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The causes and consequences of asexuality in Daphnia: insights into the evolution of sex

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The causes and consequences of asexuality in *Daphnia*: insights into the evolution of sex

by

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The causes and consequences of asexuality in *Daphnia*: insights into the evolution of sex

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DECLARATION OF CO-AUTHORSHIP/ PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare this thesis incorporates the outcome of a joint research undertaken in collaboration with A.R. Omilian, S.S. Schaack, A. Seyfert, E. Choi, M. Lynch, and D.J. Innes under the supervision of Dr. M.E. Cristescu. This collaboration is covered in Chapter 2, 3 and 4 of the thesis. The key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily in assistance of collecting data and in an advisory capacity.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-authors to include the above materials in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

II. Declaration of Previous Publication

This thesis includes three original papers that have been previously published/will be submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	S. Xu, A.R. Omilian, and M.E. Cristescu (2011) High rate of large-scale hemizygous deletions in asexually propagating *Daphnia*: implications for the evolution of sex". *Molecular Biology and Evolution* 28: 335-342	Published
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Chapter 4	S. Xu, D.J. Innes, M. Lynch, and M.E. Cristescu. Obligate asexuality in *Daphnia*: the joint roles of hybridization and meiosis suppression loci	To be submitted to *Evolution*

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ABSTRACT

Understanding the causes and consequences of asexuality is essential for addressing the question why sexual reproduction is predominant in nature. Here I used *Daphnia* as a model system to investigate causes and genetic consequences of asexual reproduction. First I used *Daphnia* mutation-accumulation (MA) lines to show the high frequency of large-scale deletions during asexual reproduction (6.7×10^{-5} locus^{-1} ·generation^{-1}) and significant amount of ameiotic crossing over and gene conversion (3.3×10^{-5} locus^{-1} ·generation^{-1}). The high rate of deletions suggests the genomic instability and irreversible process of mutation accumulation. However, it remains unclear how ameiotic recombination can affect the evolution of asexual taxa. Furthermore, I investigated the mitochondrial DNA mutation rates and spectra for the MA lines originated from asexual and sexual ancestors to test the theoretical predication that loss of sex would result in a much higher mutation rate. The nearly complete mitochondrial genome sequences of 82 sexual and 47 asexual MA lines reveal high mtDNA mutation rate of 1.37×10^{-7} and 1.73×10^{-7} per nucleotide per generation, respectively. Maximum-likelihood estimates of the *Daphnia* mitochondrial effective population size revealed that between five and ten copies of mitochondrial genomes are transmitted per female per generation. However, there was no statistical significant difference between the mutation rates in sexual and asexual MA lines and the mutation spectra are highly similar. Lastly, I use microsatellite data to examine the roles of hybridization and meiosis suppression in the origin of obligately asexual *D. pulex* that are thought to be the hybrids of *D. pulex* and *D. pulicaria*. The results of phylogenetic analyses and Bayesian estimates of ancestry based on nine microsatellite loci strongly support that these hybrids originate from the crossing of F1 progeny with *D. pulex* males produced by obligately parthenogenetic clones carrying genetic elements conferring meiosis suppression. Overall, this

thesis improves our understanding of how abandoning sex can affect the genomic stability and mutation rate in asexual taxa and provides a solid framework for future investigations on the cellular mechanisms and genetic consequences of obligate asexuality in *Daphnia*.

DEDICATION

To my wife, Li, and our daughters, Zexuan and Yushan

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CHAPTER 1. INTRODUCTION

The evolution of sex is one of the most puzzling questions in evolutionary biology (Williams 1975; Maynard Smith 1978; Bell 1982). Sexual reproduction is predominant in nature, with ~ 0.1% of named animal species (White 1978) and less than 1% of 250, 000 angiosperm species being asexual (Asker and Jerling 1992; Whitton et al. 2008). Asexual taxa usually occupy tip positions on the tree of life and rarely constitute taxonomic units higher than species, despite a few notorious exceptions such as Bdelloid rotifers that have existed for millions of years without sex (Judson and Normark 1996; Butlin et al. 1998; Mark Welch and Meselson 2000). The widespread occurrence of sex in nature indicates its evolutionary advantages compared to asexual reproduction. However, in contrast to asexual reproduction, sexual reproduction is thought to be a much more costly process and may incur some immediate disadvantages. Sexual reproduction has a two-fold demographic disadvantage, i.e., asexuals can produce twice as many offspring as sexuals because of the absence of males (Maynard Smith 1978). Sexual reproduction breaks down the association of alleles that have been built up by past selection due to meiotic recombination and segregation. Consequently, the sexually produced offspring can have different genotypes that may not fit the current environment. Moreover, sex may involve other costs such as the time and energy spent in seeking mates, the risk of predation when seeking mates, and the risk of sexually transmitted disease, to name just a few. It is therefore difficult to understand why sexuals rather than asexuals are the majority rule in higher animals and plants.

Numerous (over 20) hypotheses have been proposed over the past several decades to explain the predominance of sex in nature (Kondrashov 1993). These theories can be broadly classified into two categories: the mutational and ecological hypotheses (Hurst and Peck 1996). The mutational hypotheses suggest that sex can help purge deleterious mutations (e.g., the deterministic mutational hypothesis, Kondrashov 1988), whereas the ecological hypotheses propose that the higher genetic

diversity released by sex can facilitate escape from parasites (e.g., the red queen hypothesis, Morran et al. 2011) or limits competition with relatives. However, none of them have been universally accepted, and all of them are facing difficulties in certain cases. For example, one of the oldest, and most widely accepted ideas that sex creates genetic variation needed by natural selection (Weismann 1889) may not work in some situations, because sex need not increase variation all the time and the variation generated by sex often reduces fitness in offspring (see Otto 2009).

Examining the causes and consequences of asexuality provides an important way to gain further insight into the evolutionary forces maintaining sexual reproduction (Schön et al. 2009). The loss of sex has several evolutionary consequences. Consistent with the theoretical prediction that sex and recombination facilitate the removal of deleterious mutations, asexual reproduction has been shown to lead to the accelerated accumulation of deleterious mutations in non-recombining regions of the genome and in nuclear and organelle genomes for asexual species (e.g. Charlesworth and Charlesworth 2000; Bachtrog and Charlesworth 2002; Lynch 1997; Henry et al. in press; Paland and Lynch 2006). Asexual populations can be used as a benchmark to test whether sex can facilitate adaptation. In fact, several studies have demonstrated that asexuals cannot adapt as rapidly as sexuals (e.g. Colegrave 2002; Colegrave et al. 2002; Goddard et al. 2005; Cooper 2007). Understanding the biogeography of obligate parthenogenetic species is also critical for investigating the role of ecological conditions in the evolution of sex (e.g. geographic parthenogenesis, Hörandl 2009). Assessing the population genetics for asexual species reveals the interactions among selection, genetic drift, and mutations and also provides insights into the benefits of genetic diversity (Halkett et al. 2005; Vrijenhoek 1998). Furthermore, investigations into those "scandalous" ancient asexual taxa (Judson and Normark 1996) have discovered peculiar ways of genetic exchange, e.g. horizontal gene transfer in Bdelloid rotifers (Galdyshev et al. 2008), that may

have played a critical role in their millions years of existence after abandoning sex (Mark Welch et al. 2009).

In our conventional understanding concerning asexuality, there are many important assumptions that are central to theories on the evolution of sex. Nonetheless, these assumptions have rarely been empirically examined. In this thesis, I use a population genomic approach to examine several important questions concerning the causes and genetic consequences of obligate asexuality in *Daphnia* (Crustacea, Anomopoda). There are several unique advantages of using *Daphnia* as a model system to investigate the causes and consequences of asexuality. First, *Daphnia* typically reproduce by cyclical parthenogenesis (i.e. the alternation of sex and asexual reproduction), with some species and/or populations having lost the ability to engage in sexual reproduction. Thus, *Daphnia* provide a platform for contrasting the genetic consequences of sexual and asexual reproduction. Second, recently evolved obligate asexual lineages provide an easily accessible system for testing the current evolutionary tenets concerning the consequences of asexuality. Third, the availability of the full genome sequence of *Daphnia* (Colbourne et al. 2011) and the development of a wide array of genomic tools (e.g. Colbourne et al. 2004) will greatly facilitate empirical investigations.

The three main issues that I aim to address concern the ameiotic recombination rate in *Daphnia* (Chapter 2), the rate and molecular spectrum of mutations in the mitochondrial genomes of asexual (i.e. obligately parthenogenetic) and sexual (i.e. cyclically parthenogenetic) *Daphnia* (Chapter 3), and the causes of obligate asexuality and the genes that are involved in obligate asexuality in *Daphnia* (Chapter 4).

1.1 RECOMBINATION RATE IN ASEXUAL GENOMES

One of the most important population-genetic consequences of asexuality is that the amount of recombination is reduced to a negligible level in evolutionary long term. Thus, asexual species are

thought to be impoverished of evolutionary potential due to their incapability of quickly creating new gene combinations (Maynard Smith 1978). Asexuals are also thought to represent evolutionary dead ends (Maynard Smith 1978) because an irreversible accumulation of deleterious mutations will occur in the absence of recombination. This process is best described by Muller's ratchet (Muller 1964; Felsenstein 1974). Without recombination, individuals with the minimum number of mutations in a population cannot be reconstituted when lost due to either recurring deleterious mutations or chance events (i.e., genetic drift), thereby leading to a fitness decline in the long term. In support of this view, there has been extensive documentation of the accumulation of deleterious mutations in non-recombining genomic regions such as Y chromosomes (Charlesworth and Charlesworth 2000; Bachtrog and Charlesworth 2002), in organelle genomes (Lynch 1997; Lynch and Blanchard 1998), and asexual species (Henry et al. in press; Paland and Lynch 2006; Neiman et al. 2010).

However, some recent findings have strongly challenged this well-established evolutionary tenet. In a recent mutation-accumulation (MA) study, Omilian et al. (2006) screened asexually reproducing *Daphnia* MA lines at various coding and noncoding loci and found that the rate of ameiotic recombination inferred directly from the frequency of loss of heterozygosity (LOH) events was about 10^{-4} incidents $\cdot locus^{-1} \cdot generation^{-1}$. Thus, the rate of recombination appeared to be orders of magnitude higher than that of mutation, which was measured to be on the order of 10^{-9}~10^{-8} $bp^{-1} \cdot generation^{-1}$from similar long term MA experiments (Denver et al. 2004; Lynch et al. 2008b; Denver et al. 2009; Keightley et al. 2009). Furthermore, there has been indirect evidence of recombination during asexual reproduction. For ancient asexual taxa that have abandoned sex millions of years ago, they are expected to exhibit high DNA sequence divergence between the two alleles at a diploid locus because two chromosomes can accumulate nucleotide polymorphism independent of each other in the absence of recombination. This is known as the Meselson's effect

(Birky 1996; Judson and Normark 1996; Mark Welch and Meselson 2000) and is expected to be highly useful in proving the status of ancient asexual taxa. However, several studies examining allele divergence in ancient asexual taxa such as the Darwinulid ostracods and the aphid Tramini show no evidence of nucleotide divergence, suggesting that certain levels of recombination and/or efficient DNA repair are at work in asexual genomes (Butlin et al. 1998; Normark 1999).

Using a diploid, single-locus model, Mandegar and Otto (2007) showed that adaptation in asexual populations can be as rapid as that in sexual populations when the rate of mitotic/ameiotic recombination (10^{-4} or 10^{-5}) is much higher than the rate of mutations (e.g. 10^{-7}). Therefore, asexual taxa can gain most of the benefit of segregation via mitotic/ameiotic recombination while avoiding the costs associated with sex (Mandegar and Otto 2007). Furthermore, there has been empirical work showing that mitotic recombination can accelerate adaptation in asexual populations (Schoustra et al. 2007). In light of these interesting findings, it is thus imperative to re-examine the role of ameiotic/mitotic recombination in the evolution of asexual taxa. As an initial step, we need to establish a baseline estimate of the ameiotic/mitotic recombination rate in asexual genomes. Moreover, we need a better understanding of the interaction between mitotic recombination and mutations. DNA damage (e.g. double stand breaks) occurs frequently in organisms including both sexual and asexual species. Numerous DNA repair pathways exist to repair such DNA damage. However, some repair pathways such as non-allelic homologous recombination, single-strand annealing, and breakage induced repair can have mutagenic effect, resulting in deletions at the affected genomic regions (Helleday 2003; Hastings et al. 2009).

To address these issues, the second chapter of this thesis uses *Daphnia* mutation-accumulation lines to estimate the rate of ameiotic recombination during parthenogenesis. Moreover, this chapter estimates the rate of large-scale deletions in asexually reproducing *Daphnia* and the physical scale

of such deletions. Lastly, this chapter investigates the possible causes (e.g. unequal crossing over) of large-scale of deletions.

1.2 RATE AND MOLECULAR SPECTRUM OF MITOCHONDRIAL MUTATIONS

In asexual taxa, abandoning certain recombination activities might alter mutation rates (Lercher and Hurst 2002; Filatov and Gerrard 2003). However, none of the mutational theories of the evolution of sex take into consideration this possibility (Kondrashov 1988; Lynch et al. 1995; Barton and Charlesworth 1998). Meiosis is found to lead to a higher frequency of mutation than mitosis because many of the mutations appear to be associated with crossover events (e.g. Esposito and Bruschi 1993; Strathern et al. 1995).

In theories of the evolution of mutation rate, the absence of recombination in asexual genomes is predicted to have significant impact on mutation rate. The evolution of mutation rate is thought to be determined by the costs of exact replication, the cost of deleterious mutations, and advantages of beneficial mutations (but see Lynch 2010). Assuming that in asexual species recombination is usually negligible (Maynard Smith 1978), the cost of replication fidelity and the effects of deleterious mutation are expected to yield a low mutation rate (Dawson 1998). However, if beneficial mutations are common, mutation rates in asexual species may be higher relative to sexual species, even rising to an intolerable level that can lead to extinction (Johnson 1999; Andre and Godelle 2006; Gerrish et al. 2007). To date, few empirical studies investigate how sexual and asexual species differ in both nuclear and mitochondrial mutation rates (but see Baer et al. 2010).

Because of their well-understood genomic structure and small size, animal mitochondrial genomes provide an excellent entry point for comparing mutation rates between asexual and sexual species. For mitochondrial genomes that experience “asexual” inheritance in both asexual and sexual species, the mitochondrial-nuclear linkage in asexual species can result in reduced efficiency of selection

(Normark and Moran 2000). Asexual lineages of *Daphnia* (Paland and Lynch 2006), snails (Neiman et al. 2010), and rotifers (Barraclough et al. 2007) have been shown to accumulate deleterious mutations at a much faster rate compared to sexual lineages. Intuitively, because all the proteins involved in mitochondrial DNA replication and repair are encoded by nuclear genes, the negligible amount of recombination in asexual nuclear genomes (Maynard Smith 1978) can result in the accumulation of deleterious mutations in these genes and consequently elevated mitochondrial mutation rate. Nonetheless, little data exist contrasting the mitochondrial mutation rate in sexual and asexual species.

The mitochondrial mutation process and the transmission of mutations are also intriguing phenomena to evolutionary biologists. Despite the great utility of mitochondrial DNA (mtDNA) sequence data in population genetics and phylogenetics, key parameters describing the process of mitochondrial mutation (e.g. the rate and spectrum of mutational change) are based on few direct estimates (Denver et al. 2000; Haag-Liautard et al. 2008; Howe et al. 2010). Because germ-line cells usually harbor multiple copies of mtDNA (e.g. ~2,000 in mice primordial germ cells, Cao et al. 2007), a neutral mutation originating in a single mtDNA copy must go through a drift process to reach fixation. The question of how quickly a mutation can reach fixation depends on the effective population size of mitochondrial genomes. It is known that the mitochondrial effective population size is much smaller than the census size within a cell, due to a mtDNA bottleneck during early oogenesis and/or to the fact that some mtDNA alleles in heteroplasmic cells replicate more often than others by chance or due to selective advantages (Birky 2001). However, there are few estimates about the effective population size of mitochondrial genomes (reviewed in Lynch 2007).

To address these issues, the third chapter of this thesis directly estimates the mtDNA mutation rate and spectrum using *Daphnia pulex* mutation-accumulation (MA) lines derived from sexual (cyclically parthenogenetic) and asexual (obligately parthenogenetic) lineages. Furthermore, this

chapter uses a maximum-likelihood modeling approach to estimate the effective population size of mitochondrial genomes in *Daphnia*.

1.3 CAUSES AND GENES RESPONSIBLE FOR OBLIGATE ASEXUALITY

Transitions from sexual to asexual reproduction have repeatedly occurred in many different groups of organisms such as plants, rotifers, nematodes, and arthropods (Bell 1982). In the animal kingdom, 19 out of 34 phylum contain asexual taxa (Bell 1982). Asexuality in different phylogenetic lineages has dramatically different modes of origin including spontaneous origin, hybrid origin, contagious origin, and infectious origin (see Simon et al. 2003). Furthermore, there are a variety of cytological mechanisms responsible for asexuality in animals, such as automictic parthenogenesis, apomictic parthenogenesis, gynogenesis, and hybridogenesis (Vrijenhoek 1998; Stenberg and Saura 2009). The various origin of asexuality and cytological mechanisms can have significant impact on the genetic diversity, ecological adaptability of asexual populations/species and the outcome of competition with their sexual relatives (e.g., Howard and Lively 1994; Howard and Lively 1998). With the increasingly wide application of molecular tools in the past few decades, the origins of asexuality in a large number of taxa have been illuminated (e.g. Delmotte et al. 2003; Schwander and Crespi 2009). A robust methodology has been developed for identifying the modes of origin (e.g., hybrid origin) using phylogenetic and population genetic data (Simon et al. 2003). However, little work has been done investigating the genes/genomic regions involved in converting sexual reproduction to asexual reproduction.

An ideal system for examining the molecular mechanisms responsible for the conversion to asexual reproduction is *Daphnia pulex*. *D. pulex* and *D. pulicaria* are morphologically indistinguishable sister species, but they show distinct genotypes at the Lactate Dehydrogenase (*Ldh*) locus. Whereas *D. pulex* is homozygous for slow alleles (SS) and *D. pulicaria* is homozygous for fast alleles (FF), the hybrids are characterized by SF genotype at the *Ldh* locus (Hebert and Finston 2001). Within *D.*

pulex, asexual lineages are thought to have originated from sexual lineages multiple times (Crease et al. 1989; Hebert et al. 1989; Paland et al. 2005). These lineages have lost the ability to engage in sexual reproduction, reproducing only by obligate parthenogenesis (OP). Asexuality can spread in *D. pulex* in a contagious fashion. While lacking the ability of reproducing sexually, some asexual lineages in *D. pulex* can still produce males that carry mutations (i.e. meiosis suppression genes) that can stop meiosis during oogenesis, but not during spermatogenesis (Hebert 1981; Hebert et al. 1989). These males can mate with cyclically parthenogenetic (CP) females, spreading female asexuality by producing a mixed brood of both OP and CP individuals with SS *Ldh* genotype (Innes and Hebert 1988). Furthermore, previous studies suggest that the hybridization of male *D. pulex* carrying meiosis suppression genes and females of *D. pulicaria* (lake species) can produce F1 asexual hybrids that are found predominately in ponds in disturbed areas where forests had been cleared (Hebert et al. 1989).

A recent study by Heier and Dudycha (2009) challenged the previous view about the origin of the OP in *Ldh* SF *D. pulex* clones. This study shows that the hybrids between females of *D. pulex* and males of *D. pulicaria* produced in laboratory conditions always appear to be cyclical parthenogens, rather than obligate parthenogens. The authors further suggest that the OP hybrids originate from the crossing of F1 hybrids (i.e. cyclical parthenogens) in pond habitats with *D. pulex* males carrying genetic elements conferring obligate asexuality, i.e. meiosis suppression genes. For meiosis suppression genes, it was initially thought to be a single, dominant gene based on the 1:1 ratio of sexual and asexual offspring from crossing experiments (Innes and Hebert 1988). However, a recent study that performed genome-wide association mapping using a collection of *Ldh* SS *D. pulex* clones across North America shows that at least four genomic regions appear to be significantly associated with meiosis suppression/obligate asexuality (Lynch et al. 2008a). The fourth chapter of this thesis focuses on examining the origin of *Ldh* SF asexual *D. pulex* using microsatellite data.

Furthermore, this chapter investigates whether different genomic regions are involved in meiosis suppression and/or obligate asexuality for LDH SF individuals compared with LDH SS individuals.

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CHAPTER 2. HIGH RATE OF LARGE-SCALE HEMIZYGOUS DELETIONS IN ASEXUALLY PROPAGATING *DAPHNIA*

2.1 INTRODUCTION

Characterizing the evolutionary consequences of asexuality at the genetic level is important for understanding why sexual reproduction is predominant in nature. From a theoretical standpoint, asexual taxa are evolutionary dead ends because they are assumed to have evolutionarily negligible levels of homologous recombination (Maynard Smith 1978). Moreover, because the majority of mutations are deleterious, the mutation load for asexual taxa will irreversibly increase and lead to eventual extinction of populations (Lynch and Gabriel 1990; Lynch et al. 1993) because highly fit genotypes cannot be reconstituted without recombination (Muller 1964; Felsenstein 1974). Despite the central importance of the rates of mutation and recombination in understanding the consequences of asexuality, these basic parameters have rarely been directly estimated in asexual lineages (Omilian et al. 2006).

Mutation-accumulation (MA) experiments facilitate the study of mutations because the effects of natural selection are minimized. Replicate MA lines are propagated through regular bottlenecks each generation in a benign environment. Thus, the majority of spontaneous mutations accumulate in a neutral fashion (Keightley and Caballero 1997), thereby allowing for the direct estimation of mutation rates. In a recent study, Omilian et al. (2006) screened protein-coding and microsatellite loci across three major linkage groups in asexually reproducing *Daphnia* MA lines and found that the rate of ameiotic recombination inferred directly from the frequency of loss of heterozygosity (LOH) appeared to be orders of magnitude higher than the frequency of mutation (Denver et al. 2004; Denver et al. 2009; Keightley et al. 2009). This finding challenges the assumption that homologous recombination is evolutionarily negligible in asexual taxa. However, two important aspects of the findings of Omilian et al. (2006) need to be clarified before examining how ameiotic recombination influences the evolution of asexual taxa. First, it remains unclear whether the high rate of LOH is temporally stable or applicable to the entire *Daphnia* genome. Second, and more

importantly, it is critical to distinguish LOH events resulting from ameiotic recombination (maintaining both DNA copies of a locus) from LOH events resulting from the segmental deletion of one DNA copy of a diploid locus (hemizygosity). Non-allelic homologous recombination (e.g. unequal crossover) and various pathways for repairing DNA double strand breaks and/or stalled replication forks (e.g. single-strand annealing and breakage induced repair) can produce hemizygosity at the affected loci (Helleday 2003; Hastings et al. 2009).

In this study, we estimate the genome-wide rate of LOH events in mutation-accumulation (MA) lines of asexually propagating *Daphnia pulex* and *Daphnia obtusa* by screening microsatellite markers throughout the genome. Furthermore, we used quantitative PCR (qPCR) to determine the relative proportions of homozygous LOH events (gene conversion and cross-overs) versus hemizygous LOH events (deletions). We find that the genome-wide LOH rate is consistent with the estimate of Omilian et al. (2006). However, a large proportion (67%) of our identified LOH events resulted from deletions leading to hemizygosity, yielding a deletion rate of 6.7×10^{-5} locus^{-1} ·generation^{-1} and a recombination rate of 3.3×10^{-5} locus^{-1} ·generation^{-1}. Lastly, we examine the physical length of deletion tracts and show that they are large (2-30 kb) and span open reading frames.

2.2 MATERIALS AND METHODS

2.2.1 Mutation-accumulation (MA) lines

Single females of *D. pulex* (denoted as PX) and *D. obtusa* (OB) were isolated from temporary ponds located in Linwood, ON, Canada and Trelease Woods IL, USA, respectively. Most *Daphnia* are cyclical parthenogens, capable of both sexual and apomictic reproduction. However, obligately asexual lineages do exist (Banta 1925; Innes and Hebert 1988) and asexual reproduction in several species of *Daphnia* has been shown to be ameiotic (Schrader 1925; Zaffagnini and Sabelli 1972).

