MEI-P22 EXPRESSION IN DROSOPHILA MELANOGASTER AND D. ANANASSAE 
MALES AND FEMALES AND ITS RELATIONSHIP TO RATES OF RECOMBINATION

A thesis submitted to the
Kent State University Honors College
in partial fulfillment of the requirements
for University Honors

by
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May, 2011
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ACKNOWLEDGMENTS

It is a pleasure to thank Dr. Andrea Case and Eric Floro for their contributions in completing the experimental work of my thesis. In particular, I would like to express my sincere gratitude to my advisor, Dr. Patrick Lorch, whose guidance and support throughout the project enabled me to not only carry out the thesis, but also to develop a deeper understanding of the subject.

Mahtab A. Tehrani
CHAPTER I
INTRODUCTION

The theory of sexual selection proposed by Darwin was an attempt to explain differences in traits between males and females such as striking secondary sex traits and conspicuous traits that reduce survival (Andersson 1994). Studying these phenotypic differences and their relationship to fitness, behavior and survival has long been of interest to evolutionary biologists. One source of the variation that contributes to the evolution of these sex differences is meiotic recombination, a process that mediates genetic diversity through mixing genes inherited from parents in order to create new allelic arrangements of the genes in the progeny. By modifying rates of recombination, individuals may be able to better adapt to their environment or be more successful at mating. This thesis is an attempt to develop tools that will allow testing of the hypothesis that sexual selection lowers rates of recombination in males in order to make them better at attracting and competing for mates. In order to better understand the potential role of sexual selection and its underlying genetic basis, I begin by explaining some of the background of this theory, as well as an overview of how it may interact with the meiotic recombination process. At the conclusion of this chapter the underlying hypothesis and questions to be answered through the experimental work of this thesis will be explored in greater detail.

Sexual vs. Natural Selection

As stated by Charles Darwin (1859), sexual selection is an evolutionary phenomenon that is based on competition between individuals of one sex for access to the
opposite sex for mating. While natural selection involves competition among individuals over certain resources in their environment, such as food or shelter, sexual selection involves competition over mates. Sexual selection is thus a subset of natural selection, which can cause exaggeration of traits that make bearers more successful at competing for mates.

Individuals of every species are comprised of certain phenotypic traits that have evolved over time in order to help these individuals better adapt to their environments and improve their chances of survival and reproduction. For instance, in certain habitats, characteristics such as possession of longer beaks in birds may enable them to find food more easily than birds with smaller beak size. Thus the long beaked individuals have gained an evolutionary advantage, allowing them to win the competition with the small beaked birds over food. This is an example of how natural selection enables individuals to survive through adaptation. While Darwin's 1859 theory of natural selection seemed to hold true in a majority of cases, it still did not explain the conspicuous male traits that had persisted over generations, even though they seemed to reduce survival of individuals that carried them. Traits such as elaborate tails in male peacocks appeared to be costly to these individuals, as they would decrease the chances of survival by limiting their efficiency in accessing natural resources or escaping predators. In an attempt to solve this apparent inconsistency in his theory, Darwin postulated the theory of sexual selection: “Sexual selection...depends, not on a struggle for existence, but on a struggle between the males for possession of the females...” (Darwin 1859). Therefore, development of particularly attractive phenotypes in one sex, as proposed by Darwin, is the result of the action of sexual selection, which enables the individuals of that sex to be more successful in the mating process, and thus be more likely to produce more offspring.

Meiotic Recombination and the Role of mei-P22 in crossing over
Survival of individuals is ensured by sexual reproduction, the process of uniting an egg and a sperm (gametes) in order to produce a zygote, which has the potential to form a fetus. Gamete formation in Eukaryotes takes place as a result of meiosis, during which the diploid (2n) number of chromosomes is reduced in half, thus producing gametes, each having n chromosomes. The diploid number of chromosomes is later restored as egg and sperm come together to form the zygote. Early in meiosis, homologous chromosomes, each inherited from one parent, can pair up in the cell and exchange segments of DNA in a process known as meiotic recombination. Meiotic recombination serves a very significant evolutionary role. As segments of DNA from parents are exchanged, new allelic combinations form which will result in a unique genetic makeup for the resulting gamete. This phenomenon will contribute to genetic variation, which will further be enhanced by the union of sperm and egg during reproduction.

