# INVESTIGATION OF THE INHERITANCE OF RAPD LOCI IN Daphnia pulex



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# INVESTIGATION OF THE INHERITANCE OF RAPD LOCI IN *DAPHNIA PULEX*

by

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# ABSTRACT

Inheritance and linkage of RAPD (randomly amplified polymorphic DNA) genetic markers were assessed in *Daphnia pulex*. Genetic markers are a useful addition to studies in areas of *Daphnia* biology, such as in investigations of population genetic structure and in estimating levels of gene flow between populations, that have been largely dominated by allozyme analyses. *Daphnia pulex* are freshwater Cladocerans that are ideal organisms for breeding studies because of their cyclical parthenogenetic mode of reproduction. Therefore, the inheritance of the genetic markers, generated using the RAPD molecular technique, can be examined in parent and progeny. In this study, crosses were made among ten clones from two *Daphnia* populations in southern Ontario. The percentage of hatched eggs in the $F_1$ ranged from 0-44.4%, with a survival rate to the production of first brood ranging from 40-100%. Four of the 18 interclonal crosses had sufficient $F_1$ sample sizes for examination using the RAPD technique. Eighty-one RAPD loci, both monomorphic and polymorphic, were scored (present or absent for each individual) and fifty of these loci were unique (some RAPD loci were present in individuals in more than one cross). Eighty-two percent of the RAPD loci were inherited according to the Mendelian segregation ratios of 1:1 or 3:1. Twenty-six of 29 loci conforming to 1:1 ratios were further examined for linkage to each other, but no linkage was found. However, linkage as tight as approximately $r = 0.30$ may not be detected because of small sample size (N =21- 47). Overall, the RAPD technique was successful in generating 46 segregating genetic markers for *Daphnia pulex*. Additional markers that

are shown to be inherited in a Mendelian fashion may lead to detection of a marker linked to a gene of interest in *Daphnia*, such as the meiosis suppressor gene, and in future studies of variation in natural populations of *Daphnia*.

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# CHAPTER ONE

## INTRODUCTION

### 1.1 Molecular Overview

Molecular markers can reveal numerous sites of variation at the DNA sequence level and in many cases this variation is not expressed in the phenotype and may be nothing more than a single nucleotide difference in a gene or a piece of repetitive DNA (Jones et al., 1997). The main advantage of molecular genetic markers is that they are much more numerous than morphological genetic markers (Jones et al., 1997). Genetic markers generated for the Cladoceran, *Daphnia*, would be very useful in a number of areas in *Daphnia* biology that presently pose problems for researchers.

The generation of genetic markers is achieved through standard molecular techniques at the protein and DNA levels, such as allozyme electrophoresis, RFLPs (restriction fragment length polymorphisms), microsatellites, mitochondrial DNA and RAPDs (randomly amplified polymorphic DNA). These markers allow investigators the means to determine population genetic structure in *Daphnia*, as well as estimate levels of gene flow between *Daphnia* populations (Saunders, 1995). Additional genetic markers would contribute to more detailed studies of the clonal structure in *Daphnia* populations than previously studied using allozymes. For example, high clonal diversity over microgeographical scales was encountered in *Daphnia pulex* which exhibits obligate parthenogenesis over much of its range (Hebert and Crease, 1980). The initial surveys

revealed a total of 22 allozyme clones in 11 populations with up to 7 genotypes coexisting in a single pond. More recent studies of *Daphnia pulex* using mitochondrial DNA and allozyme markers revealed many additional clones and demonstrated that obligate parthenogenesis had a polyphyletic origin from cyclical parthenogenesis within this species (Crease et al., 1989; Hebert et al., 1989).

Despite the extensive studies using *Daphnia* species with respect to the variation in its breeding systems and how it has evolved, there remain many unanswered questions surrounding this topic. Specifically, there are two modes of reproduction in *Daphnia*, cyclical and obligate parthenogenesis. The latter incorporates strict asexual reproduction without any recombination (Innes, 1989) while the former involves asexual as well as sexual reproduction and therefore the opportunity exists for recombination. There is an additional aspect of cyclical parthenogenesis, where a population may consist of some clones capable of producing males (male producers - MP), while other clones do not produce males (non-male producers - NMP) (Innes and Dunbrack, 1993). In both forms, the resting eggs produced by cyclically parthenogenetic females will require fertilization by a male.

Some areas for future research of *Daphnia*, using genetic markers, include identification of genetic markers that can be further tested for linkage to genes controlling traits of interest such as the meiosis supressor gene in the obligate parthenogenetic *Daphnia*, the non-male producing gene in cyclical parthenogenetic *Daphnia*, and the identification of a genetic marker linked to recessive deleterious alleles known to be

present in *Daphnia* as a result of inbreeding depression in both obligate and cyclical parthenogenetic *Daphnia* (Innes and Hebert, 1988; Innes and Dunbrack, 1993; Innes, 1989).

The non-male producing capability of a cyclical parthenogen is a fascinating occurrence which has not been studied in great detail, but Innes and Dunbrack (1993) have shown, in their genetic analysis, that sex allocation with respect to the presence or absence of male production has a large genetic component. Such a life history trait would conceivably be better understood if its heritability was thoroughly examined. For example, if a genetic marker was identified as closely linked to the non-male producing gene then sampled clones could be screened for the presence of the non-male producing marker instead of closely examining broods for the presence of males over lengthy periods of time.

The difficulties in understanding the life history variation in *Daphnia*, such as non-male production mentioned above, suggests that a linkage map would be an invaluable tool in further studies of *Daphnia* genetics. Methods for generating numerous markers are becoming more accessible in the production of linkage maps, especially for many plant species that are of agronomic importance. Such a map for *Daphnia* would enable traits to be associated with specific marker loci, allowing for subsequent investigation of traits in breeding studies or trait selection.

An initial exploration of the RAPD (Randomly Amplified Polymorphic DNA) method for generating variable loci was undertaken with *Daphnia pulex*. *Daphnia* is ideal

for this kind of investigation, since matings can be set up between and within clones that are genetically different or identical, respectively. The potential to perform matings allows the determination of whether RAPD marker loci are inherited in any predictable genetic manner from parent to progeny in *Daphnia*, which is essential for their use as genetic markers.

### 1.2 Background and Distribution of *Daphnia*

*Daphnia* are freshwater crustaceans of the order Cladocera. Recent phylogenetic studies of the genus, in North America, have led to the renaming of three subgenera including *Ctenodaphnia*, *Hyalodaphnia* and *Daphnia* (Colbourne and Hebert, 1996). *Daphina* reproduce by parthenogenesis and they are found worldwide. In particular, *D. pulex*, according to Hebert (1995), has a very broad distribution throughout Canada and the United States. In Canada, the northern limit of the distribution is near the boreal forest-tundra transition and is commonly found in all areas south of this, except in the Atlantic provinces. In the United States, *D. pulex* is also quite common except in the states along the Atlantic coast and the southernmost states (Hebert, 1995). *Daphnia pulex* is commonly found in forest ponds, prairie potholes and rock pools and appears to be limited to clear-water habitats (Hebert, 1995). There is great variation among the size of *D. pulex* females, ranging in length from 1.1mm (in ponds) up to 1.7mm (in laboratory) and exceeding upwards of 3mm in the early spring (Hebert, 1995) when investment in parthenogenetic reproduction is probably at its highest.

Throughout Canada, more than 90% of the *Daphnia* in pond habitats are members

of the *pulex* group, and most populations studied reproduce by obligate parthnogenesis, but cyclical parthenogenetic populations appear to persist in the mid-west United States (Hebert, 1987). Since *D. pulex* is the most commonly distributed species throughout North America, it is often misidentified amid other members of the *pulex* group but there are morphological distinctions which help differentiate *D. pulex* from the others (Hebert, 1995). The two species, *D. pulex* and *D. pulicaria*, are by far the most difficult to distinguish and can produce $F_1$ hybrids, therefore additional analysis with allozymes is necessary (Hebert, 1995).

### 1.3 Reproduction in *Daphina*

Typically the life cycle of *Daphnia*, in a temporary pond, will begin in the spring when the pond is replenished with water after the winter season. Female *Daphnia* emerge from protective structures, ephippia, each of which contain two resting eggs that were produced the previous year through sexual reproduction (Figure 1.1A). The females which emerged are referred to as "ex-ephippial females". When these females reach reproductive maturity they will begin to reproduce parthenogenetically. Parthenogenetic eggs are released into the brood chamber, which is the space between the upper side of the body and the dorsal part of the carapace of the female (Zaffagnini, 1987) (Figure 1.1B). These eggs are produced ameiotically (without meiosis), develop immediately and are carried in the brood chamber until they are fully developed. The young swim out of the brood chamber shortly before the females' moult. The parthenogenetic offspring will be genetically identical to the female parent that produced it (Hebert and Ward, 1972;

Lynch 1983, 1984; Hebert, 1987). Adult females continue to release broods of mostly females, increasing the population density, until the conditions in the temporary pond deteriorate. Deteriorating conditions include overcrowding, changes in photoperiod and low food levels in the pond. When these conditions are approaching, female *Daphnia* begin to release broods of males, (Figure 1.1C), with increased frequency and females begin to enter into a sexual stage where they release haploid resting eggs into their brood chamber (Figure 1.1D). After the resting eggs are fertilized by a male, the brood chamber is modified into an ephippium (Hebert, 1980) that is shed through moulting of the female. The ephippial structure and its resting eggs, representing the diapause stage of *Daphnia,* are capable of withstanding freezing and dessication (Zaffagnini, 1987) until favorable conditions return in the spring and diapause is broken. The resting eggs are the primary dispersal stage for *Daphnia* and populations will rely on the production of resting eggs to ensure recruitment from year to year, especially in temporary habitats (Hebert, 1978).

Variations exist in the mode of reproduction in *Daphnia,* consisting of either cyclical or obligate parthenogenesis. The first of two phases for cyclical parthenogens include an asexual reproductive phase, with parthenogenetically produced offspring. The parthenogenetic broods may be either all female or all male, although some mixed broods have been reported (Barker and Hebert, 1986; Zaffagnini, 1987). A sexual phase follows, where females release a haploid resting egg into the brood chamber, fertilized by males. These males may be from the same clone or from a genetically different clone than the female. Therefore, the sexual phase permits an opportunity for genetic recombination in

*Daphnia* populations.

The obligate parthenogenetic individuals have continuous asexual reproduction, which is the main difference from cyclical parthenogens. Both modes of reproduction have similar aspects of life history stages, for example, the release of parthenogenetic offspring by ex-ephippial females. The most striking difference occurs when environmental conditions deteriorate and the obligate female releases diploid resting eggs, as opposed to haploid resting eggs in cyclical parthenogens, into her brood chamber due to suppression of meiosis (Innes and Hebert, 1988). The resting eggs will diapause and develop normally without any genetic contribution from any other individual. The potential for recombination does not exist for the obligate asexuals.

Cyclical parthenogenesis is the dominant and ancestral mode of reproduction in cladoceran crustaceans and the transition has been made by some species to obligate asexuality (Hebert, 1987). The only species in the subgenus *Daphnia*, known to reproduce in this way, are *D. pulex*, *D. pulicaria*, and *D. middendorffiana* (Cerny and Hebert, 1993). Innes and Hebert (1988) have attributed this transition in breeding system to a sex-linked meiosis suppressor of asexuality via interbreeding. The growing evidence of introgression between these species supports the conclusion that the breeding system transitions in all three species owe their origin to the diffusion of a single mutation across species boundaries (Cerny and Hebert, 1993). The breeding system variation in the *Daphnia pulex* group is both dynamically and structurally complex. The exploration of such complexity seems likely to contribute to the understanding of the evolutionary fate

of asexuals (Cerny and Hebert, 1993).

Cyclical and obligate parthenogenetic populations of *Daphnia* are distributed worldwide. The North American populations of *Daphnia*, especially in the Great Lakes area in particular, include individuals reproducing by both obligate and cyclical parthenogenesis (Hebert and Crease, 1980, 1983; Hebert and Loaring, 1986). In the United States and England it has been revealed that a higher incidence of cyclical parthenogens exits (Lynch, 1983; Innes et.al, 1986).

### 1.4 Genetic Variation in *Daphnia*

*Daphnia* are considered by some to be parthenogenetically reproducing organisms heading towards an evolutionary dead-end. For example, Grebelnyi (1996) states that the suppression of recombination (parthenogenesis) gives a certain competitive advantage at first and it also decreases the evolutionary potential of a species because of the reduction in diversity. In actual fact, these organisms maintain a high level of variation, heterozygosity and diversity with respect to their genetic composition (Hebert and Crease, 1983; Innes et al., 1986; Mort, 1991). Historically, cyclical parthenogenesis has generated much interest in regards to its effect on patterns of genetic variation in populations in nature (Hebert, 1987).

There is considerable variation among members of the genus *Daphnia* with respect to their life history (Lynch, 1980; Schwartz, 1984), which could give way to intraspecific variation in these characters. Studies that have shown evidence of intraspecific variation in gene control of an array of characters, such as competitive

ability (Loaring and Hebert, 1981; Weider, 1985), temperature tolerance (Carvalho, 1987) and reproductive behaviour (Ferrari and Hebert, 1982). Focal studies then switched to single-locus variation (allozyme) instead of polygenic traits.

Studies with allozyme analysis of genetic variation in natural populations of *D. pulex* have exhibited interesting patterns. Lynch (1983) examined an intermittent pond and discovered that the population was in agreement with Hardy-Weinberg expectations. He suggested that, if composite genotypes within individual clonal groups mate randomly, they will not experience differential selection or introgression from other clonal groups and will have similar timing mechanisms for ephippial hatching. Then, genotype frequencies should be in agreement with Hardy-Weinberg expectations. Similarly, Hebert et al. (1988) found that some pond populations of *D. pulex*, in the Great Lakes watershed, showed genotype frequencies congruent with Hardy-Weinberg expectations and it was therefore assumed that these populations were reproducing via cyclical parthenogensis. In comparison, Hebert et al. (1988) also found that other pond populations of *D. pulex* largely deviated from Hardy-Weinberg expectations and were considered to be reproducing by obligate parthenogenesis.

Hebert (1987) summarized work that had been done on populations of *Daphnia magna*, in England, that showed two distinct types of populations. Those populations, living in intermittent habitats, revealed stable genotypic frequencies that were in good agreement with Hardy-Weinberg expectations and, by contrast, permanent populations showed both rapid shifts in genotypic frequencies and gross deviations from Hardy-

Weinberg expectations. In addition to these results, findings in lake populations, presented by Mort and Wolf (1985,1986), have shown a population structure for lake populations similar to those of intermittent populations of *D. magna*, with the genotypic frequencies remaining fairly stable and ordinarily in Hardy-Weinberg agreement. This result is intuitively unexpected since it would be presumed that lake populations reflect the same genetic patterns as permanent pond populations as opposed to reflecting genetic patterns observed in intermittent ponds. In any case, Hebert (1987) concluded that the genotypic composition of most cladoceran populations closely resembles that of sexually reproducing organisms, and essentially there is a great deal of genetic variation among *Daphnia* populations, contrary to what is expected for an organism reproducing via parthenogenesis.