PX was determined to be an obligate parthenogen, whereas OB was determined to be a cyclical parthenogen, following established methods for breeding system determination (Innes, Schwartz, and Hebert 1986).

Daphnia were maintained under standard conditions at 20 ℃ and fed *ad libitum* with a suspension of vitamin-fortified *Scenedesmus obliquus*. Mutation-accumulation (MA) lines were initiated from 48-50 single progeny derived from a single stem mother from both PX and OB, and maintained following previously described methods (Lynch 1985). Briefly, a single randomly chosen daughter was transferred to a new beaker every asexual generation (10-12 and 8-10 days for PX and OB, respectively) while two females of the same brood were transferred into separate vessels to serve as back-ups, in case the focal individual died without producing female offspring. Back-ups were used in 15-20% of the transfers, usually because the focal individual produced only resting eggs (PX) or male offspring (OB). The use of back-ups potentially leads to a downward bias in our loss of heterozygosity (LOH) rate estimates, because LOH that is lethal or substantially retards the production of immediately-developing female offspring will be underrepresented. The MA lines screened in this study (20 lines for PX and 28 for OB) were propagated for an average of 116 and 190 generations respectively (supplementary Table S2.1). These MA lines represented a subset of the lines used by Omilian et al. (2006) at about 75 (PX) and 107 (OB) generations but included all of the lines that survived the long-term MA experiment.

2.2.2 DNA extraction, PCR, and genotyping

Genomic DNA of 5 to 10 adult individuals for each MA line was extracted using a cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). We screened microsatellite markers that constitute the framework for the 12 linkage groups of *Daphnia pulex* (Cristescu et al. 2006). We excluded microsatellite loci that were known to be homozygous in the

stem mother because they are uninformative for revealing LOH events. A total of 141 microsatellite markers were genotyped for the PX (*D. pulex*) lines, while 95 out of these 141 microsatellite markers were also screened for the OB (*D. obtusa*) lines. According to the *D. pulex* genome annotation (wfleabase.org), 65 of 141 microsatellite loci screened were located in protein-coding regions (48 in exons and 17 in introns). In order to avoid null alleles, markers that showed inconsistent amplification patterns across lines were excluded. All LOH events were confirmed with a second independent PCR using a different Taq polymerase. PCR reactions and genotyping followed methods in Cristescu et al. (2006).

2.2.3 Quantitative microsatellite analysis

Quantitative microsatellite analysis (QuMA) has been successfully applied to detect changes in DNA copy numbers in clinical genetic analyses (Ginzinger et al. 2000; Nigro et al. 2001; Suzuki et al. 2004). We quantified the DNA copy number of the 24 LOH events for 15 loci in the PX MA lines relative to heterozygous microsatellite loci using QuMA. Omilian et al. (2006) reported that one of their MA lines (LIN6, designated as PX6 herein) experienced a long tract of LOH that spanned more than half of chromosome three. Although PX6 was not part of our mutation and recombination rate estimates, we determined the DNA copy number of all 14 microsatellite loci located on this particularly long LOH tract. For QuMA, PCR reactions of a given LOH locus (referred to as the test locus) and a confirmed heterozygous locus across all MA lines (referred to as the reference locus) for the LOH MA line and two independent normal (calibrator) MA lines were performed in triplicate on the ABI 7500 Real-Time PCR system (Applied Biosystems). The microsatellite locus d088 was chosen as the reference locus because it appears to be heterozygous in all the PX MA lines (i.e. DNA copy number is 2) and its amplicon (~150 bp) is desirable for qPCR experiments.

The thermocycling regime consisted of a 10 min incubation at 95 °, followed by 40 cycles of 15 sec at 95 °, and 1 min at 60 ° with a single fluorescent reading taken at the end of each cycle. Specificity of amplification in qPCR experiments was confirmed by a dissociation analysis at the end of each run. The 20-µl PCR reactions consisted of 10 µl SYBR® Premix Ex Taq (Takara Bio USA), 0.2 µM of each primer, 0.4 µl Rox II dye, ddH_2O, and 1 -5 µg DNA template. The amplification efficiency (E) for all the primer sets used in the actual qPCR experiments was calculated using the software LinRegPCR (Ruijter et al. 2009) to assure that the E values for the test and reference loci were approximately the same (>85%). The E value for each primer pair was also confirmed by performing qPCR experiments following standard procedures (user bulletin no. 2, ABI 7700 SDS, Applied Biosystems). We used the Sequence Detection Software (v.1.2.3, Applied Biosystems) to estimate the number of PCR cycles (C_T) required for the fluorescence to reach a threshold above background for the test and reference reactions that were performed in triplicate for each DNA sample.

For each DNA sample, the average of C_T values for the test locus and the reference locus was subtracted to obtain ΔC_T [ΔC_T = mean of C_T (test locus) – mean of C_T (reference locus)]. Relative copy number at each test locus in the LOH sample was then calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = ΔC_T (LOH sample) – the average of ΔC_T for two calibrator samples. The relative copy number multiplied by 2 yields the copy number of the test locus in the LOH sample (copy number = $2 \times 2^{-\Delta\Delta C_T}$) (Ginzinger et al. 2000).

To determine whether the relative copy number of a test locus was significantly different from that of the reference locus, a tolerance interval (TI) was calculated using the pooled standard deviation (SD) of ΔC_T values for the test and reference locus on 38 calibrator DNA samples using the following formula: TI = 2 × 2 to the power ± {2.46 × the square root of

$[\sum_i (n_i - 1) \times SD_i^2 / \sum_i (n_i - 1)]\}$, where n_i is the number of calibrators analyzed per microsatellite and 2.46 was the two-sided tolerance limit factor for the total degrees of freedom ($\sum_i (n_i - 1) = 38$). The relative copy number of a normal sample will be within $2 \pm$ TI 95% of the time with 95% confidence. Based on this TI, DNA copy numbers less than 1.20 were considered deletions, whereas copy numbers greater than 3.33 were considered duplications.

2.2.4 Calculation of the rates of LOH, deletion and ameiotic recombination

The rate of LOH (locus^{-1} ·generation^{-1}) was calculated following the method in Omilian et al. (2006) using the equation $\lambda = h/(L \cdot i \cdot T)$, where *h* is the number of observed LOH events, *L* is the number of MA lines, *i* is the number of total informative (heterozygous in the stem mother) loci, and *T* is the average number of generations for MA lines. The rates of ameiotic recombination and deletion were calculated with *h* representing the number of homozygous or hemizygous loci, respectively. To obtain a deletion/recombination rate per nucleotide, *h* is the number of base pairs that experienced hemizygosity or homozygosity, and *i* is the total number of informative base pairs (Table 2.1). Given that all deletions and crossover/gene conversions span the entire length of the marker, the per locus and per bp expression are equivalent. However, we caution that the deletion rate per nucleotide is not directly comparable with point mutations rates; the deletion rate per nucleotide takes into account the physical length of the deleted genomic regions rather than the number of mutation events.

2.2.5 Characterization of physical lengths of hemizygous deletions

Physical lengths of hemizygous deletions were quantified for four loci, which included two loci (d050 and d083) that showed LOH in three or more PX MA lines (supplementary Table S2.1), and two loci (d078 and d054) that appeared to be on a long LOH tract in PX6 likely spanning one arm

of chromosome three (Fig. 2.2). A chromosome walking strategy was employed to estimate the physical lengths of hemizygous deletions. Genomic sequences of up to 100 kb surrounding focal LOH loci were downloaded from wFleaBase (wfleabase.org). Quantitative PCR primers (supplementary Table S2.2) were designed using PrimerQuest (www.idtdna.com/scitools/applications/primerquest) to amplify short fragments of DNA (~100 bp), which were 2-3 kb upstream or downstream of a focal LOH locus. QuMA analyses were performed to test the DNA copy number of these flanking markers. If these markers were found to be hemizygous, new markers distal to the previous markers were selected for further analysis until diploid markers were detected. The physical lengths of deletions were calculated as the distance (basepairs) between the two hemizygous markers at the ends of the deletion tracts, which is an underestimate of the true length of deletions.

2.3 RESULTS

2.3.1 Genome-wide LOH rate

We screened 141 microsatellite markers in 20 MA lines of *Daphnia pulex* (PX) and 95 microsatellite markers in 28 lines of *Daphnia obtusa* (OB). One hundred and eight microsatellites in *D. pulex* and 34 microsatellites in *D. obtusa* were heterozygous in the founder of the MA lines and were therefore informative for detecting LOH events (Table 2.1). In total, 24 LOH events were detected for 15 microsatellite markers in 20 PX MA lines over an average of 116 generations, whereas 41 LOH events for 15 microsatellite markers occurred in 28 OB MA lines over an average of 190 generations (Table 2.1 and supplementary Table S2.1). The rate of LOH events was 9.58×10^{-5} (PX) and 2.27×10^{-4} (OB) events $\cdot$locus^{-1} $\cdot$generation^{-1}, respectively (Table 2.1). Given that LOH rates from microsatellite loci and protein coding loci (Omilian et al. 2006) are consistent (1.7×10^{-4} locus^{-1} $\cdot$generation^{-1}), we suggest that the microsatellite-based LOH rates are representative of the entire genome.

The number of LOH events for each individual MA line ranged from zero to nine. A maximum of three non-consecutive LOH events on the same chromosome occurred in the PX MA lines (Fig. 2.1). The lack of a genetic linkage map for *D. obtusa* prevented us from determining the genomic location of LOH events in the OB MA lines. Hotspots for LOH events, defined as loci that displayed LOH in three or more individual MA lines, were also identified: d050 (7 lines) and d083 (3 lines) for PX; and d024 (6 lines), d027 (3 lines), and d068 (17 lines) for OB.

2.3.2 Rate of hemizygous deletions in D. pulex

Following the tolerance interval (TI) for QuMA analysis, LOH loci with DNA copy numbers less than 1.20 should be regarded as hemizygous. In total, 16 out of 24 LOH events appeared to be hemizygous. Seven microsatellite loci experienced hemizygosity, and the loci d050 and d083 were found to be hemizygous in multiple MA lines (Fig. 2.1). The rate of hemizygous deletions in the *D. pulex* MA lines is 6.7×10^{-5} locus^{-1} ·generation^{-1} (Table 2.1). Based on the *Daphnia* genome annotation (wfleabase.org), all hemizygous loci span open reading frames (supplementary Table S2.4) and thus the deletions are likely deleterious. We also investigated an unusually long LOH tract spanning a string of 14 microsatellite loci. Of these, two internal loci (d054 and d078) were found to be hemizygous while the rest maintained a copy number of two (Fig. 2.1 and supplementary Table S2.3). This finding indicates that the long LOH tract is likely due to a crossover event followed by internal hemizygous deletions.

2.3.3 Physical lengths of hemizygous deletions in D. pulex

Quantitative PCR (qPCR) experiments on linked markers in the regions located near focal hemizygous loci revealed that the physical lengths of deletion tracts for markers d050, d083, d054, and d078 ranged from ~2 to 30 kb (Fig. 2.2 and Table 2.2). Deletion tracts of dramatically different lengths were detected among individual MA lines at locus d050. For example, ~2 kb deletions were

observed in the PX35 and PX43 lines, while PX2, PX5, and PX32 experienced deletion tracts of 25 - 30 kb. Deletions for locus d083 were similar in length (~23 kb) for both MA individuals PX41 and PX43. Although markers d054 and d078 are adjacent in PX6, their deletion tracts were separated by an internal diploid segment; the physical lengths for d054 and d078 were ~9 kb and ~13 kb, respectively. All deletions span open reading frames (supplementary Fig. S2.1).

2.4 DISCUSSION

In this study, we used asexually reproducing *Daphnia* MA lines maintained for more than 100 generations to investigate the rate of hemizygous deletions and homologous recombination (ameiotic gene conversion and crossover) and their relative contribution to loss of heterozygosity (LOH) in the *Daphnia* nuclear genome. The genome-wide rates of LOH at microsatellite loci in the *D. pulex* mutation-accumulation (MA) lines (9.58×10^{-5} locus^{-1} ·generation^{-1}) and the *D. obtusa* MA lines (2.27×10^{-4} locus^{-1} ·generation^{-1}) are consistent with the previously estimated LOH rate (~10^{-4}) for microsatellites and protein-coding regions in lines from the same MA experiment but at a much earlier stage of MA (75 and 107 generations, respectively) (Omilian et al. 2006). Thus, the LOH rate appears to be temporally consistent in apomictic *Daphnia* germ-line cells. The LOH rate in *D. obtusa* MA lines is approximately twice that in *D. pulex*. Because LOH can lead to the unmasking of deleterious alleles that have little phenotypic effect in a heterozygous state, a higher genetic load in the obligate asexual stem mother for *D. pulex* MA lines could lead to fewer viable offspring with LOH loci compared to the *D. obtusa* lines that were derived from a cyclical parthenogenetic mother. Our direct estimates of deletion and recombination rate via qPCR experiments show that only 33% of the observed LOH events were likely due to crossover and gene conversion events, while the remaining 67% of events were likely due to large-scale deletions.

2.4.1 High rate of hemizygous deletions

We find a deletion rate of 6.7×10^{-5} locus^{-1} ·generation^{-1} in *D. pulex* MA lines (Table 2.1). When deletion hotspots are excluded (i.e. d050), the deletion rate is 3.8×10^{-5} locus^{-1} ·generation^{-1}. The physical scale of genomic regions that experience deletions is roughly 10^3 or 10^4 higher than that for point mutations based on estimates (10^{-9}- 10^{-8}) from previous MA experiments of *Drosophila melanogaster* and *Caenorhabditis elegans* (Denver et al. 2004; Denver et al. 2009; Keightley et al. 2009). Because previous MA studies primarily use sequencing technologies that cannot reveal DNA copy number polymorphisms (but see Lynch et al. 2008), these estimates are based mainly on point mutation or indels of a few basepairs. Although our deletion rate is based on microsatellite markers, it is unlikely that the high deletion rate in *Daphnia* is due to the fragile nature of microsatellites as protein-coding loci sequenced in an earlier study were as equally likely to experience LOH events as microsatellite loci. Therefore, our results strongly suggest that segmental deletions represent an important component in the molecular spectrum of spontaneous mutations and deserve further attention.

The *Daphnia* deletion rate is about one order of magnitude higher than the rate of large-scale deletions per cell division in *S. cerevisiae* (Lynch et al. 2008) and the rate of large-scale deletions per generation in *D. melanogaster* (Watanabe et al. 2009). The extremely high rate of large-scale hemizygous deletions in the *Daphnia* nuclear genome is consistent with the high level of copy number polymorphisms (duplications and deletions) observed in natural populations of flies (Dopman and Hartl 2007) and mice (Graubert et al. 2007). Although detecting duplications is beyond the scope of our work, segmental duplications might also occur at a high rate in the *Daphnia* genome. About 20% of the 27,000 gene predictions obtained from the *Daphnia* genome sequence appear to be tandem duplicates (wfleabase.org). The joint impact of segmental duplications and deletions on the evolution of asexual taxa remains largely unknown, but the extraordinarily high rate of deletion in our dataset suggests that these phenomena deserve further attention.

Our data suggest that some genomic regions are more vulnerable to deletions than others. For example, deletion tracts of three different lengths involving locus d050 in five different MA lines indicate that multiple deletion events affected the same chromosomal region across different MA lines (Fig. 2.2). Given our rate of deletions per locus in *D. pulex* MA lines (6.7×10^{-5}), and assuming a Poisson distribution of deletions, the chance of observing 7 deletions (supplementary Table S2.3) involving locus d050 is extremely small (2×10^{-19}). The non-random distribution of the observed deletions raises questions about the genomic background in which deletions occur. It is known that recombination between non-allelic homologues can produce genomic rearrangements including deletions, duplications, inversions, and translocations (Mieczkowski, Lemoine, and Petes 2006). Furthermore, a number of studies have confirmed the role of repetitive genomic elements such as tRNA genes (Dunham et al. 2002), long terminal repeats (Diogo et al. 2009), transposable elements (Mieczkowski, Lemoine, and Petes 2006; Lynch et al. 2008; Watanabe et al. 2009), major repeat sequences (Lephart and Magee 2006), microsatellites (Bena et al. 2010), and segmental duplications (Sharp et al. 2005) in mediating chromosome rearrangements including deletions. We note here that microsatellites may facilitate deletions, but as we mentioned earlier this is not likely to be the case with our data. Because the *D. pulex* genome is particularly rich in tandem gene duplications (see above), we suggest that they could mediate non-allelic homologous recombination that results in hemizygous deletions. Screening for flanking tandem genes in the 50 kb genomic regions surrounding the characterized deletion tracts in wFleaBase (wfleabase.org) revealed two tandem genes in a 10 kb window that flank the deletion tract observed in PX35 and PX43 (supplementary Fig. S2.2). Further work is necessary to elucidate the mechanism underlying the observed segmental deletion events.

2.4.2 Rate of ameiotic recombination

It is generally accepted that LOH is often the result of recombination such as crossover and/or gene conversion without the overall loss of genetic material (Luo et al. 2000; Blackburn et al. 2004; Andersen et al. 2008). However, only 33% of our observed LOH events are likely due to typical crossover and gene conversion events. Our estimate of the rate of ameiotic recombination in *Daphnia*, 3.3×10^{-5} locus^{-1} generation^{-1}, is similar to the recombination rates in other asexually reproducing eukaryotes. For example, the rate of mitotic recombination is approximately 0.8×10^{-4} per generation in *Saccharomyces cerevisiae* (Mandegar and Otto 2007), 9×10^{-4} per generation (2.82×10^{-6} events kb^{-1} generation^{-1} on a 325-kb interval) in *Candida albicans* (Lephart and Magee 2006), and 10^{-5} to 10^{-2} per generation in *Aspergillus niger* (Debets et al. 1993). However, our rate is much lower than the rate of recombination (2×10^{-2} - 6×10^{-2} events/generation) of the ribosomal DNA in the same *D. obtusa* MA lines, which is at the high end of rDNA recombination rate (10^{-5} - 10^{-2} events/generation) estimated from various species (McTaggart et al. 2007). This significant rate difference could reflect either our underestimated rate of ameiotic recombination as some hemizygous deletions are likely a result of unequal crossing over, or a much higher recombination rate for rDNA than for other genomic regions. It is generally recognized that rDNA tends to experience high levels of unequal crossing over and gene conversion due to its repetitive nature (McTaggart et al. 2007).

2.4.3 Implications for the evolution of sex

The high rates of ameiotic recombination and segmental deletions in the *Daphnia* nuclear genome have strong implications for the evolution and maintenance of sex. Using a diploid, single-locus model, Mandegar and Otto (2007) showed that adaptation in asexual populations can be as rapid as that in sexual populations when the rate of mitotic/ameiotic recombination (10^{-4} or 10^{-5}) is much higher than the rate of mutations (e.g. 10^{-7}). Therefore, asexual taxa can gain most of the benefit of segregation via mitotic/ameiotic recombination while avoiding the costs associated with sex

(Mandegar and Otto 2007). This hypothesis provides a plausible explanation for the existence of putative ancient asexual taxa such as the Darwinulid ostracods and the aphid tribe Tramini and could also explain why the Meselson effect (i.e. increased allelic divergence with age due to the lack of recombination) is absent in these lineages (Butlin, Schön, and Martens 1998; Normark 1999). However, since the *Daphnia* deletion rate is approximately twice as high as the ameiotic recombination rate, we suggest that the long-term survival of asexual *Daphnia* lineages is likely to be negatively affected by the high frequency of deletions. Moreover, the possibility that mitotic/ameiotic recombination could lead to large-scale deletions should also be considered when examining the role of recombination in the evolution of asexual taxa.

It is often argued that most mutations detected in MA lines are deleterious since the mean fitness of the lines declines over time (Bataillon 2000). Given the *Daphnia* genome size of ~200 Mb, the high segmental deletion rate (5.5×10^{-5} bp^{-1} ·generation^{-1}) indicates that on average one or two DNA segments spanning a total of 11 kb are deleted per genome per generation. Moreover, considering an average gene size of two kb, and an intergenic size of four kb (wfleabase.org), we suggest that about one or two genes per generation could experience large-scale hemizygous deletions. Deleterious mutations are of central importance for several hypotheses on the evolution and maintenance of sex (Kondrashov 1988). For example, the deterministic mutation hypothesis predicts that, with a deleterious mutation rate greater than one per genome per generation, sex can be maintained by its capacity to purge deleterious mutations (Kondrashov 1988). However, comparative analyses of DNA base substitutions failed to detect a genome deleterious mutation rate higher than one per generation in a range of animal species (Keightley and Eyre-Walker 2000; Cutter and Payseur 2003). The high rate of deletions in our *Daphnia* MA lines strongly suggests that segmental deletions should be taken into account as an important type of mutation when evaluating the role of deleterious mutations in the evolution of sex. It is likely that selection against deleterious mutations

can provide an advantage that offsets the inherent costs of sexual reproduction. Furthermore, our study clearly shows the dynamic nature of asexual genomes that experience unexpected high rates of mutation and recombination and the mutagenic consequences of ameiotic recombination.

2.5 REFERENCES

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Table 2.1. Summary of loss of heterozygosity information in *Daphnia pulex* (PX) and *Daphnia obtusa* (OB) MA lines. The length of loci represents the sum of fragment size for the homozygous/hemizygous LOH loci.

Species	***Daphnia pulex***	***Daphnia obtusa***
No. of mutation lines	20	28
Average number of generations	116	190
No. of informative (heterozygous) loci	108	34
Total length of informative loci	28159 bp	8617 bp
No. of LOH events observed	24	41
LOH rate ($locus^{-1} \cdot generation^{-1}$)	9.58×10^{-5}	2.27×10^{-4}
No. of homozygous LOH loci	8	N/A
Total length of homozygous LOH loci	2519 bp	N/A
No. of hemizygous LOH loci	16	N/A
Total length of hemizygous LOH loci	3565 bp	N/A
Segmental deletion rate ($locus^{-1} \cdot generation^{-1}$)	6.7×10^{-5}	N/A
Segmental deletion rate ($bp^{-1} \cdot generation^{-1}$)	5.5×10^{-5}	N/A
Ameiotic recombination rate ($locus^{-1} \cdot generation^{-1}$)	3.3×10^{-5}	N/A
Ameiotic recombination rate ($bp^{-1} \cdot generation^{-1}$)	3.9×10^{-5}	N/A

Table 2.2. Summary of the hemizygous deletion tracts in the *Daphnia pulex* mutation-accumulation (MA) lines, with genomic coordinates (wfleabase.org) and lengths (basepairs) of the affected genomic regions.

Locus	MA line	Genomic location	Length (bp)
d054	PX6	scaffold_62:48915-61803	12889
d078	PX6	scaffold_62:121813-130992	9180
d050	PX2	scaffold_63:448169-472883	24715
d050	PX5, PX32	scaffold_63:448169-477951	29782
d050	PX35, PX43	scaffold_63:448169-450291	2123
d083	PX41, PX43	scaffold_17:891889-914491	22603

Supplementary Table S2.1 Summary of the detected loss of heterozygosity events in the mutation-accumulation lines of *Daphnia pulex* (PX) and *Daphnia obtusa* (OB). The numbers in parentheses indicate the number of generations for a given MA line.

