring of heterozygotes may be overrepresented in Daphnia populations because of reduced fitness of homozygous individuals (Ebert *et al.* 2002; Haag *et al.* 2002). This could increase the effective recombination rate because crossing-over results in recombination only in double-heterozygous individuals.

Between-species divergence: *D. magna* and *D. pulex* are among the most distantly related species within the genus Daphnia; previous estimates on the basis of a mitochondrial molecular clock suggested a divergence time of ~200 MY (Colbourne and Hebert 1996). We estimated divergence time as $76 \times 2N_{pulex}$ generations. Assuming $2N_{pulex} = 10^6$, the divergence time estimate becomes 76×10^6 generations, which, assuming 10 generations per year (sexual and asexual generations combined, as also assumed by Paland *et al.* 2005), is only 7.6 MY. Many factors could contribute to the large discrepancy between these estimates. If the mutation and recombination rates for the studied genes are lower than we have assumed, our estimates of N_e, and consequently divergence times, would be larger. However, a very low mutation rate of $\mu \approx 5 \times 10^{-10}$ would be needed to yield an estimate of 200 MY. This seems unlikely, given that microsatellite mutation rates in Daphnia are similar to those in *C. elegans* and *D. melanogaster* (Seyfert *et al.* 2008). Second, there may be fewer than 10 generations per year, but values that would reconcile the date estimates (less than one generation per year) are inconsistent with the biology of the species (time to first reproduction is 10–15 days at 20°, Ebert 2005). A third possibility is population subdivision, which increases intraspecific polymorphism and decreases interspecific divergence estimates by a factor of $1 - F_{ST}$ (Ingvarsson 2004). Assuming $F_{ST} = 0.3$ increases the divergence time estimate to 15.6 MY. A very high degree of population differentiation would, however, have to be assumed to yield an estimate of 200 MY, and this is incompatible with the high recombination estimates.

Finally, despite correcting for multiple hits, we may have underestimated the divergence between the two species. However, under our assumptions of mutation rates and the number of Daphnia generations per year, the previous estimate of 200 MY would require ~20 substitutions per site since the two species became reproductively isolated. Our estimates of divergence times for the other two species pairs (with much lower raw divergence estimates than for *D. magna* and *D. pulex*, so that multiple hits are much less likely) are also much lower than previous estimates on the basis of mitochondrial sequences (Colbourne and Hebert 1996).

Alternatively, the earlier studies might have overestimated the age of the genus Daphnia. As for our estimates, multiple assumptions were necessary, most notably a constant mitochondrial rate (per year) of nucleotide substitution among animals. Yet, even among mammals this rate can vary by two orders of magnitude (Nabholz *et al.* 2008) and it is also highly variable among insects (Oliveira *et al.* 2008). If substitution rates were higher than assumed, this may have led to an overestimation of divergence time. Additionally, polymorphism in the ancestral species could contribute to a potential overestimation of divergence times if the two daughter species received highly divergent copies (Lynch and Jarrell 1993). Finally, because of the linkage of the whole mitochondrial genome, background selection and hitchhiking potentially contribute further difficulties in the interpretation of mitochondrial divergence times (Bazin *et al.* 2006; Oliveira *et al.* 2008). Until the relevant data are available for Daphnia, the discrepant divergence time estimates cannot currently be fully explained. However, our data suggest that the genus Daphnia may be considerably younger than previously thought. This might also change earlier conclusions that pairs of morphologically similar Daphnia species have diverged for tens of millions of years

without noticeable morphological change (COLBOURNE and HEBERT 1996).

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Nucleotide Polymorphism and Within-Gene Recombination in *Daphnia magna* and *D. pulex*, Two Cyclical Parthenogens