### 1.5 Molecular Studies in *Daphnia* - RAPD potential

Approaches have now become available that combine molecular and quantitative genetic techniques with ecology and they have led to some interesting findings and generated further questions concerning genetic variation in *Daphnia*. For example, Beaton and Hebert (1988) revealed through DNA quantification studies that there is a high incidence of endopolyloidy in *Daphnia* cells. Hebert (1987) has suggested that this could occur as a direct consequence of genome miniaturization and may reflect the need to amplify certain segments of the genome. In addition, the haploid genome size of members of the *Daphnia* genus represents one of the smallest arthropod genomes (Hebert, 1987) and Cavalier-Smith (1985) has stated that this genome miniaturization is

likely to be the product of selection for a rapid developmental rate.

Other molecular studies include analysis of mitochondrial DNA variation by Crease et al. (1989) that demonstrated a polyphyletic origin for obligate asexuality in *D. pulex*. Crease et al. (1990) also examined the geographic structure of *D. pulex* from the central United States with respect to allozyme and mitochondrial DNA variation. They found that cyclical parthenogens are one of the most extremely subdivided species to date and the population subdivision for the mitchondrial genome increases approximately three times as rapidly with distance as does that for nuclear genes, which is slower then the neutral expectation. Giebler et al (1997) have reconstructed species phylogenetic relationships of four species and four interspecific hybrids in the *Daphnia longispina* complex. Schwenk et al. (1996) similarly identified species and interspecific hybrids within the *Daphnia galeata* complex across Europe, using a combined approach of nuclear randomly amplified DNA (RAPD), mitochondrial restriction fragment length polymorphism analysis and morphology.

Randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McCelland, 1990) is a technique which has been used since the early 1990's in many different applications to study genetic variation. Williams et al. (1990) described a clear and simple process based on the PCR (polymerase chain reaction) amplification of genomic DNA with single 10-mer primers of arbitrary nucleotide sequence. When the primer is short (ten bases) there is a higher probability that, scattered throughout the genome, are small complementary inverted repeats in close proximity to one another to

serve as priming sites (Hadrys et al., 1992). Essentially the genome is scanned by the primers for the small inverted repeats and these intervening DNA segments of variable length are amplified (Hadrys et al.,1992).

The RAPD technique provides a means of revealing polymorphisms in the absence of specific nucleotide sequence information that function as genetic markers [and are detectable upon examination of an agarose gel stained with ethidium bromide]. The polymorphisms revealed by RAPDs are proposed to occur, in some instances, through only a single base change in genomic DNA (Williams et al., 1990), while other sources of polymorphisms may be due to deletions of a priming site, insertions distancing the priming sites too extensively for amplification to occur, or insertions that will change the size of the DNA fragment but still permit its amplification. RAPD markers are often found to be inherited in a Mendelian fashion (Williams et al., 1990). However, inheritance should be tested in each study. RAPD markers are usually dominant because if at least one chromosome has the primer site, a DNA fragment will be amplifed. With dominant markers it is impossible to distinguish whether a fragment of DNA is amplified from a heterozygous locus (one copy) or homozygous locus (two copies) (Lynch and Milligan, 1993). The possibility also exists for detecting co-dominant RAPD markers, observed as differing sized fragments amplified from the same locus, but this occurs rarely (Hadrys et al., 1992).

Over the last decade, the RAPD procedure has been used extensively in studies dealing with gene mapping (Reiter et al., 1992; Sobral and Honeycutt, 1993; Lodhi et al.,

1995; Promboon et al., 1995; Verhaegen and Plomion, 1996; Pessino et al., 1997; Subudhi et al., 1997; Alstrom-Rapaprt et al., 1998;), pedigree analysis (Levitan and Grosberg, 1993; Jones et al., 1994; Frey and Frey, 1995; Bishop et al., 1996), taxonomy issues (Yang and Quiros, 1993; Novy et al., 1994; Van de Ven and McNicol, 1995), quantitative trait loci (Chalmers et al., 1993; Dirlewanger et al., 1996; Verhagen et al., 1997) examining interspecific hybridization (Schwenk et al., 1996), phylogenetic analysis (Smith et al., 1994) and in studying genetic variation in natural populations (Okamura et al., 1993; Fukatsu and Ishikawa, 1994; Plomion et al., 1995; Caccone et al., 1997; D'Amato and Corach, 1997). Additional studies have used RAPDs in combination with other characters when examining genetic variation, such as RAPDs and morphological markers (Kleinhofs et al., 1993; D'ennequin et al., 1997), RAPDs and RFLPs (restriction fragment length polymorphisms) (Jermstad, 1994; Becker and Heun, 1995; Lu et al., 1996; Kaga et al., 1996; Loarce et al., 1996; Nilsson et al., 1997; Jean et al., 1997; Svitashev et al., 1998), RAPDs and mitochondrial DNA (Aagaard et al., 1995; Simon et al., 1996; Dhar et al., 1997), RAPDs and microsatellite markers (Ender et al., 1996; Sun et al., 1997) as well as RAPDs and isozymes (Lin and Ritland, 1996; Corre et al., 1997; Ayres and Ryan, 1997; Buso et al., 1998). These studies have shown that RAPD markers are useful in many applications.

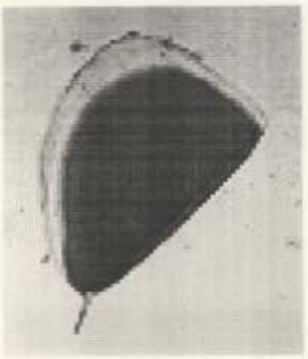
The potential usefulness of RAPDs brings with it some disadvantages and thus some studies over the last few years have felt it important to examine such subject matter. For example, competition occurring in the amplification of all RAPD products was

examined as a source of error in the RAPD analysis by Hallden et al. (1996) and they discovered that some primers and bands are more liable to errors than others. Rieseberg (1996) considered homology among RAPD fragments in interspecific comparisons for three species of wild sunflowers. Penner et al. (1993) attempted reproducibility of RAPD analysis among laboratories, finding that temperature profiles used is largely the determining factor, and the inheritance of RAPD bands was examined using $F_1$ hybrids of corn (Heun and Helentjaris, 1993) and it was found that a majority of the fragments produced are inherited in a Mendelian fashion.

**1.6 Objectives**

The objective of this study was to address the questions of whether loci, generated by the RAPD technique (representing genetic markers), are polymorphic and conform to Mendelian segregation ratios when inherited in *D. pulex* $F_1$ progeny. If this was the case for some loci, then these loci would be examined to determine whether any of the markers were linked to each other. The goal of this research was to determine the overall usefulness of the RAPD technique for generating marker loci, and how many of the loci detected would be useful and feasible in the final analysis of testing for linkage.

**Figure 1.1** Life history stages of *Daphnia pulex*. A) an ephippium, the diapausing stage, B) an asexual female with a pathenogenetic brood, C) a male, and D) a sexual female with two diapausing eggs in the brood chamber which will be molted as an ephippium.



A

B

C

D

# CHAPTER TWO

## METHODS AND MATERIALS

### 2.1 *Daphnia* Sampling

Sample collections were taken from two *D. pulex* populations in southern Ontario in the Spring of 1996. One population was Long Point 8A (LP8A population), a pond in a woodlot surrounded by agricultural land, and the other population was located in a flooded woodlot next to Disputed road (DISP population) (see Innes, 1991 for site location). Collections were taken with a plankton net (~250 μm size) and the *Daphnia* samples placed in plastic containers with their respective pond water.

### 2.2 Maintenance of *Daphnia* cultures

In the laboratory, 96 brood carrying females from each of the two populations were randomly isolated into individual 150mL plastic cups containing artificial zooplankton media (Hebert and Crease, 1980) in order to establish clones. A clone is considered established when the original female has released many healthy parthenogenetic broods. Healthy clones were then transferred into 1000 mL jars and fed approximately 20mL of aquarium-cultured algae (*Ankistrodesmus* and *Scenedesmus*) daily. All clones (from both the DISP and LP8A populations) were checked for male production and their allozyme genotypes were determined at three loci (Pgm, phosphoglucomutase; Pgi, phosphoglucose isomerase; Amy, amylase) according to the methods described in section 2.8.

The detection of males in a clone led to this clone being designated as a male

producer (MP) while the remaining clones were designated non-male producers (NMP's) (Larsson, 1991; Innes and Dunbrack, 1993, Innes, 1997). The clones designated as non-male producers were checked periodically for presence of males over a several month period. The genotypes of each clone were determined for three allozyme loci (Pgm, Pgi, Amy) (Table 2.1) using the methods described in section 2.8.

**2.3 Determination of Suitable Clones for Mating Experiments**

There are two methods by which *Daphnia pulex* clones can reproduce. These methods include cyclical parthenogenesis or obligate parthenogenesis. The intraclonal and interclonal matings cannot be performed with obligately parthenogenetic reproducing clones. Obligate parthenogens can be identified because they release eggs into their ephippia in the absence of males (Innes et al., 1986; Hebert et al., 1989).

Females were chosen from *Daphnia* clones originating from both DISP and LP8A populations. The females were placed individually into conditions known to stimulate them to become sexual (induce them to produce ephippial eggs), such as short day photoperiod (Korpelainen, 1989; Carvalho and Wolf, 1989) and were monitored for the presence of eggs in the ephippium. In the absence of males, if the females formed an ephippia without eggs, then these clones were cyclically parthenogenetic and appropriate for the intraclonal and interclonal mating experiments (Table 2.1).

**2.4 Intraclonal Matings**

Fourteen of the male producing cyclical *D. pulex* clones were chosen for the intraclonal matings. The matings involved females and males from a single healthy clone in

an individual jar (1L) containing artificial zooplankton media. These jars were placed in conditions known to induce male production and ephippial formation, including short day photoperiod (8 L:16 D , 15°C) and crowding (Banta, 1939; Stross and Hill, 1965 & 1968; Korpelainen, 1989; Larrson, 1991; Spaak, 1995; Deng, 1996). The matings were monitored for the production of ephippia and the presence of eggs in the ephippium. Ephippia indicate that the females are sexual and the presence of eggs verify that the females were mating with males from its clone. Thus, the resulting eggs would be the product of an intraclonal mating or of a self mating (Innes, 1989). For each of the fourteen matings, ephippia were collected and a random sample opened and checked for the presence of eggs. Ephippia from every intraclonal mating (~ 6910 total) were placed in an eppendorf tube wrapped in tinfoil and stored in the dark at 4°C for a minimum of four weeks, imitating the photo-refractory phase of the diapause period in *D. pulex* (Stross, 1966, 1969 & 1971).

**2.5 Interclonal Matings**

Eighteen crosses between five non-male producing (NMP) (female parent) and five male producing (MP) (male parent) clones of *D. pulex* were set up in duplicate and triplicate (Table 2.2). The number of replicates depended on availability of sexually mature females from the non-male producing clone and of males present in the male producing clones. A female was designated as suitable for the interclonal mating experiment if its brood chamber was empty and its ovaries were enlarged along the length of the digestive tract. Males were chosen if they were sexually mature (Winsor, 1997). The mating set-up involved isolating 15-30 females from a NMP clone and 10-15 males from a MP clone in a 150 mL cup

containing 50:50 (synthetic zooplankton media: conditioned media). Conditioned media was obtained by pouring off media from jars of healthy *Daphnia pulex* clones through a plankton netting essentially removing all individuals and ephippia, then diluting this media 50 per-cent with synthetic zooplankton media (Hebert and Crease, 1980; Lynch *et al.*, 1986).

All cups containing males and females for each mating were placed under specific conditions known to stimulate the females to become sexual. Such conditions include a short day photoperiod of 8 L: 16 D at a temperature of 15°C and crowding (Banta, 1939; Stross and Hill 1965 & 1968; Stross, 1969; Korpelainen, 1986). Crosses were checked daily for the occurrence of a mating. The female showed early signs of ephippial formation and the resting eggs were visibly present in the brood chamber. These mated females were placed in a separate 150 mL plastic cup with other previously mated females from the same cross. Here the ephippium was shed from the female through molting. At a later date, when the ephippia numbers were sufficiently large from each cross, they were collected. The collected ephippia (~2361 total) were placed in separate eppendorf tubes, wrapped in tinfoil, labeled and stored in the dark for four weeks at 4°C. These conditions have been known to be similar to the photo-refractory phase of diapause in *D. pulex* (Schwartz and Hebert, 1987).

**2.6 Hatching of *Daphnia pulex***

The hatching method was identical for both intraclonal matings and interclonal matings. After four weeks, under simulated diapausal conditions, the experimentally

produced ephippia were removed and opened with dissecting pins, generally releasing two eggs each. Eggs from a single mating were placed in small labeled petri dishes (50 eggs per dish) containing synthetic zooplankton media and kept in an incubator at 15°C, 40mm from a 24 hr light source (40W). These conditions are similar to the photo-stimulatory phase that is known to break diapause for *D. pulex* (Schwartz and Hebert, 1987). Eggs in the petri dishes were checked daily for development and any abnormal eggs were removed. Development to the hatching of a neonate takes an average four to five days. Once the *Daphnia* started to hatch out and were swimming around in the petri dish, these neonates were removed and placed in a 150 mL cup containing zooplankton media and labeled for the specific mating. A single cup would contain hatchlings from each mating, but just prior to the release of a first brood, approximately one week after hatching, each $F_1$ progeny was placed into a separately labeled cup to permit establishment of a single clonal lineage through parthenogenetic reproduction.

### 2.7 The $F_1$ Progeny from Intraclonal and Interclonal Matings

In order to examine the progeny with respect to allozyme and RAPD genotypes, the hatched $F_1$ progeny from the intraclonal and interclonal matings must first reproduce parthenogenetically to establish themselves as a clonal lineage. If an $F_1$ clone did not reproduce parthenogentically, it would be included in the hatching data , but the clone could not be used in further analysis. A number of individuals from each of the $F_1$ clones did establish themselves as a clonal lineage for both the intraclonal and interclonal matings. Samples from each established clone were collected, placed in an eppendorf tube and frozen

at -70°C for later allozyme analysis. Live *Daphnia* from both the intraclonal and interclonal matings were taken for DNA extraction. The production of males by the $F_1$ was noted in the interclonal matings only.

**2.8 Allozyme Characterization**

Both intraclonal and interclonal $F_1$ individuals were examined for one to three allozyme loci (Pgm, Pgi and Amy) using the standard allozyme electrophoresis methods outlined in Hebert and Beaton (1989). Allozyme variation, corresponding to several loci, provided genetic markers for crossing experiments (Innes and Hebert, 1988). Previous crossing experiments and observations on the segregation of these allozymes confirmed the genetic basis of this variation (Innes et al., 1986). Parents of the intraclonal matings must have heterozygous allozyme loci in order for that locus to show segregation when examined electrophoretically. Therefore the segregation expected in the $F_1$ progeny would follow a 1:2:1 Mendelian ratio for a heterozygous clone.

Genotypes of all $F_1$ progeny were determined for matings that would be expected to show segregation based on the genotype of the parents. The allozyme genotypes that were expected to show a 1:1 segregation (heterozygote crossed with a homozygote) in the progeny for each of the interclonal matings were examined using the Chi-square test for independence. If one of these parents, in a particular cross, had a double heterozygous genotype for two allozymes, then the genotypes of the offspring could also be tested for any evidence of linkage.