Locus	Chromosome	Mutation-accumulation lines	Genotypic change
Daphnia pulex			
d015	II	PX2 (26)	413/432→432
d016	II	PX2 (26)	178/180→180
d041	III	PX45 (134)	231/235→231
d047	II	PX35 (124)	223/225→223
d050	II	PX2 (26), PX5 (55), PX17 (106), PX20 (139), PX32 (151), PX35 (124), PX43 (157)	191/192→191
d057	IV	PX43 (157)	462/464→462
d082	III	PX49 (137)	302/318→302
d083	X	PX20 (139), PX41 (115), PX43 (157)	267/271→271(PX20 and 41) 267/271→267(PX43)
d094	III	PX45 (134)	273/282→282
d118	IX	PX41 (115), PX45 (134)	118/120→120
d145	IX	PX20 (139)	264/267→264
d147	III	PX45 (134)	285/289→289
d148	I	PX31 (150)	222/224→222
d171	IX	PX31 (150)	216/224→216
d198	VI	PX20 (139)	623/631→623

Daphnia obtusa			
d013	N/A	OB22(130), OB 28 (198)	306/310→310
d021	N/A	OB22 (130)	218/222→ 222
d024	N/A	OB2 (198), OB12(198), OB21 (187), OB30 (203), OB38 (186), OB50 (201)	254/260→254 (OB 2 and 21) 254/260→260 (OB12, 30, 38, and 50)
d027	N/A	OB25 (198), OB26 (156), OB32 (156)	144/150→150
d030	N/A	OB 28 (198), OB41(188)	183/184→184,183/184→183
d068	N/A	OB2 (198), OB4 (204), OB8 (197), OB12 (198), OB13 (204), OB14 (200), OB16 (198), OB17 (203), OB20 (202), OB25 (198), OB26 (156), OB28 (198), OB32 (156), OB41 (188), OB47 (198), OB48 (204), OB50 (201)	333/356→356
d071	N/A	OB7 (195)	134/146→134
d078	N/A	OB22 (130)	198/201→201
d148	N/A	OB26 (156)	222/224→222
d155	N/A	OB22 (130)	162/168→162
d171	N/A	OB7 (195), OB22 (130)	216/226→216
d180	N/A	OB22 (130)	178/179→179
d182	N/A	OB22 (130)	276/282→276
d184	N/A	OB22 (130)	215/219→219

Supplementary Table S2.2 Quantitative PCR primers for markers selected to determine the lengths of the hemizygous deletion tracts in *Daphnia pulex* mutation-accumulation lines.

Locus	Forward primer	Reverse primer
A	TAAATAATGCGCGCGGCACAACAC	ATAGGCACCAGCCAACGAGAGAAA
B	TTAACTGAGTGTCAACCGAGCCGA	ATTCGAATTCTCGCGAGTGGACCAAC
C	CTGGCGTGTCCTTGCCTCATTTAT	AAAGGAGCGCCTTACGATTAGGGT
D	CACCACCTTCATCGCACTCTCAAA	AATGAACAACGGAGCTGCCAAACC
E	TATTCGTGAGCCGAGCCAAGAGAA	TCGAATAATATGATGGCGGCGATG
F	TATAACCAACCGCCCACCCTCTTT	ATTAGACGCAGGACGGCCAATAGT
G	AAATGCGGCCGTACAAAGTCAACC	TCTTGGACAACACACACGCACAAC
H	ACACACATGCATAATACACGCGCC	CCCGCCTTTCCATTAGCGTTTGTT
I	AAATTGGACGAGTGTCGTCTGCTG	CCGCATGGATTATGACGGTTATCACA
J	TCAACGTCGAGATGCACAAACAGG	TCTGACACGCGACGGTGTTACAAT
K	TTCAGTCCGTCTCTTGTCTGTG	TCTTTCTTCCTTTCAGTCTCGTTTCT
L	TCCCATCGTGGAAAGGTGTTCTCT	GCCCATAAACGGAAGGGAGGGTATAA
M	GCTGTTCTTTCATCTCGGCAACATGC	TCTCGTCCTGTTTGCTCGATACCT
N	TCTTAATGGCCCTTCAGCCGTCTT	TTTGTCTTGCGCTCACTTTGGC
O	AATTCCGGCTGAGTGCCTAGTTTG	CGCTCTCTCCAACTCATTATCGACCA
P	TGCGGATGCTGTGTCCAGTTCTAA	TGATAGATTGCACGGGCTGGATGT
Q	CCAGGACCATTCAAATGCTAACAAG	TCCTCCTTTCCGCCATCCATC
R	AGAGTAGACAGAAAGGGTCTCAAAT	ACGACTCCTATTGCCACTTCATC
S	AATAATCCCGCGTGGTATGTGGAG	GGAAGGACGCCATTGATTCTGGAT
T	CACCAGTTGAGCTACTTTCCAGGT	AGTCGCCGGGTACATTTCTACA
U	CGACAACAATACGCCAGAGGTCTA	CTCCTCCATCAGCACGATGTCAAT

Supplementary Table S2.3 DNA copy numbers for microsatellite markers that showed loss of heterozygosity (LOH) in *Daphnia pulex* (PX) mutation-accumulations lines. According to the tolerance interval for the quantitative PCR experiment, copy numbers less than 1.20 indicate deletions, whereas copy numbers greater than 3.33 represent duplications.

Locus	Linkage Group	MA Line	DNA copy number
d015	II	PX2	2.45
d016	II	PX2	2.45
d041	III	PX45	2.48
d047	II	PX35	1.50
d050	II	PX2	0.83
d050	II	PX5	0.94
d050	II	PX17	0.92
d050	II	PX20	0.75
d050	II	PX32	0.65
d050	II	PX35	0.64
d050	II	PX43	0.71
d057	IV	PX43	0.68
d082	III	PX49	1.58
d083	X	PX20	0.65
d083	X	PX41	0.59
d083	X	PX43	0.41
d094	III	PX45	0.91

d118	IX	PX41	0.99
d118	IX	PX45	0.67
d145	IX	PX20	2.18
d147	III	PX45	2.53
d148	I	PX31	0.53
d171	IX	PX31	0.87
d198	VI	PX20	2.18
d003	III	PX6	1.57
d041	III	PX6	1.54
d054	III	PX6	0.91
d059	III	PX6	1.71
d062	III	PX6	1.84
d066	III	PX6	1.81
d075	III	PX6	2.63
d078	III	PX6	1.18
d082	III	PX6	1.68
d094	III	PX6	1.54
d108	III	PX6	2.20
d122	III	PX6	2.17
d136	III	PX6	2.20
d147	III	PX6	2.03

Supplementary Table S2.4 Summary of the hemizygous microsatellite loci in the *Daphnia pulex* mutation-accumulation (MA) lines, with genomic coordinates and the corresponding gene prediction model (wfleabase.org).

Locus	Genomic location	Gene prediction	Position
d050	scaffold_63: 450076 – 450291	JGI_V11_299755	Exon
d054	scaffold_62: 51713 – 51836	DP_DPGIL_00020047	Exon
d057	scaffold_8: 2259957 – 2260245	DP_DPGIL_SNO_00005118	Exon
d078	scaffold_62: 124649 – 124936	DP_DPGIL_00020065	Intron
d083	scaffold_17: 914491 – 914609	JGI_V11_240913	Exon
d094	scaffold_2: 3137984 - 3138447	JGI_V11_233379	Exon
d118	scaffold_9: 1369547 - 1369704	DP_DPGIL_SNO_00005425	Exon
d148	scaffold_61: 198594 - 198861	JGI_V11_251861	Intron
d171	scaffold_9: 848517 - 848851	JGI_V11_313559	Intron

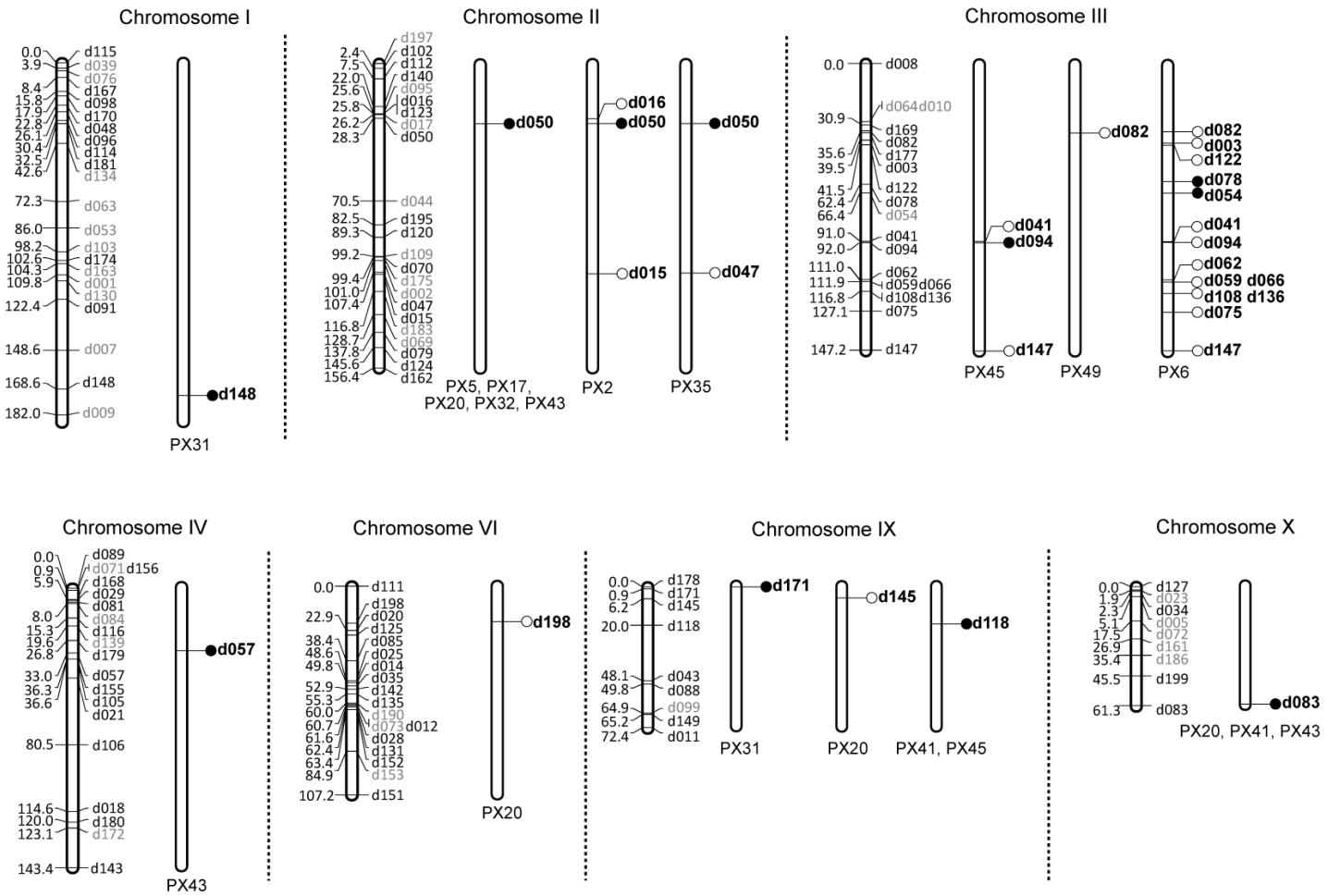


Fig. 2.1 Map locations for homozygous loss of heterozygosity (LOH) loci (open circles) and hemizygous LOH loci (black circles) in the *Daphnia pulex* mutation-accumulation (MA) lines. Only 7 of 12 screened chromosomes contain markers that underwent LOH. The ancestral diploid state for each chromosome is shown on the left of the map, with heterozygous loci in black type and homozygous loci in gray type. Numbers on the left indicate map distances (centimorgans), and numbers to the right indicate marker names. LOH profiles are shown to the right of the linkage map for each chromosome, with the mutation-accumulation (MA) line showing that profile indicated below.

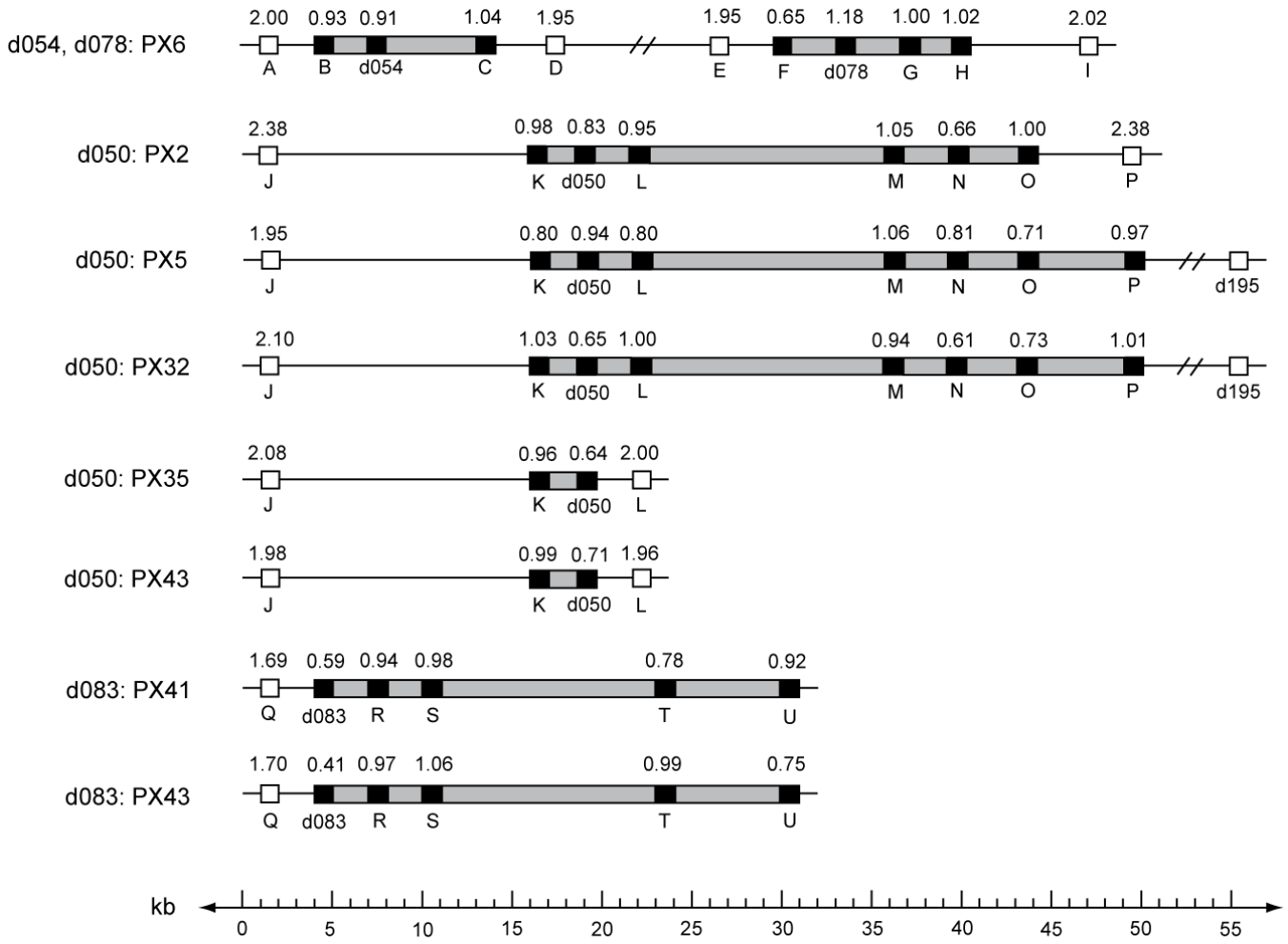
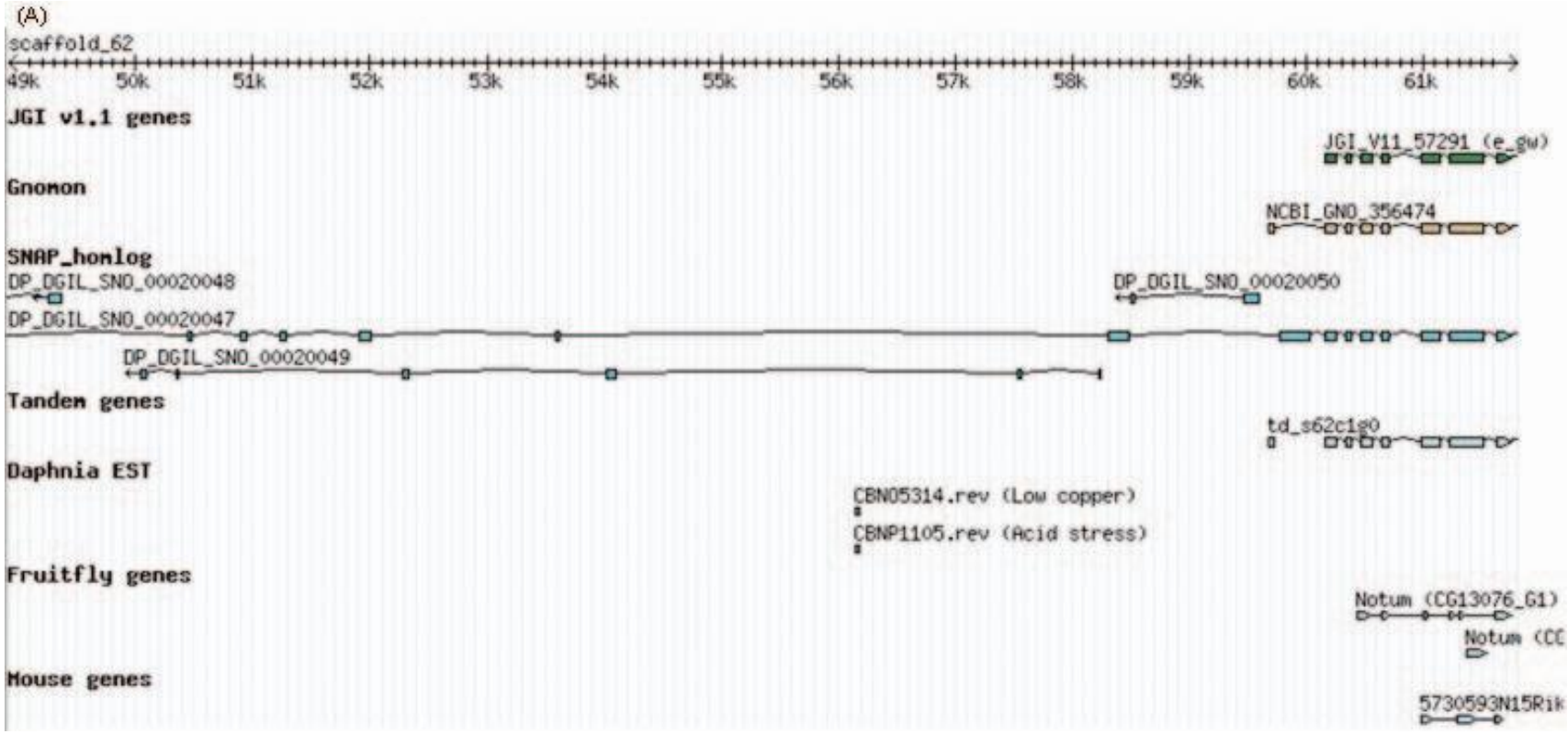
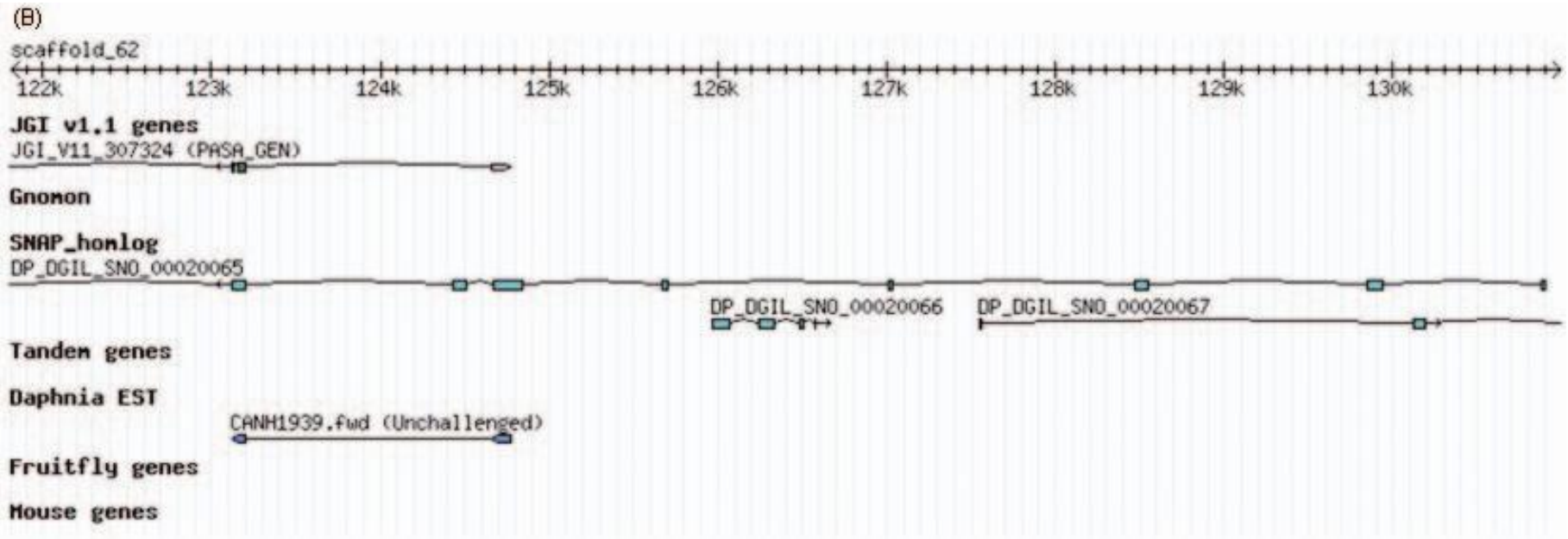


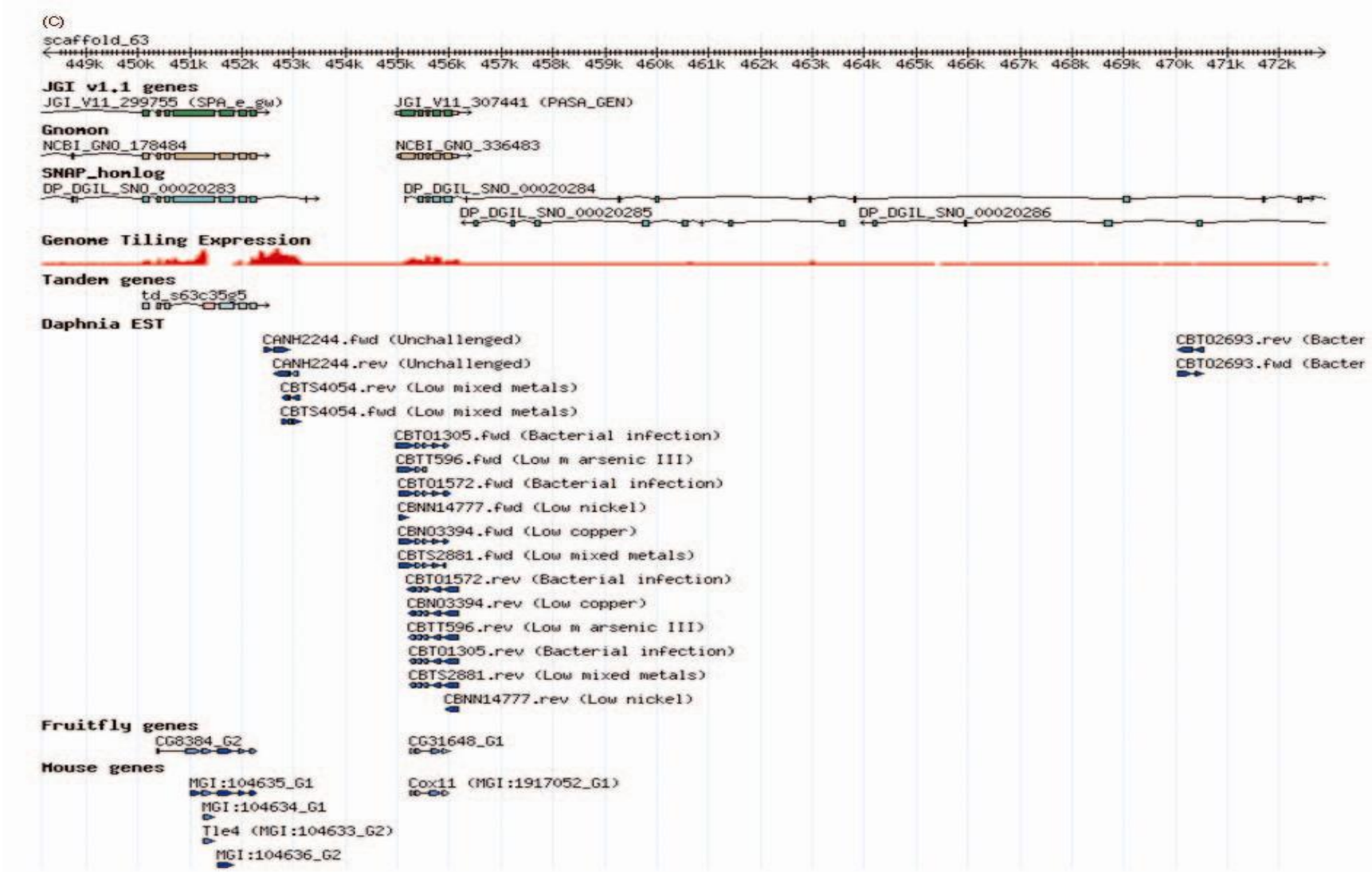
Fig. 2.2 Physical map of the hemizygous deletion tracts encompassing the loci d054, d078, d050, and d083 that experienced LOH in multiple mutation-accumulation lines. Letters denote the markers used in quantitative PCR experiments (Table S2.2). Black boxes represent hemizygous markers, whereas open boxes denote diploid markers. Grey areas indicate the inferred deletion tracts. Numbers above boxes indicate the DNA copy number for each marker tested. The marker d195 is a heterozygous marker (i.e. DNA copy number is 2) according to the genotyping results, although no quantitative PCR experiments have been performed using this locus.