Like all other cells in an organism, cells committed to differentiating into gametes undergo the regular phases of the cell cycle starting with the initial growth phase. Following this preliminary growth of the cell components, synthesis of new DNA takes place. During this phase the genetic material is replicated. DNA replication will generate an identical copy of each chromosome that will join as sister chromatids at the centromere region. After the cell has successfully passed initial checkpoints and undergone DNA replication, it will undergo a secondary growth phase setting the stage for the cell division process. At this step, somatic cells will normally undergo mitosis, a division resulting in generation of two daughter cells with genetic make-up that is identical to the parent cell. However, this will not be the case in gamete production. Gametes are the outcome of meiosis, which differs from mitosis in that the daughter cells have a genetic make-up distinct from that of the parent cell. This genetic diversity is essential for sexual reproduction and persistence of species (Trivers 1988). In order to mediate such diversity, paired homologous chromosomes, inherited from both parents, undergo a series of
structural changes that will result in generation of new allelic combinations through the process of meiotic recombination described above.

During the Prophase stage of meiosis homologous chromosomes pair up to exchange genetic material. In this process, chromosomes undergo a series of changes in their structure that are mediated by a number of proteins and gene products. The prophase of meiosis itself divided into multiple stages on the basis of the changes made to the chromosomal structure. Formation of Double Strand-Breaks (DSB) on homologous chromosomes has been observed to be correlated with pairing, and these breaks lead to initiation of the recombination process. It is also worth noting that not all pairing processes are DSB-dependent (Mehrotra and McKim 2006). However, both classes of pairing will eventually lead to formation of the Synaptonemal Complex (SC), a large protein structure that lies in between the paired chromosomes and connects them along their lengths to allow for recombination to take place (Page et al. 1998). Repair of DSBs will lead to crossovers between chromosome copies derived from each parent, and these crossovers produce meiotic recombination (Mehrotra and McKim 2006). Several genetic and epigenetic factors are known to have important roles in initiation of DSBs. For example, in Saccharomyces cervisiae, at least 10 gene products are known to play a role in DSB formation during meiosis (Liu et al. 2002). In higher eukaryotes, few homologs of these DSB-inducing genes have been identified. Among these genes is mei-P22. It has been found to play a role in initiation of DSBs in Drosophila melanogaster. Liu et al. (2002) present two lines of evidence for the role of mei-P22: one is a lack of meiotic recombination in mei-P22 mutants and the other is localization of MEI-P22 foci on meiotic chromosomes that suggests this protein may have a close relationship with DSB formation and the SC (Liu et al. 2002). This makes it a good marker of recombination events.
Hypothesis

In 75 species where both sexes experience some recombination, 45 cases show more female than male recombination, 21 cases show more male than female recombination and 9 cases show no overall sex difference (Lorch 2005). Therefore, while males and females both undergo such genetic exchange in most species studied, males tend to have lower rates of recombination. Exceptions to this pattern are generally associated with situations where sexual selection may act less strongly on males (Trivers 1988). In the widely studied fly genus Drosophila, male recombination occurs only in one species, D. ananassae (Matsuda et al. 1993).

This research is part of a broader attempt to test whether sexual selection is causing different rates of recombination in males and females as a result of certain genotypes being associated with more frequent mating (Trivers 1988). For instance, if the possession of longer antlers by a male of a certain species is attractive to a female, and if co-regulation of genes found near each other (cis-regulation) is important to producing long antlers, males with lower rates of recombination will be more likely to deliver their successful DNA sequence intact to their progeny. To test this hypothesis in the broader project, meiotic recombination rates in males and females will be monitored in populations of Drosophila ananassae manipulated to either experience or not experience sexual selection.

In a well-studied and closely related Drosophila species, D. melanogaster, only females undergo recombination during meiosis, while in several strains of D. ananassae, both males and females undergo recombination (Matsuda et al. 1993). Results obtained from this research will allow us to use what is known about recombination in females D. melanogaster to design molecular tools for quantifying recombination in D. ananassae. Ultimately, these tools will be used to compare sex differences in rates of recombination
in laboratory populations of D. ananassae kept for many generations in conditions either with or without sexual selection.

In order to measure sex differences in recombination rates, a way is needed to quantify these rates throughout the experiment. As discussed earlier, the mei-P22 gene is among the essential genes, expressed during meiosis, that allow the crossing over that mediates recombination in D. melanogaster females. Expression of MEI-P22 can be used in future experiments to estimate recombination rates in D. ananassae while using D. melanogaster as a control. Ultimately, this can be accomplished through developing immune stains for this protein so that DSB formation events can be identified and counted in meiotic chromosome squashes. A crucial preliminary step is to demonstrate that male D. ananassae with meiotic recombination express MEI-P22. This is the goal of the project I describe below.