**2.9 DNA Extraction (Isolation of DNA)**

For RAPD analysis, total genomic DNA from *D. pulex* was isolated by a CTAB (cetyltrimethylammonium bromide) mini-mini prep extraction protocol, a variation of the original procedure from Zolan and Pukkila (1986). Fresh tissue was homogenized in a 2X CTAB Extraction Buffer (1.4 M NaCl, 100 mM TRIS-pH8, 20 mM EDTA, 2% CTAB, 0.2% mercaptoethanol) in a 1.5 microfuge tube. The mixture was incubated at 60°C for at least 45 minutes and then 350µL of Chloroform:Isoamyl alcohol (24:1, vol./vol.) was added. The mixture was centrifuged (13000 rpm, 10 min, 25°C) and the upper aqueous layer containing DNA was transferred to a new tube. The nucleic acid was precipitated by addition of 350µL cold 100% isopropanol at room temperature for 10 minutes, then centrifuged (13000 rpm, 2 min, 25°C) and the supernatant was pipetted off. The pellet was washed twice with 500µL of fresh cold 70% ethanol and centrifuged (13000 rpm, 2 min, 25°C) after each wash. The supernatant was discarded and the pellet dried in a dessicator at 37°C until the ethanol had evaporated. The pellet of DNA was resuspended in 20µL $dH_2O$ either overnight at 4°C, or incubated at 60°C for 10 minutes.

**2.10 PCR Amplification Conditions**

Amplification reactions were performed in volumes of 20µL containing 10X thermophilic buffer (50 mM KCL, 10 mM Tris-HCL pH 9 at 25°C) (Promega); 2mM MgCl; 2 mM each of dCTP, dGTP, dATP, dTTP (Pharmacia Biotech); 0.2 mM of primer (DNA synthesis lab, University of Calgary); 0.12-2.3 ng of genomic DNA (DNA fluorometer, model TKO 100); and 0.6 unit of *Taq* DNA polymerase (Promega). The reaction mixtures

were kept on ice and overlain with a drop of light mineral oil (Sigma). Amplifications were performed in a Perkin Elmer 480 Thermal cycler, preheated to 95°C, and programmed for 42 cycles of 94°C for 1 min; 36°C for 1 min; 72°C, with a ramp of 50 secs, for 1 min; and an autoextension of 3 seconds per cycle; with a final extension of five minutes at 72°C. Eight primers were used in each set of PCR reactions to generate markers for the parental and the $F_1$ offspring of the intraclonal and the interclonal individuals (Table 2.4). At least one negative control tube was included per primer, with each of these PCR amplifications. The control tubes contained the PCR reaction mixture without the addition of genomic DNA.

**2.11 Electrophoresis of PCR Product**

The entire amplification product (20μL) was electrophoresed in 1.2% agarose gels (Sigma) in 0.5X TBE (44.5 mM Tris base, 44.5 mM boric acid, 1 M EDTA pH 8) buffer for approximately 2 hours at 99-101 volts. Two reference standards of a 1 Kb ladder (Life Technologies) were run on each end-well of the agarose gels. Generally the PCR products from each amplification were run on an agarose gel which had two tiers of fourteen wells (24 samples per gel and 4 standard ladders). The resulting PCR samples loaded onto a gel were usually all $F_1$ progeny from a single mating, amplified by a single primer. Following electrophoresis, the RAPD fragments were visualized by staining the gel with ethidium bromide for fifteen minutes, destaining for thirty minutes and photographing the gel under ultraviolet light with 665 Polaroid film. Each photograph contained a single gel with 24 $F_1$ progeny from a single mating that could be scored using the photograph. A limited number

of gels did contain the parents of the cross, although their presence was not crucial for the scoring of the RAPD bands. The scoring of the RAPD loci involved comparing individuals and determining the "presence" or "absence" of a band on a gel. A RAPD locus was defined as a band amplified by a single primer at a specific locality, measured in base pairs, on a gel.

### 2.12 Characterization of the PCR Products in $F_1$ Progeny

The Polaroid photograph of each gel was scanned on a flatbed desk scanner, saved as a "tif" file and analyzed using Pro-RFLP molecular weight software, version 1.37 (DNA Proscan Inc., Nashville, TN). The Pro-RFLP program permits the unknown molecular weight bands (each RAPD locus), generated by RAPD primer, to be scored according to its migration on the gel in alignment with the molecular weight standard. The scheme for the naming of each RAPD locus on the gels includes the number of the primer which amplified the band (locus) followed by the size of the marker in basepairs. For example, 153-748 is a locus that has been amplified by RAPD primer 153 and is 748 basepairs in length.

### 2.13 Analysis of RAPD Loci in the $F_1$ Progeny

It is known that the RAPD markers are dominant and result in identification of only two phenotypes. For example, the genotypes could be designated as PP or PA, resulting in the "presence" of a band at a RAPD locus; and the AA genotype resulting in the "absence" of a band at a RAPD locus.

As a result of meiosis, two alleles at a locus should segregate with equal frequencies into the gametes. If the alleles at one locus are $P_1$ and $A_1$, then the gametes of a diploid heterozygous individual will be half $P_1$ and half $A_1$. Similarly, at a separate locus, alleles $P_2$

and $A_2$ will show equal segregation into gametes.

The two RAPD phenotypes resulting from an interclonal mating are either the "presence" of a band or the "absence" of a band at a RAPD locus. Three different outcomes are expected at each RAPD locus. These outcomes include the presence of a band for all $F_1$ progeny from the crosses: $P_1P_1 \times P_1P_1$, $P_1P_1 \times P_1A_1$ and $P_1P_1 \times A_1A_1$, a 3:1 ratio for the presence of a band for the $F_1$ progeny of the cross $P_1A_1 \times P_1A_1$ and a ratio of 1:1 for the presence of a band for the $F_1$ progeny of the cross $P_1A_1 \times A_1A_1$.

In an individual containing two heterozygous loci ($P_1A_1$ at one locus and $P_2A_2$ at another locus) that are unlinked (on separate chromosomes), the alleles will undergo independent segregation and give four possible gametes. For example, $P_1A_1$ $P_2A_2$ will produce the gametes $P_1P_2$, $A_1P_2$, $P_1A_2$ and $A_1A_2$. When a parent of this genotype is mated with a parent that is homozygous recessive at these two loci, $A_1A_1$ and $A_2A_2$ respectively, then the genotypes can be inferred in the phenotypes of the $F_1$ progeny. This type of cross is referred to as a testcross. The resulting $F_1$ progeny from such a testcross will consist of the following (phenotypes) genotypes:

**Parental generation**: $P_1A_1P_2A_2 \times A_1A_1A_2A_2$

| | | Locus 1 | Locus 2 |
|---|---|---|---|
| **$F_1$ generation**: | $P_1A_1P_2A_2$ | presence | presence |
| | $P_1A_1A_2A_2$ | presence | absence |
| | $A_1A_1P_2A_2$ | absence | presence |
| | $A_1A_1A_2A_2$ | absence | absence |

The four phenotypes will occur in equal frequencies in the progeny if the loci are unlinked. If the loci are linked, the recombinant phenotypes will only arise when crossing over occurs

between the linked loci, and their frequency will be less than 50 %. In order to determine the parental types from recombinant types it has to be determined whether the alleles of each locus are in the coupling or the repulsion phases. For example, if the loci are in the coupling phase then the parental genotypes expected would be: $P_1P_2/A_1A_2$ crossed with $A_1A_1/A_2A_2$. If the loci are in the repulsion phase then the parental genotypes expected would be: $P_1A_2/A_1P_2$ crossed with $A_1A_1A_2A_2$.

**2.14 Analysis**

Hatching data were tested for differences within intraclonal matings and also between the intraclonal and interclonal matings using the F-statistic one-way ANOVA. Male production was examined for a Mendelian mode of inheritance (NMP's crossed with MP's) using a Chi-square statistic (Microsoft Excel, 1994). Each RAPD and allozyme locus was tested for fit to 1:1 and 3:1 ratios using $X^2$ tests. Those loci with an $\alpha$ value greater than the adjusted $\alpha$ value for the 1:1 tests were assumed to conform to Mendelian inheritance and were included in further $X^2$ tests for linkage. The $\alpha$ value was corrected using the Bonferroni approach (Sokal and Rohlf, 1995), where an experimentwise error rate $\alpha$ must be obtained when tests are carried out as suggested by the outcome of the overall analysis. Therefore, each individual test must be carried out at a critical probability of $\alpha' = \alpha/k$ (Bonferroni) where k is the number of intended tests (Sokal and Rohlf, 1995).

**Table 2.1** List of the male producing (MP) and non-male producing (NMP) *Daphnia pulex* clones, collected in the Spring of 1996, from two populations (DISP and LP8A) in southern Ontario, used in the interclonal and/or intraclonal matings.

| | Allozyme Genotypes: | | | | |
|---|---|---|---|---|---|
| **Clone:** | **Pgm** | **Pgi** | **Amy** | **Type:** | **Mating:** |
| DISP 1-1 | MF | SM | FF | MP | Intraclonal |
| DISP 1-4 | MF | SM | FF | MP | Intraclonal |
| DISP 1-7 | FF | MM | FF | MP | Both |
| DISP 2-5 | FF | MM | FF | MP | Intraclonal |
| DISP 2-17 | SM | SS | FF | MP | Both |
| DISP 2-18 | SM | MM | FF | MP | Both |
| DISP 2-15 | SS | SS | FF | NMP | Interclonal |
| DISP 2-14 | MF | SM | FF | NMP | Interclonal |
| DISP 3-27 | FF | MM | SF | NMP | Interclonal |
| LP8A 1-1 | MF | MM | FF | MP | Intraclonal |
| LP8A 1-3 | MF | FF | SS | MP | Intraclonal |
| LP8A 1-7 | MM | SM | SF | MP | Intraclonal |
| LP8A 1-9 | MM | SM | SF | MP | Intraclonal |
| LP8A 1-18 | MM | MF | SF | MP | Intraclonal |
| LP8A 2-11 | MM | MF | SF | MP | Intraclonal |
| LP8A 2-14 | FF | MM | SF | MP | Both |
| LP8A 3-17 | MM | SM | SS | MP | Both |
| LP8A 3-2 | MM | MM | SF | NMP | Interclonal |
| LP8A 2-6 | MM | MM | SF | NMP | Interclonal |

**Table 2.2** List of *Daphnia pulex* interclonal matings, involving non-male producing clones crossed with male producing clones. Clones listed on the left are the non-male producing female parent that are crossed with the male parent from the male producing female clones listed on the right.

| Non-Male Producers (Female parent) : | Male Producers (Male parent) : | Number of Replicates: |
|---|---|---|
| LP8A 2-6 | DISP 1-7 | 3 |
| | DISP 2-17 | 3 |
| | DISP 2-18 | 2 |
| | LP8A 2-14 | 3 |
| LP8A 3-2 | DISP 2-17 | 3 |
| | DISP 1-7 | 3 |
| DISP 2-14 | DISP 1-7 | 3 |
| | DISP 2-17 | 2 |
| | LP8A 2-14 | 3 |
| | LP8A 3-17 | 2 |
| DISP 2-15 | DISP 1-7 | 2 |
| | DISP 2-17 | 2 |
| | DISP 2-18 | 2 |
| | LP8A 2-14 | 3 |
| DISP 3-27 | DISP 1-7 | 2 |
| | DISP 2-17 | 2 |
| | DISP 2-18 | 2 |
| | LP8A 3-17 | 2 |

**Table 2.3** The 10-mer primer sequences used to generate various RAPD loci in the $F_1$ progeny of the interclonal matings .

| PRIMER | SEQUENCE (5' - 3') |
|---|---|
| 34 | CCG GCC CCA A |
| 40 | TTA CCT GGG C |
| 66 | GAG GGC GTG A |
| 115 | TTC CGC GGG C |
| 153 | GAG TCA CGA G |
| 154 | TCC ATG CCG T |
| 169 | ACG ACG TAG G |
| 177 | TCA GGC AGT C |

**Figure 2.1**. An agarose gel stained with ethidium bromide showing the reproducibility of the RAPD bands using RAPD primer #115 during PCR. The 1Kb standard marker (M) is in the far left and right lanes, while the negative control (C) is in lane nine. Lanes onethrough four and five through eight are individuals from two separate clones, DISP 1-7 and LP8A 2-6, respectively. Arrows point to specific bands unique to each of the four individuals from the two clones.

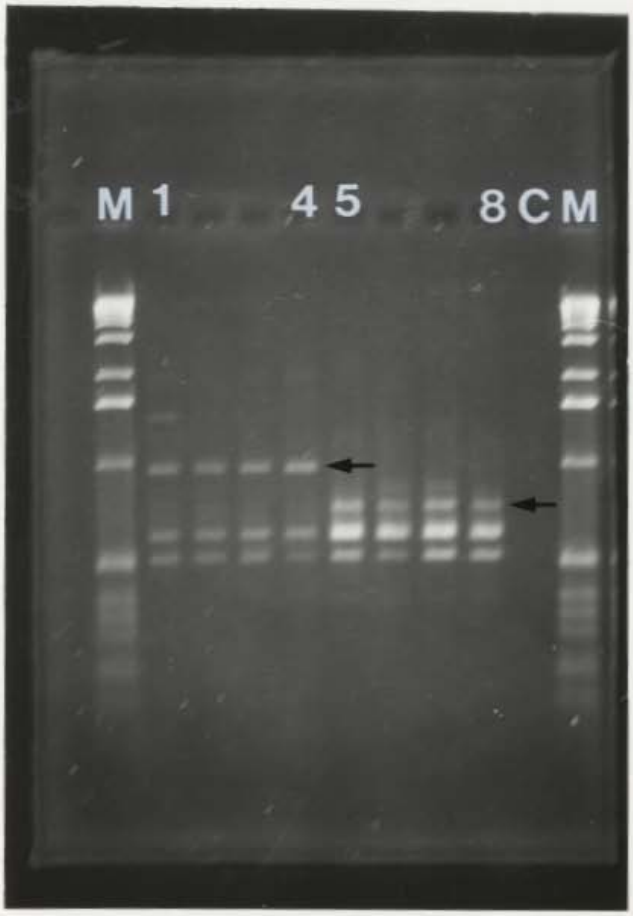
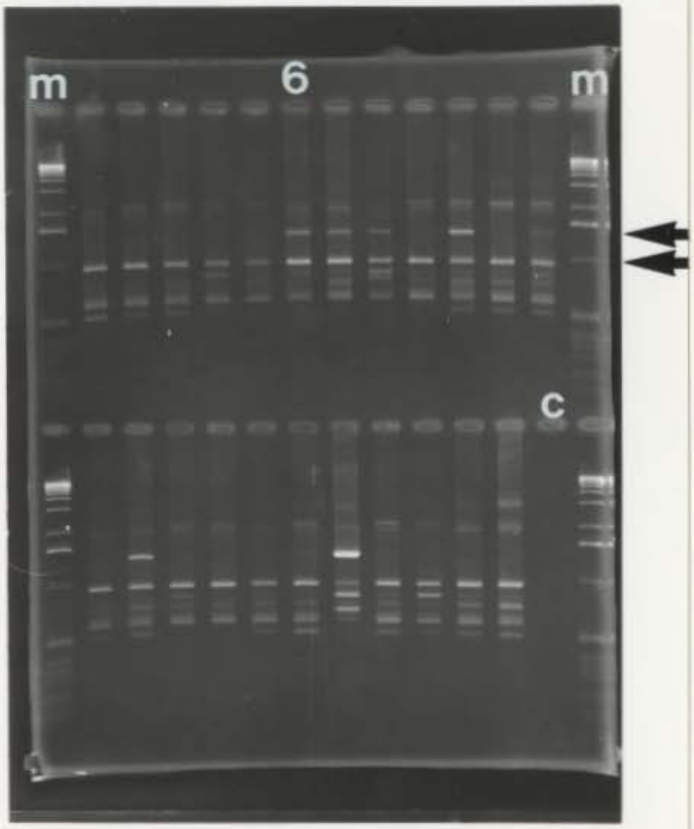

M 1
4 5
8 C M

**Figure 2.2.** A two-tiered agarose gel stained with ethidium bromide showing the polymorphic loci (upper arrow) and monomorphic loci (lower arrow) detectable in the $F_1$ progeny, from the cross of LP8A 2-6 x DISP 1-7, with primer #115. The 1Kb standard marker (m) is present in the far left and right lanes, while the negative control (c) is present in lane 24.


m
6
m
c

# CHAPTER THREE

## RESULTS

### 3.1 Hatching Success

*Daphnia pulex* neonatal hatchings usually occurred within four to five days of exposure to the photo-stimulus. Hatching percentages did not differ between the intraclonal and the interclonal matings (F=0.816, df=1, 30, P=0.374). Hatch rate percentages averaged 20.3% (range: 5.3% - 63.4%) for the intraclonal matings (Table 3.1) and 14.3% (0% - 44.4%) for the interclonal matings (Table 3.2). A comparison of the hatching percentages among the intraclonal matings did not show a significant difference between the DISP and LP8A populations (F=1.412, df =1, 12, P=0.258).