Supplementary Fig. S2.1 Genomic annotation tracks for the hemizygous regions in the *Daphnia pulex* mutation-accumulation lines. Images are retrieved from wFleaBase (wfleabase.org). (A) The deletion tract involving d050 in PX6. (B) The deletion tract involving d078 in PX6. (C) The deletion tract involving d050 in PX2. (D) The deletion tract involving d050 in PX5 and PX32. (E) The deletion tract involving d050 in PX35 and PX43. (F) The deletion tract involving d083 in PX41 and PX43.

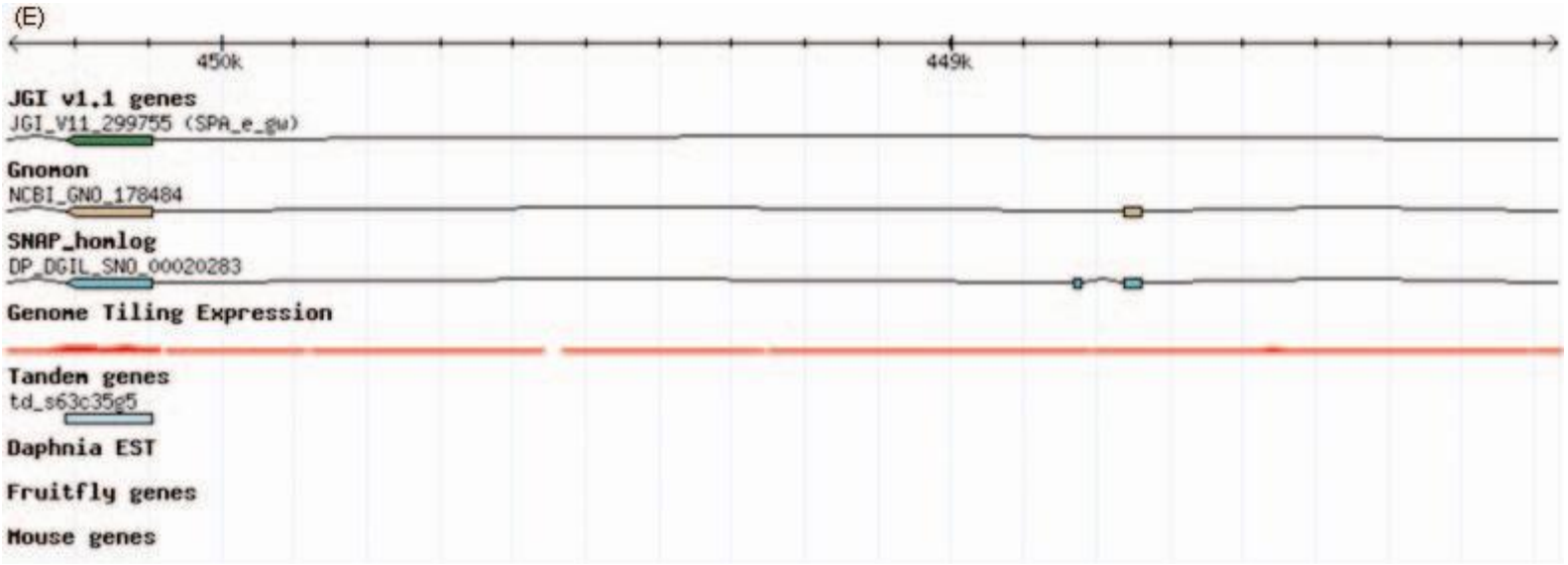


Supplementary Fig. S2.1 Continued.

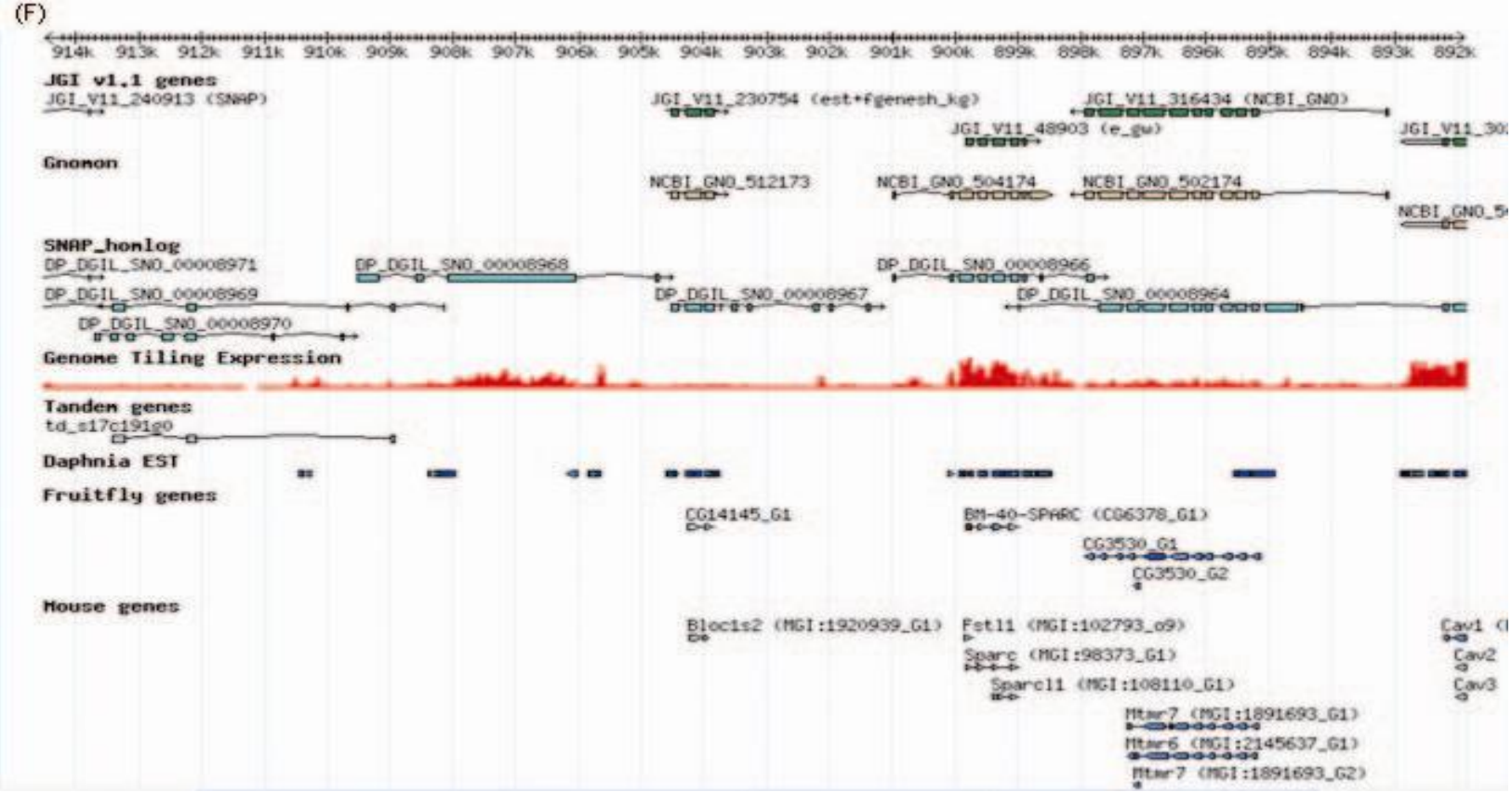


Supplementary Fig. S2.1 Continued.

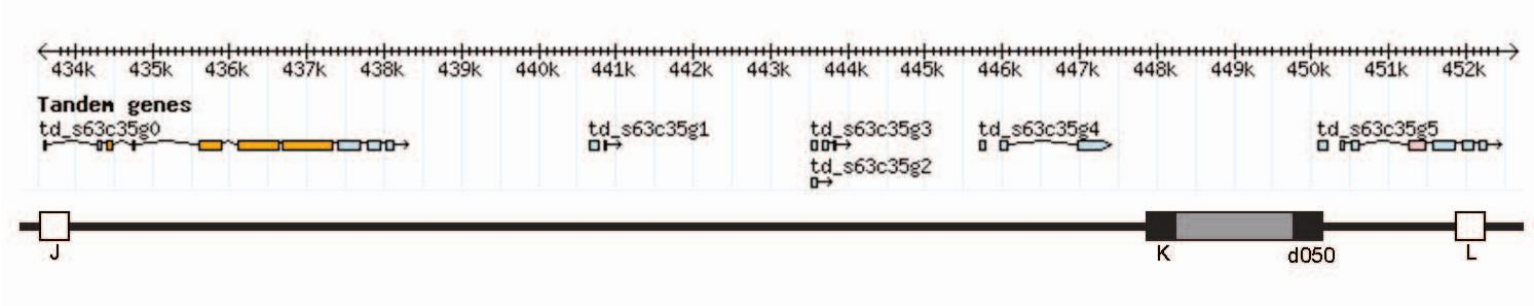
Supplementary Fig. S2.1 Continued.



Supplementary Fig. S2.1 Continued.



Supplementary Fig. S2.1 Continued.



Supplementary Fig. S2.2. The hemizygous deletion tracts in PX35 and PX43 are flanked by two tandem genes. The markers K and d050 are hemizygous, whereas J and L have two DNA copies**.**

CHAPTER 3. HIGH MUTATION RATES IN THE MITOCHONDRIAL GENOMES OF *DAPHNIA PULEX*

3.1 INTRODUCTION

Our understanding of the process of mutation in the mitochondrial genome remains limited (Denver et al. 2000; Paland 2004; Haag-Liautard et al. 2008; Howe et al. 2010), despite its wide application in evolutionary genetics. Studies of population differentiation (Avise 2000), population/species divergence time (Knowlton and Weigt 1998), species identification (Hebert et al. 2003), forensic medicine (Ivanov et al. 1996), and human disease (Taylor and Turnbull 2005) often requires a rigorous understanding of the mitochondrial DNA (mtDNA) mutation rate and spectrum. The lack of data concerning mtDNA mutation process and the less well-characterized population genetic environment of mitochondria compared to nuclear genomes leaves the evolution of mitochondrial genomes poorly understood.

In most metazoans, mtDNA shows an elevated mutation rate compared to nuclear DNA, likely due to less efficient DNA repair, a more mutagenic local environment (putatively caused by oxidative radicals), and an increased number of replications per cell division (Birky 2001; reviewed in Lynch 2007). This high mutation rate may result in accelerated mutation accumulation via Muller's ratchet (Muller 1964; Felsenstein 1974), i.e., the inability to re-constitute genomes with fewer mutations due to the absence of recombination in mtDNA (but see Piganeau et al. 2004), which may eventually affect the long term survival of populations and/or species (Loewe 2006). Mitochondrial genomes are thought to undergo severe within-individual bottlenecks during transmission (Rand 2001), further reducing the efficacy of selection against mildly deleterious mutations as they accumulate (e.g., Hasegawa et al. 1998). Thus, to understand the evolution of mitochondrial genomes in the face of high rates of deleterious mutation requires detailed data on the mtDNA mutation process and characterization

of the population-genetic environment of the mitochondrial genomes in various phylogenetic lineages.

Two approaches are generally used for estimating germ-line mtDNA mutation rates. One category of studies employs phylogenetic methods that measure substitutions at silent sites between a pair of species with known or estimated divergence dates based on, for example, geological evidence (reviewed in Lynch 2007). This approach likely provides downwardly biased estimates because it assumes the neutrality of silent sites (which may be influenced by codon bias) and does not always take into account the presence of mutation hotspots that result in multiple substitutions. These two factors can result in large disparities between rates of phylogenetic divergence and actual mutation rates (reviewed in Lynch 2007). To minimize these biases, another category of studies directly estimates germ-line mutation rates of mtDNA using long-term mutation accumulation (MA) lines. These MA lines are generally propagated in benign environments and bottlenecked at each generation by randomly picking one/a pair of the offspring from either clonal/hermaphroditic broods or from full-sib matings. This results in an extremely low effective population size for each MA line, thus reducing the efficiency of natural selection to a minimum and allowing the majority of mutations, except those having extreme effects, to accumulate over time in a neutral manner (Keightley and Caballero 1997).

To date, the direct estimates of mtDNA mutation rates have been limited to a few model species: *Caenorhabditis elegans* (Denver et al. 2000), *C. briggsae* (Howe et al. 2010), *Drosophila melanogaster* (Haag-Liautard et al. 2008), and *Saccharomyces cerevisiae* (Lynch et al. 2008). From these studies, the mtDNA mutation rate (including both base-substitutions and indels) appears to be on the order of 10^{-8} - 10^{-7} per site per generation, which is hundreds of times higher than previous phylogenetic based estimates (Denver et al. 2000). Upon closer examination,

however, the spectrum and rate of mtDNA mutation vary widely across species. For example, the mtDNA mutation rate in *C. elegans* (1.6×10^{-7} per site per generation) is approximately two times higher than in *D. melanogaster* (7.8×10^{-8} per site per generation). In *D. melanogaster*, concordant with the nearly universal A/T bias in mitochondrial genomes, 82% of the base substitutions appear to be G→A (Haag-Liautard et al. 2008). In contrast, only 15% of base substitutions in *C. elegans* increase the A + T content of the mitochondrial genome (Denver et al. 2000), and all the base-substitution mutations in *S. cerevisiae* appear to be A/T→G/C (Lynch et al. 2008), despite the strong A/T bias in their mitochondrial genomes. Because germ-line cells usually harbor multiple copies of mtDNA (e.g., ~2,000 in mice primordial germ cells, Cao et al. 2007), a neutral mutation originating in a single mtDNA copy must go through a drift process to reach fixation. Consistent with this scenario, extensive heteroplasmic base-substitution mutations are found in *D. melanogaster*. However, little heteroplasmy is observed in *C. elegans* (Denver et al. 2000), suggesting a lower mitochondrial effective population size in *C. elegans* compared to *D. melanogaster* (Haag-Liautard et al. 2008). Furthermore, the *C. briggsae* mitochondrial genome is characterized by a high rate (~0.001 - 0.0013 per site per generation) of heteroplasmic large deletions that encompass hundreds of basepairs, indicating the unique attributes of the DNA replication and repair machinery in this species (Howe et al. 2010).

Although the differences in mtDNA mutation patterns between distantly related taxa are starting to emerge, how closely related species and intraspecific populations/strains vary in their mitochondrial mutation patterns remains poorly understood (Howe et al. 2010). The evolution of mutation rate is thought to be determined by the costs of exact replication, the cost of deleterious mutations, and advantages of beneficial mutations (but see Lynch 2010). Given that in asexual species recombination is usually negligible, the cost of replication fidelity and the effects of

deleterious mutation are expected to yield a low mutation rate (Dawson 1998). However, if beneficial mutations are common, mutation rates in asexual species may be higher relative to sexual species, even rising to an intolerable level that can lead to extinction (Johnson 1999; Andre and Godelle 2006; Gerrish *et al.* 2007). To date, few empirical studies investigate how sexual and asexual species differ in both nuclear and mitochondrial mutation rates (but see Baer et al. 2010). *Daphnia pulex,* a freshwater microcrustacean that typically reproduces by cyclical parthenogenesis (i.e., clonal reproduction with annual bouts of sex), but in which numerous obligate asexual lineages have arisen (Hebert et al. 1989), provides a great system to examine mtDNA mutation rates in sexual and asexual lineages. For mitochondrial genomes that experience "asexual" inheritance in both asexual and sexual species, the mitochondrial-nuclear linkage in asexual species can result in reduced efficiency of selection (Normark and Moran 2000). Asexual lineages of *Daphnia* (Paland and Lynch 2006), snails (Neiman et al. 2010), and rotifers (Barraclough et al. 2007) have been shown to accumulate deleterious mutations at a much faster rate compared to sexual lineages. Furthermore, the mitochondrial-nuclear linkage is important in determining the mtDNA mutation rate because all the proteins and enzymes involved in mtDNA replication and repair are encoded by nuclear genes.

In this study, we describe the mtDNA mutation process in *D. pulex* MA lines, and compare rates among those derived from one cyclically parthenogenetic (hereafter, sexual) and two obligately parthenogenetic (hereafter, asexual) individuals. By direct sequencing of more than 1.6 million mtDNA nucleotides in 84 sexual and 47 asexual *Daphnia pulex* MA lines, our results show that the per generation mtDNA mutation rates in this species (1.37×10^{-7} per nucleotide per generation for the sexual clone, and 1.40×10^{-7} and 2.28×10^{-7} for the two asexual clones, respectively) are among the highest rates reported. Although the mean mtDNA mutation rates are

different, no statistically significant difference was detected between the mutation rate for sexual and asexual ancestors of the MA lines and their mutation spectrum appears to be very similar. Furthermore, the effective population size of mitochondrial genomes in *D. pulex* is estimated to range between 5 and 10 copies per generation.

3.2 MATERIALS AND METHODS

3.2.1 Mutation accumulation (MA) lines of Daphnia

The mtDNA genome was sequenced for three sets of MA lines of *Daphnia pulex*. The first set of MA lines was derived from a sexual individual from the Slimy Log population (abbreviated S, n = 82 lines), collected in Oregon. The other two sets of MA lines originated from two obligate asexual individuals collected from temporary ponds in Linwood, Ontario, Canada (abbreviated A1, n = 26 lines) and Barry County, Michigan (abbreviated A2, n = 21 lines), respectively. The protocols for maintaining *Daphnia* MA lines are described in Lynch (1985). Briefly, all lines were maintained in a benign laboratory environment and asexually propagated. The generation time for all of these MA lines is 10-13 days on average. Every generation, each line was bottlenecked by randomly picking a single individual to continue the line. Simultaneously, two additional females of each brood were maintained as backups in case the focal individual died or produced no offspring. The occasional use of backups causes differences in the number of generations for the lines derived from the same individual. The average number of generations for S and A lines was 61 and 100 (116 for A1 and 81 for A2), respectively.

3.2.2 PCR and sequencing protocols

A total of 21 - 24 partially overlapping fragments of mtDNA were amplified (average length ~750 bp). DNA from ~10 individuals from each MA line was extracted using a cetyltrimethylammonium bromide method (Doyle and Doyle 1987). PCR reactions used 18 μl

Eppendorf PCR master mix, 0.5 μM primers, and 10 to 100 ng DNA template. The following thermocycling regime was used for all PCR reactions: 4 min at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 51^{o}C for 30 sec, and extension at 72^{o}C for 1-2 minutes. Sequencing was performed with ABI BigDye ® Terminator v3.1 on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). All putative substitutions and indel mutations were confirmed by sequencing with both forward and reverse primers. Assuming a high sequencing error rate of 10^{-4} (Tindall and Kunkel 1988) and the same error in both sequencing reactions, an error rate of ~0.33×10^{-8} is expected for mutations confirmed on both strands at a given site. With this conservative estimate, only ~0.005 mutations would be expected to be due to sequencing error in the total pool of ~1.65 million basepairs that we examined. Our assumption of a negligible sequencing error rate was supported by the fact that our sequences showed high Phred scores ranging between 38 and 60 (corresponding to a sequencing error rate between ~10^{-4} - 10^{-6}).

3.2.3 Detection and quantification of heteroplasmy

Sequences for each amplified fragment were aligned against the complete *D. pulex* mitochondrial genome sequence (Genbank accession no. AF117817, Crease 1999) using CodonCode Aligner 2.0.6 (CodonCode Aligner Corporation, MA). Homoplasmic mutations were scored by eye, whereas the mutation detection function in CodonCode Aligner was used for identifying heteroplasmic mutations. This software implements the algorithms of Polyphred (Nickerson et al. 1997) that uses drops in ancestral peak intensity and rise of secondary peak as evidence for heteroplasmic mutations. The option of low sensitivity was selected to minimize the false positive rate. Each candidate mutant was confirmed by manually checking the trace files sequenced from both directions.

Because many mutations were heteroplasmic, we used the method developed by Haag-Liautard et al. (2008) to estimate the mutation frequencies based on a peak height comparison of DNA trace files. This method takes into account the effect of the preceding base on peak heights of the wild type and mutant alleles. Assuming P is the base preceding the heteroplasmic site, X is the wild type nucleotide, and Y is the mutated nucleotide, the nearest nucleotide combinations of PX and PY (reference sites) were searched within 100 bp flanking the heteroplasmic site. The frequency of mutants at the site in question (D_{mut}) was defined as (H_{mut} / H'_{mut}) / (H_{mut} / H'_{mut} + H_{wild} / H'_{wild}), where H_{mut} and H_{wild} represent the peak height of the mutated and wild type nucleotide, respectively, and H'_{mut} and H'_{wild} denote the peak height of X and Y at the reference sites, respectively. This method has been shown to yield estimates of heteroplasmy that are very consistent with a pyrosequencing approach (Haag-Liautard et al. 2008).

3.2.4 Estimation of mutation rate and mitochondria effective population size

Assuming a neutral evolution model for mtDNA, the fates of newly arisen mutants are solely determined by genetic drift and the fixation rate at drift-mutation equilibrium is proportional to the mutation rate (Haag-Liautard et al. 2008). The probability of ultimate fixation of a neutral mutation (i) is its current frequency within a MA line. Thus, the mutation rate (per nucleotide site per generation) was calculated using the equation $\mu = \sum_i D_i/(LnT)$, where D_i is the frequency of a mutant allele, L is the total number of MA lines, n is the number of nucleotides surveyed, and T is the average number of generations. Nonparametric bootstrap technique (10, 000 replicates) was used to calculate a 95% confidence interval (CI) for the average mutation rate as implemented in the R language package boot (www.r-project.org). To test whether the mtDNA mutation rate differs among the three sets of MA lines, we employed nonparametric bootstrap (10, 000 replicates) to examine if the difference between the mean mutation rates of any two sets

of MA lines is significantly different from zero, the difference being considered significant if the 95% CI for the difference between means did not contain zero.

Furthermore, we used a maximum-likelihood (ML) modeling method (Haag-Liautard et al. 2008) to infer the mtDNA mutation rate as well as the effective population size of mitochondrial genomes transmitted to each generation. Briefly, under the assumption of neutrality, this method estimates the mutation rate from the proportion of unmutated sites, and the effective number of mitochondrial genomes (N_e) is modeled using a haploid Wright-Fisher transition matrix method. For both sexual and asexual (A1 and A2 combined) MA lines, analyses were performed with base-substitutions, indels, and the combined dataset of substitutions and indels, respectively.