The experimental work of this thesis represents a preliminary stage of the overall project to quantify expression patterns of mei-P22. The thesis work involves two main steps. One is to design PCR primers for mei-P22 and to test them. The second step is to use these primers in an RT-PCR reaction in order to test for expression patterns of mei-P22 in males and females of D. ananassae along with those of D. melanogaster species as a control. The experiment is based on the prediction that mei-P22 expression will be seen in females of both species, along with males of the D. ananassae strains with male recombination. This outcome would support the possibility that male D. ananassae use MEI-P22 to control crossing over, which would allow immunostains for this protein to be used as a marker of recombination for the broader project. If the predicted result is not achieved, it could indicate that male D. ananassae do not use this gene during crossing over, and, in fact, use an entirely different mechanism for recombination control. Either way, the result will be of interest to those studying recombination.
CHAPTER II
MATERIALS AND METHODS

Identification of mei-P22 and Synthesis of Primers

In order to ensure that the mei-P22 sequence was what we expected based on published sequence for mei-P22 (http://www.flybase.org), as well as to test the viability of our primers we began by designing and testing primers on samples of genomic DNA from each species.

The mei-P22 gene sequences for each species were obtained from the FlyBase website, a database of Drosophila genes for the 12 species with sequenced genomes. The mei-P22 gene is 996 base pairs in length and contains no introns (Figure A-1). It is referred to in FlyBase by the symbol Dmel\mei-P22 (CG14827, FBgn0016036) and is a protein-coding gene from D. melanogaster, whose sequence location is listed as 3L:7237488..7238483. The putative homolog of the gene in D. ananassae was obtained using the NCBI (National Center for Biotechnology Information) Nucleotide Blast (nblast) tool. Using this program, the mei-P22 sequence of D. melanogaster was pasted in the search box, the nucleotide collection database was selected, and the program was optimized for “somewhat similar sequences” under the program selection menu. This selection was due to a low degree of homology between the gene sequence in D. melanogaster and its putative homolog in D. ananassae as indicated by the corresponding low maximum identity value of only 71%. The putative homolog for mei-P22 in D. ananassae is referred to by the symbol Dana\GF10572 (GF10572, FBgn0087613). It is a protein-coding gene. Its sequence location is scaffold 13337:14694582..14695553.
After the mei-P22 sequences in both species were obtained, a region of the gene with greatest homology between the two species needed to be identified in order to serve as the binding region for primers. In order to find the region of greatest homology, the ClustalW2 sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the sequences from both D. melanogaster and D. ananassae. Based on the alignment results, the last 252 base pairs of the gene were selected for the primer designing step as this region showed the highest degree of homology among the two species. This region of the gene was selected to design the primers using the NCBI Primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Figure 1 shows the location and orientation of the primers relative to the template sequence of mei-P22. The desired length of the PCR product was set to 200-300 bp, long enough to measure gene expression later. The program also checked that the primers should not bind to other regions to ensure specificity of the primers. The remaining settings were left as the default. The primer pair sequences are shown in Figure A-3.

Figure 1. Relative Location of mei-P22 Primers. Relative location of left and right primer products (L and R) relative to the template sequence for mei-P22.

About 50 D.ananassae flies from strains labeled as 24 (stock number 14024 0371.24 from the Drosophila Species Stock Center in San Diego, CA; http://stockcenter.ucsd.edu) and
20 (14024 0371.20), and the same amount from each of the strains of D. melanogaster numbered as 56 (14021 0231.56) and 68 (14021 0231.68) were used for the extraction. Flies were frozen in the -80 °C freezer until needed. We used DNA extraction protocol number 4 from Drosophila: A Practical Guide (Roberts 1998), using the QIAamp Mini Kit (QiaGen # 51304). Flies were ground in 1.5 ml tubes in liquid nitrogen and we added an RNase step for each sample by adding 4 µl of stock RNase A, diluted with 16 µl of distilled water. To visualize the extracted DNA, the samples were run on a 0.5 X agarose gel containing 1 µl of a 1% Ethidium bromide solution as shown in Figure 2.

Figure 2. DNA Extraction. Samples from extraction run on a .5X agarose gel at 94 volts. Samples from left to right: Dmel 56, Dmel 68, Dana 20, Dana 24. The Middle Range ladder from well # 6 is also visible.
Test of Primers and DNA Sequencing

Several attempts were made to carry out a PCR reaction that would give the desired strong bands at around 200 bp in size. Unfortunately, even though every reaction gave the desired bands at around 200 bp, slightly shorter secondary bands were also observed whose origin was not clear (Figure 3). In order to remove the secondary bands multiple PCR reactions were carried out with one variable modified each time, including running a temperature gradient PCR at a different temperature range, increasing the extension time of the reaction and a touchdown PCR. Eventually, the only remaining option was to cut the desired first bands out of the gel and purify them.