### 3.2 Survival of the $F_1$ Progeny

The mean survival of progeny from the intraclonal matings (9.15% ± SE 2.68) was significantly less than the mean survival of progeny from the interclonal matings (76.4% ± SE 6.78 ), (F=76.44, df =1, 28, P< 0.001) (Table 3.1 and 3.2). Among the intraclonal matings the DISP and LP8A populations did not differ significantly with respect to mean survival (F=0.06, df =1, 12, P =0.81) (Table 3.1) .

### 3.3 Male production in the Progeny of the Interclonal Matings

The $F_1$ progeny from the interclonal non-male producers (NMP) mated with the male producers (MP), were monitored for the production of males. Some $F_1$ progeny in ten of the eighteen matings produced males (Table 3.2). Two of these ten matings, (DISP 3-27 x DISP 1-7 and DISP 2-15 x DISP 2-18), had only 3 and 6 progeny, respectively and were not

considered in any further statistical tests. The percentage of male production ranged from 8.2% - 45.4% (average 24.9%) in the remaining eight matings. The percentage of non-male producing $F_1$ clones in each mating ranged from 33% - 100%, with the average of non-male producing progeny in a cross being 83.4%.

To determine whether male production in the $F_1$ progeny from the interclonal matings had a simple genetic interpretation, the ratio of male producers to non-male producers per cross was tested for 1:1 (NMP:MP) segregation using the Chi-square test for independence, similar to the tests performed by Innes and Dunbrack, 1993. In this study it was found that six out of the eight crosses deviated significantly from the expected 1:1 segregation ratio, (Table 3.3).

### 3.4 Segregation of Enzyme Loci for the Intraclonal $F_1$ Progeny

Four of the eighteen intraclonal matings had sufficient numbers of $F_1$ progeny to test for fit to a 1:2:1 segregation ratio (Table 3.4). The results did not deviate from the expected ratio for any of the intraclonal matings except in clone LP8A 3-17 for the Pgi locus. For this clone all of the $F_1$ progeny were heterozygous and neither of the two expected homozygous genotypes were detected.

### 3.5 Segregation of Enzyme Loci for the Interclonal $F_1$ Progeny

The allozyme genetic markers confirmed the success of the crosses of the NMP x MP. Parents homozygous for different alleles at a locus always produced progeny heterozygous at that locus. Five of the thirteen tests for the interclonal matings deviated significantly from the expected Mendelian ratios ($P< 0.05$), (Table 3.5). Notably, both

matings of DISP 2-15 x DISP 2-18 and LP8A 2-6 x DISP 2-18 were parental crosses with the genotype of SS crossed with SM, respectively, for the Pgm locus. For each of these crosses only one genotype of two classes was detected. The heterozygous genotype was present for the mating of DISP 2-15 x DISP 2-18, and only the homozygous genotype was present for the mating of LP8A 2-6 x DISP 2-18.

Two enzyme loci (Pgm and Pgi) from the interclonal mating of DISP 2-14 x DISP 1-7 were heterozygous in the DISP 2-14 parent and homozygous in the DISP 1-7 parent. These loci did not depart from the 1:1 Mendelian ratio (Table 3.5), therefore these loci could be tested for evidence of linkage. Although the sample size was small (n =15), it was found that the loci did not significantly deviate from the 1:1:1:1 ratio expected for unlinked loci.

### 3.6 RAPD Loci in the $F_1$ Progeny

There were a total of 81 RAPD fragments scored among all the $F_1$ progeny genotypes from four interclonal matings (Tables 3.6-3.9). Each of the 81 RAPD loci can be categorized into one of four groups; 1. band present in both parents that does not show segregation in the progeny, 2. band present in both parents that does show segregation in the progeny, 3. band present in one parent that does show segregation in the progeny and 4. band present in one parent that does not show segregation in the progeny (Table 3.10). Of all 81 RAPD fragments, the total number of segregating loci in the $F_1$ progeny was 56 (69.1% of all RAPD loci scored), the remaining 25 loci were present in one or both parents and all progeny. Fifty of the RAPD loci scored (representing potential genetic markers) were unique among all crosses, with 21 loci represented in two or more separate crosses,

amplified by the same RAPD primer (see Appendix I).

At least one non-segregating locus was amplified with each RAPD primer in each of the interclonal matings. Interestingly, there were only two situations where one parent had a band at a RAPD locus (the other parent did not show a band, in other words the parent was homozygous recessive for that locus - PP x AA) and the presence of that band did not vary among any of the $F_1$ progeny. Among the four interclonal matings, homozygous (non-polymorphic) RAPD loci were amplified for each primer consistently in each cross. Three homozygous loci was the average per primer in the $F_1$, ranging from 1 homozygous locus for primers 40 and 115, up to 6 homozygous loci in primer 153 (Table 3.6 -3.9).

The number of loci that conformed to the 3:1 segregation ratio was 17 of 18 loci (94.4%) (Table 3.11). The number of loci conforming to the 1:1 segregation ratio was 29 out of 38 loci (76.3%) (Table 3.10). Therefore, only loci conforming to genetic ratios could be used to test for linkage. The 29 RAPD loci that conformed to the expected Mendelian ratio of 1:1 ranged from 428 base pairs (bp) up to 1728 bp in length, with an overall average of 906 bp.

### 3.7 Segregation and Linkage Analysis among RAPD Loci

The criterion required to tests for linkage among loci included segregating according to a 1:1 ratio for individual loci and also a cross of a double heterozygote with a double recessive homozygote. This decreases the number of potential tests, despite the overall number of loci initially scored and the number of individual loci conforming to the 1:1 segregation ratios. For example, in one of the four interclonal matings (DISP 2-15 x DISP 2-

17) where two RAPD loci conform to the expected 1:1 Mendelian ratio, these loci could not be used in further linkage tests because the loci were not present appropriately in the parents (Table 3.11).

Twenty-six out of the 29 RAPD loci, which fit the Mendelian ratio of 1:1 for segregation, could be used in analysis for linkage (Table 3.11), resulting in 68 combinations to test for linkage. All 68 combinations originated from only three of the four interclonal matings, LP8A 2-6 x DISP 1-7, LP8A 3-2 x DISP 1-7 and LP8A 2-6 x DISP 2-17, resulting in loci that were possible for usage in linkage tests. The test for linkage involved one of the parents in a single cross having the presence of any two RAPD loci while the other parent has an absence for both loci. The expected progeny would have one of four possible genotypes. If the loci were in coupling phase, then the two parental genotypes would have a presence for both loci or an absence for both loci, while the two recombinant genotypes would consist of a presence for one of the loci and an absence for the other. For example, if the alleles at each of two loci are "P" and "A" and these loci are linked, then the arrangement for alleles on each chromosome in the coupling phase are "$P_1P_2/A_1A_2$" while the arrangement for alleles on chromosomes in the repulsion phase would be "$P_1A_2/P_2A_1$". These arrangements will be reflected in the expected genotypes and in turn the phenotypes, when offspring are examined.

Linkage among loci was tested by determining if each of the four expected genotypes occurred in equal frequencies fitting a 1:1:1:1 ratio with an adjusted $\alpha$ value of 0.00074, which would be the case if there was no linkage (Table 3.12-3.16). Only one of

the 68 pairwise combinations of loci, when tested for linkage, were found to be significant. These loci were 40-855 and 66-666, Table 3.14.

### 3.8 Segregation and Linkage Analysis among RAPD and Enzyme Loci

Testing linkage among RAPD and enzyme loci was similar to the test for linkage among RAPD markers. In a mating, if one parent is heterozygous at a RAPD locus ($P_1A_1$) and also at an enzyme locus, while the other parent is homozygous at the same RAPD and enyme locus, then linkage could be tested. Three enzyme loci from the non-male producing clones crossed with the male producing clones that did not deviate from the expected 1:1 Mendelian ratio could therefore be used in the linkage analysis. All the enzyme and RAPD loci were tested for the goodness of fit to a 1:1:1:1 Mendelian ratio, the $\alpha$ value adjusted to 0.01. None of the five pairwise combinations possible involving the allozyme markers and the RAPD loci showed a significant deviation from the expected ratio (Table 3.17).

**Table 3.1** The hatching, survival to first brood and DNA extracted clones from intraclonal matings of *Daphnia pulex*. Survival to first brood was calculated as a percentage of the number of eggs hatched.

| **Clone:** | **Eggs :** | **Eggs Hatched (%):** | **Survival to first brood %:** |
|---|---|---|---|
| DISP 1-1 | 550 | 62 (11.3) | 16.13 |
| DISP 1-4 | 300 | 21 (7.0) | 0 |
| DISP 1-7 | 1200 | 83 (6.9) | 4.82 |
| DISP 2-5 | 750 | 204 (27.2) | 15.69 |
| DISP 2-17 | 410 | 34 (8.3) | 2.94 |
| DISP 2-18 | 900 | 98 (10.9) | 11.22 |
| LP8A 1-1 | 300 | 59 (19.7) | 0 |
| LP8A 1-3 | 300 | 16 (5.3) | 0 |
| LP8A 1-7 | 500 | 86 (17.2) | 23.26 |
| LP8A 1-9 | 500 | 317 (93.4) | 5.36 |
| LP8A 1-18 | 300 | 29 (9.7) | 0 |
| LP8A 2-11 | 300 | 122 (40.7) | 0 |
| LP8A 2-14 | 300 | 55 (18.3) | 20.0 |
| LP8A 3-17 | 300 | 24 (8.0) | 29.17 |
| **Averages:** | 493.6 | 86.43 (20.26) | 9.15 |

**Table 3.2** Hatching percentages, survival to first brood, male production and the number of clones for which DNA was extracted for the interclonal matings of *Daphnia pulex*. Non-male producing (NMP) clones mated with male-producing (MP) clones. The $F_1$ male production was determined for clones that survived to first brood.

| **Mated clones: (NMP x MP)** | **Eggs:** | **Eggs hatched (%):** | **%Survival to first brood:** | **DNA Extracted # of clones:** | **$F_1$ male-producers** | |
|---|---|---|---|---|---|---|
| | | | | | **MP** | **NMP** |
| LP8A 2-6 x DISP 1-7 | 266 | 79 (29.7) | 92.4 | 73 | 6 | 67 |
| LP8A 2-6 x DISP 2-17 | 313 | 99 (31.6) | 96.0 | 95 | 18 | 77 |
| LP8A 2-6 x DISP 2-18 | 179 | 7 (3.9) | 71.4 | 5 | 0 | 5 |
| LP8A 2-6 x LP8A 2-14 | 256 | 0 (0) | - | - | - | - |
| LP8A 3-2 x DISP 2-17 | 59 | 7 (11.9) | 57.1 | 4 | 0 | 4 |
| LP8A 3-2 x DISP 1-7 | 104 | 30 (28.8) | 90.0 | 27 | 3 | 24 |
| DISP 2-14 x DISP 1-7 | 116 | 23 (19.8) | 100 | 23 | 2 | 21 |
| DISP 2-14 x DISP 2-18 | 96 | 12 (11.5) | 100 | 12 | 1 | 11 |
| DISP 2-14 x LP8A 2-14 | 98 | 2 (2.0) | 100 | 2 | 0 | 2 |
| DISP 2-14 x LP8A 3-17 | 30 | 5 (16.7) | 40.0 | 2 | 0 | 2 |

Table 3.2 continued.

| Mated clones: (NMP x MP) | Eggs: | Eggs hatched (%): | %Survival to first brood: | DNA Extracted # of clones: | $F_1$ male-producers MP | NMP |
|---|---|---|---|---|---|---|
| DISP 2-15 x DISP 1-7 | 234 | 5 (2.1) | 0 | 0 | 0 | 0 |
| DISP 2-15 x DISP 2-17 | 360 | 124 (34.4) | 79.8 | 99 | 45 | 54 |
| DISP 2-15 x DISP 2-18 | 278 | 7 (2.5) | 85.7 | 6 | 1 | 5 |
| DISP 2-15 x LP8A 2-14 | 344 | 26 (7.6) | 69.2 | 18 | 4 | 14 |
| DISP 3-27 x DISP 1-7 | 121 | 5 (4.1) | 60.0 | 3 | 2 | 1 |
| DISP 3-27 x DISP 2-17 | 75 | 5 (6.7) | 80.0 | 4 | 0 | 4 |
| DISP 3-27 x DISP 2-18 | 80 | 0 (0) | - | - | - | - |
| DISP 3-27 x LP8A 3-17 | 63 | 28 (44.4) | 100 | 28 | 12 | 26 |
| **Averages:** | 170.7 | 25.5 (14.3) | 76.35 | 25.06 | 5.88 | 19.81 |