3.3 RESULTS

We directly sequenced a total of 1,653,703 bp of mtDNA from 129 MA lines, covering ~87% (13410 bp) of the 15,333-bp *Daphnia* mitochondrial genome for sexual and ~77% (11789 bp) for asexual MA lines. We detected 12 mutations in the sexual lines and 11 mutations in the pooled dataset of asexual (A1 and A2) lines (Table 3.1 and supplementary Figure S3.1), yielding an overall mutation rate of 1.37×10^{-7}, (95% confidence interval (CI) 0.64 - 2.18 $\times 10^{-7}$) and 1.73×10^{-7} (0.83 - 2.71 $\times 10^{-7}$) per nucleotide per generation for sexual and asexual *D. pulex*, respectively (Table 3.2). Mitochondrial DNA mutation rate for the A1 lines was 1.40×10^{-7}(0.41 - 2.53 $\times 10^{-7}$), whereas that for the A2 lines was 2.28×10^{-7} (0.58 - 4.07 $\times 10^{-7}$). The mutation rate becomes a bit lower (1.30×10^{-7}) for the sexual lines if we consider the same regions that were sequenced in asexual lines. There was no significant difference in mutation rate between the sexual lines and pooled asexual (A1 and A2) lines. Pairwise comparisons among the three sets of MA lines also revealed no statistical difference in their mtDNA mutation rates. The maximum-likelihood (ML) estimate of the mtDNA mutation is largely consistent with our direct estimation,

suggesting a mutation rate of 1.51×10^{-7} for sexual and 1.75×10^{-7} for asexual lines (Table 3.2). Nine of the 23 *de novo* mutations detected (39%) appeared to be heteroplasmic (Table 3.1), with the frequency of mutants ranging between 0.22 and 0.78. Moreover, ML estimates of the effective mitochondrial population size using substitutions, indels, and the combined dataset for both species consistently suggested that 5 to 10 copies of mitochondria genomes were transmitted per female *Daphnia* per generation (Table 3.2).

3.3.1 Base substitutions

The sexual and asexual lines respectively showed three base substitutions (Table 3.1) that appeared to be heteroplasmic with mutant frequency ranging between 0.42 and 0.78. For sexual lines, the single-base substitutions consisted of one transition (A→C) and two transversion (G→T and G→A) events and resulted in amino acid changes (Table 3.1). For asexual lines, two transitions (G→A and A→G) and one transversion (T→A) were observed; however, none of these base substitutions produced amino acid changes (Table 3.1). The base substitution rate for sexual lines was 2.0×10^{-8}, whereas that for asexual lines was 4.3×10^{-8}.

3.3.2 Homopolymer mutations

Both the sexual and the asexual lines showed a large proportion of indels (75% and 73%, respectively) among the detected mutations. Except for one deletion event, all indels occurred in homopolymeric regions that resided in both protein-coding and non-coding regions, which is consistent with the expectation that new mutations occurring in these MA lines were not eliminated by selection. A similar proportion of indels in asexual (2 out of 8 indels) and sexual lines (1 out of 9 indels) affected protein-coding regions. All the indels in the coding region appeared to be segregating at relatively low frequency, ranging between 0.22 and 0.44, whereas all the indels in non-coding regions appeared to be fixed. Mutation hotspots were identified in

both lineages in the D-loop region. At site 14766, four of the sexual MA lines (S-11, 51, 68, and 67) showed a deletion in a homopolymeric run of nine Cs, whereas two asexual MA lines (A2-4 and A2-17) had a 1-bp insertion and a 2-bp insertion of C, respectively. Ancestral sequences were checked to ensure that these mutations were not due to sorting of ancestral heteroplasmy. At site 14840, in the homopolymeric run of nine/ten Ts, the MA lines S-32 and A2-12 appeared to have a 1-bp insertion, whereas lines, A1-9, A1-16, A1-18, and A2-18 showed a 1-bp deletion.

3.4 DISCUSSION

The individual effects of mutation, natural selection, tight linkage, and uni-parental inheritance on the evolution of mitochondrial genomes are usually difficult to tease apart. Nonetheless, mutation accumulation (MA) experiments alleviate the effects of natural selection, allowing us to examine the *Daphnia* mtDNA mutation process largely unbiased by natural selection. Furthermore, the comparison between MA lines initiated from sexual and asexual ancestors offers insight into how ancestral reproductive strategy may influence the mutation propensity of *D. pulex* clones during parthenogenesis.

3.4.1 Mutation rate and spectrum in Daphnia *mtDNA*

Our results show that the overall mtDNA mutation rate (including both base substitutions and indels) in sexual (1.37×10^{-7} per nucleotide per generation) and asexual MA lines (1.73×10^{-7}) are similar to that for *C. elegans* (1.6×10^{-7}) but significantly higher than *D. melanogaster* (7.8×10^{-8}). To better understand the mutagenic cellular environment in different species, it is useful to compare the mtDNA mutation rate on a per germ-cell division basis. Previous studies (Lynch et al. 2008) have revealed that the per cell division rates of *S. cerevisiae* (1.29×10^{-8}) and *C. elegans* (1.16×10^{-8}) are significantly higher than that of *D. melanogaster* (0.17×10^{-8}) and humans (0.21×10^{-8}). The high per generation mutation rate in *Daphnia* could be either the result

of a high number of germ cell divisions or simply due to a high mutational susceptibility. However, this hypothesis cannot be currently tested given the limited information on the gametogenesis of *Daphnia*.

The base-substitution mutation rate for *Daphnia* (Table 3.2) is significantly lower than that of *C. elegans* (9.7×10^{-8}), *C. briggsae* (7.2×10^{-8}), and *D. melanogaster* (6.2×10^{-8}) but higher than that of *S. cerevisiae* (1.2×10^{-8}). Results from recent MA studies demonstrate that the mtDNA mutation rate is several fold higher than the nuclear rate for species such as *D. melanogaster* (10-fold difference, Haag-Liautard et al. 2007; Haag-Liautard et al. 2008), *C. elegans* (7-fold difference, Denver et al. 2000; Denver et al. 2004a), and *S. cerevisiae* (37-fold difference, Lynch et al. 2008). Consistent with this overall pattern, the ratio between mtDNA and nuclear mutation rate in *Daphnia* MA lines (~10^{-9}, Lucas J.I., personal communication) is ~10.

The ratio of indel to base substitution mutations in *D. pulex* mtDNA is ~2.3, which is significantly higher than that for *C. elegans* (0.62; Denver et al. 2000), *S. cerevisiae* (0.58; Lynch et al. 2008), *D. melanogaster* (0.28; Haag-Liautard et al. 2008), and *C. briggsae* (1.20; Howe et al. 2010). The high frequency of indels observed in both sexual and asexual MA lines is driven by a few mutation hotspots at homopolymer sites. As a consequence of slippage during replication, homopolymers are known to be especially vulnerable to insertion-deletion mutations (Denver et al. 2004b). For example, four independent sexual MA lines (S-11, S-51, S-67, and S-68) showed a deletion at position 14766 at a homopolymeric run of nine Cs (Table 3.1). Alternatively, the large proportion of indels may be due to the fact that our direct sequencing approach is likely more sensitive for detecting indels than low-frequency base substitution variants. This is because base substitutions originating on a single mtDNA molecule must persist through a transitional period of heteroplasmy prior to reaching fixation during which they may

often segregate at low frequencies. With the lowering cost of next-generation sequencing and the development of statistical methods for analyzing high-coverage sequence data (Lynch 2008), mutation rate estimates that include low-frequency genetic variants will improve.

3.4.2 Intraspecific comparison of mutation rate and spectrum

Although the average mtDNA mutation rate for asexual lines (1.73×10^{-7}) is ~27% higher than for sexual lines (1.37×10^{-7}), no statistically significant difference was detected among the estimates of the three sets of MA lines. The patterns of indels between sexual and asexual MA lines are also not statistically different (P = 0.3348, Fisher's exact test). Variation of the mtDNA mutation rate has been observed in different strains of *D. melanogaster* (Haag-Liautard et al. 2008), although the difference was not strongly supported by maximum-likelihood modeling. For *C. elegans* and *C. briggsae*, mtDNA base-substitution rates are highly similar, although *C. briggsae* experiences a much higher rate of large-scale deletions in mitochondrial genomes (Howe et al. 2010). Theoretical models on the evolution of nuclear mutation rate with different reproductive modes focus on the indirect selection pressure on mutator alleles generated through linkage disequilibrium with deleterious/beneficial mutations (e.g. Johnson 1999). It is unclear how the linkage between nuclear and mitochondrial genomes influences the mtDNA mutation rate. A fundamental assumption of theoretical investigation is the absence of recombination in asexual taxa. However, recent studies have shown significant amounts of ameiotic recombination (crossing over and gene conversion) in asexual *Daphnia*, which are orders of magnitude higher than mutation rate (Omilian et al. 2006; Xu et al. 2011). The similarity of mutation rates in asexual and sexual *D. pulex* lineages shown in this study seems to suggest either that these obligately parthenogenetic lineages are not old enough to evolve a lower mutation rate than the nuclear genomes or that ameiotic recombination might play a role in the regulation of mutation

rates in asexual lineages. Further theoretical work is necessary to demonstrate how ameiotic recombination impacts the evolution of mutation rate in asexuals.

The *Daphnia* mitochondrial genome is characterized by an A + T content of 62.3% (Crease 1999). However, because of the small number of base-substitution mutations observed in this study (i.e. 3 base-substitutions for S and A lines, respectively), it remains challenging to determine whether the A + T content bias is due to mutation bias or due to selection. Previous investigations have shown contrasting patterns of base substitutions in MA lines of different species, indicating that different evolutionary forces are responsible for the universal compositional bias of A/T in mitochondrial genomes. In *D. melanogaster* (A/T composition 82%) and *C. briggsae* (A/T composition 75%), 86% and 87% of base substitutions increase A/T content, suggesting that substitution bias is likely driving the nucleotide composition bias. In contrast, *C. elegans* (A/T composition 76%) and *S. cerevisiae* (A/T composition 84%) show 75% and 67% of base substitutions increase G+C content, indicating that natural selection has strongly shaped the mitochondrial nucleotide composition in these species (Denver et al. 2000; Lynch et al. 2008).

3.4.3 Mitochondrial effective population size

An important parameter for the mitochondrial population genetic environment is the effective population size. The mitochondrial effective population size is much smaller than the census size within a cell, due to a mtDNA bottleneck during early oogenesis and/or to the fact that some mtDNA alleles in heteroplasmic cells replicate more often than others by chance or due to selective advantages (Birky 2001). To date, it has been shown that the effective number of mitochondrial genomes is approximately 10 in mice and humans (Jenuth et al. 1996; Marchington et al. 1997) and 2 in cows (Ashley et al. 1989), implying that heteroplasmy likely

lasts only a few generations. In *Drosophila*, the effective number of mitochondria ranges between 545-700 per generation (Solignac et al. 1984), whereas crickets have a mitochondrial effective population size of 87-395 (Rand and Harrison 1986), indicating that heteroplasmy may be more common in these species. Our ML estimate of the effective number of mitochondrial genomes in the germline cells of *Daphnia* (5 to 10 copies) implies a short number of generations (10-20) for a heteroplasmic mutation to reach fixation. However, this estimate is probably downwardly biased because missing low-frequency mutants can lead to underestimates using this method (see Haag-Liautard et al. 2008). Our data contribute to the growing literature of the rate and spectrum of mitochondrial mutation, a key parameter in understanding the genetic change within and between species. However, future studies are needed to further investigate the variation of mtDNA mutation rates with breeding systems to test theories about the evolution of mutation rates.

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Table 3.1. List of the mtDNA mutations detected in *Daphnia pulex* mutation accumulation lines. Bold letters indicate mutation sites.

Line	Generation	Position	Mutation	Effect	Frequency	Context
S-109	62	4401	G→A	Met>Ile	0.50	GATACCCTTTAT**G**GTTCTTATCGAA
S-60	59	8298	A→C	Met>Val	0.80	GGGGGGGCAGCT**A**TATTACTAATAG
S-58	62	8841	A(8)→A(7)	frame shift	0.44	GCCCT**A8**GTAGTCAAGACT
S-35	63	9165	G→T	Tyr>Asn	0.42	TCAAATAGATAA**G**TATCTTAGAAAG
S-13	59	12300	C(7)→C(8)	frame shift	0.22	AGAAT**C7**AAGCCTACTTTAT
S-78	63	14411	C(3)→C(2)	small rRNA	1	AAAAATTTCA**C3**TATCAACAAAAT
S-21	61	14716	AC deletion	D-loop	0.76	AAGTATTTAGCG**AC**CGCTTTGAAATT
S-11	54	14766	C(9)→C(8)	D-loop	1	TTTTGAGGGGAT**C9**TAGAACCCCCCC
S-51	61	14766	C(9)→C(8)	D-loop	1	TTTTGAGGGGAT**C9**TAGAACCCCCCC
S-67	57	14766	C(9)→C(8)	D-loop	1	TTTTGAGGGGAT**C9**TAGAACCCCCCC
S-68	51	14766	C(9)→C(8)	D-loop	1	TTTTGAGGGGAT**C9**TAGAACCCCCCC
S-32	90	14840	T(9)→T(10)	D-loop	1	ATCTCTTTTTTAT**T9**AGATTTATAATT
A1-1	110	7216	G→A	synonymous	0.78	TAGAAATAAATA**A**TAACAGTGCTGA
A1-12	146	11696	C(6)→C(7)	frame shift	0.23	ACAATCA**C6**AAAAAAATGACAC

A2-13	113	12661	A→G	Large rRNA	0.58	AACAGACTTTCC**A**ACAAAACTTCTG
A1-18	111	12949	T→A	Large rRNA	1	CCCAAACGCATT**T**AAGCTTTTTCAC
A2-4	85	14766	C(8)→C(9)	D-loop	1	TTTCGAGGGGAT**C8**TAGAACCCCCCCC
A2-17	102	14766	C(8)→C(10)	D-loop	1	TTTCGAGGGGAT**C8**TAGAACCCCCCCC
A1-9	130	14840	T(10)→T(9)	D-loop	1	AATCTCTTCTTTA**T10**AGATTTATAATT
A1-16	125	14840	T(10)→T(9)	D-loop	1	AATCTCTTCTTTA**T10**AGATTTATAATT
A1-18	111	14840	T(10)→T(9)	D-loop	1	AATCTCTTCTTTA**T10**AGATTTATAATT
A2-12	104	14840	T(10)→T(11)	D-loop	1	AATCTCTTCTTTA**T10**AGATTTATAATT
A2-18	80	14840	T(10)→T(9)	D-loop	1	AATCTCTTCTTTA**T10**AGATTTATAATT

Table 3.2. Approximate and maximum-likelihood (ML) estimates of mutation rates (per site per generation) and ML estimates of the effective population size of mitochondrial genomes (N_e) per generation.

Mutation type	**No. of mutations**	**μ (approx) × 10^{-8}**	**μ (ML) × 10^{-8}**	**N_e (ML)**
S lines (61 generation)				
Base-substitution	3	2.0	2.7	10
Indel	9	11.7	12.2	5
All events	12	13.7	15.1	10
A lines (100 generation)				
Base-substitution	3	4.3	4.8	5
Indel	8	13.0	12.7	5
All events	11	17.3	17.5	5
A1 lines (116 generation)				
All events	6	14.0	N/A	N/A
A2 lines (81 generation)				
All events	5	22.8	N/A	N/A

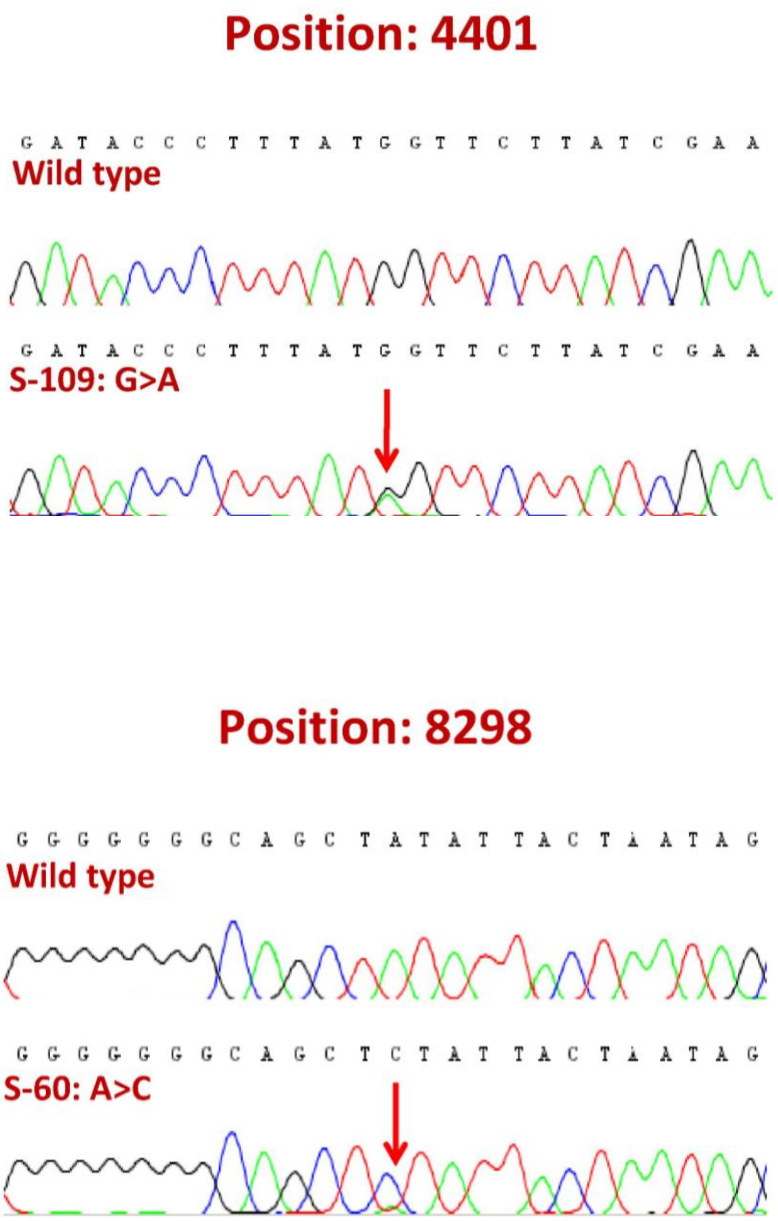
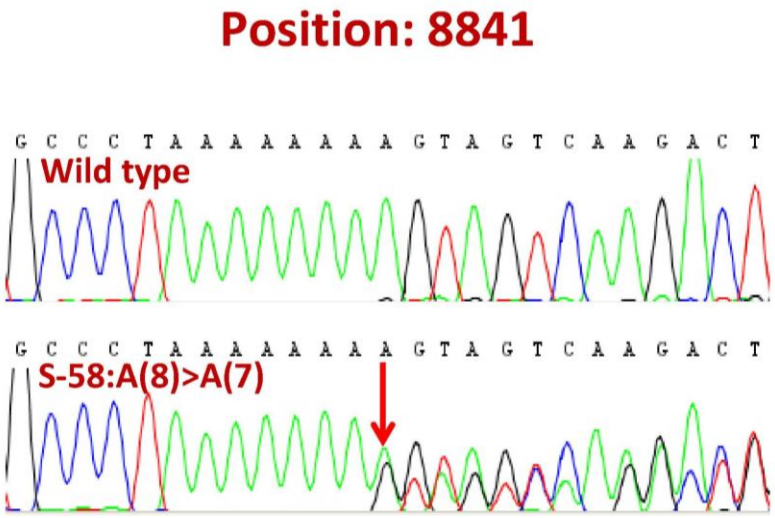
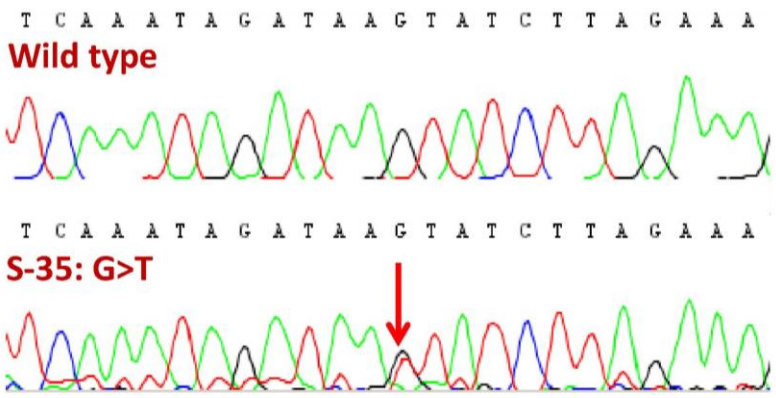


Fig. 3.1 DNA sequence trace files showing mutants and wild types.





