Genetics: Published Articles Ahead of Print, published on January 6, 2011 as 10.1534/genetics.110.124743

Diversity Enhancing Selection Acts on a Female Reproductive Protease Family in Four Sub-Species of *Drosophila mojavensis*

Erin S. Kelleher[1,2*], Nathaniel L. Clark[2] and Therese A. Markow[1,3]

1. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721
2. Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853
3. Section of Ecology, Behavior and Evolution, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093

*to whom correspondence should be addressed:

esk72@cornell.edu

Phone: (607) 254-4569

Fax: (607) 255-6249

Research Institution: University of Arizona

Submission Type: Research Article

Key Words: Gene Duplication, Protease, Reproductive Protein, Female Reproductive Tract, Sexual Conflict

Running Head: Female Reproductive Proteases in *Drosophila*

Abbreviations:

MYA = Million Years Ago

MRCA = Most Recent Common Ancestor

ABSTRACT

Protein components of the *Drosophila* male ejaculate are critical modulators of reproductive success, several of which are known to evolve rapidly. Recent evidence of adaptive evolution in female reproductive tract proteins suggests this pattern may reflect sexual selection at the molecular level. Here we explore the evolutionary dynamics of a five-paralog gene family of female reproductive proteases within geographically isolated sub-species of *D. mojavensis*. Remarkably, four of five paralogs show exceptionally low differentiation between sub-species and unusually structured haplotypes that suggest the retention of old polymorphism. These gene genealogies are accompanied by deviations from neutrality consistent with diversifying selection. While diversifying selection has been observed amongst the reproductive molecules of mammals and marine invertebrates, our study provides the first evidence of this selective regime in any *Drosophila* reproductive protein, male or female.

INTRODUCTION

The molecular interface that underlies sexual reproduction is extraordinarily dynamic. In a host of biologically diverse organisms, proteins with reproductive functions evolve rapidly through changes in coding sequence, (Reviewed in SWANSON and VACQUIER 2002A; 2002B; CLARK *et al* 2006; PANHUIS *et al* 2006), lineage-specific gene duplications, evolution of novel proteins, and regulatory changes (BEGUN and LINDFORS 2005; MUELLER *et al* 2005; BEGUN *et al* 2006; FINDLAY *et al* 2008). Within species, reproductive proteins show evidence of two contrasting selective regimes. Reduced polymorphism or elevated divergence at many reproductive protein loci indicates they have experienced positive directional selection (LEE *et al* 1995; AGUADÉ 1998; 1999; CLARK *et al* 2007; CALKINS *et al* 2007). In contrast, other reproductive proteins exhibit remarkable intraspecific diversity generated by balancing selection (METZ and PALUMBI 1996; GASPER and SWANSON 2006; LEVITAN and FERRELL 2006; HAMM *et al* 2007, CLARK *et al* 2009), alternative splicing (MOY *et al* 2008; SPRINGER *et al* 2008), copy number variation (DOPMAN and HARTL 2007), and gene conversion (KELLEHER and MARKOW 2009).

The exceptional dynamics of reproductive proteins are thought to result from sexual selection that occurs after copulation or gamete release. (Reviewed in SWANSON and VACQUIER 2002A; 2002B; CLARK *et al* 2006; PANHUIS *et al* 2006). Sperm competition predicts an ongoing arms race between the abundant gametes of multiple males to achieve fertilization of more limited female gametes (BIRKHEAD and MØLLER 1995). Cryptic female choice, a post-copulatory equivalent of traditional sexual selection, suggests that females can bias fertilization success towards certain males based on qualities of the male ejaculate (EBERHARD 1996). Finally, sexual conflict, or a difference in the reproductive interests of the two sexes, proposes that an evolutionary arms race between males and females over control of reproductive outcomes can cause rapid evolution of the proteins involved (PARKER 1979). Although both cryptic female choice and sexual conflict predict a role for female reproductive proteins in driving the dynamics of their male interactors, when compared to the extensive literature on the evolution of male reproductive molecules, examination of the female side remains lacking.

The promiscuous mating system and unique reproductive biology of the cactus breeding fruit fly, *Drosophila mojavensis*, presents an exciting system in which to explore the evolution of female reproductive proteins. These females remate daily (Reviewed in MARKOW 1996), and broods of wild caught females have been demonstrated to have up to 6 different sires, implying strong post-copulatory sexual selection (GOOD *et al* 2006). *Drosophila mojavensis* females, furthermore, are known to incorporate male-derived molecules into somatic tissues and oocytes (MARKOW and ANKNEY 1984), a nutritional benefit to copulation that dramatically contrasts the 'cost of mating' incurred by the females of their congener *D. melanogaster* (CHAPMAN *et al* 1995; PITNICK and GARCÍA-GONZÁLEZ 2002; KUIJPER *et al* 2006; BARNES *et al* 2008). Finally, *D. mojavensis* females exhibit an insemination reaction, an opaque mass of unknown composition that occupies the uterus after every copulation (PATTERSON 1946, ALONSO-PIMENTEL *et al* 1994). This reaction mass is thought to protect the male's nutritional investment from cuckoldry by competing ejaculates (MARKOW and ANKNEY 1984; 1988;

Pitnick *et al* 1997), and furthermore, may coevolve antagonistically between the sexes (Knowles and Markow 2001).

Our previous Expressed Sequence Tag (EST) analysis of the lower reproductive tracts *D. arizonae*, a closely related sister species of *D. mojavensis* (MRCA ~ 0.7 MYA, Reed *et al* 2007, Matzkin 2008), identified 241 candidate female reproductive tract proteins that potentially interact with the male ejaculate (Kelleher *et al* 2007). The most intriguing of these candidates were five recently duplicated endoprotease families, exclusively expressed in the lower female reproductive tract (Kelleher *et al* 2007). The current study explores the evolutionary history of one of the two largest protease families in four ecologically and geographically isolated sub-species of *D. mojavensis*; Baja Peninsula (*D. m. baja)*, Catalina Island (*D. m. wrigleyi*), Mainland Sonora (*D. m. sonorensis*), and the Mojave Desert (*D. m. mojavensis*) (Pfeiler *et al* 2009). The family encodes five serine-endoprotease paralogs (Figure 1, protease gene family 1, Kelleher *et al* 2007) all of which contain signal peptides suggesting they are secreted into the female reproductive tract lumen, creating the potential for biochemical interaction and coevolution with male seminal proteins (Kelleher *et al* 2007). Consistent with a hypothesis of molecular coevolution, the protease gene family examined here exhibits evidence of positive selection at some codons, as inferred from divergence between species and paralogs (Kelleher *et al* 2007).

Previous studies have demonstrated that the four sub-species of *D. mojavnesis* are genetically and morphologically differentiated (Machado *et al* 2007; Reed *et al* 2007; Matzkin 2008; Reviewed in Pfeiler *et al* 2009), and that male and female contributions to reproductive outcomes are coadapted within them (Knowles and Markow 2001;

PITNICK *et al* 2003; KNOWLES *et al* 2005; KELLEHER and MARKOW 2007). Consistent with these phenotypic observations, we find considerable evidence for population-specific selection in the female reproductive proteins examined here. We furthermore find that in contrast to *D. melanogaster*, which exhibits a high frequency of positive directional selection amongst its female reproductive tract proteins (SWANSON *et al* 2004; PANHUIS and SWANSON 2006; LAWNICZAK and BEGUN 2007), *D. mojavensis* female reproductive tract proteins display deviations from neutrality consistent with balancing selection. We discuss our results in terms of sexual selection theory and the unique reproductive biology of *D. mojavensis.*

MATERIALS AND METHODS:

Fly Strains:

D. mojavensis were collected from Catalina Island (2001), Mojave Desert (2002), Baja Peninsula (2002), and Mainland Sonora (2007) by J. Bono, L. Reed, and L. Matzkin. *D. arizonae,* the sister species of *D. mojavensis,* were collected in Tucson, Arizona (2000) by L. Matzkin. All flies used in population analyses were maintained as isofemale lines. Between 7 and 8 isofemale lines were sampled from each population for each locus. A third, closely related species, *D. navojoa*, was obtained from the Tucson *Drosophila* Stock Center.

Loci and Primer Design:

The genomic arrangement and phylogenetic relationships of the protease gene family examined in this study are presented in Figure 1. Although the genes remain unannotated, our previous study showed that they were expressed in *D. arizonae*, and that orthologous sequences were present in the *D. mojavensis* genome (KELLEHER *et al* 2007). Intron-exon splice sites were inferred from *D. arizonae* ESTs in KELLEHER *et al* (2007). For simplicity, we refer to these genes as female reproductive protease A-E, or FRP-A-E, where FRP-A corresponds to Dari\anon-EST:Kelleher9, FRP-B corresponds to Dari\anon-EST:Kelleher8, FRP-C corresponds to Dari\anon-EST:Kelleher7, FRP-D corresponds to Dari\anon-EST:Kelleher6, and FRP-E corresponds to anon-EST:Kelleher-5. *D. mojavensis* (http://rana.lbl.gov/drosophila/) orthologs further were aligned to available *D. arizonae* ESTs to generate paralog-specific primers that amplified the majority of the coding sequence for each locus. All paralogs were reciprocally monophyletic (not shown). Further, heterozygosity in sampled isofemale lines was quite low, except for flies that recently had been introduced to the lab (<5 generations). We are confident, therefore, that each set of primers amplified a unique genomic location.

Sequencing:

Genomic DNA was isolated from whole flies using the DNeasy Kit (Qiagen) according to manufacturer instructions. Standard PCR was performed using internal, paralog-specific primers (Figure 1). All sequencing was performed on an ABI 3700 DNA sequencer with Big Dye Terminator chemistry. Primers and PCR conditions are available from the authors upon request. Base-calling and assembly were performed in Sequencher 4.8.