Figure 3. PCR. 1X gel run at 82 volts with DNA samples from 30 µl, temperature gradient PCR. Samples in wells 1-4 (56, 68, 20,24) taken from row A at 67 °C. Samples in wells 5-8 with the same order from row D at 65.3°C. The same Middle Range ladder was used.
A total of 3 µl of the DNA template along with 3 µl of each of the left and the right primer were placed in each 0.2 ml reaction tube. 2.4 µl of dNTPs, 2.4 µl MgCl2, 0.3 µl of Taq polymerase and 6 µl of 5X Taq buffer, along with 9.9 µl of distilled water were added to make up the 30 µl reaction. Reactions were carried out in strip tubes using a temperature range of 62-67°C, and the products from rows A (67°C) and D (65.3°C) were run on a 1X agarose gel at 82 Volts as shown in Figure 3. Samples from the middle row (row D) were selected for the next step. The total remaining samples (~27 µl) from row D were run on a 1X concentrated gel at 72 Volts. The first bands were carefully cut out of the gel under the UV light and purified using the QIAquick gel extraction kit protocol and the QIAquick PCR Purification Kit (QiaGen # 28104). As the final step, in order to elute the DNA and increase the final yield, 30 µl of buffer EB was added to the center of the QIAquick membrane, the column was let stand for 1 min, then centrifuged for 1 min. The purified DNA was run on a 1X gel at 82 Volts for ~50 min. (Figure 4)

The tubes containing the final diluted DNA samples along with the diluted primers were sealed according to the guidelines of the Ohio State Plant Microbe Genomics Facility (http://www.biosci.ohio-state.edu/~pmgf/services_dna_sequencing.html) and sent off to this facility for sequencing.

Total RNA Extraction

Total RNA preparation was required to provide RNA template for mei-P22, so that it could eventually be used in an RT-PCR reaction to examine the expression of the gene in males and females. To this end, the RNA extraction protocol was used from Drosophila: A Practical Guide (Roberts 1998) and the extraction was carried out using the RNeasy Mini Kit (Cat. No. 74104) along with on-column DNase Digestion procedure using the RNase-Free DNase Set (Cat. No.79254). DNase digestion was necessary because
Figure 4. Gel-Purified PCR. 1X concentrated gel run at 82 Volts with the four purified samples originally taken from row D of the PCR machine. Samples in order from left to right: Dana 24, Dana 20, Dmel 68, Dmel 56 with the expected length of approximately 200 bp. and the Middle Range ladder are visible.

Preliminary analyses showed evidence that RT-PCR was priming from DNA rather than the RNA template, as intended. Since our mei-P22 has no introns, it is not possible to detect this by choosing primers that span an intron. As a pre-requisite to RNA extraction, males and females within each of the four strains needed to be separated after raising them on a yeast diet for a period of up to four days prior to extraction, to increase the final RNA yield. Flies from both species and sex were stored separately at -80 °C before use in the RNA extraction.

About 30 mg of flies from each species were ground in Liquid Nitrogen before 350
µl of RLT buffer containing β-Mercaptoethanol was added to each 30 mg sample. In order to eliminate the possibility of DNA contamination during the following RT-PCR reaction, an additional DNA digestion step was performed after the samples were initially added to the RNeasy spin columns. This step would ensure that the primers should solely prime the RNA strands and not the template DNA. Steps D1 through D4 of the DNase Digestion protocol (RNeasy Mini Kit Manual, p. 70) were carried out to remove any possible DNA contamination in the samples. After applying the samples to RNeasy spin columns and centrifugation, the DNase digestion step was carried out instead of performing the first wash step. 10 µl of DNase I stock solution was added to 70 µl of buffer RDD. The solution was mixed by gently inverting the tube and a brief centrifugation. The DNase I incubation mix was then added directly to the spin column membrane and placed on the benchtop for 15 mins. 350 µl of buffer RW1 was added to the spin column and the samples were centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. Lastly, the membrane was washed with two 15 µl volumes of RNase-free water and spun at 10,000 rpm for 1 min after each wash. The final volume was 30 µl per sample.