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TABLE S1

Primers and Amplicons

Species	Locus	Primer name	Amplicon			Forward primer (5'->3')	Reverse primer (5'->3')
			Length	Start[a]	End[a]		
D. magna	*Eif2γ*	Dm_Eif2γ_1	615	316	744	TGCGCTTCAAAAATGAGTTG	TGAGCCGAGATTGGGATAAC
D. magna	*Eno*	Dm_Eno_1	633	420	860	ATTGCTGATTTGGCTGGAAC	CAATTGAGACCATGGGGAAC
D. magna	*Gapdh*	Dm_Gapdh_1	442	218	589	CAATGGTCACCACATTCAGG	AATATTTTGGGCAGCACCAC
D. magna	*Got*	Dm_Got_1	571	12	442	GCGATAATCATGGCTCCTTC	GCGGTAGGAACGATAACTGG
D. magna	*Got*	Dm_Got_2	686	360	829	GTTGGAGCTGAATTCCTTGC	AGTGATTTGCGAACGAACAG
D. magna	*Got*	Dm_Got_3	486	816	1234	GACTTTTGTTGCGAAAGATCG	TGCACGTGGTATTTTGCTTC
D. magna	*Ldh*	Dm_Ldh_1	416	-60[b]	204	TCAGCCATAGCAGACGTTTC	TTATGGACGAAAGCCAAACC
D. magna	*Ldh*	Dm_Ldh_2	588	117	566	GGCATGTCTATCGCCTTTTG	CACCGTGTTCCCCAATAATC
D. magna	*Ldh*	Dm_Ldh_3	413	514	788	ACGTGGATTCTGCTCGATTC	TCCGCGTGTTCTTCAGTATG
D. magna	*Ldh*	Dm_Ldh_4	500	671	1050[b]	GATGCTGGCATGGAGTCAG	CAAGCATTTGACACTGTGATTG
D. magna	*Mpi*	Dm_Mpi_1	649	138	622	GCTGAACTGTGGATGGGAAC	CGAACTAAGAGACGGGATGC
D. magna	*Mpi*	Dm_Mpi_2	511	518	>809[c]	GTGTGAGGCGCTAGTCAAGG	TTAGGCTCAGCTGGTCATCC
D. magna	*Pgi*	Dm_Pgi_1	576	-13[b]	375	TGCTCGAAAGTCACGTAAAAC	TGAGCAAGGACTGCATTGAC
D. magna	*Pgi*	Dm_Pgi_2	568	254	677	CGCAAGGTGCAAGAATCTC	GGAACCAGGACTTTGCTGAC
D. magna	*Pgi*	Dm_Pgi_3	636	564	975	CCCAACGTCCATTTCGTATC	TCGCCATAAAGGACTCCAAG
D. magna	*Pgi*	Dm_Pgi_4	641	881	1319	CGCTCTCAGTATTGGCTTCC	CGTCGGCTGATTTTCCTTTC
D. magna	*Pgi*	Dm_Pgi_5	580	1208	1505	GCTCATTCACCAGGGAACTC	TCCCAAATAATTCCTTGAACG
D. magna	*Pgi*	Dm_Pgi_6	490	1424	1693[b]	CAAAGTGTTCGAAGGCAACC	TCGCTGGTTCTACTCGGTTC
D. magna	*Usp*	Dm_Usp_1	504	196	555	ACAATTCCAATGGCTCCAAG	TTGCACTCGACACGTTTCTC
D. pulex	*Eif2γ*	Dp_Eif2γ_1	1311	97	1407	GCTTCCACGTTAAAAATGTCG	GACTCGGCGACTGAGAGC
D. pulex	*Eno*	Dp_Eno_1	994	131	1124	TGGAGCTTCCACTGGTATCC	GAAGGAATCCTCGGTCTCG
D. pulex	*Gapdh*	Dp_Gapdh_1	767	38	804	TTAACGGATTTGGTCGTATCG	TCGCAGTAGCCCAAAATACC
D. pulex	*Got*	Dp_Got_1	881	306	1186	AAAGAAGGGAGGGCTACAGG	CACTTCATGGATGCACTTGG
D. pulex	*Ldh*	Dp_Ldh_1	946	15	960	CTCTGTCGACACCGTCTCC	TGACTGAACTTCGTTTAAAGTTGC
D. pulex	*Mpi*	Dp_Mpi_1	981	90	1070	CTTTCGCAAGGAAACTCAGC	CCCTGCAGGATCAAAAGG
D. pulex	*Pgi*	Dp_Pgi_1	1469	92	1560	AAGGAAAAGATTTGAACATTCACC	GCCTTGGCTAATTGCTTACC

[a]relative to *D. pulex* coding sequence