Polymorphism Analyses:

Haplotypes were phased in Arlequin (http://lgb.unige.ch/arlequin/software/), and a single haplotype for each individual was retained for subsequent analyses. Polymorphism analyses, estimation of population parameters, and tests of selection were performed in DNAsp (ROZAS *et al* 2003). Sample sizes, sequence lengths and estimates of polymorphism are presented in supplementary table 1. Significance of site frequency spectra statistics and measurements of linkage disequilibrium were assessed by coalescent simulations under the conservative assumption of no recombination. For tests requiring an outgroup, one or more *D. arizonae* orthologs were used for FRP-A, FRP-B, and FRP-C. For FRP-D we used an allele of FRP-E as the outgroup, and vice versa, due to uncertainty surrounding the identity of the *D. arizonae* ortholog. Using closely related paralogs as an outgroup sequence, in place of sibling species, is known to be a conservative approach for McDonald-Kreitman tests (MCDONALD and KREITMAN 1991; THORNTON and LONG 2005; THORNTON 2007). We note, however, that using the putative *D. arizonae* ortholog had no effect on the outcome of the test. Tests were polarized with the appropriate sequence from *D. navojoa.*

Gene conversion was detected by GENECONV within an alignment of all unique haplotypes for all paralogs using the method of SAWYER (1989). Briefly, gene conversion tracts between pairs of sequences are identified by considerable stretches of complete identity interspersed between two regions of considerable mismatch, or one region of mismatch and the end of the alignment. Statistical significance of these fragments is determined by permutation tests. Neighbor-joining gene trees (SAITOU and NEI 1987) were constructed in Paup*4.0b10 (SWOFFORD 2000).

Three Dimensional (3D) Modeling:

Serine endoprotease catalytic sites (Reviewed in POLGAR 2005), and protease inhibitor sites (Reviewed in SRINIVASAN *et al* 2006), as well as previously identified Bayes Empirical Bayes positively selected sites (KELLEHER *et al* 2007; YANG *et al* 2005), were mapped to a predicted 3D model for FRP-C obtained from Swiss-Model (SCHWEDE *et al* 2003).

To test for an association between positively selected sites and protease inhibitor sites, we implemented a permutation analysis previously described in CLARK *et al* (2007). Briefly, the distance from each selected site to the nearest inhibitor site was calculated, and its mean value compared to a distribution of distances between random pairs of sites. Buried, core sites with 10% or less surface exposure per residue, as calculated by GETAREA (FRACZKIEWICZ and BRAUN 1998), were not considered for random sets. This exclusion makes the test more conservative, because these sites evolve slowly relative to surface sites and rarely are inferred as positively selected. Statistical significance was determined as the fraction of random permutations with a mean distance equal to or lower than the observed mean distance between selected and inhibitor sites.

RESULTS:

Multiple Paralogs Evolve Non-Independently Through Gene Conversion:

Gene conversion and non-allelic homologous recombination result in non-independent evolutionary histories of paralogous loci. Describing this process is critical, as it leads to complex genealogies and unusual patterns of polymorphism not seen for single copy genes (INNAN 2003A; THORNTON 2007). In our FRPs, gene conversion tracts between pairs of paralogous haplotypes were identified as fragments of complete identity flanked by regions of significant mismatch, using the method of SAWYER (1989). No evidence of gene conversion was observed between FRP-A and any other paralog, indicating this locus evolves independently (Figure 2). In contrast, there is evidence of gene conversion in at least one pairwise comparison between all other paralogs in the examined gene family (Figure 2). The lack of detectable gene conversion between FRP-A and the other paralogs may suggest that gene conversion does not occur, but could also indicate that it is deleterious. Indeed, all sampled haplotypes of this paralog exhibit the same replacement changes in the three residues of the catalytic triad (reviewed in POLGAR 2005), suggesting it has acquired a divergent, non-proteolytic function. In contrast, all other paralogs have retained a catalytic triad, indicating that their biochemical functions may be more similar.

In terms of both tract length and frequency, the most extensive gene conversion was observed between paralogs FRP-C and FRP-D. These paralogs are neither physically adjacent, nor are they genetically more similar to each other than to the remainder of the gene family. Conversely, minimal gene conversion was observed between adjacent paralogs, or between genetically similar pairs FRP-D and FRP-E, and FRP-B and FRP C.

There is no evidence, therefore for an association between phylogenetic or physical distance and gene conversion.

Examination of the frequency that a given site is found within a significant fragment reveals that gene conversion is non-randomly distributed along the chromosome (Figure 3). Gene conversion is frequent in the 5' end of the gene, peaks near the center, and is entirely absent from the 3'end. Intriguingly, previously described selected-sites (KELLEHER *et al* 2007) are highly concentrated at the 3' end of the gene (Figure 3), suggesting a possible negative-association between gene conversion and adaptive evolution. Consistent with this hypothesis, we observed that positively selected sites exhibited a significantly lower frequency of gene conversion than non-selected sites (Wilcoxon Rank Sum Test, p = 0.0016).

Female Reproductive Proteases Exhibit Unusually Low Population Structure:

A previous examination of genome-wide variation in *D. mojavensis* shows that haplotypes are structured between the four geographically isolated subspecies, *D. m. baja*, *D. m. wrigleyi*, *D. m. sonorensis*, and *D. m. mojavensis* (MACHADO *et al* 2007, Figure 4A). Specifically, a concatenated genealogy of 10 nuclear loci revealed a well-supported (>86%) reciprocally monophyletic clade for each sub-species (MACHADO *et al* 2007). To explore the relationships between female reproductive protease haplotypes sampled here, gene genealogies were constructed for each of the five paralogs (Figure 4B-F). Well-supported clades (bootstrap values >95%) containing individuals from multiple sub-species were observed for FRP-A, FRP-C, FRP-D, and FRP-E, providing little evidence for a geographical structuring of sampled haplotypes. The genealogies of FRP-A and FRP-D are particularly unusual, as haplotypes sort into two divergent clades, suggesting ancestral polymorphism. Recombination, or gene conversion between paralogs is expected to lengthen terminal branch lengths and reduce internal branch lengths (SCHIERUP and HEIN 200). Thus, while gene conversion may affect the phylogenetic structure of duplicated loci, it is unlikely to give rise to the deep bifurcations we observe in FRP-A and FRP-D. To determine if these results represent a genuine difference from the neutral loci examined in MACHADO *et al* (2007), we constructed gene genealogies for each of the 10 loci sampled in that study individually. No well-supported clades (>95%) grouping haplotypes from multiple sub-species were observed (not shown), indicating that the genealogies of the duplicated proteases examined here are distinct from those of putatively neutral loci.

To further quantify the disparity in population structure between neutral loci and our female reproductive proteases, we compared F_{ST} values between the two gene classes (Table 1). For all six pairwise combinations of sub-species, the mean F_{ST} value for FRPs was lower than that of putatively neutral loci (MACHADO *et al* 2007), although the difference was only significant for three of these comparisons (Table 1). Furthermore, 21 individual combinations of loci and population pairs exhibit F_{ST} values that are significantly lower than the neutral distribution from MACHADO *et al* (2007), 13 of which remain significant after correction for multiple testing (Table 1). Finally, the global F_{ST} value across all four subspecies was significantly lower than those of neutral loci for every FRP examined except FRP-A (Table 1). Collectively these comparisons suggest that FRPs exhibit reduced population structure, consistent with a regime of natural selection that acts to maintain genetic variation within sub-species.

Female Reproductive Protein Haplotypes Exhibit Non-Neutral Levels of Linkage Disequilibrium:

To further characterize the unusual haplotype structure of FRPs, we estimated linkage-disequilibrium within each locus and sub-species, using *Zns* (KELLY 1997) and *Za* (ROZAS *et al* 2001) (Table 2). *Zns* measures the correlation of pairwise-linkage disequilibrium, r^2, for all polymorphic sites within a locus (KELLY 1997), while *Za* estimates the correlation in r^2 values between adjacent polymorphic sites only (ROZAS *et al* 2001). For both statistics, values that approach 1 indicate high linkage disequilibrium and low asymmetry in the frequency of polymorphic sites, a pattern that could result either from cryptic population structure, or from natural selection acting on a polymorphism that is closely linked to neutral sites in this region. For example, alcohol dehydrogenase (Adh), a frequently cited example of balancing selection in *D. melanogaster* (HUDSON *et al* 1987) exhibits significantly elevated values of *Zns* within the region that has undergone non-neutral evolution (KELLY 1997). After correction for multiple testing, four of five paralogs exhibit a degree of linkage disequilibrium that is inconsistent with a standard neutral model in at least one sub-species, as assessed by coalescent simulations (Table 2). These significant values may result from natural selection, as no evidence for cryptic population structure within sub-species has been seen for other loci (MACHADO *et al* 2007; REED *et al* 2007; MATZKIN 2008).

If gene conversion introduces multiple linked polymorphisms within the same conversion tract, it could lead to strong linkage disequilibrium between polymorphic sites and significant values of *Zns* and *Za*. Because no gene conversion was detected between FRP-A and any other paralog, this phenomenon cannot explain the degree of linkage disequilibrium observed at this locus. To elucidate the contribution of gene conversion to linkage disequilibrium at the other four loci, we identified sites with evidence of gene conversion within each population and excluded them from the analysis. While in some cases, significant linkage disequilibrium was no longer detected when sites of gene conversion were excluded, the majority of values remained significant (Table 2). We cannot, however, rule out the possibility that GENECONV failed to detect older gene conversion events that nonetheless contribute to linkage disequilibrium.