Visualization By Means of Gel Electrophoresis

In order to verify that the extracted RNA was present and not degraded, a 60 ml 1X concentrated MOPS gel was set up (based on notes from Dr. Andrea Case). The gel was made by using 0.72 g of agarose and dissolving it in 52.5 ml of RNase-free water along with 6 ml of 10X MOPS. The solution was then microwaved for 45 seconds and 1.5 ml of Formaldehyde was added to the mix. The gel was let sit for 30 min to solidify. In the meantime, the RNA samples were diluted in RNase-free water to obtain a total of 4-6 µg in 12 µl solution. Some samples were used undiluted due to very low concentrations. A master mix of loading cocktail was also prepared in hood by adding 3 µl 10X MOPS,
6 µl Formaldehyde, 16.5 µl Formamide and 0.1 µl of 1% Ethidium Bromide per sample. To load the samples onto the gel, 23 µl of the loading cocktail was added to each 12 µl of RNA sample and was incubated at 70˚C for 10 minutes, followed by a 5 min chilling on ice. 3 µl of 6X loading dye was added to each sample, vortexed very gently and loaded onto the Formaldehyde gel. 1X MOPS was used as running buffer and the samples were run at 70 Volts for 2:00 hours. After completion, the gel was soaked briefly in de-ionized water to remove the formaldehyde since it fluoresces under the UV. If this gel produced distinct bands at around 2 and 4 kb (expected size of 18S and 28S ribosomal subunits for Drosophila) we proceeded to the next step.

RT-PCR

The QIAGEN OneStep RT-PCR kit was used for the reverse transcription PCR reaction. Template RNA and kit materials were thawed and placed on ice. In order to control for DNA and other sources of contamination in the reaction tubes, three sets of controls were prepared to accompany the samples. The Adh (Alcohol Dehydrogenase) positive control consisted of the Adh primers designed as described above specifically for each of the two species. The primers for both species were designed in such a way that they either spanned exon-intron or exon-exon boundaries. Therefore, we expected to see no bands for samples containing Adh primers if they primed from RNA. In other words, if bands were seen, the only possible template for Adh primer was DNA. The expected Adh product for D. melanogaster was 700 bp and for D. ananassae samples was 516 bp. The primer sequences are shown in Figure A-4. It was important to include a positive control for every sample in order to ensure that the RT-PCR settings were working correctly and to increase our confidence that missing mei-P22 bands were due to lack of RNA template in males. A negative control was used containing all the reaction components with the
exception of the RNA template. This kind of control was necessary to indicate whether there was any other source of RNA or DNA contamination in the reaction tubes. It was expected we would not see any bands in the lanes corresponding to this treatment. One negative control was used per master mix type, that is, one for every set of Adh and mei-P22 primers for every species. The last control was to test for DNA contamination. This treatment involved inserting reaction tubes into the PCR machine at the end of the initial reverse transcription step during the RT-PCR reaction. In doing so the reactions would not be exposed to the action of the reverse transcriptase. Any bands in these gel lanes would indicate that a PCR reaction had taken place due to presence of DNA in the reactions. The PCR machine had already been pre-heated to 50°C. It was programmed as shown in Table 1.

Table 1. RT-PCR Program and Conditions

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>30 min 50°C</td>
<td></td>
</tr>
<tr>
<td>Initial PCR activation</td>
<td>15 min 95°C</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 min 94 °C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>1 min 50 °C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>1 min 72 °C</td>
<td></td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>10 min 72 °C</td>
<td></td>
</tr>
</tbody>
</table>

Products from the RT-PCR reaction were visualized on .5X agarose gels containing 1 μl of 1% Ethidium Bromide solution to observe expression patterns.
Figure 5. RT-PCR. 1X concentrated gel run at 94 Volts with RT-PCR Dana (a) and Dmel (b) samples also containing the Adh, DNA contamination and negative controls. The Middle Range ladder is also visible.
CHAPTER III

RESULTS

Identification of mei-P22 and Sequencing Results

Following the initial DNA extraction and a gradient PCR reaction with our designed primers, we were able to obtain DNA bands of the approximate 200 bp length that were purified from the gel and sent off to the Ohio State Plant Microbe Genomics Facility for sequencing. We used Sequencher (Version 4.9) to visualize the sequences that we received from the sequencing facility. After trimming the sequences of badly supported sequence on the ends, the “assemble automatically” option in the program was used, and the sequences obtained from the left and right primers for each species were aligned with the reference sequence, and they matched almost exactly. The sequence of the PCR products for both species has the expected length of 200 bp for D. ananassae and 210 bp for D. melanogaster matching the reference sequence in each case.