**Table 3.3** The $F_1$ progeny of *Daphnia pulex* (from non-male producing clones mated with male producing clones) tested using the Chi-square statistic to fit a 1:1 Mendelian ratio of NMP's to MP's

| **Cross: (NMPxMP)** | **Observed: (NMP:MP)** | **Expected: (NMP:MP)** | **P-value:** |
|---|---|---|---|
| LP8A 2-6 x DISP 1-7 | 67: 6 | 36.5: 36.5 | <0.001 |
| LP8A 2-6 x DISP 2-17 | 77: 18 | 42.5: 42.5 | <0.001 |
| LP8A 3-2 x DISP 1-7 | 24: 3 | 13.5: 13.5 | <0.001 |
| DISP 2-14 x DISP 1-7 | 21: 2 | 11.5: 11.5 | <0.001 |
| DISP 2-14 x DISP 2-18 | 11: 1 | 6: 6 | <0.005 |
| DISP 2-5 x DISP 2-17 | 45: 54 | 49.5: 49.5 | >0.05 |
| DISP 2-5 x LP8A 2-14 | 4: 22 | 13: 13 | <0.001 |
| DISP 3-27 x LP8A 3-17 | 12: 16 | 14: 14 | >0.05 |

**Table 3.4** Chi-square tests of allozyme genotypes from the $F_1$ progeny of the intraclonal matings.

| Clone: | Locus (genotype): | Number of individuals: | Ratios: | Genotypes: | | | $X^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|
| | | | | SS | SF | FF | | |
| DISP 2-5 | Amy (SF) | 36 | Exp: | 9 | 18 | 9 | 5.944 | 0.0512 |
| | | | Obs: | 7 | 25 | 4 | | |
| LP8A 1-7 | Amy (SF) | 16 | Exp: | 4 | 8 | 4 | 5.375 | 0.0681 |
| | | | Obs: | 5 | 11 | 0 | | |
| | | | | SS | SM | MM | | |
| | Pgm (SM) | 12 | Exp: | 3 | 6 | 3 | 4.000 | 0.1353 |
| | | | Obs: | 0 | 8 | 4 | | |
| LP8A 1-9 | Pgi (SM) | 16 | Exp: | 4 | 8 | 4 | 1.000 | 0.6065 |
| | | | Obs: | 5 | 6 | 5 | | |
| LP8A 3-17 | Pgi (SM) | 7 | Exp: | 1.8 | 3.4 | 1.8 | 7.412 | 0.0246 |
| | | | Obs: | 0 | 7 | 0 | | |

**Table 3.5** Chi-square tests of the resulting allozyme genotypes from the *Daphnia pulex* interclonal matings for deviations from expected Mendelian ratios.

| Cross: (NMPxMP) | Locus (genotype): | Number of individuals: | | Genotypes: | | $X^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| | | | | SS | SM | | |
| DISP 2-15 x DISP 2-17 | Pgm (SS*SM) | 96 | Exp: | 48 | 48 | 1.500 | 0.221 |
| | | | Obs: | 42 | 54 | | |
| DISP 2-15 x DISP 2-18 | Pgm (SS*SM) | 7 | Exp: | 3.5 | 3.5 | 7.000 | 0.008 |
| | | | Obs: | 0 | 7 | | |
| | | | | FF | SF | | |
| DISP 2-15 x LP8A2-14 | Amy (FF*SF) | 15 | Exp: | 7.5 | 7.5 | 8.067 | 0.004 |
| | | | Obs: | 13 | 2 | | |
| DISP 2-14 x DISP 1-7 | Pgm (MF*FF) | 15 | Exp: | 7.5 | 7.5 | 0.600 | 0.439 |
| | | | Obs: | 9 | 2 | | |
| | | | | SM | MM | | |
| | Pgi (SM*MM) | 15 | Exp: | 7.5 | 7.5 | 1.667 | 0.197 |
| | | | Obs: | 5 | 10 | | |
| DISP 2-14 x DISP 2-18 | Pgi (SM*MM) | 10 | Exp: | 5 | 5 | 3.600 | 0.058 |
| | | | Obs: | 8 | 2 | | |
| | | | | MM | SM | | |
| DISP 3-27 x LP8A3-13 | Pgi (MM*SM) | 24 | Exp: | 12 | 12 | 0.667 | 0.414 |
| | | | Obs: | 14 | 10 | | |

Table 3.5 continued.

| Cross: (NMPxMP) | Locus (genotype): | Number of individuals: | | Genotypes: | | $X^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| | | | | SF | SS | | |
| DISP 3-27 x LP8A 3-13 | Amy (SF*SS) | 24 | Exp: | 12 | 12 | 8.167 | 0.004 |
| | | | Obs: | 19 | 5 | | |
| | | | | SF | FF | | |
| LP8A 2-6 x DISP 1-7 | Amy (SF*FF) | 74 | Exp: | 37 | 37 | 0.667 | 0.642 |
| | | | Obs: | 39 | 35 | | |
| | | | | MM | SM | | |
| LP8A 2-6 x DISP 2-17 | Pgm (MM*SM) | 99 | Exp: | 49.5 | 49.5 | 4.455 | 0.035 |
| | | | Obs: | 39 | 60 | | |
| | | | | SF | FF | | |
| | Amy (SF*FF) | 100 | Exp: | 50 | 50 | 1.440 | 0.230 |
| | | | Obs: | 56 | 44 | | |
| | | | | MM | SM | | |
| LP8A 2-6 x DISP2-18 | Pgm (MM*SM) | 5 | Exp: | 2.5 | 2.5 | 5.000 | 0.025 |
| | | | Obs: | 5 | 0 | | |
| | | | | SF | FF | | |
| LP8A 3-2 x DISP 1-7 | Amy (SF*FF) | 27 | Exp: | 13.5 | 13.5 | 1.815 | 0.178 |
| | | | Obs: | 10 | 17 | | |

**Table 3.6** The RAPD loci scored for the interclonal mating of LP8A 2-6 (NMP) crossed with DISP 1-7 (MP). Notation for each locus includes the RAPD primer and the length of the fragment in basepairs; for example, 153-766 means that primer 153 amplified a product that was 766 basepairs long. The allele producing a band is denoted by "P" and the allele that does not produce a band is denoted "A". The adjusted $\alpha = 0.0036$.

| Locus (base pairs) : | Inferred Parental Genotype: | | $F_1$ Progeny Genotype : | $\chi^2$ significance: |
|---|---|---|---|---|
| | LP8A2-6 | DISP1-7 | | |
| 153-1536 | P_ | P_ | 46 P_ | - |
| 153-1018 | P_ | P_ | 46 P_ | - |
| 153-1688 | AA | PA | 14 PA: 32 AA (1:1) | 0.0080 |
| 153-1270 | PA | AA | 34 PA: 12 AA (1:1) | 0.0012* |
| 153-1113 | AA | PA | 31 PA: 15 AA (1:1) | 0.0183 |
| 153-766 | AA | PA | 25 PA: 21 AA (1:1) | 0.5553 |
| 153-656 | AA | PA | 19 PA: 27 AA (1:1) | 0.2382 |
| 153-477 | AA | PA | 34 PA: 12 AA (1:1) | 0.0012* |
| 34-2130 | AA | PP | 47 PA | - |
| 34-656 | P_ | P_ | 47 P_ | - |
| 34-1747 | AA | PA | 13 PA: 34 AA (1:1) | 0.0022* |
| 34-1505 | AA | PA | 21 PA: 26 AA (1:1) | 0.4658 |
| 34-992 | PA | AA | 23 PA: 24 AA (1:1) | 0.8840 |
| 34-727 | AA | PA | 37 PA: 10 AA (1:1) | 0.0000* |
| 34-561 | PA | PA | 34 P_: 13 AA (3:1) | 0.6737 |
| 154-1131 | P_ | P_ | 33 P_ | - |
| 154-616 | AA | PA | 17 PA: 16 AA (1:1) | 0.8618 |

Table 3.6 continued.

| Locus (base pairs) : | Inferred Parental Genotype: LP8A2-6 | DISP1-7 | $F_1$ Progeny Genotype : | $\chi^2$ significance: |
|---|---|---|---|---|
| 66-1681 | P_ | P_ | 44 P_ | - |
| 66-1419 | P_ | P_ | 44 P_ | - |
| 66-666 | AA | PA | 30 PA: 14 AA (1:1) | 0.0159 |
| 66-428 | AA | PA | 24 PA: 20 AA (1:1) | 0.5465 |
| 66-1192 | PA | AA | 29 PA: 15 AA (1:1) | 0.0348 |
| 169-540 | AA | PP | 23 PA | - |
| 169-1192 | PA | PA | 18 P_: 5 AA (3:1) | 0.0324 |
| 169-933 | PA | AA | 13 PA: 10 AA (1:1) | 0.2230 |
| 115-1045 | AA | P P | 45 PA | - |
| 115-1505 | AA | PA | 15 PA: 30 AA (1:1) | 0.0253 |
| 115-880 | AA | PA | 22 PA: 23 AA (1:1) | 0.8815 |
| 115-761 | PA | PA | 34 P_: 11 AA (3:1) | 0.9314 |
| 115-554 | PA | PA | 30 P_: 15 AA (3:1) | 0.1967 |

**Table 3.7** The RAPD loci scored for the interclonal mating of LP8A 2-6 (NMP) crossed with DISP 2-17 (MP). Notation for each locus includes the RAPD primer and the length of the fragment in basepairs; for example, 40-989 means that primer 40 amplified a product that was 989 basepairs long. The allele producing a band is denoted by "P" and the allele that does not produce a band is denoted "A" . The adjusted $\alpha = 0.0042$.

| Locus (base pairs) : | Inferred Parental Genotype: | | $F_1$ Progeny Genotype : | $\chi^2$ significance: |
|---|---|---|---|---|
| | LP8A 2-6 | DISP 2-17 | | |
| 40-1453 | P_ | P_ | 42 P_ | - |
| 40-989 | PA | PA | 28 P_: 14 AA (3:1) | 0.2123 |
| 40-855 | PA | AA | 12 PA: 30 AA (1:1) | 0.0055 |
| 66-1681 | P_ | P_ | 41 P_ | - |
| 66-1419 | P_ | P_ | 41 P_ | - |
| 66-1192 | PA | AA | 30 PA: 11 AA (1:1) | 0.0030* |
| 66-666 | PA | AA | 15 PA: 26 AA (1:1) | 0.0858 |
| 66-533 | PA | PA | 28 P_: 13 AA (3:1) | 0.3213 |
| 34-656 | P_ | P_ | 45 P_ | - |
| 34-992 | PA | PA | 32 P_: 13 AA (3:1) | 0.5469 |
| 34-561 | PA | AA | 30 PA: 15 AA (1:1) | 0.0253 |
| 34-433 | AA | PA | 31 PA: 14 AA (1:1) | 0.0011* |
| 34-2550 | AA | PA | 30 PA: 15 AA (1:1) | 0.0253 |
| 34-2130 | AA | PA | 29 PA: 16 AA (1:1) | 0.0526 |
| 169-540 | AA | PP | 42 PA | - |
| 169-1825 | PA | PA | 15 P_: 27 AA (3:1) | 0.0000* |
| 169-1585 | PA | PA | 36 P_: 6 AA (3:1) | 0.1088 |

**Table 3.8** The RAPD loci scored for the interclonal mating of DISP 2-15 (NMP) crossed with DISP 2-17 (MP). Notation for each locus includes the RAPD primer and the length of the fragment in basepairs; for example, 153-766 means that primer 153 amplified a product that was 766 basepairs long. The allele producing a band is denoted by "P" and the allele that does not produce a band is denoted "A". The adjusted $\alpha = 0.0042$.

| Locus (base pairs) : | Inferred Parental Genotype: DISP 2-15 | DISP 2-17 | $F_1$ Progeny Genotype : | $\chi^2$ significance: |
|---|---|---|---|---|
| 153-1536 | P_ | P_ | 43 P_ | - |
| 153-1018 | P_ | P_ | 43 P_ | - |
| 153-766 | PA | PA | 26 P_: 16 AA (3:1) | 0.0645 |
| 153-656 | PA | PA | 28 P_: 15 AA (3:1) | 0.1345 |
| 153-578 | PA | PA | 25 P_: 18 AA (3:1) | 0.0107 |
| 177-901 | P_ | P_ | 41 P_ | - |
| 177-1346 | PA | AA | 31 PA: 10 AA (1:1) | 0.0010* |
| 177-1230 | PA | PA | 30 P_: 11 AA (3:1) | 0.7868 |
| 177-823 | PA | AA | 20 PA: 21 AA (1:1) | 0.8759 |
| 177-460 | PA | PA | 36 P_: 5 AA (3:1) | 0.0583 |
| 169-1585 | P_ | P_ | 35 P_ | - |
| 169-1825 | PA | PA | 37 P_: 7 AA (3:1) | 0.1660 |
| 169-506 | AA | PA | 38 PA: 8 AA (1:1) | 0.0000* |
| 34-656 | P_ | P_ | 45 P_ | - |
| 34-1747 | AA | PA | 37 PA: 7 AA (1:1) | 0.0000* |
| 34-1534 | AA | PA | 25 PA: 20 AA (1:1) | 0.4561 |
| 34-850 | PA | PA | 34 P_: 11 AA (3:1) | 0.9314 |

**Table 3.9** The RAPD loci scored for the interclonal mating of LP8A 3-2 (NMP) crossed with DISP 1-7 (MP). Notation for each locus includes the RAPD primer and the length of the fragment in basepairs; for example, 154-1728 means that primer 154 amplified a product that was 1728 basepairs long. The allele producing a band is denoted by "P" and the allele that does not produce a band is denoted "A". The adjusted $\alpha = 0.0042$.

| Locus (base pairs) : | Inferred Parental Genotype: | | $F_1$ Progeny Genotype : | $\chi^2$ significance: |
|---|---|---|---|---|
| | LP8A 3-2 | DISP 1-7 | | |
| 154-1131 | P_ | P_ | 18 P_ | - |
| 154-1728 | AA | PA | 10 PA: 8 AA (1:1) | 0.6374 |
| 153-1536 | P_ | P_ | 21 P_ | - |
| 153-1018 | P_ | P_ | 21 P_ | - |
| 153-1192 | PA | AA | 17 PA: 4 AA (1:1) | 0.0046 |
| 153-766 | AA | PA | 13 PA: 8 AA (1:1) | 0.2752 |
| 153-656 | PA | PA | 16 P_: 5 AA (3:1) | 0.8997 |
| 34-992 | P_ | P_ | 22 P_ | - |
| 34-727 | PA | AA | 12 PA: 10 AA (1:1) | 0.6698 |
| 66-1681 | P_ | P_ | 21 P_ | - |
| 66-1419 | P_ | P_ | 21 P_ | - |
| 66-1192 | PA | AA | 13 PA: 8 AA (1:1) | 0.2752 |
| 66-1045 | PA | AA | 8 PA: 13 AA (1:1) | 0.2752 |
| 66-918 | PA | AA | 14 PA: 7 AA (1:1) | 0.1266 |
| 66-666 | AA | PA | 7 PA: 14 AA (1:1) | 0.1266 |
| 66-533 | PA | PA | 18 P_: 3 AA (3:1) | 0.2568 |
| 66-428 | AA | PA | 9 PA: 12 AA (1:1) | 0.5127 |