Polymorphism and Divergence Analyses:

One explanation for the unusual patterns of haplotype structure and linkage disequilibrium observed in the female reproductive proteases examined here is that these loci are subject to balancing selection. Two statistics were used to detect skews in the site-frequency spectra indicative of non-neutral evolution. Tajima's *D* (TAJIMA 1989) detects an excess of intermediate or low frequency polymorphisms suggestive of balancing or directional selection, respectively. Similarly, Fu and Li's *F* (FU and LI 1993) identifies an excess of old polymorphisms indicative of balancing selection by assigning variation to branches on a gene genealogy. Although the site-frequency spectra can be affected by demographic processes, these statistics tend to be slightly negative and close to zero at putatively neutral loci for all four races of *D. mojavensis* (MACHADO *et al* 2007; KELLEHER and MARKOW 2009). Significantly positive or negative values, therefore, cannot be attributed to demographic history.

There was a general trend towards positive values of *D* and *F* amongst the five paralogs (Table 3). This result is not unexpected, as gene conversion is predicted to create a marginally positive skew in the site frequency spectra of duplicate loci (INNAN 2003A; THORNTON 2007). Significantly positive values do not result from neutral gene conversion, however, as an over-estimation of the variance makes these statistics quite conservative for duplicate loci undergoing concerted evolution (INNAN 2003A; THORNTON 2007). In four combinations of sub-species and loci, FRP-A (*D. m. mojavensis*), FRP-C (*D. m. sonorensis*), FRP-D (*D. m. baja*), and FRP-E (*D. m. wrigleyi*), these statistics indicated a significant excess of intermediate-frequency or old polymorphisms (Table 3), suggesting that selection acts to retain genetic variation relative to a neutral model. Although no tests remain significant after a conservative Bonferroni correction for multiple measures, this frequency of rejections of the null hypothesis is unlikely to occur by chance (cumulative binomial p = 0.048). All values remained significant when sites undergoing gene conversion were excluded from the alignment (Table 3), further confirming that the observed deviations from neutrality are not the result of gene conversion.

We furthermore examined the relationship between polymorphism within subspecies of *D. mojavensis* to divergence from *D.arizonae* using the McDonald Kreitman (MK) test, (MCDONALD and KREITMAN 1991). Positive directional selection is predicted to result in significant excess of replacement divergence (MCDONALD and

KREITMAN 1991). In contrast, an excess of replacement polymorphism may indicate balancing selection, segregation of mildly deleterious variation, or recently relaxed constraint (NACHMAN 1998). FRP-A exhibited an excess of replacement polymorphism in *D. m. mojavensis* in a standard MK test, a deviation from neutrality that is consistent with balancing selection (Table 4). While this value must be interpreted with caution, as it does not remain significant after correction for multiple testing, it is consistent with the observed excess of old polymorphisms of this same locus (Table 3). Interestingly, lineage-specific tests at this locus also exhibited a high frequency of non-synonymous polymorphisms, but lacked sufficient fixed differences on the *D. mojavensis* branch to reject neutrality (Table 3).

In contrast to the FRP-A, as well as the other deviations from neutrality observed in this study, a modified McDonald-Kreitman test comparing polymorphism within populations for FRP-D and FRP-E, to divergence between these two paralogs (see materials and methods), revealed an excess of replacement divergence in FRP-D for *D. m. baja* and *D. m. sonorensis* (Table 5). This value remains significant in *D. m. baja* after correction for multiple testing. This signature of directional selection was inconsistent with the presence of two differentiated haplogroups in both of these sub-species (Figure 4, Table 2), as well as with the excess of both intermediate-frequency and old polymorphism in *D. m. baja* (Table 3). Although these deviations from neutrality may appear contradictory, it is important to remember that they are sensitive to different evolutionary signatures and time-scales. While site-frequency spectra may suggest recent selective events at a given locus, MK tests will be more sensitive to the history of the locus since its divergence from the outgroup. Deviations from neutrality in opposite directions, therefore, may indicate that the evolutionary history of these loci has been more complex than simple models of directional or diversifying selection.

Selected Sites are Structurally-Associated with Determinants of Protease Inhibitor Susceptibility:

The five paralogs examined in this study are serine endoproteases. Serine endoprotease activity in *D. arizonae* female reproductive tracts is negatively regulated by mating, suggesting susceptibility of female proteases to inhibitors in the male ejaculate (KELLEHER and PENNINGTON 2009). If female proteases interact and coevolve with male protease inhibitors, selected sites are predicted to cluster near residues that determine susceptibility to protease inhibitors. Consistent with this hypothesis, selected sites (KELLEHER *et al* 2007) often are observed to be closely associated with sites important to protease inhibitor susceptibility and resistance (Figure 5, Reviewed in SRINIVASAN *et al* 2006). We furthermore observed that 3 of 14 selected sites also are determinants of inhibitor susceptibility, a marginally significant excess (Fisher's Exact Test p = 0.058).

Examining the distance between selected sites and functional sites in three-dimensional space presents a more robust test for an association between site classes (CLARK *et al* 2007). We therefore compared the average pairwise distance between each selected site and the closest protease inhibitor interaction site to 10^6 sets of randomly sampled sites. Selected sites are significantly closer to protease inhibitor interaction sites than expected by chance (p = 0.02220), indicating that these two groups of sites are physically associated within the structure of the protein.

DISCUSSION:

Although there is considerable empirical evidence that male reproductive proteins experience both directional and diversifying selective regimes in natural populations, the degree to which these patterns extend to their female interactors, as predicted under models of intersexual coevolution, remains largely unexplored. The exceptional mating system and reproductive biology of *D. mojavensis*, in conjunction with physiological evidence of intersexual coevolution, presents a particularly compelling system for examining the evolution of female reproductive tract proteins. The female reproductive tract gene family examined here exhibits a history of directional selection, balancing selection, and gene conversion, as well as excess of amino acids substitution at functional sites of protein-protein interaction, results strongly suggestive of molecular coevolution. Evidence of diversifying selection is particularly intriguing, as the role of selection in maintaining the exceptional genetic variation observed at some reproductive protein loci often remains unresolved.

Female Reproductive Proteases in *D. mojavensis* may experience diversity-enhancing selection.

Previous population surveys of *D. melanogaster* female reproductive tract proteins (SWANSON *et al* 2004; PANHUIS and SWANSON 2006; LAWNICZAK and BEGUN 2007), as well as a recent study of a second family of female reproductive proteases in *D.*

mojavensis (KELLEHER and MARKOW 2009)*,* report a high frequency of positive directional selection. While an excess of replacement divergence at FRP-D in *D. m. baja* and *D. m. sonorensis* suggest a role for directional selection in the evolution of the female reproductive proteins examined here, the majority of deviations from neutrality we observe point to balancing selection as a comparatively more significant force in guiding the evolutionary history of this gene family. All five proteases exhibited non-geographic haplotype structure, and significantly lower F_{ST} values than neutral loci, as expected if natural selection acts to maintain genetic variation within isolated sub-species. Four of five loci exhibit significant linkage disequilibrium consistent with positive directional or diversifying selection, and site frequency spectra tests at four different loci exhibit an excess of intermediate-frequency or old polymorphisms, suggesting selective retention of genetic variation relative to a neutral model. McDonald Kreitman tests at one locus, furthermore, exhibit an excess of replacement polymorphisms consistent with balancing selection.

The evidence of diversifying or balancing selection presented here, is an unexpected evolutionary pattern in light of evidence for ejaculate-female coadaptation within sub-species of *D. mojavensis*. Crosses between these sub-species exhibit significant differences from within sub-species crosses in egg size (PITNICK *et al* 2003), mated female desiccation resistance (KNOWLES *et al* 2005), and the size and duration of the insemination reaction (KNOWLES and MARKOW 2001), predicting that the biological molecules that underlie these male by female interactions must also diverge rapidly between populations. While it remains unknown if any of the FRPs examined here play a direct role in ejaculate-coadaptation, it is clear that deviations from neutrality often are confined to a single race or group of races, as expected if each race experiences its own unique coevolutionary trajectory. Thus, our data suggest that reproductive divergence between isolated populations of *D. mojavensis* may not always be underscored by simple directional selection at interacting loci.

Gene Conversion, Diversifying Selection and Adaptive Evolution:

Divergence between paralogous members of a multigene family is postulated to result from an antagonistic process between the diversifying force of selection and the homogenizing force of gene conversion. All paralogs examined in this study, except FRP-A, exhibited considerable evidence for interlocus gene conversion, suggesting the process of diversification between paralogs is not yet complete (Figure 2). While the frequency of gene conversion showed no clear relationship with phylogenetic or physical distance (Figure 2), we observed a strong negative association between gene conversion and sites inferred to have undergone adaptive evolution (Figure 3). These results are consistent with a model where gene conversion interferes with the process of adaptive evolution, or may be costly in genetic regions that have experienced positive selection since gene duplication.

In contrast, a different female reproductive protease gene family examined in KELLEHER and MARKOW (2009) revealed no evidence for an antagonistic relationship between gene conversion and adaptive evolution. This same gene family exhibited several indicators of relaxed purifying selection at individual loci, suggesting some paralogs within it may be functionally redundant (KELLEHER and MARKOW 2009). We furthermore found minimal evidence for diversifying selection within individual paralogs (KELLEHER and MARKOW 2009). Thus, the strength of diversifying selection may be an important difference in the selective regimes experienced by these two families.

Balancing Selection and Gene Duplication:

Diversifying selection amongst *D. mojavensis* female reproductive proteins presents a stark contrast to the preponderance of directional selection observed amongst the female reproductive proteins of its congener *D. melanogaster* (SWANSON *et al* 2004; PANHUIS and SWANSON 2006). This selective regime is reminiscent of another emergent pattern of reproductive protein evolution in the *D. mojavensis* lineage: gene duplication (KELLEHER *et al* 2007; WAGSTAFF and BEGUN 2007; ALMEIDA and DESALLE 2008; 2009; KELLEHER and MARKOW 2009). Although there are a few reports of lineage-specific duplications in *D. melanogaster* seminal fluid proteins (CIRERA and AGUADÉ 1998, FINDLAY *et al* 2008), recent duplicates occur with high frequency amongst both male seminal proteins (WAGSTAFF and BEGUN 2007; ALMEIDA and DESALLE 2008), and female reproductive tract proteins (KELLEHER *et al* 2007; KELLEHER and MARKOW 2009; KELLEHER and PENNINGTON 2009) in the *repleta* species group.