RT-PCR Results

Following an RNA extraction from males and females of both species and its visualization by means of Gel Electrophoresis, distinct bands of ribosomal RNA were obtained for each of the samples. This result allowed us to proceed with an RT-PCR reaction. The D. melanogaster- 68 male RNA sample was eliminated due to suspicion of it being contaminated by RNA from a different sample. The remaining samples were used in the RT-PCR reaction and the products were run on a 0.5X gel along with a middle
range ladder. Even though bands of about 200 bp were observed in lanes corresponding to the mei-P22 treatments, almost all lanes with both mei-P22 and Adh primers as well as DNA contamination controls also contained secondary bands of about 200 bp in size, which were not expected. In addition, at least one of the negative control treatments (that corresponding to D. melanogaster) also contained a band in the 200 bp region. The results showed evidence of a DNA contamination despite the use of DNase treatment during the RNA extraction step and possibly also contamination of a mastermix with fly RNA or DNA. This summer, I plan to repeat this procedure until we can eliminate contamination to test whether mei-P22 is being expressed in males and females in the pattern expected.
CHAPTER IV
DISCUSSION

The experimental work of this thesis was designed to provide the lab with tools for quantifying rates of recombination in males and females of two Drosophila species for a broader selection project. The mei-P22 gene is known to play a major role in the meiotic recombination process in Drosophila females, through initiation of DSB formation. It was selected as a marker for recombination. Among the many species of Drosophila only one, D. ananassae, has been known to show recombination in males. Using this particular species of flies and comparing it with the well-studied D. melanogaster could provide the lab with a way to study the effects of sexual selection on rates of recombination and help test the hypothesis that sexual selection plays a role in lowering rates of recombination in males.

It is also worth noting that not all strains of D. ananassae have shown male recombination. Consequently, we contacted a Dr. Muneo Matsuda in Japan, who possesses strains of D. ananassae known to have shown recombination in males, and asked him to send samples for us to use. However, due to difficulties in communicating between the labs, strict customs rules and regulations, and now the earthquake, we have been unable to obtain strains with male recombination. For the purposes of working out the techniques described here, we have used strains of D. ananassae that we obtained from the U.S. stock center. It is not known whether they have male meiotic recombination.

As a necessary first step of the experiment the sequence of mei-P22 in D. melanogaster and that of its putative homolog in the D. ananassae species were identified.
In order to design primers, these sequences were compared to each other to find a region of greatest homology between the two. The last 252 bp region was identified as having the highest homology between the two species and was used to design primers. Following a DNA extraction and a PCR reaction using the designed primers, the products of the PCR reaction were run on a gel, the samples were purified from the gel and sent off for sequencing. The results of DNA sequencing confirmed that the sequences of mei-P22 in both species and within all strains had a high degree of homology with those available on databases. These results allowed us to verify that the correct sequence of mei-P22 was being studied and that the designed primers were functional and ready to be used in the RT-PCR reaction.

As the second main step of the experiment RNA was extracted from males and females of each species. In general, even though the RNA yields from females typically fell within an acceptable concentration range, those obtained from males were significantly lower. In order to eliminate this problem, flies were raised on yeast for a period of 4 to 6 days. Even after feeding flies on the yeast diet, the RNA yields, especially for males, did not improve substantially. As a next step, we used an alternative RNA extraction protocol that eliminated the use of QIAshredder spin columns, which were used in initial attempts. This change could potentially prevent loss of significant amounts of RNA through the use of the shredders. Despite our efforts to increase male RNA yields, even the alternative RNA extraction protocol did not seem to increase male yields significantly, although female concentrations of RNA more than doubled in some cases. Another significant step of the RNA extraction was to add a DNase treatment to the extraction procedure in order to remove any traces of DNA and thus to ensure that primers were using RNA as their template and not DNA during the following RT-PCR reactions. Unfortunately, this step does not seem to have had the expected effect, and further work is necessary to obtain DNA-free RNA extracts.
In order to test whether the mei-P22 gene was expressed in males and females, the RNA samples were used in an RT-PCR reaction along with our designed primers. A crucial step of the RT-PCR experiment was to add a set of controls to the reaction to test for DNA and other sources of contamination, as well as to ensure that the reaction setup and the procedure functioned properly. After visualization of the RT-PCR results, bands were observed in both DNA contamination and in some of the negative control lanes. This was an indication that our samples and possibly some reagents were most likely contaminated. The lanes corresponding to both mei-P22 and Adh positive controls also contained secondary bands of 200 bp size that could not be explained. We suspect that this last result might have been cross contamination between primers. Even though we had verified functionality of the mei-P22 primers and that the correct sequence of the gene was being used, the results indicate the possibility of several technical errors. Given the challenges posed by the molecular biology techniques throughout the experiment and after running out of time, we were not ultimately able to test the expression patterns of males and females of the two species.