**Table 3.10** Summary of segregating RAPD loci from the progeny of the interclonal matings.

| Cross: | | Parent Genotypes: | | Loci conforming to ratios: | | | |
|---|---|---|---|---|---|---|---|
| ♀ | ♂ | | | # loci | 1:0 | 1:1 | 1:3 |
| **LP8A 2-6** | **DISP 1-7** | PP | P_ | 6 | 6 | - | - |
| n = 47 | | AA | PA | 17 | - | 13 | - |
| | | AA | PP | 3 | 3 | - | - |
| | | PA | PA | 4 | - | - | 4 |
| **LP8A 2-6** | **DISP 2-17** | PP | P_ | 4 | 4 | - | - |
| n = 45 | | AA | PA | 7 | - | 5 | - |
| | | AA | PP | 1 | 1 | - | - |
| | | PA | PA | 5 | - | - | 4 |
| **DISP 2-15** | **DISP 2-17** | PP | P_ | 5 | 5 | - | - |
| n = 43 | | AA | PA | 5 | - | 2 | - |
| | | AA | PP | 0 | - | - | - |
| | | PA | PA | 7 | - | - | 7 |
| **LP8A 3-2** | **DISP 1-7** | PP | P_ | 6 | 6 | - | - |
| n = 21 | | AA | PA | 9 | - | 9 | - |
| | | AA | PP | 0 | - | - | - |
| | | PA | PA | 2 | - | - | 2 |

**Table 3.11** Summary of all the variable RAPD loci from the interclonal $F_1$ progeny and the proportion of the RAPD loci that could be used in tests for linkage. The segregating loci are those which were present in one or both parents while being absent in some of the $F_1$ progeny.

| CROSS: | Number of segregating loci: (total loci) | Loci conforming to 3:1 ratio: (% of 3:1) | Loci conforming to 1:1 ratio: (% of 1:1) | Tests for linkage possible: | Total number of loci pairs tested for linkage (%): |
|---|---|---|---|---|---|
| LP8A 2-6 x DISP 1-7 | 21 (30) | 4 (100) | 13 (76.5) | 78 | 48 (61.5) |
| LP8A 2-6 x DISP 2-17 | 12 (17) | 4 (80.0) | 5 (71.4) | 10 | 4 (40) |
| DISP2-15 x DISP 2-17 | 12 (17) | 7 (100) | 2 (40.0) | 1 | 0 (0) |
| LP8A 3-2 x DISP 1-7 | 11 (17) | 2 (100) | 9 (100) | 36 | 16 (44.4) |
| Total : | 56 (81) | 17 (94.4) | 29 (76.3) | 125 | 68 (54.4) |

**Table 3.12** Joint segregation analysis for RAPD loci in the DISP 1-7 parent from the mating of DISP 1-7 x LP8A 2-6. The adjusted level of significance is $\alpha = 0.00074$ for linkage.

| Parents: | Locus (bp): | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A2-6 | 153-766 153-656 | PAPA | 12 | 11.5 | 2.572 | 0.471 |
| | | AAPA | 13 | 11.5 | | |
| | | PAAA | 7 | 11.5 | | |
| | | AAAA | 14 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-766 34-1505 | PAPA | 10 | 11.5 | 1.478 | 0.687 |
| | | AAPA | 15 | 11.5 | | |
| | | PAAA | 10 | 11.5 | | |
| | | AAAA | 11 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-766 66-428 | PAPA | 13 | 11 | 0.909 | 0.823 |
| | | AAPA | 9 | 11 | | |
| | | PAAA | 12 | 11 | | |
| | | AAAA | 10 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-766 115-761 | PAPA | 12 | 11 | 0.546 | 0.909 |
| | | AAPA | 12 | 11 | | |
| | | PAAA | 9 | 11 | | |
| | | AAAA | 11 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-656 34-1505 | PAPA | 9 | 11.5 | 2.910 | 0.412 |
| | | AAPA | 9 | 11.5 | | |
| | | PAAA | 12 | 11.5 | | |
| | | AAAA | 16 | 11.5 | | |

Table 3.12 continued

| Parents: | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 153-656 | 66-428 | PAPA | 10 | 11 | 3.091 | 0.378 |
| | | | AAPA | 7 | 11 | | |
| | | | PAAA | 15 | 11 | | |
| | | | AAAA | 12 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-656 | 115-761 | PAPA | 8 | 11 | 1.273 | 0.736 |
| | | | AAPA | 11 | 11 | | |
| | | | PAAA | 13 | 11 | | |
| | | | AAAA | 12 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-1688 | 153-1113 | PAPA | 11 | 11.5 | 12.61 | 0.006 |
| | | | AAPA | 3 | 11.5 | | |
| | | | PAAA | 20 | 11.5 | | |
| | | | AAAA | 12 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-1688 | 153-766 | PAPA | 7 | 11.5 | 8.565 | 0.052 |
| | | | AAPA | 7 | 11.5 | | |
| | | | PAAA | 18 | 11.5 | | |
| | | | AAAA | 14 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-1688 | 153-656 | PAPA | 8 | 11.5 | 14.35 | 0.002 |
| | | | AAPA | 5 | 11.5 | | |
| | | | PAAA | 11 | 11.5 | | |
| | | | AAAA | 22 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-1688 | 34-1505 | PAPA | 9 | 11.5 | 12.09 | 0.007 |
| | | | AAPA | 5 | 11.5 | | |
| | | | PAAA | 11 | 11.5 | | |
| | | | AAAA | 21 | 11.5 | | |

Table 3.12 continued

| Parents: | | | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|---|
| DISP 1-7 | x | LP8A 2-6 | 153-1688 | 154-616 | PAPA | 4 | 8 | 5.000 | 0.191 |
| | | | | | AAPA | 6 | 8 | | |
| | | | | | PAAA | 11 | 8 | | |
| | | | | | AAAA | 11 | 8 | | |
| DISP 1-7 | x | LP8A 2-6 | 153-1688 | 66-666 | PAPA | 8 | 11 | 12.55 | 0.006 |
| | | | | | AAPA | 6 | 11 | | |
| | | | | | PAAA | 21 | 11 | | |
| | | | | | AAAA | 9 | 11 | | |
| DISP 1-7 | x | LP8A 2-6 | 153-1688 | 66-428 | PAPA | 9 | 11 | 6.545 | 0.088 |
| | | | | | AAPA | 5 | 11 | | |
| | | | | | PAAA | 15 | 11 | | |
| | | | | | AAAA | 15 | 11 | | |
| DISP 1-7 | x | LP8A 2-6 | 153-1688 | 115-761 | PAPA | 10 | 10.5 | 9.786 | 0.022 |
| | | | | | AAPA | 4 | 10.5 | | |
| | | | | | PAAA | 12 | 10.5 | | |
| | | | | | AAAA | 18 | 10.5 | | |
| DISP 1-7 | x | LP8A 2-6 | 153-1113 | 153-761 | PAPA | 20 | 11.5 | 9.994 | 0.017 |
| | | | | | AAPA | 10 | 11.5 | | |
| | | | | | PAAA | 5 | 11.5 | | |
| | | | | | AAAA | 11 | 11.5 | | |
| DISP 1-7 | x | LP8A 2-6 | 153-1113 | 153-656 | PAPA | 16 | 11.5 | 8.783 | 0.032 |
| | | | | | AAPA | 14 | 11.5 | | |
| | | | | | PAAA | 3 | 11.5 | | |
| | | | | | AAAA | 13 | 11.5 | | |

Table 3.12 continued

| Parents: | Locus (bp): | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 153-1113 34-1505 | PAPA | 14 | 11.5 | 6.348 | 0.096 |
| | | AAPA | 17 | 11.5 | | |
| | | PAAA | 6 | 11.5 | | |
| | | AAAA | 9 | 11.5 | | |
| DISP 1-7 x LP8A 2-6 | 153-1113 154-616 | PAPA | 12 | 8 | 4.000 | 0.261 |
| | | AAPA | 8 | 8 | | |
| | | PAAA | 4 | 8 | | |
| | | AAAA | 8 | 8 | | |
| DISP 1-7 x LP8A 2-6 | 153-1113 66-666 | PAPA | 21 | 11 | 12.37 | 0.006 |
| | | AAPA | 9 | 11 | | |
| | | PAAA | 7 | 11 | | |
| | | AAAA | 7 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-1113 66-428 | PAPA | 18 | 11 | 7.636 | 0.050 |
| | | AAPA | 12 | 11 | | |
| | | PAAA | 6 | 11 | | |
| | | AAAA | 8 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 153-1113 115-761 | PAPA | 15 | 11 | 5.999 | 0.112 |
| | | AAPA | 15 | 11 | | |
| | | PAAA | 6 | 11 | | |
| | | AAAA | 8 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 34-1505 66-428 | PAPA | 13 | 11 | 3.454 | 0.327 |
| | | AAPA | 6 | 11 | | |
| | | PAAA | 11 | 11 | | |
| | | AAAA | 14 | 11 | | |

Table 3.12 continued

| Parents: | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 154-616 | 153-766 | PAPA | 8 | 8 | 0.222 | 0.969 |
| | | | AAPA | 8 | 8 | | |
| | | | PAAA | 9 | 8 | | |
| | | | AAAA | 7 | 8 | | |
| DISP 1-7 x LP8A 2-6 | 154-616 | 153-656 | PAPA | 3 | 8 | 6.250 | 0.100 |
| | | | AAPA | 13 | 8 | | |
| | | | PAAA | 8 | 8 | | |
| | | | AAAA | 8 | 8 | | |
| DISP 1-7 x LP8A 2-6 | 154-616 | 34-1505 | PAPA | 11 | 8.25 | 1.788 | 0.618 |
| | | | AAPA | 6 | 8.25 | | |
| | | | PAAA | 7 | 8.25 | | |
| | | | AAAA | 9 | 8.25 | | |
| DISP 1-7 x LP8A 2-6 | 154-616 | 115-761 | PAPA | 13 | 8 | 5.750 | 0.124 |
| | | | AAPA | 4 | 8 | | |
| | | | PAAA | 6 | 8 | | |
| | | | AAAA | 9 | 8 | | |
| DISP 1-7 x LP8A 2-6 | 154-616 | 66-428 | PAPA | 11 | 7.5 | 3.333 | 0.343 |
| | | | AAPA | 4 | 7.5 | | |
| | | | PAAA | 7 | 7.5 | | |
| | | | AAAA | 8 | 7.5 | | |
| DISP 1-7 x LP8A 2-6 | 66-666 | 153-766 | PAPA | 13 | 11 | 7.274 | 0.064 |
| | | | AAPA | 9 | 11 | | |
| | | | PAAA | 17 | 11 | | |
| | | | AAAA | 5 | 11 | | |

Table 3.12 continued

| Parents: | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 66-666 | 153-656 | PAPA | 9 | 11 | 13.09 | 0.004 |
| | | | AAPA | 9 | 11 | | |
| | | | PAAA | 21 | 11 | | |
| | | | AAAA | 5 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 66-666 | 34-1505 | PAPA | 17 | 11 | 11.37 | 0.011 |
| | | | AAPA | 2 | 11 | | |
| | | | PAAA | 13 | 11 | | |
| | | | AAAA | 12 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 66-666 | 154-616 | PAPA | 14 | 7.5 | 11.87 | 0.008 |
| | | | AAPA | 1 | 7.5 | | |
| | | | PAAA | 9 | 7.5 | | |
| | | | AAAA | 6 | 7.5 | | |
| DISP 1-7 x LP8A 2-6 | 66-666 | 66-428 | PAPA | 19 | 11 | 10.73 | 0.013 |
| | | | AAPA | 12 | 11 | | |
| | | | PAAA | 4 | 11 | | |
| | | | AAAA | 9 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 66-666 | 115-880 | PAPA | 15 | 11 | 6.545 | 0.088 |
| | | | AAPA | 5 | 11 | | |
| | | | PAAA | 15 | 11 | | |
| | | | AAAA | 9 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 153-761 | PAPA | 10 | 11 | 1.818 | 0.611 |
| | | | AAPA | 12 | 11 | | |
| | | | PAAA | 14 | 11 | | |
| | | | AAAA | 8 | 11 | | |

Table 3.12 continued

| Parents: | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 115-1505 | 153-656 | PAPA | 10 | 11 | 0.909 | 0.823 |
| | | | AAPA | 12 | 11 | | |
| | | | PAAA | 9 | 11 | | |
| | | | AAAA | 13 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 153-1688 | PAPA | 9 | 11 | 7.277 | 0.064 |
| | | | AAPA | 13 | 11 | | |
| | | | PAAA | 5 | 11 | | |
| | | | AAAA | 17 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 153-1113 | PAPA | 19 | 11 | 11.64 | 0.009 |
| | | | AAPA | 3 | 11 | | |
| | | | PAAA | 11 | 11 | | |
| | | | AAAA | 11 | 11 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 34-1505 | PAPA | 11 | 11.25 | 0.422 | 0.936 |
| | | | AAPA | 11 | 11.25 | | |
| | | | PAAA | 10 | 11.25 | | |
| | | | AAAA | 13 | 11.25 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 154-616 | PAPA | 11 | 8 | 2.500 | 0.475 |
| | | | AAPA | 7 | 8 | | |
| | | | PAAA | 5 | 8 | | |
| | | | AAAA | 9 | 8 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 | 66-666 | PAPA | 14 | 10.5 | 4.667 | 0.198 |
| | | | AAPA | 7 | 10.5 | | |
| | | | PAAA | 14 | 10.5 | | |
| | | | AAAA | 7 | 10.5 | | |

Table 3.12 continued

| Parents: | Locus (bp): | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 2-6 | 115-1505 66-428 | PAPA | 15 | 10.5 | 3.333 | 0.343 |
| | | AAPA | 7 | 10.5 | | |
| | | PAAA | 9 | 10.5 | | |
| | | AAAA | 11 | 10.5 | | |
| DISP 1-7 x LP8A 2-6 | 115-1505 115-761 | PAPA | 13 | 11.25 | 1.133 | 0.770 |
| | | AAPA | 10 | 11.25 | | |
| | | PAAA | 9 | 11.25 | | |
| | | AAAA | 13 | 11.25 | | |
| DISP 1-7 x LP8A 2-6 | 115-761 34-1505 | PAPA | 13 | 11.25 | 1.133 | 0.769 |
| | | AAPA | 9 | 11.25 | | |
| | | PAAA | 10 | 11.25 | | |
| | | AAAA | 13 | 11.25 | | |
| DISP 1-7 x LP8A 2-6 | 115-761 66-428 | PAPA | 15 | 11 | 4.238 | 0.087 |
| | | AAPA | 5 | 11 | | |
| | | PAAA | 8 | 11 | | |
| | | AAAA | 14 | 11 | | |

**Table 3.13** Joint segregation analysis for RAPD loci in the LP8A 2-6 parent from the mating of LP8A 2-6 x DISP 1-7. The adjusted level of significance is $\alpha = 0.00074$ for linkage.

| Parents: | | | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|---|
| LP8A2-6 | x | DISP 1-7 | 34-992 | 169-933 | PAPA | 10 | 8.75 | 3.914 | 0.856 |
| | | | | | AAPA | 8 | 8.75 | | |
| | | | | | PAAA | 10 | 8.75 | | |
| | | | | | AAAA | 7 | 8.75 | | |
| LP8A2-6 | x | DISP 1-7 | 66-1192 | 34-992 | PAPA | 18 | 11 | 8.909 | 0.031 |
| | | | | | AAPA | 11 | 11 | | |
| | | | | | PAAA | 4 | 11 | | |
| | | | | | AAAA | 11 | 11 | | |
| LP8A2-6 | x | DISP 1-7 | 66-1192 | 169-933 | PAPA | 15 | 8 | 8.750 | 0.033 |
| | | | | | AAPA | 7 | 8 | | |
| | | | | | PAAA | 4 | 8 | | |
| | | | | | AAAA | 6 | 8 | | |