It is exciting to speculate that the selective forces that underlie gene duplication and balancing selection may not be independent. Several models have suggested that if a balanced polymorphism is maintained by overdominant selection, a gene duplication event that unites two functionally diverged alleles on the same chromosome will immediately experience a selective advantage due to heterosis (SPOFFORD 1969, OHNO

1970, OTTO and YONG 2002; WALSH 2003; PROULX and PHILLIPS 2006). Balancing selection and gene duplication, therefore, may be iterative steps in the diversification of *D. mojavensis* FRPs.

Biochemical Basis of Molecular Coevolution

The biochemical underpinnings of intersexual coevolution within races of *D. mojavensis* remain largely unexplored. It is compelling, however, that the female reproductive tracts of *D. arizonae,* the close sister-species to *D. mojavensis* (MRCA = 0.7 MYA REED *et al* 2007; MATZKIN 2008), exhibit exceptional levels of proteolytic activity that are negatively regulated by mating (KELLEHER and PENNINGTON 2009). While this negative regulation could be transcriptional, it also is possible that female proteases are negatively regulated by known proteases inhibitors in the *D. mojavensis* male ejaculate (WAGSTAFF and BEGUN 2005; KELLEHER *et al* 2009). Consistent with the hypothesis that female proteases interact and coevolve with protease inhibitors, our structural analysis revealed that previously inferred selected sites are clustered with sites that determine susceptibility to protease inhibitors (Figure 5). A similar result was observed in KELLEHER and MARKOW (2009), suggesting that molecular coevolution with protease inhibitors may be a general property of female reproductive proteases in *D. mojavensis*.

Conclusion:

Evolutionary dynamics of *D. mojavensis* female reproductive tracts proteins, both in terms of balancing selection and accelerated gene duplication (KELLEHER *et al* 2007;

KELLEHER and MARKOW 2009; KELLEHER and PENNINGTON 2009), present a dramatic contrast to patterns of directional selection and the paucity of gene duplication observed in *D. melanogaster* (SWANSON *et al* 2004; PANHUIS and SWANSON 2006; LAWNICZAK and BEGUN 2007). These two congeners also have dramatically different mating systems and levels of female promiscuity, as well as considerably disparate reproductive physiologies. Our data suggest these reproductive traits may leave signatures in the evolutionary histories of the proteins involved, and create a framework for comparing the dynamics of reproductive proteins between closely related organisms.

Acknowledgements:

The authors would like to acknowledge Michael Nachman for generous use of equipment, Willie Swanson for helpful discussion, and Jeremy Bono, Stephen Schaeffer and five anonymous reviewers for comments on the manuscript. This research was funded by a National Science Foundation Doctoral Dissertation Improvement Grant to E.S.K., and the Center for Insect Science and the University of Arizona. E.S.K. was supported by an NSF-IGERT research traineeship in Evolutionary, Functional and Computational Genomics at the University of Arizona, a Dissertation Fellowship from the American Association of University Women, a post-doctoral fellowship from the Cornell Center for Comparative and Population Genomics, and an NIH-NRSA post-doctoral fellowship (GM090567). N.L.C. was supported by an NIH-NRSA post-doctoral fellowship (GM084592).

REFERENCES

AGUADÉ, M., 1998 Different forces drive the evolution of ACP26Aa and ACP26Ab accessory gland genes in the *Drosophila melanogaster* species complex. Genetics. **150:** 1079–1089.

AGUADÉ, M., 1999 Positive selection drives the evolution of the Acp29AB accessory gland protein in *Drosophila*. Genetics **152:** 543–551.

ALMEIDA, F. C., and R. DESALLE, 2008 Evidence of adaptive evolution of accessory gland proteins in closely related species of the *Drosophila repleta* group. Mol. Biol. Evol. **25:** 2043–2053.

ALMEIDA, F. C., and R. DESALLE, 2009 Orthology, Function, and Evolution of Accessory Gland Proteins in the *Drosophila repleta* Group. Genetics **181:** 235–245.

ALONSO-PIMENTEL, H., TOLBERT, L. P., and W. B. HEED, 1994 Ultrastructural examination of the insemination reaction in *Drosophila.* Cell Tissue Res. **275:** 467–479.

BARNES, A. I., WIGBY, S., BOONE, J. M., PARTRIDGE, L., and T. CHAPMAN, 2008 Feeding, fecundity and lifespan in female *Drosophila melanogaster*. Proc Biol Sci. **275:**1675–1683.

BEGUN, D. J., and H. A. LINDFORS, 2005 Rapid evolution of genomic Acp complement in the melanogaster subgroup of *Drosophila*. Mol. Bio.l Evol. **22:** 2010–2021.

BEGUN, D. J., LINDFORS, H. A., THOMPSON, M. E., and A. K. HOLLOWAY, 2006 Recently evolved genes identified from *Drosophila yakuba* and *D. erecta* accessory gland expressed sequence tags. Genetics **172:** 1675–1681.

BIRKHEAD, T. R., and A. P. MØLLER (eds), 1998 Sperm Competition and Sexual Selection. London, UK: Academic Press.

CALKINS, J. D., EL-HINN, D., and W. J. SWANSON, 2007 Adaptive evolution in an avian reproductive protein: ZP3. J. Mol. Evol. **65:** 555–563.

CIRERA, S., and M. AGUADÉ, 1998 Molecular evolution of a duplication: the sex-peptide (Acp70A) gene region of *Drosophila subobscura* and *Drosophila madeirensis*. Mol. Biol. Evol. **15:** 988–996.

CHAPMAN, T., LIDDLE, L. F., KALB, J. M., WOLFNER, M. F., and L. PARTRIDGE, 1995 Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature **373:** 241–244.

CLARK, N. L., J. E. AAGAARD, and W. J. SWANSON, 2006 Evolution of reproductive proteins from animals and plants. Reproduction **131:** 11–22.

CLARK, N. L., G. D. FINDLAY, X.YI, M. J. MACCOSS, and W. J. SWANSON, 2007 Duplication and selection on abalone sperm lysin in an allopatric population. Mol. Biol. Evol. **24:** 2081–2090.

CLARK, N. L., GASPER, J., SEKINO, M., SPRINGER, S. A., AQUADRO, C. F., and W. J. SWANSON, 2009 Coevolution of interacting fertilization proteins. P. L. o. S. Genetics **5:** e1000570.

DOPMAN, E. B., and D. L. HARTL, 2007 A portrait of copy-number polymorphism in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. **104:** 19920–19925.

EBERHARD, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton, New Jersey: Princeton University Press.

FINDLAY, G. D ., X. YI, M. J. MACCOSS, and W. J. SWANSON, 2008 Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. P. L. o. S. Biol. **6:** e178.

FRACZKIEWICZ, R., and W. BRAUN, 1998 Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. J. Comp. Chem. **19:** 319.

FU, Y. X., and W. H. LI 1993. Statistical tests of neutrality of mutations. Genetics **133:** 693–709.

GASPER J., and W. J. SWANSON, 2006 Molecular population genetics of the gene encoding the human fertilization protein zonadhesin reveals rapid adaptive evolution. Am. J. Hum. Genet. **79:** 82–30.

GOOD, J. M., ROSS, C. L., and T. A. MARKOW, 2006 Multiple paternity in wild-caught *Drosophila mojavensis*. Mol Ecol. **15:** 2253–2260.

INNAN, H., 2003A The coalescent and infinite-site model of a small multigene family. Genetics **163:** 803–810.

KELLEHER, E. S., and T. A. MARKOW, 2007 Reproductive Tract Interactions Contribute to Isolation in *Drosophila*. Fly **1:** 33–37.

KELLEHER, E. S., W. J. SWANSON, and T. A. MARKOW, 2007 Gene Duplication and Adaptive Evolution of Digestive Proteases in *Drosophila* Female Reproductive Tracts. P.L.o.S. Genet. **3:** 1541–1549.

KELLEHER, E. S., and T. A. MARKOW, 2009 Duplication, Selection and Gene Conversion in a *Drosophila mojavensis* Female Reproductive Protein Family. Genetics. **181:**1451–1465.

KELLEHER, E. S., WATTS, T. D., LAFLAMME, B. A., HAYNES, P. A., and T. A. MARKOW, 2009 Proteomic analysis of *Drosophila mojavensis* male accessory glands suggests novel classes of seminal fluid proteins. Insect Biochem. Mol. Biol. **39:** 366–371

KELLEHER, E. S., and J. E. PENNINGTON, 2009 Protease gene duplication and proteolytic activity in Drosophila female reproductive tracts. Mol. Biol. Evol. 2009 **26:** 2125–2134.

KELLY J. K., 1997 A test of neutrality based on interlocus associations. Genetics 1997 **146:** 1197–1206.

KNOWLES, L. L., and T. A. MARKOW, 2001 Sexually antagonistic coevolution of a postmating prezygotic reproductive character in desert *Drosophila*. Proc. Nat. Acad. Sci. U. S. A. **98:** 8692–8696.

KNOWLES, L. L., HERNANDEZ, B. B., and T. A. MARKOW, 2005 Non-antagonistic interactions between the sexes revealed by the ecological consequences of reproductive traits. J. Evol. Biol. **18:** 156–161.

KUIJPER, B., STEWART, A. D., and W. R. RICE, 2006 The cost of mating rises nonlinearly with copulation frequency in a laboratory population of *Drosophila melanogaster*. J. Evol. Biol. **19:** 1795–1802.