Further Study

The technical challenges faced prevented us from either confirming or ruling out the idea that mei-P22 is expressed in males of D. ananassae. For now this prevents us from using this as a marker of recombination for a broader project yet to be carried out by the lab. However, the work of this thesis did provide me a great deal of experience in working with Drosophila DNA and RNA. This work also laid important groundwork on which future tests of this and other genes involved in recombination in Drosophila can be based. Future work will focus on solving the technical difficulties we encountered and completing the test of the prediction that a gene like mei-P22 is expressed in males with recombination. If the mei-P22 homolog is not expressed in male D. ananassae that
undergo recombination, the same methods can be used to test whether other genes known to regulate recombination in females are expressed in males. Ultimately this will allow the lab to identify genes that can be used as a marker of recombination in future experiments.
REFERENCES


APPENDIX

Figure A-1. Top block displays the decorated FastA sequence of mei-P22 in Drosophila melanogaster obtained from FlyBase (a; single exon shown in capital letters). Putative mei-P22 homolog in D. ananassae obtained from NCBI GenBank using the nblast tool is shown in b.

a

>3L:7237488,7238483 ATGGACAGGAACAGTTGTCCCGAGTCCGGCCTCCACC
TACTGCTCTTTATGGGTGGCTCCCAAGCAGCCTCCCCCATTTGCGGCGATCACAGCGCCTCATCGACAAGGAAAACCGGGATCTGCAGAAGATTCCGCCCAAAAGTCTTTCTCCTCAGCTATTTGAAACACTGAAGATGATTACAGTAAGACTATTTGTTGCAGCAAGGGAAAGGTGGTTCCACCTGAAGCTGCAGCTAAGTTGGTGCCCGTGAGATCC
TCGGATAATCTAGTCATCTTTGATCTCCCGGAAAGTTGTCCCCTTGAAAACTTCCAATAGAATCGTTCACCTGAAGGCCTTCATATAAGATTATCCACCTCAAAAGTTAGATGAAGTTGAAAAGACCGTAAAGAAGGGAATAATATTCAGAAAGAAGTT
CATCAAAGGAATTCCAAAGGGAGAACGATTCAATGCACTCCCAAAAAACGTGGTCGAAAACCAAAGCAACCAGCTAAGAAACTGCAGTCAAGAATTTCCACCGATCTGCTAGGAAGCACACCATCGCCATCCAAGCTGCCAAATATCCCGCCCATT
TTGTTCATCAACTTCCGCCCCTGAGGATTCAGCTGCTGCTGCCACTCGAAGAGAT
CCTTATCTGCTCCAGCTATGCAACTTTAGCGATCTACAGCCTACACCCGATGAGAT
>gi|194752595:14694582-14695553 Drosophila ananassae strain TSC#14024-0371.13 scaffold_13337, whole genome shotgun sequence

ATGAATATAACATCACCAGACCTCGAGTGTCCTCAGCAGCTACGTATCAGAGTTGG
AGCAAGAGTATGACTTCTCCAGAAGACGACGATCTCTGAGGCTGATCGAAAAGG
AGAGCCCGTCTTGTGACCGATTCTTTCCGAAGATGCATCCCGCAATTGTGTTGG
CGAAAACCTCAGGATGAGGATAGTCAGAATCTTCAGAATATCGTGGCCAGCAGCTATGCCCCGACCA
AATTGTACATCTTAAAGGATGATAGGGAAGCAAGTCCCTCGGAAAAGATATTACGT
TTCAAACCAGGATGAAGTTCACTCTCTCACTCGGACCGACTTAGGATTTCTACATCTGA
AGCAGGGAAGAGACGAAAAGAAGTGACCATCTTAAAGCATCACAAGGCTGCTA
ATAAATCTGTAAATCGGAAGAGAAGTGACTTCGACCAGCTACTCAGCAGCAGTACCATCTAAAGC
GACGAAAGAGAAGCCACCTGCTGAAGCCCATCACATCATCACCTCTCCAAAACAA
TGGACCAGCCATTCTTGTGACACATCACCCTCTACATCACATCCGACGCCATAAACCATTGGCGACTCCACCAGTTCGTCTTCAAGAACTAAGTCCCAC
ACGACCAGCCCTTTGTGACCTCACCACAGACGACTTCTACCTGCTGAACGTGGAGTTGAGTCGCC
TGCGAAGTGTTTTGGACATAACAGCGACTGACACACGAGCAGATCGTCCAAT
TCCCGATAGCTCCATGACAATCTCCTTGTGAGCAAGCGATTCATCTTGCCGAGATATTTGGA
ACCAAAATATGACDCCACTTTAATGTGAGTGGGACGAGCAGTACATTCTAA
TTGAAGAAACATCTTCCCGCATTGGCACCATGCTGAACGTGAAGTTGAGTTCGACC
TGCGAAGTGTGTTTTGGACATCAACACAGCGACTGACACACAGCAGATCGTCCAAT
TCCCGATGAAAAAGGATAGCCAAAGTCCACACTTACCTAACTTAACCTAA
Figure A-2. Sequences of D. melanogaster and D. ananassae mei-P22 left and right primer pairs were obtained using the NCBI primer-BLAST tool (5’ to 3’).