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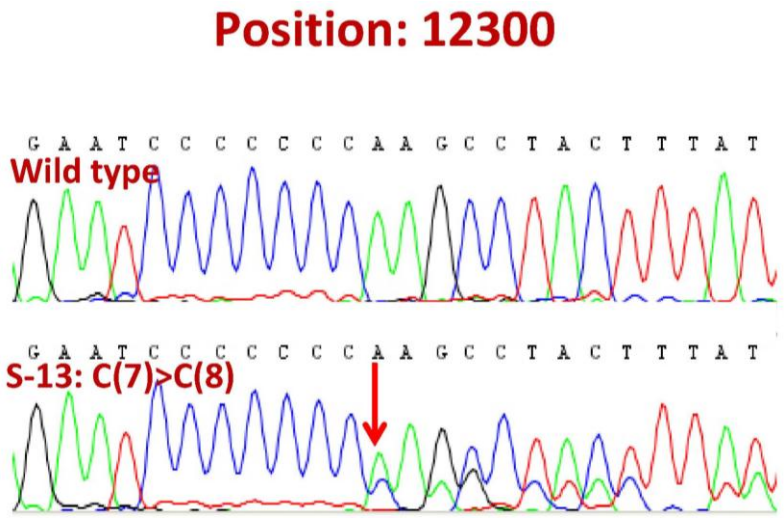

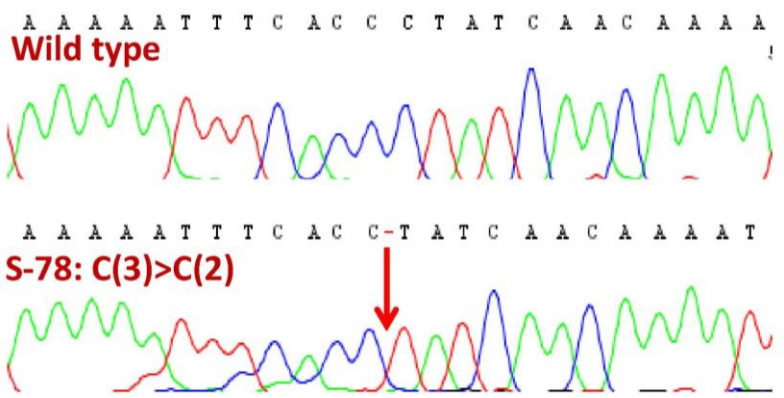

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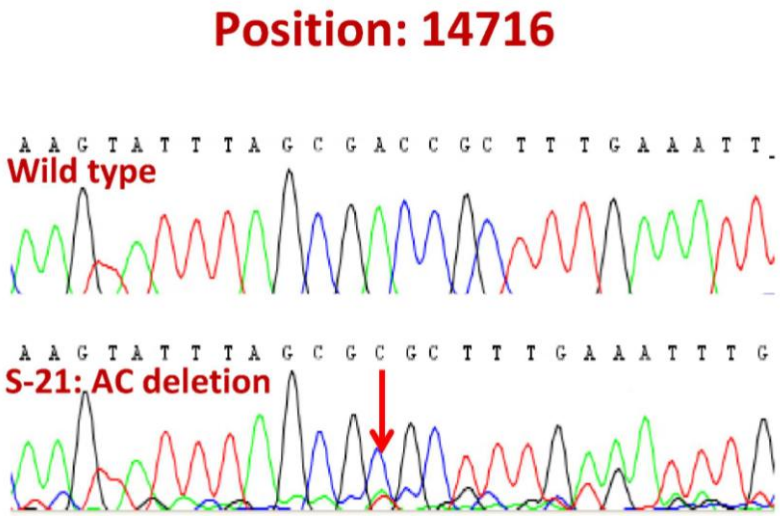

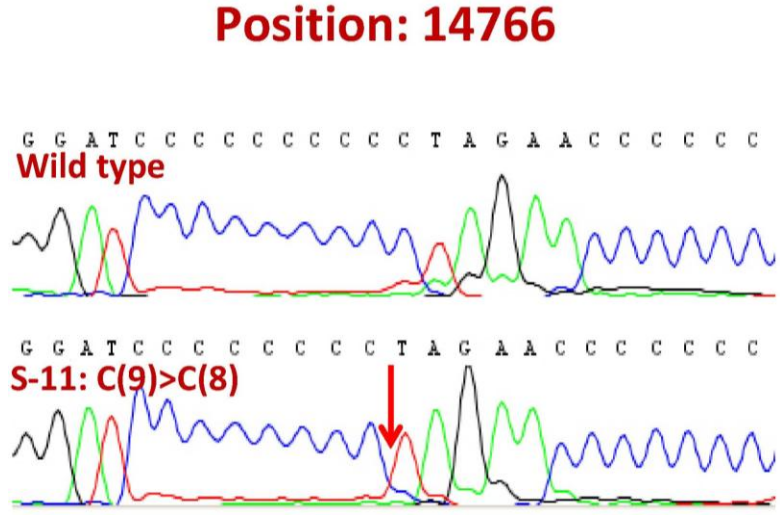

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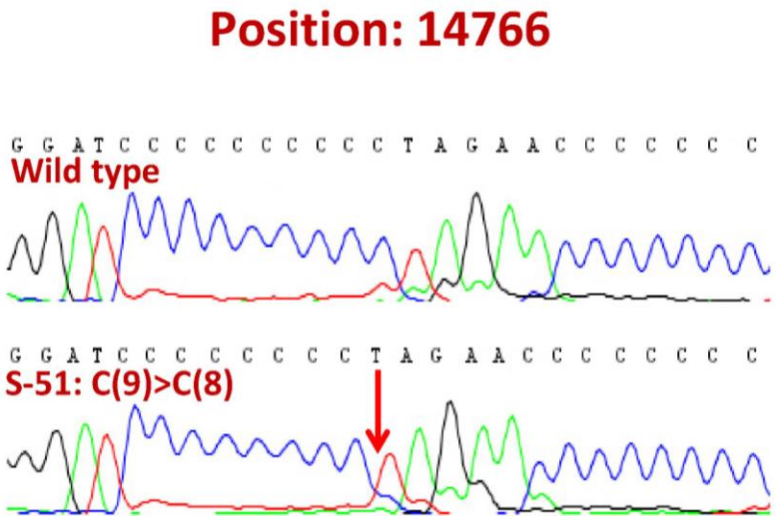

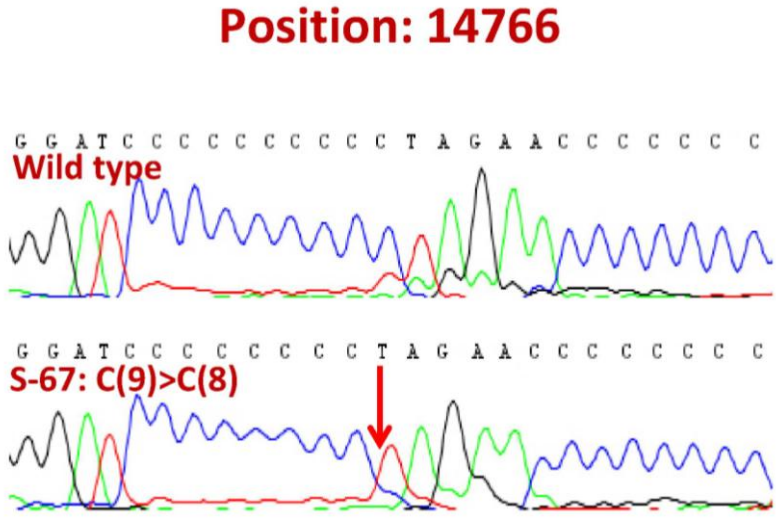

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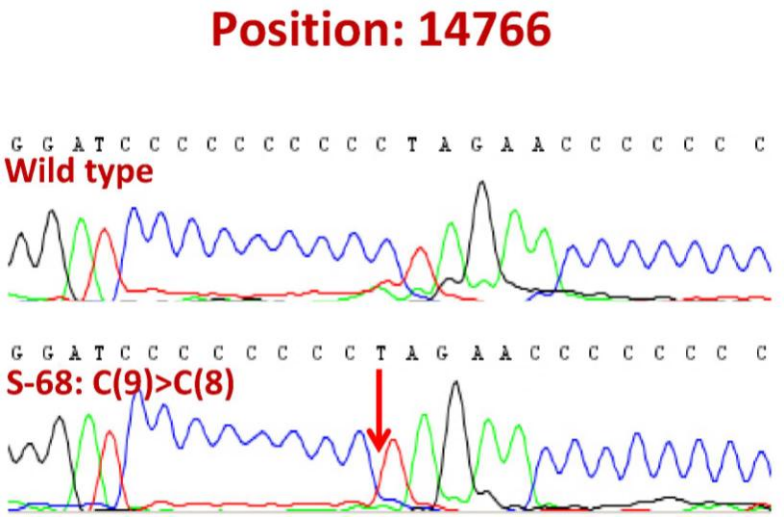

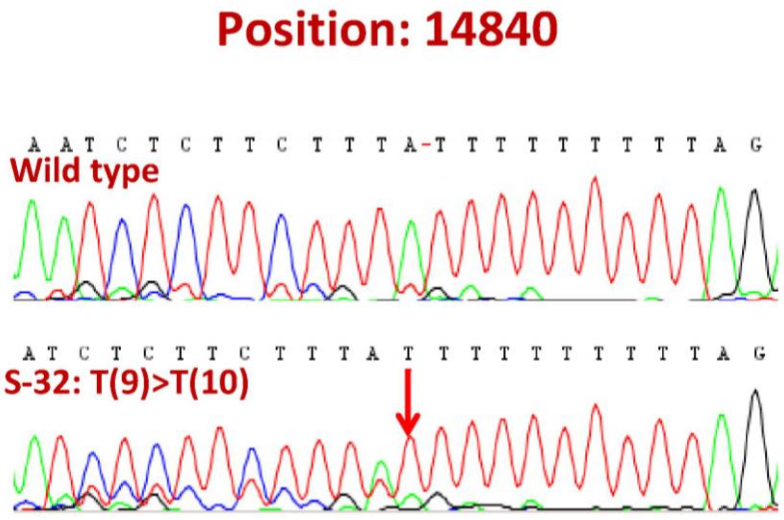

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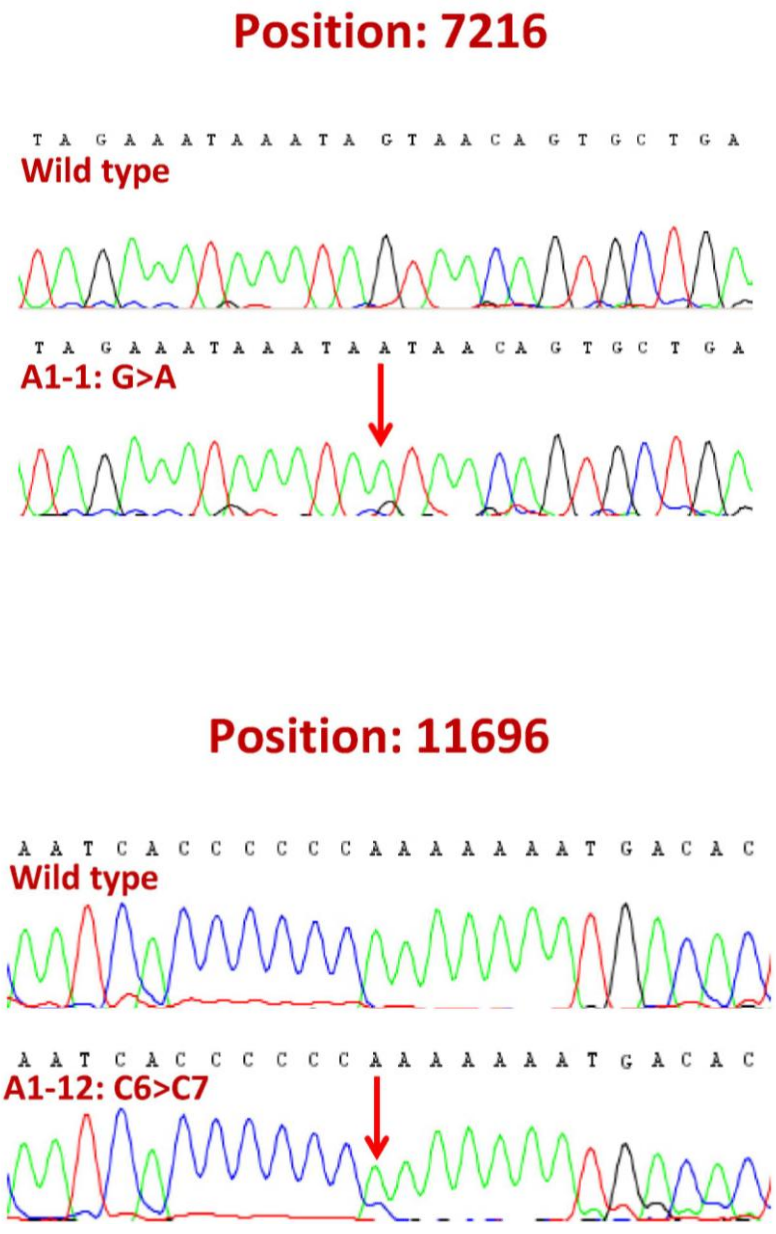

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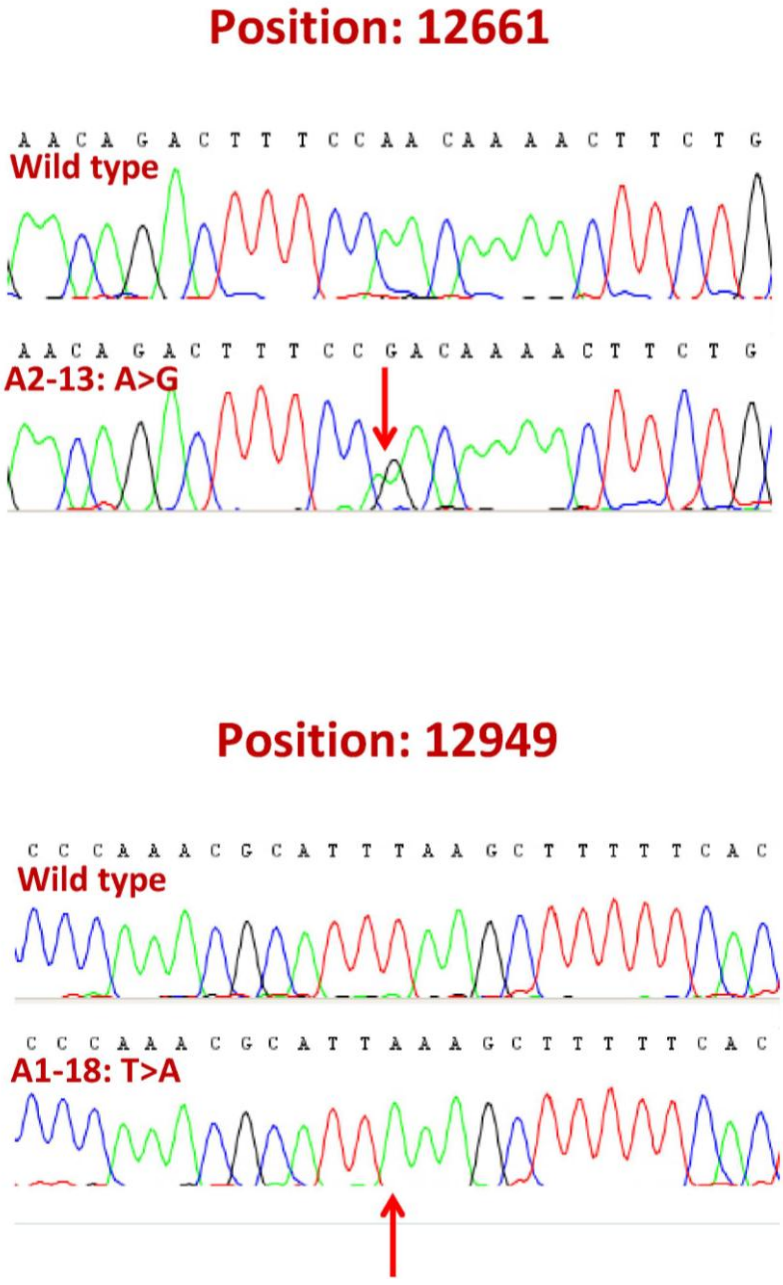

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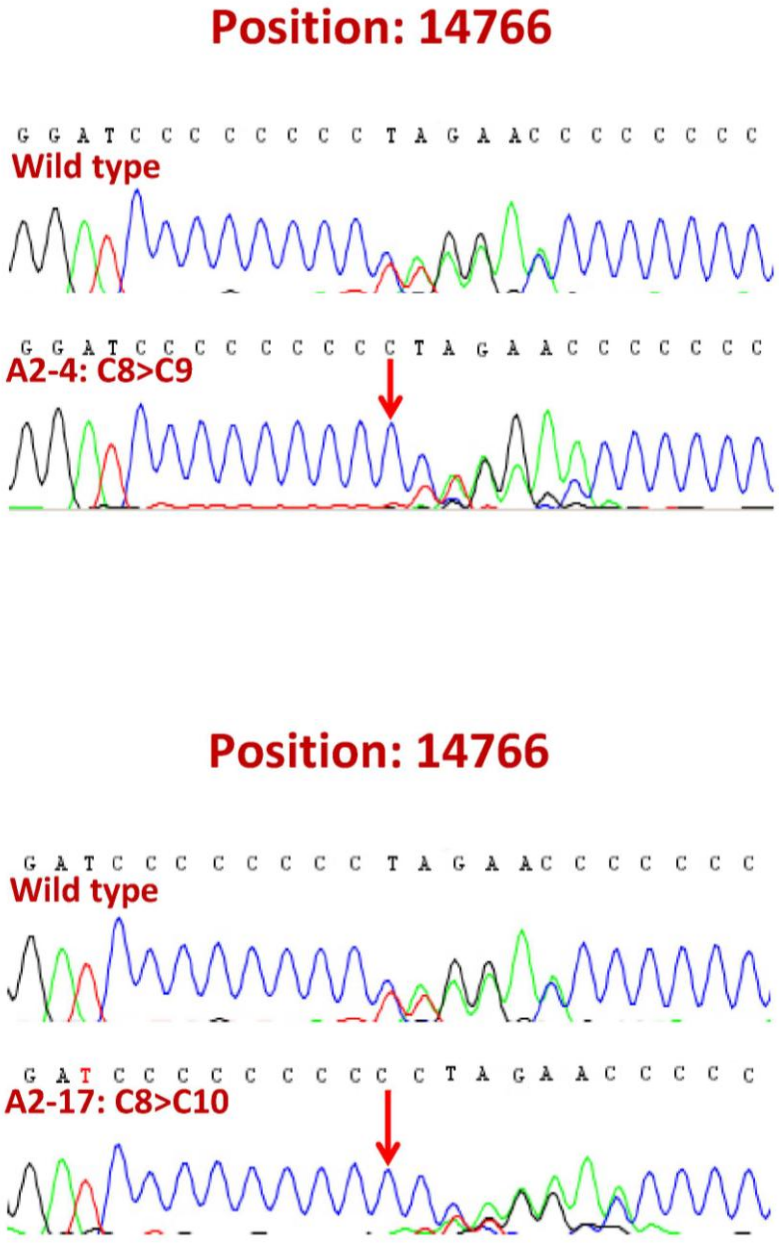

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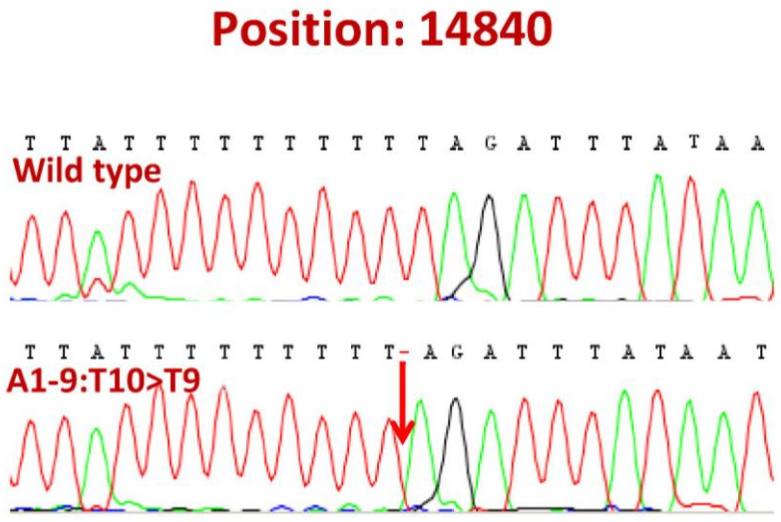

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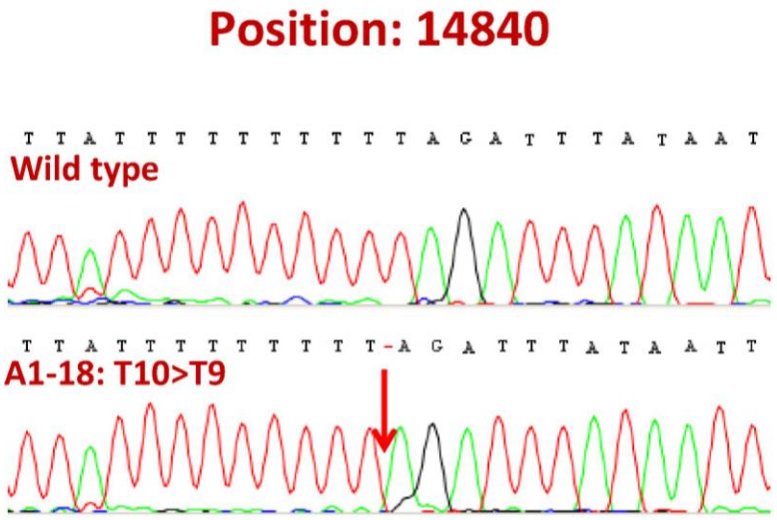

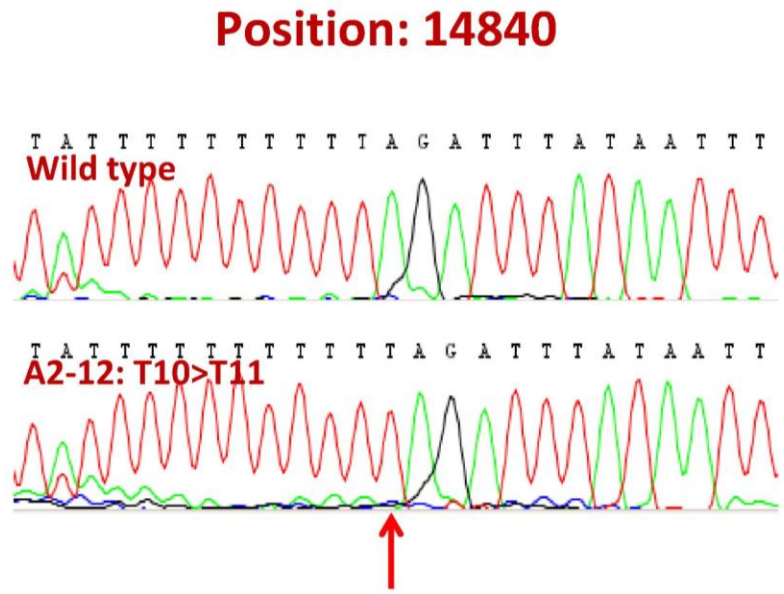

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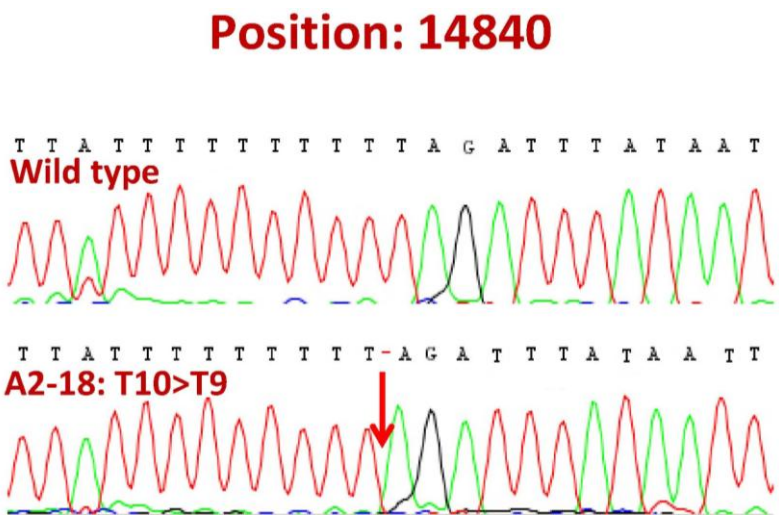

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CHAPTER 4. OBLIGATE ASEXUALITY IN *DAPHNIA*: THE JOINT ROLES OF HYBRIDIZATION AND MEIOSIS SUPPRESSION LOCI

4.1 INTRODUCTION

Sexual reproduction is the predominant reproductive mode in eukaryotes, whereas asexual taxa are rare and believed to represent evolutionary dead ends (Maynard Smith 1978). It is generally thought that sexual reproduction evolved once in the early ancestor of eukaryotes, with parthenogenesis evolving multiple times subsequently across different phylogenetic lineages (Bell 1982). Extensive research has focused on the long-term and immediate costs and benefits of sex, providing insight into how sex is maintained (Kondrashov 1993; Otto 2009). In contrast, little empirical work has considered the molecular mechanisms underlying the conversion of ancestrally sexual reproduction to a derived state of parthenogenesis (Lynch et al. 2008). As a result, our understanding of the genetic diversity, ecological adaptability of asexual populations/species and the outcome of competition with their sexual relatives is still very limited (Howard and Lively 1994; Howard and Lively 1998).

Asexuality in animals has at least four different origins, i.e., spontaneous origin, hybrid origin, contagious origin, and infectious origin (Simon et al. 2003). Spontaneous asexuality may occur through mutations in the genes involved in sexual reproduction and meiosis, e.g., the loss of sex in monogonont rotifers (Serra and Snell 2009). Interspecific hybridization causes asexuality when normal meiosis is disrupted due to genomic incompatibility between parental species (White 1978; Vrijenhoek 1998). Hybridization has been shown to be responsible for the origin of asexual lineages in vertebrates, snails, crustaceans, and insects such as aphids, weevils, and grasshoppers (reviewed in Simon et al. 2003). Contagious asexuality often results from the transmission of genetic elements that confer asexuality during sexual reproduction via environmentally induced males of obligate parthenogenetic clones, e.g., *Daphnia* (Innes and Hebert 1988). Furthermore, parasite-induced asexuality is commonly observed in haplodiploid

organisms such as wasps and mites infected by *Wolbachia*, a member of the Proteobacteria (reviewed in Simon et al. 2003).