**Table 3.14** Joint segregation analysis for RAPD loci in the DISP 2-17 parent from the mating of LP8A 2-6 x DISP 2-17 as well as RAPD loci from the LP8A 2-6 parent from the mating of LP8A 2-6 x DISP 2-17. For both parents, the level of significance is $\alpha = 0.00074$ to test for linkage.

| Parents: | | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|
| DISP 2-17 x | LP8A2-6 | 34-2550 | 34-2130 | PAPA | 18 | 11.25 | 7.711 | 0.052 |
| | | | | AAPA | 12 | 11.25 | | |
| | | | | PAAA | 10 | 11.25 | | |
| | | | | AAAA | 5 | 11.25 | | |
| LP8A 2-6 x | DISP 2-17 | 40-855 | 66-666 | PAPA | 8 | 9.25 | 21.27 | 0.000* |
| | | | | AAPA | 3 | 9.25 | | |
| | | | | PAAA | 5 | 9.25 | | |
| | | | | AAAA | 21 | 9.25 | | |
| LP8A 2-6 x | DISP 2-17 | 40-855 | 34-561 | PAPA | 10 | 10.25 | 11.39 | 0.001 |
| | | | | AAPA | 2 | 10.25 | | |
| | | | | PAAA | 17 | 10.25 | | |
| | | | | AAAA | 12 | 10.25 | | |
| LP8A 2-6 x | DISP 2-17 | 34-561 | 66-666 | PAPA | 8 | 10.25 | 7.878 | 0.049 |
| | | | | AAPA | 18 | 10.25 | | |
| | | | | PAAA | 7 | 10.25 | | |
| | | | | AAAA | 8 | 10.25 | | |

**Table 3.15** Joint segregation analysis for RAPD loci in the DISP 1-7 parent from the mating of DISP 1-7 x LP8A 3-2. The adjusted level of significance is $\alpha = 0.00074$ for linkage.

| Parents: | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|
| DISP 1-7 x LP8A 3-2 | 154-1728 | 153-766 | PAPA | 7 | 4.25 | 2.529 | 0.467 |
| | | | AAPA | 3 | 4.25 | | |
| | | | PAAA | 3 | 4.25 | | |
| | | | AAAA | 4 | 4.25 | | |
| DISP 1-7 x LP8A 3-2 | 154-1728 | 66-666 | PAPA | 1 | 4.5 | 5.556 | 0.135 |
| | | | AAPA | 8 | 4.5 | | |
| | | | PAAA | 5 | 4.5 | | |
| | | | AAAA | 4 | 4.5 | | |
| DISP 1-7 x LP8A 3-2 | 154-1728 | 66-428 | PAPA | 7 | 4.5 | 9.111 | 0.028 |
| | | | AAPA | 3 | 4.5 | | |
| | | | PAAA | 0 | 4.5 | | |
| | | | AAAA | 8 | 4.5 | | |
| DISP 1-7 x LP8A 3-2 | 153-766 | 66-666 | PAPA | 4 | 5 | 2.800 | 0.423 |
| | | | AAPA | 8 | 5 | | |
| | | | PAAA | 3 | 5 | | |
| | | | AAAA | 5 | 5 | | |
| DISP 1-7 x LP8A 3-2 | 153-766 | 66-428 | PAPA | 7 | 5 | 3.25 | 0.423 |
| | | | AAPA | 5 | 5 | | |
| | | | PAAA | 2 | 5 | | |
| | | | AAAA | 6 | 5 | | |
| DISP 1-7 x LP8A 3-2 | 66-666 | 66-428 | PAPA | 3 | 5.25 | 2.810 | 0.421 |
| | | | AAPA | 4 | 5.25 | | |
| | | | PAAA | 6 | 5.25 | | |
| | | | AAAA | 8 | 5.25 | | |

**Table 3.16** Joint segregation analysis for RAPD loci in the LP8A 3-2 parent from the mating of LP8A 3-2 x DISP 1-7. The adjusted level of significance is $\alpha = 0.00074$ for linkage.

| Parents: | | | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|---|
| LP8A 3-2 | x | DISP 1-7 | 153-1192 | 34-727 | PAPA | 9 | 5 | 7.600 | 0.055 |
| | | | | | AAPA | 7 | 5 | | |
| | | | | | PAAA | 2 | 5 | | |
| | | | | | AAAA | 2 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 153-1192 | 66-1192 | PAPA | 12 | 5 | 14.80 | 0.002 |
| | | | | | AAPA | 5 | 5 | | |
| | | | | | PAAA | 1 | 5 | | |
| | | | | | AAAA | 2 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 153-1192 | 66-1045 | PAPA | 6 | 5 | 12.40 | 0.006 |
| | | | | | AAPA | 11 | 5 | | |
| | | | | | PAAA | 2 | 5 | | |
| | | | | | AAAA | 1 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 153-1192 | 66-918 | PAPA | 11 | 5 | 11.20 | 0.011 |
| | | | | | AAPA | 5 | 5 | | |
| | | | | | PAAA | 3 | 5 | | |
| | | | | | AAAA | 1 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 34-727 | 66-1192 | PAPA | 7 | 5 | 1.952 | 0.572 |
| | | | | | AAPA | 4 | 5 | | |
| | | | | | PAAA | 6 | 5 | | |
| | | | | | AAAA | 3 | 5 | | |

Table 3.16 continued

| Parents: | | | Locus (bp): | | Progeny: | Observed: | Expected: | $\chi^2$ value: | p-value: |
|---|---|---|---|---|---|---|---|---|---|
| LP8A 3-2 | x | DISP 1-7 | 34-727 | 66-1045 | PAPA | 5 | 5 | 1.200 | 0.753 |
| | | | | | AAPA | 6 | 5 | | |
| | | | | | PAAA | 3 | 5 | | |
| | | | | | AAAA | 6 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 34-727 | 66-918 | PAPA | 7 | 5.25 | 2.429 | 0.488 |
| | | | | | AAPA | 4 | 5.25 | | |
| | | | | | PAAA | 7 | 5.25 | | |
| | | | | | AAAA | 3 | 5.25 | | |
| LP8A 3-2 | x | DISP 1-7 | 66-1192 | 66-1045 | PAPA | 5 | 5 | 2.714 | 0.423 |
| | | | | | AAAP | 8 | 5 | | |
| | | | | | PAAA | 3 | 5 | | |
| | | | | | AAAA | 4 | 5 | | |
| LP8A 3-2 | x | DISP 1-7 | 66-1192 | 66-918 | PAPA | 8 | 5.25 | 3.548 | 0.312 |
| | | | | | AAPA | 5 | 5.25 | | |
| | | | | | PAAA | 6 | 5.25 | | |
| | | | | | AAAA | 2 | 5.25 | | |
| LP8A 3-2 | x | DISP 1-7 | 66-1045 | 66-918 | PAPA | 6 | 5.25 | 3.548 | 0.312 |
| | | | | | AAPA | 2 | 5.25 | | |
| | | | | | PAAA | 8 | 5.25 | | |
| | | | | | AAAA | 5 | 5.25 | | |

**Table 3.17** Tests for linkage among the RAPD fragments and the allozyme markers. Each RAPD and allozyme marker is heterozygous in one parent and homozygous recessive in the other parent at those respective loci. $\chi^2$ tests were performed at the $\alpha$=0.01 significance level.

| Cross: | Primer/ Allozyme: | | Locus (bp/ genotype): | | Progeny genotype: | Observed: | Expected: | $\chi^2$ value: | P-value: |
|---|---|---|---|---|---|---|---|---|---|
| DISP2-17x DISP 2-15 | 34 | Pgm | 1534 | SM | PASM | 9 | 9.75 | 0.282 | 0.963 |
| | | | | | PASS | 9 | 9.75 | | |
| | | | | | AASM | 11 | 9.75 | | |
| | | | | | AASS | 10 | 9.75 | | |
| DISP2-15x DISP 2-17 | 177 | Pgm | 823 | SM | PASM | 7 | 8.75 | 1.457 | 0.692 |
| | | | | | PASS | 7 | 8.75 | | |
| | | | | | AASM | 11 | 8.75 | | |
| | | | | | AASS | 10 | 8.75 | | |
| LP8A2-6 x DISP 1-7 | 34 | Amy | 992 | SF | PASF | 13 | 11.75 | 1.255 | 0.740 |
| | | | | | PASS | 9 | 11.75 | | |
| | | | | | AASF | 11 | 11.75 | | |
| | | | | | AASS | 14 | 11.75 | | |
| LP8A2-6 x DISP 1-7 | 169 | Amy | 933 | SF | PASF | 9 | 9 | 0.889 | 0.700 |
| | | | | | PASS | 11 | 9 | | |
| | | | | | AASF | 10 | 9 | | |
| | | | | | AASS | 6 | 9 | | |
| LP8A2-6 x DISP 2-17 | 66 | Amy | 666 | SF | PASF | 7 | 9.75 | 6.231 | 0.101 |
| | | | | | PASS | 6 | 9.75 | | |
| | | | | | AASF | 16 | 9.75 | | |
| | | | | | AASS | 10 | 9.75 | | |

# CHAPTER FOUR

## DISCUSSION

### 4.1 Hatching of Diapausal Eggs

Within four or five days exposure to photo-stimulus conditions, hatching of *Daphnia pulex* diapausal eggs began in both populations. This finding was similar to those of Schwartz and Hebert (1987), Innes and Hebert (1988), Innes (1989) and Larsson (1991), where it was commonly found that *Daphnia* neonates begin to emerge from eggs after five days at 14 or 21°C. The hatching percentages were similar for both the intraclonal and the interclonal matings under the same conditions, and agreed with Innes and Dunbrack (1993). Schwartz and Hebert (1987), found that there was no difference in hatching requirements between sexual and asexual species and they concluded that, when populations are from the same geographic location, regardless of species or type of reproduction this would be the case.

Hatching of eggs did not occur from two interclonal matings, involving four different clones. The difficulty in hatching resting eggs has undoubtedly been the largest drawback with respect to broad-ranging genetic studies on the Cladocera (Schwartz and Hebert, 1987).

### 4.2 Survival of the $F_1$ clones

Low survival of the intraclonal matings to the $F_1$ clones did not permit investigation of the potential RAPD locus inheritance and furthermore permitted only limited allozyme segregational analysis. In this study there were no differences found in a comparison of the

mean survival of the progeny from the intraclonal matings from either the DISP or the LP8A populations, although significant differences were found when comparing the mean survival of progeny of the intraclonal and interclonal matings. This is in agreement with results found by Innes (1989) in the species *Daphnia obtusa*. He found that the progeny from the interclonal matings had significantly greater survival compared to the progeny from the intraclonal matings for the species. Innes (1989) suggested that the higher survival for the outcrossed (interclonal) progeny compared to the inbred (intraclonal) progeny may indicate the presence of genetic load in his sampled clones of *D. obtusa*. In addition, Innes and Dunbrack (1993) found that, for the *D. pulex* clones studied, the progeny from the intraclonal matings (inbred) had a significantly higher proportion of deaths during development than did progeny from the interclonal (outcrossed) matings, and the average survival was almost twice that of the intraclonal matings. The present study on *D. pulex* supported this with the survival of the intraclonal matings significantly less than interclonal matings, being on the average a 1:8 difference.

Genetic load can be estimated when expressed in terms of lethal equivalents (Morton et al., 1956). The zygote lethal equivalent can be calculated as -4ln (R) where R is equal to the proportion of selfed (intraclonal) progeny divided by the proportion of outcrossed (interclonal) progeny surviving. Innes (1989) found that for *D. obtusa* from four clones which were both selfed and outcrossed that the estimate of lethal equivalents per zygote was 0.9 to 5.2 . The average survivorship of outcrossed and selfed progeny produced an estimate of about 3 lethal equivalents per zygote of *D. obtusa* clones (Innes, 1989). The

data from the Innes and Dunbrack (1993) would yield as an estimate of approximately 3 lethal equivalents per zygote of clones of *D. pulex*, using the calculation by Morton, (1956). In contrast the lethal equivalent estimate calculated for the present study on clones of *D. pulex* would result in an estimate of 6.8 lethal equivalents per zygote. This is much larger than the estimate from the data of Innes and Dunbrack (1993) for *D. pulex* and Innes (1989) for *D. obtusa*. The estimate is also much larger than the range estimated by Innes (1989) for *D. obtusa*. An explanation for this occurrence might be because of the average age of the clones used in this present study. These had been kept in laboratory conditions for close to two years since their original sampling date. Evidence from Banta (1939) and Hebert (1978) suggested that genetic load does exist in natural populations of *Daphnia laevis* and they suggested that an increase in genetic load will result from an increase in the number of continuous parthenogenetic generations, without periods of genetic recombination .

**4.3 Male Production**

Male production in the progeny was observed in half of the interclonal matings between male producers (MP) and non-male producers (NMP). The ratio of NMP to MP was not in equal proportions, but rather the numbers deviated significantly from a 1:1 ratio in all but two crosses. In each cross at least 55 % of the parthenogenetic progeny in the $F_1$ were NMP. This was similar to the percentages found by Innes and Dunbrack (1993), where 58% of the progeny from outcrossed matings were found to be non-male producers.

Innes and Dunbrack (1993) concluded that the ability to produce males was under genetic control in *D. pulex*, but the exact basis of this genetic control was unclear. In their study the occurrence of both types of females (NMP and MP) among the $F_1$ progeny of NMP females ruled out strict cytoplasmic control of male production but was consistent with simple single-gene control. The results of the present study do not support a single-gene basis of genetic control of male production in *D. pulex*. Although the present study was not specifically designed to test the genetic basis of male production, it did provide further insights into variation in male production, and showed it is more complicated than a single-gene control. Innes and Dunbrack (1993), and this study, both provide evidence that there was a significant excess of non-male producing clones in the matings. An explanation provided by Innes and Dunbrack (1993) suggests evidence of variation in the relative investment in male offspring by the parent female or variation in the response of the female parent to conditions favoring male production. Similarly, Larrson (1991) found variation in male production among progeny from a cross between a clone not observed to produce males and a male producing clone. Larrson (1991) found that four of the $F_1$ progeny produced males while two showed no male production when exposed to male-stimulating conditions. These results are similar to those of Innes and Dunbrack (1993) and the present study where the ability to produce males varies among clones of *Daphnia pulex* exposed to the same conditions in the laboratory.

**4.4 Segregation of Enzyme Loci**

Intraclonal and interclonal matings of sexual clones generally did not result in a

distortion of segregating ratios among the progeny. The low survival observed from hatching to the release of first brood for the intraclonal matings might result, in part, from incompatibility of certain genotypic combinations. Genotypic incompatibility in the intraclonal matings would be expressed as deviations in the expected proportion of genotypes at marker loci within each cross, if the incompatibility were linked to the enzyme markers (Innes and Hebert, 1988). $F_1$ progeny from five of the intraclonal matings were examined and only one of these deviated from the expected genetic ratios. Interestingly, the deviation detected in the progeny possessed the heterozygous genotype and none of the other homozygous classes were represented. This could be interpreted as an example of inbreeding depression where there may be a superiority of heterozygotes over homozygotes at individual loci that are affected (Charlesworth and Charlesworth, 1987), but it appears that in the low numbers of progeny examined in this study (and therefore limited information), that for the majority of cases the incompatibility among the genomes involved in these matings did not result in any strong deviations from the expected ratios for the enzyme marker loci (Innes and Hebert, 1988).