D. melanogaster

Dmel-meiP22-1L: Forward primer  CCTTGAACTTCTCGCTGTCC  Plus

Dmel-meiP22-2R: Reverse primer  GAAAGCGCAGGATTTGCTC  Minus

D. ananassae

Dana-meiP22likeScaf13337-1L: Forward primer CATGACAATCTCCTTGAGCG  Plus

Dana-meiP22likeScaf13337-1R: Reverse primer  CGATCTGCTCGTGTCAGT  Minus
Figure A-3. Sequences of D. melanogaster and D. ananassae Adh left and right primer pairs were obtained using the NCBI primer-BLAST tool (5’ to 3’).

a. D. melanogaster Primers:
L: 5’-GCGTACATAGCCGAGATCGCG-3’ R: 5’-TAATGGGGGCGCA
GTTCGCCCAG-3’Revcomp R: CTGGCGAAACTGGCCCATTA
Left primer is in intron_CG3481:1 above R
spans an exon/exon boundary CG3481:3 to CG3481:4

Predicted product (700bp): GCGTACATAGCCGAGATCGCG
TAACGGTAGATAATGAAAAAGCTACGTAACCGAAGCTCTTGCTGTACCGA
TCTTCCTATAAAATACGGGGGCCGACACGAAACTGGGAACACCAACTAACCGGA
GCCCTCTTCCAATTGAAACAGATCGAAAGGGCTGCTAAAGCAAAAAAGAAA
GTCACCATGTCGTTTACTTTTGAACACAAAGGAGGCTGCTAAAGCAGTTGATTTTCGTTGACCGGT
CTGGGAGGGCATTGGTCTGGACACCAGCAAGGAGCTGCTCAAGCGCGATCTG
AAGAACCTGCGTGCCTGCAGCCGTGGAACCCCGGCTGCCATTGCCGAC
CTGAAAGCAGACTCAAATCCAAAGGTACCGTAACCTTCTTACTACCCCTATGATGTG
ACCGTTCGCCCAGTGGCAGACCAACAAAGGCTTCAAGACCATCTTCGCCCCAG
CTGAGAACCGTCGATGACCTGTAGTCAACCGGAGCTGGAATGGATGCTGAC
CAGATCGAGGCACCGACTGCAACTGACTGGCGTCGTTCCAGACACCAGCAG
ACCGCCATTCTGGACTTCTGGGACACAGCGAAGGCGGTTGAGGTGATC
ATCTGCAAAGATTGGATCGTCCGACTGCTATGATCCGTCCCGCCGATGG
GTCTACTCCGGCGACCAAGGGCGGCCCTGGTGTCAACTTCACCAGCTCCCTGGCG
AAAAGCTGGCCCATTA
b. D. ananassae Primers: L: 5’-CAACAAGAACGTGGTCTTCGTTGG
C-3’R: 5’-GCAGTCACCCGCTGGTGATGGG-3’ Revcomp R: CCCATC
ACCGAGGTACCGCG Left primer is in first exon dana_G
LEANR_15653:1 above R is in exon 2 dana_GLEANR_1565
3:2 and spans the exon/intron/exon boundary

Predicted product (516bp from DNA and 454 from RNA):
CAACAAGAACGTGGTCTTCGTTGGCTGGCCTGGGAGGCATTGGCCTGGACACC
ACCAAGGAGCTGCTCAAGCGCGACCTGAAGAACAATCTGGTATCCTGGACCGCA
TCGACCAACCGGTACCGTCATTTGCGGAGCTGAAGAACAATCTGGTATCAACG
CGTCGCTCTACCTTACTCGGTACTGACCGGATCCATTACCGAGACCAAGAAG
CTGCTGAAAGACCAGTCTCGACAAGCTGAAGACCCGTGGACATCCTGATCAACG
GAGCTGGCATCTCGTGATGACCACCAGATCGAGCGACCACCACCCGCTCAACTA
CAGGGTCTGTTGAAACCAACCACCCGCTCTCTGACTGTTCTGGGACAAAGCGC
AAGGGCGGACAGGGTGGCATCATCTGCAACATTGGCTCCGTAACCGGCTTCA
ACGCCATCTTACAGGTGGCTCCGTCTACTCTGGACCCACCCAGGCTGCTTCA
CTTCA CCAGCCTCCCTAGGCAAATCTGGCTTCCATCACCGAGGTACCGCG