An ideal system for examining the molecular mechanisms responsible for the conversion to asexual reproduction is the *Daphnia pulex* species complex (Crustacea, Anomopoda), which contains multiple asexual lineages derived from sexual ancestors (Crease et al. 1989; Hebert et al. 1989; Paland et al. 2005). *Daphnia* species typically reproduce by cyclical parthenogenesis, i.e., alternation of asexual and sexual reproduction. Under favourable environmental conditions, females produce diploid eggs apomictically that directly develop into daughters. Environmental cues signalling deteriorating conditions (e.g., food shortage, high population density, and/or photoperiod changes) trigger the production of males and haploid eggs by females (Innes and Hebert 1988). Fertilization of the haploid eggs results in dormant eggs deposited in a protective structure (ephippium) that can hatch after extensive periods of dormancy, up to two hundred years (e.g., Decaestecker et al. 2007). Interestingly, some lineages in the *Daphnia pulex* complex have lost the ability to engage in sexual reproduction, reproducing by obligate parthenogenesis (OP). Nonetheless, some of these lineages are still able to produce males. These males can mate with CP females and can pass along a set of genes that can suppress meiosis in females, thus converting some of the offspring to OP (Innes and Hebert 1988; Paland et al 2005).

This study focuses on examining the genetic mechanisms responsible for the obligate parthenogenesis in *Daphnia* clones that are thought to be the hybrids of *D. pulex* and its sister species *D. pulicaria* (Cristescu et al. in press). Although morphologically indistinguishable, *D. pulex* and *D. pulicaria* occupy largely distinct habitats and can be identified by diagnostic alleles of the a few allozyme loci such as the *lactate dehydrogenase* (*Ldh*) locus and by microsatellite markers (Cristescu et al. in press). *D. pulex* usually live mainly in ephemeral pond and can be

occasionally found in some permanent fishless habitats, whereas the primary habitat for *D. pulicaria* is the permanent, stratified lakes with high fish predation (Crease et al. 1997; Pfrender et al. 2000). Whereas cyclically parthenogenetic *D. pulex* are homozygous for the slow allele (SS) of *Ldh*, *D. pulicaria* appear to be homozygous for the fast allele (FF) (Hebert et al. 1989; Pfrender et al. 2000). Across the North American distribution of *D. pulex*, populations in northeast part of the continent harbour exclusively OP clones, whereas CP populations are found mainly in the mid-west and western US (Hebert and Finston 2001). The hybrids of *D. pulex* and *D. pulicaria* are common in temperate regions of North America and are found predominately in ponds in disturbed areas where forests have been cleared (Hebert and Crease 1983). These hybrids are characterized by a heterozygous (SF) genotype at the *Ldh* locus and invariably appear to be obligately asexual in nature (Hebert and Finston 2001). Interestingly, the Great Lakes watershed represents a contact zone between the OP and CP populations, i.e., many ephemeral ponds are found to contain a mixture of OP (both *Ldh* SS and SF genotypes) and CP individuals (Hebert and Finston 2001). Thus, these mixed ponds represent great opportunities to investigate the molecular mechanisms underlying obligate parthenogenesis in *Ldh* SF clones because of possible interbreeding between CP and OP clones.

It has been assumed that an important mechanism responsible for asexuality in *D. pulex* involves the transmission of genetic elements (e.g., obligate asexuality/meiosis suppression genes) within *D. pulex* via males arising from obligately parthenogenetic clones (Innes and Hebert 1988). While lacking the ability to reproduce sexually, some *D. pulex* lineages can still produce males that carry the genes that can suppress meiosis during oogenesis, but not during spermatogenesis (Hebert 1981; Hebert et al. 1989). These males can mate with cyclically parthenogenetic (CP) females, spreading female asexuality by producing a mixed brood of OP and CP individuals with

SS *Ldh* genotype (Innes and Hebert 1988). Thus, asexuality can spread in a contagious fashion, which probably accounts for the independent origin of multiple asexual lineages of *D. pulex* (Hebert et al. 1989; Paland et al. 2005). Initially, it was hypothesized that a single, dominant gene is responsible for meiosis suppression/obligate asexuality in *Daphnia* (Innes and Hebert 1988). However, a recent study that performed genome-wide association mapping using a collection of *Ldh* SS *D. pulex* clones across North America shows that, instead of a single meiosis suppression gene, four genomic regions may be associated with meiosis suppression/obligate asexuality (Lynch et al. 2008).

Meiosis suppression is also thought to be responsible for the obligate parthenogenesis in clones that appear to be of hybrid origin (*Ldh* SF clones; Hebert et al. 1989). Different from many hybrid asexual species (e.g., lizards, Avise et al. 1992), it is considered unlikely that the OP in these *Ldh* SF hybrids is a result of hybridization (i.e., genomic incompatibility) between the parental species. Heier and Dudycha (2009) have shown that the F1s of *D. pulex* females and *D. pulicaria* males in laboratory conditions are viable cyclical parthenogens. The OP in these hybrids (*Ldh* SF) was originally suggested to result from the transmission of meiosis suppression elements via male *D. pulex* produced OP clones mating with female *D. pulicaria* (Hebert et al. 1989). Nonetheless, this hypothesis seems unlikely as recent molecular studies indicate that *D. pulex* represent the maternal genotype of wild hybrids (e.g., Crease et al. 1989; Cristescu et al. in press). Furthermore, Heier and Dudycha (2009) performed crosses between females of normal CP *D. pulex* and males of CP *D. pulicaria* in laboratory conditions and showed that the F1s (*Ldh* SF) invariably appear to be cyclical parthenogens, rather than obligate parthenogens. This is distinct from the field observation that *Ldh* heterozygote genotype is always obligately asexual. Thus, Heier and Dudycha (2009) suggest that the OP hybrids might originate from the crossing

of F1 hybrids (i.e., cyclical parthenogens) in pond habitats with *D. pulex* males carrying meiosis suppressors. As a result, contrary to conventional understanding, it raises the possibility that OP clones with *Ldh* SS genotypes can appear as a consequence of the backcrossing. Thus, these OP clones are also genetically more similar to *D. pulicaria* (i.e., 25% genome from *D. pulicaria*) than pure OP *Ldh* SS *D. pulex* (i.e., 100% genome from *D. pulex*) (Heier and Dudycha 2009). However, this scenario remains hypothetical and needs to be further tested.

In this study, we specifically use microsatellite data to test whether OP clones with the *Ldh* SF genotype result from the crossing of F1s of *D. pulex* and *D. pulicaria* with male *D. pulex* that carry meiosis suppression elements from OP lineages. As a consequence, asexual offspring with *Ldh* SS genotypes can appear. These *Ldh* SS asexual clones should be genotypically different (e.g., more similar to *Ldh* SF OP clones) from the asexual *Ldh* SS individuals that arise due to the intraspecific spread of meiosis suppressors in *D. pulex*. Thus, the hypothesis of Heier and Dudycha (2009) predicts that there should be two different groups of *Ldh* SS asexual clones (i.e., one group genotypically more similar to *D. pulicaria*, the other more similar to *D. pulex*). Furthermore, we specifically test whether the diagnostic alleles associated with *Ldh* SS OP clones are in association with *Ldh* SF clones. Although several major genomic regions have been identified for meiosis suppression/obligate asexuality (Lynch et al. 2008), this was only done for OP clones of *Ldh* SS genotype. It remains uncertain whether *Ldh* SF OP clones have the same set of genes suppressing meiosis.

4.2 MATERIALS AND METHODS

4.2.1 Sampling and sexuality test

Individuals of *D. pulex* were collected from four ephemeral ponds (Canard 1, 1B, 2, and 3) located within a 5-km radius of Windsor, Ontario, Canada. These ponds are usually filled with water by snow melt in early spring and dry up by early summer. The collected individuals were brought to the laboratory, isolated, and maintained in 25-ml beakers with filtered river water at 20 ℃. Clonal lines were fed *ad libitum* with suspension of *Scenedesmus obliquus*. Three to five parthenogenetic offspring descended from each individual were used for sexuality tests. The sexuality tests involved examining whether, in the absence of males, diapausing embryos were present in ephippia. Consistent results from at least three consecutive rounds of production of ephippial embryos for each individual were used to determine its reproductive mode. The presence of embryos in ephippia indicate OP (asexual), whereas the absence of embryos suggests that individuals are CP (sexual) and fertilisation by sperm is necessary for the production of diapausing embryos (Innes and Hebert 1988). In total, 98 asexual isolates and 86 sexual isolates were identified and used in the subsequent genetic analyses. To investigate the relationship of *Ldh* SF OP individuals with CP *D. pulex* and *D. pulicaria*, we incorporated into our dataset 51 CP clones of *D. pulex* from the Disputed Pond in Windsor and Solomon pond in Michigan, USA and 217 *D. pulicaria* clones from three lakes (Three Lakes II, Lawrence, and Warner) from Michigan (Cristescu et al. in press).

4.2.2 Molecular protocols and data analyses

DNA of 5-10 parthenogenetic offspring of each clone was extracted using a cetyltrimethylammonium bromide method (Doyle and Doyle 1987). The *Ldh* genotype for each OP clone was examined using allele-specific PCR. The *Ldh* slow allele was amplified using the forward primer 5'-GAGCGATTTAACGTTGCGCCC-3' and the reverse primer 5'-GGACGACTTGTGTGTGAATTTG-3', whereas the fast allele was amplified with the forward

primer 5'-GAGCGATTTAACGTTGCGCCT-3' and the reverse primer 5'-GGACGACTTGTGTGTGAATTTC-3'(Cristescu et al. in press). To determine the genotype of a clone, two PCR reactions were run to amplify the slow and fast alleles, respectively. For each PCR reaction, individuals with *Ldh* genotypes (SS or FF) determined by allozyme screening were used as control samples to monitor whether there was cross-amplification between the slow and fast alleles. The PCR cycling regime consisted of 2 min denaturing at 94 ℃ and 35 cycles of 30 sec denaturing at 94 ℃, 30 sec annealing at 58 ℃, and 30 sec extension at 72 ℃. The PCR product for each reaction was checked on a 1.5% agarose gel to determine the *Ldh* genotype. The presence of amplicon for both slow and fast reactions indicates SF heterozygotes, whereas the presence of a band for slow/fast reaction alone is recognized as SS/FF homozygotes. The *Ldh* genotypes for a subset of samples (n = 30) examined in this study were confirmed by allozyme screening, confirming the robustness of this method.

Microsatellite markers from the identified hotspots that are involved in meiosis suppression/obligate asexuality (Lynch et al. 2008) were genotyped for both sexual and asexual clones. These 34 markers were shown to be significantly associated with obligate asexuality (Lynch et al. 2008), and mainly reside in the four linkage groups V, VIII, IX, and X. In addition to these diagnostic markers, all 20 markers mapped in the linkage group I were also genotyped, because previous studies showed that this linkage group is involved in the production of males, which is an essential feature for the contagious spread of asexuality (Colbourne, unpublished data). PCR reactions and genotyping followed methods in Cristescu et al., (2006). The asexual individuals were divided into two groups based on their *Ldh* genotypes (SS and SF). G_{ST} (Nei 1973) between sexual (*Ldh* SS) and asexual clones (*Ldh* SS or SF genotypes) was calculated for all markers in the software DISPAN (https://homes.bio.psu.edu/people/faculty/nei/software.htm).

To investigate the origin of SF obligately asexual clones, we included a set of 268 CP *D. pulex* and *D. pulicaria* isolates from two sexual ponds (Disputed and Solomon ponds), and three stratified lakes from Michigan (Cristescu et al. in press). We used nine microsatellite markers (a subset of the total 54 markers) to construct a neighbour-joining tree based on pairwise allele-shared distances (D_{AS}) between all genotypes in the software Populations 1.2 (http://bioinformatics.org/~tryphon/populations/). These nine neutral markers are expected to provide a good estimate of the relationships among the species/groups in question because they are evenly distributed across the entire genome of *D. pulex*, with about one marker on each linkage group. We also performed a Bayesian assignment test in the software Structure (Pritchard et al. 2000) to assign a relative probability that each OP clone belongs to either CP *D. pulex* (*Ldh* SS) or *D. pulicaria* (*Ldh* FF). All OP clones were analyzed as unknowns under an admixture ancestry model. Burn-in and run lengths were set to 100,000 and 1, 000,000, respectively. For the microsatellite markers associated with asexuality, we searched the 200 kb flanking region (wfleabase.org) centered at the focal locus to identify possible genes that are involved in obligate asexuality.

4.3 RESULTS

4.3.1 Overall patterns of microsatellite diversity

Two of the Canard ponds (1 and 3) contained both sexual and asexual individuals, whereas pond 1B was purely asexual and pond 2 was purely sexual. Past studies revealed that the frequency of sexual and asexual lineages in the Canard ponds can change dramatically from year to year (Innes, unpublished data). Allelic diversity at the 54 microsatellite loci ranged from 4 to 17 alleles per locus, with 571 alleles identified across all loci. The mean number of alleles per locus

for the ponds ranged from 1.13 to 7.07 (Table 4.1). All Canard populations analysed deviated significantly from Hardy-Weinberg equilibrium over all microsatellite loci (Table 4.1).

4.3.2 Microsatellite neighbour-joining tree and Bayesian estimate of ancestry

The neighbour-joining tree based on a subset of nine microsatellite markers showed that the CP *D. pulex* and *D. pulicaria* formed distinct clusters (Fig. 4.1A). The majority of OP *Daphnia* including both SS and SF *Ldh* genotypes formed another clade in between the pond and lake clade, suggesting their similar distance from these two clades and their hybrid origin (called the hybrid clade hereafter). However, many *Ldh* SS asexual individuals cluster within the *D. pulex* clade. The results of the Bayesian assignment test were largely consistent with those of the phylogenetic analysis (Fig. 4.1B). *Ldh* SF hybrid clones had similar probability of ancestry from *D. pulex* and *D. pulicaria* genetic backgrounds. Some *Ldh* SS OP clones appear to be derived from almost pure (>95%) *D. pulex* genome background, whereas other *Ldh* SS OP clones have ~25-40% similarity with *D. pulicaria* genome.

4.3.3 Meiosis suppression loci and diagnostic alleles

We estimated the population subdivision between the *Ldh* SS/SF asexual individuals and sexual individuals in the pond using G_{ST} (supplementary Tables S4.1 and S4.2). The average G_{ST} between asexual (*Ldh* SS) with sexual individuals was 0.085 (SE = 0.009), which is consistent with the previous G_{ST} estimate based on genome-wide microsatellite markers for contrasting SS asexual and sexual *D. pulex* (Lynch et al. 2008), whereas the average G_{ST} for comparing *Ldh* SF asexual with sexual individuals was 0.156 (SE = 0.012). Markers with G_{ST} values higher than the average values are considered candidates associated with obligate asexuality (supplementary Fig. S4.1). Furthermore, the alleles of these candidate markers were compared between asexual and sexual individuals to determine diagnostic alleles (Table 4.2). For the regions on linkage group V,

VIII, IX, and X that appeared to be associated with asexuality in a previous study (Lynch et al. 2008), our results showed that all contain microsatellite markers that are highly differentiated between asexual (either *Ldh* SS or SF genotypes) and sexual individuals (Table 4.2). Furthermore, the markers d039, d063, and d091 on linkage group I appeared to be highly diagnostic in both comparisons (Table 4.2), indicating their involvement in obligate asexuality. However, a few markers on linkage group I (i.e., d076, d001, d148, d188, and d138) and d113 on linkage group VIII only appeared to be diagnostic when contrasting SF asexual and sexual individuals, suggesting that these alleles likely arose in a *D. pulicaria* genetic background but are not necessarily involved in obligate asexuality.

4.3.4 Meiosis suppression genes

The search in the 200-kb regions centered at the identified diagnostic markers revealed many genes involved in DNA replication, repair, recombination, cell cycle, spindle formation, and chromatin assembly (supplementary Table S4.3). For example, the newly identified diagnostic marker (d039) on linkage group I is closely located to Transcription factor IIIC, which has functional roles in DNA replication, repair, and recombination. Linkage group IX, a large part of which is associated with meiosis suppression, contains many genes that are responsible for meiotic cell division, recombination, and sister chromatid cohesion (supplementary Table S4.3).

4.4 DISCUSSION

Previous studies have suggested that meiosis suppression mediated by hybridization is responsible for the origin of obligate parthenogenesis (OP) in *D. pulex* with *Ldh* SF genotypes. However, the two competing hypotheses of Hebert et al. (1989) and Heier and Dudycha (2009) about the hybridization process remain to be tested. Furthermore, if *Ldh* SF OP *D. pulex* clones have a similar mechanism for meiosis suppression as in *Ldh* SS OP *D. pulex*, we expect the

diagnostic markers that were identified in *Ldh* SS asexual *D. pulex* to be also associated with *Ldh* SF asexual clones. To address these issues, we performed population genetic analyses and association studies with sexual and asexual *D. pulex* individuals coexisting in a small geographic region.

4.4.1 Hybridization and obligate asexuality

Prior hypothesis suggested that *Ldh* SF hybrids could be the inheritance of meiosis suppressor from a male *D. pulex* hybridizing with a female *D. pulicaria* (Hebert et al. 1989). This hypothesis is inconsistent with the observation that the *D. pulex* is the maternal genotype of hybrids (Cristescu et al. in press). The hypothesis that *Ldh* SF asexual clones arising from the backcrossing of F1 sexual hybrids (i.e., offspring of female *D. pulex* mating with male *D. pulicaria*) with males of *D. pulex* carrying meiosis suppressors (Heier and Dudycha 2009) generates two predictions. First, *Ldh* SF asexual individuals should show the highly diagnostic alleles for obligate asexuality. Second, the asexual offspring from backcrossing could carry either *Ldh* SS or SF genotypes. Thus, there should be two types of asexual individuals with SS *Ldh* genotypes, with some of them originating from the pond sexual *D. pulex* due to the intraspecific spread of meiosis suppression and others genotypically more similar to the *Ldh* SF asexual individuals due to backcrossing. Our neighbour-joining tree based on nine microsatellite markers reveals three distinct clades, the CP *D. pulex* with some *Ldh* SS asexuals, *D. pulicaria* with no asexuals, and the hybrid clade of both *Ldh* SS and SF asexuals. The Bayesian estimates of ancestry consistently support the mixed ancestry of individuals assigned to the hybrid clade. A large proportion of *Ldh* SF asexual individuals (Table 4.2) carry the highly diagnostic alleles for meiosis suppression/obligate asexuality (Lynch et al. 2008). Most importantly, these results strongly support the scenario envisaged by Heier and Dudycha (2009). The hybrid clade contains

all the *Ldh* SF asexual clones and the nearly half of the *Ldh* SS asexuals, whereas the other *Ldh* SS asexuals cluster with the sexual *D. pulex* individuals (Fig. 4.1). This observation is consistent with previous work showing that OP *D. pulex* clones are derived from distinct genetic backgrounds/lineages (Crease et al. 1989). The Bayesian estimates of ancestry also show that some *Ldh* SS asexual clones have ~25-40% similarity to *D. pulicaria* genome (Fig. 4.1), largely consistent with the prediction that backcrossing offspring is genotypically 25% similar to *D. pulicaria*. It should be noted that some of these *Ldh* SS backcrossing might be offspring from two consecutive rounds of backcrossing generation, with a higher similarity (~50%) with *D. pulex*. Furthermore, the introgression mediated by the F1 hybrids of *D. pulex* and *D. pulicaria* has produced dramatic genetic differentiation between asexual *Ldh* SF and *Ldh* SS sexual *D. pulex* individuals, with markers d076, d001, d148, d188, d138, d113 likely carrying alleles that originated from a *D. pulicaria* genetic background. This hypothesis needs to be tested in a wide geographic range to determine whether it is the only mechanism underlying OP in *Ldh* SF *D. pulex* clones.

4.4.2 Meiosis suppression

Our results confirm that the majority of diagnostic alleles for asexuality (Lynch et al. 2008) from linkage groups V, VIII, IX, and X are highly indicative of obligate parthenogenesis in both SF and SS asexual individuals. Furthermore, we show that linkage group I likely carries genes that underlie obligate asexuality. Although these regions contain genes that are involved in cell cycle, cell division, transcription, DNA repair, and chromosome structure, some of them may not directly responsible for meiosis suppression. Instead, some of these genomic regions likely contain genetic elements underlying other essential features of obligate asexuality such as the production of males and the activation of unfertilized diploid resting eggs. The cell division of

germ-line cells in obligate asexual *Daphnia* is apomictic, involving the suppression of meiosis I of a normal meiotic division and only a single maturation division (Schrader 1925; Zaffagnini and Sabelli 1972; Hiruta et al. 2010). However, this is distinct from mitotic cell division because a polar body, a distinguishing feature of meiosis, is emitted at the end of the maturation division (Schrader 1925; Zaffagnini and Sabelli 1972). CP *Daphnia* possess the genetic machinery for both apomictic division and meiotic division, with environmental cues triggering the switch between them. Meiosis suppression can be due to a dominant mutation in one of the key genes initiating meiosis, as indicated by a previous study in which nearly half of the F1 progeny between males from OP clones and CP females was obligate asexual (Innes and Hebert 1988). In the plant literature, there have been reports of meiosis becoming ameiotic due to spontaneous mutations in major genes. In maize, the mutants of the *ameiotic* 1 gene show phenotypes of equational division (Pawlowski et al. 2009). In *Arabidopsis*, a homologue of ameiotic 1, SWI1/DYAD affects the key events for meiosis such as sister chromatid cohesion and recombination (Siddiqi et al. 2000; Mercier et al. 2001; Agashe et al. 2002; Mercier et al. 2003). The mutants of *swi1/dyad* genes show abnormal meiosis, with equational-like segregation of chromosomes (Agashe et al. 2002; Mercier et al. 2003). For obligate asexual *D. pulex*, a recent study (Eads et al., unpublished manuscript) shows that all OP clones carry an allele of the *Rec*8 B locus, which encodes the meiotic cohesin REC8, that contains a transposable element insertion upstream and a frameshift mutation, both of which are completely absent from sexual lineages. It is thus hypothesized that obligate asexuality in *Daphnia* is initiated by the abrogation or modification of REC8 function, likely due to the response triggered by the inserted transposable element at the post-transcriptional level (Eads et al., unpublished manuscript).

Despite the growing knowledge of the candidate regions associated with obligate asexuality, little work has been done on the actual function of candidate genes in these regions, many of which have only been putatively annotated. To further understand the origin of obligate asexuality, it is first necessary to sequence the DNA of the genomic regions associated with obligate asexuality and then examine the phenotypes of these diagnostic alleles. With the sharply reduced cost of next-generation sequencing, it has become possible to sequence the whole genomes of a collection of OP clones across a broad geographic range (e.g., North America) to characterize genotypes associated with OP. Investigation of the phenotypes is also becoming feasible with the development of several cell-biological methodologies in *Daphnia* such as embryonic cell culture (Robinson et al. 2006), electroporation of foreign DNA (Watanabe et al. 2010), and RNAi (Kato et al. 2011). For example, we can perform specific gene silencing using the RNAi technique to better understand the functions of particular genes in ameiotic division of germ line cells, which will ultimately lead to the discovery of the mutations causing the loss of sex and the resultant evolutionary innovations in asexual lineages.

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Table 4.1 Number of individuals sampled (N), mean number of alleles (Na), expected heterozygosity (He), observed heterozygosity (Ho), and the P value of the exact test of Hardy-Weinberg equilibrium for the sampled sites based on 54 microsatellite loci.

Site	Sexuality	N	Na	He	Ho	P
Can 1	asexual	19	3.91	0.62	0.51	<0.0001
	sexual	6	1.13	0.19	0.09	0.0199
Can 1B	asexual	21	3.37	0.54	0.70	<0.0001
Can 2	sexual	10	3.30	0.54	0.26	<0.0001
Can 3	asexual	58	7.07	0.71	0.58	<0.0001
	sexual	70	6.19	0.61	0.45	<0.0001

Table 4.2 Highly diagnostic microsatellite markers based on G_{ST} values for obligate asexuality. Asterisk indicates the markers identified by Lynch et al. (2008) and confirmed in this study.