LAWNICZAK, M. K., and D. J. BEGUN, 2007 Molecular population genetics of female-expressed mating-induced serine proteases in *Drosophila melanogaster*. Mol. Biol. Evol. **24:** 1944–1951.

LEE ,Y. H., OTA, T., and V. D. VACQUIER, 1995 Positive selection is a general phenomenon in the evolution of abalone sperm lysin. Mol. Biol. Evol. **12:** 231–8.

Levitan, D. R., and D. L. Ferrell, 2006 Selection on gamete recognition proteins depends on sex, density, and genotype frequency. Science **312:** 267–269.

Machado, C. A., L. M. Matzkin, L. K. Reed, and T. A. Markow, 2007 Multilocus nuclear sequences reveal intra- and interspecific relationships among chromosomally polymorphic species of cactophilic *Drosophila*. Mol. Ecol. 2007 **16:** 3009–3024.

Markow, T.A., and P.F. Ankney, 1984 *Drosophila* Males Contribute to Oogenesis in a Multiple Mating Species. Science **224:** 302–303.

Markow, T.A., and P.F. Ankney, 1988 Insemination Reaction in *Drosophila* found in species whose males contribute material to oocytes before fertilization. Evolution **42:** 1097–1101.

Markow, T. A., 1996 Evolution of *Drosophila* mating systems. Evol. Biol. **29:** 73–106.

Matzkin, L. M., 2008 The molecular basis of host adaptation in cactophilic *Drosophila*: molecular evolution of a glutathione S-transferase gene (GstD1) in *Drosophila mojavensis*. Genetics. **178:** 1073-1083.

McDonald, J. H., and M. Kreitman, 1991 Adaptive protein evolution at the Adh locus in *Drosophila*. Nature **351:** 652-654.

Metz, E. C., and S. R. Palumbi, 1996 Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. Mol. Biol. Evol. **13:** 397–406.

Moy, G. W., S. A. Springer, S. L. Adams, W. J. Swanson, and V.D. Vacquier, 2008 Extraordinary intraspecific diversity in oyster sperm bindin. Proc. Natl. Acad. Sci. U. S. A. **105:** 1993–8.

Mueller, J. L., Ravi Ram, K., McGraw, L. A., Bloch Qazi, M. C., Siggia, E. D., Clark, A. G., Aquadro, C. F., and M. F. Wolfner, 2005 Cross-species comparison of Drosophila male accessory gland protein genes. Genetics **171:** 131–43.

Nachman, M. W., 1998 Deleterious mutations in animal mitochondrial DNA. Genetica **102/103:** 61–69.

Ohno, S, 1970 Evolution by gene duplication. Springer-Verlag, New York.

Otto, S. P., and P. Yong, 2002 The evolution of gene duplicates. Adv. Genet. **46:** 451–483.

Panhuis, T. M., and W. J. Swanson, 2006 Molecular evolution and population genetic analysis of candidate female reproductive genes in *Drosophila*. Genetics **173:** 2039–2047.

Panhuis, T. M., N. L. Clark, and W. J. Swanson, 2006 Rapid evolution of reproductive proteins in abalone and *Drosophila*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **361:** 261–268.

Parker, G. A., 1979 Sexual selection and sexual conflict, pp. 123–166 in *Sexual selection and reproductive competition in insects*, edited by M. S. Blum and N. A. Blum. Academic Press, London, U. K.

Patterson, J. T., 1946 A new type of isolating mechanism *in Drosophila*. Proc. Nat. Acad. Sci. U. S. A. **32:** 202–208.

Pfeiler, E., Castrezana, S., Reed, L. K. and T. A. Markow, 2009 Genetic, ecological and morphological differences among populations of the cactophilic *Drosophila* *mojavensis* from southwestern USA and northwestern Mexico, with descriptions of two new subspecies. Journal of Natural History **43:** 923-938.

PITNICK, S, SPICER, G. S., and T. A. MARKOW, 1997 Phylogenetic examination of female incorporation of ejaculate in *Drosophila*. Evolution **51:** 833–845.

PITNICK, S., and F. GARCÍA-GONZÁLEZ, 2002 Harm to females increases with male body size in *Drosophila melanogaster.* Proc. Biol. Sci. **269:** 1821–1828.

PITNICK, S., MILLER, G. T., SCHNEIDER, K., and T. A. MARKOW 2003 Ejaculate-female coevolution in *Drosophila mojavensis*. Proc Biol Sci. **270:** 507–512.

POLGAR, L., 2005 The catalytic triad of serine peptidases. Cell. Mol. Life. Sci. **62:** 2161–2172.

PROULX, S. R., and R. C. PHILLIPS, 2006 Allelic divergence precedes and promotes gene duplication. Evolution **60:** 881–892.

REED, L. K., NYBOER, M., and T. A. MARKOW, 2007 Evolutionary relationships of *Drosophila mojavensis* geographic host races and their sister species *Drosophila arizonae.* Mol. Ecol. **16:** 1007–1022.

ROZAS, J., GULLAUD, M., BLANDIN, G., and M. AGUADÉ, 2001 DNA variation at the rp49 gene region of *Drosophila simulans*: evolutionary inferences from an unusual haplotype structure. Genetics **158:** 1147–1155.

ROZAS, J., SÁNCHEZ-DELBARRIO, J. C., MESSEGUER, X., and R. ROZAS, 2003 DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19:** 2496–2497.

SAITOU, N., and M. NEI, 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406–425.

SAWYER, S., 1989 Statistical tests for detecting gene conversion. Mol Biol Evol **6:** 526–538.

SCHWEDE, T., KOPP, J., GUEX, N., and M. C. PEITSCH, 2003 SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. **31:** 3381**–**3385.

SPOFFORD, J. B., 1969 Heterosis and evolution of duplications. Am. Nat. **103:** 407–432.

SPRINGER, S. A., MOY, G. W., FRIEND, D. S., SWANSON, W. J., and V. D. VACQUIER 2008. Oyster sperm bindin is a combinatorial fucose lectin with remarkable intra-species diversity. Int. J. Dev. Biol. **52:** 759-768.

SRINIVASAN, A., GIRI, A. P., and V. S. GUPTA, 2006 Structural and functional diversities in *lepidopteran* serine proteases. Cell. Mol. Biol. Lett. **11:** 132–154.

SWANSON W. J., and V. D. VACQUIER, 2002A The rapid evolution of reproductive proteins. Nat. Rev. Genet. **3:** 137–144.

SWANSON W. J., and V. D. VACQUIER, 2002B Reproductive Protein Evolution. Annu. Rev. Ecol. and Syst. **33:** 161-179.

SWANSON, W. J., WONG, A., WOLFNER, M. F., and C. F. AQUADRO, 2004 Evolutionary expressed sequence tag analysis of *Drosophila* female reproductive tracts identifies genes subjected to positive selection. Genetics **168:** 1457–1465.

SWOFFORD, D. L., 2000 PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

TAJIMA F., 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:** 585–595.

THORNTON, K., and M. LONG, 2005. Excess of amino acid substitutions relative to polymorphism between X-linked duplications in *Drosophila melanogaster*. Mol. Biol. Evol. **22:** 273-284.

THORNTON, K. R., 2007 The neutral coalescent process for recent gene duplications and copy-number variants. Genetics **177:** 987–1000.

WAGSTAFF, B. J., and D. J. BEGUN, 2005 Molecular population genetics of accessory gland protein genes and testis-expressed genes in *Drosophila mojavensis* and *D. arizonae.* Genetics **171:** 1083–1101.

WAGSTAFF, B. J., and D. J. BEGUN, 2007 Adaptive evolution of recently duplicated accessory gland protein genes in desert *Drosophila.* Genetics **177:** 1023–1030.

WALSH, B., 2003 Population-genetic models of the fates of duplicate genes. Genetica **118:** 279–94.

YANG, Z., 1997 PAML: A program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. **13:** 555–556.

YANG, Z., WONG, W. S., and R. NIELSEN, 2005 Bayes empirical bayes inference of amino acid sites under positive selection. Mol. Biol. Evol. **22:** 1107–1118.

Table 1. Comparison of FST values for neutral and FRP loci.

	wr.* and *mo.	***ba.* and *wr.***	***so.* and *wr.***	***ba.* and *mo.***	***so.* and *mo.***	***so.* and *ba.***	**All**
neutral mean FST	0.60 (±0.24)	0.44 (+0.32)	0.42 (+0.32)	0.46 (+0.25)	0.53 (+0.19)	0.19 (+0.21)	0.44 (+0.24)
FRP-A	0.45^{*}	0.13^{**}	0.45	$0.12^{***\dagger}$	$-0.02^{***\dagger}$	0.11	0.3^{**}
FRP-B	$0.26^{***\dagger}$	0.32	0.23	0.19^{**}	$0.13^{***\dagger}$	0.02^{**}	$0.22^{**\dagger}$
FRP-C	0.67	$0.08^{**\dagger}$	0.45	0.28	$0.25^{***\dagger}$	0.14	0.26
FRP-D	$0.00^{***\dagger}$	0.30	0.25^{*}	0.30	$0.25^{***\dagger}$	$0.00^{**\dagger}$	$0.18^{***\dagger}$
FRP-E	$0.26^{***\dagger}$	0.25	0.65	$-0.05^{***\dagger}$	$0.26^{***\dagger}$	0.23	0.2^{*}
FRP mean Fst	0.33 $(\pm0.25)^{*}$	0.21 (±0.11)	0.41 (±0.17)	0.16 $(\pm0.14)^{*}$	0.18 $(\pm0.12)^{***\dagger}$	0.11 (± 0.094)	0.24 $(\pm0.05)^{*\dagger}$

Neutral mean FST is calculated from loci in MACHADO *et al* (2007). Values in parentheses represent the standard deviation. For individual loci and and sub-species we determined if FST values were significantly different from the neutral distribution using a Z-test. We furthermore compared the distribution of FST values between neutral and FRP loci using a two-sample t-test. All FST values were calculated in DnaSP (ROZAS *et al* 2003). *wr.* denotes *D. m. wrigleyi*. *ba.* denotes *D. m. baja*. *so.* denotes *D. m. sonorensis*. *mo.* denotes *D. m. mojavensis.* * denotes $p < 0.05$. ** denotes $p < 0.01$. *** denotes $p < 0.001$. † denotes values that remain significant after a conservative Bonferroni correction for multiple measures.