Thirteen tests for segregation of alleles in $F_1$ clones from the interclonal matings were examined and there were five which deviated from the normal segregation ratios. Of the five progeny genotypes that deviated, three showed heterozygte excess. This finding is similar to the case from the intraclonal matings where the superiority of heterozygotes over homozygotes at individual loci may have been affected (Charlesworth and Charlesworth, 1987).

There was a single incident where linkage among two enzyme loci could be tested (Pgm and Pgi) from the interclonal mating progeny. There was no significant deviation from expected ratios, all expected genotypes were represented in equal frequencies, although low sample size limits the ability to detect linkage. Unfortunately there were no instances where the Pgm and Amy enzyme loci could be tested for linkage, as Hebert (1985) found evidence for linkage among these two loci for *Daphnia wankeltea* (subgenus Ctenodaphnia), through a selfed seven locus heterozygote and then by observing two-locus associations in the progeny. Hebert (1985) found that only Pgm and Amy were significantly associated with a recombination fraction estimated at 0.11. Innes (1989) also found these loci to be linked in his mating experiments among three pairs of enzyme loci, Got, Amy and Pgm for *D. obtusa*. Recombination estimates between Amy and Pgm were found to be 0.15.

**4.5 Segregation of RAPD loci**

The RAPD technique was successful in generating a total of 81 loci from 8 primers, 46 of which were polymorphic. Therefore, on average, 5.75 polymorphic loci were generated per RAPD primer. This finding is in mid-range when compared to findings of other RAPD studies. For example, on the lower end of the range, the number of polymorphic loci generated per RAPD primer was only 1 for a study involving peach (Chaparro et al., 1994), 1.1 in a study on sweet cherry (Stockinger et al., 1996), and 1.6 in a barley (*Hordeum vulgare* L.) investigation (Tinker et al., 1993). Other studies show average values similar to this study. These include 5.75 RAPD loci per primer for a project involving a shrub, *Haloragodendron lucasii*, (Sydes and Peakall, 1998) and 5.9

loci per primer for *Brassica* (Kresovich et al., 1992). Some studies show greater averages of polymorphic loci per RAPD primer, including a study on *Eucalyptus* revealing 7.5 loci per primer (Verhaegen and Plomion, 1996), a hydrozoan study which had 10 polymorphic loci per primer (Levitan and Grosberg, 1993) and even 12 polymorphic loci per primer were found in a study on the bean plant, *Phaseolus vulgaris* (Bai et al., 1997). Ideally, an investigator, when deciding on a molecular technique to use to generate genetic markers, would be more inclined to choose a technique that revealed a large number of polymorphic loci per primer. Therefore studies that reveal a large number of polymorphisms per primer (as found using the RAPD technique) and have the primer sequences listed that were used, have laid the ground work for other investigators who may wish to take the technique and apply it in a new way when studying that organism or even a closely related one.

The number of fragments that can be theoretically expected from one primer in an amplification reaction with 100% homology, and such a case occurs with RAPDs, can be calculated from primer length and the complexity of the genome, assuming that the nucleotides are present in equal proportions (Weising et al., 1995). Also, Weising et al. (1995) suggest that the distance between both priming sites, when only a single primer is used, should not exceed a few kilobases, since smaller fragments are more efficiently amplified than larger ones. Williams et al. (1993) gave the equation:

$$\mathbf{b = (2000 \times 4^{-2n}) \times C}$$

where **b** is the expected number of fragments per primer, **n** is the primer length in

nucleotides, and **C** is the complexity of the organism (for example, the genome size in base pairs per haploid genome). In this case, *Daphnia*, a crustacean, has an estimated genome size of 1 x $10^6$ Kb (Primrose, 1995), and therefore, 1.82 fragments per 10-nucleotide (RAPD) primer are expected to have 100% homology between primer and the template DNA (all ten bases of the RAPD primer paired with the complementary ten bases of the DNA template). This estimate not only indicates the total expected number of polymorphic bands, but includes monomorphic bands as well. However, it does not take into account the numerous amplified bands that have less than 100% homology.

The result of many investigations, according to Weising et al. (1995), suggest that the number of fragments per primer is in fact largely independent of the genome complexity of the investigated organism and that, for example, plants with large genomes (conifers) did not exhibit more complex RAPD fragment patterns than plants with comparatively small genomes, such as the tomato. It also seems that the ploidy level, at least in plants, does not appear to influence the number of fragments produced per primer (Weising et al., 1995). This independence of RAPD fragment number from genome size may be explained by mismatch and primer competition as suggested by Weising et al. (1995). They state that the outcome of the amplification reaction is determined, in part, by competition for priming sites in the genome, but primers preferably bind to target sites with a higher degree of homology where a higher number is more likely to be available in a genome that is more complex.

The number of polymorphic bands generated per primer is important, but what is

even more crucial is the number of polymorphic bands generated that ultimately conform to expected segregation ratios. In order for a genetic marker, such as a RAPD locus, to be useful, each RAPD locus must show Mendelian inheritance. When using the RAPD technique to generate markers, the ability to perform crosses with the study organism and observe the inheritance of a RAPD marker is an invaluable asset (Avise, 1994; Levitan and Grosberg, 1993). This was the case for the current *D. pulex* study where it was determined from the crosses performed that only 17.86% (10 out of 56) of the polymorphic RAPD loci generated did not conform to expected Mendelian ratios.

This value of 17.86% for *Daphnia* is at the lower end of the values obtained for organisms in other studies (for example, plants) for the percentage of polymorphic loci not conforming to expected segregation ratios. For instance, Echt et al. (1992) studying diploid alfalfa detected 9 out of 37 RAPD loci (24%) that had significant deviations from expected Chi-square values. Stockinger et al. (1996) found a value of 24% in sweet cherry. Ronald et al. (1997) found 29% (9 out of 17) for oat, Nilsson et al. (1997) detected 34% (84 of 244) in sugar beets, and Footlad et al. (1995) found 41% (48 out of 118) in a study of a peach and almond cross. There are studies, however, where low percentages of deviant polymorphic RAPD loci were found. For example, in a study using hydrozoans by Levitan and Grosberg (1993), it was found that only 4 of 133 (3%) of the RAPD loci did not conform to expected segregation ratios, but even more interesting, a study conducted by Tinker et al. (1993) on barley, found that none of the 31 RAPD loci deviated significantly from expected Mendelian ratios.

The overall number of RAPD bands that were found to be polymorphic in the present study was 46 of 56 loci (82%), with the lengths of fragments ranging from 428 - 1728 base pairs. This value is comparable to other studies. For example, in a study on striped bass by Bielawski and Pumo (1997), 31 of 51 (60.8%) of the bands were polymorphic, ranging in size from 160 - 3500 base pairs. An investigation of sugar beet by Nilsson et al. (1997) found 65% of the bands to be polymorphic. Interestingly, Gillies et al. (1997) examined Spanish cedar and discovered that 97 of 106 bands (91.5%) were polymorphic, with the fragments being 300 - 3900 base pairs in size. There are investigations where little or no polymorphisms were found using the RAPD technique. For example, Palacios and Gonzalez-Candelas (1997) examined the rare and endangered *Limonium cavanillesii* and found that 131 fragments, 300 - 3500 base pairs in size, were all monomorphic, showing no variability among samples. Similarly, Mosseler et al. (1992) studied the genetic diversity in red pine (*Pinus resinosa* Ait.) on the island of Newfoundland compared to mainland Canada and discovered that for the 69 RAPD primers used, the species was largely monomorphic.

The analysis of DNA fragment segregation in the progeny is extremely valuable for studies of genetic relatedness For example, Weising et al. (1995) state that in the study of an organism, segregation analysis is regarded as a starting point and only those fragments showing the expected Mendelian inheritance should be included in calculations of relatedness. They go on to state that high levels of linkage between bands may distort the results and therefore such loci should be avoided in studies of genetic relatedness.

Although non-Mendelian segregation ratios have frequently been reported for RAPD fragments, deviations from Mendelian segregation have also been reported for other markers such as RFLPs and allozymes, especially in connection with interspecific crosses, backcrosses, or self-incompatibility genes (Weising et al., 1995).

Heun and Helentjartis (1993), suggested an explanation, specifically for RAPDs, for the occurrence of non-Mendelian segregation. Since the RAPD technique utilizes template DNA and many cycles of amplification, the fragments that are amplified during RAPD analysis probably represent the most successful products among many more competing candidates for amplification. Hence, Heun and Helentjaris (1993) state that one may be concerned that the overall genetic background could determine which candidates are actually amplified and that marker results would only reflect the sequence or annealing at a single locus but be influenced by other regions of the genome. In other words, one might expect that RAPDs would be subject to "epistatic" effects not usually associated with other molecular markers (Heun and Helentjaris, 1993). In this case, a specific fragment, present in two individuals, could be amplified in one genetic background but not in another because of competition from other unlinked sites. Therefore a conclusion that could open to misinterpretation may be drawn from the data concerning this specific locus in both backgrounds (Heun and Helentjaris, 1993).

Hallden et al. (1996) performed a study on competition, occurring in the amplification of all RAPD fragments, as a source of error in RAPD analysis and they found that the overall conclusion that could be drawn was that certain primers and bands

are more liable to errors than others. For example, it was observed that the overall error rate per heterozygous situation was 14%. Therefore, given a randomly chosen heterozygous situation that correctly shows an expected band, the probability that another heterozygous situation for the same primer is correct is 90%, whereas given an observed error, the probability that another heterozygous situation is correct is only 62%. The corresponding probabilities at the band level are 93% and 44%, respectively, which means that the degree of dependence within bands is greater than within primers. Hallden et al. (1996) discovered that the occurrence of genotyping errors increases with the number of polymorphisms. They also found that competition resulting in the generation of errors is not a specific feature of a certain set of PCR conditions. Error frequency seemed to be independent of the type of template DNA and a tenfold change in DNA concentration did not influence the final amount of PCR product under specified amplification conditions. The actual DNA sequence rather than the sequence copy number seems to be the important determinant for successful amplification (Hallden et al., 1996).

Reproducibility and reliability of RAPD bands has been a concern of many investigators. The problems of achieving reliability are usually eliminated once optimal conditions, under which amplifications are efficient and consistent, have been determined for a specific primer and these conditions should be strictly followed (Ellsworth et al., 1993; van Oppen et al., 1996). A study was performed in 1993 that specifically dealt with reproducibility of RAPD bands, where five primers were used in six laboratories in

North America. It was found by Penner et al. (1993), that the reproducibility of results among the laboratories was affected by two factors. First, different labs amplified different size ranges of DNA fragments and therefore small and large polymorphic fragments were not always reproduced. Secondly, Penner et al. (1993), found that although reproducible results were obtained with four primers, reproducible results were not obtained with the fifth primer using the same reaction conditions. Overall, it was suggested that if the temperature profiles (especially the annealing temperature) inside the tubes are identical among the labs, then the RAPD fragments are more likely to be reproducible (Penner et al., 1993).

The polymorphic RAPD loci that did segregate according to Mendelian ratios in *D. pulex* were further examined for linkage to each other. There were a total of 26 loci that could be examined in 68 linkage tests, using the Chi-square test for independence. No linkage was found among the RAPD loci, nor was there any linkage found between the allozyme and RAPD loci. Linkage of some loci was expected since the diploid number of chromosomes is 24 for *D. pulex*. However, because of the small sample size, linkage as tight as $r = 0.30$ (the recombination fraction) could be undetected (Maliepaard et al., 1997).

### 4.7 Summary

This study has provided 46 RAPD loci (representing genetic markers) that can be useful in further studies on *D. pulex*. These loci have been shown to have a Mendelian inheritance, which is of vital importance if these loci are to be further examined as

genetic markers within and among different *Daphnia* clones. The loci are useful as genetic markers, and with addition of other genetic markers, may aid investigators with various aspects of *Daphnia* biology, such as studies of natural variation in populations. In addition, twenty-five monomorphic markers were also scored and can be useful in studies comparing *D. pulex* with other *Daphnia* species.

# CHAPTER FIVE

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# APPENDIX I

Appendix I. List of the RAPD loci scored by each primer among all the *Daphnia pulex* crosses. The RAPD locus is denoted by the primer and the basepair length of the fragment. A locus is characterized as: "N" no segregation at the locus, "S" segregation at the locus according to a genetic ratio, and "S*" segregation at the locus but not according to a genetic ratio.

| RAPD Locus (primer-bases) | LP8A 2-6 x DISP 1-7 | LP8A 2-6 x DISP 2-17 | DISP 2-15 x DISP 2-17 | LP8A 3-2 x DISP 1-7 |
|---|---|---|---|---|
| 153-1536 | N | - | N | N |
| 153-1018 | N | - | N | N |
| 153-1688 | S | - | - | - |
| 153-1270 | S* | - | - | - |
| 153-1113 | S | - | - | - |
| 153-766 | S | - | S | S |
| 153-656 | S | - | S | S |
| 153-477 | S* | - | - | - |
| 153-578 | - | - | S | - |
| 153-1192 | - | - | - | S |
| 34-2130 | N | S | - | - |
| 34-656 | N | N | N | - |
| 34-1747 | S* | - | S* | - |
| 34-1505 | S | - | - | - |
| 34-992 | S | S | - | N |
| 34-727 | S* | - | - | S |
| 34-561 | S | S | - | - |
| 34-433 | - | S* | - | - |
| 34-2550 | - | S | - | - |
| 34-1534 | - | - | S | - |
| 34-850 | - | - | S | - |
| 154-1131 | N | - | - | N |
| 154-616 | S | - | - | - |
| 154-1728 | - | - | - | S |
| 169-540 | N | N | S* | - |
| 169-1192 | S | - | - | - |
| 169-933 | S | - | - | - |
| 169-1825 | - | S* | S | - |
| 169-1585 | - | S | N | - |

Appendix I cont.

| | | | | |
|---|---|---|---|---|
| 66-1681 | N | N | - | N |
| 66-1419 | N | N | - | N |
| 66-666 | S | S | - | S |
| 66-428 | S | - | - | S |
| 66-1192 | S | S* | - | S |
| 66-533 | - | S | - | S |
| 66-1045 | - | - | - | S |
| 66-918 | - | - | - | S |
| 115-1045 | N | - | - | - |
| 115-1505 | S | - | - | - |
| 115-880 | S | - | - | - |
| 115-761 | S | - | - | - |
| 115-554 | S | - | - | - |
| 40-1453 | - | N | - | - |
| 40-989 | - | S | - | - |
| 40-855 | - | S | - | - |
| 177-901 | - | - | N | - |
| 177-1346 | - | - | S* | - |
| 177-1230 | - | - | S | - |
| 177-823 | - | - | S | - |
| 177-460 | - | - | S | - |