Marker	Allele	SF asexuals	SS asexuals	Sexuals
Linkage group I				
d039	252	43/50	11/17	0/55
d063	152	53/59	9/21	1/72
d091	279	31/38	4/12	0/65
Linkage group V				
d160*	166	39/45	5/18	0/43
Linkage group VIII				
d117	190	48/52	8/17	0/66
d077	235	28/29	5/8	0/60
Linkage group IX				
d043*	164	44/52	11/17	0/67
9-8*	130	51/63	18/28	0/76
13-5*	200	36/49	9/14	8/57
13-14*	113	59/62	23/25	1/68
99-50*	163	46/59	15/19	2/68
99-52*	163	25/31	8/12	5/61
d118	120	59/60	22/26	6/69
d145	264	26/34	6/14	0/53
Linkage group X				
d005*	302	33/36	6/10	0/57

Supplementary Table S4.1 G_{ST} values for contrasting *Ldh* SS obligately parthenogenetic *Daphnia* individuals and cyclically parthenogenetic individuals.

Marker	G_{ST}
Linkage group I	
d039	0.138
d167	0.029
d170	0.033
d076	0.035
d098	0.066
d096	0.065
d181	0.060
d048	0.055
d193	0.050
d091	0.103
d101	0.049
d103	0.066
d148	0.038
d009	0.067
d163	0.027
d063	0.082
d001	0.066
d188	0.054
d130	0.025
d138	0.066
Linkage group II	
d104	0.059
d070	0.062
Linkage group III	
d049	0.056
Linkage group V	
d160	0.104
Linkage group VI	
d144	0.048
Linkage group VIII	
d077	0.091
d117	0.111
d150	0.055
d068	0.063
d045	0.059
d113	0.063
d165	0.018
d192	0.030

d065	0.062
d121	0.074
Linkage group IX	
d178	0.045
d171	0.094
d149	0.055
d118	0.312
d088	0.049
d043	0.084
d011	0.134
13-5	0.158
9-3	0.097
9-42	0.042
13-48	0.0162
9-8	0.180
9-41	0.036
13-14	0.155
99-50	0.278
d145	0.094
99-52	0.265
Linkage group X	
d005	0.104

Supplementary Table S4.2 G_{ST} values for contrasting *Ldh* SF obligately parthenogenetic *Daphnia* individuals and cyclically parthenogenetic individuals.

Marker	G_{ST}
Linkage group I	
d039	0.215
d167	0.046
d170	0.121
d076	0.169
d098	0.120
d096	0.078
d181	0.139
d048	0.097
d193	0.126
d091	0.338
d101	0.137
d103	0.077
d148	0.166
d009	0.120
d163	0.081
d063	0.220
d001	0.385
d188	0.167
d130	0.091
d138	0.217
Linkage group II	
d104	0.037
d070	0.074
Linkage group III	
d049	0.097
Linkage group V	
d160	0.302
Linkage group VI	
d144	0.122
Linkage group VIII	
d077	0.170
d117	0.217
d150	0.084
d068	0.139
d045	0.116
d113	0.158

d165	0.087
d192	0.050
d065	0.122
d121	0.077
Linkage group IX	
d178	0.057
d171	0.161
d149	0.110
d118	0.443
d088	0.062
d043	0.211
d011	0.134
13-5	0.209
9-3	0.148
9-42	0.066
13-48	0.081
9-8	0.234
9-41	0.075
13-14	0.152
99-50	0.303
d145	0.197
99-52	0.279
Linkage group X	
d005	0.177

Supplementary Table S4.3 Candidate genes within 200-kb window around highly diagnostic microsatellite markers associated with obligate parthenogenesis in *Daphnia*. Gene models derived the JGI model V1.1 are based on homology and EST evidence, available at the JGI Joint Genome Institute website (http://genome.jgi-psf.org/Dappu1/Dappu1.home.html) or at wFleabase.org. Putative functions are based on experimental evidence from orthologous proteins from other animals. Asterisks indicate data compiled from Lynch et al. (2008).

Marker	Gene Model	Genomic Coordinates	Putative Gene Identity	Function
Linkage group I				
d039	321692	scaffold 42: 619281-627536	Transcription factor IIIC box B binding (alpha) subunit	DNA replication, repair, and recombination
d063	303537	scaffold_211: 50751-53545	APC subunit 7	Triggers the transition from metaphase to anaphase by tagging specific proteins for degradation
Linkage group V				
d160	111902*	scaffold 89: 553765-550713	Bunched / Propero	Transcription factor
	255908*	scaffold 89: 319988-321864	Pre-initiation complex. subunit CDC6	DNA replication, meiotic spindle formation

	255909	scaffold 89: 322246- 324815	E3 ubiquitin ligase	spindle formation
Linkage group VIII				
d117	301492	scaffold_136: 247290-249547	Gamma tubulin	microtubule-based movement
d113	263816	scaffold_199: 13944-14267	N/A	Transcription factor
	332621	scaffold_199: 14333-14821	N/A	Transcription factor.
	65078	scaffold_199: 21748-23215	N/A	Transcription factor
Linkage group IX				
d171	388094*	scaffold 9: 784938-780905	RecQ4	DNA helicase
	237228	scaffold_9: 723391-727605	Beta-tubulin folding cofactor D	Microtubule destabilizing protein

	237236	scaffold_9: 746264-747156	SWI-SNF chromatin-remodeling complex protein	
	98365	scaffold_9: 783754-784938	RecQ4	DNA helicase
d043	309028*	scaffold 9: 2240502-2242184	Suppressor of Ty7	Bromodomain transcription factor
	309099*	scaffold 9: 1434540-1437384	Polo kinase	Meiosis: cell cycle, chromosome segregation
	237516*	scaffold 9: 2052925-2057290	Histone tail methylase	Chromatin modification
9-8	98516	scaffold_9: 1777149-1778625	Chromodomain-helicase DNA-binding protein	Chromatin assembly
13-5	301396	scaffold_13: 1815997-1817177	Eco	Maintains cohesion between sister chromatids during DNA replication

	239425	scaffold_13: 1836864-1838305	Heterochromatin-associated protein HP1 and related CHROMO domain proteins	Chromotin assembly and disasembly
99-50/99-52	309445*	scaffold 99: 359023-358355	Chromatin assembly complex protein 1	Chromatin remodeling
	309441	scaffold_99: 215388-216912	HMG box-containing protein	
	328341*	scaffold_99: 273639-274611	TFB1	Nucleotide excision repair factor, DNA repair
d118	98470	scaffold_9: 1452051-1454749	CBF1	Recombination signal binding protein
13-14	314978	scaffold 13: 262990-263929	Mucin 91C	SWI-SNF chromatin-remodeling complex protein
	47502	scaffold 13: 267323-268837	Fen 1	DNA repair
	314983	scaffold 13: 311045-311905	Chromatin remodeling complex WSTF-ISWI. large subunit	DNA replication
d099	223147*	scaffold 13: 1428805-1430623	Pelota	Meiotic cell division

	209823*	scaffold 13: 1466635-1463326	Lissencephaly	Oogenesis, cell cycle
	100009*	scaffold 13: 1398656-1403706	Ovo/bunched	Transcription factor
	301396*	scaffold 13: 1817177-1815997	Esco1 / Ctf7	Sister chromatid cohesion
Linkage group X				
d005	306332*	scaffold 49: 566487-567436	Gonadal protein	Gametogenesis
	249597*	scaffold 49: 596296-616051	CHX / Dsx	Transcription factor
	306333*	scaffold 49: 437808-441321	Vid-21	Chromatin modification, Histone acetylation, DNA repair

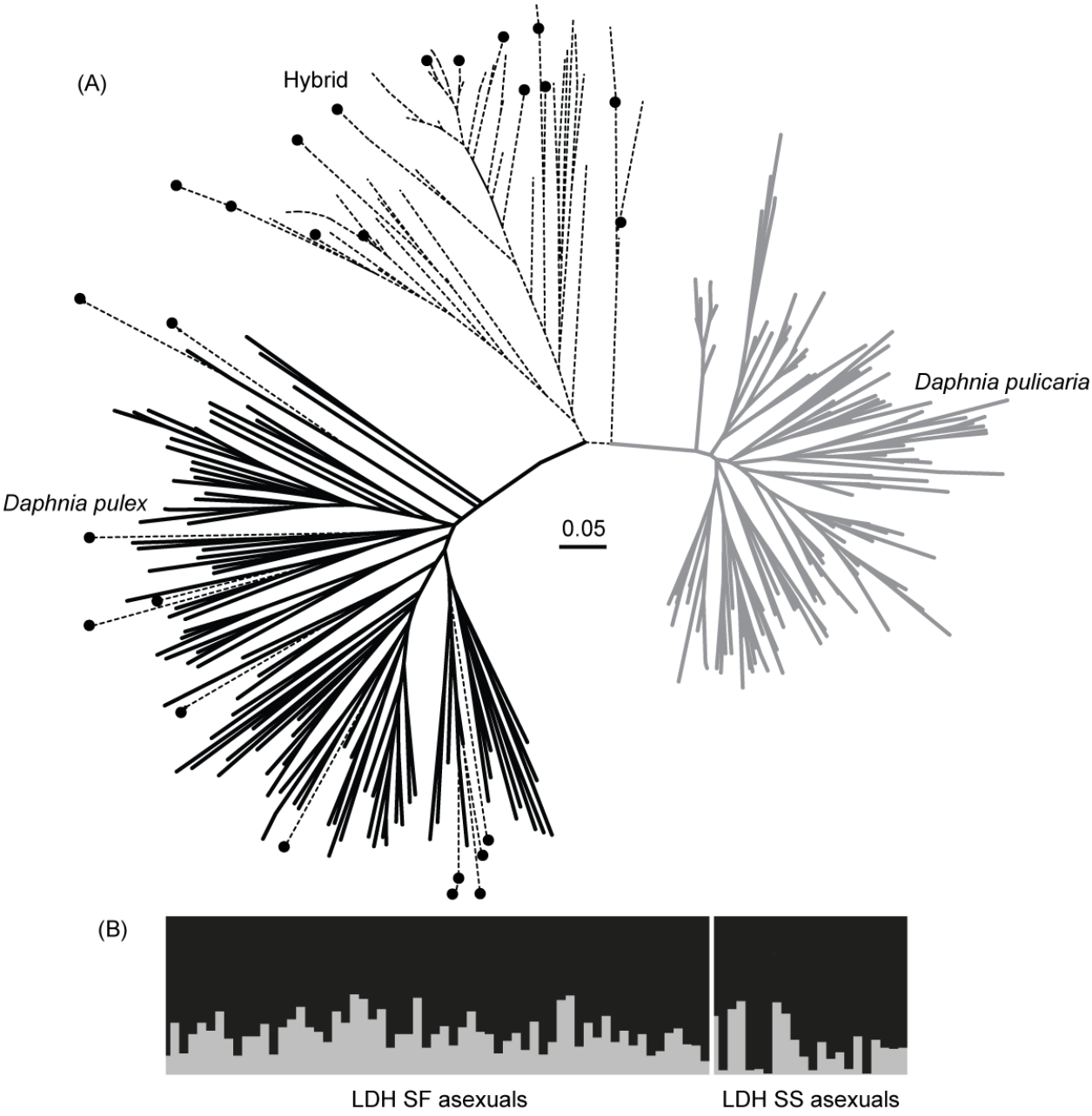
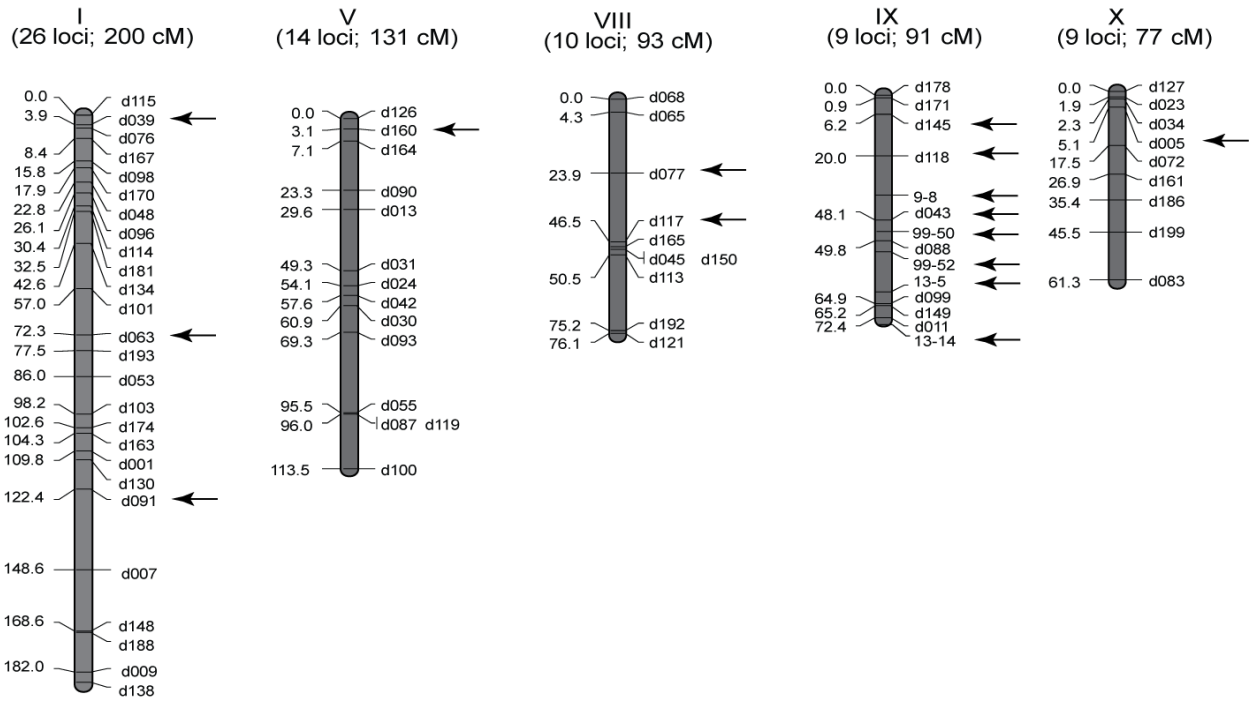


Fig. 4.1. Neighbour-joining (NJ) tree based on nine microsatellite markers (A) and Bayesian estimates of ancestry for asexual *D. pulex* individuals (B) based on the same set of markers. Dashed lines in the NJ tree designate obligately parthenogenetic individuals (both *Ldh* SS and SF), with the black circles designating *Ldh* SS asexual individuals, whereas grey lines and black lines represent cyclically parthenogenetic *D. pulicaria* (lake) and *D. pulex* (pond) individuals, respectively. Each bar of the Bayesian estimates of ancestry represents an individual, with the black and grey colour representing the probability that an individual is derived from a *D. pulex* and *D. pulicaria* genetic background, respectively.



Supplementray Fig. S4.1 Diagnostic microsatellite loci for obligate asexuality on linkage group I, V, VIII, IX, and X. Arrows indicate diagnostic loci. For each linkage group, Kosambi centimorgans are shown on the left, while marker names are shown on the right. Diagnostic markers on linkage group IX (i.e., 9-8, 13-5, 13-14, 99-50, and 99-52) are mapped based on their physical positions without showing map distance because they were not included in the linkage map construction (Cristescu et al. 2006).

CHAPTER 5. GENERAL DISCUSSION

A critical aspect in understanding the evolution of sex is to explain why sexual, rather than asexual, reproduction is dominant in nature, despite the fact that sexual reproduction has been converted to asexual reproduction many times in a vast number of phylogenetic lineages (Bell 1982). Do asexual taxa have some inherent genetic characteristics that will lead to their evolutionary failure in the long term? Negligible amount of recombination is thought to be at least partially responsible for the short evolutionary existence of lineages that have abandoned sex. Although mitotic recombination is an indispensable component of the DNA repair mechanism and of ubiquitous occurrence (Hartl and Jones 2009), it is generally thought that the frequency of these mitotic crossover or gene conversion events are so low that the evolutionary significance is negligible. Absence of segregation and recombination is thus the one of the most distinct characteristic for asexuals compared to sexuals. In theoretical modeling of asexual evolution, the assumption of no recombination is almost universally followed (but see Mandegar and Otto 2007). However, this assumption has been strongly challenged by the work of Omilian et al. (2006) that show ameiotic recombination in asexually reproducing *Daphnia* is on the order of 10^{-4} per locus per generation, which is thousands of times higher than the estimates of mutation rates. As a follow-up work, Chapter 2 of this thesis directly estimates the rate of recombination and large-scale deletions in asexually propagating *Daphnia*. Although ~67% of the Loss of Heterozygosity (LOH) events are due to deletions (i.e. hemizygosity), the rate of ameiotic recombination (crossover and gene conversion) is on the order of 10^{-5} per locus per generation.

These results are very intriguing in a theoretical context. Mitotic/ameiotic recombination (gene conversion and crossover) often result in LOH events. This is similar to the effect of segregation in sexual taxa, which creates the fittest homozygous genotypes via the mating of heterozygous

genotypes. It has been shown that the benefits of segregation could overcome the two-fold cost of sex (Kirkpatrick and Jenkins 1989) by facilitating adaptation. However, in a recent model that incorporates mitotic recombination in asexual taxa, asexuals can adapt as fast as sexuals, especially when beneficial alleles are partially recessive (Mandegar and Otto 2007). This means that, despite the lack of meiosis, asexuals can enjoy the benefits of "segregation" via mitotic recombination. More intriguingly, prior simulation studies have shown that very little sex/recombination is required for most of its evolutionary benefits (Pamilo et al. 1987; Charlesworth et al. 1993; Green and Noakes 1995). For example, Charlesworth et al. (1993) found that with a recombination rate of 10^{-5} per locus per generation, which is the same as the rate of ameiotic recombination in asexually reproducing *Daphnia* (see Chapter 2), the accumulation of deleterious mutations via Muller's ratchet is effectively countered. Moreover, a recombination rate of 10^{-4} per locus per generation can alleviate the selective interference that can increase the fixation probability of deleterious mutations. Considering mitotic/ameiotic recombination is likely common to many asexuals (see Mandegar and Otto 2007), it becomes more puzzling why sex is so common in nature.

It is likely that some important properties of recombination have been overlooked in the current theories on the evolution of sex. Based on the findings of Chapter 2, I suggest that one potential candidate is the mutagenic effects of recombination. Recombination (crossover and gene conversion) is essential for repairing DNA damages (e.g. double strand breaks) that occur during DNA replication in both sexual and asexual reproduction. There is little reason to suggest that closely related sexual and asexual species differ significantly in the amount of DNA damage that occurs in their genomes, unless they show great variation in their intra-cellular mutagenic environment (e.g. amount of oxidative stress) or some DNA repair pathways have been lost due

to mutations. Nonetheless, the way that DNA damage is repaired can be very different in sexual and asexual species. Recombination can be divided into homologous and non-homologous recombination, depending on the template used for DNA repair (Hastings et al. 2009). Homologous recombination occurs when DNA damage is repaired using homologous sequence in the same chromosomal position in the sister chromatids or homologous chromosome, whereas non-homologous recombination involves the correction of DNA damages using homologous sequence from different positions of chromosome (Hastings et al. 2009). For asexual species, although homologous recombination can occur, the possibility of non-homologous recombination is likely much higher. This is likely a consequence of aborting meiosis (e.g. the absence of homologous chromosome pairing) in apomictic parthenogenesis and the increasing divergence between homologous chromosomes that accumulate nucleotide substitutions (Meselson's effect) in the absence of recombination. Non-homologous recombination is known to often result in changes of DNA copy numbers, i.e. duplications and deletions (Hastings et al. 2009). In Chapter 2, I show that 67% of the LOH events in asexually reproducing *Daphnia* are due to deletions and provide preliminary evidence that they might be due to unequal crossover between tandem duplicates. Because the observed deletions cover protein-coding regions and span from 2-30 kb, they probably have deleterious fitness effects. To date, no theory of the evolution of sex has taken into account the mutagenic effects of recombination. Whether the deleterious effects of these mutations arising from recombination events outweigh the benefits associated with recombination in asexuals needs to be rigorously examined in future studies.

The significant amount of recombination in asexual *Daphnia* also has important implications for the evolution of mutation rates in sexuals and asexuals. Similar to the theories of the evolution of sex, the absence of recombination is invariably assumed when investigating the evolutionary

forces regulating the change of mutation rate in asexuals (e.g. Dawson 1998; Johnson 1999). To test the current theories, I directly estimated the mitochondrial DNA (mtDNA) mutation rate in mutation-accumulation (MA) lines of *Daphnia* originated from asexual and sexual ancestors. This is the first attempt to compare mutation rates and spectrum between sexuals and asexuals based on direct estimation. In Chapter 4, I show that no statistically significant difference in mtDNA mutation rate exist between asexual and sexual MA lines. I suggest that future work should examine the role of ameiotic recombination in the evolution of mutation rate in asexuals. Furthermore, although the linkage between mitochondrial and nuclear genomes in asexuals greatly impedes the efficiency of selection in mitochondrial genomes, e.g. 4 times more deleterious mtDNA mutations accumulated in asexual than in sexual *Daphnia* (Paland and Lynch 2006), it remains unclear how the mito-nuclear linkage may affect the mtDNA mutation rate.

MtDNA mutations are also of great interest to biologists because of their extremely wide evolutionary utility and roles in human disease (Taylor and Turnbull 2005). The direct estimates of the mutation rate and spectrum in *Daphnia* are extremely relevant to a broad range of evolutionary biologists using mitochondrial DNA markers to address important evolutionary questions. Furthermore, I also examined other important, but less well understood aspects of mtDNA mutation process, heteroplasmy and the effective population size of mitochondria in *Daphnia*. The estimated small (5-10 copies) effective population size of mitochondrial genomes in a female *Daphnia* indicates a rapid sorting of heteroplasmy in their mitochondrial genomes. However, the extensive heteroplasmy observed in these *Daphnia* MA lines suggests that this is likely an underestimate.

Contagious asexuality (i.e. the spread of asexuality by males carrying meiosis suppressing genes) is a very intriguing evolutionary phenomenon in the sense that it provides an excellent

opportunity to investigate the genes that are responsible for converting sexual reproduction to asexual reproduction. *Daphnia pulex* is one of the first well-established examples of contagious asexuality (Innes and Hebert 1988). Previous work has established that four major genomic regions are responsible for meiosis suppression/obligate parthenogenesis in the *Daphnia* genome (Lynch et al. 2008). Nonetheless, this is only done for asexual *D. pulex* clones with *Ldh* (Lactate Dehydrogenase) slow-slow (SS) genotypes (Lynch et al. 2008). The main reason for excluding asexual *D. pulex* clones with slow-fast (SF) *Ldh* genotypes is that the origin of these *Ldh* SF clones is not well understood, which can impede the search for genes underlying meiosis suppression/obligate parthenogenesis. In Chapter 5, I use a population genetic approach to show that these *Ldh* SF clones originate via the crossing of F1 hybrids of *D. pulex* and *D. pulicaria* with male *D. pulex* carrying meiosis suppressors. Genomic regions on linkage groups V, VIII, IX, and X that previously have been identified to be associated with meiosis suppression/obligate parthenogenesis are confirmed in *Ldh* SF clones. In addition, part of linkage group I also appears to be associated with obligate parthenogenesis. Candidate genes that might be involved in meiosis suppression/obligate parthenogenesis have been proposed. With these data, future work can focus on investigating the specific roles of these candidate genes in obligate parthenogenesis.

The body of work presented in this thesis and previous work (e.g. Paland and Lynch 2006) have shown that *Daphnia* are a wonderful system to study the maintenance of sex. Cyclical parthenogens such as *Daphnia* alternate between asexual and sexual reproduction, enjoying the benefits of both reproductive strategies. As suggested by the "balance argument" (Williams 1975; Maynard Smith 1978), the existence of a sexual phase after several rounds of parthenogenesis suggests that sex must have large short-term evolutionary advantages (e.g. increase in mean offspring fitness). However, these benefits of sex remain elusive to evolutionary biologists after

decades of research, and sex may often prove to be disadvantageous (e.g. Allen and Lynch 2008; Lynch and Deng 1994; Becks and Agrawal 2011). With a large array of molecular tools developed and well characterized whole-genome sequence (Colbourne et al. 2011), *Daphnia* provides a powerful system in empirically uncovering the genetic consequences of asexuality and the short-term evolutionary advantages of sexuality. Future work should fully take advantage of this model system to shed more light on the evolution of sex.

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