Table 2. Linkage Disequilibrium.

	All		***no conversion***	
	Zns	***Za***	***Zns***	***Za***
FRP-A				
D. m. baja.	.71	0.81^{**}	NA	NA
D. m. wrigleyi	0.42	0.52	NA	NA
D. m. sonorensis	0.47	0.53	NA	NA
D. m. mojavensis	0.81^{*}	$0.90^{**\dagger}$	NA	NA
FRP-B				
D. m. baja	0.62	0.74	0.49	0.60
D. m. wrigleyi	0.77^{**}	0.96^{**}	0.78	0.92^{*}
D. m. sonorensis	0.39	0.6	0.35	0.67
D. m. mojavensis	$.77^{*}$	$0.97^{**\dagger}$	0.75	0.95^{**}
FRP-C				
D. m. baja.	0.38	0.54	0.38	0.54
D. m. wrigleyi	NA		NA	NA
D. m. sonorensis	0.73	0.75	0.71	0.69
D. m. mojavensis	NA		NA	NA
FRP-D				
D. m. baja.	0.91^{**}	$0.96^{***\dagger}$	$0.90^{***\dagger}$	$0.96^{***\dagger}$
D. m. wrigleyi	NA	NA	NA	NA
D. m. sonorensis	0.67	0.80*	0.39	0.53
D. m. mojavensis	NA	NA	NA	NA
FRP-E				
D. m. baja.	0.85^{**}	0.85^{**}	0.84^{**}	0.85^{**}
D. m. wrigleyi	0.71	$0.95^{**\dagger}$	NA	NA
D. m. sonorensis	0.38	0.47	NA	NA
D. m. mojavensis	0.91^{**}	$0.93^{**\dagger}$	NA	NA

Zns (Kelly 1997) and *Za* (Rozas 2001) were calculated for all combinations of subspecies and loci in DnaSP, and statistical significance was determined by coalescent simulations under the conservative assumption of no recombination (Rozas and Rozas 1995). Values for all sites, as well as values where sites with evidence for gene conversion are reported. NA indicates the statistic was incalculable, or there was no evidence for gene conversion. * denotes p < 0.05. ** denotes p < 0.01. *** denotes p < 0.001. † denotes values that remain significant after a conservative Bonferroni correction for multiple measures.

Table 3. Site Frequency Spectra.

	Tajima's D	***Fu and Li's F***	***Fay and Wu's H***
FRP-A			
D. m. baja.	-0.28	0.21	-6.93
D. m. wrigleyi	0.69	1.49	0.19
D. m. sonorensis	0.42	0.52	0.14
D. m. mojavensis	1.30	1.625*	-3.71
FRP-B			
D. m. baja.	-0.76(-0.88)	-0.63(-0.67)	-5.71(4.95)
D. m. wrigleyi	0.47(0.61)	0.87(0.88)	-2.61(-2.48)
D. m. sonorensis	0.5(0.72)	0.88(0.93)	1.52(1.33)
D. m. mojavensis	0.46(0.618)	1.05(0.59)	-5.14(-1.86)
FRP-C			
D. m. baja.	-1.3(-1.07)	-1.7(-1.05)	-2.48(-3.49)
D. m. wrigleyi	0.21	-0.18	0.38
D. m. sonorensis	1.57*(1.65*)	1.66(1.41)	-0.91(-0.05)
D. m. mojavensis	-1.36	0.02	0.01
FRP-D			
D. m. baja.	1.60*(1.58*)	1.67(1.63*)	-1.10(-1.24)
D. m. wrigleyi	-1.01	-1.34	0.24
D. m. sonorensis	0.42(-1.11*)	0.61(-0.98)	-0.24(-4.19)
D. m. mojavensis	0.42	0.47	-1.5
FRP-E			
D. m. baja.	1.19(1.04)	1.55(1.51)	1.62(-1.048)
D. m. wrigleyi	1.52*	1.84*	2.52
D. m. sonorensis	-0.99	-0.51	-5.71*
D. m. mojavensis	0.82	0.75	3.24

Tajima’s *D* (TAJIMA 1989) Fu and Li’s *F* (FU and LI 1993) were calculated for all combinations of races and loci in DnaSP and statistical significance was assessed by coalescent simulations under the conservative assumption of no recombination (ROZAS *et al* 2003). Values in parentheses are generated from samples in which haplotypes with evidence for gene conversion are excluded. * denotes p < 0.05.

Table 4. MK-Tests of FRP-A.

		Standard MK Test			Lineage-Specific MK Test		
		Poly.	**Fixed**	**Test**	**Poly.**	**Fixed**	**Test**
D. m. baja	*S*	19	7	*G-test*	12	1	*FET*
	N	26	6	NS	19	1	NS
D. m. wrigleyi	*S*	10	9	*G-test*	6	2	*FET*
	N	8	5	NS	1	4	NS
D. m. sonorensis	*S*	17	7	*G-test*	10	1	*FET*
	N	32	5	NS	25	1	NS
D. m. mojavensis	*S*	11	8	*G-test*	7	2	*FET*
	N	26	5	*	19	1	NS

Silent or non-coding (*S*) and replacement (*N*) variation within sub-species of *D. mojavensis* were compared to divergence from the *D. arizonae* ortholog. *Drosophila mojavensis* lineage-specific tests were polarized with *D. navojoa* outgroup. Significance was assessed by Fisher's exact test (FET) or a G-test when appropriate. Poly. denotes polymorphic. * denotes p < .05.

Table 4. Modified MK-Tests of FRP-D and FRP-E.

		FRP-D Lineage-Specific MK Test			**FRP-E Lineage-Specific MK Test**		
		Poly.	**Fixed**	**Test**	**Poly.**	**Fixed**	**Test**
	S	20	3	*G-test*	16	12	*G-test*
D. m. baja	*N*	8	11	**	25	9	NS
	S	0	13	*FET*	7	11	*G-test*
D. m. wrigleyi	*N*	1	16	NS	9	12	NS
	S	27	3	*G-test*	3	12	*FET*
D. m. sonorensis	*N*	23	13	**†	8	6	*p=.06*
	S	0	11	*FET*	18	10	*G-test*
D. m. mojavensis	*S*	1	14	NS	21	9	NS

Silent or non-coding (*S)* and replacement *(N)* variation within sub-species of *D. mojavensis* were compared to divergence from opposing paralog, as in THORNTON and LONG (2005). The *D. mojavensis* lineage-specific tests were polarized with *D. navojoa* outgroup, and the type of contingency test is indicated. Poly. denotes polymorphic. * denotes $p < 0.05$. ** denotes $p < 0.01$. † denotes values that remain significant after a conservative Bonferroni correction for multiple measures.

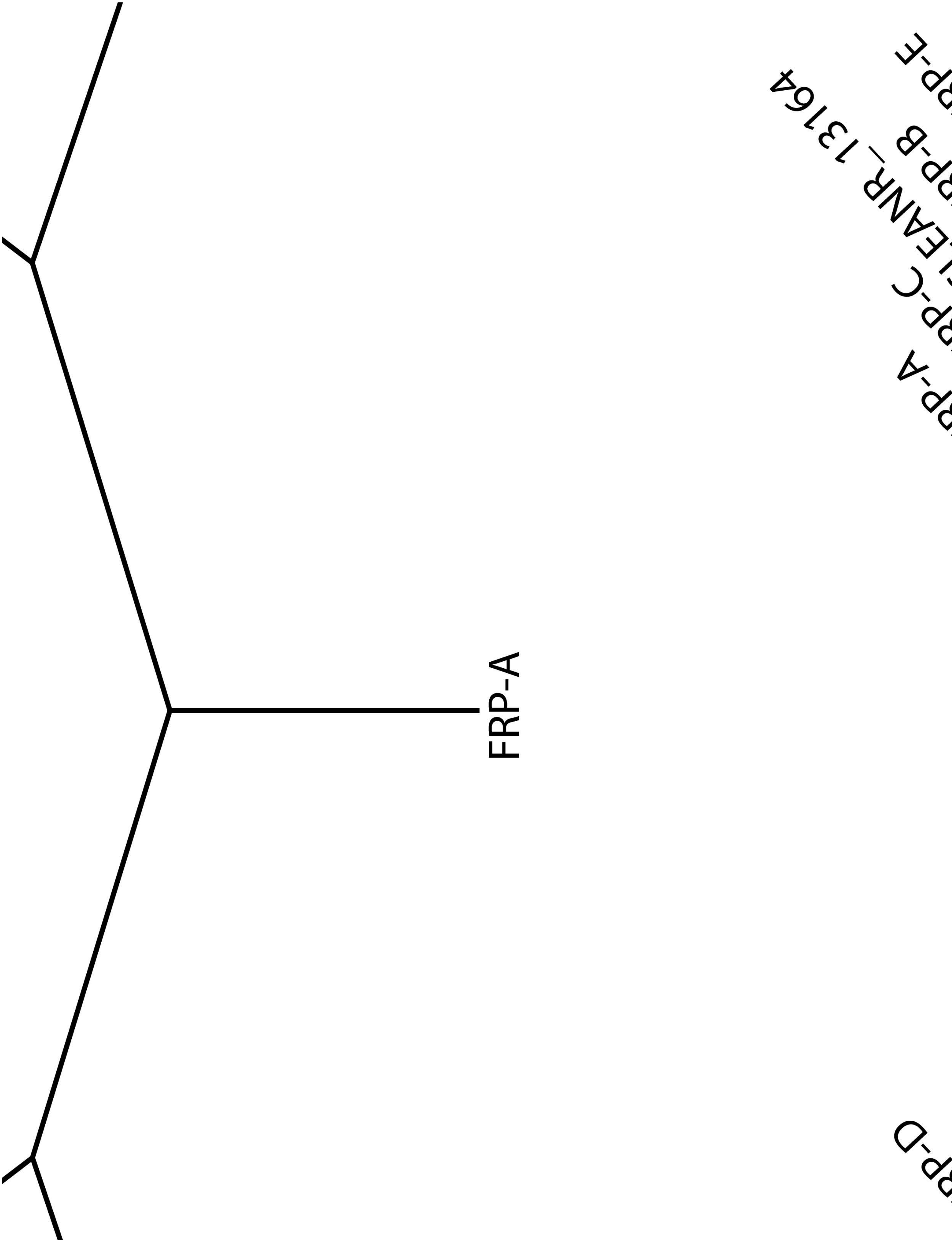
Figure 1. Phylogenetic Relationships and Genomic Arrangement of the Paralogs Examined in this Study. A) Evolutionary relationships between paralogs adapted from KELLEHER *et al* (2007) B) Genomic arrangement of the protease gene family on *D. mojavensis* chromosome 4 (scaffold_6680 bp 10216565-10169309, http://rana.lbl.gov/drosophila/). White blocks indicate individual exons of duplicated paralogs, while grey blocks indicate individual exons of flanking and interspersed coding sequences. Arrows indicate the direction of transcription.

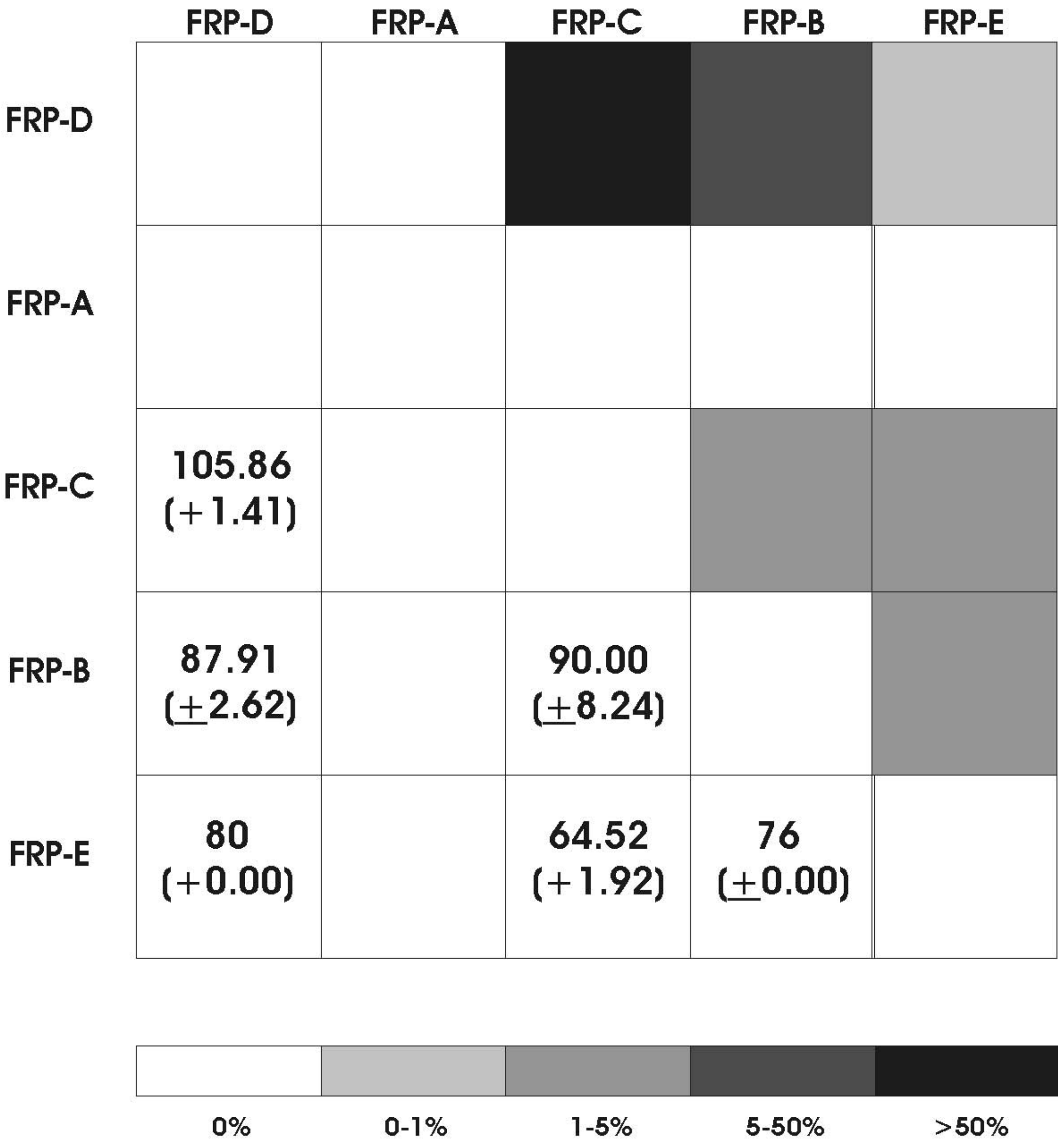
Figure 2. Ectopic Recombination. An alignment of all unique haplotypes was used to detect significant fragments of complete identity in GENECONV, based on the method of SAWYER (1989). The percentage of pairwise comparisons between paralogs that show evidence of gene conversion is indicated by gray shading in the upper right. The average length of identified conversion tracts between paralogs, and the standard deviation of this estimate, are indicated in the lower left.

Figure 3. Sliding Window Analysis of Gene Conversion. Y-axis denotes the percentage of haplotypes from the full alignment, including all haplotypes of all subspecies and all paralogs, that show evidence of gene conversion at a particular site. The intron-exon structure is shown to scale along the X-axis, with a total length of ~1 kb. Black arrows denote selected sites from KELLEHER *et al* (2007).

Figure 4. Female Reproductive Proteases Exhibit Unusual Haplotype Structures. A) Geographic distribution and collection sites of four isolated subspecies of *D. mojavensis*: *D. m. baja* (Baja Peninsula, grey star). *D. m wrigleyi* (Catalina Island, grey circle), *D. m. sonorensis* (Mainland Sonora, black star), and *D. m. mojavensis* (Mojave Desert, black circle). Gene genealogies of FRP-A (B), FRP-B (C), FRP-C (D), FRP-D (E), and FRP-E (F). All genealogies were inferred by neighbor-joining in Paup*4.0b10(SWOFFORD 2000). Symbols indicate sub-species of origin, and number of symbols indicates individual sampled alleles that correspond to that haplotype. Bootstrap values are indicated.

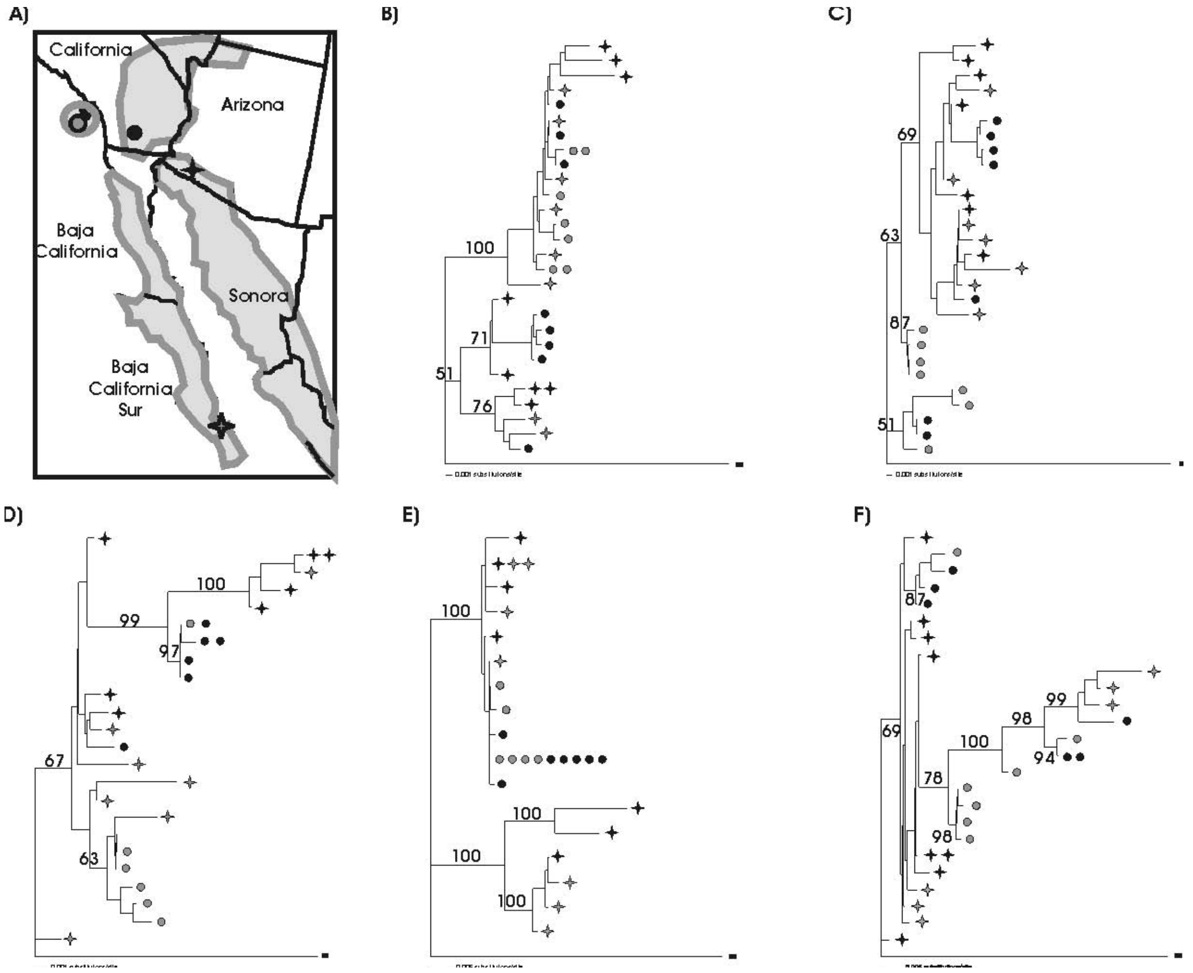
Figure 5. Predicted 3D Structure of FRP-C. Bayes Empirical Bayes selected sites identified under M8 (YANG 1997; YANG *et al* 2005) were identified in KELLEHER *et al* (2007). Sites that are determinants of protease inhibitor susceptibility are shown in white (Reviewed in SRINIVASAN *et al* 2006). Sites in grey comprise the catalytic triad (Reviewed in POLGAR 2005). Selected sites 124, 244, and 246 also are determinants of inhibitor susceptibility.

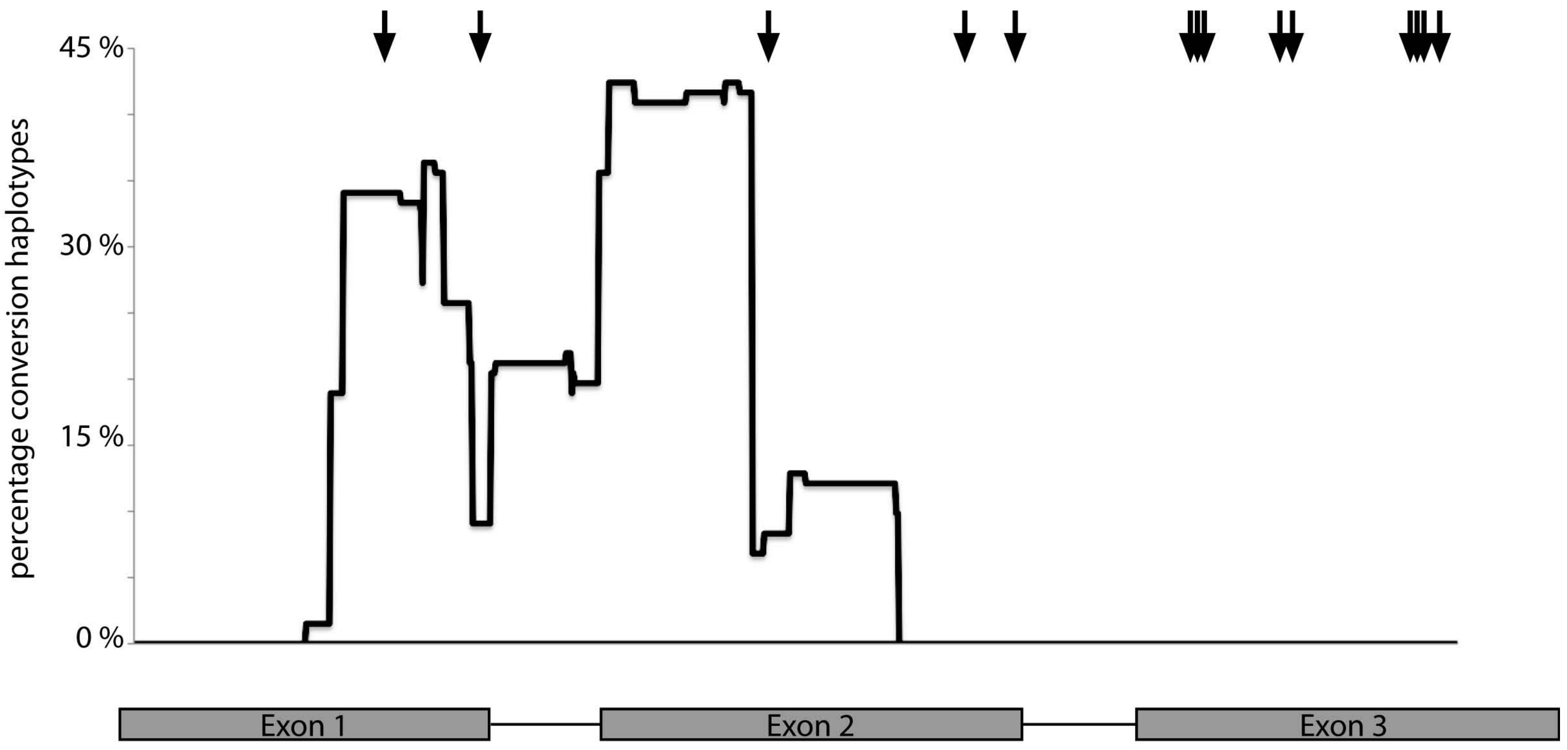

FRP-A
RP-E
RP-B
EANR_13164
RP-C
RP-A
RP-D

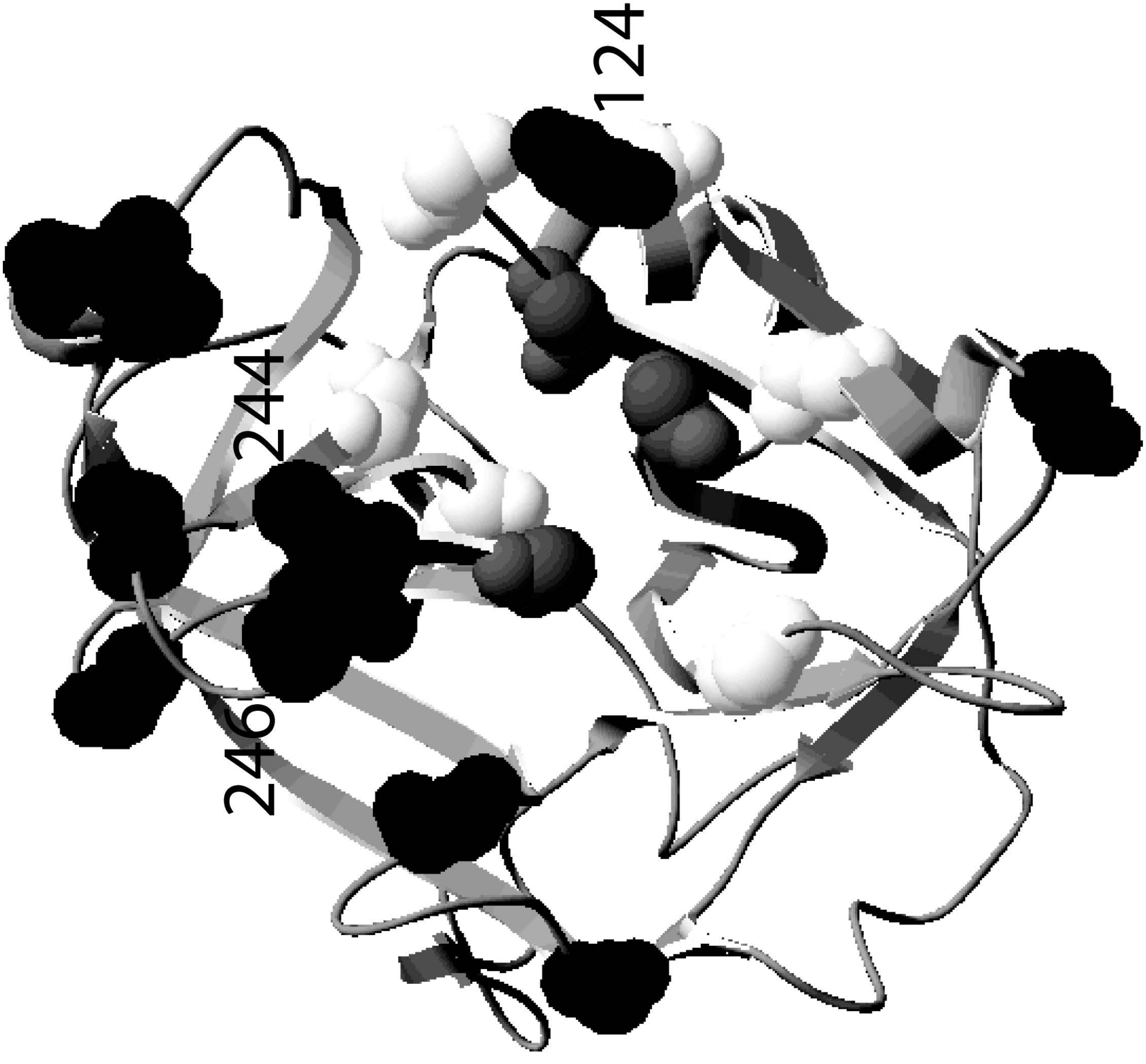


	FRP-D	FRP-A	FRP-C	FRP-B	FRP-E
FRP-D			■	▓	░
FRP-A					
FRP-C	105.86 (+1.41)			▒	▒
FRP-B	87.91 (±2.62)		90.00 (±8.24)		▒
FRP-E	80 (+0.00)		64.52 (+1.92)	76 (±0.00)	

□	░	▒	▓	■
0%	0-1%	1-5%	5-50%	>50%


A)
California
Arizona
Baja California
Sonora
Baja California Sur
B)
100
71
51
76
0.001 substitutions/site
C)
69
63
87
51
0.001 substitutions/site
D)
100
99
97
67
63
E)
100
100
100
100
F)
87
69
99
98
100
94
78
98


45 %
30 %
15 %
0 %
percentage conversion haplotypes
Exon 1
Exon 2
Exon 3


124
244
246